Heat Shock Transcription Factor 1 Is Activated as a Consequence of Lymphocyte Activation and Regulates a Major Proteostasis Network in T Cells Critical for Cell Division During Stress

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Heat shock transcription factor 1 is activated as a consequence of lymphocyte activation and regulates a major proteostasis network in T cells critical for cell division during stress

Siva K. Gandhapudi,*1 Patience Murapa,*2 Zachary D. Threlkeld,∗,3 Martin Ward,* Kevin D. Sarge,† Charles Snow,* and Jerold G. Woodward*

Heat shock transcription factor 1 (HSF1) is a major transcriptional regulator of the heat shock response in eukaryotic cells. HSF1 is evoked in response to a variety of cellular stressors, including elevated temperatures, oxidative stress, and other proteotoxic stressors. Previously, we demonstrated that HSF1 is activated in naïve T cells at fever range temperatures (39.5˚C) and is critical for in vitro T cell proliferation at fever temperatures. In this study, we demonstrated that murine HSF1 became activated to the DNA-binding form and transactivated a large number of genes in lymphoid cells strictly as a consequence of receptor activation in the absence of apparent cellular stress. Microarray analysis comparing HSF1+/+ and HSF1−/− gene expression in T cells activated at 37˚C revealed a diverse set of 323 genes significantly regulated by HSF1 in nonstressed T cells. In vivo proliferation studies revealed a significant impairment of HSF1−/− T cell expansion under conditions mimicking a robust immune response (staphylococcal enterotoxin B-induced T cell activation). This proliferation defect due to loss of HSF1 is observed even under nonfebrile temperatures. HSF1−/− T cells activated at fever temperatures show a dramatic reduction in cyclin E and cyclin A proteins during the cell cycle, although the transcription of these genes was modestly affected. Finally, B cell and hematopoietic stem cell proliferation from HSF1+/− mice, but not HSF1−/− mice, were also attenuated under stressful conditions, indicating that HSF1 is critical for the cell cycle progression of lymphoid cells activated under stressful conditions. *The Journal of Immunology, 2013, 191: 4068–4079.

Heat shock transcription factor 1 (HSF1) is a major transcriptional regulator of the eukaryotic cellular heat shock response and is evoked by a variety of stress stimuli, including elevated temperatures (1–3), radiation (4), oxidative stress (5), toxic chemicals (6, 7), infectious agents (8, 9), and other proteotoxic stressors. Upon sensing stress, HSF1 is rapidly converted from an inactive monomeric form to a trimeric DNA-binding form in the nucleus, which then interacts with DNA sequences carrying inverted repeat nGAAn sequences named heat shock elements (HSEs), and regulates target gene expression. The most widely studied genes regulated by HSF1 encode the heat shock proteins (HSPs). HSPs serve a variety of critical functions within the cell, acting as chaperones, assisting in correct protein folding, and helping to target damaged or unfolded proteins to the proteasome for degradation.

Although initially studied in the context of the heat shock response, HSF1 is now considered to be part of a larger network of protein homeostasis or proteostasis (10–13). The proteostasis network is ancient and evolutionarily conserved and consists of various cellular pathways dedicated to maintaining protein homeostasis in both “normal” and stress conditions. These include degradative pathways such as the ubiquitin proteasome system and the endoplasmic reticulum–associated degradation systems, post-translational modification, including phosphorylation, acylation, and oxidation, and protein synthesis/folding/unfolding, including ribosomes, HSF1, and the unfolded protein response (10, 12). Thus, HSF1 can be considered as one of the important sensors of proteostasis, with the capability of regulating a series of genes necessary to maintain proper proteostasis. It is clear that the needs of proteostasis will differ between cell types and between different environmental conditions. So far, the role of proteostasis in the immune system is poorly understood.

In yeast, the single HSF gene is required for viability, probably because of the requirement of HSF to maintain basal HSP activity (14, 15). In Drosophila, HSF mutants are lethal, but conditional knockouts show that it is mainly required for early embryogenesis and is dispensable for viability of the adult (16). In mammals,
there are several forms of HSF, with HSF1 serving the major stress-responsive function. HSF1 knockout mice exhibit embryonic lethality, but on a mixed genetic background, viable knockout mice are obtained (17). These mice, although capable of surviving to old age in laboratory conditions, are generally smaller than HSF1+/+ mice and show an increased susceptibility to stress, including heat and oxidative stress. Interestingly, these mice also show increased lethality to endotoxin (17) and infection with Listeria (18). In the latter case, we have shown that the increased lethality is at least in part due to an overproduction of TNF-α, in agreement with other studies showing HSF1 to be a negative regulator of TNF-α (19, 20). HSF1 also plays important roles in lung protection (7), neurogenesis (21), proliferation (22), apoptosis (23), cell cycle (24, 25), and carcinogenesis (26).

Although HSF1 affects a variety of cellular processes, a particularly striking common thread is the role of HSF1 in cellular proliferation. HSF1 knockdown or knockout has profound effects on cell cycle progression in both yeast and mammalian cells. In most cases, the requirement for HSF1 becomes more acute as the stress level increases. In Drosophila and mice, the requirement for HSF1 is most acute in early embryogenesis (24, 27). Female HSF1−/− mice are sterile owing to a requirement for maternal HSF1 for cell division of the early preimplantation fertilized oocyte (24). The critical importance for HSF1 in cell division is underscored by the finding that HSF1−/− mice are highly resistant to carcinogenesis (26, 28). Virtually all tumors and cell lines show constitutively active HSF1, and it appears that continual activation of HSF1 is necessary for most tumors to progress to high levels of mitotic activity.

With respect to the immune system, HSF1−/− mice are defective in cross-presentation of Ag, probably due to the fact that HSF1 is required for the inducible form of HSP70, known to be important in cross-presentation (29–31). HSF1−/− mice have normal lymphoid architecture and cellularity, indicating that HSF1 has no critical role in lymphoid development. However, we and others have shown clear defects in induced immune responses in HSF1−/− mice. For example, induced Ab responses are compromised in HSF1−/− mice (32), and in vitro proliferation of T cells from HSF1−/− mice is defective at elevated temperatures in the fever range in response to TCR triggering (22). These studies suggest that HSF1 serves an important role in proteostasis within the immune system.

