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# Spleen melanomacrophage centers response of Nile tilapia during *Aeromanas hydrophila* and *Mycobacterium marinum* infections

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ABSTRACT

In order to understand the pathophysiology of melanomcrophage centers (MMCs) formation during the tilapia defense response to bacterial infections, the present study evaluated the response, in terms of area, number and pigment constitution, of splenic MMCs of *Oreochromis niloticus* subjected to intraperitoneal (i.p.) infection with *Aeromonas hydrophila* and *Mycobacterium marinum*. Eighty-four fish (396.9 ± 21.0 g) were randomly distributed into twelve plastic tanks (300 L), to constitute three treatments with 28 animals each: control group (inoculated with PBS); Infected with *A. hydrophila* ($1 \times 10^7$ UFC $mL^{-1}$); Infected with *M. marinum* ($1 \times 10^6$ UFC $mL^{-1}$). The spleen was collected in seven fish per treatment on the 3rd, 7th, 14th and 21st day post-infection (DPI). The results revealed the participation of MMCs in the defense response of tilapia during bacterial infection by *A. hydrophila* and *M. marinum*, since there was an increase in the number and size of these cell aggregates. Variation of pigment accumulation with significant increase of hemosiderin, in infected tilapias by *A. hydrophila*, bacteria responsible for causing hemolytic anemia in fish was also found. On the other hand, *M. marinum*-infected tilapia had high amount of melanin in MMCs. In general, mycobacterial infections are notoriously difficult to treat, being characterized as a chronic disease. These findings demonstrate different strategies of fish response during the evolution of these bacterial diseases.

## 1. Introduction

Melanomacrophage centers (MMCs) are groupings of pigment-containing cells that are generally found inside the endothelial reticulum of the matrix of hematopoietic cells in teleost fish [1]. Despite their name the dominant pigment in these cells is lipofuscin [2], a non-degradable metabolite of unsaturated fatty acid peroxidation which accumulates in non-dividing cells in the absence of sufficient vitamin E [3]. The second most frequently observed pigment is melanin, which has been thought to be derived from exogenous sources [4] and/or be generated within the cells [5]. Hemosiderin, the least common of the pigments, is a form of intracellular iron storage which forms during the breakdown of hemoglobin, and serves as an intermediate step in the recycling of iron [6].

The macrophages are normally closely packed to form large aggregates and become bigger after phagocytic activity on heterogenous material such as cell particles, melanin pigment, hemosiderin granules and residues of lipofuscin [4], and on lipid droplets, protein aggregates and mucopolysaccharides [7]. The morphological appearance of MMCs may vary depending on species [8]. Their appearance can also differ within the same species, depending on the organ [9] and physiological conditions such as age [10], nutritional condition [11], and tissue type [6].

Several studies have suggested that the general function of MMCs is to destroy, detoxify or recycle endogenous and exogenous substances [7], such as discarded material originating from erythrocytic and cell metabolic activity [12]. In addition, MMCs perform an important role in response to foreign bodies, including infectious agents [13,14] or immune state [15].

However, it has been demonstrated that small circulating lymphocytes migrate to MMCs, which suggests that there is local interaction between the immune system cells and the antigens in immune response.

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There is evidence that suggests that MMCs are the main localities for long-term antigen retention, which favors interaction between these and lymphoid cells [16]. Other studies have demonstrated an association between the appearance of MMCs and high numbers of resistant intracellular bacteria, such as mycobacteria and parasites such as *Myxobolus* spp [8].

Increased numbers of MMCs were observed in the spleen and kidneys sixteen weeks after the intraperitoneal injection of a vaccine against *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar*), along with the presence of immune complexes and the production of high levels of antibodies. This suggests that antigen retention and the consequent activation of macrophages in MMCs is important for immunological memory [17] functioning as a kind of equivalent to germinal centers [18]. Furthermore, histopathology is an important biomarker and monitoring tool for observing the vital organs [19]. We used *A. hydrophila* and *M. marinum* by the fact that most of the fish diseases cause acute and chronic manifestation leading to important inflammatory response that compromise the fish defense system.

The present study evaluated the response, in terms of area, number and pigment constitution, of splenic MMCs of Nile tilapia subjected to infection with *A. hydrophila* and *M. marinum*, causative agents of aeromonosis and mycobacteriosis, respectively.

