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Heat shock factor 1 drives regulatory T-cell induction to limit murine intestinal inflammation

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Abstract

The heat shock response is a critical component of the inflammatory cascade that prevents misfolding of new proteins and regulates immune responses. Activation of clusters of differentiation (CD)4^+ T cells causes an upregulation of heat shock transcription factor, heat shock factor 1 (HSF1). We hypothesized that HSF1 promotes a pro-regulatory phenotype during inflammation. To validate this hypothesis, we interrogated cell-specific HSF1 knockout mice and HSF1 transgenic mice using *in vitro* and *in vivo* techniques. We determined that while

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AUTHOR CONTRIBUTIONS

EDZ and CBC provided significant contributions to the conception, implementation, analysis of the work as well as drafting, revision, and review of the manuscript. All authors substantially contributed to the acquisition, analysis, and interpretation of data. All authors approved the final version of this manuscript to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

DECLARATION OF COMPETING INTEREST

The authors have no competing interests to declare.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used ChatGPT in order to enhance the readability and clarity of our results section. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

APPENDIX A. SUPPLEMENTARY MATERIAL

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HSF1 expression was induced by anti-CD3 stimulation alone, the combination of anti-CD3 and transforming growth factor β, a vital cytokine for regulatory T cell (Treg) development, resulted in increased activating phosphorylation of HSF1, leading to increased nuclear translocation and binding to heat shock response elements. Using chromatin immunoprecipitation (ChIP), we demonstrate the direct binding of HSF1 to *foxp3* in isolated murine $CD4^+$ T cells, which in turn coincided with induction of FoxP3 expression. We defined that conditional knockout of HSF1 decreased development and function of Tregs and overexpression of HSF1 led to increased expression of FoxP3 along with enhanced Treg suppressive function. Adoptive transfer of $CD45RB^{High}$ CD4 colitogenic T cells along with HSF1 transgenic $CD25^+$ Tregs prevented intestinal inflammation when wild-type Tregs did not. Finally, overexpression of HSF1 provided enhanced barrier function and protection from murine ileitis. This study demonstrates that HSF1 promotes Treg development and function and may represent both a crucial step in the development of induced regulatory T cells and an exciting target for the treatment of inflammatory diseases with a regulatory T-cell component.

Significance statement: The heat shock response (HSR) is a canonical stress response triggered by a multitude of stressors, including inflammation. Evidence supports the role of the HSR in regulating inflammation, yet there is a paucity of data on its influence in T cells specifically. Gut homeostasis reflects a balance between regulatory clusters of differentiation $(CD)4^+$ T cells and pro-inflammatory T-helper (Th)17 cells. We show that upon activation within T cells, heat shock factor 1 (HSF1) translocates to the nucleus, and stimulates Treg-specific gene expression. HSF1 deficiency hinders Treg development and function and conversely, HSF1 overexpression enhances Treg development and function. While this work, focuses on HSF1 as a novel therapeutic target for intestinal inflammation, the findings have significance for a broad range of inflammatory conditions.

INTRODUCTION

Intestinal immune homeostasis is critical to the maintenance of general health. Mice and humans with a defect in the *foxp3* gene, a transcription factor essential to development of regulatory T cells (Tregs), develop a wide range of autoimmune and inflammatory pathologies including enteritis, defining Tregs as crucial for maintenance of intestinal homeostasis. Inflammatory bowel disease (IBD), characterized by dysregulation of intestinal homeostasis, currently affects approximately 1.5 million people in the United States, with a third of cases presenting in children[1]. Current therapies induce remission in less than 50% of IBD patients[2,3], defining an unmet need for new therapeutics in IBD. Despite the significant clinical impact of IBD, its etiology remains unclear. IBD is a chronic inflammatory condition thought to reflect a failure of the intestinal immune system to adequately regulate itself. Tregs act to modulate this overactive immune response; therefore, a better understanding of the Treg's role in the maintenance of homeostasis in the intestinal mucosa will undoubtedly inform the goal of developing novel therapeutics for IBD.

Inducible heat shock proteins (HSPs) are activated under stress to a cell; these stresses include chemicals, heat, hypoxia, and exposure to cytokines[4]. Previous studies using inhibitors of histone deacetylase (HDAC) in mice to enhance Treg function and attenuate murine colitis suggest a role for HDAC6-dependent regulation of HSP90[5]. HSPs, once

expressed, act as chaperones to either allow for normal protein folding or to stabilize the protein and avoid degradation. Transcriptional activation of the HSR requires the release of HSF1 from its chaperone HSP90, post-translational modification, and trimerization of HSF1. Previous work has demonstrated that HSF1 and HSPs are involved in the repression of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)α and interferon (IFN)γ[6–8], and activation of the anti-inflammatory gene IL-10[9,10]. In genome-wide association studies, HSF1 has been associated with IBD[11,12], generally as a protective factor.

Tregs are one of the cell types that provide tight regulation of the inflammatory response crucial to maintaining intestinal homeostasis. Multiple animal studies indicate that Tregs modulate the immune response in normal intestinal mucosa and thereby prevent colitis development[13,14]. Breakdown of this tolerance to luminal antigens plays a pivotal role in IBD development[15]. FoxP3 is the 'lineage-specifying' transcription factor specifically expressed by $CD4^+$ $CD25^+$ Tregs[16,17] and *essential* for Treg development and maintenance[18,19]. Defects in the *foxp3* gene predispose both mice and humans to intestinal inflammation along with a range of other autoimmune inflammatory pathologies, providing evidence of its importance for intestinal immune homeostasis[14]. Treg-restricted, high-level expression of FoxP3 confers a major component of the Treg transcriptome, including high expression of suppressor genes and repression of effector cytokines of Th1, Th2, and Th17 lineages[20,21]. FoxP3 stabilizes the Treg lineage, directly and indirectly, by regulating distinct cell surface and signaling molecules and interacting with a multitude of transcription factors. However, the concept that FoxP3 is the "sole requisite" transcription factor to define the Treg lineage has been challenged. While FoxP3 is *indispensable* for the majority of the Treg transcriptional and functional landscape, FoxP3 transduction by itself does not completely recapitulate the Treg transcriptional profile[22]. Despite the fact that conversion of naïve $CD4^+CD25^{Neg}$ T cells into $CD4^+$ $CD25^+$ Tregs is broadly mediated via transforming growth factor β (TGFβ)-dependent FoxP3 induction[23,24], FoxP3 expression is stringently regulated with many transcription factor binding sites on both the promoter and enhancer regions of the gene, including multiple putative HSF1 binding sites. Suppressive function of Tregs is attributed to multiple mechanisms and ascribed to the broad categories of contact-dependent (surface molecules: Cytotoxic T Lymphocyte Antigen protein 4 (CTLA4), Programmed Cell Death protein 1PD1, Lymphocyte Activation Gene 3 (Lag3), and Glucocorticoid-induced TNF Related Protein GITR) and contact-independent mechanisms (IL-10, IL-35, TGFβ, or via Cyclic adenosine Monophosphate (cAMP)[25,26]. HSF1 is known to affect IL-10 expression and may also play a role in the expression of these other suppressive factors.

Considerable evidence supports the role of the HSR in inflammation, yet there is a paucity of data on its influence on T cells. The HSR is regulated by heat shock factors (HSF1–4), which are transcription factors induced by various stressors, including heat, cold, and cytokines such as TGFβ[27]. Studies suggest that HSF1 expression is activated by T-cell receptor (TCR) stimulation[28]. HSF1 activation can be divided into multiple steps, including (1) release from HSP90; (2) post-translational modification, e.g. phosphorylation; (3) trimerization; (4) translocation into the nucleus; and (5) deoxyribonucleic acid (DNA) binding. Upregulation of HSPs occurs via HSF1 binding to heat shock elements (HSE) (5′-nGAAn-3′)[29]. Beyond a critical function in the HSR, HSF1 has been shown to regulate

the expression of genes not typically associated with heat shock. HSF1 appears to play an anti-inflammatory role by repressing transcription of the pro-inflammatory genes *TNFα*, *IL-1β*[8,30], and *IL-6*[31,32], and enhancement of IL-10 expression[33], indicating that HSF1 is an essential inflammatory regulator. TGFβ has been identified as a cytokine that activates HSF1 and promotes DNA binding[34].

We have demonstrated that activation of the HSR is one of these critical regulatory mechanisms[5,35–37], and our studies indicate that the transcription factor HSF1, in particular, plays a pivotal role in regulating Treg cells. To date we have identified that HSF1 is important for Treg function; we will now expand our studies to gain deeper insight into the molecular mechanisms underpinning this role.

We propose that activation of the HSR leads to upregulation of HSF1 preferentially expressed on $CD4^+$ $FoxP3^+$ Tregs as a protective pathway to limit inflammation. Translocation of HSF1 to the nucleus drives a transcriptional program that enhances Treg induction and function resulting in decreased pro-inflammatory cytokine production and attenuation of disease severity in animal models. Using a combination of whole-body knockout, cell-specific deletion and gain-of-function mutant mice, we have identified a critical role for HSF1 in gut homeostasis and as a potential novel therapeutic target for the treatment of both small and large intestinal inflammation.

RESULTS

Activation of the heat shock pathway promotes Treg induction

Previously, we showed that blocking HSP90 triggers HSF1 activation, leading to HSF1 nuclear translocation and the expression of multiple HSPs, FoxP3, and IL-10[35]. Now, we have found that subjecting isolated $CD4^+$ T cells to a 39 °C heat shock for 30 minutes[28] induces FoxP3, a Treg-associated transcription factor (Fig. 1A), and the anti-inflammatory cytokine IL-10 (Fig. 1B). This heat shock also increases *foxp3* messenger ribonucleic acid (mRNA), resulting in higher FoxP3 protein levels after a 3-day incubation, as detected by flow cytometry (Figs 1C and 1D). In contrast, another cellular stress pathway involving hypoxia-inducible factor (Hif1α) and its target genes remains unchanged (Supplementary Fig. 1), highlighting the specificity of HSF1 activation. Notably, $CD4^+$ $FoxP3^+$ Tregs express higher levels of Hif1α and related genes compared to $CD4^+$ Th0 cells, suggesting Hif1α's involvement in Treg development but no activation of this pathway by heat shock (Supplementary Figs 1A and 1B).

