Epigenetic drug screen identified IOX1 as an inhibitor of Th17-mediated inflammation through targeting TET2

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Summary

Background Targeting helper T cells, especially Th17 cells, has become a plausible therapy for many autoimmune diseases.

Methods Using an *in vitro* culture system, we screened an epigenetics compound library for inhibitors of IFN-γ and IL-17 expression in murine Th1 and Th17 cultures.

Findings This identified IOX1 as an effective suppressor of IL-17 expression in both murine and human $CD4^+$ T cells. Furthermore, we found that IOX1 suppresses *Il17a* expression directly by targeting TET2 activity on its promoter in Th17 cells. Using established pre-clinical models of intraocular inflammation, treatment with IOX1 *in vivo* reduced the migration/infiltration of Th17 cells into the site of inflammation and tissue damage.

Interpretation These results provide evidence of the strong potential for IOX1 as a viable therapy for inflammatory diseases, in particular of the eye.

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Keywords: Th17; IOX1; Epigenetic drug; TET2

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Research in context

Evidence before this study

Differentiation of CD4$^+$ T cells is recognized to involve global alterations in histone modifications such as H3K4me3 and H3K27me3, as well as DNA methylation at Th subset specific gene loci including *Ifng*, *Il17a*, and *Foxp3*. Therefore, targeting the epigenetic machinery contributing to the pathogenic activation of CD4$^+$ T cells may offer new therapeutic strategies for fighting autoimmune diseases. Previous work from our laboratory demonstrates how the DNA methyltransferase inhibitor Zebularine and the Bromodomain and Extra-Terminal Protein Inhibitor OTX015 can both suppress Th17 mediated inflammation. However, systemic toxicity and low specificity in patients limits the potential for clinical application of these drugs.

Added value of this study

Our study demonstrates that IOX1 suppresses *Il17a* expression directly by targeting TET2 activity on its promoter in Th17 cells. Treatment with IOX1 in EAU could reduce the migration/infiltration of Th17 cells into the site of inflammation and tissue damage.

Implications of all the available evidence

This study supports the strong potential for IOX1 as a viable therapy for inflammatory diseases, in particular of the eye.

Introduction

CD4$^+$ T helper (Th) cells are critical in host defense against invading pathogens. However, their aberrant activation underlies the pathogenesis of many autoimmune diseases affecting multiple organ systems, including joints (eg, rheumatoid arthritis), the brain (eg, multiple sclerosis), skin (eg, psoriasis), the gut (eg, Crohn's disease), pancreas (eg, type I diabetes) and the eye (eg, uveitis).[1] Naïve CD4$^+$ T cells can differentiate into a diverse repertoire of Th lineages including Th1, Th2, Th17, Treg, and Tfh, which differ in phenotype and function. Ultimately, these subsets are determined through coordinated regulatory mechanisms in response to extrinsic factors (cytokines within the microenvironment) and intrinsic regulation (master transcriptional regulators and chromatin accessibility). Increased understanding of transcriptional and epigenetic control of Th differentiation now offers the therapeutic opportunities to modulate CD4$^+$ T cell mediated inflammation associated with autoimmune conditions.[2]

The dynamic nature of the epigenome permits direct manipulation of epigenetic machinery to alter disease-associated pathogenic chromatin states.[3] As an epigenetically regulated process, differentiation of CD4$^+$ T cells is recognized to involve global alterations in histone modifications such as H3K4me3 and H3K27me3,[4] as well as DNA methylation at Th subset specific gene loci including *Ifng*, *Il17a*, and *Foxp3*.[5] The H3K27me3 demethylase JMJD3[6] and DNA demethylases TET2/TET3[5] are recognized activators of IL-17 expression and evidence suggests H3K9me3 may also regulate the expression of IL-17.[7] Therefore, targeting the epigenetic machinery contributing to the pathogenic activation of CD4$^+$ T cells may offer new therapeutic strategies for fighting autoimmune diseases. Previous studies report histone deacetylase (HDAC) inhibitors,[8,9] bromodomain antagonists,[10–14] and histone H3K27me3 demethylase inhibitors[15] can reduce CD4$^+$ T cell mediated inflammation. Similarly, previous work from our laboratory demonstrates how the DNA methyltransferase inhibitor Zebularine[16] and the Bromodomain and Extra-Terminal Protein Inhibitor OTX015[17] can both suppress Th17 mediated inflammation. However, systemic toxicity and low specificity in patients limits the potential for clinical application of these drugs.[18,19]

In the current study, we used an *in vitro* culture system to screen an epigenetics compound library for candidate inhibitors of IFN-γ and IL-17 cytokine expression in murine Th1 and Th17 cultures. Our further *in vivo* analysis confirmed the anti-inflammatory effect of IOX1 in Th17-mediated ocular inflammation models.

Methods

Human peripheral blood CD4$^+$ T cell isolation and treatment

CD4$^+$ T cells were obtained by negative selection from the peripheral blood of healthy controls (HCs) (N = 6; 5 female and 1 male; average age of 32.83) following informed consent in accordance with National Health Service Research Ethic Committee approved protocols at the University Hospitals Bristol Foundation Trust, United Kingdom (04/Q2002/84). The written informed consents were obtained from all study participants. CD4$^+$ T cells were obtained by incubating up to 80 ml uncoagulated peripheral blood with RosetteSep Human CD4$^+$ T cell Enrichment Cocktail (STEMCELL Technologies, Canada) and Ficoll–Paque PLUS (GE Healthcare, USA) according to the manufacturer's instructions. Enriched CD4$^+$ T cells were removed from the density gradient and washed in RPMI-1640 (Thermofisher, USA) supplemented with 10% fetal calf serum (Gibco, USA). The purity of human CD4$^+$ T cells was greater than 95%.

CD4$^+$ T cells were stimulated with Dynabeads Human T-Activator CD3/CD28 (RRID: AB_2916088, Thermofisher Scientific, USA) and seeded in 96 well

microplates (0.1 million cells/well) with or without DMSO or indicated concentrations of IOX1 in complete RPMI1640 medium. Cells were stimulated for 4 h with PMA and ionomycin prior to harvest at day 5 for proliferation and intracellular cytokine staining assays.

Mice

Female C57BL6/J, B10.RIII, or B6.Cg(TcraTcrb) 425Cbn/J (OT-II) mice (8–10 weeks) were purchased from Charles River, UK or Guangdong Medical Laboratory Animal Center (China). The $CD4^{Cre}Tet2^{f/f}$ mice were a gift from Prof. Guoqiang Chen in Shanghai Jiaotong University. All animals were housed in SPF facilities at University of Bristol or Zhongshan Ophthalmic Center. All animal experiments were approved by the Institutional Animal Care and Use Committees of University of Bristol (PPL 30/3281) or Zhongshan Ophthalmic Center (2018-159). All animal work was performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Materials

Epigenetic Compound Library with 128 compounds (Cat#L1900) was purchased from Selleck, USA. All compounds were originally in DMSO solution with a stock concentration of 10 mM and were further diluted to working solution as indicated in each experiment. Murine anti-CD3 (Thermo Fisher Scientific Cat# 16-0031-85, RRID: AB_468848), anti-CD28 (Thermo Fisher Scientific Cat# 16-0281-86, RRID: AB_468923), anti-IL-4 (Thermo Fisher Scientific Cat# 16-7041-95, RRID: AB_2573101), and anti-IFN-γ (Thermo Fisher Scientific Cat# 16-7312-38, RRID: AB_2573112) antibodies for cell culture were purchased from eBioscience, USA. The murine anti-IL-17 (Thermo Fisher Scientific Cat# 12-7177-81, RRID: AB_763582), anti-IFN-γ (Thermo Fisher Scientific Cat# 40-7311, RRID: AB_2621469), anti-CD3 (100221, RRID: AB_2057374), anti-CD4 (Thermo Fisher Scientific Cat# 25-0041-82, RRID: AB_469576 and BioLegend Cat# 100433, RRID: AB_893330), anti-CD44 (BioLegend Cat# 103024, RRID: AB_493687), and anti-CD62L (104411, RRID: AB_313098) antibodies for FACS analysis or sorting were purchased from Biolegend or eBioscience, USA. Human anti-IL-17 (BioLegend Cat# 512306, RRID: AB_961394), anti-IFN-γ (BioLegend Cat# 502526, RRID: AB_961355) antibodies for FACS were purchased from Biolegend, USA. Recombinant murine mIL-2 (Cat#212-12, PeproTech, USA) was purchased from PeproTech, USA. Recombinant murine mIL-6 (Cat#406-ML, R&D Systems, USA), mIL-12 (Cat#419-ML, R&D Systems, USA), and recombinant human hTGF-β1 (Cat#240-B, R&D Systems, USA) were purchased from R&D Systems, USA. The anti-TET2 (Cell Signaling Technology Cat# 45010, RRID: AB_2799277), anti-TET3 (Cat#ab139311, Abcam, UK), anti-JMJD3 (Cell Signaling Technology Cat# 3457, RRID: AB_1549620), and anti-KDM3A (Proteintech Cat# 12835-1-AP, RRID: AB_1072935) antibodies were purchased from CST, USA; Abcam, UK; Proteintech, USA, respectively.

