Bacterial Heat Shock Protein 60, GroEL, Can Induce the Conversion of Naïve T Cells into a CD4⁺ CD25⁺ Foxp3-Expressing Phenotype

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Key Words

GroEL • Gut microflora • Heat shock protein • Mucosal immunology • Regulatory T cells

Abstract

Recent publications report that heat shock proteins (HSPs) can endow regulatory responses to the systemic immune system when administered via the mucosal route, leading to an amelioration of atherosclerosis and allergy. However, it remains poorly understood if HSP antigens exist in the luminal contents of the gastrointestinal tract and which types of HSP induce regulatory responses. Here we addressed these problems, considering that numerous gut microflora and foods are natural sources of HSPs. SDS-PAGE and immunoblotting with the anti-HSP60 antibody demonstrated the intact and degraded forms of HSP60 mainly in appendix and large intestine of the gastrointestinal tract. No reactivity with this antibody was observed for any of the luminal contents derived from germ-free animals, suggesting gut microflora to be a source of the intestinal HSPs because of lack of HSPs in animal chow diet. GroEL, a typical member of bacterial HSP60, showed a tendency to stimulate splenocytes in germ-free mice, compared to that in conventional mice, sug-

gesting that resident commensal bacterial GroEL may stimulate HSP-reactive T cells as regulatory cells in conventional animals. Importantly, GroEL, but not mouse-derived HSP60, caused naïve T cells to differentiate into CD4⁺ CD25⁺ Foxp3⁺ T cells, indicating that the production of regulatory T cells depends on the type of HSP. Thus, HSPs derived from commensal microbes can be utilized to stimulate immunoregulatory pathways for the maintenance of intestinal homeostasis.

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Introduction

Molecular chaperones of the heat shock protein (HSP) family are evolutionarily conserved proteins that modulate a variety of intracellular functions including the folding of proteins, the assembly and disassembly of multimeric proteins, the translocation of proteins across membranes, the degradation of proteins and signal transduction [1, 2]. Besides their chaperone activities, HSPs are involved in the regulation of innate immunity and mucosal immunity. Several lines of evidence [3, 4] have demonstrated *Mycobacterium tuberculosis*-derived HSP (Mtb

HSP) to be important immunogenic antigens in infections. Mtb Hsp70 was found to stimulate both in vivo and in vitro antigen-primed and to a lesser extent naïve PBMCs to produce inflammatory chemokines such as MIP-1 α , MIP-1 β and RANTES, that attract antigen-presenting cells (APCs) and effector T and B cells [5]. Mtb Hsp70 was also characterized to function as an adjuvant in stimulating host immune responses [6, 7]. A role for HSPs as antigens in inflammatory responses was reported in rats with heat-inactivated mycobacteria-induced adjuvant arthritis [8] and in pathogenesis of atherosclerosis [9, 10].

The regulatory functions of HSPs against inflammatory diseases have been focused on ever since immunization with a recombinant mycobacterial HSP60 was found to protect against adjuvant-induced arthritis [8]. HSP-reactive T cells still remain in a normal condition because of insufficient deletion in the thymus of T cells reactive to self-HSP antigens [11]. Since a lack of central tolerance needs to be compensated for peripheral tolerance, HSPs should be a driving force in generating regulatory responses [12, 13]. In fact, several reports have shown HSP-reactive T cells to have an immunoregulatory phenotype, indicating that HSPs, particularly, HSP60 and HSP70, constitute a group of autoantigens with the potential to trigger immunoregulatory pathways, which can suppress immune responses that occur in human inflammatory diseases, such as rheumatoid arthritis [8, 14], diabetic mellitus type 1 [11, 15], atherosclerosis [16] and allergy [17]. The intragastric administration or nasal immunization of HSPs was effective for preventive and therapeutic interventions of atherosclerosis in animal models, suggesting that mucosal immunization of HSPs is valuable for the regulation of chronic inflammation [18].

Numerous commensal bacteria exist in the gastrointestinal tract in animals. Also, the food that passes through the tract originates from living organisms. This implies that mucosal surfaces are constitutively or periodically exposed to plenty of major cellular components, HSPs, as an immunoregulator in oral route. Stimulation by exogenous HSPs in the gastrointestinal tract may activate HSP-reactive T cells to induce peripheral tolerance. From the viewpoint of the immunological cross-reactivity between microbial HSPs and mouse-derived HSPs, in the present study, we examined if HSP antigens exist in the luminal contents of the gastrointestinal tract using the immunoblotting procedure with an HSP60-specific antibody. Using in vitro differentiation assay, we also examined which types of HSP have the ability to induce regulatory responses.