We employed the $TNF^{\Delta ARE/+}$ mouse model of Crohn's disease, a model in which $CD4^+$ effector T cells play a central role[38]. These mice are heterozygous for a TNFα mutation which lacks the AU-rich element (ΔARE) controlling mRNA stability, leading to over-stabilized TNFα mRNA and systemic elevation of TNFα protein[39]. This results in the spontaneous development of chronic transmural ileitis reminiscent of human Crohn's disease in its histological features and highlights the pivotal role played by TNFα in IBD pathogenesis[38,39]. In our study, we found a five-fold increase in heat shock transcription factor HSF1 mRNA expression in intestinal $CD4^+$ T cells isolated from inflamed $TNF^{\Delta ARE/+}$ mice compared to controls (Fig. 1E). HSF1 was preferentially expressed by

Tregs relative to total $CD4^+$ T cells, a pattern maintained during inflammation (Fig. 1E). This heightened expression was particularly noticeable in ileal Tregs of $TNF^{\Delta ARE/+}$ mice (Fig. 1F). The increased HSF1 mRNA was consistent with an increased expression of HSF1 protein in permeabilized Tregs from the spleen, mesenteric lymph node (MLN) and ileal lamina propria (LP) of $TNF^{\Delta ARE/+}$ mice relative to controls (Fig. 1G). Taken together, these findings suggest that HSF1 plays a role in promoting Treg induction in response to intestinal inflammation.

Deficiency in HSF1 impairs Treg development and function

Analysis of colonic FoxP3+ Tregs in $HSF1^{-/-}$ knockout mice showed a significant reduction compared to wild-type (WT) controls in the colonic LP associated with an increase in $CD4^+$ $CD25^+$ $FoxP3^+$ cells in the spleen and draining lymph node (Figs. 2A and 2B). This decrease in LP Treg expression coincided with reduced Treg induction in response to stimulation conditions (anti-CD3/CD28, TGFβ, IL-2; Fig. 2C). Notably, heat shock treatment did not enhance Treg development in $HSF1^{-/-}$ mice, unlike in WT Th0 cells (Supplementary Fig. 1). This decline in FoxP3 induction coincided with decreased Treg suppressive function. Co-culturing $CD4^+$ $CD25^{Neg}$ T cells with Tregs from both WT and $HSF1^{-/-}$ mice resulted in increased responder T cell proliferation, indicating impaired Treg suppressive function across all tested concentrations (Fig. 2D). These findings suggest that HSF1 plays a central role in regulating Treg development and function, with the HSR promoting Treg development in an HSF1-dependent manner.

Cell-specific HSF1 deletion impairs Treg development which is restored by HSF1 overexpression

We used loss-of-function mutant $HSF1^{Fl/Fl}$ $CD4^{Cre/+}$ mice to investigate the cell-intrinsic role of HSF1 in Treg development. $HSF1^{Fl/Fl}$ $CD4^{Cre/+}$ mice displayed a deficit in both Treg frequency (Fig. 3A) and FoxP3 protein abundance (measured as mean fluorescence intensity (MFI); Fig. 3D) compared with Cre^+ controls. We then examined Treg numbers in $HSF1^{Fl/Fl}$ $FoxP3^{Cre/+}$ mice to assess HSF1's role in maintaining Treg characteristics given that knockout would occur in cells with existing expression of FoxP3. Under these conditions, $HSF1^{Fl/Fl}$ $FoxP3^{Cre/+}$ mice also displayed a significant decrease in Treg frequency (Fig. 3B) and protein expression (Fig. 3E) in both the MLN and LP relative to controls. Additionally, we investigated HSF1 overexpression in HSF1Tg mutant mice, which resulted in increased Treg frequency (Fig. 3C) and higher FoxP3 protein expression (Fig. 3F).

Under *in vitro* conversion conditions, both $HSF1^{Fl/Fl}$ $CD4^{Cre}$ and $FoxP3^{Cre}$ displayed decreased Treg induction while overexpression in $HSF1^{Tg}$ naïve T cells increased Treg induction relative to WT controls (Figs. 3G and 3H). Similarly, the capacity for heat shock to increased Treg conversion in WT cells, was lost in both $HSF1^{Fl/Fl}$ $CD4^{Cre}$ (Fig. 3I) and $FoxP3^{Cre}$ (Fig. 3J) but was further potentiated by HSF1 overexpression (Fig. 3K) consistent with a direct role for HSF1 in Treg conversion during stress conditions. Similar to conversion, Treg suppressive function was decreased in both $HSF1^{Fl/Fl}$ $CD4^{Cre}$ (Fig. 3L) and $FoxP3^{Cre}$ (Fig. 3M) while HSF1 overexpression in $HSF1^{Tg}$ Tregs coincided with an increased suppressive function relative to WT controls (Fig. 3N). In summary, these findings highlight HSF1's cell-intrinsic role in regulating Treg induction and potentially stability.

Furthermore, HSF1 overexpression has therapeutic potential for IBD by increasing Treg frequency and function under both stressed and non-stressed conditions.

T-cell activation triggers HSF1 nuclear translocation and binding to the Foxp3 promoter

In isolated $CD4^+$ T cells, we observed a significant rise in HSF1 protein expression upon TCR activation (Fig. 4A). Furthermore, HSF1 activation, evidenced by increased phosphorylation at serine 326, occurred under Treg induction conditions (IL-2, TGFβ, anti-CD3, and anti-CD28), similar to heat shock (Fig. 4B). Western blot analysis of nuclear HSF1 showed a clear increase in total HSF1 protein when stimulated with IL-2 alone or in combination with TGFβ (Fig. 4C). Notably, this increase was observed in two bands, with TGFβ causing a shift from the higher to the lower band (Fig. 4C). We further examined this by performing Western blot under non-denaturing conditions, revealing increased trimerization of HSF1 in nuclear extracts under Treg conversion conditions (Fig. 4D). These steps are crucial for HSF1's activation as a transcription factor. To confirm the functional significance of enhanced HSF1 activation, trimerization, and nuclear translocation, we used Jurkat T cells transfected with an HSE promoter reporter luciferase construct, which supported increased HSF1 activity under Treg conversion conditions (Fig. 4E). Cytospin analysis also showed greater focal nuclear HSF1 expression in response to Treg induction conditions (Fig. 4F). Finally, using chromatin immunoprecipitation (ChIP), we assessed HSF1 binding to the foxp3 gene. We found increased binding of HSF1 to the foxp3 promoter (3x, 5x) and enhancer regions (CNS2, 3x, and CNS3 2x) under Treg conversion conditions (Fig. 4G). Overall, these findings indicate that the steps necessary for boosting HSF1 function, including induction, phosphorylation, translocation, trimerization, and binding, are all induced by Treg culture conditions.

HSF1 deficiency heightens colitogenic potential in the adoptive transfer colitis model

Given the diminished capacity of T cells from $HSF1^{Fl/Fl}$ $CD4^{Cre+}$ mice to acquire a regulatory phenotype and suppress effector T cell proliferation, *in vitro*, we next examined the *in vivo* consequences using the adoptive transfer model of colitis. Naïve WT $CD4^+$ $CD45RB^{High}$ T cells were injected intraperitoneally (i.p.) into lymphopenic $Rag1^{-/-}$ recipients either alone or with a 1:4 ratio of Tregs from either $CD4^{Cre+}$ or $HSF1^{Fl/Fl}$ $CD4^{Cre+}$. Mice were then monitored for the development of colitis. Mice receiving sufficient WT Tregs (white triangles) to limit colitogenic T cell development experienced significantly less weight loss (Fig. 5A). Conversely, impaired Tregs from $HSF1^{Fl/Fl}$ $CD4^{Cre+}$ mice (black squares) failed to restrain colitis development, leading to worsening weight loss and exacerbated histological signs of inflammation (Figs. 5B and 5C). Analysis of colonic tissue explant supernatants showed a concurrent decrease in the anti-inflammatory cytokine IL-10 and an increase in the pro-inflammatory cytokines IL-17 and IFNγ (Fig. 5D). The worsening colitis when co-transferring Tregs from $HSF1^{Fl/Fl}CD4^{Cre+}$ mice correlated with a decrease in $CD4^+$ $FoxP3^+$ Treg and $CD4^+$ $FoxP3^+$ $IL\text{-}10^+$ Treg frequency with no significant change in $CD4^+IL\text{-}17^+$ T-cell frequency (Figs. 5D–H). Similar findings were obtained when Tregs from $HSF1^{Fl/Fl}$ $FoxP3^{Cre+}$ were co-transferred (Supplementary Fig. 2). In these mice, the floxed allele is removed later in Treg development, partially mitigating the global effect on all $CD4^+$ T cells seen with the $CD4^{Cre}$ strain. In line with previous *in vitro* results, the HSF1 deficiency resulted in impaired Treg suppressive function *in vivo*, exacerbating colitis.

Overexpression of HSF1 reduces spontaneous murine ileitis

Given that loss of HSF1 worsens intestinal inflammation, we next sought to determine if increased HSF1 expression would attenuate a gene-targeted chronic murine ileitis model. We generated $TNF^{\Delta ARE/+}HSF1^{Tg}$ mice and examined the impact of HSF1 overexpression on the development of ileitis. Histological evaluation of $TNF^{\Delta ARE/+}$ mice crossed to $HSF1^{Tg}$ demonstrated a significant reduction in histological evidence of inflammation with HSF1 overexpression (Figs. 6A and 6B) scored by a trained pathologist blinded to the study design. Flow cytometric evaluation of inflammatory cell infiltrate within the ileum further demonstrated an increase in $CD4^+$ $FoxP3^+$ Tregs (Figs. 6C and 6D) including IL-10-positive Tregs (Figs. 6C and 6E) but not $CD4^+$-$FoxP3^{Neg}IL\text{-}10^+$ cells (Supplemental Fig. 6). The decreased inflammation also restored $CD4^+$ $IL\text{-}17^+$ T-cell frequency back to levels within the ileum of healthy controls (Fig. 6F). $TNF^{\Delta ARE/+}$-$HSF1^{Tg}$ mice displayed a decrease in intestinal barrier permeability seen by FITC dextran flux relative to $TNF^{\Delta ARE/+}$ mice (Fig. 6G), a surrogate marker for attenuation of inflammation. We next opted to examine Treg suppressive function *in vitro* given that Tregs from inflamed mice display impaired suppressive function (Fig. 6H) and consistent with findings from $HSF1^{Tg}$ mice, $TNF^{\Delta ARE/+}HSF1^{Tg}$ Tregs displayed enhanced Treg suppressive function compared to inflamed controls (Fig. 6I). In summary, these data support a role for HSF1 expression in driving enhanced Treg suppressive function and point to its therapeutic potential in multiple distinct IBD models.

