Article

Expression of toll-like receptors (TLR2 and TLR4) in the eyes of mice intranasally infected with *Acanthamoeba* sp.

Karolina Kot [1], Danuta Kosik-Bogacka [1,*], Natalia Łanocha-Arendarczyk [1], Agnieszka Wojtkowiak-Giera [2] and Agnieszka Kolasa-Wołosiuk [3]

[1] Department of Biology and Medical Parasitology, Pomeranian Medical University in Szczecin, 70-204 Szczecin, Poland; kotkarolina17@gmail.com (K.K.); nlanocha@pum.edu.pl (N.Ł.-A.)
[2] Department of Biology and Medical Parasitology, Poznan University of Medical Sciences, Fredry 10, 61-701 Poznan, Poland; awojtko@ump.edu.pl
[3] Department of Histology and Embryology, Pomeranian Medical University in Szczecin, 70-204 Szczecin, Poland; agnieszka.kolasa@pum.edu.pl
* Correspondence: kodan@pum.edu.pl; Tel.: +48-91-466-1672

Abstract: Toll-like receptors (TLRs) play a key role in the innate immune response to numerous pathogens, including *Acanthamoeba* sp. The aim of this study was to determine the expression of TLR2 and TLR4 in the eyes of mice following intranasal infection with *Acanthamoeba* sp. Amoebae used in this study were isolated from the bronchial aspirate of a patient with acute myeloid leukemia (AML) and atypical symptoms of pneumonia. We found statistically significant differences in the expression of TLR2 and TLR4 in the eyes of immunocompetent mice at 8, 16, and 24 days post *Acanthamoeba* sp. infection (dpi) compared to control. Immunosuppressed mice showed significant differences in the expression of TLR2 at 16 and 24 dpi compared to uninfected animals. Our results indicate that TLR2 and TLR4 are upregulated in the eyes of mice in response to *Acanthamoeba* sp. We suggest that it is possible for trophozoites to migrate through the optic nerve from the brain to the eyes.

Keywords: *Acanthamoeba* sp.; eyes; toll-like receptor 2 (TLR2); toll-like receptor 4 (TLR4)

1. Introduction

Parasitic infections can initiate both specific and non-specific immune responses. Toll-like receptors (TLRs) are a family of membrane receptors that play a key role in the non-specific immune response, recognizing pathogen-associated molecular patterns (PAMPs) common to most pathogenic microorganisms. Recognition and binding of PAMPs leads to dimerization or oligomerization of TLRs and recruitment of intracellular signaling molecules, including myeloid differentiation primary response 88 (MyD88), Toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif containing protein (SARM) [1,2]. The myeloid differentiation primary response gene (88) (MyD88) is a universal adaptor molecule in inflammatory pathways downstream of all TLRs, except TLR3. Binding of MyD88 to TLR leads to the activation of MAP kinases, including extracellular signal-regulated kinases ERK1/2 and transcription nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB), which controls the expression of genes encoding pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin-2 (IL-2) [3]. TIRAP mediates transmission of TLR2 and TLR4 signals and activates the MyD88-dependent pathways. TRIF binds to TLR3 and TLR4, activating an alternative pathway that leads to the

activation of NF-κB, MAP kinases, and interferon regulatory factor 3 (IRF3), which regulates the expression of type I interferons (IFN), mainly IFN-α [3].

Acanthamoeba sp. can be found in a wide variety of environments: soil, dust, air, natural water, tap water, drinking and bottled water, seawater, swimming pools, sewage, sediments, air-conditioning units, dental treatment units, hospitals and dialysis units, eyewash stations, and contact lenses. They also often infect bacterial, yeast, and mammalian cell cultures [4]. In earlier studies, antibodies for *Acanthamoeba* antigens were found in 50% to 100% of studied populations, indicating that contact with these pathogens is common [5,6]. *Acanthamoeba* sp. are the causative agents of granulomatous amoebic encephalitis (GAE), a fatal disease of the central nervous system (CNS), and amoebic keratitis (AK), a painful vision-threatening disease of the eyes [7,8]. Several species of *Acanthamoeba*, including *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. rhysodes*, *A. griffini*, *A. quina*, and *A. lugdunensis*, have been reported to cause AK [9]. AK is most common in contact lens wearers (>8%), occurring when lenses are stored contrary to the recommendations of doctors and manufacturers, as well as in patients with mechanical corneal damage [10,11]. Studies of *Acanthamoeba* keratitis found that the host immune system prevents the spread of amoebae into the eyeball and other organs through the recruitment of neutrophils [12-14]. However, there is some data showing infection of the eyeball in hosts with disseminated acanthamoebiasis [15].

The receptor responsible for immune recognition of *Acanthamoeba* sp. is TRL4 [16]. Based on *in vitro* and *in vivo* studies, it was found that TLR-4 is expressed during amoebic infection in AK. Activation of TLR4 stimulates the pathways TLR4- MyD88- NF-κB and TLR4-ERK1/2 to induce the secretion of inflammatory cytokines, including chemokine ligand 2 (CXCL2), IL-8, TNF-α, and IFN-α [16-18]. TLR activation also plays a significant role in directing helper T (Th) lymphocyte differentiation. The presence of TLR ligands mainly initiates a Th1 response, but may also lead to the appearance of induced regulatory T lymphocytes [19]. Recent studies on the AK mouse model showed that amoebic invasion induces Th17 lymphocytes and Treg lymphocytes in the cornea [20]. In our earlier studies on generalized acanthamoebiasis, induction of Th1, Th2, and Th17 expression was seen in immunocompetent hosts in the late stages of *Acanthamoeba* sp. infection, whereas in hosts with suppressed immunity, we observed a strong Th1 response, without Th17 [21].

Previous studies on the role of TLRs in acanthamoebiasis have focused mainly on the expression or activation of TLRs in the corneas of people with AK, after contact lenses with clinical isolates of *Acanthamoeba* were placed onto the center of corneas or the *Acanthamoeba* solution was applied to previously scratched corneas [16,18]. In this study, mice were infected intranasally with *Acanthamoeba* sp. Following infection, we determined the expression of TLR2 and TLR4 in eye structures.

