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STAT6$^{-/-}$ mice exhibit decreased cells with alternatively activated macrophage phenotypes and enhanced disease severity in murine neurocysticercosis

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ABSTRACT

In this study, using a murine model for neurocysticercosis, macrophage phenotypes and their functions were examined. *Mesocestoides corti* infection in the central nervous system (CNS) induced expression of markers associated with alternatively activated macrophages (AAMs) and a scarcity of iNOS, a classically activated macrophage marker. The infection in STAT6$^{-/-}$ mice resulted in significantly reduced accumulation of AAMs as well as enhanced susceptibility to infection coinciding with increased parasite burden and greater neuropathology. These results demonstrate that macrophages in the helminth infected CNS are largely of AAM phenotypes, particularly as the infection progresses, and that STAT6 dependent responses, possibly involving AAMs, are essential for controlling neurocysticercosis.

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1. Introduction

Neurocysticercosis (NCC) is the most common parasitic disease worldwide which affects the central nervous system (CNS). The disease occurs as a result of infection of the brain with the larval stage of the tape worm parasite *Taenia solium* (*T. solium*) (Davis and Kornfeld, 1991; Nash et al., 2006; Sciutto et al., 2007; Terrazas, 2008; White, 2000). Interestingly, in NCC humans remain asymptomatic for long periods of time (months to years). The infection becomes apparent upon degeneration of larvae caused by either therapeutic treatment or normal attrition resulting in severe headaches, epilepsy, intracranial hypertension, focal deficit, and/or cognitive impairment (Nash et al., 2006). These disease symptoms are frequently associated with an intense immune response induced by the dead parasite (Correa et al., 1985; Grewal et al., 2000). The precise immune response induced in the context of the CNS during NCC has been addressed mainly via analysis of helper T cell (Th) responses. This response is characterized by an overt Th1 phenotype (Restrepo et al., 1998), or a mixed Th1, Th2, and Th3 phenotype depending upon the absence or presence of granuloma formation and the stage of the granulomatous response (Restrepo et al., 2001). However, evidence regarding the appropriate immune responses required in controlling the infection or the clinical outcome of NCC is unknown.

The ability to elicit an immune response to infections is orchestrated, in large part, by specialized innate cell types such as macrophages. However, less attention has been paid to identifying the role of macrophages in the CNS microenvironment during NCC. In this context, macrophages activated during Th1-type responses, such as elicited by bacterial and viral infections, exhibit an inflammatory phenotype and produce pro-inflammatory cytokines, oxygen and nitrogen radicals (Nathan and Shiloh, 2000). In contrast, macrophages during helminth parasitic infection have an alternatively activated phenotype (Kreider et al., 2007). These AAMs exhibit an anti-inflammatory and a protective role in conjunction with the induced Th2-type response (Kreider et al., 2007; Noel et al., 2004). AAMs are thought to be involved in tissue repair and remodeling at the site of injury (Gordon, 2003; Mosser, 2003). This is of considerable importance during helminth infection as large metazoan parasites can cause extensive damage as they pass through tissue, releasing proteolytic enzymes that damage cells and tissue (Kreider et al., 2007). At the same time, AAMs largely fail to produce nitric oxide (NO) due to their induction of arginase (Gordon, 2003). This has led to the speculation that impairment of microbial killing functions of these cells can increase host susceptibility to infection. Correlatively, several studies have demonstrated that STAT6 knockout (KO) mice, which typically exhibit a defect in AAM associated responses as well as upregulated classically activated macrophage (CAM) associated responses, display enhanced anti-microbial immunity in a variety of parasitic disease models (Reyes and Terrazas, 2007). This suggests a role for STAT6 and AAMs in facilitating the establishment of a chronic infection (Reyes and Terrazas, 2007). In a contrasting situation, STAT6 associated responses are thought to be required for the expulsion of helminth parasites (Finkelman et al., 2004; Gause et al., 2003; Urban et al., 1998; Voehringer et al., 2007). Thus, an important question is whether CNS helminth infections such as in NCC induce AAMs in the brain microenvironment.

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In an experimental murine model for NCC developed in our laboratory (Cardona et al., 1999), mice intracranially (i.c.) inoculated with *Mesocestoides corti* (*M. corti*) metacestodes display an inflammatory CNS immune response which is similar in nature to human NCC (Cardona et al., 2003, 1999; Cardona and Teale, 2002; Mishra et al., 2009). In this study, analysis of the expression of AAM associated molecules and iNOS in the CNS of *M. corti* infected mice was performed. Additionally, the effect of the absence of AAM development/accumulation on the subsequent susceptibility and immunopathology in infected STAT6$^{-/-}$ mice was compared with WT mice. The evidence indicates that the absence of STAT6 mediated responses results in increased disease severity possibly through the lack of AAMs.

2. Materials and methods

2.1. Mice

Female STAT6$^{-/-}$ on C57BL/6 background, wildtype (WT) C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Female BALB/c mice were used in this study for parasite maintenance and were obtained from the National Cancer Institute animal program (Bethesda, MD). All animal experiments were conducted under the guidelines of the IACUC, UTSA, University of Texas System, the U.S. Department of Agriculture and the National Institutes of Health.

