Intestinal tuft cells in IBS patients: Relationship to mucosal IgE-expressing mast cells

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Content

Acknowledgements

Starting in 2019, a certain part-time job offer piqued my interest and I ended up working with cryosectioning and staining small intestine biopsies in the lab. As a natural consequence of patiently preparing 100+ biopsies for diagnostic analysis, I chose this master thesis assignment in order to make use of the huge sample material collected for research purpose. For this I would like to thank Prof. Trond S. Halstensen for giving me this opportunity to expand my horizons in this field and for always being motivated for a discussion be it weekdays or vacation. This work would not have been possible without his constant guidance and support.

I would also like to thank Solveig Stig for patiently teaching me the ropes in the lab and all the technical skills required to perform my job.

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Introduction

The prevalence of allergy, including food allergy, has been rising for the past few decades particularly in the Western countries, USA and Europe, but also in developing countries. Studies have shown that approximately 3-8% of children are affected by food allergy in the west (1). The tendency for an individual to produce IgE following exposure to allergens is known as “atopy”, while the production of allergen specific IgE itself is known as sensitization. The clinical manifestation of allergies is generally perceived as the results of high levels of allergen-specific IgE and its clinical consequence, allergy, but the aetiology behind these allergic reactions is rather complex. As allergies has become more and more of a concern among the general populace, it is of great interest to understand more of the mechanisms behind the tendency to develop allergic reactions. One particular newcomer in this growing field is the tuft cell located in the epithelial lining in all mucosal surfaces including the gastrointestinal tract (GI), which may stimulate IgE-mediated reactions in the gastrointestinal tract.

Although approximately 20% of the adult population report to have foods intolerance or “allergies”, only ~10% of these react upon the allegeable food substance when tested in a double blinded placebo-controlled food challenge, the golden standard for verifying food allergies. However, there are other mechanisms behind food reactions besides the classical IgE mediated allergies, as both non-absorbable carbohydrate-mediated irritable bowel syndromes (IBS, see later), T cell mediated gliadin reactions in celiac disease, and alterative mast cell activations in IBS subgroups may result in food reactions.

In this study we aimed to examine whether these tuft cells may be involved in the underlying mechanisms of food mediated gastrointestinal symptoms or not, and if so, to what degree. We furthermore correlated tuft cell densities to IgE-positivity on mast cells and non-mast cells.

The innate and adaptive immune system

The human immune system consists of two subsystems that cooperate in defending the body from infections and diseases [figure 1]. The innate immune system provides an inherited immediate immune response to a generalized spectrum of pathogens. White blood cells, also known as leukocytes, are the immune cells tasked with protecting the body. While these leukocytes orchestrate complex immune responses against foreign, potential dangerous antigens in both systems, the innate immune system also includes physical and chemical barriers that removes potential pathogenic agents through epithelial desquamation and secretion of antimicrobial peptides. Innate immune cells are stationed in all peripheral tissue and are activated once the physical barriers are damaged or when a pathogen has penetrated said barrier. The immune cells use pattern recognition receptors (PRRs) to identify classes of infectious agents by their pathogen-associated molecular patterns (PAMPs). Innate immune cells promote inflammation at the site of infection and recruit and activate adaptive immune cells. The innate immune cells include neutrophils, mast cells (MCs), macrophages, eosinophils, basophiles, neutral killer cells (NK) and the more newly added innate lymphoid cells (ILC). Dendritic cells (DC) also serve as an innate immune cell, but their function is more to serve as a bridge between the innate and the adaptive immune system.

The adaptive immune response includes specialized leukocytes called lymphocytes that provides a memory-based learning protocol to induce stronger and more specific immune responses. Due to time needed to tailor the immune response towards a specific pathogen, there is a delay between first antigen exposure and the adaptive immune response towards it. The adaptive immune response consists of T and B cells and their respective effector cells.

The function of dendritic cells

DC is an antigen-presenting cell (APC) with tendril like appendages, located beneath the body surfaces. They are one of the first cells to interact with external antigens using toll-like receptors (TLR) and/or other PRR to identify PAMPs. They process antigens from peripheral tissues, undergo maturation and then present antigenic peptides to immature naïve T cells (T_h0) in the lymph node, making them essentially a cell that travels between the innate and adaptive immune system. The antigens are presented to T cells as peptides loaded in human major histocompatibility complex (MHC), the human leucocyte antigen (HLA), along with upregulation of co-receptors that are essential for T cell activation.

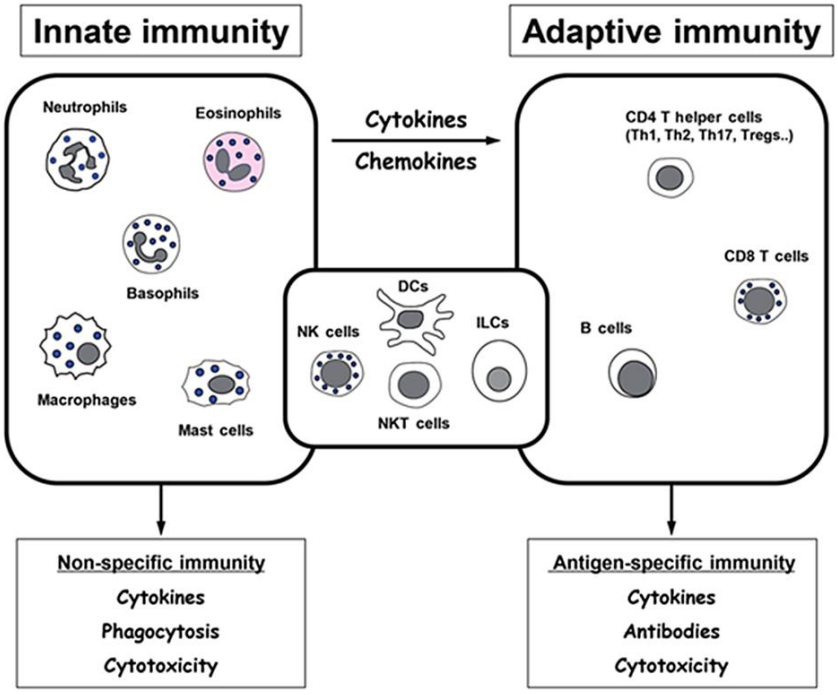

Figure 1

A schematic view of cells in the innate and adaptive immune system including the following effects of the immune responses in both subsystems. Figure adopted from Bonamichi, B. and J. Lee (2017) and modified by H. Yi under creative commons license.

Dendritic cells are categorized into several subsets with further specialized functions. The main subsets include plasmacytoid DC (pDC), conventional DC-type 1 (DC1) and conventional DC type 2 (DC2) (2)[figure 2]. DC may also derive from monocytes during inflammation. Some DCs may express the high affinity IgE receptor FcεRI, as reviewed by Shin et al. (3), and become IgE^{+}. Three different markers were used to identify DC-subtypes, whereas conventional DC2 express CD1c, monocyte-derived DC express DC-SIGN (**D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin, CD209) and mature DC express DC-LAMP (**L**ysosome-**A**ssociated **M**embrane glyco**P**rotein 3, alias LAMP3, CD208).

