

Gut T cell receptor- $\gamma\delta^+$ intraepithelial lymphocytes are activated selectively by cholera toxin to break oral tolerance in mice

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Accepted for publication 25 November 2014

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Summary

The gut immune system is usually tolerant to harmless foreign antigens such as food proteins. However, tolerance breakdown may occur and lead to food allergy. To study mechanisms underlying food allergy, animal models have been developed in mice by using cholera toxin (CT) to break tolerance. In this study, we identify T cell receptor (TCR)- $\gamma\delta^+$ intraepithelial lymphocytes (IELs) as major targets of CT to break tolerance to food allergens. TCR- $\gamma\delta^+$ IEL-enriched cell populations isolated from mice fed with CT and transferred to naive mice hamper tolerization to the food allergen β -lactoglobulin (BLG) in recipient mice which produce anti-BLG immunoglobulin (Ig)G1 antibodies. Furthermore, adoptive transfer of TCR- $\gamma\delta^+$ cells from CT-fed mice triggers the production of anti-CT IgG1 antibodies in recipient mice that were never exposed to CT, suggesting antigen-presenting cell (APC)-like functions of TCR- $\gamma\delta^+$ IELs. In contrast to TCR- $\alpha\beta^+$ cells, TCR- $\gamma\delta^+$ IELs bind and internalize CT both *in vitro* and *in vivo*. CT-activated TCR- $\gamma\delta^+$ IELs express major histocompatibility complex (MHC) class II molecules, CD80 and CD86 demonstrating an APC phenotype. CT-activated TCR- $\gamma\delta^+$ IELs migrate to the lamina propria, where they produce interleukin (IL)-10 and IL-17. These results provide *in-vivo* evidence for a major role of TCR- $\gamma\delta^+$ IELs in the modulation of oral tolerance in the pathogenesis of food allergy.

Keywords: cholera toxin, intraepithelial lymphocytes, oral tolerance

Introduction

The human gut is exposed to large amounts of foreign antigens, including food proteins known to be potentially allergenic. In consequence, the gut immune system developed oral tolerance to these exogenous but harmless and essential proteins. However, in an atopic individual, a breakdown in oral tolerance can result in immunoglobulin (Ig)E-mediated food allergy [1]. Adverse immune responses to foods affect approximately 5% of young children and 3–4% of adults in westernized countries, and tend to increase in prevalence. Severe allergic reactions to foods (anaphylaxis) are potentially fatal, and to date no cure is available for the disease [2]. To understand more clearly the mechanisms involved in tolerance breakdown that leads to food allergy, a mouse model of food allergy eliciting an immune response as well as symptoms similar to humans was developed and studied by several groups, including ours [3–5]. In this model, cholera toxin (CT) a bacterial protein from *Vibrio*

cholerae, is used as an adjuvant to break tolerance and induce a T helper type 2 (Th2) response, with generation of IgG1 and IgE antibodies specific to food allergen. It has been described previously that CT, through its B subunit (CT-B), displays a high affinity for GM1 gangliosides present ubiquitously on membranes of various cells, including T cells [6]. However, to display toxicity, CT has to be subjected to endocytosis and to enter the endoplasmic reticulum via retrograde vesicular traffic before the A subunit is delivered to cell cytosol and, in turn, activates fluid secretion resulting in diarrhoea. The adjuvant function of CT has been studied *in vitro* in intestinal epithelial cell (IEC) lines (IEC-17 and IEC-6). In the latter cells, CT did not modulate major histocompatibility complex (MHC) class II molecule expression, but promoted proinflammatory cytokine [e.g. interleukin (IL)-1 β and IL-6] production [7,8]. Also *in vitro*, CT was shown to be mitogenic for both T cell receptor (TCR)- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ intestinal intraepithelial T cells (IELs) [9]. *In-vivo* studies in CD4^{-/-}

and CD8^{-/-} mice suggested that CD8⁺ T cells are dispensable to CT adjuvant effect [10].

The gut immune system, also referred to as gut-associated lymphoid tissue (GALT), comprises lymphoid aggregates and diffusely distributed lymphoid cells. Within the GALT, T lymphocytes are at the prime line of contact with potentially harmful proteins (e.g. pathogens), but also harmless proteins such as foods. Lymphocytes are found at various locations in the gut mucosa. T cells resident in the epithelium, i.e. the IELs, have a specific phenotype; they are predominantly CD8 $\alpha\alpha$ ⁺ cells and display a $\gamma\delta$ TCR. These cells may produce various cytokines, including IL-17, IL-10 and interferon (IFN)- γ [11–13]. In addition, human and bovine peripheral blood, as well as mouse spleen and lymph node TCR- $\gamma\delta$ ⁺ T cells, display antigen-presenting functions upon activation *in vitro* [14–16], suggesting an important role of TCR- $\gamma\delta$ ⁺ cells in innate immunity. Because they are located at the interface of the gut lumen and the GALT, TCR- $\gamma\delta$ ⁺ IELs are likely to be key cells in the regulation of the immune response to CT. This study was undertaken to investigate IEL response after *in-vivo* exposure to CT and to determine how this might affect oral tolerance.

Methods

Ethics statement

The protocol was approved by Committee of the Ethics of Animals Experiment of the University of Geneva and the Veterinary Office of Geneva (permit number 1054/3309/2R). All experiments were carried out in strict accordance with their recommendations.

Mice

C3H/HeOuJ females were purchased from Charles River Laboratories (L'Arbresle, France) and were housed at the Animal Facilities of the University of Geneva, School of Medicine. Animals were used between 4 and 5 weeks of age and were fed with standard mice pellets without milk proteins.

Antibodies, reagents and medium

Anti-TCR- β (H57-97), anti-TCR- δ (GL3), anti-CD8 α (53-6-7), anti-CD8 β (H35-17-2), anti-CD11c (HL3), anti-CD25 (3C7), anti-CD44 (IM7), anti-CD45RA (14-8), anti-CD69 (H1-2F3), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-MHC class II (M5), anti-IL-4 (11B11), anti-IL-10 (JES5-16E3), anti-IL-17 (TC11-18H10) and anti-IFN- γ (XMG1-2) were from BD Pharmingen (Franklin Lakes, NJ, USA), anti- $\alpha 4\beta 7$ (Act-1) was from Leukosite (Cambridge, MA, USA) and α IEL $\beta 7$ (2G5) from Immunotech (Marseille, France) anti-CCR7 (4B12) was from Biolegend (San Diego, CA, USA) and anti-CCR9 (242503) from R&D Systems (Minne-

apolis, MN, USA). 7-amino-actinomycin D (7-AAD) was from Sigma (St Louis, MO, USA).

CT was from List Biological Laboratories (Campbell, CA, USA); CT-B and dextran 40 s coupled to Alexa 488 and ovalbumin (OVA) coupled to fluorescein isothiocyanate (FITC) were from Invitrogen (Paisley, Scotland, UK). BLG was from Sigma and alum from Serva (Heidelberg, Germany).

RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) and Hanks's balanced salt solution (HBSS) medium were supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 100 μ g/ml gentamicin, 15 mM HEPES pH 7.4 and 10% heat-inactivated fetal calf serum (FCS). In addition, DMEM was supplemented with 2×10^{-5} M 2-mercaptoethanol, 1% non-essential amino acids and 1 mM sodium pyruvate (all reagents from Sigma).

In-vivo priming, adoptive transfer and induction of oral tolerance and β -lactoglobulin (BLG) specific response

Mice were primed by oral administration of 10 μ g of CT alone or for capture experiments in association with 10 μ g of antigen coupled to indicated fluorophore in a solution containing 0.2 M of NaHCO₃, pH 9 (Fig. 1). Mice were then killed at the indicated time and their intestinal lymphoid cells isolated.

