Halofuginone-Induced Amino Acid Starvation Regulates Stat3-Dependent Th17 Effector Function and Reduces Established Autoimmune Inflammation

Thaddeus J. Carlson, Alex Pellerin, Ivana M. Djuretic, Catherine Trivigno, Sergei B. Koralov, Anjana Rao and Mark S. Sundrud

J Immunol 2014; 192:2167-2176; Prepublished online 31 January 2014;
doi: 10.4049/jimmunol.1302316
http://www.jimmunol.org/content/192/5/2167

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/01/31/jimmunol.1302316.DCSupplemental

References
This article cites 47 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/192/5/2167.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Halofuginone-Induced Amino Acid Starvation Regulates Stat3-Dependent Th17 Effector Function and Reduces Established Autoimmune Inflammation

Thaddeus J. Carlson,* Alex Pellerin,* Ivana M. Djuretic,* Catherine Trivigno,† Sergei B. Koralov,‡,1 Anjana Rao,‡,2 and Mark S. Sundrud*,†,‡

The IL-23 pathway is genetically linked to autoimmune disease in humans and is required for pathogenic Th17 cell function in mice. However, because IL-23R–expressing mature Th17 cells are rare and poorly defined in mice at steady-state, little is known about IL-23 signaling. In this study, we show that the endogenous CCR6+ memory T cell compartment present in peripheral lymphoid organs of unmanipulated mice expresses Il23r ex vivo, displays marked proinflammatory responses to IL-23 stimulation in vitro, and is capable of transferring experimental autoimmune encephalomyelitis. The prolyl-tRNA synthetase inhibitor halofuginone blocks IL-23–induced Stat3 phosphorylation and IL-23–dependent proinflammatory cytokine expression in endogenous CCR6+ Th17 cells via activation of the amino acid starvation response (AAR) pathway. In vivo, halofuginone shows therapeutic efficacy in experimental autoimmune encephalomyelitis, reducing both established disease progression and local Th17 cell effector function within the CNS. Mechanistically, AAR activation impairs Stat3 responses downstream of multiple cytokine receptors via selective, posttranscriptional suppression of Stat3 protein levels. Thus, our study reveals latent pathogenic functions of endogenous Th17 cells that are regulated by both IL-23 and AAR pathways and identifies a novel regulatory pathway targeting Stat3 that may underlie selective immune regulation by the AAR. The Journal of Immunology, 2014, 192: 2167–2176.

Th17 cells accumulate at mucosal surfaces where they regulate inflammation and immunity via the production of IL-17A, IL-17F, and IL-22 (reviewed in Ref. 1). They are also widely involved in the pathogenesis of many common autoimmune disorders, including rheumatoid arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease. Thus, understanding points of Th17 control is important for the rational design of new and more selective autoimmune therapies.

Th17 cells differentiate from naive precursors following TCR stimulation in the presence of IL-6, together with TGF-β or IL-1β (2–4). These signals converge to induce expression of the ROR, Rorc (RORγt), in a Stat3-dependent manner (5, 6). RORγt and Stat3, in turn, synergize to activate Il17a gene expression and locus remodeling (7). Accordingly, T cells lacking Rorc or Stat3 display impaired Th17 differentiation in vitro, and these cells fail to induce Th17-associated pathologies when adoptively transferred in vivo (3, 5). Numerous other transcription factors and signaling pathways have been implicated in the control of Th17 differentiation in recent years (reviewed in Refs. 8, 9).

Much less is known about the regulation of pre-established memory Th17 cells and their effector functions, although this is undoubtedly an important aspect of clinical autoimmunity. Local cytokine production by mature Th17 cells within autoimmune lesions is both a biomarker for, and a proximal cause of, tissue damage (1, 10). IL-23 is a Stat3-activating cytokine that acts on memory Th17 cells to enforce expression of inflammatory cytokines, including IL-17A, IL-17F, and IL-22, and it is necessary for the pathogenic function of murine Th17 cells in vivo (4, 11, 12). Moreover, genome-wide association studies have linked polymorphisms in the Il23r gene to several human autoimmune disorders, including Crohn’s disease, psoriasis, psoriatic arthritis, ankylosing spondylitis, spondyloarthropathies, and Behçet’s disease (13–16).

Despite their established roles in autoimmune pathogenesis, the pathways that regulate IL-23 signaling in Th17 cells are unknown. In large part, this is because IL-23R is only expressed on proinflammatory subsets of mature Th17 cells, which are thought to be extremely rare in mice at steady-state (17). The few endogenous Th17 cells that are present in unmanipulated mice reside in portions of the gut and develop in response to colonization by specific microbiota (3, 18, 19), although it is unclear whether these or other endogenous Th17 cell populations express IL-23R and have pathogenic potential (18, 20). Because of these logistical issues, Th17 cell responses to IL-23 have only been studied in T cell populations generated in vitro or through analysis of Il23r−/− mice, where direct molecular analysis of IL-23–mediated signal transduction is not possible (12, 21, 22).

