Food-sensitized pediatric patients show colonic cow's milk protein-specific Th2 cells

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February 22, 2024

Abstract

Background: Manifestations of food allergies vary among patients, being the most common gastrointestinal, skin and respiratory symptoms. This study aimed to identify and characterize polyp Th2 cells as a critical component of the allergic reaction. We previously characterized colorectal polyps in patients sensitized to food allergens as inflammatory, with a Type 2-dominated stroma, with elevated IL-13, IL-4 and locally synthesized IgE. Methods: We isolated milk-specific T cells from tissue and blood of food-sensitized patients (n=10) and obtained cell lines and cell clones. Proliferation, specificity, cytokine secretion, gut homing markers and TCR nature were analyzed after antigen recall; CCL25 was assessed in polyps and surrounding tissue. Results: Lamina propria and peripheral T cells responded similarly to cow´s milk proteins, showing similar cell proliferation index and Th2 cytokine release *in vitro*. All CD4 $^{+}$ T cells expressed the membrane T $_{\alpha\beta}$ receptor and secreted higher IL-13 amounts than unstimulated cells, whereas IFN-γ secretion remained unchanged. Remarkably, the gut homing chemokine receptor CCR9 was augmented in cow's milk-specific peripheral and lamina propria T cells, and CCL25 was found to be expressed in the inflammatory polyp tissue and not in the adjacent mucosa. Conclusion: We isolated and characterized cow´s milk-specific lamina propria CD4 $^{+}$ TCR$\alpha\beta$ $^{+}$ Th2 cells from colonic inflammatory polyps. The CCR9 expression on polyp cells and the increased production of tissue CCL25 might facilitate T cells' recruitment, which are key players in the allergic reaction to promote local IgE synthesis. Our findings may be critical to proposing rational and novel T cell-targeted immunotherapies.

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Running title: Th2 colonic T-cells in food sensitized patients

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ABSTRACT (n=249)

Background : Manifestations of food allergies vary among patients, being the most common gastrointestinal, skin and respiratory symptoms. This study aimed to identify and characterize polyp Th2 cells as a critical component of the allergic reaction. We previously characterized colorectal polyps in patients sensitized to food allergens as inflammatory, with a Type 2-dominated stroma, with elevated IL-13, IL-4 and locally synthesized IgE.

Methods : We isolated milk-specific T cells from tissue and blood of food-sensitized patients (n=10) and obtained cell lines and cell clones. Proliferation, specificity, cytokine secretion, gut homing markers and TCR nature were analyzed after antigen recall; CCL25 was assessed in polyps and surrounding tissue.

Results : Lamina propria and peripheral T cells responded similarly to cow´s milk proteins, showing similar cell proliferation index and Th2 cytokine release *in vitro* . All $CD4^+$ T cells expressed the membrane $T_{\alpha\beta}$ receptor and secreted higher IL-13 amounts than unstimulated cells, whereas IFN-γ secretion remained unchanged. Remarkably, the gut homing chemokine receptor CCR9 was augmented in cow's milk-specific peripheral and lamina propria T cells, and CCL25 was found to be expressed in the inflammatory polyp tissue and not in the adjacent mucosa.

Conclusion : We isolated and characterized cow´s milk-specific lamina propria $CD4^+$ $TCR\alpha\beta^+$ Th2 cells from colonic inflammatory polyps. The CCR9 expression on polyp cells and the increased production of tissue CCL25 might facilitate T cells' recruitment, which are key players in the allergic reaction to promote local IgE synthesis. Our findings may be critical to proposing rational and novel T cell-targeted immunotherapies.

AKCNOWLEDGEMENTS

GHD, RC, MCA and CIM are researchers of CONICET, and KEC and MI are fellows of CONICET.

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2018-2479) and UNLP (grant 11/X695) to GHD and PICT 2016-0648 to CIM.

