Regulatory T cells and cellular environments in pediatric immune-mediated gastrointestinal diseases

Ph.D. thesis

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1. Introduction

The gastrointestinal disorders form a large group of chronic childhood diseases. These diseases are divided into three subgroups: those are the consequence of developmental deficiencies, malnutrition syndromes and chronic inflammatory ones. The main causes of chronic inflammation are infections and autoimmune processes. The pathomechanism of infections are mostly well known, the duration is acute and therapy is supportive or in a small part antibiotics are given. The autoimmun disorders are long-lasting chronic processes and the main goal of the treatment is the modulation of immune response. Only parts of the pathomechanism are described but grooving body of evidence suggests how the dysregulation of immune response is involved.

We plan to immunophenotype the investigated diseases and to check whether the available therapy influence the possible alterations in immune response, or not. The three investigated diseases are Crohn's disease (CD), celiac disease and allergic colitis (AC) and develop in different period of childhood. According to the recent hypotheses regulatory T cells (Tregs) play a central role the inhibition of abnormal immune activation. Therefore the measurement of the Treg prevalence was the main goal of our work. However, immune cells are working together and forming a complicated network with lymphocyte activation in the centrum. Indeed, we investigated not only the prevalence of Tregs but that of their cellular environments.

Our aim was to develop and test method which represents an easy-to-use and also not stressful technique. The measurements with flow cytometry (FACS) requires only small amount of blood and peripheral sample taken during routine blood tests are widely acceptable for patients.

2. Aims

Our aim was to develop a FACS method that require only small amount of blood from patients and available to investigate Treg cells and their cellular environment. We also aimed to immunophenotype pediatric gastrointestinal disorders using this method. The investigated method was suitable to assess several other immunmediated diseases (see related publications).

2.1. Crohn's disease

During our prospective study our aim was to characterize the prevalence of both the adaptive and innate immune cells in newly diagnosed, therapy-naïve CD patients; in conventional therapy treated (steroid, azathioprine (AZT), 5-aminosalycilate (5-ASA)) and biological therapy (infliximab, IFX) patients and to test whether abnormalities of immune phenotype are normalized with the improvement of clinical signs and symptoms of disease.

2.2. Celiac disease

We characterized the prevalence of major interacting members of the adaptive and innate immune system in peripheral blood of newly diagnosed children with celiac disease and tested its alteration with the improvement of clinical signs after the introduction of gluten free diet (GFD).

2.3. Allergic colitis

The objective of our study was to investigate the prevalence of adaptive immunity in infants with allergic colitis and test the alteration with the cessation of AC signs and symptoms.

3. Patients and methods

3.1. Patients

3.1.1. Crohn's disease

We enrolled the following patient groups into our study: *First group* 14 therapy-naïve CD children. No drug was prescribed for these patients at the time of CD diagnosis. The diagnosis of CD was established by means of 'The Porto criteria'; disease activity was determined according to the Pediatric Crohn's Disease Activity Index (PCDAI). *Second group:* during conventional treatment (steroid, azathioprine (AZT) and 5-aminosalicylate (5-ASA)), 10 children responded forming the remission group. Clinical remission was defined as a PCDAI < 10. *Third group:* IFX therapy (5 mg/kg IFX at weeks 0, 2, and 6) was started in 12 CD children who failed to respond to conventional therapy forming the 'relapsed group'. Non-responsiveness was defined as moderately increased PCDAI (PCDAI > 30) in patients under conventional therapy. *Fourth group:* fifteen age- and gender-matched children with functional abdominal pain served as controls. All patients and controls were diagnosed, treated and followed up in the Outpatient Clinic of the First Department of Pediatrics, Semmelweis University between September 2007 and August 2009. The Institutional Ethical Committee approved our study; written parental informed consent was obtained.

Together with other routine blood sampling 6 mL of lithium-heparin anticoagulated blood was taken from therapy-naïve patients at the time of diagnosis, at the time of first remission in the remission group and at the initiation of IFX therapy, and then 2 and 6 weeks later in the relapsed groups.