Clues from the human immune system provide insights into the potential identity of IL-23–responsive Th17 cells. Human peripheral blood contains a larger reservoir of endogenous Th17
cells than do the peripheral lymphoid organs of wild-type mice. Human Th17 cells are characterized by the expression of IL-17A, as well as by the expression of the Th17-associated chemokine receptor CCR6 (23–25). More recent evidence suggests that human CCR6+ memory T cells can express Th17-lineage markers, including Rorc and Il23r, independent of IL-17A expression (25). Thus, endogenous IL-23–responsive Th17 cells in mice may be underestimated by analyses of IL-17A expression alone.

In this study, we show that steady-state expression of CCR6 in unmanipulated healthy mice discriminates an endogenous Th17 cell compartment that includes IL-23–responsive memory Th17 cells. These cells, like their human counterparts, express Rorc and Il23r ex vivo and upregulate IL-17A, IL-17F, and IL-22 expression upon stimulation in vitro. As predicted by their responsiveness to IL-23, we show that endogenous mouse CCR6+ memory Th17 cells have latent pathogenic functions and induce experimental autoimmune encephalomyelitis (EAE) following transfer into lymphopenic recipients. In leveraging this platform to investigate IL-23 signaling in Th17 cells, we describe a novel link between the IL-23 and amino acid starvation response (AAR) pathways.

The AAR pathway is an evolutionarily conserved, cytoprotective stress response that is activated by unaminoacylated (uncharged) tRNA molecules (reviewed in Ref. 26). AAR activation is controlled by binding of uncharged tRNAs to the protein kinase Gcn2, which, in turn, phosphorylates eIF2α to promote translation of the functional open reading frame of the stress-activated transcription factor Atf4. Whereas eIF2α phosphorylation limits amino acid demand by reducing protein synthesis, Atf4 increases amino acid supply by inducing transcription of gene products involved in amino acid transport and biogenesis (27, 28). The AAR pathway is activated physiologically when amino acid concentrations are limiting, but it also can be induced pharmacologically via treatment of cells with tRNA synthetase inhibitors, such as the plant natural product derivative and prolyl-tRNA synthetase inhibitor, halofuginone (HF) (29, 30).

We showed previously that HF blocks Th17 differentiation in vitro and protects mice from developing IL-17A–associated autoimmune pathology in vivo (31). We further demonstrated that HF activates the AAR by directly binding to and inhibiting the enzymatic function of the mammalian prolyl-tRNA synthetase, EPRS (29, 31). In this study, we show that HF-induced AAR activation selectively blocks IL-23–mediated Stat3 signaling and downstream induction of inflammatory cytokines in endogenous CCR6+ Th17 cells; acts therapeutically in vivo, limiting both experimental autoimmune encephalomyelitis (EAEC) following i.c. injection of B. pertussis toxin on days 0 and 1, (0.5 mg/kg) or vehicle (DMSO) diluted in PBS was injected i.p. daily (6 μg/mouse; ~0.3 mg/kg), beginning at the onset of clinical symptoms. Disease was scored daily as follows: 0, asymptomatic; 1, limping; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete paralysis of one or more limbs; and 5, moribund state. Cell analyses were performed after 1 wk of treatment by isolating lymph nodes (axillary, inguinal, brachial, and cervical), spleen, and CNS. For EAE-transfer experiments, 105 day 5–expanded naive, CCR6+ memory, or CCR6+ memory T cells were injected i.p. into Rag1−/− mice.

Cell culture

T cells were magnetically isolated from pooled lymph node and spleen cells, using CD4+ negative isolation kits from either EasySep or Miltenyi Biotech, according to the manufacturer’s instructions. naïve (CD45.2+CD44+CD62Lhi) or memory (CD45.1+CD62Llo) T cells were further sorted for CCR6 expression by FACS sorting (FACS Aria II; BD). Cells were ≥98% pure following sorting, as determined by FACS analyses.

Mice were housed in specific pathogen–free barrier facilities at Tempero with protocols approved by respective animal care and use committees.

Materials and Methods

Mice

Mice were housed in specific pathogen-free barrier facilities at the University of Pennsylvania. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17A–IRE- eGFP-transgenic reporter mice were purchased from Biogenetix (Worcester, MA) and were bred to 2D2 TCR-transgenic mice at The Jackson Laboratory. IL-23R–GFP reporter mice were licensed from Prof. Vijay Kuchroo (Brigham and Women’s Hospital; Boston, MA) and bred at The Jackson Laboratory.

