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Phagocytosis in *Mesocestoides vogae*-induced peritoneal monocytes/macrophages via opsonin-dependent or independent pathways

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Summary

Intraperitoneal infection with larvae of cestode *Mesocestoides vogae* offers the opportunity to study dynamic changes in the proportion and functions of individual cell types under a direct influence of parasites. The phagocytic activity is one of the basic effector functions of professional phagocytes and receptor-mediated uptake is a central in implementation of inflammatory responses. Present study extends information on this issue by exploring several phagocytosis pathways in *M. vogae*-induced myelo-monocytic cells. In addition, we analyzed proportions of morphologically distinct phenotypes within macrophage compartments after oral inoculation of larvae to mice. In gradually elevated population of peritoneal exudate cells, monocytes/ macrophages and giant cell were dominant cell types from day 21 p.i. Phagocytic activity of these cells had biphasic behaviour for both opsonin-dependent and independent pathways, whereas uptake by multinucleated macrophages was profoundly reduced. Highly elevated proportions of activated phagocytic cells were found from day 7 to 14 p.i., regardless particle type (latex beads, HEMA, liposomes) and opsonisation. Source of opsonins used for coating of liposomes suggested higher expression of complement receptors than Fc receptors on these cells, although the uptake of non-opsonized liposomes had different kinetics and was very high by activated cells early p.i. Present data indicate that early recruited macrophages/monocytes attain pro-inflammatory functions as indicated by highly elevated phagocytosis of immunologically inert particles as well as opsonized liposomes what is down-regulated once larvae start to proliferate in the peritoneal cavity, suggesting the role of parasite-derived molecules in modulation of this key phagocytes function.

Keywords: phagocytosis; *Mesocestoides vogae*; mice; latex beads; HEMA particles; liposomes; opsonization

Introduction

Macrophages are innate immune cells that play a key role in tissue homeostasis, presentation of foreign and self-antigens following infection and injury, pathogen clearance, resolution of inflammation and wound healing (Ruckerl & Allen, 2014; Hume, 2008; Murray & Wynn, 2011). During infections immature monocytes are released from bone marrow into circulation, migrate into peripheral tissues. Following antigen stimulation they differentiate into mature macrophages and/or dendritic cells and replenish resident cell population (Mantovani *et al*., 2007). Macrophages are strategically located throughout the body and express a multitude of receptors on their surfaces that detect “non-self” signals which are later eliminated via phagocytosis. Phagocytosis is the process that cells

have evolved to internalize large particles such as mineral debris, which they store, or apoptotic cells and pathogens, which they have the capacity to kill and degrade upon activation (Janeway & Travers, 1994). The recognition of phagocytic targets is mediated by specific receptors on phagocytes that either recognize serum components (opsonins) bound to the particles or directly identify molecular determinants on the target. Opsonized pathogens are recognized and internalized by receptors at the Fc portion of immunoglobulins, iC3b complement and/or fibronectin (Reichner *et al*., 2001; Ezekowitz *et al*., 1984; Niedergang & Chavrier, 2004), whereas effector cells bind to the surface of opsonised multicellular parasites and phagocytosis is restricted to the glycocalyx surface and tegumental glycoconjugates (Walbaum *et al*., 1994; Alvarez *et al*., 2008). Activation of cells usually occurs upon recognition of Pathogen or Damage-Associated Molecular Pattern (PAMP/DAMP) via different receptors such as Toll-like receptors (TLRs) on their surface (Murray & Wynn, 2011). Many studies using various particular assays systems concluded that the interplay between phagocytic receptors (which initiate and assist in the mechanism of phagocytosis) and Pattern Recognition Receptors (PRRs) (which detect "non self" or "damage" structures) is very complex (Desjardins & Griffiths, 2003; Stuart & Ezekowitz, 2005). Importantly, receptors not only trigger an engulfment but also act to define the pro-inflammatory or anti-inflammatory cell phenotype. Macrophage mannose receptor and dectin-1 receptor are probably the best characterized PRRs recognizing carbohydrate structures and plasticity of their expression reflects cell activation phenotypes (Stein *et al*., 1992; Saijo & Iwakura, 2011). The scavenger receptor A (SR-A) and macrophage receptor with collagenous structure (MARCO) are two well-characterized non-opsonin phagocytic receptors present on myeloid cells (Peiser *et al*., 2002; Kraal *et al*., 2000). Class B scavenger receptor type-I is responsible for binding of apoptotic, necrotic cells and cell debris after recognition of phosphatidylserine on dying cells (Osada *et al*., 2006).

Populations of macrophages are typically classified as M1 or classically activated, versus M2 or alternatively activated what make distinction between cytotoxic and regulatory activity, respectively (Edwards *et al*., 2006; Mosser & Edwards, 2008). Classically activated macrophages (M1) are generally formed in response to the IFN-γ and TNF-α and are characterized by the expression of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, promotion of Th1 response and strong microbicidal and tumoricidal activity. Cytokines such as IL-4 and IL-13 are important regulatory cytokines in Th2 associated immunity, and promote development of alternatively activated macrophages (M2). These cells appear to be involved in immunosuppression, tissue repair and remodelling, tumor progression and also have immunoregulatory functions (Gordon, 2003; Martinez *et al*., 2009; Mosser & Edwards, 2008). Importantly, the M2 macrophages are associated with helminth infections and type 2 immunity, where incredible variability in species- and stage-specific patterns of responses can be observed (Kreidr *et al*., 2007; Ruckerl & Allen, 2014). The M2 macrophages phagocytic ability differs depending on the particle and receptor involved (Wolf & Underhill, 2014) and some authors demonstrated that IL-4 cytokine reduces phagocytosis in M2 cells (Varin *et al*., 2010; Moreno *et al*., 2007).