## 2. Materials and methods

### 2.1. Experimental design

To evaluation the MMCs, eighty-four male and female juvenile specimens of Nile tilapia (396.9 ± 21.0 g total weight and 28.5 ± 2.1 cm total length), were randomly distributed into twelve plastic tanks (300 L), with water supplied from an artesian well and with supplementary aeration at a flow of 1 L $min^{-1}$. They were fed commercial feed (3% of biomass, 28% of GP and 4000 kcal of GE $kg^{-1}$). Water quality was maintained within the adequate range for fish comfort [20] (dissolved oxygen = 5.6 ± 0.5 mg $L^{-1}$; temperature = 25.1 ± 1.8 °C; potential of hydrogen ions (pH) = 7.31 ± 0.4; and electric conductivity = 116.3 ± 12.5 µS $cm^{-1}$), evaluated with a YSI Model MPS 556 probe. After conditioning for seven days, the fish were anesthetized in an alcoholic solution of benzocaine (0.1 g $mL^{-1}$) (1:10000 anesthesia/water) to minimize suffering during the study procedures. All animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the Ethics Committee for the Use of Animals (CEUA) (Protocol n° 2013/01838-8) of São Paulo State University.

### 2.2. Bacteria inoculation

The control group (n = 28) were inoculated intraperitoneally (i.p.) with 1 mL of cold PBS. The groups stimulated with *A. hydrophila* (n = 28) (GenBank accession number: MH305534.1) and *M. marinum* (n = 28) (CEPANZO/OPAS/OMS, Argentina) kindly donated by Dra. Eliana Roxo, Biological Institute of São Paulo (Process TTM n° 016/2013/IB) were inoculated in the same manner as the control group with 1 mL of a previously determined sublethal dose of bacterium in sterile PBS ($1 \times 10^7$ UFC $mL^{-1}$ and $1 \times 10^6$ UFC $mL^{-1}$, respectively). To collect the spleen, the fish were killed by means of immersion in an aqueous solution of benzocaine (1:500 v/v). Seven fish per treatment were sampled at 3rd, 7th, 14th and 21st days post-infection (DPI).

### 2.3. Histopathology

After collection, the material was fixed in Bouin solution for 6 h then washed in 70% alcohol. The usual histopathological methods were followed to prepare paraffinized sections with a thickness of 5 µm, which were mounted on slides. These were stained with hematoxylin-eosin (HE), Ziehl-Neelsen (ZN), toluidine blue, Perls' (identification of hemossiderin - blue ferric pigmentand and melanin), Schmorl's (lipofuscina - brown pigment). The mounted slides were examined under light microscopy (Olympus BX 51) and were photographed (Olympus DP73).

### 2.4. Morphobiometric and pigment evaluation

For morphometric evaluation of the MMCs, five fields stained with toluidine blue were randomly selected on each slide and a total of 140 readings per treatment were performed at 200 x magnification. After each field had been photographed (Olympus DP73), the area was measured (µ$m^2$) (cellSens v.1.5 software) and the number of MMCs per field was counted. The methodology employed in morphometric determination was used to determine the pigments contained in the MMCs. The percentage of each pigment was analyzed by assigning a score where 0 = macrophages without pigments: 1 = 1–20%; 2 = 21–50%; 3 = ≥ 51 macrophages with pigments (lipofuscin, hemosiderin and melanin).

### 2.5. Statistical analysis

All data was statistically analyzed using a 'Split-plot design' [seven slides x five fields x four times], in accordance with [21]. The analysis of variance for comparing the different experimental groups was carried out by applying a General Linear Model (GLM) Procedure (SAS Institute Inc., 2001). Significant differences ($p < 0.05$) were estimated based on the Tukey test [22].

## 3. Results

Perls' staining demonstrated that the MMCs in all the groups contained hemosiderin and melanin (Fig. 1), while Schmorl's staining indicated lipofuscin (Fig. 2). Acid-alcohol resistant bacillus (BAAR) was not observed with ZN staining.