2.2. Antibodies

Phycoerythrin (PE)-conjugated anti-mouse CD11b (Mac-1), biotinylated anti-mouse CD11b, biotinylated anti-mouse CD11c and anti-mouse iNOS antibodies were purchased from BD PharMingen (San Diego, CA). AAMs in the brain were detected by using purified anti-mouse YM1 (ECF-L) (R&D Systems, USA), anti-mouse Fizz1 (RELMα) (Abcam, Cambridge, MA) or anti-mouse arginase 1 (ARG-1) (Santa Cruz Biotechnology, CA, USA). Purified anti-mouse mannose receptor 1 (MR1) and anti-mouse macrophage galactose-type C-type lectins (MGL1/2) were purchased from R&D System. For indirect immunofluorescence, appropriate fluorescent conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA) were used. Biotinylated primary antibodies were detected using Alexa Fluor 488-labeled streptavidin (Molecular Probes, USA).

2.3. Murine model of neurocysticercosis

In this study we used a mouse model of NCC developed in our laboratory (Cardona et al., 1999; Cardona and Teale, 2002). *M. corti* tetrathyridia (immature stage) were maintained by serial intraperitoneal (i.p.) inoculation of 8- to 12 weeks-old female BALB/c mice. Tetrathyridia were aseptically harvested, and murine NCC was induced by i.c. injection of 50 μl of HBSS containing approximately 40 organisms into 5 weeks-old mice under short-term anesthesia using a 50 μl mixture of ketamine HCL and xylazine (30 mg/ml ketamine and 4 mg/ml xylazine) in phosphate buffered saline (PBS) intramuscularly, as described previously (Cardona et al., 1999). Mock infected control mice were similarly injected with 50 μl sterile HBSS alone. At indicated times post-inoculation, anaesthetized animals were perfused through the left ventricle with 10 ml cold PBS, and brains were harvested to analyze parasite burden and various immune parameters.

2.4. RNA isolation and real-time PCR analysis

To determine the gene expression of YM1, Fizz1, ARG-1 and iNOS in murine NCC, brains were removed from infected and vehicle control mice at 1 week and 2 weeks post infection (p.i.). Animals were perfused with PBS prior to sacrifice to avoid RNA contamination from blood cells. Brains were immediately removed after perfusion. Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturers' instructions, and cDNA was prepared from 1 μg total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems). The cDNA derived from brains of mock and parasite-infected mice were loaded onto microfluidic cards preloaded with fluorogenic probes and primers for custom-designed Taq-Man Low Density Arrays (Applied Biosystems, CA, USA) for AAM and CAM markers and housekeeping genes β-actin, ribosomal 18 S, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). These cards were then loaded for thermal cycling on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Analyses of gene expression were determined using the ABI Prism 7900 Sequence Detection System software (Applied Biosystems). The target expression levels were normalized to levels of the house keeping genes 18S, β-actin and GAPDH in the same sample. Expression of each specific gene in infected samples was determined as fold change over that in control samples as calculated by using the formula $2^{-(\Delta\Delta Ct)}$.

2.5. Histology and immunofluorescence staining

The brains were immediately removed from perfused animals, embedded in O.C.T. resin (optimal cutting temperature), and snap frozen. Serial horizontal cryosections, 10 μm in thickness, were placed on silane prep slides (Sigma-Aldrich, St. Louis, MO). One in every four slides was fixed in formalin for 12 min at room temperature and stained with hematoxylin and eosin (H&E), as described previously (Mishra et al., 2009). The remainder of the slides were air-dried overnight and fixed in fresh acetone for 20 s at room temperature. Acetone-fixed sections were wrapped in aluminum foil and stored at −80 °C or processed immediately for *in-situ* immunofluorescence (IF) microscopy analysis as previously described (Alvarez and Teale, 2007a,b; Cardona et al., 2003; Mishra et al., 2006).

2.6. Quantification of expression levels of immune mediators

Sections of brains from mock infected and NCC mice (WT and STAT6$^{-/-}$) were stained with anti-YM1, anti-ARG-1, ant-Fizz1, anti-iNOS, anti-MR1, and anti-MGL1/2 antibodies followed by labeled secondary antibodies to determine potential differences in infection induced expression levels between WT and STAT6$^{-/-}$ mice. Some brain sections were additionally stained with antibodies to the macrophage marker CD11b to assess co-localization with YM1, Fizz1, ARG-1, MR1, MGL1/2, and iNOS. Protein level expression of these molecules was determined by capturing images at 20× magnification in the brain of mock infected and NCC animals using identical camera settings so that the number and intensity of pixels would reflect differences in protein expression (Mishra et al., 2008, 2009). The area (number of pixels) and fluorescence intensity (average intensity of pixels) of the staining was measured from ten images captured in brain areas containing infiltrating immune cells. This was done using the imaging software IP lab 4.0 (BD Biosciences Bioimaging, Rockville MD). The relative expression of YM1, Fizz1, ARG-1, iNOS, MR1, and MGL1/2 was calculated by multiplying the number of pixels (area) by the average intensity of pixels.

2.7. Statistical analysis

Statistical analysis was performed with a Student's *t*-test using SIGMA PLOT 8.0 (Systat Software, San Jose, CA). A p value less than 0.05 was considered to be statistically significant. Statistical differences in mortality of parasite-infected WT and STAT6$^{-/-}$ mice were analyzed using Kaplan–Meier survival analysis.

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