Materials and Methods

Materials

Mouse HSP60 recombinant protein (MO60) and *M. tuberculosis* HSP65 (Mtb65) were purchased from StressGen Biotechnologies Corp. Recombinant GroEL, a typical bacterial HSP60 derived from *Escherichia coli*, was prepared as described previously [19]. The purity of the proteins was checked by SDS-PAGE and found to be over 97%. The protein concentration was measured with bicinchoninate method (Protein Assay Bicinchoninate kit; Nacalai Tesque). Other chemicals of guaranteed grade were obtained from Nacalai Tesque and Wako Pure Chemical Industries Ltd.

The contamination of protein solutions by endotoxins was reduced by a phase separation technique using the detergent Triton X-114, as reported previously [20]. In brief, Triton X-114 was added at a final concentration of 1%, and the solutions were mixed well and then stood in an ice bath for 10 min. The solutions were warmed to 37°C, whereupon two phases formed. The upper aqueous layers containing detoxified protein samples were collected by centrifugation at 12,000 g for 10 s. Removal of the endotoxin was achieved with five cycles of this phase separation. Finally, the samples were subjected to gel filtration with NAP-5 (GE Healthcare) to remove the detergent.

The antibodies used in the present study were as follows: anti-HSP60 monoclonal antibody (clone LK-2; StressGen), horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz); biotinylated anti-mouse CD3 ϵ (clone 145-2C11; eBioscience), FITC-conjugated anti-CD4 (clone GK1.5; eBioscience), anti-CD16/CD32 (clone 93; eBioscience), PE-conjugated anti-CD25 (clone PC61.5; eBioscience), allophycocyanin-conjugated anti-mouse CD45 (clone 30-F11; BioLegend), allophycocyanin-conjugated anti-Foxp3 (clone JFK-16s; eBioscience) and fluorochrome-conjugated streptavidin (eBioscience).

SDS-PAGE and Western Blotting

Samples equivalent to 5 μ g of protein were electrophoresed on a 7.5% SDS-polyacrylamide gel by the standard method used by Laemmli [21] and then transferred to a PVDF membrane (Bio-Rad Lab) in the same way as described by Hirano [22]. HSPs were probed with the monoclonal antibody raised against human HSP60 (clone LK-2) that cross-reacts with bacterial homolog, and the blot was developed with a peroxidase-conjugated goat antibody in the presence of the TMB substrate (Nacalai Tesque).

Animals

Female mice of the BALB/c, C57BL/6, C3H/HeJ and ICR strains at 6–8 weeks of age were purchased from Japan SLC Inc. Mice were housed in a conventional room at 22°C, under 12-hour cycles of light and dark. Germ-free (GF) mice of the IQI strain were purchased from CLEA Japan Inc. All experiments were approved by the Animal Experimentation Committee at Kyoto University and performed according to institutional guidelines.

To collect luminal contents in gastrointestinal tracts, mice were cervically dislocated, and their small and large intestines as well as appendices were excised. The small intestines were cut into proximal and distal parts. The luminal contents of each tissue were flushed out, so as not to be contaminated by epithelial cells, with 1.5 ml of sterile PBS containing 2 mM EDTA and protease inhibitor cocktail (Nacalai Tesque) to inhibit protein degradation

by digestive enzymes, and the suspensions were immediately frozen in liquid nitrogen and stored at -80°C until used. An aliquot of the suspensions was centrifuged at $4,000\text{ g}$ for 30 min to remove debris. The supernatants were passed through a $0.22\text{-}\mu\text{m}$ filter membrane by centrifugation at $12,000\text{ g}$ for 5 min at 4°C to obtain the intestinal luminal samples for measurement of protein concentration. The remaining portion of the suspensions was homogenized and centrifuged at $12,000\text{ g}$ for 5 min at 4°C to obtain the supernatants for SDS-PAGE analysis of luminal HSPs.

