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ACTIVATION OF THE ADENOSINE A2A RECEPTOR ATTENUATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND IS ASSOCIATED WITH INCREASED INTRACELLULAR CALCIUM LEVELS

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Abstract—Multiple sclerosis (MS) is a common autoimmune disease that inevitably causes inflammatory nerve demyelination. However, an effective approach to prevent its course is still lacking and urgently needed. Recently, the adenosine A2A receptor (A2AR) has emerged as a novel inflammation regulator. Manipulation of A2AR activity may suppress the MS process and protect against nerve damage. To test this hypothesis, we treated murine experimental autoimmune encephalomyelitis (EAE), a model for MS, with the selective A2AR agonist, CGS21680 (CGS). We evaluated the effects of CGS on the pathological features of EAE progression, including CNS cellular infiltration, inflammatory cytokine expression, lymphocyte proliferation, and cell surface markers. Treatment with CGS significantly suppressed specific lymphocyte proliferation, reduced infiltration of $CD4^+$ T lymphocytes, and attenuated the expression of inflammatory cytokines, which in turn inhibited the EAE progression. For the first time, we demonstrate that CGS can increase the intracellular calcium concentration ($[Ca^{2+}]i$) in murine lymphocytes, which may be the mechanism underlying the suppressive effects of CGS-induced A2AR activation on EAE progression. Our findings strongly suggest that A2AR is a potential therapeutic target for MS and provide insight into the mechanism of action of A2AR agonists, which may offer a therapeutic option for this disease. © 2016 Published by Elsevier Ltd on behalf of IBRO.

Key words: multiple sclerosis, adenosine A2A receptor, experimental autoimmune encephalomyelitis, intracellular calcium.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease that is considered to have $CD4^+$-mediated autoimmune pathogenesis. Murine experimental autoimmune encephalomyelitis (EAE), an animal model of MS, is a T cell-mediated autoimmune disease that can be induced by immunization with myelin protein peptides and used to assess the efficacy of putative therapeutic agents (Paterson, 1986; Dittel, 2008). The $CD4^+$ T helper (Th) cell plays a key role in autoimmune disease (Dittel, 2008). Studies show that the balance of Th1, Th2, and regulatory T (Treg) cells plays a role in EAE pathogenesis (Chen et al., 2006). When the Th1:Th2 cell ratio shifts to a predominantly Th2 profile, the effects of proinflammatory Th1 cytokines, such as IFN-γ and TNF-α, are countered, and the severity of autoimmune disease is alleviated (Yoles et al., 2001; Weaver et al., 2005). Recent reports show that IL-17, a proinflammatory cytokine released from Th17 cells, also plays an important role in the pathogenesis of allergic and autoimmune diseases (Nakae et al., 2003a,b; Mu et al., 2009), whereas Treg and Th2 cytokines play an anti-inflammatory role and maintain tolerance to self-antigens. Therefore, a shift from a Treg/Th2 profile to a Th1/Th17 profile may be responsible for the development and/or progression of EAE.

Adenosine released from metabolically active cells and generated extracellularly by degradation of released adenosine triphosphate (ATP) regulates the function of immune cells and other cell types (Erdmann et al., 2005; Haskó and Pacher, 2008). Through its binding to the adenosine A2A receptor (A2AR), adenosine can modulate numerous cellular functions and protect cells and tissues from inflammation. Therefore, adenosine is said to "put the brake on inflammation" (Kirkpatrick, 2002). The importance of A2AR in mediating this negative feedback loop has been demonstrated by the finding that A2AR knockout mice lack the ability to control inflammation,

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Abbreviations: A2AR, adenosine A2A receptor; CFA, complete Freund's adjuvant; CGS, CGS21680; CRAC, Ca^{2+} release-activated calcium; EAE, experimental autoimmune encephalomyelitis; FITC, fluorescein isothiocyanate; MS, multiple sclerosis; PBS, phosphate-buffered saline; PE, phycoerythrin; SCH, SCH58261.

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which leads to extensive and fatal tissue destruction (Ohta and Sitkovsky, 2001). The expression of A2AR is the highest in immune cells, including monocytes and macrophages (Khoa et al., 2001), lymphocytes (Koshiba et al., 1999; Apasov et al., 2000; Armstrong et al., 2001), dendritic cells, neutrophils, natural killer cells (Williams et al., 1997; Harish et al., 2003; Raskovalova et al., 2005), and natural killer T cells (Lappas et al., 2006).