Experimental autoimmune encephalomyelitis

Mice were immunized in the dorsal flanks by s.c. injection of 200 μg MOG35–55 peptide, emulsified in IFA (Difco) and supplemented with 5 mg/ml heat-killed Mycobacterium tuberculosis strain H37Ra (Difco). Mice were injected i.p. with 300 ng Borrelia pertussis toxin on days 0 and 1, (0.5 mg/kg) or vehicle (DMSO) diluted in PBS was injected i.p. daily (6 μg/mouse; ~0.3 mg/kg), beginning at the onset of clinical symptoms. Disease was scored daily as follows: 0, asymptomatic; 1, limping; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete paralysis of one or more limbs; and 5, moribund state. Cell analyses were performed after 1 wk of treatment by isolating lymph nodes (axillary, inguinal, brachial, and cervical), spleen, and CNS. For EAE-transfer experiments, 105 day 5–expanded naive, CCR6+ memory, or CCR6+ memory T cells were injected i.p. into Rag1−/− mice.

Cell isolation

T cells were magnetically isolated from pooled lymph node and spleen cells, using CD4+ negative isolation kits from either EasySep or Miltenyi Biotech, according to the manufacturer’s instructions. naïve (CD45.2+CD44+CD62Lhi) or memory (CD45.1+CD62Llo) T cells were further sorted for CCR6 expression by FACS sorting (FACS Aria II; BD). Cells were ≥98% pure following sorting, as determined by FACS analyses.

Real-time qPCR

Total RNA was isolated from flash-frozen T cell pellets using an RNeasy Plus Mini kit (Qiagen). A total of 1–10 ng RNA, quantified by a NanoDrop spectrophotometer (Thermo Scientific), was used for cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems). TaqMan quantitative PCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystems). All TaqMan primer/probe sets were from Applied Biosystems, and assay IDs were: Mm00437762_m1, Mm01261222_m1, Mm00519943_m1, Mm00439619_m1, Mm00521423_m1, Mm00444241_m1, Mm01268754_m1, Mm01168134_m1, Mm01290062_m1, and Mm01219775_m1.

Quantitative PCR

Total RNA was isolated from flash-frozen T cell pellets using an RNeasy mini kit (Qiagen). A total of 1–10 ng RNA, quantified by a NanoDrop spectrophotometer (Thermo Scientific), was used for cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems). TaqMan quantitative PCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystems). All TaqMan primer/probe sets were from Applied Biosystems, and assay IDs were: Mm00437762_m1, Mm01261222_m1, Mm00519943_m1, Mm00439619_m1, Mm00521423_m1, Mm00444241_m1, Mm01268754_m1, Mm01168134_m1, Mm01290062_m1, and Mm01219775_m1.
Immunoblotting

Cultured T cells were harvested at the indicated time points, washed once in PBS, and lysed on ice at 5–10 × 10^6 cells/ml in Laemmli sample buffer (Bio-Rad) supplemented with protease inhibitor tablets (Roche) and phosphatase inhibitors, as described (31). Whole-cell lysates, cleared by centrifugation at 12,000 × g for 10 min at 4°C, were quantified by Bradford Assay and stored at −80°C. 10–20 μg total protein was resolved via SDS-PAGE. Protein was transferred to nitrocellulose membranes, blocked, and blotted with primary and secondary Abs, per the manufacturer’s instructions. Abs against phospho-Stat3 (9138), total Stat3 (9139), and IgG (1482) were from Cell Signaling. Abs against Stat1 (sc-346), Stat4 (sc-486), Stat5 (sc-835), Stat6 (sc-1689), and β-actin (sc-1616) were from Santa Cruz Biotechnology. Anti-mouse, anti-goat, and anti-rabbit secondary Abs were from GE Life Sciences. Western blots were visualized using the ECL Western blotting System (GE Life Sciences) on a FluorChem Q (Cell Biosciences).

Results

Characterization of endogenous IL-23–responsive Th17 cells in mice

Endogenous Th17 cells in humans and mice are defined by ex vivo expression of IL-17A, and these cells uniformly express the inflammatory chemokine receptor CCR6 (17, 23, 24). However, human CCR6+ memory T cells can express other Th17-lineage markers independent of ex vivo IL-17A expression (25). We reasoned that CCR6 expression might similarly discriminate IL-23–responsive Th17 memory T cells in mice independent of IL-17A. We confirmed that unmanipulated wild-type mice, on either the C57BL/6 or BALB/c background, possessed a small, but readily detectable, population of CD4+CD25+CCR6+ memory T cells in spleen and lymph nodes (Fig. 1A). CCR6 expression was restricted to the CD62LlowCD44hi effector/memory T cell compartment and was observed, on average, on 5–10% of these T cells. Sorted splenic and lymph node CCR6+ memory T cells, but not naive or CCR6− memory cells, expressed Rorc and Il23r, as determined by qPCR (Fig. 1B). Selective expression of Il23r was also evident by GFP expression in a portion of endogenous CCR6+ memory T cells from Il23r+/eGFP reporter mice (32) (Fig. 1C). In contrast to naive or CCR6− memory T cells, ex vivo–stimulated CCR6+ memory T cells expressed characteristic Th17 cytokines (Il17a, Il17f, Il22), as well as the Th17-associated chemokine Ccl20 (Fig. 1D). CCR6− memory T cells preferentially expressed Ifng and Csf2 (the gene encoding GM-CSF) (Fig. 1D), whereas Il2 and Tnf were similarly expressed in CCR6+ and CCR6− memory subsets (data not shown). We confirmed that IL-17A was selectively

![Figure 1](http://www.jimmunol.org/Downloaded_from/...)

