Small-Molecule Antagonist of VLA-4 (GW559090) Attenuated Neuro-Inflammation by Targeting Th17 Cell Trafficking across the Blood- Retinal Barrier in Experimental Autoimmune Uveitis

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Research

Keywords: Integrin (α4β1, VLA-4), uveitis, experimental autoimmune uveitis, leukocyte migration, Th17 cells, inflammatory monocytes/macrophages

DOI: https://doi.org/10.21203/rs.3.rs-65522/v1

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Abstract

Background: The integrin VLA-4 (α4β1) plays an important role in leukocyte trafficking. This study investigated the efficacy of a novel topical α4β1 integrin inhibitor (GW559090, GW) in a mouse model for non-infectious posterior uveitis (experimental autoimmune uveitis; EAU) and its effect on intraocular leukocyte subsets.

Methods: Mice (female; B10.RIII or C57Bl/6; aged 6-8 weeks) were immunized with specific interphotoreceptor retinoid-binding protein (IRBP) peptides to induce EAU. Topically administered GW (3, 10, and 30 mg/ml) were given twice daily either therapeutically once disease was evident, or prophylactically, and compared with vehicle-treated (Veh) and 0.1% dexamethasone-treated (Dex) controls. Mice were sacrificed at peak disease. The retinal T cell subsets were investigated by immunohistochemistry and immunofluorescence staining. The immune cells within the retina, blood and draining lymph nodes (dLNs) were phenotyped by flow cytometry. The effect of GW559090 on non-adherent, adherent and migrated CD4+ T cell subsets across a central nervous system (CNS) endothelium was further assayed in vitro and quantitated by flow cytometry.

Results: There was a significant reduction in clinical and histological scores in GW10 and Dex treated groups as compared to controls either administered therapeutically or prophylactically. There were fewer CD45+ leukocytes infiltrating the retinae and vitreous fluids in the treated GW10 group (P < 0.05). Immunofluorescence staining and flow cytometry data identified decreased levels of retinal Th17 cells (P ≤ 0.001) in the GW10 treated eyes, leaving systemic T cell subsets unaffected. In addition, fewer Ly6C+ inflammatory monocyte/macrophages (P = 0.002) and dendritic cells (P = 0.017) crossed the BRB following GW10 treatment. In vitro migration assays confirmed that Th17 cells were selectively suppressed by GW559090 by adhering to endothelial monolayers.

Conclusions: This α4β1 integrin inhibitor may exert a modulatory effect in EAU progression by selectively blocking Th17 cell migration across the blood-retinal barrier without affecting systemic CD4+ T cell subsets. Local α4β1 integrin-directed inhibition could be clinically relevant in treating a Th17-dominant form of uveitis.

Background

Non-infectious posterior uveitis is an inflammatory disease targeting the uveal tract in the eye by leukocytes that infiltrate the retinal tissues. It has the potential to threaten vision and accounts for 10–15% of patients who are blind, especially affecting people of working age [1, 2]. It is considered to be an immune-mediated disorder based on clinical treatment responses to corticosteroids and immunosuppressants, the ability of adoptively transferred retinal antigen-specific CD4+ T cells to induce an experimental model of autoimmune uveitis (EAU), and histology obtained from postmortem uveitis eyes [3, 4]. The process is accompanied by evidence of blood-retinal-barrier (BRB) breakdown [5]; leukocytes infiltrating the anterior and/or posterior chamber and vitreous; immigration of retinal CD4+ T
cells, mainly Th1 (secreting IFNγ and expressing Tbet) and Th17 (secreting IL-17A and expressing RORγt) [6]; structural damage mediated via cytokines released by infiltrating leukocytes, including activated macrophages and neutrophils [7].

Recruitment of effector CD4+ T cells to an immune privileged site involves co-ordination of adhesion molecules expressed by circulating leukocytes and by endothelial cells within the blood vessels [8, 9]. The specific cell migration process is largely mediated by leukocyte functional antigen-1 (LFA-1) / intercellular adhesion molecule-1 (ICAM-1) and very late activation antigen-4 (VLA-4) / vascular cell adhesion molecule-1 (VCAM-1) interactions [10, 11]. There is evidence of increasing expression of ICAM-1 and VCAM-1 in studies of human uveitis retinal specimens and from EAU retinal tissues [5, 9] and enhanced VCAM-1 expression specifically in focal areas of the retinal postcapillary venule where inflammation initiates and when EAU is evident [5, 12]. However, in vitro studies suggested that lymphocyte migration is more dependent on LFA-1/ICAM-1 than VLA-4/VCAM-1 interactions [13, 14]. The discrepancy may be due to the fact that the VLA-4/VCAM-1 axis participated in migration only under inflamed conditions [13]. Indeed, targeting VLA-4 or LFA-1 molecules systemically has previously been shown to barricade lymphocytes from crossing the BRB and ameliorate the clinical signs of EAU, indicating the importance of the VLA-4/VCAM-1 pathway in inflamed retinae [15–20]. Whether there is a preferential use of specific integrins by Th1 cells versus Th17 cells has not been addressed within the uveitogenic CD4+ T cell subsets.

The side effects of systemic administered integrin inhibitors has been reported with long term use, for example, infection and progressive multifocal leukoencephalopathy in Natalizumab (humanized α4 antibody) treating multiple sclerosis patients [21]. Recently, a topical α4β1 (VLA-4, CD49d/CD29) integrin inhibitor (GW559090) was shown to be clinically efficacious in a mouse model of dry eye disease [22–24]. It has been reported to exert its clinical effect through its high affinity binding to the α4β1/VLA-4 integrin and specific blockade of cell interaction to VCAM-1 [22]. In this study, we investigated the effect of topically applied GW559090 in EAU, and the potential cellular mechanisms involved.

