IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

TNF α Regulates SIRT1 Cleavage during Ocular Autoimmune Disease

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Elevated tumor necrosis factor (TNF) α levels are associated with chronic autoimmune diseases in which effects of TNF α on immune cells are multiple and complex. Analysis of uveitis in mice exhibiting severe autoimmune inflammation, resulting in a destructive subtotal loss of photoreceptors, revealed the presence of high plasma levels of TNF α and a significant population of CD4⁺TNF α ⁺ cells in the periphery and the eye at peak disease (TNF α ^{hi}). We have shown previously by pharmacological activation that the deacetylase Sirtuin 1 (SIRT1) has an anti-inflammatory role in a less severe, TNF α ^{lo} model of uveitis. We now show that SIRT1 activation fails to clinically suppress severe TNF α ^{hi} disease, whereas glucocorticoid treatment is successful. TNF α has been reported to mediate cleavage and inactivation of SIRT1 during inflammation, and at peak disease we observed both full-length and cleaved SIRT1 in draining lymph node cells. *In vivo* systemic TNF α blockade suppressed severe ocular disease and restricted SIRT1 cleavage in the periphery, maintaining full-length active SIRT1 protein. When combining a suboptimal TNF α blockade with SIRT1 activation, a synergistic suppression of severe disease compared with TNF α blockade alone occurred. Our data suggest a new role for TNF α in exacerbating the severity of autoimmune disease by regulating SIRT1 cleavage in draining lymph node effector cells. SIRT1 activation may be effective as an adjunctive treatment for inflammatory conditions not fully controlled by TNF α inhibitors. (*Am J Pathol* 2015, 185: 1324–1333; <http://dx.doi.org/10.1016/j.ajpath.2015.01.017>)

Tumor necrosis factor (TNF) α is a proinflammatory cytokine required for mounting the host response to infection, but whose excessive production can result in many chronic autoimmune pathological features, such as rheumatoid arthritis, type 2 diabetes, and psoriasis.¹ Although TNF α can be produced by several cell types, including macrophages, T cells, natural killer cells, and neutrophils,² the two distinct receptors for TNF α are ubiquitously expressed and downstream signaling of TNF α results in broad effects, such as activation, proliferation, or apoptosis, depending on the specific environment and cell type.³ Therapeutic agents that block TNF α activity are widely used in clinical practice for the treatment of chronic inflammatory conditions, including uveitis. Although effective, the immunomodulatory consequences of TNF α blockade are still poorly understood, particularly with regard to predicting and refining patient outcomes. Recent published

data suggest that TNF α can paradoxically promote immunosuppression during chronic inflammation.⁴

The NAD⁺-dependent histone deacetylase Sirtuin 1 (SIRT1) has been shown to be immunosuppressive,^{5–8} and we have recently demonstrated that its activation *in vivo* using the small-molecule SIRT1 activator SRT2379⁹ protects against

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autoimmune ocular inflammation in mice.¹⁰ This protection occurs, in part, by suppression of proinflammatory cytokine secretion, such as interferon (IFN)- γ , IL-17A, and IL-6, and suppression of IL-2–driven T-cell proliferation via the perturbation of STAT5 signaling.¹⁰ In addition, SIRT1 has also been shown to mediate immune suppression via targeting of histones and numerous proteins, including p53, AP-1, RelA/p65, and STAT3.^{11–13} Recently, TNF α signaling in osteoarthritic chondrocytes has been shown to result in cleavage of the 110-kDa full-length SIRT1 to a 75-kDa form that no longer retains deacetylase activity but can block apoptosis, resulting in the breakdown of cartilage-specific extracellular matrix, as observed in rheumatoid arthritis.¹⁴ These findings raise important questions about the impact of TNF α on SIRT1 in lymphocytes during autoimmune disease.

