

# Non-IgE Mediated Food Allergy

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**Abstract:** Adverse reactions to dietary proteins (DPs) can impose a significant impact on one's daily life and can even affect the 'life style' of an entire family. Adverse reactions to DPs may or may not be immune-mediated. The immune-mediated adverse reaction to food is defined as food allergy (FA) which is roughly divided into IgE mediated or non-IgE mediated FA. As opposed to IgE mediated FA, NFA primarily affects the GI mucosa. In addition, there is far less of an understanding of NFA than IgE-mediated FA and its clinical relevance is likely under-estimated in most cases. This is partly due to delayed onset of symptoms and subsequent difficulty in making the clinical association between offending food and clinical symptoms. The lack of easily accessible diagnostic measures also contributes to the problem.

The gut mucosal barrier is thought to have developed to execute an immensely difficult task; digestion and absorption of nutrients without provoking immune responses and cohabiting with commensal flora in a mutual beneficial relationship, while maintaining an immune defense against pathogenic microbes. The gut mucosal immune system accomplishes this task partly by establishing tolerance to macronutrients with potent immunogenicity. Immune tolerance to macronutrients (DPs) is maintained in part by active suppressive mechanisms involving antigen (Ag)-specific regulatory T (Treg) cells. This active immune tolerance state appears to be affected by various environmental factors such as change in commensal flora.

In the first few years of life, humans gradually develop an intricate balance between tolerance and immune reactivity in the gut mucosa along with a tremendous expansion of gut associated lymphoid tissue (GALT). Not surprisingly, both IgE and non-IgE mediated food allergy (FA) is frequently seen during this period. The most common causative DPs for non-IgE mediated food allergy (NFA) are those contained in infant formulas (cow's milk and soy proteins). Unlike IgE mediated FA, NFA is rarely life-threatening. However, NFA to DPs can cause significant morbidity in rapidly growing infants and young children. A better understanding of pathogenesis of NFA is crucial for timely management of NFA in this vulnerable population.

This review discusses the gut mucosal immune system in the first few years of life including genetic/environmental factors affecting the development of mucosal immune system and pathogenesis of NFA in association with clinical/laboratory findings.

## INTRODUCTION

During the first years of life, the human body has to develop a gut mucosal immune system that enables us to maintain immune homeostasis with commensal flora and digest huge amounts of macronutrients without provoking immune reactions, all of which must be accomplished while maintaining effective immune defense against pathogenic microbes. This is not an easy task, and appears to be an error-prone process. In fact, many infants have adverse reactions to macronutrients such as dietary proteins (DPs) when they are initially introduced, typically manifested as gastrointestinal (GI) symptoms (diarrhea, indigestion, bloating, loose stool, etc). This likely reflects the fact that young infants are not well equipped for establishing oral tolerance to common DPs. In this review, we discuss 1) components of the mucosal immune system associated with the development of food allergy, 2) proposed mechanisms of tolerance induction and immune homeostasis, 3) factors influencing immune homeostasis, and 4) pathogenesis of NFA and clinical/laboratory findings.

## I. Intestinal Mucosal Immune System

The structures essential for composing the mucosal immune system include the epithelial cell monolayer and GALT, primarily composed of Peyer's patches (PP) and lamina propria (LP).

The epithelium: The intestinal epithelial barrier is functionally immature in newborn infants which results in higher intestinal permeability than in adults. In addition, there are significant differences in gastric acidity, activity of digestive enzymes, intestinal motility, and amounts and contents of mucous [1]. It has been hypothesized that higher intestinal permeability and less intestinal motility is beneficial during the newborn period, enabling translocation of antibodies (Abs) and other macromolecules contained in the human breast milk [1]. The glycosylation patterns of oligosaccharides expressed on epithelial cells also change over time; fetal sialylation is followed by fucosylation and galactosylation in humans [1, 2]. Changes in glycosylation patterns make the intestinal epithelial cells less susceptible to complement-mediated cell lyses and also appear to affect colonization patterns of microbes. Other molecules are also important for the barrier function. For example, impaired intestinal epithelial barrier function in patients with protein-losing enteropathy was implicated with the specific loss of heparan sulfate proteoglycans from the basolateral surface of intesti-

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nal epithelial cells [3, 4]. The importance of heparan sulfate proteoglycans was further supported in rodent models deficient of these molecules [5].

Apart from the barrier functions, epithelial cells also exert important innate defense. For example, anti-microbial peptides which are mainly produced by Paneth cells clustered in the bottom of the intestinal crypts facilitate innate immune defense [6]. This is also true for respiratory epithelial cells that produce IFN- $\beta$ , a critical factor for innate defense against viral pathogens [7]. However, in colon where commensal microbes inhabit at high concentrations, colonic epithelial cells are thought to contribute to the maintenance of colonic homeostasis. In rodents, it was shown that colonic epithelium expresses Toll like receptor (TLR) 9 on the cell surface [8, 9]. Apically expressed TLR9 is reported to compromise the inflammatory cascade induced basolaterally by several TLRs [8, 9]. The importance of TLR9 mediated inhibition is supported by the fact that TLR9 deficient mice exhibited more severe epithelial ulceration in colitis induced by dextran sodium sulfate [8].

