

Research Article

Concurrent exposure to thermal stress and oral Ag induces intestinal sensitization in the mouse by a mechanism of regulation of IL-12 expression

TAO LIU,¹ BIN-QUAN WANG,¹ CHANG-SHENG WANG¹ and PING-CHANG YANG²

¹Institute of Allergy, Shanxi Medical University, Taiyuan, Shanxi, China and ²Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Summary The mechanism of food allergy remains unclear. The absorption of intact protein Ag into the intestinal tissue is a prerequisite in the development of intestinal sensitization. Previous studies indicate that thermal stress compromises the intestinal barrier function. Mice were concurrently exposed to thermal stress and oral Ag. Intestinal sensitivity, levels of serum-specific IgE, IL-4 and INF- γ were assessed. Intestinal dendritic cell, Th1 and Th2 functions were determined. The mice that were treated with thermal stress and oral Ag showed high levels of serum Ag-specific IgE, intestinal mast cell activation in response to oral Ag challenge, suppression of IL-12 expression in the intestinal dendritic cells, inhibition of T-bet expression and Th1 function and marked increases in (GATA)₃ expression and Th2 function. Mice exposed to thermal stress alone or oral Ag alone did not show any signs of the intestinal sensitization. Pretreatment with IL-12 inhibited the intestinal sensitization induced by the concurrent exposure to thermal stress and Ag gavage. We conclude that although Ag absorption is essential, Ag absorption alone is insufficient; other accessory factors that can disturb the local immune homeostasis are also required for the induction of intestinal sensitization. The present study illustrates that concurrent exposure to thermal stress and oral Ag can prove to be a factor in the induction of intestinal sensitization by a mechanism of regulating IL-12 expression.

Key words: cytokine, dendritic cell, food allergy, intestine, T cell.

Introduction

Approximately 4–8% of children and 1–2% of adults have food allergy.^{1,2} It is not clear how an individual develops sensitivity to a particular Ag. Generally, Ag seen in the gut lumen induce active suppression of immune/inflammatory responses, known as oral tolerance, rather than a detrimental reaction.³ Oral tolerance is not completely understood, but probably involves Ag processing by mucosal cells. Both epithelial and dendritic cells (DC) are identified as important cells in this process.⁴ So far, no definitive therapies are available for food allergy and the only effective remedy is the avoidance of offending food in the diet.

An association between stress and mucosal sensitization has drawn an attention.⁵ A link between psychological stress and promoting intestinal Th2 response leading to intestinal sensitization was identified recently.⁶ High ambient temperature or high body temperature is a type of stress that affects human life frequently as, for example, during strenuous exercise, fever in the period of diseases or therapies resulting in body high temperature, while working in high-temperature environments without appropriate ventilation etc.^{7–10} Studies suggest

that thermal stress increases intestinal epithelial permeability resulting in the absorption of macromolecular substances, such as intact food Ag.¹¹ The absorbed Ag have the potential to contact the immune cells in the lamina propria and to induce inappropriate immune reactions such as food allergy.

IL-12 is a heterodimeric cytokine produced primarily by macrophages and DC in both innate and adaptive immune responses. IL-12 is formed by a 35 kDa chain (p35 or IL-12 α) and a 40 kDa chain (p40 or IL-12 β).¹² IL-12 β is encoded by the IL-12 β gene on the chromosome 5q31–33, a region that has been shown to be linked to allergy.¹² IL-12 is a key factor in the production of Th1 cytokines and the suppression of the differentiation of Th2 cells, which control humoral immunity in the production of IgG1, IgE and IgA.¹³ It is reported that the suppression of IL-12 results in suppression of Th1¹⁴ and upregulation of Th2 cytokines that play a critical role in the pathogenesis of allergic diseases including food allergy.¹⁵ However, our knowledge about the regulation of IL-12 in the body is still limited. Ahlers *et al.* reported that thermal stress decreased the serum levels of IL-12 as well as IFN- γ .¹⁶ The fact gives us a hint that thermal stress might subsequently repress Th1 immunity and increase Th2 immunity, which might contribute to the development of sensitization in the body. This postulate is supported by a recent study where Hino *et al.* used extrinsic IL-12p40 and successfully blocked Ag-specific, Th2-type responses that prevented allergic diarrhoea and inhibited ovalbumin (OVA)-specific IgE synthesis.¹⁷

Correspondence: Dr Ping-Chang Yang, HSC-3N5C, McMaster University, 1200 Main Street West, Hamilton, ON, Canada L8N 3Z5. Email: yangp@mcmaster.ca

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The T-bet (T-box expressed in T cells) and GATA binding protein (GATA)₃ are the master transcription factors in the Th1/Th2 cell immunity.^{18,19} T-bet is expressed in Th1 cells and its expression is mediated mainly by a signal transducer and activator of transcription (STAT)-4-dependent pathway. (GATA)₃ is expressed during Th2 differentiation through pathways that involve the IL-4-dependent activation of the STAT-6 and the expression of (GATA)₃ is critical for Th2 cell development.^{19,20} In addition, (GATA)₃ directly transactivates the expression of genes for IL-5 and IL-13 in Th2 cells.²¹

Based on our previous studies in food allergy^{22–24} and studies carried out by others,²⁵ Ag have to be transported into the intestinal mucosa to induce the local sensitization. In general, the intestinal epithelial barrier restricts intact protein uptake and transport, which is one of the means of preventing the intestine from sensitization to food Ag. Our recent finding indicates that stress can markedly compromise the intestinal barrier function and facilitate intact protein Ag absorption.^{6,26}

In this study, we have suggested that the concurrent exposure to thermal stress and food Ag could be a factor inducing intestinal sensitization. The specific aims of this study included the following. In a mouse model, we would determine whether (i) thermal stress increases a model Ag horseradish peroxidase (HRP) absorption; (ii) thermal stress affects intestinal DC and CD4⁺ T-cell functions; and (iii) the concurrent exposure to thermal stress and food Ag induces intestinal sensitization. The results showed that the concurrent exposure to thermal stress significantly compromises the mouse intestinal barrier functions and induces macromolecular protein Ag absorption; thermal stress suppresses the intestinal DC IL-12 expression and subsequently, induces skewed intestinal Th2 polarization and intestinal sensitization.

