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Oolong tea is useful for lens cleansing in transnasal small-caliber esophagogastroduodenoscopy

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Background and study aims: Unsedated transnasal small-caliber esophagogastroduodenoscopy (EGD) has been used to examine the upper gastrointestinal tract with proven feasibility and tolerability. However, a limitation of transnasal EGD is the poor lens-cleansing function of the scope due to the small-caliber water-jet nozzle. Therefore, this trial was designed to evaluate the cleansing effect of oolong tea for transnasal small-caliber EGD.

Patients and methods: Oolong tea (O), barley tea (B), and distilled water (W) were prepared as washing solutions for endoscopic lenses. Study I: after the lenses were soiled by lard oil, they were washed with one of the three washing solutions, and the image quality of photographs was judged. Study II: 982 patients who were due to undergo transnasal EGD were enrolled and randomly assigned to the O-, B-, or W-groups. The level of lens cleansing, the overall time required for endoscopy, and the volume of washing solution used were measured.

Results: Study I: the image quality of photographs taken with lenses washed with oolong tea was significantly superior to that associated with other solutions. Study II: the level of lens cleansing in the O-group was significantly superior to that of the B- and W-groups ($P < 0.001$). The volume of solution used for lens cleansing in the O-group was significantly smaller than that in the W-group ($P < 0.05$). Endoscopic examination times in the O-group were shorter than those in the B- and W-groups ($P < 0.05$).

Conclusions: In transnasal small-caliber EGD, oolong tea instead of water as a washing solution for endoscopic lens cleansing is useful to maintain good visibility.

Introduction

Esophagogastroduodenoscopy (EGD) has been widely recognized as a screening modality for the diagnosis of upper gastrointestinal disease. Transnasal EGD with a small-caliber endoscope has been reported to decrease choking sensations and gagging episodes [1–4], and to be less stressful to the cardiovascular system compared with EGD by conventional transoral procedures for unsedated patients [5–9]. Therefore, transnasal EGD has become widely used in clinical practice instead of conventional transoral EGD. However, poorer optical quality due to the limited size of the charge-coupled device is one of the limitations of the small-caliber endoscope. Moreover, weak spraying of washing solution and aspiration of fluid contents related to the small diameter of the water-jet nozzle result in an inferior lens-cleansing function [10–13], which may prolong endoscopic examination time, increase stress both in patients and endoscopists, and possibly result in poor diagnostic accuracy [2, 14–17]. Recently, it has been reported that the saponins present in oolong tea show surfactant actions, resulting in a good cleansing effect of oil soiling [18, 19]. Therefore, oolong tea has recently been tested as a possible lens-cleansing solution in endoscopic procedures [20]. However, there is still little information on the effect of oolong tea for EGD, though it may improve image quality in small-caliber endoscopy. To evaluate the efficacy of oolong tea as a lens-washing solution for transnasal EGD with a small-caliber endoscope, we performed basic and clinical studies.

Patients and methods

A basic in vitro study (Study I) and a randomized prospective clinical study (Study II) were carried out to evaluate the efficacy of oolong tea for cleansing the lens of small-caliber endoscopes used in transnasal EGD.

Study I

The cleansing effect on oil-soiled lenses of small-caliber endoscopes was evaluated by examining the image quality of the endoscopic photographs after endoscopic lens-cleansing procedures. Three washing solutions, oolong tea (O-group), barley tea (B-group), and distilled water (W-group), were used for two kinds of endoscopes, the GIF-XP260N (Olympus Medical Systems Co., Tokyo, Japan) and the EG-530N (Fujinon Toshiba ES Systems Co., Ltd., Tokyo, Japan). The distal end diameters of the scopes were 5.0 mm and 5.9 mm, respectively. The diameter of the forceps channel was 2.0 mm in both endoscopes. The tip of the endoscope was immersed in 37 °C lard oil for 3 seconds, and photographs of the test chart were taken after a lens-cleansing procedure that consisted of injection of air and subsequent washing solution from the endoscopic jet nozzle for 3 seconds each. We selected lard oil as the soiling material because of its turbid and sticky characteristics. The photographs were taken after every soiling and cleansing. Twenty soiling and cleansing procedures of the lenses of two kinds of endoscopes were repeated in three kinds of cleansing solutions. The quality and clarity of twenty photographs taken after the use of each cleansing solution were evaluated. Photographs were taken at a distance of 2 cm from the chart. The image quality of these photographs was evaluated by expert endoscopists who had no prior knowledge of the washing solution used. The quality of lens cleansing was categorized into five levels: level 5 (all of the field is clear), level 4 (between levels 5 and 3), level 3 (approximately 80% of the field is clear), level 2 (between levels 3 and 1), level 1 (over 50% of the field is unclear). Representative photographs of each group are shown in ● Fig. 1.

Three endoscopic lens-cleansing solutions were selected: ready-made oolong tea (Suntory oolong tea, Suntory Holdings Ltd., Osaka, Japan), barley tea (Ito En Ltd., Tokyo, Japan), and distilled water. These were placed in a covered washing solution tank by the medical support staff; endoscopists were blinded to the solutions used. A full tank of washing solution can be used for one day. The concentrations of oolong tea and barley tea used in this study did not allow endoscopists to discriminate the tea from distilled water in terms of its color during endoscopies.

Study II

The study for the clinical evaluation of the efficacy of oolong tea for lens cleansing was a randomized, controlled, prospective study. Patients (n = 982) who were due to undergo transnasal EGD for abdominal symptoms or for an annual medical check between July and December 2008 in Izumo City General Medical Center were enrolled. Patients who underwent upper gastrointestinal tract surgery, such as gastrectomy or esophagectomy, were excluded from the present study. Patients were randomly assigned to one of six groups: the O-, B-, and W-groups with either the GIF-XP260N or EG-530N endoscope. The following three aspects of the endoscope study were evaluated: the total procedure time required for the endoscopic examination, the volume of washing solution used during the examination, and the cleansing level of the endoscopic lens. The volume of washing solution used during the examination was measured after the endoscopic study by measuring the volume of washing solution remaining in the bottle. Immediately after EGD, the endoscopists judged the level of lens cleansing during the procedure. The lens-cleansing results were categorized into five levels: level 5 (fully satisfied with the quality of the image), level 4 (between levels 5 and 3), level 3 (possible to diagnose but with some difficulties), level 2 (between levels 3 and 1), level 1 (impossible to provide an accurate diagnosis). All transnasal EGD procedures were performed by three expert endoscopists with a board certification from the Japan Gastroenterological Endoscopy Society. The present study was a double-blind study, as the identity of the washing solution, oolong tea, barley tea or water, was concealed from the endoscopists as described for Study I.

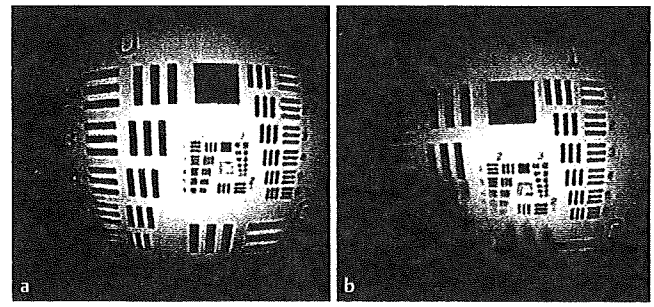

Fig. 1 Photographs of the test chart with the GIF-XP260N endoscope, the lens of which was soiled by lard oil, taken after 20 procedures of lens cleansing. **a** Level 4 lens cleansing. **b** Level 2 lens cleansing.

Nasal anesthesia was carried out by spraying a solution of 0.4% lidocaine/0.5% naphazoline into the nostril. The patients were then instructed to inhale 2% lidocaine jelly (Xylocaine jelly, AstraZeneca Co., Osaka, Japan), followed by the insertion of a 20-Fr or 18-Fr catheter covered with lidocaine jelly to the deeper nasal cavity for 5 minutes. All EGDs were done with the patient in the left lateral recumbent position with neither the administration of scopolamine butylbromide nor glucagon. The enrolled patients did not receive any premedication or sedation before or during endoscopy.

This prospective randomized study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Izumo City General Medical Center. Written informed consent was obtained from all participants.

Statistical analysis

The primary outcome parameter in Study II was the amount of washing solution used during endoscopic procedures. In a pilot study using a XP260N endoscope, the amount of washing water used in one endoscopic study was 20.8 ± 9.0 mL (mean ±SD, n = 30). A 20% decrease in requirement for washing solution (4 mL) was considered to be a clinically relevant difference. The sample size of at least 130 patients per group was calculated to be necessary for a statistically significant difference between groups when a test power was over 80% at the 0.05 significant level in a two-tailed test.

Statistical analysis of the comparative study for each group was investigated with the chi-squared, Mann–Whitney U, and Kruskal-Wallis rank tests. The analysis by the latter test was done only when the Friedman test showed significant differences. Categorical data were compared with either Student's *t*-test or Welch's test. When unequal variances were found in the analyzed data, a significant difference was statistically calculated by Welch's test instead of Student's *t*-test. *P*-values of less than 0.05 were considered to be significant. All statistical analyses were performed using Statistical Analysis Software (SPSS, version 12.0 for the PC, SPSS Japan Inc., Tokyo, Japan).

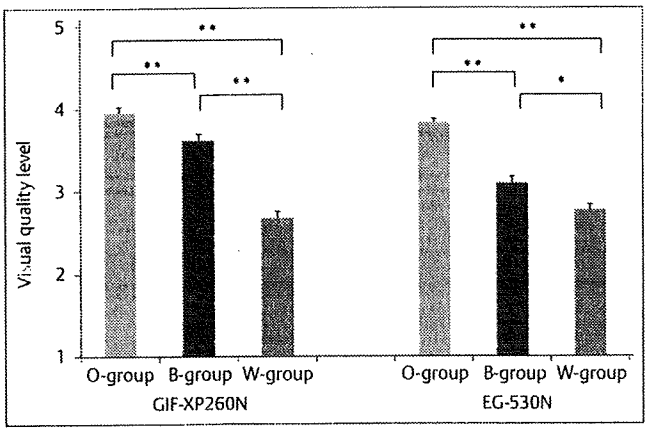

Fig. 2 In the study using GIF-XP260N and EG-530N endoscopes, the effects of cleansing on the image quality of a lard oil-soiled lens are shown. The cleansing effect of oolong tea (O-group) was statistically superior to barley tea (B-group) and distilled water (W-group). Barley tea was statistically superior to distilled water.*$P < 0.01$; **$P < 0.001$. Bars show standard errors.

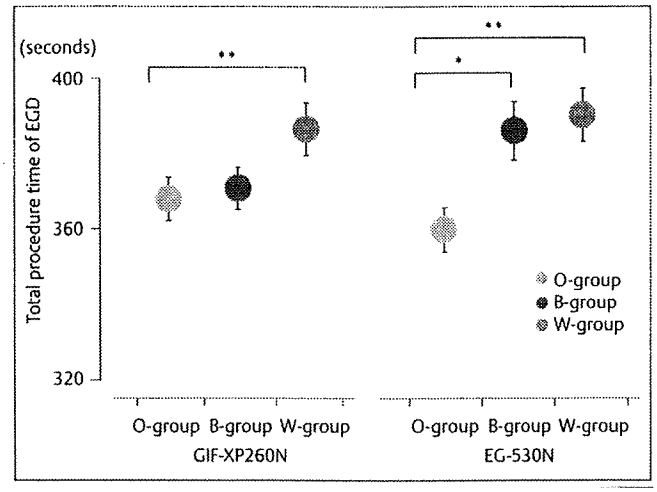

Fig. 3 The time required for the total esophagogastroduodenoscopy procedure. With both GIF-XP260N and EG-530N, the effect of the oolong tea (O-group) was statistically significantly different from that of water (W-group). With EG-530N, the oolong tea was statistically significantly different from the barley tea (B-group). *$P < 0.05$. **$P < 0.01$. Bars show standard errors.