3.1.2. Celiac disease

We enrolled ten children in our study (4 boys and 6 girls, age 3 [2-5] years), duration of major clinical symptoms 2 [2-4] months leading to the establishment of the diagnosis of celiac disease. The diagnosis of celiac disease was made according to the criteria of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition and according to Marsh criteria.Transglutaminase (TG) IgA antibody levels were above 200 U/l and histological investigation of biopsy samples demonstrated subtotal or total villous atrophy of intestinal

mucosa with increased number of intraepithelial lymphocytes (>40%) in all patients. Strict GFD was introduced in all patients diagnosed celiac disease. The clinical symptoms of enrolled children fully resolved after 3 [2.5-4] months of GFD and TG IgA levels returned to normal (10-40 U/l). As controls, 15 children (6 boys and 9 girls, age 3 [2-6] years) with functional non-organic abdominal pain were recruited. All patients and controls were diagnosed, treated and followed up in the Outpatient Clinic of the First Department of Pediatrics, Semmelweis University between December 2007 and December 2009. The Institutional Ethical Committee approved our study; written parental informed consent was obtained.

Six mls of lithium-heparin anticoagulated venous blood were taken from controls during their routine assessment and from patients at the time of diagnosis and at the second visit, when clinical symptoms were resolved.

3.1.3. Allergic colitis

During a 16-month period (September 2006 - January 2008) at the Outpatient Clinic of the First Department of Pediatrics, Semmelweis University, 34 infants with HC (age, median [range]: 5 months [4 days – 7.5 months]) were evaluated. After exclusion of infection and fissures, adherence to maternal elimination diet (i.e. exclusion of cow milk and eggs from diet) was advised. After 1 month HC persisted in 11 infants who were still breast-fed; of those, one infant was diagnosed with Crohn's disease with colonoscopy. The remaining 10 infants with HC (age, median [range]: 4.5 [1.0 – 6.5] months) were enrolled to the study. In these patients AC was established according to characteristic signs on colonoscopy (i.e. lymphoid nodular hyperplasia or aphthous ulceration), elevated number of eosinophils in colonic biopsy specimens (> 6 per one high power field, HPF), and cessation of HC after long-lasting maternal diet (milk, egg) and or the introduction of elemental L-amino acid formula (Neocate; SHS Int, Liverpool, UK) with no recurrence after long-term follow-up (13-24 months).

2 ml lithium-heparin anticoagulated blood was taken at the time of diagnosis. All ten infants were switched to elemental L-amino-acid based formula or solids. The second blood sample was taken when symptoms resolved (after median [range]: 2 [1 – 3] months). A fecal occult blood test (HSV10; Diagnosticum Zrt, Budapest, HU) was used to verify the complete

absence of blood in stool. Ten age- and gender-matched, and breast-fed matched healthy infants with functional abdominal pain were used as controls; blood sampling was done simultaneously with routine laboratory measurements. The Institutional Ethical Committee approved our study; written parental informed consent was obtained.

3.2. Methods

3.2.1. Flow cytometry

We collected peripheral blood from patients and healthy controls (BD Vacutainer, Beckton Dickinson & Co, Plymouth, UK). The sample was store on room temperature max 12h. Plasma was separated with centrifugation and stored for chip assay. From whole blood, PBMCs were separated with gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Surface markers (6B11, CCR4, CD3, CD4, CD8, CD11c, CD14, CD25, CD45RA, CD45RO, CD62L, CD69, CD123; CD161, CXCR3, HLA-DR and Lin-1, BD Biosciences Pharmingen, San Diego, CA, USA; TLR-2 and TLR-4, eBioscience, San Diego, CA, USA) and intracellular FoxP3 assay was used (eBioscience, San Diego, CA, USA) according to the manufacturers' protocols. We identified the members of adaptive immunity, such as helper T (Th; $CD4^+$), cytotoxic T (Tc; $CD8^+$), Th1 ($CXCR3^+$), Th2 ($CCR4^+$), naïve (i.e. $CD45RA^+$), effector/memory ($CD45RO^+$), early activation marker expressing ($CD25^+$ and $CD69^+$), late activation marker positive ($CD62L^+$ and HLA-DR^+) and regulatory T lymphocytes ($CD4^+CD25^{hi}FoxP3^+$). Simultaneously, we identified the members of innate immunity including natural killer cells (NK; $CD3^-CD161^+$), natural killer T cells (NKT; $CD3^+CD161^+$), invariant NKT (iNKT; $CD3^+6b11^+$), dendritic cells (DCs; $Lin1^-$HLA-DR^+), mieloid DC (mDC;. $CD11c^+$), plasmocytoid DC (pDC; $CD123^+$) and peripheral monocyte cells ($CD14^+$), along with the TLR-2 and TLR-4 expressing antigen presenting cells (APCs), namely DCs and monocytes.

