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Suppressor of cytokine signaling 2 modulates the immune response profile and development of experimental cerebral malaria

Fatima Brant [a,b,1], Aline S. Miranda [a,b,1], Lisia Esper [a,b], Melisa Gualdrón-López [b], Daniel Cisalpino [c], Danielle da Gloria de Souza [c], Milene Alvarenga Rachid [d], Herbert B. Tanowitz [e], Mauro Martins Teixeira [a,b], Antônio Lucio Teixeira [a,b], Fabiana Simão Machado [a,b,*]

[a] Program in Health Sciences: Infectious Diseases and Tropical Medicine, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[b] Department of Biochemistry and Immunology, Institute of Biological Science, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[c] Department of Microbiology, Institute of Biological Science, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[d] Department of Pathology, Institute of Biological Science, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[e] Department of Pathology and Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

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ABSTRACT

Plasmodium falciparum infection results in severe malaria in humans, affecting various organs, including the liver, spleen and brain, and resulting in high morbidity and mortality. The *Plasmodium berghei* ANKA (PbA) infection in mice closely recapitulates many aspects of human cerebral malaria (CM); thus, this model has been used to investigate the pathogenesis of CM. Suppressor of cytokine signaling 2 (SOCS2), an intracellular protein induced by cytokines and hormones, modulates the immune response, neural development, neurogenesis and neurotrophic pathways. However, the role of SOCS2 during CM remains unknown. SOCS2 knockout ($SOCS2^{-/-}$) mice infected with PbA show an initial resistance to infection with reduced parasitemia and production of TNF, TGF-β, IL-12 and IL-17 in the brain. Interestingly, in the late phase of infection, $SOCS2^{-/-}$ mice display increased parasitemia and reduced Treg cell infiltration, associated with enhanced levels of Th1 and Th17 cells and related cytokines IL-17, IL-6, and TGF-β in the brain. A significant reduction in protective neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), was also observed. Moreover, the molecular alterations in the brain of infected $SOCS2^{-/-}$ mice were associated with anxiety-related behaviors and cognition impairment. Mechanistically, these results revealed enhanced nitric oxide (NO) production in PbA-infected $SOCS2^{-/-}$ mice, and the inhibition of NO synthesis through L-NAME led to a marked decrease in survival, the disruption of parasitemia control and more pronounced anxiety-like behavior. Treatment with L-NAME also shifted the levels of Th1, Th7 and Treg cells in the brains of infected $SOCS2^{-/-}$ mice to the background levels observed in infected WT, with remarkable exception of increased $CD8^{+}IFN^{+}$ T cells and inflammatory monocytes. These results indicate that SOCS2 plays a dual role during PbA infection, being detrimental in the control of the parasite replication but crucial in the regulation of the immune response and production of neurotrophic factors. Here, we provided strong evidence of a critical relationship between SOCS2 and NO in the orchestration of the immune response and development of CM during PbA infection.

1. Introduction

Malaria is an overwhelming disease caused by apicomplexan parasites of the genus *Plasmodium* ssp. and is responsible for killing approximately 500,000 people each year world-wide (WHO, 2013). The primary victims of this disease are sub-Saharan African children under five years old (Hansen, 2012; Riley and Stewart, 2013; WHO, 2013), who present severe syndromes, including severe anemia, cerebral malaria (CM), acute respiratory distress and liver disease (Haque et al., 2011a).

CM is a life-threatening neurological syndrome affecting children and non-immune adults, and individuals who survive CM commonly exhibit residual neurological and cognitive abnormalities (Idro et al., 2006; Linares et al., 2013; Miranda et al., 2013).

* Corresponding author at: Federal University of Minas Gerais – Institute of Biological Sciences, Department of Biochemistry and Immunology – Bloco O4, 190, Av. Antônio Carlos, 6627 – Pampulha, 31270-901 Belo Horizonte, MG, Brazil.
E-mail address: machadofs@icb.ufmg.br (F.S. Machado).
[1] These authors contributed equally to this work.

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CM is a complex and multifactorial syndrome (Schofield and Grau, 2005), and the mechanisms underlying the cerebral damage are not fully understood. It has been suggested that the sequestration of parasitized erythrocytes (iRBC) to the cerebral vasculature, an overly vigorous immune response to parasite products and increased permeability of the blood-brain barrier (BBB) contribute to the development of CM (Nacer et al., 2012; Pamplona et al., 2009; Schofield and Grau, 2005).

Several immune mediators, including the pro-inflammatory cytokines TNF, IFN-γ, IL-1β and IL-6 (Wunderlich et al., 2012), contribute to the pathogenesis of severe malaria in both humans (Lyke et al., 2004) and rodents (Wunderlich et al., 2012). Previous reports have suggested that in addition to cytokines, nitric oxide (NO) and neurotrophic factors, such as brain derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF) and nerve growth factor (NGF), might also contribute to the pathogenesis of CM (Gazzinelli et al., 2014; Jeney et al., 2014; Linares et al., 2013). Neurotrophic factors are a large group of growth factors essential for physiological functioning and the development of the nervous system (Allen et al., 2013). BDNF levels are reduced in the brains of humans with neurodegenerative diseases, such as Alzheimer disease (Zuccato and Cattaneo, 2009). Importantly, recent studies have demonstrated that decreased levels of BDNF during malaria infection might contribute to cerebral damage (Comim et al., 2012; Linares et al., 2013). Additional studies have also shown a high concentration of BDNF and NGF associated with a reduction in disease severity in human and murine CM (Serghides et al., 2014). Nitric oxide plays an important role in CNS functions, such as leukocyte adhesion, synaptic transmission and cellular defense (Calabrese et al., 2007). During malaria infection, the low bioavailability of NO has been associated with CM pathogenesis (Gramaglia et al., 2006), and previous studies have indicated that the administration of NO might suppress the development of CM (Cabrales et al., 2011b; Jeney et al., 2014; Ong et al., 2013).