Material and methods

Animals

BALB/c mice, 8–10 weeks of age of mixed sex were maintained in the Experimental Animal Center at Shanxi Medical University. The mice were maintained at 20°C on a 12:12 h light : dark cycle. They were fed ad libitum commercial mouse chow and were provided with environmental enrichment (plastic rolls). On the day of each experiment the animals were weighed, then carried to the laboratory in a transport cage. All experiments were conducted in the morning to minimize any effects of diurnal variation. The experiments were approved by the local Animal Use and Care Committee.

Thermal stress protocol

Water baths were used to create a heating environment. An aluminium box equipped with a thermometer inside was floated on the water. The mice were placed inside the box for 1 h where the temperature was maintained at 43°C throughout the experimental period.

Sensitization protocol

The thermal-stress antigen (TS-Ag) group mice were intragastrical, delivered with a bolus of horseradish peroxidase (HRP, type II, Sigma-Aldrich, St Louis, MO, USA; a model Ag) (500 µg/mouse) and pertussis toxin (Sigma) 50 ng/mouse in 0.5 mL saline i.p. just before the thermal stress session (TS-Ag group). Fifteen days after the gavage, mice were killed by cervical dislocation. Retro-orbital blood

was obtained for serum IgE determination. Intestinal segments were removed from the jejunum and pieces were immediately fixed for morphological studies, frozen for mRNA measurements or prepared for functional studies, as described shortly.

Control groups

Naive mice, which were not exposed to either thermal stress or HRP gavage were used as baseline control group. Mice of the sham stress group were treated in the same way as the TS-Ag group, except for being treated with room temperature. The TS group mice were treated with thermal stress without HRP gavage. Because extrinsic IL-12 can efficiently induce bioactivity (such as hyperalgesia) in the body, mice in IL-12 group were pretreated with recombinant murine IL-12 (200 ng/mouse, i.p.) just before HRP gavage and were then subjected to thermal stress.²⁷ The purpose of the pretreatment with extrinsic IL-12 was to compensate for the thermal stress caused IL-12 suppression. Ag group mice were treated with HRP gavage without thermal stress.

Determination of intestinal sensitization

The mice were challenged with 500 µg HRP (in 0.5 mL saline) by gavage 14 days after the sensitization. Just before killing the mice, the retro-orbital blood was collected under general anaesthesia. Serum HRP-specific IgE titres were determined using standard procedures, as described in a previous report.²⁸ Values that were greater than 1:16 were accepted as positive. Stool samples were collected from the mice by opening the intestine after killing them. Total proteins were extracted from the stool samples and IgE levels were measured with ELISA. Diarrhoea was selected as a clinical symptom in response to the Ag challenge that was determined by visually monitoring mice for up to 1 h following the intragastric challenge.²⁹ Mice showing profuse liquid stool were recorded as diarrhoea-positive animals. Mast cell activation is one of the pathological features of type I allergic reaction and was determined by observing the intestinal mast cell degranulation and the histamine release. The mice were killed by cervical dislocation 60 min after the challenge. The intestinal segments were excised and processed for electron microscopy.^{22,23} Mast cell granules were categorized into the intact and the degranulated granules.^{22,23} Histamine levels were determined in the intestinal tissue with the method reported previously.³⁰

Determination of the paths of Ag absorption

The samples used for mast cell degranulation observation were also used to locate HRP products in the intestinal mucosa with electron microscopy. Intestinal mucosal image windows were randomly selected in the intestinal mucosa under an electron microscope with the reported methods.^{22,31} The HRP uptake in the intestinal mucosa was quantitatively evaluated with computerized image processing technique.³² HRP products in the paracellular spaces were defined as high electron density substances as compared with the normal paracellular spaces (empty).³²

Thermal stress suppresses IL-12 expression in the intestine

The intestinal DC were the main source of IL-12 and were purified with magnetic assisted cell sorting (MACS; Miltenyi Biotec, Belgisch, Germany).³³ The purified intestinal CD11c⁺ DC were cultured with RPMI-1640 (Biochem 21, Shenzhen, China) in the presence of LPS (50 µg/mL) for 24 h. The supernatants and DC were collected. The levels of IL-12p70 protein in the supernatants were evaluated with

ELISA kits (Genetimes Technology, Shanghai, China), following the manufacturer's instruction. The total RNA was extracted from the DC and subjected to RT-PCR to evaluate IL-12p35 and IL-12p40 mRNA expression. The primers used in the experiments were cited in previous reports.³⁴ The procedures of RNA extraction and RT-PCR were referred to the previous reports.²⁴