Murine $CD4^+$ T cell culture

Murine lymph nodes and spleens were dissected and processed into single cell suspension. Total $CD4^+$ T cells were isolated using CD4 (L3T4) Microbeads (Cat#130-117-043, Miltenyi Biotec, Germany) according to the manufacturer's instruction. Alternatively, $CD62L^+CD44^-$ naïve $CD4^+$ T cells were FACS sorted using the BD Aria Sorter. The achieved purity of $CD4^+$ T cells was greater than 95%. Isolated $CD4^+$ T cells were seeded into 96-well plates (0.1 million cells/well) pre-coated with 5 μg/ml anti-CD3 and 2 μg/ml anti-CD28. The following conditions were used to polarize helper T cells: Th1 (20 ng/ml mIL-12 and 100 ng/ml anti-IL-4), Th17 (20 ng/ml mIL-6, 1 ng/ml hTGF-β1, 100 ng/ml anti-IL-4, and 100 ng/ml anti-IFN-γ), and iTreg (50 ng/ml mIL-2 and 10 ng/ml hTGF-β1). All cells were cultured with RPMI-1640 containing 10% FCS, Pen/Strep, and 2-mercaptoethanol in a humidified incubator containing 5% CO_2 at 37 °C. In screening experiments, 128 epigenetic compounds were added into the cultures at the beginning of cell polarization in concentrations decided by published data or preliminary experiments. In OTII experiments, splenocytes from C57BL6/J mice were collected and irradiated as APCs, $CD4^+$ T cells from C57BL6 OT-II Tg mice were isolated and seeded with APCs (1CD4:10APC) under polarizing culture conditions (Th1: 4 ng/ml mIL-12 and 4 μg/ml anti-IL-4 mAb; Th17: 4 μg/ml anti-IL-4 mAb,1.2 ng/ml hTGF-β,4 ng/ml mIL-6, 2 ng/ml mIL-1α, 4 μg/ml anti-IL-12 mAb and 8 μg/ml Anti-IFN-γ mAb) and OVA peptide (2 μg/ml).

Flow cytometry

The LIVE/DEAD fixable dead cell stain kit (Cat#L34962, Thermofisher Scientific, USA) was used to exclude dead cells. After incubated with 24G2 cell supernatant, cells were stained with the primary antibodies against cell surface markers, followed by intracellular cytokine staining using the protocol as previously described.[16] All samples were analyzed using the BD LSR II or BD LSR Fortessa X-20 (BD Bioscience, USA) flow cytometers and the FCS data were analyzed with FlowJo v10 (Tree Star Inc, USA). An overview of flow cytometry gating strategy is depicted in Fig. S1. Briefly, lymphocytes and singlets were gated based on side scatter (SSC), forward scatter (FSC), FSC-area (FSC-A) and FSC-height (FSC-H). Then live cells were gated as unstained live and stained dead cells. CD4 positive or CD4 and CD45

double positive cells were selected to analyze the intracellular staining.

RNA-seq analysis

Total RNA from IOX1 or DMSO treated Th17 cells (N = 2) was extracted with the MasterPure Complete DNA and RNA Purification Kit (Epicentre, UK) according to the manufacture's instruction. A total of 100 ng RNA was sonicated into fragments of 300–400 base pairs using Bioruptor PLUS (Diagenode, Belgium). The mRNA library construction was prepared using VAHTS mRNA-seq V3 Library Prep Kit for Illumina (Vazyme, Nanjing, China) following the manufacture's protocol and were sequenced on the Illumina HiSeq2500 sequencer with HiSeq SR Cluster Kit V4 and HiSeq SBS Kit V4 50 cycle kit (Illumina). The initial processing was performed by CASAVA (v1.8.2). Sequencing reads were then subjected to quality control processed by FastQC (v0.11.5) and trimmed by Cutadapt (v1.9.1). Quality controlled reads were then analyzed for differentially expressed genes (DEG) using DEseq2 and for pathway analysis using Ingenuity Pathway Analysis (IPA).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from induced Th1 or Th17 cells using RNAzol RT RNA Isolation Reagent (GeneCopoeia, USA). cDNA was then reverse transcribed using HiScript III RT SuperMix for qPCR (Vazyme, China) after treated with DNA wiper. The qPCR was performed with ChamQ SYBR Color qPCR Master Mix (Vazyme, China) on the Light Cycler 480 instrument (Roche, Switzerland). The following primer pairs were used: murine GAPDH-F: TGGCCTTCCGTGTTCCTAC; murine GAPDH-R: GAGTTGCTGTTGAAGTCGCA; murine IFN-γ-F: ATGAACGCTACACACTG CATC; murine IFN-γ-R: CCATCCTTTTGCCAGTTCCTC; murine IL-17A-F: TTTA ACTCCCTTGGCGCAAAA; murine IL-17A-R: CTTTCCC-TCCGCATTGACAC; murine CCL20-F: GTGGGTTTCA-CAAGACAGATGGC; murine CCL20-R: CCAGT TCTGCTTTGGATCAGCG.

ELISA

Supernatants were collected from the DMSO or IOX1 (20 μM) treated Th17 cell cultures at 72 h and were analyzed with the CCL20 ELISA kit (R&D Systems) according to the manufacturer's instructions.

Bisulfite sequencing

DNA was extracted from cultured Th17 cells using Epicenter MasterPure Purification Kit (EpiCentre, USA), and was then converted with EpiTect Bisulfite Kits (Qiagen, Germany) according to the manufacturer's instruction. After the conversion, DNA was purified and eluted into 20 μl elution buffer. The *Il17a* promoter sequence was PCR amplified, TA-cloned, and sequenced for more than 30 clones. The following primer pairs were used: Bisulfite-F: TAAATATTAA-TAGGTTTTTTGATAATATGT; Bisulfite-R: AATAAAA-CTCTCCCTAAACTCATATTTA.

Chromosome immunoprecipitation (ChIP)

The ChIP assay was performed using the ChIP-IT Kit (Active Motif, USA). In brief, IOX1 or DMSO treated Th17 cells were fixed with fixation buffer containing 1% formaldehyde at room temperature for 15 min. The nuclear DNA-protein complex was then extracted and sonicated using Bioruptor PLUS (Diagenode, Belgium). Samples were incubated with the anti-TET2 antibody at 4 °C overnight. Antibody-DNA complexes were then captured by protein G agarose beads. The immunoprecipitated DNA was then purified and quantified by real-time PCR. The following primer pairs were used: CpG-6-8-F: TCTGCCCTTCCCATCTAC; CpG-6-8-R: TCCCTGGACT-CATGTTTG; CpG-5-F: TGCTCTCTAGC-CAGGGAA; CpG-5-R: ATGGGAAGGGC-AGAAGTT.

Cellular thermal shift assay (CETSA)

The CETSA was performed as described previously.[20] Briefly, total $CD4^+$ T cells were isolated and polarized under Th17 condition for 3 days. IOX1 (20 μM) was added into the culture 4 h prior to harvest. Following harvesting, cells were washed in PBS in room temperature twice and resuspend in PBS containing proteinase inhibitor in 200 μl PCR tubes. Cells were then heated to different temperatures and snap frozen in liquid nitrogen. Extracted protein from treated cells was then load on 7.5% 15 well mini-gels in Bio-Rad Mini-PROTEAN Tetra system and analyzed using antibodies against JMJD3, KDM3A, TET2, and TET3.

Biolayer interferometry (BLI)

BLI is a label-free technique that monitors light intensity interference to analyze biomolecular interactions. In our study, we immobilized his-tagged TET2 (ab271754, Abcam, USA) on a Ni-NTA sensor (18-5101, Fortebio, Pall Life Sciences, Menlo Park, USA) at the concentration of 20 μg/ml in PBS. IOX1 was reconstituted with DMSO and further diluted with 1% DMSO-PBST. The concentrations of IOX1 were 15 μM, 7.5 μM, 3.75 μM, 1.88 μM, and 0.9375 μM. The baseline was established for 60s, then the sensor with or without immobilized TET2 was immersed into wells containing serial concentrations of IOX1 to obtain the association signal. Then dissociation curve was obtained by dipping the sensor back into PBST buffer. With FortéBio's Data Analysis software 10.0, the equilibrium dissociation constant (KD) was evaluated.

Chemical-immunoprecipitation-PCR assay

Our method was adopted from a previous study by Anders et al.[21] Briefly, biotinylated IOX1 was

synthesized by Dangang Peptides Inc. (China). The liquid chromatograph-mass spectrometer and Nuclear Magnetic Resonance were used to verify the product. Total $CD4^+$ T cells were isolated and polarized under the Th17 condition for 3 days. Twenty μM IOX1-biotin or 20 μM biotin was added into the Th17 cells 3 h before harvesting cells. The cells were then fixed with fixation buffer containing 1% formaldehyde at room temperature for 15 min and the fixation was stopped by adding stop buffer for 5 min. After sonication using Bioruptor PLUS (Diagenode, Belgium), all samples were incubated with streptavidin beads (MyOne Streptavidin T1, Invitrogen) in 4 °C overnight, washed with PBS containing 0.1% BSA, and eluted with elution buffer from ChIP kit mentioned above at 70 °C. The immunoprecipitated DNA was then purified and quantified by real-time PCR.

Experimental autoimmune uveoretinitis (EAU)

On Day 0, B10.RIII mice were immunized subcutaneously with 100 μL of recombinant human interphotoreceptor retinoid binding protein (IRBP) peptide (161–180) (1 mg/mL) emulsified 1:1 (v:v) in complete Freund's adjuvant containing 2.5 mg *Mycobacterium tuberculosis* complete H37Ra (BD Biosciences). After immunization, each mouse was injected with 1 μg of Bordetella pertussis toxin (Tocris Bioscience) intraperitoneally. Intraperitoneal administration of IOX1 (12.5 mg/kg) or DMSO solution was performed twice daily post-immunization. At peak disease (Day 14), mice were sacrificed, and tissues collected for *ex-vivo* analysis. To assess the clinical score of EAU, we used either a TEFI system[22] or a Micron IV murine fundus camera (Phoenix Research Labs, USA) and contemporaneous Spectralis-optical coherence tomography (OCT) scanning.[23] The clinical scores were given by two independent experienced observers in a blindfold manner based on the criteria for EAU scoring as described previously.[22,23]

Uveitis induced by adoptive transfer of $CD4^+$ T cells

EAU was induced in the donor mice as described above. On Day 11, spleens and lymph nodes from donor mice were harvested and single cell suspensions prepared. About 7.5×10^6 cell/Flask were cultured in 25 ml complete RPMI1640 media supplemented with 20 ng/ml murine IL-23 and 10 μg/ml hIRBP161-180 peptide, with DMSO or IOX1 (20 μM). At 24 h, an additional 10 ml of fresh media supplemented with murine IL-2 (final concentration 10 ng/ml) was added to each flask. Blasting $CD4^+$ T cells were separated using CD4 (L3T4) Microbeads (Miltenyi Biotec, Germany) after 72 h, and the cell number was calculated. A total of 2 million $CD4^+$ T cells (in 200 μl PBS) was adoptively transferred into each naïve recipient mouse intraperitoneally.