The intravascular accumulation of monocytes in several organs, including the brain, liver, lung and spleen, has been observed in severe malaria (Amante et al., 2010; Haque et al., 2011b). In the brain, the accumulation of $CD4^+$ and $CD8^+$ T cells and sequestration of iRBCs play important roles in the pathogenesis of experimental cerebral malaria (ECM) (Baptista et al., 2010; Claser et al., 2011; Hansen, 2012; Haque et al., 2011b,c; McQuillan et al., 2011). Importantly, regulatory T cells (Tregs) participate in the regulation of the immune response against *Plasmodium* ssp., although their role during CM remains controversial (Amante et al., 2007; Hansen, 2012; Vigario et al., 2007).

Suppressor of cytokine signaling (SOCS) proteins are a family of intracellular molecules constitutively expressed in several cells/tissues, including hepatocytes, T cells, macrophages, dendritic cells, leukocytes, neurons, and the liver and lungs (Palmer and Restifo, 2009; Rico-Bautista et al., 2006). SOCS2 inhibits growth hormone (GH) signaling and regulates the protein levels of other SOCS members, such as SOCS1 and SOCS3, potentiating the signaling regulated by these SOCS proteins (Piessevaux et al., 2006; Tannahill et al., 2005). SOCS2 expression is induced through several mediators, such as IFN-γ, GH, IL-2, IL-6 and lipoxin $(LXA)_4$ (Machado et al., 2006; Palmer and Restifo, 2009; Piessevaux et al., 2006). The SOCS2 expression induced through LXA_4 is dependent on aryl hydrocarbon receptor (AhR) activation (Machado et al., 2006), an intracellular receptor with transcription factor activity important for the modulation of the immune response and the development of malaria (Brant et al., 2014). In addition, AhR modulates SOCS2 expression during *Toxoplasma gondii* infection (Machado et al., 2006). Importantly, during *T. gondii* and *Trypanosoma cruzi* infections in mice, the absence of SOCS2 results in injury in the CNS and heart, respectively (Esper et al., 2012; Machado et al., 2006), indicating an important function for this protein in the control of immune responses during infection. Moreover, SOCS2 plays an important role in the regulation of neuron embryonic development via the inhibition of GH signaling (Turnley et al., 2002) and the regulation of neurotrophin signaling through tyrosine kinase A (TrkA) receptor (Uren and Turnley, 2014). Considering the important function of SOCS2 in the immune response and the regulation of neuron development, the aim of the present study was to investigate the role of SOCS2 in the development of severe malaria in a mouse model.

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with the Brazilian guidelines on animal work and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal ethics committee of the Universidade Federal de Minas Gerais (CEUA/UFMG) approved all experimental procedures, including euthanasia and fluid and organ removal (Permit Number: 202/10). All animal experiments were planned to minimize suffering.

2.2. Parasite, infection and disease development

Wild-type (WT) C57BL/6 female mice (6–8 weeks old) were obtained from the Animal Care Facilities of Universidade Federal de Minas Gerais, Minas Gerais, Brazil. SOCS2 knockout mice ($SOCS2^{-/-}$) (Metcalf et al., 2000) were a kind gift from Dr. Warren S. Alexander (the Walter and Eliza Hall Institute of Medical Research, Australia). The mice were maintained under pathogen-free conditions at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais.

Blood stages of *Plasmodium berghei* ANKA (PbA), constitutively expressing green fluorescent protein (*P. berghei* ANKA-GFP) (15cy1 clone) (Neres et al., 2008), a kind gift from Dr. Claudio Marinho (Universidade de São Paulo), were stored in liquid nitrogen. The mice were intraperitoneally (i.p.) infected with 10^5 PbA-infected red blood cells suspended in PBS. The percentage of parasitemia was quantified based on the GFP frequency in whole blood using flow cytometry as previously described (Brant et al., 2014). Briefly, a drop of whole blood from the tails of PbA-infected or uninfected mice was collected directly into a polystyrene tube containing 2 ml of PBS for flow cytometry analysis. Parasitemia was evaluated from the 3rd until the 7th day after infection (dpi). Each sample was examined using a flow cytometer (FACS CANTOII, Becton Dickinson, San Jose, CA). The GFP frequency was measured using argon laser (488 nm), and the acquisition was processed using Diva software (Becton Dickinson, San Jose, CA). The erythrocyte population was identified based on morphological characteristics in dot plot graphic (FSCxSSC), and subsequently analyzed for the presence of GFP. A minimum of 100,000 gated events in erythrocyte populations were acquired for analysis (Brant et al., 2014). The mice were observed daily for parasitemia, weight loss and survival. The clinical signs of CM were assessed daily using the rapid murine coma and behavior scale (Carroll et al., 2010). For the hematocrit determination, samples of blood were collected into heparinized capillary tubes at 5 dpi and centrifuged for 10 min in a hematocrit centrifuge (HT, São Paulo, Brazil).

2.3. L-NG-nitroarginine methyl ester (L-NAME) treatment

Nitric oxide (NO) inhibition in WT and $SOCS2^{-/-}$ mice was achieved through gavage with L-NAME (SIGMA, St. Louis, Missouri,

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