3.2.2. Cytokine chip

Serum was isolated and stored at -70 °C until analysis and cytokine measurements were undertaken within 1 month. For cytokine measurements, the Bio-Plex™ multiplex system

(Human Cytokine Kit [Cat. No. 171A11171], Bio-Rad Inc., Hercules, CA, USA) was used, and IFNγ an IL-4 cytokine levels were defined. The measurements were done in Central Laboratory of Semmelweis University. The manufacturer's instructions were followed during the measurements.

3.2.3. Statistical analyses

As our data did not followed Gaussian distribution, non-parametric test were used. Unpaired two sample comparisons were done with Mann-Whitney test, while paired analyses with Wilcoxon test. Three-, or more groups were investigated with Friedman test, post hoc tests were done according to Dunn's. Correlations were analyzed with Spearman test, while the contributing factors were analyzed with multiple regression models. The analyses of contingency tables were done with Fisher's exact test and chi-square on larger table. A P-value < 0.05 was considered to be statistically significant. (Statistica 8, Statsoft, Tulsa, OK, USA).

4. Results

4.1. Crohn's disease

First, we compared the immune phenotype in therapy-naïve CD patients with that of healthy controls. In CD children, the prevalence of activated T cells (i.e. $CD4^+CD25^+$ cells) increased. At the same time, the prevalence of T cells with Th1 commitment (i.e. $CD4^+CXCR3^+$ cells) decreased resulting in a skewness of Th1/Th2 to Th2. The prevalence of memory (i.e. $CD4^+CD45RO^+$) cells increased and, therefore, a shift in the naïve/memory ratio toward memory cells was observed. The prevalence of regulatory T (i.e. $CD4^+CD25^{hi}FoxP3^+$) cells was comparable between the two groups.

Striking differences in cell prevalence values of innate immunity were obtained between therapy-naïve CD and healthy children. The occurrence of NK and NKT cells (marked as $CD3^-CD161^+$ and $CD3^+6b11^+$, respectively) was lower in CD children. Interestingly, the prevalence of the APCs investigated differed largely between CD and healthy children. DCs (i.e. those with $Lin1^-HLADR^+$ expression) were more prevalent and, within DC cells, the myeloid DCs (mDCs, i.e. $CD11c^+$ cells) were more prevalent, while plasmocytoid DCs (pDCs, i.e. $CD123^+$ cells) were less frequent in CD than in healthy children. This leads to skewness of mDCs in the mDC/pDC ratio. The prevalence of peripheral monocytes (i.e. $CD14^+$ cells) also increased in therapy-naïve CD patients. In addition, the prevalence of DC cells and monocytes expressing Toll-like receptor (TLR) 2 and 4 was also increased in CD. Of note, 4 of the 14 therapy-naïve CD children did not respond to conventional therapy. Their immune phenotype at the therapy-naïve phase did not differ from those children who responded to conventional therapy.

In the second phase of our study, we prospectively tested the alteration in cell prevalence values during therapy. At the time of first remission with conventional therapy, the Th1/Th2 ratio shifted to Th1 and normalized along with activated T cell prevalence. Memory T cells remained elevated, while all the other cell types of adaptive immunity were comparable to that measured before therapy. For innate immune cells, NK and NK-T remained lower than normal and total DC prevalence remained higher than the control. However, mDC and pDC ratios, total monocyte prevalence and cells expressing TLR2/TLR4 receptor values (including monocytes and DCs) were normal.

