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Research Paper

Amelioration of progressive autoimmune encephalomyelitis by epigenetic regulation involves selective repression of mature neutrophils during the preclinical phase

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ABSTRACT

We have recently demonstrated that treatment of NOD mice with the epigenetic drug Trichostatin A (TSA) ameliorated myelin peptide induced progressive experimental autoimmune encephalomyelitis (P-EAE). Protection was accompanied by induction of antigen-specific T-cell tolerance in the periphery and reduced influx of T cells into the spinal cord. In this investigation, we examined whether the epigenetic drug could impact the innate immune system as well. Whereas the mature (MHC class II^+) $CD11b^+Ly\text{-}6G^+$ neutrophils expanded substantially in the peripheral lymphoid compartment during the preclinical phase, the MHC class II^+, $CD11b^+Ly\text{-}6C^+$ mature monocytes increased modestly throughout the disease course. Amelioration of the clinical disease by TSA treatment was accompanied by diminished abundance of $CD11b^+Ly\text{-}6G^{dim}$ activated neutrophils in secondary lymphoid organs and their influx into the spinal cord without affecting monocytes. Interestingly, the co-inhibitory ligand $CD274^+$ ($PD\text{-}L1^+$) but not $CD275^+$ ($ICOS\text{-}L^+$), $CD39^+$ or $CD11c^+$ dendritic cells were decreased in the peripheral lymphoid compartment of drug treated mice. Thus, in addition to myelin-specific T cell tolerance induction observed previously, selective repression of mature neutrophils and $PD\text{-}L1^+$ cells is critically involved in the epigenetic regulation of P-EAE.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is initiated by the activation of myelin-specific $CD4^+$ T lymphocytes in the peripheral lymphoid organs. Activated T cells infiltrate the central nervous system (CNS) and cause neuronal damage (reviewed by Dendrou et al., 2015). The CNS-resident microglia, as well as the infiltrating macrophages and dendritic cells have been implicated in the reactivation of T cells in the CNS leading to perpetuation of neuronal degeneration (Mildner et al., 2009). However, the roles of the innate immune cells such as macrophages and neutrophils in EAE remain contentious. Both pathogenic (Takahashi et al., 2007; King et al., 2009; Mildner et al., 2009) and protective roles (McColl et al., 1998; Saiwai et al., 2013; Slaney et al., 2013) have been attributed to $Ly\text{-}6C^+$ monocytes in monophasic EAE models. On the other hand, most studies attributed a pathogenic role for neutrophilic granulocytes. Thus, depletion of neutrophils by treatment with the monoclonal antibody RB6-8C5 (McColl et al., 1998; Carlson et al., 2008; Steinbach et al., 2013) or 1A8 (Yi et al., 2012) afforded protection against monophasic EAE.

Myeloid derived suppressor cells (MDSCs) are immature cells with suppressive potential that can differentiate into monocytic (M-MDSCs) or granulocytic (G-MDSCs) types (Peranzoni et al., 2010; Bronte et al., 2016). Although MDSCs primarily arise in the context of tumors resulting in immunosuppression, their role in neurodegeneration remains contentious. Putative G-MDSCs were either shown to exacerbate (Yi et al., 2012) or protect against monophasic EAE (Ioannou et al., 2012). In both cases, G-MDSCs were characterized by the co-expression of the common myeloid CD11b integrin and the granulocytic marker, Ly-6G. Since the maturation status of these cells was not defined in these studies, it is difficult to ascertain whether the contrasting effects of the Ly6-G-expressing cells on EAE could be attributed to mature neutrophils or the immature G-MDSCs with suppressive potential.

Abbreviations: CNS, central nervous system; DMSO, dimethyl sulfoxide; EAE, experimental autoimmune encephalomyelitis; G-MDSC, granulocytic myeloid derived suppressor cells; HDAC, histone deacetylase; MHC, major histocompatibility complex; M-MDSC, monocytic myeloid derived suppressor cells; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NOD, non-obese diabetic; P-EAE, progressive experimental autoimmune encephalomyelitis; TSA, Trichostatin A

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Importantly, it is unclear whether neutrophils play a key role in the induction of progressive EAE (P-EAE) (Jayaraman et al., 2017) and can be modulated by epigenetic drug treatment.

Immunization with the myelin oligodendrocyte glycoprotein 35–55 ($MOG_{35–55}$) peptide induced prolonged EAE in 100% of autoimmune prone female NOD mice (Slavin et al., 1998; Basso et al., 2008; Hidaka et al., 2014; Dang et al., 2015; Jayaraman et al., 2017). The disease progressed without prominent remission resembling primary, progressive multiple sclerosis (MS) and hence termed P-EAE (Basso et al., 2008; Jayaraman et al., 2017). The NOD mouse model also shares certain unique features with progressive MS including demyelination and axonal loss (Slavin et al., 1998; Basso et al., 2008; Hidaka et al., 2014; Dang et al., 2015; Jayaraman et al., 2017) and astrogliosis (Dang et al., 2015). Thus, the NOD mouse model offers a unique opportunity to study certain pathological events associated with MS not found in frequently studied monophasic and relapsing-remitting EAE models (Dang et al., 2015; Procaccini et al., 2015).