Statistics

The statistical analyses were performed with Prism Graphpad 7.0 (GraphPad Software, USA). Mann–Whitney U, Kruskal–Wallis or two-way ANOVA tests were used accordingly.

Role of funders

The funders had no role in study design, collection, analysis, or interpretation of data or in the writing of the publication.

Results

In vitro screening of epigenetic drugs to suppress IFN-γ and IL-17A expression in $CD4^+$ T cells

Evidence from our previous investigations further highlights how epigenetic regulation plays a key role in the differentiation of different $CD4^+$ T helper subsets. To assess which molecular components of the epigenetic machinery to target in order to inhibit inflammatory cytokine expression in Th subsets, we screened a commercially available "Epigenetics Compound Library" (purchased from Selleck in 2015) for their potential effects in the proliferation and differentiation of Th1, Th17, and Treg cells. The compound library comprises 128 inhibitors of different epigenetic enzymes and signaling molecules including Histone Deacetylase (HDACs), JAKs, Histone demethylases, Histone Acetyltransferases (HATs), DNA and Methyltransferase (DNMTs) (Fig. 1a). The murine Th1, Th17, and inducible Treg (iTreg) culture system was used to screen the effects of these 128 compounds on Th cell differentiation and viability (Fig. 1a). Two doses (named low and high doses) were selected for each compound based on previous application found in relevant literature (the highest dose used in previous literature was defined as the "high" dose and 1/10 of the high dose was defined as the "low" dose in our assays) or from preliminary results we carried out in the murine Th cell cultures (Table S1). Over 4000 cultures were performed to characterize the responses of Th1, Th17, and iTreg cells to all 128 compounds. The signature cytokine IFN-γ and IL-17, as well as transcription factor Foxp3, detected by FACS staining, were used as the differentiation markers of Th1, Th17, and iTreg, respectively. As shown in Fig. 1b and Table S2, eleven compounds (i.e. the selective Aurora A inhibitor Alisertib) at high dose were found to promote the frequency of IFN-γ^+ cells by at least 2 fold in Th1 cultures, while 52 compounds (i.e. the JAK2 inhibitor CEP-33779) were found to reduce the frequency of IFN-γ^+ cells by at least 2 fold in Th1 cultures. On the other hand, ten compounds (i.e. the PARP inhibitor AZD2461) at high dose were found to promote the frequency of IL-17^+ cells by at least 2 fold in Th17 cultures, while 42 compounds (i.e. the bromodomain-containing proteins inhibitor Molibresib) were found

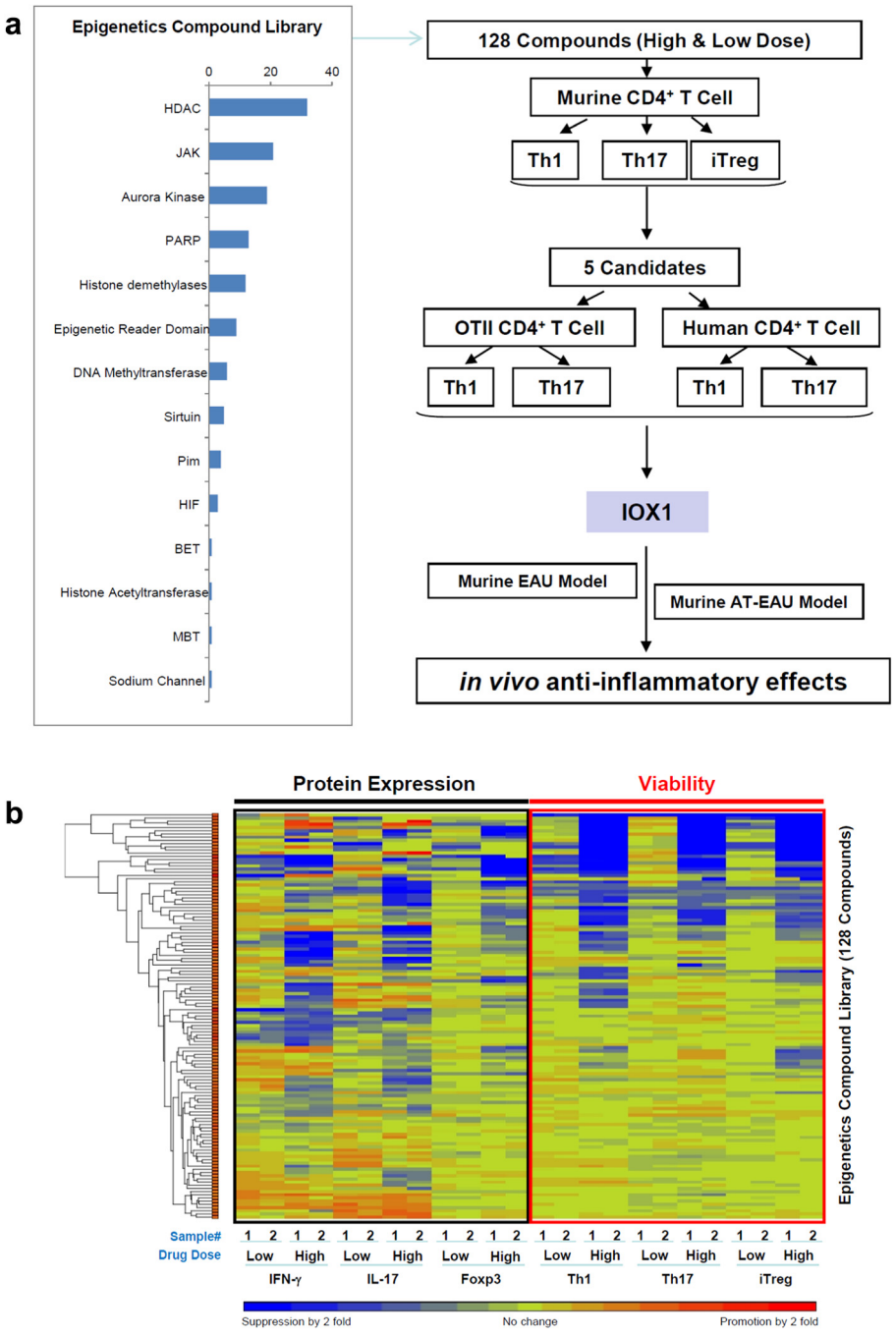

Fig. 1: **Screening of a "Epigenetics Compound Library" for anti-inflammatory molecules targeting helper T cells.** (**a**) Schematic view of the *in vitro* compound screen and *in vivo* confirmation process that identified IOX1 as an inhibitor of Th17 cells. (**b**) left panel: hierarchical clustering of the fold changes in cytokines (IFN-γ and IL-17) and Foxp3 positive cell frequencies in Th1, Th17, and iTreg cells in response to low or high dose drug treatments; right panel: hierarchical clustering of the viability changes of Th1, Th17, and iTreg cells in response to low or high dose drug treatments. Sample#: the replicates in each drug dose used in screening.

to reduce the frequency of IL-17$^+$ cells by at least 2 fold in Th17 cultures. None of the compounds we tested was able to promote Foxp3 expression in iTreg cells. Importantly, among those inhibitors of Th1 (52) and Th17 (42) differentiation, we found ~62% caused significant cell death (at least 20% reduction in cell

viability). Among these 36 compounds that did not significantly affect T cell viability, we found five ones (INO-1001, AZD1208, IOX1, OTX015, and Tubastatin A) that demonstrated equivalent inhibitory effects in suppressing both IFN-γ and IL-17 expression in Th1 and Th17 cells, respectively (Fig. S2a). Therefore, our results suggest that the suppression of key inflammatory cytokine expression in Th1 and Th17 cells often associates with compromise of cell viability in response to epigenetic drugs.

The Th1 and Th17 cultures using the antigen-specific OVA stimulated OTII system, as well as the peripheral blood total $CD4^+$ T cells from healthy volunteers were used to examine whether the five candidate compounds were still able to control the expression of IFN-γ and IL-17 *in vitro* (Fig. 1a). As shown in Fig. S2b and c, none of the five compounds suppressed IFN-γ expression in Th1 cells, while IOX1 and OTX015 suppressed the expression of IL-17 in Th17 cells. Moreover, an inhibition of both IFN-γ and IL-17 were observed in Th1 and Th17 cells in response to the stimulation of high doses of AZD1208, IOX1, and OTX015 (Fig. S2d and e). These data demonstrated that IOX1 and OTX015 might be candidates for inhibitors of Th1 and Th17 differentiation.

