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Reduction of inflammation and preservation of neurological function by anti-CD52 therapy in murine experimental autoimmune encephalomyelitis

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ABSTRACT

Alemtuzumab, a monoclonal antibody directed against human CD52, is used in the treatment of MS. To characterize the impact of anti-CD52 administration, a monoclonal antibody to mouse CD52 (anti-muCD52) was generated and evaluated in EAE mouse models of MS. A single course of anti-muCD52 provided a therapeutic benefit accompanied by a reduction in the frequency of autoreactive T lymphocytes and production of pro-inflammatory cytokines. Examination of the CNS revealed a decrease in infiltrating lymphocytes, demyelination and axonal loss. Electrophysiological assessment showed preservation of axonal conductance in the spinal cord. These findings suggest that anti-CD52 therapy may help preserve CNS integrity.

1. Introduction

Multiple sclerosis (MS) is a chronic, autoimmune disorder of the central nervous system (CNS) leading to demyelination and associated neurological symptoms.

The exact etiology of MS remains unclear but is generally believed to involve a combination of genetic and environmental factors that lead to the development of CNS autoimmunity and progression of disease in susceptible individuals. Experimental and clinical data suggest that in MS, peripheral activation of T cells reactive against CNS antigens results in upregulation of adhesion molecules and ability of the T cells to cross the blood–brain barrier. Once in the CNS, autoreactive T cells are activated locally by CNS antigens and cause inflammation and damage through the release of pro-inflammatory cytokines and chemokines that recruit and activate other effector cells (e.g. macrophages) (Friese et al., 2014). B lymphocytes are also postulated to participate in disease pathogenesis through the production of autoantibodies, presentation of CNS antigens to T lymphocytes and/or production of cytokines (Krumbholz et al., 2012). But perhaps the strongest evidence for a pathogenic role of lymphocytes in MS is the observed efficacy of disease-modifying therapies targeted at T and/or B cells.

Over the past few years, a number of therapies designed to inhibit the proliferation, function or migration of T lymphocytes have been approved for the treatment of RRMS (Ransohoff et al., 2015). Recent clinical trial data with B cell depleting agents (rituximab, ocrelizumab) also suggest that targeting B lymphocytes may provide a clinical benefit thus supporting a role of B lymphocytes in MS disease pathogenesis (Castillo-Trivino et al., 2013). Alemtuzumab, a humanized monoclonal antibody that targets both T and B lymphocytes was recently approved in over 40 countries. Alemtuzumab is specific for CD52, an antigen present at high levels on the surface of T and B lymphocytes and at lower levels on other immune cells (Hale et al., 1990; Rao et al., 2012). Pivotal phase 3 trials conducted in treatment-naïve relapsing–remitting (RRMS) patients (CARE-MS I) and in RRMS patients who relapsed on prior therapy (CARE-MS II) indicated that alemtuzumab administered as two annual courses of treatment (12 mg intravenously on 5 consecutive days at study start and 3 days a year later) provided superior efficacy compared to interferon-β1a (IFN-β1a) administered three times per week (44 μg subcutaneously) (Cohen et al., 2012; Coles et al., 2012a). In addition, long-term follow-up of patients from a phase 2 study (CAMMS223) suggested that alemtuzumab provides a durable

Abbreviations: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; mu, murine; SMEP, spinal motor evoked potential.

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benefit as it lowered the risk of sustained accumulation of disability by 72% and the rate of relapse by 69% compared to IFNβ-1a out to 4 years after the last alemtuzumab treatment (Coles et al., 2012b). The mechanism by which alemtuzumab exerts its therapeutic effect in MS is not fully understood but may involve rebalancing of the immune system through the depletion and repopulation of lymphocytes. In clinical studies, the administration of alemtuzumab results in rapid depletion of lymphocytes from the circulation which may reduce the inflammatory processes associated with MS (Cox et al., 2005; Thompson et al., 2010). This is followed by a prolonged period of lymphocyte repopulation and clinical research findings suggest that the number, proportions and properties of lymphocyte subsets are altered during the repopulation process. An enrichment in regulatory T (Treg) cells as well as a shift in the $CD4^+$ T cell cytokine profile, characterized by a decrease in the production of pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, have been observed in repopulating lymphocytes post-alemtuzumab (Cox et al., 2005; Zhang et al., 2013). Such changes may lead to a rebalancing of the immune system that persists long after clearance of the antibody and which may contribute to the observed long-lived therapeutic benefit in MS patients.

While studies in MS patients have allowed characterization of the impact of alemtuzumab on blood lymphocyte populations and on CNS parameters via imaging, mouse models afford the opportunity to collect tissues not accessible in humans and allow for the conduct of additional in vivo assays to further our understanding of the mechanism of action and effects of anti-CD52 treatment. In previous studies, we took advantage of the ability to perform immunological challenges and collect lymphoid organs in the mouse to conduct an in-depth characterization of the functionality of the immune system following treatment with anti-CD52 (Hu et al., 2009; Turner et al., 2013). In the current studies, the impact of anti-CD52 therapy was evaluated in mouse models of experimental autoimmune encephalomyelitis (EAE) with a particular focus on the CNS compartment.