HSF1 overexpression reduces colitogenic potential in the adoptive transfer colitis model

We next opted to focus on the impact of HSF1 overexpression on Tregs using the adoptive transfer colitis model. In previous *in vitro* experiments we noted that $HSF1^{Tg}$ Tregs are more suppressive, therefore in this model, naïve $CD4^+$ $CD45RB^{High}$ T cells were co-transferred with a suboptimal concentration of Tregs (1:8 ratio) isolated from gut-draining lymph nodes, which is insufficient to prevent colitis onset so that there would be a measurable effect in these experiments when comparing WT versus $HSF1^{Tg}$ Tregs. While WT Tregs could not prevent weight loss or inflammation at this ratio, co-transfer of $HSF1^{Tg}$ Tregs successfully protected mice from weight loss (Fig. 7A) and histological signs of inflammation (Figs. 7B and 7C). The frequency of LP $CD4^+$ T cells decreased with $HSF1^{Tg}$ Treg co-transfer (Fig. 7D), with the remaining cells showed an increase in $IFN\gamma^+$ T cells (Fig. 7E), but not $IL\text{-}17^+$ T cells (Fig. 7F). This protection correlated with a greater than twofold increase in Tregs in the colonic LP, as identified by flow cytometry (Fig. 7G), which was not observed in the MLN. Additionally, there was an increase in anti-inflammatory $FoxP3^+$ $IL\text{-}10^+$ double-positive $CD4^+$ T cells (Fig. 7H) in both the MLN and LP. These findings suggest that elevated HSF1 expression in Tregs could potentially serve as a therapeutic target for mitigating intestinal inflammation.

DISCUSSION

Lost tolerance to luminal antigens plays a central role in IBD development[40]. Defective *foxp3* gene expression predisposes both mice and humans to intestinal inflammation along with a range of other autoimmune inflammatory pathologies, providing evidence of its importance for intestinal immune homeostasis[14]. FoxP3 is the master regulating

transcription factor specifically expressed by $CD4^+$ $CD25^+$ Tregs[16,17] and essential for Treg development and maintenance[19,41,42]. Treg-restricted, high-level expression of FoxP3 confers a major component of the Treg transcriptome, including high expression of suppressor genes and repression of effector cytokines of Th1, Th2, and Th17 lineages[16,17,20,21]. FoxP3 also stabilizes Treg lineage, directly and indirectly, by regulating distinct cell surface and signaling molecules and interacting with a multitude of transcription factors. Promoting FoxP3 expression and therefore Treg stability and suppressive function represents an attractive therapeutic target for chronic inflammatory diseases including but not limited to IBD.

A key component of the inflammatory cascade is the HSR which serves as a checkpoint for inflammation and coordinates the large increase in protein production associated with an inflammatory response. The HSR can be induced by non-pyrogenic stimuli including both inflammation-associated cytokines TNFα[43] and TGFβ[27] which mediate a response through the induction of transcription factors including HSF1. TGFβ has been shown previously to upregulate HSF1 in human breast cancer via induction of FAM3C protein[44]. In turn, FAM3C is upregulated under inflammatory conditions such as alveolar echinococcosis[45] Within the colon, FAM3C has been linked to Treg infiltration and increased risk of colorectal cancer[46]. As such, FAM3C may have a role in mediating TGFβ-induced regulation of HSF1 in Tregs in this study. Previous intestinal inflammatory studies have demonstrated the induction of HSF1 associated with ischemia-reperfusion injury but not with ischemia alone[47]. Consistent with these results, we describe induction of HSF1 in response to chronic murine ileitis using the $TNF^{\Delta ARE/+}$ Crohn's disease model in our data. As with the study above, this induction was independent of inflammation-associated hypoxia as has been previously suggested[48], as the hypoxia-associated transcription factor HIF1α and its downstream target GLUT1 were unchanged. Nonetheless, HIF1α has previously proven important in IBD pathogenesis[49] through modulation of Treg function. The increased expression of HSF1 may also be partly due to an increase in acetylation as seen during chemically induced acute colitis[50]. While acetylation of the DNA binding domain of HSF1 can block HSF1 function, acetylation of the central regulatory domain blocks HSF1 degradation which may account for the increased expression seen in our studies[51].

Interestingly, the increase in HSF1 was preferentially seen in $CD4^+$ $FoxP3^+$ Tregs, a key cell subset responsible for promoting gut homeostasis. We propose that similar to adenosine signaling via the AdoraA2B receptor, HSF1 acts during inflammation to limit overzealous immune activation[52,53]. Furthermore, HSF1 deficiency led to impaired induction of Tregs in response to TGFβ which can activate HSF1 and promote DNA binding[34] and TCR stimulation which is also associated with activation of HSF1[28]. This is consistent with previous studies demonstrating a role for HSPs targeted by HSF1, including DnaJ, HSP60, and HSP70, in the induction of Tregs and promoting Treg function through increasing IL-10 production[54–56]. Loss of HSF1 also resulted in impaired Treg suppressive function consistent with a role for HSF1 in enhancing IL-10 production, a key immunosuppressive cytokine[33]. Taken together these data support our hypothesis that induction of HSF1 in response to inflammation promotes Treg induction, and functions as a negative feedback inhibitory mechanism to limit excessive inflammation. We have further outlined this hypothesis in a schematic diagram below (Supplemental Fig. 3).

In order for HSF1 to drive transcription, it must undergo a tightly regulated sequence of events including upregulation, phosphorylation, nuclear translocation, and trimerization[57] prior to binding to HSE on target genes[29]. We noted, by Western blot, that anti-CD3 or anti-CD3/CD28 stimulation of naïve $CD4^+$ T cells increases the expression of HSF1, which correlates with previous studies in naïve $CD4^+$ T cells[28]. We have previously reported on the role played by HSP90 in forming an inhibitory complex with HSF1 and sequestering it in the cytoplasm, thereby preventing this cascade[35,58]. HSP90 inhibition enhanced Treg suppressive function *in vitro*[5] and increased FoxP3 expression[35] in an HSF1-dependent manner. This is also consistent with studies demonstrating enhanced Treg function in $HDAC6^{-/-}$ mice coinciding with increased HSP90 acetylation, impaired HSP90 function[5], and the greater release of HSF1. As noted in other cell types, we note that blockade of the HSP90 activates HSF1, drives it into the nucleus of Tregs, and increases the expression of multiple HSPs as well as FoxP3 and IL-10[35]. Nuclear translocation of HSF1 has been identified as mediated by extracellular regulated kinase/mitogen-activated protein ERK (MEK) and c-jun N-terminal kinase pathways[59].

HSF1 binding is highly sensitive to post-translational modification with phosphorylation of serine-121 by ERK1, preventing HSF1 function, while phosphorylation at serine-326 activates HSF1[60]. The combination of TCR and TGFβ appears to promote phosphorylation of serine residues at position 326 along with trimerization and nuclear translocation. To further define the impact of apparent HSF1 signal activation, we turned to an HSE-binding luciferase assay. We determined in Jurkat cells (human $CD4^+$ T cell line) that TGFβ stimulates HSF1 binding to the HSE, whereas TCR stimulation alone decreased luciferase production. Finally, TGFβ and TCR stimulation together induced recovery of luciferase production, suggesting that TGFβ drives HSF1 binding to the HSE and that while activation of the T cell increases HSF1 expression it is likely inactive until TGFβ is added. The induction of HSF1 binding to the HSE combined with our initial data demonstrating impaired FoxP3 induction in the absence of HSF1 prompted us to identify HSE elements on the *foxp3* gene. We note that there are multiple HSE DNA binding sites within the *foxp3* promoter and enhancer regions in both mice and humans. Yet, presence does not predict the effect, thus determining if HSF1 binds requires further evaluation. We found differential binding of HSF1 at multiple sites on the *foxp3* gene, including the promoter, CNS2 (a region that has been identified as critical for maintaining Treg cell lineage and sustaining FoxP3 expression during mature Treg division[61]), and CNS3 which can potently increase the frequency of peripheral Tregs[62]. The multitude of HSF1 binding sites on *foxp3* noted by our ChIP studies is consistent with other findings that HSF1 acts to partially open chromatin to allow binding of other activators or repressors of transcription and may act as a chromatin remodeling factor in the transcription of heat shock as well as other genes[32,63]. These data are consistent with a direct cell-intrinsic role for HSF1, through directly binding to HSE on *foxp3* to promote Treg induction and enhance their function.

At this point in our studies, we wished to understand the impact of HSF1 deficiency on intestinal inflammation. Previously insufficient expression of HSF1 in idiopathic arthritis has been implicated in disease pathogenesis[64] and $HSF1^{-/-}$ mice are more susceptible to chemically induced colitis[12]. $HSF1^{-/-}$ mice express a mutant HSF1 protein in which the HSE-binding domain has been deleted along with a portion of the oligomerization domain,

thereby silencing HSF1 signaling[65]. Given the clear role of HSE binding on *foxp3* in driving Treg induction, we chose to focus on the adoptive transfer colitis model in which $HSF1^{-/-}$ Tregs failed to restrain inflammation, unlike their control counterparts. While *in vitro* assays suggest that $HSF1^{-/-}$ Tregs are functionally impaired, a deficiency in gut homing may also contribute to the outcome of both these studies and the decrease in intestinal Treg frequency seen in $HSF1^{-/-}$ mice. This apparent disruption in the ability of T cells to migrate to the gut may explain why there is variation between the effects of deleting HSF1 using $CD4^{Cre}$ or $FoxP3^{Cre}$ recombinase strains. In the case of $FoxP3^{Cre}$, there is no decrease in LP Tregs because HSF1 is deleted at a later stage of T cell development, potentially allowing gut homing to proceed normally and resulting in higher levels of Tregs. On the other hand, when HSF1 is deleted early with $CD4^{Cre}$, it impairs gut homing, which has more significant impact on Tregs due to their limited ability to proliferate. This ultimately leads to a shift in the frequency of intestinal Tregs. This may not be the whole story, however, given that short-term experiments with $HSF1^{-/-}$ mice looking at acute kidney ischemia renal injury demonstrated that Treg trafficking was enhanced in $HSF1^{-/-}$ mice[66]. Interestingly, $HSF1^{-/-}$ mice do not spontaneously develop inflammation as seen with *foxp3* deficiency, but instead, require an inflammatory trigger to expose the differential function of the Tregs. This is consistent with an inducible rather than constitutive role for HSF1 in regulating Treg development and function making it an even more attractive therapeutic target.

In light of the impaired Treg function seen with a loss-of-function mutation of HSF1, we next sought to determine if overexpression of HSF1 can enhance Treg function and attenuate intestinal inflammation. Precedent does exist for this expectation as enhancement of HSF1 protects against chemically induced colitis[12]. Similarly, pre-induction of the HSR can protect against acute murine colitis[67], and while not identified at the time, this effect is likely in part driven by HSF1[57]. HSF1 overexpression has also been shown to be protective in a murine model of Alzheimer's disease in which it phenocopied the effect of chronic rapamycin treatment[68], a compound that is also known for expanding Tregs[69]. Not surprisingly, therefore, HSF1 overexpression enhanced Treg suppressive function both *in vitro* and *in vivo* in line with our overarching hypothesis. Perhaps more critical is that HSF1 overexpression was protective in multiple IBD models suggesting that this approach may have a broad impact across the spectrum of IBD.