To study the *in-vivo* functions of intestinal lymphoid cells, IELs or lamina propria lymphocytes (LPLs) from primed or naive mice were isolated as described below. Five $\times 10^6$ IELs or LPLs or 10^7 total T cells in 200 μ l of phosphate-buffered saline (PBS) were transferred to naive mice by intravenous (i.v.) injection into the tail vein (Fig. 1). Due to technical limitations, isolation procedures resulted in 50% purity for TCR- $\gamma\delta$ ⁺ cells in the IEL and LPL cell fractions.

Twenty-four h after adoptive transfer, a group of mice was exposed continuously to 2 mg/ml of BLG in their drinking water for 3 weeks (Fig. 1), a procedure leading to oral tolerization [5]. Mice were then sensitized twice 1 week apart by intraperitoneal (i.p.) injection of 10 μ g Al(OH)₃ (alum) and 10 μ g BLG in 0.2 ml PBS. Mouse sera were collected before cell transfer, before the first sensitization and 1 and 2 weeks after the second sensitization.

Isolation of lymphocytes

Venous blood was collected in 250 U/ml heparin (Roche, Mannheim, Germany). Lymphocytes were enriched by Lympholyte M (Cederland, Hornby, Canada) gradient centrifugation, as described by the manufacturer, for 20 min at 600 g at room temperature. After killing the mice with CO₂, the small intestine was excised and LPLs and IELs were isolated using modified methods, as described previously

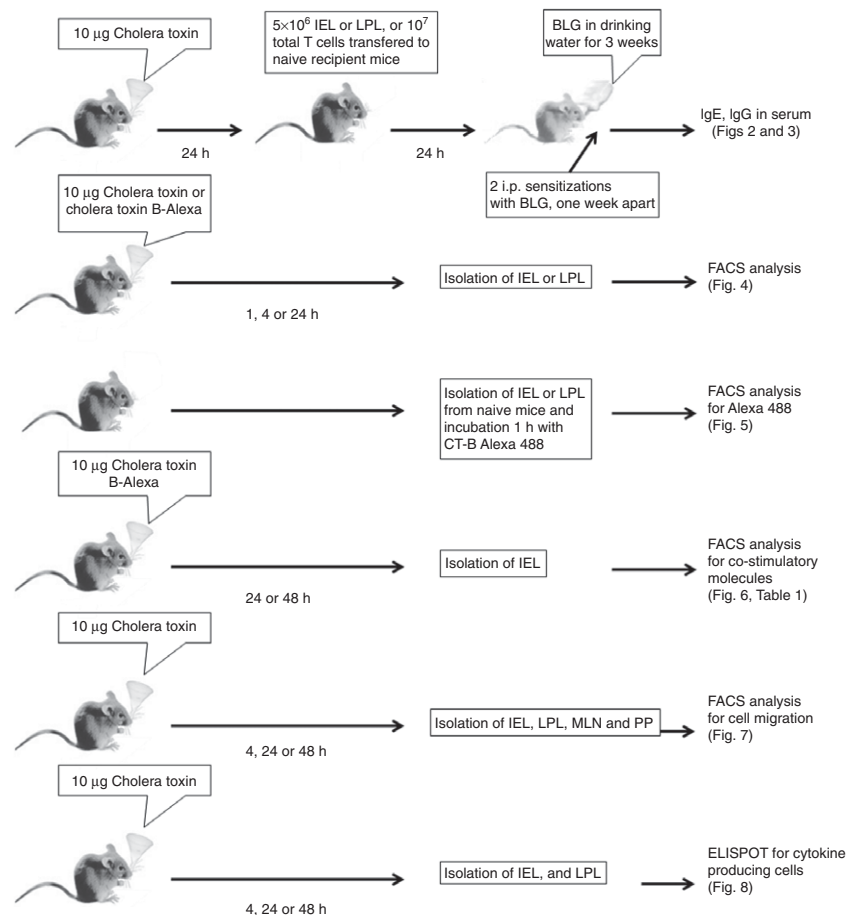


Fig. 1. Schematic representations of the experiments described in the study. Details in Material and methods; results in the indicated figures.

[17]. Briefly, fat tissue was removed, PP were excised mechanically and the gut was flushed extensively with complete HBSS. Intestinal pieces were opened longitudinally and cut into 5-mm pieces. Tissue pieces were incubated in calcium- and magnesium-free complete HBSS containing 2 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiotreitol (Sigma) for 30 min at 37°C with magnetic stirring prior to being vigorously vortexed. Intestinal IELs were obtained by filtration through 250-µm nylon gauze. The remaining preparation was washed three times with complete RPMI, and intestinal pieces for LPL isolation were subsequently incubated twice with magnetic stirring for 30 min at 37°C in complete RPMI, supplemented with 100 U/ml collagenase D (Roche). Cells were separated twice from tissue debris by purification through a 250-µm nylon filter and filtration procedure through a nylon wool column.

Each cell population was washed twice and lymphocytes were enriched by discontinuous 15/30/44% Percoll (Bioscience, Uppsala, Sweden) on a Lympholyte M gradient for 20 min at 600 g at room temperature. Lymphocytes were harvested from the Percoll 44% Lympholyte M interface.

Measurement of CT- and BLG-specific antibodies in mouse serum

Sera were obtained by tail vein bleeding. BLG-specific antibody titres were measured by a method adapted from Adel-Patient *et al.* [18]. Briefly, Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated for 18 h at room temperature with 250 ng/well streptavidin (Fluka, St Louis, MO, USA), followed by overnight incubation at 4°C with 10 mg/ml of polyvinylpyrrolidone K25 (Fluka). One µg/well of biotinylated BLG was incubated for 3 h at room temperature. The sera were diluted in various concentrations in enzyme-linked immunosorbent assay (ELISA) buffer (PBS containing 10% horse serum) and incubated 3 h at 37°C. Corresponding monoclonal rat anti-mouse IgE (BD Pharmingen) or polyclonal goat anti-mouse IgG1 peroxidase-labelled antibodies (Southern Biotechnologies, Birmingham, AL, USA) at 1/1000 dilution in ELISA buffer were added for 90 min at 37°C. IgE were then detected with sheep anti-rat peroxidase-labelled antibody (Serotec, Kidlington, UK) at 1/1000 in ELISA buffer for 60 min at 37°C. Antibody titres were determined by the addition of *ortho*-phenylenediamine and H₂O₂ (Sigma), and absorbance

was measured at 490 nm on a plate reader (Molecular Device Corporation, Menlo Park, CA, USA). Results were analysed with the SoftMax™ software (Molecular Device Corporation) and normalized as arbitrary units, with pooled sera from BLG and alum-immunized mice used as a reference serum.

For the measurement of CT-specific antibodies, Maxisorp microtitre plates (Nunc) were coated with 100 µl/well of 1 µg/ml CT in PBS and incubated overnight at room temperature. Wells were blocked with 200 µl/well of a solution of 10 mg/ml polyvinylpyrrolidone K25 for 2 h at 37°C. Measurement of CT-specific antibody titres was performed as described above for antibodies to BLG.

In-vitro binding and endocytosis assay

Freshly isolated IELs and LPLs (10⁶ cells/well) were incubated in cDMEM with various concentrations of CT-B-Alexa 488 for titration studies, or 0.1 µg/ml of CT-B-Alexa 488, OVA-FITC or dextran 40s-FITC for endocytosis studies. Plates were incubated on ice or at 37°C in 5% CO₂ for 1 h. Cells were washed extensively with ice-cold PBS–2% FCS, and analysed by flow cytometry.

Flow cytometry. In order to block Fc receptors, cells were incubated for 20 min on ice with purified rat IgG (Sigma) prior to incubation with coupled antibodies for 30 min on ice and washed twice, followed by immediate surface-marker expression analysis.