IOX1 suppression of IL-17 expression in $CD4^+$ T cells *in vitro*

Further investigations using OTX015 (a new BET inhibitor) demonstrated *in vitro* suppression of both proliferation and subset-dependent proinflammatory cytokine expression in murine and human $CD4^+$ T cells, including selective suppression of IL-17 in human memory $CD4^+$ cells.[17] Then we focused on evaluation of IOX1, a potent and broad-spectrum inhibitor of 2OG oxygenases, including the JmjC demethylases and DNA demethylases (Fig. 2a). Initially, we examined whether IOX1 could suppress Th1 and Th17 subsets induced from total $CD4^+$ T cells isolated from mouse lymphoid tissue. Cells were polarized into Th1 or Th17 subsets in the presence or absence of IOX1 at two doses (10 and 20 μM) for 72 h (Fig. 2b–d and S3a–e). This highlighted how IOX1 has a more profound effect on IL-17 expression from induced Th17, than on IFN-γ from Th1 cells, albeit without any impact on cell viability or proliferation of each subset. These results were also confirmed using assays in which FACS sorted naïve $CD62L^+CD44^-$ T cells were polarized in the presence of IOX1 (Fig. S4a–d). In other experiments, using polarized transgenic OT-II $CD4^+$ T cells activated with their cognate $OVA_{323–339}$ peptide, IOX1 treatment only suppressed antigen specific Th17 differentiation without affecting Th1 differentiation (Fig. S4e). The different effects of IOX1 on Th1 differentiation compared with total $CD4^+$ T cells isolated from mouse lymphoid tissue may associate with the difference between antigen-nonspecific vs antigen-specific Th1 responses. To test the potential to translate these murine observations to human disease, we also applied IOX1 on human $CD4^+$ cells. IOX1 suppressed, in a dose-dependent manner, both T cell proliferation and the frequency of IFN-γ^+ and IL-17^+ subsets in the mixed population of human cells (Fig. 2e and f). Collectively, these results indicated that IOX1 treatment *in vitro* exerts anti-inflammatory effects on T helper cell populations, preferentially inhibiting Th17 cells.

IOX1 suppresses *Il17a* expression by targeting TET2 protein

To understand at the molecular level how IOX1 treatment modulates Th17 cells, next we performed RNA-seq analysis to profile the genome-wide expression changes of Th17 cells, polarized from total $CD4^+$ T cells, in response to the treatment of IOX1 (20 μM) for 72 h. As shown in the Volcano plot (Fig. 3a), the expression of 36 genes were significantly downregulated ($P < 0.05$, fold change>2, DEseq2) by IOX1 in Th17 cells, while only 7 genes were upregulated by IOX1 (Table S3). The expression of Th17 signature cytokines *Il17a* and *Ccl20* were both significantly suppressed by IOX1 (Fig. 3a). With qPCR, we also verified that IOX1 could effectively suppress the mRNA expression of *Ifng, Il17a* as well as *ccl20* in murine induced Th17 cells (Fig. S5d–f). However, none of transcription factors promoting Th17 differentiation such as *Rorc*, *Rora*, *Stat3*, or *Irf4* were altered in response to IOX1 treatment. Further gene ontology studies using Ingenuity Pathway Analysis revealed that pathways such as IL-17A signaling and inflammasome were significantly enriched among the 43 IOX1 response genes (Fig. 3b), while diverse upstream regulators potentially were responsible for the regulation of IOX1 response genes (Fig. 3c). Therefore, our transcriptomic analysis suggests that IOX1 may directly regulate expression of IL-17 in Th17 cells.

To understand the molecular mechanism by which IOX1 suppressed *Il17a* expression, we next investigated the potential target(s) of IOX1 in Th17 cells. As an inhibitor of 2OG oxygenases, potential targets of IOX1 include both histone and DNA demethylases. Demethylases play pivotal roles in mediating the demethylation of methylated DNA and histone marks, including H3K4me3, H3K9me3, and H3K27me3, which collectively coordinate the epigenetic regulation of Th17 differentiation.[2] Since the H3K27me3 demethylase JMJD3[6] and DNA demethylases TET2/TET3[5] are recognized activators of IL-17 expression and evidence suggests H3K9me3 may also regulate the expression of IL-17,[7] we examined whether IOX1 targeted histone demethylase JMJD3, KDM3A, as well as DNA demethylase TET2/TET3 to suppress *Il17a* expression in Th17 cells. Cellular thermal shift assays[20] were performed to determine whether IOX1 directly interacts with these

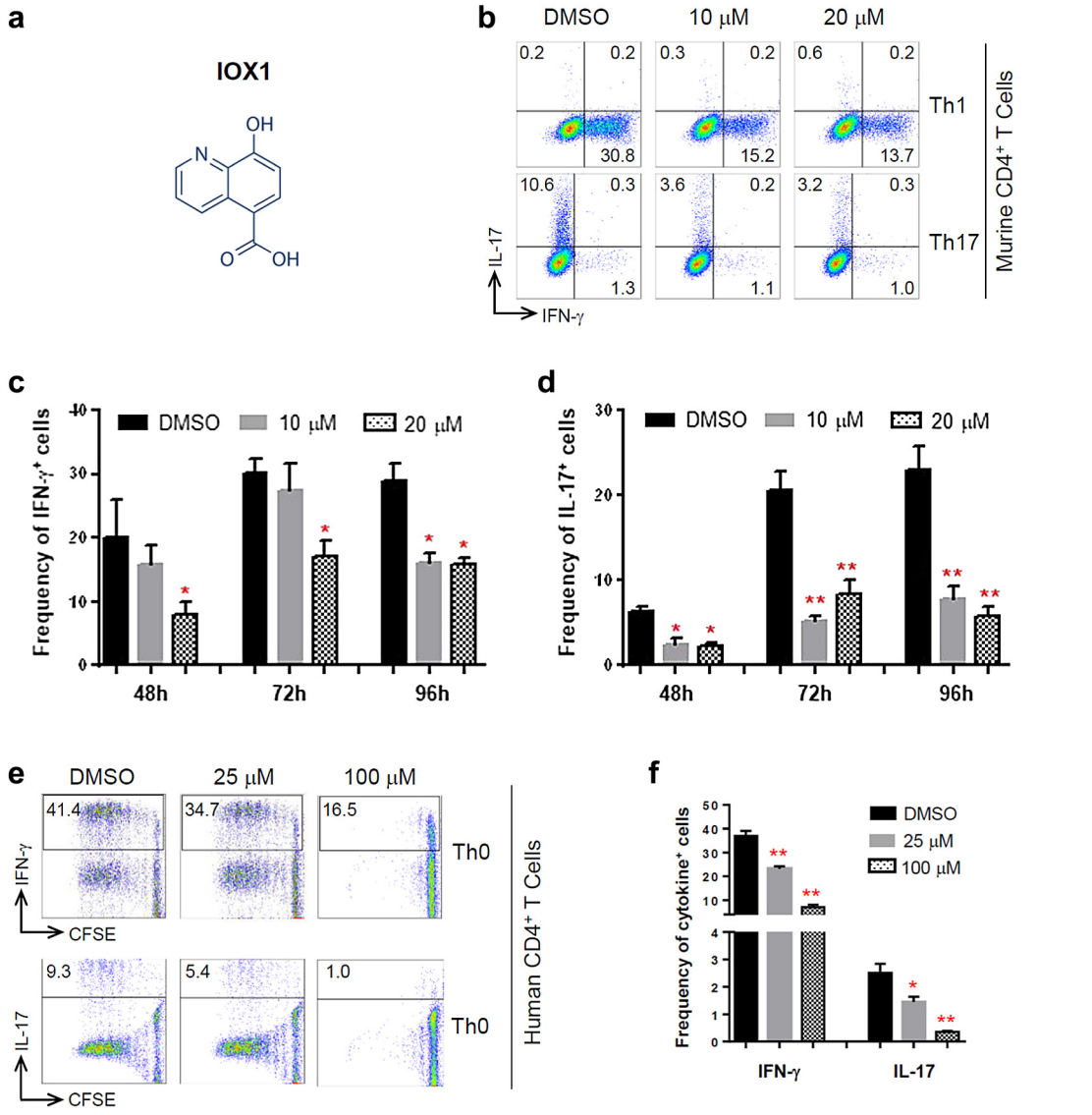

***Fig. 2:* Effects of IOX1 on murine and human CD4⁺ T cells *in vitro*.** (**a**) Structural representation of IOX1. (**b**) Representative FACS plots showing IFN-γ and IL-17 expression in induced Th1 and Th17 subsets derived from activated murine CD4⁺ T cells, in the presence or absence of IOX1. Summary graphs of IFN-γ⁺ and IL-17⁺ cell frequencies in murine Th1 (**c**) and Th17 (**d**) cultures (N = 6 for each condition). (**e**) Representative FACS staining of IFN-γ, IL-17, and CFSE in peripheral human CD4⁺ T cells stimulated by anti-CD3/CD28 coated beads in response to indicated doses of IOX1 treatments at Day 5. (**f**) Summary of IFN-γ⁺ and IL-17⁺ cell frequencies from human CD4⁺ T cell cultures following anti-CD3/CD28 stimulation in the presence of IOX1 (25 vs 100 μM) at Day 5 (N = 6 for each condition). (*P < 0.05, **P < 0.01, Mann–Whitney U test).

target proteins. As shown in Fig. 3d and e, no interactions were identified between IOX1 and JMJD3, KDM3A, or TET3, while potential interaction between IOX1 and TET2 was suggested. To further verify potential interaction between TET2 and IOX1, biolayer interferometry was used to show the binding kinetics and affinity between these two molecules. We found that IOX1 in PBST could bind to TET2, with an equilibrium dissociation constant (KD) of 1.20E-05 M (Fig. 3f and Table S4). The binding affinity between IOX1 and TET2 was higher than a previously identified TET2 interactor Bocat339,[24] which had an KD of 5.10E-05 (Table S5). These results indicate IOX1 functions through interaction with the TET2 DNA demethylase in Th17 cells.

To confirm TET2 was the target of IOX1 in Th17 cells, the DNA demethylation status of *Il17a* promoter might be altered in response to IOX1 treatment,

we performed bisulfite sequencing assays to determine the frequency of demethylated DNA on all 12 CpG sites present on the *Il17a* promoter (–600 to +200 bp of the transcription start site) (Fig. 4a). The results demonstrate that IOX1 significantly reduced the frequency of demethylated DNA on CpG sites 7 and 8 (Fig. 4b). Moreover, chromatin immunoprecipitation (ChIP) demonstrated that TET2 is directly bound to the CpG-6-8 site (Fig. 4c), while chemical-immunoprecipitation (Chem-IP) assays (the chemical structure of biotinylated IOX1 was shown in Fig. 4e) confirm IOX1 binds the CpG-6-8 site of the *Il17a* promoter (Fig. 4d). Taken together, these results indicate IOX1 directly interacts with TET2 on the *Il17a* promoter, which may counteract the DNA demethylase function of TET2.

