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Novel insights into the peritoneal inflammation of rainbow trout (*Oncorhynchus mykiss*)

Tomáš Korytář [a], Joanna Jaros [a], Marieke Verleih [b], Alexander Rebl [b], Günter Kotterba [a], Carsten Kühn [c], Tom Goldammer [b], Bernd Köllner [a,*]

[a] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Immunology, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[b] Leibniz Institute for Farm Animal Biology (FBN), Institute of Molecular Biology, 18196 Dummerstorf, Germany
[c] Landesforschungsanstalt für Landwirtschaft und Fischerei Mecklenburg-Vorpommern (LFA-MV), Institut für Fischerei, Born, Germany

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ABSTRACT

The peritoneal cavity has been extensively used as a laboratory model of inflammation in many species, including the teleost fish. Although, the peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*) was previously shown to contain a resident population of leukocytes, closer information about their exact composition and their functional response to pathogens is still missing. In the presented work, flow cytometric analysis using monoclonal antibodies was performed to characterize this cell population and evaluate its traffic during the first 72 h after antigenic stimulation and infection with *Aeromonas salmonicida*. Obtained results indicate that the unstimulated peritoneal cavity represents rather a lymphoid niche, dominated by the IgM^+ B cells. Expectedly, the composition changed rapidly after stimulation, which resulted in two complete changes of dominant cell type within first 72 h post injection. While the first stage of inflammation was dominated by myeloid cells, lymphocytes predominated at the later time points, with IgM^+ B cells representing more than two thirds of all cells. Later, the infection experiment elucidated the peritoneal infection and identified the key differences to the antigenic stimulation. Additionally, the data indicate that the resolution of the inflammation depends more on the bacterial clearance by myeloid cells than on regulation by lymphocytes. Taken together, obtained results represent the first complete description of the immune reaction protecting the peritoneal cavity of the fish and shed some light on the conservation of these processes during the evolution.

1. Introduction

Peritoneal leukocytes comprise a unique mixture of macrophages, monocytes, neutrophils and lymphocytes defending the body cavity [1]. Based on the easy collection and quantification, peritoneal cavity has been employed for decades as a model of inflammation in numerous animal species including fish [2–10]. Earlier studies on the composition of resident leukocytes in rainbow trout revealed the presence of both lymphocytes and myeloid cells, the former comprise the predominant cell type [3]. However, further research in different fish species indicated that the proportion between lymphocytes and myeloid cells can differ and is often species specific. Thus, the number of myeloid cells and lymphocytes is equal in barramundi (*Lates calcarifer*) [7], while in Sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*), the myeloid cells dominate the cavity and lymphocytes represent only a small fraction [5]. Although the consequences of different composition of resident leukocytes remain unclear, injection of stimulants induces a rapid reaction in all species and recruits a high number of cells of myeloid origin within few hours [9,11–14]. These cells possess all weapons necessary for the elimination of potential pathogens, including phagocytosis, production of reactive oxygen species, and cytotoxic ability and actively contribute to clearance of pathogens from the cavity [4,7,11,15–18]. Once the antigen is cleared, the number of myeloid cells decreases and lymphocytes appear. However, in contrast to the well-studied role of myeloid cells, the role of lymphocytes was neglected during previous studies, and their contribution to the resolution of inflammation was rarely discussed. Furthermore, not much is known about the antigen processing at the site of inflammation and the development of the adaptive immunity by B and T lymphocytes. As a results, the precise kinetic of lymphocytes and their composition after the stimulation was never studied in teleost, even though lymphocytes represent the predominant population of resident leukocytes [3]

* Corresponding author. Tel.: +49 383517 1208; fax: +49 383517 1226.
E-mail address: bernd.koellner@fli.bund.de (B. Köllner).

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and their increasing number was previously observed early after stimulation in gilthead Sea bream [18]. Such lack of information is particularly striking considering that the majority of fish vaccines is still delivered by intra-peritoneal injection and the peritoneal niche is intensively used also for the delivery of other substances [19–23]. Thus, a detailed study of the cellular and molecular processes in the stimulated peritoneal cavity would not only benefit to the current knowledge of the local immune system, but its applications could also improve the vaccine formulations and be particularly useful for applied science.

Aeromonas salmonicida, the causative agent of furunculosis, is a commonly used model for studies of host pathogen interaction in salmonids [24–31]. Despite a substantial scientific effort, the infection route of *A. salmonicida* is still not fully described although it was proposed that *A. salmonicida* enters the body through the skin, epithelium of gills or gastrointestinal tract [32–37]. Despite these findings, the peritoneal challenge is still the predominant method of bacterial delivery in controlled experiments and bath or co-habitation challenges are only exceptionally used [24,38].