2. Materials and methods

2.1. Anti-mouse CD52 monoclonal antibody

Anti-mouse CD52 (muCD52) monoclonal antibodies were generated by immunizing human CD52 knock-in/murine CD52 knockout mice. Animals received three subcutaneous (s.c.) immunizations with a muCD52 peptide (GQATTAASGTNKNSTSTKKTPLKS) conjugated to keyhole limpet hemocyanin (KLH) emulsified in Titermax Gold (Sigma-Aldrich; St. Louis, MO) followed by intraperitoneal (i.p.) immunization with wild type C57BL/6 mouse splenocytes as a source of naturally occurring CD52. Four days prior to the collection of spleen cells, animals were rechallenged i.p. with muCD52-KLH peptide and the following day with the same peptide-KLH intravenously (i.v.). Spleen cells were fused to SP0/2 cells to generate monoclonal antibodies, serum titers assessed by peptide and/or muCD52 fusion protein-based ELISA, and clones screened for muCD52 specificity by flow cytometry. The characteristics of the clone selected for these studies are described in Section 3.1.

2.2. Experimental autoimmune encephalomyelitis (EAE) mouse models

All experimental procedures were approved by and performed in accordance with the Genzyme Institutional Animals Care and Use Committee (IACUC) and conducted in Genzyme's Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. EAE was induced by immunizing C57BL/6ByJ and SJLByJ mice (Jackson Laboratories Bar Harbor, ME) s.c. with 200 μg of MOG_{35-55}, MOG_{1-121}, or $PLP_{131-153}$ peptides emulsified in complete Freund's adjuvant (Chondrex, Redmond, WA) containing 4 mg/ml *Mycobacterium tuberculosis* (Stromnes and Goverman, 2006). Pertussis toxin (200 ng: Sigma-Aldrich; St. Louis, MO) was given i.v. on days 0 and 2. Clinical symptoms of EAE were evaluated using a 5 point scale as follows: 0 no clinical disease, 1.0 complete tail paralysis, 2.0 tail paralysis with hind limb weakness, 3.0 hind limb paralysis, 4.0 hind limb paralysis and fore limb weakness, 5.0 moribund/death. Vehicle (phosphate-buffered saline: ThermoFisher Scientific Inc., Waltham, MA) or anti-muCD52 treatment was given s.c. for five consecutive days as described in each individual figure legend. A dose level of 10 mg/kg of anti-muCD52 was selected based on its ability to achieve levels of lymphocyte depletion and kinetics of repopulation comparable to those observed with alemtuzumab administration in human CD52 transgenic mice (Hu et al., 2009; Turner et al., 2013) and patients.

2.3. Flow cytometry analysis of immune cell populations and cytokine expression

Quantitation of lymphocyte populations from the spleen and peripheral blood was performed by flow cytometry analysis of single cell suspensions as previously described (Turner et al., 2013). Isolation of resident CNS (brain and spinal cord) lymphocytes was performed as described (Rothhammer et al., 2011). Briefly, CNS tissue from perfused animals was digested with collagenase and DNAse followed by mononuclear cell separation by Percoll density gradient centrifugation (Sigma-Aldrich; St. Louis, MO). Mononuclear cells were collected and used for flow cytometry analysis. Staining of immune cells was performed by incubating 1×10^6 cells with fluorescently-labeled antibodies against mouse cell surface markers including CD4 (RM4-5), CD8 (53–6.7) CD44 (1M7), CD62L (Mel-14), CD25 (PC61.5), FR4 (12A5), B220 (RA3-6B2), NK1.1 (PK136), and, GR-1 (RB6.8C5) purchased from Ebioscience (San Diego, CA) or BD Bioscience (San Jose, CA). Peripheral blood was evaluated by staining 50 μl of whole blood with the indicated antibodies followed by removal of contaminating red blood cells with FACs lysis buffer (BD Bioscience) as described by the manufacturer. Fluorescence intensities were determined using an LSR-II flow cytometer (BD Bioscience) and data analysis was performed using Flowjo software (Treestar Inc., Ashland, OR).

Quantitation of absolute numbers of specific cell populations in the spleen and CNS was determined by multiplying the percentage of the FACS-identified cellular population by the total number of cells present in each organ. Numbers of specific cell populations per microliter of peripheral blood were quantified using Countbright Absolute Counting Beads (Invitrogen Life Technologies, Grand Island, NY) as described by the manufacturer (number of cells/μl = number of cells counted / number of beads counted × number of beads added). Percent control for all tissues was calculated by dividing the number of total cells for each population by the mean total number of the same cellular population from the vehicle-treated control group [total cells in test animal/mean of total cells in control group × 100].

Intracellular cytokine staining was performed by culturing splenocytes in vitro with 1 μg/ml of MOG_{35-55} peptide for 5 h in the presence of GolgiStop (BD Bioscience; San Jose, CA) as described by the manufacturer. Cells were co-stained with anti-CD4, anti-IFNγ and anti-IL-17 antibodies (Ebioscience, San Diego, CA).

Levels of cytokine release were evaluated by culturing splenocytes in vitro for 48 h with 1 μg of MOG_{35-55} peptide or irrelevant peptide ($Ova_{323-339}$). Supernatants were then collected and analyzed for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFNγ and TNFα using an Aushon multiplex array (Aushon Biosystems, Billerica, MA) following the manufacturer's instructions.

2.4. Immunohistochemistry

Spinal cord tissue samples were processed and sectioned by Mass Histology (Worcester, MA). Tissues were processed using a standard protocol consisting of formalin fixation and paraffin embedding, followed by cutting of 5 μm thick sections. Hematoxylin and eosin (H&E) staining was performed on deparaffinized and rehydrated sections. Hematoxylin and