Identification of novel genes and miRNAs related to the pathogenesis of Inflammatory Bowel Disease by using modified sample collection

Abstract of Ph.D. thesis

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I. INTRODUCTION AND AIMS

Inflammatory Bowel Disease (IBD) is a group of multifactorial disorders characterized by chronic inflammation along the digestive tract. IBD associates with several genetic factors, however twin studies showed low concordance between these factors and manifestation of the disease (Bernstein and Shanahan 2008). The main conclusion from the whole genome association studies is that genetic factors does have an effect through those genes that are responsible for the interaction between host and environment (e.g. NOD2, FUT2). Importance of the microbiome and lifestyle is indisputable in the pathogenesis of IBD, but these circumstance cause disease merely in genetically susceptible persons (Bernstein and Shanahan 2008).

Chronic inflammation significantly reduces the quality of life and increases the risk of colorectal cancer of IBD patients. Understanding of the background molecular mechanism/s of IBD and development of novel therapies are important, because incidence of IBD is increasing worldwide. In line with this the aims of this study were:

1. The refinement of the available *in vivo* rat model of Inflammatory Bowel Disease, in which IBD conditions can be precisely modelled.
2. Transcriptome analysis of samples derived from *in vivo* system and subsequent validation of significantly altered mRNAs and miRNA precursors relevant in IBD pathogenesis by QPCR and by immunofluorescent labelling.
3. The *in silico* examination of functional relationships between significantly altered transcripts and determination of molecular patterns/signaling pathways of inflammation by pathway analysis software.
4. The identification of miRNA/mRNA target pairs with potential role in inflammatory processes in IBD patients.

II. MATERIALS AND METHODS

In vivo rat model and sample collection

To investigate molecular background of IBD we used 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced rat model of experimental colitis. Male Wistar rats were randomly divided into two groups: first group served as control and the second group was induced by TNBS (colitis-induced) based on the method described by Morris et al (Morris, Beck et al. 1989). 72 hours after the treatment all animals were sacrificed and distal colons were removed. In the case of the control group, samples were taken from random colon sections; samples from colitis-induced animals were

taken from inflamed colon region as well as from non-adjacent uninflamed region. All samples were kept in TRIzol reagent (Thermo Fisher) at -80°C.

IBD patients

Colonic biopsies were obtained from 15 consenting patients with IBD (10 females and 5 males; median age 41 years, standard deviation 10,91 year) undergoing colonoscopy for diagnostic purposes approved by Hungarian Medical Research Council (ETT) Medical Research Council's Committee of Scientific and Research Ethics (TUKEB). Human colonic biopsies were obtained by experienced gastroenterologists at the 1st Department of Internal Medicine, University of Pécs in accordance with the guidelines set out by the Medical Research Council of Hungary. Sample collection and classification was performed according to disease status of patients, active/relapsing or inactive/remission phase. Furthermore, samples from relapsing patients were subdivided as uninflamed or inflamed according to the status of colon tissue.

Extraction of total RNA

Samples from rat colons were homogenized in TRIzol reagent by ULTRA-TURRAX T-18 (IKA) instrument as described previously (Boros, Csatari et al. 2017). 0,1 ml of chloroform (Sigma-Aldrich) was added to 0.3 ml homogenized sample with vigorous vortexing. Samples were centrifuged at 13000 rpm for 10 minutes. Total RNA was than extracted from the upper aqueous phase. RNeasy Plus Mini Kit (Qiagen) was used to purify total RNA from rat colon samples according to the manufacturer's protocol. Total RNA from human biopsies was isolated using NucleoSpin RNA Kit (Macherey-Nagel) according to the manufacturer's protocol. The quality and the quantity of the extracted RNAs were determined by TapeStation (Agilent) and Qubit Fluorometer (Thermo Fisher).