To validate whether IOX1 directly targets TET2, its capacity to suppress IL-17 expression in TET2 deficient ($CD4^{cre}$ mediated conditional deletion of *Tet2* in T cells) Th17 cells was assessed. As shown in Fig. 4f and g, Th17 polarization is significantly inhibited both in $CD4^+$ T cells and TET2 deficient $CD4^+$ T cells. Furthermore, the average fold reduction of $IL\text{-}17^+$ cells in response to IOX1 treatment was reduced 3 fold in TET2 deficient Th17 cells (Fig. 4h). In addition to *Il17a*, our earlier RNA-seq analysis and qPCR revealed that expression of *Ccl20* was also significantly decreased in Th17 cells in response to IOX1 treatment (Fig. 3a and S5f). To demonstrate whether this effect was directly mediated through IOX1 interaction with TET2, we assayed the culture supernatants for CCL20 protein levels. As shown in Fig. 4i and j, in TET2 deficient Th17 cells, IOX1 treatment led to a 2-fold reduction in CCL20 protein expression, confirming the direct role for TET2 in mediating IOX1 inhibition of IL-17 expression.

IOX1 controls intraocular inflammation *in vivo*

To assess *in vivo* efficacy of IOX1 as a potential treatment for autoimmune disease, we employed the established experimental autoimmune uveoretinitis (EAU) model of ocular inflammation.[25] Using the highly susceptible B10.RIII strain, in which the immunizing regimen produces consistent moderate disease severity,[22] we assessed whether systemic IOX1 administration could modulate disease outcome. Groups of immunized mice were treated via daily intraperitoneal injections (see methods section) with IOX1 or control (DMSO), and clinically monitored until peak disease (Day 13 post-immunization). The IOX1 treatment regimen did not impact the overall health or behavior of animals, with no deviation in mean weights between the experimental groups (Fig. S6a). Clinical assessment fundal images and optical coherence tomography (OCT) scans were scored to evaluate the severity of intraocular inflammation at different time-points during EAU. As shown in Fig. 5a–c, DMSO treated mice exhibit typical clinical disease features, with initial signs of inflammation evident between Day 7 and Day 11, with rapidly progressing vasculitis and vitreous infiltrate to peak disease at Day 13. In contrast, mice that received IOX1 treatment exhibit significantly reduced clinical disease severity. To assess the efficacy of IOX1 treatment on local and systemic immunity, single cell suspensions from the eyes, eye draining lymph nodes, inguinal lymph nodes, and spleens were prepared for FACS analysis of $CD4^+$ T cell subsets. By Day 14, IOX1 treatment significantly reduced the absolute number and frequency of intraocular $CD4^+$, $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}17^+$, and $CD4^+IFN\text{-}\gamma^+IL\text{-}17^+$ T cell populations (Fig. 5c–h). In contrast, analysis of secondary lymphoid tissues demonstrated that IOX1 treatment only impacted the frequency of $CD4^+IL\text{-}17^+$ T cells present in the inguinal lymph nodes (Fig. S7a–o). As shown in Fig. 3a and S5f, the IOX1 treatment also significantly reduced the expression of *Ccl20*, which mediates the Th17 cell migration.[26] These data suggest that systemic administration of IOX1 can limit the migration and infiltration of antigen-specific effector populations to the eye, without any significant alteration to the systemic pool of $CD4^+$ T cell populations.

Whilst induction of EAU using active immunization generates robust intraocular inflammation, it relies on the use of adjuvants (Complete Freund's adjuvant and pertussis toxin), which have detectable effects on disease phenotype.[27] Additionally, we recognized that our systemic IOX1 administration approach may also influence the severity of intraocular inflammation via regulation of other populations of immune cells. To refine our capability to delineate IOX1 specificity for $CD4^+$ T cells, independently from any innate immune influences of the induction method, we elected to use an adoptive transfer EAU model.[28] In this model, following systemic transfer of purified donor autoantigen-specific T cells into naïve recipients, initial signs of ocular inflammation are evident (Day 5) rapidly progressing to a peak of severe clinical disease (Day 10), which then develops into persistent uveitis. Adapting the standard protocol, donor antigen-specific spleen and lymph node cells were harvested and *in vitro* cultured in media supplemented with IOX1 or DMSO for two days, before transfer of purified $CD4^+$ T cells populations into naïve recipient mice (Fig. 6a). Clinical assessment demonstrates mice receiving control (DMSO) treated cells develop expected disease onset (Day 5) and progressive inflammation, whereas disease severity in response to IOX1 treated $CD4^+$ T cells was delayed (Day 7), with an overall significantly reduced level of intraocular inflammation (Fig. 6b and c). Reduced clinical disease severity in mice transferred with IOX1 treated cells was associated with significant reduction in the numbers of infiltrating ocular $CD4^+$ T cells, specifically Th17 cells (Fig. 6d–i), and reduced presence of Th17 cells in the eye draining lymph nodes (Fig. S8a–o). This data supports the

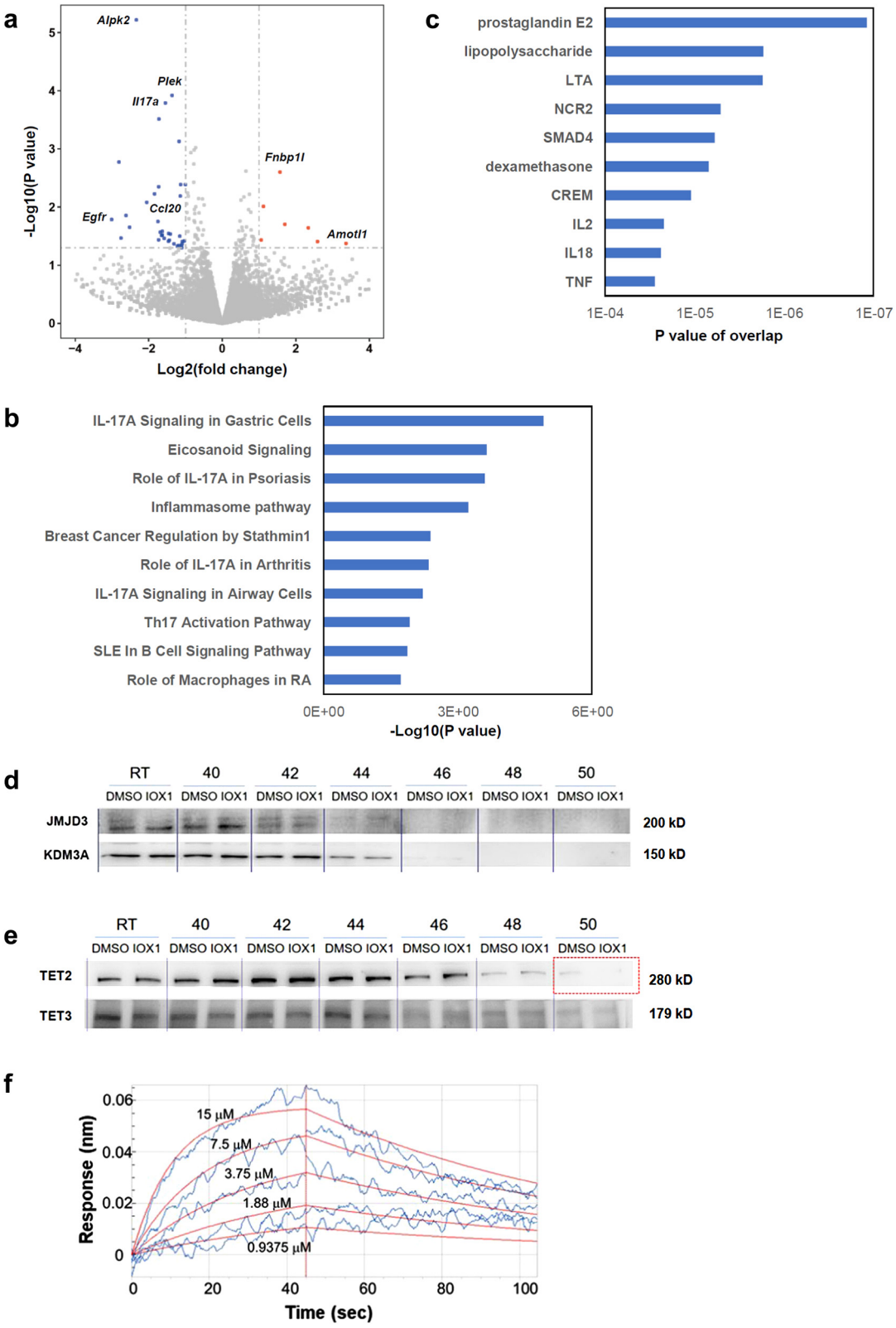

***Fig. 3*: IOX1 regulated gene programs in Th17 cells.** (**a**) Differentially Expressed Genes (DEG) changed by at least two fold with a $P < 0.05$ (DEseq2) in response to IOX1 treatment represented in Volcano plot. (**b**) Pathways enriched in IOX1 induced DEGs, analyzed using IPA.

concept that IOX1 treatment can control Th17 mediated inflammation *in vivo*.