2. Results

2.1. Expression of tlr2 and tlr4 genes

There were significant differences in the levels of mRNA expression of both TLR2 and TLR4 in immunocompetent mice (group A) at 8, 16, and 24 days post *Acanthamoeba* sp. infection, and in the expression of TLR2 in immunosuppressed mice (AS) between 16 and 24 dpi.

In immunocompetent mice (group A), TLR2 mRNA expression significantly increased at 8, 16, and 24 dpi compared to the uninfected controls (group C) ($p<0.01$, Fig. 1). At 24 days post *Acanthamoeba* sp. infection, TLR2 mRNA expression decreased compared to 8 dpi ($p<0.05$). In mice treated with the immunosuppressive drug (group AS), TLR2 mRNA expression increased significantly at 24 dpi ($p<0.01$). We found statistically significant differences in the expression of TLR2 between the uninfected mice (group C) and those observed at 16 ($p<0.05$) and 24 ($p<0.01$) days post *Acanthamoeba* sp. infection (group AS). Moreover, statistically significant differences in expression were observed between immunocompetent (group A) and immunosuppressed (group AS) mice at 0 ($p<0.05$), 8 ($p<0.05$), and 24 ($p<0.01$) days post *Acanthamoeba* sp. infection.

The expression of TLR4 in immunocompetent mice (group A) was increased at 8 ($p<0.05$), 16 ($p<0.05$), and 24 ($p<0.01$) days post *Acanthamoeba* sp. infection compared to uninfected mice (group C) (Fig. 2). Statistically significant differences were observed in the immunocompetent (group A) mice between 8 dpi and 24 dpi, and between 16 dpi and 24 dpi ($p<0.01$). In mice treated with the

immunosuppressive drug, no statistically significant differences were found in the level of TLR4 expression between uninfected mice (group CS) and those infected with *Acanthamoeba* sp. (group AS). Comparison of TLR4 expression levels between immunocompetent (group A) and immunosuppressed (group AS) mice showed a statistically significant difference only at 24 days post *Acanthamoeba* sp. infection ($p<0.01$).

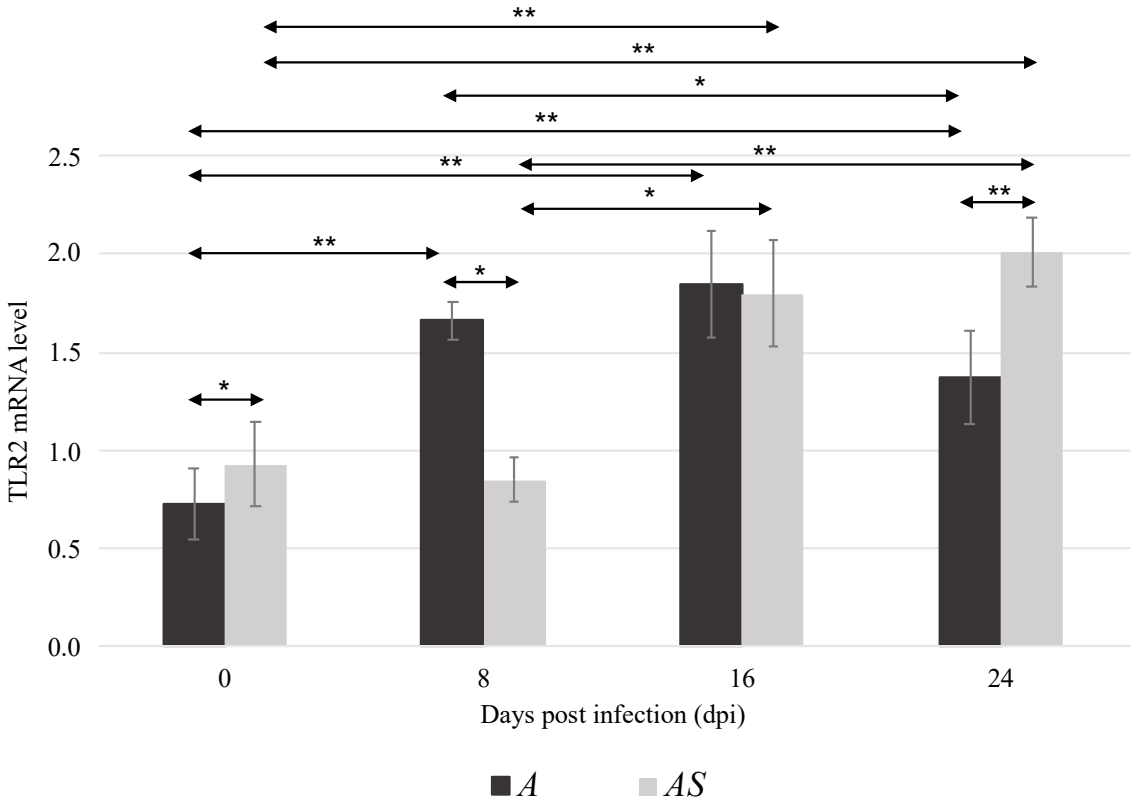

Figure 1. The mRNA expression of the *tlr2* gene in the eyes of uninfected (0 dpi) and infected mice at 8, 16, and 24 days post *Acanthamoeba* sp. infection (dpi), according to the immunological status of hosts (A - immunocompetent mice; AS - immunosuppressed mice). The data represent mean ±SD; *$p<0.05$, **$p<0.01$, compared with control value from uninfected mice (Mann–Whitney U test).