We show herein that in a severe model of autoimmune ocular inflammation with extensive tissue destruction, the dominant CD4⁺ T-cell population infiltrating the eye at disease peak expresses TNF α . CD4⁺TNF α ⁺ cells also dominate in peripheral lymphoid tissues at disease peak, where cleavage and inactivation of SIRT1 are observed. Systemic administration of the immunosuppressive SIRT1 activator SRT2379 fails to prevent disease development, except in combination with clinically suboptimal TNF α blockade, suggesting TNF α may have a role in exacerbating and maintaining chronic inflammation via the cleavage of SIRT1 in peripheral lymphocytes. The data shown are important for the application of SIRT1 activators as a therapy for the treatment of systemic autoimmune disease. The potential synergy of combining SIRT1 activators and TNF α antagonists could prove useful to suppress inflammatory conditions not fully controlled by TNF α inhibitors as well as facilitating reduced dosing of TNF α inhibitors and consequently, a decrease of commonly seen adverse effects of TNF α blockade, such as *Mycobacterium tuberculosis* reactivation.

Materials and Methods

Mice

B10.RIII mice were derived originally from The Jackson Laboratory (Bar Harbor, ME) and were obtained from the breeding colony at GlaxoSmithKline (Stevenage, UK). On arrival at the UCL Institute of Ophthalmology (London, UK), all mice were housed in individually ventilated cabinets in specific pathogen-free conditions with continuously available water and food for the duration of all experimental autoimmune uveoretinitis (EAU) studies, according to UK Home Office Regulations. All animal studies were ethically reviewed and performed in accordance with Animals (Scientific Procedures) Act 1986 and the GlaxoSmithKline Policy on the Care, Welfare, and Treatment of Animals. Female mice immunized for EAU disease induction were aged between 5 and 8 weeks. Treatment of animals also conformed to The Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Reagents

Human interphotoreceptor retinoid-binding protein (IRBP)_{161–180} (SGIPYIISYLHPGNTILHVD) was obtained from Cambridge Peptides (Cambridge, UK) and stored lyophilized at 4°C until use. Complete media consisted of Iscove's Modified Dulbecco's Medium (Sigma, Gillingham, UK) supplemented with 10% fetal calf serum, 0.05 mmol/L 2-mercaptoethanol (Sigma), 0.1 mmol/L nonessential amino acids, 1% minimal essential medium essential vitamin mixture, 100 U/mL penicillin-streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Loughborough, UK). Water-soluble dexamethasone (Dex; D2915; Sigma) was dissolved in phosphate-buffered saline. SRT2379 is a small-molecule activator of SIRT1 developed by GlaxoSmithKline (formerly Sirtris Pharmaceuticals Inc, Cambridge, MA), which has been characterized for use *in vivo* dissolved in 2% hydroxypropylmethylcellulose/0.2% dioctyl sodium sulfosuccinate, referred to as vehicle. The soluble TNF α receptor–Ig fusion protein (sTNFR-Ig) was a gift from Dr. Herman Waldmann (University of Oxford, Oxfordshire, UK).

EAU Induction

B10.RIII mice were immunized s.c. in the flank with 300 μ g IRBP_{161–180} in phosphate-buffered saline emulsified with complete Freund's adjuvant (Sigma) supplemented with 1.5 mg/mL *M. tuberculosis* complete H37 Ra (Difco Microbiology, Voigt Global Distribution, Lawrence, KS) (1:1 v/v). The mice also concurrently received 0.4 μ g *Bordetella pertussis* toxin (Sigma) i.p.

Fundus Imaging

The topical endoscopic fundus imaging (TEFI) system¹⁵ was used for routine *in vivo* imaging of mouse retinal fundus. TEFI images were clinically scored using a scoring system modified from Copland et al¹⁵ (Supplemental Table S1).

Histology

At day 14 after EAU induction, enucleated eyes were fixed in 4% glutaraldehyde for 2 hours, followed by overnight fixation in 10% formalin. Eyes were then processed, orientated, and embedded for paraffin wax sectioning. Eyes were serially cut into sections (7 to 12 μ m thick), stained with eosin, counterstained with hematoxylin, and scored for disease, as previously published.¹⁶

Ocular Dissection and Single-Cell Preparation

Enucleated eyes were dissected in 100 μ L of cold Dulbecco's modified Eagle's media. After incision at the limbus with a 29-gauge needle, a circumferential cut around the eye following the limbus was made. Iris was dissected away, releasing anterior chamber infiltrating cells into the dissection media. The retina and vitreous were then removed carefully from the eye cup,

leaving the sclera/retinal pigment epithelium/choroid intact. The dissection media containing anterior fluid, vitreous, and retina were then pipetted up into a 1.5-mL Eppendorf tube and mechanically disrupted by rapping 10 times for a single-cell suspension, followed by centrifugation through a single well of a 96-well, 60- μ m cell strainer plate (Millipore, Watford, UK). The resulting cell pellets were resuspended and stained for flow cytometry, as described below.

