Int J Clin Exp Med 2013;6(2):98-104
www.ijcem.com /ISSN:1940-5901/IJCEM1212015

Original Article

Toll-like receptor 4, F4/80 and pro-inflammatory cytokines in intestinal and mesenteric fat tissue of Crohn's disease

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Received December 15, 2012; Accepted January 11, 2013; Epub January 26, 2013; Published February 6, 2013

Abstract: Introduction: Crohn's disease (CD) is a chronic intestinal ailment with a multifactorial etiology, whose incidence has increased during the last three decades. Recently, a role for mesenteric fat has been proposed in CD pathophysiology, since fat hypertrophy is detected nearby the affected intestinal area; however, there are few studies on this aspect. Aim: To evaluate inflammatory activity in intestinal mucosa and mesenteric fat tissue of patients with CD and controls. Materials and Methods: Ten patients with ileocecal CD and 16 patients with non-inflammatory disease (control groups) were studied. The specimens were snap-frozen and the expression of TLR-4, F4/80, IL1-β and IL-6 were determined by immunoblot of protein extracts. TLR4 RNA level were measured using RT-PCR. The t Test was applied ($p<0.05$). The local ethical committee approved the study. Results: The intestinal mucosa of CD group had significantly higher protein levels of TLR-4, F4/80, IL-1β and IL-6 than the controls. The gene expression of TLR4 was lower in the intestinal mucosa of CD compared to the control group. Regard the mesenteric fat tissue, there was no statistical difference related to TLR-4, F4/80, IL-1β and IL-6 proteins expression. Conclusions: These findings may result from an up-regulation of macrophage activation and intracellular pathways activated by bacterial antigens, which are more important in intestinal mucosa than fat tissue in CD patients. This may represent an anomalous regulation of innate immunity and could contribute to the production of proinflammatory mediators and disease development.

Keywords: Crohn's disease, inflammatory bowel disease, innate immunity, cytokines

Introduction

The increasing incidence of inflammatory bowel disease (IBD), particularly Crohn's disease (CD) and ulcerative colitis (UC), has raised queries regarding the pathogenesis and factors potentially involved in the onset of these diseases. CD and UC are multifactorial chronic intestinal diseases, which occur mainly in the second and third decades of life [1].

Macrophages are key cells involved in IBD, since they are part of the innate immune system at the interface with the external environment, and are responsible for the release of various cytokines such as TNF-α and IFN-γ. Once released, these proinflammatory cytokines induce the activation of their respective receptors triggering the activation of an intracellular signaling cascade ultimately leading to the activation of nuclear transcription factors NF-KB and STAT-1, which control the transcription of inflammatory factors [2, 3].

The membrane receptors TLRs (Toll-like receptors) that mediate the recognition of antigens of the intestinal lumen as lipopolysaccharide (LPS), peptideoglycan (PGN) or flagellin (Flag) were also associated with CD due to the activation of NF-KB via MyD88, thereby increasing the production of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and susceptibility to invasion by pathogens in the lamina propria, thus perpetuating the inflammatory process [4,

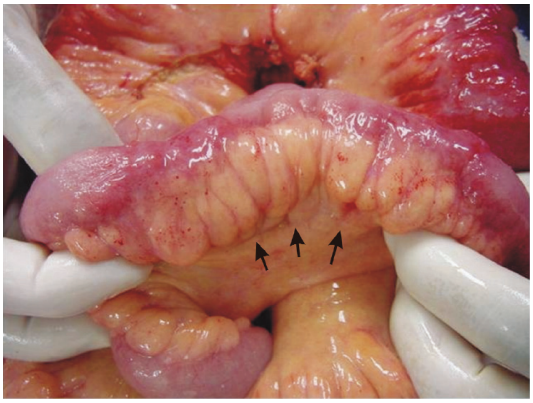

Figure 1. Surgical aspects of creeping fat in ileum with Crohn's disease.

5]. In addition, the intestinal epithelium is able to express various TLRs, especially TLR4, verified in IBD [6, 7].