**GALT:** The LP located beneath the intestinal epithelium serves as a meshwork of connective tissue containing of plasma blasts, T cells, dendritic cells (DCs), mast cells, macrophages, and granulocytes (neutrophils and eosinophils). IgM plasma cells are dominant during the newborn period upon exposure to luminal Ags [7]. These are gradually taken over by IgA plasma cells approximately by 6 months of age. The presence of abundant mast cells in the LP during infancy and early childhood is thought to be important for mucosal immune defense against extra-cellular parasites. In addition, mast cells are also indicated to have a role in development of tolerance induction against macronutrients [10].

The PPs are lymphoid aggregates composed of B cell follicles, surrounding interfollicular CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as other lineage cells (DCs, macrophages, etc) intervening beneath the follicle-associated epithelium. Development of B cell follicles are dependent on exposures to intestinal antigens (Ags) typically derived from the gut commensal flora. TNF- $\alpha$  and expression of TNF- $\alpha$  receptors are also important for development of PPs [11]. DCs are abundant in the subepithelial dome of PPs and thought to be composed of various subsets [12]. Activated DCs can migrate to mesenteric lymph nodes (MLNs) or LPs and can exert stimulatory and modulatory actions [13].

**Processing and presentation of luminal Ags:** Luminal Ags can be sensed by the gut immune system *via* several routes. Food Ags that have escaped intestinal digestion are taken up by microfold cells (M cells) which are thought to be specialized for Ag sampling when present as particulate Ags [12, 14]. M cells deliver these luminal Ag to DCs that are present abundantly in the subepithelial dome region of PPs. DCs present in the gut mucosa can also directly sample luminal Ags by extending dendrites in the gut lumen without disrupting the tight junction of the intestinal epithelial cells [14, 15]. M cells are considered to be important for Ag presentation and tolerance induction, however, their precise function in Ag processing and Ag presentation is not well understood.

Soluble dietary Ags can also be taken up by epithelial cells through endocytosis. Endo-cytosed macromolecules are either digested or exocytosed to the extracellular space where they are taken up by Ag presenting cells (APCs). The intestinal epithelial cells expressing major histocompatibility complex (MHC) II molecules may also function as APC for endocytosed Ags, preferentially activating CD8<sup>+</sup> T suppressor cells and facilitating immune homeostasis in the gut [16, 17].

Presentation of luminal Ags are affected by the dose of Ag and presence or absence of adjuvant effects. High doses of Ag are predominantly presented by DCs, macrophages, and B cells locally but also by systemic sites. In contrast, low doses of Ag are predominantly presented by DCs of the PP and MLN. Pathogenic bacteria produce microbial by-products which can stimulate innate immune cells including the gut APCs and serve as the major source of adjuvant [18, 19]. In contrast, commensal flora often down-regulate APC activation [15].

**T cell subsets:** To maintain intestinal homeostasis, multiple subsets of T cells are present in the intestinal mucosa including effector subsets of T-helper (Th) cells including Th1, Th2, and Th17 cells, regulatory T (Treg) cell subsets, and CD8<sup>+</sup> T cells. The functions of effector T cells are suboptimal in neonates and most of them are naive T cells which are sensitized with exposure to gut luminal Ags. Th1 and Th2 cells are already well described elsewhere and Treg cells were discussed in detail in the next section. Thus we briefly discuss about Th17 cells.

Th17 cells are another subset of T cells that have been recently identified. They are characterized by production of distinct cytokines (IL-17A, IL-17F, IL-22) and were shown to be important for immune defense against fungal and certain bacterial pathogens [20-22]. IL-17A enhances T cell priming and activate innate immune cells (fibroblasts, macrophages, endothelial cells, and epithelial cells) to produce inflammatory mediators including IL-1, IL-6, TNF- $\alpha$ , and iNOS, resulting in neutrophilic inflammation [21, 22]. The key differentiation factors for Th17 cells are reported to be IL-6 and IL-1 $\beta$  in humans [23, 24] and recent studies also indicate a role of IL-23 [24]. Mature Th17 cells become responsive to IL-23 which serves as a survival factor for committed Th17 cells, while IL-27 suppresses the development of Th17 cells [25, 26]. Given its pro-inflammatory nature, Th17 cells have been shown to exert detrimental effects in autoimmune conditions including multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (IBD) [21, 22]. However, the role of Th17 cells in FA is unknown.