Discussion

Anti-Th17 therapies

It is well recognized that dysregulated Th17 cell responses contribute to the immunopathogenesis of multiple inflammatory and autoimmune diseases, including autoimmune uveitis.[1] Therefore, targeting Th17 cells as well as those molecules mediating the differentiation and inflammatory functions of these cells is an attractive therapeutic approach for autoimmune disease. Whilst biologic agents (monoclonal antibodies or recombinant proteins) targeting IL-17 and IL-23, as well as inhibitors of RORγt have shown potent efficacy in treating diseases such as psoriasis and rheumatoid arthritis, this potential has not been realized in other conditions such as uveitis and Crohn's disease.[29–31] In our current study, the screening strategy we employed identified IOX1 as a direct suppressor of *Il17a* expression, without significant alteration of RORγt expression. In addition, IOX1 blocked the expression of Th17 specific chemokine *Ccl20* (Fig. 3a and S5f), which correlated with the reduction of Th17 migration to the diseased sites *in vivo* (Figs. 5, 6, S7h, and S8c). Therefore, our data highlights IOX1 as an effective anti-Th17 therapeutic agent to control Th17 cell migration and function.

TET2 as a potential drug target of IOX1 in Th17 cells

The Ten-Eleven-Translocation (TET) proteins TET1, TET2, and TET3 catalyze 5-methylcytosine (5 mC) conversion to 5-hydroxymethylcytosine (5hmC) to mediate DNA demethylation process.[32] Our previous study demonstrates how the TET2 protein, which is highly expressed in activated helper T cells, promotes DNA demethylation and expression of inflammatory cytokines in Th1 and Th17 subsets.[5] Therefore, targeting TET2 may function to modulate cytokine expression in Th1 and Th17 cells. The therapeutic potential for inhibitors of TET proteins has until now not been fully appreciated or developed to modulate TET-mediated immunoregulation. Our study has identified IOX1 as the potential small molecule that suppresses Th17 function via targeting TET2 binding on the *Il17a* promoter. Recognizing that IOX1 is a general 2OG enzyme inhibitor which can also target other histone and DNA demethylases, we examined whether IOX1 directly interacted with demethylases that potentially activate *Il17a* expression, including the H3K27me3 demethylase JMJD3, H3K9me3 demethylase KDM3A, and DNA demethylase TET3. Our results demonstrated no interaction with IOX1 (Fig. 3d and e), however it may be that IOX1 suppresses Th17 cells through targeting other 2OG enzymes. IOX1 is the most potent broad-spectrum inhibitor of 2OG oxygenases, including the JmjC demethylases. More than 30 human JmjC oxygenases have been identified,[33] and further studies are needed to elucidate the potential targets of IOX1 in Th17 cells.

In Th17 cells, transcriptional regulation of *Il17a* is largely orchestrated by a group of transcription factors including RORγt, RORα, IRF4, Batf, c-Maf, c-Rel, and AHR[2]. Nonetheless, bioinformatic analyses of genes altered in response to IOX1 treatment in Th17 cells did not reveal any enrichment of these transcription factors' downstream genes (Fig. 3c). Importantly, *Il17a* and *Ccl20* are among the top 6 ranked genes directly regulated by TET2 in Th17 cells,[5] and were also regulated by IOX1 treatment (Figs. 3a and S5d and f). Moreover, we have demonstrated the direct interaction between TET2 and IOX1 (Fig. 3e and f), association of TET2 and IOX1 on the promoter of *Il17a* in Th17 cells (Fig. 4c and d), and the dependency of IL-17 and CCL20 suppression in IOX1 stimulated Th17 cells on TET2, as revealed in experiments using TET2 deficient cells (Fig. 4f–j). Taken together, our data suggest that IOX1 suppresses *Il17a* expression through targeting TET2 activity in Th17 cells.

Epigenetic therapy for inflammatory diseases

Epigenetic reprogramming is known to play an important role in the pathogenesis of autoimmune disease. Remarkably, ~60% of likely causal variants for 21 autoimmune diseases, including Behcet's disease, map to enhancer-like elements, with preferential correspondence to stimulus-dependent $CD4^+$ T-cell enhancers that respond to immune activation by increasing histone acetylation and transcribing noncoding RNAs.[34] A single-cell multi-omic study of PBMCs demonstrates that the T cells in Vogt-Koyanagi-Harada patients exhibited activation phenotypes, with compositional and epigenomic alterations.[35]

Epigenetic therapy has proven effective for the treatment of multiple cancers[36] and a growing number of reports also demonstrate potential benefits in the treatment of inflammatory and autoimmune diseases.[12,15,37,38] However, there are still no FDA approved epigenetic drugs on the market for use as immunosuppressants. Our screening experiments compared a selection of epigenetic drug candidates that function in different ways to modify chromatin

(**c**) Potential upstream regulators of IOX1 induced DEGs were analyzed using IPA. Th17 cells were collected at 72 h. The CETSA was performed to analyze the potential association between IOX1 and JMJD3, KDM3A (**d**) and between IOX1 and TET2, TET3 (**e**). (**f**) The response curve of TET2 and IOX1 in biolayer interferometry assay. The red rectangle highlights the changes identified in the CETSA of TET2.

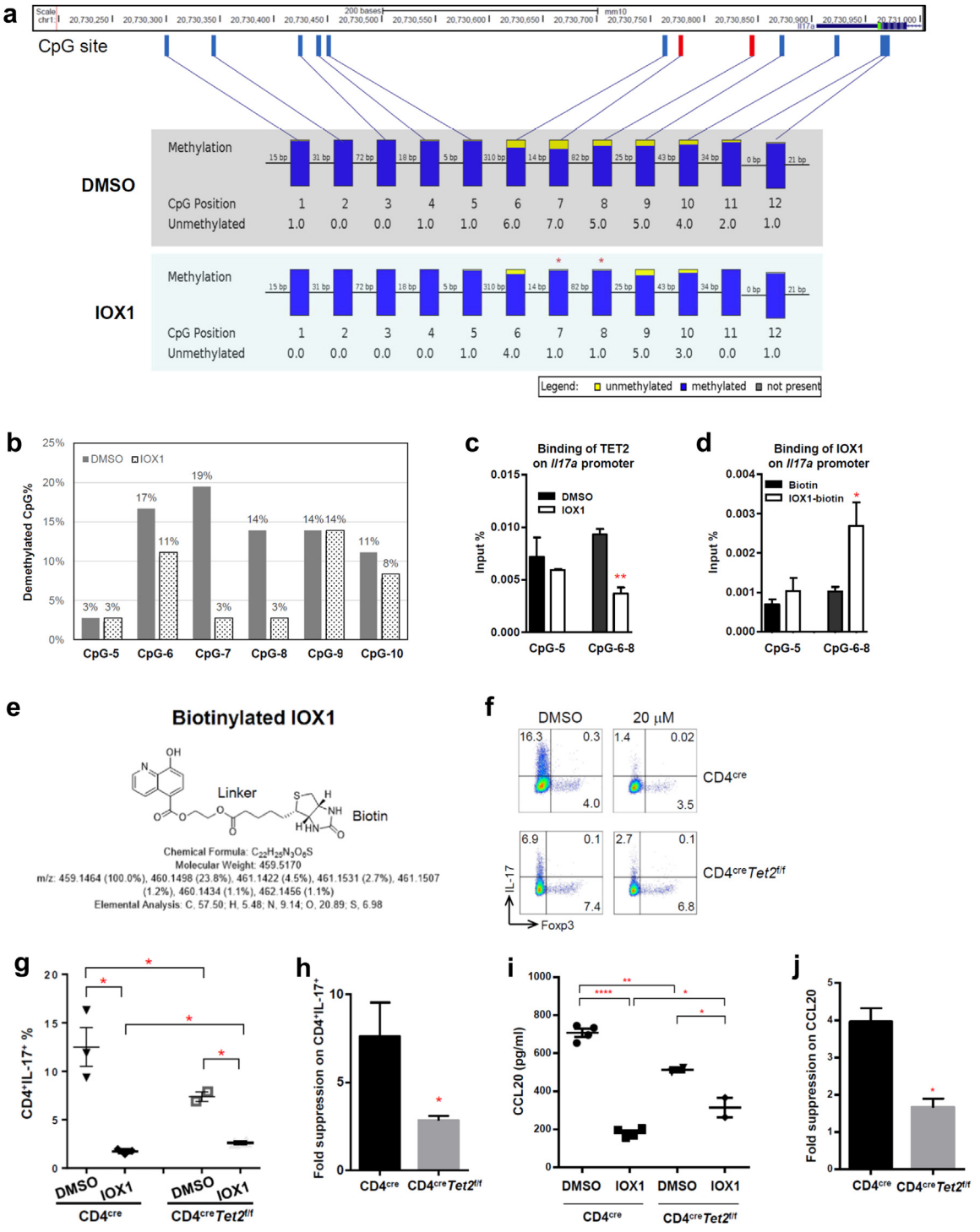

Fig. 4: **IOX1 regulates *Il17a* expression through direct association with TET2 on the *Il17a* promoter.** (**a**) DNA methylation status was assayed using bisulfite sequencing analysis of named CpG sites (#1–12) on the proximal promoter of *Il17a* in murine total CD4 polarized Th17 cells. A total of 36 clones from four independent experiments were sequenced. The methylation status of CpG site #7 and #8 were statistically different between DMSO and IOX1 treated Th17 cells (Fisher exact test, *P < 0.05). (**b**) Summary of the frequencies of demethylated CpG sites on the *Il17a* promoter. (**c**) Binding of TET2 on the CpG-5 and CpG-6-8 sites of *Il17a* promoter in response to IOX1 treatment, assayed by ChIP. (N = 3) (**d**) Binding of IOX1 to the CpG-5 and CpG-6-8 sites of *Il17a* promoter assayed by Chem-IP. (N = 2) (**e**) The chemical structure of biotinylated IOX1. (**f**) Representative FACS analysis of IL-17 expression in response to IOX1 (20 μM) in WT ($CD4^{Cre}$) and TET2 deficient