Results

Study I

The results of the in vitro study on the cleansing effects on the endoscope lens after lard oil soiling are shown in ○ **Fig. 2**. The image quality of the photographs taken by GIF-XP260N and EG-530N was consistently better in the O-group than in the other groups ($P < 0.001$). The B-group also showed a statistically better cleansing effect than the W-group ($P < 0.01$) for both endoscopes.

Study II

Characteristics of the enrolled patients in each group are shown in ○ **Table 1**. The O-, B-, and W- GIF-XP260N groups contained 162, 155, and 160 patients, respectively, and the O-, B-, and W-EG-530N groups contained 165, 172, and 168 patients, respectively. No statistically significant differences were found among groups for categorical data such as age, sex, or number of cases of biopsy or chromoendoscopy. In this study, no critical complications or difficulties with endoscopic systems were experienced. Moreover, no adverse interaction was found between oolong or barley tea and the disposable materials, such as the biopsy forceps and the spraying catheter.

The total procedure time for the endoscopic examination of each group is shown in ○ **Fig. 3**. The times necessary for EGD with GIF-XP260N (mean value ± SE) were 368.1 ± 5.7, 370.9 ± 5.6, and 386.8 ± 7.1 seconds in the O-, B-, and W-groups, respectively; those with EG-530N were 360.1 ± 6.0, 386.3 ± 7.8, and 390.5 ± 7.1 seconds, respectively. There was a statistically significant difference in the procedure time between the O- and W-groups with both kinds of endoscopes.

The volumes of washing solution used for lens cleansing in each group with both kinds of endoscopes are shown in ○ **Fig. 4**. The volumes (mean value ± SE) were 11.8 ± 0.6, 13.0 ± 0.6, and 20.4 ± 1.9 mL in the O-, B-, and W-groups with GIF-XP260N, respectively; with EG-530N, the volumes were 12.0 ± 0.7, 12.5 ± 1.0, and 13.4 ± 0.9 mL, respectively. With GIF-XP260N, the volumes used in the O- and B-groups were significantly smaller than the volume of water used in the W-group ($P < 0.05$). However, there were no significant differences between the three kinds of washing solutions with EG-530N.

The levels of lens cleansing are shown in ○ **Fig. 5**. Mean satisfactory levels with GIF-XP260N (mean value ± SE) were 3.7 ± 0.1, 2.9 ± 0.1, and 2.5 ± 0.1 in the O-, B-, and W-groups, respectively; with EG-530N, they were 4.1 ± 0.1, 2.7 ± 0.1, and 2.6 ± 0.1, respectively. With both types of endoscope, the lens-cleansing level in the O-group was statistically significantly superior to that in the remaining two groups ($P < 0.001$). With GIF-XP260N, the level in the B-group was also significantly superior to that in the W-group ($P < 0.05$).

Table 1 Characteristics of the enrolled patients in Study II.

	GIF-XP260N			EG-530N		
	Oolong tea (n = 162)	Barley tea (n = 155)	Distilled water (n = 160)	Oolong tea (n = 165)	Barley tea (n = 172)	Distilled water (n = 168)
Age, years	54.7 ± 15.2	58.8 ± 14.5	56.4 ± 13.5	58.9 ± 15.4	58.1 ± 14.7	56.4 ± 14.4
Sex						
Male	71 (43.8)	67 (43.2)	78 (48.7)	72 (43.6)	105 (61.0)	81 (48.2)
Female	91 (56.2)	88 (56.8)	82 (51.3)	93 (56.4)	67 (39.0)	87 (51.8)
Endoscopic procedures						
Biopsy, n (%)	13 (8.0)	21 (13.5)	20 (12.5)	27 (16.4)	29 (16.9)	21 (12.5)
Chromoendoscopy, n (%)	14 (8.6)	30 (19.4)	25 (15.6)	36 (21.8)	35 (20.3)	40 (23.8)
Past history of gastrectomy, n (%)	1 (0.6)	8 (5.1)	3 (1.9)	7 (4.2)	4 (2.3)	2 (1.2)

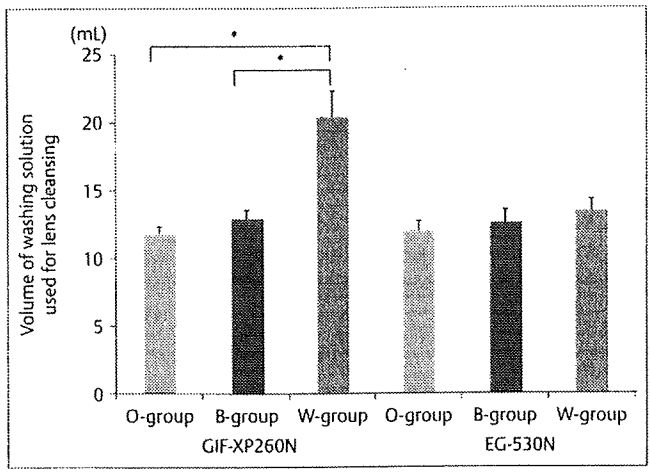

Fig. 4 The solution volume used for lens cleansing during the total esophagogastroduodenoscopy procedure. With GIF-XP260N, the volumes of oolong (O-group) and barley (B-group) tea used were significantly smaller than the volume of distilled water used (W-group) ($P < 0.05$), although no differences were found with EG-530N. *$P < 0.05$. Bars show standard errors.

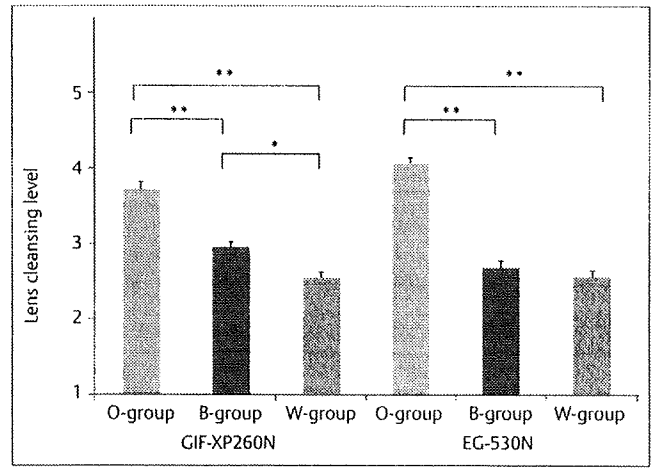

Fig. 5 The level of lens cleansing at esophagogastroduodenoscopy. The cleansing level of oolong tea (O-group) was significantly higher than that of the barley tea (B-group) and distilled water (W-group) with both types of endoscopes ($P < 0.001$). With GIF-XP260N, barley tea also showed a significantly higher level than distilled water ($P < 0.05$). *$P < 0.05$; **$P < 0.001$. Bars show standard errors.

Discussion

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources. They are also found in particular abundance in various plant species, for example, some kinds of tea, including oolong tea. They are phenomenologically classified into an amphipathic glycosides group and produce soap-like foaming when shaken in aqueous solutions. They are structurally composed of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. Surfactants such as saponin are also contained in soaps and detergents, and help to clear oil-soiled clothes, kitchenware, etc. [21–23]. Oolong tea is rich in saponin and has been used for cleaning oil-soiled window glasses in Japan. Thus, oolong tea possesses a cleansing effect for oily soil based on its surfactant action.

Mucous soiling can be removed and cleansed by water jet from the endoscopic nozzle because mucus is hydrophilic. The oily soiling from residual food or body composition is difficult to remove by water jet alone. Therefore, we chose oily soiling but not mucous soiling in Study I. In the present in vitro study with an oily-soiled endoscope, we confirmed that oolong tea significantly improved the lens-cleansing effect of small-caliber endoscopes compared with barley tea, which contains a smaller amount of saponin, and distilled water. This effect of oolong tea is considered to be useful for clinical practice by transnasal small-caliber endoscopy.

In the clinical practice of transnasal EGD with a small-caliber endoscope, endoscopists have experienced some difficulties with longer examination times, a poor image quality or lens soiling, although patient tolerability and acceptability are proven. In particular, the time required for lens cleansing occupied a large part of the examination, and is often a source of irritation for the endoscopist. In the present clinical study, the use of oolong tea brought higher satisfaction with the level of lens cleansing. The superiority of oolong tea as a lens-washing solution is the most important observation in this study. The difference in procedure time and volume of washing solution may not be very relevant clinically. In our opinion, however, these are valid surrogate markers for a better endoscopic view provided by using oolong tea as a lens-washing solution. Also, no complications or difficulties were associated with the use of oolong tea in this study. Oolong tea contains caffeine, but cardiopulmonary adverse effects were not observed, as the volume of oolong tea used in each endoscopy examination was only 10–20 mL. Moreover, oolong tea jetted from the endoscopic nozzle was clear but not brownish in color, and it did not disturb the endoscopic observation. The concentrations of oolong tea and barley tea used in this study did not allow the endoscopists to discriminate oolong tea from barley tea or distilled water during the endoscopic procedures.

The influence of oolong tea on the endoscopic system and the instruments is not yet completely known. However, as oolong tea is a standard beverage supplied in a plastic container that can be stored for months, it is reasonable to assume that oolong tea will not have a negative impact on the endoscopic system or the instruments, especially after complete post-procedure washing. There was, indeed, no adverse effect on endoscopic systems or instruments during our 8-month study period.

In conclusion, oolong tea is superior to water as a washing solution for lens cleansing and helps to maintain good visibility during EGD with a transnasal small-caliber endoscope.

Competing interests: None

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Helicobacter pylori Promotes the Production of Thymic Stromal Lymphopoietin by Gastric Epithelial Cells and Induces Dendritic Cell-Mediated Inflammatory Th2 Responses▽

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Helicobacter pylori **colonizes the stomach and induces strong, specific local and systemic humoral and cell-mediated immunity, resulting in the development of chronic gastritis in humans. Although *H. pylori*-induced chronic atrophic gastritis is characterized by marked infiltration of T helper type 1 (Th1) cytokine-producing CD4$^+$ T cells, almost all of the inflamed gastric mucosae also contain focal lymphoid aggregates with germinal centers. In addition, typical *H. pylori*-induced chronic gastritis in children, called follicular gastritis, is characterized by B-cell follicle formation in the gastric mucosa. The aim of this study was to examine whether thymic stromal lymphopoietin (TSLP), an epithelial-cell-derived cytokine inducing a dendritic cell (DC)-mediated inflammatory Th2 response, is involved in Th2 responses triggering B-cell activation in *H. pylori*-induced gastritis. Here, we show that *H. pylori* triggered human gastric epithelial cells to produce TSLP, together with the DC-attracting chemokine MIP-3α and the B-cell-activating factor BAFF. After DCs were incubated with supernatants from *H. pylori*-infected epithelial cells, the conditioned cells expressed high levels of costimulatory molecules, such as CD80, and triggered naïve CD4$^+$ T cells to produce high levels of the Th2 cytokines interleukin-4 and interleukin-13 and of the inflammatory cytokines tumor necrosis factor alpha and gamma interferon. In contrast, after incubation of the supernatants with the neutralizing antibodies to TSLP, the conditioned DCs did not prime T cells to produce high levels of Th2 cytokines. These results, together with the finding that TSLP was expressed by the epithelial cells of human follicular gastritis, suggest that *H. pylori* can directly trigger epithelial cells to produce TSLP. It also suggests that TSLP-mediated DC activation may be involved in Th2 responses triggering B-cell activation in *H. pylori*-induced gastritis.**

Helicobacter pylori (*H. pylori*) infection in the stomach induces chronic gastritis associated with the development of peptic ulcer diseases, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma in humans (3, 8, 35). Although *H. pylori*-induced chronic atrophic gastritis is characterized by marked infiltration of T helper type 1 (Th1) cytokine-producing CD4$^+$ T cells (3, 8, 35), almost all of the inflamed gastric mucosae also contain focal lymphoid aggregates with germinal centers (5, 11). In addition, typical *H. pylori*-induced chronic gastritis in children, called follicular gastritis, is characterized by B-cell follicle formation in the gastric mucosa (10, 11, 34). Th2 responses triggering B-cell activation appear to be involved in the development of lymphoid aggregates with germinal centers. However, molecular mechanisms to induce Th2 responses triggering B-cell activation are not clear.