Flow Cytometry

Cells were incubated with purified rat anti-mouse CD16/CD32 (Fc γ III/II receptor) monoclonal antibody (mAb; Mouse Fc Block; eBioscience, Hatfield, UK), followed by incubation with fluorochrome-conjugated mAbs against cell surface markers on ice. Multiparameter flow cytometry was performed using a combination of mAbs with different fluorochromes. The following mouse-specific mAbs were purchased from eBioscience: α CD4-phosphatidylethanolamine (PE), α CD4-PECy7, α IFN γ -allophycocyanin, α CD11b-phycoerythrin/Cy7, α CD45—fluorescein isothiocyanate, α TNF α —fluorescein isothiocyanate, α TNF α -eFluor450, and the fixable viability dye eFluor 780. α Ly6G-allophycocyanin (clone 1A8) was purchased from Biolegend (London, UK), and the following were purchased from BD Biosciences (Oxford, UK): α IL-17—PE and Foxp3-Alexa 488. Cells were washed twice in staining buffer (phosphate-buffered saline with 0.1% bovine serum albumin and 0.05% azide) after incubation with fluorochrome-conjugated mAbs.

Intracellular IFN γ and IL-17 were detected after 4 hours of activation with 50 ng/mL 4 β -phorbol 12-myristate 13-acetate, 500 ng/mL ionomycin, and 1 mg/mL brefeldin (all from Sigma) in complete Iscove's Modified Dulbecco's Medium. Mouse cells were incubated with Mouse Fc Block and incubated with PE-Cy7—conjugated anti-CD4 mAb, before permeabilization using the BD Perm/Fix kit (BD Biosciences), according to the manufacturer's instructions, and intracellular staining with the appropriate mAbs. Cells were washed before analysis.

Transcription factor analysis was performed using the Foxp3 Cell Staining Kit (BD Biosciences), according to the manufacturer's instructions. Relevant isotype control mAbs were purchased from BD Biosciences and eBioscience and combined as fluorescence minus one controls. Cells were acquired on a BD FACSCalibur using CellQuest (BD Cytometry Systems, Oxford, UK) and a BD LSRFortessa-x20 using FACSDiva (BD Cytometry Systems). Single-stained beads (OnceComp eBeads; eBioscience, London, UK) of all fluorochromes were used to generate appropriate compensation matrices, and fluorescence minus one controls were used for positive gating. Flow cytometric analyses were performed using FlowJo Software (TreeStar, Ashland, OR).

Western Blot Analysis

Western blot detection of both full-length and cleaved SIRT1 was performed on 70 μ g total protein cell extract

from mouse dLN cells pooled from three animals. Cells were lysed in radioimmunoprecipitation assay lysis buffer containing 1:100 freshly added protease inhibitor cocktail (Sigma) with shaking for 20 minutes at 4°C. Proteins were separated using 9% SDS-PAGE and blotted to Immobilon-P transfer membrane (Millipore), followed by detection with α SIRT1 N-terminal polyclonal antibody (07-131; Millipore). This was followed by incubation with secondary Goat α -Rabbit-HRP (7074; Cell Signaling Danvers, MA) and subsequent development with an ECL Plus detection kit (Amersham, GE Healthcare, Little Chalfont, UK). ImageJ software (NIH, Bethesda, MD) was used for quantification.

Cytokine Detection

Cell supernatant cytokines were determined by use of TNF α Quantikine enzyme-linked immunosorbent assay (R&D Systems Europe Ltd, Abingdon, UK) and multiplex bead array using a Luminex 100 System (Austin, TX) and the type 17 helper T-cell (Th17) six-plex kit (Bio-Rad, Hemel Hempstead, UK), following the manufacturer's instructions as described.¹⁰ The data were analyzed using the xPONENT version 3.1 analysis software (Luminex).