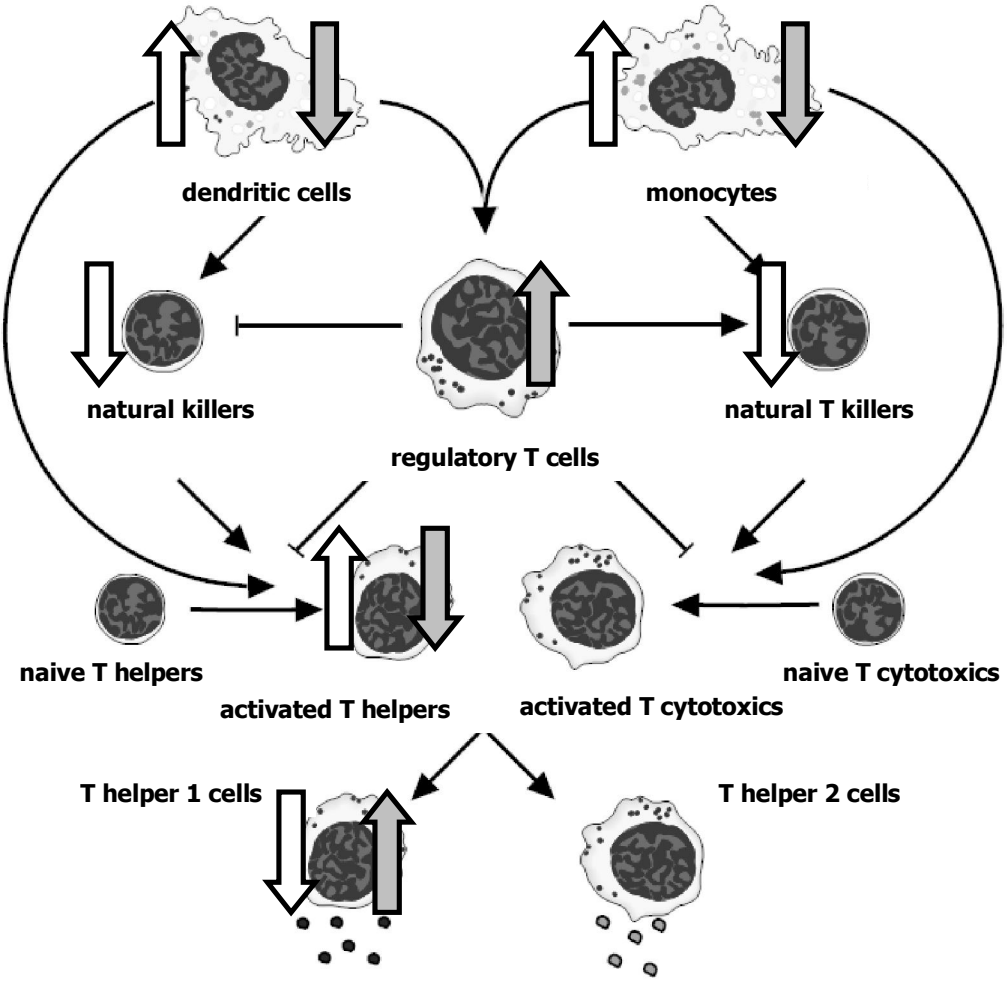

Figure 1. Summary of the peripheral immunophenotye in Crohn's disease. *White arrows represent the alterations in untreated patients compared to healthy controls, while gray arrows mean the effect of conventional or infliximab therapy in comparisons with untreated patients.*

In children who relapsed with conventional therapy, immune phenotype again became comparable to that in therapy-naïve CD. Therefore, we measured lower Th1, increased activated T, higher DC and higher macrophage prevalence as well as higher TLR2 and TLR4 expression in comparison to controls. In addition, the prevalence of mDCs, simultaneously with TLR2 and TLR4 expressing DCs was higher in relapsed than in remitted CD. During IFX therapy, immune cell prevalence was measured at two time points (i.e. 2 and 6 weeks after the initiation of therapy). Th1, activated T and Treg prevalence increased significantly by week 6 of therapy. Total DC, mDC, pDC, total monocyte, along with TLR2 and TLR4 expressing DC and macrophage prevalence were normal at this time.

4.2. Celiac disease

Major differences in the prevalence of cells of adaptive immune system were observed between newly diagnosed children with celiac disease and controls. These include lower than normal prevalence of $CD4^+$, and $CD4^+$ cells expressing late activation markers (i.e. CD62L), while that of early cells (i.e. CD69) was higher in celiac disease. In addition, Th1 cell populations were smaller in celiac disease in comparison to controls. The prevalence of Tregs was comparable.