**FIGURE 1.** The endogenous CCR6+ memory T cell population in mice contains IL-23–responsive Th17 cells. (A) Ex vivo CCR6 expression determined by FACS analysis in CD4+ naive or memory T cell subsets from spleen and peripheral lymph nodes of wild-type C57BL/6 or BALB/c mice. Naive and memory T cell subsets were distinguished within CD4+CD25− T cells based on expression of CD44 and CD62L (left panels). Data represent more than five independent staining experiments on each mouse strain. (B) Expression of Rorc (RORγt) and Il23r mRNAs in FACS-sorted naive or CCR6+ or CCR6− memory T cells (see Supplemental Fig. 1 for FACS sorting/gating strategy). Rorc and Il23r gene expression was normalized to h2M and is presented as mean differential (fold-change) expression ± SD from triplicate samples. Data represent three qPCR experiments. (C) GFP expression in naive or CCR6− or CCR6+ memory T cells, as determined by FACS analysis of GFP levels in CD4+ T cells isolated from Il23r+/eGFP reporter mice. IL-23–GFP expression is shown in cells gated as in (A). Data represent five experiments. (D) Cytokine and chemokine gene expression determined by qPCR in FACS-sorted naive or CCR6− or CCR6+ memory T cells as in (B). RNA was isolated from cells stimulated ex vivo with PMA and ionomycin. Data are normalized and presented as fold change in triplicate samples, as in (B). Data represent three experiments. (E) GFP expression in naive or CCR6− or CCR6+ memory T cells determined by FACS analysis in CD4+ T cells isolated from Il17a-eGFP-transgenic reporter mice. IL-17A–GFP expression is shown in cells gated as in (A). Data represent five experiments. (F) FACS-sorted CCR6− or CCR6+ memory T cells were activated with anti-CD3/CD28-coated beads, with or without IL-23. Cells were restimulated with PMA and ionomycin on day 2 for intracellular cytokine staining and FACS analysis.
expressed by a portion of ex vivo–stimulated CCR6+ memory T cells via intracellular cytokine staining (data not shown) and by analysis of GFP expression in endogenous naive or memory T cell subsets from IL-17A–GFP–transgenic reporter mice (Fig. 1E).

To test the impact of IL-23 on endogenous memory T cell cytokine production, CCR6+ and CCR6− memory cells were FACS sorted from wild-type mice (Supplemental Fig. 1), and these cells were stimulated with CD3/CD28 for 2 d, with or without IL-23. Upon restimulation and intracellular cytokine staining, only CCR6+ memory cells produced IL-17A, consistent with our ex vivo analyses, whereas both CCR6+ and CCR6− memory subsets produced IFN-γ (Fig. 1F). CCR6+ memory T cells activated in the absence of IL-23 produced IL-17F, but they expressed little to no IL-17F or IL-22 (Fig. 1F). Addition of IL-23 to CCR6+ memory T cells induced marked increases in IL-17A levels, and more cells now also expressed IL-17F and IL-22 (Fig. 1F). Consistent with their lack of Il23r expression, IL-23 had no effect on cytokine expression in CCR6− memory T cells; IL-23 also failed to influence GM-CSF or IFN-γ production by CCR6+ memory T cells (Fig. 1F). Thus, as in humans, CCR6 expression broadly discriminates endogenous mouse memory T cells with Th17 characteristics and can be used to isolate IL-23–responsive Th17 cells from mice at steady-state.

In vivo pathogenic functions of endogenous CCR6+ Th17 cells

IL23R polymorphisms are linked to human autoimmune disease (13–15). Further, Il23r expression is both indicative of, and required for, pathogenic Th17 cell function in mouse models of autoimmunity (2, 4, 11, 12). Thus, our findings that endogenous mouse CCR6+ memory T cells express Il23r ex vivo and display functional responses to IL-23 stimulation in vitro suggested that at least a subset of these cells have pathogenic potential. To test the proinflammatory functions of endogenous CCR6+ Th17 cells in vivo, we isolated these cells, as well as naive and CCR6− memory subsets from IL-17A–GFP–transgenic reporter mice, and these cells were stimulated with CD3/CD28 for 2 d, with or without IL-23. Upon restimulation and intracellular cytokine staining, only CCR6+ memory cells produced IL-17A, consistent with our ex vivo analyses, whereas both CCR6+ and CCR6− memory subsets produced IFN-γ (Fig. 1F). CCR6+ memory T cells activated in the absence of IL-23 produced IL-17F, but they expressed little to no IL-17F or IL-22 (Fig. 1F). Addition of IL-23 to CCR6+ memory T cells induced marked increases in IL-17A levels, and more cells now also expressed IL-17F and IL-22 (Fig. 1F). Consistent with their lack of Il23r expression, IL-23 had no effect on cytokine expression in CCR6− memory T cells; IL-23 also failed to influence GM-CSF or IFN-γ production by CCR6+ memory T cells (Fig. 1F). Thus, as in humans, CCR6 expression broadly discriminates endogenous mouse memory T cells with Th17 characteristics and can be used to isolate IL-23–responsive Th17 cells from mice at steady-state.

