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Pulmonary *Aspergillus fumigatus* infection in rats affects gastrointestinal homeostasis

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ABSTRACT

Microbiota inhabiting mucosal tissues is involved in maintenance of their immune homeostasis. Growing body of evidence indicate that dysbiosis in gut influence immune responses at distal sites including lungs. There are also reports concerning gut involvement with pulmonary injury/inflammation in settings of respiratory viral and bacterial infections. The impact of infections with other microorganisms on gut homeostasis is not explored. In this study, the rat model of sublethal pulmonary infection with *Aspergillus fumigatus* was used to investigate the effect of fungal respiratory infection on gut immune-mediated homeostasis. Signs of intestinal damage, intestinal and gut-draining lymphoid tissue cytokine responses and gut bacterial microbiota diversity were examined. Intestinal injury, inflammatory cell infiltration, as well as increased levels of intestinal interferon-γ (IFN-γ) and interleukin-17 (IL-17) (as opposed to unchanged levels of anti-inflammatory cytokine IL-10) during the two-week period depict intestinal inflammation in rats with pulmonary *A. fumigatus* infection. It could not be ascribed to the fungus as it was not detected in the intestine of infected rats. Increased production of pro-inflammatory cytokines by major gut-draining mesenteric lymph nodes point to these lymphoid organs as places of generation of cytokine-producing cells. No changes in spleen or systemic cytokine responses was observed, showing lack of the effects of pulmonary *A. fumigatus* infection outside mucosal immune system. Drop of intestinal bacterial microbiota diversity (disappearance of several bacterial bands) was noted early in infection with normalization starting from day seven. From day three, appearance of new bacterial bands (unique to infected individuals, not present in controls) was seen, and some of them are pathogens. Alterations in intestinal bacterial community might have affected intestinal immune tolerance contributing to inflammation. Disruption of gut homeostasis during pulmonary infection might render gastrointestinal tract more susceptible to variety of physiological and pathological stimuli. Data which showed for the first time gut involvement with pulmonary infection with *A. fumigatus* provide the baseline for future studies of the impact of fungal lung infections to gut homeostasis, particularly in individuals susceptible to these infections.

1. Introduction

At late seventies the term "common mucosal immunological system" (CMIS) was coined to describe similarities in lymphoid tissues associated with bronchus and intestine (McDermott and Bienenstock, 1979). From then mechanisms underlying immune responses to pathogens in the gastrointestinal and respiratory tract have been established (Mowat and Agace, 2014; Sato and Kiyono, 2012). Growing body of evidence suggests that two mucosal sites communicate and interact in health and disease what brought suggestion that mucosal immune system is system wide-organ (Gill et al., 2010). Pulmonary involvement seen in patients with chronic gastrointestinal diseases such

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as inflammatory bowel disease and irritable bowel syndrome as well as association of gastrointestinal symptoms observed in patients with pulmonary infections depict cross-talk between gastrointestinal and pulmonary defences (Samuelson et al., 2015; Tulic et al., 2016).

Much interest in investigation of gut and lung communication was given to gut microbiota (Dickson et al., 2013). The use of animals treated with antibiotics or which differ in intestinal microbiota composition showed that gut microbiota is essential for promoting optimal pulmonary response to allergens (Kim et al., 2014), against viruses (Abt et al., 2012; Ichinohe et al., 2011; Wu et al., 2013) and bacteria (Chen et al., 2011; Fagundes et al., 2012; Gauguet et al., 2015; Schuijt et al., 2016).

Changes in gut microbiota were also found to be involved in settings of pulmonary immune responses. Infection of mice with Influenza A (IA) (Deriu et al., 2016; Groves et al., 2018; Wang et al., 2014; Yildiz et al., 2018; Zhang et al., 2015) or respiratory syncytial (RSV) viruses (Groves et al., 2018) resulted in altered diversity of gut bacterial microbiota and was associated with intestinal injury and inflammation. Presence of the virus in the intestine was responsible for tissue injury only in one study (Zhang et al., 2015). Intestinal injury is thought to be induced by virus-specific T cells recruited from the lung (Wang et al., 2014), though innate pulmonary responses might be responsible judging by data which showed that administration of agents which induce pulmonary inflammation such are poly I:C (Deriu et al., 2016) and LPS (Sze et al., 2014) also affected gut microbiota. Alterations of gut microbiota were detected in patients (Luo et al., 2017) and mice with pulmonary *M. tuberculosis* infection (Winglee et al., 2014).

Gut microbiota has the potential to influence host response to fungi as well. Treatment of mice with antibiotics and oral *C. albicans* thereafter, prime mice for allergic T-cell based response to *Aspergillus* (Noverr et al., 2004). More recently, the influence of intestinal bacterial microbiota on the generation of pulmonary IL-17 response to *A. fumigatus* was noted in mice (McAleer et al., 2016). There are, however no data concerning the effects of pulmonary fungal infections on gut homeostasis.