In the MMCs stained with toluidine blue (Fig. 3), it was observed that on the 3rd DPI there was a statistical difference in the areas of the MMCs. The areas were higher in the *M. marinum* treatment than the control, while there was no statistical difference between the *A. hydrophila* infected tilapia and both treatments (*M. marinum* or the control). There was no significant difference ($p > 0.05$) in the number of MMCs 3 DPI (Table 1). For the other periods of 7, 14 and 21 DPI, tilapia infected with *A. hydrophila* and *M. marinum* presented significant increase ($p < 0.05$) in the number and area of MMCs when compared to animals from control group, although there was no difference between

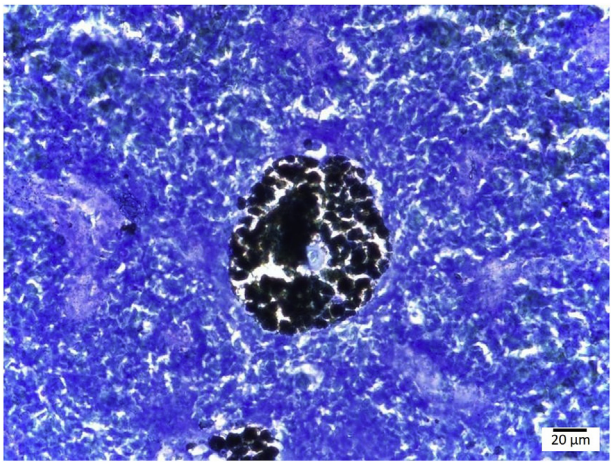


**Fig. 1.** Photomicrograph of spleen. MMC positive to hemosiderin (intense blue) in *Oreochromis niloticus*. Stained with Perls'. Bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

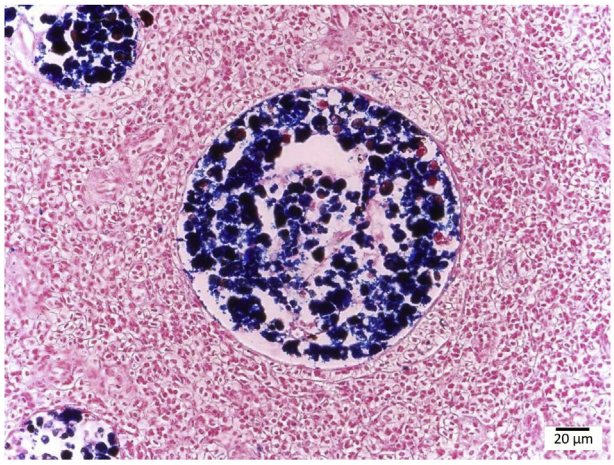


**Fig. 2.** Photomicrograph of spleen. MMC of *Oreochromis niloticus*. Positive to lipofuscin (brown), with few melanin (black). Stained with Schmorl's. Bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

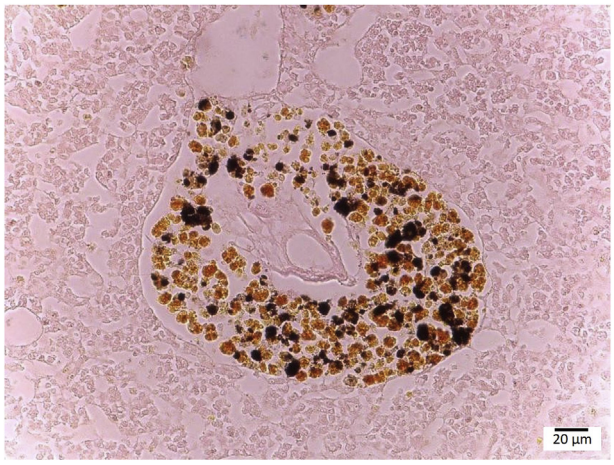


**Fig. 3.** Photomicrograph of spleen. MMC of *Oreochromis niloticus*. Stained with toluidine blue. Bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the two infected groups.

In analysis of each treatment over time, it was observed that in the control group there was no significant difference in the number and area of MMCs (Table 1). There was an increase in the area of the MMCs following treatment with *A. hydrophila* and *M. marinum*, mainly in the 14 and 21 DPI. In terms of the number of MMCs, infected fish presented significant increase ($p < 0.05$) during the experimental period, except on the 3rd DPI. *M. marinum* infected fish showed increased area of MMCs after 14 and 21 DPI.

In the analysis of MMCs pigment content (Table 2), control fish presented higher level of lipofuscin ($p < 0.05$) when compared to infected fish with *A. hydrophila* and *M. marinum*. Hemosiderin levels showed no significant difference between the two infected groups, although there was a significant ($p < 0.05$) increase when compared to control fish. Tilapia infected with *M. marinum* showed higher levels ($p < 0.05$) of melanin pigment in the MMCs when compared to control and infected fish with *A. hydrophila* (Table 2). No statistical difference was observed for these pigments over time among the experimental periods, except hemosiderin and melanin for control fish at the 3rd DPI.