**The effects of innate immunity:** Innate immune defense is initiated by sensing pathogen derived molecular patterns (PAMPs) *via* specific receptors called pattern recognition receptors (PRRs). TLRs are the first family of PRRs characterized [18]. TLRs and other PRRs initiate the first line immune defense by activating innate immune cells. PRRs mediated signaling also activates APCs by up-regulating expression of MHCs/co-stimulatory molecules, augmenting the Ag processing, and facilitating migration of DCs to the lymphoid organs [18]. PAMPs are a major source of adjuvants in the gut lumen. PRRs can also sense molecular patterns derived from injured tissue (so-called danger-signals) and can

initiate immune defense, a process which appears to be vital for wound healing [27].

On the other hand, induce suppressive effects in the intestine where cohabitation with microflora is required. LPS (endotoxins) are produced by both pathogenic microbes and commensal floras and sensed by TLR4. Interestingly, in the small intestine where bacterial density is low, intestinal epithelial cells appear to respond to LPS [28, 29]. However, in the colon where bacteria exist at high density, colonic epithelial cells are much less responsive to LPS, rendering the state of endotoxin tolerance [15, 18]. They also become less responsive to other TLR mediated signaling (cross-tolerance). TLR expression on macrophages and DCs in the small intestine is also down-regulated under normal conditions, preventing excessive inflammatory responses [30, 31]. As noted previously, apically expressed TLR9 also mediates inhibitory signaling in colonic epithelium [8, 9].

The above-described innate immune defense is genetically pre-determined and full term babies are fully capable of mounting innate immune defense. However, Ag-specific, adaptive immune responses are slow to develop. T cells in newborns are mostly naïve T cells which require more potent co-stimulatory signaling for activation than memory or effector T cells. By adulthood, half of the circulating T cells become memory T cells. During the first few months of life a rapid expansion of Ag-specific T and B cells occurs [32]. During this period, the subtle changes in innate immune responses can significantly affect development of GALT and may make certain individuals more prone to aberrant responses to DPs. However, such aberrant responses can be compensated by immuno-suppressive molecules contained in the breast milk and other environmental factors.

## II. Intestinal Homeostasis – Tolerance Induction and Maintenance

Oral tolerance can be induced by a high single dose of Ag and also by low doses of Ag given repeatedly. Mechanisms of tolerance induction differ significantly in these two conditions. High dose tolerance is thought to be mediated by T cell anergy and deletion, while low dose tolerance requires active immuno-suppression exerted by Treg cells. In NFA to milk protein, the importance of milk-protein specific Treg cells has been reported [33].

**Treg cells:** Mounting evidence indicates that oral tolerance is mediated by active immune-suppression in the gut. The major cells involved in this process are Treg cells. Thymus derived, naturally occurring Treg (nTreg) cells appears vital for maintaining self-tolerance in the periphery as evidenced in IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, x-linked syndrome), a rare primary immunodeficiency caused by Foxp3 deficiency [34, 35]. These patients suffer from early-onset, organ-specific autoimmunity and severe allergic inflammation.

In the gut mucosa, other types of regulatory T cells appear to play a crucial role in tolerance induction and maintenance against luminal Ags. They are called induced or adaptive Treg (aTreg) cells which are derived from naïve T cells in the periphery upon Ag exposure in the presence of IL-2 and TGF- $\beta$  [36]. Treg cells are characterized by expression of CD4, CD25<sup>high</sup>, Foxp3, CTLA-4 (cytotoxic T-lymphocyte associated Ag 4), and GITR (glucocorticoid-induced TNF receptor-

related protein). Several studies revealed the importance of aTreg cells in oral tolerance and it has also been shown that Ag-specific aTreg cells are induced in allergen immunotherapy in the lung [37]. As discussed previously, the presence of milk-protein specific aTreg cells has been observed in children who have outgrown NFA against cow's milk protein (CMP) [33].

In addition to the Foxp3<sup>+</sup> aTreg cells, the Foxp3<sup>-</sup> IL-10<sup>+</sup> T regulatory type 1 (Tr-1) cells are also thought to have derived from naïve T cell upon Ag exposure [38]. These cells are initially reported in SCID patients following hematopoietic stem cell transplantation and thought to be important for regulating systemic inflammation [39]. However, they are also reported to be abundant in the intestine and possibly have a role in down-regulation of inflammatory responses triggered by microbiota [38].