**Material And Methods**

**EAU induction and treatment**

Female B10.RIII mice, aged 6 to 8 weeks, were obtained from an in-house colony and female C57BL/6 mice were purchased from Charles River Laboratories (n = 5–8 per experimental group). All studies were conducted according to the UK Home Office Regulations on the Care, Welfare and Treatment of Laboratory Animals and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Visual Research.

EAU was induced by immunization of naive mice with subcutaneous 400 μg human IRBP peptides (IRBP161-180 for B10.RIII mice; IRBP1-20 for C57BL/6 mice) in complete Freund’s adjuvant (CFA; Sigma, Gillingham, UK; 1:1 vol/vol) supplemented with 1.5 mg/ml Mycobacterium tuberculosis H37 Ra (Difco Microbiology, Voigt Global Distribution, Lawrence, KS, USA). All mice simultaneously received 0.4 μg
*Bordetella* pertussis toxin (Sigma-Aldrich, UK) intraperitoneally. Mice receiving CFA in PBS alone were used as controls.

Eyedrops (5µL) containing α4β1 integrin antagonist GW559090 ((S)-3-(4-((4-carbamoylpiperidine-1-carbonyl)oxy)phenyl)-2-((S)-4-methyl-2-(2-(otolyloxy)acetamido)pentanamido) propanoic acid, kindly provided by GSK) solubilized in phosphate buffered saline (PBS, pH = 7) were given twice daily in both eyes either prophylactically (from days 0 to 14 post EAU induction) or therapeutically (from days 10 to 18 post EAU induction). The concentrations of GW559090 were selected based on previously published data [22, 23]. GW559090 was solubilized in PBS and used at a final concentration of 3 mg/ml (GW₃), 10 mg/ml (GW₁₀) or 30 mg/ml (GW₃₀) and compared to vehicle treated controls (Veh) or 0.1% dexamethasone eye drops (Maxidex®, Alcon, UK; Dex) as treatment controls. In some experiments, 10 mg/ml GW559090 was applied to the right eye only (GW₁₀R) to evaluate whether it had a sympathetic effect on the untreated left eye (GW₁₀L). Mice were sacrificed for further investigation when those in Veh group reached peak disease (day 14 in B10.RIII mice, day 18–21 for C57BL/6 mice).

**Disease Grading**

Dilated ocular examination was performed using retinal funduscopy (Micron III; Phoenix Research Laboratories, Pleasanton, CA, USA) on days 10, 14 and/or day 18–21 after disease induction. Pupils were dilated with topical 2.5% phenylephrine and 1% tropicamide, and the corneas protected with 0.2% carbomer eye gel (Bausch & Lomb Ltd, UK). The clinical grading was performed by two independent experts [25]. In summary, optic disc neuropathy, vasculitis, retinitis, and structural damage were scored separately in each eye, from 0 (no disease) to 5 (severe inflammation) with half-point increments and then scores were summed to generate the clinical score of each eye on a scale from 0–20. The clinical score attributed to each mouse corresponds to the mean of the scores from both eyes.

**Immunohistochemistry Staining For Cd45 Cells**

Eyes were collected and fixed *in situ* with 4% glutaraldehyde for 1 hour, followed by overnight fixation in 10% formalin, and embedded in paraffin (Sigma Aldrich, UK). A series of four anterior-posterior sections passing through the optic nerve with 4 µm thick sections were prepared and stained with hematoxylin and eosin (H&E). The results were graded by two independent, masked observers [26].

For immunohistochemistry staining, the sections were blocked with 5% goat serum in PBS for 1 hour at room temperature, and then incubated with primary rabbit polyclonal antibody to CD45 (1:200; ab10558; Abcam, Oxford, UK) at 4°C overnight. After washing, the sections were incubated with biotinylated anti-rabbit IgG Ab (Vectastain Elite ABC HRP Kit, Abcam, UK) for 1 hour followed by visualization with a brick-red indicator product (NovaRED™, Vector Laboratories, UK). Stained sections were scored [27, 28]. In brief, cellular infiltrates (0–30) were scored within the ciliary body, vitreous, vessels, rod outer segments, and choroid, whereas the structural score (0–12) was based on the rod outer segments, neuronal layers, and
retinal morphology. Both scores were added for a final score for each eye, and mean scores from both eyes calculated for each mouse (0–42).

**Immunofluorescence Staining Locating Immune Cells Within The Eye**

Ocular anterior-posterior sections were blocked with 5% bovine serum albumin (BSA) in TBS for 2.5 hours, then incubated overnight at 4°C with the following primary antibodies in different combinations: CD4 (rat; 1:100; Santa Cruz Biotechnology, USA), Tbet (rabbit; 1:100; Santa Cruz Biotechnology, USA), RORγt (goat; 1:100; Santa Cruz Biotechnology, USA), FoxP3 (rabbit; 1:100; Abcam, USA), and Iba-1 (myeloid cell marker; mouse; 1:100; Santa Cruz Biotechnology), diluted in TBS supplemented with 1% BSA. After three washes in TBS, sections were incubated in the dark for 2 hours with species-specific secondary antibodies coupled to different fluorochromes as indicated in the data: Alexa Fluor 488, 555, and 633-conjugated secondary antibody (1:200; Molecular Probes, Eugene, OR, USA). After three final washings in PBS (20 min each), sections were mounted with Vectashield antifade mounting medium containing DAPI (Thermo Fisher Scientific, UK).

