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Serum IL-6, IL-23 profile and Treg/Th17 peripheral cell populations in pediatric patients with inflammatory bowel disease

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IL-6 and IL-23 are both pleiotropic cytokines involved in the regulation of the immune response, inflammation, and hematopoeisis. They also could mediate effector cells and tolerance mediated by cells with regulatory function. Inflammatory bowel disease (IBD) is associated with a reduced ratio of Treg cells ato Th17 effector cells in peripheral blood and is characterised by a pro-inflammatory cytokine microenvironment which supports the continued generation of Th17 cells. It is well described in adults but little is known in a pediatric population. This study was aimed to investigate the role of IL-6, IL-23 and its association with Treg and Th17 subsets in pediatric IBD patients. Peripheral blood mononuclear cells from patients and controls were stimulated with PMA, ionomycin, and brefeldin A. The frequencies of $CD4^+Foxp3^+$ cells, and $CD4^+IL17a^+$ cells were analyzed by flow cytometry. The serum level of IL-6 and IL-23 was determined by Elisa kit. The mRNA expression of Foxp3, IL-17a, IL-6 and IL-23 was detected by real-time quantitative PCR. The ratio of Treg/Th17 decreased in pediatric IBD patients, and it strongly correlated with IL-6 and IL-23. The present study provides a quantitative analysis regarding the Th17/Treg cell balance in peripheral blood of children with IBD and its association with serum IL-6 and IL-23 level.

1. Introduction

Inflammatory bowel diseases (IBD) comprise several chronic disorders affecting the intestinal mucosa, with ulcerative colitis (UC) and Crohn's disease (CD) as distinct disease entities (Yu et al. 2016). IL-6 is a pleiotropic cytokine involved in the regulation of the immune response, inflammation and hematopoiesis (Bobby et al. 2014). Unlike many cytokines, IL-6 can be detected in the serum, although baseline levels are low in the absence of inflammation. The elevated levels of IL-6 were found in autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, diabetes, multiple sclerosis, and asthma (Tanaka et al. 2014). IL-6 may also contribute to malignancies such as multiple myeloma and colon cancer (Barbosa et al. 2016). Interleukin (IL)-23 belongs to the IL-12 family of cytokines and consists of the two subunits p19 and p40 (Peng et al. 2016). IL-23 is mainly produced by macrophages and DCs, and through its interaction with the IL-23 receptor (IL-23R), composed of the IL-12Rβ1 unit and the specific IL-23R unit, plays a central role in inflammation including the induction of Th17 cells. The cytokine IL-23 is produced by dendritic cells, macrophages, and Th17 pro-inflammatory cells, which results in increased IL-17 production (Wines et al. 2016).

Recently it has been shown that IL-6 is involved in the regulation of a balance between two T cell subsets that play a pivotal role in inflammatory and autoimmune diseases. These are IL-17-producing Th17 cells that contribute to the progression of inflammation and $Foxp3^+$ T regulatory cells which are natural suppressors that control overactive cells (Zhang et al. 2016). IL-23 is thought to be involved in the promotion of Th17 cell polarization (Moutsopoulos et al. 2012). A balance between Th17 and Treg subsets is crucial for immune homeostasis, however it is shown to be impaired in various clinical disorders. However, the role of IL-6 and IL-23 which contribute to the balance between Treg and Th17 cells is still unclear.

The aim of this study was to determine the impaired quantitative as well as qualitative properties of $Foxp3^+$ Tregs in IBD children. We developed a stringent Flow based staining and gating strategy to accurately enumerate the Treg and Th17 cells using Foxp3 and IL17a as the defining markers. These impairments were probably dependant on an ongoing inflammatory response in these children. In addition, serum IL-6, IL-23 levels in patients with IBD which may play an important role in pathogenesis of pediatric IBD have also been investigated. Our current work shows the association between IL-6, IL-23 serum levels and Treg/Th17 subsets in IBD children patients. It supports the view that targeting IL-6/IL-23 signaling may improve the treatment of autoimmune and chronic inflammatory diseases.