The metacestode stage of cestode *Mesocestoides vogae* (syn. *M. corti*) is characterized by the rapid asexual multiplication of the larvae (tetrathyridia) in the liver and peritoneal cavity of vertebrate hosts (Specht & Voge, 1965; Crosbie *et al*., 1998) and the murine infection has often been used as an experimental model for medically important larval tapeworm infections in immunological and pharmacological studies (i.e. Hrčková & Velebný, 2014; O´Connell *et al*., 2009; Maggiore & Elissondo, 2014). The increasing number of larvae in the liver and the peritoneal cavity is associated with massive accumulation of inflammatory cells in the site of infection (O´Connell *et al*., 2009). Recently we have shown (Vendelova *et al*., 2015) that macrophages from this infection site express M2 markers, namely the FIZZ-1, RELM-α (Ym-1) and Arg-1 (Chang *et al*., 2001; Raes *et al*., 2002) and study of their gene activation revealed dynamic changes in their expression as infection has been progressing. Similarly, we found that *M. vogae* derived antigens can directly induce M2-like phenotype *in vitro*.

In the present study we monitored distribution of myelo-monocytic cell phenotypes in the peritoneal cavity of mice with *M. vogae* infection within the period of 5 weeks. In addition we evaluated capacity of these cells for ex vivo phagocytosis of inorganic particles and liposomes, either non-opsonized or opsonized, to assess impact of infection.

Material and Methods

Animals and Infection

ICR mice (male, 6 – 8-weeks old) were obtained from the own breeding at Animal Unit of the Institute of Parasitology, Slovak Academy of Sciences. Animals were housed under the pathogen-free conditions in a room with 12 hr light/dark cycle, temperature 22 ± 2 ºC. Food and water were provided *ad libitum* and all experiments were carried out according to the principles described by the Law No. 23/2009 of the Slovak Republic for the Care and Use of Laboratory Animals. All procedures involving mice were approved by the ethic committee of the State Veterinary Administration of Slovak Republic. Infection with *M. vogae* tetrathyridia is maintained in ICR mice by the serial passage of larvae obtained from the peritoneal cavity of mouse with 4 – 5 months old infection. For all experimental infections, mice were inoculated with 60 – 70 larvae in PBS by the oral gavage.

Preparation of M. vogae products

M. vogae tetrathyridia were cultured in serum-free medium containing 100U/ml of penicillin, 100 μg/ml of streptomycin and 50 μM 2-mercaptoethanol. The culture supernatant was collected every 24 hr, sterilized through 0.22 μm pore-size filter (Sartorius, Germany), concentrated and buffer exchanged with the phos-

phate-buffered saline (PBS) through 3-kDa column (Merck Millipore, USA) and termed as excretory/secretory products (ES). To obtain somatic homogenate (MvH) *M. vogae* tetrathyridia from *in vitro* culture were extensively washed and mechanically squeezed with glass slides in cold PBS. The supernatant from homogenized larvae was collected and sterile-filtered through 0.22 µm pore-size filter. Protein concentration was determined by Bradford protein assay using a commercial reagent (BioRad, USA). Both parasite preparations were shown to be endotoxin-free.

Experiments and isolation of peritoneal exudate cells
Larvae and peritoneal exudate cells (PEC) were obtained from peritoneal cavities of mice by the lavage into RPMI-1640 medium (Biochrom-Merck, Germany) on days 7, 14, 21, 28, 35 post infection (p.i.) in the first experiment (n=5/day) and on days 3, 7, 10, 14, 21 and 35 p.i. in the second experiment (n=4/day). Following red cells lysis with 1.4 % NH_4Cl at room temperature for 10 min, peritoneal cells were washed with RPMI-1640 medium and counted. Cells without serum addition were re-suspended in RPMI-1640 medium with antibiotics (100 U/ml of penicillin, 100 µg/ml of streptomycin) (Biochrom-Merck, Germany) to obtain the final dilutions used in the particular phagocytosis assay. Second experiment was carried out twice, firstly to study phagocytosis of HEMA particles and secondly, the uptake of liposomes. Detailed protocols are described later. Peritoneal cells from pathogen-free ICR mice served as the naive control (N/0).

Cell Staining
PEC isolated from mice on selected days p.i. in the first experiment were used also for preparation of cell smears. Following fixation in 70 % methanol in PBS (pH 6.8), cells were stained with May-Grünwald/Giemsa stains according to the standard protocol. Leukocyte differential counts were determined by scoring 500 cells on each slide at 1000x magnification and peritoneal cells from 4 – 5 mice per experimental day were used for enumeration of leukocyte subpopulations.

