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Deacetylase Inhibition Increases Regulatory T Cell Function and Decreases Incidence and Severity of Collagen-induced Arthritis

Sandra J. Saouaf^{*}, Bin Li^{*}, Geng Zhang^{*}, Yuan Shen^{*}, Narumi Furuuchi^{*}, Wayne W. Hancock^{*,†}, and Mark I. Greene^{*,‡}

^{*}Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, 19104-6082, USA

[†]Department of Pathology and Laboratory Medicine, Joseph Stokes, Jr. Research Institute, Children's Hospital of Philadelphia, PA, 19104-4318, USA

Abstract

Collagen-induced arthritis (CIA) is an established mouse model of disease with hallmarks of clinical rheumatoid arthritis. Histone/protein deacetylase inhibitors (HDACi) are known to inhibit the pathogenesis of CIA and other models of autoimmune disease, although the mechanisms responsible are unclear. Regulatory T cell (Treg) function is defective in rheumatoid arthritis. FOXP3 proteins in Tregs are present in a dynamic protein complex containing histone acetyltransferase and HDAC enzymes, and FOXP3 itself is acetylated on lysine residues. We therefore investigated the effects of HDACi therapy on regulatory T cell function in the CIA model. Administration of an HDACi, valproic acid (VPA), significantly decreased disease incidence ($p < 0.005$) and severity ($p < 0.03$) in CIA. In addition, VPA treatment increased both the suppressive function of CD4⁺CD25⁺ Tregs ($p < 0.04$) and the numbers of CD25⁺FOXP3⁺ Tregs *in vivo*. Hence, clinically approved HDACi such as VPA may limit autoimmune disease *in vivo* through effects on the production and function of FOXP3⁺ Treg cells.

Keywords

T cell; suppression; autoimmunity; rheumatoid arthritis

Introduction

Collagen induced arthritis (CIA) is an established mouse model of human rheumatoid arthritis (Kannan et al. 2005). Immunization with heterologous collagen type II in susceptible mouse strains results in autoantibody production to murine collagen type II, followed by autoantibody binding to joint cartilage, activation of the complement cascade, and inflammation resulting in tissue destruction (Joe et al. 1999). Although the pathogenesis of CIA involves autoantibodies, disease induction requires the interaction of CD4⁺ T cells and B cells, as well

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[‡]**Correspondence:** Mark I. Greene, Pathology and Laboratory Medicine, University of Pennsylvania, 252 John Morgan, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, USA; Phone: 1 215 898 2847; Fax: 1 215 898 2401; greene@reo.med.upenn.edu.

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as the production of multiple cytokines. Both MHC and non-MHC genes are implicated in disease susceptibility (Kannan et al. 2005). Regulatory T cells (Treg) are important to the pathogenesis of CIA given exacerbation of disease following their depletion (Morgan et al. 2003; Frey et al. 2005), and data showing that adoptive transfer of CD4⁺CD25⁺ Treg cells can cure CIA (Frey et al. 2005; Morgan et al. 2005).

Histone/protein deacetylase inhibitors (HDACi) can mitigate disease in models of autoimmune disease including CIA, experimental autoimmune encephalomyelitis, and systemic lupus erythematosus (Chung et al. 2003; Nishida et al. 2004; Reilly et al. 2004; Camelo et al. 2005; Garcia et al. 2005; Lin et al. 2007). HDACi can also decrease tumor cell proliferation through modulation of gene expression and induction of cell cycle arrest (Buglio et al. 2008; Jones et al. 2008), and the same mechanisms may apply in autoimmune models (Chung et al. 2003; Nishida et al. 2004; Camelo et al. 2005). We have shown that FOXP3, HDACs, histone acetyltransferases and additional proteins form a large macromolecular complex in Treg cells (Li et al. 2006; Li et al. 2007). FOXP3 is the master regulatory gene responsible for Treg control of CD4⁺ effector T cells (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). Using the HDACi valproic acid (VPA), we have now examined the effects of modulating HDAC activity on Treg cell function in the CIA model of autoimmune disease. Our results indicate that deacetylase inhibition by VPA enhances Treg function, increases the number of FOXP3⁺ Treg cells, and limits autoimmune disease *in vivo*.