Images from at least three separate fields per retinal section (X 400) were captured using a Zeiss LSM 700 (or 710) confocal microscope. Staining patterns were observed to be consistent between consecutive single-, double-, or triple-stained sections using ZEN 2.1 software (Carl Zeiss, Oberkochen, Germany). ImageJ software (http://imagej.nih.gov/ij/) was used for counting cells in the images. These images were used for counting populations of CD4+Tbet+, CD4+RORγt+, CD4+Tbet+RORγt+, CD4+Foxp3+ and Iba-1+ cells in eye tissue. For cell scoring within the anterior chamber, total cells from three non-overlapping images were counted; for scoring cells in inner retina and vitreous, cell counts were from 5 images to cover the whole eye. Cells were verified with DAPI and counted within the anterior chamber, ciliary body, vitreous, vessels, retinal layers and choroid, and were summed up for a final score. The mean percentages of CD4+ T cell subsets in different experimental groups were calculated.

**Identification of T cell subsets and myeloid populations by flow cytometry**

Single-cell suspensions were prepared from retinal tissues, dLNs and blood and investigated by flow cytometric staining for their expression of CD4-FITC (RM4-5) and CD11b-PE (M1/70) (myeloid cell marker; eBioscience); CD11c-BV786 (HL3), Ly6G-PE-CF594 (IA8), CD45-BV605 (30-F11), FoxP3-PE/Cy5 (FJK-16 s), Ly6C-PerCP/Cy5.5 (HK 1.4), IFNy-PE-Cy7 (XMG1.2), IL-17A-APC (TC11-18H10.1) and CD64-BV421 (X54-5/7.1; all BD Biolegend). In experiments investigating the myeloid population, data were obtained from 2–3 eyes pooled from the same treatment group in order to have sufficient cell numbers for analysis. For intracellular cytokine staining, cultures were treated for 4 hours with PMA (50 ng/mL) and ionomycin (1 µg/mL), and additional brefeldin A (1 µg/mL; all Sigma-Aldrich) for final 1 hour. Cells were incubated with relevant surface antibodies and a Live/Dead fixable dye (Molecular Probes; Life Technology, Paisley, U.K.) for 30 min at 4°C, before resuspending in FACS buffer and fixed. Cells were then
permeabilized using fixation/perm buffer (Fix and Perm cell permeabilization kit; eBioscience, La Jolla, CA, USA) for 10 min. Transcription factor expression was determined using the Foxp3 cell Staining Kit (BD Biosciences), according to the manufacturer’s instructions. Relevant isotype control mAbs (BD Biosciences and eBioscience) were combined as fluorescence minus one (FMO) controls. Up to one million live cells per sample were acquired on a BD FACSCalibur using CellQuest (BD Cytometry Systems, Oxford, UK) or a BD LSR Fortessa-x20 using FACSDiva (BD Cytometry Systems). Isotypes and FMO controls were used for accurate gating. Compensation matrices were performed using OneComp Beads (eBioscience, UK). Flow cytometric data were analyzed using FlowJo software (Version 9.6.4; Tree Star). Debris and doublets were excluded, and live cells were gated before further analysis.

**In vitro leukocyte migration assays**

Human microvascular endothelial cells (HMEC; a gift from Professor P. Turowski UCL Institute of Ophthalmology, London, UK) were cultured with EGM2-MV (Endothelial cell growth medium-2 bullet kit, Lonza, USA) with supplements and the culture medium replaced weekly. Transmigration of lymphocytes was assayed using 6.5 mm Transwell inserts with a 3 µm pore size in a 24 well plate (Corning, Sigma-Aldrich, UK). HMEC cells were seeded on fibronectin-coated filters at a density of 3 × 10^5 cells and stimulated with human IFNγ (200 IU/ml, PeproTech, UK) 3 days before the assay [29].

Venous blood was obtained from healthy volunteers and collected in EDTA (3 mmol/L). Human buffy coat leukocytes were isolated by density gradient centrifugation by layering over Histopaque (Sigma Aldrich, UK) and centrifugation at 300 g to obtain mononuclear leukocytes. Mononuclear lymphocytes were cultured at 1 × 10^6/ml in T cell medium (RPMI 1640 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 (all Sigma Aldrich). Lymphocytes were stimulated weekly with phytohaemagglutinin (PHA; 3 µg/ml) and human rIL-2 (50 IU/ml; PeproTech, UK) added every 3 days for a week before the assay [30], and lymphocytes were re-stimulated with PHA 24 hours before the assay. This methodology has proven to allow to keep the effector T cells in culture for a maximum of 4 weeks, when their viability is reduced [31, 32]. Cells were pre-treated with 10, 100, 1000 µg/ml GW559090 or Saline (Sal) as controls for 2 hours prior to assay.

To assess migration, 2 × 10^6 lymphocytes were pelleted by centrifugation, re-suspended in 100 µl fresh T cell medium and placed into each Transwell insert. The lower wells were filled with 500 µl of T cell medium. Following incubation for 18 hours at 37°C, not adherent, adherent and migrated lymphocytes were carefully harvested from each compartment and stained for CD4-FITC (OKT4), RORγt-APC (AFKJS-9), Tbet-PE (eBio4B10) (all from eBioscience), and FoxP3-BV711 (236AIE7; BD Biosciences) and acquired for flow cytometry, as described above. In some experiments, additional markers for CXCR3-BUV395 (1C6/CXCR3), CCR6-BV421 (G034E3), IFNγ-BV605 (4S.B3; BD Biosciences) and IL-17A -PE (eBio64DEC17; eBioscience) were used. BD Liquid Counting Beads (BD Biosciences) was used to evaluate the cell numbers within each chamber, according to the manufacturer’s protocol.
Statistics

Results are presented as the mean ± SEM for at least 5 data points and mean ± SD for 3 data points. For comparing means from two groups, \( P \) values were calculated by the unpaired two-tailed Student \( t \)-test. For comparison of means from three or more groups for two factors, two-way ANOVA was used to reveal differences in the data sets, followed by Dunn’s or Tukey’s multiple comparison post hoc test. Statistical analyses were performed using GraphPad Prism 6 software. \( P \) values < 0.05 were considered statistically significant.