In this study, we demonstrate that HSF1 becomes activated in T cells shortly after TCR ligation, even at normal temperatures. This in turn activates a genetic program, regulating transcription of >200 genes, including a signature of proteostasis-related genes. We further demonstrate that HSF1 is necessary for optimal T cell proliferation at normal temperatures in the presence of robust stimuli in vivo and in vitro. These results suggest that HSF1 is a critical proteostasis regulator in lymphoid cells, one function of which is to maintain optimal proliferation in the face of proteotoxic stress.

Materials and Methods

Mice

HSF1−/− mice on the 129Sv/BALB/c mixed background were colonized from a breeding pair provided by Ivor Benjamin (University of Utah, Salt Lake City, UT) and maintained by breeding heterozygotes as described previously (22). HSF1+/+ mice and HSF1−/− mice were derived from the same breeding colony and identified by PCR. DO11.10 and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions at the Division of Laboratory Animal Resources, University of Kentucky Medical Center (Lexington, KY). All animal experiments have been approved by the University of Kentucky Institutional Animal Care and Use Committee.

Lymphocyte cultures

T cells were purified from spleens and/or peripheral lymph nodes using either anti-CD3 magnetic beads or Pan T Cell Isolation Kit and the autoMACS (Milteny Biotech, Cambridge, MA), according to the manufacturer’s protocol. B cells were purified by depletion of T cells with a mixture of anti-T cell Ab and complement as described previously (33). Unless specified otherwise, cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mM glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 50 μM 2-mercaptoethanol, and 100 U/ml streptomycin (complete RPMI medium, or cRPMI medium). Hematopoietic bone marrow cells were isolated by removing bone marrows from femurs of HSF1−/− or HSF1+/+ mice. The single-cell suspensions were then washed with 1× HBSS and resuspended in cRPMI medium.

In vitro proliferation assays

For proliferation assays, 2 × 10^5 spleen cells were cultured alone or with staphylococcal enterotoxin B (SEB) (20μg/ml) (Sigma Aldrich, St. Louis, MO) or anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in 96 well plates for 72 h at indicated temperatures. H-thymidine (1 μCi/ml) was added to each culture during the last 18h and proliferation was determined by scintillation counting. Alternatively, spleen cells or purified T cells obtained through anti-CD3 magnetic bead purification were labeled with CFSE as previously described (34) and cultured in 24-well plates at 1 × 10^6 cells per well in medium alone or with SEB or anti-CD3/CD28 under the conditions described. Following activation, the cells were harvested, stained and analyzed using FACScan flow cytometer. For coculture studies, flow through cells (untouched negative fraction) obtained during the T cell purification step were cultured at indicated ratios with APCs. For antigen stimulation studies, CFSE labeled bone marrow cells were added to individual wells of 24 well plates (“bottom well”) in 1ml of cRPMI media with or without SEB or anti-CD3/CD28. For the “top well,” an equal number of spleen cells was then added to a 10mm tissue culture insert containing a 0.4μM membrane bottom (Nalgé Nunc International, Rochester, NY) placed inside an individual well of the 24 well plate. Following activation, the cells in bottom wells were harvested and analyzed by flow cytometry after staining the cells with PE conjugated anti-Vb8.1, 8.2, and 8.3 (eBioscience, San Diego, CA). Following this step, cells were fixed, permeabilized, and stained with anti-BrDU Ab using a BrDU staining kit (BD Pharmingen). The Vb8 T cell expansion was then analyzed using a FACScan flow cytometer by gating on CD3+ cells and analyzing Vb8-, BrDU+ cells within this gate.

Native gel shift assay

Native gel protein extracts were prepared by lysing cells as described previously (35, 36). Briefly, cells pellets were rapidly frozen in liquid nitrogen and then suspended in lysis buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. The lysates were then centrifuged at 15,000 rpm for 1 h at 4°C and the supernatants were stored at ~80°C until further analysis. For the EMSA, 5 μg extract was mixed with 0.1 ng [32P]-labeled HSE oligonucleotide probe in a 25 μl reaction mixture containing 0.5 μg poly(dI-dC), 2 μg BSA, 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol. The reaction mixture was incubated for 30 min at 25°C, and a dye solution (50% glycerol and 100 μM bromophenol blue) was added and directly loaded onto a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. The gels were run for 2.5 h at 130 V, dried, and subjected to autoradiography. [32P]HSE oligonucleotides were prepared by 5’ end-labeling one strand with T4 kinase as described previously (35).
Western blot analysis

Cells were washed once with 1× PBS, lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris [pH 8.0]) containing protease inhibitor mixture (Thermo Scientific, Waltham, MA) and centrifuged at 13,000 rpm for 15 min. Protein concentrations in the supernatants were determined using the Bradford method (Thermo Scientific). The supernatants were then boiled for 5 min in 1× SDS loading buffer and loaded directly onto an 8% SDS-polyacrylamide gel and subjected to electrophoresis at 120 V for 90 min. The proteins were then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk (Sigma-Aldrich, St. Louis, MO) and probed at 4°C overnight with protein-specific primary Abs: rabbit polyclonal anti-cyclin D2 (sc-452; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin E1 (sc-481), cyclin A2 (sc-751), p27, and monoclonal mouse anti–β-actin as a loading control. Blots were then washed and developed with HRP-conjugated goat anti-rabbit secondary Ab or goat anti-mouse secondary Ab and the signal was detected using ECL detection system (Thermo Scientific).

Microarray analysis

Purified T cells from pooled spleen and lymph nodes of HSF1−/− or HSF1+/+ mice were activated with plate-bound anti-CD3 in 5 ml at either 37°C or 40°C. Following activation, cells were harvested, washed with 1× PBS, and centrifuged. RNA from the cell pellets was isolated using an RNeasy kit (Qiagen, Valencia, CA) with a DNA clean-up step included in the protocol. RNA quality and quantity were measured using spectrometry and a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For each treatment, equal amounts of RNA isolated from mice (n = 3) were pooled and stored at −80°C until further analysis. The total RNA (2.5 μg) was then used to transcribe to biotinylated cRNA by the Microarray Core Facility at University of Kentucky and was hybridized to the whole mouse genome microarray chip (Affymetrix mouse genome 430 2.0) using two chips for each RNA sample. Hybridization of cRNA on to the chips was carried out for 16 h and was then labeled using streptavidin–anti-biotin and streptavidin–PE. The chips were then scanned using Affymetrix GC3300 7G scanners and the data were recorded for further analysis.