Reverse transcription and quantitative real-time PCR (QPCR)

Reverse transcription was performed by SuperScript VILO Master Mix (Thermo Fisher) according to the manufacturer's instructions. mRNA and pri-miRNA expression was measured by quantitative real-time PCR using the StepOne PCR Systems (Thermo Fisher). SybrGreen technology based QPCR reactions were performed by SYBR Select Master Mix (Thermo Fisher) with specific exon spanning primer sets; while TaqMan technology based reactions were performed by TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays (Thermo Fisher). The ratio of each gene relative to the 18S rRNA was calculated using the $2^{-\Delta\Delta CT}$ method.

miRNA reverse transcription and QPCR

Reverse transcription of miRNAs was performed by TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher). For measurement of miRNA expression levels TaqMan Universal PCR Master Mix (Thermo Fisher) and miRNA specific assays (Thermo Fisher) were used according to the manufacturer's instructions. The ratio of each miRNA relative to the endogenous U6 or RNU48 was calculated using the $2^{-\Delta\Delta CT}$ method.

Whole transcriptome analysis by RNA-Seq and bioinformatics

RNA-Seq was performed by SOLiD total RNA-Seq Kit (Life Technologies), according to the manufacturer's instructions and sequenced on SOLiD 5500xl instrument using the 50-base sequencing chemistry. Raw sequence data was size-selected discarding reads shorter than 50 bp. CLC Genomic Workbench tool (Qiagen) was used to obtain gene expression estimates (mapped read counts) for each annotated gene in all samples. Genes were filtered based on their expression levels keeping only those features that were detected by at least 5 mapped reads in at least 25% of the samples included in the study. After data normalization, genes showing at least two-fold gene expression change with an FDR value below 0.05 were considered as significant. Multi-dimensional scaling was also applied to visually summarize gene expression profiles revealing similarities between samples and unsupervised cluster analysis was carried out on the normalized data.

Pathway analysis

For the interpretation of transcriptomic data and for the definition of functional relationship between significantly changed molecules we used Ingenuity Pathway Analysis (IPA) application (http://www.ingenuity.com). Database of IPA (*Knowledge Base*) is based on published experimental results. Significantly activated canonical pathways in the *in vivo* model of experimental colitis were determined by core analysis function of IPA by using *log fold change* and *false discovery rate* values.

Tissue sections and immunofluorescent labelling

Rat colons were embedded in Technovit 7100 and tissue sections of 7 μm thickness were cut with Reichert Jung 1140 Autocut microtome for immunofluorescent staining. Tissue sections were than blocked for 20 minutes in PBS (Gibco) containing 5 % fetal bovine serum (FBS; Gibco) and 0,1% TritonX (Sigma). Next, sections were overnight incubated with mouse anti-Axl (Santa Cruz) primary antibody (1:100 dilution), then washed three times with PBT (PBS containing

TritonX) for 10 min each and were incubated with FITC conjugated anti-mouse IgG (Sigma) secondary antibody (1:250 dilution) for 90 minutes and washed three times with PBT (10 minutes each). Negative controls consisted of incubation with isotype matched control (Sigma). Finally, samples were washed with PBS containing 4',6-diamidino-2-phenylindole (DAPI; 1:10000 dilution) and mounted in Citifluor mounting media (Citifluor). Samples were analyzed using epifluorescent illumination of the Axiovision Z1 Fluorescent Microscope (Zeiss) and images recorded by Axiovision software.

III. RESULTS AND DISCUSSION

1. In the case of the widely used 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced rat model of IBD the intracolonically administered TNBS destroys the integrity of intestinal epithelia and generates sporadically located lesions. Hence, inflamed and phenotypically normal or uninflamed tissue regions alternate along the colons, similarly to that observed in the digestive tract of IBD patients. After the TNBS treatment, the ratio of the morphologically distinguishable inflamed and uninflamed tissues is different among biological replicates. Consequently, the conventionally - that is longitudinally - achieved sample collection from the colons may lead to heterogeneous sample population. To investigate the transcriptional changes after TNBS induction in different regions of the colon, we took samples from both the inflamed and the uninflamed tissue areas and compared them to untreated control samples. To demonstrate global gene expression alteration between inflamed and uninflamed tissue regions following TNBS treatment, whole transcriptome analysis was used by applying RNA-Seq. Next, multidimensional scaling (MDS) was performed to visually summarize gene expression profiles revealing similarities and differences between samples. *In silico* performed MDS of individual transcriptomic data clustered samples into control, TNBS treated uninflamed and TNBS treated inflamed groups. In addition, heatmap representation of significantly altered transcripts confirmed differences between inflamed and uninflamed samples of TNBS treated animals; in contrast, control and uninflamed samples proved to be very similar at global transcriptomic level. Based on two independent methods TNBS treated uninflamed and inflamed regions of the colon significantly differ from each other at transcriptional level. These data justify the modified sample collection applied for TNBS induced rat model of experimental colitis.