In the view of above cited studies which showed the impact of pulmonary viral and bacterial infections on gut homeostasis, we hypothesized that pulmonary immune response to fungi might affect gut homeostasis as well. Among variety of rat models of aspergillosis (Chandenier et al., 2009; Desoubeaux and Cray, 2017) we used rat model of pulmonary *Aspergillus fumigatus* infection in immunocompetent rats (El-Muzghi et al., 2013; Mirkov et al., 2015) to investigate the effect of *A. fumigatus* infection on gut homeostasis. Signs of intestinal damage, intestinal and gut-associated lymphoid tissue cytokine responses as well as gut bacterial microbiota diversity were examined at different time points following infection, shown previously by us to be accompanied with marked changes in fungal load resulting from inflammatory/immune activity in the lungs (El-Muzghi et al., 2013; Mirkov et al., 2015). Data that were obtained showed for the first time that lung infection with *A. fumigatus* is associated with intestinal damage and pro-inflammatory cytokine response in small intestine and major-gut draining (mesenteric) lymph nodes. Decrease in diversity of intestinal bacterial microbiota was observed during pulmonary infection, with appearance of new bacterial species, not noted in non-infected controls, what might have contributed to intestinal inflammation. Disruption of gut homeostasis during pulmonary infection might render gastrointestinal tract more susceptible to variety of physiological and pathological stimuli.

2. Material and methods

2.1. Rats

Male Dark Agouti (DA) rats, ten to twelve weeks old, bred and conventionally housed at the Institute for Biological Research “Sinisa Stankovic” (Belgrade, Serbia) were used in the study. Animals were fed commercial chow and received no medication prior experimental procedure. Four individuals were assigned per group for each time point in two independent experiments. All animal procedures were complied with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee of the Institute for Biological Research “Sinisa Stankovic”.

2.2. Fungal culture conditions and infection

Fungal culture conditions and infection were as previously described (Mirkov et al., 2015). In brief, conidia of human isolate of *Aspergillus fumigatus* [grown on Sabouraud maltose agar (SMA, Torlak, Belgrade, Serbia) for seven days] (Booth, 1971) were obtained by flooding the surface of agar slants with pyrogen-free sterile physiological saline (Hemofarm, Vrsac, Serbia). 10^7 conidia of *A. fumigatus* in 0.2 ml of saline were injected into the trachea of anesthetized rats (Zoletil 100, Virbac, Carros, France) by tracheostomy. Control (sham infected) rats received saline only. Infection caused no death of experimental animals. Presence of *A. fumigatus* in lungs was checked using quantitative colony forming units (CFU) assay as previously described (Mirkov et al., 2015).

2.3. Sacrificing of animals and tissue samples collection

Animals were sacrificed at days 1, 3, 7 and 14 post infection. Blood was withdrawn from the abdominal aorta with a syringe containing 5 U/ml of heparin. Following centrifugation, plasma was collected for interleukin-6 (IL-6) measurements.

Samples of small intestine collected at necropsy and washed through the lumen with ice-cold non-pyrogenic physiological saline were used for histological analysis and preparation of tissue homogenates. Major gut-draining mesenteric lymph nodes (mLN), harvested from prepared mesentera, and spleens (removed, blotted dry and weighed) were used for cell suspension preparation.

2.4. Histology

Samples of small intestine were fixed in 4% formaldehyde (pH 6.9), and embedded in paraffin wax for sectioning at 5 μm. Samples of intestine with longitudinally cut crypts were stained with hematoxylin and eosin (H&E), analyzed subsequently using Coolscope digital light microscope (Nikon Co, Tokyo, Japan) and inflammation was scored according to criteria defined for mouse models of intestinal inflammation (Erben et al., 2014). Inflammatory cell infiltrate was classified as minimal (< 10%), mild (11–25%), moderate (26–50%) or marked (> 51%) depending on leukocyte density of lamina propria area infiltrated. Reduction of goblet cell numbers relative to baseline goblet cell numbers per crypt was classified as minimal (< 20%), mild (21–35%), moderate (36–50%) or marked (> 51%).

2.5. Preparation of intestinal homogenates

Tissue samples were homogenized by IKA T18 Basic Homogenizer (IKA Works Inc., Wilmington NC, USA) in ten volumes of sucrose buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA, 250 mM sucrose) on ice, homogenates were sonicated and then centrifuged (1000 × *g* for 20 min, at 4 °C). Obtained supernatants were used for cytokine measurements.

2.6. Gut-draining lymph nodes and spleen cell isolation and culture

Cell suspensions were prepared by mechanical teasing of tissue over nylon mesh (70 μm nylon, BD Bioscience, Bedford, USA) and suspended in RPMI-1640 culture medium (Biowest, Nuaillé, France) supplemented with 2 mM glutamine, 20 μg/ml gentamicin (Galenika a.d., Serbia) with

Table 1
Body weight and pulmonary response following intra tracheal *A. fumigatus* administration.f.

	Days post infection							
	1		3		7		14	
	Sham	Infected	Sham	Infected	Sham	Infected	Sham	Infected
Body weight (g)	213.9 ± 23.2	207.7 ± 12.6	209.8 ± 23.9	202.1 ± 20.8	225.1 ± 11.8	217.7 ± 24.1	220.6 ± 24.4	215.6 ± 19.8
Lungs								
Neutrophils ($\times 10^6$/g tissue)	0.08 ± 0.04	0.43 ± 0.26*	0.23 ± 0.21	0.76 ± 0.89*	0.27 ± 0.23	0.72 ± 0.43*	0.18 ± 0.09	0.35 ± 0.09*
[a]IFN-γ (pg/ml)	1275.9 ± 247.7	1396.6 ± 224.1	1030.0 ± 184.3	1732.4 ± 527.9*	1247.1 ± 277.6	1758.6 ± 349.1*	1271.3 ± 652.3	1396.6 ± 51.7
[a]IL-17 (pg/ml)	20.8 ± 9.7	38.9 ± 9.8*	55.0 ± 7.1	79.2 ± 14.5*	45.7 ± 14.3	69.8 ± 13.3**	60.0 ± 7.2	62.9 ± 17.3
[a]IL-10 (pg/ml)	532.5 ± 181.8	593.8 ± 38.2	550.9 ± 51.9	592.2 ± 131.9	752.9 ± 155.8	707.0 ± 301.6	656.5 ± 144.9	734.6 ± 138.2

Values are the mean ± SD of individual samples from two independent experiments with four individuals per group for each time post infection per experiment.
[a] Measured in lung homogenates.
* Significantly different at $p < 0.05$ vs sham infected animals.
** Significantly different at $p < 0.01$ vs sham infected animals.