In humans, an epithelial-cell-derived cytokine, thymic stromal lymphopoietin (TSLP), activates CD11c$^+$ myeloid dendritic cells (DCs), and activated DCs strongly upregulate the expression of costimulatory molecules, such as CD80 and CD86 (23, 38, 43, 44). TSLP-activated DCs promote CD4$^+$ T cells to differentiate into inflammatory Th2 cells that produce interleukin-4 (IL-4), IL-5, IL-13, and tumor necrosis factor alpha (TNF-α) while downregulating IL-10 and gamma interferon (IFN-γ) (23, 44). Interestingly, TSLP primes DCs to produce large amounts of IL-12 following CD40 ligand stimulation. In addition, DCs activated with TSLP and CD40 ligand induce the differentiation of naïve CD4$^+$ T cells into effectors producing both Th1 and Th2 cytokines. These findings suggest that IL-12-mediated negative regulation of Th2 responses is not effective in TSLP-induced Th2 inflammation and that it leads to a mixed Th1 and Th2 profile (42).

Here, we show that *H. pylori* triggered human gastric epithelial cells to produce TSLP. Such cells produced a DC-attracting chemokine, macrophage inflammatory protein 3α (MIP-3α), and a B-cell-activating factor belonging to the TNF family (BAFF). After DCs were incubated with supernatants from *H. pylori*-infected epithelial cells, the conditioned cells expressed high levels of costimulatory molecules and triggered naïve CD4$^+$ T cells to produce IL-4 and IL-13 with the inflammatory cytokines TNF-α and IFN-γ. In addition, TSLP was expressed by the epithelial cells of human follicular gastritis. These results suggest that *H. pylori* can directly trigger epithelial cells to produce TSLP and that TSLP-mediated DC activation may be involved in Th2 responses triggering B-cell activation in *H. pylori*-induced gastritis.

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MATERIALS AND METHODS

***H. pylori* and *H. felis*.** *H. pylori* TN2GF4, isolated from a Japanese patient with a duodenal ulcer, was donated by M. Nakao (Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan). It was maintained as described previously (26). The inoculated *H. pylori* strain, TN2GF4, was CagA and VacA positive, as described previously (46). *Helicobacter felis* (ATCC 49179) was purchased from the American Type Culture Collection (Rockville, MD). The bacteria were grown in brucella broth at a titer of 1×10^8 organisms/ml. The bacterial suspension was stored at −80°C until it was used.

Gastric epithelial cell culture. Upon 80% confluence of the human gastric cancer cell line passages 20 to 30, AGS, MKN28, MKN45, MKN74, and KATOIII cells were trypsinized (trypsinethylenediaminetetraacetic acid; Gibco, Taastrup, Denmark). These cells were reseeded at 5.0×10^5 cells per well in six-well plates and maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Sigma, St. Louis, MO), penicillin G, and streptomycin (Gibco). Six hours after being seeded, the cells were washed with phosphate-buffered saline (PBS) and stimulated for 12 to 36 h in the presence of live *H. pylori* or *H. felis* at 1 cell per 150 bacteria or at the indicated cell/bacterium ratio. In some experiments, cells were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* (1 μg/ml; Sigma) and cultured in a Transwell (Corning, NY).

Real-time quantitative RT-PCR. Real-time quantitative reverse transcription (RT)-PCR was performed as described previously (45). Gastric epithelial cells were frozen in RNAlater (Qiagen, Valencia, CA) and stored at −80°C until they were used. Total RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative reactions were performed with an ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: GAPDH, 5′-CCACATCGCTCAGACACCAT-3′ and 5′-GGCAACAATATCCACTTTACCAGAGT-3′; TSLP, 5′-CCCAGGCTATTCGGAAACTCAG-3′ and 5′-CGCCACAATCCTTGTAATTGTG-3′; and BAFF, 5′-ACCGCGGGACTGAAAATCT-3′ and 5′-CACGCTTATTTCTGCTGTTCTGA-3′.

Cytokine production. After 24 h of culture of gastric epithelial cells under the conditions described above, culture supernatants were collected and analyzed with protein enzyme-linked immunosorbent assay (ELISA) kits for TSLP, MIP-3α, MIP-1α, MIP-1β, and monocyte chemoattractant protein 1 (MCP-1) (all from R&D Systems).

DC purification and culture. This study was approved by the Institutional Review Board for Human Research at the Graduate School of Medicine, Kyoto University. Peripheral blood mononuclear cells (PBMCs) were obtained from adult buffy coats of healthy donors (kindly provided by the Kyoto Red Cross Blood Center, Kyoto, Japan). $CD11c^+$ DCs were isolated from PBMCs as described previously (43). $CD11c^+$ lineage$^-$ cells were isolated with a FACS Aria (BD Biosciences, San Jose, CA) to >99% purity. $CD11c^+$ DCs were cultured immediately after being sorted in RPMI 1640 medium supplemented with 5% human AB serum (Sigma), penicillin G, streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate (Gibco BRL) (referred to as complete medium). Cells were seeded at a density of 1×10^6/ml in round-bottom 96-well plates in the presence of 15 ng/ml of TSLP (R&D Systems, Minneapolis, MN) or 50 μl of supernatant from the *Helicobacter*-colonized gastric epithelial cells. For neutralization of TSLP, supernatants from *H. pylori*-colonized gastric epithelial cells were incubated with 20 μg/ml of anti-human TSLP (R&D Systems). After 24 h of culture, viable DCs were counted by trypan blue exclusion of dead cells.

Analysis of cell surface markers of DCs. To determine the cell surface markers characteristic of activated DCs, DCs were incubated with various stimuli for 24 h. The DCs were subsequently stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (Immunotech). Finally, they were analyzed with a FACS Calibur (BD Biosciences).

DC–T-cell coculture. After 24 h of culture, $CD11c^+$ DCs were collected and washed three times to remove any cytokines. Viable DCs were counted by trypan blue exclusion of dead cells. $CD4^+$ $CD45RA^+$ naive T cells were isolated from PBMCs using a FACS Aria to reach >99% purity, as described previously (43). The remaining DCs were cocultured with 2.5×10^4 freshly purified allogeneic naive $CD4^+$ T cells in round-bottom 96-well culture plates in complete medium. The cells were cultured in triplicate at a DC/T-cell ratio of 1:5. After 7 days of culture, viable cells were counted by trypan blue exclusion of dead cells.

T-cell cytokine production. DC-primed $CD4^+$ T cells were collected on day 7 of the coculture, washed twice, and restimulated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) plus 2 μg/ml ionomycin (Sigma) in flat-bottom 96- or 48-well plates at a concentration of 1×10^6 cells/ml. After 3.5 h, brefeldin A (Sigma) was added at 10 μg/ml. After 2.5 h, cells were collected and stained for cell surface molecules. The cells were fixed and permeabilized using a Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, An Der Grub, Austria) and stained with phycoerythrin-conjugated monoclonal antibodies (MAbs) to IL-4, IL-13, TNF-α, and FITC-conjugated anti-IFN-γ (all from eBioscience). The stained cells were analyzed on a FACS Calibur.

Gastric mucosa samples. Samples of mucosa in biopsy specimens were obtained from inflamed gastric mucosae in three patients with *Helicobacter*-induced follicular gastritis (male/female ratio, 1/2; mean age [range], 37.3 [32–44] years). Samples of healthy controls were taken from three patients with duodenal ulcers (male/female ratio, 2/1; mean age [range], 37.7 [35–43] years) in whom the absence of inflammation had been histopathologically confirmed.

Histological and immunohistological analyses. Samples of mucosa in biopsy specimens were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin for histopathology. Fluorescence immunohistology was performed on frozen sections as described previously (18, 43, 45). In brief, 6-μm sections were cut from tissue blocks of frozen mucosal samples onto glass slides. The sections were air dried for 30 min, fixed in acetone for 5 min, and blocked with phosphate-buffered saline containing 10% nonfat dried milk for 30 min. The sections were stained with anti-human TSLP (43, 45), anti-CD11c (BD Pharmingen), or anti-DC-LAMP (Immunotech) antibodies (Abs) for 1 h, followed by staining using FITC-conjugated anti-immunoglobulin Abs. After the final wash, the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope.

Statistical analysis. Statistical analysis was performed by the Student *t* test for pairwise comparisons and analysis of variance with the Tukey-Kramer test for multiple comparisons. *P* values below 0.05 were considered significant.

RESULTS

***H. pylori* colonization induces TSLP expression in human gastric epithelial cells.** To test whether *H. pylori* colonization can induce expression of TSLP in gastric epithelial cells, various human gastric epithelial cell lines, such as AGS, MKN28, MKN45, MKN74, and KATOIII cells, were cultured for 24 h with *H. pylori*. Expression levels of mRNA encoding TSLP were measured using real-time quantitative RT-PCR. In contrast to the gastric epithelial cells not exposed to *H. pylori*, gastric epithelial cells—MKN28, MKN45, and MKN74—upregulated TSLP expression after *H. pylori* colonization (Fig. 1A). TSLP expression enhanced by *H. pylori* colonization was detectable when we used low epithelial cell/bacterium ratios (e.g., 1 cell per 70 to 100 bacteria) (Fig. 1B); it reached a maximal level at 1 cell per 150 to 200 bacteria and did not increase at ratios of 1 cell per 300 bacteria or higher. Because 150 to 200 bacteria can actually adhere to gastric epithelial cell membranes (6), the condition of epithelial cells expressing TSLP by *H. pylori* might be similar to mucosal lesions of *H. pylori*-infected gastritis patients. Next, we investigated the time course of *H. pylori*-induced TSLP expression (Fig. 1C). Upregulation of TSLP expression was induced after 12 h of *H. pylori* colonization and was sustained after 24 h of colonization.

Direct contact of *H. pylori* triggers human gastric epithelial cells to produce TSLP. *H. felis* is a gastric *Helicobacter* that colonizes the stomachs of laboratory mice, dogs, and cats and can induce active chronic gastritis that mimics the pathological features observed in *H. pyroli*-induced gastritis in humans (4, 9, 19, 20, 29). Next, we examined whether *H. felis* colonization can induce expression of TSLP in gastric epithelial cells. MKN28 and MKN45 cells were cultured for 24 h with *Helicobacter* bacteria, and the expression levels of mRNA encoding TSLP were measured using real-time quantitative RT-PCR. TSLP cytokine production in the culture supernatant was as-

