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Selective CB2 receptor activation ameliorates EAE by reducing Th17 differentiation and immune cell accumulation in the CNS

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ABSTRACT

CB2, the cannabinoid receptor expressed primarily on hematopoietic cells and activated microglia, mediates the immunoregulatory functions of cannabinoids. The involvement of CB2 in EAE has been demonstrated by using both endogenous and exogenous ligands. We showed previously that CB2 selective agonists inhibit leukocyte rolling and adhesion to CNS microvasculature and ameliorate clinical symptom in both chronic and remitting-relapsing EAE models. Here we showed that Gp1a, a highly selective CB2 agonist, with a four log higher affinity for CB2 than CB1, reduced clinical scores and facilitated recovery in EAE in conjunction with long term reduction in demyelination and axonal loss. We also established that Gp1a affected EAE through at least two different mechanisms, *i.e.* an early effect on Th1/Th17 differentiation in peripheral immune organs, and a later effect on the accumulation of pathogenic immune cells in the CNS, associated with reductions in the expression of CNS and T cell chemokine receptors, chemokines and adhesion molecules. This is the first report on the in vivo CB2-mediated Gp1a inhibition of Th17/Th1 differentiation. We also confirmed the Gp1a-induced inhibition of Th17/Th1 differentiation in vitro, both in non-polarizing and polarizing conditions. The CB2-induced inhibition of Th17 differentiation is highly relevant in view of recent studies emphasizing the importance of pathogenic self-reactive Th17 cells in EAE/MS. In addition, the combined effect on Th17 differentiation and immune cell accumulation into the CNS, emphasize the relevance of CB2 selective ligands as potential therapeutic agents in neuroinflammation.

1. Introduction

The cannabinoid system consists of cannabinoid receptors and both exogenous and endogenous receptor ligands. Most cannabinoid actions are mediated through the classical CB1 and CB2 receptors, with a preferential but not exclusive distribution in the CNS and periphery, respectively [reviewed in [1–4]]. The exogenous ligands include natural (plant derived) ligands and synthetic CB1/CB2 agonists and inverse agonist/antagonists [2,4]. The best characterized endocannabinoids include anandamide (AEA) and 2-arachydonoylglycerol (2-AG), both partial CB1/CB2 agonists [4,5].

The CB1 receptors are located primarily in the CNS at neuronal terminals and regulate neurotransmitter release and psychoactivity. In contrast, the CB2 receptors are expressed primarily on immune cells in the periphery, and during neuroinflammation on activated microglia in the CNS [2,4]. Changes in cannabinoid receptor expression and in endocannabinoid levels have been reported in several pathological conditions, leading to proposed roles of CB1/CB2 receptors in various diseases [reviewed in [1]]. The therapeutic potential of CB2 receptor signaling in a wide array of diseases has been reviewed recently [2]. Important considerations for developing highly selective CB2 receptor agonists include absence of psychoactive effects, sustained anti-inflammatory activity, tissue/cell protection, lack of cardiovascular adverse effects, and efficacy in several disease models, including models of neuroinflammation [1,2].

Multiple sclerosis (MS) is a neuroinflammatory disease characterized in terms of pathology by the presence of CNS inflammatory infiltrates, demyelination and axonal damage, and in terms of symptoms by sensory and motor impairment, ataxia, spasticity,

Abbreviations: APC, antigen-presenting cell; BBB, blood brain barrier; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CNS, central nervous system; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; FACS, fluorescence activated cell sorter; LPS, lipopolysaccharide; MNCs, mononuclear cells; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, phosphate buffered saline; PT, pertussis toxin; qRT-PCR, quantitative RT-PCR; TCR, T cell receptor.

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and cognitive impairment [6]. In MS patients, the majority of lesions (plaques) are present in the brain periventricular white matter, cerebellum, brainstem and optic nerves, and in many cases also in the spinal cord. Studies using the experimental autoimmune encephalomyelitis (EAE) animal model established a sequence of events initiated by activation of myelin-specific CD4 T cells in the periphery, followed by extravasation and reactivation within the CNS, and ultimately leading to potent inflammatory events which result in neuronal and oligodendrocyte cell death [reviewed in [7,8]]. Both myelin-specific Th1 and pathogenic Th17 cells were shown to induce EAE upon adoptive transfer, with the Th17/Th1 ratio being a determining factor for lesion localization [reviewed in [8]].

CB1 and CB2 receptors were shown to play a role in EAE [reviewed in [5,9]]. Both CB1-deficient and CB2-deficient mice developed more severe EAE [10–13], and cell specific receptor knock-outs established the protective effect of neuronal CB1 and of T cell CB2 receptors [10]. The major effect of neuronal CB1 appears to be the dampening of TNFα potentiation of glutamate induced excitatory postsynaptic currents [13], whereas CB2 signaling has a general anti-inflammatory effect, affecting immune cell activation and traffic to the CNS [4,10,11,14].

We reported previously that systemic administration of the selective CB2 receptor ligand O-1966 attenuated both chronic and remitting-relapsing EAE and reduced rolling and adhesion of endogenous leukocytes to pial microvasculature [15]. However, the mechanisms involved in the protective effect of CB2 signaling in EAE, especially in terms of pathogenic Th1/Th17 differentiation and function, have not been elucidated. Here we used the recently developed, highly selective, CB2 agonist Gp1a (N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide) to investigate the protective mechanisms involved in early and delayed Gp1a administration in EAE, and its effects on the in vivo and in vitro Th1/Th17 differentiation.