Although there is phenotypic variation in surgical specimens from CD patients, macroscopic aspects are notorious, especially with regard to thickening of the mesenteric fat next to the affected intestinal area [8-10]. As macrophages and epithelial cells, adipocytes from normal individuals are able to synthesize various proinflammatory and anti-inflammatory cytokines, and fat hormones. Indeed, adipocytes can express TLR4 for the recognition of local or systemic bacterial antigens, and can express CD14 protein that assists in binding of LPS to TLR4 [11, 12].

There have been few studies of mesenteric fat in CD discussing this aspect [13-18]. Therefore, in order to compare the inflammatory activity in fat and intestinal tissue between CD patients and controls we employed assays to determine the expression of proteins related to innate immune system (F4/80, TLR4) and of pro-inflammatory proteins.

Materials and methods

Mucosal biopsies were taken from 10 patients with ileocecal CD [median age 34.9 (range, 14-60) years; male 50%; female 50%]. The biopsies from intestinal mucosa and mesenteric fat tissue of patients with CD were designated as ICD and FCD groups respectively. The presence of disease activity was assessed by colonoscopy before surgery and all patients had Crohn's disease activity index (CDAI) [19] more than 250 points. The controls groups were composed of eight patients who underwent to retosigmoidectomy for non-inflammatory disease (megacolon), with normal distal ileum (ileum mesenteric fat tissue control – FC group) [median age 55.6 (range, 39-70) years; male 62.5%; female 37.5%], and eight patients with normal ileocolonoscopy (intestinal tissue control – IC group) [median age 50.4 (range, 33-60) years; male 37.5%; female 62.5%]. The features of mesenteric fat are shown in **Figure 1**.

The study was performed in accordance with the Declaration of Helsinki, and was approved by the local Ethical Committee. All biopsies were taken after informed consent from the patients. The study was carried out at the State University of Campinas, Colorectal Surgery Unit, and at the Cell Signaling Laboratory of the Department of Internal Medicine.

Biopsies from the mucosa of the terminal ileum and from mesenteric fat tissue near the intestinal affected area were snap-frozen in liquid nitrogen and stored at -80°C until use.

Western blotting analysis

For total protein extract preparation, the fragments were homogenized in solubilization buffer at 4°C [1% Triton X-100, 100mM Tris-HCl (pH 7.4), 100mM sodium pyrophosphate, 100mM sodium fluoride, 10mM EDTA, 10mM sodium orthovanadate, 2.0mM phenylmethylsulfonyl fluoride (PMSF), and 0.1mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 sec. The material was centrifugated (20 min at 110000 rpm at 4°C). The protein concentrations of the supernatants were determined by the Bradford dye binding method [20]. Aliquots of the resulting supernatants containing 50μg total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with BSA (bovine serum albumin), and blotted with anti-F4/80, anti-TLR-4, IL-1β and IL-6 antibodies [21].

Reagents for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Richmond, CA). Phenylmethylsulfonyl fluoride, aprotinin, Triton X-100, Tween 20, glycerol were from Sigma (St. Louis, MO). Nitrocellulose paper (BA85, 0.2μm) was from Amersham (Aylesbury,