Materials and Methods

Collagen induced arthritis model

CIA was induced in DBA/1 male mice at age six to eight weeks by intradermal injection of 100 µg of chicken type II collagen (Sigma) in complete Freund's adjuvant on day 0 followed by intraperitoneal (i.p.) injection of 100 µg collagen in PBS on day 21 (Murali et al. 2005). VPA (400 mg/kg) or PBS was administered i.p. daily, starting on day 21 of the study. Clinical disease was scored every 2–3 days using established criteria (Kim et al. 2002). Paws were scored individually as: 0, normal; 1, mild inflammation (redness and swelling) of a single area (toes, midfoot, or ankle); 2, mild to moderate inflammation of 2 areas (toes, midfoot, or ankle); 3, moderate to severe inflammation involving entire paw – toes, midfoot, and ankle; 4, severe inflammation of entire paw resulting in ankylosis and loss of joint movement. The score for each paw was added such that each mouse scored between 0–16. This animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

Histology

For histological examination of joints, mice were sacrificed at day 60 of the study, paws were collected and fixed in Histochoice (Amresco), decalcified in 7% NH₃OH, 0.38M EDTA, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin/eosin. Tissue sections were scored in a blinded manner by a pathologist using the histologic scale of 0 (normal), 1 (mild), 2 (moderate) and 3 (severe) for each of inflammation, synovial hypertrophy, cartilage damage and bone destruction.

Immunopathology

Additional paraffin sections of joints harvested for histology at day 60 of the study (n=4/group) were dewaxed, stained by immunoperoxidase (Tao et al. 2007) using mAbs to murine leukocyte cell markers (BD Biosciences), FOXP3 (FJK-16s, eBioscience) and anti-acetylated lysine (7F8, Cayman) or isotype controls, plus an immunoperoxidase Envision kit (Dako), and counterstained with hematoxylin.

Treg assay

CD4⁺CD25⁺ Treg cells from CIA-induced mice were isolated on day 60 of the study using a magnetic bead isolation kit (Miltenyi) according to manufacturer's instructions. CD4⁺CD25⁺ T effector (Teff) cells from syngeneic naïve mice without induced disease were also isolated using magnetic beads. Purity of >92% was routinely obtained as shown by flow cytometry. Treg and Teff cells were co-cultured for 72 hours in 96-well plates pre-coated with anti-CD3ε mAb (145-2C11, Biolegend, 1 µg/ml). The ratio of Treg cells to Teff cells was 1:2 Treg:Teff cells/well, using 0.02×10^6 Treg/well and 0.04×10^6 Teff/well in 200 µl assay media (RPMI plus 10% FCS, penicillin, streptomycin, L-glutamine, 1mM sodium pyruvate, 2×10^{-5} M β-mercaptoethanol and 0.1mM non-essential amino acids, 0.01M HEPES buffer). Controls included 0.04×10^6 Teff and 0.02×10^6 Treg cells cultured separately on anti-CD3ε mAb coated plates. Cells were cultured in triplicate wells at 37° C in 7% CO₂. During the last 12–18 hours of culture, cells were pulsed with 1µCi of tritiated thymidine/well and proliferation levels were determined using scintillation counting of incorporated tritiated thymidine.

Flow cytometry

Whole splenocytes from CIA-induced mice were collected on day 60 of the study and were stained for cell surface CD4 and CD25 using 0.5mg anti-CD4 FITC-conjugated GK1.5 mAb (BD Pharmingen) and 0.5mg anti-CD25 PE-conjugated PC-61 mAb (Southern Biotech). After washing, cells were permeabilized for intracellular staining of FOXP3 using FJK-16s mAb conjugated to APC, or isotype control Rat IgG2a-APC (eBioscience). Flow cytometry was performed on a FACSCalibur (Becton Dickinson) in the University of Pennsylvania flow cytometry core facility, and analyzed with CELLQUEST software (Becton Dickinson).

Statistical analyses

Data are expressed as a mean $\pm$ SEM. Analysis of differences between groups was performed using a two-tailed t-test. For statistical analysis of disease incidence a Chi square test for independence was used.

