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Involvement of Heat Shock Protein A4/Apg-2 in Refractory Inflammatory Bowel Disease

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Background: Expression of heat shock protein A4 (HSPA4, also called Apg-2), a member of the HSP110 family, is induced by several forms of stress. The physiological and pathological functions of HSPA4 in the intestine remain to be elucidated.

Methods: We assessed HSPA4 expression and function by generating HSPA4-deficient mice and using 214 human intestinal mucosa samples from patients with inflammatory bowel disease (IBD).

Results: In the colonic mucosa of patients with IBD, a significant correlation was observed between the expression of HSPA4 and antiapoptotic protein Bcl-2, a T-cell–derived cytokine IL-17 or stem cell markers, such as Sox2. In refractory ulcerative colitis, a condition associated with increased cancer risk, expression of HSPA4 and Bcl-2 was increased in inflammatory cells of colonic mucosae. HSPA4 was overexpressed both in cancer cells and immune cells of human colorectal cancers. Patients with high expression of HSPA4 or Bmi1 showed significantly lower response rates upon subsequent steroid therapy as compared with patients with low expression of each gene. HSPA4-deficient mice exhibit more apoptosis and less expression of IL-17/IL-23 in inflammatory cells and less number of $Sox2^{+}$ cells after administration of dextran sodium sulfate than control mice. Transduction of $HspaA4^{+/-}$ bone marrow into wild-type mice reduced the immune response.

Conclusions: Upregulation of Bcl-2 and IL-17 by HSPA4 would control apoptosis of inflammatory cells and immune response in the gut, which might develop treatment resistance in IBD. HSPA4 and Bmi1 would be a useful biomarker for refractory clinical course and a promising approach for a therapeutic strategy in patients with IBD.

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Key Words: Bcl-2, Bmi1, Sox2, IL-17, treatment resistance

Inflammatory bowel disease (IBD) is a chronic disease characterized by intestinal inflammation. Both entities of IBD, Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a marked infiltration of inflammatory cells into the affected mucosa, but the pathogenesis of IBD remains poorly understood. The clinical features of IBD, its histological findings, and the efficacy of immunosuppressive treatment, however, indicate the involvement of immunological mechanisms, although the initial development of IBD may be triggered by bacterial, viral, environmental, or other factors. It has been suggested that the induction of apoptosis in various effector cells may be a relevant therapeutic mechanism in IBD. In fact, different drugs used for treating IBD, such as anti-TNF, have the capacity to induce apoptosis in T cells or monocytes in vitro and in vivo.[1] In contrast, defective apoptosis of inflammatory cell populations regulated by Bcl-2 seems to be a relevant pathogenetic mechanism in IBD.[2,3]

Stem cells, characterized by their ability to self-renew indefinitely and produce progeny capable of differentiating and repopulating tissue-specific lineages, are critical for maintaining normal tissue homeostasis.[4] The intestine is a highly dynamic organ, with proliferative epithelial cells invaginating into the mesenchyme to form flask-shaped crypts and differentiated cells projecting into the lumen. Sox2, a member of the Sox HMG box family of transcription factors, is required in the early stages of

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embryonic development to maintain the pluripotency and self-renewal of embryonic stem (ES) cells.[5] Sox2 controls cellular self-renewal through Bmi1, a polycomb complex transcriptional repressor that is known to be important for maintaining stemness.[6]

Heat shock protein A4 (HSPA4, also called Apg2 [ATP and peptide-binding protein in germ cells-2]), a member of the HSP110 family, is expressed in various organs[7] and is inducible under various conditions, including oncogenic stress.[8,9] In this study, we examined whether HSPA4 is involved in intestinal inflammation by using human tissue samples and HSPA4-deficient (*HspA4*$^{-/-}$) mice. HSPA4 expression was increased in inflammatory cells of refractory patients with IBD. Upregulation of Bcl-2 and IL-17 expression by HSPA4 would reduce apoptosis of inflammatory cells and augment immune response in the gut. Expression of HSPA4 was correlated with resistance to treatment in human IBD. HSPA4 might be involved in the pathogenesis of refractory IBD. To the best of our knowledge, this is the first report on HSPA4 as a possible regulator of intestinal homeostasis and treatment resistance in IBD.

PATIENTS AND METHODS

Human Tissue Sample

At the Hospital of Kinki University between January 2011 and October 2013, 214 samples from the intestinal mucosa of patients with IBD (29 with CD and 185 with UC) and 30 samples from the normal colonic mucosa of patients without IBD were obtained endoscopically. The subjects comprised 30 cases in remission and 155 patients with active UC including 66 refractory patients. Twenty-nine CD cases comprised 13 colitis, 8 ileocolitis, and 8 ileitis. Tissue samples were collected from involved areas of the intestine. Active inflammation was defined as a Mayo endoscopic score ≥2 in UC cases and a presence of ulcer in CD cases or a presence of symptoms. Refractory IBD patients were defined as those who had been suffering from active IBD for more than 6 months when biopsy samples were obtained. Regarding this, 66 colonic mucosal samples of refractory UC patients were not always obtained before the initiation of treatment. By contrast, among 185 samples of patients with UC, 23 colonic mucosal samples were obtained from patients with UC before the initiation of steroid therapy, and the response to steroid was evaluated prospectively. Colorectal cancer tissue samples were obtained from 32 patients undergoing colorectal resection, including 8 cases with IBD-associated cancer. Informed consent concerning the utilization of the tissue samples for the analysis was obtained from all the patients. All study protocols conformed to the ethical guidelines of the Declaration of Helsinki (1975) and were approved by the appropriate institutional review boards.