Measurement of Cell Proliferation

Spleen tissue was mashed between two pieces of frosted glass, and red blood cells were hemolyzed with ACK lysing buffer (Lonza Walkersville Inc.) to obtain single cell suspensions after extensive washing with PBS. Aliquots of splenocytes (20×10^4 cells/well) from ICR or IQI mice were cultured at 37°C for 96 h in a final volume of $100\text{ }\mu\text{l}$ in the presence or absence of a variety of HSPs at the concentration of $20\text{ }\mu\text{g/ml}$. After centrifugation at 900 g for 5 min, the cells were cultured with WST-8 reagent (Dojindo) in complete RPMI-1640 medium at 37°C for an additional 2 h. Cell proliferation was assessed by measuring the absorbance at 450 nm of the culture supernatants showing the formation of a soluble formazan dye. The magnitude of the proliferative response is expressed as stimulation index defined as the ratio of the mean increase in the absorbance of triplicate cultures with antigen to that in the absence of HSPs in each group.

Measurement of Cytokine IL-10

Small intestinal tissues were excised from mice and removed of Peyer's patches, and cut longitudinally after the removal of blood vessels and fat tissue. After mucus was removed from inside the tract by tapping on paper towel, tissues were washed with fresh PBS and divided into pieces approximately 5 mm long. The pieces were shaken at 37°C for 20 min twice in the presence of Hank's balanced salt solution containing 5% FBS (complete HBSS), 5 mM EDTA and 0.5 mM DTT to remove epithelial cells, and then digested at 37°C for 45–60 min with continuous stirring in the presence of 1 mg/ml collagenase type I and $30\text{ }\mu\text{g/ml}$ DNase I in complete HBSS. The digests were filtered through $40\text{-}\mu\text{m}$ cell strainers, and the filtrates were centrifuged to obtain cell pellets. The cell pellets were washed with PBS, suspended in complete HBSS solution containing 27% percoll (GE Healthcare), and centrifuged at 650 g for 20 min to remove fat layers. For the analysis of lamina propria (LP) leukocytes, cells were suspended in complete HBSS containing 40% percoll, layered over a 70% percoll solution, and centrifuged at 650 g for 20 min to collect the low-density fraction as LP leukocytes at the interface with a density of between 1.058 and 1.093 g/ml .

Aliquots of leukocytes (1×10^6 cells) from LP tissues were cultured at 37°C for 24 h in complete RPMI-1640 medium. The amount of the cytokine IL-10 in the culture supernatants was determined using a commercially available ELISA kit provided by eBioscience according to the manufacturer's directions. After the addition of sulfuric acid to stop enzymatic reactions, absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad).

In vitro Differentiation Assay

To obtain leukocytes from mesenteric lymph nodes (MLNs), organs isolated from C57BL/6 mice were cut finely with scissors

and digested at 37°C for 30–40 min with continuous stirring in the presence of 1 mg/ml collagenase type I (Wako Pure Chemical Industries Ltd.) and $30\text{ }\mu\text{g/ml}$ DNase I (Roche) in RPMI 1640 medium containing 5% FBS (complete RPMI-1640). The digests were passed through $40\text{-}\mu\text{m}$ cell strainers and washed twice with an extensive volume of PBS. Single-cell suspensions of MLNs were manually enriched for naïve T cells by twice selecting magnetically labeled cells with a $\text{CD4}^+ \text{CD62L}^+$ T cell isolation kit II (Miltenyi Biotec GmbH). APCs were prepared from single-cell suspensions of MLNs by positive selection with a Pan-DC purification kit (Miltenyi Biotec GmbH). MACS-enriched DCs and $\text{CD4}^+ \text{CD62L}^+$ T cells were co-cultured for 6 days at a 1:10 ratio (5×10^5 T cells) in the presence of HSP60 at the concentration of $20\text{ }\mu\text{M}$ in RPMI-1640 medium containing 10% FBS, $20\text{ }\mu\text{M}$ 2-mercaptoethanol, 5 ng/ml recombinant murine IL-2 and antibiotics. Fresh RPMI-1640 medium was added every 2 days. On day 6, cells were stained with antibodies to CD4 and CD25 antigens. Foxp3⁺ cells were detected on a FACSCalibur with antibody to Foxp3 after fixation and permeabilization with a kit for intracellular staining provided by eBioscience in accordance with the manufacturer's instructions.

Flow Cytometry

Aliquots of 5×10^5 cells were incubated at 4°C for 30 min with $0.5\text{ }\mu\text{g}$ of various fluorochrome-conjugated, or biotinylated, antibodies against cell surface markers in the presence of anti-CD16/CD32 antibody to inhibit non-specific binding to the cell surface. After being washed, the cells were incubated at 4°C for 10 min with fluorochrome-conjugated streptavidin when secondary staining was needed. Fluorescence was analyzed for living cells gated in the presence of 7-AAD using a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (Tomy Digital Biology Co. Ltd). Detection of the intracellular transcription factor Foxp3 was performed basically according to the manufacturer's instructions (eBioscience).