Phagocytosis of latex beads
In the first experiment latex (polystyrene) beads of diameter 1.1 µm (Sigma, USA) were used without pre-opsonisation. PEC collected from each animal at indicated time points were washed and viable cells were counted using a Neubauer chamber under trypan blue exclusion.
Viable cells were re-suspended at the concentration of $1x10^6$/ml in complete RPMI medium (RPMI 1640, Biochrom-Merck, Germany) with 10 % heat-inactivated foetal calf serum (Biochrom-Merck, Germany), 100 U/ml of penicillin (Sigma, USA), 100 µg/ml of streptomycin (Sigma, USA), 2 mM L-glutamin (Sigma, USA) and 50 µM 2-mercaptoethanol (Sigma, USA). A 0.5 ml of cell suspension was placed into round-bottom polystyrene tubes (in duplicates). Then 5 µl of latex beads containing approximately $5x10^6$ particles (diluted 1:10 in complete RPMI medium) were added into cell suspensions. Phagocytosis was assessed after incubation at 37 °C with gentle agitation in water bath for 1hr. Then the reaction was stopped and non-phagocytized particles were removed by extensive washing in cold PBS. Cell smears stained with May-Grünwald/Giemsa stains were prepared and examined under a microscope (Olympus, Czech Republic). Phagocytosis rate was calculated by counting at least 300 random myelo-monocytic cells per sample and from 2 replicates per condition/day. The results were expressed as cells with phagocytic capacity (percentage) that ingested more than 3 latex beads, where total number of counted cells were used to obtain an average number of latex beads/cell. Negative control cells where treated on ice.
To determine whether the larval antigens themselves can influence phagocytosis of latex beads, PEC pooled from naïve ICR mice were used in the same dilution in complete RPMI 1640 medium. Cells were first treated with antigens: ES (20 µg/ml) or McH (20 µg/ml) or positive control LPS (0.1 µg/ml, E. coli 0127:B8, Sigma, USA) or medium alone for 2 hr at 37 °C. Alternatively, cells were pre-treated with parasite antigens 2 hr prior to addition of LPS. After that latex beads were added to treated cell suspensions and assayed as described above.

Preparation of [^{3}H]-cholesterol labelled liposomes
Liposomes are widely used as tailor-made delivery vesicles prepared from various phospholipids and other substances, acting as carriers of water- and lipid-soluble compounds for pharmaceutical, cosmetic and biochemical purposes (Ulrich *et al*., 2002). Since these amphiphilic phospholipids are natural components of mammal´s bodies they represent universal and non-toxic lamellar vesicles suitable also for the study of cell functions. In this study multilamellar negatively charged liposomes were prepared as described previously (Hrčková & Velebný, 1995) from dipalmitoylphosphatidycholine (DPPC), cholesterol (Chol) and dicetylphosphate (DCP) in molar ratio 7:2:1. All lipids were purchased from Serva Fine Biochemica GmbH and Co. (Germany). [^{3}H]Cholesterol (specific activity 555 MBq/mg; radioactivity concentration 37 MBq/ml) was purchased from Amersham (UK). Labelling of the lipid phase was done by adding 7.9 µl (0.297 MBq) of [^{3}H] cholesterol solution per 100 mg of liposomal lipids dissolved in the organic solvents. Incorporation of radioactive label was measured before and after dialysis against PBS (pH 7.2) in aliquots of liposome samples using Rackbeta 1217 scintillation counter (LKB, UK). The mean radioactivity per mg/liposomal lipids was calculated from 4 aliquots and expressed for 1 µg of liposomal lipids. Size distribution of liposomal particles was determined on Coulter Counter Model D (Coulter Electronics, UK) and expressed as numbers of particles in 1 µl what allowed us to calculate the proportion of liposomal fractions (%) from the total number of particles. Liposome suspension was stored under nitrogen atmosphere at 4 °C.

Phagocytosis of liposomes
The same liposomal suspension was used throughout the experi-

ment. The size distribution analysis revealed that the most frequent liposome particles were of diameter < 0.8 μm (45.0 ± 4.8 %) and in interval between 0.8 to 1.9 μm (38.2 ± 5.9 %). *In vitro* assays were carried out with cell suspensions isolated from the peritoneal cavities of mice on selected days p.i. in the second experiment. Cells were washed and diluted to the concentration of $1x10^6$ cells/ml in serum-free medium. Then 1 ml of cell suspensions was plated into 24 well-plate in quadruplicates and incubated for 4 hr at 37 °C to allow monocytes/macrophages attachment. Non-adherent cells were removed by washing with PBS and phagocytic assay was performed in 0.5 ml serum-free RPMI medium. Aliquots of liposomal suspensions were incubated for 30 min at 37 °C in the equal volume of one of following mice sera to allow opsonisation with serum components. Treatment A represented incubation of liposomes in mouse serum obtained from pathogen-free mice of ICR strain, further termed "normal" serum and without heat-inactivation of complement. Treatment B was performed using heat-inactivated-normal mouse serum (heated at 55 °C for 30 min) and treatment C represented liposomes incubated in hyper-immune serum isolated from mice with chronic *M. vogae* infection. Liposomal suspensions (untreated or opsonised) were added to the wells with adherent cells without washing step at concentration of 60 μg of liposomal lipids/well and cultivated for another 2 hr at 37 °C. Then cells were washed 3 times in cold PBS and lysed with 1 ml of 10 mM NaOH/0.1 %Tritox X-100 (v/v) overnight at 4 °C. Cell lysates (20 μl) were used for protein determination using Bradford protein reagent (Bio-Rad, USA) and in the rest of samples radioactivity was measured. Finally, proportion of liposomes taken up by macrophages was calculated as ng of liposomal lipids per μg of cell proteins.