Therefore, we tested whether HF-induced AAR activation also regulates IL-23 responses in CCR6+ memory Th17 cells. FACS-sorted CCR6+ or CCR6− memory T cells were activated with anti-CD3/anti-CD28 for 2 d, with or without IL-23, and these cells were further treated with either HF or vehicle (DMSO). HF treatment of CCR6+ memory T cells selectively impaired IL-23–dependent induction of IL-17A, IL-17F, and IL-22 but had no effect on the baseline expression of these cytokines in the absence of IL-23 (Fig. 3A, Supplemental Fig. 2). Further, HF did not affect expression of IFN-γ, GM-CSF, or TNF-α, which were expressed in both CCR6+ and CCR6− memory T cells in an IL-23–independent manner (Fig. 3B, Supplemental Fig. 2A). The effect of HF treatment on IL-23–induced IL-17A expression in CCR6+ memory T cells was dose dependent at concentrations (5–20 nM) that had little to no effect on the basal expression of IL-17A in CCR6+ memory cells or IFN-γ production by CCR6+ or CCR6− memory T cells (Fig. 3C, data not shown). Moreover, HF-mediated inhibition of IL-23–inducible IL-17A expression was
abolished following addition of excess l-proline to culture medium (Fig. 3D), confirming that HF modulates IL-23 signaling through inhibition of its cognate receptor, the prolyl-tRNA synthetase EPRS, and subsequent activation of the AAR pathway (29, 30). These data establish that HF-induced AAR activation selectively represses proinflammatory IL-23 responses in mature Th17 memory cells.

HF treatment inhibits Stat3 tyrosine (Y705) phosphorylation during Th17 cell differentiation (31). Because IL-23 also activates Stat3, and Stat3 is critical for the proinflammatory effects of IL-23 on Th17 cells (34, 35), we next determined the effects of IL-23 and HF-induced AAR activation on Stat3 phosphorylation in endogenous memory T cell subsets. CCR6+ or CCR6− memory T cells were activated as above, with or without IL-23, and treated with vehicle (DMSO) or HF (20 nM). Cells were washed to remove HF and restimulated with PMA and ionomycin on day 2 for analyses. Mean percentage (± SD) of IL-17A+, IL-17F+, and IL-22+ cells (A) or IFNγ+, GM-CSF+, and TNFα+ cells (B) from three or four biological replicates. (C) Dose response of HF on IL-17A or IFN-γ expression by CCR6+ or CCR6− memory T cells, respectively. Mean normalized percentages (± SD) of cytokine-positive cells from biological duplicates relative to vehicle-treated cultures are shown. IL-23–induced IL-17A expression by CCR6+ cells, after subtracting baseline IL-17A expression, in cells stimulated without IL-23. (D) Mean (± SD) IFN-γ expression in CCR6+ (left panel) or IL-17A expression in CCR6− (right panel) memory T cells from three independent experiments in cells stimulated with anti-CD3/CD28 plus IL-23 for 2 d, with or without 20 nM HF and or 50 mM l-proline. Controls for HF or l-proline were equal volumes of DMSO or sterile water, respectively. Cytokine expression was determined by intracellular cytokine staining on day 2 following PMA + ionomycin restimulation. (E) Stat3 (Y705) phosphorylation determined by phospho-specific intracellular staining and FACS analysis in CD3/CD28-stimulated CCR6− (left panel) or CCR6+ (right panel) memory T cells cultured for 2 d with or without IL-23 and 20 nM HF. Unstimulated cells (no anti-CD3/anti-CD28; resting) were used to determine background. Data represent three experiments. (F) Phospho-Stat3 MFI from three experiments (± SD); background staining in unstimulated cells was subtracted. *p < 0.05, **p < 0.01, paired Student t test.

HF restricts established inflammation and modulates local Th17 cell function in EAE