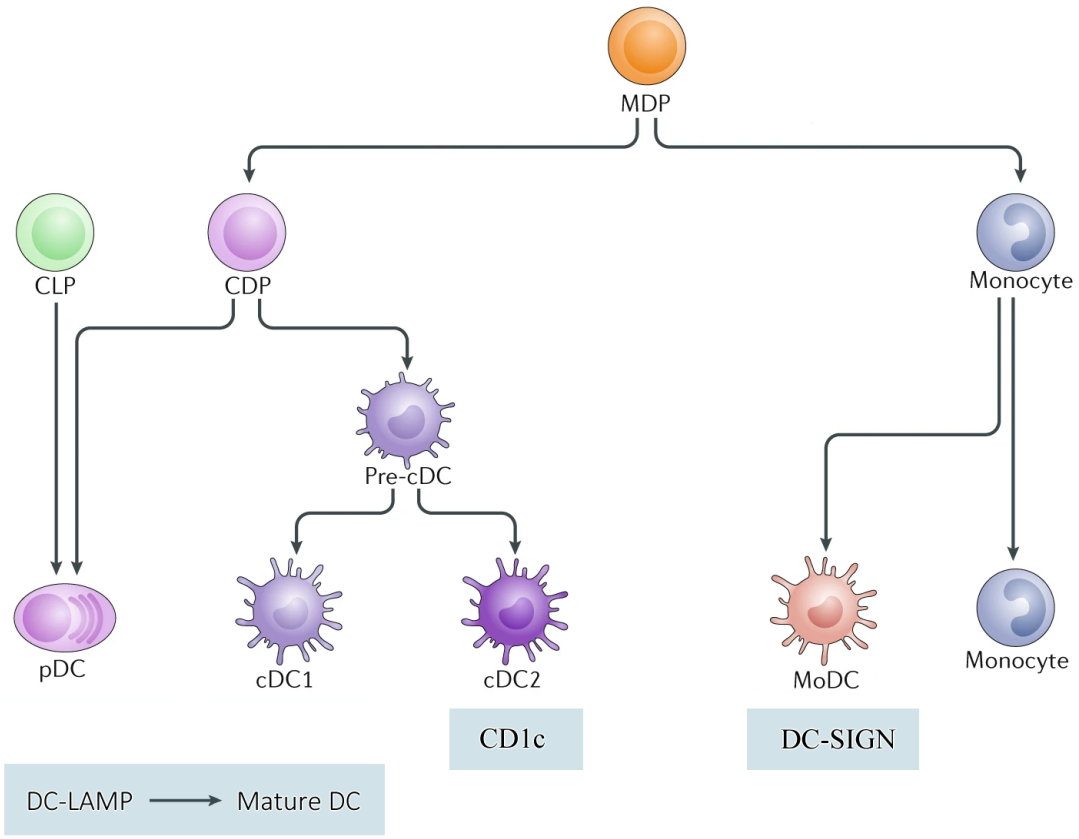

Figure 2

An overview of dendritic cell (DC) subset lineages and the respective markers used in this study. Whereas both the conventional (c) DC type 1 (cDC1) and 2 (cDC2) express CD1c, the **Mo**nocyte-derived **DC** (MoDC) express DC-SIGN and mature DCs, express in general, DC-LAMP. Abbreviations: MDP: **M**yeloid **M**acrophage DC **P**rogenitor; CDP: **C**ommon **D**C **P**recursor; CLP: **C**ommon **L**ymphoid **P**rogenitor; pDC, **p**lasmacytoid **DC**; **c**ommon **DC** type **2**: cDC2; DC-SIGN, **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin; DC-LAMP: **L**ysosome-**A**ssociated **M**embrane glyco-**P**rotein 3. Figure from Eisenbarth (2018) (4) and modified by H. Yi.

The function of T cells

T cells or Thymus derived lymphocytes are essential adaptive immune cells which precursor originates from the bone marrow and matures in the thymus before becoming T_h0 that detect peptides associated with HLA-class I ($CD8^+$ T cells) or class-II molecules ($CD4^+$ T cells). These immature T cells may differentiate into several functional subsets [figure 3]. The $CD4^+$ T helper cells are further divided into several functional subtypes including T_h1, T_h2 and T_h17 depending on the cytokine signalling the APC produces during the naïve T cell differentiation process. The main role of $CD4^+$ T "helper" cells is to facilitate a proper response against targets

expressing a specific peptide sequence (T cell epitope). This process includes regulation of other immune cells responses, such as macrophages or B cells. Cytotoxic $CD8^+$ T cells directly target and kill cancer and infected cells through recognizing specific intracellular oligopeptides mounted on the HLA-class-I complex, peptides that derives from the cellular protein synthesis machinery. The intensity of the immune response is controlled by both natural and induced regulatory T cells (Tregs) that recognize self-peptides derived from normal proteins and therefore represent normal cellular function, in contrast to e.g., a virus infected cell that produce foreign viral proteins. These Tregs may dampen ongoing immune responses to limit tissue destruction and putative fatal organ failure and/or prevent autoimmune diseases.

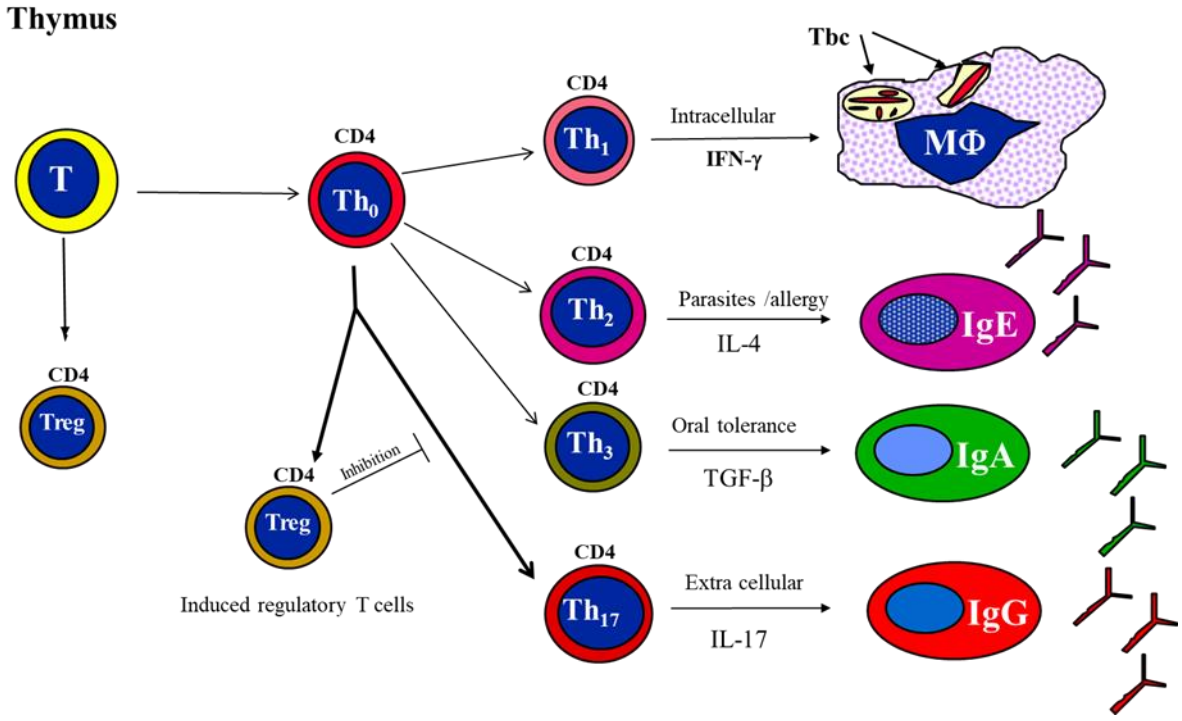

Figure 3

Naïve $CD4^+$ T cells from the thymus (yellow) are differentiated into effector T cells with specific function and cytokine productions. Illustration by Halstensen (2021).

The function of B cells

B cells or B lymphocytes make up the other half of the adaptive immune system and their main role is to differentiate into plasma cells that produce antigen-specific immunoglobulins (Ig) alias antibodies. In order to adapt to different harmful substances, the B cells produce a multitude of diverse antibodies with various affinities through a process called V(D)J

recombination during their development in the bone marrow. These membrane anchored IgM (mIgM) functions as the B cell receptor (BCR) in naïve B cells that are released into the blood stream. Once a naïve B cell has been activated by antigen induced mIgM crosslinking, it proliferates into IgM producing plasma cell and to $mIgM^+$ B cells that are clonally expanded by follicular dendritic cells in the germinal centres of lymphoid follicles. During this cell expansion and affinity maturation, the B cells mutate their antigen-binding regions producing a variety of binding affinities that compete for the antigen in a Darwinian manner: "survival of the fittest" (somatic hyper mutation and affinity maturation). Only the B cells harbouring the highest affinity BCR survive and become the founders of a new round of clonal expansion. T cells in the follicles controls this process and induces B cell class switching in order to determine which immunological effector functions binding of the Ig should induce. There are five main antibody isotypes, and six subtypes, with different properties, inducing different immunologically pathways, including IgE which will be of interest in the current study.

The function of mast cells

Mast cells are granulated immune cell present in the peripheral tissue close to the surface beneath barriers that express the high IgE affinity receptors, FcεRI. IgE-producing plasma cells may be located in the bone marrow or in the peripheral tissue, secreting IgE into the blood stream where it finds its way to the FcεRI on mast cells throughout the body. Antigen induced crosslinking of IgE induces mast cell activation and degranulation, releasing histamine, tumour necrosis factor (TNF- α), prostaglandin D2 (PGD_2), leukotriene C_4 ($LTC_{4)}$ and cytokines (IL-4, IL-5, IL-6 etc.). This process mediates a classical allergic response in the body, also known as an atopic inflammation. One of histamine's main function is to increase vascular permeability, which is necessary for the recruitment of other immune cells to the site of inflammation.