To measure intracellular cytokines, cells were incubated for 4 h in cDMEM in the presence of Golgi-plug (BD Pharmingen) at 37°C in 5% CO₂. Cells were then fixed and permeabilized in Cytofix/Cytoperm (BD Pharmingen) for 20 min on ice and washed twice with Perm/Wash buffer (BD Pharmingen). Staining was performed with directly coupled antibodies in Perm/Wash buffer on ice for 30 min. Detection of apoptotic cells was performed with an annexin V detection kit (BD Pharmingen), according to the manufacturer's instructions.

Multi-parameter analysis was acquired on a flow cytometer (FACSCalibur™; BD Bioscience Europe, Erenbodegen, Belgium) and analysed with CellQuest™ software (BD Bioscience). Dead cells were excluded from the analysis after staining with 7-AAD. To analyse T lymphocyte subpopulations we gated on CD3⁺ cells. To analyse the CD8αα⁺ subpopulation, we excluded CD8β⁺ cells.

Statistical analysis

Data were expressed as mean ± standard error of the mean (s.e.m.). Experiments were repeated three times with three pooled mice per experiment. Statistical significance between groups was analysed using the Wilcoxon signed-rank test for non-parametric unpaired data.

Results

Cells isolated from the GALT and activated *in vivo* by CT break oral tolerance and induce an antibody-specific response to CT

IELs and lamina propria lymphocytes (LPLs) are in contact with foreign antigens early in the immune response to foreign proteins in the gut, and might possess the ability to facilitate an antigen-specific immune response. In order to study the *in-vivo* function of IELs, mice were primed by feeding with CT, and thereafter IELs and LPLs were harvested and transferred into naive mice. Recipient mice were administered BLG, a common food allergen, in their drinking water for 3 weeks, and then sensitized twice i.p. with BLG in alum. Sera were collected at different times to measure BLG-specific antibody titres. IgG1 and IgE antibodies specific to BLG were observed in mice that received cells from CT-fed mice in contrast with mice that received cells from untreated mice (Fig. 2a,b). The absence of a BLG-specific IgG1 antibody response despite oral administration of BLG in mice receiving cells from naive mice confirmed the induction of oral tolerance by continuous BLG feeding in this group. Anti-BLG IgE antibodies were scarcely detected in serum, due to their localization within tissues. However, despite low titres, cells from CT-fed mice were more effective than cells from naive mice for inducing anti-BLG IgE antibodies in recipient mice (Fig. 2b). These experiments suggest that *in-vivo* preactivation of cells from the GALT by CT gave them the capacity to modulate the local immune response and to induce a Th2-type antibody response. Both IELs and LPLs displayed the ability to induce the production of IgG1 and IgE antibodies to BLG (Fig. 2c,d), although IELs were more potent than LPLs to induce an inflammatory response. These data suggest that IELs and LPLs from CT-fed mice were able to break oral tolerance to BLG. Moreover, cells isolated from the GALT of CT-activated mice and transferred to naive mice induced the production of detectable, low-titre anti-CT IgG1 antibodies in the recipient mice, although the latter were never exposed to CT (Fig. 3a). Anti-CT IgE antibodies were not detected (not shown); these observations suggest that CT-primed GALT cells play an essential priming role in the antigen-specific immune response in the gut. As observed with mice fed BLG, the transfer of IELs induced higher titres of CT-specific IgG1 antibody than LPLs (Fig. 3b).

TCR-γδ⁺ T lymphocytes from the GALT bind CT *in vivo*

Passive transfer of GALT cells from CT-fed mice into naive mice induced a Th2 response in recipient animals, suggesting that CT durably affects TCR-γδ⁺ cell functions. In order to determine whether CT might affect GALT cell activity directly, we assessed the interaction of CT with GALT cells

Fig. 2. Lymphoid cells isolated from intestinal epithelium and lamina propria of cholera toxin (CT)-fed mice reduce oral tolerance induced by food antigen administration. (a,b) Mice were fed or not with 10 μ g CT. After 24 h, cells isolated from the lamina propria and the intestinal epithelium of naive (open circles) or CT-fed mice (closed circles) were transferred to naive mice. After adoptive transfer, mice received β -lactoglobulin (BLG) in their drinking water (tolerization procedure) for 3 weeks (days 1–21), followed by two intraperitoneal (i.p.) sensitizations with BLG and Alum (days 21 and 28). (c,d) Mice were fed with 10 μ g CT. After 24 h, isolated lamina propria lymphocytes (LPLs) (squares) or intraepithelial lymphocytes (IELs) (triangles) were transferred into naive mice that were then treated as described in (a,b). BLG-specific immunoglobulin (Ig)G1 (a,c) and IgE (b,d) titres were measured in mouse serum at the indicated time after adoptive transfer. Mean $\pm$ standard error of the mean of antibody titres expressed in arbitrary units (AU); a representative experiment out three with six recipient mice per groups is presented. * $P < 0.05$.

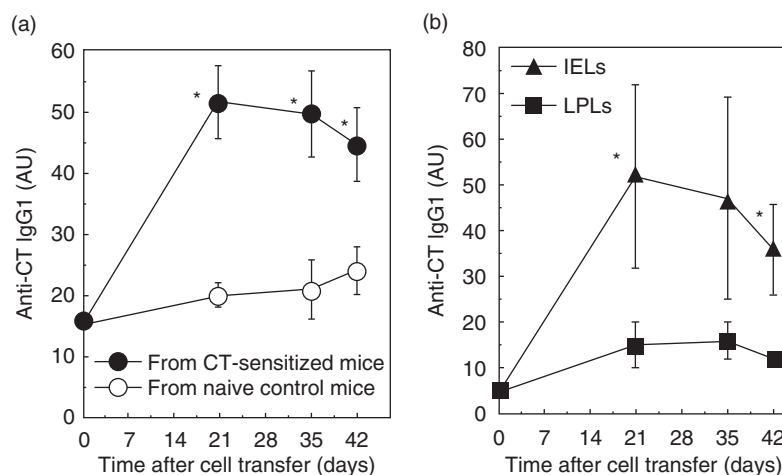
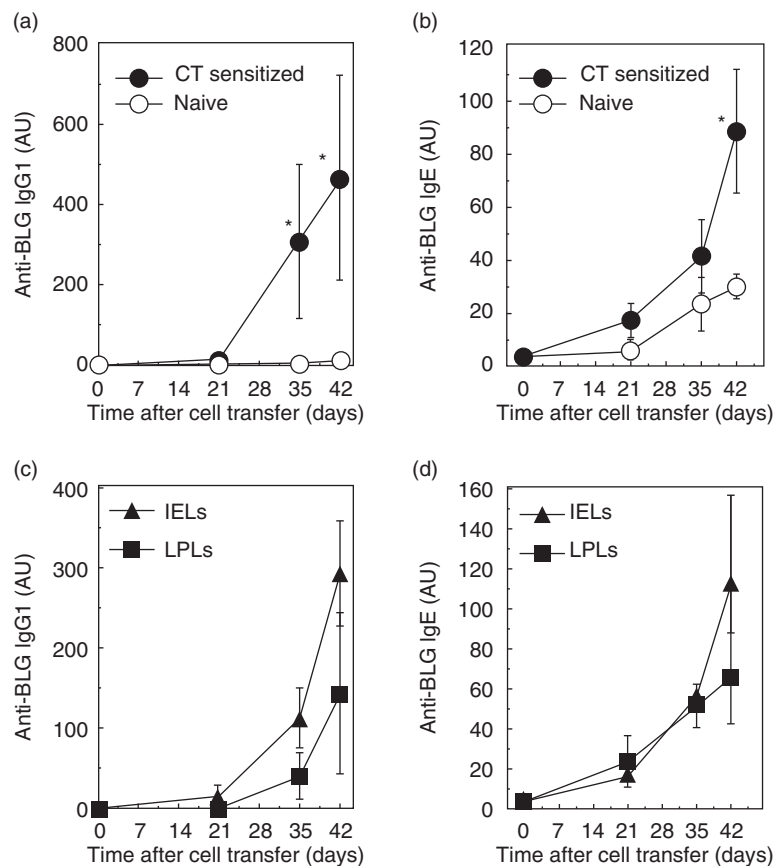


Fig. 3. The transfer of lymphoid cells from the intestinal epithelium of cholera toxin (CT)-fed mice induces the production of CT-specific immunoglobulin (Ig)G1 in the recipient CT-naive mice. (a) Mice were fed (closed circles) or not (open circles) with 10 μ g CT and killed 24 h later. Lamina propria and intestinal epithelium cells were transferred into CT-naive mice to measure CT-specific IgG1 titres in the serum at the indicated time after transfer. (b) Mice were fed with 10 μ g CT. After 24 h, isolated lamina propria lymphocytes (LPLs) (squares) or intraepithelial lymphocytes (IELs) (triangles) were transferred into CT-naive mice and CT-specific IgG1 titres measured in serum at the indicated time. Mean $\pm$ standard error of the mean of antibody titres expressed in arbitrary units (AU); an experiment with six CT-naive recipients mice per groups is represented. Experiments were done three times. * $P < 0.05$.