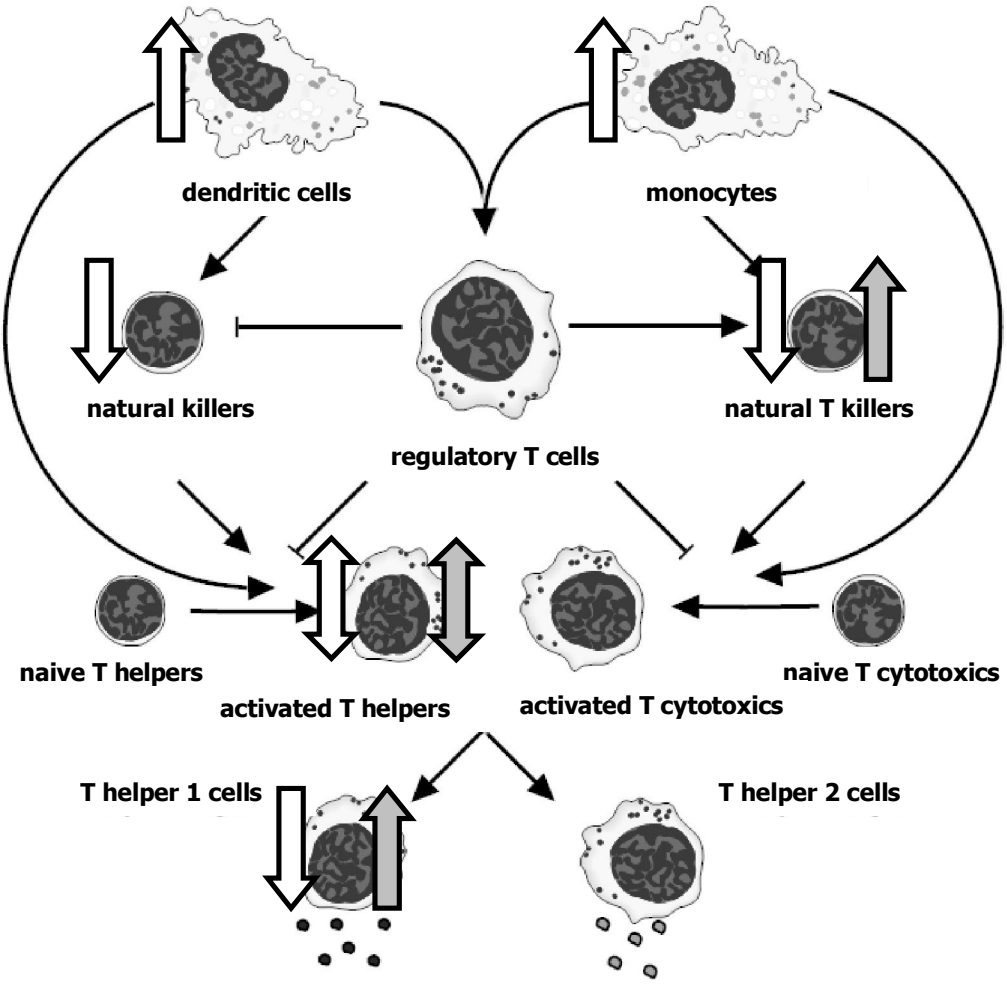

Figure 2. Summary of the peripheral immunophenotye in celiac disease. *White arrows represent the alterations in untreated patients compared to healthy controls, while gray arrows mean the effect of gluten free diet in comparisons with untreated status.*

The phenotype of innate immunity was also abnormal in celiac disease as NK, NKT and iNKT cells were less prevalent in newly diagnosed patients with celiac disease than in controls. On the other hand, DCs, particularly mDCs were more prevalent in celiac disease than in healthy control subjects. APCs expressing TLR-2 and TLR-4 receptors were also more prevalent in celiac disease.

After introduction of GFD and cessation of celiac disease symptoms the immune phenotype started to normalize. CD4 lymphocytes' prevalence increased along with that of Th1 committed CD4 cells. The prevalence of $CD4^+$ cells expressing early and late activation markers (i.e. $CD69^+$ and $CD62L^+$, respectively) tended to normalize.