2. Investigations and results

2.1. General characteristics of each analyzed group

Fourteen children with Crohn's disease (CD) (median age 13.8 years), 12 children with ulcerative colitis (UC) (median age 14.3 years), and 11 control patients (median age 15.2 years) were investigated. Of the total 26 IBD biopsies collected, 9 were from patients with moderate disease activity, 8 had mild disease activity and 9 had inactive disease, based on standard criteria using clinical, radiological, endoscopic, and histopathological findings in accordance with Porto criteria (Kocsis et al. 2008).

2.2. $CD4^+Foxp3^+$ regulatory T cells in peripheral blood of pediatric IBD patients

Compared to the healthy individuals from the control group, the analysis of Tregs in peripheral blood of pediatric CD and UC patients revealed a lower percentage of $CD4^+Foxp3^+$ regulatory T cells (P=0.0027, P=0.0025 respectively) as well as a decreased absolute number of $CD4^+Foxp3^+$ regulatory T cells (P=0.0084, P=0.0049 respectively). Moreover, the pediatric CD patients showed higher $CD4^+Foxp3^+$ regulatory T cell levels compared with the UC patients, but it is no significant differences.

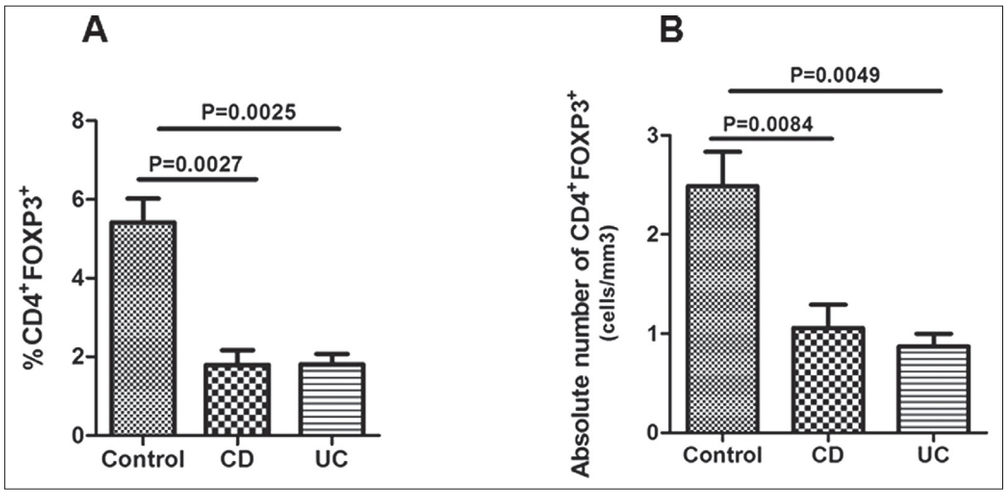

Fig. 1: Comparison between percentage (A) and absolute number (B) of CD4+Foxp3+ T cells in peripheral blood of pediatric IBD patients and control group. Fresh, resting PBMCs from pediatric IBD patients and healthy individuals were stained with antibodies against CD4 and Foxp3 molecules and analyzed using flow cytometry.