Statistical Analysis

Data were analyzed using the following statistical tests, as described in the figure legends for each individual experiment: unpaired two-tailed *t*-test, one-way analysis of variance multiple comparisons, two-way analysis of variance, *U*-test, and the Kruskal-Wallis test, all using Prism software version 5.0 (Graphpad Software, La Jolla, CA).

Results

Severe Ocular Inflammation in *B10.RIII* Mice Peaks at Day 14 and Is Dominated by Neutrophils and CD4⁺TNF α ⁺ Cells Infiltrating the Eye

In the *B10.RIII* colony, induction with 300 μ g IRBP_{161–180} and 0.4 μ g of pertussis toxin results in ocular disease, which, by fundal examination (TEFI), manifests at day 8 with mild optic disc swelling, peaks at day 14 with anterior synechiae and foci of preretinal hemorrhage, and after acute disease inflammation, persists until day 95, where the presence of scar tissue can be observed (Figure 1A and Supplemental Figure S1A). At day 14, EAU examination of the cornea and anterior chamber revealed the presence of erythrocytes (Supplemental Figure S1, B and C), and examination of dissected whole retina compared to complete Freund's adjuvant controls (Supplemental Figure S1, D and E) revealed patches of hemorrhage and patches of retinal pigment epithelial cells adherent to the retina (Supplemental Figure S1E).

Histological processing of eyes at day 14 EAU compared to eyes from complete Freund's adjuvant

Figure 1 Severe ocular inflammation in *B10.RIII* mice peaks at day 14 with complete photoreceptor outer segment loss and is dominated by neutrophils and CD4⁺ tumor necrosis factor (TNF) α cells infiltrating the eye. **A**: Mean clinical scores from topical endoscopic fundus imaging: from day 4 to day 95 of experimental autoimmune uveoretinitis (EAU). **B–H**: Representative histology at day 14 of EAU and complete Freund's adjuvant (CFA) immunized eyes. EAU eye (**B**), with magnification (**C**) of the anterior chamber. **Arrows** show inflamed ciliary body and anterior synechiae. **D**: Magnification of EAU retina. **Arrows** indicate vessel hemorrhage and exudate. **E**: Magnification of retina/vitreous with erythrocyte leakage. **F**: CFA control (Ctrl) eye. **G**: Magnification of CFA retina. **H**: Histology scores for EAU disease at day 14, average of two blinded scorers (P.J.G. and S.Y.). **I**: Combined total CD45⁺ ocular infiltrate broken down into myeloid and CD4⁺ cells by subset. **J**: Spread of absolute counts of ocular infiltrating CD45⁺ leukocytes at peak disease by subset. **K**: Representative flow cytometry plots showing the gating strategy for leukocyte staining. **L**: CD4⁺ T-cell subsets infiltrating the whole eye at peak disease after *ex vivo* restimulation with 4 β -phorbol 12-myristate 13-acetate/ionomycin with brefeldin, followed by intracellular cytokine staining, with representative flow cytometry plots showing gating. Data are given as means $\pm$ SEM (**I–K**). *n* = 6 eyes (**H**); *n* = 10 eyes (**I, J**, and **L**). Original magnifications: $\times 20$ (**C, D**, and **G**); $\times 40$ (**E**). C, choroid; Cm, cornea; FSC, forward scatter; G, ganglion layer; IFN, interferon; L, lens; P, photoreceptor layer; SSC, standard saline citrate; V, vitreous.

immunized control mice revealed the near-total loss of photoreceptors with massive retinal exudate (Figure 1, B and D) and confirmed the presence of anterior synechiae (Figure 1C) and retinal hemorrhage with erythrocytes observed in the vitreous (Figure 1, D and E) as compared to complete Freund's adjuvant control eyes (Figure 1, F and G). Histological scoring of sections showed that day 14 EAU eyes were at the top of our scoring spectrum, reflecting the targeted immune response resulting in near-total loss of the photoreceptors, which express the target antigen IRBP (Figure 1H).

Analysis of the ocular infiltrate at the peak of disease by flow cytometry revealed a substantial infiltration of leukocytes

composed of myeloid cells and CD4⁺ T cells with a dominant CD11b⁺Ly6G⁺ neutrophil population (Figure 1, I and J).