Microarray data analysis

The Affymetrix mouse genome 430 2.0 GeneChip has 45,101 probe sets, of which 27,518 probe sets were selected for further analysis after removing 17,583 probe sets that were always absent. A two-way ANOVA with repeated measures test was used to identify the probe sets with significance in expression. Probe set hybridization levels with a p value of ≤0.01 between HSF1−/− and HSF1+/+ at 37°C and HSF1−/− and HSF1+/+ at 40°C and a fold change of at least 0.5 on a log 2 scale between the treatments were selected for further analysis. A complete listing of the gene expression data has been deposited with the Gene Expression Omnibus data repository (accession no. GSE41005; available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41005). Surprisingly, most of these genes (228) were regulated by HSF1 at 37°C. Additionally, 156 of those genes showed higher expression in HSF1−/− cells, suggesting negative regulation by HSF1. There were subsets of genes regulated by HSF1 only at 37°C or only at 40°C, indicating that temperature has a critical modulating effect on genes regulated by HSF1 (Fig. 1). For those genes regulated by HSF1 at both 37°C and 40°C, the effect was generally greater at 40°C, consistent with the higher levels of HSF1 activation at 40°C (36).

The genes most significantly regulated by HSF1 are grouped by function (Supplemental Table I). Many of these genes are involved in the stress response, apoptosis, cell cycle, inflammation, signal transduction, transcription and translation, and metabolism. Chaperones, including HSPs, are the best known targets of activated HSF1 that this activation was accompanied by the induction of HSP70i protein (the product of the nearly identical hspal and hspaib genes), indicating that the acquisition of HSF1 DNA-binding activity was accompanied by transcriptional activity. The lack of HSP70i protein in HSF1−/− cells supports the notion that HSP70i is strictly dependent on HSF1 (17, 22). We also noted that HSP70i protein was induced upon T cell activation at 37°C and more strongly at 39.5°C, in an HSF1-dependent manner (22). These results indirectly suggest that HSF1 is capable of transactivating target genes following T cell activation in the absence of thermal stress.

To determine the gene program regulated by HSF1 in freshly activated T cells, we performed a microarray analysis. We used a whole mouse genome microarray to identify the genes differentially regulated in anti-CD3–activated T cells from HSF−/− and HSF1+/+ mice after 5 h of activation at normal (37°C) or febrile (40°C) temperatures. We chose 5 h of T cell activation to allow for HSF1 to transactivate gene expression of target genes, but to limit the influence of secondary transactivation by genes not directly regulated by HSF1. Of 27,518 probes that were always present across the treatment groups, 323 probe sets showed a differential regulation between HSF1−/− and HSF1+/+ at either physiological (37°C) or febrile temperatures (40°C) based on the selection criteria of a minimum of log2 0.5-fold change and p value of <0.01. A complete listing of the gene expression data has been deposited with the National Center for Biotechnology Information’s Gene Expression Omnibus data repository (accession no. GSE41005; available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41005).

Results

HSF1 transactivates a proteostasis network in T cells

In previous studies, we have demonstrated that HSF1 lacked DNA-binding activity and was inactive in resting spleen cells or purified resting T cells at 37°C, but it was activated to the DNA-binding form upon incubation at 39.5°C for 1 h (22, 36). We also showed that this activation was accompanied by the induction of HSP70i protein (the product of the nearly identical hspal and hspaib genes), indicating that the acquisition of HSF1 DNA-binding activity was accompanied by transcriptional activity. The lack of HSP70i protein in HSF1−/− cells supports the notion that HSP70i is strictly dependent on HSF1 (17, 22). We also noted that HSP70i protein was induced upon T cell activation at 37°C and more strongly at 39.5°C, in an HSF1-dependent manner (22). These results indirectly suggest that HSF1 is capable of transactivating target genes following T cell activation in the absence of thermal stress.

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FIGURE 1. Venn diagram representing the probe sets that showed differential regulation between HSF1−/− and HSF1+/+ mice. Probe sets that had a p value of ≤0.01 and fold change of log2 0.5 in at least one of the two conditions (37°C or 40°C) were chosen for representation. The p values were calculated using two-way ANOVA of mean intensities of probe sets among the treatments. Up arrows indicate genes expressed at higher levels in HSF1−/− T cells compared with HSF1+/+ T cells, and therefore negatively regulated by HSF1. Down arrows indicate genes expressed at lower levels in HSF1−/− T cells compared with HSF1+/+ T cells, and therefore positively regulated by HSF1.
HSF1. As expected, expression of several of the HSPs was significantly lower in activated HSF1−/− T cells at febrile temperatures, indicating positive regulation. Interestingly, all of these HSPs were also positively regulated by HSF1 at 37˚C. Of the 323 genes regulated by HSF1, the Hspa1a and Hspa1b genes, encoding the inducible HSP70 proteins, showed greatest reduction in expression in the HSF1−/− T cells compared with HSF+/+, indicating strong positive regulation.

Many of the genes regulated by HSF1 in T cells fit the proteostasis signature common to a wide range of organisms (Table I) (12). These include genes involved in the heat shock response, ubiquitin pathway, and the unfolded protein response. Thus, activation of T cells through TCR ligation activates a proteostasis network through the action of HSF1.

### Activation of lymphocytes resulted in the activation of HSF1 to the DNA-binding form in the absence of apparent cellular stress

HSF1 is constitutively expressed in an inactive form within the nucleus of virtually all cell types. Upon sensing stress, HSF1 is rapidly converted to the DNA-binding form and, when properly phosphorylated, regulates gene expression. This conversion can be readily detected by EMSA using labeled HSE oligonucleotides (38). We have previously demonstrated that resting T cells had no detectable HSF1 DNA-binding activity at 37˚C, but they were very sensitive to HSF1 activation, only requiring a temperature increase of 2.5˚ to 39.5˚C (36). In our microarray analysis, the T cells were activated with anti-CD3 for 5 h, and in that case, we observed that most HSF1-dependent genes were regulated at 37˚C. These results suggest that T cell activation alone may result in the conversion of HSF1 to the DNA-binding form.