Phagocytosis of HEMA particles

Phagocytic activity of naive and post-infection peritoneal monocytes/macrophages was measured by HEMA (2-hydroxymethyl methacrylate polymer) particles with diameter of 1.2 μm (USOL, Czech Republic). The method, originally described by Toman and Pšikal (1985) for evaluation of blood monocytes phagocytosis was used with following modifications. Peritoneal cells were diluted in RPMI serum-free medium to the concentration of $1x10^6$ cells/ml, then 200 μl of cell suspensions were placed into round-bottom polystyrene tubes (in duplicates) and 50 μl of particles suspension (in PBS) were added to each tube. Cells were incubated at 37 °C in water bath with gentle agitation for 1 hr and then processed as it is described for assay with latex beads. Mean phagocytic activity was calculated after counting 300 cells per sample and assayed in duplicates for each mouse (n=8). Results are expressed as phagocytic activity (PA) representing percentage of cells containing more than 3 HEMA particles from total counted cells. Index of phagocytic activity (IPA) was calculated as the average number of phagocytosed particles per phagocytosing cells from total particles counted.

Statistical analysis

All data were analyzed with GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) by non-parametric one-way ANOVA for data sets obtained at the same experimental settings (phagocytosis of latex beads and HEMA, non-opsonized liposomes) following by Tukey post-hoc test. Differences in uptake of opsonised liposomes were calculated by two-way ANOVA and Bonferroni post-hoc test and are indicated in section Results. All results are expressed as means ± standard deviation (SD) and P levels are indicated in the text.

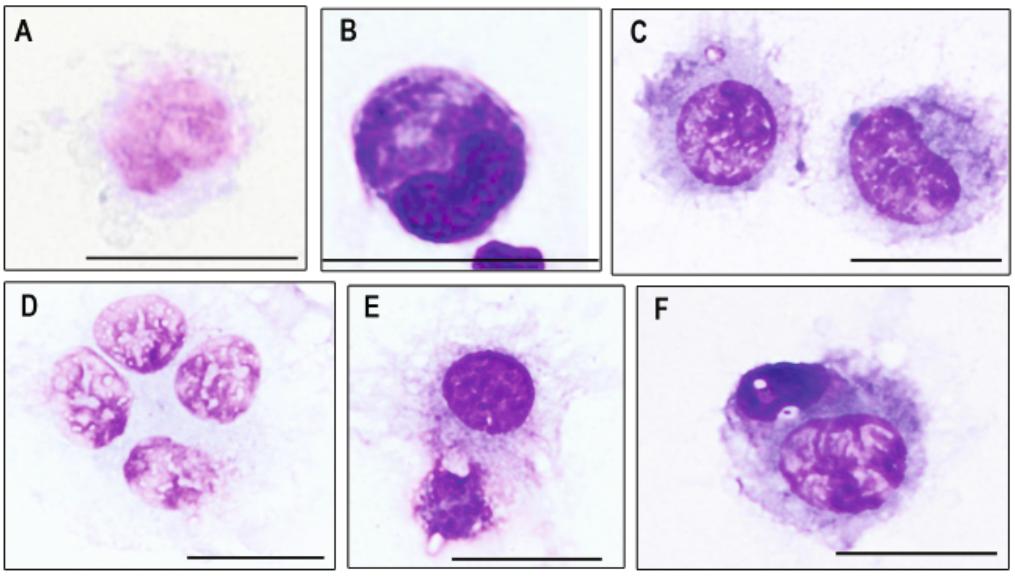

Fig. 1. Microscopic immages of myelo-monocytic cells from the peritoneal cavities of mice with *M. vogae* infection. Peritoneal cells were isolated on selected days and cell smears were stained with May-Grünwald/Giemsa stains. (A) resident macrophage from non-infected mouse, (B) monocyte, day 7 p.i., (C) inflammatory macrophages, day 35 p.i., (D) multinucleated "giant" macrophage, (E) macrophage attached to polymorphonuclear cell initiating its phagocytosis (F). Scale bar = 20 μm

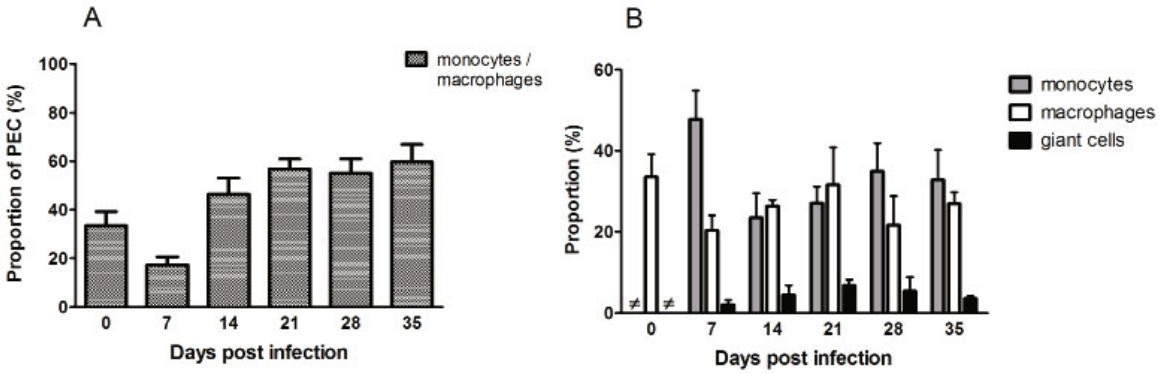

Fig. 2. Proportion (%) of monocytes/macrophages/giant cells in the peritoneal cavities of non-infected and *M. vogae* infected ICR strain of mice. (A) proportions of macrophage–like cells from total isolated peritoneal exudate cells (PEC) within 5 weeks p.i., (B) proportion of morphologically distinct phenotypes of macrophages/ monocytes/giant cells from total macrophage-like cells. Data shown for day 0 represent peritoneal cells from non-infected mice