Analysis of whole ocular infiltrating CD4⁺ T-cell subsets by intracellular cytokine staining demonstrated the previously reported and expected presence of IFN γ ⁺ (mean, 10.6%; SD, $\pm 7.1\%$) and IL-17A⁺ (mean, 4.4%; SD, $\pm 2.5\%$) cells, but the dominant population of T cells expressed TNF α (mean, 74.9%; SD, $\pm 11.4\%$) (Figure 1L) and, hence, we refer to these mice, in terms of the T-cell response during uveitis, as TNF α ^{hi}.

Analysis of ocular infiltrating CD4⁺ T cells at peak disease in mice, previously published by us, where IFN γ ⁺ cells

Figure 2 Severe CD4⁺ tumor necrosis factor (TNF) α ⁺-driven experimental autoimmune uveoretinitis (EAU) disease is associated with SIRT1 cleavage in draining lymph nodes. **A:** Flow cytometry analysis of *ex vivo* CD4⁺ T-cell subsets by intracellular cytokine staining from naïve animals or animals with peak EAU disease. **B:** Western blot of SIRT1 from draining lymph node cells pooled from three animals comparing complete Freund's adjuvant control animals with peak EAU disease animals. **C:** Plasma cytokine levels. Data are given as means $\pm$ SEM (**A** and **C**). $n = 4$ animals (**A** and **C**). IFN, interferon.

and IL-17A⁺ cells make up a mean of 19.3% (SD, $\pm$ 16.6%) and 15.5% (SD, $\pm$ 9.8%), respectively,¹⁰ revealed that TNF α ⁺ cells constitute only 31.1% (SD, $\pm$ 2.1%) of the CD4⁺ population. Hence, we term TNF α ^{lo} mice, with respect to the T-cell phenotype during disease (Supplemental Figure S2). We have access then to two *B10.RIII* colonies that manifest as TNF^{hi} and TNF^{lo} EAU disease with differential severity following an identical disease induction protocol (Supplemental Figure S2). Analysis of SIRT1 protein by Western blot analysis at peak EAU disease revealed minimal cleavage of SIRT1 in TNF^{lo} mice (Supplemental Figure S2); hence, we have used these two different manifestations of autoimmune pathology to interrogate the effect of TNF on SIRT1 function.

Severe CD4⁺TNF α ⁺-Driven EAU Disease Is Associated with SIRT1 Cleavage in Draining Lymph Nodes

Analysis of peripheral lymphoid tissue in *B10.RIII* mice revealed a dominant CD4⁺TNF α ⁺ population in cells restimulated from both naïve and peak EAU disease mice, with an IL-17A population also participating in disease (Figure 2A). TNF α has recently been shown to regulate cleavage of SIRT1 within chondrocytes during inflammation¹⁴; hence, we investigated SIRT1 in the periphery of the EAU mice. Analysis of SIRT1 in dLN cells revealed only full-length active SIRT1 in naïve animals and the presence of both full-length and the inactive cleaved form of SIRT1 in cells from mice at peak disease (Figure 2B). Analysis of plasma cytokines in peak EAU mice revealed high levels of circulating systemic TNF α and the presence of IL-1 β and low levels of IL-6 (Figure 2C).

Prophylactic Systemic SIRT1 Activation *in Vivo* Fails to Prevent Disease Development during TNF α ^{hi} Severe EAU Disease

We have previously shown suppression of milder expressed ocular inflammatory disease in *B10.RIII* mice using the

SIRT1 activator SRT2379¹⁰; hence, we investigated the ability of SRT2379 to suppress severe TNF α ^{hi} disease. We predicted that, in the presence of high TNF α levels, the action of the SIRT1 activator would be negated. *B10.RIII* mice administered with 10, 30, and 100 mg/kg SRT2379 by once-daily oral gavage from the time of immunization until peak EAU had histological disease scores indistinguishable from vehicle-treated mice (Figure 3, A and B), whereas systemic Dex by daily i.p. injection from the day of EAU induction, which has been shown to suppress disease in mice,¹⁷ significantly reduced disease ($P < 0.0001$, $n = 6$). Corticosteroid treatment prevented extensive structural changes to the retina, limiting changes to cellular infiltration in the vitreous, perivascular cuffing, and mild retinal folding (Figure 3B).