5% (v/v) heat-inactivated fetal calf serum (Biowest, Nuaillé, France) (complete medium) and voriconazole (5 μg/ml) (Pfizer PGM, France). Cell number was determined by improved Neubauer hemocytometer, and number of viable cells, determined by trypan blue exclusion assay exceeded 90%.

For cytokine production, cells (1×10^6/well) were cultured in complete medium in 96-well plates for 48 h.

2.7. ELISA

Cytokine concentrations were determined using commercially available ELISA sets for rat IFN-γ, IL-6 and IL-10 (R&D Systems, Minneapolis, USA) and mouse IL-17 cross-reactive with rat IL-17 (eBioscience Inc., San Diego, CA, USA) according to manufacturer's recommendations. Cytokine titer was calculated by a reference to a standard curve constructed using known amounts of recombinant cytokines.

2.8. Denaturing gradient gel electrophoresis (DGGE) analysis and DGGE fragments' sequencing

Denaturing gradient gel electrophoresis (DGGE) analysis was carried out as previously described (Lukic et al., 2013), with minor modification. Extraction of bacterial DNA from gut samples was done using the ZP Genomic DNA™ – Tissue MiniPrep (Zymo Research, Irvine, California, United States). PCR with isolated genomic DNA as a template was performed with the universal 16S rRNA gene-targeting primers U968-GC-f (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401-r (5′ GCG TGT GTA CAA GAC CC) (amplify the V6 to V8 regions of the bacterial 16S rRNA) (Zoetendal et al., 1998) (Invitrogen, Thermo Phisher Scientific, Carlsbad, California, United States). Cloning of PCR fragments of interest, transformation of the competent DH5α cells and analysis of selected transformants was performed as previously described (Ninkov et al., 2016) except that pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Thermo Scientific, Vilnius, Lithuania), was used for cloning experiments. The vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Recircularized pJET1.2/blunt vector molecules lacking an insert express a lethal restriction enzyme, which kills the host *E. coli* cell after transformation. The insert-containing pJET1.2/blunt vectors were sequenced with pJET1.2/blunt-specific primers SO501-pJET1.2-f (5′ CGA CTC ACT ATA GGG AGA GCG GC 3′) and SO511-pJET1.2-r (5′ AAG AAC ATC GAT TTT CCA TGG CAG 3′) (Macrogen Europe Service, Amsterdam, the Netherlands). Sequence annotation and database searches for sequence similarities were performed using BLAST program (NCBI; www.ncbi.nlm.nih.gov).

2.9. Determination of A. fumigatus in intestine

Presence of *A. fumigatus* in intestine was determined by PCR for the fungus detection according to Ferrer et al. (2001), using primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′), complementary to the end of 18S rDNA, and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′), complementary to the beginning of 28S rDNA for the first round amplification and the primers ITS86 (5′ GTG AAT CAT CGA ATC TTT GAA C 3′), complementary to the 5.8S rDNA region together with ITS4 primer for the second round of amplification (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The PCR amplicons obtained were purified using kit (Thermo Scientific, Lithuania) and sequenced (Macrogen, Amsterdam, the Netherlands). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

2.10. Data presentation and statistics

Results were pooled from two independent experiments with 4 animals per group per experiment for each time point and expressed as mean values ± S.D.. Data were analyzed using a Mann-Whitney *U* test (STATISTICA 7.0, StatSoft Inc., Tulsa, Oklahoma, USA). *p*-values less than 0.05 were considered significant.

3. Results

3.1. Pulmonary infection of rats with A. fumigatus: general information

No differences in body weight were observed between sham infected and rats administered with *A. fumigatus* throughout the investigated period (Table 1). In line with our previously published data, pulmonary *A. fumigatus* infection induced lung leukocyte infiltration, predominantly neutrophils, (starting from day one) and proinflammatory cytokine lung response (IFN-γ from post infection day three to day seven; IL-17 from day one to day seven).

3.2. Intestinal injury in rats with pulmonary A. fumigatus infection

Histological examination of intestine revealed edema of *lamina propria* (expansion of *lamina propria* and villouses) which was caused by a pronounced dilatation of lymph vessels (asterisk). Number of goblet cells (arrows) from the epithelial cell layers was moderately to markedly reduced especially at the top of the villous, while number of mononuclear cells was moderately increased (circles), starting from day 1 post infection (p.i.) (Fig. 1B) in comparison with sham infected