Intestinal Ag-specific T-cell proliferation

The intestinal CD4⁺ T cells were purified from the separated lamina propria mononuclear cells by MACS (Mitenyi Biotech).³³ A batch of the separated CD4⁺ T cells were cultured in RPMI-1640 in the presence of specific Ag HRP (10 µmol), brefeldin A (10 µg/mL, Sigma-Aldrich) and ionomycin (300 ng/mL, Jingmei Biotech, Shenzhen, China) for 4 days. The cells were fixed and incubated with FITC-labelled anti-IL-4 and phycoerythrin (PE)-labelled anti-IFN-γ antibodies. The positively stained cells were counted by flow cytometry. The Th1 (IFN-γ) and Th2 (IL-4) cytokines in the supernatants were measured with ELISA kits following manufacturer's instruction. Irradiated spleen cells (2×10^5 cells/well) and CD4⁺ cells (2×10^5 cells/well) were added to 96-well plates in the presence of Ag HRP (20 µg/mL). Cells were cultured for 72 h at 37°C with [³H]thymidine added (3.7×10^{10} Bq/well) for the last 16 h. Total [³H]thymidine incorporation was measured as an indicator of cell proliferation. The supernatants were collected for cytokine evaluation. A portion of the cells were collected for flow cytometry and western blot assay. Nuclear protein of the Ag-stimulated CD4⁺ cells were prepared using the Nuclear Extraction kit (Genetimes Technology). Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were probed with a 1/1000 dilution of a (GATA)₃ or T-bet mAbs (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Membranes were probed with a 1/2000 dilution of a HRP conjugated second antibody (Biochem 21, Shanghai, China). Membrane blots were then exposed to enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Piscataway, NJ, USA) and observed using radiographs. Densitometric analysis was carried out using ImageJ software (NIH, Bethesda, MD, USA) and normalized with the integrated control beta-actin blots.

Thermal stress increases corticosterone secretion

Mice were subjected to thermal stress (or sham stress) for 1 h. The retro-orbital blood was collected to determine the levels of corticosterone (CS) in the serum with the method of radioimmunoassay.

Statistics

Data are presented as mean ± SD. Differences between groups are analysed with Student's *t*-test. A *P*-value <0.05 was considered to be significant.

Results

Mouse intestines were sensitized by the concurrent exposure to thermal stress and Ag

The sensitization status of the mice was determined by three parameters: (i) Ag-specific IgE in the serum; (ii) allergic diarrhoea; and (iii) mast cell degranulation in response to the specific Ag challenge. The passive cutaneous anaphylaxis (PCA) results indicated that all the mice exposed to thermal stress and Ag concurrently (TS-Ag group) showed positive reactions (the dilution of the serum >1:16) whereas none of the mice in the

control groups or IL-12 group showed positive PCA reactions. Total IgE levels in the stool samples were significantly higher in TS-Ag group (366.3 ± 29.8 ng/g) than in naive group (29.6 ± 21.8 ng/g) and in sham-stressed group (36.9 ± 31.8 ng/g). The presence of Ag-specific IgE antibodies in the circulation is a standard method to document sensitization in both human being and animals these mice were considered sensitized.¹ All the mice of TS-Ag group had diarrhoea three to five times after the oral challenge with Ag-specific HRP. The mice in control groups did not have diarrhoea. Mouse colonic stool observations are listed in Table 1. No solid stool was observed in the colon of TS-Ag group, whereas solid stool was observed in almost all the mice in the rest of the five groups. Also, we did not find loose stool in their colon. The IL-12 group mice also did not show any signs of diarrhoea after the oral HRP challenge. As depicted in Figure 1 (a–c), the mast cells have markedly degranulated in the intestine of the TS-Ag group; the rate of degranulation was significantly higher in the TS-Ag group compared with the control groups and the IL-12 group. Higher levels of histamine were detected in the intestinal tissue of the TS-Ag group after the Ag challenge compared with the control groups and the IL-12 group (Fig. 2).

Ag uptake and transport across the intestinal epithelium are upregulated by thermal stress

There were only few HRP-containing endosomes of small size in the apical region of the intestinal epithelial cells in the control mice (Fig. 3a). A large number of HRP endosomes of large size were distributed in both apical and basal region of the epithelial cells of the TS, TS-Ag and IL-12 groups. Some HRP endosomes appeared in the subepithelial region, in the lamina propria and inside the phagocytes (Fig. 3b). The epithelial paracellular spaces were observed under an electron microscope (Fig. 3b). HRP products were also identified in the paracellular spaces of the epithelium: TS group, 356 out of 1034 (34.4%); TS-Ag group, 312 out of 968 (32.2%); and IL-12 group, 275 out of 889 (30.9%). No HRP products were observed in the naive and sham-stressed mice. All the mice (including the naive mice that were not exposed to HRP at the first time) were re-exposed to HRP by gavage 14 days after the sensitization. A large number of HRP endosomes of large size were also found in the epithelium as well as in the lamina propria of the TS-Ag group mice, but not in the paracellular spaces (Fig. 3c). Only a few HRP endosomes were found in the apical region of the epithelium of the control and IL-12 mice. To show Ag (HRP) absorption in the intestine after the thermal stress treatment, HRP was quantified in the intestinal tissue. The results showed that no HRP was detected in the naive and TS groups. Thermal stress

Table 1 Stool observations in mouse colon

Groups	Stool		
	Solid	Soft	Liquid
Naive	10	0	0
Sham	10	0	0
TS	9	1	0
Ag	10	0	0
TS-Ag*	0	3	7
IL-12	10	0	0