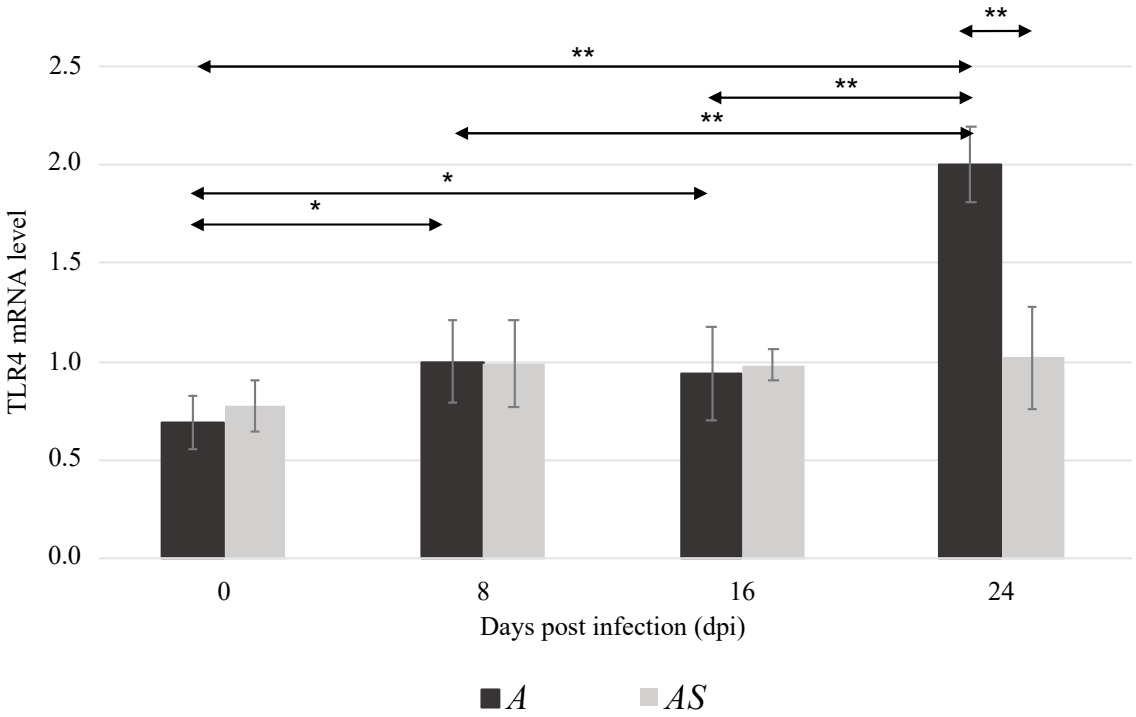

Figure 2. The mRNA expression of the *tlr4* gene in the eyes uninfected (0 dpi) and infected mice at 8, 16, and 24 days post *Acanthamoeba* sp. infection (dpi), according to the immunological status of hosts (A - immunocompetent mice; AS - immunosuppressed mice). Data represent mean ±SD; *p<0.05, **p<0.01, compared with control value from uninfected mice (Mann–Whitney U test).

2.2. Immunohistochemical (IHC) reaction

In uninfected immunocompetent (group C) mice, immunohistochemical detection of TLR2 was mainly localized to the corneal epithelium (Fig. 3A, black arrows), Bowman's membrane (Fig. 3A, red arrows), and corneal endothelium (Fig. 3A, blue arrows). TLR2 expression in *Acanthamoeba* sp.–infected immunocompetent (group A) mice was detected in the same structures as in the control group. The highest expression was observed at 8 dpi (Fig. 3E).

Brown pigmentation indicating TLR4 immunohistochemical staining was observed in the corneal epithelium (black arrows), Bowman's membrane (red arrows), corneal stroma (white asterisks), and corneal endothelium (blue arrows). The level of expression of TRL2 in uninfected immunosuppressed (group CS) mice was comparable to that of uninfected immunocompetent (group C) controls. In *Acanthamoeba* sp.–infected immunosuppressed (group AS) mice, TLR2 was expressed in all corneal layers, with the highest expression levels observed at 16 dpi. In immunosuppressed (group AS) mice at 8 and 24 days post *Acanthamoeba* sp. infection, the corneal endothelium (Fig. 3G and Fig. 3O) and Descemet's membrane (Fig. 3O) were found as TLR2-negative at 24 dpi.

In the retinae of immunocompetent (group A) and immunosuppressed (group AS) mice, TLR2 expression was observed in the optic nerve fiber layer (white arrows), the inner and outer plexiform layers (green and orange arrows, respectively), and the rods and cones layer (pink arrow) (Fig. 3). TLR2 immunoexpression was most intense at 8 and 16 days post *Acanthamoeba* sp. infection (Fig. 3F and Fig. 3J). Changes in immunoexpression of TLR2 were also observed in the visual part of the retina in immunosuppressed mice (group AS). The highest TLR2 expression levels were observed at 24 dpi (Fig. 3P).

Expression of TLR4 in the corneas of uninfected immunocompetent (group C) mice was similar to that of TLR2. Similarly, the highest TLR4 expression levels were seen at 24 dpi (Fig. 4). TLR4 expression at 24 dpi was observed in all corneal layers, including the corneal epithelium (black arrow), Bowman's membrane (red arrow), corneal stroma (white star), posterior limiting

(Descemet's) membrane (yellow arrow), and corneal endothelium (blue arrow). In immunosuppressed mice (group CS), the weakest expression of TLR4 was observed at 8 dpi (Fig. 4G) and the strongest at 16 dpi (Fig. 4K) and 24 days post amoeba infection (Fig. 4O). In the cornea, expression of both TLR2 and TLR4 was found only in the perinuclear space.

In the visual part of the retina in the eyes of immunocompetent and immunocompetent mice, expression of TLR4 was observed in the optic nerve fiber layer (white arrow), the inner plexiform layer (green arrow), the outer plexiform layer (orange arrow), and rods and cons layer (pink arrow) (Fig. 4). In immunocompetent hosts, the intensity of the immunohistochemical reaction increased 8 days after *Acanthamoeba* sp. infection (Fig. 4F), then fell at 16 dpi (Fig. 4J) and increased again, reaching a maximum at 24 dpi (Fig. 4N). Brown pigmentation indicating immunohistochemical detection of TLR4 in the visual part of the retina of immunosuppressed animals (groups AS or CS) was different than that observed for TLR2. The weakest expression of TLR4 was observed in the control group (C) and was visible only in the layer of rods and cones (Fig. 4D). The intensity of TLR4 expression increased with the duration of infection (Fig. 4H, L, P). No TLR2-positive or TLR4-positive cells were found in the ganglion cell layer or in the inner or outer nuclear layers in either immunocompetent or immunocompetent mice.