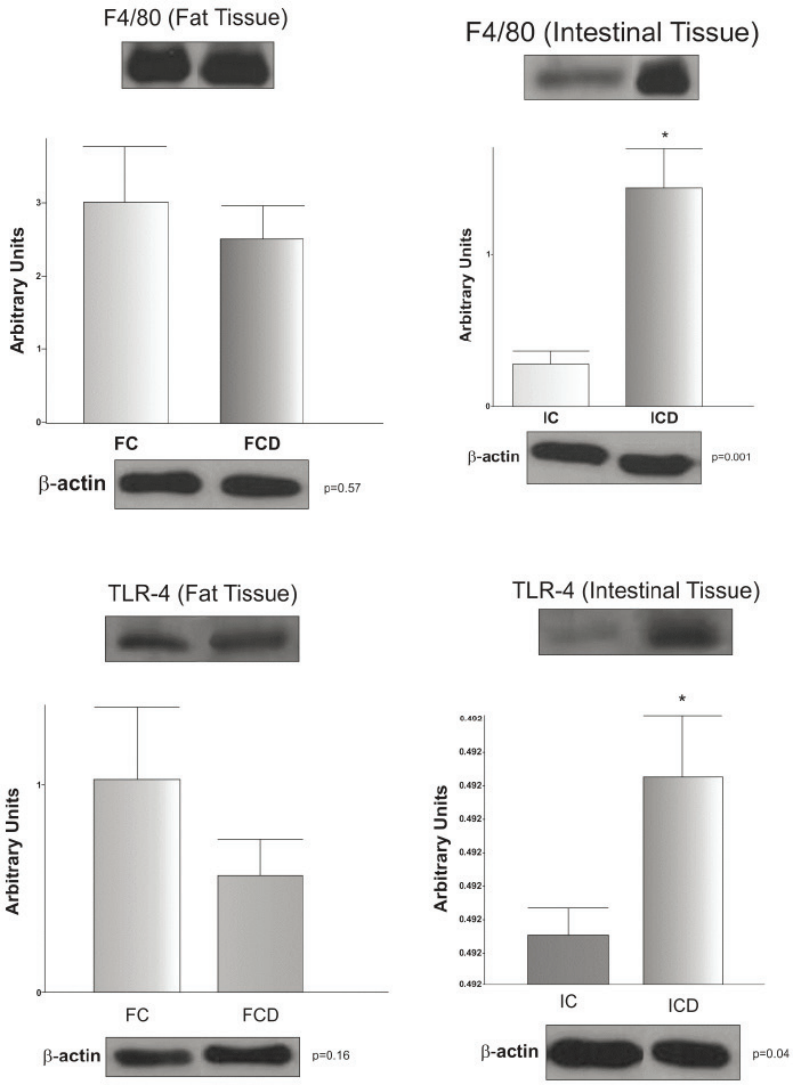

Figure 2. Representative Western blot analyses and determination (mean and standard deviation) of F4/80 and TLR4 proteins expressions in fat tissue of the control (FC) and Crohn's disease (FCD) groups, and in intestinal tissue of the control (IC) and Crohn's disease (ICD) groups. For illustration purpose each line band represents one patient. For all conditions, n=10, *p<0.05 vs Control.

UK). The anti-F4/80 (sc-71085, rat monoclonal), anti-TLR4 (sc-10741, rabbit polyclonal), anti-IL-1β (sc-1252, goat polyclonal) and anti-IL-6 (sc-1266, goat polyclonal) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protein molecular weight was assessed by the PageRulerTM from Fermentas (MD, USA).

The signal was detected by chemiluminescent reaction (SuperSignal®West Pico Chemilumine -scent Substrate from Pierce Biothecnology, Inc. Rockford).

All numerical results are expressed as the mean ± SD of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Gel-Pro Analyzer 6.0 software (Exon-Intron Inc., Farrell, MD). The readings of the bands were standardized according to the beta-actin expression.

RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. RNA purity and concentration were determined by UV spectrophotometry at 260nm. RNA was treated with RNase-free Dnase (RQ1 RNase-free Dnase, Promega), then reverse transcribed using oligo (dT) primers and reverse transcriptase (RevertAid™ Kit, Fermentas). The reaction mixture (20μl) was incubated at 42°C for 60 min, then 10 min at 70°C, and cooled on ice. RT-PCR was performed on resulting cDNA, using the manufacturer's protocol, in a 25μl reaction volume per capillary. Gene-specific primers (Applied Biosystems™) were: Hs00152939 (TLR4); NM_002046.3 (GAPDH). RT-PCR amplification consisted of an initial denaturation step (50°C for 2 min and 95°C for 10 min), 40 cycles of denaturation (95°C for 15s), annealing (53°C for 20s) and extension (72°C for 20s), followed by a final incubation at 60°C for 1 min. All measurements were normalized by the expression of GAPDH gene, considered as a stable housekeeping gene. Gene expression was determined using the delta-delta Ct method: $2^{-\blacktriangle\blacktriangle CT}$ ($\blacktriangle\blacktriangle CT=[Ct(target\ gene) - Ct(GAPDH)]_{patient}$ –