Results

The CIA model of rheumatoid arthritis was used to assess the effect of HDACi on autoimmune disease. CIA was induced in DBA/1 mice by intradermal administration of type II collagen on day 0, followed by intraperitoneal injection of type II collagen on day 21. VPA (400 mg/kg) or PBS was administered daily, starting on day 21 of study. Disease was monitored by scoring clinical signs every 2–3 days, from day 28 until the end of the study on day 60. Disease incidence was decreased from 79% of CIA-induced mice receiving PBS to 31% of CIA-induced mice in the VPA administered group (Table I) ($p < 0.005$). The mean clinical score of CIA-induced mice treated with VPA was decreased throughout the study, resulting in a 92% decrease in clinical disease severity of VPA-treated mice on day 60, with VPA-treated mice having a mean clinical score of 0.33 ± 0.22 and PBS-treated mice 4.27 ± 1.40 ($p < 0.03$) (Fig. 1).

Joint destruction in CIA is marked by synovial hyperplasia, pannus formation and cartilage and bone destruction. Histologic analysis of proximal interphalangeal (PIP) joints of CIA-induced mice were performed on day 60 of the study. Sections from VPA treated CIA-induced mice reveal well preserved PIP joints with negligible inflammation, synovial hypertrophy, cartilage damage or bone destruction. Sections from control PBS treated CIA-induced mice, however, show severe arthritis, marked erosion of articular surfaces, cartilage breakdown, synovial hypertrophy and inflammatory cell infiltration (Fig. 2A). Histologic sections were scored in a blinded manner according to the severity of inflammation, synovial hypertrophy,

cartilage damage and bone destruction. In all cases VPA treatment significantly decreased the histopathologic score associated with CIA (Fig. 2B).

Treg cells of patients with rheumatoid arthritis are defective in their suppression of CD4⁺ T effector cells (Ehrenstein et al. 2004; Valencia et al. 2006). We have shown that FOXP3 function in regulatory T cells is affected by the activity of histone acetyltransferase and HDAC enzymes (Li et al. 2007; Tao et al. 2007). We assessed the effect of modulating HDAC activity on Treg function, using the HDACi, VPA. Daily treatment with VPA increased acetylation of proteins isolated from splenocytes of CIA-induced mice compared to CIA-induced mice treated with PBS (not shown). CD25⁺CD4⁺ splenocytes from CIA-induced mice treated with VPA or PBS were isolated and co-cultured with CD25⁻CD4⁺ T effector cells from naïve mice on anti-CD3 mAb coated plates. Tregs from PBS-treated, CIA-induced mice inhibited the proliferation of CD25⁻CD4⁺ T effector cells to TCR signals by 31%, whereas Treg cells from VPA-treated, CIA-induced mice decreased the same proliferative response by 60% (Fig. 3). This indicates that treatment with HDACi, VPA, can increase the function of Treg cells from CIA-induced mice, resulting in improved clinical disease. In similar studies in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis, VPA treatment resulted in greatly improved clinical disease and increased Treg function (not shown).

To further investigate the effect of HDACi treatment on the regulatory T cell subset, we examined the number of FOXP3⁺ T cells in VPA versus PBS treated CIA-induced mice. VPA treatment of CIA-induced mice resulted in a 39% increase in splenic CD4⁺FOXP3⁺ T cells and a 42% increase in the splenic CD25⁺ FOXP3⁺ T cell subset compared to CIA-induced mice treated with PBS (Fig 4. and Table II). HDACi treatment not only improved Treg function, but also promoted the expansion of FOXP3⁺ regulatory T cells *in vivo* in disease-induced mice.

Further histologic analysis of PIP joints revealed a marked reduction of leukocytes in the joints and peri-articular tissues of CIA-induced mice treated with VPA as compared to PBS control (Fig. 5a, b). Despite the reduced number of leukocytes, recruitment of FOXP3⁺ mononuclear cells to peri-articular tissues in PIP joint sections from VPA treated mice was observed (Fig. 5e, f). FOXP3⁺ cells comprised 20–30% of the residual peri-articular infiltrate in VPA-treated mice. In addition, compared with PBS-treated mice, VPA therapy was associated with increased staining for acetylated lysine, consistent with inhibition of HDAC activity *in vivo* (Fig. 5c, d).

Taken together, treatment of CIA-induced mice with VPA, a clinically approved HDACi, greatly improves the clinical disease state by increasing both the function and number of Treg cells.

