SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

NOME E COGNOME: _____Alessia Di Silvestre____

UNIVERSITÀ: __Università di Trieste____

DIPARTIMENTO (in caso di borsa per soggiorno all'estero specificare l'ente presso cui si è svolta la ricerca):

University of Birmingham - Institute of Inflammation and Ageing - College of Medical and Dental Sciences

TUTOR (in caso di borsa per soggiorno all'estero specificare il tutor dell'ente presso cui si è svolta la ricerca):

______Professor Andy Clark____

TIPOLOGIA DI BORSA RICEVUTA: _______Borsa soggiorno all'estero_______________________________

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): ___Finale_________________________________

TITOLO DELLA RELAZIONE: ______TTP phosphorylation in the development of inflammatory bowel disease and rheumatoid arthritis____

RELAZIONE:

Background and Aim

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing disorders of the gastrointestinal tract that are characterized by intestinal inflammation and epithelial injury. Cytokines seem to have a crucial role in the pathogenesis of progressive and destructive forms of IBD (Neurath, 2014). In particular cytokines production is under the control of the signaling cascades involving the MAPK-activated protein kinase 2 (MK2) that has also a role in mediating IBD (Broom et al., 2009).

The mRNA-binding protein tristetraprolin (TTP) binds to adenosine/uridine-rich elements in the 3' untranslated region of target mRNAs (Ross et al., 2016). TTP recognizes the conserved *cis*-acting pentameric sequence AUUUA and recruits the Ccr4/Caf1/Not deadenylation complex that shortens the poly (A) tail of target mRNAs resulting in suppression of translation and in a rapid degradation of the transcript. TTP target mRNAs are mostly pro-inflammatory cytokines, for example TNF, *IL3*, *IL2*, *IL1b*, *IL6*, *IL8* and many others studied principally in macrophages of different tissues (Brooks et al., 2013). TTP has a complex regulation: the MK2 phosphorylates serines 52 and 178 of murine TTP (60 and 186 of human TTP), protecting it from destruction by the proteasome and impairing its ability to recruit deadenylases, resulting in a stabilization of its mRNA targets and in an increase of transcripts of most pro-inflammatory cytokines (Ross et al., 2016). In a recent work the expression of TTP protein was found to be significantly elevated in synovial tissue of patients with rheumatoid arthritis compared with non-inflamed controls, as a result of a phosphorylated TTP (Ross et al., 2016).

The aim of this study is to investigate if the complex activity of TTP could influence also the pathogenesis of IBD.

Methods

Samples

Eleven IBD paediatric patients (5 UC and 6 CD) were enrolled at diagnosis at the Paediatric Clinic of IRCCS Burlo Garofolo in Trieste. Ethics Committee approval was obtained. The parents of all the participating children gave written informed consent before the study began. For each patient, during a colonoscopy, two biopsies (inflamed and non-inflamed) were collected. TRIzol was used for protein extraction.

Since TTP has been shown to be a very stable protein in macrophages (Cao et al., 2004), human macrophages were differentiated from peripheral blood mononuclear cells of healthy donors from the Queen Elizabeth Hospital of Birmingham. Human macrophages were maintained for 5 days in RPMI-1640 Medium with GM-CSF (50ng/ml), 5% fetal bovine serum, 1% penicillin-streptomycin and L-glutamine. At day 6, $1x10^6$ cells were stimulated for 60 and 120 min with 10 ng/ml LPS and lysed in lysis buffer.

Paraffin-embedded sections of inflamed colon biopsies from three IBD adult patients (2 UC and 1 CD) were recruited from Human tissue Biorepository Center of University of Birmingham.

Western Blot

Biorad Protein Assay (Bradford Assay) was used to quantify the amount of proteins in each sample.
A protein amount of 50 µg per well was loaded on SDS-PAGE gels and probed with primary antibodies, immunoreactive proteins were visualized with HRP-coupled secondary antibodies and chemiluminescence reagents (Bio-Rad, Pierce, or Cell Signaling Technology). Primary antibodies used: anti-tristetraprolin antibody [1A2] (Ab 119779) and the rabbit anti-TTP antiserum that was purified in a previous work (Mahtani et al., 2001). Blots were visualized using the ChemiDoc MP Imaging System.

Immunofluorescence

Paraffin-embedded slides were stained for confocal microscopy. Primary antibodies were added in PBS and slides incubated overnight at 4 °C. Slides were washed in PBS and stained with secondary antibodies in PBS containing 10 µg/ml Hoechst 33342. Slides were then washed in PBS and mounted with ProLong Diamond Antifade. Images were obtained using a 10x or 20x objectives on an LSM 510 microscope (Zeiss), and processed using the Zeiss LSM Image Examiner software.