Results

HSP60-Like Antigens in the Gastrointestinal Tract Are Derived from Gut Microflora

First, we estimated the amounts of proteinaceous soluble compounds in four areas of the gastrointestinal tract: the proximal half of the small intestine, the distal half of the small intestine, the large intestine and the appendix. The protein concentration was estimated to average 6.9, 5.1, 3.8 and 2.9 mg/ml , respectively, in conventional mice of the BALB/c strain (fig. 1a). Similar observations were made in the C57BL/6 and C3H/HeJ strains, but the corresponding values were 6.0, 6.0, 13.7 and 7.1 mg/ml in GF mice of the IQI strain. The difference in estimates between the conventional and GF groups indicates a more progressive digestion of proteins and peptides by gut microflora as the luminal contents pass down the tract.

Fig. 1. HSP60-like antigens in the gastrointestinal tract are derived from gut microflora. **a** Amounts of proteinaceous compounds in four areas of the gastrointestinal tract. In five strains of mice, luminal samples were collected from the following areas: the proximal half of the small intestine (P), the distal half of the small intestine (D), the large intestine (L) and the appendix (A). **b** Reactivity of anti-HSP60 monoclonal antibody LK-2. Cross-reactivity was observed for purified GroEL derived from *E. coli* (EC), but not for cell lysates obtained from *L. acidophilus* (LA).

Any reactivity of this antibody was not detected in chow diets used for both conventionally reared (Conv) and GF mice. **c** Existence of HSP60-like antigens in the gastrointestinal tract of conventionally-reared mice, regardless of mouse strain. HSP60-like antigens were absent in the gastrointestinal tract of GF mice. HSP60 and related products were probed with anti-HSP60 monoclonal antibody followed by the development with a peroxidase-conjugated goat anti-mouse antibody.

Next we checked the reactivity of the monoclonal antibody LK-2 to probe HSPs in the luminal contents. As shown in figure 1b, this antibody reacted not only with mouse HSP60 but also substantially with a bacterial HSP60, GroEL derived from *E. coli*. However, the cross-

reactivity with GroEL derived from *Lactobacillus acidophilus* was not observed. This suggests that this antibody used can recognize HSP60 derived from, not all bacterial phylotypes, but a limited population of gut microflora. When the luminal contents were probed with

Fig. 2. Bacterial GroEL exhibits stimulatory effect in GF mice. Aliquots of splenocytes from conventional (Conv) ICR or IQI GF mice were cultured in the presence or absence of a variety of HSPs. Cell proliferation is expressed as stimulation index, and each symbol denotes a stimulation index derived from individual mouse, compared with the control group in the absence of HSP (w/o) in each strain.

anti-HSP60 antibody, a band with a molecular size of 60 kDa was clearly detected mainly in the appendix and large intestine, regardless of mouse strain (fig. 1c). A few faint bands with a molecular size below 60 kDa were also observed in the appendix, although the pattern of the bands differed in individual strain, suggesting that GroEL-like antigens are susceptible to proteolytic digestion by pancreatic or bacterium-derived proteases. In contrast, no band for an HSP60-like antigen was observed in the luminal contents throughout the gastrointestinal tract of GF mice (fig. 1c) and in the animal chow diets used (fig. 1b). These results demonstrated that HSP60-like antigens in the gastrointestinal tract are derived from gut microflora.

Bacterial GroEL Exhibits Stimulatory Effect in GF Mice

We examined whether self- or non-self-HSPs have stimulatory effects on splenocytes from conventional and GF mice using mouse-derived MO60 and bacterium-derived GroEL. MO60 exhibited significant stimulatory effect on splenocytes from conventional mice, but GroEL did not substantially stimulate them (fig. 2). In contrast, in GF mice, GroEL showed a tendency to stimulate splenocytes, whereas a stimulatory effect of MO60 was low-

ered compared to that in conventional mice. GF mice positively responded to Mtb65 antigen derived from an inexperienced bacteria, *M. tuberculosis*. These results suggest the existence of HSP-reactive T cells under the physiologically naïve condition.

Bacterial GroEL Potentially Converts Naïve T Cells into Regulatory Cells

We evaluated the effect of gut microflora on the production of cytokine IL-10 as an index of gastrointestinal environments. A significant amount of IL-10 was detected in the culture supernatant of leukocytes prepared from LP tissues of conventional mice, but not GF mice (fig. 3a), suggesting that the gut microflora provide an environment favorable to the formation of regulatory T (Treg) cells.