2. To quantify transcriptomic data we compared gene expression values of sample groups to each other. The expression of ~3800 transcripts changed significantly in at least one of the three different comparisons. We next performed *in silico* pathway analysis by using IPA in order to better understand results obtained by RNA-Seq and to identify possible relationships between molecules with altered expression. Functional analysis of our data sets revealed significant alteration of canonical pathways at the site of inflammation related to communication between immune cells, immune cell recruitment, signaling through pattern recognition receptors and regulation of inflammatory response. Notably, we observed significant alteration of pathways and molecules promoting the infiltration of leukocytes, namely agranulocytes and granulocytes from the blood to the inflamed tissues; in line with this, we detected the activation of NF-κB signaling that interconnects several inflammation regulating pathways.
3. Innate immune response is induced by the recognition of microbial components or molecules derived from damaged host cells by pattern recognition receptors (PRR) (Akira, Uematsu et al. 2006). Significant activation of PRR signaling and altered expression of TLR and NLR receptors were observed in the TNBS treated inflamed rat colons. The firstly identified IBD susceptibility loci was in an NLR receptor: NOD2 recognizes bacterial cell wall component peptidoglycan and induces the expression of proinflammatory genes (Hugot, Chamaillard et al. 2001, Ogura, Bonen et al. 2001, Elinav, Strowig et al. 2011). According to the transcriptome analysis, expression of Nod2 was significantly upregulated in the inflamed colon regions but remained unaltered in the uninflamed samples. QPCR validation of the expression profile of Nod2 - and another 37 genes - supported the results obtained by RNA-Seq. Inflammasome complex forming NLR receptors (e.g. NLRP3) have a role in the maturation of proinflammatory cytokines (e.g.: IL1β, IL33). We have determined that the expression of Nlrp3, Il1β and Il33 significantly increased in the inflamed tissues; in contrast, the expression of the anti-inflammatory NLRP6 and its regulator, PPARγ significantly decreased. In conclusion, gene expression pattern of PRRs is characterized by the elevated level of proinflammatory genes and reduced expression of anti-inflammatory molecules in the TNBS treated inflamed rat colon samples.
4. Ligand binding of PRRs induce the expression of chemotactic molecules, such as cytokines and chemokines leading to the infiltration of immune cells to the damaged tissues (Feghali and Wright 1997). The expression of well-known markers of inflammation - such as Tnfα,

Il1β, Il6, Il10, Ccl3 and Cxcl1 - significantly increased in the inflamed colon samples, but remained unaltered in the uninflamed regions. We have next proved the relevance of our results obtained from rat model by using samples taken from IBD patients. The expression of proinflammatory cytokines significantly increased in the inflamed colons of patients as compared both to the inactive and to the active-uninflamed samples: these data corroborated those obtained from rat model. The importance of cytokines are known in the progression of IBD, for example anti-TNF treatment can moderate symptoms, but it cannot cure IBD (Silva, Ortigosa et al. 2010).

After the examination of expression pattern of genes with known impact on IBD pathogenesis in TNBS induced rat model of experimental colitis, and validation of our results in samples originated from IBD patients, we next aimed to identify novel molecules with altered expression in IBD.

5. Increasing evidence suggests that malfunction in the negative regulation of immune response leads to robust and prolonged inflammation. TAM receptors (TYRO3, AXL and MERTK) are known negative regulators of inflammation (Rothlin, Ghosh et al. 2007). We have determined that the expression of Tyro3 and Mertk decreased in the inflamed colon regions; in contrast, both mRNA and protein level of Axl significantly increased in the TNBS treated inflamed rat samples. In addition, we also detected elevated AXL gene expression in the colonic lesions of IBD patients. At the site of inflammation, pleiotropic AXL may have a role as a negative regulator of innate immunity as well as phagocytic receptor in the reconstruction of tissue homeostasis (Lemke and Rothlin 2008). In addition, as the inducer of epithelial-mesenchymal transition (EMT) Axl may also enhance the risk of colorectal cancer in IBD patients (Asiedu, Beauchamp-Perez et al. 2014).