2. Materials and methods

2.1. Mice

Six to eight weeks old C57BL/6 mice (H-2^b) and C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J (MOG_{35-55} specific TCR) were purchased from Jackson Laboratory (Bar Harbor, ME). Transgenic mice were bred and maintained in the Temple University School of Medicine (TUSM) animal facility (Philadelphia, PA) under pathogen-free conditions. CB2R $(Cnr2)^{+/-}$ mice on C57BL/6 background were originally obtained from the NIH and bred in our TUSM facility to generate $Cnr2^{-/-}$ and $Cnr2^{+/+}$ littermates. All mice used in experiments were between 6 and 10 weeks of age. Mice were handled and housed in accordance with the guidelines of the Temple University Animal Care and Use Committee.

2.2. Reagents

Recombinant murine IL-12, IL-6, IL-1β were purchased from Peprotech Inc (Rocky Hill, NJ). Gp1a was purchased from Abcam (Cambridge, MA). Lipopolysaccharide (LPS) (*Escherichia coli* O55:B5), pertussis toxin (PT), streptavidin-peroxidase, phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). Neutralizing anti-mouse IFNγ and APC-conjugated anti-mouse INFγ were purchased from eBioscience (San Diego, CA). FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse IL17, APC-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse CD3, recombinant mouse IFNγ, capture and biotinylated anti-mouse IFNγ, GolgiPlug, Cytofix/Cytoperm, Perm/Wash buffer and TMB Substrate Reagent Set were purchased from BD PharMingen (San Diego, CA). 7-AAD Viability Staining Solution was purchased from Biolegend (San Diego, CA). CellTrace™ CFSE Cell Proliferation Kit, MOG_{35-55}, 1×HBSS, 10×HBSS and Trizol reagent were purchased from Invitrogen Corporation (Carlsbad, CA). Capture and biotinylated anti-mouse IL17 and recombinant mouse IL17, recombinant TGFβ were purchased from R&D Systems (Minneapolis, MN). DNase I grade II and Liberase TL were purchased from Roche (Indianapolis, IN). Ketamine HCl was purchased from Fort Dodge Animal Health (Fort Dodge, IA). Xylazine was purchase from Butler Animal Health Supply (Dublin, OH). 0.5 M EDTA was purchased from Promega Corporation (Madison, WI). Percoll was purchased from GE Healthcare (Piscataway, NJ). *Mycobacterium tuberculosis* H37 RA was purchased from Difco (Detroit, MI). CD4+ CD62L+ T cell isolation kit II was purchased from Miltenyi Biotec (Auburn, CA). The BrdU flow kit was purchased from BD PharMingen.

2.3. EAE induction and treatment

C57BL/6 mice were immunized with 100 μg MOG_{33-55} peptide emulsified in complete Freund's adjuvant containing 2 mg/ml of *Mycobacterium tuberculosis* H37 RA, s.c. on day 0 and 100 ng pertussis toxin (PT) was administered i.p. on day 0 and day 2. Mice were treated with Gp1a (5 mg/kg in PBS) or vehicle (PBS) via tail vein injection, twice per week. The treatment started from day 0 or day 7 depending on the experiments. Clinical scores were as follows: (0) no overt signs of disease; (1) limp tail or hind limb weakness but not both; (2) limp tail and hind limb weakness; (3) partial hind limb paralysis; (4) complete hind limb paralysis; (5) moribund state, euthanized.

2.4. Isolation of mononuclear cells from central nervous system (CNS)

C57BL/6 mice were immunized as described before. Mice were anesthetized with 20 μl of mix of ketamine HCl and xylazine and perfused through the left cardiac ventricle with 30 ml of HBSS containing 2 mM EDTA. The brain was dissected and spinal cord was flushed out with HBSS. CNS tissue was digested with 10 ml HBSS containing DNAse I (0.1 mg/ml for brain and 0.05 mg/ml for spinal cord) and Liberase (0.05 mg/ml for brain and 0.025 mg/ml for spinal cord) for 45 min at 37 °C with shaking, followed by blocking solution (10% FCS, 10 mM EDTA in HBSS). The tissue was pelleted and resuspended in 10 ml of 30% isotonic Percoll (diluted with 10× HBSS and distilled water), underlaid with 5 ml of 70% isotonic Percoll. Mononuclear cells were isolated from the 30/70 interphase after gradient centrifugation. Cells were washed with RPMI 1640 medium. FACS analysis was undertaken to characterize mononuclear cells and detect IFNγ and IL17 producing CD4 T cells.

2.5. In vivo CD4 T cell differentiation

C57BL/6 mice were immunized as described before. On day 11 the spleens were harvested. Splenocyte single cell suspensions from individual mice were prepared after erythrocyte lysis. Splenocytes (5×10^6 cells/ml) were restimulated with 50 μg MOG_{35-55} in RPMI 1640 medium supplemented with 10% FBS and L-glutamine. Cells were collected and subjected to qRT-PCR after 48 h of culture to detect transcription factor (*Tbx21, Rorc, Foxp3, and GATA3*) expression. After 72 h of MOG_{35-55} activation, the cells were restimulated with PMA and ionomycin in the presence of GolgiPlug in order to measure intracellular cytokine production by FACS.