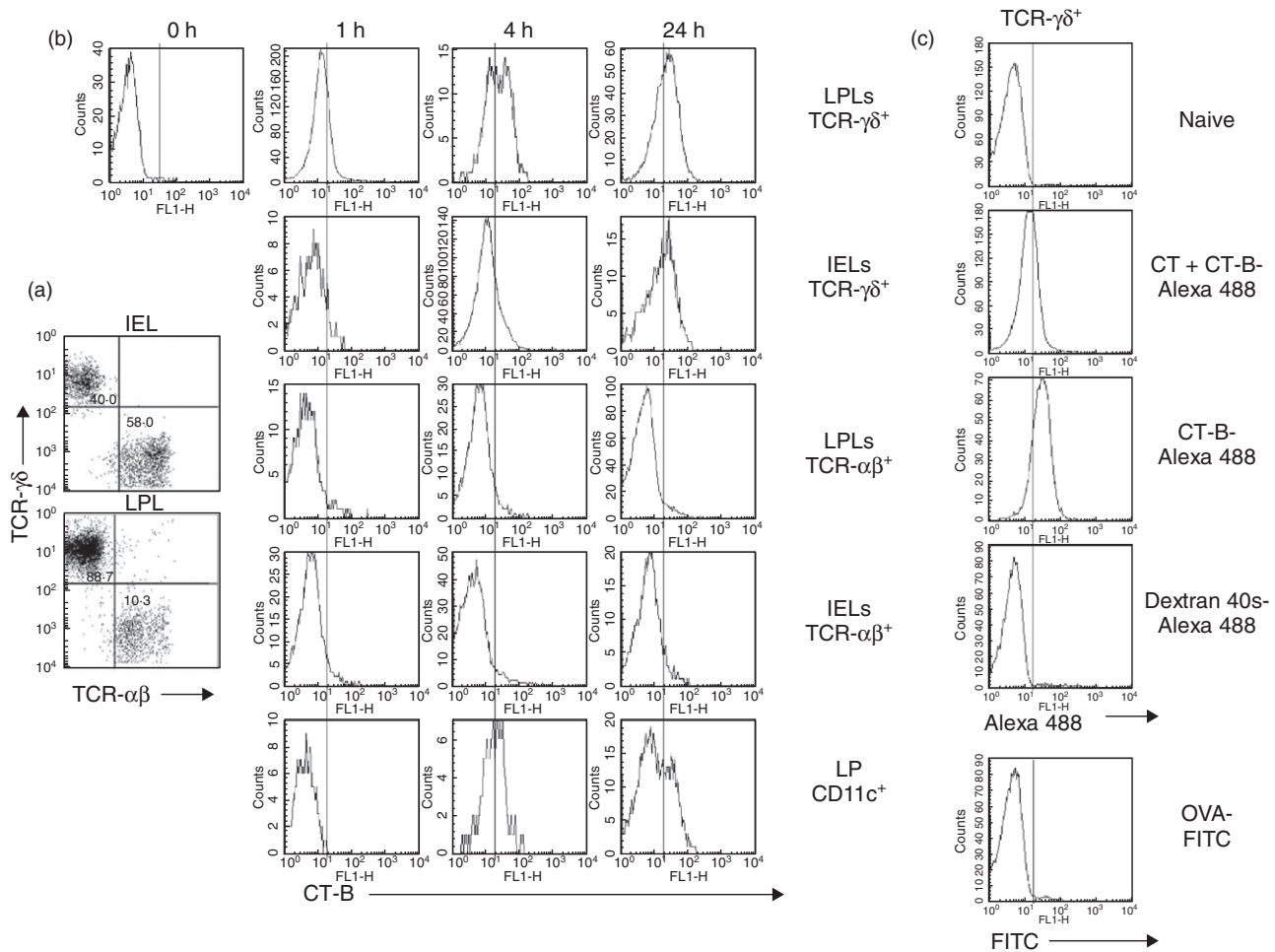


Fig. 4. Cholera toxin (CT) administered *in vivo* is bound by T cell receptor (TCR)- $\gamma\delta^+$ cells. (a) Isolated cell fractions from intraepithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) populations are shown on the lower left. (b) Mice were fed with 10 μ g CT and CT-B Alexa 488 and killed after 1, 4 and 24 h to isolate LPLs and IELs. The fluorescence of Alexa 488 positive cells among TCR- $\alpha\beta^+$, TCR- $\gamma\delta^+$ and CD11c $^+$ cells was measured. (c) Mice were killed 4 h after feeding with 10 μ g CT-B-Alexa 488 in the presence or absence of 10 μ g CT (second and third panel), dextran-Alexa 488 or ovalbumin-fluorescein isothiocyanate (OVA-FITC) (as indicated). Isolated TCR- $\gamma\delta^+$ IELs were analysed for fluorescence of Alexa 488 or FITC. (a,b) Flow cytometry analysis representative of three different experiments is presented. Pooled cells from three mice were used for each experiment. Experiments were performed three times.

in vivo. Mice were fed with a mixture of CT and CT-B coupled to Alexa-488 (CT-B-Alexa 488) and killed after 1, 4 or 24 h. The presence of CT-B-Alexa 488 in TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ LPLs and IELs was analysed by flow cytometry. An enhanced mean fluorescence intensity (MFI) was observed in TCR- $\gamma\delta^+$ T cells isolated from the lamina propria and gut epithelium (Fig. 4a), suggesting that they interacted durably with CT-B-Alexa 488. CT-B-Alexa 488 was already detected in TCR- $\gamma\delta^+$ 1 h after oral administration of CT. Noticeably, TCR- $\gamma\delta^+$ cells displayed the same capacity to retain CT than CD11c $^+$ cells from the lamina propria, whereas TCR- $\alpha\beta^+$ T cells no longer interacted with CT-B-Alexa 488 after *in-vivo* administration and cell isolation (Fig. 4b). *In-vivo* oral administration of CT in combination with other antigens coupled to fluorophores, i.e. dextran-Alexa-488 or OVA-

FITC, did not induce a shift of MFI in TCR- $\gamma\delta^+$ lymphocytes (Fig. 4c), suggesting that these antigens did not interact durably with GALT cells. Moreover, the increase of MFI was more important when CT-B-Alexa 488 was administered to mice in the absence of whole CT, suggesting that the CT-B subunit competed with CT to bind the same receptor on TCR- $\gamma\delta^+$ cells.

