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FELLOWSHIP FINAL REPORT

The impact of IFNγ signaling in the pulmonary damage during *Plasmodium berghei* ANKA infection

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ABSTRACT

Malaria is one of the most important parasitic infection in the world. Cerebral and pulmonary complications may occur after infection and are often lethal. Immune response plays an important role in controlling malaria infection; however, excessive inflammatory response can lead to severe disease. The present work aims to decipher the cellular and molecular events associated with brain and pulmonary pathology in response to blood stage Plasmodium berghei ANKA (PbA) infection. PbA infection in C57BL/6 wild-type (WT) mice induces experimental cerebral malaria (ECM), associated with strong pro-inflammatory response, brain damage, as well as paralysis, coma early death (around day 7 p.i.). Interestingly, IFNγ receptor deficient mice (IFNγR1$^{-/-}$, C57BL/6 background) are resistant to ECM and died at a later time-point, due to the hyperparasitaemia and severe anemia. Here, we addressed the impact of IFNγR1 deficiency in the development of pulmonary damage during PbA infection. At day 7 post-infection, the broncho-alveolar lavage (BAL) allowed the quantitative analysis of total cells and proteins in the broncho-alveolar space of the animals. In addition, histological analysis and Western blot were performed to compare the cerebral and pulmonary compartments. As compared to PbA-infected WT mice, the histological sections confirmed a less intense accumulation of leukocytes as well as an absence of hemorrhages in the brains of IFNγR1$^{-/-}$ mice. In addition, the quantification of pro-apoptotic proteins (Granzyme B and cleaved caspase-3) in olfactory bulbs showed lower levels in IFNγR1$^{-/-}$ mice. While IFNγR1 deficient mice were fully resistant to brain pathology, those mice were partially protected for pulmonary damage, as observed by the levels of Granzyme B and cleaved caspase-3 in the lung parenchyma, leukocyte number in the broncho-alveolar space and pulmonary edema.

1- Introduction

Plasmodium parasites annually infect hundreds of millions of people and complications known as severe malaria lead to half a million deaths. Severe malaria includes cerebral malaria (CM), acute respiratory distress syndrome (ARDS), and severe anemia [1]. Experimental malaria models are relevant to study severe malaria-associated syndromes, respecting the

complexity and diversity of those manifestations [2].

CM is characterized by neurological manifestations, such as paralysis, seizures and coma. These complications are due to the adhesion of a large number of infected red cells (iRBC) and leukocytes to the endothelium of the cerebral capillaries [3]. Previous studies show that, in a mouse model of C57BL/6 mice infected with *Plasmodium berghei* ANKA (PbA), a strong pro-inflammatory response and leukocyte accumulation in the brain are responsible for the development of dysfunctions of the cerebral endothelium. C57BL/6-infected mice display neurological symptoms between 6-10 days after infection [4] [5]. In addition, during severe forms of malaria, large quantities of interferon-γ (IFNγ) has a pro-inflammatory role, notably by inducing the TH1 response and being responsible for the damage of blood-brain barrier and the pulmonary endothelium, characteristic of severe cerebral and pulmonary malaria [6] [7] [8].

The development of malaria associated-ARDS is still poorly understood. This manifestation is characterized by the presence of pulmonary edema, congestion of the pulmonary capillaries, the presence of leukocytes in the lumen of the lungs and the production of pro-inflammatory cytokines [9] [10] [11] [12].

This study aimed to evaluate the immune response during PbA infection in the context of the IFNγ signaling, as well as the consequences of this deficiency on pathophysiology in the brain and lungs.

2- Experimental details

C57BL/6 Wild type (WT) mice (Janvier) and IFNγR$^{-/-}$ (on C57BL/6 genetic background) were used in this study. Mice were infected with *Plasmodium berghei* ANKA (PbA) clone 15cy1, expressing the Green Fluorescent Protein (GFP) constitutively. Mice were infected via intraperitoneal injection of 10^5 parasitized erythrocytes and cumulative ECM incidence after infection was reported [7].

Parasitemia was assessed in the peripheral blood using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA) and FlowJo software (TreeAge Software, Inc, Williamstown, MA, USA).

Bronchoalveolar lavage (BAL) was performed as previously described. Differential cell counts were performed by counting an average of 250 cells on Cytospin preparations (Shandon CytoSpin3, Thermo Scientific, Illkirch, France) after May-Grünwald-Giemsa (MGG) staining (Diff Quick, Medion Diagnostics, Düdingen, Switzerland) according to manufacturer's instructions. Protein content was measured using Pierce 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA).

After euthanasia, mice were perfused intracardiacally to remove circulating red blood cells and leukocytes. Brain and lungs were immediately removed and fixed in 10% buffered formalin for histological analysis. Longitudinal sections (4µm) were stained with hematoxylin and eosin (H&E), which were evaluated. Sections were captured with a digital camera (Optronics DEI-470) connected to a Nikon eclipse 80i microscope.

Protein concentrations in brain and pulmonary samples were determined by using Pierce BCA protein assay (ThermoFisher Scientific). 20 µg of proteins were denatured by boiling (95 °C, 5 min) in reducing SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Amersham, UK). The membranes were blocked 2 hours in 5% Blotting-Grade Blocker (BioRad, France), washed three time in Tris-Buffered saline (TBS)- 0,1% Tween 20 and incubated with primary mouse anti-cleaved caspase-3 and anti-Granzyme B antibody in TBS-BSA (Bovine Serum Albumine) 1%- azide 0,5 mM overnight at 4°C. Membranes were then washed three times in TBS-0,1% Tween 20 and incubated with the appropriate second antibody conjugated to

horseradish peroxidase (HRP) two hours at Room Temperature (RT). Membranes were incubated with mouse anti-actin HRP-conjugate (Sigma-Aldrich) in 5% Blotting-Grade Blocker in TBS-0,1% Tween 20 for 2 hours at RT. Detection was performed with ECL Western-blotting Detection Reagent (GE Healthcare). The intensity of bands revealed was quantified using a densitometric software (ImageJ software version 1.45; NIH, Bethesda, MD), and the values were normalized to the values of beta-actin in the same sample.