6. Most of the transcripts with significantly altered expression identified by RNA-Seq was detected in the TNBS treated inflamed colon samples (more than 2600), while in the uninflamed samples only 113 genes showed altered expression. Importantly, the expression pattern of molecules responsible for the protection of the epithelia falls within the latter group: we have detected their significant induction the TNBS treated uninflamed rat samples. Epithelial cells are covered by mucous gel layer that is created by mucins. We have identified Muc2 with significantly elevated expression in the uninflamed rat colon samples. Although previous studies suggested the impact of MUC2 on IBD patients, they failed to detect any alteration in its expression, probably due to the fact that they did not

separated the inflamed and uninflamed regions of IBD patients in relapsing phase (Larsson, Karlsson et al. 2011). Similarly to the expression observed in the *in vivo* rat model, we have shown that the expression of MUC2 indeed differs between the uninflamed and inflamed colon regions of relapsing IBD patients with significantly elevated expression in the intact tissues. This elevated expression of MUC2 in the uninflamed tissues probably has a role in the protection against further tissue damages. Glycosyltransferases also have a role in the protection of epithelia by the synthesis of carbohydrate chains of glycocalyx (Pu and Yu 2014). Similarly to the expression of Muc2, the amount of A4gnt, B4galnt2, Fut4 and Fut9 mRNAs also increased in the TNBS treated uninflamed tissue regions; in contrast, the expression of Fut1 and Fut2 significantly decreased in the inflamed rat samples. Elevated expression pattern of genes responsible for the glycosylation in the uninflamed tissues may protect epithelial cells against hazards and may promote the maintenance of the tissue homeostasis.

7. Importantly, we detected a number of genes with significantly altered expression in the TNBS induced *in vivo* model of experimental colitis that are related to both inflammation and tumorigenesis. Investigation of these genes is particularly important because long-lasting inflammation highly increases the risk of colorectal cancer in IBD patients (Saleh and Trinchieri 2011). During tumor progression epithelial-mesenchymal transition (EMT) is a key process that facilities metastasis formation (Lamouille, Xu et al. 2014). Upon EMT, epithelial cells lose their epithelial characteristics, cell-cell connections disintegrate and cells motility enhances. We have determined that the expression of EMT activating genes significantly increased both in the inflamed colon samples of TNBS treated rats as well as in IBD patients. The expression of growth factors (FGF2, FGF7, EGR1), signal transducer molecules (NOTCH2, JAK2, HIF1α), EMT inducing transcriptional factors (ZEB2, SNAI1), extracellular matrix remodeler MMP9 and mesenchymal markers (VIM and LOX) are all upregulated in the inflamed colon tissues; in contrast, the expression of the epithelial marker CDH1 is decreased.
8. Besides protein coding mRNAs we observed significant alteration in the expression of miRNA precursors, the pri-miRNAs by using RNA-Seq. Biogenesis of miRNAs is very complicated and regulated by different enzymes, hence we decided to monitor the expression of mature miRNAs derived from pri-miRNAs by QPCR. We observed the global suppression of miRNAs: 27 out of 28 miRNAs (e.g. miR-192, -200b, -375, -143) showed

downregulated expression profile. Most of these miRNAs have targets among EMT inducing genes (Zaravinos 2015). For instance, there is a negative feedback loop between miR-200b and ZEB2: they inhibit the expression of each other (Bracken, Gregory et al. 2008). In addition, ZEB2 is also regulated by miR-192 that is repressed by EMT activating TWIST1 transcriptional factor (Wu, Rupaimoole et al. 2016). Reduced expression of miR-192 was observed both in inflamed colon regions of rats and IBD patients, where the amount of its targets EGR1 and FGF2 is upregulated. EMT inducer SNAI1 inhibits the expression of miR-375 (Ding, Xu et al. 2010, Xu, Jin et al. 2014) that results in the elevation of the amount of JAK2. Significantly decreased expression of miR-199a correlated with the upregulation of its targets SNAI1 (Suzuki, Mizutani et al. 2014) and AXL (Mudduluru, Ceppi et al. 2011); their inverse expression pattern was observed both in inflamed rat and human colon samples. Finally, miRNAs miR-30 and miR-107 are regulators of EMT through the inhibition of VIM (Liu, Chen et al. 2014) and HIF1α (Yamakuchi, Lotterman et al. 2010): we again detected their mutually exclusive expression pattern. In conclusion, all of the miRNA-mRNA target pairs in the inflamed colon regions changed reciprocally: the expression of miRNAs was significantly reduced while the amount of their target mRNAs was elevated; this pattern may further enhance EMT in the inflamed colon tissues.