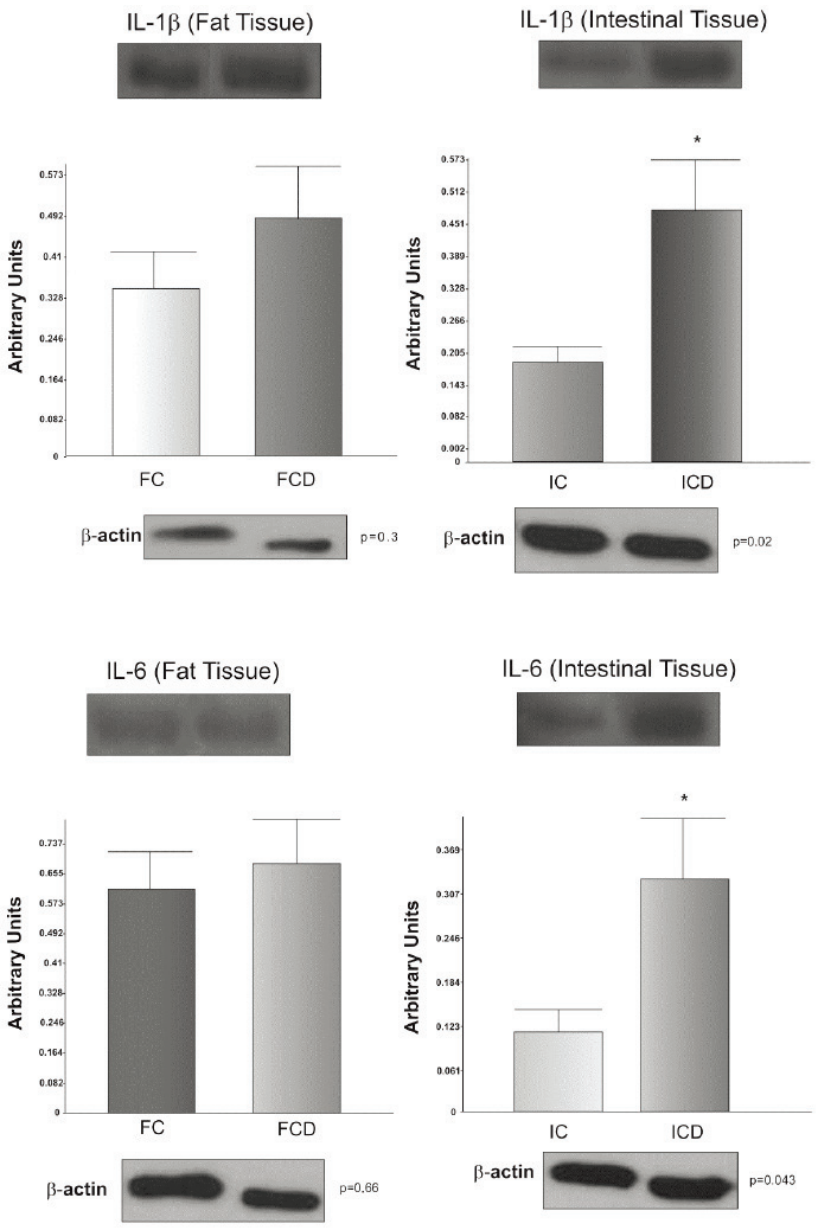

Figure 3. Representative Western blot analyses and determination (mean and standard deviation) of IL-1β and IL-6 proteins expressions in fat tissue of the control (FC) and Crohn's disease (FCD) groups, and in intestinal tissue of the control (IC) and Crohn's disease (ICD) groups. For illustration purpose each line band represents one patient. For all conditions, n=10, *p<0.05 vs Control.

[Ct(target gene) – Ct(GAPDH)]$_{control}$). Real-time data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems).

Statistical analyses

Data were analyzed by t Test, comparing mesenteric fat tissue of CD group (FCD) and its respectively fat control group (FC); and comparing, separately, intestinal tissue of CD group (ICD) and its respectively intestinal control group (IC). The level of significance was set at $p<0.05$.

Results

Patients with CD had significantly higher levels of TLR4 and F4/80 (marker of macrophage activation) in intestinal mucosa (ICD) when compared to intestinal tissue control group (IC) ($p<0.05$). However, the comparison of local levels of these proteins in mesenteric fat tissue of CD (FCD) and controls (FC) revealed that they were similar among the groups ($p>0.05$) (**Figure 2**).

With regard to IL-1β and IL-6 expressions, there were higher levels in ICD group than in the control group (IC) ($p<0.05$). The expressions of the cytokines in mesenteric fat tissue were similar among the groups ($p>0.05$) (**Figure 3**).