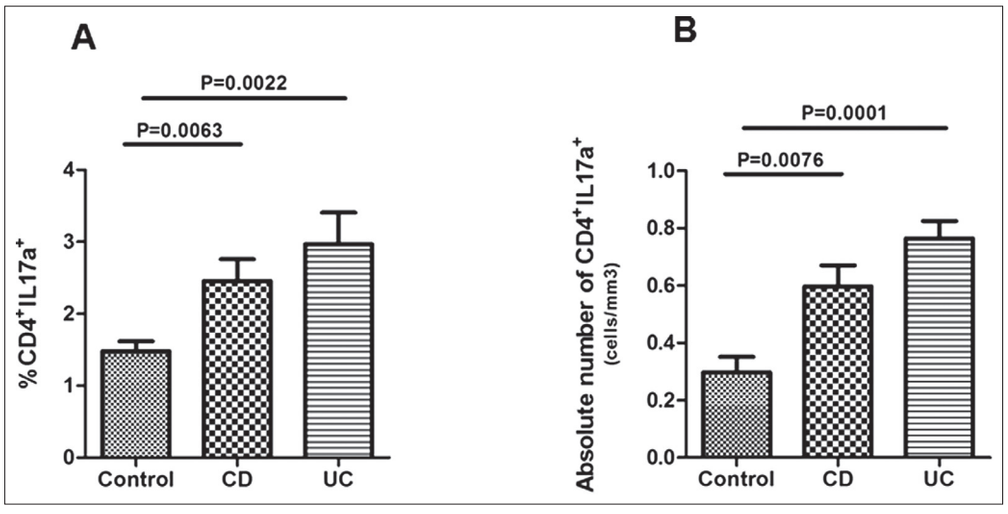

Fig. 2: Comparison between percentage (A) and absolute number (B) of CD4+IL17a+ T cells in peripheral blood of pediatric IBD patients and control group. Fresh, resting PBMCs from pediatric IBD patients and healthy individuals were stained with antibodies against CD4 and IL-17a molecules and analyzed using flow cytometry.

2.3. $CD4^+IL\text{-}17a^+$ Th17 cells in peripheral blood of pediatric IBD patients

Th17 immunity is associated with inflammatory and autoimmune diseases and plays an important role in the pathogenesis of IBD. Therefore, this cell subset in peripheral blood of pediatric IBD patients and healthy individuals were analyzed. When comparing $CD4^+IL\text{-}17a^+$ cell numbers as well as the expression of IL-17a among $CD4^+IL\text{-}17a^+$ cells between IBD and healthy group, we found that IBD patients had a higher frequency as well as the absolute number of $CD4^+IL\text{-}17a^+$ Th17 cells than their healthy counterparts. In the pediatric CD patients, a 1.7-fold increase in $CD4^+IL\text{-}17a^+$ Th17 cells was observed (P = 0.0063) compared to controls, while a 1.9-fold increase in $CD4^+IL\text{-}17a^+$ Th17 cells was observed in UC patients (P =0.0022). Interestingly, the absolute number of $CD4^+IL\text{-}17a^+$ regulatory T cells showed the same trend. The pediatric CD and UC patients also showed a significant increase compared to the control group (P=0.0076, P=0.0001 respectively).

2.4. Serum IL-6 and IL-23 level are increased in the pediatric IBD patients

The cytokine distribution in blood serum is presented in Fig. 3, the levels of IL-6 and IL-23 were significantly increased in the IBD group compared to the control group. It was found that IL-6 was increased in both CD (P = 0.0036) and UC (P= 0.0014), and IL-23 was expressed at significantly higher levels in the serum of CD (P = 0.0293) and UC (P = 0.0443) patients. But there was no significant difference in the levels of IL-6 and IL-23 expressed in the serum of CD patients and UC patients.

2.5. Expression of IL-17a, Foxp3, IL-6 and IL-23 is increased in the intestinal mucosa of pediatric IBD patients

As mentioned, IL-6 and IL-23 are cytokines that have impact on Tregs as well as Th17 cells. In addition, the elevated levels of those cytokines were observed by other authors in IBD individuals. To further explore the diversification of Th17 with cytokines,