In SIRT1 activator-treated mice, we observed significantly reduced CD45⁺ ocular infiltrate with 100 mg/kg SRT2379 of 38% compared to vehicle ($P = 0.0499$) (Figure 3, C and D); however, ocular infiltrating CD11b⁺Ly6G⁺ and CD11b⁺Ly6G⁻ were unchanged (Figure 3E). The overall number of CD4⁺ cells was reduced by 51% when compared to vehicle ($P = 0.0199$) (Figure 3F).

Analysis of CD4⁺ T-cell subsets revealed no specific effects of SRT2379 on proportions of intracellular cytokine expression in ocular infiltrating CD4⁺ T cells (Supplemental Figure S3). We published previously that SIRT1 activation with SRT2379 abolished IL-17A and IL-6 production by dLN cells from TNF^{lo} EAU mice.¹⁰ Analysis of cytokine production by *ex vivo* cultured dLN cells from TNF α ^{hi} SRT2379-treated EAU mice, unstimulated or restimulated with 20 μ g/mL IRBP₁₆₁₋₁₈₀ peptide for 48 hours, showed significant, but limited, suppression of IL-17A levels at the 100 mg/kg dose, whereas significant suppression of IL-6 was observed at both doses irrespective of stimulation (Figure 3G). SIRT1 activator efficacy of the same batch of compound was tested contemporaneously in the TNF^{lo} mice throughout the period of the study with the TNF^{hi} mice, to ensure that the observed decreased compound activity did not account for the lack of efficacy in the TNF^{hi} mice.

Systemic Blockade of TNF α during EAU Suppresses Disease and Restricts SIRT1 Cleavage in Peripheral Lymphoid Tissue

Given our finding that during TNF α^{hi} EAU, SIRT1 is cleaved in the periphery and SIRT1 activator treatment is limited, we sought to test the notion that SIRT1 activator-mediated immune suppression could be restored on

neutralization of systemic TNF α . To accomplish this, we required the means of attenuating systemic TNF α levels such that full-length SIRT1 would be protected from cleavage, without the complete abrogation of disease. Blockade of TNF α during EAU using a recombinant sTNFR-Ig has been shown to be successful in preventing and reducing the severity of pathology.¹⁸ It has been proposed that the main action of sTNFR-Ig is via modulation

Figure 4 Systemic blockade of tumor necrosis factor (TNF) α during experimental autoimmune uveoretinitis (EAU) suppresses disease and restricts SIRT1 cleavage in peripheral lymphoid tissue. **A:** Representative topical endoscopic fundus images of animals at day 12 EAU treated at day 9 with a single i.p. injection of control (Ctrl) IgG or varying doses of recombinant soluble TNF α receptor–Ig fusion protein (sTNFR-Ig), as indicated, with mean clinical scores. **B:** Quantification of the CD45⁺ ocular infiltrate at day 14 by flow cytometry. **C:** Serum levels of TNF α in peak EAU mice treated as indicated. **D:** Western blot of SIRT1 on 70 μ g total protein from draining lymph node cells pooled from five peak EAU animals comparing control IgG treatment with indicated doses of sTNFR-Ig treatment. **E:** Combined quantification of two Western blots from two independent experiments using ImageJ analysis. Data are given as means $\pm$ SEM (**A–C** and **E**). $n = 8$ eyes (**B**); $n = 4$ animals (**C**). * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. CL, cleaved; FL, full length.

of T-cell phenotype, function,¹⁹ and neutralization of T-cell–produced soluble TNF α and transmembrane TNF α , acting to minimize the activation of infiltrating inflammatory monocytes and macrophages.²⁰ Treatment of the severe TNF α ^{hi} EAU mice with sTNFR-Ig resulted in a dose-titrated reduction in disease, as observed by TEF1 clinical examination and scoring (Figure 4A) and quantification of CD45⁺ ocular infiltrate (Figure 4B). Analysis of systemic TNF α levels revealed that the 0.005 mg/kg dose resulted in suppression, but not complete abrogation, of TNF α compared to control IgG treatment, whereas at the 5 mg/kg dose, TNF α was not detected (Figure 4C). We next assessed the effect of TNF α blockade on SIRT1 cleavage. Analysis of SIRT1 by Western blot analysis in dLN cells from EAU mice treated with sTNFR-Ig revealed a predominantly intact, full-length SIRT1 was present, with minimal SIRT1 cleavage product

observed in the 5 mg/kg sTNFR-Ig–treated group as compared to cells from control IgG-treated mice with partially cleaved SIRT1 present at the lower 0.005 mg/kg sTNFR-Ig–treated group (Figure 4, D and E).

