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Basic

Research Article

Functionally distinct IFN-γ$^+$IL-17A$^+$ Th cells in experimental autoimmune uveitis: T-cell heterogeneity, migration, and steroid response

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Immunopathogenic roles for both Th1 (CD4$^+$IFN-γ$^+$) and Th17 (CD4$^+$IL-17A$^+$) cells have been demonstrated in experimental autoimmune uveitis (EAU). However, the role for Th17/Th1 (CD4$^+$ T cells co-expressing IFN-γ and IL-17A) cells in EAU is not yet understood. Using interphotoreceptor retinoid-binding protein peptide-induced EAU in mice, we found increased levels of Th17/Th1 cells in EAU retinae (mean 9.6 ± 4.2%) and draining LNs (mean 8.4 ± 3.9%; $p = 0.01$) relative to controls. Topical dexamethasone treatment effectively reduced EAU severity and decreased retinal Th1 cells ($p = 0.01$), but had no impact on retinal Th17/Th1 or Th17 cells compared to saline controls. Using in vitro migration assays with mouse CNS endothelium, we demonstrated that Th17/Th1 cells were significantly increased within the migrated population relative to controls (mean 15.6 ± 9.5% vs. 1.9 ± 1.5%; $p = 0.01$). Chemokine receptor profiles of Th17/Th1 cells (CXCR3 and CCR6) did not change throughout the transendothelial migration process and were unaffected by dexamethasone treatment. These findings support a role for Th17/Th1 cells in EAU and their resistance to steroid inhibition suggests the importance of targeting both Th17 and Th17/Th1 cells for improving therapy.

Keywords: experimental autoimmune uveitis · IL-17A$^+$IFN-γ$^+$ · steroid resistance · T cell migration · Th17/Th1 cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Noninfectious posterior uveitis, an immune-mediated disease involving the uveal tract of the eye, is characterized by leukocyte infiltration and structural damage. Patients are at risk of visual impairment and blindness. The immunopathogenic mechanism is thought to involve activated CD4$^+$ T cells, which migrate across the blood-retinal barrier, activating local myeloid cells, initiating tissue destruction, angiogenesis, and disruption of immunoregulation within the retinal microenvironment [1]. Both interleukin 17 (IL-17) producing helper T cells (Th17 cells) and interferon gamma (IFN-γ) producing helper T cells (Th1 cells) have been shown to be increased in retinal tissues [2] and are thought to be important in the immunopathogenesis of posterior uveitis in man. Evidence to support this comes from the experimental model of

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experimental autoimmune uveitis (EAU), where both Th1 and Th17 cells can induce EAU in adoptive transfer experiments [3]. These T cells are characterized by their specific cytokine-producing signature, as well as their distinctive transcription factor expression: RORγt and T-bet for Th17 and Th1 cells, respectively [4].

Although initial studies concluded that the phenotypes of these cells are distinct, more recent reports show that $CD4^+$ T cells can co-express markers from different phenotypes [5]. It has been reported that Th17 cells have substantial plasticity and readily acquire the ability to produce IFN-γ in addition to IL-17 [6–8]. This subset, so-called Th17/Th1 cells, is thought to originate from Th17 cells, facilitated through IL-12 and IL-23 stimulation [6]. In recent human studies, there is new evidence that Th17/Th1 cells could further progress to a fully differentiated Th1 phenotype ($CD161^-CCR6^+$ nonclassical Th1 cells) by losing IL-17 secretion, controlled by the transcription factor Eomes [9, 10]. Human Th1 cells are traditionally considered less plastic, although a reverse plasticity from Th1 to Th17 cell trans-differentiation in response to TGF-β and IL-6 has been reported [11–13]. Trans-differentiation of $CD4^+$ T cells into functionally distinct subsets can be crucial in balancing the protective and pathogenic features of the immune response [14]. The detection of Th17/Th1 cells has been associated with Th17-mediated diseases, for example in juvenile idiopathic arthritis [15], graft versus host disease [16], and in skin lesions in Behçet's disease [17]. In diabetes, Th17/Th1 cells have been associated with β-cell autoantibody activity in human and animal models of type I diabetes mellitus and was considered as a biomarker for disease progression [18]. Similarly in experimental models of disease, Th17/Th1 cells have been reported in EAE [19] and dry eye disease [20]. In addition, in a Th17-dominant mouse model of inflammatory bowel disease, Th17 cells deficient in IFN-γ failed to induce intestinal inflammation, confirming Th17/Th1 cells are required for Th17-induced colitis [21].

In this study, we investigated the function of Th17/Th1 cells in EAU and human PBMCs. We first determined if Th17/Th1 cells are sensitive to dexamethasone treatment. Second, we assessed the ability of effector $CD4^+$ T cell subsets to migrate across CNS microvascular endothelium monolayers in vitro. To our knowledge, this is the first time that functional aspects of Th17/Th1 cells in noninfectious inflammatory eye disease (EAU) are described.

Results

$CD4^+IL\text{-}17A^+IFN\text{-}\gamma^+$ (Th17/Th1) cells were increased in inflamed retinae, and in dLNs at peak EAU

To compare $CD4^+$ T cell subsets in inflamed EAU with control (nonimmunized) mice, single cells were isolated from retinae, draining LNs (dLNs), and peripheral blood, and immunophenotyped by flow cytometry (Fig. 1A). In control mice, there was a minimal presence of $CD4^+$ T cells (0.05 ± 0.03%; $n = 3$) in retinal tissues, but the percentages increased significantly in EAU retinae (4.6 ± 1.6%; $n = 6$; Fig. 1B). Within the live cell population, Th1, Th17, as well as Th17/Th1 cells were detectable in EAU retinae (1.05 ± 0.90% in Th17, 0.09 ± 0.08% in Th1, and 0.35 ± 0.2% in Th17/Th1 cell, respectively), while there were barely cells detected in the controls (Fig. 1C and D). Increases in both Th17/Th1 and Th1 cells were also observed in EAU dLNs relative to controls (Fig. 1E). However, the proportions of Th17/Th1 cells detected in the blood were similar between controls and EAU (Fig. 1F).

Intraocular Th1, but not Th17/Th1, cell downregulation by corticosteroids

We investigated the therapeutic effect of topical dexamethasone on Th17/Th1 cells in EAU in comparison with saline-treated EAU controls. After signs of EAU were detected at day 10 postimmunization, dexamethasone treatment over 7 days reduced clinical severity scores (11.6 ± 4.1 vs. 5.9 ± 1.8; Fig. 2A), and reduced cellular infiltration within the retinae (Fig. 2B). There were also fewer $CD45^+CD4^+$ T cells found intraocularly in the dexamethasone-treated group (Fig. 2C). In both retinae and dLNs, dexamethasone-treated mice showed a significantly decreased percentage of Th1 cells (36.5 ± 25.9% to 11.3 ± 2.7% in the retinae; 21.4 ± 8.2% to 15.0 ± 0.9%, in the dLNs), but dexamethasone treatment had no effect on levels of Th17 or Th17/Th1 cells (Fig. 2D–E).

