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Short communication

Testing effects of glatiramer acetate and fingolimod in an infectious model of CNS immune surveillance

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ABSTRACT

Immune surveillance of the CNS is critical for preventing infections; however, there is no accepted experimental model to assess the risk of infection when utilizing disease-modifying agents. We tested two approved agents for patients with multiple sclerosis (MS), glatiramer acetate and fingolimod, in an experimental model of CNS immune surveillance. C57BL/6 mice were infected with the ME49 strain of the neuroinvasive parasite *Toxoplasma gondii* (*T. gondii*) and then treated with GA and fingolimod. Neither treatment affected host survival; however, differences were observed in parasite load and in leukocyte numbers in the brains of infected animals. Here we demonstrate that this model could be a useful tool for analyzing immune surveillance.

1. Introduction

Currently there is no universally accepted experimental model to assess CNS immune surveillance. The lack of such a model is a substantial shortfall in drug development as it allows for the approval of pharmacological agents with unknown effects on CNS infections and neoplastic growth.

We recently developed and tested an experimental model of CNS immune surveillance using the protozoan *Toxoplasma gondii* (*T. gondii*). *T. gondii* is a highly prevalent obligate intracellular pathogen. Like other mouse strains, the C57BL/6 mouse strain is susceptible to the ME49 strain of *T. gondii.* Infection of C57BL/6 mice with *T. gondii* strain ME49 is characterized by a rapid expansion of tachyzoites in the host, which differentiate into bradyzoites and form tissue cysts predominantly in the CNS. The development of tissue cysts defines the chronic stage of the infection. Depletion of either $CD4^+$ or $CD8^+$ T-cell results in reactivation of the parasite, and is associated with rapid mortality of infected animals in this mouse strain (Gazzinelli et al., 1992). The host survival of C57BL/6 mice following an infection with the ME49 strain of *T. gondii* is well established (Gazzinelli et al., 1992, 1991; Yarovinsky et al., 2006).

We infected C57BL/6 mice with the ME49 strain of *T. gondii* and further treated with two pharmacological agents, glatiramer acetate (GA) and fingolimod. GA is approved for the treatment of patients with relapsing–remitting multiple sclerosis (RRMS) and in patients who experience an initial clinical relapse with MRI findings that are compatible with a diagnosis of MS. GA reduces the frequency of disease relapses (La Mantia et al., 2010), and may decrease disease progression. The mechanism of action of GA is still not fully understood. However, immune responses under GA are shifted from a pro-inflammatory Th1 cytokine profile to an anti-inflammatory Th2 cytokine profile (Duda et al., 2000; Neuhaus et al., 2000). GA also inhibits the activation and proliferation of encephalitogenic T cells, and the modulation of antigen presenting cells (Weber et al., 2004, 2007). GA may also have neuroprotective properties through the induction of brain-derived neurotrophic factor (BDNF) by T cells and other cells within the CNS (Ziemssen et al., 2002; Aharoni et al., 2005). Currently, GA is the only therapeutic intervention that does not require any laboratory testing while patients are being treated (Rommer et al., 2013). Fingolimod is an agent with a relatively novel mode of action that is currently approved by the FDA to treat patients with RRMS (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/022527s000lbl.pdf). In clinical studies fingolimod was efficient in reducing the rates of relapse and MRI evidence of inflammatory lesions (Brinkmann et al., 2010). Fingolimod binds to sphingosine-1-phospate (S1P) receptors on the surface of leukocytes causing the receptors to be internalized thereby inhibiting

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migration. Consequently, these leukocytes are unable to egress from lymphatic tissues (Brinkmann et al., 2002). Neoplastic growths and possible risk of progressive multifocal leukoencephalopathy (PML) have been reported by the FDA (http://www.fda.gov/Drugs/DrugSafety/ucm366529.htm).

Here, we assessed the feasibility of a *T. gondii* infectious model of CNS immune surveillance to further test these pharmacological agents and their potential role on leukocyte homeostasis and immune surveillance of the CNS.

2. Methods

2.1. Infection of mice with T. gondii

Four groups of five eight-week-old female C57BL/6 mice were infected intraperitoneally with 20 cysts per mouse of the avirulent type II *T. gondii* strain ME49. Recipients were left untreated, treated daily with subcutaneous injections of 150 μg of GA, oral fingolimod (0.3 mg/kg/day), or both for another 50 days. Mice were monitored for survival.

2.2. FACS analysis

After 30 days post-infection, or at time of death, mice were perfused via the left ventricle with cold PBS and brains were harvested. Brains were pressed through a 70-μm nylon mesh cell strainer. Brain cells from all mice in each experimental group were pooled and processed as previously described (Cravens et al., 2011, 2013). In brief, brain cells were washed twice in 37% Percoll and CNS mononuclear cells were isolated by centrifugation at 2118 ×*g* for 15 min at 22 °C over a 30/70% Percoll gradient. The interphase cells were collected, washed with 0.5% BSA/PBS, resuspended in complete RPMI 1640 and counted. The following monoclonal antibodies were used: anti-CD3-AF700 (17A2), anti-CD45-PE-Cy7 (30-F11), anti-CD4-APC (RM4-5), anti-CD8-Pacific Orange (MCD0830), anti-Gr-1-APC-Cy-7 (RB6-8C5), and anti-CD11c-Pacific Blue (N418). Cells were resuspended in staining buffer (4% FCS and 0.1% sodium azide in PBS) and Fc receptors were blocked with anti-CD16/32 (BD Biosciences) for 15 min at 4 °C before staining with mAbs for 30 min at 4 °C. Cells were then washed, resuspended in staining buffer, and fixed in 1% paraformaldehyde. Up to 500,000 events were acquired on a FACSAria (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.3. Quantification of parasite cysts

To quantify *T. gondii* cysts in the brain, we removed brains after 30 days of infection or at time of death and parasite load was determined by the number of cysts in the brain (Yarovinsky et al., 2005).