A role of Th17 cells has been noted in pathogenesis of certain autoimmune diseases including inflammatory bowel disease (IBD). Interestingly, recent studies revealed reciprocal differentiation of Treg vs Th17 cells in both rodents and humans. High concentration of IL-6 appears to override differentiation signal by TGF- $\beta$  preferring Th17 differentiation, while retinoic acid, a metabolite of vitamin A, inhibits IL-6 driven Th17 cell differentiation and greatly augments Treg cell differentiation in the presence of TGF- $\beta$  [22, 40-41]. These findings indicate a critical importance of retinoic acid metabolites in maintaining the gut immune homeostasis. TGF- $\beta$  also acts as isotype switching factor for induction of IgA<sup>+</sup> B cells and secretory IgA augments intestinal immune homeostasis [42]. Interestingly, in a rodent model of asthma induced by sensitization of ovalbumin (OVA) *via* airway, breast feeding attenuated airway inflammation by providing TGF- $\beta$  and vitamin A, subsequently inducing OVA-specific Treg cells [43].

In addition to Treg cells, other cells can also contribute tolerance induction in the gut mucosa. These include liver-associated NK1.1<sup>+</sup> T cells and  $\gamma\delta$ T cells. However, the role of these cells in food allergy is not well understood. Given these findings, one can imagine that the subtle change in mucosal intestinal environment may greatly affect the body's reactivity to food proteins in the first year of life when oral tolerance has yet to be established.

## III. Factors Affecting Intestinal Immune Homeostasis

### Genetic Factors

Innate immune defense differs from adaptive immunity in that it is genetically pre-determined and post-natal regulation is not likely to occur. This renders genetic variation (polymorphism) more influential in clinical manifestations. The importance of polymorphism in innate immunity has been shown in IBD patients.

Another family of PRRs identified following TLRs is a nucleotide oligomerizing domain (Nod) like receptor (NLR) family [18, 19, 44]. Nod1 [also called as caspase recruitment domains 4 (CARD4)] expressed on intestinal epithelial cells recognize peptidoglycans (PGN) that contain meso-diaminopimelic acid (meso-DAP) [45]. DAP is unique to PGN from Gram-negative [G(-)] bacteria and certain G(+) bacteria. Thus Nod1 plays a crucial role in innate immune defense against invasive intestinal bacteria such as *Shigella flexneri* and entero-invasive *Escherichia coli* [46, 47]. Several Nod1

polymorphisms have been reported to be associated with higher risk of atopic eczema, asthma, and higher serum levels of IgE [48, 49]. These results indicate that Nod1 polymorphism may also be associated with IgE mediated food allergy.

In contrast, Nod2 (CARD15) recognizes a component of PGN, muramyl dipeptide (MDP), common to most bacterial PGN [18-19, 44]. Intracellular Nod2 is highly up-regulated in monocytes and Paneth cells and can also be induced on intestinal epithelial cells by TNF- $\alpha$  and IFN- $\gamma$  [50, 51]. Genetic variation in Nod2 is associated with increased susceptibility to various inflammatory conditions, most notably Crohn's disease (CD) at least in Caucasians [18, 19]. Human CD-associated Nod2 variants exhibit reduced or loss of activity in cell lines and it is assumed that a deficit in sensing bacterial presence may trigger an abnormal inflammatory responses as observed in a germ-free mice [53, 54].

Other multiple genetic factors are now implicated with development of IBD [52] and possibly in other inflammatory conditions. Recent genome wide analysis will likely further shed a light for the effects of genetic factors on gut mucosal immunity.

#### Environmental factors

**Commensal flora:** Evidence is now accumulating supporting the beneficial effects of commensal flora on mucosal immune responses. Lack of commensal flora or TLR4 signaling augments allergic responses to food Ags in both animal models and humans [55]. Moreover, others reported restoration of oral tolerance in germ-free mice by feeding the mice LPS, a TLR4 agonist [56]. This finding appears to be associated with the fact that LPS exposure enhances the suppressive activity of TLR4<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>low</sup>CD25<sup>+</sup> Treg cells [57]. Prolonged exposure to LPS or TLR2 stimulants leads to tolerance and cross-tolerance to other PAMPs in intestinal epithelial cell lines [58]. Likewise, other TLR agonists also appear to be important in maintaining proper communication between the gut mucosal immune system and commensal intestinal flora, limiting excessive inflammation as elegantly shown by J. Lee *et al* [8, 9, 18, 59]. The above described studies in animal models indicate importance of TLR mediated signaling triggered by commensal flora in prevention of intestinal injury and maintenance of gut mucosal homeostasis.

In addition to anti-inflammatory effects of the commensal flora mediated by the host-immune system, recent studies have revealed direct anti-inflammatory actions exerted by commensal flora. For example, *B. thetaiotaomicron* was shown to restrict the signaling induced by flagellin, a TLR5 agonist, and flagellated pathogens. These microbes block downstream signaling associated with NF- $\kappa$ B activation by promoting the nuclear export of transcriptionally active RelA [60]. This effect of *B. thetaiotaomicron* may partly explain why the gut tolerates large amounts of flagellated, potentially inflammatory commensal bacteria. In addition, other commensal bacteria were shown to block the activation of NF- $\kappa$ B by inhibiting I $\kappa$ B- $\alpha$  ubiquitination [61].