To address this question, T cells were activated by a variety of stimuli and whole-cell native protein extracts from these cells were subjected to EMSA analysis using a [32P]-labeled HSE oligonucleotide probe. There was no visible retarded band in resting T cells, but a prominent retarded band was present after 20 h of anti-CD3 activation at 37˚C or 40˚C (Fig. 2A). The prominent retarded band was largely absent in anti-CD3, 37˚C activated T cells from HSF1−/− mice, indicating that the retarded species was specific for HSF1. Some weak residual binding was observed in activated HSF1−/− T cells that is likely due to weak binding of the other constitutive HSF species to the HSE probe (39, 40). To determine whether Ag-specific T cell activation would also activate the DNA-binding activity of HSF1, spleen cells from DO11.10 TCR transgenic mice were activated with OVA323–339 for 20 h. As we saw in anti-CD3-stimulated T cells, a prominent retarded band corresponding to HSF1 was present in OVA-activated spleen cells at both 37˚C and 40˚C (Fig. 2B). SEB stimulation of HSF1−/− spleen cells also resulted in a retarded band that was absent in HSF1−/− spleen cells. Heat-shocked Jurkat cells, widely used as a control for HSF1 activation, produced a retarded band migrating to the same position. Thus, T cell activation through the TCR results in prominent HSF1 activation to the transcriptionally active DNA-binding form in the apparent absence of external stress. Nevertheless, in both cases, more HSF1 binding was present at 40˚C, indicating a synergy between cellular activation signals and mild temperature stress for the activation of HSF1.

The previous experiments showed that HSF1 was activated after 20 h of T cell activation. To determine how rapidly HSF1 was activated following T cell activation, DO11.10 T cells were activated with OVA323–339 for 2, 4, or 6 h and HSF1 DNA binding was evaluated. Within 4 h of T cell activation, prominent HSF1 DNA-binding activity was present and was not further increased at 6 h (Fig. 2C). Thus, we conclude that activation of HSF1 DNA binding occurs rapidly, between 2 and 4 h, following T cell activation. To determine whether early TCR signaling events were required for HSF1 activation, we activated purified T cells with PMA and ionomycin. This mitogen provides full T cell activation signals by directly activating protein kinase C and opening calcium channels, thus bypassing proximal TCR signaling events. Again, prominent HSF1 DNA-binding activity was induced within 5 h, indicating that cellular activation events downstream of protein kinase C are involved in activation of HSF1 (Fig. 2D).

Because HSF1 was activated rapidly in T cells through general mitogenic signals, and given the known importance of HSF1 in cellular proliferation, we reasoned that HSF1 would be activated in other lymphoid cell types receiving proliferative signals. To test

### Table I. Proteostasis signature genes regulated by HSF1 in T cells

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<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Log2 (KO37/WT37)</th>
<th>p Value</th>
<th>Log2 (KO40/WT40)</th>
<th>p Value</th>
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<td>AHA1, activator of heat shock 90-kDa protein</td>
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</tr>
<tr>
<td>Heat shock protein 1, θ</td>
<td>Hspa1b</td>
<td>-0.54</td>
<td>0.025</td>
<td>-0.68</td>
<td>0.009</td>
</tr>
</tbody>
</table>
Thus, HSF1 activation is associated with proliferative stimuli in both T and B lymphocytes in the absence of thermal stress.

**HSF1 is required for optimal proliferation of T cells in vivo at nonfebrile temperatures**

In our previous studies, we demonstrated that proliferation of purified T cells from HSF1<sup>−/−</sup> mice was severely affected upon activation with plate-bound anti-CD3 in vitro under a stress paradigm that did not affect wild-type T cells (40°C and low CO<sub>2</sub> concentration, or [CO<sub>2</sub>]). Nevertheless, comparable proliferation was observed in both HSF1<sup>+/+</sup> and HSF1<sup>−/−</sup> T cells at 37°C (22).

An experiment reproducing these results is shown in Supplemental Fig. 1A. Therefore, it was important to understand how loss of HSF1 affected T cell proliferation under more physiologic conditions in vivo. An ideal model system would be to create HSF1<sup>−/−</sup>, TCR transgenic mice. However, we and others have found that viable homozygous HSF1<sup>−/−</sup> pups cannot be obtained after backcrossing to either BALB/c or C57BL/6 mice, and the line must be maintained on a mixed BALB/c and 129 background (17), precluding the use of adoptive transfer studies. To evaluate in vivo T cell proliferation in this mixed background strain, we used an SEB-mediated T cell activation model. SEB is a superantigen that cross-links MHC class II molecules on the APCs to V<sub>β</sub>-chains of the TCR and results in T cell activation and proliferation of all T cells expressing appropriate V<sub>β</sub>-chains in an Ag-independent manner (41). SEB was injected i.v. into HSF1<sup>+/+</sup> or HSF1<sup>−/−</sup> mice followed by an i.p. injection of BrdU to label dividing cells.

We monitored the division of V<sub>β8</sub>-bearing T cells, which represents the major V<sub>β</sub> population of T cells in SEB mice, by flow cytometry after staining with anti-V<sub>β8</sub> and anti-BrdU Abs. Administration of SEB to the HSF1<sup>−/−</sup> mice induced a significant expansion of V<sub>β8</sub> T cells in vivo during the 48-h period (Fig. 3A). Additionally, these mice showed significant enlargement of spleen and lymph nodes (visual observation and data not shown) compared with control mice after SEB injection. In contrast, HSF1<sup>+/+</sup> mice had similar lymph node enlargement, but the spleens showed much less enlargement and remained significantly smaller compared with HSF1<sup>−/−</sup> spleens, which was reflected in total cell counts (Supplemental Fig. 1B). Analysis of the proliferating V<sub>β8</sub> T cells in the HSF1<sup>−/−</sup> mice confirmed the visual observation showing that SEB-induced expansion was significantly impaired in the spleen, but not lymph nodes, of HSF1<sup>−/−</sup> mice. We observed a marked reduction in the percentages (Fig. 3A) and total number of V<sub>β8</sub><sup>+</sup> and V<sub>β8</sub><sup>+</sup>BrdU<sup>+</sup> cells in spleen but not in lymph nodes (Fig. 3B, 3C). Thus, HSF1<sup>−/−</sup> T cells show an impaired proliferation in response to SEB in spleen but not in lymph nodes.