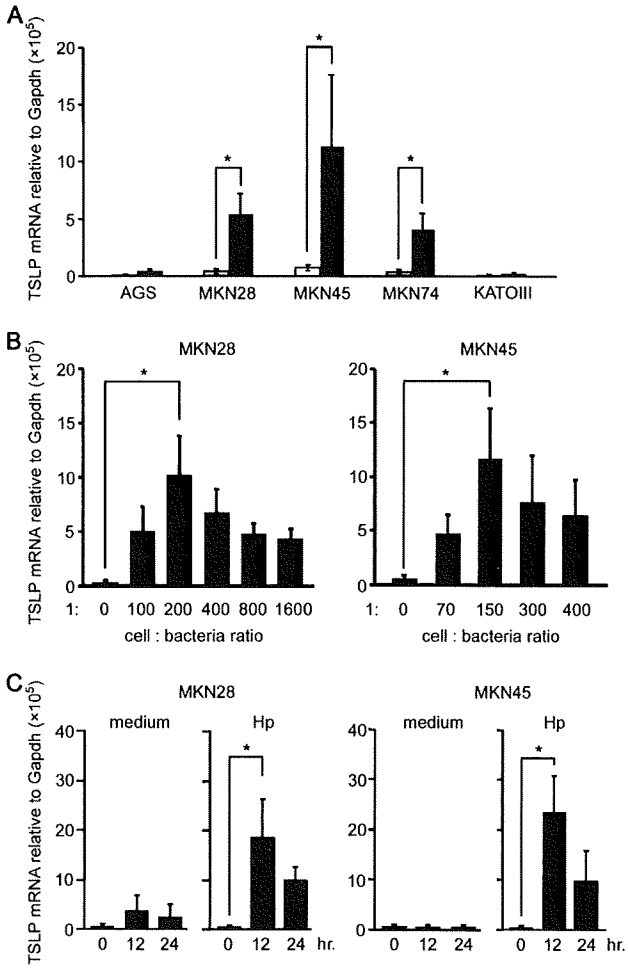

FIG. 1. TSLP expression in gastric epithelial cells after *H. pylori* colonization. (A) Various human gastric epithelial cell lines were cultured for 24 h with (closed bars) or without (open bars) *H. pylori* at 1 cell per 150 bacteria. (B) MKN28 and MKN45 cells were cultured for 24 h with *H. pylori* at the indicated cell/bacterium ratios. (C) MKN28 and MKN45 cells were cultured for the indicated times with *H. pylori* at 1 cell per 150 bacteria. Expression levels of mRNA encoding TSLP were measured using real-time quantitative RT-PCR. The data represent the means of three independent experiments. The error bars represent standard deviations (SD). *, $P < 0.05$.

sessed by protein ELISA. In contrast to the exposure to *H. pylori*, gastric epithelial cells did not upregulate TSLP expression after *H. felis* colonization (Fig. 2A and B). It seemed likely that one mechanism by which *H. pylori*, but not *H. felis*, could mediate induction of TSLP expression was by the secretion of specific proinflammatory factors. However, when gastric epithelial cells were separated from *H. pylori* by culture in a Transwell, induction of TSLP expression was not observed (Fig. 2A and B). Indeed, one of the proinflammatory factors, the Toll-like receptor 4 ligand LPS (from *Escherichia coli*), also failed to induce TSLP expression in gastric epithelial cells (Fig. 2A and B). Taken together, these data suggest that direct contact of *H. pylori* with human gastric epithelial cells is essential for induction of TSLP expression in these cells.

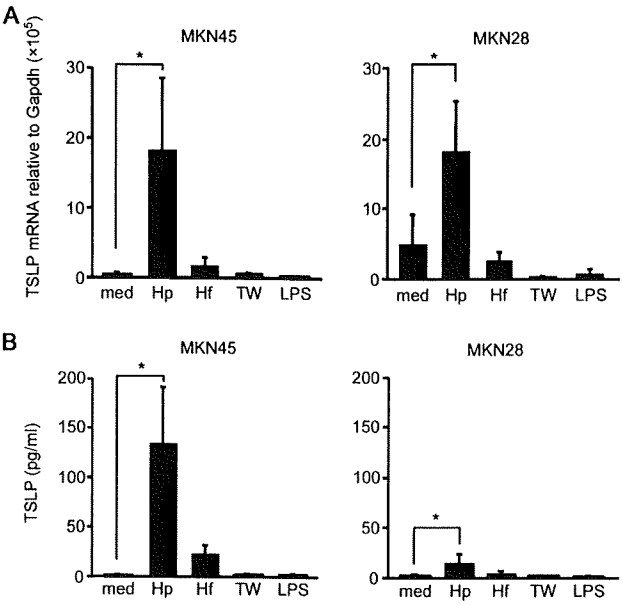

FIG. 2. TSLP expression in gastric epithelial cells, depending on direct contact with *H. pylori*. MKN45 and MKN28 cells were cultured for 24 h in the presence of 1 μg/ml LPS, *H. felis* (Hf), or *H. pylori* at 1 cell per 150 bacteria with (TW) or without (Hp) using Transwell, or medium alone (med). The expression levels of TSLP mRNA were measured using real-time RT-PCR (A) or those of TSLP protein in the culture supernatant were measured by protein ELISA (B). The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

Direct contact of *H. pylori* triggers human gastric epithelial cells to produce MIP-3α. Chemokine production by epithelial cells plays a critical role in the migration of immune cells in inflamed mucosal lesions, and *H. pylori* infection induces upregulation of MIP-3α gene expression in gastric epithelial cells in humans and mice (26, 49). Next, we examined whether *H. pylori* colonization can induce production of chemokines attracting myeloid lineage cells, such as MCP-1 (also called CCL-2), MIP-1α (CCL3), MIP-1β (CCL4), and MIP-3α (CCL20), together with TSLP, in gastric epithelial cells. MKN45 cells were cultured for 24 h with *Helicobacter* bacteria, and chemokine production in the culture supernatant was assessed by protein ELISA. In contrast to MCP-1, MIP-1α, or MIP-1β, production of MIP-3α was strongly upregulated after colonization by *H. pylori*, but not *H. felis*, in gastric epithelial cells (Fig. 3). In addition, when gastric epithelial cells were separated from *H. pylori* by culture in a Transwell, no induction of MIP-3α production was observed (Fig. 3). Moreover, LPS (from *Escherichia coli*) failed to induce MIP-3α production in gastric epithelial cells (Fig. 3). Taken together, these data suggest that direct contact of *H. pylori* with human gastric epithelial cells is also essential for induction of MIP-3α production in these cells.

TSLP-containing supernatants from *H. pylori*-colonized gastric epithelial cells enhanced surface CD80 expression in DCs. Human $CD11c^+$ blood immature DCs respond to the stimulation of TSLP, and TSLP stimulation enhances CD80 expression in DCs (23, 44). To examine whether TSLP-containing supernatants from the *H. pylori*-infected gastric epithelial cells

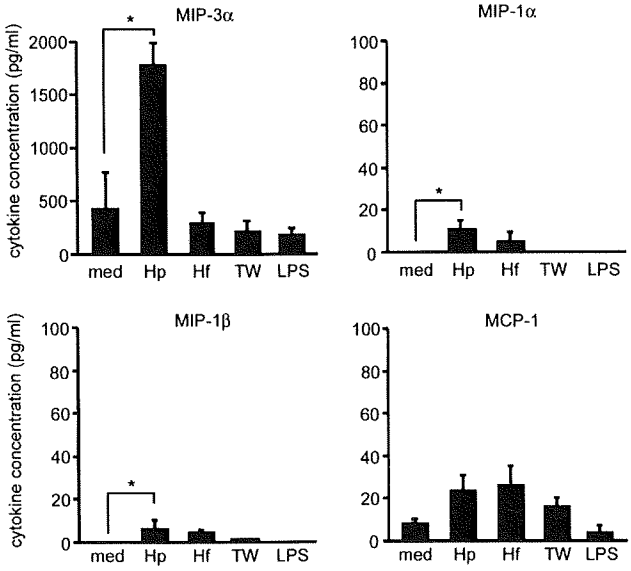

FIG. 3. Chemokine production by gastric epithelial cells, depending on direct contact with *H. pylori*. The cells were cultured as described in the legend to Fig. 2. MIP-3α, MIP-1α, MIP-1β, and MCP-1 were measured in the culture supernatant by protein ELISA. The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

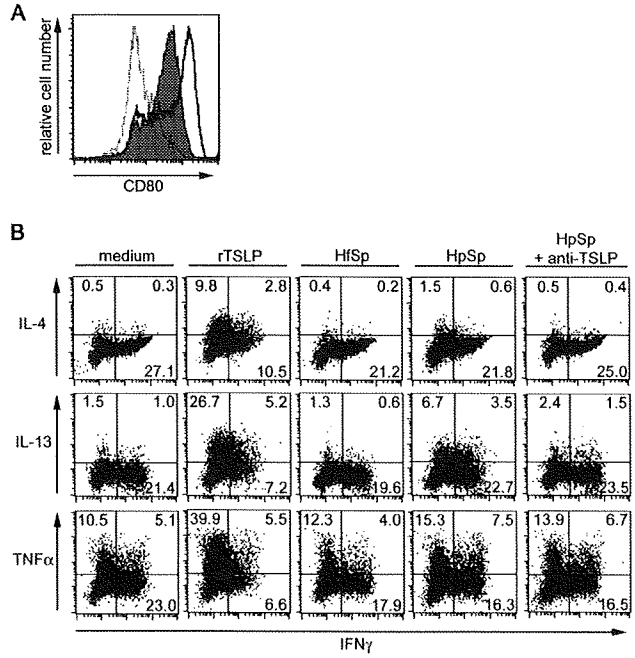

FIG. 4. CD80 expression on myeloid DCs and the cytokine-producing capacity of $CD4^+$ T cells are expanded by activated DCs. (A) Purified $CD11c^+$ myeloid DCs were cultured with recombinant TSLP (solid line, open histogram) or supernatants from MKN45 cells with (filled histogram) or without (dotted line, open histogram) exposure to *H. pylori* at 1 cell per 150 bacteria. After 24 h of incubation, the surface expression of CD80 on DCs was determined by flow cytometry. The data represent one of three experiments. (B) Purified DCs were cultured with rTSLP or supernatants from MKN45 cells exposed to *H. felis* (HfSp), *H. pylori* (HpSp) at 1 cell per 150 bacteria, or medium alone (medium). For neutralization of TSLP, supernatants were incubated with anti-human TSLP (20 μg/ml) (HpSp + anti-TSLP). After 24 h of incubation, DCs were cocultured with allogeneic naïve $CD4^+$ T cells at a 1:5 ratio of DCs to T cells. After 7 days of coculture, T cells were restimulated for 6 h with PMA plus ionomycin, and production of indicated T-cell-derived cytokines was determined by intracellular cytokine staining. Shown are dot blot profiles of the indicated cytokine-producing cells. The numbers indicate the percentages of cells in each quadrant. The data represent one of three independent experiments.

enhanced cell surface expression of CD80 in DCs, we isolated blood myeloid DCs and incubated them with supernatants from the *H. pylori*-infected gastric epithelial cells. After 24 h of incubation, we analyzed the surface expression of CD80 on DCs by flow cytometry. Recombinant TSLP protein induced upregulation of surface CD80 expression in DCs (Fig. 4A). Notably, after DCs were incubated with supernatants from *H. pylori*-infected epithelial cells, the conditioned cells also enhanced surface CD80 expression (Fig. 4A).

DCs conditioned by TSLP-containing supernatants prime naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines. TSLP-activated DCs induce differentiation of inflammatory Th2 cells, and DCs activated with TSLP and CD40 ligand induce the differentiation of effectors producing both Th1 and Th2 cytokines (23, 42, 44). Next, we conditioned DCs with TSLP-containing supernatants from the *H. pylori*-colonized gastric epithelial cells and cocultured these DCs with allogeneic naïve $CD4^+$ T cells at a 1:5 ratio of DCs to T cells. After 7 days of coculture, we evaluated the cytokine production capacity using intracellular cytokine staining of primed T cells restimulated with PMA plus ionomycin. This staining demonstrated that DCs conditioned with supernatants from gastric epithelial cells without *Helicobacter* colonization (medium) primed $CD4^+$ T cells to produce IFN-γ and TNF-α, but not IL-4 or IL-13 (Fig. 4B). In contrast, DCs activated with recombinant TSLP protein (rTSLP) primed $CD4^+$ T cells to produce IL-4, IL-13, and TNF-α as expected. Although DCs incubated with supernatants from *H. felis*-colonized gastric epithelial cells (HfSp) did not change the Th1 cytokine production profile of cocultured $CD4^+$ T cells, DCs incubated with supernatants from *H. pylori*-colonized gastric epithelial cells (HpSp) did change it to a mixed Th1 and Th2 profile in which cocultured T cells produced IL-4 and IL-13, together with IFN-γ and TNF-α. After incubation of the TSLP-containing supernatants with neutralizing antibodies to human TSLP, however, the conditioned DCs (HpSp plus anti-TSLP) did not prime T cells to produce high levels of IL-4 and IL-13. These data suggest that DCs conditioned with supernatants from the *H. pylori*-colonized gastric epithelial cells promote naïve $CD4^+$ T cells to differentiate into effectors producing both Th1 and Th2 cytokines and that TSLP in the supernatants is responsible for the conditioning of DCs to prime $CD4^+$ T cells to produce Th2 cytokines.