Given the important beneficial effects of commensal flora, any disruption of the normal commensal flora will greatly affect intestinal immune homeostasis and may result in disruption and/or failure of tolerance induction to non-pathogenic luminal Ags. Such conditions likely occur following frequent oral antibiotics and severe gastro-enteritis, common conditions

encountered in the first few years of life. In fact, it is not surprising for pediatricians to observe temporary 'apparent' adverse reaction to common DPs following prolonged antibiotics and acute viral gastroenteritis.

**Probiotics:** When mucosal immune responses are persistently dysregulated, aberrant immune responses to benign luminal Ags will likely develop, leading to chronic gut inflammation. In such pathogenic conditions, commensal bacteria may provide beneficial effects. VSL#3® (a high concentration of probiotic preparation consisted of eight freeze-dried bacterial species found in normal human intestinal flora –4 strains of lactobacilli, three strains of bifidobacteria, and *Streptococcus salivarius subsp. Thermophilus*) has been developed for the treatment of ulcerative colitis (UC), pouchitis, and irritable colon syndrome. The results of several clinical studies are encouraging, supporting its favorable therapeutic effects with little adverse reactions [62-64]. It was also shown that VSL#3 and lactobacilli induce  $\beta$  defensin 2 in human epithelial cell line (Caco-2 cells) [65]. VSL#3 is also reported to upregulate alkaline sphingomyelinase in the intestinal mucosa in UC patients, rendering attenuation of gut inflammation; sphingomyelinase hydrolyzes sphingomyelin and induces epithelial apoptosis [66]. In children with CMP allergy and atopic dermatitis, concurrent administration of probiotics (Lactobacillus species) with a hypoallergenic formula yielded a statistically more significant improvement in eczema and a decrease in fecal  $\alpha$ 1-antitrypsin levels than the infants treated with the formula only [67]. These results also support the beneficial effects of probiotics in food protein-induced GI inflammation.

**Micronutrients:** As indicated previously, recent studies revealed a novel role of retinoic acid, a metabolite of retinol (vitamin A), for gut mucosal immune system. Namely, it was shown that as compared to spleen DCs, lamina propria DCs augment de novo generation of Foxp3<sup>+</sup> Treg cells and their actions are dependent on TGF- $\beta$  and retinoic acid [40-41, 68]. In contrast, IL-6 inhibits the effects of retinoic acid on Treg cell differentiation, while retinoic acid antagonizes the augmenting effects of IL-6 on Th17 differentiation [40-41, 68]. Retinoic acid was also known to imprint gut-homing specificity on T cells [69]. Kang *et al* reported that retinoic acid induces Foxp3<sup>+</sup> Treg cells expressing gut-homing receptors at high levels in both humans and mice [70].

Another emerging important regulator in mucosal immunity is adenosine which is likely derived from injured cells/tissues and also can be generated from extracellular nucleotides by ectoenzymes CD39 and CD73 [71]. It has been shown that that Treg cells express CD39 and CD73 and adenosine induces Treg cells to produce suppressive cytokines (IL-10 and TGF- $\beta$ ). These findings also indicate regulatory roles of Treg cells in non-pathogen triggered tissue injury [71, 72]. Adenosine is also reported to exert anti-inflammatory effects independent of Treg cells<sup>71</sup>. These findings indicate importance of micronutrients in the gut mucosal homeostasis, especially in the first few years of life.

#### IV. Non-IgE Mediated Food Allergy (NFA)

Food allergens causing immune mediated allergic reactions are typically categorized as Class I and Class II antigens. Class I allergens are water-soluble glycoproteins that are stable to heat, acid, and digestive enzymes and most food allergens belong to this class. Class II allergens are

those causing oral mucosal irritation through sensitization by homologous components of plant allergens *via* airway mucosa [73].

Food allergy or adverse immune reaction to DPs is classified into two major categories; IgE mediated and non-IgE mediated FA. Class II allergens cause oral allergy syndrome [73] and Class I allergens cause most of the FA affecting the GI tract. In general, during the first few years of life, NFA is likely more common than potentially life-threatening IgE-mediated FA due to the immature gut mucosal immune system as detailed earlier in this review. However, this may not be well appreciated by general practitioners due to the lack of an easily appreciable causal relationship between offending food and clinical features. That is, in contrast to easily appreciable IgE-mediated FA, NFA symptoms can occur hours later, rendering clinical diagnosis difficult and making parents often frustrated, even though presence of NFA has been described in the literature for more than 60 years.