TCR- $\gamma\delta^+$ GALT cells bind and internalize CT-B *in vitro*

The ability of IELs to induce an immune response after CT activation suggests that CT might be internalized and/or presented by the cell. To determine whether CT was internalized by TCR- $\gamma\delta^+$ cells or remained bound at the cell surface, we incubated isolated, naive IELs for 1 h with

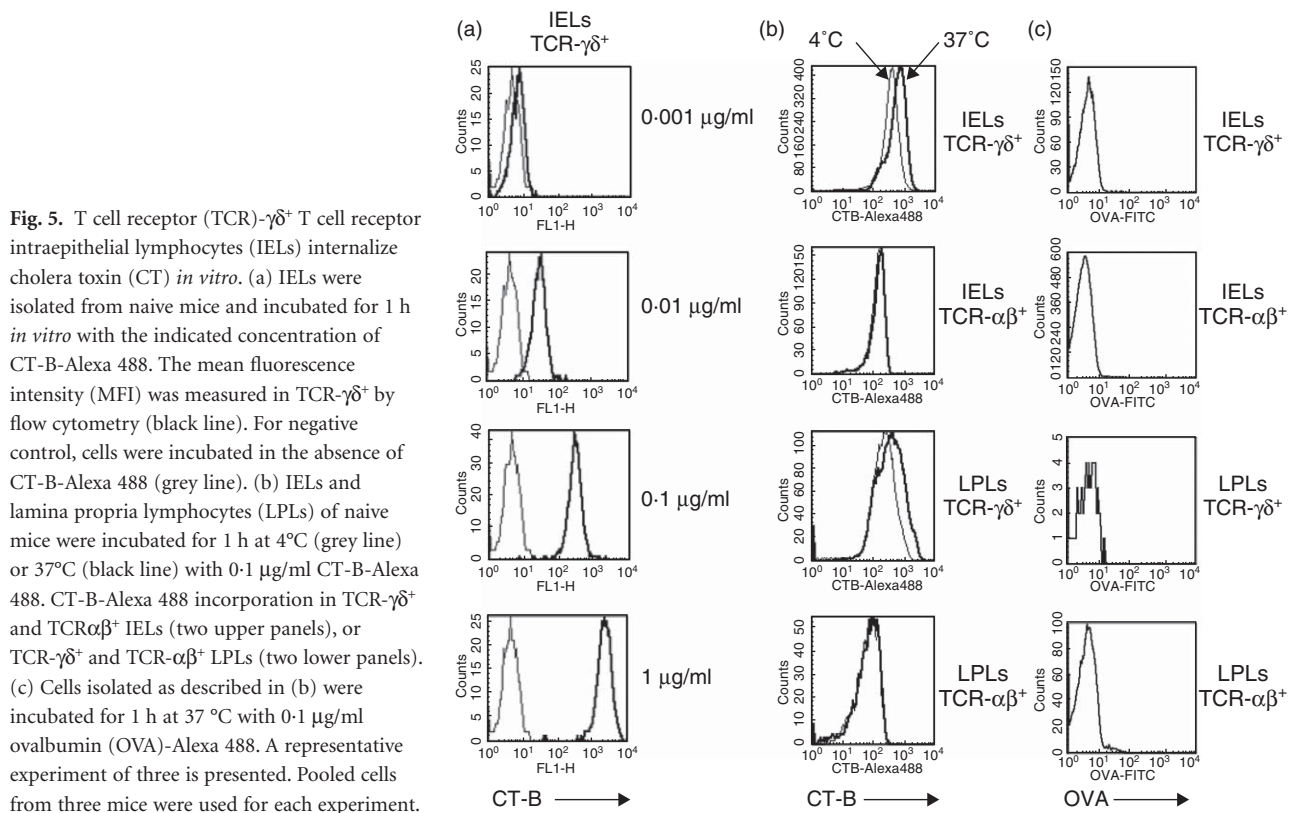


Fig. 5. T cell receptor (TCR)- $\gamma\delta^+$ T cell receptor intraepithelial lymphocytes (IELs) internalize cholera toxin (CT) *in vitro*. (a) IELs were isolated from naive mice and incubated for 1 h *in vitro* with the indicated concentration of CT-B-Alexa 488. The mean fluorescence intensity (MFI) was measured in TCR- $\gamma\delta^+$ by flow cytometry (black line). For negative control, cells were incubated in the absence of CT-B-Alexa 488 (grey line). (b) IELs and lamina propria lymphocytes (LPLs) of naive mice were incubated for 1 h at 4°C (grey line) or 37°C (black line) with 0.1 $\mu\text{g/ml}$ CT-B-Alexa 488. CT-B-Alexa 488 incorporation in TCR- $\gamma\delta^+$ and TCR- $\alpha\beta^+$ IELs (two upper panels), or TCR- $\gamma\delta^+$ and TCR- $\alpha\beta^+$ LPLs (two lower panels). (c) Cells isolated as described in (b) were incubated for 1 h at 37°C with 0.1 $\mu\text{g/ml}$ ovalbumin (OVA)-Alexa 488. A representative experiment of three is presented. Pooled cells from three mice were used for each experiment.

increasing concentrations of CT-B-Alexa 488. As shown in Fig. 5a, a dose-dependent enhancement of MFI was observed in TCR- $\gamma\delta^+$ cells. Because CT internalization was studied mainly in cells capable of endocytosis, e.g. non-immune epithelial cells and cell lines [19–21], we determined whether GALT T cells were able to internalize CT. To this aim, isolated IELs and LPLs from naive mice were incubated with 0.1 $\mu\text{g/ml}$ CT-B-Alexa 488 at either 4°C to hamper, or at 37°C to allow, internalization. In TCR- $\gamma\delta^+$ cells incubated at 37°C, MFI was enhanced slightly compared to MFI in cells incubated at 4°C (Fig. 5b). This effect was specific to TCR- $\gamma\delta^+$, as it was not observed in TCR- $\alpha\beta^+$ cells (Fig. 5b). This result suggests that, *in vitro*, CT-B-Alexa 488 interacted with the cell surface, a small part of it being internalized by TCR- $\gamma\delta^+$ cells but not by TCR- $\alpha\beta^+$ cells. Comparable results were obtained with IELs and LPLs, suggesting that the ability to internalize CT was a characteristic of TCR- $\gamma\delta^+$ cells independently of their location. Binding and internalization was specific to the toxin, as it was not observed with the food antigen OVA (Fig. 5c). These results demonstrate that TCR- $\gamma\delta^+$ lymphocytes localized in the intestinal epithelium and the lamina propria specifically bound and internalized CT-B. By extension, we hypothesized that *in-vivo* CT was likely to bind to and be internalized by both IELs and LPLs TCR- $\gamma\delta^+$ cells to directly affect their phenotype.

Oral administration of CT induces an antigen-presenting cell (APC)-like phenotype on TCR- $\gamma\delta^+$ T lymphocytes

The above results demonstrate that TCR- $\gamma\delta^+$ GALT lymphocytes and particularly IELs of CT-fed mice were able to break oral tolerance and to trigger a Th2 response in recipient mice (see Figs 2c,d and 3b) and that TCR- $\gamma\delta^+$ cells displayed similar ability to CD11c⁺ dendritic cells (DCs) to interact with CT (Fig. 4a). Consequently, we sought to ascertain whether *in-vivo*-activated IELs were expressing APC co-stimulatory molecules. We explored the activation phenotype of TCR- $\gamma\delta^+$ cells after oral administration of CT by analysing isolated IELs and LPLs for the expression of surface markers of T lymphocyte activation (CD25, CD69, CD44 and CD45), chemokine receptors and integrins implicated in intestinal homing (CCR7, CCR9, $\alpha 4\beta 7$ and $\alpha \text{IEL}\beta 7$), as well as molecules involved in antigen presentation (MHC class II molecules, CD80 and CD86). Both TCR- $\gamma\delta^+$ and TCR- $\alpha\beta^+$ LPLs and IELs constitutively expressed significant levels of CD25, CD69, CCR7, CCR9 and $\alpha \text{IEL}\beta 7$ that were not affected significantly by CT feeding. The expression of $\alpha 4\beta 7$, CD44 and CD45 was low, and did not change after CT feeding (not shown). In contrast, the expression of MHC class II molecules, CD80 and CD86, was enhanced in TCR- $\gamma\delta^+$ IELs and LPLs of CT-fed mice;

Table 1. Modulation of surface marker expression on T cell receptor (TCR)- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) after *in-vivo* CT administration.