Key words: Food Allergy, T lymphocytes, Colorectal polyps, Cow´s milk proteins

INTRODUCTION

Cow´s milk allergy (CMA) is one of childhood´s most prevailing food allergies[1,2]. The continuous epidemiological change of allergic reactions, particularly those against food, has intensified the interest in understanding the immunopathogenesis of these inflammatory disorders to design novel disease-modifying immunotherapies. During an allergic reaction to food, the skin, gastrointestinal tract, respiratory and cardiovascular system can be affected. Over past decades, the worsening of allergic responses has led to an increase in the incidence of anaphylactic shocks in the pediatric population, highlighting the need to develop therapies to overcome the immunological tolerance breakdown observed in patients. Milk-induced inflammatory changes were observed in the colonic mucosa, leading to different clinical disorders[3]. Duodenal milk-specific T cells displaying a type-2 cytokine expression pattern were described for non-IgE-mediated hypersensitivity mechanisms in food-related inflammatory disorders[4]. However, less is known about IgE-mediated allergic responses in the human gut, which may be involved in the occurrence of the systemic hypersensitivity reactions of anaphylaxis. We recently showed that juvenile polyps (JP) could be associated with an inflammatory response

to food allergens. JP showed a prominent cell infiltration with a high frequency of recruited eosinophils, inflammatory cytokines including IL-4 and IL-13, CMP-specific IgE and active IgE synthesis in germinal centers[5,6]. This study shows the presence of milk-specific T cells in the inflammatory tissue, which might be critical to understanding the rationale for a disease-modifying treatment that could mitigate mucosal inflammation. $CD4^+$ effector T cells can exert a pivotal role in the induction and regulation of IgE synthesis and eosinophil attraction through the secretion of different interleukins. It has been described that the Th2 cells that drive an IgE-mediated allergic reaction are a heterogeneous cell population that can have different roles depending on the immunopathogenesis of the individual inflammatory disorders[7]. A novel T cell-targeted immunotherapy design should be focused on any particular Th2 subset to improve its safety and efficacy.

There is scarce information about the complexity of T cells in the human gut, specifically during an allergic response to food allergens in the large bowel. One of the current challenges is the low frequency of food allergen-specific T cells and ethical limitations to obtaining gut biopsies from food-sensitized or allergic patients. The present study reports for the first time the establishment of primary cultures and cloning expansion of cow´s milk protein (CMP)-specific T cells from juvenile colorectal polyps of IgE-sensitized pediatric patients. Intestinal lamina propria T cells and peripheral blood lymphocytes responded to CMP allergens, showed a Th2 phenotype, and increased gut homing receptor CCR9 upon antigen recall, while polyp tissue expressed high levels of the CCR9 ligand CCL25.

MATERIALS AND METHODS

Patients and biological samples

Patients with rectal bleeding were included in the study (n=10). Peripheral blood, juvenile colorectal polyps and surrounding colonic biopsies were taken from pediatric patients that underwent a screening colonoscopy due to painless rectal bleeding at the Gastroenterology Unit of the Children´s Hospital Sor María Ludovica of La Plata. The average age of patients was 4.50 yo (IQR= 3.00-6.25 yo), and 50% of males (n=5). Food sensitization was assessed by means of clinical history (table 1), skin prick test (SPT) and serum IgE tests at the Allergy Unit. SPT was carried out with commercial extracts of CMP, fish, egg, peanut, soy, and wheat (Alergo Pharma, Buenos Aires, Argentina), histamine phosphate (10 mg/ml) and saline solution as controls. A skin reaction with a flare >4 mm was considered positive. Serum total and food-specific IgE (peanut, soy, and cow´s milk) were assessed by ELISA, according to Docena *et al.* [8].

Peripheral blood samples from patients without signs of CMA were analyzed as controls.

The study protocol was analyzed and approved by the Ethics Committee of the Children´s Hospital of La Plata (#389-2014 and #389-2018) and patient parents gave written informed consent in all cases.

Generation of T cell lines from juvenile polyps or colonic biopsies :