In our previous in vitro studies and as shown in Supplemental Fig. 1A, HSF1<sup>−/−</sup> cells stimulated with anti-CD3 or anti-CD3/CD28 showed impaired proliferation only at 39°C (22). Thus, we hypothesized that the impaired T cell proliferation observed in vivo in response to SEB was a consequence of an increase in body temperature due to the effects of SEB. Using implanted temperature transponders, we directly tested whether SEB administration would significantly raise mouse body temperature. To our surprise, we did not see any significant change in core body temperature of either wild-type or HSF1<sup>−/−</sup> mice injected with SEB during a 48-h period (Supplemental Fig. 2). In contrast, we readily detected fever range temperatures in mice infected with *Listeria* (18). Thus, the impaired proliferation of V<sub>β8</sub> T cells in HSF1<sup>−/−</sup> mice occurred under nonfebrile temperatures.

These data suggested that unlike in vitro anti-CD3 stimulation, in vivo SEB stimulation imparted a sufficient level of stress to impair the proliferation of HSF1<sup>−/−</sup> T cells at normal temperatures within the spleen microenvironment, but not in lymph nodes. To
determine whether HSF1−/− T cells were impaired in vitro in response to SEB, we activated spleen and lymph node cells separately with SEB at indicated concentrations and measured the Vβ8+ T cell proliferation using CFSE dilution and thymidine incorporation assays. Both spleen and lymph node cells were impaired in proliferation when stimulated with SEB at 37°C and to a greater extent at 40°C (Fig. 4A, 4B, Supplemental Fig. 1C). Thus, SEB stimulation of T cells in vitro revealed a defect in proliferation of HSF1−/− cells that anti-CD3 stimulation did not. This suggested the possibility that SEB stimulation resulted in a heightened level of stress compared with anti-CD3 stimulation sufficient to impair proliferation of the HSF1−/− T cells. To test this, spleen cells from HSF1+/+ or HSF1−/− mice were labeled with CFSE, split in half, and stimulated with either anti-CD3 or SEB. The proliferation of the Vβ8+ T cells in the culture was then evaluated by flow cytometric analysis of CFSE dilution in the live Vβ8+ T cell population. Consistent with our previous report and the data shown in Supplemental Fig. 1A, the HSF1−/− cells showed no impairment in proliferation when stimulated with anti-CD3/anti-CD28. In contrast, only the HSF1+/+ cells showed robust proliferation in response to SEB, revealing a striking qualitative difference between SEB stimulation and anti-CD3 stimulation of T cells in vitro (Fig. 4C). Thus, proliferation of HSF1−/− T cells in response to SEB is impaired both in vitro and in vivo. However, in vivo, this impairment was only observed in the spleen, but not in the lymph node microenvironment.

The defective proliferation of HSF1−/− T cells in response to SEB stimulus is T cell–intrinsic

When anti-CD3 was used as a stimulus, we were able to use purified T cells and demonstrate that the impaired proliferation of HSF1−/− T cells under stressful conditions is T cell–intrinsic (22). In contrast, when SEB was used as a stimulus, cultures necessarily included APCs. Thus, it is possible that the impaired proliferation was due to lack of HSF1 in the APCs and not to a T cell defect.

To determine whether APCs play a role contributing to the defective proliferation of HSF1−/− T cells in response to SEB, we labeled purified T cells with CFSE and stimulated them with SEB in a coculture system containing APCs from HSF1+/+ or HSF1−/− mice added at equal ratios to the T cell cultures. Cells were harvested 3 d after activation and CFSE dilution profiles for CD4+, Vβ8+ T cells were analyzed by flow cytometry. As shown previously, HSF1+/+ T cells proliferated vigorously to SEB at both 37°C and 40°C, and this proliferation was the same whether the APCs were from HSF1+/+ or HSF1−/− mice (Fig. 5A). Also as expected, HSF1−/− T cells were impaired in their response to SEB, and this impairment was apparent whether the APCs were from HSF1+/+ or HSF1−/− mice. There was a modest enhancement of proliferation in HSF1−/− T cells in the presence of HSF1+/+ APCs (heterogeneous coculture) compared with HSF1−/− APCs (homogeneous coculture). However, this difference was small compared with the proliferation of HSF1+/+ T cells, and it was not apparent at 40°C. Thus, the impaired proliferation in response to SEB is due to a lack of HSF1 in the T cell and not due to changes in APC function.

Because T cell proliferation depends, in part, on autocrine growth factors such as IL-2 and IL-15, and can be negatively impacted by inhibitory factors such as IL-10, we investigated whether soluble factors released as a result of APC/T cell interactions during SEB stimulation affected the proliferation of HSF1−/− T cells. To test this, we used a transwell system where spleen cells from HSF1+/+ and HSF1−/− mice were cultured in the same well but separated by a permeable membrane. CFSE-labeled spleen cells from HSF1+/+ or HSF1−/− mice were added to bottom wells of a 24-well plate and unlabeled spleen cells from HSF1+/+ or HSF1−/− mice, respectively, were added to the transmembrane inserts on the top to make homogeneous or heterogeneous transwell cultures. The cultures were then stimulated with SEB for 72 h at 37°C or 40°C. Following the activation, cells in the bottom well were harvested and CFSE profiles of CD4+, Vβ8+ gated T cells were analyzed by flow cytometry. HSF1−/− T cells proliferated poorly compared with HSF1+/+ T cells, and this proliferation was not rescued by soluble factors from HSF1+/+ cells (Fig. 5B).

Similarly, the normal proliferation of HSF1+/+ T cells was not inhibited by soluble factors from HSF1−/− cells. Thus, the proliferation defect in HSF1−/− T cells is not simply due to a lack of autocrine growth factors such as IL-2, nor to an overproduction of an inhibitory factor such as IL-10. Collectively, the above studies indicate that the impaired proliferation of HSF1−/− T cells in response to SEB is T cell–intrinsic, resulting in heightened sensitivity to SEB-induced stress.

