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Increased Expression of TLR4 and TLR7 but Not Prolactin mRNA by Peripheral Blood Monocytes in Active Celiac Disease*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. Celiac disease (CD) is an organ-specific autoimmune disease, and both adaptive and innate immunity are involved in its development.

Objectives. The aim of the study was to determine whether the markers of intestinal mucosal inflammation in CD can be detected in peripheral blood monocytes (PBMs), and whether the immune properties of PBMs change as the clinical signs and symptoms of CD improve after the introduction of a gluten-free diet (GFD). The focus was on changes in mRNA expression of selected toll-like receptors (TLR2, TLR4, TLR7), stress cytokine prolactin (PRL), and pro- and anti-inflammatory cytokines (TNF- α , IL-6, IL-12, IL-10) in PBMs.

Material and Methods. The study involved 20 CD patients diagnosed according to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition criteria and Marsh criteria: 10 recently-diagnosed cases (rCD) and 10 on a GFD for a minimum of one year. The control group comprised 10 age- and sex-matched healthy volunteers. PBMs from peripheral blood specimens were separated using immunomagnetic CD14+ beads. Total RNA was isolated using a standard commercial kit. Cytokine and TLR mRNA levels were quantified by relative qPCR with *PGK1* as a reference gene.

Results. Significantly higher expression of TLR4 and TLR7 mRNA was observed in PBMs from rCD patients compared to the healthy controls (1.63 times higher; $p < 0.05$). TLR7 mRNA levels in rCDs were also significantly elevated in comparison to the CD-GFD patients (2.11 times higher; $p < 0.01$). TNF- α mRNA expression tended to be higher in both groups of patients; by contrast, in IL-6 mRNA, a trend to a fourfold decrease was detected in PBMs from the CD-GFD subjects. IL-10, IL-12 and PRL levels did not differ among the groups.

Conclusions. The data suggest that the inflammatory process in rCD intestinal mucosa and submucosa reflecting enterocyte damage can be detected in PBMs in peripheral blood. Further, the cytokine and TLR expression profile in PBMs alters after one year of GFD treatment (*Adv Clin Exp Med* 2016, 25, 5, 887–893).

Key words: celiac disease, monocytes, cytokines, prolactin, innate immunity.

Celiac disease (CD) is defined as the loss of oral tolerance to gluten in genetically predisposed individuals; it is an organ-specific autoimmune disease with a prevalence of about 1% in Europe [1]. Clinical manifestation of the gluten-induced inflammatory response

in celiac mucosa is highly variable and can occur both in childhood and in adulthood. The only possible treatment of CD is a lifelong gluten-free diet (GFD).

Imbalance in the innate immune mechanisms is known to contribute to the pathogenesis of CD.

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Innate immunity can be activated by lipopolysaccharides and peptidoglycans derived from rod-shaped bacteria detected in the intestinal biopsy of CD patients but not healthy subjects [2]. These bacterial products are recognized by monocytes carrying specific toll-like receptors (TLRs) and CD14 receptors [3]. Similarly, viral infection, especially gastrointestinal viral infection, may serve as a trigger of celiac disease [4]. TLR7 and TLR8 recognize viral ssRNA, and proper control of TLR7 expression has been proved to be necessary to limit autoimmunity [5]. Increased TLR2, TLR3 and TLR4 expression has been detected in duodenal biopsies from child CD patients when compared to non-celiac tissues [6]. Activation of TLR2, TLR4 and TLR7 leads, among other things, to expression of pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-12 [3].

Besides pattern recognition receptors (PRRs) such as TLRs, peripheral blood monocytes (PBMs) produce immunomodulatory cytokines, including prolactin (PRL). Prolactin increases the progression of the immune response in autoimmune diseases [7] and stimulates production of cytokines and chemokines, such as TNF- α , IFN- γ , IL-2, IL-10 and IL-12 [8, 9]. Serum hyperprolactinemia has been observed in a large number of autoimmune diseases, including CD [10], whereas a reduction in PRL levels detected both in serum from children with multiorgan failure [11] and in PBMs derived from septic hemato-oncological patients [12] correlated with failure of the immune response.