**P* < 0.05, compared with naive group by χ^2 test.

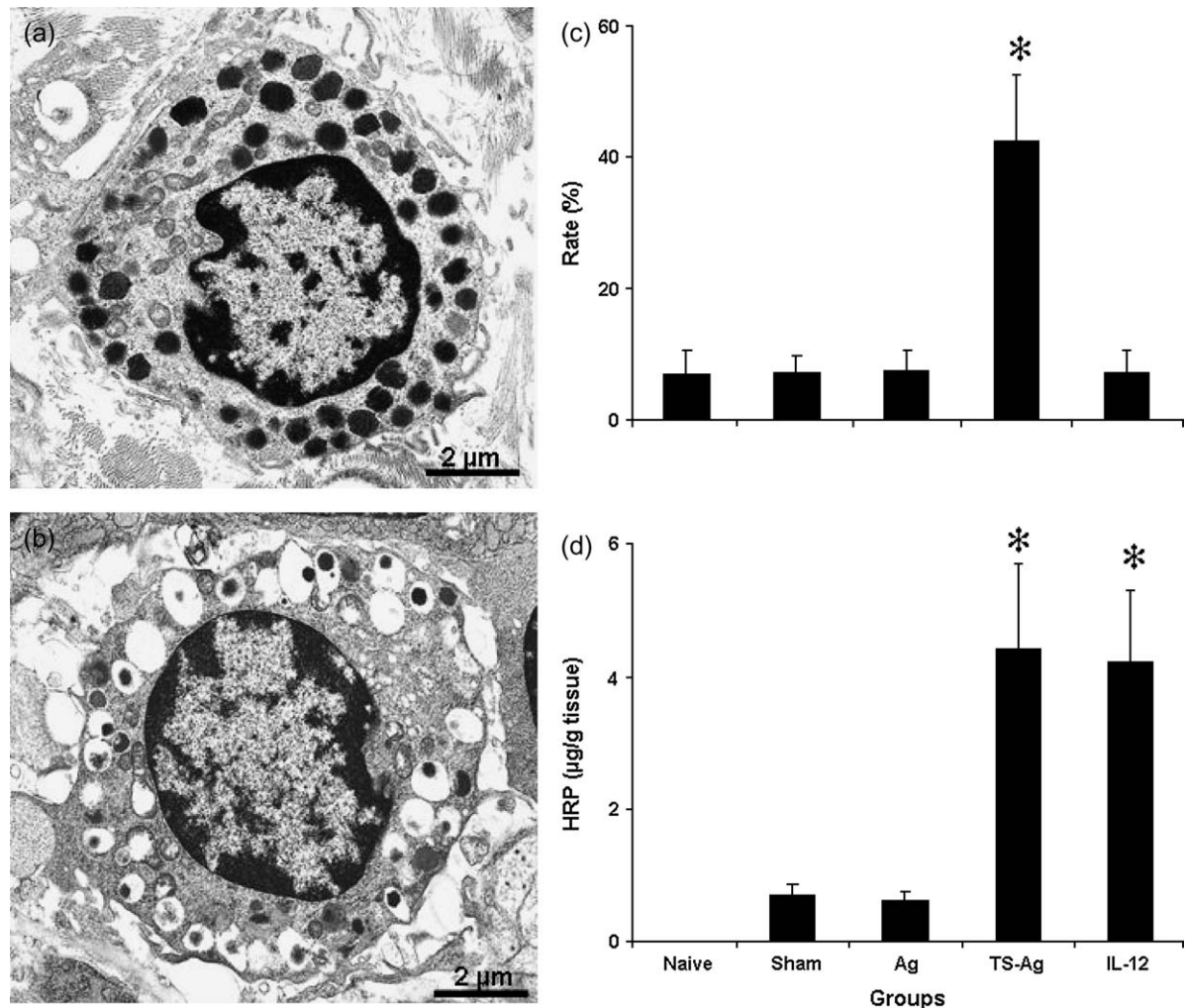


Figure 1 Intestinal mast cell degranulation and Ag absorption. After oral Ag horseradish peroxidase (HRP) challenge, the intestinal tissue was processed and observed with an electron microscope. Intestinal absorbed HRP was quantified with enzyme assay. (a) A representative mast cell electron photomicrograph from the mice treated with HRP gavage alone and (b) a mast cell image from the mice treated with thermal stress and HRP gavage concurrently. Mast cell degranulation was determined as (i) decrease in granular density or (ii) losing granular content manifesting as empty rings around the remains of granular matrices. (c) Rate of intestinal mast cell degranulation of the mice in different groups. Bars show the rate of mast cell degranulation. The rate was calculated with the degranulated granules divided by the number of total granules. Data are expressed as mean $\pm$ SD from 30 mast cells in each group. * $P < 0.05$, compared with the naive group, and (d) bars show the absorbed HRP levels in the mouse intestinal tissue. Data are expressed as mean $\pm$ SD. * $P < 0.05$, compared with the Ag group (naive, mice with no treatment; sham, mice treated with sham stress; Ag, mice were treated with HRP gavage alone; TS-Ag, mice were exposed to thermal stress and HRP gavage concurrently; IL-12, mice were pretreated with IL-12 and then treated with thermal stress and HRP gavage concurrently).

significantly increased HRP absorption in TS-Ag and IL-12 groups as compared with Ag group and sham group (Figs 1d,3d).

Thermal stress attenuates the IL-12 expression in the intestinal DC

In the present study, the intestinal sensitization was observed in the mice exposed to thermal stress and oral Ag, but not in the mice exposed to oral Ag alone. A possible mechanism of this phenomenon is that thermal stress affects Th1/Th2 function. IL-12 plays a critical role in facilitating Th1 cell development and inhibits Th2 responses.³⁵ As expected, reduction of IL-12

mRNA expression in the separated DC was observed in the TS-Ag and TS mice at both IL-12p35 (Fig. 4a) and IL-12p40 (Fig. 4b) subunits. IL-12p70 protein was also significantly suppressed in TS and TS-Ag groups (Fig. 4c). Pretreated with extrinsic IL-12, the mice did not show any sign of intestinal sensitization although they underwent the same procedures as the mice of TS-Ag mice did.

Thermal stress modulates the Th1/Th2 functions in the intestinal mucosa

After the thermal stress treatment, the intestinal CD4⁺ T cells were separated and cultured in the presence of the specific Ag