The function of eosinophils

Eosinophils are granulated immune cells specialized to fight parasitic infections. They are released into the bloodstream after maturation in the bone marrow and migrate predominantly to sites of atopic inflammation where they may paralyze parasites by their neurotoxins or induce tissue damage during an allergic response.

IgE mediated immune response

Sentinel cells close to the surface barrier, e.g. dendritic cells (DC) and innate lymphoid cells (ILC), may recognize pathogens through their PAMPS, which classically induce the C-C

chemokine receptor type 7 (CCR7) upregulation that allow these cells to enter the lymph node. After receptor mediated antigen internalization, the DC cleave the antigenic proteins into smaller 7-12 amino acid long oligopeptides that is loaded into the human leucocyte antigen (HLA) groove. The peptide-HLA-complex is then presented to both memory and naïve T cells in the intrafollicular area of the lymph node, resulting in activation of T cells with the appropriate T cell receptor (TCR). ILC, and to some extent the DC itself, uses PPR signals to define which cytokine array they should produce in order to promote differentiation of naïve T cell into its proper effector subset. The DC uses the membrane co-factors CD80 or CD86 to bind the T cell's CD28 in order to test the T cells cytokine production program and to change it, if needed, and to induce clonal T cell expansion.

Should the immune system misinterpret a harmless antigen as belonging to a parasite, it becomes an allergen as the APC induces T helper 2 cell (T_h2) activation. T_h2 cells produce high quantity of IL-4 that promotes the allergen specific naïve B cells to shift to IgE producing plasma cell precursors. IgE-producing plasma cells release allergen-specific IgE into the blood circulation where it eventually binds to the FcεRI on mast cells (and other FcεRI bearing cells), resulting in a classical allergic response. Due to the role of these T_h2 cells, this pathway is generally termed a type 2 immune response and is primarily an anti-parasitic reaction. This immune response to non-parasitic, harmless allergens has become so prevalent to the point that allergies and asthma is now considered one of the most common health issues in children and young adults. Although there are several hypotheses to why this is occurring, the exact mechanism is still unknown. One widespread hypothesis that has been put forward is the "hygiene hypothesis", which links the absence of bacterial challenges during childhood to the development of allergies. Another hypothesis connects allergies to the modern urban lifestyle, which includes the indoor climate and exposure to artificial materials and environmental toxins.

The role of IgE

Though IgE may cause allergic reactions, its intended purpose is to function as a defence mechanism against parasitic infections like helminth. The purpose of the classical allergic symptoms like itching, sneezing, increased mucous production in the airways, is to physically expel the foreign organism. In the cases of a parasitic gastrointestinal (GI) infection, the common symptoms include abdominal pain, vomiting and diarrhoea. Allergic reactions are therefore the result of dysregulated anti-parasitic defence mechanism. While atopy is defined as a genetic tendency to develop allergic diseases such as allergic rhinitis, asthma and atopic

dermatitis (eczema), the current study introduces the term “intestinal atopy” according to immunohistofluorescence staining for IgE on mast cells in the jejunal mucosa.

The function of ILC2

Type 2 innate lymphoid cells (ILC2) were discovered around two decades ago and is today known as one of the main sources of IL-5 and IL-13, which are type 2 immune response promoting interleukins (5). T_h2 cells and ILC2 co-operate and amplify each other’s effects during parasitic infections or allergic reactions as they play similar roles, albeit in different areas. While T_h2 cells generally reside in peripheral blood and lymphoid tissue, ILC-2 is localized under mucosal surfaces similar to DCs where they function as early responders to a type 2 immune response. Cytokines that is known to activate ILC2 include IL-33, IL-25 and TSLP as well as T cell derived IL-2, IL-4, IL-7 and IL-9. (5) Notably, tuft cell is the sole source of IL-25 in the small intestines. (6)

Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a fairly common gastrointestinal disorder, affecting approximately 11% of the global population (7). The condition is commonly diagnosed after excluding other potential gastrointestinal diseases, such as celiac disease and inflammatory bowel disease, and is based on symptoms like diarrhoea, constipation and general abdominal discomfort. Though IBS can be a complex syndrome with fluctuating symptoms and unclear aetiologies, the introduction of the Rome I criteria in 1992 and currently Rome IV in 2016, has facilitated clinical sorting and research communication. One key element is the four IBS-subgroups: IBS-C (constipation predominant), IBS-D (diarrhoea predominant), IBS-M (mixed) and IBS-U (unsubtyped) (8). While IBS is not commonly associated with atopic conditions, there has been studies showing correlation between colonic mucosal mast cell densities and visceral IBS related hypersensitivity (9).

Intestinal epithelial cell lineage

The small intestine surface consists of specialized epithelial cell-coated finger like structures known as villi that protrude towards the lumen. Intestinal crypts are located between the villi and expands inwards towards lamina propria. Intestinal epithelial cells (IEC) are constantly renewed with a lifespan of 3-5 days. Six IEC types differentiate from crypt base columnar cells (CBC) that are stem cells at the base of the intestinal crypts [figure 4]. The dominating IEC is the absorptive enterocyte that makes out the basis of the intestinal lining. Then there are the three secretory IEC types: goblet-, enteroendocrine-, and Paneth cells; the specialized

membrane (M) cells within the dome of Peyer's patches and finally the occasional tuft cells scattered between enterocytes throughout the gastrointestinal tract. The lineage specificity is regulated by Notch signalling and expression of the two transcription factors: hairy and enhancer of split-1 (HES1) and atonal homolog 1 (ATOH1). Deletion of the latter results in increased enterocyte, and impaired secretory IEC differentiation (10). In contrast to the other secretory IEC, tuft cell differentiation is independent of the transcription factors: Neurogenin-3 (Neurog3), SRY-box transcription factor 9 (SOX9), zinc finger protein Gfi-1 (GFI1), and SAM pointed domain-containing Ets (SPDEF) (11). There are still many unknown factors involved in tuft cell differentiation, and it is still unsettled whether tuft cells are to be considered

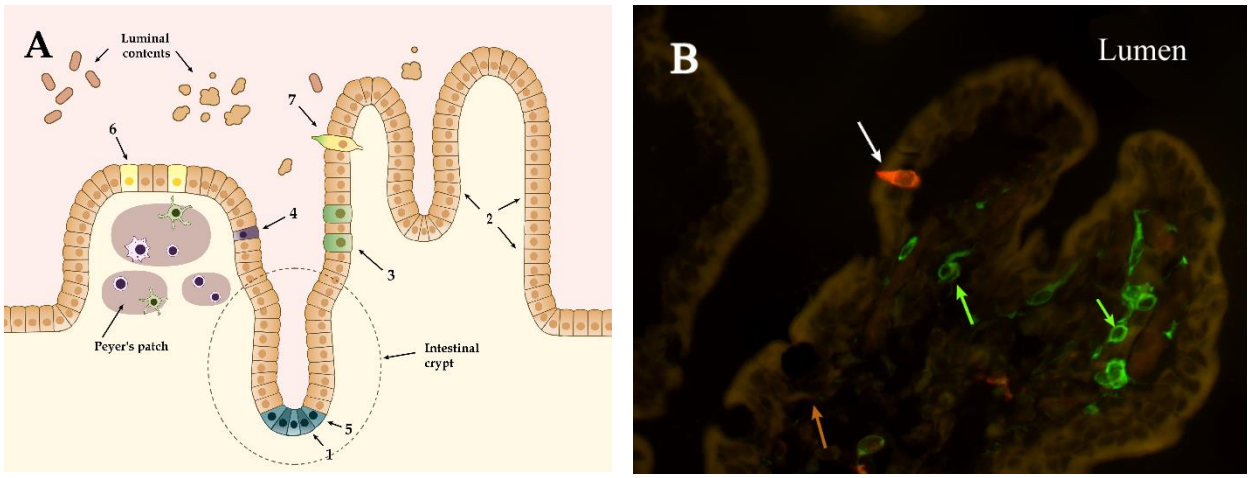

Figure 4

A) A schematic view of the intestinal epithelial cell lining. Six different specialized epithelial cell types originating from the crypt base columnar cells (1). Although absorptive enterocytes are the dominating epithelial cells (2), the secretory intestinal epithelial cells, goblet-(3), enteroendocrine- (4) and Paneth cells (5) are scattered through the epithelial lining and/or crypts, whereas the specialized M cells (6) is located in the dome of the Peyer's patches. Only few tuft cells (7) are observed, normally. Illustrated by H. Yi (2021).