Epigenetics refers to changes in gene expression without altering the DNA sequence that can result in phenotypic variations with identical genotypes (Allis and Jenuwein, 2016). Acetylation of histones mediated by histone acetyl transferases has profound influence on gene transcription (Brandl et al., 2009), which is opposed by histone deacetylases (HDACs). Trichostatin A (TSA) derived from *Streptomyces platensis* is the most potent of all HDAC inhibitors (de Ruijter et al., 2003). TSA administration inhibited P-EAE in NOD mice when given prophylactically or therapeutically (Jayaraman et al., 2017). This was accompanied by increased histone H3 acetylation and reduced inflammation, demyelination and axonal damage in the spinal cord. Interestingly, TSA compromised the ability of T lymphocytes in the peripheral lymphoid organs to proliferate and produce IFN-γ, IL-17A, and GM-CSF when challenged with the immunizing antigen, indicating induction of antigen-specific T cell tolerance. Generation of $CD4^+CD44^+$ memory cells but not the $CD4^+FoxP3^+$ or $CD4^+CD62L^+$ T regulatory cells was impaired in drug treated mice. Importantly, TSA treatment reduced the influx of T cells producing IFN-γ, IL-17A, and GM-CSF into the spinal cord (Jayaraman et al., 2017). These data demonstrate pronounced effects of TSA on the adaptive immune system.

In this investigation, we determined the effects of TSA on the innate immune system. We report herein that mature neutrophils and not MDSCs expanded transiently in the secondary lymphoid organs of mice undergoing P-EAE. Drug treatment reduced the clinical symptoms and neutrophils in the peripheral lymphoid compartment and their infiltration into the spinal cord. Interestingly, cells expressing CD274 (PD-L1) but not CD275 or CD39 were diminished by the drug treatment. These data demonstrate the differential influence of TSA on innate immune cells, which may have implications to MS treatment.

2. Methods

2.1. Experimental design

The Office of Animal Care and Institutional Biosafety of the University of Illinois at Chicago approved the animal protocol. Experiments were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. EAE induction and evaluation

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*. Mice were immunized s.c with 100 μg of mouse $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK, Tocris Bioscience) emulsified in complete Freund's adjuvant with an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) (Fisher Scientific), as described (Jayaraman et al., 2017). Mice also received 300 ng of Pertussis toxin (List Biological Laboratories) i.v on the day of immunization and 2 days later. TSA (Sigma) was dissolved in dimethyl sulfoxide (DMSO), diluted in PBS and the desired amount (500 μg/Kg body weight) was injected s.c in a final volume of 0.2 ml three times a week, as we described earlier (Patel et al., 2011; Jayaraman et al., 2013; Jayaraman et al., 2017). The duration of drug treatment in individual experiments is indicated in the Results section. Controls received the same amount of DMSO diluted in 0.2 ml of PBS. EAE score was 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 (Jayaraman et al., 2017).

2.3. Flow cytometry

Separate sets of mice from various experiments were sacrificed at specific time points. A single cell suspension of splenocytes and draining lymph node cells or leukocytes from the spinal cord was prepared as described (Jayaraman et al., 2017) and subjected to phenotypic analysis by flow cytometry. The following antibodies were used: anti-CD11b (clone M1/70, eBioscience) conjugated with PE-Cyanine5, anti-Ly-6C-FITC (clone HK1.4, eBioscience), anti-Ly-6G-FITC (clone 1A8, BD Pharmingen), anti-Ly-6G (Gr-1) Alexa Fluor488 (clone RB6-8C5, eBioscience), PerCP eFluor719-CD274 (clone M1H5, eBioscience), Alexa Flour488-CD115 (AFS98, eBioscience), FITC-CCR2 (clone 475,301, R & D Systems), and APC-CD11c (clone N418, eBioscience). Dual expression of Ly-6C and Ly-6G was detected among $CD11b^+$ cells by staining with anti-Ly-6G-FITC and brilliant violet 421 conjugated anti-mouse Ly-6C (clone HK1.4, BioLegend) along with PE-Cy5-CD11b. Cells were also incubated with purified rat anti-mouse CD275 (ICOS-L, clone HK5.3, eBioscience), anti-CD39 (clone Duha 39, BioLegend) or anti-F4/80 (clone C1:A31, Serotec) followed by staining with goat anti-rat DyLight 488 (Catalog # 405409, BioLegend) or PE-goat anti-rat antibody (catalog # SC 3740, Santa Cruz). To detect NOD-specific MHC class II determinant $I\text{-}A^{g7}$, cells were incubated with purified mouse anti-rat RT1B (clone OX-6, BD Pharmingen) followed by rat anti-mouse antibody conjugated with V450. Live leukocytes were selected based on forward and side scatter properties and analyzed on a BD Fortessa flow cytometer. Flow cytometry data were analyzed using the FlowJo (Treestar) software.

2.4. Confocal imaging

Splenocytes were stained with anti-CD11b-PerCP-Cy5, anti-Ly-6C-FITC or anti-Ly-6G-FITC. Cytospin preparations were air-dried, fixed with absolute methanol, stained with Hoechst and imaged using Zeiss LSM 710 confocal microscope. Images were acquired using Zen software and analyzed using Adobe Photoshop Elements (9.0).

2.5. Statistical analysis

Statistical significance of clinical scores was assessed by Mann Whitney *U* test as reported (Jayaraman et al., 2017). Flow cytometry data were analyzed for statistical significance using two-tailed unpaired Student's *t*-test (GraphPad Prism 6.0).

3. Results

3.1. TSA treatment ameliorated P-EAE

We have recently reported that immunization of female NOD mice with the mouse $MOG_{35–55}$ peptide induced discernible clinical disease as early as 14 days followed by a substantial increase in the clinical score around 28 days. The disease persisted with similar intensity as long as observed, 115 days (Jayaraman et al., 2017). Mice were treated prophylactically with TSA by s.c injections on alternate days starting from the day of immunization and until 11, 14, 23 and 45 days and