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The epigenetic drug Trichostatin A ameliorates experimental autoimmune encephalomyelitis *via* T cell tolerance induction and impaired influx of T cells into the spinal cord

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ABSTRACT

Multiple sclerosis is a T cell mediated chronic demyelinating disease of the central nervous system. Although currently available therapies reduce relapses, they do not facilitate tolerization of myelin antigen-specific T lymphocytes to ensure prolonged protection against multiple sclerosis. Here, we show that treatment of NOD mice with the histone deacetylase inhibitor, Trichostatin A affords robust protection against myelin peptide induced experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Protection was accompanied by histone hyperacetylation, and reduced inflammation and axonal damage in the spinal cord. Drug treatment diminished the generation of $CD4^+$ memory T cells and induced tolerance in $CD4^+$ T cells recognizing the immunizing myelin peptide. During the early immunization period, $CD4^+$ T cells producing GM-CSF + IFN-γ, GM-CSF + IL-17A, as well as those expressing both IL-17A + IFN-γ (double-producers) were detected in the secondary lymphoid organs followed by the appearance of cells producing IFN-γ and GM-CSF. On the other hand, IFN-γ producing Th1 cells appear first in the spinal cord followed by cells producing IL-17A and GM-CSF. Treatment with Trichostatin A substantially reduced the frequencies of all T cells secreting various lymphokines both in the periphery and in the spinal cord. These data indicate that epigenetic modifications induced by histone hyperacetylation facilitates T cell tolerance induction in the periphery leading to reduced migration of T cells to the spinal cord and mitigation of neuronal damage and improved clinical outcome. These results suggest that epigenetic modulation of the genome may similarly offer benefits to multiple sclerosis patients *via* abrogating the function of encephalitogenic T lymphocytes without exerting severe side effects associated with currently used disease-modifying therapies.

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1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) (Dendrou et al., 2015; Weissert, 2013). Several disease-modifying therapies reduce relapses in MS patients but are associated with significant side effects including fatalities (Wingerchuk and Carter, 2014; Martin et al., 2016). Although anti-virals such as interferon (IFN)-β-1a and 1b are well tolerated, they are inefficient in affecting Th17 cells (Axtell et al., 2013), which are found abundantly in the blood and cerebrospinal fluid of MS patients during clinical exacerbations (Matusevicius et al., 1999). Natalizumab, a humanized mAb against widely expressed α4 integrin, a part of the VCAM-1 cognate ligand, and Dimethyl fumarate are associated with progressive, fatal multifocal leukoencephalopathy in John Cunningham virus seropositive patients (Weissert, 2013; Wingerchuk and Carter, 2014; Martin et al., 2016). Fingolimod that targets sphingosine-1-phosphate receptor and blocks T cell transmigration into the CNS results in cardiac complications, reactivation of varicella zoster and herpes simplex virus, and exacerbation of MS and experimental autoimmune encephalomyelitis (EAE), the mouse model of MS (Mohammad et al., 2014). Other severe adverse side effects include leukemia and cardiovascular contradictions (Fingolimod), teratogenic risk (Teriflunomide), and secondary autoimmune diseases (Weissert, 2013; Wingerchuk and Carter, 2014; Martin et al., 2016). Although these non-specific immunomodulatory therapies target activation of

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; CNS, central nervous system; ConA, Concanavalin A; DMSO, dimethyl sulfoxide; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; GM-CSF, granulocyte macrophage-colony stimulating factor; HDAC, histone deacetylase; IL-17A, interleukin 17A; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NOD, non-obese diabetic; PMA, phorbol myristic acetate; PP-EAE, primary progressive EAE; TCR, T cell receptor; Th, T helper; Treg, T regulatory; TSA, Trichostatin A.

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immune cells in the periphery and their entry into the CNS, they hardly impinge upon the function of encephalitogenic T lymphocytes or resident inflammatory component in the CNS. Thus, therapies that can induce antigen-specific tolerance in encephalitogenic T lymphocytes are an unmet need for MS treatment.

Many clinical trials had been conducted in attempts to induce T cell tolerance in MS patients (Lutterotti and Martin, 2014; Steinman, 2015). These include administration of synthetic peptides corresponding to T cell epitopes mapped within myelin components such as myelin basic protein, proteolipid proteins, and myelin oligodendrocyte glycoprotein (MOG). Bovine myelin, a bacterial plasmid encoding whole human myelin basic protein and altered ligand peptide, a peptide derivative of myelin basic protein that was modified at the T cell receptor (TCR) contact region were also used for tolerance induction. Other strategies included the TCR vaccination constituting attenuated autologous antigen-specific T cells or peptides from the complementarity determining region 2 or 3 of myelin reactive T cells and autologous peripheral blood mononuclear cells chemically coupled with myelin peptides. None of these maneuvers induced T cell tolerance as assessed by the ability of peripheral blood T cells to proliferate and produce IFN-γ in response to a challenge with the corresponding immunizing peptide *in vitro*. Importantly, they also did not improve the clinical outcome in MS patients. Although transient $CD4^+$ T cell deletion in conjunction with the administration of antigen (spinal cord homogenate) coupled splenocytes late during EAE in Biozzi ABH mice suppressed relapses, it failed to prevent the long-term neurodegeneration caused by axonal damage (Pryce et al., 2005; Hampton et al., 2013). Thus, an effective antigen-specific T cell tolerance-inducing strategy is required to protect against relapses and minimize long-term neurological deficits in EAE and in MS patients.