While our experiments identify a protective role for HSF1 in intestinal inflammation through enhancement of Treg development and function, additional anti-inflammatory mechanisms have also been described for HSF,1 which may undoubtedly impact our findings in the $TNF^{\Delta ARE/+}$ model in particular. $HSF1^{-/-}$ peritoneal macrophages express significantly higher levels of the pro-inflammatory cytokine TNFα upon lipopolysaccharide stimulation[12]. Similarly, HSF1 inhibits NFκB activity both *in vitro* in RAW 264.7 macrophage cell line[70] and *in vivo* in cadmium-induced lung injury[71]. Similarly, we have shown that inhibition of the C-terminus of HSP90 which can antagonize HSF1, leads to attenuation of intestinal inflammation through inhibition of NFκB[36]. Whereas induction of HSF1 appears to play an anti-inflammatory role by repressing transcription of pro-inflammatory genes TNFα, IL-1β[8,30], and IL-6[31,32]. Another potential contributing mechanism may be through attenuation of apoptosis. The barbiturate thiopental which can inhibit T-cell function does so in part by induction of HSF1 phosphorylation and DNA

binding leading to the production of HSP70 and conferring protection against staurosporine-induced apoptosis[72]. While our data points to a direct role for HSF1 in regulating Treg suppressive function and thereafter pro-inflammatory cell proliferation, HSF1 null mice have also been shown to express higher inflammatory cytokine production and lower autophagy activity in response to endotoxemia[73] consistent with an additional potential mechanism of action of HSF1. Overexpression of HSF1 in a murine model of Huntington's disease resulted in attenuation of apoptosis and production of reactive oxygen species consistent with a proposed immunosuppressive role[74].

Finally, the enhanced barrier function seen in $TNF^{\Delta ARE/+}$-$HSF1^{Tg}$ mice may not simply be a consequence of reduced intestinal inflammation. Rather it is due to the capacity for HSF1 to bind to occludin promoter regions[75], a critical tight junction protein controlling intestinal epithelial barrier integrity. The capacity to enhance intestinal barrier function may be causative in reducing the severity of ileitis by limiting antigen exposure. Once again, it is not surprising that in the absence of inflammatory stimulus driving upregulation of HSF1 in the $HSF1^{Tg}$ mice, there was no intrinsic difference in intestinal barrier permeability[75]. This recapitulates findings regarding Treg development, suggesting that HSF1 represents an inducible response to inflammation without strong constitutive activity, making it a highly attractive target for therapeutic intervention.

In conclusion, our studies highlight the role of HSF1 in promoting gut homeostasis through Treg induction, enhancement of Treg function, and suppression of pro-inflammatory cytokines. The same signals of TCR and TGFβ which promote Treg induction also drive upregulation, activation, and translocation of HSF1 leading to DNA binding as well as targeting multiple promoters and enhancer regions on the *foxp3* gene. The impact of HSF1 on Treg and epithelial barrier function appears to be restricted to the inflammatory context making this pathway a candidate for future pharmacological intervention with HSF1 activators such as celastrol and HSF1A[76,77].

METHODS

Mice

C57Bl/6 (000664), $HSF1^{-/-}$ mice (C;129-*Hsf1*^{tm1Ijb}/J, Jackson Laboratories, Bar Harbor, ME, USA), $Rag1^{-/-}$ (2216), *foxp3*$^{YFP\text{-}Cre}$ ($FoxP3^{Cre}$; 016959), *CD4*^{Cre} ($CD4^{Cre}$; 017336), were obtained from Jackson laboratories. $HSF1^{Tg}$ mice were kindly provided by Anson Pierce[65]. The B6.129S-Tnf^{tm2GKl}/Jarn strain ($TNF^{\Delta ARE/+}$; MGI:3720980) was generated by continuous backcrosses between heterozygous $TNF^{\Delta ARE/+}$ on a mixed background[39] to C57BL6/J mice[38] and kept under specific-pathogen-free conditions. Experimental animals were heterozygous for the ΔARE mutation ($TNF^{\Delta ARE/+}$) or homozygous WT, which served as controls. At 8–12 weeks of age, mice that are heterozygous for the $TNF^{\Delta ARE/+}$ mutation reach peak transmural intestinal inflammation and as such, our studies were focused on mice at this age. WT littermates served as non-inflamed controls. $HSF1^{loxp/loxp64}$ mice were crossed with *CD4*^{Cre} and *foxp3*$^{YFP\text{-}Cre}$ mice to generate a conditional knockout mouse lacking HSF1 on total $CD4^+$ T cells or on $FoxP3^+$ Tregs, respectively. Mice were kept under specific pathogen-free conditions, and fecal samples were negative for *Helicobacter* species,

protozoa, and helminths. All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Colorado Denver.

Lymphocyte isolation

Splenocytes, MLN, and LP mononuclear cells were isolated as previously described[36,78]. Single-cell suspensions were obtained by forcing the spleen and MLN against a 70 μm cell strainer. Red blood lysis was performed using ammonium chloride lysing reagent (ACK Lysis Buffer, Invitrogen Carlsbad, CA, USA). Intestinal segments were opened along the mesentery and rinsed of luminal contents with PBS before cutting into 1 cm sections in Phosphate Buffered Saline (PBS) containing 15 mM HEPES and 1 mM EDTA with vigorous agitation on a vortex mixer. The tissue was then passed through a 70 μm tissue strainer and the process was repeated until the wash remained clear. The remaining LP was digested in 1 mg/ml Collagenase Type VIII (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes in an orbital shaker at 270 rpm and 37 °C. Tissues were vortexed briefly and filtered to remove any remaining undigested material and cells were counted prior to flow cytometric evaluation. Cells from indicated compartments were incubated with fluorescent rat anti-mouse antibodies for T-cell subset evaluation. Intracellular cytokine staining was performed by stimulating cells for 5 hours with 20ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-AldrichSt. Louis, MO, USA) and 1μg/mL calcium ionomycin (Sigma-Aldrich) in the presence of 3μg/mL brefeldin-A (Sigma-Aldrich St. Louis, MO, USA), followed by permeabilization and staining with antibodies against CD4 (GK1.5), CD25 (PC61.5), IL-17A (17B7), IFNγ (XMG1.2) and FoxP3 (FJK16S) (BioLegend, San Diego, CA, USA). Live cells were identified using Live/Dead Fixable Aqua dye (Invitrogen Carlsbad, CA, USA). FoxP3 staining was performed according to the manufacturer's instructions (FoxP3/Transcription Factor Staining Buffer Kit, eBiosciences, San Diego, CA, USA). Cells were washed and fixed with 2% paraformaldehyde and then analyzed using the FACS® Canto II system (Becton-Dickinson, Franklin Lakes, NJ, USA). Post-analyses were performed using FlowJo software (Tree Star Inc. Ashland, OR, USA).

RNA preparation and quantification of mRNA expression

Total RNA was isolated from ileal tissue or T-cell subsets using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) and converted to complementary DNA (cDNA) using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA200–500 ng of total RNA, final reaction volume 20 μl). Relative quantitation of mRNA expression was performed using Taqman Gene Expression Assays and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Polymerase chain reaction assays for HSF1 (Mm1201402_m1), IL-10 (Mm99999062_m1), FoxP3 (Mm00475156_m1), HIF1α (Mm00468869_m1), GLUT1 (Mm00441480_m1), HSP90a (Mm00658568_gH) and HSP90b (Mm00833431_g1) were carried out, with ribosomal 18s as an endogenous multiplexed control.

Conversion assays

Magnetically isolated naïve $CD4^+$ $CD25^{Neg}$ T cells isolated from the spleen were incubated for 72 hours in complete media (RPMI-1640 + L-glutamine, Corning Inc. Corning NY, USA; supplemented with 10 % FBS, 100 IU penicillin and 100 μg/ml streptomycin;

Invitrogen, Carlsbad, CA, USA) in the presence of IL-2 (5 IU/ml;), plate-bound anti-CD3 (1 μg/ml; BioLegend, San Diego, CA, USA), soluble anti-CD28 (37.15; 1 μg/ml; BioLegend, San Diego, CA, USA) and TGFβ (5ng/ml; R&D Systems, Minneapolis, MN, USA). The expression of FoxP3 was measured by flow cytometry.

In vitro suppression assays

Treg suppression assays were performed as previously described[79]. Briefly, $CD4^+CD25^+$ Tregs and $CD4^+$ $CD25^{Neg}$ effector T cells were magnetically isolated from murine splenocytes using the EasySep Mouse CD25 Regulatory T Cell Positive Selection Kit II (STEMCELL Technologies, Vancouver BC, Canada). 5×10^5 CellTrace Violet-labeled (Invitrogen, Carlsbad, CA, USA) $CD4^+$ $CD25^{Neg}$ WT effector T cells per well were stimulated with anti-CD3 mAb (1mg/mL, BioLegend, San Diego, CA, USA) in the presence of 7.5×10^5 irradiated syngeneic antigen presenting cells and varying ratios of Tregs isolated from C57/Bl6, $HSF1^{-/-}$, $HSF1^{Tg}$, $TNF^{\Delta ARE}$, $HSF1^{Tg}/TNF^{\Delta ARE/+}$, $CD4^{Cre}$ or $HSF1^{Fl/Fl}/CD4^{Cre}$ mice; suppression of proliferation was determined by the profile of dividing effector cells at 72 hours.

HSF1 protein Western blot

Nuclear and cytoplasmic protein extracts were isolated after 6 hours using the NE-PER extraction kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Western blots of HSF1 (4356, 1:500, Cell Signaling, Danver, MA, USA) using both denaturing and non-denaturing conditions were performed on 4%–15% gradient precast gels (Bio-Rad). Followed by a 2-hour incubation at room temperature with a goat anti-rabbit antibody (1:1000 Thermo Fisher Scientific, Rockland, IL, USA). Tata binding protein (TBP) detection was performed with mouse anti-TBP (Abcam, Waltham, MA, USA) followed by 2-hour incubation with goat anti-mouse (1:1000, Thermo Fischer Scientific, Rockland, IL, USA). TBP served as loading control for nuclear extracts. Blots were then exposed to enhanced chemiluminescence substrate (Clarity Western ECL Substrate; Bio-Rad, Herules, CA, USA, or SuperSignal West Femto Substrate, Thermo Fisher Scientific, Rockland, IL, USA) for 5 minutes and imaged using the Bio-Rad ChemiDoc™ MP system (Hercules, CA, USA). For [$pSer^{326}$]HSF1 WB cells were lysed in RIPA buffer after treatment for either 30 minutes at 39 °C (heat shock) or directly placed for 4 hours with or without anti-CD4 and TGFβ and anti [$pSer^{326}$]HSF1 polyclonal antibody (Enzo Life Sciences, Farming-dale, NY, USA) was used to probe for phosphorylated protein.