Generation of *HspA4*$^{-/-}$ Mice and Treatment

An 8-kb fragment of the *HspA4* gene including the translation initiation site was replaced with the targeting construct, which contained the PGK-Neo gene cassette flanked by loxP sites for positive selection. This construct additionally contained the Diphtheria toxin-A gene fragment (MC1-DTA) at the 3′-end to facilitate counter selection. The targeting construct was introduced by electroporation into E14 ES cells. G418-resistant colonies were screened by Southern blot hybridization. The ES cell clones containing the targeting event were injected into C57BL/6 blastocysts, and chimeras were derived. Chimeric male mice harboring heterozygous ES cells were crossed with C57BL/6 females to achieve germline transmission of the targeted *HspA4* allele. To genotype the mice, genomic DNAs were extracted from tails and analyzed by PCR. To discriminate between wild-type (WT) and mutated alleles, the following sets of primers were used: for wild-type loci, 5′-accttctgaggccagtttcctgt-3′ and 5′-taccagagctctgtggcaccaa-3′ and for targeted locus, 5′-ctcgtctgatcacgggaagtgag-3′ and 5′-ctgctaaagcgcatgctccaga-3′ (see Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A632). *HspA4*$^{-/-}$ mice were produced in collaboration with Dr. S. Itohara at the Riken Brain Science Institute (Wako, Japan) and deposited in the Laboratory Animal Resource Bank, National Institute of Biomedical Innovation (Osaka, Japan). Because of a decrease in expected Mendelian breeding ratios, we used *HspA4*$^{+/-}$ mice in some experiments.

Sex- and age-matched C57BL/6 WT and *HspA4*$^{-/-}$ mice (8–12 weeks old) received 2.5% (wt/vol) dextran sodium sulfate (DSS; molecular weight, 36–50 kDa; MP Biomedicals, Solon, OH) in their drinking water. After euthanization, the colon was excised from the ileocecal junction to the anus, cut open longitudinally, and prepared for histological evaluation. Isolation of epithelial and lamina propria cells was performed as described previously.[10] The isolated cells were sorted using immunomagnetic beads coated with monoclonal antibodies against CD4 and CD11b (MACS Beads; Miltenyi Biotec, Bergisch Gladbach, Germany) with the help of a separation column and a magnetic separator from the same company in accordance with the manufacturer's recommendations for isolating murine macrophages and T cells, respectively. Bone marrow (BM) transplantation experiments were performed as previously described, with slight modifications.[11] BM from the tibia and femur was washed twice in Hanks' balanced salt solution, and 10^7 BM cells were injected into the tail vein of lethally irradiated (11 Gy) recipient mice. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. The Medical Ethics Committee of Kinki University Faculty of Medicine approved this study.

Cell Culture

Mouse embryonic fibroblast cell lines of *HspA4*$^{-/-}$ genotype (Apg-2-KO/3T3) were established by the so-called "3T3 protocol."[12] To produce isogenic stable cell lines, the Flp-In System was used as described.[13] Apg-2-KO/3T3 cells were first transfected with pFRT/lacZeo to generate the Flp-In host cell lines, and HspA4 cDNA was cloned into the pcDNA5/FRT vector (Life

Technologies, Carlsbad, CA). Then the Flp-In host cell lines were cotransfected with pOG44 (Life Technologies) and the constructed plasmid or parent vector, and the stable transfectants were selected with hygromycin. Expression of HSPA4 was confirmed by Western blotting using anti-HSPA4 antibody (see Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A633). Jurkat cells, an immortalized line of human T lymphocytes, were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum, containing penicillin (100 U/mL) and streptomycin (100 mg/mL), and transfection of siRNA of HSPA4 (Santa Cruz, Dallas, TX) was carried out using Lipofectamine RNAiMAX reagent (Life Technologies). The number of viable cells was estimated by MTT assay.

Biochemical and Immunochemical Analyses

Real-time qPCR, immunoblotting, and immunohistochemistry were performed as previously described.[14] Primer sequences are given in Table, Supplemental Digital Content 3, http://links.lww.com/IBD/A634. The following antibodies were used: anti-β-actin, anti-HSPA4 (Sigma-Aldrich, Tokyo, Japan) and anti-Bcl-2, anti-Bcl-xL, anti-HSPA4, anti-Sox2, anti-E-cadherin (Cell Signaling, Danvers, MA). Immunohistochemistry was performed using ImmPRESS reagents (Vector Laboratory, Burlingame, CA) according to the manufacturer's recommendations. Apoptosis in paraffin-embedded sections was measured by immunofluorescent TUNEL staining using the In Situ Apoptosis Detection Kit (Takara, Tokyo, Japan) and a confocal laser microscope (Fluoview FV10i, Olympus) according to the manufacturer's instructions. Nuclei were stained with 4,6′diamidino-2-phenylindole (DAPI) to count the total number of cells per crypt. A minimum of 10 crypts with normal morphology were counted per section, and TUNEL-positive crypts were counted in 100 randomly selected crypts. In a double immunofluorescence staining using an anti–E-cadherin antibody, TUNEL-positive cells were counted in 5 randomly selected views. Caspase-8 and caspase-9 activity was measured using Caspase-8 and Capase-9 Assay kits (MBL, Woburn, MA).

Statistical Analysis

Differences were analyzed by Student's *t* test, and relationship between the expression of several genes was analyzed by Spearman rank correlation test. To compare variables of more than 2 conditions, analysis of variance with post hoc Tukey–Kramer honestly significant difference multiple comparison was applied. *P* values <0.05 were considered significant.

RESULTS

Significant Correlation Between the Expression of HSPA4 and Bcl-2 or Stem Cell Markers in the Colonic Mucosa of Patients with IBD

HSPA4 expression correlated significantly with Bcl-2 and Bmi1, showing a linear coefficient of 0.52 and 0.61, respectively in the colonic mucosa of patients with UC (Fig. 1A, B). Furthermore, HSPA4 expression correlated with several stem cell markers, such as Sox2 and Lgr5, with linear coefficients of 0.52 and 0.50, respectively (Fig. 1C, D). Gankyrin, an ankyrin-repeat oncoprotein, is known to increase stemness factor expression and mediate stem cell expansion in colorectal carcinogenesis.[15] We previously reported that cold-inducible RNA-binding protein (Cirp), a stress-response protein, exhibited antiapoptotic activity in mouse fibroblasts.[16] HSPA4 expression correlated significantly with gankyrin and Cirp in patients with UC (Fig. 1E, F). There was moderate, but significant, correlation between HSPA4 and Bcl-xL or cytokines, such as IL-17, IL-23, IL-6 and TNF-α (data not shown).