Combining TNF α Blockade with SIRT1 Activation Results in Synergistic Suppression Compared to TNF α Blockade Alone

Because neutralization of TNF α resulted in prevention of SIRT1 cleavage in dLN cells during EAU, we sought to recover SIRT1 activator–mediated immune suppression via combination with TNF α blockade. For this, we chose the 0.005 mg/kg dose of sTNFR-Ig, having shown significant suppression of serum TNF α levels with this dose and protection of full-length SIRT1 without abolishing disease.

Indeed, the combination of TNF α blockade with 100 mg/kg systemic SRT2379 treatment during EAU resulted in synergistic suppression of CD45⁺ ocular infiltrate over that observed with sTNFR-Ig treatment alone (Figure 5A). We also observed significant reduction of CD4⁺TNF α ⁺ T cells, as determined by intracellular cytokine staining, compared with the sTNFR-Ig alone treated group (Figure 5B). Of the infiltrating CD45⁺ cells observed in the combination treated group, cellular proportions were changed, with a marked reduction in neutrophils (Figure 5C). Histological analysis of combination treated eyes revealed that the reduction in cellular burden was sufficiently lower than a threshold required to manifest tissue damage (Figure 5D), with a mean disease score of 1 compared with the sTNFR-Ig—treated group, which maintained disease scores with a mean of 3 (Figure 5E).

Discussion

TNF α is a pleiotropic cytokine that plays central roles in both driving and regulating multiple autoimmune diseases,^{1,21} including human uveitis.²² Indeed, an increased risk of uveitis has been shown to correlate with polymorphisms that result in increased TNF α production.²³ Although anti-TNF α agents have proved successful in rheumatoid arthritis and uveitis, where high systemic TNF α levels contribute to disease progression,²⁴ a paradox regarding the precise role of TNF α remains in the context of immune-mediated tissue damage, with data both for^{25–27} and against^{4,28,29} the proinflammatory role of TNF α . Recent reports have described how inflammatory cues via TNF α result in cleavage of the deacetylase SIRT1 within osteoarthritic chondrocytes, resulting in joint damage,¹⁴ and we have shown recently that SIRT1 activation is immunosuppressive in T cells during autoimmune disease. Herein, we used a TNF α ^{hi} severe model of autoimmune ocular inflammation in mice to interrogate the impact of high TNF α levels on SIRT1 function during autoimmunity. We show that TNF α determines disease severity, in part, through initiating cleavage of SIRT1 in lymphocytes, rendering prophylactic SIRT1 activator administration ineffective at suppressing disease.

Our data reveal that severe autoimmune ocular pathology associates with a high TNF α profile, suggesting a role for CD4⁺ TNF α ⁺ cells in driving autoimmune disease. The severity, pathological features, and retinal damage observed at peak disease are greater than those reported in other *B10.RIII* mouse colonies in the literature.^{30–33} *B10.RIII* mice are naturally high Th1/Th17 responders³⁴; however, we show that TNF α -producing CD4⁺ cells dominate both the ocular infiltrate and peripheral lymphoid tissue at peak disease, with a striking elevation of serum TNF α . TNF α expression in ocular infiltrate is a consistent feature of EAU, with TNF α acting to trigger macrophage expression of nitric oxide synthase 2—producing nitric oxide and superoxides, which lead to tissue destruction.³⁵ Although TNF α is produced by many cell types, T cell—derived TNF α (relatively

Figure 5 Combining tumor necrosis factor (TNF) α blockade with SIRT1 activation results in synergistic suppression compared with TNF α blockade alone. **A:** Quantification of CD45⁺ ocular infiltrate at peak disease by flow cytometry. **B:** Quantification of the CD4⁺TNF α ⁺ ocular infiltrating cells at peak disease by flow cytometry. **C:** Leukocyte subset proportions of total CD45⁺ infiltrate. **D:** Representative histology of peak disease eyes from soluble TNF α receptor—Ig fusion protein (sTNFR-Ig) day 9—treated animals combined with either oral gavage treatment with vehicle or SRT2379 from day 0 to peak. **E:** Mean histology disease scores of two independently blinded scores (P.J.G. and S.Y.). Data are given as means $\pm$ SEM (**A–C** and **E**). $n = 6$ to 8 eyes per group (**A** and **B**); $n = 6$ eyes (**E**). * $P \leq 0.05$, ** $P \leq 0.01$.