HSF1−/− T cells show dysregulated cyclin expression when activated with anti-CD3 under stressful conditions

We have demonstrated that HSF1−/− T cells have a defect in cell cycle progression under two different experimental conditions,
both of which create an apparent stress on cells. In the case of anti-CD3 stimulation, the block in proliferation was apparent at elevated temperatures and low [CO₂]. In the case of SEB stimulation, the block in proliferation occurred at 37°C. Thus, it is likely that the underlying mechanism is similar: in the absence of HSF1, T cell proliferation is hypersensitive to proteotoxic stress. Collectively, our data in this study and from previous studies (22) point to an intrinsic block in T cell cycle progression. To further understand the mechanism of this block in cell cycle, we stimulated purified T cells with anti-CD3 under benign (37°C) or stressful (40°C and 2% [CO₂]) conditions and visualized the appearance of key cell cycle–related proteins by Western blot. We chose these conditions because anti-CD3 stimulation allows the use of purified T cells and stimulates most T cells in the culture. Furthermore, the stress paradigm used (40°C, 2% [CO₂]) induces a profound block in HSF1−/− T cell proliferation but does not impair HSF1+/+ T cell proliferation.

T cell proliferation is initiated by membrane-proximal signaling events, which ultimately trigger a MAPK cascade, NFAT, and NF-kB, which, in turn, activate transcription of a variety of genes required for cellular activation and proliferation. Entry into the cell cycle is initiated by expression of cyclin D2. Immunoblot analysis revealed no marked changes in the expression of cyclin D2 in the lysates from HSF1+/+ or HSF1−/− T cells activated with anti-CD3 at either stressful or nonstressful conditions (Fig. 6A). This confirmed our previous conclusion that early activation events were not affected in HSF1−/− T cells (22). In contrast, whereas the expression of cyclin E1 and cyclin A2 was comparable between lysates from HSF1−/− and HSF1+/+ T cells at nonstressful conditions, the expression of these cyclins was significantly reduced in HSF1−/− T cells activated under stressful conditions (Fig. 6A). Additionally, we also observed increased levels of p27 protein in lysates from HSF1−/− T cells activated at stressful conditions, whereas no p27 was observed in these conditions in the lysates from HSF1+/+ T cells.

To determine whether the lack of cyclin E and A in HSF1−/− cells was reflected at the transcriptional level, we carried out semiquantitative PCR analysis of cyclins in activated T cells. The mRNA expression analysis revealed a 43% reduction in the levels of cyclin A2 mRNA and a 25% reduction in the levels of cyclin E1 mRNA in HSF1−/− T cells compared with HSF1+/+ T cells activated under stressful conditions (Fig. 6B). This reduction in mRNA levels appears to be much less than the almost complete elimination of protein seen in Fig. 6A. These results suggest that the reduction of cyclins E and A in stressed HSF1−/− cells may be due to a combined reduction in gene transcription as well as a decrease in protein stability.

**Loss of HSF1 also affected the proliferation of activated B cells and bone marrow hematopoietic cells activated under stressful conditions**

Our results suggest that the proliferation block in HSF1−/− T cells activated under stressful conditions is due to reduced expression of important cell cycle proteins. Because this pathway is common to all proliferating cells, we postulated that other cell types would show the same blockage in proliferation under similar stress. Hence, we evaluated proliferation of B cells and differentiating bone marrow monocytic cells derived from HSF1+/+ or HSF1−/− mice. Purified resting B cells from HSF1+/+ or HSF1−/− mice were activated with either LPS or anti-IgM and anti-CD40 and monitored for proliferation after incubation at either nonstressful or stressful conditions (Fig. 7A). As we observed for T cells, there was significant impairment in B cell proliferation in the HSF1−/− B cells compared with HSF1+/+ B cells when activated with either LPS or anti-IgM and anti-CD40 under stressful conditions. These results were also consistent with our previous gel-shift analysis (Fig. 2), which showed activation of HSF1 DNA-binding activity in B cells upon stimulation with LPS. We also cultured CFSE-labeled bone marrow cells isolated from HSF1−/− or HSF1+/+ mice in the presence of GM-CSF and monitored the proliferation...
Purified T cells from HSF1+/+ or HSF1-/- mice were CFSE labeled and mixed with unlabeled APCs (flow-through fraction obtained during T cell purification) from HSF1+/+ or HSF1-/- spleens at a 1:1 ratio and activated with 20 μg/ml SEB in 24-well plates for 3 d at the indicated temperatures. Cells were then labeled with anti-Vβ8, and anti-CD3 and the CFSE fluorescence of the Vβ8+, CD3+ T cells was analyzed. Histograms represent CFSE profiles of control, unstimulated (top row), or SEB-stimulated T cells in homogeneous cocultures (HSF1+/+ T cells and HSF1-/- APCs or HSF1-/- T cells and HSF1+/+ APCs) (center row) or heterogeneous cocultures (HSF1+/+ T cells and HSF1+/+ APCs or HSF1-/- T cells and HSF1+/+ APCs) (bottom row). (B) Spleen cells from HSF1+/+ or HSF1-/- mice were CFSE labeled and activated with SEB in 24-well transwell plates for 3 d at indicated temperatures. Histograms represent CFSE profiles of control, unstimulated (top row) or SEB-stimulated homogeneous transwell cultures (HSF1+/+ or HSF1+/+ spleen cells in both top and bottom wells) (center row) or heterogeneous transwell cultures (HSF1+/+ spleen cells in the bottom wells and HSF1-/- cells in the top wells and vice versa) (bottom row). After 3 d, cells were harvested from the bottom well of each transwell, washed, and labeled with PE-conjugated anti-Vβ8 and allophycocyanin-conjugated anti-CD3, and the CFSE fluorescence of the Vβ8+CD3+ T cells was analyzed. Numbers in each histogram represent the percentage of total gated live cells (average of three replicates) that have undergone cell division (M1 gate, left line). Histograms are representative of data from three repeated experiments with three replicates in each treatment, and cells obtained from two mice per strain were pooled in each experiment.