9. Besides the EMT inhibiting miRNAs, we identified other miRNAs with altered expression which are related to posttranscriptional regulation of inflammatory genes. For example, we detected the elevated expression of miR-223 in the inflamed colons of both rats and IBD patients. This miRNA is expressed by immune cells infiltrating to the damaged tissues where it inhibits chemotactic (CXCL2 and CCL3) and proinflamamtory (TNFα, IL1β, IL6 and NLRP3) molecules (Poon, Palanisamy et al. 2017).

   Finally, the common target of IBD therapies is TNFα that is stored as inactive pro-TNFα in the cytoplasm until it is converted to active form by matrix metalloproteinases, such as MMP13 (Vandenbroucke, Dejonckheere et al. 2013). Both in the inflamed colon samples of TNBS treated rats as well as in IBD patients, elevated expression of TNFα associated with increased MMP13 mRNA amount. Importantly, we detected significantly decreased expression of miR-27b that posttranscriptionally regulates MMP13 (Akhtar, Rasheed et al. 2010). Since TNFα is a popular target of many inflammatory disorders including IBD miR-27b may be a potential therapeutic target as its ectopic overexpression may indirectly reduce the level of TNFα.

In the life of IBD patients inactive and active disease phases fluctuate, while the localization of inflamed and uninflamed colon regions also alternate along the digestive tract. This associates with the fluctuation of inflammation and the altered expression of EMT regulating genes and miRNAs. Long lasting chronic inflammation can destroy the balance of this complex regulatory system that may be responsible for the enhanced risk of colorectal cancer in IBD patients. Circulating miRNA carrying exosomes and miRNA expressing blood cells (Poon, Palanisamy et al. 2017) may reach and exert their effects on cells both in the inflamed and uninflamed regions of the gastrointestinal tract and delay or obstruct the reconstruction of damaged tissue homeostasis.

Colon tissue is composed of several cell types and all of them contribute differently to the maintenance of homeostasis or to the regulation of immune response. In the present work, we monitored the actual gene expression alteration of a heterogenic cell population present in the uninflamed or inflamed regions of rat and human tissue samples. Facilitated by the rapid development of single-cell sequencing methods we plan to increase the resolution of our transcriptome analysis by applying single-cell RNA-Seq (scRNA-Seq) and determine the gene expression alteration of specific cell types in the inflamed vs. uninflamed colon tissues.

Global expressional reduction of miRNAs in the inflamed colons presumes the existence of a common regulatory mechanism. We plan to examine this observation by applying miRNA-Seq method at the level of mature miRNAs. Our working hypothesis is that under physiological conditions the constitutively expressed miRNAs inhibit their proinflamamtory target genes; in contrast, the global reduction of miRNAs during inflammation leads to the synchronized elevation of their targets that generates robust inflammatory response.

IV. LIST OF PUBLICATIONS

MTMT ID: 10059533

PUBLICATIONS RELATED TO THE Ph.D THESIS

Éva Boros, Marianna Csatari, Csaba Varga, Balázs Balint, István Nagy: **Specific Gene- and MicroRNA-Expression Pattern Contributes to the Epithelial to Mesenchymal Transition in a Rat Model of Experimental Colitis**. MEDIATORS OF INFLAMMATION Paper 5257378. 9 p. (2017)
(IF: 3.232)

Tóth EJ, Boros É, Hoffmann A, Szebenyi C, Homa M, Nagy G, Vágvölgyi C, Nagy I, Papp T: **Interaction of THP-1 monocytes with conidia and hyphae of different *Curvularia* strains**. FRONTIERS IN IMMUNOLOGY 8: Paper 1369. 9 p. (2017)
(IF: 6.429)