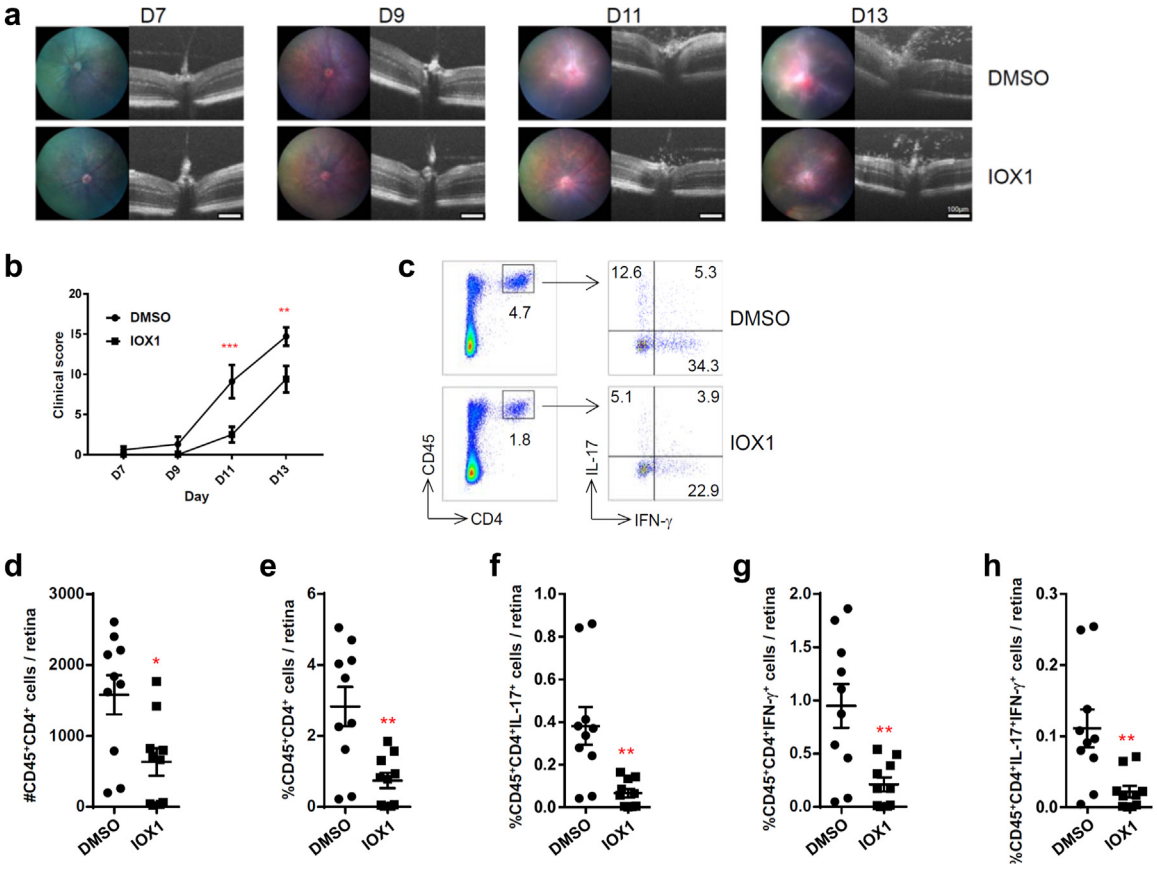

Fig. 5: **The effects of IOX1 on murine EAU.** (**a**) Representative fundus images and corresponding OCT scans showing the severity of ocular inflammation on Days 7, 9, 11, and 13 post immunization. Scale bar = 100 μm. (**b**) Summary of the EAU clinical scores (N = 10 for each of DMSO and IOX1 treated mice). (**c**) Representative FACS staining of Th1 and Th17 populations from a single retina. (**d**) Graph showing the absolute number of retinal $CD45^+CD4^+$ cells per eye. (**e**) The frequency of retinal $CD45^+CD4^+$ cells among all live cells from each retinal tissue. (**f**) The frequencies of retinal $CD45^+CD4^+IL\text{-}17^+$ cells among all live cells from each retinal tissue. (**g**) The frequencies of retinal $CD45^+CD4^+IFN\text{-}\gamma^+$ cells among all live cells from each retinal tissue. (**h**) The frequencies of retinal $CD45^+CD4^+IL\text{-}17^+IFN\text{-}\gamma^+$ cells among all live cells from each retinal tissue. (N = 10 for each of DMSO and IOX1 treated mice). (*P < 0.05, **P < 0.01, Mann–Whitney U test).

structures in helper T cells, aiming to find the best Th17 suppressors in terms of balancing the effectiveness of IL-17 suppression and cellular toxicity. Compared to previously known inhibitors of IL-17 mediated inflammation such as JQ1 and Tofacitinib,[18] IOX1 presented similar efficacy with less cellular toxicity. The results indicated that IOX1 exerts anti-inflammatory effects on T helper cells without significantly affecting T cell viability (Fig. S3) and the body weight of mice (Fig. S6) at the concentration of IOX1 (20 μM) we used, suggesting the limited cytotoxicity of IOX1 on T cells and limited systemic toxicity of IOX1 in mice. However, the concentration or dose of IOX1 is still a very complex issue that needs further in-depth study.

Therefore, this study identifies IOX1 as a strong candidate therapeutic agent for intraocular inflammation. During non-infectious uveitis, activated T cells produce cytokines and chemokines that comprising among others upregulation of adhesion molecules such as P selectin, ICAM-1 and VCAM-1 and downregulation

($CD4^{Cre}Tet2^{f/f}$) Th17 cells. (**g**) Summary of IOX1 induced IL-17 suppression in WT ($CD4^{Cre}$) and TET2 deficient ($CD4^{Cre}Tet2^{f/f}$) Th17 cells (N = 3). (**h**) The fold suppression of $IL\text{-}17^+$ cell frequencies induced by IOX1 in WT ($CD4^{Cre}$) and TET2 deficient ($CD4^{Cre}Tet2^{f/f}$) Th17 cells (N = 3). (*P < 0.05, **P < 0.01, Mann–Whitney U test). (**i**) Summary of IOX1 induced suppression of CCL20 expression (in culture supernatants, measured by ELISA) in WT ($CD4^{Cre}$) (N = 4) and TET2 deficient ($CD4^{Cre}Tet2^{f/f}$) (N = 3) Th17 cells. (**j**) The fold suppression of CCL20 expression induced by IOX1 in WT ($CD4^{Cre}$) (N = 4) and TET2 deficient ($CD4^{Cre}Tet2^{f/f}$) (N = 3) Th17 cells. (*P < 0.05, Mann–Whitney U test).