TLR4 gene expression was lower in ICD group when compared to control ($p<0.05$), and no statistically significant differences were detected when the mesenteric fat tissue groups were compared ($p>0.05$). **Figure 4** illustrate these findings.

Discussion

The role of mesenteric fat tissue neighboring the intestinal area affected by CD remains unclear. There are few studies evaluating this question by analyzing biopsies of patients with CD and comparing to the samples of mesen-

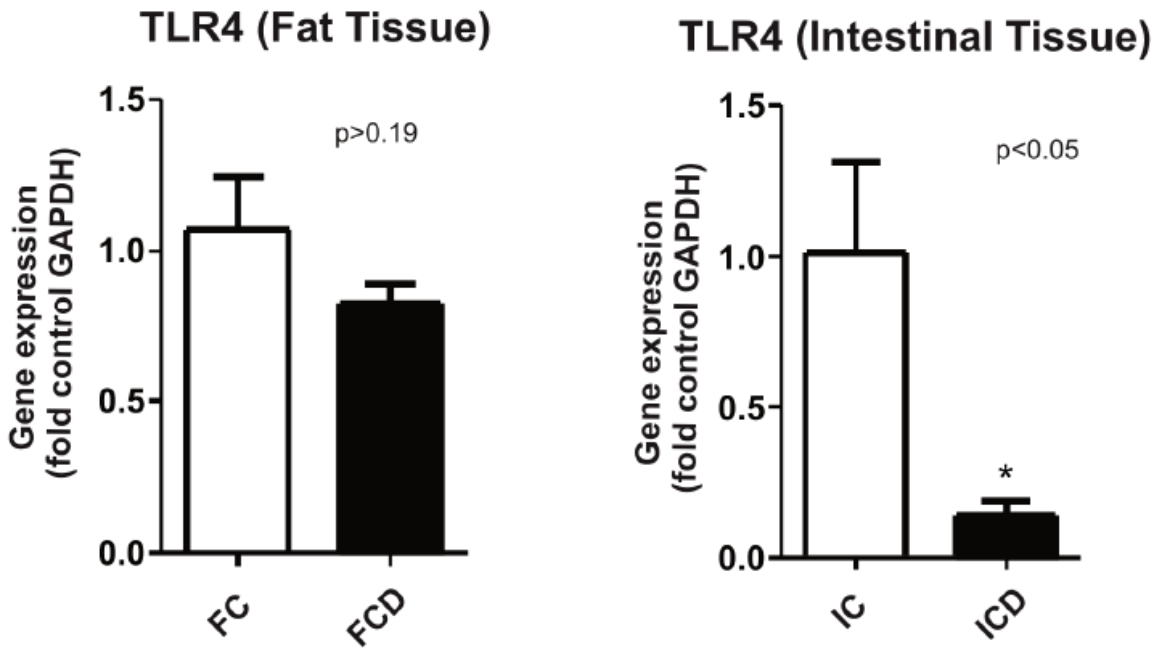

Figure 4. Results of TLR4 gene expression, determined by RT-PCR. For FCD Group, n=10; for FC Group, n=8, *p<0.05 vs control.

teric tissue of controls who were submitted to surgery for other reasons, excluding IBD. Most of these studies measured hormones released by the adipocytes, such as adiponectin and leptin, proinflammatory cytokines and growth factor expressions [22-25].

In this study, we evaluated the expression of proteins related to innate immune system, particularity the macrophage activation (F4/80) [26] and the ability of bacterial antigens recognition (TLR4) [27] to evaluate these important aspects in intestinal and mesenteric fat tissue in patients with active CD, comparing to controls. Indeed, we evaluated proinflammatory cytokines in the same conditions. Even noticed that was fat hypertrophy near to the intestinal affected area by CD during the surgical procedure of all patients involved in this study, there were no difference of the evaluated proteins between mesenteric tissue of CD and the control. However, the protein expressions of F4/80 and TLR4, but not in RNA expression to this receptor, were extremely higher in intestinal mucosa of CD group when compared to the control of terminal ileum.