2.4. Statistical analysis

Correlations between continuous and categorical variables were assessed using the Mann–Whitney *U*-test. The means of two normally distributed samples were compared by Student's *t*-test. All other statistical comparisons between groups were examined using one-way multiple range analysis of variance (ANOVA) for multiple comparisons or Student–Newman–Keuls multiple comparison test. A P-value <0.05 was considered significant.

3. Results

Neither treatment group had a significant adverse effect on host survival (Fig. 1a). Compared to untreated mice, the number of parasites in the brains of GA-treated animals was not increased (Fig. 1b). In contrast, the number of parasite cysts more than doubled in fingolimod-treated animals. Interestingly, addition of GA to fingolimod reduced the number of brain cysts back to baseline (Fig. 1b). The addition of GA to fingolimod reverses a minor decrease of CNS leukocyte numbers seen with fingolimod monotherapy (Fig. 2). The combination also appears to result in an expansion of some myeloid subsets (Fig. 2).

4. Discussion

Infection of C57BL/6 mice with the ME49 strain of *T. gondii* is a valid experimental model to test CNS immune surveillance for MS pharmacotherapies. C57BL/6 mice are susceptible to *T. gondii* infection and develop encephalitis. Innate and adaptive immune responses against the pathogen are well characterized in this mouse strain, and the survival rate is established. Parasite cysts accumulate within the brain, and there is an association between parasite numbers and clinical outcomes. Thus, the effect on any pharmacological intervention on survival, parasite numbers, and the composition on immune cells can be assessed.

In preliminary experiments, we confirmed that GA is a safe agent that does not alter survival of experimental animals, and that is not associated with an expansion of parasite cysts in the brain. Fingolimod, which was designed to sequester T cells into lymph nodes and away from the CNS, also did not adversely affect host survival. However, fingolimod led to an increase in CNS parasite load, which was reversed through the combination of GA and fingolimod.

These results are perhaps not surprising, given that GA has an outstanding track record with regard to safety, and as stated above, is currently the only agent that does not require any safety monitoring. Also, GA was shown to expand the $CD8^+$ T cell compartment (Karandikar et al., 2002; Racke et al., 2010), which is critical in host defense against *T. gondii* infection (Gazzinelli et al., 1992, 1991), and which may explain a reversal of high parasite counts in the brain under fingolimod when GA was added.

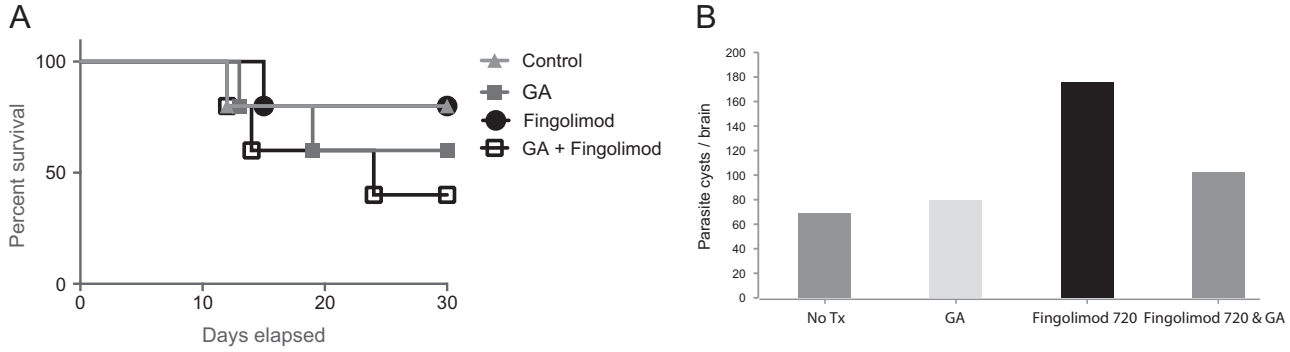

Fig. 1. A. Treatment with GA or fingolimod, or both had no adverse effect on host survival. GA = Glatiramer acetate. Mice were infected with 20 cysts per mouse of ME49 and treated with 150 μg of GA s.c., oral fingolimod (0.3 mg/kg/day), or both and monitored. B. Compared to untreated mice, the number of parasites in the brains of Glatiramer acetate (GA)-treated animals was not increased after 30 days. In contrast, the number of parasite cysts more than doubled in fingolimod-treated animals. Interestingly, addition of GA to fingolimod reduced the number of brain cysts back to baseline.