NFA to CMP was first described in 1940's as infants with bloody diarrhea that resolved following elimination of CMP from the diet [74]. Since then, infants whose non-IgE mediated immune reactivity to CMP and soy have been described with a wide range of clinical features – so-called food protein induced enterocolitis syndrome (FPIES) [75]. The immune reactivity to offending food (mainly milk and soy) was typically evaluated by the resolution of GI symptoms and recurrence of symptoms following oral challenge. It should be noted that most patients reveal negative skin test reactivity and absence of food-allergen specific IgE [75, 76]. FPIES is typically manifested as frequent vomiting in early infancy but diarrhea and loose stool generally become more common in late infancy. In severe cases of FPIES, clinical features may resemble sepsis with lethargy, hypotension, dehydration, abdominal distention, and acidemia [77, 78]. These patients may also reveal hypoalbuminemia and failure to thrive. In severe patients with FPIES, reintroduction of offending food can cause shock, despite absence of IgE antibodies [77]. Although it can be potentially life-threatening, these instances are rare and the prognosis of FPIES is generally excellent with resolution of symptoms in several weeks following implementation of the elimination diet (ED). It is also known that many infants with NFA to CMP and soy eventually outgrow this condition that may be associated with establishment of oral tolerance in the gut mucosal immune system.

### **Pathogenesis of NFA**

**Intestinal barrier function:** As opposed to IBD, there appears to be no characteristic histological finding in the intestinal mucosa in patients with FPIES. Due to chronic malabsorption, villous atrophy may be found but histological findings varied considerably in literatures, mostly describing non-specific inflammatory changes [75-76]. There can be no appreciable epithelial lesion by light microscopic examination in some FPIES patients.

Even so, intestinal barrier function may be affected in FPIES patients. This was shown as changes in intestinal permeability [79, 80]. Commonly used markers for assessing intestinal permeability include mannitol and lactulose. Mannitol is thought to pass through aqueous pores in the enterocyte membrane *via* diffusion while lactulose, a larger sugar

molecule than mannitol, permeates through the intercellular spaces. Increased recovery of lactulose is considered to reflect altered tight junctions and cell extrusion zones of epithelium while decreased recovery of mannitol is thought to be associated with reduced cell surface areas and pore numbers. In children with FPIES, increased recovery of lactulose and decreased passage of mannitol in correlation with histologically defined severity of inflammation has been reported [80]. Moreover, these changes were all normalized after implementation of an ED [80]. These results indicate that inflammation caused by immune reactivity to DPs could cause altered barrier functions by disrupting epithelial tight junctions.

**Immune reactivity:** Immune reactivity to DPs observed in FPIES were initially addressed by exploring presence of IgG or IgA antibodies and also proliferation of T cells in response to offending DPs. However, these parameters were shown to be of little clinical value and recent studies are focused on the role of Ag-specific T cells and their production of TNF- $\alpha$ . It has been shown that PBMCs from FPIES produce various proinflammatory cytokines including TNF- $\alpha$  which altered epithelial barrier capacity when tested using a monolayer of HT29 cl.19A epithelial cells *in vitro* [81]. The authors report that intact CMP revealed superior capacity of stimulating PBMCs from FPIES patients than processed CMP with regard to TNF- $\alpha$  production [82, 83]. They also report a significant decrease of TNF- $\alpha$  production following the implementation of an ED [82, 83]. These findings support a role for lymphocyte responses and subsequent TNF- $\alpha$  production in development of NFA. A critical role of TNF- $\alpha$  in NFA was further supported by the findings of increased amounts of fecal TNF- $\alpha$  and presence of TNF- $\alpha$  in the duodenal biopsy specimens in infants with FPIES [80, 84]. Motrich *et al* also reported that CMP stimulated TNF- $\alpha$  production is the most reliable marker of NFA as compared to other markers tested [85].

Clinical findings generally suggest that infants with FPIES outgrow NFA status and become tolerant to offending food. If this is the case, we must then ask how tolerance is induced in these infants. The study by Karlsson *et al* indicates a role of Ag-specific CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in tolerance induction in children with NFA to CMP [33]. In this study, they have evaluated children outgrowing NFA to CMP following a period of being on dairy-free diet in comparison with children with active NFA to CMP. The results revealed higher frequency of circulating CD4<sup>+</sup>CD25<sup>+</sup> Treg cells specific for CMP ( $\beta$ -lactoglobulin) in children outgrowing NFA to CMP. Their results also suggest that the suppressive action of CMP-specific Treg cells was exerted partly by direct cell-cell contact and partly by production of TGF- $\beta$ 1. In contrast, IL-10 production by PBMCs was higher in children with active NFA to CMP. It is of note that TGF- $\beta$ 1 is shown to play a role in epithelial barrier function preventing antigenic macromolecule penetration [86] and one report indicates down-regulation of type 1 TGF- $\beta$ 1 receptor expression in the duodenum obtained from FPIES children [87].