The central role of IL-23 in autoimmune pathogenesis (36), along with our studies showing that HF regulates proinflammatory IL-23 responses in mature endogenous Th17 cells, suggested that HF...
may have therapeutic effects on established autoimmune inflammation. To test this, wild-type or IL-17A–GFP mice were immunized with MOG33–55/CFA to induce EAE; animals were injected daily with HF or vehicle (DMSO) beginning at disease onset, and disease progression was monitored over a 10-d period. Notably, administration of HF rapidly and significantly attenuated the progression of pre-established EAE symptoms (Fig. 4A). To assess whether the therapeutic efficacy of HF was associated with perturbed T cell effector function, we determined the cytokine expression profiles of memory T cells isolated from MOG33–55/CFA-immunized IL-17A–GFP reporter mice following treatment with DMSO or HF. HF had no effect on cytokine expression (IL-17A, IFN-γ) in CD4+CD44hi effector/memory T cells in nontarget organs, such as the spleen or draining lymph nodes (Fig. 4B–D). In contrast, HF suppressed the level of IL-17A expression in IL-17A+ cells in the CNS (as seen by reduced mean fluorescence intensity [MFI]) and modestly, but consistently, reduced the proportion of CNS-infiltrating memory T cells that expressed IL-17A (Fig. 4B, 4C). Consistent with the strongest effect of HF treatment was on the level of IL-17A expression per cell, and not the frequency of cells expressing IL-17A, absolute numbers of Th17 cells within the CNS (and peripheral lymphoid organs) of HF-treated mice were not significantly different from vehicle-treated animals (Fig. 4C). In contrast, HF had no effect on the expression levels, percentages, or absolute numbers of IFN-γ-expressing effector/memory T cells (Fig. 4B, 4D). Therapeutic HF treatment did not influence the infiltration of total or effector/memory CD4+ T cells into the CNS (Supplemental Figs. 3, 4) or recruitment/localization of other immune cell types, including B cells, NK cells, or neutrophils (Supplemental Figs. 3, 4A). Thus, therapeutic efficacy of HF in MOG/CFA-induced EAE is specifically associated with reduced IL-17A expression by Th17 cells in the CNS. Interestingly, in contrast to MOG/CFA-induced EAE, therapeutic HF dosing in Rag1–/– mice injected with endogenous 2D2 TCR-transgenic CCR6+ effector/memory T cells did not prevent the progression of clinical EAE symptoms (Supplemental Fig. 4B, 4C).

Cytokine-independent regulation of Stat3 by the AAR

Stat3 is necessary for Th17 cell development and is important for IL-23-mediated inflammatory function of mature Th17 cells (5–7, 12, 34). Because both our previous (31) and current findings indicate that HF-dependent inhibition of Th17 responses is associated with decreased Stat3 activation, we sought to determine the mechanism by which AAR activation regulates Stat3 in T cells. We used naive CD4+ T cells for these experiments because of the limiting numbers of endogenous effector/memory T cells in unmanipulated mice; we first asked whether HF treatment regulates Stat3 responses in a cytokine-specific manner. This was not the case, because HF treatment reduced both IL-6- and IL-27-mediated Stat3 (Y705) phosphorylation in naive T cells (Fig. 5A). Specifically, and consistent with the effects of HF on Stat3 activation in naive CD4 T cells treated with TGF-β plus IL-6 (31), HF treatment reduced the levels of sustained phospho-Stat3 in cells treated with IL-6 or IL-27 alone (e.g., 12–24 h after adding ligand), without affecting Stat3 phosphorylation within the first 2 h of adding ligand (Fig. 5A). As expected, sustained Stat3 phosphorylation in IL-6- or IL-27-stimulated naive T cells treated with HF was restored by adding excess proline (data not shown), confirming that, as with IL-23 stimulation, HF regulates IL-6- and

FIGURE 4. HF treatment reduces established EAE disease and inhibits Th17 effector function in the CNS. (A) Mean clinical EAE score ± SD in wild-type C57BL/6 mice immunized with MOG33–55 and treated daily with HF (0.3 mg/kg; n = 43) or vehicle (DMSO; n = 40) beginning at the onset of disease symptoms. (B) Cytokine expression in ex vivo–isolated and PMA-ionomycin-stimulated mononuclear cells from the spleen, peripheral lymph nodes (pLNs), or brain and spinal cord (CNS) of vehicle-treated (EAE score = 3) or HF-treated (EAE score = 2) mice with active EAE on day 6 postdisease onset/HF treatment. Data shown are on CD45+TCRβ+CD4+CD44hi-gated memory T cells. Data represent two independent experiments, with each including analysis of three mice/group. Analysis of IL-17A (C) or IFN-γ (D) expression by CD4+CD44hi effector/memory T cells from spleen, pLNs, or CNS of six DMSO- or HF-treated Il17a-eGFP reporter mice with active EAE on day 6 postdisease onset/HF treatment. MFI of IL-17A (GFP) or IFN-γ (intracellular cytokine staining) expression in effector/memory cells gated as positive for cytokine expression (top panels). Percentage of effector/memory cells from each organ displaying detectable IL-17A or IFN-γ expression (middle panels). Absolute numbers of IL-17A+ or IFN-γ+ effector/memory T cells, calculated as follows: total number of mononuclear cells recovered × % of live lymphocytes × % of CD4+CD44hi effector/memory T cells × % of cytokine-positive cells (bottom panels). All data are mean ± SD from six mice/group across two independent experiments. *p < 0.05; **p < 0.01, ***p < 0.001, paired Student t test.
IL-27–mediated Stat3 activation via inhibition of EPRS and activation of the AAR pathway.