The aim of the present study was to determine whether the local inflammatory processes in the intestines of active CD cases can be detected in PBMs in the peripheral blood. Therefore, the expression levels of pro-inflammatory cytokines TNF- α , IL-6 and IL-12, pattern recognition receptors TLR2, TLR4, TLR7, immunostimulatory cytokine prolactin and the mRNA level of anti-inflammatory cytokine IL-10 were measured in CD patients and healthy subjects. A further aim was to investigate whether the immune properties of PBMs change as the clinical signs and symptoms of CD improve after the introduction of a GFD.

Material and Methods

The Subjects

The study involved 20 female CD patients (mean age 30.40 ± 1.37 years) from the Kralovske Vinohrady Faculty Hospital in Prague, the Czech Republic ($n = 10$), and from the General University Hospital in Prague, the Czech Republic ($n = 10$), diagnosed according to the criteria of the European Society for Pediatric Gastroenterology,

Hepatology and Nutrition [13] and the Marsh criteria [14]. Among these patients, 10 were recently-diagnosed cases with active disease (rCD; mean age 31.3 ± 2.38 years), and 10 patients (mean age 29.5 ± 1.45 years) had been on a gluten-free diet for a minimum of one year (CD-GFD).

The control group comprised 10 age- and sex-matched healthy volunteers (mean age 29.5 ± 1.55 years) who met the following criteria: no known autoimmune disorder, and a minimum of two weeks after complete clinical recovery from any infectious disease.

From each healthy subject and CD patient, 8 mL of citrate-treated peripheral blood samples were collected, along with her past history and informed consent. The samples were processed within 2 h of collection.

Monocyte Isolation and RNA Extraction

Peripheral blood monocytes were isolated using Dynabeads[®] CD14 positive immunomagnetic separation (Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, USA). Total RNA was isolated from the separated cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Prague, the Czech Republic).

Quantitative Real-time PCR

The extracted RNA was reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA). TNF- α , IL-6, IL-10, IL-12A, TLR2, TLR4, TLR7 and PRL mRNA were quantified using the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) and TaqMan-based gene expression Assays on Demand (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Waltham, USA). The human phosphoglycerate kinase-1 (*PGK1*) gene served as a reference gene. The $2^{-\Delta C_t}$ method was used for relative quantification, and the detected mRNA values were reported in arbitrary units (AU). In order to present data with a better resolution, values in the PRL and IL-6 assays were multiplied by 10^6 , and values in all the other assays were multiplied by 10^3 .

Statistical Analysis

GraphPad Prism 5.00.288 software (GraphPad Software Inc., La Jolla, USA) was used for the statistical analysis. To test for normality the D'Agostino-

Pearson normality test was performed. The data in the text are presented as medians with 25% and 75% percentiles; in charts, the data are presented as dot plots with a median and interquartile range. Data involving two groups were compared using the Mann-Whitney rank-sum test; data involving three groups were compared using the Kruskal-Wallis test. A *p*-value lower than 0.05 was regarded as statistically significant. The study was not powered for the multitude of statistical tests performed; therefore, the results should be regarded as exploratory ones.

Results

No change in TLR2 mRNA expression between the active CD cases and the healthy controls was detected; only a trend towards an increase in TLR2 mRNA expression in the rCD patients in comparison with the CD-GFD patients was observed (rCD: median 272.5 AU, 239.1–304.7; CD-GFD: median 157.3 AU, 99.11–331.7; healthy controls: median 237.9 AU, 197.1–303.2; *p* = ns.). As for TLR4, monocytes from the rCD patients expressed 1.63 times higher mRNA levels than PBMs from the healthy controls (rCD: median 130 AU, 100.2–204.9; healthy controls: median 79.96 AU, 68.12–117.1, *p* < 0.05), but did not differ in TLR4 mRNA expression from the CD-GFD monocytes (median 108.5 AU, 81.95–123.5, *p* = ns.; Fig. 1A). Similarly, expression of TLR7 mRNA in PBMs from the rCD patients was higher than TLR7 mRNA expression from both the CD-GFD subjects (2.11 times higher, *p* < 0.01) and the healthy