Numerous studies have shown that A2ARs are the primary adenosine receptors that mediate lymphocyte responses (Day et al., 2004; Odashima et al., 2005a; Blackburn et al., 2009; Lau et al., 2009; Alam et al., 2009). When activated, adenosine receptors can down-regulate inflammation and protect against tissue damage (Link et al., 2000; Ohta and Sitkovsky, 2001). Many studies have suggested that adenosine can inhibit cytokine production *in vitro* and *in vivo* via stimulating A2ARs. Schnurr et al. (2004) have found that adenosine significantly inhibits the production of IFN-α, IL-6, and IL-12 by activated plasmacytoid dendritic cells via A2ARs. Lappas et al. (2005) have reported that A2AR activation in $CD4^+$ T cells inhibits IFN-γ release. Another study has demonstrated that A2AR activation exerts strong inhibitory actions on both Th1 and Th2 cells during early and late stages of lymphocyte activation (Csóka et al., 2008). The A2AR agonist, ATL313, can inhibit lymphocyte proliferation through its actions on both T lymphocytes and APCs and can significantly reduce IFN-γ release in a dose-dependent manner (Sevigny et al., 2007). A2AR stimulation in human Th cells isolated from blood or gastric biopsy specimens efficiently suppresses IL-2, IFN-γ, and TNF-α production (Alam et al., 2009).

Lukashev et al. (2003) have shown that A2ARs are variably expressed in T cell subsets and may regulate cytokine production in activated T lymphocytes; however, they focused only on Th1 and Th2 cells. A2AR expression in Th17 and Treg subsets was not measured. In MS and EAE, T cells are predominately of the Th1 or Th17 phenotype, as indicated by their ability to generate IFN-γ and IL-17, but less IL-4 (a Th2 cytokine). Recent reports have indicated that loss of the A2AR exacerbated EAE pathology in mice (Yao et al., 2012; Wang et al., 2014). In addition, Mills et al. (2008, 2012) reported that A2AR lymphocyte expression is essential for limiting the severity of EAE, and they also demonstrated that A2AR (−/−) lymphocytes are more proliferative and produce more IFN-γ than their wild-type counterparts. These results indicate that the A2AR plays a key role in regulating the lymphocyte inflammatory responses.

Calcium signals are essential for diverse cellular functions, including differentiation, effector function, and gene transcription. In the brain, adenosine A1, A2B, and A3 receptors can promote inositol triphosphate receptor-regulated calcium release from intracellular stores (Doengi et al., 2008). In lymphocytes and other cell types, store-operated Ca^{2+} entry through Ca^{2+} release-activated calcium (CRAC) channels is the main mechanism for entry of extracellular Ca^{2+} across the plasma membrane (Lewis, 2001). Opening of CRAC channels leads directly to sustained increase of $[Ca^{2+}]i$, which, in turn, affects T cell functions that have long-term consequences, including lymphocyte proliferation and the differentiation of naïve T cells into various effector or memory T cells.

The aims of our study were to determine whether a relationship exists between A2AR expression and the severity of EAE, to evaluate the effects of an A2AR agonist on the development and progression of EAE, and to elucidate a plausible mechanism that would explain the effects of the A2AR agonist on EAE. We hypothesized that stimulation of A2AR activity in mice would result in inhibition of EAE development and/or progression and alleviation of disease signs.

EXPERIMENTAL PROCEDURES

Induction and clinical assessment of EAE

Female C57BL/6 mice, 6–8- week-old, were purchased from the Peking Vital River Laboratory Animal Ltd. (Peking, China). All animals were bred and maintained in accordance with the guidelines for the Care and Use of Laboratory Animals published by the China National Institute of Health. Mice in the EAE group were immunized subcutaneously with 200 μg of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$, MEVGWYRSPFSRVVHLYRNGK, AC Scientific, China) peptide emulsified in incomplete Freund's adjuvant (IFA, St. Louis, MO, USA) that contained 250 μg of *Mycobacterium tuberculosis* H37RA extract (Difco, Detroit, MI, USA). Each mouse received 200 ng of pertussis toxin (Campbell, CA, USA) in 200 μl of phosphate-buffered saline (PBS) intravenously on day 0 and day 2 of immunization. The control group was immunized with complete Freund's adjuvant (CFA) alone without $MOG_{35–55}$ peptide and no follow-up intravenous PT administration. Animals in all groups were weighed at the beginning of the experiment and every day thereafter until the end of the study period (28 days postimmunization).

Mice were monitored daily after immunization. The clinical signs of EAE were assigned using the following scoring system: 0, normal mouse without signs of disease; 1, limp tail; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis and/or forelimb paralysis; and 5, moribund state or death due to EAE. Mean clinical scores for each group were calculated daily by adding the scores of individual mice (including mice not developing signs of EAE) and dividing by the number of mice in the group. Average day of onset was calculated by adding the first days of clinical signs of individual mice and dividing by the number of mice in the group.

Treatment with the CGS21680

2(4-((2-Carboxymethyl)phenyl)ethylamino)-5′-Nethylcarboxamidoadenosine (CGS-21680) was obtained from Tocris, Bristol, UK. CGS-21680 was dissolved in 10% DMSO and diluted in 0.9% NaCl for i.p. On the day of immunization, the mice were arbitrarily assigned into four groups: CFA (control), EAE, CGS-1, and CGS-5.