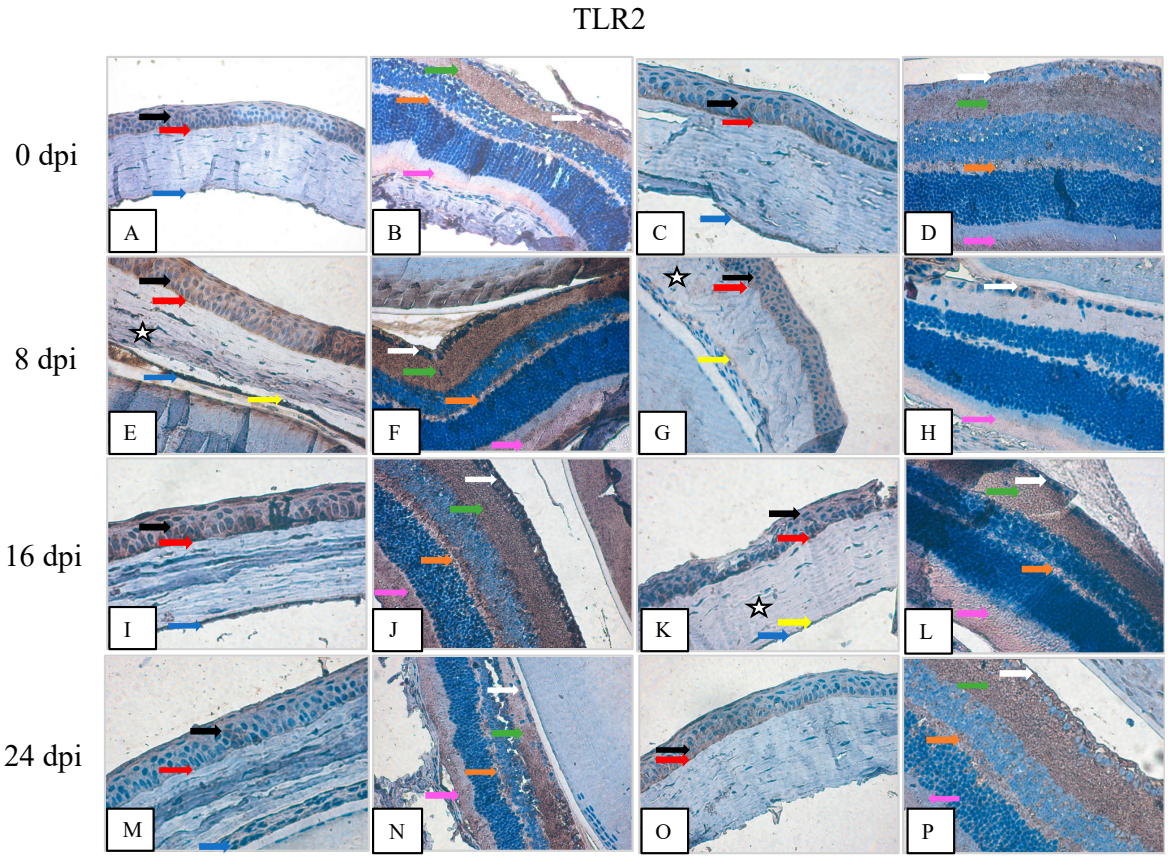

Figure 3. Immunohistochemical staining with primary anti-TLR2 antibodies in the corneas (A, C, E, G, I, K, M, O) and retinae (B, D, F, H, J, L, N, (P) of immunocompetent (A, B, E, F, I, J, M, N) and immunosuppressed mice (C, D, G, H, K, L, O, P) from control groups (0 dpi) and at 8, 16, and 24 days post *Acanthamoeba* sp. infection (dpi). Magnification x40 (black arrows - corneal epithelium; red arrows – external limiting membrane, Bowman's membrane; white stars – stroma of cornea, substantia basalis; yellow arrows – Descemet's membrane; blue arrows - corneal endothelium; white arrows – optic nerve fiber layer; green arrows - inner plexiform layer; orange arrows - outer plexiform layer; pink arrows - rods and cones layer.

Peer-reviewed version available at BioMed Research International 2019; doi:10.1155/2019/1401894