Interestingly, the abnormalities in innate immune system characteristic for newly diagnosed celiac disease were maintained after GFD; only the prevalence of TLR-4 expressing APCs normalized.

Our exploratory analysis revealed important correlations between TG IgA levels and immune phenotype including the prevalence of $CD8^+$, $CD4^+CD69^+$, $CD4^+$HLA-DR^+ cells, pDCs and TLR-2 expressing monocytes in patients with CD on GFD.

4.3. Allergic colitis

At the baseline the prevalence of Tregs was lower in AC than in controls. Simultaneously, the $CD4^+CD45RA^+/CD4^+CD45RO^+$ ratio was higher, and the prevalence of $CD4^+CD25^+$ cells was lower in AC. The $CD4^+CXCR3^+/CD4^+CXCR4^+$ and IFNγ/IL-4 ratios were lower in AC patients compared to the controls.

We also tested the alteration of these parameters after the resolution of AC. We found that the prevalence of Tregs increased to normal at this time. Although other cell types tested did not change significantly during the therapy in comparison with baseline cell prevalence values, the $CD4^+CXCR3^+/CD4^+CXCR4^+$ and IFNγ/IL-4 ratios and cell prevalence data measured at symptom cessation were not significantly different from that in the controls.

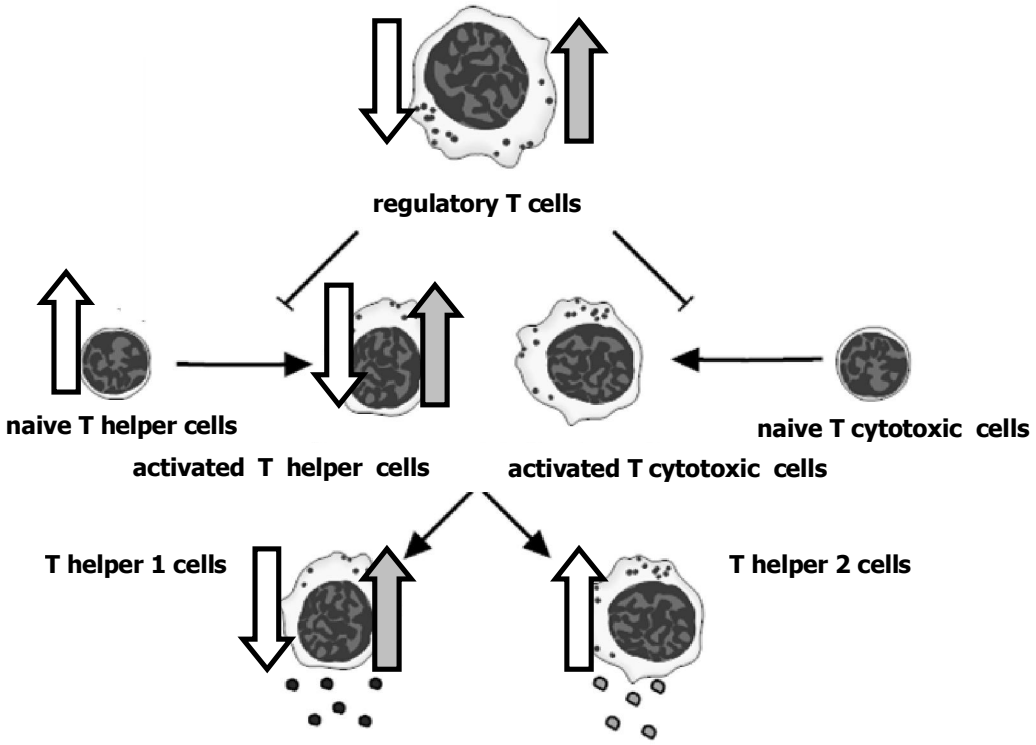

Figure 3. Summary of the peripheral immunophenotye in allergic colitis. *White arrows represent the alterations in untreated patients compared to healthy controls, while gray arrows mean the effect of elementary formula in comparisons with untreated status.*

5. Conclusions

(1) In newly-diagnosed pediatric Crohn's patients both the members of adaptive and innate immunity were affected. We observed a shift in Th1/Th2 ratio toward Th2 in children with therapy-naïve CD. Also the prevalence of effector and activated T lymphocytes increased in this population. The APCs, namely DCs and monocytes were more prevalent and their Toll-like receptor expression is upregulated in untreated patients.