IL-17/IFN-γ expression by Th17/Th1 cells correlated with Tbet/RORγt expression

Immunofluorescence staining of retinal tissue sections, identifying $CD4^+$ T-cell subsets by their transcription factor expression, revealed that $CD4^+$ T cells infiltrated the retinal layers and the vitreous space in EAU. There were no $CD4^+$ T cells observed in control tissues (data not shown). Using serial retinal sections from peak EAU, we observed $CD4^+Tbet^+ROR\gamma t^+$ (nonclassical Th1 or Th17/Th1) cells in EAU eyes (Fig. 3A). We identified Th17/Th1 cells mainly located in close proximity to the optic nerve, and within the retina. By retinal single cell flow cytometry, we demonstrated that the level of $CD4^+Tbet^+ROR\gamma t^+$ expression correlated with Th17/Th1 cytokine expression ($R^2 = 0.54$, $p = 0.02$; Fig. 3B).

A further correlation analysis was done comparing the presence of $CD4^+IL\text{-}17A^+IFN\text{-}\gamma^+$ (Th17/Th1) cells with Th1 and Th17 cells in the inflamed retinal tissues from EAU eyes (Fig. 3C). There was a strong positive correlation between Th17/Th1 cells and $CD4^+IFN\text{-}\gamma^-IL\text{-}17A^+$ (Th17) cells ($R^2 = 0.94$, $p < 0.001$) and a negative correlation between Th17/Th1 cells and $CD4^+IFN\text{-}\gamma^+$ $IL\text{-}17A^-$ (Th1) cells ($R^2 = 0.54$, $p < 0.001$). The results suggested that single IFN-γ expressors mostly do not coexist with double cytokine expressors, whereas single IL-17A expressors are likely to coexist with double cytokine expressors.

Lastly, cells were identified based on their intracellular cytokine profiles and then analyzed for expression of RORγt and Tbet (Fig. 3D–F). Using this approach, the percentages of single RORγt expression were higher than dual RORγt and Tbet expression in Th17/Th1 cells, and the intensity of RORγt is stronger than Tbet in Th17/Th1 cells (Fig. 3E and F). In addition, the mean