Polyps and biopsies were collected on RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics, and a cell suspension was obtained as described in Curciarello *et al.* , 2021[9]. Briefly, the epithelial layer was removed with EDTA (Sigma-Aldrich, MO, USA) and DTT (Sigma-Aldrich, MO, USA) and the remaining tissue was minced and digested with type 1A collagenase (Sigma-Aldrich, MO, USA) and DNAse (Roche, ThermoFisher, MA USA). Cells were filtered, washed and cultured in serum-free Ultraculture medium (LONZA, Basel, Switzerland), supplemented with 2 mM glutamine (LONZA, Basel, Switzerland), 20 µM 2-mercaptoethanol (DTT, Sigma-Aldrich, MO, USA), and Antibiotic-Antimicotic (Gibco, Texas, USA). To obtain allergen-specific lamina propria T cell lines (LPTCL), cells were stimulated with skimmed cow's milk powder (1 mg/mL that contains 350 µg/mL of CMP, La Serenísima, Argentina) at 37°C, 5% CO_2. Four days later, cells were treated with recombinant human (rh) IL-2 (10 U/ml, Preprotech, NJ, USA), rhIL-7 (10 ng/mL, Preprotech, NJ, USA) and rhIL-15 (10 ng/mL, Preprotech, NJ, USA). After 5 days, viable T-cell blasts were enriched by Ficoll-Paque^{TM} (GE, Healthcare, Life Sciences, Sweden) gradient and then incubated in 96-well round-bottom cultures plates in Ultraculture medium (Lonza, Basel, Switzerland), supplemented with cytokines, 10% human AB^+ plasma and irradiated PBMC. Cell expansion was repeated twice a week until number of cells was obtained, thus generating LPTCL.

Generation of peripheral T cell lines :

PBMC were isolated from peripheral blood using a Ficoll-Paque^{TM} (GE, Healthcare, Life Sciences, Sweden) density gradient. Peripheral blood T cell lines (PBTC) were obtained and expanded as described previously[10].

Generation and characterization of T cell clones

Allergen-specific lamina propria T cell clones (LPTCC) and peripheral blood T cell clones (PBTCC) were obtained by the limiting dilution technique[10]. Briefly, 500 cells of LPTCL or PBTCL were resuspended in serum-free Ultraculture medium and supplemented with 300x10^6 irradiated PBMCs, 10% human plasma AB$^+$, 4000 U rhIL-2 (plus 10 ng/mL IL-7 and IL-15 for LPTCC) and 1 µg/ml phytohemagglutinin-L (PHA) (Sigma, MO, USA). The cloning mixture was distributed in U-bottomed 96-well plates and incubated. Cells were observed overtime at 14, 21 and 28 days, and wells with proliferation were expanded following the procedure described for LPTCL and PBTCL. Once every two expansions, cells were restimulated with CMP and irradiated-syngeneic Epstein-Barr virus immortalized cells (EBV).

For TCR characterization, 10^5 cells were stained with anti-CD3-APC (BD Pharmingen, CA, USA), anti-TCR α/β-APC and anti-TCR δ/γ-percp-cy5.5 (BioLegend, CA, USA) or the appropriate isotype controls and analyzed by flow cytometry in a FACS Aria Fusion (BD, NY, USA). Alternatively, TCR α and β chains were amplified by RT-PCR using specific primers as in Heufelder*et al.* [11]. Briefly, cells from 5 wells were harvested and total RNA was extracted using Total RNA Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Amersham Palace, Little Chalfont, UK) according to the manufacturer's protocols. RNA was then reverse transcribed using random primers and M-MLV Reverse transcriptase (Invitrogen, CA, USA). PCR was performed using a stepwise program of 30 cycles (95°C for 1 min, 62°C for 1 min, 72°C for 1 min) followed by a 7-min extension at 72°C. The amplification products were visualized in 3 % agarose gels.

Generation of Epstein-Barr Virus (EBV)-immortalized B cell lines

PBMCs from patients were transformed with EBV-containing culture , obtained from exponentially growing B95-8 marmoset cells (containing EBV) according to Biddison *et al* .[12] .

Allergen-specific proliferation and characterization assays

T cell lines and T cell clones were rested for 10 days, and then 10^5 cells were labeled with CFSE (Sigma-Aldrich, MO, USA), co-cultured with syngeneic fresh 10^5 irradiated immortalized B cells, and stimulated with CMP. Controls were included with or without PHA (2.5 µg/mL, Sigma-Aldrich, MO, USA). Cells were maintained in serum-free AIMV medium (Thermofisher Scientific, MA, USA) for 4 days, supernatants were harvested for cytokine quantification and cells were stained with anti-CD3-APC (BD Pharmingen, CA, USA) by flow cytometry (FACSC BD, NJ, USA and FACS Aria Fusion, BD, NJ, USA). For the T cell proliferation assay, anti-CD4-APC (BD Pharmingen, CA, USA) was included in the cytometry analysis. The proliferation index was calculated as in Munson (Munson, 2010). The frequency of CCR9$^+$ T cells was assessed using an anti-CD199-PE monoclonal antibody (, CA, USA). Appropriate isotype controls were used and data were analyzed using FlowJo software (BD, NJ, USA).