underexplored in autoimmunity) has been shown to be nonredundant in protecting from high bacterial load,^{36,37} and to contribute to liver damage during hepatitis² and lung inflammation.³⁸ It is possible that the CD4⁺TNF α ⁺ cells observed in ocular inflammation have a high level of transmembrane TNF α , which can bind to TNF α receptors on monocytes, resulting in monocyte activation and further TNF α production.³⁹

We have shown previously the immunosuppressive effects of SIRT1 during mild non-TNF α -dominant EAU in *B10.RIII* mice.¹⁰ SIRT1 activator administration to TNF α ^{hi}

severe EAU mice did not result in prevention of disease. Although infiltrating CD4⁺ T-cell numbers were reduced, infiltrating CD11b⁺ cells, which ultimately mediate tissue destruction, were unchanged compared to vehicle controls. Although IL-6 levels produced by *ex vivo* dLN cultures were suppressed, IL-17A levels in response to antigen stimulation were not abrogated by SIRT1 activator administration in TNF α ^{hi} severe EAU mice, as previously reported,¹⁰ suggesting that myeloid cells are more sensitive to SIRT1 activation than T cells in the context of TNF α -mediated partial SIRT1 cleavage. This lack of suppression of IL-17, a known neutrophil chemoattractant, may explain why CD11b⁺Ly6G⁺ infiltrating cell numbers were unchanged after SIRT1 activator treatment.

Glucocorticoids remain first-line therapy for posterior uveitis,⁴⁰ and Dex has been shown to curtail Th1-dominant autoimmune disease⁴¹ by suppressing TNF α -induced granulocyte adhesion⁴² and directly targeting TNF α production in the retina.⁴³ Effective prevention of disease by Dex implies that the lack of immunosuppression by SIRT1 activation may result from changes to SIRT1 during disease and exposure to high levels of TNF α and suggests that Dex effectively targeted the dominant autoreactive CD4⁺TNF α ⁺ cells.

The cleavage of full-length SIRT1 to an inactive form has been shown to be mediated by TNF α in chondrocytes,¹⁴ with an anti-apoptotic role for the cleaved 75-kDa fragment.⁴⁴ Our data support the observation that cleavage of SIRT1 in dLNs occurs during TNF α ^{hi} disease; however, whether the 75-kDa SIRT1 fragment is protective against apoptosis in lymphocytes remains to be determined. That some full-length SIRT1 protein remains in dLNs may explain the limited suppression of ocular infiltrate in the absence of disease prevention following SIRT1 activator administration. Systemic blockade of TNF α during EAU reveals both the crucial role of TNF α in driving disease in this mouse model of autoimmunity and the impact of TNF α on SIRT1 cleavage. Indeed, suboptimal attenuation of TNF α levels with respect to suppression of disease and serum TNF α levels still prevented SIRT1 cleavage such that combined treatment with a SIRT1 activator resulted in superior suppression of disease over TNF α blockade alone. The implication is that systemic TNF α levels may regulate the balance between full-length active and cleaved inactive SIRT1 and, in doing so, determine the threshold above which SIRT1 activation would no longer be effective at suppressing autoimmune disease.

In summary, SIRT1 activator treatment for autoimmunity may be most effective in combination with anti-TNF α therapy or in inflammatory conditions in which TNF α is not a dominant contributor to the disease process and, hence, full-length SIRT1 remains intact. Indeed, it is common clinical practice to combine immunosuppressive drugs in the treatment of human uveitis; however, caution must be exercised regarding any potential adverse effects of SIRT1 activator therapies, which may impede p53 activation.⁴⁵ We highlight the interplay between TNF α and the function of SIRT1 within peripheral lymphocytes during developing

ocular autoimmune disease and emphasize the importance of characterizing the level of TNF α present during autoimmune disease and inflammation for the successful future use of sirtuin-activator therapies.

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Supplemental Data

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