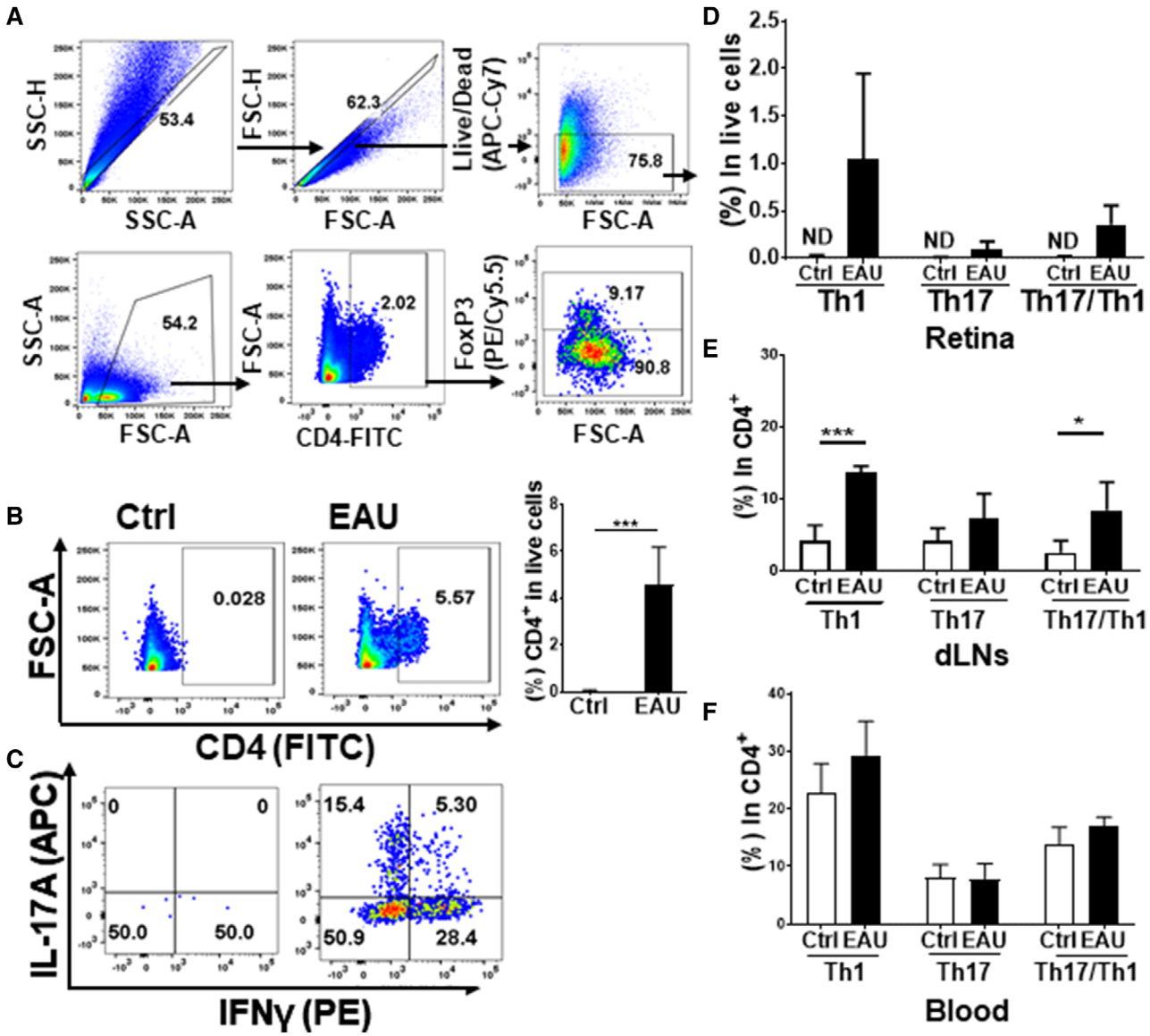

Figure 1. Increased levels of retinal $CD4^+IFN\text{-}\gamma^+IL\text{-}17A^+$ (Th17/Th1) cells and $CD4^+IFN\text{-}\gamma^-IL\text{-}17A^+$ (Th17) cells in EAU. (A) Demonstration of the gating strategy for the flow cytometric analysis of mouse $CD4^+$ T-cell subsets from retina, blood, and dLNs. In this example, a single cell suspension was prepared from the single retina of a peak EAU mouse and stained with fluorochrome-conjugated antibodies against CD4 (FITC), FoxP3 (PE/Cy5.5), IL-17A (APC), and IFN-γ (PE) based on surface and intracellular staining protocols. Doublets were excluded by stringent gating on SSC-H x SSC-A and FSC-H x FSC-A; live cells were selected with live/dead staining (APC-Cy7), and then lymphocytes identified by their scatter properties (FSC-A x SSC-A plot). (B) Retinal $CD4^+$ T cells were significantly upregulated in peak EAU mice (closed bars; $n = 5$), compared to controls (open bars; $n = 3$) and representative flow cytometry plots are shown. (C) Dot plots to identify $CD4^+$ T cells subsets using IL-17A and IFN-γ in the retinae (D), dLNs (E), and blood samples (F). All graphs represent mean scores ± SD for five mice per EAU group and three mice per control group from one experiment. These observations were confirmed in a total of three independent experiments using B10.RIII EAU. Mean ± SD. $^*p < 0.05$, $^{***}p < 0.001$, using Student's *t*-test.

fluorescence intensity (MFI) and the percentage of expression of RORγt and Tbet expression were unaffected by dexamethasone treatment (Fig. 3E and F).

EAU effector Th17/Th1 cells migrated in vitro

To determine whether Th17/Th1 cells are capable of migrating across endothelial cells of the blood-retinal barrier (Fig. 4A), we performed in vitro transendothelial migration assays. A mixed population of $CD4^+$ T cells activated in vitro by a pan T-cell activator (PHA) and IL-2 were cocultured with primary activated microvascular CNS endothelial cells. At the end of the assays, cells in each fraction (nonadherent (N), adherent (A), and migrated (M)) were counted for cell yield (Fig. 4B). A mean of 56.2 ± 9.0% live cells remained adhered to the endothelial monolayer, while 5.1 ± 0.4% cells had migrated through the endothelium (Fig. 4C). Th1 cells were detected at low levels (<20%) in all three fractions.

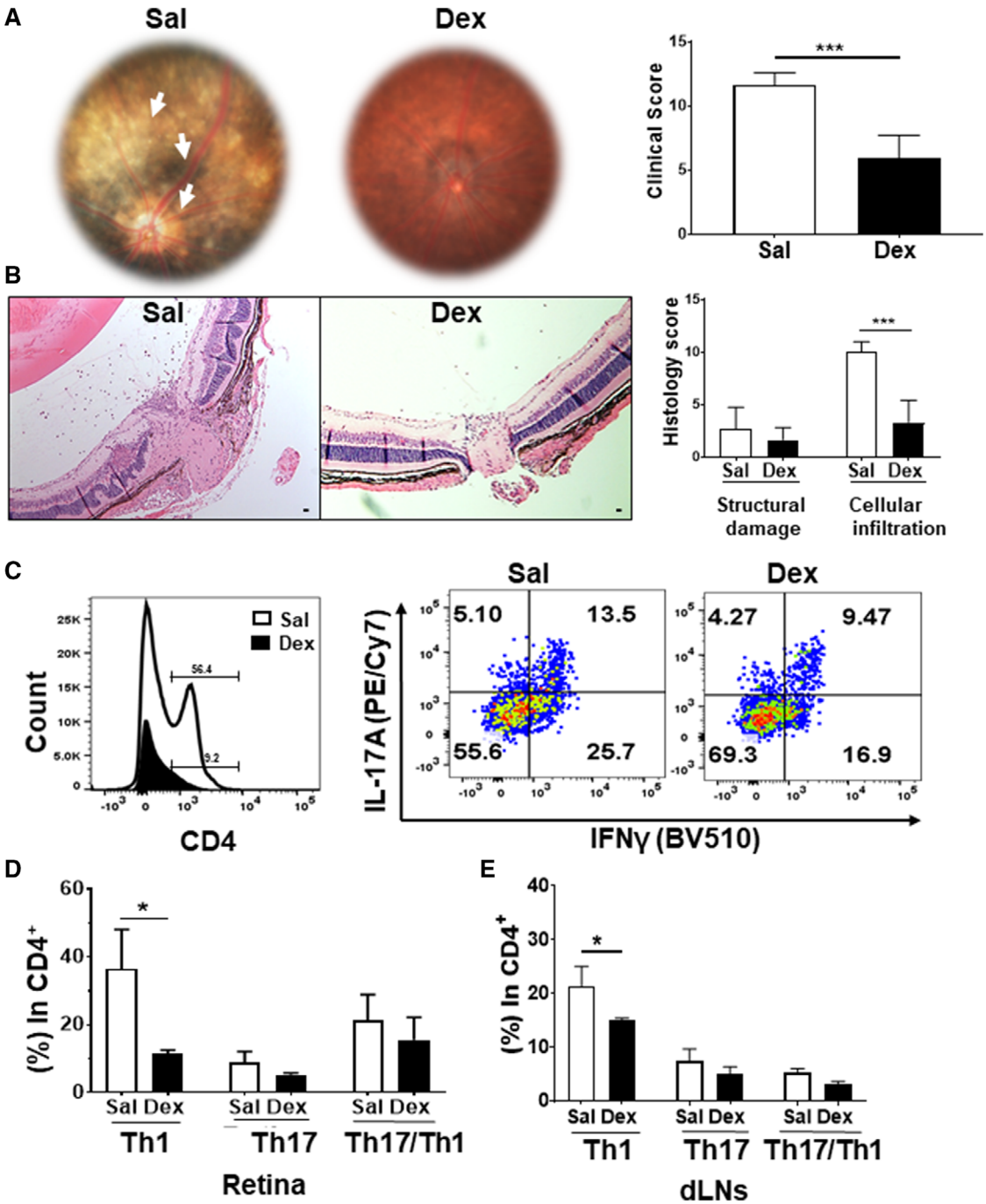

Figure 2. Dexamethasone treatment effectively prevents the progression of EAU, and mainly suppressed Th1 cells intraocularly. (A) Central 50° of retinal fundus images were captured by Micron III fundus camera (Phoenix Laboratories), which revealed retinal inflammation and vessel cuffing (arrows), reflecting the EAU clinical score at peak disease and following seven daily treatments with dexamethasone. (B) H&E staining of retinal sections of dexamethasone eyedrop treated (Dex) and control eyes (Sal). Histological staining illustrated cellular infiltrations, structural folding of the retina, and infiltrating leukocytes within the vitreous in Sal group and following Dex treatment. Scale bars represent 100 μm and 200× magnification. The average cellular infiltration score was 10.0 ± 0.6 and 3.3 ± 1.2 in Sal control and Dex group ($p = 0.0006$). The average structural damage score in Sal and Dex group was 2.7 ± 1.2 and 1.7 ± 0.7, respectively. (C) Intraocular flow cytometry depicting $CD4^+$ T cells in Sal and Dex groups from single live cell region. (D–E) Distribution of Th1, Th17, and Th17/Th1 cells in the retina (D) and draining lymph nodes (dLNs, E). All bar plots are expressed as mean ± SEM. (A–E) There were five mice per group. Data were shown as one representative from three independent experiments using C57Bl/6 EAU. $^*p < 0.05$, $^{***}p < 0.001$, using Student's *t*-test.