**Table 1**
Mean values[a] and analysis of variance (ANOVA)[b] of the measurements of area (µm[b]) and number of MMCs[c] present in spleen of *Oreochromis niloticus*.

| Period (DPI) | Treatment | Area of MMC | Number of MMCs |
|---|---|---|---|
| 3 | Control | $248.69^{Ba}$ | $2.16^{Aa}$ |
| | *A. hydrophila* | $299.82^{ABb}$ | $3.20^{Ab}$ |
| | *M. marinum* | $319.62^{Ab}$ | $3.08^{Ab}$ |
| 7 | Control | $252.88^{Ba}$ | $2.68^{Ba}$ |
| | *A. hydrophila* | $298.53^{Ab}$ | $6.16^{Aa}$ |
| | *M. marinum* | $294.10^{Ab}$ | $7.76^{Aa}$ |
| 14 | Control | $260.90^{Ba}$ | $3.48^{Ba}$ |
| | *A. hydrophila* | $427.32^{Aa}$ | $6.68^{Aa}$ |
| | *M. marinum* | $425.11^{Aa}$ | $6.44^{Aa}$ |
| 21 | Control | $261,57^{Ba}$ | $2.92^{Ba}$ |
| | *A. hydrophila* | $442,18^{Aa}$ | $7.00^{Aa}$ |
| | *M. marinum* | $436,04^{Aa}$ | $6.62^{Aa}$ |
| Treatment | | 103.15** | 174.84** |
| Time | | 56.70** | 78.18** |
| Treatment x Time | | 5.68** | 10.25** |
| CV | | 26.22% | 26.60% |

[a] Mean values (n = 35): means with at least one letter in common did not differ between each other according to Tukey's test ($P < 0.05$). Lowercase letters in the columns compare the treatments within each experimental period, while uppercase letters in the lines compare the different experimental periods within each treatment.

[b] Value of F = 6.67; Probability of significance of F = < 0.0001; Coefficient of variation = 9.12%.

[c] Mean MMCs counts in 35 fields/fish/treatment at 200 x magnification.

**Table 2**
Mean values[a] and analysis of variance (ANOVA)[b] of scores of pigments contained in MMCs in spleens of *Oreochromis niloticus*.

| Period (DPI) | Treatment | Lipofuscin | Hemosiderin | Melanin |
|---|---|---|---|---|
| 3 | Control | $1.82^{Aa}$ | $0.31^{Bb}$ | $0.34^{Bb}$ |
| | *A. hydrophila* | $0.46^{Bb}$ | $2.17^{Aa}$ | $0.60^{Ba}$ |
| | *M. marinum* | $0.73^{Ba}$ | $2.06^{Aa}$ | $1.77^{Aa}$ |
| 7 | Control | $1.49^{Aa}$ | $0.68^{Ba}$ | $0.65^{Bab}$ |
| | *A. hydrophila* | $0.98^{Ba}$ | $2.07^{Aa}$ | $0.77^{Ba}$ |
| | *M. marinum* | $0.71^{Ba}$ | $1.76^{Aa}$ | $1.71^{Aa}$ |
| 14 | Control | $1.58^{Aa}$ | $0.63^{Ba}$ | $0.79^{Ba}$ |
| | *A. hydrophila* | $0.77^{Bab}$ | $2.04^{Aa}$ | $0.68^{Ba}$ |
| | *M. marinum* | $0.76^{Ba}$ | $1.55^{Aa}$ | $1.46^{Aa}$ |
| 21 | Control | $1.60^{Aa}$ | $0.60^{Ba}$ | $0.82^{Ba}$ |
| | *A. hydrophila* | $0.58^{Bb}$ | $2.12^{Aa}$ | $0.84^{Ba}$ |
| | *M. marinum* | $0.96^{Ba}$ | $1.85^{Aa}$ | $1.41^{Aa}$ |
| Treatment | | 136.24** | 373.02** | 135.97** |
| Time | | $0.26^{NS}$ | $1.19^{NS}$ | $1.40^{NS}$ |
| Treatment x Time | | 5.69** | 4.57** | 5.01** |
| CV | | 66.13 | 45.23 | 71.63 |

[a] Mean values (n = 35): means followed by the same letter did not differ for the Tukey-Kramer test ($P < 0.05$); lowercase letters compare treatments within each experimental period; uppercase letters compare different experimental periods within each treatment.