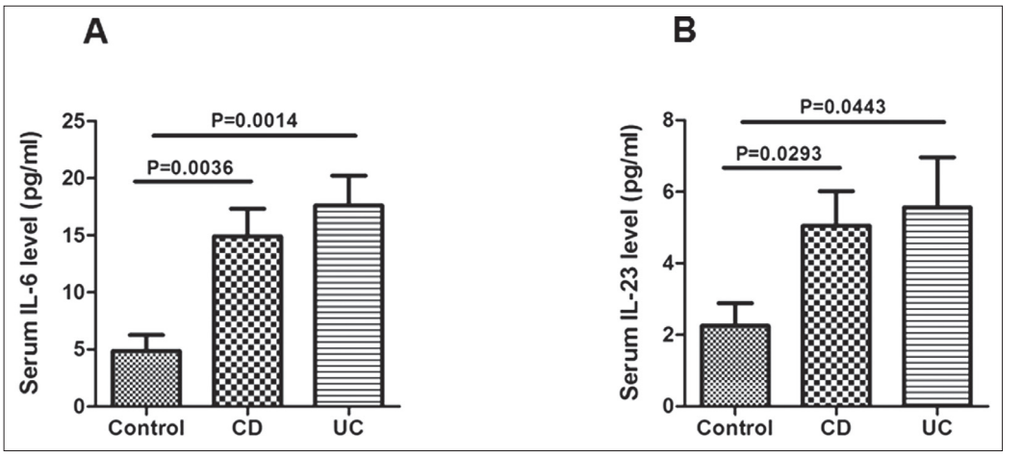

Fig. 3: Bar graphs show the serum levels (pg/ml) of IL-6 (A) and IL-23 (B).

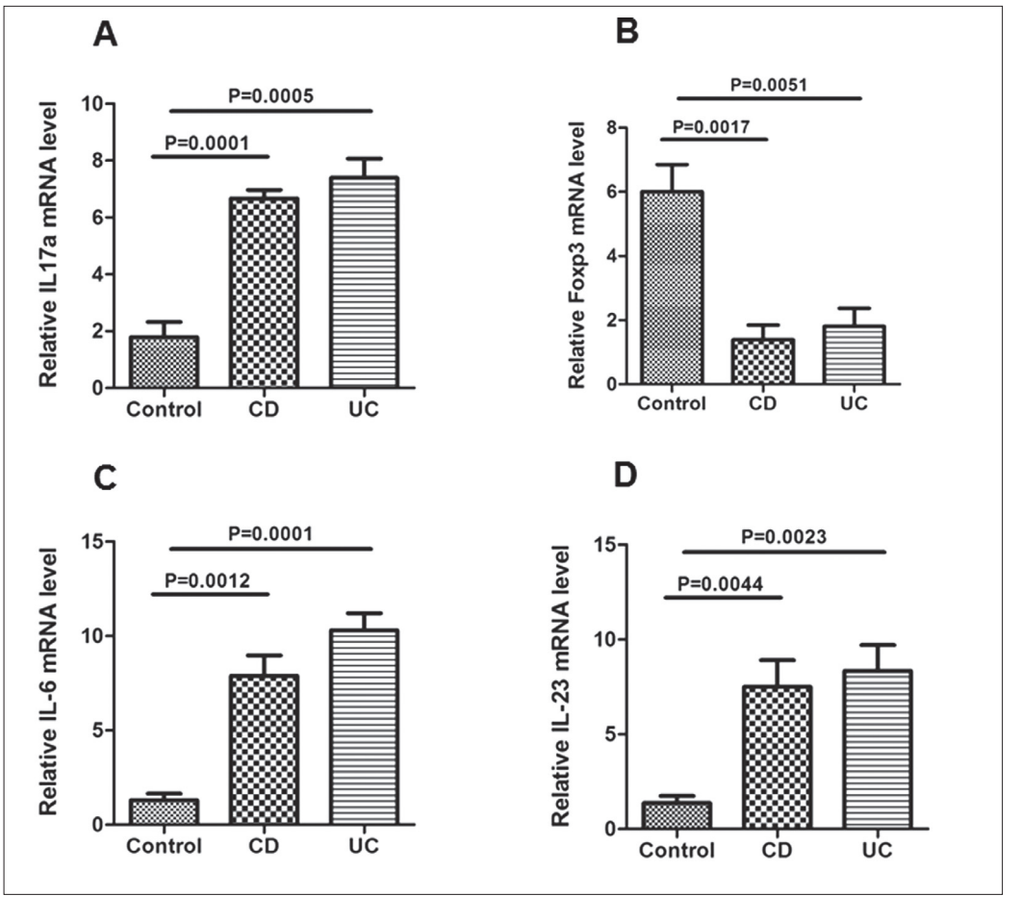

Fig. 4: RNA was extracted from intestinal biopsies with IL-17a (A), Foxp 3 (B), IL-6 (C), and IL-23 (D) were determined by real-time RT-PCR and normalized to GAPDH expression.