	TCR- $\alpha\beta^+$				TCR- $\gamma\delta^+$			
	LPLs		IELs		LPLs		IELs	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MHC-II	1.5	1.2	0.9	1.5	5.2*	6.6*	4.2*	5.4*
CD80	1.2	1.8	1.3	1.8	5.1*	4.2*	3.8*	2.1*
CD86	1	1.9	1	0.9	22.3*	10.1*	8.9*	2.2*

Results presented as mean fluorescence intensity (MFI) ratio of cell surface molecule expression in T cells isolated at the indicted time after cholera toxin (CT) feeding *versus* T cells from naive mice; * $P < 0.05$. MHC = major histocompatibility complex.

the expression of the latter molecules was not affected in TCR- $\alpha\beta^+$ cells (Table 1). These results suggest that CT feeding triggered a cell phenotype that usually characterizes APCs in GALT TCR- $\gamma\delta^+$ T cells only, the TCR- $\gamma\delta^+$ cell phenotype remaining unchanged.

To ascertain that up-regulation of MHC class II molecules, CD80 and CD86, was due to *in-vivo* interaction of CT with TCR- $\gamma\delta^+$ cells, we investigated the expression of the latter molecules in cells positive for CT-B-Alexa 488 after feeding mice with CT-B-Alexa 488. MHC class II molecules, CD80, and CD86, were up-regulated in TCR- $\gamma\delta^+$ IELs positive for CT-B-Alexa 488 compared with TCR- $\gamma\delta^+$ IELs from naive mice (Fig. 6). These results suggest that the uptake of CT *in-vivo* triggered TCR- $\gamma\delta^+$ IELs to express surface molecules specific to APC.

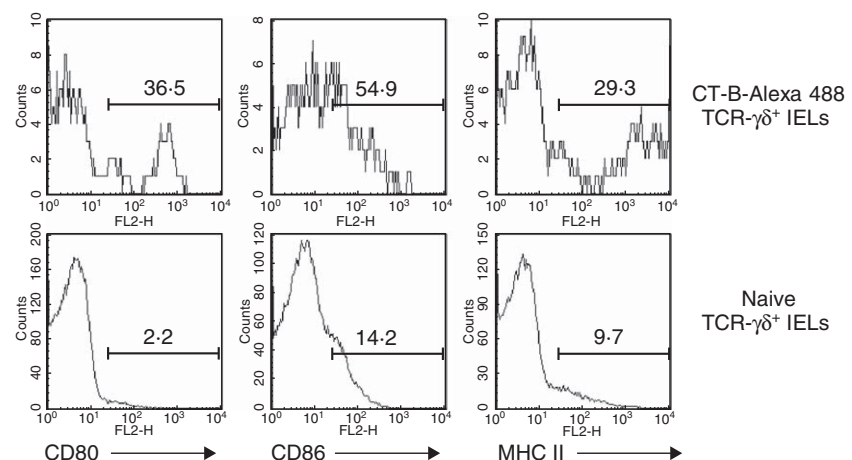
Oral administration of CT induces the migration of TCR- $\gamma\delta^+$ T lymphocytes from the intestinal epithelium to the lamina propria

To function as APCs, activated IELs would have to migrate to lymphoid structures in order to interact with and activate T cells. We therefore explored the *in-vivo* migratory capacity of CT-primed IELs. Mice fed with 10 μ g of CT were killed after 4, 24 and 48 h to analyse cells by flow cytometry. As shown in Fig. 7a, CD3 $^+$ cell frequency decreased rapidly in intestinal epithelium after CT administration and remained

low for at least 48 h. The decrease of CD3 $^+$ cell frequency was accompanied by a decrease of TCR- $\gamma\delta^+$ cell frequency, whereas TCR- $\alpha\beta^+$ cell frequency was proportionally enhanced (Fig. 7b). This suggests that TCR- $\gamma\delta^+$ were diminishing in number in the gut epithelium after mice were fed with CT.

To assess whether the diminution of TCR- $\gamma\delta^+$ IELs in gut epithelium was due to migration of cells to other compartments, we investigated the effect of CT feeding on the frequency of TCR- $\gamma\delta^+$ cells among CD3 $^+$ cells in different intestinal compartments. To this aim, 4 h after mice were fed with CT, we isolated lymphocytes from the lamina propria, Peyer's patches and mesenteric lymph nodes. In addition, cells were isolated from spleen and peripheral blood. Cell populations were first gated for the expression of TCR- δ (TCR- $\gamma\delta^+$ cells), and then for the expression of CD8 α (CD8 $\alpha\alpha^+$ cells). As expected, CT administration diminished the frequency of TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ IELs in treated mice compared to naive mice IELs (Fig. 7c). TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells were not detected in the mesenteric lymph nodes, spleen and blood, suggesting a preferential localization in the mucosal compartment of the intestine of either CT-fed or naive mice (Fig. 7d). Indeed, after CT feeding, the frequency of TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells decreased from $21.0 \pm 4.0\%$ to $11.1 \pm 1.9\%$ in the epithelium, but increased from $2.5 \pm 1.5\%$ to $6.4 \pm 2.0\%$ in the lamina propria, and tended to increase from $2.6 \pm 1.5\%$ to $4.6 \pm 2.0\%$ without

Fig. 6. T cell receptor (TCR)- $\gamma\delta^+$ intraepithelial lymphocytes (IELs) which interacted with cholera toxin (CT) *in vivo* display an antigen-presenting cell (APC) phenotype. Mice were fed or not with 10 μ g CT-B-Alexa 488 and killed 24 h later. TCR- $\gamma\delta^+$ IELs positive for CT-B-Alexa 488 were analysed by flow cytometry for their expression of CD80, CD86 and major histocompatibility complex (MHC) II molecules as indicated. A representative experiment of three is presented. Pooled cells from three mice were used for each experiment.



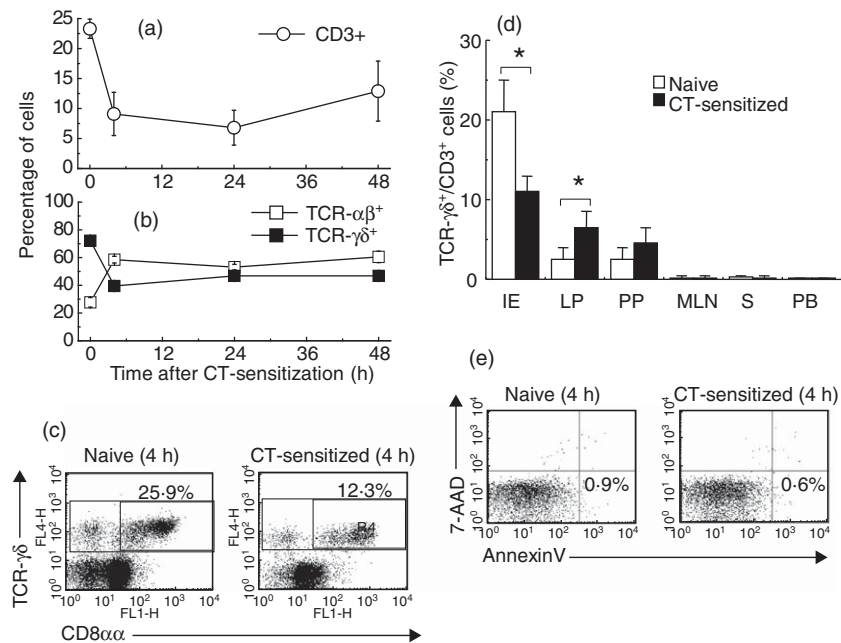


Fig. 7. Cholera toxin (CT) administration decreases the number of T cell receptor (TCR)- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells in the intestinal epithelium compartment. (a) Mice were fed with 10 μ g CT, killed at the indicated time, and the percentage of CD3⁺ cells in intestinal epithelium was measured by flow cytometry (upper panel). (b) The percentage of TCR- $\gamma\delta^+$ (closed squares) and TCR- $\alpha\beta^+$ (open squares) among CD3⁺ cells of intestinal epithelium were analysed by flow cytometry. (c) Percentage of TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ intraepithelial lymphocytes (IELs) 4 h after feeding with 10 μ g CT. (d) Percentage of CD3⁺TCR- $\gamma\delta^+$ /total CD3⁺ cells isolated from various locations 4 h after administration of 10 μ g CT; intestinal epithelium (IE), lamina propria (LP), Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen (S) and peripheral blood (PB), * $P < 0.05$. (e) Viability of TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells isolated from the epithelium. Pooled cells from three mice were used for each experiment. Experiments were performed three times.