B) A single tuft cell, which is immunohistofluorescence labelled for prostaglandin-D synthase (PGDS) (red) and COX-1 (green) in a double labelling experiment (appear light red/yellowish, white arrow) can be seen between the enterocytes. Goblet cells are easily identified by their empty cytosols (orange arrow). Several COX-1^{+}, but $PGDS^{neg}$ cells are observed in the intestinal lamina propria (green arrows).

as secretory cells or not (11, 12) as several research groups have contradictory results. However, it is well accepted that tuft cells are post-mitotic cells originating from Leucine-rich repeat-containing G-protein coupled Receptor 5 (LGR_5) expressing CBC cells like the other IECs according to Gerbe, F. et al. (2011) (11).

The function of Tuft cells

Tuft cells were discovered decades ago but only recently were their functions better understood. Tuft cells have a distinct morphology and location as elongated cell located in the epithelial lining with its namesake "tuft" in the form of microvilli protruding from the apical tip into the lumen. The consensus, formed by previous studies, is that tuft cells collaborate with ILC2 to create a feed-forward signalling circuit to expel parasites (6). Tuft cells express taste G protein-coupled receptors (GPCR) with the taste specific G-protein, gustducin and member five of the transient receptor potential cation channel subfamily M (TRPM5), which plays a chemosensory function in protozoa and helminth detection. Another similar receptor is the succinate receptor 1 (SUCNR1) that detects helminth-derived succinate and induces tuft cells to produce the T_h2-instructor cytokine, IL-25, essential for ILC2 activation. A positive feedback circuit is then established as IL-25 induce ILC2 to produces IL-13, which skews epithelial cell differentiation toward tuft and goblet cells. (13) [figure 10]

Tuft cell has, as all IEC, a high turnover rate with a 3–5-days lifespan. Therefore, any changes in tuft cell density will be observable shortly after changes in tuft cell differentiation occur. This is illustrated in mice where experimental parasite infection causes an increase in intestinal tuft cell densities few days after infection (6, 13, 14). Although tuft cells have an established role in type 2 immunity, there is little evidence for its involvement in allergic reactions. As the production of allergen specific IgE may be considered as a dysregulated anti-parasitic defence system, it makes one ponder whether tuft cells are involved in regulating gastrointestinal allergic reactions as well.

However, tuft cell activation may also be involved in non-allergic food reactions in non-atopic individuals. Most individuals with food reactions do not have any detectable allergen-specific IgE, although their symptoms may be similar to classical IgE-mediated food allergies. Moreover, patients suffering from irritable bowel syndrome (IBS) often conceives that they have food allergies and/or suffer from coeliac disease. The aetiology of non-IgE mediated food sensitivities/intolerance and the cause of IBS are poorly understood. With the chemosensory

tuft cells spanning across the gastrointestinal epithelial lining and its involvement in amplify atopic immune responses, these cells may play a role in both these conditions.

Tuft cell's role in type 2 immunity

Tuft cells express enzymes required for prostaglandin D_2 (PGD_2) production, which is a major prostanoid with an established role in mediating allergic responses (15, 16). PGD_2 is derived from the eicosanoid biosynthesis pathway thorough COX-1 and/or COX-2 catalysing reactions that produces prostaglandin H2 (PGH2) that become converted to PGD_2 by the key enzyme, prostaglandin D synthase (PGDS). [figure 5]. While several cells like T_h2 and DCs may produce PGD_2, mast cells remain the dominating producer during allergic reactions. Studies suggests that PGD_2 promotes T_h2 cell activity through the PGD_2 receptors, D prostanoid receptor (DP) and the chemoattractant receptor homologous molecule 2 (CRTH2), which DC express. PGD_2 stimulated DC has been demonstrated in several studies to downregulate T_h1 immune activity through different proposed mechanisms. This includes the inhibition of DC migration to the lymph node and the inhibition of T_h1-promoting cytokines, leading to the immune system favouring T_h2 differentiation. While the function of CRTH2 on DC are less understood, CRTH2 promote T_h2 cell chemotaxis towards the PGD_2 source, abundant in locations with high MC activity (17).

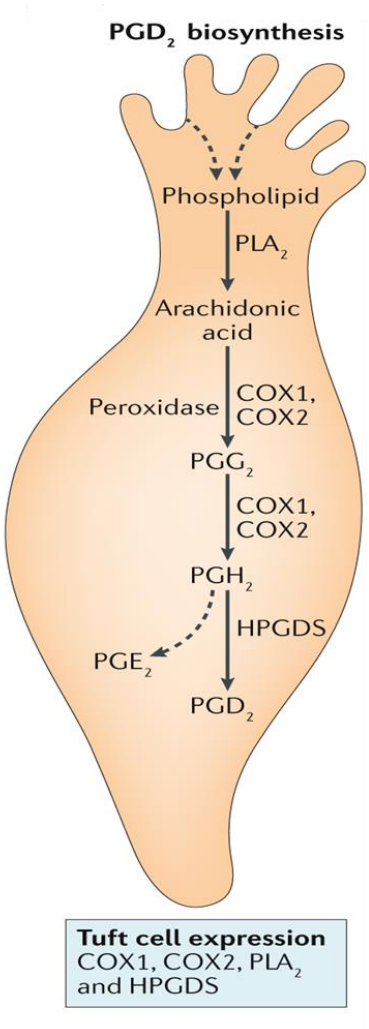

Figure 5

Tuft cells express the necessary enzymes for the conversion of arachidonic acid into prostaglandin D2 (PGD_2). Cyclooxygenase 1 (COX1) and the hematopoietic prostaglandin D synthase (HPGDS) may support the constitutive activity of this pathway in tuft cells. Abbreviations: PGG_2: prostaglandin G_2; PGH_2: prostaglandin H_2; PGE_2: prostaglandin E_2; PLA2: phospholipase A2. Figure from Schneider et al. (2019) (15) and modified by Yi.

Tuft cell are involved in anti-parasitic, T_h2 type immune reactions.

The aim of his project was to examine tuft cell's association with a subpopulation of non-allergic IBS patients with increased densities of IgE positive lamina propria MC in the duodenum (Halstensen et al., unpublished observations). For this study, we have termed increased MC density with increased IgE positivity as "intestinal atopy". The aim was also to analyse the functional status of the tuft cells through different markers. Both the adaptive and innate immune system contributes to the overall type 2 immune responses against helminth and protozoa, but only the adaptive side of the response (T_h2/IgE pathway) appear to be strongly connected to allergic reactions. Moreover, succinate produced by non-parasites could potentially activate tuft cells and induce increased intestinal motility leading to diarrhoea prone IBS (D-IBS). It could hypothetically also trigger food antigen specific IgE production and increased FcεRI expression on intestinal MC, explaining the increased intestinal IgE^+ MC seen in subgroups of IBS patients.

Materials and methods

Selection

Gastroduodenal endoscopy-collected small duodenal biopsies were taken from adult IBS patients referred to an expert clinic at Lovisenberg Diaconal Hospital (LDS). The patients were selected from 340 IBS patients as to contain 25 patients with "intestinal atopy" (increased duodenal MC densities and increased IgE staining intensity) and 20 patients with almost no intestinal IgE^+ mast cells (previous diagnostic observation).

Multi-colour immunohistofluorescence

Immunohistofluorescence is a well-established labelling method utilizing primary antibodies (Ab) to specific antigens for detecting cell phenotypes and tissue structures in tissue-sections by using fluorochrome-labelled secondary Ab to detect the binding pattern in a florescence microscope.

Tissue storage and preparation

Small intestine biopsies were stored at -20C in RNA-later to stabilize and protect cellular RNA and thereafter moved to a modified periodate-lysine-paraformaldehyde (PLP) solution for fixation overnight. Calcium containing tris-buffered saline (TBS) were added to PLP instead of phosphate buffered saline (PBS) to prevent epithelial detachment. The biopsies were then

20% sucrose infiltrated for cryoprotection before embedded in optimal cutting temperature (OCT, Fisher) for further storing at -75^{o}C. The cryosectioned biopsies (4um) were mounted on polysine slides (Thermo Scientific, USA) with three parallel sections on each slide labelled a, b and c.