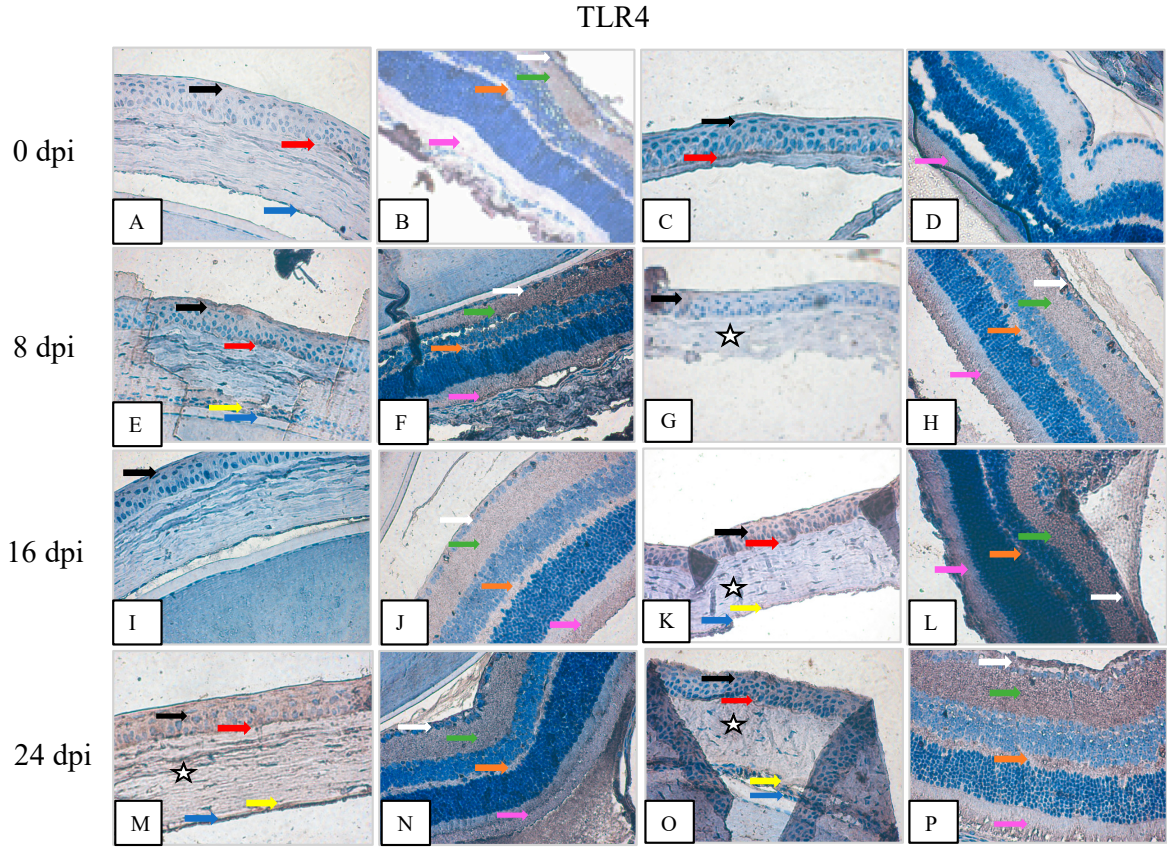

Figure 4. Immunohistochemical staining with primary anti-TLR4 antibodies in the corneas (A, C, E, G, I, K, M, O) and retinae (B, D, F, H, J, L, N, (P) of immunocompetent (A, B, E, F, I, J, M, N) and immunosuppressed mice (C, D, G, H, K, L, O, P) from control groups (0 dpi) and at 8, 16 ,and 24 days post *Acanthamoeba* sp. infection (dpi). Magnification x40 (black arrows - corneal epithelium; red arrows – external limiting membrane, Bowman's membrane; white stars – stroma of cornea, substantia basalis; yellow arrows – Descemet's membrane; blue arrows - corneal endothelium; white arrows - optic nerve fiber layer; green arrows - inner plexiform layer; orange arrows - outer plexiform layer; pink arrows - rods and cones layer.

3. Discussion

Many authors have reported an important role for TLRs in protective immunity against *Acanthamoeba* infection. For instance, in a study where mice were infected with *Acanthamoeba* sp. isolated from a patient with *Acanthamoeba* keratitis and from environmental water samples, TLR2 and TLR4 showed increased expression in pneumocytes, interstitial cells, and epithelial cells of the bronchial tree [22]. Increased TLR2 and TLR4 expression was also observed in neurons, glial cells, and endothelial cells within the neocortex [23]. Derda et al. [22] observed that in the lungs of mice infected with *Acanthamoeba* sp., the expression of TLR2 was higher than the expression of TLR4. Moreover, the authors observed increased expression of TLR2 and TLR4 from 2 to 30 days post *Acanthamoeba* sp. infection.

Experimental models of amoebic corneal inflammation are created by introducing trophozoites through intracorneal or intraconjunctival injection, deposition into the conjuctival cul-de-sac, or topical application to an abraded corneal surface [24]. In human and laboratory animals corneal epithelial cell lines, it was found that TLR4 plays a major role in corneal inflammation caused by *Acanthamoeba* sp. infection [16-18]. *In vitro*, immunological interactions between this amoeba and the corneal epithelium and corneal stroma were also found to increase expression of TLR2 [17]. However, on repeating the experiment in the rat model, no increase in TLR2 expression at either the mRNA or protein level [18] was found. In the present study, more statistically significant differences in mRNA-level expression were observed for TLR2 than TLR4 in the eyeballs of mice inoculated with

Acanthamoeba. Johnson et al. [25] demonstrated previously that the activation of TLR2 by certain ligands induced neutrophil recruitment and increased corneal thickness. Our histological examination of the cornea did not reveal inflammatory cells in studied mice; however, we observed an increased corneal thickness due to an increase in the number of layers of stratified nonkeratinized squamous epithelium.

The level of mRNA expression of both TLR2 and TLR4 in immunocompetent animals was significantly higher at 8, 16, and 24 dpi compared to the control group. In immunocompromised mice, statistically significant differences in TLR2 expression compared to uninfected animals were seen at 16 and 24 dpi. In terms of TLR4 receptor expression, among immunosuppressed by methylprednisolone mice, no statistically significant differences were found in comparison to levels at 0 dpi. Jin et al. [26] showed that hydrocortisone increased expression of TLR2 and TLR4 in human corneal epithelial cell lines (HCEC), while in human corneal fibroblasts (HCF), expression of these receptors was decreased. The authors suggest that topical treatment with steroids may promote opportunistic corneal infections by inhibiting the release of the innate immune response mediators through the interaction of TLR with this glucocorticosteroid. Hara et al. [27] showed that dexamethasone can increase HCEC susceptibility to viral infections by altering signaling pathways of Toll-like receptors. In our study, mice were immunosuppressed by administration of the glucocorticosteroid methylprednisolone. Statistically significant differences were observed between the expression of TLR2 and TLR4 in immunocompetent and immunosuppressed mice. TLR2 expression was higher in the AS group compared to the A (unsuppressed) group and increased with long-term amoeba infection (24 dpi). Regarding to TLR4, the expression of this receptor in immunosuppressed mice was reduced at 24 dpi compared to the immunocompetent group.

Differences between the levels of expression observed in this paper and those of other authors' papers may result from differences in the method of infection, type of immunosuppressant, our performance of analyses in all structures of the eye, and/or differences between amoeba strains were used.

Injection of amoeba trophozoites into the anterior chamber of the eye did not cause morphological changes in the retina or posterior chamber of the eye [28]. However, in another study, an injection of *Acanthamoeba* into the corneal stroma led to severe encephalitis in some animals [29]. This is explained by the belief that amoebae can migrate along corneal nerves [30]. Chandra et al. [15] described the case of a patient with a normal immune response diagnosed with meningitis caused by *Acanthamoeba* sp., in whom examination of the posterior part of the eyeball revealed subarachnoid inflammation surrounding the optic nerve. Moreover, trophozoites of *Acanthamoeba* sp. were found in the perioral space and optic nerve. In this study, *Acanthamoeba* sp. were reisolated from the eyeballs and optic nerves of mice infected with amoebae.