***H. pylori*-colonized gastric epithelial cells sequentially upregulate TSLP and B-cell-activating factor BAFF.** BAFF (also called BLyS) is a powerful regulator of B-cell biology (2, 17, 33, 39). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). Next, we tested whether *H. pylori* colonization on gastric epithelial cells can upregulate BAFF gene expression in gastric epithelial cells. Such cells were cultured with *H. pylori*, and the

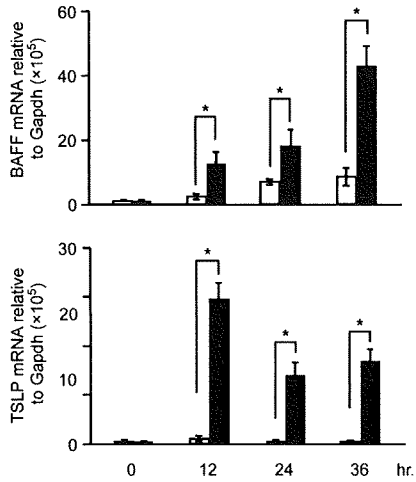

FIG. 5. TSLP and BAFF expression in gastric epithelial cells after *H. pylori* colonization. MKN28 cells were cultured for the indicated times with (closed bars) or without (open bars) *H. pylori* at 1 cell per 150 bacteria. The expression levels of mRNAs encoding TSLP and BAFF were measured using real-time quantitative RT-PCR. The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

expression levels of mRNA encoding BAFF were measured using real-time quantitative RT-PCR. In contrast to gastric epithelial cells not exposed to *H. pylori*, gastric epithelial cells that were exposed upregulated BAFF expression (Fig. 5). Although induction of TSLP expression peaked at 12 h after *H. pylori* colonization, induction of BAFF expression was detected after 12 h of *H. pylori* colonization and increased even after 36 h. These findings suggest that *H. pylori* colonization induces upregulation of TSLP and subsequently of BAFF expression in gastric epithelial cells.

Expression of TSLP is induced in mucosal lesions from *H. pylori*-infected gastritis patients. Finally, we evaluated the expression of TSLP in mucosal lesions from patients with *H. pylori*-infected follicular gastritis in which B-cell activation, including Th2 responses, was apparently involved. Frozen sections of mucosal lesions from follicular gastritis were stained with anti-TSLP antibodies. As shown in Fig. 6, immunoglobulin isotype control antibodies did not produce any positive staining, and there was no detectable immunostaining for TSLP in normal gastric mucosa. However, we found anti-TSLP staining of epithelial cells in the inflamed gastric mucosa from patients with *H. pylori*-infected follicular gastritis. TSLP expression was associated with the presence of $CD11c^+$ DCs within the inflamed gastric mucosa and was also associated with the presence of DC–lysosome-associated membrane protein (DC-LAMP, which is a DC activation marker)-positive cells. These data suggest that expression of TSLP is enhanced in mucosal lesions from *H. pylori*-induced follicular gastritis patients, but not in normal gastric mucosa, and also that TSLP expressed by gastric epithelial cells may play an important role in DC-mediated T-cell activation of a process related to Th2 inflammation in *H. pylori*-induced chronic gastritis.

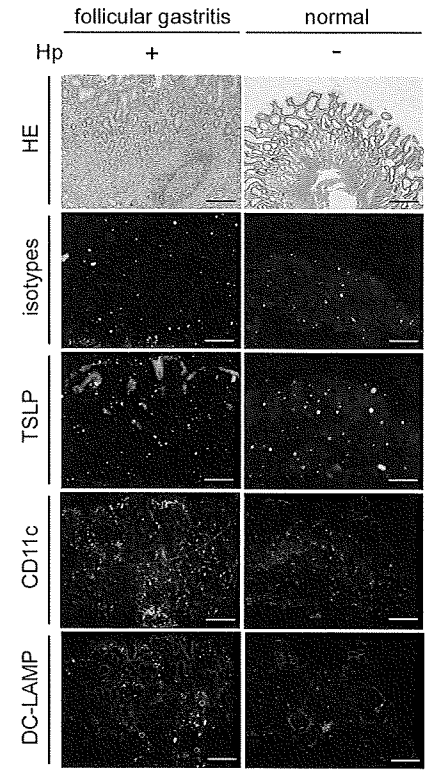

FIG. 6. Immunohistological staining of gastric mucosa. *H. pylori* (Hp)-infected inflamed mucosa containing a lymphoid follicle from a patient with follicular gastritis (left column) and non-*H. pylori*-infected normal mucosa (right column) were stained with hematoxylin and eosin (HE), immunoglobulin isotype control antibodies (isotypes), anti-human TSLP (TSLP), anti-CD11c (CD11c), or anti-DC-LAMP (DC-LAMP). All scale bars, 100 μm.

DISCUSSION

In the present study, we demonstrated that *H. pylori* triggered human gastric epithelial cells to produce TSLP, together with the DC-attracting chemokine MIP-3α and B-cell activating factor BAFF. DCs conditioned by *H. pylori*-infected epithelial cells expressed high levels of costimulatory molecules and primed naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines.

H. pylori-induced atrophic gastritis is characterized by marked infiltration of $CD4^+$ T cells that produce IFN-γ (12, 14). Development of *H. pylori*-induced atrophic gastritis is severely impaired in mice lacking $CD4^+$ T cells or IFN-γ production (7, 32). In addition, development of Th1 cell-mediated atrophic gastritis is also severely impaired in *Helicobacter*-infected Peyer's patch-null mice, which normally develop well-organized lymphoid organs, except for Peyer's patches. In these mice, the marked colonization of bacteria in the gastric mucosa can be detected (18, 25).

Although the gastric mucosa originally does not have a lymphoid apparatus, *H. pylori* infection triggers the development of MALT-like structures consisting of lymphoid aggregates and organized B-cell follicles in patients with *H. pylori*-induced gastritis (10, 11, 34). High-level expression of B-cell-activating chemokine 1 (BCA-1) (also called CXCL13) and its receptor,

CXC chemokine receptor (CXCR) 5, is observed in lymphoid aggregates and in the mantle zone of secondary lymphoid follicles in *Helicobacter*-induced follicular gastritis, suggesting that the chemokine-chemokine receptor interaction triggers the recruitment of lymphocytes (24). However, the mechanisms underlying the triggering of B-cell activation, including Th2 responses apparently involved in *H. pylori*-induced chronic gastritis, are not fully understood.

Chronic inflammatory processes in both autoimmunity and infection are characterized by the infiltration of a variety of immune cells, such as T cells, macrophages, and DCs, but also B cells and plasma cells. These cellular elements often organize the *de novo* formation of B-cell follicles and T-cell areas (1). In mouse models of *H. pylori*-induced follicular gastritis, the inflamed gastric mucosa contains B-cell follicles with germinal centers, T-cell areas, and high endothelial venules (28, 31). On the other hand, the constitutive expression of BAFF in secondary lymphoid tissues is essential for sustaining the long-term survival of mature B cells *in vivo* (17, 33). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). In addition, a recent human study suggested an association between circulating levels of BAFF in ectopic germinal centers of the salivary gland in primary Sjögren's syndrome (16, 37). Taken together, these results suggest that TSLP, together with BAFF, produced by *H. pylori*-infected gastric mucosa leads to inflammatory Th2 responses and maintenance of B-cell activation.

TSLP is involved in a variety of immune responses in the mucosal immune system in humans and mice. Epithelial cells in the tonsils and intestines release TSLP (13, 30, 38, 43, 48, 50). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). TSLP-conditioned DCs induce homeostatic noninflammatory Th2 responses and produce a proliferation-inducing ligand (APRIL) (13, 30, 43, 50). This results in enhancement of IgA2 class switching by intestinal epithelial cells under physiological conditions. In addition, TSLP-mediated signaling plays critical roles in host-protective Th2 cytokine-dependent immunity to the intestinal nematode pathogen *Trichuris* (40, 50). TSLP is involved in the regulation of Th1-type inflammation in a mouse model of colitis (40). We showed that *H. pylori*, one of the major pathogens in the human gastrointestinal tract, triggered gastric epithelial cells to produce TSLP, implying that TSLP may be crucial in a variety of immune responses in the gastrointestinal tract, including the stomach.

In mice, TSLP appears to suppress Th1 responses by acting on DCs in the mucosal immune system. In infection of mice by *Trichuris*, neutralization of TSLP or deletion of the TSLP receptor (TSLPR) in normally resistant mice resulted in defective expression of Th2 cytokines and persistent intestinal infection (40). In the intestinal inflammation in these mice, expression of inflammatory cytokines, such as IFN-γ, IL-17, and IL-12/23p40, was abundant. Neutralization of IFN-γ rescued the Th2 response and restored antiworm immunity in TSLP-deficient mice. In humans, although TSLP-activated DCs promote $CD4^+$ T cells to differentiate into inflammatory Th2 cells, TSLP primes DCs to produce large amounts of IL-12 following CD40 ligand stimulation. DCs activated with TSLP and CD40 ligand induce the differentiation of naïve $CD4^+$ T cells into effectors producing both Th1 and Th2 cytokines (23, 42, 44). These data suggest that, in the human system, induction of Th1 responses mediated by IL-12 is not suppressed under TSLP-induced Th2 inflammation and that IL-12-mediated negative regulation of Th2 responses is not effective in TSLP-induced Th2 inflammation. In this study, DCs conditioned by *Helicobacter*-infected epithelial cells primed naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines. Taken together, although Th1 cytokine-producing $CD4^+$ T cells markedly infiltrate inflamed mucosa in *Helicobacter*-induced chronic gastritis, these Th1 cytokines may not suppress Th2 cytokine production by TSLP-conditioned DCs.

Several recent studies have shown that microorganism-derived stimulation and/or proinflammatory cytokines upregulate expression of both TSLP and MIP-3α in human epithelial cells, and these upregulations are directly controlled by NF-κB (15, 21, 22, 36). In addition, although it is unclear whether BAFF expression is under the direct control of NF-κB, a recent study demonstrated that Toll-like receptor 3 ligand poly(I · C) induced upregulation of TSLP and subsequently of BAFF production in human tonsillar epithelial cells (48). In this study, we demonstrated that *H. pylori* triggered human gastric epithelial cells to produce TSLP, together with MIP-3α and BAFF. Because *H. pylori* products induce NF-κB activation in gastric epithelial cells (8, 27, 41, 47), *H. pylori*-induced NF-κB activation in gastric epithelial cells may directly and/or indirectly upregulate the expression of TSLP, MIP-3α, and BAFF in these cells.

In conclusion, we have demonstrated that *H. pylori* triggered gastric epithelial cells to produce TSLP, MIP-3α, and BAFF and that DCs conditioned by *Helicobacter*-infected epithelial cells triggered differentiation of T cells with a mixed Th1 and Th2 profile. These results, and the finding that TSLP was expressed by the epithelial cells of human follicular gastritis, suggest that *H. pylori* can directly trigger epithelial cells to produce TSLP and that TSLP-mediated DC activation may be involved in Th2 responses triggering B-cell activation in *H. pylori*-induced gastritis.