reaching statistical significance in Peyer's patches. Additional experiments at 24 and 48 h did not detect TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells in all other compartments explored (including spleen and peripheral blood), ruling out a delayed migration beyond the GALT (not shown). The diminution of TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cells in the intestinal epithelium was not due to cell death, as demonstrated by measuring the frequency of 7AAD-D/annexin V-positive cells (Fig. 7e). Taken together, these data demonstrate that the reduction of TCR- $\gamma\delta^+$ and, more specifically, TCR- $\gamma\delta^+$ CD8 $\alpha\alpha^+$ cell frequency in the intestinal epithelium was due to cell migration from the epithelium to the lamina propria and Peyer's patches.

Oral administration of CT induces the production of IL-10 and IL-17 by TCR- $\gamma\delta^+$ LPLs

Activated T cells usually express a specific pattern of cytokines, which are characteristics of their polarization. In order to assess the pattern of cytokine production of activated TCR- $\gamma\delta^+$ cells, mice were killed at different times (4, 24 and 48 h) after CT feeding. The production of IFN- γ , IL-4, IL-10 and IL-17 by freshly isolated GALT T cells was measured by flow cytometry after 4 h incubation in the absence of stimulus at 37°C with Golgi plug to block

cytokine secretion. Although CT is defined as a Th2 pathway mucosal adjuvant, IL-4 was not detected in LPLs or IELs after *in-vivo* activation by CT (Fig. 8, all parts). Furthermore, IFN- γ , IL-10 and IL-17 were hardly detected, with the exception of TCR- $\gamma\delta^+$ LPLs, which expressed both IL-10 and IL-17 (Fig. 8a). In the latter cells, the production of IL-10 and IL-17 reached a peak 4 h after CT administration and then decreased slowly (Fig. 8a). This suggests that CT-activated TCR- $\gamma\delta^+$ cells are primed to produce cytokines related to a Th2, but also a Th17 inflammatory-type response contributing to a breakdown of oral tolerance.

Discussion

This study demonstrates that gut TCR- $\gamma\delta^+$ lymphocytes are triggered towards an APC-like phenotype upon *in-vivo* activation by CT. Once activated by CT, GALT TCR- $\gamma\delta^+$ lymphocytes isolated from CT-fed mice no longer play a part in the regulation of tolerance, but contribute to tolerance breakdown. CT-activated TCR- $\gamma\delta^+$ IELs display an APC-like phenotype and migrate from intestinal epithelium to lamina propria, where they are triggered to produce IL-10 and IL-17. The ability of CT-activated TCR- $\gamma\delta^+$ lymphocytes to break oral tolerance is long-lasting, as they are able to hamper tolerization to a food antigen (BLG) after

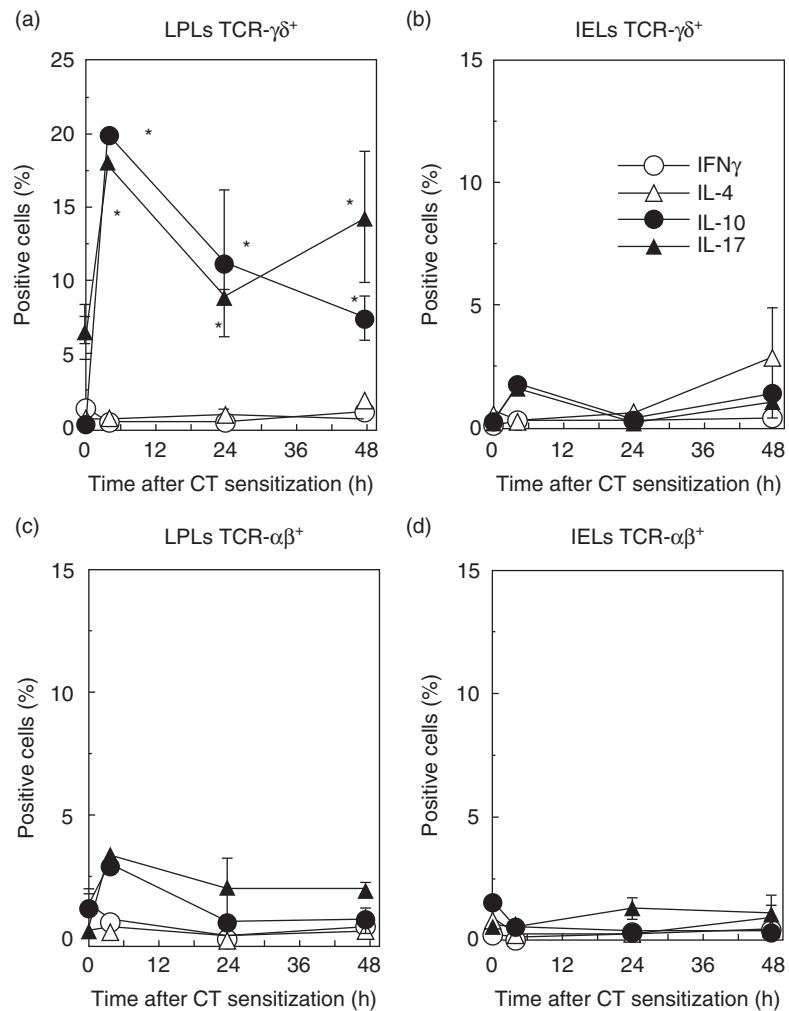


Fig. 8. Cytokine production by T cell receptor (TCR)- $\gamma\delta^+$ (a,b) or TCR- $\alpha\beta^+$ (c,d) lamina propria lymphocytes (LPLs) (a,c) or intraepithelial lymphocytes (IELs) (b,d). Mice were sensitized by oral gavage with cholera toxin (CT), killed at different times and IELs or LPLs were isolated and characterized by flow cytometry for intracellular cytokine production. * $P < 0.05$. Mean $\pm$ standard error of the mean for a total of three experiments with three pooled mice per groups are represented.

adoptive transfer into naive mice in which they induce a Th2 immune response to both BLG and CT. The effects of CT were directed specifically to TCR- $\gamma\delta^+$ cells, as TCR- $\alpha\beta^+$ cells were not able to internalize CT.