we determined the mRNA expression of IL-17a, Foxp3, IL-6 and IL-23 in the intestinal mucosa of pediatric IBD patients and healthy controls by real time RT-PCR. It was found that IL-17a was increased in both pediatric CD (P = 0.0001) and UC (P= 0.0005), but Foxp3 was expressed at significantly lower levels in the mucosa of CD (P = 0.0017) and UC (P = 0.0051) patients. Moreover, IL-6 and IL-23 were expressed at high levels in the intestinal mucosa of both pediatric CD and UC patients compared to the controls.

3. Discussion

In this study, we have shown that the dysregulated balance of Th17 and Tregs in pediatric patients with IBD may partly depend on impaired IL-6 and IL-23 signalization. As the surrogate markers of Treg and Th17, we demonstrated the expression of Foxp3 and IL-17a in the intestinal mucosa. We found the higher levels of this cytokine in serum of pediatric IBD patients in comparison to healthy controls. IL-6 and IL-23 were found to be associated with

a lower frequency of $CD4^+Foxp3^+$ Tregs as well as lower intensity of Foxp3 expression in these cells. On the other hand, the levels of IL-6 and IL-23 were in positive relation with IL-17a produced by Th17 cells, indicating that both IL-6 and IL-23 participate in the development and perpetuation of IBD through regulating the balance of Treg and Th17 cells.

Recently, there has been reported that IL-6 exerts its signaling effect involving in binding to IL-6R receptor on different cell types (Azevedo et al. 2011). Meanwhile, IL-6 transsignaling via soluble IL-6 receptor blocks the expression of Foxp3 which correlates with loss of Tregs suppressive function (Eikawa et al. 2010). In addition, the recent studies on mice as well as on patients with rheumatoid arthritis showed that blocking the IL-6 receptor with a monoclonal antibody resulted in decrease in the percentage of Th17 cells and an increase in the percentage of Treg cells. As another pivotal cytokine in the progress of IBD, IL-23 has also been confirmed to play a very important role in the balance of Treg and Th17 cells (Feng et al. 2011). IL-23 is a member of the IL-12 cytokine family and it is mainly secreted by activated macrophages and dendritic cells (DCs). Blocking IL-23, which is required for Th17 expansion and maintenance, has also been attempted as a potential therapeutic strategy for autoimmune diseases (Segal 2009). The levels of IL-17 and IL-23 are elevated in the serum and intestinal mucosa of patients with IBD, and these levels positively correlate with the disease severity. There are other pieces of support for the strategic role of IL-23 in several autoimmune diseases including IBD. For example, naïve T cells do not express the receptor for IL-23 (IL-23R), but this receptor is expressed on activated Th17 cells. Furthermore, IL-23 is required for the amplification and stabilization of Th17 cells. Sustained IL-23 signaling in T cells is of importance for maintaining ongoing inflammation (Astry et al. 2015). Differentiated Th17 cells are maintained and expanded primarily by IL-23. Subsequent studies showed that IL-23 promotes the expansion of the novel Th17 population characterized by the production of IL-17a and other related proinflammatory cytokines.

In summary, our data demonstrate the increased frequency of $CD4^+IL17a^+$ cells and its association with essential T cell subsets in paediatric patients with IBD as well as a decreased frequency of $CD4^+Foxp3^+$ cells, suggesting that IBD is characterised by an imbalance between Th17 effector cells and Treg cells, with elevated Th17 cells and a cytokine microenvironment exists that promotes Th17 development. In addition to Treg and Th17 cells, the frequency of IL-6 and IL-23 is also aberrant in pediatric patients with IBD. CD and UC children patients show relative higher levels compared to the healthy controls. Therefore, therapeutic approaches that aim to re-establish the balance of Treg and Th17 cells by regulating the expression of IL-6 and IL-23 may prove effective in the treatment of IBD. Future studies are needed to show if blockade of IL-6 and IL-23 signaling has a beneficial effect on Treg subsets in pediatric IBD patients.