To address the specificity of proliferating cells, TCL were rested for 10 days in triplicate with whole CMP (1 mg/mL), purified α-casein, β-casein, ϰ-casein, and β-lactoglobulin (50 µg/mL) (Sigma-Aldrich, MO, USA) or medium. Wells contained 10^5 mucosal or peripheral blood cells number of irradiated syngeneic EBV-treated cells in AIMV medium. After 80h of incubation, cells were pulsed with 1 µCi of^{3}H-thymidine (Amersham Biosciences, UK) per well for 16 h. Then, cells were harvested and counted in a liquid counter. The stimulation index (SI) was calculated as the ratio between counts per minute (cpm) of stimulated to unstimulated cells. A value above unstimulated media + 10 SD was considered positive.

Real-time RT-PCR analysis

RNA was extracted from 6 wells of CMP-expanded PBTCL and TCC. Isolated RNA was then reverse-transcribed, as indicated before. Real-time quantitative PCR (RT-qPCR) for CCR4, CCR9 and cutaneous

lymphocyte antigen (CLA) was done using specific primers and SYBR Green PCR Master Mix (BioRad, Hercules, CA, USA) and the iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Gene expression was normalized to β-actin and data were analyzed using the comparative threshold (Ct), expressed as relative quantitation of gene expression (2^{-Ct}). Primers sequences used are shown is Supplementary Table 1. To evaluate tissue CCL25 expression levels, a polyp fragment or surrounding tissue biopsy were minced using a T10 Basic Ultra Turrax 230V homogenizer before RNA extraction, which was performed as indicated above.

Quantification of cytokines. Type-1 and type-2 cytokines were quantified in PBTCL and LPTCL supernatants by ELISA using commercial kits and following manufacturer's instructions (human IL-13 -Invitrogen, Thermo-Fisher, MA, USA and human IFN-γ-ImmunoTools, Germany).

Statistical analysis . All statistical analyses were carried out using GraphPad Prism 8 software. The significance of the difference was determined using an independent-sample t -test or ANOVA. Ap -value $<$ 0.05 was considered statistically significant.

RESULTS

Proliferation and cytokine production of peripheral blood and lamina propria T cells

Patients showed clinical history associated with allergy or atopy (Table 1), a median (IQR) of IgE concentration in serum of 152.2 IU/mL and 100% (10/10) had CMP-specific IgE. The skin prick test was negative in all of them. TCL from peripheral blood and lamina propria of juvenile colorectal polyps were expanded by stimulation with CMP and we found that the frequency of proliferating $CD4^+CFSE^{low}$ cells was significantly higher for CMP-stimulated PBTCL and LPTCL, and PHA-stimulated cells than for non-stimulated control cells (Figure 1A and B). Fresh cells removed biopsies of the control surrounding tissue did not proliferate upon milk stimulation for 4 days, even with the addition of IL-2, IL-7 and IL-15 to the culture medium. Furthermore, T cells from the peripheral blood of control patients did not proliferate following stimulation with CMP.

To further characterize these cells, we analyzed the cytokine secretion of PBTCL and LPTCL after 3 days of CMP stimulation. We found higher values of soluble IL-13 than in resting TCL (76.64±41.6 *vs*.43.9±31.3 pg/ml for peripheral blood and 77.6±24.2 *vs.* 36.5±14.6 pg/ml for lamina propria TCL, $p<0.05$), whereas the concentration of IFN-γ remained unchanged in all PBTCL and LPTCL stimulated with CMP (Figure 1C).

In order to study whether cells from food-sensitized patients responded to individual milk proteins, we recalled PBTCL and LPTCL with α-, β-, κ-casein and β-lactoglobulin and then analyzed the proliferation index. Since CMP-specific LPTCL were difficult to maintain alive in culture compared to PBTCL, we could only analyze LPTCL from four patients. We found that the PBTCL proliferation was significantly induced with α-, β-, κ- caseins and β-lactoglobulin when compared to non-stimulated PBTCL (Figure 1D). Similar results were obtained when LPTCL were stimulated with caseins. However, we found no significant proliferation of LPTCL with β-lactoglobulin (Figure 1D). Then, individual TCL were evaluated for the allergen-specific proliferative response and we found that 7/10 PBTCL responded to the major milk allergen, α-casein, whereas 6/10 PBTCL proliferated with β-casein. Then, 8/10 PBTCL responded to κ-casein and 6/10, to β-lactoglobulin. All LPTCL proliferated in response to caseins, whereas only 2 out of them responded to β-lactoglobulin. Of note, LPTCL2 responded to all allergens tested while the corresponding PBTCL2 only responded to CMP, which means that it likely responds to other dairy allergens (Figure 1E). Similarly, protein specificity differed for PBTCL3 and -4, compared to the LPTCL from the same patient.