controls (1.63 times higher, *p* < 0.05; rCD: median 16.72 AU, 13.83–33.91; CD-GFD: median 7.94 AU, 6.07–12.48; healthy controls: median 10.24 AU, 5.26–16.61; Fig. 1B).

TNF- α expression did not differ significantly between the active CD patients, the CD patients on a GFD and the healthy controls; only a trend was detected towards increased levels in both the diseased groups as compared to the healthy control group (rCD: median 10.55 AU, 2.64–16.32; CD-GFD: median 11.06 AU, 1.94–38.15; healthy controls: median 6.99 AU, 3.36–13.46; *p* = ns.) (Fig. 2A).

There was a statistically insignificant tendency toward approximately four times lower IL-6 mRNA expression in monocytes from the CD-GFD patients in comparison to the other two groups, whereas the IL-6 mRNA levels in the active CD patients were similar to those in the healthy controls (rCD: median 130.2 AU, 7.54–344.7; CD-GFD: median 28.53 AU, 0.20–381.2; healthy controls: median 109.3 AU, 0.10–437.6; *p* = ns.; Fig. 2B).

The expression of the other cytokines tested, IL-12A, IL-10 and PRL, did not show any differences in mRNA level between ill and healthy individuals (IL-12A: rCD: median 1.37 AU, 0.23–2.24; CD-GFD: median 2.06 AU, 0.77–2.79; healthy controls: median 1.92 AU, 0.57–2.42; *p* = ns.; IL-10: rCD: median 0.73 AU, 0.53–2.23; CD-GFD: median 0.73 AU, 0.35–1.80; healthy controls: median 0.96 AU, 0.53–1.31; *p* = ns.; PRL: rCD: median 211.8 AU, 161–792.4; CD-GFD: median 243.7 AU, 0.57–581.2; healthy controls: median 176.9 AU, 67.33–358.6; *p* = ns.).