Results

Topical VLA-4 inhibitor prevented retinal inflammation

The GW559090-treated B10.RIII EAU mice demonstrated a reduction of retinal inflammatory infiltrates as compared to vehicle-treated (Veh) controls at peak disease. Retinae from Veh group became clinically inflamed and showed moderate to severe signs of EAU in histopathologic examination by H&E staining on day 14. For example, retinal structural damage, retinal vasculitis, optic neuropathy and diffuse infiltration of cells into the vitreous, uvea and retina (Fig. 1a, b). In contrast, mice treated with GW559090 topically from day 0 to 14, showed minimal disease development in the GW\(_{10}\) group (\( P = 0.005 \); Fig. 1d, e). The reduction of EAU severity was significant in GW\(_{10}\) group compared to Veh group, but the effect was not seen in GW\(_{3}\) and GW\(_{30}\) groups. In addition, there was a significant decrease in disease incidence in GW\(_{10}\) group (\( P < 0.05 \); Fig. 1c). In mice treated in just the right eye, disease was suppressed in the treated eye (GW\(_{10\text{R}}\)), but EAU continued to develop in the contralateral eye (GW\(_{10\text{L}}\); Fig. 1f).

Topical Vla-4 Inhibitor Given Therapeutically Arrested Disease Progression

EAU in C57BL/6 mice was induced as described above, and Veh groups were compared with GW\(_{10}\), GW\(_{30}\), and Dex treatment. All mice were assessed for their development of EAU at day 10 following disease induction and were confirmed to have a similar disease severity (2.9 ± 0.1). After treating the mice for 7 days, there was a decrease in disease progression in the GW\(_{10}\) group (3.5 ± 0.5, \( P < 0.0001 \)) and the Dex group (4.4 ± 0.4, \( P < 0.0001 \)) as compared to Veh group (7.6 ± 0.4; Fig. 1g, h). A decrease in disease scores involving optic disc infiltrates, subretinal infiltrates, and peri-vascular infiltrates was observed in GW\(_{10}\) group, and there was a reduced clinical severity comparable to the Dex treated group (Fig. 1h). The results were further confirmed by H&E staining (data not shown).

Cellular infiltration into the retina was preferentially blocked by GW559090

By staining retinal sections with CD45, the infiltrating leukocytes were able to be analyzed quantitatively by their location. In EAU controls, leukocytes were localized in the anterior chamber, optic nerve, vitreous
cavity and retinal layers (Fig. 2a-c). There were fewer leukocytes around the optic nerve head, in the vitreous, anterior chamber and inner retina following GW<sub>10</sub> treatment (Fig. 2d-f) and levels were significantly decreased relative to Veh controls (Fig. 2g-i). There was an overall decrease in cell numbers within the anterior chamber and inner retinal layer in all treated groups (Fig. 2h, i). In summary, a significantly reduced cellular infiltration score was observed in both GW<sub>10</sub> and GW<sub>30</sub> groups (Fig. 2j), although some retinal folding remained (Fig. 2f), and the structural damage scores in all groups were relatively low and did not differ (data not shown). Despite cellular infiltration scores being reduced in the GW<sub>30</sub> group (Fig. 2j), the cumulative disease scores revealed a significant disease reduction only in the GW<sub>10</sub> group (data not shown).

**Topical VLA-4 inhibitor targeted retinal Th17 cell infiltration in vivo**

There was a mixture of Th1 (CD4<sup>+</sup>Tbet<sup>+</sup>) and Th17 (CD4<sup>+</sup>RORγt<sup>+</sup>) cells, with Th17 cells being the predominant subset in EAU retinae (Veh group, Fig. 3a). These cells were mostly localized to the inner retinal layer and vitreous (Fig. 3a). In contrast, in the GW<sub>10</sub> treated group, there were fewer cells detected in the posterior chamber of the eye, and the dominant CD4<sup>+</sup> T cell subset was Th1. Most cells were located within the retina and there were fewer infiltrating cells observed in the vitreous cavity (Fig. 3b). There were minimal levels of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cell (Tregs) in the vitreous and posterior chamber in Veh control eyes and some Treg cells were detected in the anterior chamber, vitreous, and inner retina in the GW<sub>10</sub> eyes (data not shown). By calculating all of the CD4<sup>+</sup> T cell subsets within vitreous by immunofluorescence staining of sections, Th17 cells were mostly suppressed by treatment with GW<sub>10</sub>, whilst the proportion of Th1 cells was unaffected (Fig. 3c).