Milk-specific TCL expressed the gut home marker CCR9

We next analyzed peripheral blood TCL for T cell homing markers by RT-qPCR. Milk sensitized patients were classified according to the presence of skin signs. Three out of them had atopic dermatitis, one had urticaria related to exposure to CMP (named wsm from "with skin manifestation"), and the remaining 6 patients had no cutaneous manifestation (named wosm from "without skin manifestation"). We found that all PBTCL expressed the gut-homing chemokine receptor CCR9 (0.116±0.005 *vs* . 0.03±0.02 relative to

β-actin expression for wsm*vs* wosm, respectively). $PBTCL_{wsm}$ expressed the skin homing CLA and CCR4 transcripts (3.26±3.2 and 703±300 relative to β-actin expression). No amplification was observed for these transcripts in samples from wosm patients

We next assayed the expression of CCR9 on T cells by flow cytometry. We found that CCR9 MFI was increased in CMP-activated $CD4^+$ LPTCL, whereas a trend was observed for CMP-stimulated PBTCL (Figure 2B). Nevertheless, the analysis of $CD4^+CFSE^{low}$ proliferating T cells showed a higher frequency of $CCR9^+$ cells in PBTCL and LPTCL upon recall ($p<0.05$) (Figure 2). Interestingly, for PBTCL, we found that there was also a significant increase in the percentage of $CD4^+CFSE^{low}CCR9^-$ cells (Figure 2).

Finally, we assessed the transcript level of CCL25 in the colon and our findings showed that the CCL25 expression was significantly higher in polyp tissue than in the adjacent colonic tissue (Figure 2E).

T cell clones from peripheral blood and lamina propria of patients are Th2 and express the αβ T cell receptor

We generated several TCC from the peripheral blood and the lamina propria of polyps from 3 patients (P1-P3) and its specificity was assessed by the analysis of CMP-stimulated proliferative cells by flow cytometry. All TCC were $CD4^+$ cells and those with SI_{CMP}/SI_{Medium} >1.5 were considered positive for proliferation. As shown in figure 3A, PBTCC and LPTCC proliferated in response to CMP. We next assessed IL-13 and IFN-γ secretion from TCC by ELISA, and clones were classified based on IL-13/IFN-γ ratio as follows: >5 corresponded to Th2 cells; 0.2–5, to Th0; and <0.2, to Th1 (Figure 3B). All PBTCC obtained were Th2 cells, except one from P1 that was classified as Th0. As for LPTCC, 2234 clones from P1 were Th2 cells, 5 Th0 and 7 Th1 cells.

Finally, TCC were analyzed for TCR expression by RT-PCR and flow cytometry. We found that all PBTCC and LPTCC expressed the α and β chain transcripts and the anchored-membrane receptor (Figure 3C).

DISCUSSION

We previously demonstrated that juvenile colonic polyps from patients sensitized to food allergens presented high tissue levels of IL-4, high IL-13/IFN-γ ratio, high level of CCL26 accompanied of a dense eosinophil infiltration and IgE-producing germinal centers compared to the surrounding colonic tissue[5,6]. We also found high milk-specific IgE levels in serum and in polyp tissue, indicating that CMP might be involved in allergic inflammation. The polyp-confined Th2 environment prompted us to investigate the T cell infiltration of polyps. Cells obtained from tissues were challenged with CMP to generate specific T cell lines. Our results showed that T cells from polyps, and not those from the healthy colonic mucosa or peripheral cells from non-allergic patients, responded to milk allergens and we found activated $CD4^+TCR_{\alpha\beta}{}^+$ cells that secreted IL-13 with no IFN-γ production. Similar results were found by Beyer *et al.* for duodenal T cell lines from milk allergic patients[13].