Multi-colour immunohistofluorescence staining procedure

Blocking agent, bovine serum albumin (BSA) in PBS, were used to buffer the Ab and to prevent non-specific binding of Ab to tissue structures. The primary Ab were buffered in 1,25% BSA in PBS, and the secondary Ab were buffered in 12,5% BSA in PBS with 2% human serum. The sections were fixed in acetone (10 min) before staining procedure. After drying, primary Ab to target antigens were applied to the sections and left overnight in a humidity chamber and rinsed three times for 3 min in PBS. The PBS were rinsed off with ultrapure lab water (Milli-Q®) before applying the fluorochrome labelled secondary Ab that targeted the primary Ab. The slides were incubated with the secondary Ab in a humidity chamber for 30 minutes before repeating the washing procedure. While left to air dry for 10 minutes in the dark, the polyvinyl alcohol (PVA) was pH adjusted, and air bobbles removed before it was applied to the slides so the coverslips could adhere to the microscopic slides. The slides were then stored at 4^{o}C overnight to avoid smearing and movement of the coverslips when examined.

The three adjacent sections were labelled as following: Section a and c were double-labelled for PGDS and COX-1 as tuft cell markers, and section b was double labelled for MC with Ab to c-Kit and Ab to IgE [table 1]. All primary and secondary Ab were quality checked for unspecific labelling by using negative controls and non-immune sera from the same species as the primary Ab, and their concentrations were adjusted accordingly to each batch of antibodies.

Labelling analysis and cell markers

The slides were analysed in a Zeiss fluorescence microscope. tuft cells were reliably identified by PGDS and COX-1 co-expression along with its epithelial location and unique, elongated morphology. Separate fluorescence filter blocks were used to evaluate single labelling intensity in particular when there was uncertainty whether a cell was double positive or not (normally when one marker is expressed weakly).

The biopsies were analysed for tuft cell and MC densities and the IgE positivity was scored from 0 to 3+ and used in calculation of intestinal atopy scores. [Figure 6]. We also selected biopsies with “intestinal atopy” that had many IgE^{+} non-MC in an attempt to identify the non-

MC cell type(s). Staining for IL-25 and the tuft cell marker doublecortin like kinase 1 (DCLK1) were also attempted, but produced no results.

Counting method

Tuft cells were counted via an ocular grid (10 x 23 μm^2) placed on the epithelium at 40x magnification in a Zeiss Axioplan-2 microscope with single, double and triple colour fluorochrome filter block that allows single and simultaneously examination of green, red and blue emissions. Epithelial tuft cells and intestinal crypt tuft cells and were counted separately. Morphologically unclear epithelial cells that expressed varying degrees of PGDS and COX-1 were also counted and coined as "tuft cell lookalikes". The data from sections a and b were combined and tuft cells densities were calculated per 230 μm^2 epithelium similar to MCs and other IgE positive cells in the lamina propria.

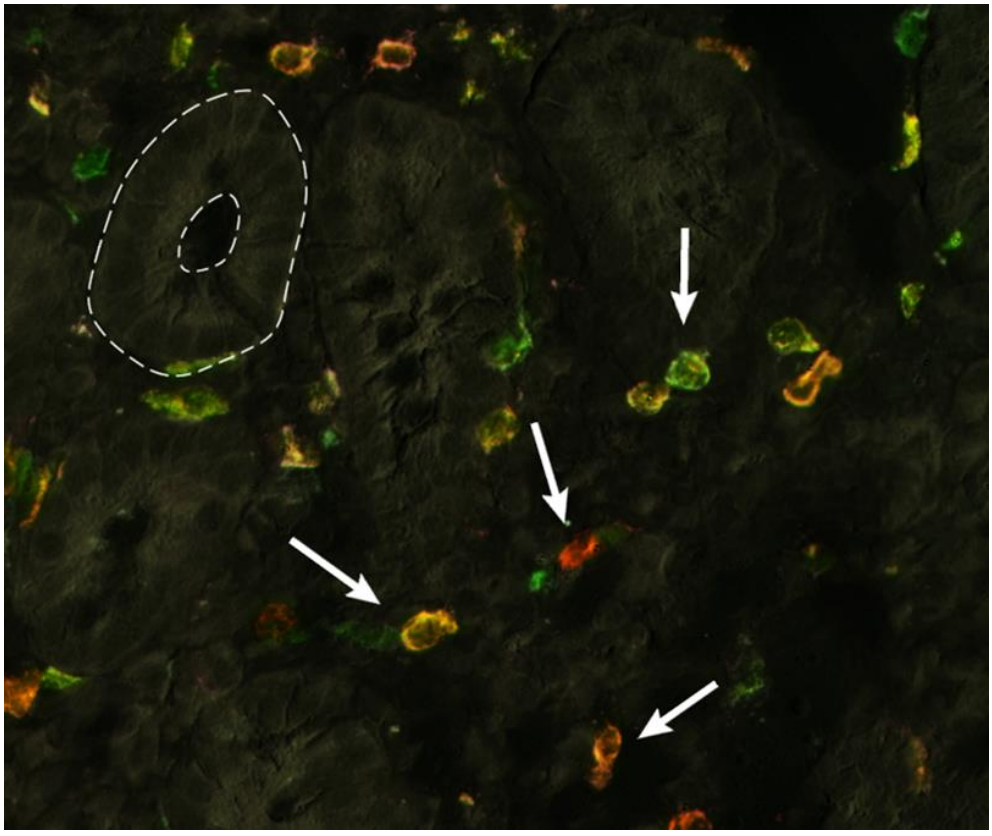

Figure 6

Immunohistofluorescence staining combined with differential interference contrast (DIC) microscopy for enhanced depth perception of the intestinal tissue structure. Intestinal crypts with acini-like structures is marked by dotted line There are several c-kit$^+$, IgE$^+$ MC cells with variable IgE-positivity in the lamina proria, producing red (almost IgE-negative), orange to yellow (IgE-strong positive) MC cells (Arrows).

Section	Primary antibodies	Secondary antibodies	Comment
a&c	Mouse monoclonal (mAb) IgG1 to Prostaglandin-D synthase (PGDS) mixed with mouse mAb IgG3 to cyclooxygenase-1 (COX-1).	Alexa-Fluor 594 conjugated goat anti-mouse IgG1 (Red) mixed with Alexa Fluor 488 conjugated goat anti-mouse IgG3 (Green).	$PGDS^{+\ tuft}$ cells express varying degree of COX-1 and become yellow in double-fluorescence.
b	IgG1 mouse mAb to human c-Kit mixed with rabbit anti human IgE (ε-chain specific).	Alexa Fluor 594 conjugated goat anti-mouse IgG1 mixed with Alexa Fluor 488 conjugated goat anti-rabbit IgG	C-kit+ MC (red) contained varying amount of IgE (green) and become variable yellow-orange in double fluorescence.

Table 1.

Antibody combinations used for two-colour immunohistofluorescence labelling.

Antibodies	Producer
Anti- PGDS mAb, clone ETC45	Gift from Dr. Kinya Nagata, BioMedical Laboratories, Matoba, Kawagoe, Saitama, Japan.
COX-1 mAb (Mouse IgG2b)	Cayman, Michigan, USA
C-kit (CD117, 104D2)	DAKO, Denmark
IgE (Rabbit anti-ε-chain)	DAKO, Denmark
Alexa Fluor 594 (goat anti-mouse IgG1)	Invitrogen, Eugene, Oregon, USA
Alexa Fluor 488 (goat anti-mouse IgG3)	Invitrogen, Eugene, Oregon, USA
Alexa Fluor 488 (Goat anti-rabbit IgG	Invitrogen, Eugene, Oregon, USA
IL-25/IL-17E mAb 68C1039.2)	Novus Biologicals, Centennial, CO, USA
DCLK1	R&D systems, Minneapolis, Minnesota, USA
CD1c	Immunotech, Beckman Coulter, Marseille, France
DC-LAMP	Immunotech, Beckman Coulter, Marseille, France
CD209 (DC-SIGN)	Immunotech, Beckman Coulter, Marseille, France

Table 2.

Table of antibodies used in immunohistofluorescence labelling experiments.

Results

Tuft cell morphology

Tuft cells become observable through immunohistofluorescence labelling for PGDS and COX-1 in the intestinal epithelium and crypts. While tuft cells express PGDS throughout the cell body, it expressed almost no COX-1 apically (the "tuft") leaving the tufts as almost single PGDS-positive [figures 7, 8]. Depending on the angle of the histological section, tuft cells were easily identified through their unique morphology and as the sole epithelial cell expressing PGDS and COX-1 in the intestinal epithelium.