We compared the ability of HSPs to convert naïve T cells into Treg cells. MACS-enriched DCs and CD4⁺CD62L⁺ T cells were co-cultured for 6 days in the presence of GroEL or MO60. After the culture, surprisingly, 3.7% of the CD4⁺-gated T cells were converted to CD25⁺Foxp3⁺ cells in the presence of GroEL, but 0% in the presence of MO60 (fig. 3b, c). These results suggest that bacterial HSPs potentially induce the conversion of naïve T cells into effector T cells with a regulatory phenotype such as Treg cells, and that this effect may depend on the type of HSP.

Discussion

The most extensive microbial community is harbored by the gastrointestinal tract of mammals after birth. The gut microbiota outnumber about 10 times the total number of our somatic and germ cells, and thus the host-microbiota relationship has so far evolved to become mutually beneficial. For example, Hrnir et al. [23] found that the presence of gut microflora derives the expansion of CD4⁺ T cells including Foxp3-expressing T cells in MLNs. In addition, the presence of gut microbiota increased the production of IL-10 and IFN- γ cytokines. Recently, Round and Mazmanian [24] reported that a prominent human commensal, *Bacteroides fragilis*, directs the development of Foxp3⁺ Treg cells with a unique inducible signature. They also identified the immunomodulatory molecule, polysaccharide A, of *B. fragilis* to mediate the conversion of CD4⁺ T cells into Foxp3⁺ Treg cells that produce IL-10 during commensal colonization. Thus, gut microbiota play a crucial role in the development of Treg cells to maintain intestinal homeostasis.

Fig. 3. Bacterial GroEL potentially converts naïve T cells into regulatory cells. **a** High level of IL-10 production by LP leukocytes of commensal microflora-resident mice. Leukocytes prepared from conventional mice (n = 3) or GF mice (n = 8) were cultured at 37°C for 24 h, and then the level of IL-10 in the culture supernatant was measured by ELISA. **b** The frequency of Treg cells generated in the in vitro differentiation assay. After 6-days' co-culture, cells were stained with antibodies to CD4 and CD25 antigens, and in-

tracellularly with an antibody to the Foxp3 antigen, and analyzed with a flow cytometer. **c** The percentage of CD25⁺ Foxp3⁺ T cells generated was estimated for the gated CD4⁺ cells, and expressed as the mean with SEM (n = 5) for the control group (light gray bar), the MO60 group (dark gray bar) and the GroEL group (black bar). Statistical difference was determined by Student's t test, and denoted by * p < 0.001, compared with the control group in the absence of HSP.

Here we focused on a common and primordial antigen, HSPs that are expressed in all living organisms and are the most conserved proteins present both in prokaryotes and eukaryotes. Assuming that HSP-reactive T cells still remain in a normal condition because of insufficient deletion in the thymus of T cells reactive to self-HSP antigens [11], we expect T cell proliferation in the presence

of HSP antigens. In GF mice that have never experienced environmental bacteria, we observed cell proliferation in the presence of HSP antigen, irrespective of the type of HSP, as shown in figure 2. In contrast, little proliferation was detected for the conventional mice in the presence of GroEL antigen. This observation implies that HSP-reactive T cells are functionally impaired in GF mice, as re-

ported previously [25], when we consider that HSPs derived from commensal bacteria should be a driving force in generating regulatory responses, since a lack of central tolerance needs to be compensated for peripheral tolerance [12, 13]. Alternatively, the conventionally reared mice are more tolerant for bacterial antigen. In other words, resident bacterial GroEL may stimulate HSP-reactive T cells as regulatory cells educated in conventional animals. Contrary to GroEL, MO60 somewhat promotes cell proliferation in conventionally reared mice. We cannot exclude the possibility of bacterial HSP60 akin to MO60 to direct the development of T cell subsets other than Treg cells. However, neither commercially available GF mice of the IQI strain nor conventionally reared mice of the ICR strain are inbred, but they are closed colonies comprised of four different genetic backgrounds. Considering that genetic background could substantially influence cellular responses, therefore, we must carefully interpret our results in the present study and are now in progress for the experiment using GF mice of inbred strain with a single genetic background.