On the other hand, previous reports showed that Peyer´s patch T lymphocytes from non-allergic individuals showed a type-1 signature upon stimulation with α1 caseins[14], or an IFN-γ-dominated response to β-lactoglobulin[15]. Our findings suggest a causal relationship between the polyps and the allergic inflammation with a similar response of intestinal and circulating milk-specific $CD4^+$ T cells. We also tested the T cell line response to milk´s allergens (α-, β- and κ-caseins and β-lactoglobulin), trying to find a link with IgE-mediated allergen sensitization[14]. We found that lamina propria T cell lines are mainly specific for caseins and only 50% of them responded to β-lactoglobulin. Nevertheless, peripheral blood T cell lines presented a more heterogeneous response. Surprisingly, the milk-specific PBTCL2 showed no proliferation response to the proteins tested, which may be due to other specificities such as α-lactalbumin, immunoglobulins and/or lactoferrin[16]. Lamina propria T cells have been described to be transcriptionally, phenotypically and functionally different from peripheral blood cells[17]. In this work, we found a broad range of milk allergen recognition with a homogeneous functional signature and no predominant differences were observed between circulating and intestinal cells. Further studies should be done to characterize the expression of tissue-resident markers on polyp T cells.

The chemokine analysis on cells showed a dominant expression of CCR9 on peripheral and tissue T cells, which suggests that these cells are programmed to home to the small bowel, while the expression of the skin-homing markers CCR4 and CLA was observed only in patients with skin manifestations. Previously, Schade *et al.* described antigen-specific T cells in food-allergic children with or without atopic dermatitis, although only patients with atopic dermatitis showed a type-2 cytokine expression[18]. In our study all TCL presented a predominant Th2 phenotype. Also, we found a differential chemokine expression pattern that regulates T cell homing. It is known that once activated, effector and memory cells migrate from lymph nodes to the effector tissues, where the antigen was first encountered[19]. For food antigens, the small intestine has been characterized as the inductive site for oral tolerance, and so T cells are imprinted with $\alpha 4\beta 7$ and CCR9 expression to home to this intestinal section[20]. The epithelial cells from the small bowel constitutively secrete CCL25, the CCR9 ligand[21], whereas in the colon, it has only been described to be induced in an inflammatory condition[22]. Collectively, our results suggest that the Th2 environment promotes the secretion of CCL25 that recruits the activated $CCR9^+$ T cells within the polyp of sensitized patients. In our study, milk-specific PBTCL and LPTCL expressed high levels of CCR9 upon antigen stimulation, and its expression was higher in CD4 polyp infiltrating T cells than in $CD4^+$peripheral T cells. Accordingly, it has been reported that CCR9 is strongly induced in mucosal inflammation[23–25]. Therefore, our findings suggest that CCR9 is expressed on circulating and tissue T cells, being the former attracted to the small and large bowel and the latter retained in the inflammatory tissue through the secretion of CCL25. Of note, none of the patients included in this study nor in our previous investigations showed small bowel-associated symptoms such as vomiting or failure to thrive, as is typically seen in the classical CMP allergy presentation in children. Another interesting observation was that upon antigen recall, lamina propria and peripheral blood $CCR9^+$ specific T cells mainly proliferated. Nevertheless, a $CCR9^-$ population was also observed to respond to CMP in peripheral blood. Further studies should be performed to characterize this population.

In conclusion, we generated CMP-specific colonic TCL and TCC from the lamina propria of juvenile polyps from CMP-sensitized patients for the first time. Together with our previous studies on juvenile polyps, our findings suggest that juvenile colorectal polyps have an allergic microenvironment and that T cells could be primed and proliferate in the follicle structures described within the polyp, and further differentiate to IL-13-producing T cells that are retained in the tissue by the local production of CCL25. These Th2 cells then contribute to the local synthesis of IgE in the polyp tissue´s active germinal centers[5].

The elucidation of the origin of the Th2 cells, which are pivotal for the induction and regulation of the development of allergy and tolerance to food allergens, is essential. Although the etiology of food allergy is not completely known, our work provides insights into how a restricted tissue can originate the conditions to promote IgE synthesis and T cells with Th2-biased cytokine secretion. These findings may be critical to designing T cell-targeted immunotherapies to restore mucosal and systemic tolerance in food-allergic patients.

Author contributions

All authors have made substantial contributions to the study:

-Conception and design of the study: GHD and CIM.