(2) The majority of alterations diminished in remitted CD. However, in patients non-responding to conventional therapy these changes in immunophenotype persist, and the prevalence of mDCs and TLR expressions remained higher.

(3) We introduced IFX in patients non-responding to conventional therapy. Both the adaptive and innate immunophenotype normalized. However, the IFX treatment increased the prevalence of the members of adaptive immunity, such as effector, Th1 and regulatory T lymphocytes.

(4) The immunophenotype in untreated celiac children exhibits several abnormalities: these include the increased activation of both adaptive and innate immunity. The prevalence of $CD4^+$ and Th1-committed lymphocytes decreased in newly-diagnosed patients. Simultaneously, the prevalence of early activation marker expressing T cells increased, while that of late activation marker expressing lymphocytes decreased.

(5) While the alterations of adaptive immunity are mainly normalized with GFD, while those of innate immunity still persist in spite of the clinical improvement of celiac disease associated signs and symptoms. Indeed, the shift toward activated and Th1 direction mainly normalized during gluten free diet. The prevalence of APCs, DCs and monocytes remained higher after GFD.

(6) Our study revealed that AC is accompanied by low Treg prevalence, an increased ratio of naïve/memory T cells, a decreased prevalence of activated T cells and a shift of $CD4^+$ T lymphocytes to Th2 direction.

(7) After introduction of elementary formula with the cessation of AC signs and symptoms these alterations ceased. Indeed, the prevalence of Treg cells normalized, and the Th1/Th2 shift also diminished. These findings indicate that an immune dysfunction may contribute to this gastrointestinal disorder in infancy.

6. Publications

6.1. Basic publications

Cseh Á, Vásárhelyi B, Szalay B, Molnár K, Nagy-Szakál D, Treszl A, Vannay Á, Arató A, Tulassay T, Veres G: „Immune phenotype of children with newly diagnosed and gluten-free diet-treated celiac disease" (Digestive Diseases and Sciences 2011;56:792-8. **IF: 2.060**)

Cseh A, Molnár K, Pintér P, Szalay B, Szebeni B, Treszl A, Arató A, Vásárhelyi B, Veres G.: „Regulatory T cells and T helper subsets in breast-fed infants with hematochezia caused by allergic colitis" (Journal of Pediatric Gastroenterology and Nutrition 2010;51:675-7. **IF: 2.180**)

Cseh A, Vasarhelyi B, Molnar K, Szalay B, Svec P, Treszl A, Dezsofi A, Lakatos PL, Arato A, Tulassay T, Veres G.: „Immune phenotype in children with therapy-naïve, remitted and relapsed Crohn's disease" (World Journal of Gastroenterolgy 2010;16:6001-9. **IF: 2.240**)

Cumulative impact factors: 6.480

6.2. Related publications

Cseh A, Bohács A, Szalay B, Losonczy G, Tulassay T, Vásárhelyi B, Tamási L: „Peripheral dendritic cells in asthma" (Journal of Investigational Allergology and Clinical Immunology 2010;20:533-5. **IF: 1.489**)

Bohács A, **Cseh A**, Stenczer B, Müller V, Gálffy G, Molvarec A, Rigó J Jr, Losonczy G, Vásárhelyi B, Tamási L: „Effector and regulatory lymphocytes in asthmatic pregnant women" (American Journal of Reproductive Immunology 2010;64:393-401. **IF: 2.451**)

Mácsai E, **Cseh A**, Budai G, Mészáros G, Vásárhelyi B, Fischer K, Szabó A, Treszl A.: „Effect of 3 months of doxazosin therapy on T-cell subsets in type 2 diabetic patients" (The Journal of International Medical Research 2009;37:1982-7. IF: **0.938**)

Cseh Á, Bohács A, Müller V, Szalay B, Losonczy Gy, Tulassay T, Vásárhelyi B, Tamási L: „Az asztma megváltoztatja a perifériás dendritikus sejtarányt" (Medicina Thoracalis 2010;LXIII:358-362.)