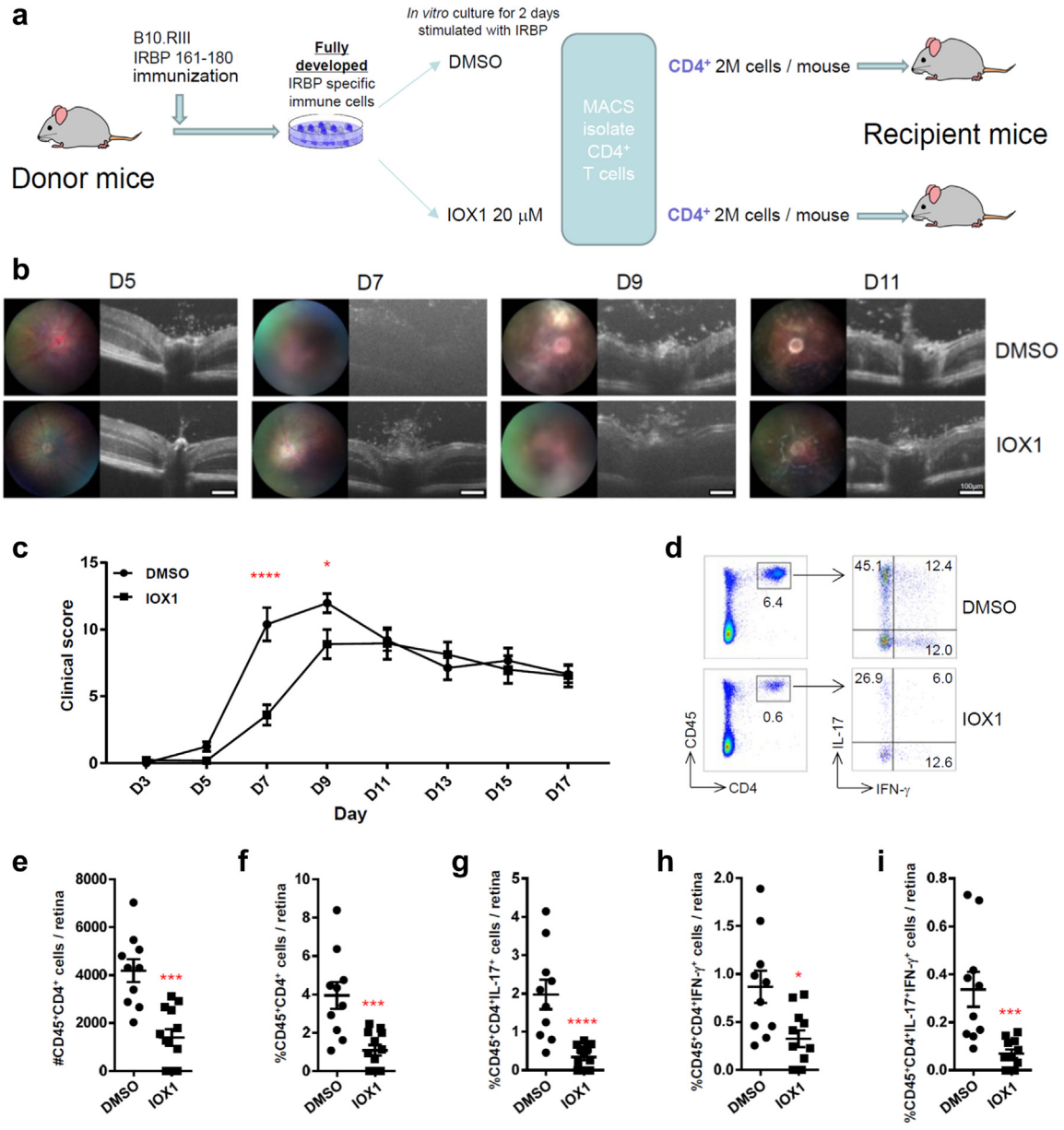

Fig. 6: **The effects of IOX1 on adoptively transferred CD4$^+$ T cell induced intraocular inflammation.** (**a**) Schematic detailing the generation and treatment of autoantigen specific CD4$^+$ cells with IOX1 or DMSO for transfer to naïve recipients. (**b**) Representative fundus image and OCT scan showing the severity of ocular inflammation on Days 5, 7, 9, and 11 post cell transfer. Scale bar = 100 μm. (**c**) Summary of the EAU clinical scores (N = 10 for each of DMSO and IOX1 treated mice). (**d**) Representative FACS staining for Th1 and Th17 populations from a single retina. (**e**) Absolute number of retinal CD45$^+$CD4$^+$ cells. (**f**) The frequencies of retinal CD45$^+$CD4$^+$ cells among all live cells from each retinal tissue. (**g**) The frequencies of retinal CD45$^+$CD4$^+$IL-17$^+$ cells among all live cells from each retinal tissue. (**h**) The frequencies of retinal CD45$^+$CD4$^+$IFN-γ$^+$ cells among all live cells from each retinal tissue. (**i**) The frequencies of retinal CD45$^+$CD4$^+$IL-17$^+$ IFN-γ$^+$ cells among all live cells from each retinal tissue. (N = 10 for each of DMSO and IOX1 treated mice). (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Mann–Whitney U test).

of tight junction proteins. This leads to the recruitment of a wide range of immune cells such as NK cells, monocytes/macrophages, NKT cells. A recent study reported that IOX1 increased the IFN-γ$^+$ NK cell ratio in PBMCs from healthy donors in the presence of IL-15.[39] Further study is warranted to elucidate the molecular mechanisms by which IOX1 epigenetically regulates the expression of these key cytokines and transcription factors in T cells and other immune cells.

Contributors

LW, RWJL, and XW conceived and designed experiments. PZ, ADD, YL, XW, RWJL, and LW supervised and coordinated the work. XH, YZ, DAC, and LPS performed most experiments and analyzed data. YL, PJPL, MS, ZZ, XW, JJL, SY, and SL provided technical assistance and performed experiments. SG, JL, and TC performed the RNA-seq experiments and carried out RNA-seq data analysis; LW, DAC and RWJL drafted the manuscript. LW, DAC and RWJL verified the data. All authors read and approved the final version of the manuscript.

Data sharing statement

Study protocol and all data collected for the study, including raw data and data analysis will be made available to others upon request. All data will be available upon publication of the manuscript, by contacting the corresponding author.

Declaration of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2022.104333.

References

1 Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol*. 2009;27:485–517.
2 Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu Rev Immunol*. 2012;30:707–731.
3 Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov*. 2012;11(5):384–400.
4 Wei G, Wei L, Zhu J, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity*. 2009;30(1):155–167.
5 Ichiyama K, Chen T, Wang X, et al. The methylcytosine dioxygenase Tet2 promotes DNA demethylation and activation of cytokine gene expression in T cells. *Immunity*. 2015;42(4):613–626.
6 Li Q, Zou J, Wang M, et al. Critical role of histone demethylase Jmjd3 in the regulation of CD4+ T-cell differentiation. *Nat Commun*. 2014;5:5780.
7 Takada I. DGCR14 induces Il17a gene expression through the RORgamma/BAZ1B/RSKS2 complex. *Mol Cell Biol*. 2015;35(2):344–355.
8 Goschl L, Preglej T, Boucheron N, et al. Histone deacetylase 1 (HDAC1): a key player of T cell-mediated arthritis. *J Autoimmun*. 2020;108:102379.
9 Regna NL, Chafin CB, Hammond SE, Puthiyaveetil AG, Caudell DL, Reilly CM. Class I and II histone deacetylase inhibition by ITF2357 reduces SLE pathogenesis in vivo. *Clin Immunol*. 2014;151(1):29–42.
10 Bandukwala HS, Gagnon J, Togher S, et al. Selective inhibition of CD4+ T-cell cytokine production and autoimmunity by BET protein and c-Myc inhibitors. *Proc Natl Acad Sci U S A*. 2012;109(36):14532–14537.
11 Hammitzsch A, Tallant C, Fedorov O, et al. CBP30, a selective CBP/p300 bromodomain inhibitor, suppresses human Th17 responses. *Proc Natl Acad Sci U S A*. 2015;112(34):10768–10773.
12 Mele DA, Salmeron A, Ghosh S, Huang HR, Bryant BM, Lora JM. BET bromodomain inhibition suppresses TH17-mediated pathology. *J Exp Med*. 2013;210(11):2181–2190.
13 Nicodeme E, Jeffrey KL, Schaefer U, et al. Suppression of inflammation by a synthetic histone mimic. *Nature*. 2010;468(7327):1119–1123.
14 Zhang W, Prakash C, Sum C, et al. Bromodomain-containing protein 4 (BRD4) regulates RNA polymerase II serine 2 phosphorylation in human CD4+ T cells. *J Biol Chem*. 2012;287(51):43137–43155.
15 Cribbs AP, Terlecki-Zaniewicz S, Philpott M, et al. Histone H3K27me3 demethylases regulate human Th17 cell development and effector functions by impacting on metabolism. *Proc Natl Acad Sci U S A*. 2020;117(11):6056–6066.
16 Zou Y, Hu X, Schewitz-Bowers LP, et al. The DNA methylation inhibitor Zebularine controls CD4(+) T cell mediated intraocular inflammation. *Front Immunol*. 2019;10:1950.
17 Hu X, Schewitz-Bowers LP, Lait P, et al. The bromodomain and extra-terminal protein inhibitor OTX015 suppresses T helper cell proliferation and differentiation. *Curr Mol Med*. 2018;18(9):594–601.
18 Lee DU, Katavolos P, Palanisamy G, et al. Nonselective inhibition of the epigenetic transcriptional regulator BET induces marked lymphoid and hematopoietic toxicity in mice. *Toxicol Appl Pharmacol*. 2016;300:47–54.
19 Nebbioso A, Carafa V, Benedetti R, Altucci L. Trials with 'epigenetic' drugs: an update. *Mol Oncol*. 2012;6(6):657–682.
20 Jafari R, Almqvist H, Axelsson H, et al. The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat Protoc*. 2014;9(9):2100–2122.
21 Anders L, Guenther MG, Qi J, et al. Genome-wide localization of small molecules. *Nat Biotechnol*. 2014;32(1):92–96.
22 Copland DA, Wertheim MS, Armitage WJ, Nicholson LB, Raveney BJ, Dick AD. The clinical time-course of experimental autoimmune uveoretinitis using topical endoscopic fundal imaging with histologic and cellular infiltrate correlation. *Invest Ophthalmol Vis Sci*. 2008;49(12):5458–5465.
23 Chu CJ, Herrmann P, Carvalho LS, et al. Assessment and in vivo scoring of murine experimental autoimmune uveoretinitis using optical coherence tomography. *PLoS One*. 2013;8(5):e63002.
24 Chua G, Wassarman KL, Sun H, et al. Cytosine-based TET enzyme inhibitors. *ACS Med Chem Lett*. 2019;10(2):180–185.
25 Caspi RR. Experimental autoimmune uveoretinitis in the rat and mouse. *Curr Protoc Immunol*. 2003:15–16 [Chapter 15].
26 Meitei HT, Jadhav N, Lal G. CCR6-CCL20 axis as a therapeutic target for autoimmune diseases. *Autoimmun Rev*. 2021;20(7):102846.
27 Caspi RR. A look at autoimmunity and inflammation in the eye. *J Clin Invest*. 2010;120(9):3073–3083.
28 Agarwal RK, Silver PB, Caspi RR. Rodent models of experimental autoimmune uveitis. *Methods Mol Biol*. 2012;900:443–469.
29 Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci*. 2014;35(10):493–500.
30 Fragoulis GE, Siebert S, McInnes IB. Therapeutic targeting of IL-17 and IL-23 cytokines in immune-mediated diseases. *Annu Rev Med*. 2016;67:337–353.
31 Chong WP, Mattapallil MJ, Raychaudhuri K, et al. The cytokine IL-17A limits Th17 pathogenicity via a negative feedback loop driven by autocrine induction of IL-24. *Immunity*. 2020;53(2):384–397.
32 Pastor WA, Aravind L, Rao A. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol*. 2013;14(6):341–356.
33 Schiller R, Scozzafava G, Tumber A, et al. A cell-permeable ester derivative of the JmjC histone demethylase inhibitor IOX1. *ChemMedChem*. 2014;9(3):566–571.

34 Farh KK, Marson A, Zhu J, et al. Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature*. 2015;518(7539):337–343.
35 Shi W, Ye J, Shi Z, et al. Chromatin accessibility analysis reveals regulatory dynamics and therapeutic relevance of Vogt-Koyanagi-Harada disease. *Commun Biol*. 2022;5(1):506.
36 Jones PA, Ohtani H, Chakravarthy A, De Carvalho DD. Epigenetic therapy in immune-oncology. *Nat Rev Cancer*. 2019;19(3):151–161.
37 Hull EE, Montgomery MR, Leyva KJ. HDAC inhibitors as epigenetic regulators of the immune system: impacts on cancer therapy and inflammatory diseases. *BioMed Res Int*. 2016;2016:8797206.
38 Tough DF, Tak PP, Tarakhovsky A, Prinjha RK. Epigenetic drug discovery: breaking through the immune barrier. *Nat Rev Drug Discov*. 2016;15(12):835–853.
39 Yao B, Yang Q, Yang Y, et al. Screening for active compounds targeting human natural killer cell activation identifying Daphnetin as an enhancer for IFN-gamma production and direct cytotoxicity. *Front Immunol*. 2021;12:680611.