-Acquisition of data: KEC, CIM, MCR, MRC, MI, EF, MCA and MG.

-Removal of polyps: LG and VB.

-Analysis and/or interpretation of data: GHD, KEC, LG, VB, and CIM.

-Drafting the manuscript: GHD and CIM.

-Revising the manuscript critically for important intellectual content: GHD, BB, RC and CIM.

-Approval of the version of the manuscript to be published: KEC, MCR, MRC, MI, EF, RC, MCA, LG, VB, MG, GHD, CIM.

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TABLE

Table 1. Demographics of pediatric patients with rectal bleeding and juvenile polyps.

Characteristic	Characteristic	Subjects (n=10)
Age (yo), median (IQR)	Age (yo), median (IQR)	4.50 (3.00 – 6.25)
Females, n (%)	Females, n (%)	5 (50)
Total IgE level (IU/mL), median (IQR)	Total IgE level (IU/mL), median (IQR)	152.20 (77.06 – 359.10)
Total IgE level > 60 UI/mL, n (%)	Total IgE level > 60 UI/mL, n (%)	9 (90)
Cow's milk protein (CMP)-specific IgE, n (%)	Cow's milk protein (CMP)-specific IgE, n (%)	**10 (100)**
	Class 1	5 (50)
	Class 2	2 (20)
	Class 3	2 (20)
	Class 4	1 (10)
SPT, n (%)		0 (0)
Dermatitis		3 (40)
Urticaria in response to CMP		1 (10)
Asthma		3 (40)
Rhinitis		4 (50)

LEGEND TO FIGURES

Figure 1. T cell response to cow's milk protein . Lamina propria- and peripheral blood-T cell lines (LPTCL and PBTCL) were stimulated with allergens and analyzed by the CFSE proliferation assay (n=10). **(A)** Cells were selected according to physical parameters, CD4 and CFSE staining ($CFSE^{low}$ are proliferating cells). **(B)** Percentage of $CD4^{+}CFSE^{low}$ cell after 3 days of culture with medium, CMP or PHA. **(C)** Quantification of Th1 and Th2 cytokines in the supernatant obtained from the previous proliferation assay. **(D)**Cell proliferation by $[^{3}H]$ thymidine incorporation in response to medium, PHA, CMP, α-, β-, ϰ-casein or β-lactoglobulin stimulation. **(E)** Summary of the results from the previous assay. Black boxes indicate that the proliferation index was higher than that of the negative control (medium). Statistical differences were calculated using paired two-tailed t-tests or ANOVA: *P<0.05, **P<0.01, ***P<0.005.

Figure 2.

Figure 3. Analysis of the response of T cell clones to cow's milk proteins . T cell clones (TCC) were obtained from juvenile polyps and peripheral blood of 3 patients (P1, P2 and P3). **(A)**Stimulation index of peripheral and lamina propria $CD4^+$ $CFSE^{low}$ after a 3-day stimulus with CMP or medium. **(B)** TCC were classified based on IL-13/IFN-γ secretion ratio. Clones with a ratio >5 were classified as Th2-like (white circles), 0.2–5 as Th0 (white squares) and <0.2 as Th1-like (black diamond). The gray box shows the Th1 clones in detail for P1. **(C)** TCRα and β were amplified by RT-PCR using specific primers. Left panel: Representative images of agarose gels are shown for P3. Membrane expression of the TCR on circulating and lamina propria T cells. Center panel: histogram comparing the expression of TCR-α/β to isotype control. Right panel: graph showing n fluorescence intensity in PBTCC (white square) or LPTCC (black squares). Statistical differences were calculated using paired two-tailed t-tests or ANOVA: *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