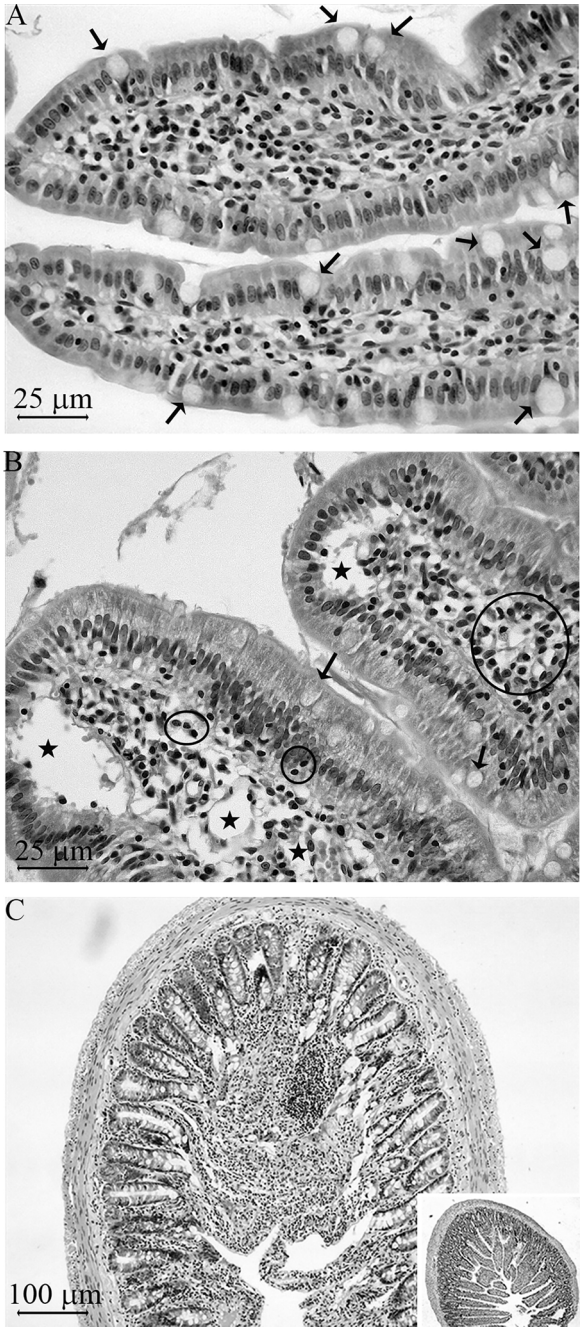

Fig. 1. Histological appearance of intestine in rats with pulmonary *A. fumigatus* infection (A) Intestine in sham infected animals (B) Edema of *lamina propria*, infiltration of mononuclear cells (circles), dilatation of lymph vessels (asterisk) and decrease in number of goblet cells (arrows) in animals with pulmonary *A. fumigatus* infection (C) Fibrosis in *lamina propria* and granulation tissue at day 14 following pulmonary infection. Insert: sham infected animals.

animals (Fig. 1A). At day 14 following pulmonary infection, fibrosis in *lamina propria* (manifested by severe focal architectural disorder: villous blunting and adhesion of adjacent villouses, loss of mucous membranes at that site, *lamina propria* filled with cellular connective tissue) and occurrence of granulation tissue were present (Fig. 1C).

3.3. Intestinal inflammation in rats with pulmonary A. fumigatus infection

Examination of cytokine content in gut revealed increased proinflammatory cytokine contents (IFN-γ and IL-17) at all time points examined (Fig. 2A,B). In contrast, increased levels of IL-10 were noted

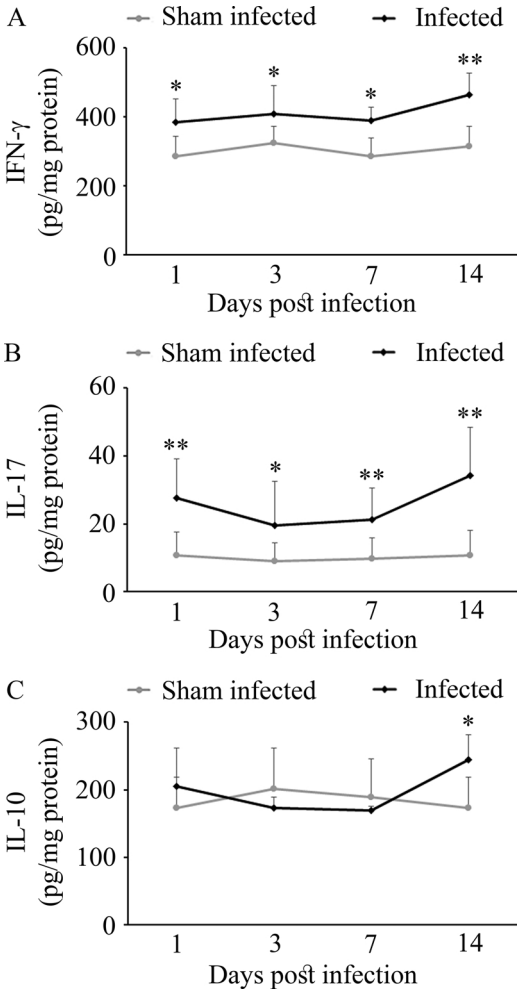

Fig. 2. Cytokine content in gut of rats with pulmonary *A. fumigatus* infection (A) IFN-γ (B) IL-17 (C) IL-10. Cytokine contents were determined in intestinal homogenates by ELISA. Values are the mean ± SD of individual samples from two independent experiments with four individuals per group for each time post infection per experiment. Significantly different at * $p < 0.05$ and ** $p < 0.01$ *vs* sham infected animals.

late following pulmonary infection (day 14 following infection) with lack of changes during the preceding period (Fig. 2C).

3.4. Gut-associated lymphoid tissue activity in rats with pulmonary A. fumigatus infection

No difference in cell numbers of gut-draining mesenteric lymph nodes (mLN) between sham-infected and infected animals was observed (Fig. 3A).