**Cytokine production profile in NFA children against CMP:** In our laboratory, we have also explored pattern of cytokine production (IFN- $\gamma$ , IL-5, IL-10, IL-12p40, IL-17A, TNF- $\alpha$ , and TGF- $\beta$ ) by PBMCs from children with NFA to CMP. We tested the profile of cytokine production by their

PBMCs in response to CMP and its major components ( $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin). We have reproduced others' results, finding an increase in production of TNF- $\alpha$  and IL-12p40 [88]. We did not observe increase in IL-17 production with CMP or candida Ag, a representative microbial luminal Ag, as opposed to IBD patients (unpublished observation). Consistent with others' report<sup>33</sup>, we observed a decline in TNF- $\alpha$  production following the implementation of ED along with decline in IL-10 production (Fig. 1). However, we did not observe an appreciable change in TGF- $\beta$  production in NFA children after resolution of GI symptoms (Fig. 1). In 5 NFA children (Age 2-5 year-old), we also tested TGF- $\beta$  and IL-10 production several months after resolution of GI symptoms, using intact PBMCs or those depleted of CD25<sup>+</sup> cells (CD25<sup>-</sup> PBMCs). Our results revealed little change in IL-10 production irrespective of presence of CD25<sup>+</sup> cells but a significant decrease in TGF- $\beta$  production by CD25<sup>+</sup> PBMCs (Fig. 2). These results indicate presence of TGF- $\beta$  producing CD25<sup>+</sup> Treg cells in the peripheral blood in children who are outgrowing NFA to CMP. IL-10 may be produced by both CD25<sup>+</sup> and CD25<sup>-</sup> PBMCs, since IL-10 produced by multiple lineage cells in this condition.

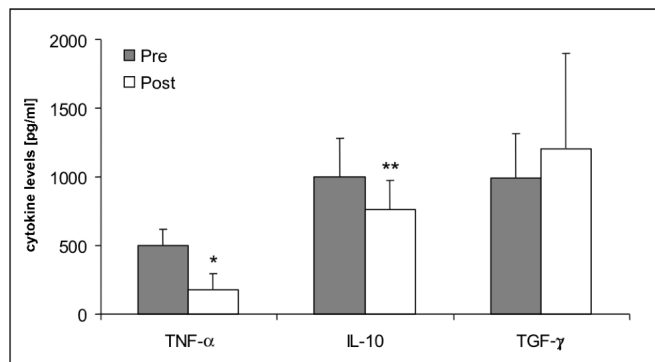


**Fig. (1).** Production of TNF- $\alpha$ , IL-10, and TGF- $\beta$  by PBMCs obtained from NFA children (N=12) before and 3-6 months after implementation of ED. PBMCs were stimulated with  $\beta$ -lactoglobulin (Sigma-Aldrich, St. Louis, Mo, USA) (10  $\mu$ g/ml) for 4 days and then the cytokine levels were measured by ELISA. The results are expressed as a mean value  $\pm$  standard deviation (SD). \*, Lower than prior to ED ( $p < 0.01$ ), \*\*, Lower than prior to ED ( $p < 0.05$ ) by paired Wilcoxin ranks test.

## DIAGNOSIS AND TREATMENT

The above described laboratory measures such as *in vitro* TNF- $\alpha$  production in response to DPs are not yet commonly available. Thus in most clinical settings, the gold standard diagnostic measure still is a trial of avoidance of suspected offending food for several weeks. It is important to note that due to chronic nature of GI inflammation, it is unlikely that GI symptoms resolve within a few days. Typically complete resolution of GI symptoms is observed 3-4 weeks after implementation of the ED. To confirm the diagnosis, a challenge test is indicated. This is rather a lengthy procedure and it would be helpful if there were practical, immediate testing measures available. Skin patch testing has been employed to detect delayed type FA for that purpose. Typically, food was applied to intact skin for 48 hrs and then 24 h after removal of food, skin reactivity was measured. Although this is still in the early stage of development, the results may be promis-

ing [89, 90]. A significant concern is that there are no standardized food proteins available for patch skin testing and clinical applicability of patch skin testing is not well established [91]. Development of practical, reliable laboratory measures for diagnosis of NFA is critical and will be very helpful for general practitioners.