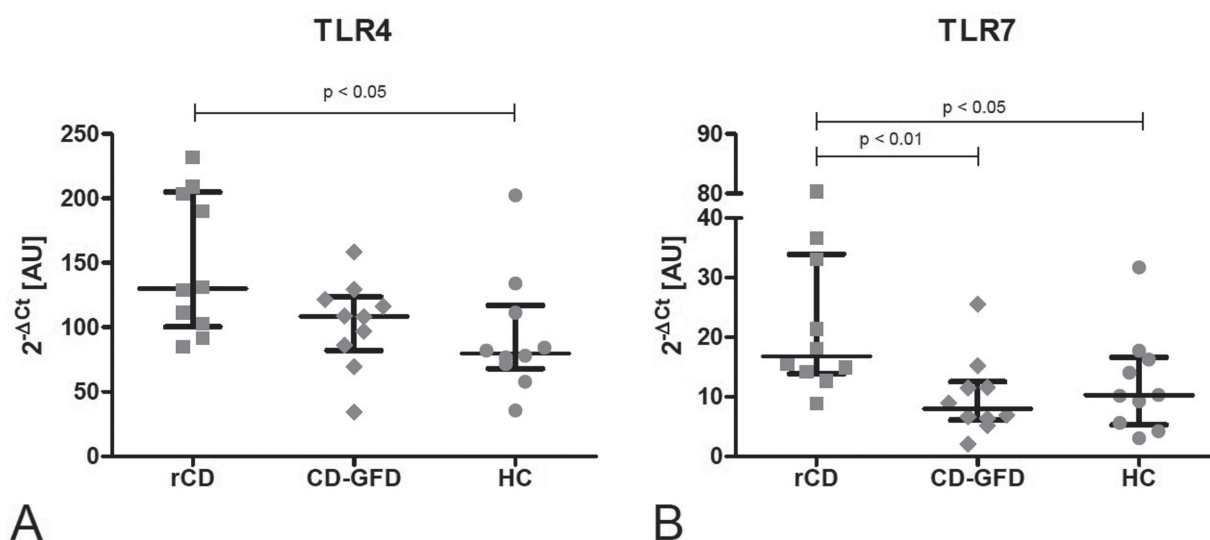


Fig. 1. A comparison of TLR4 (1A) and TLR7 (1B) mRNA levels in all the study groups

The horizontal bar in the middle represents the group median and interquartile range. HC – healthy controls; rCD – recently-diagnosed celiac disease patients; CD-GFD – celiac disease patients on a gluten-free diet for more than one year; AU – arbitrary units multiplied by 10^3 .

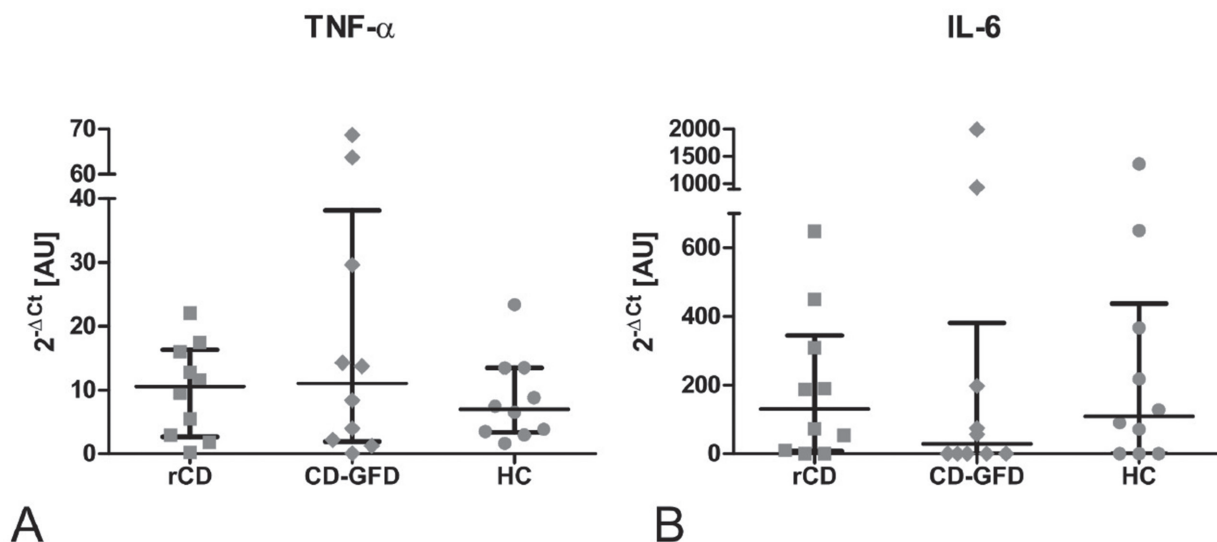


Fig. 2. A comparison of TNF- α (2A) and IL-6 (2B) mRNA levels in all the study groups

The horizontal bar in the middle represents the group median and interquartile range. HC – healthy controls; rCD – recently-diagnosed celiac disease patients; CD-GFD – celiac disease patients on a gluten-free diet for more than one year; AU – arbitrary units multiplied by 10^3 or by 10^6 .

A potential relationship was verified between the molecular markers and cytokines studied and the clinical markers of the disease. Nevertheless, there was neither any relationship nor any correlation with the following clinical and immunological data: histology grade (Marsh), anti-endomysium IgA, anti-endomysium IgG, anti-transglutaminase IgA or total IgA.