Results

Proportion of macrophages

Resident population of peritoneal macrophages in ICR mice represents around 30 – 40 % of total cell population what in numbers equals around 9 x 10^5 cells. Upon standard May-Grünwald/Giemsa staining these non-inflammatory macrophages (~15 µm in diameter) with condensed nuclei showed affinity to acidic part of stain resulting in light pink colour (Fig. 1A). Monocytes (~10 µm) could be distinguished by their affinity to "basophilic" stain compounds resulting in dark blue nuclei and light blue cytoplasm (Fig. 1B). Morphology of post-infectious macrophages was distinct as compared with residential ones judged by the size (~20 – 25 µm), big nuclei and fluffy appearance (Fig. 1C). All parameters pointed towards their activation (Adams, 1974). These cells resembled epithelioid cells with indistinct shape contour and stronger affinity to the acidic stain compounds. Epithelioid cells can form giant cells with 3 to 5 nuclei (Anderson, 2000) (Fig. 1D) and they were present within peritoneal exudates from day 7 p.i. representing around 10% of myelo-monocytic cell compartment. Differential counts of cells reveal that as infection progressed, several macrophage-like cells were detected to ingest small polymorphonuclear cell (Fig. 1E, F). In the initial phase of inflammation (day 7 p.i.) proportion (%) of monocytes/macrophages from total PEC declined in the favour of other inflammatory cells such as dendritic cells, lymphocytes and eosinophils (not shown, Vendelova *et al.*, 2015). With progressing infection and larval counts, proportion of macrophage-like cells from total PEC gradually increased (Fig. 2A). Within this cell compartment, the high numbers of monocytes seen on day 7 p.i. reflected the typical result of inflammation after their recruitment from the blood stream (Fig. 2B). Giant cells represented minor proportion of myelo-monocytic cell compartment.

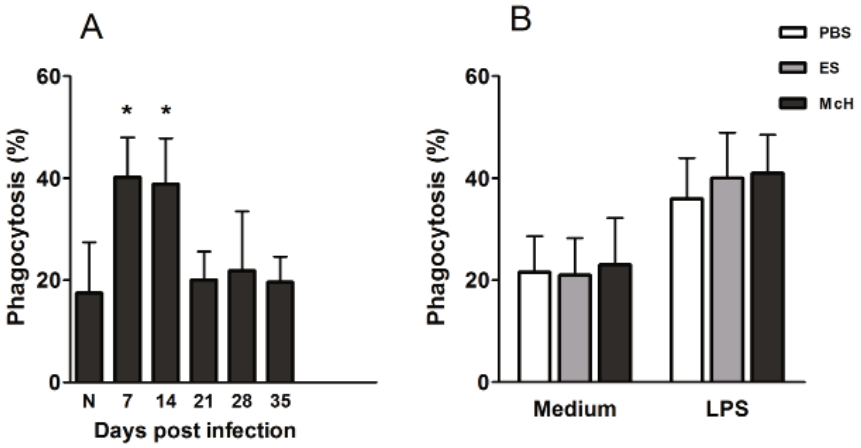

Fig. 3. Phagocytosis of latex beads by peritoneal mononuclear phagocytes. (A) *M. vogae*-induced peritoneal cells were incubated with latex beads and mononuclear phagocyte populations were then assayed for phagocytosis by microscopy and expressed as percentage of phagocytic cells. (B) Peritoneal cells were pre-treated with ES (20 µg/ml), MvH (20 µg/ml) or medium and LPS as a control before addition of latex beads. Alternatively, latex beads were added to peritoneal cells together with LPS. Data represent mean ± SD of 4-5 independent in vitro experiments. *Significant difference (P <0.05) between data for naïve and post-infectious cells

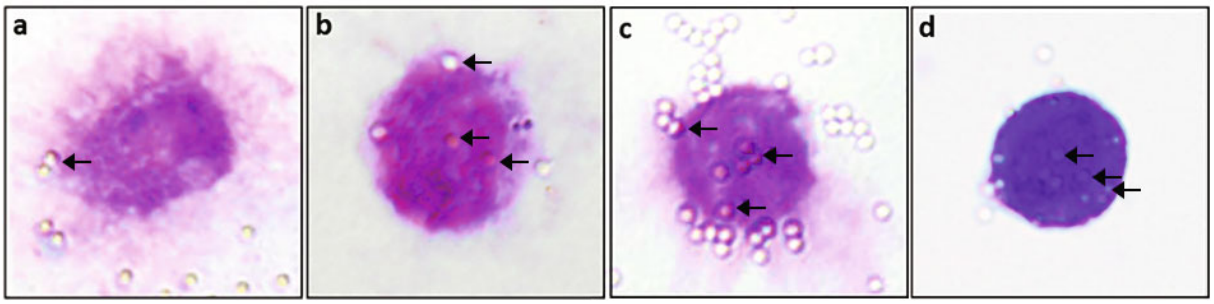

Fig. 4. Microphotographs of monocytes/macrophages after incubation with latex beads (lxb) in vitro. Representative cells stained with May Grünwald/Giemsa stains that ingested lxb. (a) 0 lxb, (b) 2 lxb, (c) 5 lxb, (d) 8 lxb. Arrowhead indicates the ingested lxb (dull appearance), arrows indicate beads outside the cell (brighter, unstained). (x1000)