Data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). Comparisons between 2 groups were performed using Mann-Whitney test, and multiple groups were compared using Kruskal-Wallis test. For survival curves, a log-rank Mantel-Cox test was used. Values of $p < 0.05$ were considered statistically significant.

3- Results and discussion

As a first approach, we aim to characterize the endothelial-leukocyte interactions in a rodent model of experimental cerebral malaria, in the context of pulmonary damage, through the involvement of IFNγ signaling.

The role of IFNγ signaling during PbA infection was assessed by survival, ECM incidence and parasitemia in infected WT and IFNγR$^{-/-}$ mice. Infected WT mice developed typical neurological symptoms of ECM including postural disorders, ataxia, loss of grip strength, progressive paralysis, coma, dying on day 7 p.i. In contrast, the majority of infected IFNγR$^{-/-}$ mice survived longer than WT mice ($p<0.05$), without signs of ECM. Parasitemia levels were similar between the groups from day 3 p.i. up to day 10 p.i.

To determine histopathological changes during PbA infection, we examined the brains and lungs of infected mice on day 7 p.i., when mice presented characteristic neurological symptoms of ECM. In infected WT mice, intravascular leukocyte accumulation and perivascular hemorrhage were common findings in the brain.

In C57BL/6-infected mice, the olfactory bulbs are mainly affected, as noticed by the sequestration of leukocytes in the brain vessels and extensive areas of hemorrhages. These findings were almost absent in infected IFNγR$^{-/-}$ mice, when compared with infected WT mice. Next, histological sections of the cerebellum of infected WT mice displayed microvascular obstruction and hemorrhage, whereas IFNγR$^{-/-}$ mice presented less intense histopathological changes. These data suggest that absence of IFNγ signaling induces less leukocyte accumulation and hemorrhage in the brain tissue during PbA infection.

During PbA infection, mice also exhibit malaria-associated lung pathology, a process dependent of macrophages and T cells. The lungs of WT-infected mice displayed congested capillaries of the alveolar septae, hemorrhage in the alveoli and interstitial edema. Finally, we investigated the effects of the IFNγ signaling disruption during malaria-associated pulmonary disease. Histopathological analysis found no significant difference between infected WT and infected IFNγR$^{-/-}$ mice. Therefore, lung pathology induced by PbA infection seems to be, at least partially, independent of IFNγ signaling.

Granzyme B and Caspase-3-mediated cellular damage occurs in ECM and are relevant for the pathogenesis of disease. First, we assessed the presence of Granzyme B as well as cleaved subunits of caspase-3 (17/19 kDa) in the olfactory bulbs taken from naïve and infected mice. First, the profile of β-actin, used as a reference, does not show any variation among the groups. In infected WT mice, densitometry measures confirmed a significant increase of Granzyme B and cleaved caspase-3 at day 7 after inoculation. The presence of those proteins was significantly lower in infected IFNγR$^{-/-}$ mice. Levels of Granzyme B and cleaved caspase-3 were also measured in pulmonary samples. We observed a significant increase for both proteins in WT-infected mice, when compared with naïve mice. Moreover, infected IFNγR$^{-/-}$ mice also showed a slight increase in Granzyme B and cleaved caspase-3 levels in the lung.

In addition, we found a significant increase in the number of total immune cells recovered in the BAL of PbA-infected mice as compared to naïve mice. However, infected IFNγR$^{-/-}$ mice presented lower levels as compared to WT PbA-infected mice.

The increase in the total number of immune cells in BAL was followed by a significant rise in the macrophage number in the BAL of infected mice. WT-infected mice also showed significant number in macrophage as compared to infected IFNγR$^{-/-}$ mice. Finally, no significant difference was observed regarding the number of lymphocytes, neutrophils and eosinophils in PbA-infected mice compared to naïve mice.

The Bradford assay in the BAL fluid allowed us to quantify the proteins and thus to highlight the presence of pulmonary edema. PbA infection induced an increase in the amount of total protein in the BAL of WT and IFNγR$^{-/-}$ mice, indicating respiratory barrier damage even in the absence of IFNγ signaling. However, the amount of protein was significantly lower in infected IFNγR$^{-/-}$ mice as compared to WT PbA-infected mice.

4- Conclusion

In general, the results obtained here showed that IFNγR$^{-/-}$ mice are resistant to the development of the ECM, but not the pulmonary form.

5- Perspectives of future collaborations with the host laboratory

My previously acquired knowledge from diverse fields allowed me additional collaborations, including one with the team led by Research Director Isabelle Couillin. They developed long-term interest and expertise in Chronic Obstructive Pulmonary Disease (COPD), associated with repeated exposure to cigarette smoke, and Idiopathic Pulmonary Fibrosis (IPF), two severe and irreversible lung disease which treatments display very limited efficacy. So far, this collaboration resulted in one publication as a co-author and at least two others are in progress.

6- Articles published in the framework of the fellowship

Nascimento M, Gombault A, **Lacerda-Queiroz N**, Panek C, Sbeity M, Bourinet M, Le Bert M, Savigny F, Riteau N, Ryffel B, Quesniaux V, Couillin I. Self-DNA release and STING-dependent sensing drives inflammation to cigarette smoke in mice. *Sci Rep.* 9(1):14848, 2019.

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