Discussion

This study examined peripheral blood, specifically monocytes, for signs of inflammatory processes taking place in the small intestine of patients suffering from active celiac disease. A further aim was to find potential markers distinguishing treated and untreated CD patients, and to determine if the treated form of CD differs in terms of PBM cytokines and PRR mRNA profile from the physiological expression in healthy individuals. Slight but significant differences in TLR4 mRNA levels were found between rCD patients and healthy subjects; and TLR7 mRNA levels were increased in rCD patients when compared with both healthy controls and with patients on a GFD (Fig. 1A, B). A similar trend was also detected with TLR2, with the highest mRNA expression in monocytes from untreated CD patients and the lowest in monocytes from CD patients on a GFD.

To date, the search for TLR2 and TLR4 expression in the small intestinal mucosa of celiac disease patients has resulted in inconsistent findings with respect to the type of change as well as disease sta-

tus [6, 15, 16]. In peripheral blood, however, relevant data are missing. A Hungarian study examining the phenotype of children with CD and how it alters upon dietary intervention indicates how important it may be to focus on peripheral and local markers that reflect events taking place at the site of inflammation [17]. Those authors found a higher prevalence of TLR2- and TLR4-expressing PBMs in newly diagnosed children when compared to controls; further, in treated patients, an increased percentage of TLR2- but not TLR4-positive monocytes was observed [17]. Regrettably, the study did not measure TLR expression levels in cells *per se*. Gliadin fragments have been reported to activate the innate immune system *via* TLR2 and TLR4 [18]; this activation could be responsible for upregulated TLR expression in wheat-consuming CD subjects. Alternatively, the increased expression of TLR2 and TLR4 in monocytes from rCD patients who have gluten in their diet may be a consequence of bacterial imbalance in the gut in CD patients [2].

In the present study, monocytes from the patients with active CD expressed significantly higher TLR7 mRNA levels than PBMs from the CD-GFD subjects and the healthy controls. The *TLR7* gene localizes within the celiac disease-associated region [19]; the function of TLR7 is to recognize viral ssRNA. There is a monocyte subtype (designated CD14^{dim}) that responds to viruses and nucleic acids *via* the TLR7-TLR8-MyD88-MEK pathway [20] and seems to be involved in viral infection, one of the risk factors for developing celiac disease [4]. In active CD, the higher expression

of TLR7 observed in the present study may thus be related to recent viral infection. Eiro et al. reported only a very low percentage of cases with positive immunostaining for TLR7 in biopsy samples from CD patients, and comparable TLR7 mRNA expression levels in duodenal tissues between CD patients and healthy controls [15]. Nonetheless, in contrast to the present study, those authors did not differentiate between newly-diagnosed and treated cases. Based on the TLR data in the present study, it may be possible to detect some changes in the gut epithelial environment in peripheral blood monocytes. This is in agreement with the recent conclusion made by Hoffmanova et al., who reported the possibility of detecting enterocyte damage using markers in the peripheral blood [21].

The mucosa of the small intestine in active CD is an inflamed environment characterized by local immune imbalance, including escalated secretion of pro-inflammatory molecules. Also, activation of TLRs has been reported to lead to the activation of transcription factors, resulting in the expression and release of inflammatory cytokines such as TNF- α , IL-6 and IL-12 [3]. In contrast to that, the present study did not find any statistically significant increase in inflammatory cytokines or PRL mRNA expression in monocytes from recently-diagnosed CD patients. The data from the present study showed just a trend toward increased levels of TNF- α in the rCD and CD-GFD patients when compared to the healthy controls; IL-6 mRNA levels were similar in the rCD patients and the healthy subjects, and surprisingly, they were approximately four times higher than in the CD-GFD subjects. In the available literature, there are no corresponding data from CD PBMs. In *in vitro* studies, elevated levels of TNF- α mRNA in PBMs have been detected after gliadin stimulation [22]. In serum and duodenal mucosa, though, the available data are conflicting [23–25]. Lahat et al. detected higher TNF- α and IL-6 mRNA expression both in intestinal mucosa and peripheral blood of active CD patients in comparison with controls. Notably, those authors proved that some rCD and CD-GFD subjects express these inflammatory cytokines neither in the intestines nor in peripheral blood [26], implying that the immune response to some triggers varies among individuals.