Confocal microscopy and histological analysis of the cornea in established cases of *Acanthamoeba* keratitis show corneal oedema, presence of inflammatory cells in the corneal stroma, trophozoites and cysts of *Acanthamoeba* sp. in all intraepithelial and stromal layers of the cornea, as well as regional stromal necrosis [18,29,31]. In a patient with *Acanthamoeba* keratitis, Kato et al. [32] observed polymorphonuclear leukocytes in the corneal stroma, an abscess in the granulation tissue at the sclera near the ciliary body, and macrophages and lymphocytes surrounding blood vessels. There was no inflammation of the retina and vascular system. In our study, despite the fact that *Acanthamoeba* sp. was administered intranasally, we found morphological changes in the eyeballs of immunosuppressed mice, including invagination of Bowman's membrane into the substantia propria of corneal stroma and an increase in the number of layers of stratified nonkeratinized squamous epithelium. Moreover, we found morphological changes of the ciliary body and dilatation of nuclear layers of retina. However, no inflammatory cells or amoeba developmental forms were found in eyeball structures.

4. Materials and Methods

4.1. Animal model

Our experimental animal model has been described in our previous research [33,34]. Adult male, Balb/c mice (~23 g, 6-10 months, Center of Experimental Medicine, Medical University in Białystok, Poland) were housed individually on a 12h:12h light/dark cycle under controlled temperature with free access to food and water.

All animal procedures were carried out in accordance with established practices for laboratory animal work according to the 'Guide for the Care and Use of Laboratory Animals.

The mice (n=96) were divided into four groups:

- group C - uninfected immunocompetent mice (control group; n=18);
- group CS - uninfected mice treated with an immunosuppressive drug (n=18);
- group A - *Acanthamoeba* sp. infected immunocompetent mice (n=30);
- group AS - *Acanthamoeba* sp. infected mice treated with an immunosuppressive drug (n=30).

Mice from groups A and AS were infected by intranasal inoculation with 3 µl of suspension containing 10-20 thousand of *Acanthamoeba* sp. strain AM22 isolated from a patient with acute myeloid leukemia (AML) and atypical pneumonia [35]. Cultures were incubated at 37 °C in NN Agar covered with a suspension of deactivated *Escherichia coli* according to standard protocol [36]. Animals from control groups (C and CS) were given the same volume of saline (3 µl 0.9% NaCl). To suppress immunity, AS and CS animals were intraperitoneally administered (i.p.) 0.22 mg (10 mg/kg body weight) methylprednisolone sodium succinate (MPS, Solu-Medrol, Pfizer, Europe MA EEIG) dissolved in 0.1 mL 0.9% saline daily for four days before inoculation with *Acanthamoeba* sp. [33,37].

Mice were sacrificed by sodium pentobarbital (Euthasol vet, FATRO) injection administered intraperitoneally at 2 mL/kg body weight. The mice were weighed, and then eyes were removed for analysis. The virulence of *Acanthamoeba* sp. was determined by the degree of infection. Eye were placed on NN agar and incubated at 41°C to assess infection intensity [38]. Observations of the culture were performed daily for 10 days using a light microscope (x10).

The study was approved by the Local Ethical Committee for Experiments on Animals (No. 29/2015 and 64/2016).

4.2. Isolation of RNA and conversion od cDNA by reverse transcription

The expression of toll-like receptor (TLR2 and TLR4) genes at the mRNA level in the eyes of mice from all groups was examined using reverse transcription polymerase chain reaction (RT-PCR). Tissues were homogenized in liquid nitrogen, and total RNA was isolated according to the manufacturer's instructions (Qiagen, Germany); more analytical procedures are given by Wojtkowiak-Giera et al. [23].

4.3. Real-time PCR

TLR 2 and TLR 4 gene expressions in the eyes was measured by quantitative real-time polymerase chain reaction (Q-PCR). Q-PCR was carried out in a LightCycler realtime PCR detection system from Roche Diagnostic GmbH (Mannheim, Germany) using SYBR Green I as detection dye and target cDNA was quantified by relative quantification using a calibrator; more analytical procedures are given by Wojtkowiak-Giera et al. [23].

4.4. Immunohistochemical staining

Samples fixed in 4% buffered formalin solution (Avantor, Poland; cat. no.:432173111) were subsequently embedded in paraffin and cut into sections of 4-µm thickness. The sectioned tissue was deparaffinized in microwave and irradiated with citrate buffer (pH 6.0) to induce epitope retrieval. After slow cooling to room temperature, slides were washed in PBS twice for 5 min and then incubated with primary antibodies overnight (4 C). Immunohistochemistry was performed using

specific primary rabbit polyclonal antibodies against TLR2 and TLR4 (Santa Cruz Biotechnology, Inc., cat. no. sc-10739 and sc-30002, respectively) in a final 1:500 dilution. Sections were stained with an avidin-biotin-peroxidase system with diaminobenzidine as the chromogen (DakoCytomation, Code K0679), performed according to staining procedure instructions included. Sections were washed in distilled H_2O and counterstained with hematoxylin. For a negative control, specimens were processed in the absence of primary antibodies. Positive staining was defined by visual identification of brown pigmentation using light microscope (Leica, DM5000B, Germany).

4.5. Statistical analysis

Statistical analysis was performed using StatSoft Statistica 10.0 and Microsoft Excel 2016. Intergroup comparisons were performed using Mann-Whitney U test. The significance level was $p<0.05$.

5. Conclusions

The present study indicates that TLR2 and TLR4 are upregulated in the eyes of mice in response to *Acanthamoeba* sp. infection. The observed changes in the expression of both toll-like receptors may confirm involvement of the innate immune system in the pathomechanism of intranasally-triggered acanthamoebiasis. The results suggest that it may be possible for trophozoites to migrate through the optic nerve from the brain to the eyeball.