In the intestinal mucosa of CD, HSPA4 expression correlated significantly with Bcl-2, Sox2, Lgr5, and IL-17, showing a linear coefficient of 0.71, 0.73, 0.42, and 0.52, respectively (Fig. 2A–D). In the colonic mucosae of Crohn's

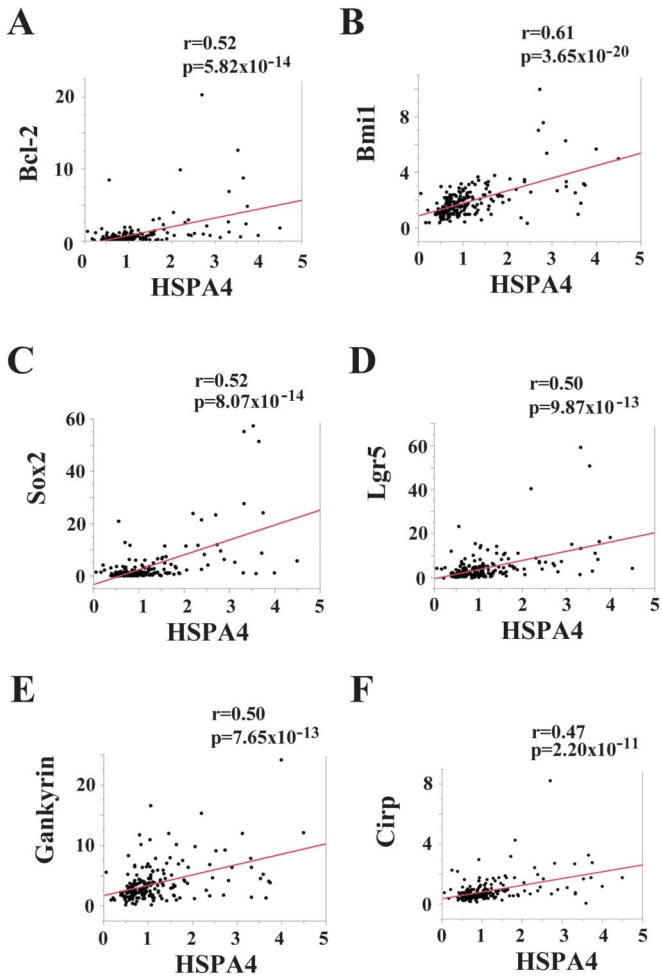

FIGURE 1. Association between HSPA4 and Bcl-2 or stem cell marker expression in the colonic mucosa of patients with UC. Scatter plot of relative mRNA levels of HSPA4 and the indicated genes (A, Bcl-2; B, Bmi1; C, Sox2; D, Lgr5; E, Gankyrin; F, Cold-inducible RNA-binding protein, Cirp) in human colonic mucosa. The mRNA level of each gene in the normal colon of an individual without UC was given an arbitrary value of 1.0.

colitis, a significant correlation was found between HSPA4 and Lgr5 or Bmi1 with a linear coefficient of 0.71 or 0.57, respectively (see Fig. A, Supplemental Digital Content 4, http://links.lww.com/IBD/A635 and data not shown). By contrast, in the ileal mucosa of patients with Crohn's ileocolitis and ileitis, no significant correlation was found between HSPA4 and Bmi1 (data not shown). The expression of Bmi1 in the ileal and the colonic mucosa might be regulated by a different mechanism. It remains to be determined why this correlation was not observed in the ileum and what factors are involved in the difference between the ileum and the colon. We have examined the expression levels of HSPA4, Bmi1, and Lgr5 in the ileal mucosa of controls, Crohn's ileitis and UC patients (see Fig. B, Supplemental Digital Content 4, http://links.lww.com/IBD/A635). In patients with Crohn's ileitis, expression levels of Lgr5 and Bmi1 were significantly reduced, whereas HSPA4 expression was increased compared with those in controls or patients with UC. Increased HSPA4 expression might affect the disease activity of CD with small intestinal involvement. Consistently, in refractory Crohn's ileitis, HSPA4 expression in the ileum was significantly higher than that in the ileum of controls (1.55 ± 0.44 versus 0.77 ± 0.12, $P = 0.033$).

Expression of HSPA4, Bcl-2, and Bmi1 Is Increased in the Intestinal Mucosa of Refractory IBD

Immunohistochemical analysis was performed to identify the cells expressing HSPA4 in human intestine. In the normal mucosa, epithelial cells but not inflammatory cells expressed HSPA4 protein, whereas in chronically inflamed mucosa, HSPA4 was expressed in inflammatory cells as well as in epithelial cells (Fig. 3A). Recent data have shown that the most powerful therapeutic approaches inhibit inflammatory cell survival by inducing apoptosis.[2,3] Correspondingly, in refractory UC, inflammatory cells express antiapoptotic Bcl-2 protein (Fig. 3A). Given the significant correlation between HSPA4 and Bcl-2 expression (Fig. 1), we next explored whether an association exists between HSPA4 expression and the clinical status of UC. HSPA4 expression levels were significantly higher in patients with refractory UC than in those with nonrefractory active UC or controls (Fig. 3B). In contrast, similar expression levels of HSPA4 were found in the colonic mucosa between patients without IBD and those with nonrefractory active UC (Fig. 3B). Increased Bcl-2 and Bmi1 expression levels were also found in the colonic mucosa of refractory UC patients compared with nonrefractory UC patients (Fig. 3B).