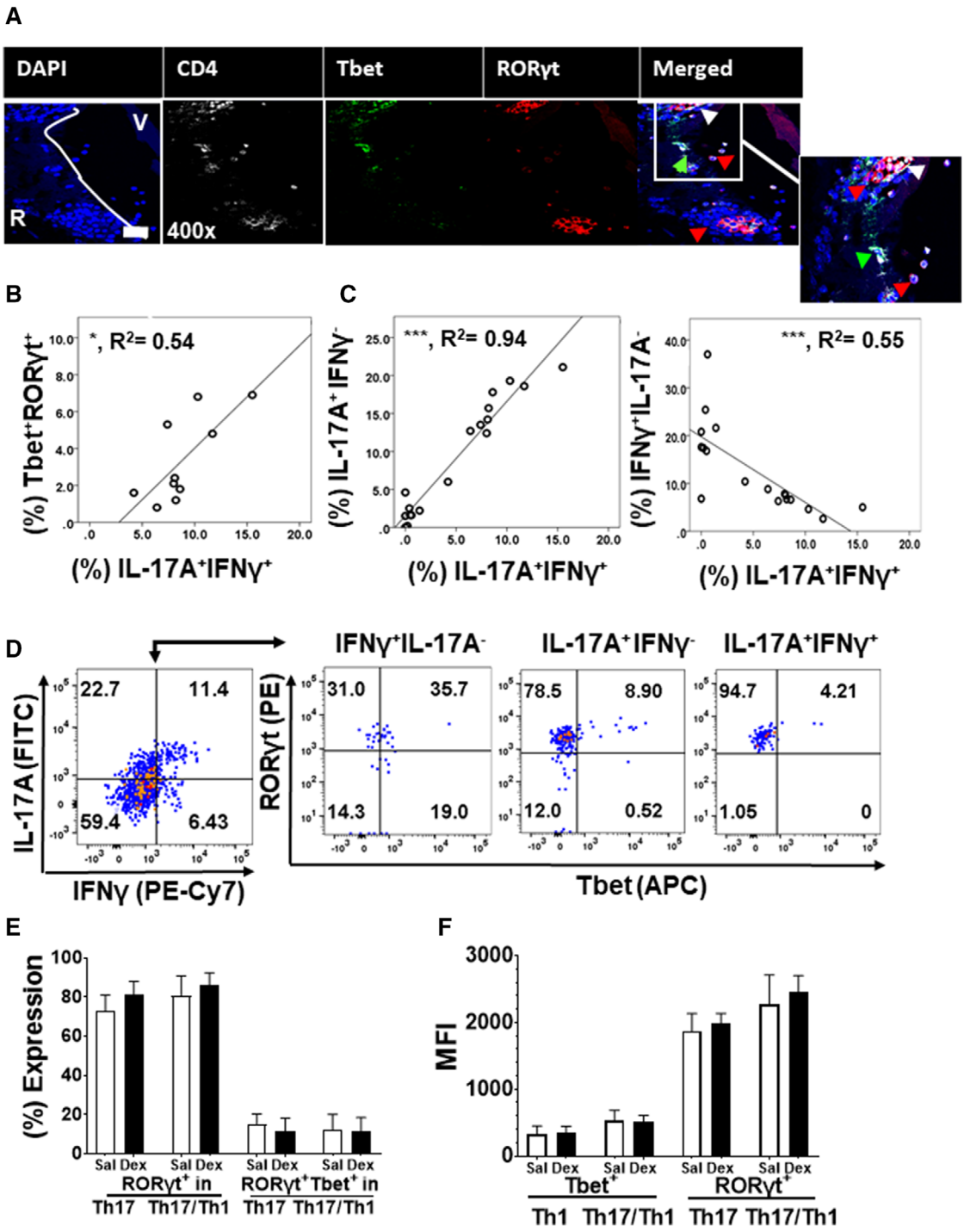

Figure 3. RORγt and Tbet expression in retinal Th17/Th1 cells in EAU. (A) Localization of T-cell subsets by four-color immunofluorescence staining targeting CD4 (gray), Tbet (green), and RORγt (red) within retinal sections during peak EAU. The histopathological changes in the Sal-treated group include accumulation of effector $CD4^+$ T cells (Th1 (green arrowhead), Th17 (red arrowhead), and nonclassical Th1 or Th17/Th1 (white arrowhead)) in the optic nerve head areas, as well as inflammatory cells migrating into the retina (R) and vitreous (V). Scale bar, 20 μm and 400× magnification. (B) Correlations of retinal T-cell subsets defined as expression of $ROR\gamma t^+Tbet^+$ with $IFN\text{-}\gamma^+IL\text{-}17A^+$ (Th17/Th1) cytokine expression. (C) Correlation between Th17/Th1 ($CD4^+IFN\text{-}\gamma^+IL\text{-}17A^+$) cells, Th1 ($CD4^+IFN\text{-}\gamma^+IL\text{-}17A^-$), and Th17 ($CD4^+IFN\text{-}\gamma^-IL\text{-}17A^+$) cells. (D) Representative flow cytometry figures to identify $ROR\gamma t^+$ and $Tbet^+$ in Th1, Th17, and $IFN\text{-}\gamma^+IL\text{-}17A^+$ (Th17/Th1) cells. (E) Expression of $ROR\gamma t^+$ and $Tbet^+$ in Th1 ($CD4^+IFN\text{-}\gamma^+IL\text{-}17A^-$), Th17 ($CD4^+IFN\text{-}\gamma^-IL\text{-}17A^+$), and Th17/Th1 cells measured by flow cytometry. (F) Median fluorescence intensity (MFI) of Tbet and RORγt in Th1, Th17, and Th17/Th1 cells (A). Data were from one individual experiment as described in Fig. 2, which comprised five mice per treatment group and was repeated for three times (B and C). Data were pooled from three individual experiments, with five mice per group. (E and F) Mean ± SD, from one experiment in three representatives with five mice per treatment group. Data in this figure were generated using C57Bl/6 EAU model. $^*p < 0.05$, $^{***}p < 0.001$, using Student's *t*-test. Correlations were calculated using Pearson's test.