4. Experimental

4.1. Subjects

Patients for this study were recruited over a period of 18 months (08/2008 to 02/2010) from Wuxi People's Hospital of Nanjing Medical University. Diagnosis of CD and UC was based on standard criteria using clinical, radiological, endoscopic, and histopathological findings in accordance with Porto criteria. Control subjects had non-inflammatory disorders or were undergoing colon cancer screening. Colon biopsies and blood samples were obtained from children with IBD and healthy controls following informed consent during colonoscopy performed for clinical care.

4.2. Sample collection

Blood samples were immediately placed on ice, clarified by centrifugation at 3000 ×g for 5 minutes at 4 °C, and kept frozen at −80 °C until assayed.

4.3. Cell isolation and culture

Venous blood samples (4–6 mL) were collected aseptically into the tubes and used to isolate peripheral blood mononuclear cells (PBMC). PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Marlow, UK). For Th17 analysis cells were suspended at a density of 2×10^6 cells/mL and cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS). Cultures were stimulated for 5 h using 50 ng/mL of phorbol myristate acetate PMA (Sigma, USA) and ionomycin (1μL/mL) (Sigma, USA) in the presence of 5 μL/mL of brefeldin A (BioLegend, USA) in 37 °C with 5% CO_2. For Treg analysis, 1×10^6 PBMCs were suspended and centrifuged at 200 × g for 5 minutes. Cell pellets were then destined for flow cytometric staining.

4.4. Flow cytometric staining and analysis

After washed with cell staining buffer, cells were stained with anti-CD4 antibody (BD, Sydney, NSW, Australia). And then the cells were washed and stained for intracellular expression of Foxp3 in case of Treg and IL-17a in case of Th17 cells after 20 min incubation at room temperature. The anti-Foxp3 and anti-IL-17a monoclonal antibodies were used for Treg and Th17 intracellular staining according to the manufacturers' suggestions (BioLegend, USA). Expression of cell surface and intracellular markers was assessed using flow cytometry (LSRII, Becton Dickinson, USA) after gating on live lymphocytes according to forward and side scatter. It was quantified as a ratio of mean fluorescence intensity for Foxp3 or IL-17a to mean fluorescence intensity (MFI) for appropriate isotype control. Data were analyzed by Flowjo software version 7.6.1.

4.5. Determination of IL-6, IL-23 levels

Serum levels of IL-6, IL-23 were measured by ELISA method (R&D Systems Inc, USA) for each sample according to the manufacturer's protocol.

4.6. Real-time PCR analysis for Foxp3 and IL-17a expression

Total RNA was isolated from intestinal biopsies using the Trizol reagent (Invitrogen, USA). One microgram of RNA was reverse transcribed to obtain complimentary DNA (cDNA) using Qiagen Quantitect Reverse transcription kit (Qiagen, Hilden, Germany). Specific PCR primer pairs for the target genes were:
GAPDH forward: 5'-GGTGGTCTCCTCTGACTTCAACA-3'
GAPDH reverse: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'
Foxp3 forward: 5'-GTGGCATCATCCGACAAGG-3'
Foxp3 reverse: 5'-TGTGGAGGAACTCTGGGAAT-3'
IL-17a forward: 5'-CAATCCCACGAAAT CCAGGATG-3'
IL-17a reverse: 5'-GGTGGAGATTCCAAGGTGAGG-3'
IL-6 forward: 5'- AAATTCGGTACATCCTCGACGG -3'
IL-6 reverse: 5'- GGAAGGTTCAGGTTGTTTTCTGC -3'
IL-23 forward: 5'-GGACAACAGTCAGTTCTGCTT-3'
IL-23 reverse: 5'-CACAGGGCTATCAGGGAGC-3'
PCR conditions for gene amplification began with a 10 min 95 °C enzyme activation step, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Expression of Foxp3, IL-17a, IL-6 and IL-23 mRNA was normalised to GAPDH expression.