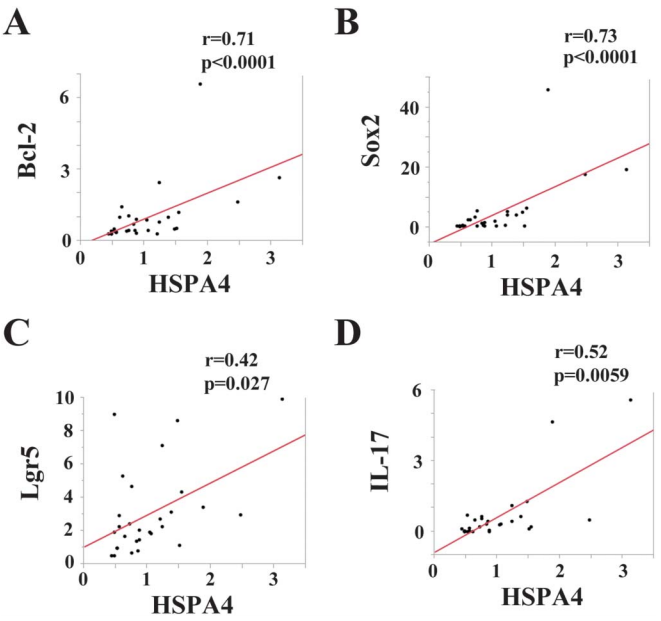

FIGURE 2. Association between HSPA4 and Bcl-2 or stem cell marker expression in the intestinal mucosa of patients with CD. Scatter plot of relative mRNA levels of HSPA4 and the indicated genes (A, Bcl-2; B, Sox2; C, Lgr5; D, IL-17) in human colonic mucosa. The mRNA level of each gene in the normal colon of an individual without UC was given an arbitrary value of 1.0.

Twenty-three colonic mucosal samples were obtained from patients with UC before the initiation of steroid therapy, and the response to steroid therapy was evaluated prospectively. Among the 23 patients with UC treated with steroid and evaluated prospectively, 8 turned out to be steroid-resistant. We then determined the correlation of the expression levels of HSPA4 and Bmi1 to the clinical outcome of steroid therapy. The expression levels of HSPA4 and Bmi1 in the colonic mucosa were 1.34 ± 0.30 and 2.65 ± 0.21 in patients without subsequent clinical response to steroid therapy, whereas we detected expression levels of 0.77 ± 0.10 and 1.57 ± 0.15 in patients with clinical response, respectively. Patients with high expression of HSPA4 or Bmi1 showed significantly lower response rates on steroid therapy as compared with patients with low expression of HSPA4 or Bmi1 (Fig. 3C). There was no significant difference in age, disease duration, serum C-reactive protein, hemoglobin, or endoscopic findings evaluated using Matts score between steroid-responders and nonresponders (see Table, Supplemental Digital Content 5, http://links.lww.com/IBD/A636). We also evaluated the expression level of HSPA4 and Bmi1 by a receiver operating characteristic analysis. The area under curve was 0.75 (95% confidence interval, 1.117–51.24) in HSPA4 expression and 0.88 (95% confidence interval 1.900–34.72) in Bmi1 expression. The receiver operating characteristic analysis identified a value ≥0.98, as an optimum cutoff point to differentiate between patients with high and low HSPA4 expression, with a sensitivity of 71.5% and a specificity of 75.0%, whereas the receiver operating characteristic analysis identified a value of ≥ 2.2, as an optimum cutoff point to differentiate between patients with high and low Bmi1 expression, with a sensitivity of 85.7% and a specificity of 87.5% for the prediction of response to steroid therapy (see Fig., Supplemental Digital Content 6, http://links.lww.com/IBD/A637).

Chronic inflammation often precedes or accompanies many types of cancers. In this study, HSPA4 was overexpressed in 29 of 32 colorectal cancer cases we examined. Moreover, HSPA4-specific signals were observed in colorectal cancer cells and inflammatory cells (Fig. 3D and see Fig., Supplemental Digital Content 7, http://links.lww.com/IBD/A638).