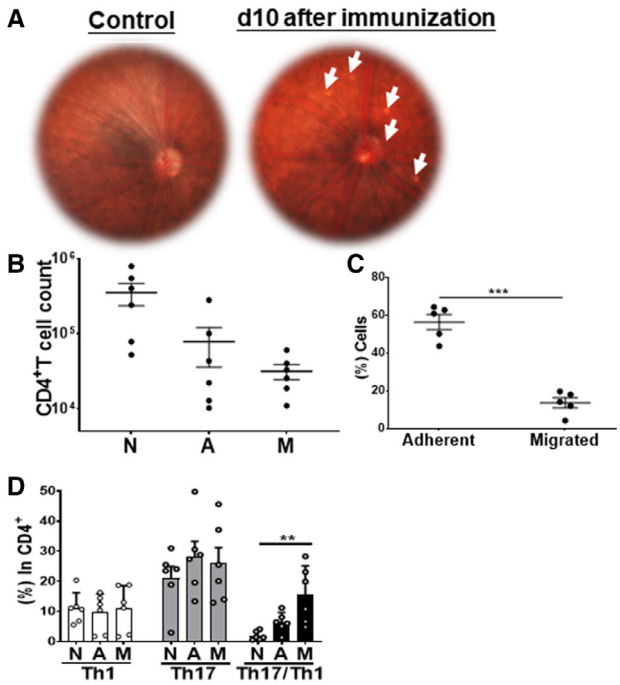

Figure 4. Adherence and migration of B10.RIII EAU $CD4^+$ T-cell subsets on CNS endothelium. (A) Central 50° of retinal fundus images was captured using Micron III fundus camera of early (d10) EAU mice, arrows indicating some vessel cuffing, retinal tissue and retinal nerve infiltration. (B) Cell counts of mouse lymphocytes on nonadherent (N), adherent (A), and migrated (M) populations from each single well were measured by counting beads using flow cytometry. (C) Percentages of $CD4^+$ T cells adhered and migrated measured by counting beads using flow cytometry. (D) Percentages of $CD4^+$ T cell subsets in nonadherent (N), adherent (A), and migrated (M) fractions analyzed by intracellular cytokine staining within $CD4^+FoxP3^-$ live cell region were calculated using counting beads by flow cytometry. Data are presented as means ± SEM from one representing three independent experiments, with six six replicate wells per group using stimulated LN cells originated from day 9 EAU mice ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.001$, using Student's *t*-test.

Th17 cells were the predominant subset and the percentages of Th17 cells in the nonadherent, adherent, and migrated fractions remained unchanged (Fig. 4D). In the Th17/Th1 subset, there was a significant increase in migrated as compared with nonmigrated (nonadherent) cells (15.6 ± 9.5 vs. 1.9 ± 1.5%; Fig. 4D). Prior to running the transmigration assays, the mixed T-cell populations were immunophenotyped (Supporting Information Fig. 1A). The migration assay was controlled by assays without activating the endothelial cells. In the absence of endothelium activation, the T-cell migration levels were minimal (data not shown).

Human Th17/Th1 cells adhered to CNS endothelium in vitro

In order to investigate the effects of dexamethasone on human Th17/Th1 cell function in vitro, migration assays using activated healthy human PBMCs were performed with activated human CNS endothelial monolayer. In the absence of endothelium activation,

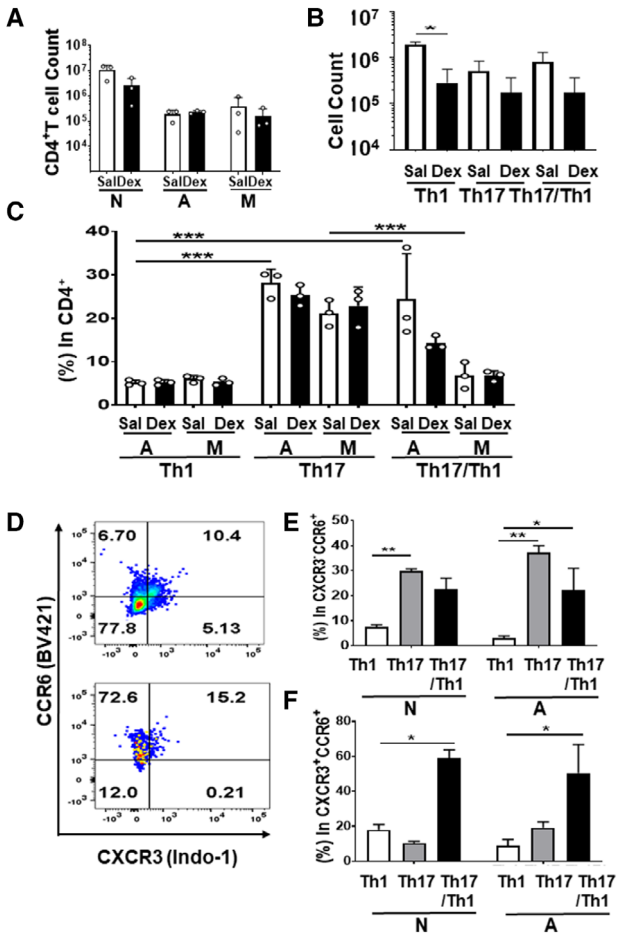

Figure 5. Migration assays with human $CD4^+$ T cell subsets. (A) $CD4^+$ T cell counts in nonadherent (N), adherent (A), and migrated (M) fractions in dexamethasone (Dex) treated as compared to saline controls (Sal) and analyzed by counting beads using flow cytometry. (B) Total cell counts of Th1, Th17, and Th17/Th1 in the nonadherent cell population were measured by intracellular cytokine staining within $CD4^+FoxP3^-$ live cell region and calculated using counting beads by flow cytometry. (C) Percentages of Th1, Th17, and Th17/Th1 cells within $CD4^+$ T cells in the adherent (A) and migrated (M) fractions comparing saline-treated EAU (Sal) with dexamethasone (Dex) were measured by intracellular cytokine staining within $CD4^+FoxP3^-$ live cell region and calculated using counting beads by flow cytometry. (D) Gating strategy to identify $CD4^+$ T-cell subsets on the basis of expression of CXCR3 and CCR6 in $CD4^+FoxP3^-$ live cells in nonadherent (N) and adherent (A) fractions, prior to identifying the intracellular cytokine profiles. (E) The expression of IFN-γ and IL-17A in $CD4^+$ T subsets characterized by CCR6 expression. (F) The expression of IFN-γ and IL-17A in $CD4^+$ T subsets characterized by CCR6 and CXCR3 co-expression. Data are presented as means ± SD from one experiment representing three independent experiments, with triplicate wells in each condition. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$, using Mann–Whitney *U* test.