This fact is interesting showing that there is up-regulating of marker of macrophage activation and in protein translation of TLR4 in mucosa of CD, more importantly than in mesenteric fat tissue. Probably, lamina propria cells in intestinal mucosa play a significant role in the beginning of this process in CD, leading the intestinal barrier more responsive to bacterial antigens, due to up-regulation of membrane receptors, such as TLR4. The activation of macrophages in intestinal tissue of CD evaluated by F4/80 expression, showed a concordance with what is described in previous work, that these cells are derived from plasma circulating monocytes, which differentiate in macrophage in the intestinal lamina propria, when it remains [28, 29]. Conversely, the most important cell derived of monocytes in the mesenteric fat tissue is the dendritic cells, and macrophages are not seen in this tissue [30]. This could explain the similar expression of F4/80 in the mesenteric tissue groups. This difference between transcription and translation found in TLR4 is not very unusual. One explanation is higher protein stability by protected ubiquitination becoming more stable and not being degraded. Furthermore, the pro-inflammatory cytokines expressions were detected in mesenteric fat tissue, but were similar to the controls, showing that the most important inflammatory process occurs in intestinal mucosa.

Because disease whose etiology is not fully elucidated, CD has been investigated in terms of molecular and genetic, in order to improve knowledge of the inflammatory pathways involved, and the mechanism of recognition of antigens in the intestinal lumen.

The present study shows that, even under inflammatory conditions, the mesenteric fat tissue of CD patients presented similar expressions of TLR4, F4/80 and pro-inflammatory cytokines, when compared with controls. It suggests that primary defects of macrophage regulation and response to bacterial antigens may occur in intestinal mucosa rather than in the adjacent fat tissue. The mesenteric fat tissue could play an important role in the maintenance of local inflammation in these patients; however, is needed more research to assess the influence of the mesenteric fat in CD and its association with the severe forms of disease as well as the role in its pathogenesis.

Acknowledgments

We thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial support.

We are grateful to Dr. Nicola Conran for English grammar review.

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References

[1] Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology 2011; 140: 1785-94.

[2] Schreiber S, Nikolaus S, Hampe J. Activation of nuclear factor Kappa B in inflammatory bowel disease. Gut 1998; 42: 477-484.

[3] Schreiber S, Rosenstiel P, Hampe J, Nikolaus S, Groessner B, Schottelius A, Kühbacher T, Hämling J, Fölsch UR, Seegert D. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. Gut 2002; 51: 379-385.

[4] Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP, Raedler A. Enhanced secretion of tumor necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patient with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993; 94: 174-181.

[5] Watanabe T, Higuchi K, Kobata A, Nishio H, Tanigawa T, Shiba M, Tominaga K, Fujiwara Y, Oshitani N, Asahara T, Nomoto K, Takeuchi K, Arakawa T. Non-steroidal anti-inflammatory drug-induced small intestinal damage is Toll-like receptor 4 dependent. Gut 2008; 57: 181-187.

[6] Cario E, Podolsky DK. Differencial alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000; 68: 7010-7017.

[7] Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002; 20: 197-216.

[8] Golder WA. The "creeping fat sign"-really diagnostic for Crohn's disease? Int J Colorectal Dis 2009; 24: 1-4.

[9] Sheehan AL, Warren BF, Gear MW, Shepherd NA. Fat-wrapping in Crohn's disease: pathological basis and relevance to surgical practice. Br J Surg 1992; 79: 955-8.

[10] Olivier I, Théodorou V, Valet P, Castan-Laurell I, Guillou H, Bertrand-Michel J, Cartier C, Bezirard V, Ducroc R, Segain JP, Portier G, Kirzin S, Moreau J, Duffas JP, Ferrier L, Eutamène H. Is Crohn's creeping fat an adipose tissue? Inflamm Bowel Dis 2011; 17: 747-57.

[11] Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated Toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. J Biol Chem 2000; 275: 24255-63.

[12] Batra A, Pietsch J, Stroh T, Fedke I, Glauben R, Okur B, Zeitz M, Siegmund B. Toll-like receptor expression and response to specific stimulation in adipocytes and preadipocytes: on the role of fat in inflammation. Ann N Y Acad Sci 2006; 1072: 407-409.