Macrophage phagocytosis of latex beads

To test non-specific phagocytic ability of mononuclear phagocytes from the site of infection, peritoneal cells isolated from uninfected and infected mice were exposed to latex beads for 1 hr. The proportion of macrophages with ingested particles (n ≥ 3) is shown in Fig. 3A, and their appearance is shown on Fig. 4 (a-d). Results demonstrate that the ability to take up beads increased at the early stage of infection up to day 14 p.i. (40.2 ± 7.8 %; 38.8 ± 9.4 %) as compared to uninfected control (17.4 ± 10.0 %), (P<0.01). Peritoneal phagocytes showed a significant decrease in phagocytic ability from day 21 p.i. as compared to day 14 p.i. However, phagocytic ability did not drop below values seen in control. In our settings we did not observe phagocytosis of latex beads by multi-nucleated giant cells (data not shown).

To determine whether larval antigens, both secreted (ES) and somatic (MvH) were interfering with the phagocytic ability of macrophage/monocytes, cells were cultured with medium and LPS in the presence or absence of *M. vogae* antigen prior to exposure to non-opsonized latex beads. The results demonstrated that pre-incubation with antigens alone did not influence phagocytosis as compared to PBS or LPS treated cells (Fig. 3B).

HEMA particles phagocytosis

In the second experiment we compared *in vitro* non-specific phagocytic activity of naive and post-infectious peritoneal monocytes/macrophages to internalize HEMA particles (USOL, Czech Republic) in serum-free medium. Results summarized in Table 1 showed that as many as 53.3 ± 3.2 % of cells from naive mice were able to phagocytose (IFA, 6.40 ± 0.9). The proportion of cells with ingested non-opsonized particles significantly increased within 14 days p.i., peaking on day 7 p.i. (88.4 ± 5.9 %) and day 14 p.i. (76.6 ± 2.9 %). In contrast to the uptake of latex beads, the uptake of HEMA particles remained significantly elevated throughout the experiment comparing to naive phagocytes (P< 0.05).

Liposomes phagocytosis

To investigate capacity of post-infectious myelo-monocytic cells for opsonin-receptors mediated phagocytosis, liposomes were pre-incubated in various mice sera and their negative surface charge allowed opsonisation with various serum components including complement and immune complexes (Chonn *et al*., 1991; Aramaki *et al*., 1994). In parallel assays we monitored the uptake of unmodified liposomes via non-opsonin receptors/endocytosis as well as

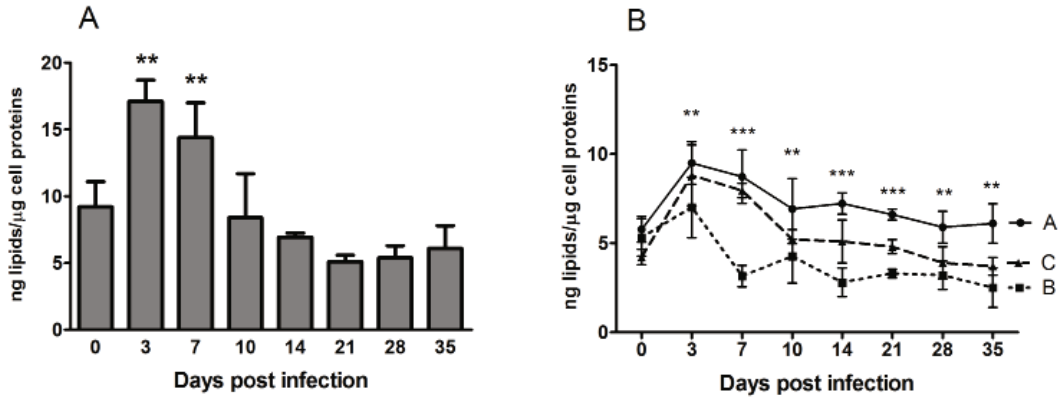

Fig. 5. Phagocytosis/ endocytosis of [^{3}H] cholesterol-labelled liposomes either non-opsonized or opsonised with serum components by peritoneal mononuclear phagocytes. Adherent monocytes/macrophages from non-infected (day 0 p.i.) and *M. vogae*-infected mice were incubated with untreated or serum pre-treated liposome suspensions (60 µg of liposomal lipids/well) and proportion of internalized particles was expressed as ng liposomal lipids/mg of cell proteins. (A) uptake of untreated liposomes by adherent monocytes/macrophages. **Significant difference (P <0.01) between uptake of naive and post-infectious phagocytes. (B) uptake of liposomes opsonised with normal mice serum (line A), with HI-normal mice serum (line B) and serum from mice with chronic *M. vogae* infection and without heat-inactivation (line C). Data are means ± SD of four in vitro tests on cells from 4 mice/day. Significant difference between treatment A and B, **(P <0.01), ***(P <0.001)