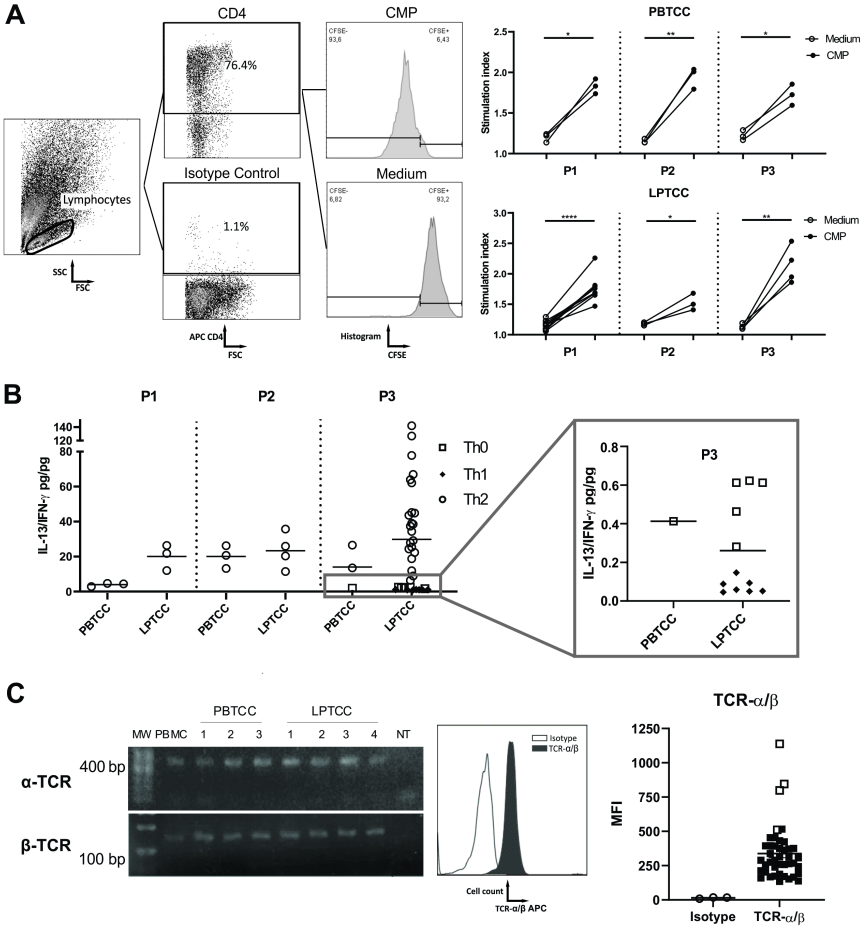

Figure 2

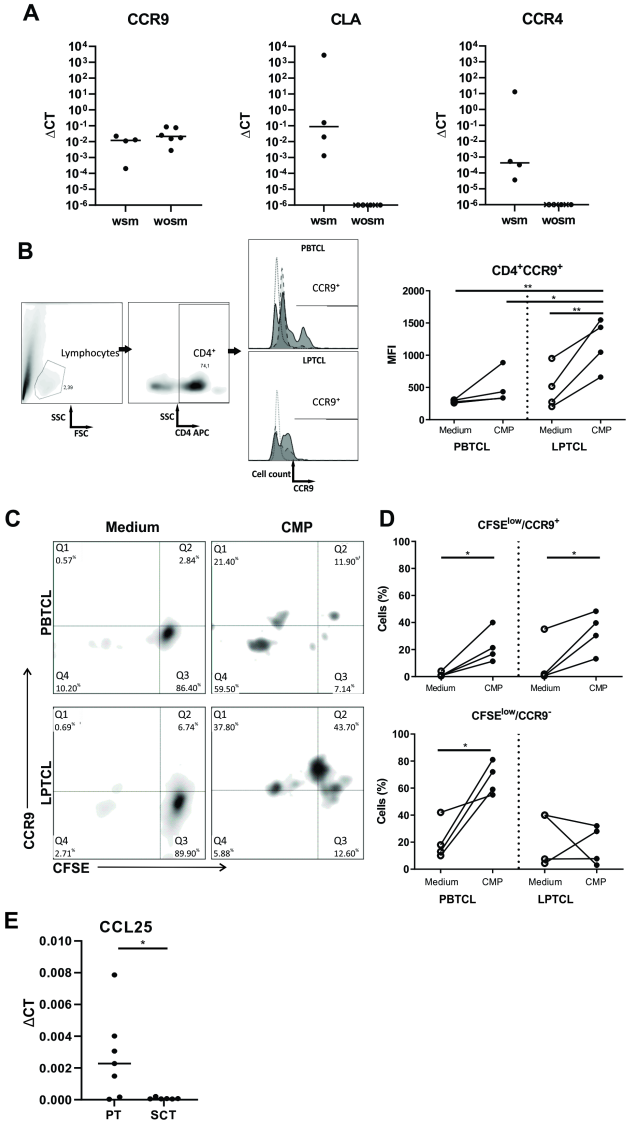

Figure 1