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Clinical and Experimental Allergy, **39**, 89–100

ORIGINAL PAPER **Basic Mechanisms in Allergic Disease**

Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions

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Clinical and Experimental Allergy

Summary

Background Human thymic stromal lymphopoietin (TSLP) is expressed in the human asthmatic lung and activates dendritic cells (DCs) to strongly induce proallergic T-helper type 2 (Th2) cell responses, suggesting that TSLP plays a critical role in the pathophysiology of human asthma. Th2 cells are predominantly involved in mild asthma, whereas a mixture of Th1 and Th2 cells with neutrophilic inflammation, probably induced by Th17, affects more severe asthmatic disease. Exacerbation of asthmatic inflammation is often triggered by airway-targeting RNA viral infection; virus-derived double-stranded RNA, Toll-like receptor (TLR)3 ligand, activates bronchial epithelial cells to produce pro-inflammatory mediators, including TSLP.

Objective Because TSLPR-expressing DCs express TLR3, we examined how the relationship between TSLP and TLR3 ligand stimulation influences DC activation.

Methods $CD11c^+$DCs purified from adult peripheral blood were cultured in TLR ligands containing media with or without TSLP and then co-cultured with allogeneic naïve $CD4^+$ T cells.

Results $CD11c^+$ DCs responded to a combination of TSLP and TLR3 ligand, poly(I : C), to up-regulate expression of the functional TSLP receptor and TLR3. Although TSLP alone did not induce IL-23 production by DCs, poly(I : C) alone primed DCs for the production of IL-23, and a combination of TSLP and poly(I : C) primed DCs for further production of IL-23. The addition of poly(I : C) did not inhibit TSLP-activated DCs to prime naïve $CD4^+$ T cells to differentiate into inflammatory Th2 cells. Furthermore, DCs activated by a combination of TSLP and poly(I : C) primed more naïve $CD4^+$ T cells to differentiate into Th17-cytokine-producing cells with a central memory T cell phenotype compared with DCs activated by poly(I : C) alone.

Conclusions These results suggest that through DC activation, human TSLP and TLR3 ligands promote differentiation of Th17 cells with the central memory T cell phenotype under Th2-polarizing conditions.

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Keywords dendritic cells, double-stranded RNA, IL-23, T cell differentiation, Th17 cells, Th2 cells, Toll-like receptor 3 ligand, TSLP

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Introduction

Human thymic stromal lymphopoietin (TSLP) activates $CD11c^+$ dendritic cells (DCs) to give rise to proallergic T cell responses [1, 2]. TSLP activates DCs to strongly up-regulate the expression of costimulatory molecules and to uniquely produce T helper (Th)2 cell-attracting chemokines, thymus and activation-regulated chemokine (TARC), and macrophage-derived chemokine (MDC) – but not the pro-inflammatory cytokines IL-1β, IL-6, IL-12, or TNF-α [3]. $CD4^+$ T cells are primed by TSLP-activated DCs to differentiate into inflammatory Th2 cells that produce the proallergic cytokines IL-4, IL-5, IL-13, and TNF-α, while down-regulating IL-10 and IFN-γ [3–5]. In addition, naïve $CD8^+$ T cells are primed by TSLP-activated DCs to produce IL-5 and IL-13, but exhibit poor cytolytic activity [6]. Moreover, human TSLP is produced by bronchial epithelial cells, bronchial smooth muscle cells, lung

fibroblasts, and mast cells [1–3]. TSLP expression is increased in asthmatic airways and correlates with the expression of Th2-attracting chemokines and disease severity [7, 8]. These data suggest that TSLP is involved in the pathogenesis of human asthma [9–12].

TSLP plays a critical role in asthmatic inflammatory responses not only in humans but also in mice and monkeys. TSLP concentrations are increased in mice with antigen-mediated airway inflammatory disease, and lung TSLP concentrations correlate with the severity of eosinophilia [13]. Furthermore, TSLPR-deficient mice develop severely attenuated antigen-mediated airway inflammatory disease [13, 14]. In the rhesus monkey model of dust-mite-induced allergic asthma, TSLP is expressed on airway respiratory cells and smooth muscle cells in sensitized monkeys [15]. Inhibition of OX40L, which is a critical downstream mediator in TSLP-mediated immune responses [4, 5], inhibits Th2 inflammation in the monkey model of asthma [15]. These data suggest that TSLP plays a critical role in the development of asthma.

Th17 cells–characterized by the production of IL-17/IL-17A, IL-17F, IL-6, TNF-α, and IL-22–mediate protection against extracellular microbes and link to the development of autoimmune diseases *in vivo* [16, 17]. TGF-β, IL-1β, IL-6, IL-22, and IL-23 play critical roles in IL-17 production in human T cells [18–21], and the IL-23–IL-17 axis is involved in human psoriasis and inflammatory bowel diseases, suggesting an important role for Th17 cytokines in chronic inflammatory diseases [17, 18, 22]. Although asthma is characterized by chronic dysregulated Th2 inflammation in the airways, recent human studies have shown that IL-17 is also expressed in the airways of patients with asthma, and IL-17 expression is increased in patients with moderate-to-severe asthma, compared with patients with mild asthma and with normal controls [23–25]. Thus, Th2 cells are predominantly involved in mild asthma, whereas a mixture of Th2 and Th1 cells with neutrophilic inflammation probably induced by Th17 cells affects more severe asthmatic disease [26]. Still, it has remained unclear how Th17 lineage commitment is mediated in Th2-predominant inflammation, such as human asthma.

Exacerbation of asthmatic inflammation is often triggered by infection of airway-targeting viruses such as the rhinovirus, coronavirus, influenza virus, respiratory syncytial virus, and adenovirus [27–29]. Bronchial epithelial cells express several Toll-like receptors (TLRs); virus-derived double-stranded RNA activates bronchial epithelial cells through TLR3 to produce pro-inflammatory mediators, including TSLP [30–32].

Because TSLPR-expressing DCs express TLR3 [3, 33, 34], in this study we examined how the relationship between TSLP and TLR3 ligand stimulation influences DC activation. We show that a combination of TSLP and TLR3 ligands activated human $CD11c^+$ DCs to produce IL-23. In addition, the addition of poly(I : C) did not inhibit TSLP-activated DCs to prime naïve $CD4^+$ T cells to differentiate into inflammatory Th2 cells. Furthermore, DCs activated by a combination of TSLP and poly(I : C) primed more naïve $CD4^+$ T cells to differentiate into Th17-cytokine–producing cells with a central memory T cell phenotype compared with DCs activated by poly(I : C) alone. These results suggest that through DC activation, human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory T cell phenotype under Th2-polarizing conditions.

Materials and methods

DC purification and culture

This study was approved by the Institutional Review Board for Human Research at the Graduate School of Medicine, Kyoto University. Peripheral blood mononuclear cells (PBMCs) were obtained from the adult buffy coat of healthy donors (kindly provided by Kyoto Red Cross Blood Center, Kyoto, Japan). $CD11c^+$ DCs were isolated from PBMCs as described previously [34, 35]. $CD11c^+lineage^-$ cells were isolated by a FACS Aria™ (BD Biosciences, San Jose, CA, USA) to reach >99% purity. $CD11c^+$ DCs were cultured immediately after being sorted in RPMI 1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 5% human AB serum (Sigma, St Louis, MO, USA), penicillin G, streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate (Gibco BRL; referred to as complete medium). Cells were seeded at a density of 1×10^6 cells/mL in round-bottomed 96-well plates in the presence of 25 μg/mL of poly(I : C) (InvivoGen, San Diego, CA, USA), 1 μg/mL of lipopolysaccharide (LPS) (Sigma) or 5 μg/mL of *Escherichia coli* RNA/LyoVec (InvivoGen), with or without 15 ng/mL of TSLP (R&D Systems, Minneapolis, MN, USA). After 24 h of culture, viable DCs were counted by trypan blue exclusion of dead cells.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with SuperScript™ II (Invitrogen, Carlsbad, CA, USA). Real-time quantitative reactions were performed with an ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)$\times10^5$. The following primers were used: *GAPDH*: 5′-CCACATCGCTCAGACACCAT-3′ and 5′-GGCAACAATATCCACTTTACCAGAGT-3′; *TSLPR*: 5′-AGAGCAGCCAGACGACATT-3′ and 5′-CAGCGACTTGCG

GTGAAAAC-3′; *IL-7Rα*: 5′-GGATGAAAACAAATGGACG CATG-3′ and 5′-GCTGCCGGTTGGAGCTTTCT-3′; *TLR3*: 5′-TGACTGAACTCCATCTCATGTCC-3′ and 5′-CCATTATGA GACAGATCTAATGTG-3′; *TLR4*: 5′-CTGCGTGAGACCAG AAAGC-3′ and 5′-TTCAGCTCCATGCATTGATAA-3′; *TLR8*: 5′-TCTGCATGAGGTTGTCGATGA-3′ and 5′-TGCGCTACC ACCTTGAAGAGA-3′; *T-bet*: 5′-AACACAGGAGCGCACTG GAT-3′ and 5′-TGCTCTCCTGGCTGCAGAC-3′; *GATA-3*: 5′-ACCGGCTTCGGATGCAA-3′ and 5′-TGCTCTCCTGGCT GCAGAC-3′; *RORc*: 5′-TTTTCCGAGGATGAGATTGC-3′ and 5′-CTTTCCACATGCTGGCTACA-3′; *CCR4*: 5′-CAGA AAAGCAAGAAGCTGCTTCTG-3′ and 5′-CGAGGGTGGTA TCTGCTATATC-3′; and *CCR6*: 5′-GGCTGCAAATTTGGG TAAAA-3′ and 5′-CACAGGAGAAGCCTGAGGAC-3′.

DC cytokine production

After a 24-h culture of DCs at a concentration of 1×10^6 cell/mL, DC culture supernatants were collected and analysed with protein ELISA kits for TARC, MDC, IL-12p70 (from R&D Systems), and for IL-1β, IL-6, IL-23p19/p40, TNF-α, and TGF-β1 (all from eBioscience, San Diego, CA, USA). Diluted supernatants (1 : 10, 1 : 10, 1 : 1, 1 : 1, 1 : 5, 1 : 1, 1 : 1, and 1 : 1) were used for ELISA for TARC, MDC, IL-12p70, IL-1β, IL-6, IL-23p19/p40, TNF-α, and TGF-β1, respectively.

Analysis of cell surface markers of DCs

To determine the cell surface markers characteristic of activated DCs, DCs were incubated with various stimuli for 24 h. DCs were subsequently stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (eBioscience). Finally, they were analysed with a FACS Calibur™ (BD Biosciences).

DC T cell co-culture

After 24 h of culture, $CD11c^+$ DCs were collected and washed three times to remove any cytokines. Viable DCs were counted by trypan blue exclusion of dead cells. $CD4^+CD45RA^+$ naïve T cells were isolated from PBMC using a FACS Aria™ to reach >99% purity, as described previously [3, 35]. The remaining DCs were co-cultured with 2.5×10^4 freshly purified allogeneic naïve $CD4^+$ T cells in round-bottomed 96-well culture plates in complete medium. Cells were cultured in triplicate at a DC : T cell ratio of 1 : 5. After 7 days of culture, viable cells were counted by trypan blue exclusion of dead cells.

T cell cytokine production

After 7 days of co-culture, DC-primed $CD4^+$ T cells were restimulated for 24 h with plate-bound 10 μg/mL anti-CD3 (UCHT1, BD Biosciences) and soluble 1 μg/mL anti-CD28 (CD28.2, BD Biosciences) at a concentration of 1×10^6 cell/mL. Cytokine production was assessed in the culture supernatant by protein ELISA for IL-10, IL-13, IL-17, IFN-γ and TNF-α (all from eBioscience), and IL-22 (from R&D Systems). Diluted supernatants (1 : 20, 1 : 2, 1 : 1, 1 : 100, 1 : 20, and 1 : 5) were used for ELISA for IL-10, IL-13, IL-17, IFN-γ TNF-α, and IL-22, respectively. For intracellular cytokine production, T cells were collected on day 7 of the co-culture, washed twice, and restimulated with 50 ng/mL PMA (Sigma) +2 μg/mL ionomycin (Sigma) in flat-bottomed 96- or 48-well plates at a concentration of 1×10^6 cell/mL. After 3.5 h, brefeldin A (Sigma) was added at 10 μg/mL. After 2.5 h, cells were collected and stained for cell-surface molecules. Cells were fixed and permeabilized using a Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, An Der Grub, Austria), and stained with phycoerythrin-conjugated mAbs to IL-13, TNF-α IL-17, and FITC-conjugated anti IFN-γ (all from eBioscience). Stained cells were analysed on a FACS Calibur.