Examination of cytokine production by mLN cells cultures revealed increased production of both IFN-γ (during first week of infection) (Fig. 3B) and IL-17 (at days 3 and 7 p.i.) (Fig. 3C). In contrast to increases in proinflammatory cytokine production, no changes in IL-10 production were observed (Fig. 3D).

3.5. Lack of the effects of pulmonary A. fumigatus infection outside pulmonary and intestinal mucosal immune system

Investigation of selected aspects of activity of spleen (as nonmucosal lymphoid tissue) and changes in plasma levels of IL-6, a sensitive indicator of inflammation at systemic level revealed no differences in cell number (Fig. 4A), spleen cells IFN-γ production (Fig. 4B) and plasma IL-6 between (Fig. 4C) sham-infected and infected rats and no time-related

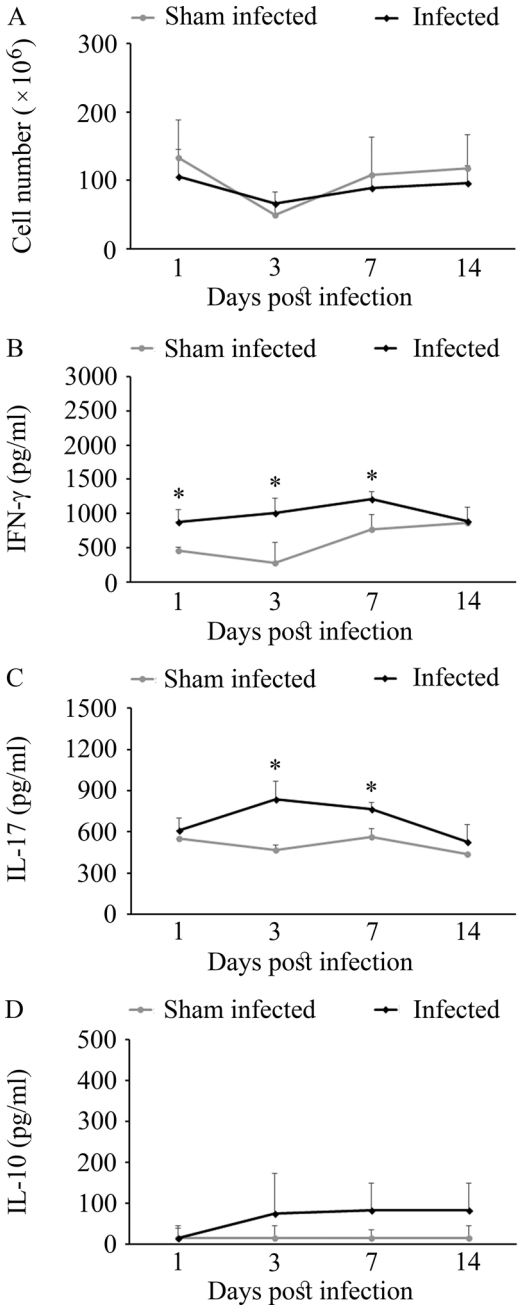

Fig. 3. Gut-associated lymphoid tissue activity in rats with pulmonary *A. fumigatus* infection (A) Number of cells in mesenteric lymph nodes (B) IFN-γ production by mLN cells (C) IL-17 production by mLN cells (D) IL-10 production by mLN cells. Values are the mean ± SD of individual samples from two independent experiments with four individuals per group for each time post infection per experiment. Significantly different at * $p < 0.05$ *vs* sham infected animals.

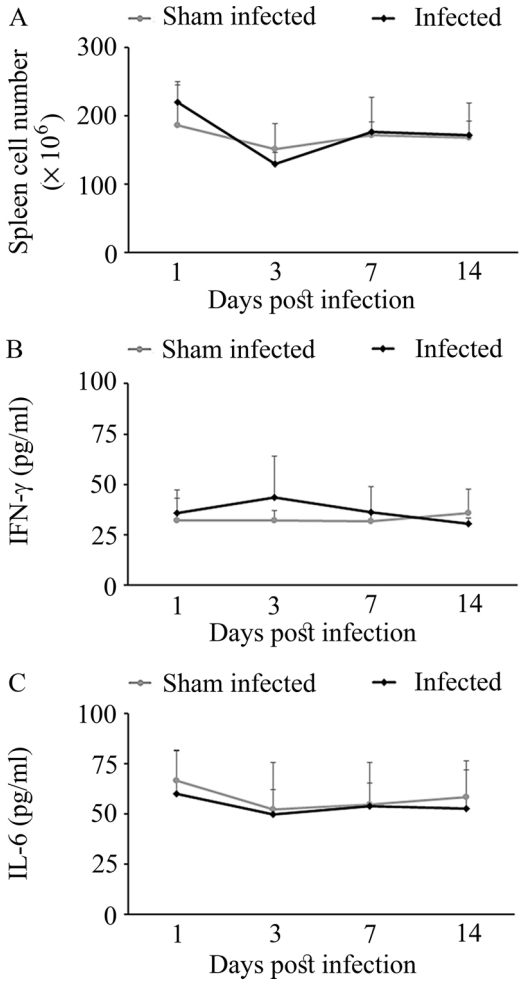

Fig. 4. Selected aspects of spleen activity and plasma IL-6 levels following intra tracheal *A. fumigatus* administration. (A) Spleen cell number. (B) IFN-γ production by spleen cells. (C) Plasma IL-6 levels. Values are the mean ± SD of individual samples from two independent experiments with four individuals per group for each time post infection per experiment.

changes in either of the parameters examined were observed.