**FIGURE 5.** Defective proliferation of HSF1−/− T cells in response to SEB stimulation is not due to a defect in APC interactions or influenced by stimulatory or inhibitory factors released from APC/T cell interactions. (A) Purified T cells from HSF1+/+ or HSF1−/− mice were CFSE labeled and mixed with unlabeled APCs (flow-through fraction obtained during T cell purification) from HSF1+/+ or HSF1−/− spleens at a 1:1 ratio and activated with 20 μg/ml SEB in 24-well plates for 3 d at the indicated temperatures. Cells were then labeled with anti-Vβ8, and anti-CD3 and the CFSE fluorescence of the Vβ8+, CD3+ T cells was analyzed. Histograms represent CFSE profiles of control, unstimulated (top row), or SEB-stimulated T cells in homogeneous cocultures (HSF1+/+ or HSF1−/− T cells and HSF1+/+ APCs or HSF1−/− T cells and HSF1+/+ APCs) (center row) or heterogeneous cocultures (HSF1+/+ T cells and HSF1+/+ APCs or HSF1−/− T cells and HSF1+/+ APCs) (bottom row). (B) Spleen cells from HSF1+/+ or HSF1−/− mice were CFSE labeled and activated with SEB in 24-well transwell plates for 3 d at indicated temperatures. Histograms represent CFSE profiles of control, unstimulated (top row) or SEB-stimulated homogeneous transwell cultures (HSF1+/+ or HSF1+/+ spleen cells in both top and bottom wells) (center row) or heterogeneous transwell cultures (HSF1+/+ spleen cells in the bottom wells and HSF1−/− cells in the top wells and vice versa) (bottom row). After 3 d, cells were harvested from the bottom well of each transwell, washed, and labeled with PE-conjugated anti-Vβ8 and allophycocyanin-conjugated anti-CD3, and the CFSE fluorescence of the Vβ8+CD3+ T cells was analyzed. Numbers in each histogram represent the percentage of total gated live cells (average of three replicates) that have undergone cell division (M1 gate, left line). Histograms are representative of data from three repeated experiments with three replicates in each treatment, and cells obtained from two mice per strain were pooled in each experiment.
study of the mechanism of HSF1 activation. This is possible because lymphocytes can be isolated as resting, nonproliferating cells, largely in the G0 stage of the cell cycle. At this stage, activated HSF1 and inducible HSP70 are almost undetectable (22, 36). Within 4 h of activation with a mitogenic signal, HSF1 DNA-binding and transcriptional activity are induced. Thus, in this model of HSF1 activation, there is no obvious stressor. However, when one considers the enormous cellular changes that must occur when a small resting lymphocyte is activated, then perhaps it is not surprising that HSF1 senses these changes as stress and becomes activated. Possibilities include changes in intracellular calcium, increases in mitochondrial activity, including the electron transport chain and ROS, increased gene transcription, and protein synthesis.

What are the consequences of HSF1 activation in T cells? In this study, we used whole genome mouse microarrays to investigate the role of HSF1 in the regulation of gene expression in activated T cells at normal and febrile temperatures. We identified a diverse set of 323 genes differentially regulated between activated T cells from HSF1+/− and HSF1−/− mice, either at normal or febrile temperatures. About 143 genes in the group showed significant differences between HSF1+/− and HSF1−/− cells both at normal and febrile temperatures, clearly indicating that these genes are influenced by HSF1-mediated gene regulation. The identification of so many genes regulated by HSF1 at 37°C in activated T cells is noteworthy because it identifies a gene program regulated by HSF1 independent of heat shock. Several of these genes are either already known to be directly or indirectly regulated by HSF1, or are known to be differentially regulated from previous microarray studies characterizing HSF1-mediated gene regulation under various conditions (23, 58–60). The inducible form of HSP70 is the most extensively studied HSF1-regulated gene, probably because it shows the greatest fold induction upon HSF1 activation and appears to be completely dependent on HSF1 for its expression (22, 61). Consistent with this, the hsp70a and hsp70b genes (the two inducible forms of HSP70) showed the largest fold difference in expression between HSF1+/− and HSF1−/− mice in our microarray analysis. This is in agreement with several reports showing that inducible HSP70 is almost exclusively regulated by HSF1 (22, 61). This result also validates the accuracy of the microarray analysis in identifying HSF1-regulated genes in T cells. Other HSPs and co-chaperones regulated by HSF1 in our microarray are known to be regulated by HSF1 in other cells (58–60). These include genes encoding HSP110, HSP90α, HSP90β, HSP40, and HSP10. Our microarray analysis identified an HSP70...
co-chaperone, Bag3, as being positively regulated by HSF1 in T cells. Bag3 is known to be regulated by HSF1 in response to heat shock and plays a role in protecting cells from apoptosis through its interaction with HSP70 (23, 62–65). Also in agreement with previous studies (59), our microarray studies showed positive regulation of genes encoding anti-apoptotic proteins such as clusterin, and caspase 6 and negative regulation of proapoptotic genes, including BCL2l11, Card6, Card11, and Traf1, by HSF1 in activated T cells. Several reports show that HSF1 plays a role in inflammation and the immune response through transcriptional regulation of genes involved in the immune response (6, 17, 32). Consistent with these data, expression of genes involved in the NF-κB pathway, including IKK2, NF-κB2, and NF-κB6, was higher in HSFF12−/− T cells, indicating negative regulation by HSF1 of such proinflammatory genes. Likewise, the proinflammatory cytokine IL-16 showed negative regulation by HSF1, whereas the Th2 cytokines IL-4 and IL-13 displayed positive regulation. Additionally, we found a large number of genes regulated by HSF1 that are involved in signal transduction, metabolism, transcription, and translation (Table I). Finally, many of the genes regulated by HSF1 in T cells fit a “proteostasis signature” of genes regulated in organism as diverse as Saccharomyces cerevisiae, C. elegans, Drosophila, and mammals (42).