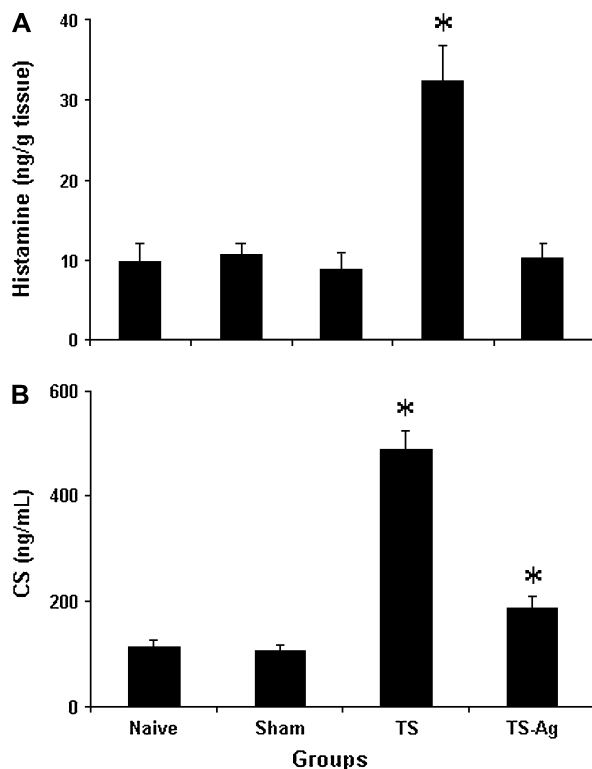


Figure 2 (a) Histamine in the intestine. Histamine levels were measured in the intestinal tissue after the challenge with horse-radish peroxidase (HRP) gavage. (b) Serum corticosterone (CS) levels. Bars show the intestinal histamine levels (mean $\pm$ SD). * P < 0.05, compared with the naive group.

HRP for 4 days. Th2 cytokine IL-4 and Th1 cytokine IFN- γ were detected in the supernatants. TS-Ag mice showed significant increase in IL-4 (Fig. 5a) and a decrease in INF- γ (Fig. 5b) compared with the other four groups. The results of [^{3} H]thymidine incorporation assay showed that the Ag-specific CD4 $^+$ T-cell proliferation enhanced significantly in the TS-Ag mice in response to the specific Ag HRP challenge, whereas the intestinal CD4 $^+$ T cells in the Ag mice did not show much difference from the naive control mice (Fig. 5c).

Thermal stress inhibits T-bet and increases (GATA) $_3$ expression in the intestinal CD4 $^+$ cells

To identify the potential molecular basis of the impaired Th1 response in thermal stress, we examined the levels of T-bet and (GATA) $_3$ in the separated CD4 $^+$ cells from the thermal stress treated mice (Fig. 6). Baseline expression levels of T-bet, (GATA) $_3$ were detected in the isolated CD4 $^+$ T cells; following stimulation with thermal stress, a significant impairment in the induction of T-bet (3.67-fold reduced) and an increase in (GATA) $_3$ (2.1-fold increased) was observed. No significant changes were found in the groups of TS, sham, Ag and IL-12.

Thermal stress increases CS secretion

Serum CS levels were determined by immunoassay. As depicted in Figure 2(b), thermally stressed mice showed significantly

higher serum CS levels in the TS group. Although the TS-Ag group showed much lower CS levels than the TS group, it was significantly higher than naive controls and sham-stressed groups.

Discussion

The main results of this study include the one that the ambient thermal stress can induce intestinal barrier dysfunction and compromise intestinal Th1/Th2 balance by a mechanism of suppressing of IL-12 production in the intestinal DC. The results show that Ag absorption alone is insufficient to initiate sensitization in the intestine; an accessory factor (s) that can drive intestinal aberrant Th2 polarization or/and suppress Th1 function is required.

One of the prerequisites of the induction of intestinal sensitization is the intact Ag absorption. The presence of food Ag in the lamina propria has the potential to interact with immune cells to induce inappropriate immune reactions in the intestine. The results show that thermal stress significantly increases mouse intestinal epithelial barrier permeability and luminal Ag absorption, which is consistent with previous studies. Prosser *et al.* reported that ^{51} Cr EDTA absorption increased significantly when rats were heated to 41.5°C.³⁶ Lambert *et al.* has observed that hyperthermia increases intestinal permeability and induces intestinal epithelial damage.³⁷ The mechanism of thermal stress disrupting the intestinal barrier function may be that the thermal stress induces intestinal vasoconstriction;³⁸ the reduction of blood supply in the intestine induces insufficient tissue oxygen delivery³⁸ and the hypoperfusion of the intestine damages the intestinal barrier function and increases epithelial barrier permeability.³⁹

The production of Ag-specific IgE is the gold standard to determine whether a subject has been sensitized to specific Ag. The results show that the TS-Ag mice produced high levels of serum-specific IgE. It is the evidence that these mice had been sensitized to the Ag HRP. The sensitized status in the mice is also confirmed by the mast cell activation manifesting extensive degranulation and histamine release in response to specific Ag challenge (Figs 2,3). Even under the physiological conditions, a small quantity of intact protein Ag is absorbed by the intestinal mucosa. These proteins are supposed to be harmless to the body.⁴⁰ We have also found that a small volume of HRP can be absorbed, as shown by naive control mice in the present study and by the rats in the previous experiments.^{20,21} Thus, it is possible that the absorbed Ag alone are insufficient and other factors are required to act synergistically in inducing intestinal sensitization. That the Ag group mice were also introduced HRP, in the same way as the TS-Ag group mice did, but none of the mice in Ag group was sensitized, indicates that the thermal stress plays a critical role in the development of the intestinal sensitization in this model.