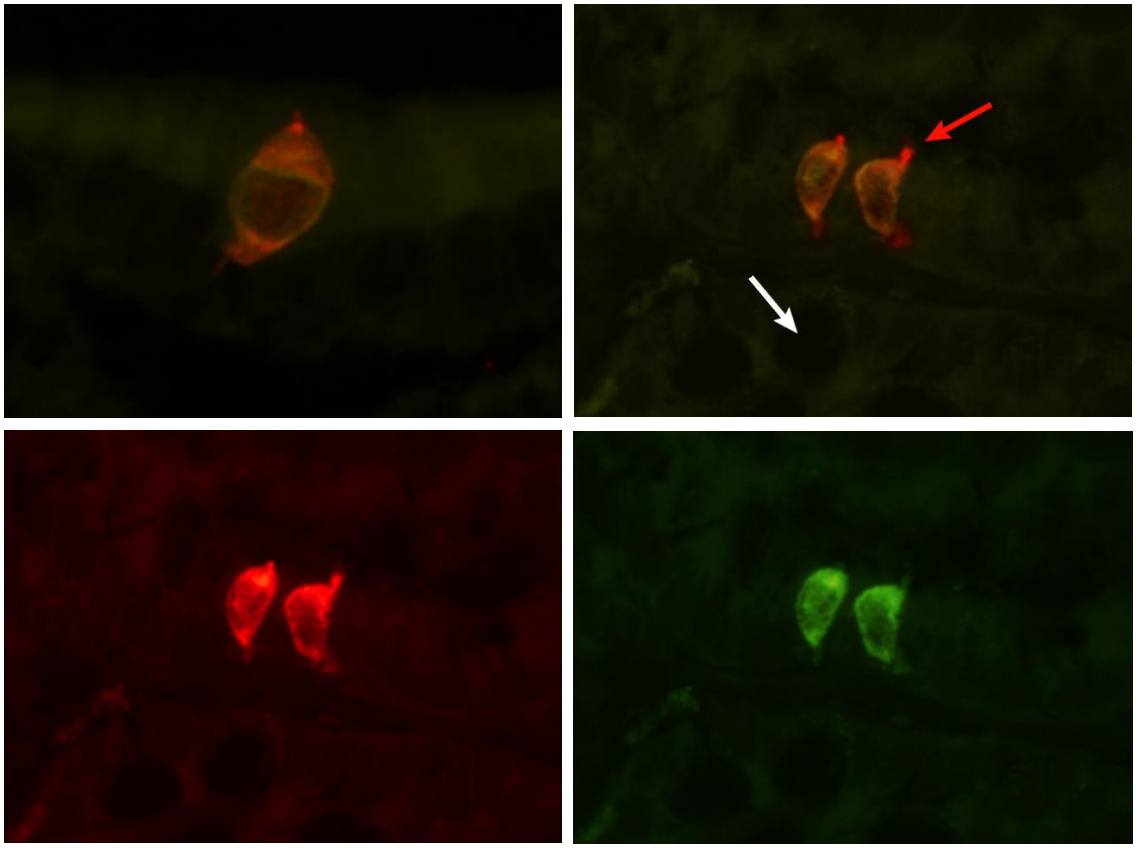

Figure 7

Immunohistofluorescence double labelling for PGDS (red) and COX-1 (green) for tuft cells, located in the intestinal epithelium. PGDS is expressed throughout the cytoplasm with considerably less COX-1 in the tufts, hence the visual stronger red PGDS-positivity at the apical tufts. This was a characteristic element of the tuft cell discovered during microscopic analysis. The apical tufts are always facing the lumen, goblet cells are commonly seen throughout the intestinal lining (white arrow).

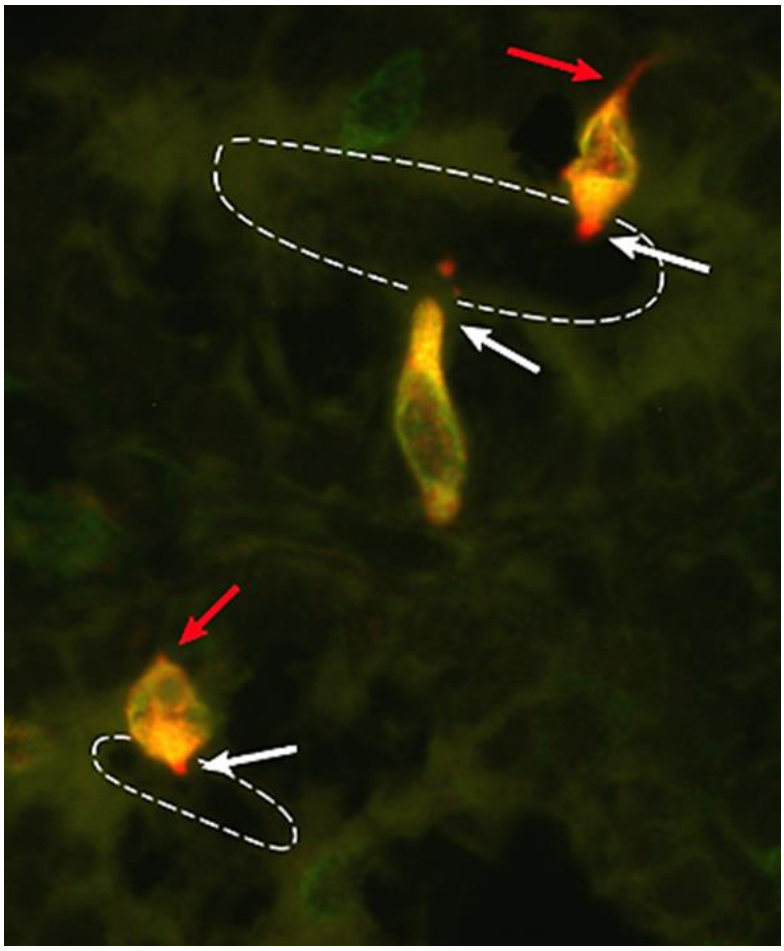

Figure 8

Three PGDS+, COX-1+ tuft cells with characteristic morphology are seen facing the the intestinal crypt lumen. The almost PGDS-single positive apical tufts (red) is towards the lumen (white arrow), but a similar "tail like" protrution is observed at det basal end of the cell (red arrow). PGDS stained microtubule like structures can be vaguely observed in the cytosol. The function of the tail like structure is currently unknown.

Tuft cell, MC and IgE count

Multiplying the intensity of the IgE positivity on c-Kit$^+$ MC with the percentage of MCs that were IgE$^+$ times the MC density produced an "intestinal atopy" score that revealed that the tuft cell density increased with intestinal atopy scores ($p < 0.05$), albeit with a rather large spread (Spearman r=0,30) as it included preselected patients with few intestinal IgE$^+$ MC as well. Tuft cell density correlated more with the "intestinal atopy" scores in the preselected patients (n=25) with many IgE$^+$ MC, using 13 MC/mm lamina propria and >40% IgE-positivity as the lower limits ($p<0.05$; Pearson r = 0,45, CI=0,067-0,72). However, some non-atopic individuals expressed high tuft cell density and several atopic samples had few tuft cells [figure 10]. The MC density was not correlated to tuft cell density by itself.

Identifying the IgE$^+$ non-mast cells.

Unknown IgE$^+$ c-Kit-negative non-MCs were observed in the lamina propria, in particular in individuals with "intestinal atopy" [figure 9]. Further phenotyping revealed that the majority of these IgE$^+$ non-MCs co-stained for CD1c and therefore represented CD1c$^+$ DC. However, as not all IgE$^+$ non-MCs expressed CD1c, it was examined whether the addition of other DC-

markers such as DC-LAMP and DC-SIGN would identify the remaining IgE^+ non-MC. The results showed that approximately 10% of the IgE^+ cells remained unknown when the MC marker, c-Kit and the DC markers were combined. When the DC markers were examined by