3.6. Lack of the presence of A. fumigatus in the intestine

No *A. fumigatus* DNA was detected in the intestine of rats infected via respiratory route.

3.7. Changes in gut microbiota in rats with pulmonary A. fumigatus infection

Examination of gut bacterial diversity in settings of pulmonary *A. fumigatus* infection revealed microbiota alteration and the presence of novel visible bands on DGGE profiles in samples from infected rats. The highest disturbance of gut microbiota was detected at the day 1 after infection (Fig. 5A). In particular, the most visible DNA bands in DGGE profile of control samples (bands 3, 4, 5, 6, 7, 8, 9) were not detected in infected rats at day 1 after infection (samples 5 and 6), while novel visible bands became visible indicating the possible presence of opportunistic bacteria (sample 6). The mild restauration of microbiota was scored on the day 3 after infection, together with the appearance of new clearly visible bands that were not detected in control DGGE profiles (Fig. 5B, bands 10, 11, 12, 13, 14, 15, 16). Finally, gut microbiota diversity was relatively normalised on the days 7 and 14 after infection and DGGE profiles resemble to the control samples. With the aim to identify the main bacterial players detected at the day 3 after infection in DA rats but not in control samples, the 11 unique DNA fragment-bands were cloned and sequenced (Fig. 5B). A complete list of identified clones is given in Table 2. Interestingly, *Streptococcus parasanguinis / castoreus / gallinaceus* (band 10) and *Allobaculum stercoricanis / Turicibacter sanguinis*-like (band 11) belonging to phylum *Firmicutes*, were detected only on day 3 after infection, while *Lactobacillus reuteri* (band 19a and 20) *and L. garvieae* (band 19b), *Mobilitalea sibirica / Extibacter muris / Lachnoclostridium phytofermentans*-like (band 21a) and *Helicobacter* sp. (band 21b) were detected on the day 7 and/or 14 after infection. *Campilobacter* sp. (bands 1, 2 and 17) and *Lactobacillus*

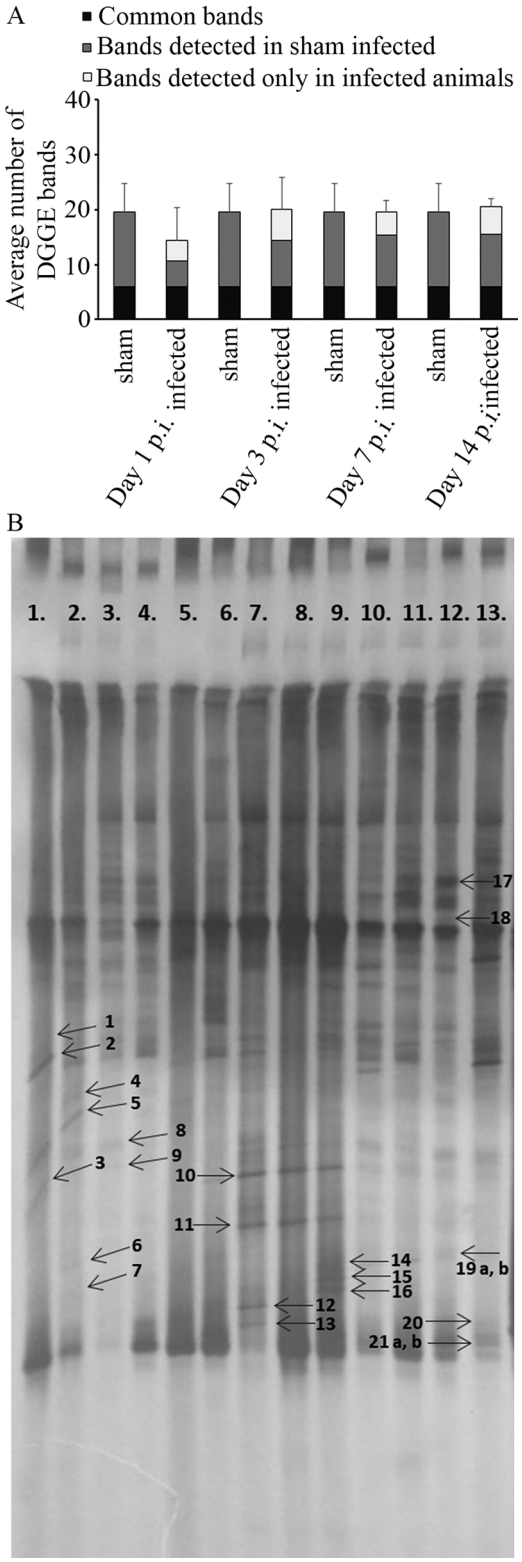

Fig. 5. Changes in gut microbiota in rats with pulmonary *A. fumigatus* infection (A) Average number of DGGE bands in sham infected and infected animals. Values are the mean ± SD of individual samples from two independent experiments with four individuals per group for each time post infection per experiment. (B) Representative DGGE profile of sham infected (samples 1–4) and infected animals at different time points following infection (day 1 p.i. samples 5 and 6; day 3 p.i. samples 7–9; day 7 p.i. samples 10 and 11; day 14 p.i. samples 12 and 13).

Table 2
Identified clones.