The immunoregulatory properties of HSPs are proposed to stem from high evolutionary conservation among species, which enables immune cross-reactivity between exogenous microbial HSPs and mouse-derived HSPs. HSPs, however, differ somewhat in structure, size and location, especially in their C-terminal regions, although they are highly conserved across species from prokaryotic bacteria to mammals. Here we used a recombinant GroEL protein derived from *E. coli* as an antigen to generate Treg cells. Considering that enormous numbers of bacteria make up commensal microflora, we expect that HSPs derived from the bacterial species other than *E. coli* have higher ability to generate Treg cells. As shown in figure 1c, the reactivity of the antibody in large intestine was lower than that in the appendix. This difference may reflect the structural diversity of the C-terminal region of HSPs among astounding numbers of bacteria colonized in large intestine, since the antibody recognizes the epitope located between amino acid residues 383 and 447 of human HSP60 [26]. Therefore, we are currently trying to compare the ability to generate Treg cells between the *E. coli* GroEL protein and intrinsic HSP60-like antigens prepared from the luminal contents of gastrointestinal tracts.

Recently, colonization of the small intestine of mice with a single commensal microbe, segmented filamentous bacterium, was found to induce IL-17-producing T cells (Th17 cells) [27]. Colonization with this microbe increased the expression of serum amyloid A, a member of

the family of acute-phase response proteins induced during infections or under inflammatory conditions. Serum amyloid A promoted the Th17 cell differentiation of naive T cells with the aid of inflammatory cytokines produced by mucosal APCs. Taken together, it is most likely that the intestinal homeostasis can be maintained in a stress signal-controlled manner.

The mucosal immune system consists of a variety of lymphocytes and accessory cells such as DCs and macrophages dispersed in gut-associated lymphoid tissues, including villi, Peyer's patches, LP and MLNs. Recent reports support that the status of APCs determines whether an antigen induces tolerance or protective immunity, depending on how it is presented to antigen-specific T cells by APCs [28, 29]. CD103⁺ DCs induced the differentiation of Treg cells via the production of the vitamin A metabolite retinoic acid, in the presence of TGF- β , which in addition results in the recruitment of T and B cells to intestinal tissues [30–32]. Intestinal macrophages were also found to display an inflammatory anergy [33] and CD11b⁺ macrophages in LP tissues induced the responses of Treg cells [34].

The bacterial component LPS usually causes inflammatory responses, including the production of the pro-inflammatory cytokines TNF- α and IL-12, whereas it urges LP macrophages to produce the regulatory cytokine IL-10, indicating a unique immunological feature of mucosal surfaces. In other words, depending on its location, even a substance that systemically evokes inflammatory responses can function as a regulator with tolerogenic properties. This hypothesis can be partly supported by the report that microbial LPS contaminants contained in sterile diet influence the development of Treg cells [23]. Furthermore, the effects of intestinal epithelial cells can be taken into consideration. Because we observed the intrinsic binding of bacterial DnaK, a typical HSP70 molecule, to mouse rectum CMT-93 carcinoma with epithelial-like morphology [unpubl. data], HSP60-like antigens may act primarily on epithelial cells rather than directly on APCs and T cells. Intestinal epithelial cells are continuously exposed to numerous products of commensal bacteria. Recently, it was suggested that the down-regulation of TLR4 gene expression contributes to the insensitivity of intestinal epithelial cells to LPS for the maintenance of intestinal homeostasis [35]. Therefore, gut microflora-derived HSPs, like LPS, may have the potential to act as a tolerogenic regulator for immune homeostasis at peripheral mucosal sites as well as a pro-inflammatory signal in the systemic immune system.

In conclusion, we demonstrated that HSP60-like antigens exist throughout the gastrointestinal tract, irrespective of mouse strain. These antigens were found to originate from gut microflora, not from the host animal, since no cross-reactivity with the anti-HSP60 antibody was observed for luminal contents derived from germ-free animals. GroEL showed a tendency to exhibit stimulatory effect in GF mice compared to that in conventional mice, suggesting the possibility that resident bacterial GroEL educates HSP-reactive T cells as regulatory cells in conventional animals, or that conventional mice are more tolerant. Importantly, we found that the bacterial GroEL, but not self-HSP60, could generate CD4⁺ CD25⁺ Foxp3⁺ T cells from naïve T cells, indicating that the production of Treg cells depends on the type of HSP. Taken together, our findings imply that the exogenous HSPs derived from

gut microflora can constitutively function as a regulatory factor to control immune homeostasis at peripheral mucosal sites, providing a link between innate and adaptive immune systems [36–38].

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