HSF1 immunofluorescence

Cytospins were generated by resuspending treated cells at 1×10^6 cells/ml and centrifuged at 1200 rpm for 5 minutes. Slides were then fixed with 4% paraformaldehyde/2% sucrose and permeabilized with 0.25% Triton X-100 in PBS. Cells were then blocked for 30 minutes at room temperature prior to staining with HSF1 Antibody (Cell Signaling, Danver, MA, USA) overnight at 4 °C. Slides were then washed repeatedly in PBS prior to staining using a PE-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas TX, USA). Cells were then counterstained with DAPI and cover-slipped with ProLong Gold antifade mounting medium (Thermo Fisher Scientific, Rockland, IL, USA).

HSE luciferase assays

Early passage Jurkat cells were plated at 0.5×10^6 cells per well in a 6-well format and were transfected using Lipofectamine LTX with 2.5 μg of DNA per well of HSE luciferase/reporter construct and Renilla luciferase. Twenty-four hours post-transfection cells were re-plated to 2.5×10^5 cells/well in a 96-well plate and treated with human recombinant TGFβ (R&D Systems, Minneapolis, MN, USA; 10 ng/ml) or anti-human CD3/CD28 dynabeads (invitrogen; 8 ml/well) or both for 24 hours. Cells were then centrifuged, supernatants removed and cells lysed in 25 μl of passive lysis buffer (Promega, Madison, WI, USA) prior to reading using the dual luciferase reporter assay system and luciferase activity measured on a GloMax-Multi Microplate Multimode Reader (Promega, Madison, WI, USA).

ChIP analysis

Naïve murine $CD4^+$ $CD25^{Neg}$ cells were isolated as described above. These T cells were incubated for 4 hours in complete media (RPMI-1640 + L-glutamine, Corning Inc., Corning, NY, USA; supplemented with 10% FBS, 100 IU penicillin and 100 μg/ml streptomycin; Invitrogen, Carlsbad, CA, USA) in the presence of IL-2 (5 IU/ml; R&D Systems, Minneapolis, MN, USA), plate-bound anti-CD3 (17A2; 1 μg/ml; BioLegend, San Diego, CA, USA) and soluble anti-CD28 (37.15; 1 μg/ml; BioLegend, San Diego, CA, USA) with or without TGFβ (5ng/ml; R&D Systems, Minneapolis, MN, USA). The cells were fixed, and protein and DNA were crosslinked using a stock concentration of 18.5% formaldehyde (Sigma-Aldrich, Burrlington, MA, USA), incubated for 6 minutes, then a 1/10 volume of 1.5 m glycine (Sigma-Aldrich, Burlington, MA, USA) was added for 5 minutes while rotating. The cells were centrifuged and then washed twice with 1 mL ice-cold PBS and the cell pellets flash-frozen in LN_2 and stored at −80 °C. Cells were lysed and nuclei and chromatin were prepared with micrococcal nuclease and then pelleted; these preparations were then sonicated for 18 cycles. DNA was purified using a spin column. Immunoprecipitation was performed with 1μL mouse anti-HSF1 (Santa Cruz Biotechnology, Dallas, TX, USA) and control was 1μL rat anti-mouse Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, TX, USA) and incubated at 4 °C for 8 hours while rotating tubes. Primers (EpiTect ChIP quantitative polymerase chain reaction Primer Assay for Mouse *foxp3*, Qiagen, Venlo, The Netherlands) were used to amplify foxp3 in 1 Kb fragments.

$CD45RB^{High}$ adoptive transfer model of murine colitis

C57BL6/J donor mice were euthanized and a single-cell suspension of untouched $CD4^+$ cells was obtained using the CD4-negative selection kit (EasySep, Stemcell Technologies, Vancouver, BC, Canada). $CD4^+$ $CD45RB^{High}$ cells were then isolated by Flourescence-activated cell sorting (FACS) using the BD FACSAria™ Cell Sorting System (BD Biosciences, Franklin Lakes, NJ, USA). 5×10^5 cells were then injected i.p. into $RAG1^{-/-}$ recipients. Mouse weight was recorded twice weekly and mice that failed to lose weight by week 4 were omitted from the study (n = 1–2 per category per experiment). As indicated, adoptive transfer experiments included either 1:4 or 1:8 $CD4^+$ $CD25^+$ magnetically sorted Tregs co-administered at time of i.p. injection. Excised colon or ilea were fixed in 10% formalin, embedded, cut into 3- to 5-μm sections, and stained with hematoxylin and

eosin. Histologic assessment of ileal inflammation was performed using a standardized semi-quantitative scoring system, as described previously[80].

Cytokine production assays

Tissue explants (0.5 cm^2) were cultured for 24 hours in Dulbecco's Modified Eagle Media (DMEM without sodium pyruvate; Cellgro, Mediatech Inc., Manassas, VA, USA) supplemented with 5 % FBS, 2 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin (Invitrogen), then culture supernatants were then analyzed for the presence of cytokines using IL-6, IFNγ, IL-10, and IL-17 Ready-Set-Go! Enzyme linked immunosorbent assay (ELISA) kits (Affymetrix eBioscience, Santa Clara, CA, USA).

Statistics

Statistical analyses were performed using Student *t* test or one-way analysis of variance with Kruskall-Wallace test. Graphs are presented as means ± SEM and were generated using GraphPad Prism software (LaJolla, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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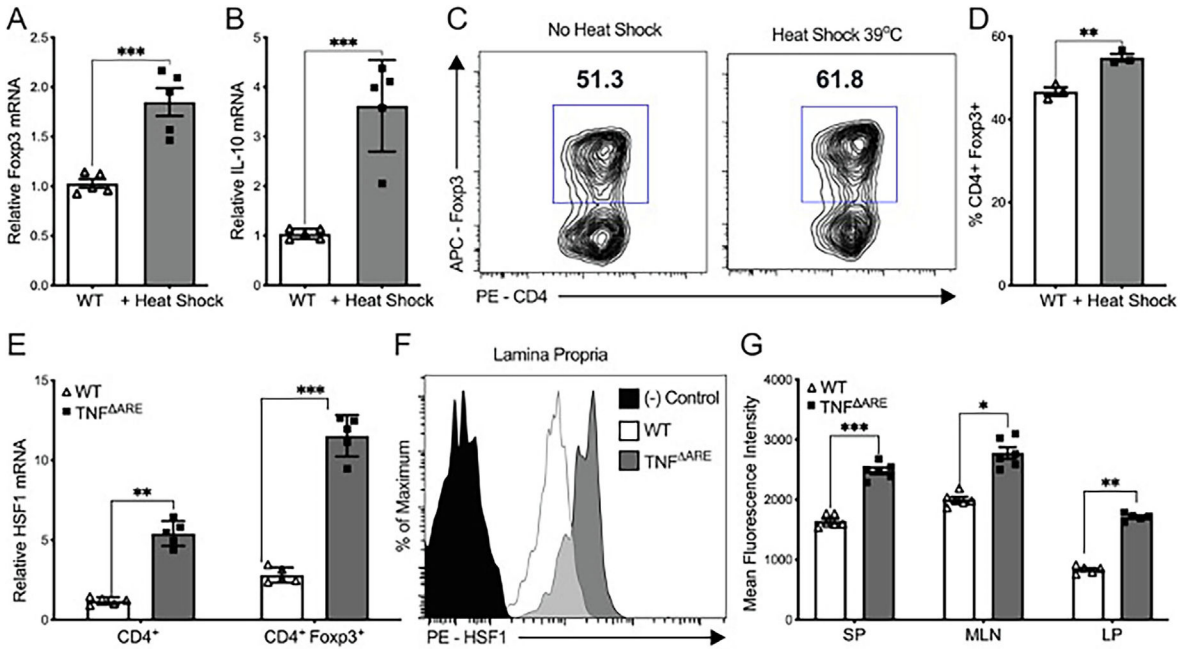

Fig. 1.

Promotion of Treg Induction by Heat Shock via Transcription Factor HSF1. Heat shock treatment at 39 °C for 30 minutes increased the expression of (A) *foxp3* and (B) *il-10* mRNA in naïve mouse $CD4^+$ T cells. This transient heat shock also increased the frequency of $CD4^+$ $Foxp3^+$ Treg induction over a 3-day period depicted in representative contour plots (C) and bar graphs (D). In the chronically inflamed intestine ($TNF^{\Delta ARE/+}$ mice), HSF1 was preferentially upregulated in $CD4^+$ T cells and Tregs at both the mRNA (E) and protein (F) levels, as determined by RT-PCR and flow cytometry. Representative histograms and (G) bar charts display the MFI of HSF1 on $CD4^+$ cells in the SP, MLN, and LP. The results are presented as mean ± SEM, n ≥ 4. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. CD = clusters of differentiation; HSF1 = heat shock factor 1; LP = lamina propria; MFI = mean fluorescence intensity; MLN = mesenteric lymph nodes; RT-PCR = Reverse transcription polymerase chain reaction; SP = spleen.