The effects of GW559090 treatment on different T cell subsets were further validated by single cell analysis in the retinae, blood and dLNIs by flow cytometry, investigating cytokine expression by Th1 (CD4<sup>+</sup>IFNγ<sup>+</sup>), Th17 (CD4<sup>+</sup>IL-17A<sup>+</sup>) in CD4<sup>+</sup>FoxP3<sup>−</sup> live cell region (Fig. 3d and supplementary Fig. 1a). The results confirmed the observations from immunofluorescence staining, that Th17 cells were predominant in Veh eyes (Fig. 3e). Within the CD4<sup>+</sup> T cell subsets, there was a decrease of Th17 cells in the GW<sub>10</sub> treated group (7.6 ± 1.7%, \( P < 0.001 \)) and the GW<sub>30</sub> treated group (5.9 ± 1.6%, \( P < 0.001 \)) and there were increased levels of Th1 cells in both the GW<sub>10</sub> treated group (21.0 ± 4.8%, \( P = 0.018 \)) and the GW<sub>30</sub> group (22.7 ± 3.1%, \( P = 0.004 \)) inside the eye as compared to Veh group (Fig. 3e). There were no significant changes in percentages of Treg cells following topical application of GW559090 in the eye (data not shown). In the draining LNs, a significantly reduced level of Th17 cells in GW<sub>10</sub> and Dex groups compared to Veh group was identified, but not for other CD4<sup>+</sup> T cell subsets (Fig. 3f-g). In addition, there was no significant change regarding Th1 and Th17 cells in the blood from mice treated topically with GW559090 (data now shown). There was an increase of Tregs in GW<sub>10</sub>, GW<sub>30</sub>, and Dex group in the blood compared to control but this effect was not found in the retinal cells (data not shown).

**Increased VLA-4 expression on all retinal CD4<sup>+</sup> T cell subsets in EAU**
VLA-4 expression and the mean fluorescence intensity were increased in all \( \text{CD}4^+ \) T cell subsets in EAU than controls in retinae and dLNs (Fig. 3h-k and supplementary Fig. 2) but not in blood (data not shown). The mean fluorescence intensity of cells expressing VLA-4 molecules on their surface did not significantly differ between Th1 and Th17 in EAU lymphocytes in both retinae and dLNs.

**Inflammatory Retinal Monocytes/macrophages Were Blocked By Gw559090 In Eau**

Since VLA-4 is also expressed by myeloid cells, the effects of GW559090 on myeloid cell expression levels were further analyzed by immunofluorescence staining using a panel of CD4, Iba-1 and DAPI. In the control Veh group, CD4\(^+\) T cells were increased within the vitreous cavity and retinal layers and Iba-1\(^+\) myeloid cells were mainly located in the inner retinal vascular areas (Fig. 4a). In GW\(_{10}\) group, of the few infiltrating cells, most were CD4\(^+\) T cells rather than myeloid cells (Fig. 4b).

Inflammatory and resident monocyte/macrophages were also investigated by flow cytometry, comparing cells isolated from retinal tissues and blood (Supplementary Fig. 1b) [33]. In the eyes, a decrease in myeloid cells (CD45\(^+\)Ly6G\(^-\)CD11b\(^+\)CD11c\(^-\)CD64\(^+\)) was only detected in the Dex group \((P = 0.027)\) but not in any of the GW559090 treated groups (data not shown). There was a decrease of CD45\(^+\)CD11b\(^-\)CD11c\(^+\) dendritic cells (DCs) in the retina in Dex and GW\(_{10}\) group \((P = 0.0002\) and 0.017 respectively, Fig. 4c-d). Within the myeloid population, however; there were decreased levels of CD45\(^+\)Ly6G\(^-\)CD11b\(^+\)CD11c\(^-\)CD64\(^+\)Ly6C\(^+\) inflammatory monocytes/macrophages, whilst levels of Ly6C\(^-\) resident monocytes/macrophages within the eyes were unchanged after GW\(_{10}\) treatment (Fig. 4e-f). There were very few eosinophils in the retinae and their presence did not alter with treatment. Similarly, GW treatment did not affect levels of neutrophil or NK cells in the eye (data not shown). In the blood, topical treatments did not have any effect on levels of activated monocytes or CD45\(^+\)CD11b\(^-\)CD11c\(^+\) DCs (data not shown). Systemic neutrophils were decreased in the Dex group but were unaffected by GW559090 (data not shown).

**Adhesion of Th17 cells was decreased by GW559090 in vitro**

To assess whether human lymphocyte migration was affected by GW559090, activated human PBMCs were pretreated with GW559090, and assayed for their ability to migrate across inflamed HMEC monolayers grown on a fibronectin-coated matrix. Cells retained their viability for 18 hours treated with 1, 10, 100 and 1000 \(\mu\text{g/ml}\) GW559090 (data not shown). Cell numbers post treatment with GW559090 in non-adherent (N), adhered (A) and migrated (M) populations relative to Saline (Sal) were calculated (Fig. 5a), and demonstrated that numbers adhering or migrated did not change significantly with treatment. However, the percentages of cells that migrated were significantly decreased if pre-treated with at least 100 \(\mu\text{g/ml}\) GW559090 (Fig. 5b). The CD4\(^+\) T cell subsets in the non-adhered, adhered and
migrated populations were further evaluated by flow cytometry and a suppression of Th17 cells adhering to activated endothelium was obtained by pretreating with 1000 µg/ml GW559090 (Fig. 5c-d).