Although Genome Wide Association Studies have implicated genes encoding human leukocyte antigens in MS pathogenesis (International Multiple Sclerosis Genetics Consortium et al., 2011), environmental factors including Epstein-Barr virus infection, smoking, and Vitamin D deficiency may have substantial influence by indirectly altering gene expression without changing the DNA sequence, termed epigenetics (Allis and Jenuwein, 2016). However, the field of epigenetics of MS is in its infancy and remains to be fully elucidated (Koch et al., 2013). A clear understanding of these mechanisms will pave the way for better manipulation of MS without causing undesirable side effects induced by currently used disease modifying agents. Accumulating evidence indicates that the histone deacetylase (HDAC) inhibitors such as Trichostatin A (TSA), originally developed for cancer treatment (Marks, 2010), and other similar drugs, Vorinostat and Valproic acid can ameliorate monophasic, self-limiting EAE in C57BL/6 mice (Camelo et al., 2005; Ge et al., 2012; Lv et al., 2012). This model is frequently used to test the efficacy of various disease manipulations because of the availability of genetically modified strains on the C57BL background. However, the pathology of monophasic EAE studied in C57BL/6 mice does not parallel that of an MS form (Slavin et al., 1998). Since each variant of EAE recapitulates some but not all features of MS, it is critical to ascertain the efficacy of HDAC inhibitors in a preclinical model that closely resembles MS. Immunization of autoimmune prone female non-obese diabetic (NOD) mice with the immunodominant $MOG_{35–55}$ peptide consistently induces EAE with a high frequency that shares unique features with MS including life long disease, prominent demyelination, axonal loss, and astrogliosis (Slavin et al., 1998; Basso et al., 2008; Pham et al., 2011; Hidaka et al., 2014). Thus, the NOD mouse model appears ideal for investigating the efficacy of HDAC inhibitors like TSA for MS treatment. In addition, we have recently shown that treatment of female NOD mice with TSA ameliorated spontaneously occurring autoimmune type 1 diabetes associated selective regulation of a set of proinflammatory genes (Patel et al., 2011; Jayaraman et al., 2013). Therefore, we envisaged that the epigenetic modulation of the genome could similarly modify the development of EAE induced by immunization with an immunodominant myelin antigen.

We demonstrate herein the utility of the HDAC inhibitor TSA to afford robust protection against primary progressive EAE (PP-EAE) in the autoimmune prone NOD strain of mouse. In addition to preventing inflammation, demyelination and axonal damage, TSA treatment also induced antigen-specific tolerance in both Th1 (T helper 1) and Th17 cells and thus ensuring irreversible inactivation of the pathogenicity of these functionally distinct subsets implicated in MS pathogenesis (Dendrou et al., 2015; Weissert, 2013; Axtell et al., 2013; Matusevicius et al., 1999). Thus, epigenetic modulation of the genome can prevent experimentally induced demyelinating disease in a preclinical model that closely resembles MS. These data suggest that similar epigenetic regulation using drugs such as TSA may alter the epigenetic landscape leading to tolerance induction in myelin reactive T lymphocytes and reduction in accrual of disabilities in MS patients.

2. Materials and methods

2.1. Animals

Six to eight wk old female NOD/ShiLtj mice purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained under specific pathogen-free conditions with standard animal chow and water *ad libitum*. All procedures in studies involving animals were approved by the Office of Animal Care and Institutional Biosafety Committee of the University of Illinois at Chicago and were conducted in accordance with the ethical standards of the institution and the National Institutes of Health guide for the care and use of animals.

2.2. EAE induction and assessment

Five mice per experimental group were randomly assigned. All mice in each experiment were littermates. Mice were injected s.c with 0.1 ml of phosphate buffered saline containing 100 μg of mouse $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK, Tocris Bioscience) and emulsified with an equal volume of complete Freund's adjuvant with an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) (Fisher Scientific), as described (Slavin et al., 1998). Mice were injected i.v with 300 ng of reconstituted lyophilized Pertussis toxin (List Biological Laboratories) on the day of immunization and two days later. The stock solution of TSA (1 mg/ml in DMSO, Sigma) was diluted 20× in phosphate-buffered saline and injected s.c on the flank at a final dose of 500 μg/kg body weight three times a week, as we described earlier (Patel et al., 2011; Jayaraman et al., 2013). Controls received 0.2 ml of 20× diluted DMSO in phosphate buffered saline. Body weight was recorded every other day and EAE score assigned as follows: 0, normal, 1, limp tail, 2, one hind limb weakness, 3, both hind limbs weakness, 4, one or both fore limb weakness, and 5, paralysis, moribund or death.

2.3. Histological analysis

At specified time points, mice were anesthetized and perfused intracardiacally with 4% paraformaldehyde. The spinal cord was dissected out, fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μM thin sections. Hematoxylin and eosin stained sections were observed for inflammation. Luxol fast blue staining was performed for assessing demyelination. Silver impregnation method was used for determining axonal integrity. Investigators unaware of treatment conditions examined the spinal cord sections from various regions of individual mice.

2.4. Western blotting

Nuclear proteins from the spinal cord were isolated by acid extraction and separated on a 15% PAGE gel, blotted onto a PVDF membrane, incubated with rabbit antibodies generated against total histone H3 and acetyl-histone H3 (Upstate) as we described (Patel et al., 2011).

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