No. of clone	Species	Identity (%)	Phylum
1	*Campylobacter volucris*	99%	*Proteobacteria*
	Campylobacter subantarcticus	99%	
	Campylobacter jejuni	99%	
2	*Campylobacter volucris*	99%	*Proteobacteria*
	Campylobacter subantarcticus	99%	
	Campylobacter jejuni	99%	
10	*Streptococcus parasanguinis*	93%	*Firmicutes*
	Streptococcus castoreus	93%	
	Streptococcus gallinaceus	93%	
11	*Allobaculum stercoricanis*	90%	*Firmicutes*
	Turicibacter sanguinis	88%	
17	*Campylobacter subantarcticus*	99%	*Proteobacteria*
	Campylobacter jejuni	99%	
18	*Lactobacillus intestinalis*	95%	*Firmicutes*
19a	*Lactobacillus reuteri*	99%	*Firmicutes*
19b	*Lactococcus garvieae*	99%	*Firmicutes*
20	*Lactobacillus reuteri*	99%	*Firmicutes*
21a	*Mobilitalea sibirica*	89%	*Firmicutes*
	Extibacter muris	89%	
	Lachnoclostridium phytofermentans	89%	
21b	*Helicobacter marmotae*	95%	*Proteobacteria*
	Helicobacter mesocricetorum	95%	
	Helicobacter rodentium	95%	

intestinalis (band 18) were found in all samples regardless *A. fumigatus* infection.

4. Discussion

The effect of lung *A. fumigatus* infection on gut homeostasis was examined in this study. Data obtained showed for the first time that pulmonary infection with the fungus is associated with intestinal damage and inflammation and was accompanied by cytokine response of major gut-draining lymph nodes. It is not result of intestinal response to *A. fumigatus*, since fungus was not detected in the gut, judging by highly sensitive and rapid method (seminested PCR specifically amplifying internal transcribed spacer 1 (ITS1) and ITS2 and 5.8S rDNA in *Aspergillus* species, allowing detection of even 1 CFU per sample) (Ferrer et al., 2001). Instead, intestinal inflammation in rats infected with *A. fumigatus* seemed associated with changes in intestinal microbiota.

Mucosal homeostasis in gut is the result of interaction of mucus layer, epithelial cells, microbiota and gut-associated lymphoid tissue and changes in almost every of the components were noted in rats with pulmonary *A. fumigatus* infection. The described microscopic changes indicate seemingly light damage to the intestinal mucous membrane and induction of mononuclear inflammation starting from day 1 p.i. Connective tissue repair on the day 14 p.i. indicates irreversible damage to the intestinal mucous membrane, which led to the architectural disorder and narrowing of the bowel at that site. Changes in gut bacterial microbiota most probably contributed to disruption of homeostasis, given data which show the impact of bacterial microbiota on local (intestinal) immune-mediated homeostasis as well as on development of disease (Hooper et al., 2012; Palm et al., 2015). Infiltrated leukocytes probably contribute to the increase in IFN-γ and IL-17 content noted in intestine. Proinflammatory *milieu* in the intestine (increased IFN-γ and IL-17 and unchanged IL-10) might have further increased effector activities of immune cells in this tissue which in turn contributed to tissue damage. Depletion of goblet cells have resulted directly from changes in bacterial microbiota in analogy with data which showed differences in the numbers of these cells in germ-free compared to conventionally raised animals (Deplancke and Gaskins, 2001) or indirectly, as a result of proinflammatory cytokine response effects as shown in mice with intestinal bacterial infection (Bergstrom et al., 2008).

Intestinal damage might have accounted for stimulation of proinflammatory cytokine responses of mLN cells. Microenvironment of mLN in basal state is normally tolerogenic and establishes firewall between this lymphoid compartment and the rest of immune system (Macpherson and Smith, 2006). Increases of production of IFN-γ and IL-17 along with lack of changes of IL-10, imply the shift from immune tolerogenic towards proinflammatory *milieu* in this lymphoid compartment. In analogy with the data showing that induction of effector (IFN-γ- and IL-17-producing) T cells in Crohn's disease (CD) occurs in mLN (Sakuraba et al., 2009), this lymphoid tissue might be envisaged as site for generation of effector cells that subsequently infiltrated gut mucosa in rats with pulmonary *A. fumigatus* infection. Proinflammatory cytokine response most probably resulted from stimulation by products acquired from injured intestine and/or stimulation with intestinal microbial antigens which gained access into lymphatics and trafficked to mLN where they primed cytokine responses (Mowat, 2003). Absence of changes in spleen activity and lack of an increase in circulating IL-6 levels indicate that immune response to *A. fumigatus* is limited to mucosal tissues (lungs and gut).