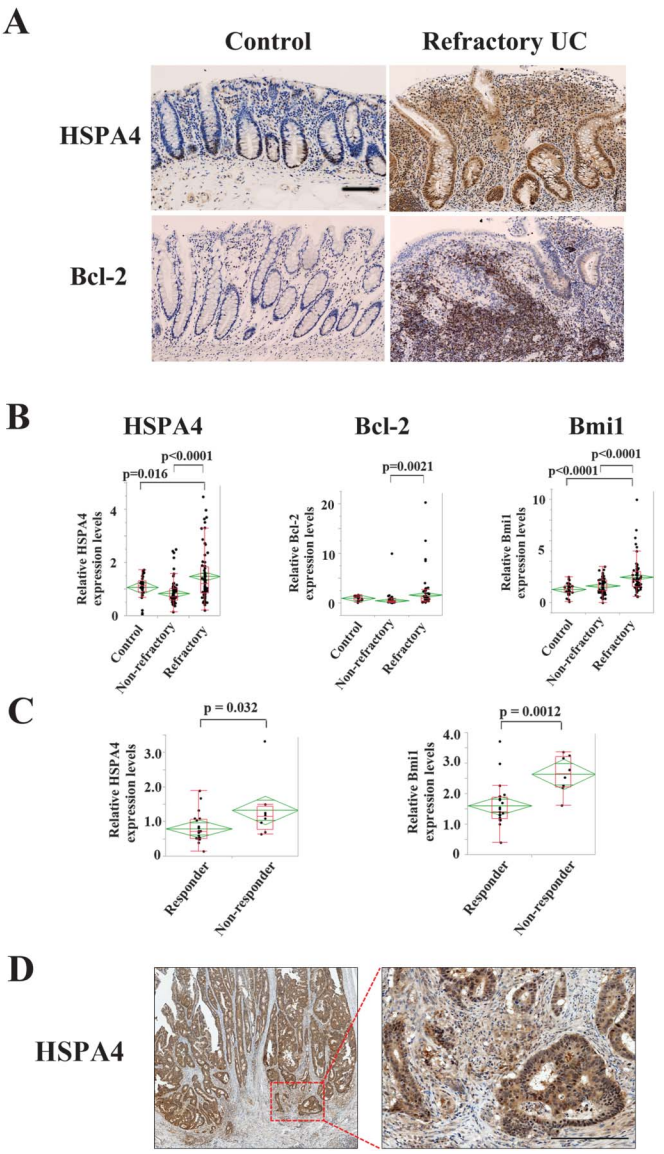

FIGURE 3. Increased expression of HSPA4, Bcl-2, and Bmi1 in the intestinal mucosa of patients with refractory IBD. A, Representative immunohistochemical images of HSPA4 and Bcl-2 in colonic tissues of patients with treatment-refractory UC and controls. Scale bar, 100 μm. B, The expression of HSPA4, Bcl-2, and Bmi1 mRNA in human colonic mucosa was analyzed by quantitative real-time PCR for patients without IBD (controls; n = 30), those with nonrefractory active UC (n = 90), and those with refractory UC (n = 66). *P* values were calculated by post hoc Tukey–Kramer honestly significant difference multiple comparison. The *F* and *P* values for the analysis of variance test are as follows: $F_{(2,179)} = 17.00$; $P < 0.0001$ (HSPA4), $F_{(2,179)} = 5.92$; $P = 0.0032$ (Bcl-2), and $F_{(2,179)} = 16.55$; $P < 0.0001$ (Bmi1). C, The expression of HSPA4 and Bmi1 mRNA in human colonic mucosa was analyzed by quantitative real-time PCR for steroid-responders and nonresponders. D, Representative immunostaining images of colorectal cancer tissues with anti-HSPA4 antibody. Scale bar, 100 μm.

Increased Susceptibility of *HspA4*$^{-/-}$ Mice to Intestinal Cell Apoptosis When Challenged with DSS

HspA4$^{-/-}$ mice were used to clarify the physiological and pathological functions of HSPA4 in vivo. They did not show gross abnormalities but produced smaller litters than WT siblings, which is consistent with the results of a previous report.[17,18] Experimental colitis was induced by treating mice with 2.5% DSS, and DSS-induced colon shortening, a marker for intestinal damage, was found to be augmented in *HspA4*$^{-/-}$ mice (Fig. 4A, B). Histological analysis revealed substantially more epithelial damage and disruption of crypt architecture in *HspA4*$^{-/-}$ mice than in WT mice (Fig. 4C). To confirm the role of HSPA4 in apoptosis, we compared apoptosis induction in DSS-treated WT and *HspA4*$^{-/-}$ mice. Gut apoptosis was 2- to 3-fold higher in DSS-treated *HspA4*$^{-/-}$ mice than in similarly treated WT mice (Fig. 4D, E).

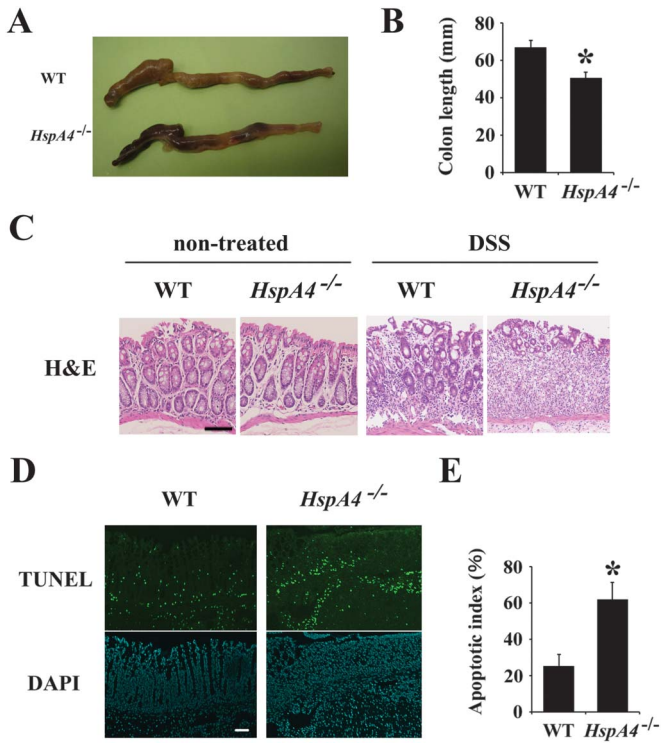

FIGURE 4. Susceptibility to intestinal cell apoptosis is increased in *HspA4*$^{-/-}$ mice challenged with DSS. WT and *HspA4* knock-out (*HspA4*$^{-/-}$) mice were treated with DSS for 7 days and euthanized. A, Colons of WT and *HspA4*$^{-/-}$ mice. B, Colon lengths after treatment with DSS. *$P < 0.05$ compared with WT mice. C, Representative photographs of H&E-stained colons of WT and *HSPA4*$^{-/-}$ mice before and 7 days after the initiation of DSS administration. Scale bar, 100 μm. D, TUNEL staining of colonic tissues from WT and *HspA4*$^{-/-}$ mice treated with DSS for 7 days. Scale bar, 50 μm. E, The apoptotic index was calculated by counting TUNEL signals in 100 crypts. DAPI: 4,6′diamidino-2-phenylindole.