Analysis of cell-surface markers of T cells

To determine the cell-surface markers characteristic of $CD4^+$ T cells, after 7 days of co-culture, DC-primed $CD4^+$ T cells were stained with FITC-conjugated anti-CD45RA, anti-CD45RO, anti-CD62L (all from eBioscience), or purified mouse anti-CCR7 (BD Bioscience) and FITC-conjugated anti-mouse IgM (AbD Serotec, Kidlington, UK), followed by allophycocyanin-conjugated anti-CD4 (eBioscience). In secondary culture, T cells were cultured for 3 days with plate-bound 10 μg/mL anti-CD3 mAb and soluble 1 μg/mL anti-CD28 mAb with 2 ng/mL IL-2 (R&D Systems). Finally, they were analysed with a FACS Calibur.

$CD4^+$ T cell purification

$CD4^+CD45RA^+$ naïve T cells and $CD4^+CD45RA^-$ memory T cells (purity, >99%) were isolated by cell sorting as described above [35]. Purified T cells were cultured at 2.5×10^6 cells/mL in complete medium. In primary culture, T cells were cultured with plate-bound 10 μg/mL anti-CD3 mAb and soluble 1 μg/mL anti-CD28 mAb in the presence of 10 ng/mL of IL-6 and IL-23, and 10 μg/mL of anti-IL-4 and anti-IFN-γ neutralizing Abs (all from R&D Systems) with or without 15 ng/mL of TSLP. After 3 days of culture, activated T cells were expanded for 4 days with 10 μg/mL plate-bound anti-CD3, soluble 1 μg/mL anti-CD28 mAbs, and 2 ng/mL of IL-2. Culture supernatants were measured by protein ELISA for IL-17 and IL-22. Diluted supernatants (1 : 10, 1 : 3) were used for ELISA for IL-17 and IL-22, respectively.

Results

TSLP and TLR3 ligands synergistically enhance the expression of their own receptors

Human CD11c$^+$ immature DCs respond to the stimulation of TLR ligands (TLRLs) and TSLP [3, 33, 34]. First, to examine the effects of TLRLs and TSLP on the expression levels of TSLPR, IL-7Rα chain, and TLRs in activated DCs, we isolated CD11c$^+$ immature DCs from blood buffy coats and cultured them for 24 h in TLRLs containing media with or without TSLP. Using real-time quantitative RT-PCR, we found that human CD11c$^+$ DCs cultured with medium alone expressed both TSLPR and IL-7Rα chain genes and that their expressions were enhanced by stimulation with TSLP and TLR3, 4, and 7/8 ligand agonist, poly(I : C), LPS, and LyoVec, respectively (Fig. 1a). TSLP did not enhance LPS- or LyoVec-induced expression of TSLPR or IL-7Rα chain genes. However, TSLP significantly up-regulated poly(I : C)-induced expression of both TSLPR and IL-7Rα chain genes (Fig. 1a). Moreover, although TSLP alone did not affect TLR3 gene expression, it significantly enhanced poly(I : C)-induced TLR3 gene expression in DCs (Fig. 1b). In contrast, TSLP did not enhance gene expression of TLR4 and TLR8 induced by LPS or LyoVec. These data indicate that TSLP had a synergistic enhancing action only with the TLR3 ligand on expression of their own receptor genes.

TSLP enhances TLR3 ligand-induced production of IL-23 by DCs

Because cytokine and chemokine production by activated DCs plays a critical role in the differentiation of primed T cells, we next assessed cytokine and chemokine production in activated DCs in the culture supernatant by protein ELISA. After 24 h of culture, TSLP-primed DCs produced high levels of TARC and MDC, while TSLP did not stimulate DC production of pro-inflammatory cytokines IL-1β, IL-6, IL-12, IL-23, or TNF-α, demonstrating one of the unique properties of TSLP in DC activation (Fig. 2a, left panels) [3, 34]. In contrast, poly(I : C) alone and LPS alone primed DCs to produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23, and TNF-α (Fig. 2a, middle panels). TSLP did not affect poly(I : C)- or LPS-induced production of IL-1β, IL-6, IL-12, or TGF-β1, whereas TSLP marginally enhanced poly(I : C)- and LPS-induced increase in TNF-α production (Fig. 2a, middle panels and 2c). However, TSLP significantly up-regulated poly(I : C)-induced increase of IL-23 production (Fig. 2a, middle panels and 2b), which can induce CD4$^+$ T cell differentiation into Th17 cells in humans [18–20]. Although TLRLs alone did not produce large amounts of TARC or MDC in DCs, TSLP strongly induced production of the Th2-cell attracting chemokines, TARC and MDC, regardless of the presence of the TLRLs (Fig 2a, lower

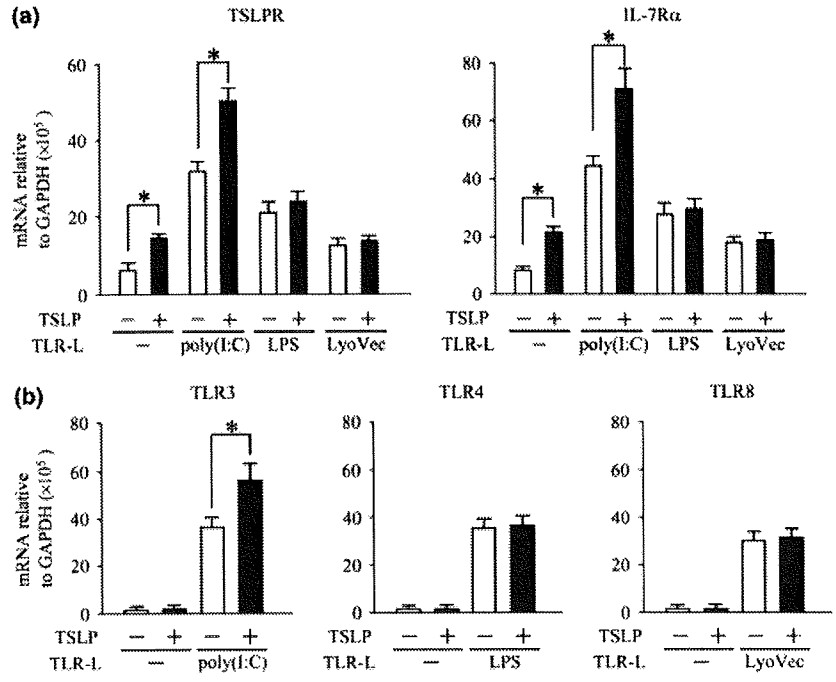

Fig. 1. TSLP and TLR3 ligands synergistically enhanced expression of their own receptors. Blood CD11c$^+$ DCs were activated by TLR-ligands, poly(I : C), LPS, or LyoVec with (closed bar) or without TSLP (open bar) for 24 h. DCs were used for real-time quantitative RT-PCR analyses for TSLPR and IL-7Rα chain mRNA expressions (a) and for TLR3, TLR4, and TLR8 mRNA expressions (b). Closed bar and horizontal error bars indicate the mean and SD of three independent experiments, respectively. Student's *t*-test for unpaired data was used to compare the values between two groups. $^*P<0.05$. TSLP, thymic stromal lymphopoietin; TLR, Toll-like receptor; DCs, dendritic cells; LPS, lipopolysaccharide.

panels). Taken together, these data suggest that TSLP may maintain its unique ability in DC activation regardless of the presence of TLRLs.

TSLP conserves its unique ability in DC activation in the presence of TLRLs

One of the characteristics of TSLP in DC activation is its capability to induce strong cell-surface expression of costimulatory molecules, such as CD80 and CD86, in DCs [3, 34, 35]. To examine whether TSLP retains this ability in the presence of TLRLs, we analysed the surface expression of CD80 on DCs by flow cytometry. Poly(I : C), LPS, LyoVec, and TSLP all stimulated surface CD80 expression in DCs (Fig. 2d). Notably, TSLP enhanced surface CD80 expression in the presence of TLRLs to an extent similar to that of TSLP alone. Another trait of TSLP stimulation in DCs is enhancement of DC survival [3, 35]. After 24 h of

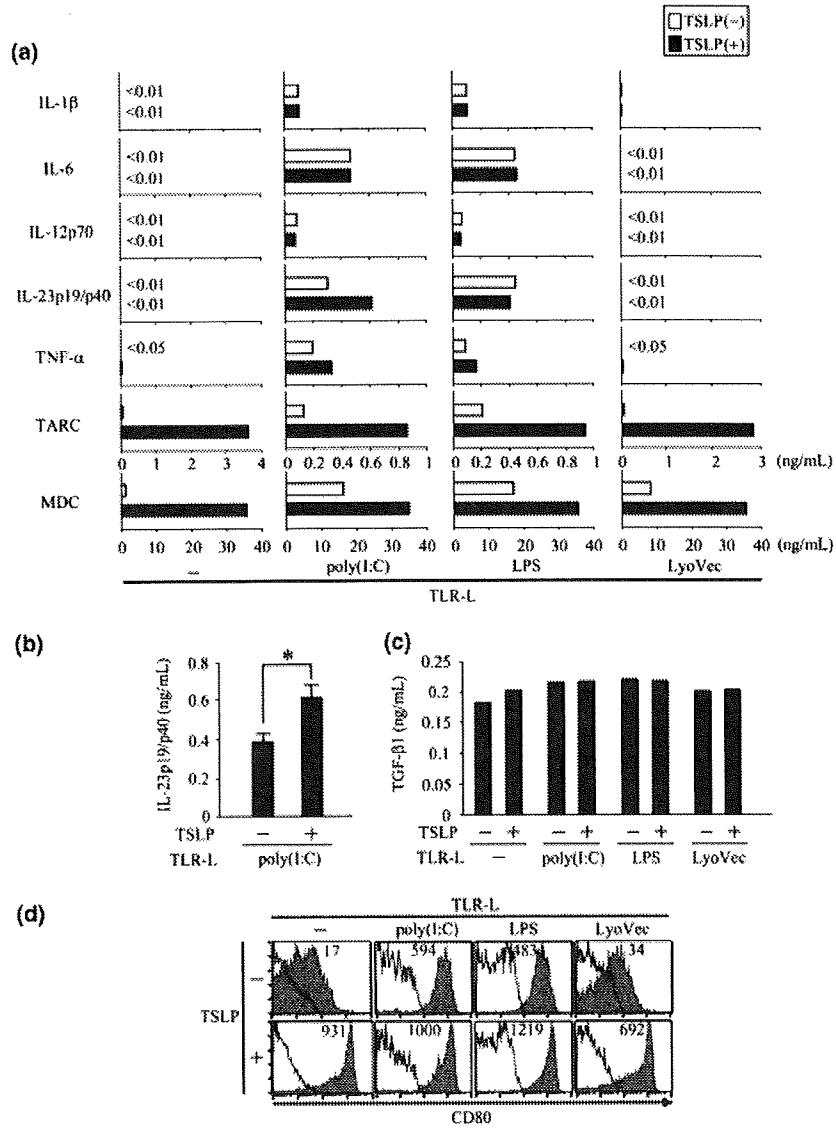

Fig. 2. TSLP conserved its unique abilities in DC activation in the presence of TRLs and enhanced TLR3 ligand induced IL-23 production. Blood $CD11c^+$ DCs were activated by TLR-ligands, poly(I : C), LPS, or LyoVec, with or without TSLP for 24 h. (a), IL-1β, IL-6, IL-12p70, IL-23p19/p40, TNF-α TARC, and MDC were measured in the culture supernatant by protein ELISA. (b), IL-23p19/p40 production measured by protein ELISA. Closed bar and horizontal error bars indicate the mean and SD of five independent experiments, respectively. Student's *t*-test for unpaired data was used to compare the values between two groups. $^*P<0.05$. (c), TGF-β1 concentration measured by protein ELISA. (d), Cell-surface marker phenotypes of DCs were determined by flow cytometry. Filled histograms represent staining of CD80; open histograms represent the isotype control. Numbers above histograms indicate the mean fluorescence intensity for CD80. Data represent one of five independent experiments. TSLP, thymic stromal lymphopoietin; TLR, Toll-like receptor; DCs, dendritic cells; MDC, macrophage-derived chemokine.

culture, TSLP augmented the number of viable DCs induced by the TLRLs (data not shown). Taken together, these data suggest that TSLP maintains its unique abilities in DC activation regardless of the presence of TLRLs.