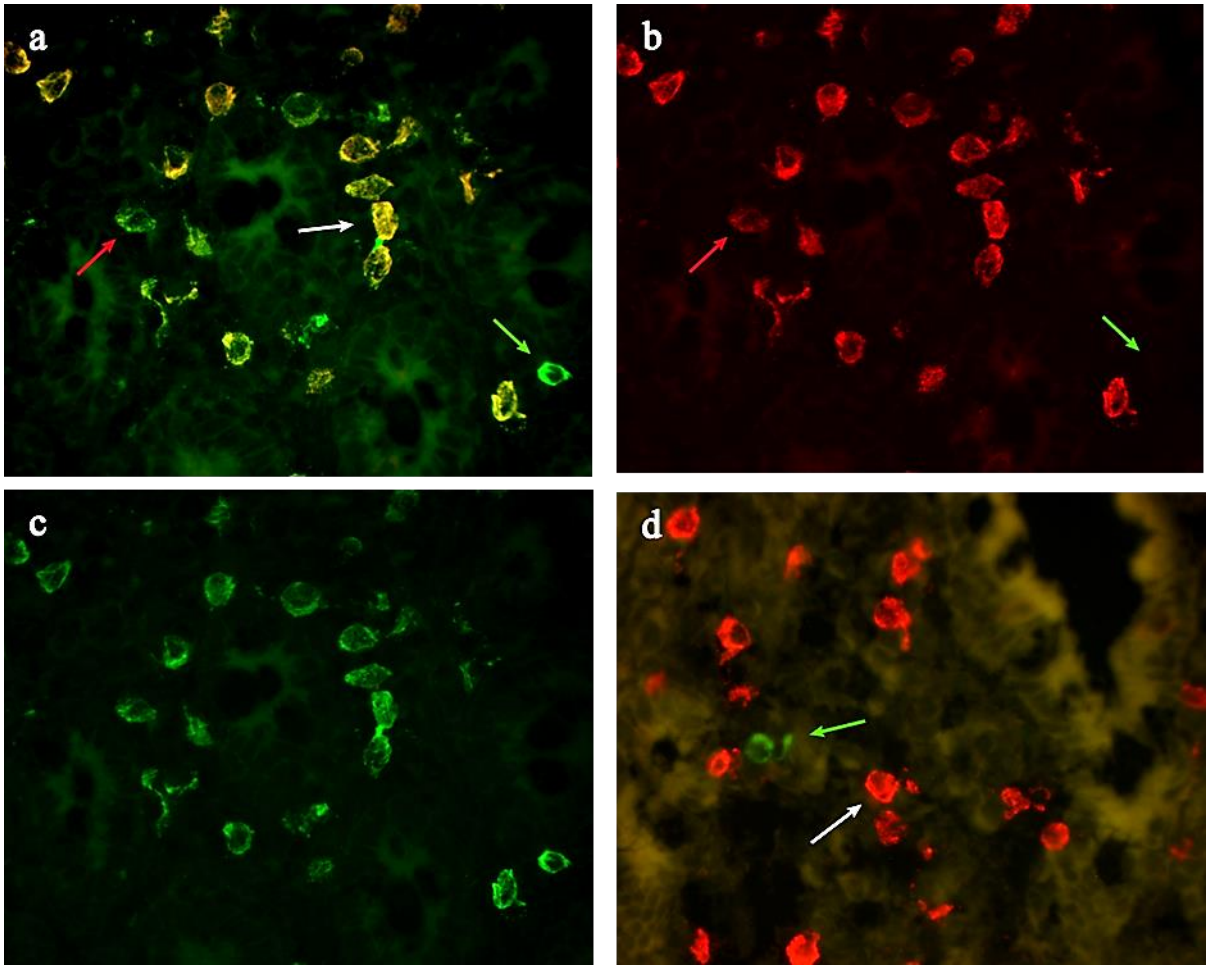

Figure 9

Immunohistofluorescent staining for C-kit (red) and IgE (green) using filter blocks to study each phenotype separately in the same section. Double exposure of $C\text{-}kit^+$, IgE^+ cells (a) show varying intensity of IgE-positivity on $c\text{-}Kit^+$ MC (yellow cells, white arrow). Weaker expression of a marker reduces the fluorescence visible to the eyes, but its presence may be confirmed through the single filters (red arrows, a, b). A single $C\text{-}kit^{neg}$, IgE^+ cell can be seen (green arrow) as confirmed by the absence of C-kit expression under the red filter (b). Figure (d) from another subject than (a, b, c) expresses no $C\text{-}kit^+$, IgE^+ double positive cells but are abundant with $C\text{-}kit^+$, IgE^{neg} cells (white arrow) most likely representing MC without IgE on the surface. A single MC^{neg}, IgE^+ cell can be observed (green arrow). Figure (a) shows the double exposure of red and green filters (yellow cells), (b) shows the single red filter for C-kit, and (c) the single green filter for IgE.

themselves it was revealed that most (~80%) of the $CD1c^+$ cells, approximately half of the $DC\text{-}LAMP^+$ cells, and 2/3 of $DC\text{-}SIGN^+$ cells expressed IgE. The c-Kit-, CD1c-, DC-LAMP- and DC-SIGN-negative IgE^+ cells may therefore have represented an unknown cell (DC?) subset not detected by these markers.

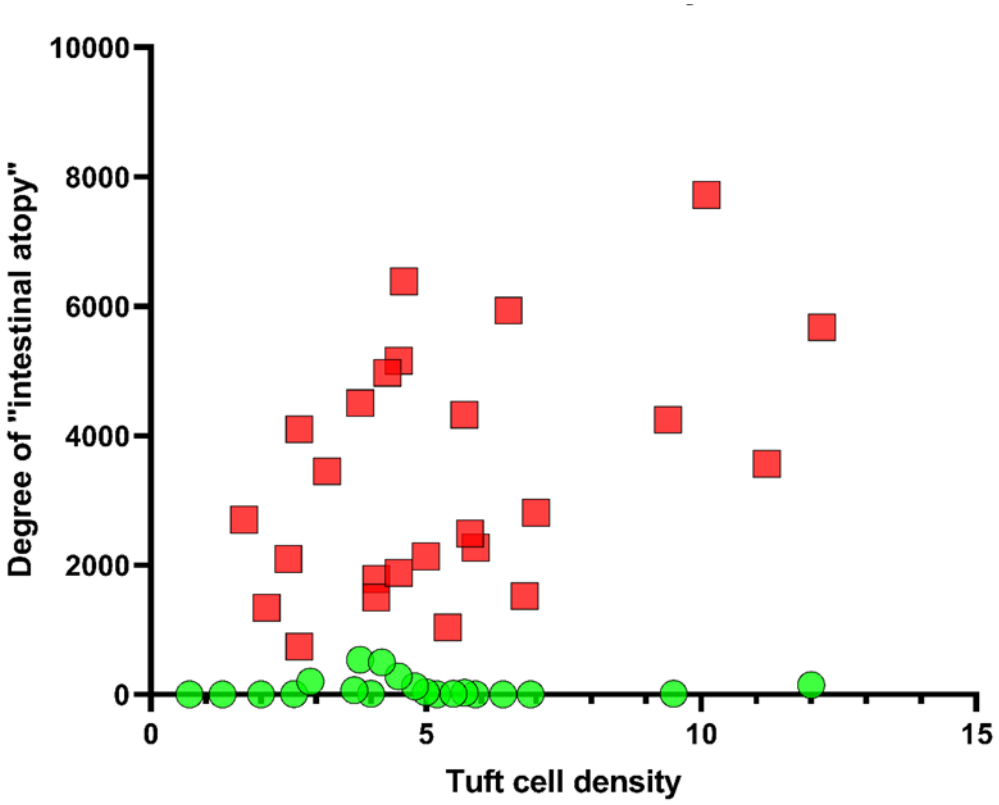

Figure 10

The tuft cell density was significantly correlated to the "intestinal atopy" score (mast cell density multiplied with the weighted IgE-positivity and the percentage IgE^+ mast cells) in IBS patients with "intestinal atopy" (red squares, $p<0.05$, spearman $r=0.45$). This in contrast to the non-atopic individuals where the tuft cell densities were unrelated to mast cell densities and their IgE-positivity (green circles).

Discussion

The tuft cell density was significantly correlated to the degree of “intestinal atopy” in the preselected “atopic” IBS patients, only [figure 10]. These findings suggested that at least two different pathways may increase tuft cell density. Murine studies have convincingly shown that experimental intestinal parasitic infections increase intestinal tuft cell density, which promotes a T_h2-type anti-parasitic immune response through its IL-25 production. This in turn activates submucosal ILC2s that respond with increased IL-13 production. This ultimately shifts the epithelial crypt cell differentiation towards the tuft cell lineage potentially creating a positive feedback loop. The increased tuft cell densities in these IBS patients with “intestinal atopy” may therefore have been secondary to the “intestinal atopy” or alternatively as a response to luminal contents. The latter suggestion is comparable to an earlier murine study by Leyva-Castillo et al. that showed that mechanical injury-induced tuft cell proliferation was essential before allergen-sensitized mice responded on allergen challenge with increased MC densities, food allergen-induced MC activation and anaphylaxis (18). This food allergy model was based on a feed forward loop in which keratinocyte dependent IL-33 contributed to the activation of ILC2 and its production of IL-13 and IL-4, which then promoted the production of IL-25 through tuft cell activation. Keratinocyte-derived IL-33 and tuft cell-derived IL-25 then cooperate in amplifying ILC2 activation and subsequently also the activation of intestinal MCs through IL-4 and/or IL-13 secretion [figure 11]. Our findings may support the possibility that tuft cells and ILC2 plays an important role in promoting intestinal MC activation, but the exact mechanism need to be further studied. Following one of the key cytokines behind ILC2 activation, epithelial IL-33 function as an alarmin and serves a role in maintaining intestinal homeostasis. Intestinal irritation through exposure to pathogens, antigens or toxin may increase epithelial IL-33 production (19). As IL-33 levels increases, accompanied tuft cell and ICL2 activation may increase MC densities in the intestinal mucosa. This also aligns with the proposed hypothesis of “low grade” inflammation and increased MC activation as an underlying cause for IBS (9, 20). By this mechanic, the activated MC will promote increased permeability and recruitment of adaptive