Changes in intestinal bacterial microbiota noted in the present study are in line with data which showed the impact of viral (Wang et al., 2014; Deriu et al., 2016; Yildiz et al., 2018; Groves et al., 2018) and bacterial (Winglee et al., 2014) pulmonary infections on intestinal bacterial community in the absence of these microorganisms in the intestine. The influence of pulmonary *A. fumigatus* infection on gut microbiota was identified as decreased gut microbiota diversity, scored on the day 1 after infection. However, on the day 3 after *A. fumigatus* infection gut microbiota dysbiosis in DA rats was accompanied with the appearance of novel visible bands on DGGE profiles indicating the outgrowth of other microbial groups, such as *Streptococcus* sp. and *Allobaculum stercoricanis / Turicibacter sanguinis*-like bacteria, in the absence of core microbiota. Moreover, the presence of bacteria belonging to family *Lachnospiraceae* (*Mobilitalea sibirica / Extibacter muris / Lachnoclostridium phytofermentans*-like) was scored on the days 7 and 14 after infection. Similar results were reported previously pointing that core microbiota in DA rats (strain used in the present study) is less consistent compared to another rat strain and more susceptible to changes mediated by alterations in immunological status (Stanisavljević et al., 2016). Interestingly, *Turicibacter* sp. was previously detected in DA rats in the peak of experimental autoimmune encephalomyelitis (EAE) and correlated with increased butyric acid, recognized for specific immunosuppressive potential (Zhong et al., 2015). On the other hands, the *Lachnospiraceae* family members were linked to the alleviation of EAE symptoms (Stanisavljević et al., 2016). Thus, it could be speculated, that the increased abundance of *Turicibacter*-like bacteria and members of family *Lachnospiraceae* in gut microbiota of DA rats after fungal infection could be a part of host defense mechanism against intestinal inflammation. Interestingly, *Lactobacillus reuteri* and *L. garvieae* were detected on the days 7 and 14 after infection, possibly as an attempt of normalization of gut microbiota composition. Data which showed changes in bacterial intestinal community in rats infected with *A. fumigatus*, are in line with those which showed alterations of gut microbiota in settings of pulmonary infection with Influenza A (Deriu et al., 2016; Groves et al., 2018; Wang et al., 2014; Yildiz et al., 2018) or respiratory syncytial (RSV) viruses (Groves et al., 2018), as well as *M. tuberculosis* infection (Winglee et al., 2014). Outgrowth of bacteria belonging to phylum *Proteobacteria* (indicated by appearance of novel visible bands on DGGE profiles) following pulmonary *A. fumigatus* infection suggest that pulmonary fungal infection, as well as inflammation caused by viruses (Deriu et al., 2016; Wang et al., 2014), increase relative abundance of *Proteobacteria.* In contrast to data obtained in Influenza and *M. tuberculosis* infection which have shown decrease in relative abundance of *Firmicutes* (Deriu et al., 2016; Groves et al., 2018; Wang et al., 2014; Winglee et al., 2014) in gastrointestinal tract, our findings indicate that fungal infection increase the abundance of bacteria belonging to that phylum.

It can be assumed that gut microbiota is influenced by pulmonary *A. fumigatus* infection through proinflammatory cytokine response since *A. fumigatus* was not detected in gut samples. In line with this assumption is current view of interactions between the intestinal bacterial microbiota and the immune system in terms of the reciprocity in these interactions (Hooper et al., 2012; Maynard et al., 2012). Because *A. fumigatus* infection induced inflammatory cytokine response in the lungs (El-Muzghi et al., 2013) and is associated with intestinal inflammation as well, one can speculate about inflammatory cross-talk in intestinal involvement with pulmonary *A. fumigatus* infection. In this regard, the influence of acute lung inflammation evoked by LPS instillation was reported recently in mice (Sze et al., 2014). Of note, similar changes (inflammatory cell infiltration and cytokine responses) were observed both in the intestine and lungs of rats with experimental ulcerative colitis (Liu et al., 2013).

The cause of disturbances in gut bacterial microbiota in our study is not known. It is not caused by changes in diet/calorific intake as no differences were observed in body mass between sham-infected and infected rats. It might, at least partly, be ascribed to the reduction of goblet cell numbers, as changes in the mucus observed in mice with respiratory viral infections were proposed as one of the mechanisms (Groves et al., 2018). Stress of infection might be contributing mechanism, as gastrointestinal tract is known as particularly responsive to different stressors (Konturek et al., 2011). Migration of virus-specific T cell from lungs to the intestine and subsequent induction of gut dysbiosis and intestinal inflammation was shown in mice with respiratory infection (Wang et al., 2014). In this respect, redistribution of *A. fumigatus*-specific T cells to the gut is noted in IL-10 knock-out mice with pulmonary infection, though such a pattern of migration was actually induced by intestinal inflammation caused by IL-10 deficiency (Rivera et al., 2009). Contribution of molecules with antibacterial properties produced by *A. fumigatus* to gut dysbiosis could not be excluded, as it was shown that metabolites of this fungi exert inhibitory effects against Gram negative (*Pseudomonas aeruginosa*, *Salmonela typhimurium*) and Gram positive bacteria (*Listeria monocytogenes*) (Hassan and Bakhiet, 2017).

Alteration of gut microbiota in settings of pulmonary infection with Influenza A (Deriu et al., 2016; Groves et al., 2018; Wang et al., 2014; Yildiz et al., 2018) or respiratory syncytial (RSV) viruses (Groves et al., 2018), as well as *M. tuberculosis* infection (Winglee et al., 2014) together with presented data indicates that inflammation in lungs might be associated with gut dysbiosis. Data showing changes in gut microbiota following pulmonary administration of agents which induce inflammation (poly I:C and LPS) support such assumption (Deriu et al., 2016; Sze et al., 2014). Mechanisms by which inflammation induced in lungs affects intestinal homeostasis are not fully understood and warrant future attention.

In conclusion, data presented in this study showed for the first time that pulmonary infection with *A. fumigatus* is associated with intestinal damage, inflammation and induction of cytokine response of major gut-draining lymph nodes. Primed mLN cells might help protection of vulnerable intestine from gut dysbiosis, but can contribute to perturbation of local immune homeostasis as well. These effects might be perceived as price which has to be paid by immunocompetent host, but disruption of gut homeostasis during pulmonary infection might render gastrointestinal tract more susceptible to variety of physiological and pathological stimuli. Observed changes provide the baseline for future studies of the impact of fungal lung infections to gut homeostasis, particularly in individuals susceptible to these infections.

5. Conflict of interest

The authors declare no conflict of interest.

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