Antibodies used for immunofluorescence

Antibody	Isotype/ Fluorophore	Final concentration / dilution
CD68 (Y1/82A)	IgG2b	1:100
TTP (8B5)	IgG1	1:50
Anti-mouse IgG2b	AlexaFluor 546	1:50
Anti-mouse IgG1	FITC	1:500

Results

Western blot (WB) analysis was performed on IBD patients biopsies and human macrophages samples using the antibodies previously described.

In whole protein lysates from IBD patients TTP expression was not detectable with none of the antibodies. On the other hand, WB analysis, with both Ab119779 and anti-TTP antiserum antibodies, on human macrophages cells showed an increase of TTP expression after 60 min of LPS stimulation respect to the unstimulated and a further increase after 120 min. These data suggest that differentiation of macrophages is necessary for the detection of TTP protein.

Preliminary data obtained from adult colon IBD biopsies using confocal microscopy need further investigation. The analysis was performed using an antibody against human macrophages (CD68) and an antibody against the TTP protein in order to demonstrate their colocalization. Experiments were performed on three IBD patients however the results obtained with TTP perfectly matched with IgG1 isotype control, as shown in the fig.1, suggesting that the signal showed by the TTP antibody is a false positive result and that further improvement of the protocol is required.

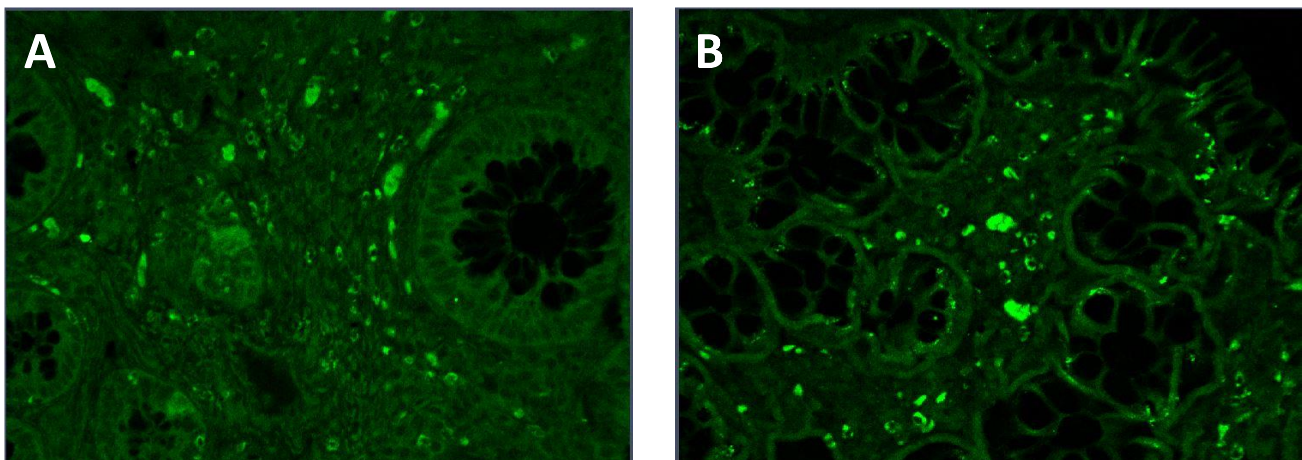

Fig.1 UC paraffin-embedded colon biopsies stained with TTP antibody IgG1 (A) and the IgG1 isotype control (B)

Conclusions

Western Blot results confirmed a role of the protein under LPS-induced inflammation and moreover suggest that isolation of macrophages from PBMC of IBD patients could be useful to clarify the role of TTP in this disease.

Preliminary data from confocal microscopy suggest that further analyses are needed. Frozen samples will be used in the future to avoid the difficulty of working with paraffin-embedded biopsies that may interfere with protein detection. In addition, different antibody dilutions will be used to improve the technique.

If the role of TTP will be confirmed in IBDs it would be interesting to study the role of the MK2 phosphorylation in the activation of the protein and then analyse tissues obtained from patients treated with anti-TNF therapeutic antibodies, to better understand the role of TTP in the regulation of TNF.

Neurath (2014). *Nat Rev Immunol.* 14, 329-42

Broom et al. (2009). *Clin Exp Immunol.* 158, 272-80

Ross et al. (2016). *Ann Rheum Dis.* 76, 612-619

Brooks et al. (2013). *Biochim Biophys Acta*. 1829, 666–679

Cao et al. (2004). *J Biol Chem*. 279, 21489–21499.

Mahtani et al. (2001). *Mol Cell Biol* 21: 6461–69.