Goal of this investigation was the description of the resident population of peritoneal leukocytes and the evaluation of its composition using a flow cytometric analysis with a unique set of established monoclonal antibodies (MAb). Their combination allowed the investigation of the proportion of IgM^+ B cells (MAb 1,14) [39], thrombocytes (MAb 42) [40], myeloid cells (MAb 21, replaced a previously published antibody [41]) and the population of T lymphocytes recognized by novel antibody MAb D30 [42]. The population of T lymphocytes could be further divided using recently published anti-CD8α MAb [43] to the population of $CD8\alpha^+$ cytotoxic T cells and $CD8\alpha^-$ T cells; presumably $CD4^+$ T helper cells. Consequently, all major populations of peritoneal leukocytes could be analysed with exception of the population of IgT^+ B cells, which is proposed to represent a unique population of trout leukocytes responsible for the adaptive immune responses on mucosal surfaces [44]. Once the composition of resident leukocytes was evaluated, a model of peritoneal inflammation with *A. salmonicida* was established based on previous publications [3,4,12,18]. Aware of the fact that *A. salmonicida* is able to survive within macrophages and can produce toxins influencing the immune effectors [45,46], we decided to evaluate the immune reaction towards the formalin-killed bacteria prior the infection experiments. In sum, our experimental results allow the first complete description of peritoneal inflammation in fish species and suggest striking conservation of these processes in evolution.

2. Material and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) was obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany and Binnenfischerei Mecklenburg GmbH Schwerin in Frauenmark, Germany. Animals were kept in 1000 l tanks at 15 °C in partially recirculating water systems and were fed with commercial dry pellets. The light period was 12 h per day and night. Fish were at age of one to two years during sampling. For the stimulation and infection experiments, fish were transferred to 300 l glass aquaria and acclimated for two weeks.

2.2. Sampling and leukocyte preparation

Blood was collected from the caudal vein using a heparinized syringe and immediately diluted in cold medium mixed Isove's DMEM/Ham's F12 (Gibco, Germany) at a ratio of 1:1. Fish organs (thymus, head kidney and spleen) were homogenized to prepare single cell suspensions. The peritoneal leukocytes (PEL) were obtained via lavage with 5 ml of ice cold PBS containing 5 mM EDTA.

The purged and opened gut was cut into small pieces and vigorously shaken for 5 min in 30 ml of cold medium to free the cells from the tissue. Gills were cut into small pieces and after brief shaking in cold medium the fragments were homogenized with Potter-Elvehjem homogenizer. The prepared single cell suspensions of gut and gills were filtered through gauze. The cells were centrifuged at 1800 RPM for 5 min to discard the excess of mucus and cell debris. The organ specific cell pellets were resuspended in 5 ml of fresh cold medium.

The cell suspensions were layered onto an isotonic Percoll gradient (Biochrom AG, Germany) ($r = 1.075$ g/ml) and centrifuged at 1800 RPM for 40 min. Cells at the Percoll/medium interphase were collected, washed with PBS, resuspended in corresponding volume of medium or PBS-EDTA to the final concentration of 4×10^6 cells/ml and kept on ice until further preparation.

2.3. Flow cytometry

Peritoneal leukocytes were resuspended in total 1 ml of PBS-EDTA for the analysis of cell number and distribution of lymphoid and myeloid populations. 100 μl of the cell suspension were diluted in 300 μl of PBS-EDTA. Cells were acquired by FACSCalibur (Becton Dickinson, Germany) on HIGH throughput for 20 s. The number of cells was counted with equation based on measurement of a dilution row of head kidney leukocytes.

The analysis of cell composition was performed using a set of monoclonal antibodies against thrombocytes (MAb42-1 [40];), IgM^+ B cells (MAb 1,14 [39];); CD8α RPE (MAb 13-2D [43];), myeloid cells (MAb21) and T cells (MAbD30, MAbD11 [42];). The new mab 21 was established immunization with a macrophage like cell line (SHK) and shows an identical reaction pattern as the earlier published mab 45 (41).

The preparation of samples was performed as follows: leukocytes at total number of 2×10^5 cells/ml were incubated for 30 min with diluted antibodies in combination for double staining. Antibodies were either directly labeled with fluorochrome or after washing with PBS. The cells were incubated for another 30 min with the corresponding mouse isotype specific FITC (Rockland, USA) and RPE (Jackson ImmunoResearch Laboratories, USA) conjugates whereas the control was only treated with secondary conjugates.

2.4. A. salmonicida for immunization and infection experiments

The *A. salmonicida* ssp. *salmonicida* wild type strain JF 2267 used for all stimulation trials was kindly provided by J. Frey, University Bern, Switzerland. The bacteria were cultivated from cryoconserved batches (MicrobankTM, PRO-LAB Diagnostics, Cheshire, UK) on Casein-peptone Soymeal-peptone slant agar (LB broth; SIFIN, Berlin, Germany) at 15 °C for 98 h and subsequent mass culture replant in LB broth (SIFIN, Berlin, Germany) for further 48 h. The initial cultures were checked for purity by Gram staining, cell morphology and motility. The bacterial suspension was concentrated by a 10 min centrifugation step at 4300 RPM and at 4 °C. The supernatant was discarded and the bacterial pellet was once washed in sterile 0.9% sodium chloride solution and diluted to 1×10^8 bacteria/ml. Each dose was controlled afterwards by counting the colony-forming units (CFU) after culturing of bacteria on CASO-Agar plates at 15 °C incubation temperature.

For the immunization experiment, bacteria were inactivated (i*A.s.*) in 1.5% PFA for 1 h. The bacteria suspension was set to the concentration of 1.6×10^9 cells/ml, frozen in 1 ml aliquots and kept