IL-12, produced by DC, is an important counter-regulatory cytokine for the development of the Th2 response. Consistent with previous reports, the present results show that thermal stress significantly suppresses IL-12 production in the intestine.¹⁶ This event may be critical in the subsequently induced intestinal sensitization because inhibition of IL-12 production suppresses the Th1 response.⁴¹ The report that intranasally given murine IL-12p70 naked DNA expression plasmids

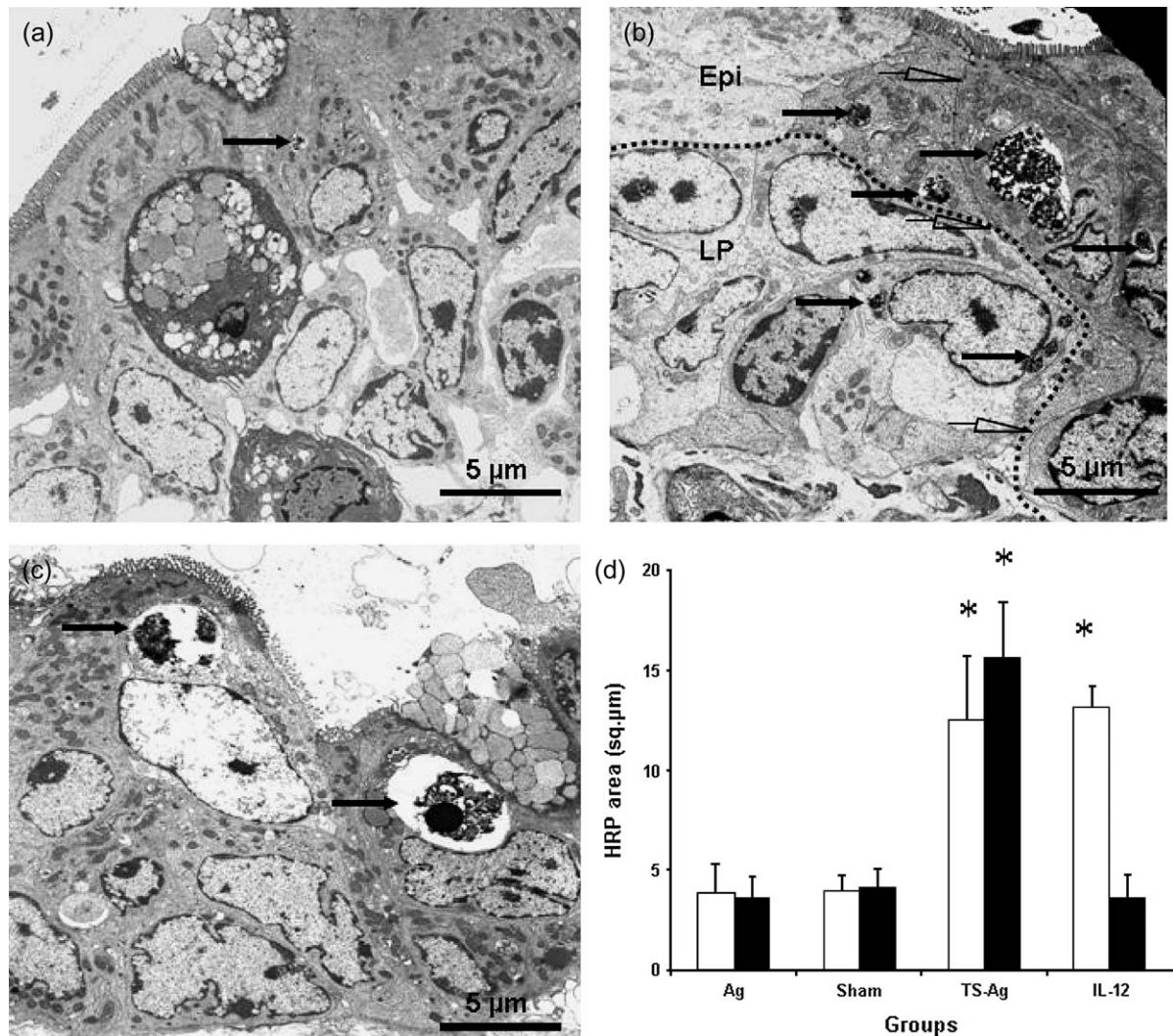


Figure 3 Antigen absorption in the intestinal mucosa. Representative electron photomicrographs of mouse intestinal epithelial cells were taken with electron microscopy. (a) Intestinal epithelium from the mice treated with horseradish peroxidase (HRP) alone. A HRP endosome (arrow) is located in the apical region of the epithelial cell. (b) Intestinal mucosa of Peyer's patch from the mice treated with thermal stress and HRP gavage. A number of HRP endosomes (arrows) are located in the intestinal epithelial cells and paracellular spaces as well as in the lamina propria. The broken line was drawn by hand indicating the basement membrane. Ep, epithelium; LP, lamina propria. (c) Two weeks after the concurrent treatment with thermal stress and HRP gavage, the mice were challenged with oral Ag HRP. HRP endosomes of large size are located in the intestinal epithelial cells. (d) Photomicrographs of the intestinal mucosa were randomly selected and taken (30 photographs per group). HRP endosome area in the photographs was quantitatively evaluated with a computerized image processing system. Bars represented the average HRP endosome area per sample image (300 μm^2). Data were presented as mean $\pm$ SD. * $P < 0.05$, compared with the group treated with HRP gavage alone. $\square$, 1st: 1 h after the first treatment; $\blacksquare$, 2nd: mice were re-challenged with oral HRP 14 days after the first treatment.

successfully prevent mice from the OVA-induced intestinal sensitization further confirms the important role of IL-12 in intestinal immune regulation.¹⁷ Two subunits of IL-12 have been identified, the IL-12p35 and IL-12p40. It has also been reported that IL-12p40 has an inhibitory effect on IL-12p70 expression to promote intestinal allergy development whereas our results show that thermal stress inhibits both IL-12p35 and IL-12p40 production resulting in intestinal sensitization in the presence of Ag OVA.⁴² Although the difference between the studies may be because of the different experimental system used, it implicates that IL-12 is involved in more compli-

cated mechanisms in the intestinal immune regulation. The mechanism of IL-12 suppression after thermal stress may be that thermal stress increases cortisol secretion⁴³ that is capable of inhibiting DC producing IL-12.⁴⁴ The present data also showed that CS levels significantly increased after thermal stress treatment.