TSLP+poly(I : C)-activated DCs [TSLP+poly(I : C)-DCs] induce strong CD4$^+$ T cell expansion

Both virus-derived TLRL stimulation and epithelial-cell-derived TSLP appear to be involved in exacerbating asthma [7, 8, 27–32]. In addition, we demonstrated in this study that TSLP+poly(I : C) stimulation resulted in unique activation of DCs (Fig. 2). Subsequently, we examined how CD4$^+$ T cells expand and differentiate by DCs activated with TSLP+poly(I : C). CD11c$^+$ DCs purified from adult peripheral blood were cultured in poly(I : C)-containing media with or without TSLP and then co-cultured with allogeneic naïve CD4$^+$ T cells at a 1 : 5 ratio of DCs: T cells. After 7 days of co-culture, CD4$^+$ T cells co-cultured with TSLP+poly(I : C)-DCs expanded significantly more than those co-cultured with DCs activated with poly(I : C) alone, suggesting that TSLP+poly(I : C)-DCs have a stronger effect on CD4$^+$ T cell expansion than do poly(I : C)-DCs (Fig. 3a).

T cells primed with TSLP+poly(I:C)-DCs show the central memory T cell phenotype

To determine the cell-surface markers characteristic of CD4$^+$ T cells primed by TSLP+poly(I : C)-DCs, we used flow cytometry to analyse naïve CD4$^+$ T cells before and after culture for 7 days with activated DCs. After isolation, naïve CD4$^+$ T cells decreased their CD62L expression, possibly due to cell isolation processes (Fig. 3b) [35]. Priming with TSLP+poly(I : C)-DCs down-regulated CD45RA and up-regulated CD45RO in CD4$^+$ T cells, acquiring a CD45RA$^-$CD45RO$^+$CD62L$^+$CCR7$^+$ central memory T cell phenotype, a phenotype similar to TSLP-DC-induced memory Th2 cells [5]. Restimulation with anti-CD3, anti-CD28 mAbs, and IL-2 for 3 days converted these T cells to a CD45RA$^-$CD45RO$^+$CD62L^{low}CCR7^{low} effector memory T cell phenotype. In addition, these effector memory T cells showed enhanced expression of CCR4, which is a receptor for the Th2 cell-attracting chemokines–TARC and MDC (Fig. 3c). These data suggest that T cells expanded with TSLP+poly(I : C)-DCs can rapidly acquire effector functions.

TSLP+poly(I : C)-DCs programme naïve CD4$^+$ T cells more to differentiate into Th17 cells, and do not alter development of the inflammatory Th2 cells

Next, we examined the cytokine-producing capacity of CD4$^+$ T cells primed by DCs. CD4$^+$ T cells were primed for 7 days by DCs activated with TSLP, poly(I : C), or a combination of both stimuli. After washing the cells, half of the expanded CD4$^+$ T cells were collected to measure the expression levels of mRNA encoding lineage-specific transcription factors such as GATA-3, RORc, and T-bet, as well as Th-17 cell-expressing CCR6 [17, 36]. The remaining CD4$^+$ T cells were restimulated for a further 24 h with anti-CD3 and anti-CD28 mAbs, and T cell-derived cytokines were measured in the culture supernatant using protein ELISA.

CD4$^+$ T cells primed with TSLP-DCs expressed a high level of GATA-3 mRNA but not RORc, CCR6, or T-bet (Fig. 4a–c) and produced only the inflammatory Th2 cytokines IL-13 and TNF-α (Fig. 4d–f). In contrast, poly(I : C)-DCs enhanced expression of all the lineage-specific transcription factors and production of various cytokines, including IL-17, IL-22, IFN-γ, and IL-10 from CD4$^+$ T cells. In comparison with priming of poly(I : C)-DCs, TSLP+poly(I : C)-DCs primed CD4$^+$ T cells to express a decreased level of T-bet (Fig. 4c) and increased levels of GATA-3, RORc, and CCR6 mRNA (Fig. 4a and b). In addition, CD4$^+$ T cells primed with TSLP+poly(I : C)-DCs enhanced the production of IL-17 and IL-22, while they reduced the production of IFN-γ and IL-10 compared with CD4$^+$ T cells primed with poly(I : C)-DCs (Fig. 4e and f). Interestingly, when compared with TSLP-DCs, TSLP+poly(I : C)-DCs failed to induce further production of IL-13 and TNF-α from primed CD4$^+$ T cells (Fig. 4d). These data suggest that TSLP+poly(I : C)-DCs prime naïve CD4$^+$ T cells more to differentiate into Th17 cells compared with poly(I : C)-DCs, and do not alter development of the inflammatory Th2 cells compared with TSLP-DCs.

TSLP+poly(I : C)-DCs reduce IL-17 and IFN-γ double-producing T cells and increase IL-17 single-producing T cells

We further evaluated cytokine production capacity using intracellular cytokine staining of primed T cells restimulated with PMA plus ionomycin. Intracellular cytokine staining of T cells demonstrated that the percentage of TNF-α-producing cells primed with TSLP+poly(I : C)-DCs was similar to those with poly(I : C)-DCs and TSLP-DCs (Fig. 5a and b, left panels). In contrast, the percentage of IL-17-producing cells primed with TSLP+poly(I : C)-DCs was greater than those with poly(I : C)-DCs and TSLP-DCs (Fig. 5a and b, middle panels). Importantly, the percentage of IL-13-producing cells primed with TSLP+poly(I : C)-DCs was greater than that primed with poly(I : C)-DCs, but similar to that with TSLP-DCs (Fig. 5a and b, right panels).

In addition, priming with TSLP+poly(I : C)-DCs increased IL-17 single-producing T cells more prominently than priming with poly(I : C)-DCs (Fig. 5c, left panels). In contrast, IL-17 and IFN-γ double-producing (Th17/Th1) cells decreased by priming with TSLP+poly(I : C)-DC as compared with those primed by poly(I : C)-DCs

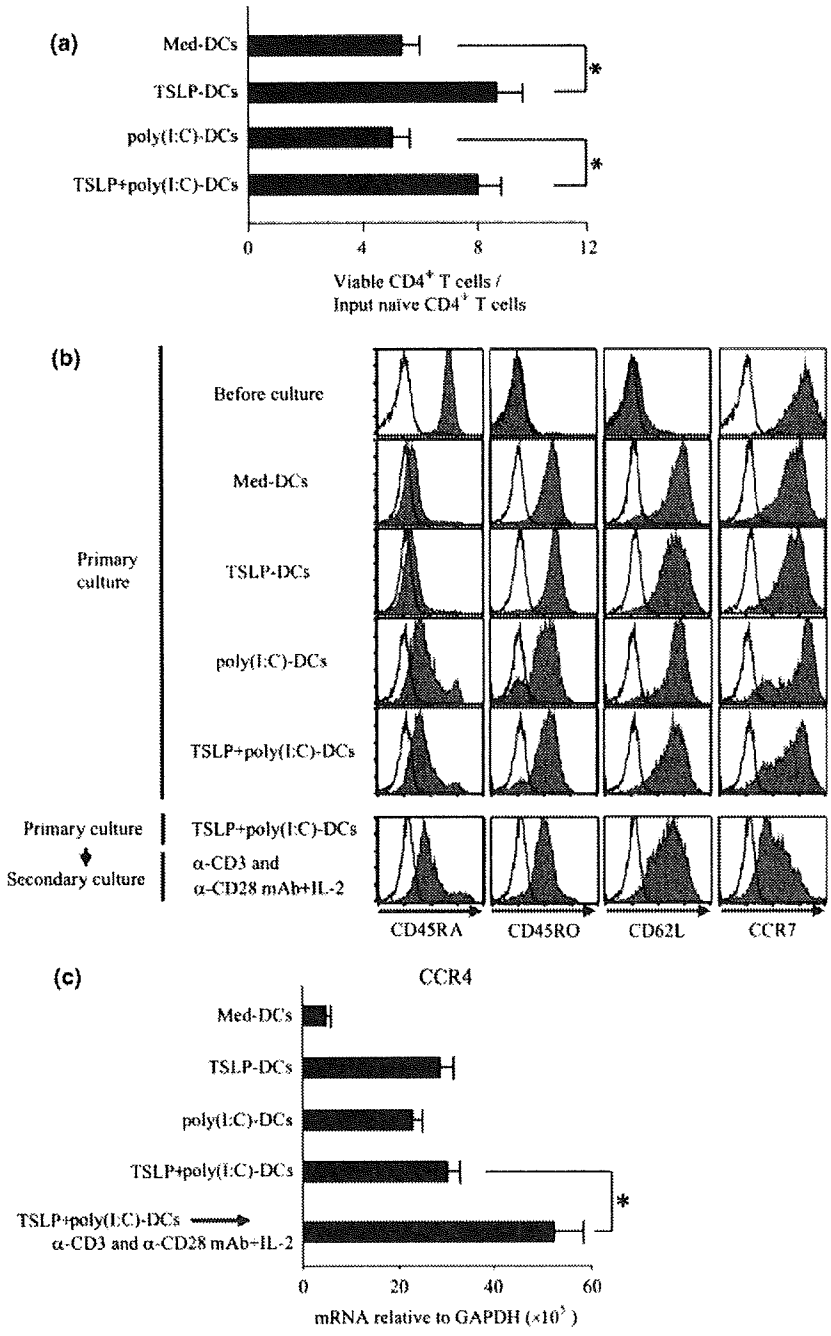

Fig. 3. TSLP+poly(I:C)-DCs efficiently induced naïve $CD4^+$ T-cell expansion and converted naïve to central memory T cells. $CD11c^+$ DCs cultured medium alone or activated with TSLP, poly(I:C), or the combination of TSLP and poly(I:C) were used to prime naïve $CD4^+$ T cells for 7 days. For secondary culture, T cells primed by TSLP+poly(I:C)-DCs were subsequently stimulated for 3 days with anti-CD3, anti-CD28 mAbs, and IL-2. (a), Fold expansion of $CD4^+$ T cells cultured with indicated DCs compared with the initial T cell number. Viable T cells were counted by trypan blue exclusion of dead cells, and cell-surface marker phenotypes were determined by flow cytometry. Closed bar and vertical error bars indicate the mean and SD of five independent experiments, respectively. Student's *t*-test for unpaired data was used to compare the values between two groups. $^*P<0.05$. (b), Cell-surface marker phenotypes of $CD4^+$ T cells before and after culture with indicated DCs. Cell-surface marker phenotypes were determined by flow cytometry. Filled histograms represent staining of T cells with indicated markers; open histograms represent isotype controls. Data represent one of five experiments. (c), CCR4 expression of various $CD4^+$ T cells. Expression level of CCR4 mRNA was measured using real-time quantitative RT-PCR. Closed bar and horizontal error bars indicate the mean and SD of three independent experiments, respectively. Student's *t*-test for unpaired data was used to compare the values between two groups. $^*P<0.05$. TSLP, thymic stromal lymphopoietin; TLR, Toll-like receptor; DCs, dendritic cells.