**Discussion**

In this study, we have investigated α4β1-dependent mechanisms involved in leukocyte infiltration into the retinal tissues before and during EAU by using GW559090 eyedrops. We also investigated the functional role of α4β1 in human CD4+ T cell transmigration using *in vitro* assays, in which VCAM-1 has previously been demonstrated to be involved only when cells are activated [13]. Both rat and human neutrophils, eosinophils, monocytes, and lymphocytes are known to express α4β1 [17]. Treatment with GW559090 before the onset of EAU resulted in a significant decrease of disease scores and a noticeable attenuation of disease severity if administered therapeutically, once EAU was detected. Our novel findings were firstly that EAU can be therapeutically downregulated by a topical α4β1 inhibitor, as seen both in the levels of retinal cell infiltration and in clinical severity scores. It preferentially downregulated Th17 cells and inflammatory monocyte/macrophages, without affecting Treg or neutrophils in the retina. We also determined that α4β1 facilitated adhesion, but not migration, of human Th17 cells to endothelial cells *in vitro*.

There is evidence for adhesion molecule expression in uveitis affecting man, where histological investigations demonstrated an increase in adhesion molecule expression in uveitis tissue specimens [9, 34, 35]. Furthermore, there is indirect evidence for the importance of adhesion molecules from studies with Behçet's disease patients. It was demonstrated that the level of ICAM-1 was increased in bloods from patients with inactive uveitis but with an active associated systemic disease more so than when disease was localized to the eye or with healthy controls. However, in patients with localized active uveitis without systemic involvement, the level of ICAM-1 and VCAM-1 in the blood was similar to controls [36]. The results suggested that the increased integrin expression levels in blood can only be detected when there is an associated systemic inflammation, although there is currently a lack of knowledge regarding integrin levels expressed in eyes from uveitis patients with localized disease. In animal studies, Xu et al. reported the distribution of P-selectin, E-selectin, ICAM-1, VCAM-1 and PECAM-1 on the retinal microvascular endothelium increased as EAU progressed [5]. VCAM-1 is however not expressed in healthy eyes, and its level is only increased after EAU has developed, especially at the site of post-capillary venules where inflammation involves cells being recruited to the retina [5, 12, 37]. Thus, blocking the VLA-4/VCAM-1 pathway appears to be a more specific way of preventing the activated leukocyte from crossing the BRB. This has also been validated by systemically administered VLA-4 inhibitors which attenuated EAU disease severity [16, 19]. Our study involves treatment, administered topically via eye drops, to achieve a localized therapeutic effect without affecting the systemic immune system, thereby avoiding the serious side effects which have occurred in multiple sclerosis patients receiving intravenous natalizumab (a humanized anti-α4 integrin antibody), in which a fatal or catastrophic re-activation of JC virus occurred within CNS glial cells, that could be the result of drug-induced systemic immunosuppression [11]. In patients with refractory uveitic macular edema, it has been reported that
weekly subcutaneous injection with an ICAM-1 inhibitor, Efalizumab (Raptiva, Genentech, San Francisco, CA, USA) could achieve therapeutic effect after 6 months of treatment and the effect persisted 6 months after discontinuing the treatment. However, a frequent report of PML and neurological symptoms in patients who have been treated with efalizumab chronically (often more than 2 years) urged the manufacturer to discontinue the commercial use of efalizumab [38].

During the development of uveitis, it is essential for lymphocytes entering the eye to first traverse the vascular endothelial cell wall and cross the BRB. This process involves adhesive interactions between the endothelial cells and lymphocytes, and their subsequent migration across the endothelium. It has been described that the migratory process is controlled by adhesion molecules which may be upregulated following cell activation and has been well characterized in CNS studies [30]. This was supported by evidence that VCAM-1 expression was only detected if the endothelium was stimulated with IFNγ in vitro [10, 14, 39]. In contrast, in the LFA-1/ICAM-1 axis, ICAM-1 is expressed at the luminal surface continuously at intercellular junctions between adjacent endothelial cells even when the endothelium was not inflamed [5, 40]. In a study investigating rat retinal endothelial cells co-cultured with mitogen-activated antigen-specific T cells, it was demonstrated that adhesion on CNS vascular EC was largely dependent on LFA-1/ICAM-1 whether expressed on active or inactive EC, and only a small portion was dependent on VLA-4/VCAM-1 when the EC was activated by IL-1β [13]. In an in vitro study using primary human brain endothelial cells co-cultured with polarized Th1 and Th2 cells originated from healthy donor cells stimulated with or without myelin basic protein, it was reported that the process was more dependent on LFA-1/ICAM-1 interactions, while blocking the VLA-4/VCAM-1 axis had no effect on trans-endothelial migration [40]. These reports indicated that LFA-1/ICAM-1 interactions play an important role in general cell migrations, whereas VLA-4/VCAM-1 interactions are only involved in specific inflammatory migratory conditions. Our results agreed with the finding that blocking VLA-4 did not affect migrating cells, but the adhesion of CD4+ T cell subsets. It has previously been reported that lymphocytes derived from active multiple sclerosis patients were more migratory to fibronectin-coated and brain-derived endothelial cell monolayers as compared to lymphocytes from non-affected individuals [41]. Our results are limited by using healthy human PBMCs activated nonspecifically with PHA. The fact that GW559090 functionally reduced adhesion but not migration of Th17 cells could perhaps be due to their not being retinal antigen-specific and therefore were less migratory.