[13] Dereumaux P, Ernst O, Geboes K, Gambiez L, Berrebi D, Müller-Alouf H, Hafraoui S, Emilie D, Ectors N, Peuchmaur M, Cortot A, Capron M, Auwerx J, Colombel JF. Inflammatory alterations in mesenteric adipose tissue in Crohn's disease. Gastroenterology 1999; 117: 73-81.

[14] Barbier M, Vidal H, Desreumaux P, Dubuquoy L, Bourreille A, Colombel JF, Cherbut C, Galmiche JP. Overexpression of leptin mRNA in mesenteric adipose tissue in inflammatory bowel diseases. Gastroenterol Clin Biol 2003; 27: 987-91.

[15] Yamamoto K, Kiyohara T, Murayama Y, Kihara S, Okamoto Y, Funahashi T, Ito T, Nezu R, Tsutsui S, Miyagawa JI, Tamura S, Matsuzawa Y, Shimomura I, Shinomura Y. Production of adi-

ponectin, an anti-inflammatory protein, in mesenteric adipose tissue in Crohn's disease. Gut 2005; 54: 789-96.
[16] Peyrin-Biroulet L, Gonzalez F, Dubuquoy L, Rousseaux C, Dubuquoy C, Decourcelle C, Saudemont A, Tachon M, Béclin E, Odou MF, Neut C, Colombel JF, Desreumaux P. Mesenteric fat as a source of C reactive protein and as a target for bacterial translocation in Crohn's disease. Gut 2012; 61: 78-85.
[17] Peyrin-Biroulet L, Chamaillard M, Gonzalez F, Beclin E, Decourcelle C, Antunes L, Gay J, Neut C, Colombel JF, Desreumaux P. Mesenteric fat in Crohn's disease: A pathogenetic hallmark or an innocent bystander? Gut 2007; 56: 577-583.
[18] Zulian A, Cancello R, Micheletto G, Gentilini D, Gilardini L, Danelli P, Invitti C. Visceral adipocytes: old actors in obesity and new protagonists in Crohn's disease? Gut 2012; 61: 86-94.
[19] Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's disease Study. Gastroenterology 1976; 70: 439-444.
[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
[21] Velloso LA, Folli F, Sun XJ, White MF, Saad MJ, Kahn CR. Cross-talk between the insulin and angiotensin signaling systems. Proc Natl Acad Sci U S A 1996; 93: 12490-12495.
[22] Paul G, Schäffler A, Neumeier M, Fürst A, Bataillle F, Buechler C, Müller-Ladner U, Schölmerich J, Rogler G, Herfarth H. Profiling adipocytokine secretion from creeping fat in Crohn's disease. Inflamm Bowel Dis 2006; 12: 471-477.
[23] Schäffler A, Fürst A, Büchler C, Paul G, Rogler G, Schölmerich J, Herfarth H. Secretion of RANTES (CCL5) and interleukin-10 from mesenteric adipose tissue and from creeping fat in Crohn's disease: Regulation by steroid treatment. J Gastroenterol Hepatol 2006; 21: 1412-1418.
[24] Erhayiem B, Dhingsa R, Hawkey CJ, Subramanian V. Ratio of visceral to subcutaneous fat area is a biomarker of complicated Crohn's disease. Clin Gastroenterol Hepatol 2011; 9: 684-687.
[25] Schäffler A, Fürst A, Büchler C, Paul G, Rogler G, Schölmerich J, Herfarth H. Vascular endothelial growth factor secretion from mesenteric adipose tissue and from creeping fat in Crohn's disease. J Gastroenterol Hepatol 2006; 21: 1419-23.
[26] Haidl ID, Jefferies WA. The macrophage cell surface glycoprotein F4/80 is a highly glycosylated proteoglycan. Eur J Immunol 1996; 26: 1139-1146.
[27] Himmel ME, Gijs H, Piccirillo CA, Steiner TS, Levings MK. The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease. Immunology 2008; 125: 145-153.
[28] Niess JH, Adler G. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol 2010; 184: 2026-37.
[29] Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, Liu K, Jakubzick C, Ingersoll MA, Leboeuf M, Stanley ER, Nussenzweig M, Lira SA, Randolph GJ, Merad M. Origin of the lamina propria dendritic cell network. Immunity 2009; 31: 513-25.
[30] Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, Pabst O. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med 2009; 206: 3101-14.