the T-cell migration levels were minimal (data not shown). Addition of dexamethasone to the PBMCs had no significant effect on the total numbers of live $CD4^+$ T cells in the nonadherent, adherent, and migrated fractions (Fig. 5A). Similarly, total numbers of Th17 and Th17/Th1 cells in the nonadherent, adherent, and migrated fractions were unaffected by dexamethasone (data not shown) and only the Th1 cells in the nonadherent fraction were significantly decreased by dexamethasone (Fig. 5B). In the absence

of dexamethasone, the percentages of Th17 and Th17/Th1 cells were significantly increased relative to the Th1 population adhering to the monolayer (Fig. 5C). Within the migrated $CD4^+$ T cells, Th17 cells were significantly increased in comparison with Th1 and Th17/Th1. Prior to running the transmigration assays, the mixed T-cell populations were immunophenotyped (Supporting Information Fig. 1B).

The nonadherent, adherent, and migrated cell populations were characterized on the basis of their expression of CXCR3 and CCR6, within the live $CD4^+FoxP3^-$ cell population. In addition, intracellular cytokine expression levels were also investigated to determine if migratory function could be selected on the basis of chemokine receptor expression (Fig. 5D). In general, expression of intracellular cytokines by $CD4^+$ T cell subsets already characterized by chemokine receptors was distinct for each fraction (Fig. 5D–F). Within the $CXCR3^-CCR6^+$ cells, both Th17 and Th17/Th1 cells were significantly enriched in the nonadherent and adherent fractions. In contrast, within the $CXCR3^+CCR6^+$ cells, Th17/Th1 cells were significantly increased relative to the other $CD4^+$ T-cell subsets in both the nonadherent and adherent fractions. The findings indicate that healthy human Th17/Th1 cells were well characterized as dual cytokine-expressing cells, which maintained their chemokine receptor expression during migration. These findings confirm that CXCR3 and CCR6 chemokine receptors differentially define Th17 and Th17/Th1 cell subsets in line with their cytokine expression.

Discussion

Depending on the local microenvironment, effector $CD4^+$ T cells can transdifferentiate into functionally distinct subsets, and this process plays a key role in balancing the protective and pathogenic features of the T-cell response. Unlike the concept of "cell polarization," which was described for naïve T cells, $CD4^+$ T-cell plasticity has been extended to mature effector cells. Among $CD4^+$ T-cell subsets, Th17 cells are a crucial example of plasticity, since these cells play an important immunopathogenic role in many autoimmune and infectious diseases in different tissues [4]. In the EAE mouse model, it has been reported that the fate of Th17 cells is influenced by different proinflammatory cytokines including GM-CSF, IFN-γ, and IL-1 in vivo, which determine distinct patterns of plasticity [19]. In a previous study using a rat model of EAU, the natural course of effector T cells was investigated, and there were equal numbers of retinal Th1 and Th17 cells at disease onset and these remained at similar levels throughout in a monophasic model. Th17/Th1 cells also appeared at disease onset and increased twofold when disease resolved. However, in a chronic relapsing rat EAU model in the same study, the distribution of T-cell subsets was different. Th17 cells played a dominant role at disease onset and declined at later stages. There were limited Th1 cells when disease began and hence these cells increased. It was concluded that Th1 cells play an important role in disease relapse. Th17/Th1 cells remained at similar detection levels throughout. Furthermore, IL-10 expression was investigated in both Th1 and Th17 cells suggesting a regulatory role [22]. Our study also demonstrated that the significant increase of Th17/Th1 phenotype in peak EAU in a monophasic mouse model was only detected in retinal tissues and not dLNs or peripheral blood [23], and suggests an important role of the tissue microenvironment in $CD4^+$ T cell plasticity. Our detection of increased levels of intraocular Th17/Th1 cells in comparison to dLNs could be as a result of the local microenvironment within the eye, which contains a range of cytokines, notably TGF-ß [24]. However, we did not investigate IL-10 expression by the T-cell subsets.

It is debatable whether Th17/Th1 cells have differentiated from Th17 or Th1 cells or vice versa. In an experimental model of inflammatory bowel disease, it was reported that Tbet and, to a lesser extent, STAT4 expression by Th17 cells is required for their transition to Th17/Th1 cells [21]. Similarly, in EAE, Tbet, Runx1, and Runx3 transcription factors are required to initiate Th17/Th1 cell differentiation from Th17 cells. In a mouse model of dry eye disease, the Th17/Th1 cell population was assumed to be derived from Th17 precursors and exerted a pathogenic role in addition to Th17 cells [20]. However, new studies provide evidence for the Th1 cells' ability to transdifferentiate into Th17 cells, under the influence of TGF-β and IL-6, upregulating expression of Runx1, and increasing accessibility of Runx1 binding sites in the RORγt promoter as well as Runx1 and RORγt binding sites in the IL-17 promoter [11–13]. Hence, there is currently no clearly defined route for differentiation toward Th17/Th1 cells, or if this subset represents a stable $CD4^+$ T-cell subpopulation. In our EAU studies, we were unable to determine whether Th17/Th1 plasticity was occurring within the eye due to the very low levels of cells present and our study was limited to investigating Th17/Th1 cells only at peak stage of disease.

In EAU, Lyu et al. reported that a selective RORγt inhibitor (TMP778) substantially reduced Th1 and $CD4^+IL\text{-}17^+IFN\text{-}\gamma^+$ cells in the retinae and reduced clinical severity in a B10.A model of developing EAU [25]. Given these results, it is tempting to speculate that Th1 and Th17/Th1 subsets play an immunopathogenic role in EAU as suggested from our findings. Furthermore, by targeting RORγt, there was a suppression of IFN-γ-producing Th1 cells, suggesting either these cells were co-expressing RORγt (nonclassical Th1 cells) or they had trans-differentiated toward Th17/Th1 cells. Our group previously reported using an epigenetic target in EAU, in which it could also inhibit interphotoreceptor retinoid-binding protein (IRBP)-specific Th17/Th1 cells in vitro. The data suggested that blocking plasticity of effector T cells may be controlled by chromatin regulators [26]. In our current study, while dexamethasone effectively downregulated EAU severity scores via a Th1 cell reduction, the disease itself was not completely suppressed. We believe that this was due to the presence of Th17 and Th17/Th1 cells, which were still detected within the retina following dexamethasone treatment. These studies indirectly suggest a pathogenic role of Th17/Th1 cells in EAU. We found a negative correlation between Th1 and Th17/Th1 cells during peak EAU within the intraocular $CD4^+$ T-cell populations, supporting the hypothesis that the reduction in the proportion of Th1 cells intraocularly is due to their switching to Th17/Th1 cells.