Driving naive CD4⁺ T cells to become Th1 cells requires the synergistic action of multiple factors. IL-12-activated STAT-4 signalling plays a critical role in inducing IFN- γ gene transcription, triggers Th1 cell differentiation and activation of STAT-1; induction of T-bet plays a decisive role in expanding

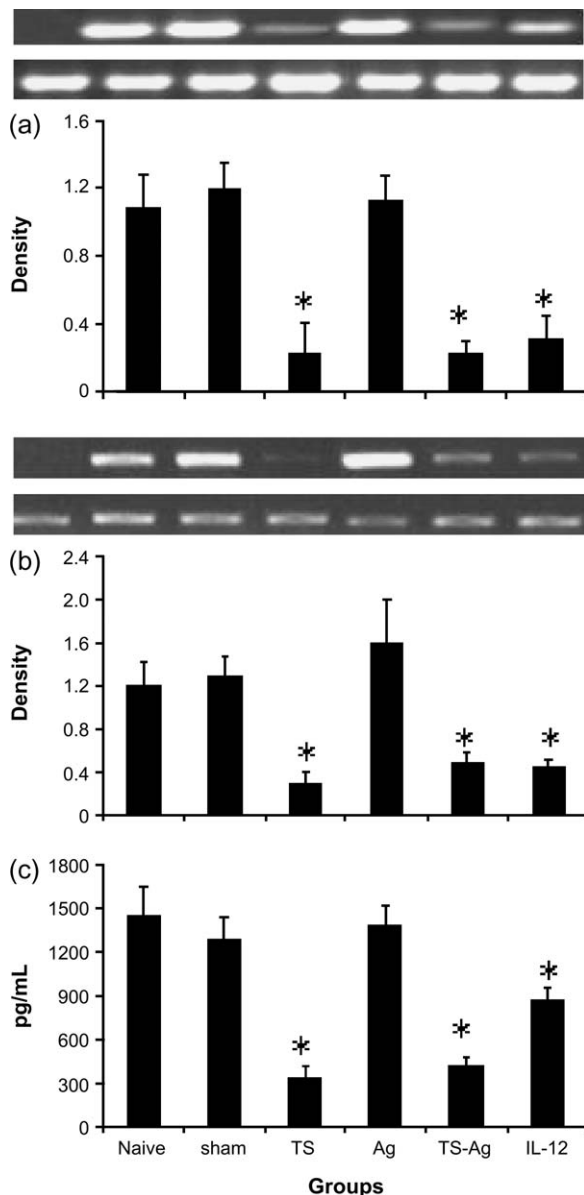


Figure 4 IL-12 expression in the intestinal dendritic cells (DC). Intestinal DC were separated and cultured in the presence of LPS overnight. IL-12 mRNA was evaluated with RT-PCR; IL-12 protein was evaluated with ELISA. (a) IL-12p35 mRNA. Upper rows: bands of IL-12p35 (a) and IL-12p40 (b); lower rows: bands of G3PDH. The first spots on upper rows are negative controls that were omitted in the reverse transcriptase. (b) IL-12p40 mRNA. Bars show band density that was normalized with the band density of G3PDH. (c) IL-12p70 protein in the supernatant was evaluated with ELISA. Bars show IL-12p70 levels. Data are expressed as mean $\pm$ SD. Each datum is from six separate experiments. * $P < 0.05$, compared with naive group.

and sustaining Th1 cell responses. Consistent with the existing theory, the present study shows that inhibition of IL-12 further results in suppression of Th1-associated transcription factor T-bet expression and IFN- γ production in the murine intestine.

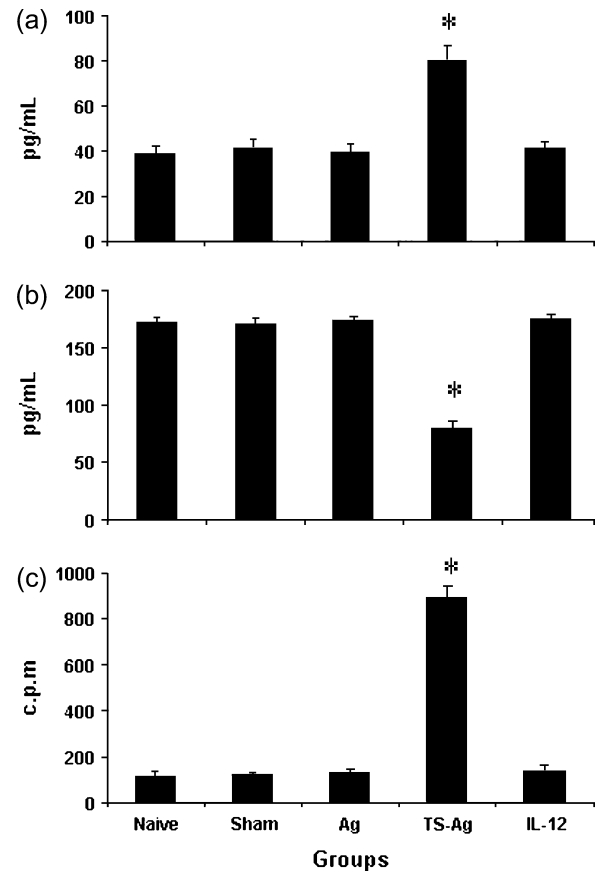


Figure 5 Ag-specific T-cell function. Intestinal CD4⁺ T cells were separated and cultured in the presence of horseradish peroxidase (HRP). Levels of IL-4 and IFN- γ in the supernatants of the CD4⁺ T cells were measured with ELISA. Bars show the levels of IL-4 (a) and IFN- γ (b) in the supernatant. (c) Freshly separated intestinal CD4⁺ cells were cultured in the presence of the specific Ag HRP. [³H]thymidine incorporation experiments were carried out to determine the Ag-specific T-cell proliferation. Bars show [³H] counts per minute (c.p.m.) of the CD4⁺ cells cultured in the presence of the specific Ag HRP. Data are expressed as mean $\pm$ SD from six separate experiments. * $P < 0.05$, compared with the naive controls.