Consistent with previous reports, we found that IL-6, when combined with anti–IL-4 and anti–IFN-γ Abs, was sufficient to induce Th17 differentiation and IL-17A expression, whereas IL-27 promoted expression of both IL-10 and IFN-γ but not IL-17A (7, 37, 38) (Fig. 5B). IL-27–induced Stat3 activation directly impacts IL-10 expression, whereas IL-27–induced IFN-γ requires Stat1 (39, 40). HF treatment attenuated both IL-6–induced IL-17A expression and IL-27–induced IL-10 expression, but it did not affect induction of IFN-γ by IL-27 (Fig. 5B), suggesting that HF selectively targets Stat3 activation. Together with our previous studies (31), these findings suggest that HF treatment selectively inhibits Stat3 activation and downstream transcriptional responses in T cells, but it does so irrespective of the activating cytokine/cytokine receptor.

Inhibition of Stat3 phosphorylation by HF also was evident by Western blot analyses of naive T cells cultured in Th17-polarizing conditions, as determined by Western blotting. Data represent three independent experiments. (D) Mean relative (fold-change) Stat3 mRNA expression ± SD from duplicate samples, as determined by qPCR in CD4+CD25+ T cells cultured for the indicated time points as in (C). Stat3 expression was normalized to b2m; data represent two experiments. (E) Effects of the indicated doses of HF on Stat protein levels in CD4+CD25+ T cells cultured for 18 h in Th17-polarizing cytokine conditions, as determined by Western blotting. Data represent two independent experiments. (F) Stat3 and β-actin protein levels, determined by Western blotting, in Th17-polarized CD4+CD25+ T cells treated with titrating concentrations of HF, as indicated, for 18 h. Some cultures were supplemented with 5× (50 mM l-proline [L-Pro]); data represent three independent experiments. (G) IκBα and β-actin protein levels in resting or CD3/CD28-stimulated CD4+CD25+ T cells cultured for 2 h, with or without titrating concentrations of MG132. Data represent two experiments. (H) Stat3, Stat1, and β-actin protein levels in CD4+CD25+ T cells activated in Th17-polarizing conditions for 18 h in the presence of titrating concentrations of HF, with or without 5 μg/ml MG132, as indicated. MG132 was added 12 h postactivation. Data represent three experiments.

but it did not affect steady-state Stat3 protein levels between 0 and 2 h post–T cell activation (Fig. 5C). In contrast to Stat3 protein, HF did not influence the levels of Stat3 mRNA; thus, the AAR regulates Stat3 at a posttranscriptional level (Fig. 5D). Further, HF treatment reduced Stat3 protein levels in differentiating Th17 cells in a dose-dependent manner and at concentrations that also regulate both endogenous CCR6+ memory cells (see above) (Fig. 5E). Again, effects of HF on total Stat3 protein levels were abolished by addition of excess proline (Fig. 5F). Importantly, the inhibition of Stat3 protein expression by low-dose HF treatment was selective, because other Stat proteins were not similarly affected (Fig. 5F).

The reduced abundance of Stat3 protein in HF-treated T cells could be due to decreased protein synthesis or to increased turnover. To address whether the AAR regulates Stat3 protein abundance via a degradation pathway, control or HF-treated Th17 cells were cultured with or without the proteasome inhibitor MG132. Although MG132 blocked CD3/CD28 stimulation–induced IκBα degradation in naive T cells (Fig. 5G), it did not restore Stat3 protein levels in HF-treated Th17 cells (Fig. 5H), indicating that HF-induced AAR activation regulates Stat3 protein abundance independent of the proteasome.
**Discussion**

We showed that the CCR6\(^+\) memory T cell compartment present in mice at steady-state contains IL-23–responsive Th17 cells with latent pathogenic functions. Like their human counterparts (23–25), a portion of murine CCR6\(^+\) memory T cells express Rorc and Il23r and produce IL-17A, IL-17F, and IL-22 following acute TCR stimulation. Further, CCR6\(^+\), but not CCR6\(^-\), memory cells display marked proinflammatory functional responses to IL-23 stimulation in vitro. Although CCR6\(^+\) memory T cells are likely heterogeneous, and not all of these cells are “pathogenic Th17 cells,” as indicated by IL-17A–GFP or IL-23R–GFP reporter gene expression in only a fraction of CCR6\(^+\) memory T cells, the frequency of CCR6\(^+\) memory cells that produce Th17 cytokines following in vitro culture, particularly in the presence of IL-23, is far greater than that predicted based on ex vivo IL-17A expression. Thus, as with human CCR6\(^+\) memory T cells, it appears that the expression of Th17 cytokines in mature CCR6\(^+\) Th17 cells can be activated as readily as it is repressed (25). Presumably, this dynamic control of Th17 cytokine expression, generally referred to as “plasticity,” depends on cues present in local microenvironments or tissues (9, 20, 41).