Éva Boros, Zoltán Kellermayer, Péter Balogh, Patrícia Sarlós, Áron Vincze, Csaba Varga and István Nagy: **Elevated expression of AXL may contribute to the epithelial-to-mesenchymal transition in Inflammatory Bowel Disease patients.** MEDIATORS OF INFLAMMATION
(Under review)

Total IF: 9.661

ACADEMIC MATERIALS RELATED TO THE SUBJECT OF THE THESIS

Boros E: **Transcriptome profiling of TNBS induced rat model of IBD**. ACTA BIOLOGICA SZEGEDIENSIS 60:(1) p. 81. (2016)

Boros E: **The expression profile of TAM and NLR receptors upon physical activity in TNBS induced colitis in rats**. 21th International Congress on Sport Science, Budapest, 2014. April 10-12., (2014)

Boros E, Strifler G, Voros A, Szasz A, Kemeny L, Varga C, Tubak V, Nagy I: **The Expression Profile of TAM and NLR Receptors upon Immune Challenge and Chronic Inflammation** IMMUNOLÓGIAI SZEMLE V:(3) p. 19. (2013) Magyar Immunológiai Társaság 42. Vándorgyűlés. Pécs, Magyarország: 2013.10.16 -2013.10.18.

OTHER ACADEMIC MATERIALS

Kellermayer Z, Vojkovics D, Kajtar B, Berta G, Nagy I, Boros E, Balint B, Schippers A, Wagner N, Balogh P: **Mouse colonic stromal cells induce a protective ILC3 phenotype in the absence of Nkx2-3 transcription factor**. ICMI 2017: 18th International Congress of Mucosal Immunology. Konferencia helye, ideje: Washington, Amerikai Egyesült Államok, 2017.07.19-2017.07.22.p. 113.

Tóth EJ, Boros É, Hoffmann A, Vágvölgyi Cs, Nagy I, Papp T: **Activation of monocytes and their production of cell signaling molecules induced by *Curvulaia* strains**. 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of abstracts.

Konferencia helye, ideje: Újvidék, Szerbia, 2016.06.02-2016.06.04. Újvidék: University of Novi Sad, 2016. p. 36. (ISBN:978-86-6253-059-2)

Toth EJ, Boros E, Hoffmann A, Vagvolgyi C, Nagy I, Papp T: **Ativation of cytokine, chemokine and chemotactic genes in THP1 cells in response to conidia and hyphase of *Curvularia* strains**. 13th European Conference on Fungal Genetics (ECFG13), Bacterial Fungal Interactions Workshop,: Abstract Book. 620 p.

Toth EJ, Boros E, Hoffmann A, Nagy I, Chandrasekaran M, Kadaikunnan S, Alharbi NF, Vagvolgyi C, Papp T: **Interaction of *Curvularia* Strains with Human THP1 Cells**. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 62:(Suppl.) p. 231. (2015); 17th International Congress of the Hungarian Society for Microbiology. Budapest, Magyarország: 2015.06.08 -2015.06.10.

Toth EJ, Hoffmann A, Boros E, Nagy I, Papp T: **Response of human macrophages for infections with *Culvularia* strains.** 17th Danube-Kris-Mures-Tisza (DKMT) Euroregional Conference on Enironment and Health. Konferencia helye, ideje: Szeged, Magyarország, 2015.06.05-2015.06.06. Szeged: University of Szeged, Faculty of Medicine, p. 72.

V. CONFLICT OF INTEREST

As a corresponding author of the following publication declare that Éva Boros Ph.D. candidate had a great contribution to the published results. Results discussed in her thesis won't be applied to other Ph.D. thesis.

Éva Boros, Marianna Csatari, Csaba Varga, Balázs Balint, István Nagy
Specific Gene- and MicroRNA-Expression Pattern Contributes to the Epithelial to Mesenchymal Transition in a Rat Model of Experimental Colitis
MEDIATORS OF INFLAMMATION, 5257378. 9 p. (2017)

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Tóth EJ, Boros É, Hoffmann A, Szebenyi C, Homa M, Nagy G, Vágvölgyi C, Nagy I, Papp T
Interaction of THP-1 monocytes with conidia and hyphae of different *Curvularia* strains
FRONTIERS IN IMMUNOLOGY, 8: 1369. 9 p. (2017)

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