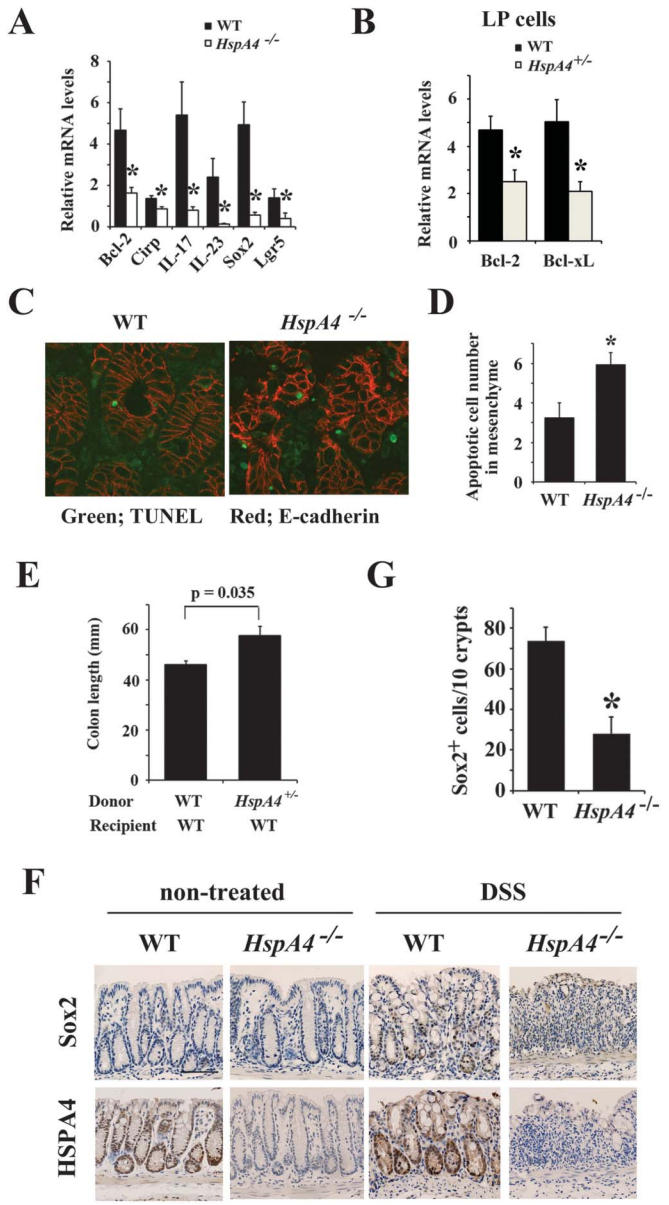

FIGURE 5. Attenuated immune response by HSPA4 deficiency in inflammatory cells. WT, heterozygous *HspA4* knock-out ($HspA4^{+/-}$) and *HspA4* knock-out ($HspA4^{-/-}$) mice were treated with DSS for 7 days and euthanized. Because $HSPA4^{-/-}$ mice produced smaller litters than WT siblings, $HspA4^{+/-}$ mice were used in some experiments. A, Relative mRNA amounts of the indicated genes in colonic tissues were determined by real-time qPCR and normalized to the levels of actin mRNA. The mRNA level of each gene in the untreated colon was given an arbitrary value of 1.0. Data are expressed as mean ± SEM (n = 6 per group). *$P < 0.05$ compared with WT mice. B, Relative levels of Bcl-2 and Bcl-xL in colonic lamina propria myeloid cells of mice treated with DSS. C, Representative images of immunohistochemical detection of E-cadherin, a marker for epithelial cells, and TUNEL staining of colonic tissues from DSS-treated mice. D, $TUNEL^+$/$E\text{-cadherin}^-$ cells were counted in 5 randomly selected views under a confocal laser microscope. *$P < 0.05$ compared with WT mice. Data are expressed as mean ± SEM. E, WT mice with WT or $HspA4^{+/-}$ BM and $HspA4^{+/-}$ mice with WT or $HspA4^{+/-}$ BM were generated by BM transplantation. *P* values were calculated by post hoc Tukey–Kramer honestly significant difference multiple comparison. F, Expression of Sox2 and HSPA4 in DSS-treated colons. Colon sections before and after DSS treatment were immunostained with the indicated antibodies. Scale bar = 100 μm. G, Quantification of $Sox2^+$ intestinal stem cells per 10 crypts. *$P < 0.05$ compared with WT mice. Data are expressed as mean ± SEM (n = 6 per group).

Attenuated Immune Response by HSPA4 Deficiency in Inflammatory Cells

Examination of the colonic lysates from WT and $HspA4^{-/-}$ mice revealed that HSPA4 deficiency resulted in decreased levels of antiapoptotic protein Bcl-2 and stress-response protein Cirp (Fig. 5A). HSPA4 is expressed both in epithelial cells and mesenchymal cells including T lymphocytes and macrophages (see Fig., Supplemental Digital Content 8, http://links.lww.com/IBD/A639). In inflammatory cells, heterozygous HSPA4 deficiency reduced Bcl-2 expression (Fig. 5B) and HSPA4 deficiency increased apoptosis (Fig. 5C, D), which would attenuate immune response in the gut. Consistently, in the intestinal tissues of DSS-treated $HspA4^{-/-}$ mice, IL-17 and IL-23 expression levels were reduced compared with those of similarly treated controls (Fig. 5A).

In short-term inflammation induced by DSS administration for 7 days, HSPA4 expression levels in inflammatory cells was much lower than that in epithelial cells (see Fig., Supplemental Digital Content 8, http://links.lww.com/IBD/A639), whereas chronic inflammation substantially upregulated HSPA4 expression in immune cells (Fig. 3A, B). These data suggest the possibility that HSPA4 in immune cells might be involved in the pathogenesis of refractory IBD. To functionally characterize the contribution of different cell populations to intestinal inflammation, we performed BM transplantation. Nontransplanted controls survived <2 weeks after irradiation, indicating there was ablation of the endogenous marrow. All $HspA4^{+/-}$ mice rescued with WT BM were dead when treated with DSS for 7 days. WT mice harboring WT BM had significantly shorter colons than WT mice harboring $HspA4^{+/-}$ BM (Fig. 5E), suggesting that HSPA4 in hematopoietic cells contributes to augmented immune response.

Given the significant correlation between expression of HSPA4 and stem cell markers in humans (Fig. 1), we speculated that HSPA4 regulates the expression of stem cell markers. HSPA4 deficiency decreased both Sox2 expression and the number of $Sox2^+$ cells in the DSS-treated colon (Fig. 5A, F, G).