Cumulative impact factors: 4.878

6.3. Other publications

Vásárhelyi B, **Cseh A**, Kocsis I, Treszl A, Györffy B, Rigó J Jr.: "Three mechanisms in the pathogenesis of pre-eclampsia suggested by over-represented transcription factor-binding sites detected with comparative promoter analysis" (Molecular Human Reproduction 2006;12:31-4. **IF: 2.760**)

Cseh Á. Oh., Rigó J. Jr. Dr.,Vásárhelyi B. Dr., Páli A. Oh., Győrffy B. Dr.: "Szerepet játszanak-e a transzkripciós faktorok az endometriózis kialakulásában?" (Magyar Nőorvosok Lapja 2007;70:183-186.)

Páli A., Arató A., Dezsőfi A., **Cseh Á**., Treszl A., Veress G., Szőnyi L.: „Wilson-kór vagy epeút-elzáródás?" (Gyermekgyógyászat 2007;58:350-353.)

Gyorffy A, Baranyai Z, **Cseh A**, Munkácsy G, Jakab F, Tulassay Z, Gyorffy B.: „Promoter analysis suggest, the implications of NFkB/C-REL transcription factors in biliary atresia." (Hepato-Gastroenterology 2008;55:1189-92. **IF: 0.680**)

Szebeni B., Sziksz E., Prókai Á., Gál K., Vannay Á., **Cseh Á**., Veres G., Dezsőfi A., Korponay Szabó I., Bodánszky H., Arató A.: "Fokozott szérum és glükokortikoid regulált kináz-1 expresszió gyermekkori cöliákiában" (Gyermekgyógyászat 2009;60:67-73.)

Cseh A, Szebeni B, Szalay B, Vásárhelyi B.: "Az Akt enzim: új terápiás célpont rákban és cukorbetegségben?" (Orvosi Hetilap 2009;150:373-8.)

Gyarmati B, Beko G, Szalay B, **Cseh A**, Vásárhelyi B, Treszl A.: „Maternal cytokine balance on 3rd postpartum day is not affected by the mode of delivery after healthy pregnancies" (The Journal of International Medical Research 2010;38:208-13. **IF: 1.068**)

Szebeni B, Vannay A, Sziksz E, Prókai A, **Cseh A**, Veres G, Dezsőfi A, Győrffy H, Szabó IK, Arató A.: „Increased Expression of Serum- and Glucocorticoid-regulated Kinase-1 in the Duodenal Mucosa of Children With Coeliac Disease" (J Pediatr Gastroenterol Nutr 2010;50:147-53. **IF: 2.180**)

Szakál DN, Gyorffy H, Arató A, **Cseh A**, Molnár K, Papp M, Dezsofi A, Veres G.: „Mucosal expression of claudins 2, 3 and 4 in proximal and distal part of duodenum in children with coeliac disease." (Virchows Archiv 2010;456:245-50. **IF: 2.336**)

Gyarmati B, Szabó E, Szalay B, **Cseh A**, Czuczy N, Toldi G, Vásárhelyi B, Takáts Z.: „Emelkedett hepcidinszint nőgyógyászati műtéteket követő harmadik napon" (Orvosi Hetilap 2010;151:1790-4.)

Molnár K, Vannay Á, Szebeni B, Sziksz E, **Cseh Á**, Bánki NF, Dezsőfi A, Arató A, Tulassay T, Veres G, Győrffy H, Lakatos PL, Papp M: „Intesztinális alkalikus foszfatáz vizsgálata krónikus bélgyulladásban (IBD) szenvedő gyermekek bélnyálkahártyájában" (Gyermekgyógyászat 2011;62:105-109.)

Gál K, **Cseh A**, Szalay B, Rusai K, Vannay A, Lukácsovits J, Heemann U, Szabó AJ, Losonczy G, Tamási L, Müller V: „Effect of cigarette smoke and dexamethasone on Hsp72 system of alveolar epithelial cells" (Cell Stress Chaperones. Epub ahead of print. DOI: 10.1007/s12192-010-0249z **IF: 3.162**)

Gyarmati B, Szabó E, Szalay B, Czuczy N, Toldi G, **Cseh Á**, Vásárhelyi B, Takáts Z: „Serum maternal hepcidin levels three days after delivery are higher compared to those measured at parturition" (The Journal of Obstetrics and Gynaecology Research. Accepted. **IF: 0.869**)

Cumulative impact factors: 24.413

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