Author Contributions: Kot Karolina, Kosik-Bogacka Danuta and Łanocha-Arendarczyk Natalia conceived and designed the research. Kot Karolina, Kosik-Bogacka Danuta and Łanocha-Arendarczyk Natalia performed the experiments; Kot Karolina, Wojtkowiak-Giera Agnieszka and Kolasa-Wołosiuk Agnieszka analyzed the data; Kot Karolina and Kosik-Bogacka Danuta contributed to writing the manuscript. All authors read and approved the final manuscript.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

A	immunocompetent *Acanthamoeb*a sp.-infected mice
AK	*Acanthamoeba* keratitis
AM 22	amoebic strain no.22
AML	Acute myeloid leukemia
AS	immunosuppressed *Acanthamoeb*a sp.-infected mice
C	immunocompetent uninfected control group mice
CNS	central nervous system
CS	immunosuppressed uninfected mice
CXCL2	Chemokine (C-X-C motif) ligand 2
Dpi	days post infection
ERK 1/2	extracellular signal-regulated kinase
GAE	granulomatous amebic encephalitis
HCEC	human corneal epithelial cell lines
HCF	human corneal fibroblasts
IFN	interferon
IHC	Immunohistochemical reaction
IL-2	interleukin-2
IL-8	Interleukin-8
IRF3	interferon regulatory factor 3
MAP	mitogen-activated protein kinases
MPS	methylprednisolone sodium succinate
NF-κB	nuclear transcription factor
MyD88	Myeloid Differentiation primary response 88
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction

RT-PCR	Reverse transcription PCR
Q-PCR	Real-time PCR
SARM	Sterile-alpha and Armadillo modif containing protein
Th	T helper cells
Th1	Type 1 T helper
Th2	Type 2 T helper
Th17	Type 17 T helper
TIR	Toll-interleukin-1 receptor
TIRAP	Toll-interleukin-1 receptor domain-containing adapter
TLR	Toll-like receptor
TLR2	Toll-like-2 receptor
TLR3	Toll-like-3 receptor
TLR4	Toll-like-4 receptor
TNF-α	tumor necrosis factor α
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon β

References

1. Mishra, B.B.; Gundra, U.M.; Teale, J.M. Toll-like receptors in CNS parasitic infections. *Curr Top Microbiol Immunol* **2009**, 336, 83-104. doi: 10.1007/978-3-642-00549-7_5.
2. Portou, M.J.; Baker, D.; Abraham, D.; Tsui, J. The innate immune system, toll-like receptors and dermal wound healing: A review. *Vascul Pharmacol* **2015**, 71, 31-36. doi: 10.1016/j.vph.2015.02.007.
3. Kawai, T.; Akira, S. TLR signaling. *Semin Immunol* **2007**, 19, 24-32.
4. De Jonckheere, J. F. Ecology of *Acanthamoeba. Rev Infect Dis* **1991,** 13, S385-S387.
5. Cerva, L. *Acanthamoeba culbertsoni* and *Naegleria fowleri*: occurrence of antibodies in man. *J Hyg Epidemiol Microbiol Immunol.* **1989**, 33, 99-103.
6. Cursons, R.T.; Brown, T.J.; Keys, E.A.; Moriarty, K.M.; Till, D. Immunity to pathogenic free-living amoebae: role of humoral antibody. *Infect Immun*. **1980**, 29, 401-407.
7. Martinez, A.J.; Visvesvara, G.S. Free-living, amphizoic and opportunistic amebas. *Brain Pathol*. **1997**, 7, 583-598.
8. Nagington, J.; Watson, P.G.; Playfair, T.J.; McGill, J.; Jones, B.R.; Steele, A.D. Amoebic infection of the eye. *Lancet* **1974**, 2, 1537-1540.
9. Schaumberg, D.A.; Snow, K.K.; Dana, M.R. The epidemic of *Acanthamoeba* keratitis: where do we stand? *Cornea* **1998**, 17, 3-10.
10. Król-Turmińska, K.; Olender, A. Human infections caused by free-living amoebae. *Ann Agric Environ Med.* **2017**, 24, 254-260. doi: 10.5604/12321966.1233568.
11. Kot, K.; Łanocha-Arendarczyk, N.A.; Kosik-Bogacka, D.I. Amoebas from the genera *Acanthamoeba* and their pathogenic properties. *Ann Parasitol* **2018**, 64, 299-308.
12. Harrison, J.L.; Ferreira, G.A.; Raborn, E.S.; Lafrenaye, A.D.; Marciano-Cabral, F.; Cabral, G.A. *Acanthamoeba culbertsoni* Elicits Soluble Factors That Exert Anti-Microglial Cell Activity. *Infect Immun* **2010**, 78, 4001-4011. doi: 10.1128/IAI.00047-10.
13. Knickelbein, J.E.; Kovarik, J.; Dhaliwal, D.K.; Chu, C.T. *Acanthamoeba* keratitis: a clinicopathologic case report and review of the literature. *Hum Pathol*. **2013**, 44, 918-922. doi: 10.1016/j.humpath.2012.10.007.
14. Neelam, S.; Niederkorn, J.Y. Pathobiology and Immunobiology of *Acanthamoeba* Keratitis: Insights from Animal Models. *Yale J Biol Med*. **2017**, 90, 261-268.
15. Chandra, S.R.; Adwani, S.; Mahadevan, A. *Acanthamoeba* meningoencephalitis. *Ann Indian Acad Neurol*. **2014**, 17, 108-112. doi: 10.4103/0972-2327.128571
16. Alizadeh, H.; Tripathi, T.; Abdi, M.; Smith, A.D. Pathogenic strains of *Acanthamoeba* are recognized by TLR4 and initiated inflammatory responses in the Cornea. *PLOS One* **2014**, 9, 3. doi: 10.1371/journal.pone.0092375
17. Ren, M.; Gao, L.; Wu, X. TLR4: the receptor bridging Acanthamoeba challenge and intracellular inflammatory responses in human corneal cell lines. *Immunol Cell Biol.* **2010**, 88, 529-536. doi: 10.1038/icb.2010.6.
18. Ren, M.Y.; Wu, X.Y. Toll-like receptor 4 signalling pathway activation in a rat model of *Acanthamoeba* Keratitis. *Parasite Immunol*. **2011**, 33, 25-33. doi:10.1111/j.1365-3024.2010.01247.x.