Inhibition of Apoptosis by HSPA4 Overexpression and Decreased Cytokine Production by HSPA4 Knock-down

HSPA4 seemed to exhibit antiapoptotic activity in the intestine (Fig. 4). Next, we assessed the antiapoptotic effect of HSPA4 using isogenic stable cell lines. An imbalance between the cellular demand for protein synthesis and the capacity of the endoplasmic reticulum (ER) in promoting protein maturation and transport can lead to the accumulation of unfolded or

malfolded proteins in the ER lumen, a condition known as ER stress.[19] Unresolved ER stress in intestinal epithelial cells, a common feature of IBD, has emerged as an important mechanism that initiates intestinal inflammation.[20] Thapsigargin, an inhibitor of the sarcoplasmic/ER Ca^{2+} ATPase pump, disrupts ER Ca^{2+} stores and triggers ER stress-mediated apoptosis.[21] In this study, overexpression of HSPA4 significantly prevented thapsigargin-induced cell death in HSPA4-deficient fibroblasts (Fig. 6A). HSPA4 also prevented cell death induced by genotoxic stress (Fig. 6B). To assess whether HSPA4 prevents apoptosis, we measured caspase-8 and caspase-9 activity. HSPA4 overexpression suppressed both caspase-8 and caspase-9 activities in thapsigargin-treated cells (Fig. 6C, D). Additionally, HSPA4 overexpression suppressed adriamycin-induced activation of caspase-8 and caspase-9 (Fig. 6C, D). The antiapoptotic protein levels of Bcl-2 and Bcl-xL, which are known to play critical roles in the caspase-dependent apoptotic pathway,[22] were upregulated by HSPA4 (Fig. 6E, F). HSPA4 overexpression increased Sox2 expression in mouse fibroblasts (Fig. 6F). In Jurkat cells, an immortalized line of human T lymphocytes, knock-down of HSPA4 enhanced cacpase-8 and caspase-9 activities (see Fig. A, B, Supplemental Digital Content 9, http://links.lww.com/IBD/A640 and data not shown) and cell death in response to ER and genotoxic stress (see Fig. C, Supplemental Digital Content 9, http://links.lww.com/IBD/A640). HSPA4 knock-down decreased expression of proinflammatory cytokines, such as IL-1β, IL-6, IL-17, and TNF-α (Fig. 6G).

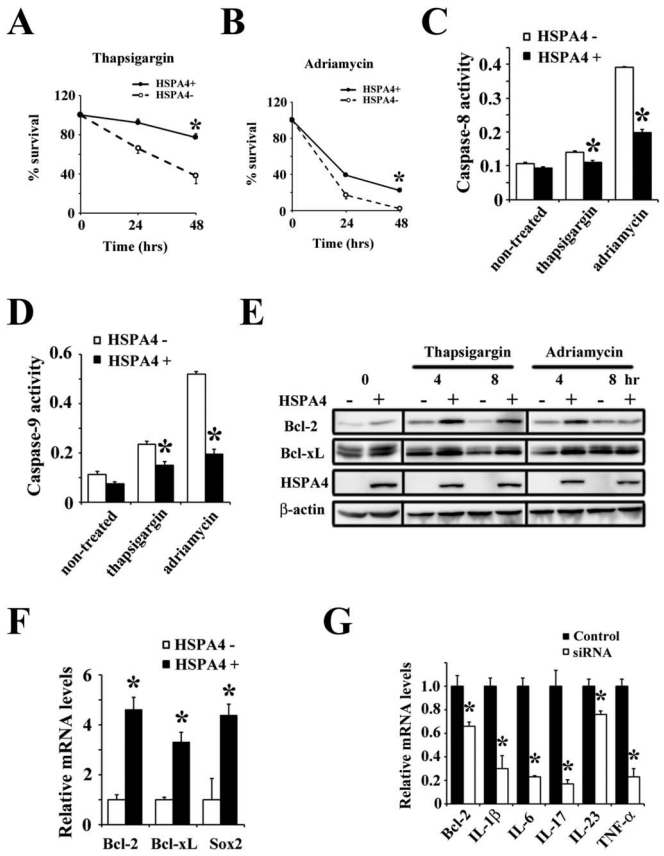

FIGURE 6. Inhibition of apoptosis by HSPA4 overexpression and decreased cytokine production by HSPA4 knock-down. A and B, *HspA4*-deficient fibroblasts with or without HSPA4 overexpression were cultured with media containing (A) thapsigargin (3 μM) or (B) adriamycin (2 μM). The number of viable cells was estimated by MTT assay at the indicated times after exposure to the stressors. C and D, *HspA4*-deficient fibroblasts with or without HSPA4 overexpression were cultured with media containing (C) thapsigargin (3 μM) or (D) adriamycin (2 μM) for 7 hours. Caspase-8 (C) and caspase-9 (D) activity was measured. Data are expressed as the mean ± SEM of triplicate experiments. *$P < 0.05$ compared with *HspA4*-deficient fibroblasts. E, *HspA4*-deficient fibroblasts with or without HSPA4 overexpression were cultured with media containing thapsigargin (3 μM) or adriamycin (2 μM) for the indicated times. Homogenates were gel-separated and immunoblotted with the indicated antibodies. F, mRNA was isolated from *HspA4*-deficient fibroblasts with and without HSPA4 overexpression. Relative mRNA levels of the indicated genes was determined by real-time qPCR and normalized to the levels of actin mRNA. Data are expressed as the mean ± SEM of triplicate experiments. *$P <$ 0.05 compared with *HspA4*-deficient fibroblasts. G, HSPA4 siRNA or control siRNA was transfected into Jurkat cells, an immortalized line of human T lymphocytes. After 24 hours, RNA was extracted. Relative mRNA levels of the indicated genes was determined by real-time qPCR and normalized to the levels of actin mRNA.