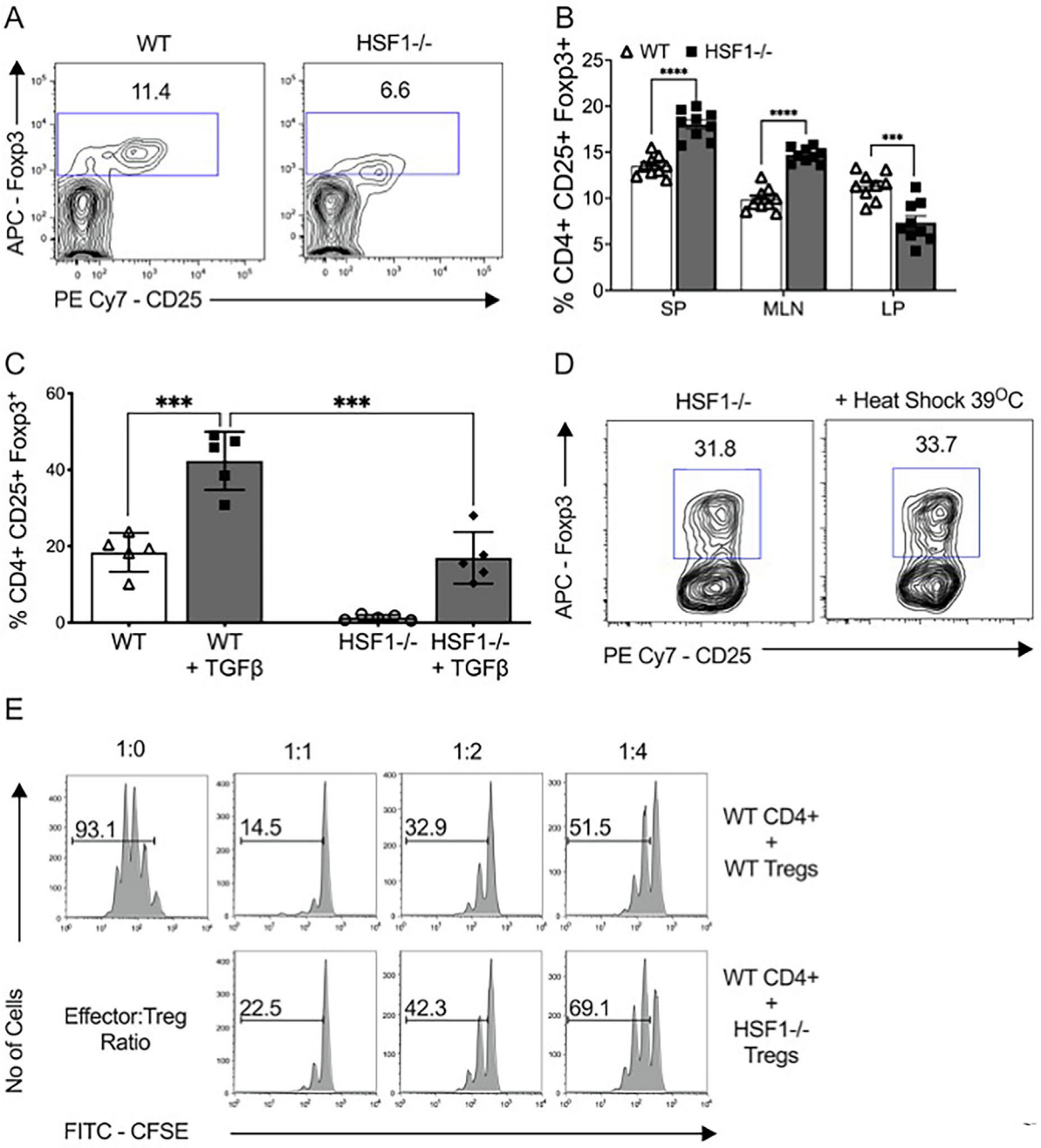

Fig. 2.

HSF1-deficient Tregs exhibit impaired function and reduced Foxp3 protein. The frequency of $CD4^+$ $FoxP3^+$ $CD25^+$ regulatory T cells (Treg) $HSF1^{-/-}$ mice was analyzed by flow cytometry. The results showed a significant decrease in Treg frequency in $HSF1^{-/-}$ mouse LP depicted in representative contour plots (A) and an increase in SP and MLN expression seen in the bar chart (B). Culturing naïve $CD4^+$ T cells from wild-type (WT) and $HSF1^{-/-}$ mice under Treg converting conditions (anti-CD3/CD28, IL-2, ±TGFβ) revealed decreased Treg induction in $HSF1^{-/-}$ cells (C) compared to WT. (D) Heat shock for 30 minutes at 39 °C had no effect on Treg conversion in naïve $HSF1^{-/-}$ T cells. To assess Treg suppressive

function Isolated $CD4^+$ $CD25^+$ Tregs from WT and $HSF1^{-/-}$ mice were co-cultured with irradiated $CD90^{Neg}$ antigen-presenting cells and fluorescently labeled effector cells at increasing ratios (E). Proliferation was stimulated with soluble anti-CD3 and measured over 72 hours. The percentage of proliferating labeled effector lymphocytes served as a surrogate marker for Treg suppressive function. Tregs from $HSF1^{-/-}$ mice displayed significantly weaker suppression of proliferation compared to WT Tregs. Results represent mean ± SEM, $n = 3$ from three independent studies. ** $p < 0.01$. CD = clusters of differentiation; HSF1 = heat shock factor 1; SEM = standard error of the mean; Treg = regulatory T cells; WT = wild-type.

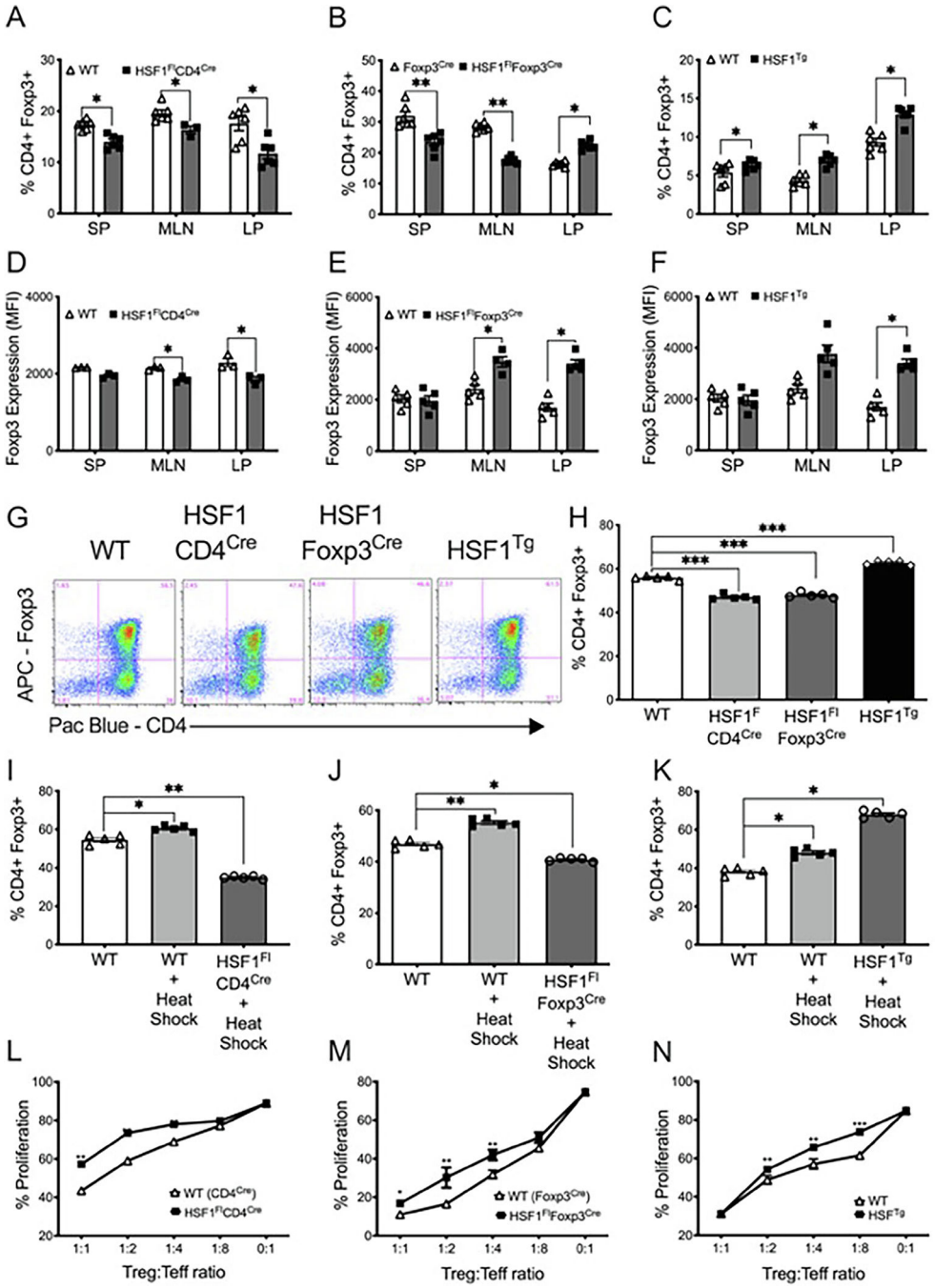

Fig. 3.

Evaluation of Conditional $HSF1^{-/-}$ mice and $HSF1^{Tg}$ mice. Flow cytometric analysis revealed a decrease in $CD4^+$ $Foxp3^+$ Treg frequency (A) in the colonic LP, MLN, and spleen of $HSF1^{Fl/Fl}CD4^{Cre/+}$. This coincided with reduced mucosal Foxp3 protein indicated by (D) reduced mean fluorescence intensity in LP and MLN. $CD4^+$ $Foxp3^+$ Tregs frequency increased in the LP of $HSF1^{Fl/Fl}FoxP3^{Cre/+}$ mice relative to controls but decreased in MLN and spleen (B). $HSF1^{Fl/Fl}FoxP3^{Cre/+}$ mice displayed a similar reduction in Foxp3 protein by MFI (E). In contrast, HSF1 overexpression in $HSF1^{Tg}$ mice coincided with increased

mucosal $CD4^+$ $Foxp3^+$ Treg frequency (C) and a concomitant increase in HSF1 MFI in both LP and MLN (F). Conversion of naïve $CD4^+$ T cells *in vitro* demonstrated that $HSF1^{Fl/Fl}$/$CD4^{Cre/+}$ and $HSF1^{Fl/Fl}$/$FoxP3^{Cre/+}$ had decreased conversion to $CD4^+$ $FoxP3^+$ cells and increased conversion in $HSF1^{Tg}$ $CD4^+$ T cells (G, H). Heat shock treatment increased the conversion of naïve $CD4^+$ cells to $CD4^+$ $FoxP3^+$ T cells in control mice ($CD4^{Cre/+}$ or $FoxP3^{Cre/+}$), while HSF1-deficiency in either (I) $HSF1^{Fl/Fl}CD4^{Cre/+}$ or (J) $HSF1^{Fl/Fl}FoxP3^{Cre/+}$ T cells coincided in decreased Treg conversion. This was reversed in $HSF1^{Tg}$ $CD4^+$ T cells, which exhibited increased conversion to $CD4^+$ $FoxP3^+$ T cells after heat shock (K). Treg suppression assays indicated reduced suppressive activity in (L) $HSF1^{Fl/Fl}CD4^{Cre/+}$ and (M) $HSF1^{Fl/Fl}FoxP3^{Cre/+}$ Tregs compared to control mice. Conversely, $HSF1^{Tg}$ Tregs (N) demonstrated increased suppressive function. Results represent mean ± SEM, $n = 3$ from three independent studies. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CD = clusters of differentiation; HSF1 = heat shock factor 1; LP = lamina propria; MFI = mean fluorescence intensity; MLN = mesenteric lymph nodes; SEM = standard error of the mean; SP = spleen; Treg = regulatory T cells.