19. Grygorowicz, M.A.; Kozłowska, E. Involvement of receptors recognizing pathogen-associated molecular patterns – TLRs in modulation of regulatory T cell CD4+CD25+FoxP3+ activity. *Post Mikrobiol* **2011**; 50: 141-154.
20. Suryawanshi, A.; Cao, Z.; Sampson, J.F.; Panjwani, N. IL-17A-mediated protection against *Acanthamoeba* keratitis. *J Immunol* **2015**, 194, 650-663. doi: 10.4049/jimmunol.1302707.
21. Łanocha-Arendarczyk, N.; Kolasa-Wołosiuk, A.; Wojciechowska-Koszko, I.; Kot, K.; Roszkowska, P.; Krasnodębska-Szponder, B.; Paczkowska, E.; Machaliński, B.; Łuczkowska, K.; Wiszniewska, B.; Kosik-Bogacka, D. Changes in the immune system in experimental acanthamoebiasis in immunocompetent and immunosuppressed hosts. *Parasit Vectors* **2018**, 11, 517. doi: 10.1186/s13071-018-3108-x.
22. Derda, M.; Wojtkowiak-Giera, A.; Kolasa-Wołosiuk, A.; Kosik-Bogacka, D.; Hadaś, E.; Jagodziński, P.P.; Wandurska-Nowak, E. *Acanthamoeba* infection in lungs of mice expressed by toll-like receptors (TLR2 and TLR4). *Exp Parasitol.* **2016**, 165, 30-34. doi: 10.1016/j.exppara.2016.02.012.
23. Wojtkowiak-Giera, A.; Derda, M.; Kolasa-Wołosiuk, A.; Hadaś, E.; Kosik-Bogacka, D.; Solarczyk, P.; Jagodziński, P.P.; Wandurska-Nowak, E. Toll-like receptors in the brain of mice following infection with *Acanthamoeba* spp. *Parasitol Res* **2016**, 115, 4335-4344.
24. van Klink, F.; Alizadeh, H.; He, Y.; Mellon, J.A.; Silvany, R.E.; McCulley, J.P.; Niederkorn, J.Y. The role of contact lenses, trauma, and Langerhans cells in a Chinese hamster model of *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci.* **1993**, 34, 1937-1944.
25. Johnson, A.C.; Heinzel, F.P.; Diaconu, E.; Sun, Y.; Hise, A.G.; Golenbock, D.; Lass, J.H.; Pearlman, E. Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol Vis Sci*. **2005**, 46, 589-595.
26. Jin, X.; Qin, Q.; Tu, L.; Qu, J. Glucocorticoids inhibit the innate immune system of human corneal fibroblast through their suppression of toll-like receptors. *Mol Vis*. **2009**, 15, 2435-2441.
27. Hara, Y.; Shiraishi, A.; Kobayashi, T.; Kadota, Y.; Shirakata, Y.; Hashimoto, K.; Ohashi, Y. Alteration of TLR3 pathways by glucocorticoids may be responsible for immunosusceptibility of human corneal epithelial cells to viral infections. *Mol Vis*. **2009**, 15, 937-948.
28. Clarke, D.W.; Niederkorn, J.Y. The immunobiology of *Acanthamoeba* keratitis. *Microbes Infec* **2006**, 8, 1400–1405.
29. Feng, X.; Zheng, W.; Wang, Y.; Zhao, D.; Jiang, X.; Lv, S. A Rabbit Model of *Acanthamoeba* Keratitis That Better Reflects the Natural Human Infection. *Anat Rec* **2015**, 298, 1509-1517. doi: 10.1002/ar.23154.
30. Alfawaz, A. Radial keratoneuritis as a presenting sign in *Acanthamoeba* keratitis. *Middle East Afr J Ophthalmol*. **2011**, 18, 252-255.
31. Füst, Á.; Tóth, J.; Simon, G.; Imre, L.; Nagy, Z.Z. Specificity of in vivo confocal cornea microscopy in *Acanthamoeba* keratitis. *Eur J Ophthalmol*. **2017**, 27, 10-15. doi: 10.5301/ejo.5000817.
32. Kato, K.; Hirano, K.; Nagasaka, T.; Matsunaga, K.; Takashima, Y.; Kondo, M. Histopathological examination of *Acanthamoeba* sclerokeratitis. *Clin Ophthalmol*. **2014**; 8: 251-253. doi:10.2147/OPTH.S54807
33. Łanocha-Arendarczyk, N.; Baranowska-Bosiacka, I.; Kot, K.; Gutowska, I.; Kolasa-Wołosiuk, A.; Chlubek, D.; Kosik-Bogacka, D. Expression and Activity of COX-1 and COX-2 in Acanthamoeba sp.-Infected Lungs According to the Host Immunological Status. *Int J Mol Sci.* **2018**, 19. doi: 10.3390/ijms19010121.
34. Łanocha-Arendarczyk, N.; Baranowska-Bosiacka, I.; Kot, K.; Pilarczyk, B.; Tomza-Marcianiak, A.; Kabat-Koperska, J.; Kosik-Bogacka, D. Biochemical Profile, Liver and Kindey Selenium (Se) Status during Acanthamoebiasis in a Mouse Model. *Folia Biologica* **2018**, 66, 33-40. doi.org/10.3409/fb_66-1.04.
35. Lanocha, N.; Kosik-Bogacka, D.; Maciejewska, A.; Sawczuk, M.; Wilk, A.; Kuźna-Grygiel, W. The occurrence *Acanthamoeba* (free living amoeba) in environmental and respiratory samples in Poland. *Acta Protozool.* **2009**, *48*, 271–279.
36. Culbertson, C.G.; Ensminger, P.W.; Overton, W.M. The isolation of additional strains FF pathogenic *Hartmanella* sp. (*Acanthamoeba*): Proposed culture method for application to biological material. *Am. J. Clin. Pathol.* **1965**, *43*, 383–387.
37. Markowitz, S.M.; Sobieski, T.; Martinez, A.J.; Duma, R.J. Experimental *Acanthamoeba* infections in mice pretreated with methylprednisolone or tetracycline. *Am. J. Pathol.* **1978**, *92*, 733–744.
38. Górnik, K.; Kuźna-Grygiel, W. Histological studies of selected organs of mice experimentally infected with *Acanthamoeba* spp. *Folia Morphol.* **2005**, *64*, 161–167.