We previously demonstrated that T cells from HSFF1−/− mice are able to proliferate normally under benign in vitro conditions of anti-CD3 stimulation at 37°C. However, when the temperature is increased to 40°C, proliferation of HSFF1−/− T cells is markedly reduced. The [CO2] in the incubator also has a modifying effect on the proliferation block at 40°C, with lower concentrations in the 2% range resulting in severe inhibition of T cell proliferation, and elevated [CO2] showing a protective effect. This CO2 effect is at least partly related to changes in intracellular ROS levels (22). These modified conditions have no deleterious effect on proliferation of T cells from HSFF1−/+ mice (22). Because HSFF1−/− mice must be maintained on a mixed genetic background to prevent embryonic lethality, we were unable to perform adoptive transfer experiments to assess T cell proliferation in vivo. Instead, we monitored Vβ8 T cell proliferation in response to SEB in individual mice. HSFF1−/− mice consistently showed reduced Vβ8 T cell proliferation in the spleen. Interestingly, there was no impairment of proliferation in lymph nodes. Because these mice were not febrile during the time frame of the experiment, it suggests that the stress resulting in the impaired proliferation of HSFF1−/− cells is derived from events other than hyperthermia. This was confirmed in in vitro experiments showing decreased proliferation of HSFF1−/− T cells in response to SEB. Both spleen and lymph node T cells from HSFF1−/− mice showed impaired proliferation in response to SEB in vitro, indicating that the normal proliferation of lymph node cells in vivo must be related to differences between the lymph node and spleen microenvironment. A particularly revealing experiment compared the expansion of the same population of Vβ8 T cells from HSFF1−/− mice stimulated by anti-CD3 with those stimulated with SEB. The fact that only SEB stimulation resulted in impaired proliferation indicates a fundamental qualitative difference between anti-CD3 and SEB stimulation, with the latter producing a sufficient level of stress on the T cell as to require HSF1 for normal proliferation.

We have previously shown that the HSF1 requirement for anti-CD3–stimulated proliferation under stress is apparent in purified T cells, indicating that the effect is T cell–intrinsic, but manifested only in the face of extrinsic stress. Because SEB-stimulated proliferation requires cross-linking MHC class II on APCs with the Vβ region of the TCR, APCs were necessarily included in these cultures. However, cross-mixing experiments between T cells and APCs from either HSFF1−/− or HSFF1−/+ mice reveal that the impaired proliferation in response to SEB is independent of the genotype of the APCs and therefore is also T cell–intrinsic. Furthermore, in transwell experiments, our data show that the impaired proliferation is not simply due to the lack of a required cytokine such as IL-2 or to the overproduction of an inhibitory cytokine such as IL-10. Collectively, these data point to stressful conditions present during SEB stimulation that, in the absence of HSF1, result in impaired T cell proliferation.

Although further studies are required to determine qualitative differences in the T cell immune responses after activation with SEB and plate-bound anti-CD3, or anti-CD3/CD28, there are several obvious differences between anti-CD3 T cell activation and SEB-mediated T cell activation that can explain the apparent increased stress level. Activation of T cells with plate-bound anti-CD3 cross-links CD3 receptors and is capable of inducing T cell activation in the absence of APCs. In contrast, activation of T cells with SEB involves cross-linking of MHC class II on APCs and TCR on T cells and also involves additional secondary signaling molecules, including CD4, CD8, and CD28 (66, 67). Thus, in addition to T cell signaling, SEB-induced T cell/APC cross-linking activates signaling pathways in MHC class II–expressing cells (68) including B cells, dendritic cells (69, 70), and macrophages (71, 72). Additionally, in vivo administration of SEB induces maturation and migration of dendritic cells in spleen (69, 70). Recently, SEB has been shown to engage CD28 in addition to MHC class II and the TCR, inducing high levels of IL-2, IFN-γ, and TNF-α (67). Because of this, SEB induces a more complete T cell response, more closely mimicking an Ag-specific response, complete with a wide range of proinflammatory cytokines (67, 73–75). Proinflammatory cytokines such as TNF-α, IL-1, and IFN-γ are known to induce NO, causing oxidative stress in a variety of cell types (76, 77). Thus, it may be that these cytokines are the source of stress resulting in impaired proliferation of SEB-stimulated HSFF1−/− T cells. Future experiments adding purified cytokines to anti-CD3–stimulated HSFF1−/− cells will address this question.

We have previously demonstrated that the impairment of T cell proliferation in HSFF1−/− T cells under stress is due to an intrinsic block in the cell cycle, localized to the G1–S phase transition (22). In this study we extend these findings by showing that stressed HSFF1−/− T cells entered the cell cycle normally, as revealed by normal expression of cyclin D2 protein. However, there was a dramatic reduction of cyclins E and A within HSFF1−/− T cells only under conditions of stress. Thus, it is likely that this disappearance of cyclins E and A is the primary reason for the block in cell cycle progression observed in stressed HSFF1−/− T cells. Transcriptional assays revealed some reduction of cyclin D and cyclin A mRNA in HSFF1−/− T cells. Transcriptional mechanisms may be responsible for the reduced cyclin E and A protein is due to a combination of transcriptional and posttranscriptional mechanisms.

The cell cycle has emerged as a process that is particularly sensitive to proteotoxic stress. The cell cycle progresses through the precise sequential orchestration of cyclins and kinases, many of which are regulated at the level of protein stability. HSF1 is known to be important in cell cycle progression in a number of systems, including yeast, Drosophila, and mammalian cells. Yeast cells carrying an HSF-null mutation show significant defects in cell cycle progression through the G2/M phase mainly due to the loss of HSP90-mediatedcdc2 protein stabilization (78). Likewise, the Hsps70 family member Hspc73 (Hspa8; heat shock cognate protein) directly interacts with p27Kip protein, suggesting a possible role of cognate HSPs in targeting these proteins to ubiquitin-
mediated protein degradation (79). p27 is an important cell cycle inhibitory protein controlling the G1/S phase transition. In agreement with these studies, our studies also showed inefficient significant downregulation of HSc73 gene expression in HSF1−/− T cells. Thus, it is possible that dysregulated p27Kip degradation observed in HSF1−/− T cells could partially affect the kinase activity of CDK4/cyclin D and CDK2/cyclin E complexes, thereby eventually affecting the transition through the G1 phase. Although the mechanism of HSF1 protection of cyclin E and A levels in the face of stress is unclear at present, our results indicate that these proteins are acutely sensitive to proteotoxic stress and that the HSF1-regulated proteostasis network is critical for maintaining normal levels of these cyclins during the cell cycle.

The defective proliferation in HSF1−/− cells provides a window on stressful situations that ordinarily are not apparent using HSF1−/− cells. Thus, this phenotype revealed a level of stress associated with SEB stimulation that was not apparent in anti-CD3 stimulation. SEB stimulation is more reflective of a “complete” immune response involving APC/T cell interaction and CD3 stimulation. SEB stimulation is more reflective of a “complete” immune response involving APC/T cell interaction and antibodies, as well as fever. Although our work identified cytokines, ROS, NO, as well as fever. Stressors could in-vivo. Stressors could in-vivo.