The ability of endogenous CCR6\(^+\) Th17 cells to respond functionally to IL-23 is particularly significant given the well-established links of IL-23 to autoimmunity (36). Indeed, and despite their presence in healthy mice, we confirmed that endogenous CCR6\(^+\) Th17 cells have pathogenic potential in vivo. The fact that CCR6\(^+\) Th17 cells, like CCR6\(^+\) memory T cells, can induce autoimmune inflammation following transfer into lymphopenic hosts, likely speaks to the importance of endogenous mechanisms of T cell tolerance, such as those provided by regulatory T cells, as well as of Ag specificity, in controlling lymphopenic hosts, likely speaks to the importance of endog-

**EAE.** HF treatment did not markedly reduce Th17 cell numbers in the CNS of mice with EAE, although the effect of HF on the level of IL-17A expression per memory cell (gated as IL-17A\(^+\)) indicates an ∼2-fold decrease in the amount of IL-17A in the CNS of sick mice. However, potentially more important is the fact that HF did not show therapeutic efficacy in EAE when disease was induced in lymphopenic Rag1\(^−/−\) animals by injection of endogenous CCR6\(^+\) effector/memory T cells from 2D2 TCR-transgenic mice. These results may suggest that the regulatory activity of HF in EAE manifest in MOG33–55/CFA-immunized wild-type mice requires regulation of other cells (e.g., de novo Th17 cell differentiation from naïve cells following disease onset). However, at least two alternative explanations maintain that the therapeutic efficacy of HF in EAE observed in intact mice is linked to regulation of the endogenous CCR6\(^+\) Th17 effector/memory cell compartment. First, HF may influence disease activity in MOG33–55/CFA-immunized mice via regulation of “bystander” Th17 cells present in inflamed CNS tissue that are not specific for myelin Ags. Alternatively, lymphopenic expansion may enable pathogenic functions of CCR6\(^+\) Th17 cells that are not present in wild-type mice following MOG33–55/CFA immunization and are not sensitive to HF and the AAR. Clearly, further insight into the settings where HF possesses or lacks immunoregulatory activity will be important to inform its potential therapeutic usefulness.

In beginning to explore the mechanism by which the AAR selectively regulates Th17 responses, we show that HF-induced AAR activation restricts Stat3-dependent T cell function downstream of multiple cytokines. In addition to repressing IL-23–mediated Stat3 activation and downstream cytokine expression in endogenous CCR6\(^+\) memory T cells, we show that HF treatment inhibits Stat3 activation in naïve T cells following IL-6 or IL-27 stimulation. In the context of IL-6 stimulation, the result of AAR activation is reduced IL-17A expression; when IL-27 is the principal activator of Stat3, the AAR represses IL-10, but not IFN-\(γ\) expression. These results are consistent with Stat3-selective regulation by the AAR; both IL-6–dependent IL-17A expression and IL-27–induced IL-10 production require Stat3, whereas IL-27 activates IFN-\(γ\) expression via Stat1 (39, 40). Further, the AAR regulates Stat3 phosphorylation indirectly, by decreasing Stat3 protein abundance. HF treatment reduced Stat3 protein levels in a dose-dependent manner, despite normal, high-level Stat3 mRNA expression. Further, AAR-dependent regulation of Stat3 was selective and did not similarly reduce the expression of other Stat proteins, consistent with the fact that HF treatment preferentially affects Stat3-dependent cytokine expression. Finally, we show that the AAR does not influence Stat3 protein levels via activation of a proteasome-mediated degradation pathway, leading us to suggest that AAR activation likely targets Stat3 protein synthesis.

Examples of transcript-selective translational control have emerged in recent years. For example, Sabatini and colleagues (45) showed that the key nutrient-sensing kinase, mTOR, preferentially regulates translation of mRNAs that encode ribosomal proteins and that contain a 5′ terminal oligopyrimidine motif. Other studies have shown that VEGFA protein synthesis is selectively regulated in monocytes by both inflammation (in the form of IFN-\(γ\)) and hypoxia (46, 47). In those instances distinct structural elements in the 3′ untranslated region of VEGFA mRNA dictate translation, where the gamma IFN–activated inhibitor of translation (GAIT) complex binds to a 3′ GAIT element in VEGFA mRNA and blocks translation initiation, whereas the hypoxia-inducible hnRNP L–DRBP76–hnRNP A2/B1 complex blocks GAIT complex binding and facilitates VEGFA mRNA translation (46). The GAIT complex may be particularly relevant to the effects of HF treatment on Stat3.
protein levels in T cells, because the RNA-binding subunit of this complex is EPRS, the cellular receptor of HF (47).

In summary, our study suggests that both the size and the proinflammatory potential of the endogenous Th17 cell compartment in mice are greater than previously recognized. This new knowledge, together with the fact that endogenous Th17 cells can be readily discriminated and isolated based on steady-state expression of CCR6, will facilitate further study of mature Th17 cell inflammatory function. As established by IL-23, Stat3, and the AAR, these pathways are likely to play important roles in persistent inflammatory responses, including those that are manifest in patients with autoimmune diseases.

Acknowledgments

We thank Drs. Deryn Utzumatz, Victor Torres, and John Cleveland, as well as members of Tempero Pharmaceuticals, Inc., for helpful discussion and critical review of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


