Journal of Neuroimmunology 220 (2010) 52–63

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Rapamycin inhibits relapsing experimental autoimmune encephalomyelitis by both effector and regulatory T cells modulation

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ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 5 January 2010
Accepted 5 January 2010

Keywords:
T regulatory cells
EAE
Multiple sclerosis
Rapamycin
FoxP3

ABSTRACT

Rapamycin is an oral immunosuppressant drug previously reported to efficiently induce naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T ($_nT_{reg}$) cells re-establishing long-term immune self-tolerance in autoimmune diseases. We investigated the effect of rapamycin administration to SJL/j mice affected by $PLP_{139-151}$-induced relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE). We found that oral or intraperitoneal treatment at the peak of disease or at the end of the first clinical attack, dramatically ameliorated the clinical course of RR-EAE. Treatment suspension resulted in early reappearance of disease. Clinical response was associated with reduced central nervous system demyelination and axonal loss. Rapamycin induced suppression of IFN-γ, and IL-17 release from antigen-specific T cells in peripheral lymphoid organs. While $CD4^+FoxP3^+$ cells were unaffected, we observed disappearance of $CD4^+CD45RB^{high}$ effector T (T_{eff}) cells and selective expansion of T_{reg} cells bearing the $CD4^+CD45RB^{low}FoxP3^+CD25^+CD103^+$ extended phenotype. Finally, the dual action of rapamycin on both T_{eff} and T_{reg} cells resulted in modulation of their ratio that closely paralleled disease course. Our data show that rapamycin inhibits RR-EAE, provide evidence for the immunological mechanisms, and indicate this compound as a potential candidate for the treatment of multiple sclerosis.

1. Introduction

Relapsing–remitting experimental autoimmune encephalomyelitis (RR-EAE) is a $CD4^+$ T_H1 and T_H17 T cell-mediated autoimmune, inflammatory and demyelinating disease of the central nervous system (CNS), used as animal model for human multiple sclerosis (MS) (Kroenke and Segal, 2007). RR-EAE is induced in susceptible female SJL/j mice by immunization with the proteolipid protein ($PLP_{139-151}$)-peptide (Kennedy et al., 1990), and the clinical course is characterized by multiple cycles of relapses and remissions. The immunoregulatory mechanisms responsible for the spontaneous recovery, which may be similar to those responsible for spontaneous remissions seen in patients with relapsing–remitting MS (RRMS), remain to date to be fully elucidated (Duplan et al., 2006; Zhang et al., 2006), but may provide novel therapeutic targets for autoimmunity.

Among the compounds with potent immunosuppressive activity aimed at inducing immune modulation and redirecting awry immune functions is rapamycin. Rapamycin is an oral immunosuppressive drug (Abraham and Wiederrecht, 1996) currently used to prevent rejection in human organ transplantation (Kahan and Camardo, 2001). In mammalian cells, rapamycin forms a complex with the intracellular immunophilin FK506-binding protein-12 (FKBP12), which blocks the activation of a serine/threonine protein kinase called mammalian target of rapamycin (mTOR), that is crucial for cell-cycle progression and protein synthesis, inhibiting antigen-induced T and B cell proliferation (Sehgal, 2003). The efficacy and safety of rapamycin have been previously evaluated in an open-label trial. Patients with clinically definitive RRMS or secondary progressive MS with relapses displayed a significant beneficial effect on the incidence of new enhancing magnetic resonance imaging lesions and number of relapses, with an acceptable risk/benefit profile (Kappos et al., 2005; Neuhaus et al., 2007). In EAE, the effect of this drug has also been examined in rat and mouse models, in which it was highly effective in preventing the onset and severity of disease (Carlson et al., 1993; Branisteanu et al., 1997). Until now, the mechanisms by which rapamycin exerts

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doi:10.1016/j.jneuroim.2010.01.001

its beneficial effect both in humans and in preclinical animal models of MS are largely unknown.

Battaglia et al. have shown that rapamycin administration does not block proliferation in all T cell subtypes, but induces selective expansion of the naturally occurring regulatory T ($_nT_{reg}$) cells subpopulation *in vitro* (Battaglia et al., 2005), and establishes long-term immune self-tolerance in NOD mice affected by type 1 diabetes (Battaglia et al., 2006). A great deal of uncertainty remains, however, about the phenotypic characterization, among the murine $CD4^+$ T_{reg} cells that express the transcription factor Forkhead box P3 (FoxP3) (Fontenot et al., 2003; Hori et al., 2003), of the specialized subsets with effective suppressive function, since recently activated T cells can also express FoxP3 (Zhou et al., 2008). Thus, several additional markers have been used to this aim. They include the historically first proposed T_{reg} marker, interleukin (IL)-2Rα chain (CD25) (Sakaguchi et al., 1995), an activation-induced cytokine receptor component apparently unrelated to the regulatory function because it does not easily discriminate T_{reg} cells from activated T cells. Furthermore, suppressive activity has been demonstrated also in CD25 negative subsets (Annacker et al., 2001; Olivares-Villagomez et al., 2000; Stephens and Mason, 2000). The cytotoxic T lymphocyte-associated antigen (CTLA)-4 (Read et al., 2000) and the integrin $\alpha_E\beta_7$ (CD103) (Lehmann et al., 2002) have been described as additional markers strictly related to a unique population of T_{reg} cells with highly potent regulatory function into sites of inflammation (Korn et al., 2007), although other studies have excluded a significant role for these molecules (Annacker et al., 2005; Levings et al., 2001; Thornton and Shevach, 1998). Finally, other investigations suggest the use of CD45RB to discriminate T_{reg} from T_{eff} cells (Powrie et al., 1994; Dardalhon et al., 2008), the latter being $CD45RB^{high}$ while those required for tolerance induction being $CD4^+CD45RB^{low}FoxP3^+CD25^+$ (Fehervari and Sakaguchi, 2004; Mason and Powrie, 1998; Powrie et al., 1993). These data, altogether, suggest that the use of additional markers may allow to distinguish more efficiently effector from regulatory T cells.