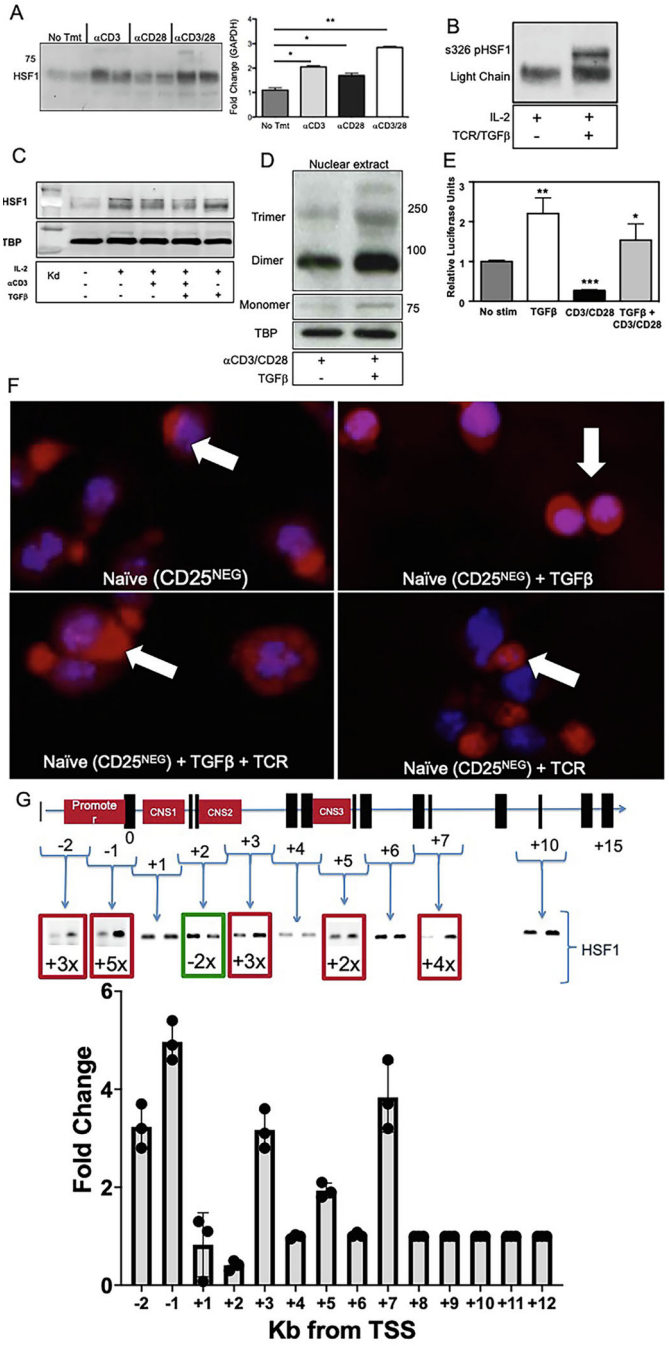

Fig. 4.

Assessment of HSF1 activation in Tregs. Naïve CD4^{+} T cells were stimulated *in vitro* with IL-2 and either plate-bound anti-CD3 or anti-CD28 antibodies for 4 hours. (A) Western blot analysis revealed increased HSF1 expression with TCR stimulation expressed relative to GAPDH as a reference. (B) Phosphorylation of HSF1 at serine 326 (s326) indicating HSF1 activation was observed with heat shock (HS), TCR stimulation, and combined TCR/TGFβ stimulation after 4 hours. (C) Western blotting of HSF1 in nuclear extracts showed increased HSF1 expression with IL-2 and TGFβ treatment for 4 hours. (D) Non-denatured Western

blotting of nuclear extracts of $CD4^+$ T cells treated with IL-2, anti-CD3, anti-CD28 with or without TGFβ demonstrated increased dimerization and trimerization of HSF1 after 4 hours. (E) Jurkat cells transfected with a heat shock element dual luciferase promoter reporter construct showed HSF1 binding at the HSE in response to Treg activation and conversion conditions. (F) Immunofluorescent evaluation of $CD4^+$ $CD25^{Neg}$ T cytospins demonstrates an increase in nuclear (4'6-diamidine-2-phenylindole (DAPI) Blue) expression of HSF1 (Red) after 4 hours Treg converting conditions. (G) ChIP assay assessed HSF1 binding to the foxp3 gene in naive $CD4^+$ $CD25^{Neg}$ T cells incubated with different culture conditions for 4 hours. DNA analysis was performed using EpiTect ChIP qPCR Primer Assay for mouse *foxp3* (Qiagen). Data represent mean ± SEM from three mice per group from three independent studies. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. CD = clusters of differentiation; HSF1 = heat shock factor 1; SEM = standard error of the mean; TCR = T cell receptor; TGFβ = transforming growth factor β

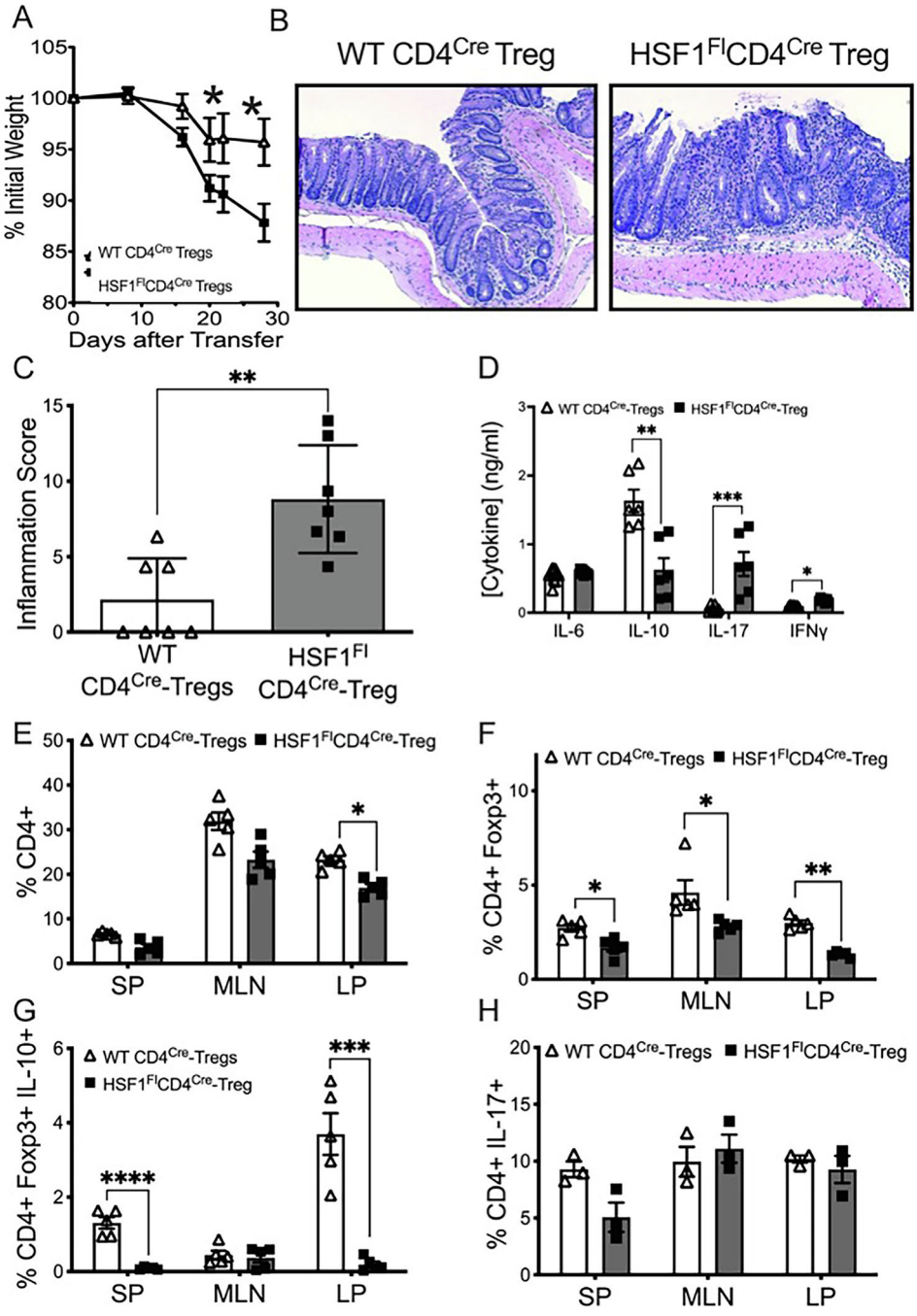

Fig. 5.

In vivo suppression of inflammation by Tregs from HSF1$^{Fl/Fl}$ CD4^{Cre} Tregs. To evaluate the effect of Tregs on inflammation, Rag1$^{-/-}$ immunodeficient mice were injected with naïve CD4^{+} CD45RB^{High} cells from WT C57BL/6 mice along with CD4^{+} CD25^{+} T cells isolated from either WT CD4$^{Cre/+}$ or HSF1$^{Fl/Fl}$ CD4$^{Cre/+}$ mice at a ratio of1:4 (Treg to CD45RB^{High} cells). Mice receiving HSF1$^{Fl/Fl}$CD4$^{Cre/+}$ Tregs experienced rapid weight loss (A), a surrogate marker of colitis and histological evidence of inflammation. Tissue histology (B) was independently scored by a pathologist blinded to the study (C). ELISA

evaluation (D) was performed on 24-hour explant cultures using colonic tissue isolated from the aforementioned experimental mice and secretion of IL-6, IL-10, IL-17, and IFNγ were assessed. Flow cytometric analysis quantified the frequency (E) of $CD4^+$ cells, (F) $CD4^+$ $Foxp3^+$ Tregs, (G) IL-10-secreting $CD4^+$ $Foxp3^+$ T and (H) IL-17-secreting $CD4^+$ cells from the colonic lamina propria, mesenteric lymph node, and spleen. The results demonstrated a significant decrease in Treg frequency in the $HSF1^{Fl/Fl}CD4^{Cre/+}$ mice. Results represent the mean ± SEM, n = 6 mice per group from three independent studies. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CD = clusters of differentiation; ELISA = enzyme-linked immunosorbent assay; HSF1 = heat shock factor 1; IFN = interferon; IL = interleukin; SEM = standard error of the mean; Treg = regulatory T cells.