Oral administration of food antigens promotes systemic tolerance that is long-lasting and affects immune responses in terms of antibody production, lymphocyte proliferation, delayed hypersensitivity and contact hypersensitivity [22]. In this context, GALT TCR- $\gamma\delta^+$ cells play an important part in the induction and maintenance of oral tolerance to allergens [23,24]. Feeding with antigens induces a state of tolerance that is associated with activation of CD8 $^+$ T cells that can transfer unresponsiveness to naive syngeneic hosts [25]. In contrast, in this study we demonstrate that CD8 $^+$ TCR- $\gamma\delta^+$ cells activated by CT not only lose this protective characteristic, but display a long-lasting allergenic function. It is likely that the CT-B subunit *per se* displays the ability to induce a bias towards an APC phenotype in TCR- $\gamma\delta^+$ cells, as IELs isolated from mice fed with CT-B-Alexa 488 in the absence of the CT-A toxin expressed CD80, CD86 and MHC class II molecules. That the CT-B subunit may affect the expression of APC-specific surface molecules was dem-

onstrated previously in different types of cell. For instance, CT-B enhances the expression of MHC class II molecules in B cells and up-regulates the levels of CD40 and CD86 in macrophages when coupled to an antigen [26,27]. Besides, APCs treated with CT increase T cell proliferative responses to anti-CD3 antibodies, suggesting that APC functions were strengthened by CT treatment [28]. More recently, it was demonstrated that the binding of CT-B to cell surface GM1 induces signal transduction in APCs *in vitro*, thus suggesting a direct effect of CT-B in target cells [29]. Together with the present results, these studies indicate that CT-B is likely to be sufficient to induce an APC phenotype in TCR- $\gamma\delta^+$ GALT cells.

The results presented here demonstrate that GALT TCR- $\gamma\delta^+$ T cells, and particularly IELs, display the ability to induce the production of IgE and IgG1 antigen-specific antibodies in recipient mice, suggesting that CT-activated TCR- $\gamma\delta^+$ IELs not only display an APC phenotype but also exhibit APC activity. Indeed, as TCR- $\gamma\delta^+$ from CT-fed mice rather display an APC- than a Th2-like phenotype, it is probable that they trigger a full acquired immune response when transferred into naive mice. Similarly, induction of a

Th2 response in recipient mice was observed after adoptive transfer of DCs from OVA-sensitized mice [30]. The extent of CT-B-Alexa 488 binding to TCR- $\gamma\delta^+$ lymphocytes was similar to that of CD11c⁺ DCs from the lamina propria, suggesting that both types of cell display comparable interaction, with CT being able to internalize it. The ability of T cells to bind CT-B with a subsequent abrogation of induction of suppressor T cells was demonstrated previously by others [31]. The latter study also demonstrated a marked depletion of IELs in epithelium, suggesting that CT administration induces migration of IELs in agreement with the present data. Furthermore, our data are in accordance with previous results, which demonstrate that CT-B inhibits mucosal Th1 cell signalling [32]. However, in contrast with the present study, the involvement of a direct effect of CT-B on TCR- $\gamma\delta^+$ IELs was not demonstrated in the latter study.

CT-B activation of GALT TCR- $\gamma\delta^+$ T cells induces the expression of molecules required for antigen presentation and subsequent T cell activation (CD80, CD86, MHC class II molecules), but does not significantly modulate typical T cell activation markers. This suggests that TCR- $\gamma\delta^+$ T cells differentiate into APCs, a mechanism that is confirmed by their potency to induce the production of IgE and IgG1 once transferred into naive mice. Similar experiments investigating the up-regulation of co-stimulatory molecules have been performed on canonical APCs, i.e. DCs and macrophages. Murine bone marrow-derived DCs activated *in vitro* by CT display higher levels of CD80 expression, and induce allogenic CTL responses [33]. In contrast, CT up-regulates CD86 expression differentially in bone marrow macrophages [34]. However, in pig alveolar macrophages treated with CT, the expression of both CD80 and CD86 is enhanced [35]. More recently, it has been demonstrated that a combination of anti-CD8 and anti-CD28 up-regulates MHC class II molecule expression in mice TCR- $\gamma\delta^+$ T cells, thus inducing an APC phenotype [16].

CT-activated TCR- $\gamma\delta^+$ IELs migrate out of the epithelium to lamina propria and Peyer's patches. The depletion of IELs in the epithelium occurs rapidly after CT administration (4 h) and lasts for at least 48 h. Migrating TCR- $\gamma\delta^+$ IELs are found mainly in the lamina propria, their increased frequency in Peyer's patches being below statistical significance at 4 h. This demonstrates that CT triggers IEL migration towards lymphoid structures with germinal centres, where they might act as APCs to trigger a Th2 response. APC migration in the gut has been demonstrated upon infection or toxin treatment. DCs migrate from the subepithelial dome to T and B cell areas of Peyer's patches within 12–24 h after administration of CT or live *Salmonella* [36]. Furthermore, feeding with a high dose of CT (50 μ g) induces migration of subepithelial DCs to interfollicular T cell areas within 24 h, i.e. migration from Peyer's patches to follicle-associated epithelium [37]. The migration of IELs has been suggested by the marked depletion of CD8⁺ cells in

the gut epithelium after intestinal administration of 10 μ g of CT that was accompanied by altered proliferation of these cells [31]. More recently, in accordance with the present results, depletion of IELs in gut tissues has been observed 3 days after initiation of food allergy with CT and peanut extract [24]. In the latter study, however, the target tissue was not identified. Here we show that IELs that interact *in vivo* with CT-B-Alexa 488 leave the gut epithelium to migrate to lamina propria and Peyer's patches.

In-vivo CT-activated TCR- $\gamma\delta^+$ IELs migrate rapidly from epithelium to lamina propria, where they are triggered to express IL-10 and IL-17. Indeed, only isolated TCR- $\gamma\delta^+$ LPLs produce cytokines in the absence of further *in-vitro* activation in contrast with TCR- $\gamma\delta^+$ IELs and TCR- $\alpha\beta^+$ T cells. Few studies have investigated cytokine production by TCR- $\gamma\delta^+$ T cells, due mainly to difficulties in activating *in-vitro* cytokine production, and to the limited viability of TCR- $\gamma\delta^+$ T cells in such conditions. In contrast with gut lymphocytes from other compartments, TCR- $\gamma\delta^+$ LPLs isolated from naive mice and activated *in vitro* produce IL-10 and IL-17 [13]. TCR- $\gamma\delta^+$ from other sources than gut are major producers of IL-17 upon infection [38–40]. The role of IL-10 has been demonstrated extensively in the regulation of inflammation in the gut [41,42]. The significance of the production of cytokines with seemingly antagonistic functions by the same types of cells remains to be determined. Also, an indirect role of TCR- $\gamma\delta^+$ T cells, unrelated to specific cytokine production by these cells, might be possible. A clue might be in the premise that in macrophages activated by CT, the enhancement of APC-specific molecules is associated with increased IL-10 production [43].

Our study presents some limitations, related mainly to the difficulty of isolating and purifying TCR- $\gamma\delta^+$ cell populations. Indeed, in the cell samples we used in our experiments, we cannot exclude a potential antigen-presenting role of other cells. However, CD11c⁺ cells were present in only very small amounts (1–3%) of the IEL cell samples; if there were any, their role as APCs was most probably only partial. Also, adoptive transfers with highly purified populations for TCR- $\gamma\delta^+$ cells were sought. However, we were not able to increase the purity of IEL cells samples, due possibly to mucus interference. TCR- $\gamma\delta^+$ cell viability was also interfering with cell transfer experiments.

Together, the present results demonstrate that TCR- $\gamma\delta^+$ T cells are a major CT target to break oral tolerance. Recent observations demonstrated the unique regulatory role of TCR- $\gamma\delta^+$ T cells in the gut [24]. More specifically, TCR- $\gamma\delta^+$ IELs have also been shown in humans to regulate inflammation in a non-IgE-mediated food hypersensitivity, i.e. coeliac disease [44]. Here, in a mouse model of IgE-mediated food allergy, we demonstrate that CT alters TCR- $\gamma\delta^+$ lymphocyte phenotype and, in turn, changes their regulatory role into an allergenic, proinflammatory role.

Acknowledgements

This study was funded by grants 310030-134904 and 310000-120318 from the Swiss National Science Foundation, the Yde Foundation and the Hans Wilsdorf Foundation.

Disclosure

None.

Author contributions

The study was initiated and designed by C. P. F., P. A. E. and D. B.; C. P. F. and K. E. A. performed the experiments, C. P. F., D. B. and P. A. E. wrote the manuscript.

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