In the present study we used rapamycin monotherapy administered to RR-EAE mice to investigate the effect of this immunomodulatory compound on the pool of endogenous T_{reg} cells and its ability to directly expand their number or preserve their suppressive function *in vivo*. Our data show that rapamycin administration inhibits the induction and the progression of established RR-EAE by effector T (T_{eff}) cells suppression and simultaneously increasing the percentage of $CD4^+CD45RB^{low}FoxP3^+CD25^+CD103^+$ T_{reg} cells.

2. Materials and methods

2.1. Mice

Female SJL/j mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Calco, Italy). Mice were housed in specific pathogen-free conditions, in roomy cages, allowing free access to food and water. All procedures involving animals were performed according to the animal protocol guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC #331) at San Raffaele Scientific Institute (Milan, Italy).

2.2. Induction and assessment of EAE

RR-EAE was induced in female SJL/j mice, as previously described (Butti et al., 2008; Pluchino et al., 2005), by two subcutaneous (s.c.) injections, seven days apart, into both flanks with 100 µl of an emulsion containing 200 µg/mouse of $PLP_{139–151}$ (Espikem, Florence, Italy) and 8 mg/ml of heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco, Lawrence, KS) in incomplete Freund's adjuvant (Sigma, St Louis, MO). In addition, mice received four i.p. injections of 500 ng of pertussis toxin (PT) (List Biological Laboratories, Campbell, CA) on the day of immunization and 24 h later. Mice were weighed and scored for clinical signs daily up to the day of sacrifice. Clinical assessment of EAE was performed according to the following scoring criteria: 0 = healthy; 1 = limp tail; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; 5 = moribund or death.

2.3. Rapamycin administration

RR-EAE female SJL/j mice were immunized as described above and randomized into different treatment groups of 10 mice each according to an early therapeutic schedule (from day 10 to day 45 post-immunization) (p.i.) and a therapeutic schedule (after the first attack of EAE to day 80 p.i.: recovery is defined as the 2nd day in which the mice have recovered at least 1 point on the disease score scale, after the EAE attack). In both cases clinical observation continued up to days 45th and 80th. Rapamycin (Sirolimus, Rapamune®; Wyeth-Europe Ltd, UK) was dissolved in a vehicle solution containing (0.2% w/v) carboxymethylcellulose sodium salt (C-5013) and (0.25% v/v) polysorbate-80 (P-8074) (Sigma, St Louis, MO), in distilled water and stored at 4 °C (39.2 °F) protected from light according to manufacturer's instructions. Rapamycin treatment consisted of i.p. or oral injections given once daily for 15 consecutive days at a dose of 1 mg/kg in a volume of 100 µl for each injection. Dosage was adjusted to the body weight. After the first 15 days of daily treatment, rapamycin administration was continued every three days until the end of the observation period (days 45th and 80th). Control mice received vehicle solution only according to the same schedules and administration routes.

2.4. Preparation of CNS mononuclear cells

At the indicated time points, mice were deeply anesthetized and perfused through the left cardiac ventricle with cold PBS. The forebrain and cerebellum were dissected and spinal cords flushed out with PBS. CNS tissue (brain and spinal cord) was cut into small pieces and digested with 2.5 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) and 1 mg/ml deoxyribonuclease (Sigma, St Louis, MO) at 37 °C for 45 min followed by mechanical disaggregation. Mononuclear cells were isolated by passing the tissue through a cell strainer (70 µm), followed by 30/40/80/100% percoll (GE Healthcare Bio-Sciences AB, Sweden) gradient centrifugation, and recovery of mononuclear cells from the 40/80% interphase.

2.5. Cell isolation and in vitro proliferation assays

Single cell-suspensions were obtained from draining lymph nodes (both inguinal, axillary and cervical) and spleens from untreated or treated mice. Triplicate cultures of (2.5×10^5/well) cells were cultured in round-bottom 96-well culture plates (Costar, Cambridge, MA) in HL1 medium, supplemented with 2% U-glutamine (Lonza, Belgium) and 50 µg/ml Gentamycin (Sigma, St Louis, MO) with serial concentrations (0, 1, 3, 10, 20 µM) of the $PLP_{139–151}$ peptide or precoated with plate-bound 2.5 µg/ml α-CD3 (clone 145-2C11) plus 5 µg/ml soluble α-CD28 (clone 37.51) mAbs (BD Biosciences, Mountain View, CA) for 72 h. To measure cell proliferation, during the last 16 h, cultures were pulsed with 1 µCi/well of [^{3}H]-labeled thymidine (Amersham, Buckingham, UK) followed by harvesting on glass fiber filters and analysis of incorporated [^{3}H]thymidine in a β-counter.

2.6. ELISA cytokines assays

Splenocytes and lymph node cells were cultivated as above. Supernatants were collected after 48 h to assess IL-17 and IFN-γ cytokine production by a mouse-specific ELISA (DuoSet) kit purchased from R&D Systems (UK) according to the manufacturer's protocol. Standards were assayed in duplicate and samples in triplicate.