Under various inflammatory conditions, LFA-1/ICAM-1 and VLA-4/VCAM-1 paired axes may participate in lymphocyte migration differently. Our results suggested that individual CD4+ T cell subsets possess differential abilities with respect to their transendothelial, despite expressing a similar increased VLA-4 expression profile in all CD4+ T cell subsets within the retina and dLNs. This is supported by a previous CNS study in which the percentage of healthy peripheral blood-derived Th1 and Th2 cells expressed similar levels of VLA-4 and LFA-1 before and after migration across adult brain-derived endothelium in vitro. However, Th2 cells were more potent for migration than Th1 cells, even in the presence of an inhibitory ICAM-1 antibody [40]. In another study investigating VLA-4 on CD4+ T cells in EAE, it was reported that Th1 cells preferentially infiltrate the spinal cord via a VLA-4-mediated mechanism, whereas
the entry of Th17 cells into the brain parenchyma occurred in the absence of VLA-4 integrins but was dependent on the expression of LFA-1 [42]. This conflicts with our findings but might reflect local barrier-specific differences. These results could also reflect tissue-specific differences in chemokine and chemokine receptor interactions in different autoinflammatory conditions. Crane et al. further supported this concept by reporting that CCR5 plays an important migratory role for antigen-specific Th1-polarized cells entering the BRB and into the retina during an inflammatory reaction rather than for rolling on the retinal vasculature during the initial stages of cell recruitment [43].

We discovered that by treating with GW559090 at the same time as inducing EAU, specific CD4⁺ T cells were blocked from crossing the BRB, thus preventing structural damage. In contrast, applying GW559090 only after signs of EAU were already detectable, GW559090 appeared to stop further cell infiltration, and low levels of disease could still be observed. It has been proposed in a time course study of EAU in B10.RIII mice, that leukocytes cross the BRB at the level of the small venules, utilize selectin and ICAM-1 before the disease appears, and then utilize VCAM-1 and PECAM-1 when the disease starts to manifest, and that these conditions were observed for EAU but not in ovalbumin-immunized mice [5]. In our study, by blocking VLA-4 preventively, the disease severity was reduced, indicating low levels of adhesion molecule upregulation were associated with a decreased severity of disease. When blocking VLA-4 only once EAU had developed, a few lymphocytes might utilize other adhesion molecules infiltrating the BRB, which could explain why the disease was not fully ablated. The interaction between VLA-4/VCAM-1 is not a sole event. It was reported for human T cells that VLA-4 integrin/VCAM-1 interaction could activate LFA-1 integrin-mediated adhesion to ICAM-1 [44]. Inhibition of both pathways systemically exerted a synergistic effect in EAU [19]. However, we did not have the opportunity to test these inhibitors in our study.

As it has been shown that, apart from CD4⁺ T cells, other cells express VLA-4, we investigated retinal myeloid cells, and found that if the α4β1 inhibitor GW559090 was given therapeutically, a decrease of Iba1-expressing myeloid cells within the retinae and vitreous cavity and a reduction in Ly6C⁺ inflammatory monocytes/macrophages could be observed. Given that macrophages and dendritic cells were not detectable in CFA controls (data not shown), it is likely that the decrease in Ly6C⁺ inflammatory monocyte/macrophage was due to an inhibition of cell infiltration into the retina by GW559090 treatment. It has been reported that Ly6C⁺ inflammatory monocyte/macrophages appeared in EAU eyes a week post immunization and are involved in the chronic tissue damage seen in the eye during the course of uveitis [45, 46]. Activated macrophages have been demonstrated to use VLA-4 adhesion molecules as their primary integrin for in vitro CNS immigration [47]. It has been reported that early introduction of anti-VLA-4 mAb systemically can prevent CD45⁺CD11b⁺ activated microglia/macrophages infiltrating the CNS along with targeting T cell infiltration to achieve a reduction in clinical severity in EAE [48]. Our study is the first to report that following GW559090, the Ly6C⁺ inflammatory monocyte/macrophages were abrogated in the retina, further supporting the CNS studies and suggesting its role to prevent further tissue damage.
**Conclusion**

In conclusion, topical GW559090 significantly ameliorated CD4⁺ T cell infiltration into the eye, especially Th17 cells, without affecting systemic CD4⁺ T cell levels. The results were further supported by *in vitro* studies, showing that Th17 cells were specifically suppressed with respect to their adhesion to endothelial cells by GW559090. In addition, Ly6C⁺ inflammatory monocyte/macrophages in the eye were also ameliorated. α4β1 integrin-directed inhibition could therefore be of potential use in Th17-dominant uveitis disease.

**Abbreviations**

A  
adhered  
ARVO  
Association for Research in Vision and Ophthalmology  
BBB  
blood-brain-barrier  
BRB  
blood retinal barrier  
CNS  
central nervous system  
EAU  
experimental autoimmune uveitis  
EAE  
experimental autoimmune encephalitis  
dlNs  
draining lymph nodes  
ICAM-1  
Intercellular Adhesion Molecule-1  
IRBP  
interphotoreceptor retinoid-binding protein  
LFA-1  
leucocyte functional antigen-1  
M  
migrated  
MS  
multiple sclerosis  
N  
non-adherent  
PBS
Declarations

Ethics approval and consent to participate

Ethics approval for all animal experiments was obtained from the animal ethics committee of UCL Institute of Ophthalmology (I1627DED3).

Consent for Publication

Not applicable

Availability of data and materials

All relevant data generated or analyzed during this study are included in this published article.

Competing interests

This work was supported in part by a research collaborative award from GSK (ALL028527), Stevenage, UK (VC) and the Biomedical Research Centre (BRC) at UCL Institute of Ophthalmology & Moorfields Eye Hospital (VC). Yi-Hsing Chen is a recipient of a studentship from Funds of Chang Gung Memorial Hospital, Taiwan. The rest of the authors declare no commercial or financial conflict of interest.

Funding

Not applicable

Authors’ contributions

YHC, JG, SL and VC designed the study. YHC and ME performed the in vivo experiments. ZC and GG assisted on flow cytometry and associated data analysis. YHC performed all other experiments. YHC, ME, SL and VC wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors acknowledge Ms. Hodan Jama for setting up the histology staining.