Table 1. Phagocytosis of HEMA particles by peritoneal mononuclear phagocytes. Peritoneal cells isolated from non-infected mice and mice inoculated orally with *M. vogae larvae* were incubated with HEMA particles and mononuclear phagocyte populations were assayed ex vivo for phagocytic activity (FA) by microscopy, which was expressed as percentage of phagocytic cells from total counted cells. Index of phagocytic activity (IPA) was calculated as the average number of phagocytosed particles per phagocytosing cells from total particles counted. Data are mean ±SD after counting 300 cells per sample and assayed in duplicates for each mouse (n=8). Significantly different values from naïve cells isolated from non-infected mice (N): *** P<0.001; ** P<0.01; * P<0.05

Days p.i.	N	3	7	10	14	21	28	35
FA	53.3 ± 3.2	82.1 ± 4.8 ***	88.4 ± 5.9 ***	69.3 ± 3.5 **	76.6 ± 2.9 **	65.0 ± 1.8 *	61.2 ± 3.1 *	62.7 ± 2.8 *
IFA	6.4 ± 0.9	10.6 ± 0.6 **	12.1 ± 1.1 ***	6.8 ± 0.3	6.9 ± 0.4	7.1 ± 1.5	7.4 ± 0.9 *	6.2 ± 2.5

the uptake of opsonized liposomes to assess this process via opsonin receptors on cells (presumably complement receptors and Fc receptors). As shown in Fig. 5A, significantly higher uptake of non-opsonized liposomes by adherent post-infectious monocytes/macrophages was recorded on day 3 and 7 p.i. (17.1 ±1.6 and 14.4 ± 2.7, resp.) in comparison with naive peritoneal macrophages (9.2 ± 1.9) (P<0.01) expressed as ng lipids/μg cell proteins. In contrast to the phagocytosis of latex beads, uptake of liposome particles declined gradually, being significantly lower than in naive macrophages from day 14 p.i. As shown on Fig. 5B, uptake of liposomes pre-incubated with different sera had different kinetic, except of similar phagocytosis of naïve macrophages. Uptake of liposomes after treatment A (fresh serum from non-infected mice) and C (chronically infected mice) was the most active in cells collected from peritoneal cavities within first seven days p.i. (9.51± 1.2 and 8.80 ± 1.7, resp.), remaining higher for liposomes coated with normal fresh serum. The significantly lower (P< 0.05) uptake of liposomes pre-incubated in HI-normal mouse serum was found when compared with uptake of liposomes incubated in normal mouse serum during the whole examined period, indicating important role of opsonin receptors.

Discussion

Intraperitoneal infection with larvae of *M. vogae* offers the opportunity to study many aspects of cestodes innate and adaptive immune responses. In particular, dynamic changes in the proportion and functions of individual cell types can be assessed. The phagocytic activity, one of the basic effector functions of professional phagocytes represented by monocytes and macrophages has not been investigated in *M. vogae*-infected peritoneal cavities. Moreover, the capacity of multinucleated macrophages "giant cells" for phagocytosis has been investigated in several granulomatous diseases (Anderson, 2000; Boros, 1989), but not yet in this model infection. Present study extends the knowledge on this issue by exploring several phagocytosis pathways in *M. vogae*-induced or recruited peritoneal myelo-monocytic cells. We also analyzed changes in their proportions from total cell numbers in the peritoneal cavities as well as the proportions of morphologically distinct phenotypes within macrophage compartments after oral inoculation of larvae to the mice. Total number of peritoneal exudates cells has increased gradually with progressing infection, representing approximately 60 – 70 million cells within the first month p.i. (Vendelova *et al*., 2015; O´Connel *et al*., 2009). Except of day 7 p.i., proportion of macrophages/monocytes elevated and they were dominant cell types from day 14 p.i. onwards. This may be the specific for this strain and route of infection. In contrast, after intraperitoneal inoculation of *M. vogae* larvae to the "high response" SJL strain of mice, Lammas *et al*. (1990) found lower proportions of macrophages in account of eosinophils, from second week p.i. We were able to distinguish between recruited monocytes and macrophages according their appearance and affinity to type and pH of dyes and on day 7 p.i. monocytes dominated among mononuclear myeloid cells. From this day giant cells, representing multinucleated macrophage phenotype typical for the granulomatous diseases (Anderson, 2000), were found in the elevated numbers. Helming and Gordon (2007) showed that giant cells arise as a result of monocytes /macrophages fusion triggered by IL-4 cytokine. We showed that their phagocytic capacity for particulate material was very low in this model infection.

Mononuclear phagocytes have a number of specialized mechanisms for the recognition and removal of inflammatory and infectious stimuli. The products of B and T lymphocytes are particularly important in this respect. It is believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulation (Goerdt & Orfanos, 1999; Mosser & Edwards, 2008). In our experiments we observed the biphasic behaviour of peritoneal monocytes/macrophages regarding their phagocytic activity *ex vivo* for all particle types and their coating. However, it is important to consider that *ex vivo* isolated cells can represent already activated cells what might lower their capacity to phagocytose. Nevertheless, activation of this cell function was observed in the increasing number of cells from the beginning of infection until days 10 – 14 day p.i. Then the proportion of phagocytosing monocytes/macrophages declined. Our previous findings using the same experimental settings showed that peritoneal macrophages/monocytes had significantly increased respiratory burst and generated high levels of superoxide anions peaking on day 14 p.i. (Velebný *et al*., 2010), following by decline. This time-dependent pattern of response coincides with up-regulation of markers characteristic for alternatively activated macrophages (M2) such as Arg-1, FIZZ-1 and Ym-1, and low expression of gene for IFN-γ, which

is crucial for classical activation of macrophages (M1) (Martinez *et al*., 2008; Fairweather & Cihakova, 2009). Rapid stimulation of IL-4 gene expression from day 14 p.i. was reported by Vendelova *et al*., 2015. Using the same murine infection, O´Connell *et al*. (2009) and Rawat *et al*. (2003) showed that IL-4, the potent activator of M2 macrophage phenotypes, was highly upregulated in *M. vogae* infection from day 14 p.i. and was necessary for survival of mice.