Discussion

The function of regulatory T cells in rheumatoid arthritis patients has been studied and reveals them to be defective in function (Ehrenstein et al. 2004; Valencia et al. 2006). Regulatory T cells from rheumatoid arthritis patients are unable to prevent release of inflammatory cytokines from effector CD4⁺ CD25⁺-T cells, they do not suppress effector T cell proliferation, and they have low levels of FOXP3 expression (Valencia et al. 2006). Given the ability of adoptively transferred regulatory T cells to mitigate CIA (Frey et al. 2005; Morgan et al. 2005), it is reasonable to propose therapy aimed at increasing Treg cell function in rheumatoid arthritis.

VPA is a clinically approved HDACi that is currently used in the treatment of epilepsy and has been shown to be a safe, effective treatment in humans (Garcia-Morales et al. 2007). Although the mechanism of action of VPA responsible for the observed decrease in seizures is not known, it may involve increased GABA-mediated neurotransmission (Rosenberg 2007). Through inhibition of deacetylation of histones, HDACi modulate gene expression. In T cells from

systemic lupus erythematosus patients, Trichostatin A, an HDACi used *in vitro*, down-regulated CD40 ligand and IL-10 expression and up-regulated IFN-gamma gene expression to reverse aberrant expression of these gene products observed in lupus (Mishra et al. 2001). Similarly, alteration of gene expression in rheumatoid arthritis and multiple sclerosis disease models has been proposed as a mechanism for HDACi-mediated disease mitigation (Chung et al. 2003; Camelo et al. 2005; Gray et al. 2006; Nakamura et al. 2008). Histone acetyltransferase and deacetylase enzymes not only modify histone acetylation, but also modify non-histone proteins such as p53 (Gu et al. 1997) and as we have shown, FOXP3 itself (Li et al. 2007; Tao et al. 2007).

Our examination of the effects of HDACi on regulatory T cell function in CIA indicates that enhanced Treg cell function is another mechanism by which the autoimmune disease state is clinically improved by HDACi. Our biochemical studies reveal that increased acetylation of FOXP3, as would result from inhibition of deacetylase activity with VPA treatment, results in increased binding of the FOXP3 repressor protein to chromatin, thus allowing regulation of key cytokine genes involved in autoimmune disease progression (Samanta et al. 2008). Additionally, increased acetylation of FOXP3 which occurs on lysine residues may have a stabilizing effect on the FOXP3 protein itself due to decreased competition of these lysine residues for ubiquitination, which would target the protein for degradation. Such a stabilization mechanism would play a role in the observed increase in the percentage of FOXP3⁺ T cells in VPA treated, CIA-induced mice (Fig. 4, Table II and Fig. 5). A precedence exists for this type of stabilization of repressor proteins as acetylation of p53 prevents degradation (Nag et al. 2007).

Use of HDACi, such as VPA, clearly improves the clinical pathogenesis of autoimmune disease. We have shown that disease pathology is mitigated by VPA treatment in CIA-induced mice through increased regulatory T cell activity and expansion of FOXP3⁺ Treg cells *in vivo*. Similarly, *in vivo* administration of HDACi recently resulted in cardiac and islet allograft survival as well as improved inflammatory bowel disease due to increased FOXP3 expression and increased regulatory T cell function (Tao et al. 2007). Additionally, HDACi treatment of NZB/W F1 mice, a model of systemic lupus erythematosus, increased the percentage of Treg cells and their FOXP3 expression level, resulting in decreased disease (Reilly et al. 2008). Clinically approved deacetylase inhibitors such as VPA may improve FOXP3 function to limit autoimmune disease *in vivo*.

Abbreviations

CIA, collagen induced arthritis; Treg, regulatory T cell; VPA, valproic acid; HDACi, histone deacetylase inhibitor; PIP, proximal interphalangeal; Teff, T effector.

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Figure 1.

Mean clinical disease score of CIA-induced mice treated with PBS or VPA. CIA was induced in DBA/1 mice as described in materials and methods. VPA (400mg/kg) or PBS was administered daily starting on day 21 and clinical disease was scored every 2–3 days starting on day 21 of the study with a score of 0–16 possible for each mouse. Values shown are mean clinical score $\pm$ SEM. Data from representative experiment are shown with PBS n=11 and VPA n=9. * $p < 0.04$ in two-tailed t-test.