As the primary function of IL-10 is containment of the immune response, abnormal levels of IL-10 can result in atypical innate immune response, autoimmune disorders, the development of cancer and allergic reactions [27]. Although some studies have reported elevated IL-10 levels in sera from patients with active celiac disease but not from those on a GFD [25], and in biopsies from recently-diagnosed CD patients with villous

atrophy and elevated lymphocyte infiltration [28], no significant changes in monocyte expression of IL-10 mRNA were observed in the present study. Perhaps it was as a consequence of this that the IL-12A mRNA expression detected in monocytes was comparable among all the tested groups, while in another autoimmune disease, systemic lupus, overexpression of IL-10 reportedly decreases production of IL-12 [29].

Several studies monitoring levels of pituitary prolactin in blood serum pointed out the still not fully explored role of PRL in autoimmune inflammation in the background of intestinal damage that is characteristic of celiac disease. Significantly higher serum PRL levels in CD patients than in controls, with a reduction after six months of a GFD [30], and serum hyperprolactinemia in patients with active disease but not in treated CD subjects [24, 31] point to the direct involvement of PRL in the pathogenesis of CD. This is supported by the fact that it has been shown that elevated serum PRL levels in CD patients correlate with disease activity indicated by the presence of malnutrition and the degree of histological damage of the intestinal mucosa [10]. PBMs circulate in blood vessels and migrate to the site of inflammation in the duodenal mucosa [32]; their signaling affects the organism both at the local and the systemic levels. PBMs are known to express PRL and its receptor; the question, however, was whether monocyte-derived PRL plays any role in the pathogenesis of CD. In immune cells, levels of PRL are in general very low [33]; on the other hand, even a minor increase may have a significant biological effect on the local environment. Nevertheless, the difference in PRL mRNA expression in PBMs from rCD patients did not reach statistical significance when compared to CD-GFD patients or healthy individuals. As expected, PBMs from healthy subjects expressed the lowest PRL mRNA levels; interestingly, a trend toward the highest levels of PRL mRNA expression could be observed in PBMs derived from treated CD patients, whose PRL mRNA levels tended to be 1.4 times higher than those detected in the healthy controls. The lack of a significant distinction in *PRL* gene expression among recent CD subjects, CD patients on a GFD and healthy controls may indicate that monocyte-derived PRL does not contribute to the hyperprolactinemia observed in autoimmune disorders, including celiac disease. Moreover, PRL expressed in monocytes does not seem to participate in the autoimmune and/or inflammatory processes in CD.

Despite histological recovery of the intestinal mucosa in celiac disease after a GFD is introduced, complete normalization of the immune milieu has

not been confirmed so far. The data from the present study indicate that full normalization of the innate immune system does not occur within a minimum of one year on a gluten-free diet. Though insignificant, the decrease in TLR2 and IL-6 mRNA expression noted in the patients on a GFD, along with TNF- α mRNA levels equally elevated as in active CD, and TLR4 mRNA levels higher than the physiological level observed in healthy individuals, imply that the immune system does not reach full recuperation after autoimmune inflammatory damage of the duodenal mucosa.

Summarizing, increased levels of TLRs (TLR2, TLR4 and TLR7) are detectable in peripheral blood monocytes from patients with active celiac disease. Based upon the data presented, PBMs do not seem to be a main source of elevated serum levels of the inflammatory cytokines TNF- α , IL-6, IL-12 and PRL. Finally, monocytic PRL probably does not play any role in the pathogenesis or progression of CD. The possible bright future for TLR4 and TLR7 as diagnostic and/or prognostic markers and perhaps good therapeutic targets in CD requires thorough validation of the results presented.

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