Two major opsonin-dependent receptors on macrophages in mice are receptors for the Fc portion of immunoglobulins (FcγRI - FcγRIII) and complement receptor (CR3), which recognize complement fragment C3bi and their ligation initiates phagocytosis of opsonised targets (Hawlisch & Köhl, 2006; Nimmerjahn & Ravetch, 2006). Antagonistic effects of IL-4 vs. IFN-γ was observed on FcRI and FcRII as well as CR3 and CR4 receptor expression (Becker & Daniel, 1990). Authors demonstrated that phagocytosis of particulate immune complexes via Fc receptors as well as production of superoxide anion (O_2^-) was inhibited by IL-4 and inhibition was reversed by IFN-γ.

In the present study opsonin-dependent phagocytosis was examined using lipid membrane particles – liposomes of various size, which exerted negative surface charge. The interaction of non-opsonized liposomes with phagocytic cells comprise the stable adsorption to the cell surface, cellular uptake of intact vesicles by energy-dependent manner and lysosomal degradation (Ahsan *et al*., 2002). Their uptake by peritoneal cells similarly showed the biphasic behaviour. The high amount of liposomes internalized within 7 days p.i. were probably used by activated phagocytes as source of phospholipids for cell membranes to form phagosomes. To support this explanation, fusion of this type of liposomes with plasma membrane followed by integration of liposomal lipids were reported by Pagano *et al*. (1974) and Martin and McDonald (1976). However, pattern of internalization is different when liposomes are coated with the specific molecules (Munn & Parce, 1982). Phagocytosis of liposomes opsonized with serum proteins (presumably complement) from pathogen-free mice was higher, although not significantly, from day 3 p.i. onwards, in comparison with uptake of liposomes coated with serum proteins from mice with chronic *M. vogae* infection. In addition to complement, it is suggested that post-infection serum contains high levels of circulating larval ES antigens, antibodies and probably also immune complexes (unpublished data). In case of *M. vogae* infection, larvae begun to release a specific antigens early p.i., what was initially showed Sogandares-Bernal *et al*. (1981), who detected circulating ES antigens already at day 2 after intraperitoneal infection. We also detected parasite ES-specific IgG antibodies in serum of mice already on day 3 after oral infection (unpublished data). We suppose that post-infectious phagocytes have higher expression of complement receptors in comparison with expression of Fc receptors, which might be partially down-regulated by the larval antigens in order to control inflammation. This larval-driven modulation of phagocytosis and associated respiratory burst in M2 macrophages might contribute to establishment of infection and alleviates the pathology in the host. Indeed, it was shown that macrophages and giant cells from granulomas loose their receptors, particularly those for IgG (Mariano *et al*., 1977). Toye *et al*. (1984) revealed that activation of complement did not play an essential role in the restriction of *M. vogae* growth in mice, indirectly supporting our findings.

We further evaluated the capacity of naive and post-infection peritoneal macrophages and monocytes to internalize non-opsonized particles. We used inert particles, namely latex beads and HEMA particles to study phagocytosis via opsonin-independent receptors. Proportion of activated cells was examined *ex vivo* after isolation of cells without additional stimulation. Capacity of naive peritoneal macrophages to engulf effectively latex beads was found in 20 % of the cells. The proportion of phagocytosing cells increased nearly twice within 14 days p.i., and then dropped to pre-infection level. Receptor-mediated uptake of uncoated latex beads by myeloid cells has been suggested but the exact mechanism is not clear and endocytosis is also taken into consideration (Pratten & Lloyd, 1986). Loke *et al*. (2007) showed that alternatively activated macrophages have enhanced phagocytosis of apoptotic neutrophils but uptake of latex beads was not influenced. Similarly, in our *ex vivo* study cells were often found to engulf neutrophils having the marks of apoptosis (Fig.1E, F). In addition, we showed that pre-treatment of cells with larval antigens had no effect on latex beads internalization, indicating that non-specific phagocytic ability was not influenced. Involvement of other or a few opsonin-independent receptors might be considered in case of phagocytosis of HEMA particles. This implies the higher number of phagocytosing cells as well as the higher mean numbers of ingested particles in comparison with uptake of latex beads. When testing phagocytosis of HEMA by macrophages/monocytes *ex vivo,* a significantly higher cell numbers with ingested particles in comparison with non-stimulated macrophages were seen during 5 weeks p.i.

In summary, the present study showed that phagocytic activity of *M. vogae*-induced peritoneal monocytes/macrophages had biphasic behaviour for both opsonins-dependent and independent pathways, whereas giant cells were very poor phagocytes. Highly elevated proportions of phagocytosing cells were found between days 10 – 14 p.i., regardless the type of particles and opsonisation. Source of opsonins used for coating the liposomes suggested higher expression of complement receptors than Fc receptors on these cells, whereas uptake of non-opsonized liposomes had different kinetics. Cells morphologically identified as monocytes, dominated among activated phagocytes.

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