The existence of certain levels of IL-12 in the tissue is required in maintaining Th1/Th2 balance.⁴⁵ A question here is whether the suppression of IL-12 expression induces the aberrant Th2 polarization in the intestine and further develops the intestinal sensitization. However, it is unlikely that the thermal stress suppressed Th1 cell reaction promotes Th2 cell differentiation naturally, given that there is no change in IL-4 synthesis and STAT-6 activation. The supportive evidence provided by the present results is that the mice that are treated with thermal stress alone have not developed intestinal sensitization.

Consistent with clinical findings in the patients with food allergy, we have also determined that the Ag-specific T-cell clones in the intestinal lamina propria of the TS-Ag mice.⁴⁶ T-cell clone expansion in response to the specific Ag stimulation has been also observed. These T-cell clones are supposed to be the Ag-specific T memory cells, which can be activated

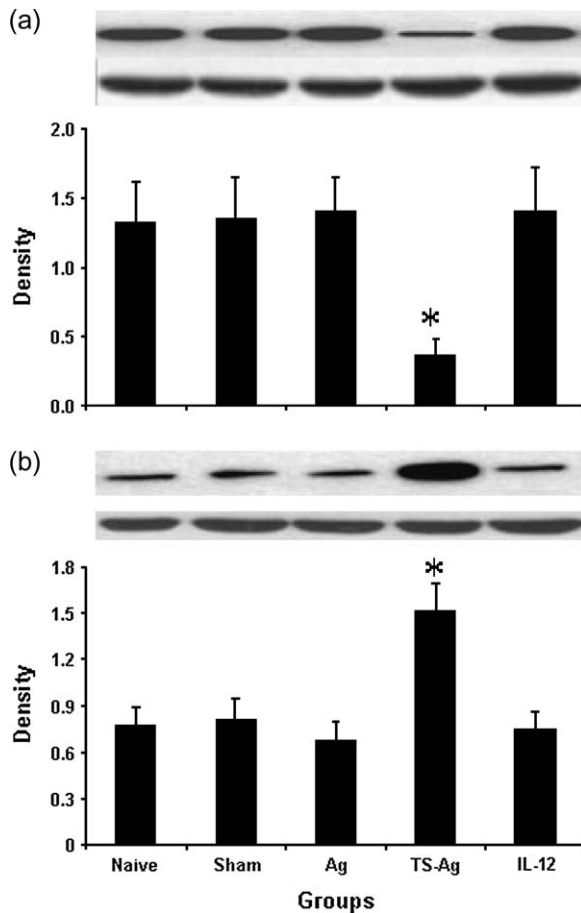


Figure 6 Th1/Th2 transcription factor expression in the CD4⁺ T cells. Separated intestinal CD4⁺ T cells were cultured in the presence of specific Ag horseradish peroxidase. T-box expressed in T cells (T-bet) (a) and GATA binding protein (GATA)-3 (b) protein expression was evaluated with western blot in the extracted nuclear proteins from the cultured cells. The upper rows of the bands are T-bet (a) or GATA-3 (b) protein; the lower rows of bands are β -actin protein. Bars show the normalized band density that is expressed as mean $\pm$ SD from six separate experiments. * $P < 0.05$, compared with the naive controls.

on re-exposure to specific Ag and are the 'root' of allergic reactions.⁴⁷

As T-bet and (GATA)₃ are considered to be counter-regulators in Th1/Th2 reactions, we examined both T-bet and (GATA)₃ expression in isolated CD4⁺ T cells in parallel. The results show that thermal stress suppresses the T-bet expression and increases the (GATA)₃ expression in these cells. A possible mechanism may be that thermal stress down-regulates the IL-12 expression in the DC; lacking IL-12 stimulation, the T-bet expression in CD4⁺ T cells is inactivated. A previous study also indicates that IL-12 is a critical factor in upregulating T-bet expression.⁴⁸ T-bet has been shown to inhibit (GATA)₃ expression.⁴⁹ The suppression of T-bet expression might enhance (GATA)₃ expression. This is in accordance with previous report; Nakamura and Hoshino has determined that (GATA)₃ expression is highly upregulated in patients with airway allergy.⁵⁰

Aberrant Th2 polarization is one of the main features of the pathogenesis of allergic diseases. The present data show that the isolated intestinal CD4⁺ T cells consist of more Th2 cells than Th1 cells and higher Th2 cytokine production in the TS-Ag mice. These features could attribute to the thermal stress induced IL-12 suppression and the subsequently induced T-bet inhibition and (GATA)₃ upregulation. Similar phenomena have been found to occur in the CD4⁺ T cells isolated from the patients with allergic disorders.⁵¹ Following Th2 cytokine overproduction, it can be inferred that Ag-specific IgE synthesis by the B cell will be increased. The present data have confirmed that there are HRP-specific IgE in the mice treated with thermal stress and HRP gavage. These Ag-specific IgE antibodies may bind to the high-affinity IgE receptors on mast cells, leading to the local tissue sensitization.⁵¹ This speculation is supported by the present results that the intestinal mast cells were activated on the specific Ag HRP challenge (Figs 1,2).

In summary, the mice exposed to thermal stress and Ag HRP concurrently became sensitized that are characterized by the results that (i) inhibition of IL-12 expression in the intestinal DC; (2) intestinal CD4⁺ T cells express less T-bet and more (GATA)₃; (3) more Th2 cells and cytokines, less Th1 cells and cytokines were determined in the isolated intestinal CD4⁺ T cells; (iv) high titres of serum HRP-specific IgE antibodies were detected in the mice exposed to thermal stress and HRP gavage; (v) the intestinal mast cells became activated in response to the HRP challenge; and (vi) none of the mice treated with thermal stress alone or HRP gavage alone showed any signs of sensitization. The present study shows that although Ag absorption is essential, other accessory factors that can disturb the immune homeostasis in local tissue are required for the development of sensitization.

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