However, there is limited evidence for nonclassical Th1 cells in mice. The functional differences between nonclassical Th1 cells and Th17/Th1 cells are poorly understood. It is believed that, following an IL-17/IFN-γ double-producing phase, Th17/Th1 cells lose their ability to produce IL-17 and become nonclassical Th1 cells expressing IFN-γ and IL-17 but only a minimal level of RORγt [11].

It was reported that in rheumatoid arthritis, human Th17/Th1 cells mediate their pathogenic roles by increasing their proliferative capacity in response to TCR signaling, cytokine polyfunctionality, and resistance to Treg suppression [27]. Human Th17/Th1 ($IL\text{-}17^+IFN\text{-}\gamma^+$) cells are distinguished from Th1 cells based on their expression of CD161, CCR6, IL-17 receptor E, *RORC*, and IL-4-induced gene 1 [7, 28, 29]. It has been demonstrated that nonclassical human Th1 cells can be distinguished from Th17/Th1 cells on the basis of their expression of CXCR4 [30]. Our in vitro data suggests that human Th17/Th1 cells can be defined by both CXCR3 and CCR6 expression, but few $CD4^+CXCR3^+CCR6^-$ or $CD4^+CCR6^+CXCR3^-$ cells also expressed both IFN-γ and IL-17, indicating a functional instability of this subset. The importance of nonclassical Th1 cells has added complexity to our understanding of the function of Th17/Th1 cells in man. For example, in the multiorgan disease sarcoidosis, which particularly affects the lungs and eyes, Kaiser et al. found that the presence of lung $CD4^+Tbet^+ROR\gamma t^+CXCR3^+CCR6^+$ T cells in sarcoidosis was associated with a favorable prognosis [31]. Our group previously compared PBMCs from healthy controls and from active or quiescent uveitis patients, all receiving systemic corticosteroids, and found that levels of peripheral blood $CD4^+Tbet^+ROR\gamma t^+$ T cells did not differ with disease status [32]. We were unable to investigate those cells within affected tissues, so it remains unclear as to their roles within the retinae. Altogether, these reports suggest that either nonclassical Th1 cells are not pathogenic or that the corticosteroids had no effect on nonclassical Th1 cells or Th17/Th1 cells.

Corticosteroids inhibit T cell migration either by binding to palindromic DNA elements or through cytoskeleton rearrangements and interactions with other transcription factors [33]. In EAU, Th1 cells are more sensitive to the suppressive effects of dexamethasone, whereas Th17 cells are steroid resistant [34]. Our finding that Th17/Th1 cells are also refractory to dexamethasone in the C57Bl/6 EAU is novel and was supported by a previous study, investigating systemic dexamethasone treatment in a B10.RIII mouse model of EAU at peak disease [34]. Steroid resistance may be due to human memory $CD4^+CCR6^+CXCR3^{hi}CCR4^{lo}CCR10^-CD161^+$ (Th17/Th1) cells, which expressed a stable level of multidrug transporter MDR1 (also known as P-glycoprotein [P-gp] and ABCB1), an ATP-dependent membrane efflux pump, which manifests as resistance to corticosteroid suppression in these cells [35].

The increase of Th17/Th1 cells in EAU retinal inflammation could be considered a selective target with biologics. A clinical trial targeting the IL-17A pathway alone (secukinumab) was ineffective in treating posterior uveitis, where Th1 and Th17/Th1 cells are also involved [36]. The anti-TNF-α mAbs etanercept and adalimumab have been shown to preferentially suppress Th17/Th1 cells and Th17 cell plasticity in vivo in juvenile idiopathic arthritis [37], and would explain the clinical benefits seen for anti-TNF therapy in posterior uveitis patients. In a future study, we would like to generate Th17/Th1 cells to assess for their ability to induce disease in adoptive transfer studies. However, we struggled to generate enough cells for adoptive transfer and obtaining a pure population is difficult. To our knowledge, there is no standard protocol for producing Th17/Th1 cells and there is no available methodology for this yet.

The development of Th17/Th1 and nonclassical Th1 cells is strictly controlled at the molecular level and sustained by epigenetic and transcriptional events. It seems that nonclassical Th1 cells are crucial for host protection, whereas Th17/Th1 cells are more pathogenic in uveitis. This was supported by the observed higher proportions of Th17/Th1 cells in dLNs and retina in diseased EAU mice, and that these cells also have migratory properties across retinal endothelium in vitro. Their resistance to dexamethasone leads us to conclude that targeting Th17/Th1 plasticity with biologics may provide a valuable tool for a potential future therapeutic strategy in posterior uveitis.

Material and methods

Animals

Female 6- to 8-week-old WT C57Bl/6 mice purchased from Charles River Laboratories, and B10.RIII mice, a colony maintained in-house, were used in this study. Animals were cared for under UK Home Office Regulations. All experiments were approved by the UCL Institute of Ophthalmology Animal Care and Use Committee and compliant with the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research.

EAU induction and scoring

EAU was induced in mice as previously described [38]. In brief, all B10.RIII mice were subcutaneously immunized with a mixture of 400 μg human $IRBP_{161\text{-}180}$ peptide emulsified in complete Freund's adjuvant (CFA; Sigma, Gillingham, UK; 1:1, v/v) with an additional 1.5 mg/mL heat-killed *Mycobacterium tuberculosis* (Difco Microbiology, Voigt Global Distribution, KS, USA). Each mouse also received 1.5 μg *Bordetella* pertussis toxin (Sigma, Gillingham, UK) intraperitoneally. For C57Bl/6 mice, $IRBP_{1\text{-}20}$ peptide was used. The development of clinical severity was scored by dilated-pupil fundus examination using Micron III retinal fundoscopy (Phoenix Research Laboratories, Pleasanton, CA, USA) and scored according to inflammatory changes to the optic disc and retinal vessels in addition to retinal lesions and structural damage using a well-defined grading system [39].