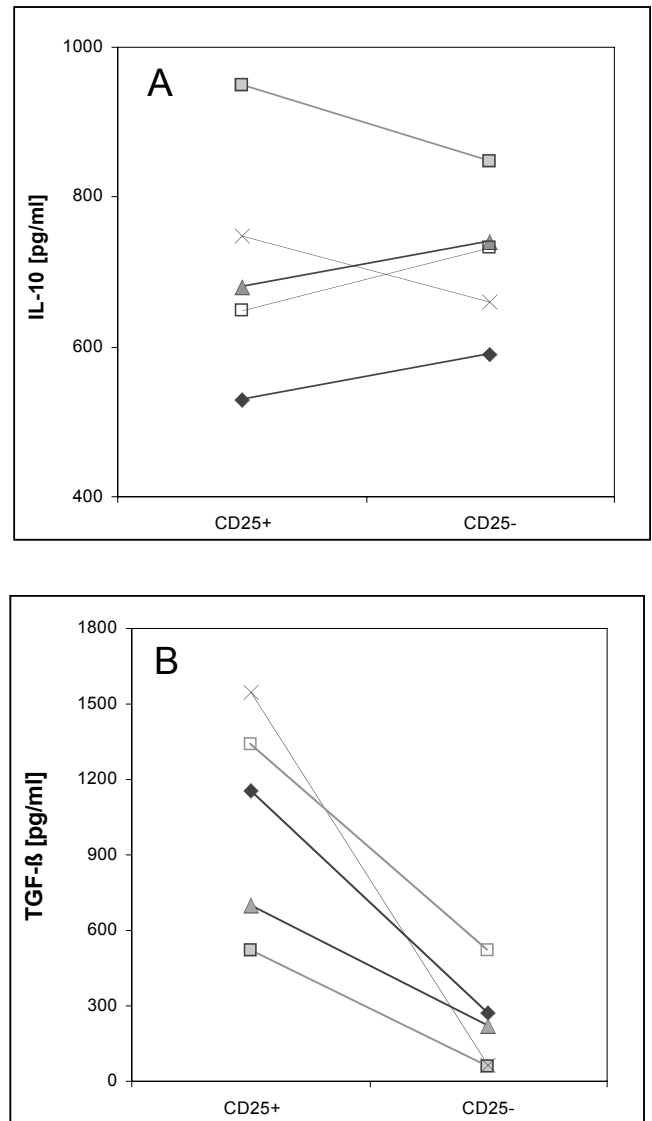


**Fig. (2).** Production of IL-10 (Panel A) and TGF- $\beta$  (Panel B) by PBMCs obtained from 5 NFA children (age 2-5 yo) more than 6 months after resolution of GI symptoms following implementation of ED. PBMCs were used with or without depletion of CD25<sup>+</sup> cells. CD25<sup>+</sup> cell depletion was performed by passing PBMCs through a column of magnetic immunoaffinity beads following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

## CONCLUSION

The recent progress regarding immune homeostasis of the gut mucosal immune system is quite exciting and will hopefully improve our understanding of the pathogenesis of NFA and our ability to diagnose NFA (FPIES) in a timely manner. In general, NFA is an easily treatable clinical entity. However, when under-diagnosed/under-treated, serious and possibly irreversible complications can occur. Increased knowledge and awareness of NFA, as well as the identifica-

tion of biomarkers and risk factors is crucial for early diagnosis and timely intervention of NFA (FPIES).

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## ABBREVIATIONS

|                        |                                               |
|------------------------|-----------------------------------------------|
| Ab                     | = Antibody                                    |
| Ag                     | = Antigen                                     |
| APC                    | = Ag-presenting cells                         |
| CARD                   | = Caspase recruitment domains                 |
| CD                     | = Crohn's disease                             |
| CMP                    | = Cow's milk protein                          |
| DCs                    | = Dendritic cells                             |
| DPs                    | = Dietary proteins                            |
| ED                     | = Elimination diet                            |
| FPIES                  | = Food-protein induced enterocolitis syndrome |
| FTT                    | = Failure to thrive                           |
| GALT                   | = Gut associated lymphoid tissue              |
| GI                     | = Gastrointestinal                            |
| IBD                    | = Inflammatory bowel disease                  |
| IFN                    | = Interferon                                  |
| LP                     | = Lamina propria                              |
| M cells                | = Microfold cells                             |
| MDP                    | = Muranyl dipeptide                           |
| meso-DPA               | = Meso-diaminopimelic acid                    |
| MHC                    | = Major histocompatibility complex            |
| MLN                    | = Mesenteric lymph node                       |
| NFA                    | = Non-IgE mediated food allergy               |
| Nod                    | = Nucleotide oligomerizing domain             |
| NLR                    | = Nod like receptor                           |
| PAMPs                  | = Pathogen associated molecular patterns      |
| PBMCs                  | = Peripheral blood mononuclear cells          |
| PP                     | = Peyer's patches                             |
| PRRs                   | = Pattern recognition receptors               |
| TGF- $\beta$ 1         | = Transforming growth factor $\beta$ -1       |
| Th                     | = T-helper                                    |
| TLR                    | = Toll-like receptor                          |
| TNF                    | = Tumor necrosis factor                       |
| TNFR II                | = Type II TNF receptor                        |
| Treg cells             | = Regulatory T cells                          |
| T <sub>H</sub> 1 cells | = Type 1 Treg cells                           |
| UC                     | = Ulcerative colitis                          |

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