[b] Value of F = 6.67; Probability of significance of F = < 0.0001; Coefficient of variation = 9.12%.

## 4. Discussion

The area of MMCs is related to various factors and conditions [23] such as the organ [9], age [10,24], nutritional condition [11], type of tissue [6], iron metabolism [12], pathological conditions [25], and acute [26] and chronic [14,27] inflammatory conditions. The pigment content of the MMCs is related to the type of tissue damage [28], and environmental pollution conditions [29,30].

Melano-macrophage centers act the focal depositories for resistant intracellular bacteria, from Which chronic infections may Develop. Melano-macrophage centers Develop focally in association with the late stages of chronic in fl ammatory response to severe tissue damage (Fig. 3) and in association with the cellular response to a variety of

infections.

In the present study, the groups treated with *A. hydrophila* and *M. marinum* stimulated an increase in the area of the MMCs in all periods in comparison with the control group, a finding also observed by Manrique et al. [14] in Nile tilapia stimulated with inoculum of BCG and the subcutaneous implant of glass slides and in *Carassius auratus* stimulated with the intracellular inoculum of coal-based paint [7]. According to Agius and Roberts [2], late stages of chronic inflammatory reaction and cellular response to a variety of infectious diseases are associated with development of MMCs which can act as focal depositories for resistant intracellular bacteria. For both treatments in the present study, it is suggested that the increased MMC activity in the spleen was stimulated by hemolysis caused by inflammation, as was also described by Roberts [8]. The pigment with the greatest presence in the MMCs of the spleen was hemosiderin, most notably in the *A. hydrophila* treatment group. This condition is related to the ability of the spleen to store iron by products from the lysis of erythrocytes caused by this bacterium [31,32] which are phagocytosed by the melanomacrophages that make up the MMCs. This pigment is normally observed in close association with lipofuscin granules [4], which explains its presence in the MMCs, especially in the control groups in each period. It is known that this pigment is the result of oxidative and polymerization processes of polyunsaturated fatty acids [33], processes considered normal in healthy animals.

Although the *M. marinum* bacterium does not directly cause cell lysis, it also caused an increase in hemosiderin pigment in comparison with control, although in smaller amounts than the *A. hydrophila* treatment. This slight increase may be influenced by the vascular alteration and necrosis caused by bacterial inflammatory stimuli [34], or most probably by the tendency of the animals to develop microcytic hypochromic anemia, as observed in *O. Niloticus* when inoculated intramuscularly with *M. marinum* [35].

*M. marinum* is a bacterium that produces chronic disease, and in some cases can lead to fulminant death [34]. When disease is chronic, normally occurs increased area, number and melanin content in MMC. This fact may be related to the ability of the melanin to absorb and neutralize free radicals and other potentially toxic cations derived from the phagocytic degradation of cellular material [5], giving the body more time to react to acute processes. On the other hand, melanin is composed of complex polymers that may have a relevant role inside the melanomacrophage as a neutralizer of the hydrogen peroxide [24] released in the fatty acid catabolism of cell membranes after phagocytosis [4]. Some authors have used MMCs as a tool to determine the state of fish hygiene [32,36,37] and as an aquatic environmental indicator [1,38–40], without considering the type of pigments contained therein. Other authors have used MMCs as an indicator of immune response [41] due to the important role they play in this type of response in some teleost fish [2,40], to the point where they seem to have an evolutionary type relationship as a precursor of germinal centers, as occurs in higher vertebrates [42]. It should be noted that, to consider MMCs as a tool to evaluate the health status of fish, joint analysis (area, number and pigment) should be taken into account. As observed in the present study, the three pigments were present in all the treatments, however the quantity ratio of each varied according to the situation, as has also been observed by other authors in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) fish [43].

The results of this study revealed the participation of MMCs in the defense response of tilapia during bacterial infection with *A. hydrophila* and *M. marinum*, since there was an increase in the number and size of these cell aggregates, as well as variation of pigment accumulation with significant increase of hemosiderin, especially in infected tilapia by *A. hydrophila*, bacteria responsible for causing hemolytic anemia in fish. *M. marinum*-infected tilapia showed high amount of melanin in MMCs. In general, mycobacterial infections are notoriously difficult to treat, being characterized as a chronic disease. These findings demonstrate different strategies of fish response during the evolution of these bacterial diseases.

## Declaration of competing interest

None.

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