Drug treatment

To investigate the effect of dexamethasone on $CD4^+$ T cell subsets, mice were treated with 5 μL dexamethasone eyedrops (0.1% Maxidex, Novartis Pharmaceuticals, UK) (Dex), compared with controls, receiving 5 μL saline eyedrops (Sal), twice daily after disease development was confirmed, usually around day 10, and was given for 7 days, until peak stage of disease. For each experiment, at least five mice were included per treatment group.

Histology

Whole eyes from EAU mice were fixed with formalin, and embedded in paraffin (Sigma-Aldrich, UK). A series of five sequential 4 μm sections were collected with an autoslide preparation system. Anterior–posterior sections were stained with H&E and graded by two independent, masked observers [40]. Sections were also stained with rat anti-mouse CD45 monoclonal antibody (Serotec, Oxford, UK), counterstained with hematoxylin (ThermoShandon, Pittsburgh, PA), and scored for inflammatory infiltrates (presence of CD45-positive cells) and structural disease (disruption of morphology) using a well-defined grading system [41].

For immunohistochemistry staining, the sections were blocked with 4% goat serum in PBS for 1 h at room temperature, and then incubated with primary rat antibody against mouse CD4, rabbit antibody against mouse Tbet, and goat antibody against mouse RORγt (all Santa Cruz Biotechnology) at 4°C overnight. After three washes with PBS/Tween 20, sections were incubated with secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit, Alexa Fluor 555-conjugated anti-rat, Alexa Fluor 633-conjugated anti-goat; Eugene, Oregon, USA) for 1 h at room temperature and counterstained with DAPI (Thermo Fisher Scientific). Images were acquired with a confocal microscope (LSM700; Zeiss).

Immunophenotyping by flow cytometry

Both human and murine-cultured or single cell suspensions collected from retina, blood, or dLNs were stained for flow cytometric analysis [42]. This study adhered to the guidelines on the use of flow cytometry in immunological studies [43]. Blood samples were collected with ethylene diamine tetraacetic acid (EDTA) anticoagulant tubes. The activation strategy was performed as follows: cells were cultured with 50 ng/mL phorbol myristate acetate (PMA) and 1 μg/mL ionomycin (Sigma-Aldrich, USA) in complete RPMI 1640 (ThermoFisher) supplemented with 10% FCS for 4 h at 37°C. Brefeldin A (5 μg/mL) was added for the last 1 h. Dead cells were excluded via staining with Live/dead fixable dead cell Stain (Molecular Probes; Life Technology, Paisley, UK) prior to surface staining or fixation (ThermoFisher). Surface markers were stained for 20 min at +4°C, with CD4 (RM4-5 mouse) and OKT4 (human; eBioscience or BD Bioscience). For staining of transcription factors, cells were first fixed for 1 h at 4°C with Foxp3 fixation/permeabilization working solution according to the manufacturer's instructions (eBioscience), washed with 1× permeabilization buffer (eBioscience), and stained for 45 min at RT with anti-Foxp3 (FJK-16s in mice studies and 236A/E7 in human studies). Cells were then fixed in Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA, USA) for 10 min at RT, washed with 1× permeabilization buffer (BD Biosciences), and intracellularly stained for 45 min at RT with anti-IFN-γ (XMG1.2 in mice studies, and 4S.B3 in human studies) and anti-IL-17A (TC11-18H10.1 in mice studies, and eBio64DEC17 in human studies). In human assays, CCR6 (G034E3) and CXCR3 (1C6/CXCR3) were used in addition. In some mice experiments, RORγt (AFKJS-9) and Tbet (eBio4B10) were also used. Samples were acquired with a Fortessa flow cytometer (BD Biosciences). Single-stained One Comp beads (eBioscience) were used to generate compensation matrices. Isotype and FMO controls were used to identify gating boundaries. Data were analyzed using FlowJo version 10 (TreeStar Inc., Ashland, OR, USA).

Cells for migration assays

Murine $CD4^+$ T cells were isolated from dLNs 10 days postimmunization from B10.RIII mice. Human PBMC (All Cells; Caltag Medsystems, Buckingham, UK) were cultured at 1×10^6/mL in culture medium (X-VIVO 20; Lonza, Cambridge, UK), supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. Lymphocytes were stimulated with phytohaemagglutinin (PHA; 3 μg/mL) and IL-2 (50 U/mL) weekly for up to three cycles prior to use. Note that 1 μM dexamethasone was added 1 day prior to assay. In human cell assays, the hCMEC/D3 cell line (a gift from P. Turowski, UCL, UK) was seeded at a density of 3×10^5 cells/transwell and cultured in EGM2-MV medium (Lonza, Slough, UK) 3–4 days prior to assay. In mouse studies, primary mouse brain endothelial cells were harvested from 10-day old naïve B10.RIII mice according to previously published methods and cultured in EGM2-MV medium (Lonza, Slough, UK) [44].

Migration assay

Transwells composed of 6.5-mm collagen-coated polytetrafluoroethylene (PTFE) membrane with 3.0 μm pore size insert (Corning, Sigma-Aldrich, UK) were positioned in a 24-well plate. EC monolayers were activated with human- or mouse-specific IFN-γ (100 IU/mL) 72 h prior to the migration assay as a published method [45]. To assay migration, 1×10^6 T cells in 100 μL fresh medium were placed into each triplicate insert. The lower wells of a 24-well plate were filled with 500 μL medium alone. Transwells were incubated for 18 h at 37°C and 5% CO_2, after which time, nonadherent (N), adherent (A), and migrated (M) T cells were recovered from each chamber and immunophenotyped for flow cytometry. Collection of cells involved careful aspiration of all medium from each chamber. The upper chamber cells were

removed and defined as nonadherent cells. The lower side of the transwell membrane was washed to dislodge those lymphocytes that had migrated through the endothelium, but not reached the lower chamber, and these were added to those collected directly from the lower chamber (M). Enzymatic digestion of the endothelium was performed to assess the remaining lymphocytes adhered to the endothelium. AccuCheck counting beads (Thermo Fisher Scientific) were included to determine cell counts.

For all assays, the integrity of the endothelial monolayers was routinely assessed by visual inspection and transendothelial electrical resistance measurements [46].

Statistical evaluation

Each experiment was repeated at least three times. Comparison of means in parametric data was analyzed by Student's *t*-tests. Nonparametric data were analyzed by the Mann–Whitney *U* test. Pearson's correlation coefficient was used for comparing two continuous variables. Values were considered statistically significant when $p < 0.05$.

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Abbreviations: **dLNs**: draining lymph nodes · **EAU**: experimental autoimmune uveitis · **IRBP**: Interphotoreceptor retinoid-binding protein

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