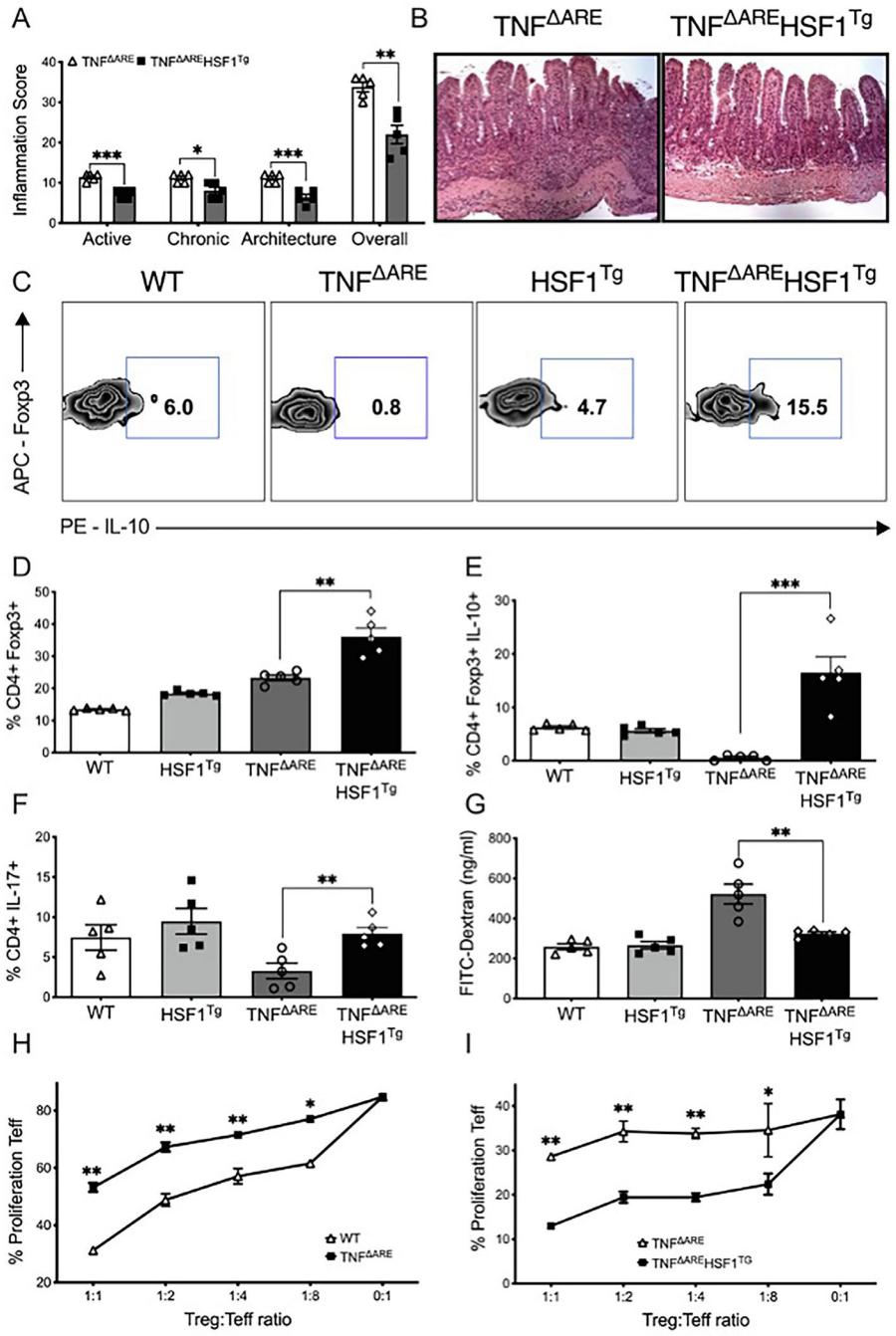

Fig. 6.

Evaluating the impact of HSF1 overexpression in an ileitis model. $TNF^{\Delta ARE/+}$ $HSF1^{Tg}$ mice were used to investigate the effects of HSF1 overexpression on ileitis in the $TNF^{\Delta ARE/+}$ model. Assessment of ileitis in mice overexpressing HSF1 reveals decreased inflammation, as indicated by (A) decreased inflammatory scoring conducted by a blinded pathologist and (B) representative histology. Flow cytometry analysis of isolated ileal lamina propria cells identifies an increase in $CD4^+$ $Foxp3^+$ T cells (C, D) IL-10-secreting $CD4^+$ $Foxp3^+$ cells (E), and IL-17-secreting $CD4^+$ cells in the $TNF^{\Delta ARE/+}HSF1^{Tg}$ ileum (F). HSF1

overexpression (G) reduced 4kd FITC dextran transport across the intestinal epithelial barrier, a surrogate marker of intestinal inflammation. Tregs from $TNF^{\Delta ARE/+}$ mice exhibit decreased suppressive function *in vitro* (H). However, Tregs from $TNF^{\Delta ARE/+}$ $HSF1^{TG}$ mice demonstrate improved suppressive function compared to Tregs from $TNF^{\Delta ARE/+}$ mice (I). Results represent mean ± SEM, n ≥ 5 mice per group from three independent studies. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. CD = clusters of differentiation; FITC = Fluorescein isothiocyanate; HSF1 = heat shock factor 1; IL = interleukin; SEM = standard error of the mean; TNF = tumor necrosis factor; Treg = regulatory T cells.

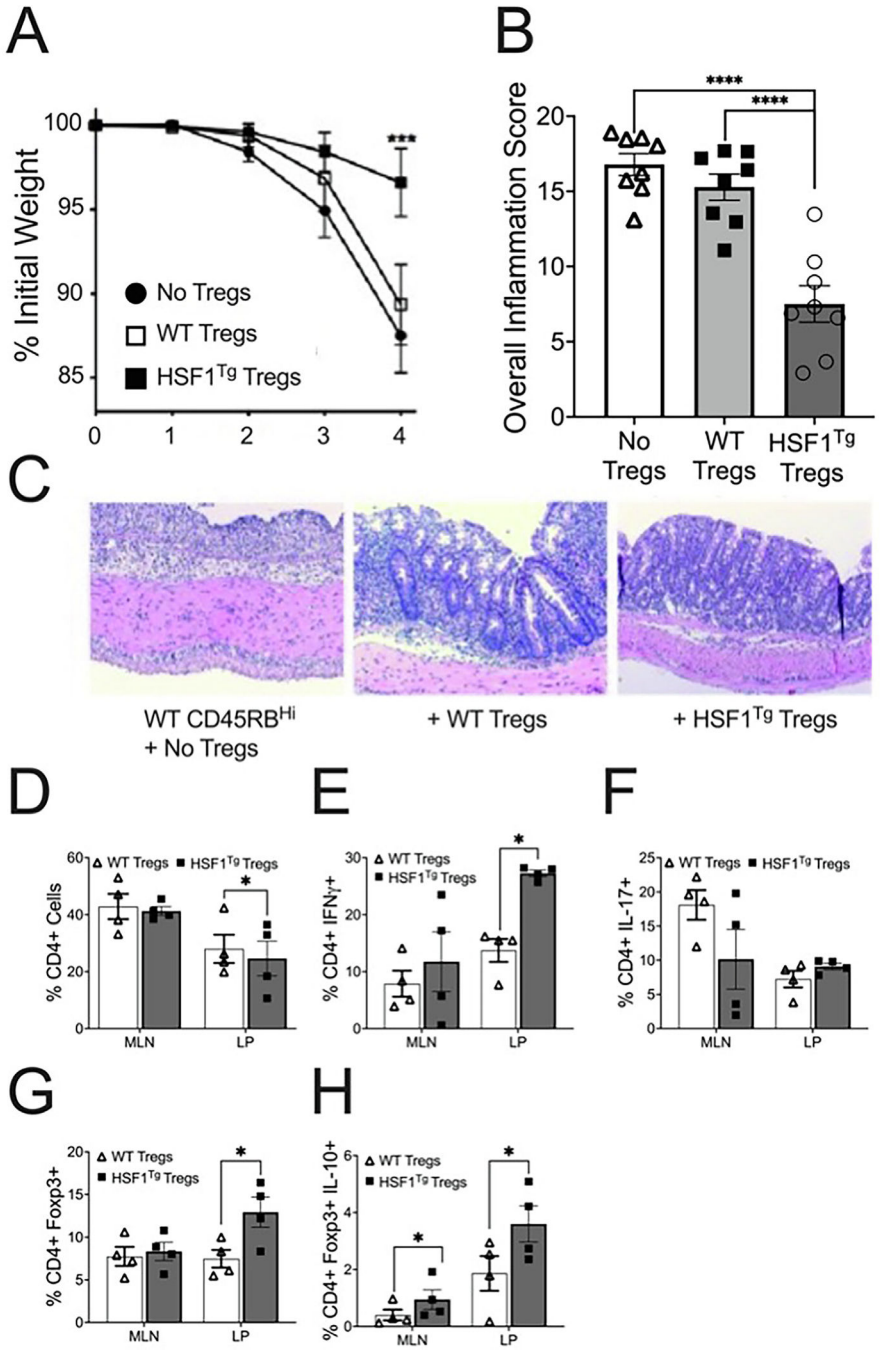

Fig. 7.

Adoptive transfer of Tregs from HSF1^{Tg} mice improves inflammation. Rag1$^{-/-}$ immunodeficient mice were co-injected with CD4^{+} CD45RB^{High} cells from WT C57BL/6 mice and CD4^{+} CD25^{+} T cells isolated from either WT CD4$^{Cre/+}$ or HSF1^{Tg} mice at a ratio of 1:8 (Treg to CD45RB^{High} cell) or no Tregs (A). Weight monitoring identified decreased weight loss in the mice treated with Tregs from the HSF1^{Tg} mice consistent with attenuation of colitis. (B) Tissue histology identified a decrease in acute and chronic inflammation in the HSF1^{Tg} Treg treated mice relative to controls resulting in improved overall inflammation

scores in the HSF^{Tg} Treg treated mice. (C) Representative H&E-stained micrographs demonstrate the architectural distortion and lymphocytic expansion in the lamina propria of the no Treg or WT Treg-treated mice but significantly less inflammation in the colon of the $HSF1^{Tg}$ Treg-treated mice. Flow cytometric analysis of the frequency of (D) $CD4^+$, (E) IFNγ-secreting, (F) IL-17-secreting, (G) $CD4^+$ $FoxP3^+$ cells and (H) $CD4^+$ $FoxP3^+$ IL-10 secreting T cells from the colonic LP and mesenteric MLN demonstrate a significant increase in Treg frequency in the $HSF1^{Tg}$ Treg treated mice across both tissues. Results represent mean ± SEM of 4–6 mice per group and from three independent studies. *$p < 0.05$. ****$p < 0.001$. CD = clusters of differentiation; HSF1 = heat shock factor 1; IL = interleukin; LP = lamina propria; MLN = mesenteric lymph nodes; SEM = standard error of the mean; Treg = regulatory T cells.; WT = Wild type.