4.7. Statistical analysis

All data were analyzed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The differences between the groups were calculated with the nonparametric *U* Mann Whitney tests. Spearman's correlations were used to compare cell frequencies with analyzed parameters. For all tests, two sided P value < 0.05 was considered statistically significant.

Conflicts of interest: None declared.

References

Azevedo A, Cunha V, Teixeira AL, Medeiros R (2011) IL-6/IL-6R as a potential key signaling pathway in prostate cancer development. World J Clin Oncol 2: 384-396.

Astry B, Venkatesha SH, Moudgil KD (2015) Involvement of the IL-23/IL-17 axis and the Th17/Treg balance in the pathogenesis and control of autoimmune arthritis. Cytokine 74: 54-61.

Bobby R, Robustelli P, Kralicek AV, Mobli M, King GF, Grötzinger J, Dingley AJ (2014) Functional implications of large backbone amplitude motions of the glycoprotein 130-binding epitope of interleukin-6. FEBS J 281: 2471-2483.

Barbosa Vendramini-Costa D, Francescone R, Posocco D, Hou V, Dmitrieva O, Hensley H, de Carvalho JE, Pilli RA, Grivennikov S (2016) Anti-inflammatory natural product goniothalamin reduces colitis-associated and sporadic colorectal tumorigenesis. Carcinogenesis pii: bgw112.

Eikawa S, Ohue Y, Kitaoka K, Aji T, Uenaka A, Oka M, Nakayama E (2010) Enrichment of Foxp3+ CD4 regulatory T cells in migrated T cells to IL-6- and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6. J Immunol 185: 6734-6740.

Feng T, Qin H, Wang L, Benveniste EN, Elson CO, Cong Y (2011) Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. J Immunol 186: 6313-6318.

Kocsis AK, Lakatos PL, Somogyvári F, Fuszek P, Papp J, Fischer S, Szamosi T, Lakatos L, Kovacs A, Hofner P, Mándi Y (2008) Association of beta-defensin 1 single nucleotide polymorphisms with Crohn's disease. Scand J Gastroenterol 43:299-307.

Moutsopoulos NM, Kling HM, Angelov N, Jin W, Palmer RJ, Nares S, Osorio M, Wahl SM (2012) Porphyromonas gingivalis promotes Th17 inducing pathways in chronic periodontitis. J Autoimmun 39: 294-303.

Peng LL, Wang Y, Zhu FL, Xu WD, Ji XL, Ni J (2016) IL-23R mutation is associated with ulcerative colitis: A systemic review and meta-analysis. Oncotarget doi: 10.18632/oncotarget.13607.

Segal BM (2009) Getting to the crux of the matter: IL-23 and Th17 cell accumulation in the CNS. Eur J Immunol 39: 1713-1715.
Tanaka T, Narazaki M, Kishimoto T (2014) IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 6: a016295.
Wines BD, Yap ML, Powell MS, Tan PS, Ko KK, Orlowski E, Hogarth PM (2016) Distinctive expression of interleukin-23 receptor subunits on human Th17 and γδ T cells. Immunol Cell Biol doi: 10.1038/icb.2016.93.
Yu Q, Mao R, Lian L, Ng SC, Zhang S, Chen Z, Zhang Y, Qiu Y, Chen B, He Y, Zeng Z, Ben-Horin S, Song X, Chen M (2016) Surgical management of inflammatory bowel disease in China: a systematic review of two decades. Intest Res 14: 322-332.
Zhang JG, Chen XJ, Liu T, Jiang SJ (2016) FOXP3+ associated with the pro-inflammatory regulatory T and T helper 17 effector cells in asthma patients. Exp Ther Med 12: 2753-2758.