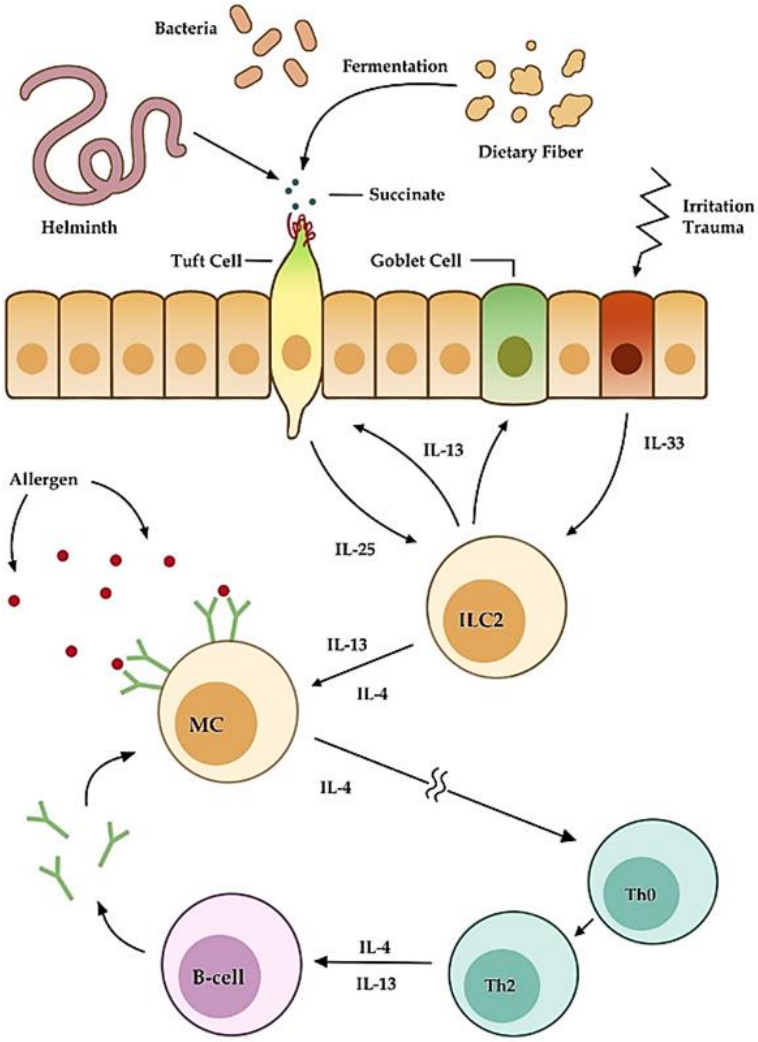

Figure 11

An overview of potential tuft cell activation pathways and its role in promoting T_h2 immune responses. tuft cell and ILC2 drives a feed forward circuit once activated. ILC2 is dependent on T_h2 cytokines including epithelium derived IL-33 and tuft cell derived IL-25 in order to activate ILC2. Likewise, increased tuft cell differentiation dependent on ILC2 derived IL-13. An alternative pathway is the succinate dependent activation of tuft cells through succinate receptor 1 (Sucnr1), based on their chemosensory function towards the intestinal lumen. tuft cells are known to respond on helminth detection, but dietary and bacteria derived succinate could also be potential triggers of tuft cell activation. – Illustrated by Yi (2021).

immune cells. However, not every sensitized individual react upon exposure to specific antigens and the amount of serum allergen-specific IgE does not seem to correlate with clinical symptoms (21). Studies have shown that high MC densities in combination with high production of antigen specific IgE is essential for the clinical symptoms to occur (18). Similarly, the tuft cell density correlated to the intestinal MC density, only when it was combined with the membrane-IgE levels.

Although both the tuft cell and MC densities may increase as a result of ILC2 activation, tuft cells and IgE^+ MCs may directly affect each other as tuft cell activation may promote MC differentiation and vice versa. However, this feed forward atopic loop may predominantly be active in genetically atopic individuals with high IgE producing capacities, whereas tuft cell

activation in patients with genetically poor IgE-producing capacities may work through other mechanisms.

As the subjects in this study had IBS, those with "intestinal atopy" may be an IBS-subgroup with atopy related food-induced abdominal symptoms. A murine study by Aguilera-Lizarraga1 et al. showed that a bacterial intestinal infection could induce dietary-antigen-specific IgE production in the intestine due to breakdown of oral tolerance (9). Their results suggested that exposure to dietary antigens during infections may induce IgE production that leads to sensitized MCs that, if activated, caused abdominal pain through histamine release.

As tuft cells has been branded as sentinels during parasitic infections and a potential first responder cells in type 2 immune responses, they may also serve a role in diet and bacterial induced inflammation. Earlier studies have demonstrated that the metabolite succinate is sufficient to trigger a T_h2 type of immune response through tuft cell activation as they expressed succinate receptor 1 (Sucnr1) (22, 23). Notability, in addition to the natural occurring succinate in the diet, it is a common food supplement. Large quantities of dietary succinate can be produced by bacterial fermentation of dietary fibres which could potentially be sensed by colonic tuft cells as reviewed by Connors et al. (24). Given that succinate is a natural occurring metabolite both produced endogenously and acquired through diet, succinate might not activate tuft cells at homeostasis, as suggested by Nadjsombati et al.(22). However, intestinal dysbiosis may affect epithelial IL-33 production, and how tuft cells perceive succinate, which together may activate ILC2 signalling to fully establish the feed forward loop in the tuft cell-ILC2 circuit.

Additionally, tuft cells express choline acetyltransferase (ChAT) constitutively, which is required to produce the intestinal motility-promoting neurotransmitter acetylcholine (Ach). Activated tuft cells may therefore affect the cholinergic neurons in the enteric nervous system and promote gastrointestinal motility and diarrhoea, similar to what overstimulation of the intestinal secretory neuronal network does (25). Though tuft cell express ChAT, it lacks elements required for vesicular ACh transport and other synaptic proteins, which questions the mechanism behind its ACh secretion (26).

Although our research shows an association between tuft cell densities and "intestinal atopy" scores, further research is required to confirm these findings as this pilot study has several limitations. All patients in this study had IBS symptoms and were selected to either have high or low levels of intestinal IgE^+ MCs. Thus, we did not have access to a proper age-adjusted control group with no intestinal symptoms. Moreover, the use of colonic biopsies might have

given a different result as IBS is primarily thought to be the result of dysbiosis in the colon, in contrast to the small intestine tissue used in this study. Compared to mice studies which utilizes whole intestine, biopsies from humans are small hence only a few tuft cells were identified in each biopsy. Tuft cells were not evenly distributed throughout the gastrointestinal tract, which can cause discrepancy between overall tuft cell and IgE^+ MC densities. As former studies have claimed that tuft cells constitutively express IL-25, it is also of concern why anti-IL-25 antibody were unable to label human duodenal tuft cells in the current study. Similarly, DCLK1, which is coined as one of the hallmark markers of tuft cell also failed to label duodenal tuft cells in contrast to previous reports. Another concern is the labelling specificity for the MC marker, c-Kit, which has been claimed to label ILC2 as well in flow cytometry (27). This may weaken the MC density count as ILC2s may have been mistakenly included as IgE-negative MCs. Finally, a single researcher counted the cells manually. While quality control was conducted by supervisor and counting method was frequently discussed, human error cannot be excluded.

Conclusion

Though our research has shown clear association between duodenal tuft cell and duodenal "intestinal atopy" in preselected "atopic" individuals, the study does not reveal whether tuft cells play a role in IBS symptomatology or not. Nevertheless, our data do suggest that non-allergic IBS patients with "intestinal atopy" express higher tuft cell load. Whether this is causal or confounding need to be explored in further studies.

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