Figure 2.

Histopathology of the proximal interphalangeal joints of CIA-induced mice. A. Photomicrographs are H&E-stained paraffin sections (original magnifications $\times 0$) of proximal interphalangeal (PIP) joints of CIA-induced mice from day 60 of the study. Sections from VPA-treated mice show well-preserved PIP joints with negligible inflammation, synovial hypertrophy, cartilage damage or bone destruction (top). Sections from control PBS-treated mice show severe arthritis, marked erosion of articular surfaces, cartilage breakdown, synovial hypertrophy and inflammatory cell infiltration (bottom). B. Histopathologic score of PIP joint sections from CIA-induced mice using a standard 0–3 histologic scale (0/normal, 1/mild, 2/moderate, 3/severe) for each of 4 points: inflammation, synovial hypertrophy, cartilage damage

and bone destruction. Data are shown as the mean histopathology score $\pm$ SEM. Statistical evaluation using a two-tailed t-test revealed ** $p < 0.003$, * $p < 0.015$ as indicated.

Figure 3.

Regulatory T cells from VPA-treated CIA-induced mice have improved function.

CD25⁺CD4⁺ Treg cells were purified on day 60 of the study from splenocytes of CIA-induced mice treated with VPA or PBS as described in material and methods and were co-cultured with CD25⁻CD4⁺ T effector cells from naïve mice at a 1:2 ratio of Treg : Teff on anti-CD3ε mAb coated plates (1μg/ml anti-CD3ε mAb) for 72 hrs. Teff and T reg cells were also cultured separately as shown. Proliferative response was measured by incorporation of tritiated thymidine during the last 12–18 hours of culture. Mean CPM are shown +/- SEM, *p<0.04 in two-tailed t-test. Percent inhibition is calculated as 100 - [(CPM PBS or CPM VPA/CPM control)*100], where control is culture containing T effector cells only.

Figure 4.

Flow cytometric analysis of splenocytes from CIA-induced mice treated with PBS or VPA. Representative dot plots of splenocytes isolated on day 60 of the study from PBS or VPA treated CIA-induced mice stained for cell surface CD4, CD25 and intracellular FOXP3 are shown.

Figure 5.

Immunoperoxidase staining of PIP joints and adjacent synovial tissue of CIA-induced mice. (a, b) Decreased recruitment of host leukocytes, as reflected in staining for the leukocyte-common antigen, CD45, in joints harvested from CIA-induced mice on day 60 of the study receiving VPA therapy. (c, d) VPA induced a marked increase in nuclear staining for acetylated lysine within the joints and associated tissues. Arrows indicate examples of positively-stained nuclei. (e, f) Inflamed joints of PBS-treated mice lacked infiltration by FOXP3⁺ cells, whereas VPA therapy was associated with recruitment of FOXP3⁺ mononuclear cells to peri-articular tissues (arrows). (Hematoxylin-counterstained paraffin sections, ×250 original magnifications, representative of data from analysis of 4 paws/group).

Table I

Effect of Valproic Acid on CIA

<u>Treatment^a</u>	<u>Disease Incidence^b</u>
PBS	79%
VPA	31%

^a) CIA was induced in DBA/1 male mice age 6–8 weeks by intradermal injection of 100 ug chicken type II collagen in CFA on day 0 followed by ip injection of 100 ug collagen in PBS on day 21. VPA (400 mg/kg) or PBS was administered daily starting on day 21 of the study.

^b) Disease incidence is calculated from 2 independent experiments with n=19 (PBS) and n=16 (VPA) calculated on day 60 of the experiment. $p < 0.005$ using the Chi square test for independence.

Table IIVPA Increases Number of FOXP3⁺ Cells

<u>Cell subset</u>	<u>% Increase^a</u>
CD4 ⁺ FOXP3 ⁺	39
CD25 ⁺ FOXP3 ⁺	42

^a) Increased percentage of cell subset in VPA treated versus PBS treated CIA mice calculated from flow cytometric data of CIA splenocytes stained for CD4, CD25 and FOXP3 as (% positive cell subset VPA-% positive cell subset PBS)/ % positive cell subset PBS × 100.