DISCUSSION

Stress triggers important adaptive responses to maintain homeostasis. The most conserved and the most ubiquitous of the stress responses is induced expression of heat shock proteins that act as chaperones against stress-induced denaturation of protein. By contrast, an increased and reduced stress response can create pathogenic conditions, such as inflammation and carcinogenesis.[23] Using the zebrafish model system, Crawford et al[24] reported that under inflammatory stress conditions within the gut, expression of HSPA4 is upregulated in a manner similar to that previously observed for mammalian HSP70. Here, we found that HSPA4 expression is increased by refractory and long-term inflammation in human colonic mucosa. Hypoxia that is enhanced in chronic inflammatory diseases including IBD was suggested to upregulate the expression of HSP110 and HSP70.[25] Given that HSPA4 is a member of the HSP110 family, this may be one explanation for HSPA4 induction by chronic inflammation. However, the exact mechanisms by which long-term inflammation upregulates HSPA4 expression remain to be unknown.

In chronically inflamed mucosa seen in refractory IBD, HSPA4 was found to be expressed in inflammatory cells and epithelial cells. In the colonic mucosa of patients with IBD, a significant correlation was observed between the expression of HSPA4 and antiapoptotic protein Bcl-2. Furthermore, HSPA4 was shown to increase Bcl-2 expression and inhibit apoptosis in inflammatory cells in murine colitis model. Given that Bcl-2–mediated apoptosis resistance in inflammatory cells has been shown to exacerbate inflammation and attenuate therapeutic efficacy in IBD,[3] HSPA4, whose expression is upregulated by

chronic inflammation, likely inhibits apoptosis in inflammatory cells and augments resistance to treatment through the upregulation of Bcl-2. Correspondingly, gene expression analysis on biopsy samples prospectively obtained before treatment from responders and nonresponders suggested that HSPA4 expression might have a predictive value for the efficacy of steroid treatment in IBD. Persistent inflammation resulting from insufficient treatment might exacerbate intestinal inflammation through the increased expression of HSPA4 in inflammatory cells.

The interleukin (IL)-23/Th17 (T-helper IL-17–producing cell) pathway has been identified to play a critical role in IBD. IL-23 has been shown to promote the expansion of a distinct lineage of Th17 cells that are characterized by production of a number of specific cytokines not produced by Th1 or Th2 cells, including IL-17A, IL-17F, IL-21, and IL-22.[26] Th17 cells are a lineage of $CD4^+$ effector T cells expressing HSPA4. In a cell line of human T lymphocytes and colonic mucosa of DSS-treated mice, HSPA4 deficiency reduced IL-17 and IL-23 expression. In the intestinal mucosa of patients with IBD, HSPA4 expression correlated with proinflammatory cytokines, including IL-17. Given the increased expression of HSPA4 in refractory IBD, HSPA4 overexpression, which results from chronic inflammation due to therapeutic resistance, would further drive resistance to therapy through the increased expression of Bcl-2 and proinflammatory cytokines, such as IL-17.

Stem cell homeostasis is required to maintain tissue viability and ensure long-term survival. Bcl-2 promotes cancer stem cell survival in response to stimuli by inhibiting the mitochondrial proapoptotic pathway,[27] whereas Bcl-2 inhibition reduces oxidative phosphorylation and selectively eradicates quiescent stem cells.[28] In this study, disruption of HSPA4 decreased the Bcl-2 expression and $Sox2^+$ cell population in DSS-induced inflamed colon. In addition, the expression of stem cell markers, such as Sox2, Lgr5, and Bmi1 correlated significantly with that of HSPA4 in the colonic mucosa of patients with IBD. These findings suggest that HSPA4 might promote survival of stem/progenitor cells through the upregulation of Bcl-2. Cancer stem cells, the microenvironment, and the immune system interact with each other through cytokines. In the context of chronic inflammation, cytokines, secreted by immune cells, activate the necessary pathways required by cancer stem cells.[29] Recent study has suggested that $IL\text{-}17^+$ cells have the capacity to induce cancer-initiating cells.[30] It should be noted that downregulation of the stem cell markers in the absence of HSPA4 might be secondary effects mediated by reduced inflammation rather than the direct effects by HSPA4 deficiency.

It is clear that chronic inflammation plays a causative role in the transition to adenocarcinoma in IBD. The association between IBD and colitic cancer is well established; the cumulative risk for developing colorectal cancer after 20 years is reported to be 7% for UC and 8% for CD.[31] Periodic colonoscopic surveillance with multiple biopsies is the conventional means of early detection of colitis-associated dysplasia/cancer. However, such surveillance programs have a number of limitations,[32] and surveillance strategies need to be appropriately personalized. Here, we showed that HSPA4 expression is increased by refractory and long-term colitis in humans, which is a high-risk factor for the development of colitic cancer. IL-23/IL-17 signaling, which is activated by HSPA4, enhances the immunosuppressive activity of regulatory T cells and reduces $CD8^+$ cells in tumor, leading to enhanced tumor initiation and promotion.[33,34] HSPA4 was overexpressed in human colorectal cancer cases, and HSPA4-specific signals were observed both in cancer cells and inflammatory cells. HSPA4 might be involved in colorectal tumorigenesis through increased expression of IL-17. The expression of Bcl-2 and Bmi1 is also increased in refractory UC. Therefore, analyzing HSPA4, Bcl-2, and Bmi1 expression levels may be effective for improving the identification of patients with IBD at high risk for colitic cancer. A future large-scale study of patients with IBD at different stages will be crucial for determining whether HSPA4 can be used as a biomarker for predicting the risk for colorectal carcinogenesis.

Bmi1-positive cells are resistant to high-dose radiation injury. After irradiation, the normally quiescent Bmi1-positive stem cells dramatically proliferate to clonally repopulate multiple contiguous crypts and villi.[35] We demonstrated that Bmi1 expression was increased by chronic inflammation in human colonic mucosae. Bmi1 might be a marker for mucosal regeneration under stress conditions.

In summary, HSPA4 induced by chronic inflammation likely inhibits the apoptosis of inflammatory cells and augments immune response through upregulating Bcl-2 and IL-17 expression, leading to treatment resistance. HSPA4 and Bmi1 would be useful biomarkers for refractory clinical course in patients with IBD.

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