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Figures
Topical administration of α4β1 VLA-4 inhibitor either at the time of EAU induction or when disease was evident (10 days post EAU induction) demonstrated a reduction of cellular inflammation in the EAU eyes. 

a-b H&E staining of eye sections including optic nerve illustrating increased numbers of cells within the vitreous, retinal folding, and disruption of the retinal layers in vehicle-treated (Veh) mice (a) than the 10mg/ml GW559090 (GW10) treated eye (b) (X200). 

c Grading of histological scores for EAU eyes treated with GW3, GW10, and GW30 and vehicle controls. 

d-e H&E staining of retinal sections of GW10 untreated left eye (d, GW10L) and treated right eyes (e, GW10R) demonstrating decreased retinal folding and fewer infiltrating cells in the treated eye (X200). 

f Comparing the histological scores for EAU mice treated preventively with 10mg/ml of GW559090 in right eye only and the untreated left eye. 

g Retinal fundus imaging of EAU eyes at peak stage of disease. 

h There were decreased perivascular infiltrates, disc infiltration, subretinal infiltrations, and vasculitis in GW10 therapeutic treatment in EAU. 

i Clinical scores after EAU signs were evident by day 10 post immunization (d 10pi) and after a further 7 days’
topical treatment with GW559090. The mice developed clinical extensive disease in Veh group, while in GW10 and Dex treatment group, a significant reduction of clinical severity was observed. Values were mean ± SEM for 5 or more data points (c, i) and mean ± SD for 3 data points in f. n=8 in preventive treatment experiment (a-e); n=3 in unilateral eye treated group (f), and n=5 in therapeutic treatment experiment (g-i). *P < 0.05, ***P < 0.001 using a two-tailed, unpaired Student’s t test.

Figure 2

Localization of leukocytes within the eyes after treating with GW559090. There was an increase in leukocytes around the optic nerve (X200; a), within the anterior chamber (X200; b), retinal layers and within the vitreous cavity (X400; c) in vehicle-treated control (Veh). In GW10 treated eyes, decreased levels of cells were detected around the optic nerve (X200; d), in the anterior chamber (X200; e), and within the vitreous (X400; f). Mean ± SEM scores were calculated for cell numbers infiltrating the vitreous (g),
anterior chamber (h) and inner retina (i) in all groups. j Cellular infiltration score. n=5 in each treatment group. *P < 0.05; **P < 0.01, using a two-tailed, unpaired Student’s t test.

Figure 3

Immunofluorescence staining and flow cytometric analysis of CD4+ T cell subsets within the retina after GW559090 treatment. a There were both Th1 (CD4+Tbet+) and Th17 (CD4+RORγt+) cells in the vitreous and inner retinal layers in Veh group. b In GW10 treated group, there were decreased numbers of CD4+ T
cells infiltrating the retina which were mainly Th1 cells. c Counting the CD4+ T cell subsets within the vitreous revealed that Th17 cells were mostly suppressed by 10mg/ml GW559090 treatment. d Representative retinal flow cytometric figures for CD4+ T cells. e Presentation of CD4+ T cell subsets in the retina in by expression of IFNγ or IL-17A. f Representative retinal flow cytometric figures for the distribution of IFNγ or IL-17A within CD4+FoxP3- live cells. g Figures represent the results of flow cytometric analysis for CD4+ T cell subsets within the dLNs. h-i VLA-4 expression in CD4+ T cell subsets within the retina (h) and dLNs (i). j-k Mean fluorescence intensity (MFI) of VLA-4 expression in Th1 and Th17 cells in eye (j) and dLNs (k). Means ± SD. n=5 in Fig. 3c, and n=3 in Fig e, g, h, i-k in each treatment group. Eye data were from single eye in a single mouse. *P < 0.05; **P < 0.01; ***P < 0.001 compared to Veh group, using two-tailed, unpaired Student’s t test.
Figure 4

Localization and effect of GW559090 treatment on myeloid cells. a There were more myeloid cells (CD4-Iba-1+ cells) present within the inner retinal layers in Veh group (X400). b There were reduced levels of CD4+ T cells observed within the vitreous and only very limited numbers of myeloid cells in the eyes of GW10 group (X400). c Representative flow cytometry figure depicting CD11b+CD11c- myeloid population and CD11b+CD11c+ dendritic cells (DC) from CD45+ cell gates in single live cells. d Level of DCs in each treatment group illustrating a decrease of DCs in the eyes treated with Dex and GW10 compared to the control. e Representative flow cytometry figures illustrating inflammatory monocytes/macrophages (Ly6C+) and residential monocytes/macrophages (Ly6C-) from CD45+CD11b+CD11C-CD64+L6G- single live cell gate. f Comparing percentage of inflammatory and residential monocytes/macrophages between treatment groups. (d, f). n=3 data points per group from pooled two eyes for flow cytometric analysis. Mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 using a two-tailed unpaired Student’s t test.
Figure 5

Effect of GW559090 on human PBMCs migration and CD4+ T cell subsets in vitro. a The leukocyte cell number in non-adherent (N), adhered (A), and migrated (M) populations in saline-treated controls (Sal) and 10, 10^2, 10^3 µg/ml of GW559090 treated groups. b Percentages of live cells adhered and migrated in each group. c-d CD4+ T cell subsets analyzed by Tbet and RORγt in CD4+FoxP3-live cells in adhered (c) and migrated (d) populations. Data were presented with mean ± SD, with triplicates in each condition. *P < 0.05, **P < 0.01, ***P < 0.001, using a two-tailed unpaired Student’s t test.

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