In vivo confocal microscopy of inflammatory cells in the corneal subbasal nerve plexus in patients with different subtypes of anterior uveitis

Andreea S Postole,1,2 Alexandra B Knoll,1,2 Gerd U Auffarth,1 Friederike Mackensen1,2

ABSTRACT
Purpose Previously we could show increased numbers and densities of dendritic-like cells (DLCs) in the subbasal nerve plexus of the central cornea in patients with herpetic anterior uveitis (HAU). Now we aimed to explore these and other inflammatory cells seen in this layer in different subtypes of anterior uveitis using in vivo confocal microscopy.

Methods Consecutive eyes of patients with different types of anterior uveitis, HAU, Fuchs’ uveitis syndrome (FUS), juvenile idiopathic arthritis (JIA) and human leucocyte antigen (HLA)-B27-related anterior uveitis were examined in vivo with the combination of Heidelberg Retina Tomograph II/III and Rostock Cornea Module. The contralateral eye was used as control. Inflammatory cells were defined on the basis of their morphology: type 1 (DLCs) and type 2 (cell bodies lacking dendrites). Frequencies were evaluated statistically in each group.

Results The difference between means of type 1 cells density of affected eyes in all four groups was significant (one-way analysis of variance (ANOVA) p=0.039). The difference between means of type 1 cell densities of affected eyes in patients with HAU (96.8±44.2 cells/mm², n=10) and that of patients with FUS (46.4±38.7 cells/mm², n=17) was significant (Tukey’s post hoc p=0.025), whereas the difference between patients with HAU and JIA (53.3±34.5 cells/mm², n=7) and patients with HAU and HLA-B27 (63.1±59.2 cells/mm², n=10) was not significant (Tukey’s post hoc p=0.181 and 0.300). In contrast, the following means resulted from the evaluation of type 2 cells: the difference between means of affected eyes in all four groups was not significant (one-way ANOVA p=0.185). Density means difference of patients with HAU (44.9±22.6 cells/mm², n=5) and that of FUS (20.0±11.0 cells/mm², n=2) and that of patients with JIA (56.0±18.3 cells/mm², n=2) and that of HLA-B27 (36.1±24.1 cells/mm², n=5) was not significant (Tukey’s post hoc p=0.302, 0.877 and 0.739). The contralateral eye of all patient groups showed also an inflammatory cell infiltrate of lesser extent.

Conclusions The high density and morphology of DLCs in the central cornea of patients with HAU and JIA (53.3±34.5 cells/mm², n=7) was significant (Tukey’s post hoc p=0.025), whereas the difference between means of affected eyes in all four groups was not significant (one-way ANOVA p=0.185). Density means difference of patients with HAU (44.9±22.6 cells/mm², n=5) and that of FUS (20.0±11.0 cells/mm², n=2) and that of patients with JIA (56.0±18.3 cells/mm², n=2) and that of HLA-B27 (36.1±24.1 cells/mm², n=5) was not significant (Tukey’s post hoc p=0.302, 0.877 and 0.739). The contralateral eye of all patient groups showed also an inflammatory cell infiltrate of lesser extent.

CONCLUSIONS
The high density and morphology of DLCs in the central cornea of patients with HAU assessed by confocal microscopy supports the clinical diagnosis of HAU especially when compared with patients with FUS but not when compared with patients with JIA or HLA-B27.

Clinical relevance This study suggests that the non-invasive confocal microscopy of the cornea is capable of supporting a clinical diagnosis in patients with uveitis.

INTRODUCTION
Dendritic-like cells (DLCs) have an important immunogenic role in different tissues, for example, cornea. They modulate the innate and adaptive immune response towards pathogenic input in different subtypes of anterior uveitis (AU).1 2

Using in vivo confocal microscopy, DLCs present as hyperreflective, branched structures in the subepithelial region (basal epithelium/Bowman’s layer or interspersed in the subbasal nerve plexus) of the cornea at a depth of 40–60 μm (for a review, see 3). For easier reading, we decided to refer to these cells as subepithelial throughout the paper even though they may branch into adjacent regions. There is increased body of evidence that even the normal cornea of healthy patients is in up to 30% endowed with these so-called resident dendritic cells (DLC) that are localised in the epithelium and stroma in low numbers.4 5 Hamrah et al showed that the immunological function of the corneal DLCs is identical to those of the active Langerhans cells (LC) in the epidermis. Furthermore, Zhivov et al analysed the distribution of DLCs in normal corneas of healthy volunteers and showed in corneal transplantation model that there are low numbers in the central cornea with increasing density towards the periphery of the cornea.1 7–9 During corneal inflammation (eg, herpetic keratitis) and irritation (eg, contact lens wear), increased numbers in the central cornea have been described.1 7 10

Recently, we showed increased DLC densities in patients with herpetic anterior uveitis (HAU) compared with patients with Fuchs’ uveitis syndrome (FUS).11 So far we do not know the pathogenetic role of these cells in uveitis, nor if they are resident or infiltrating cells. Equally open to discussion is why the cornea would be infiltrated by inflammatory cells during AU and if this would be also the case in non-infectious uveitis. The aim of this follow-up study, therefore, was to further evaluate and describe the morphology and density of cells in the subbasal nerve plexus of the central cornea of patients with HAU during intraocular inflammation and compare it with other aetiologies of AU: FUS, juvenile idiopathic arthritis (JIA) and human leucocyte antigen (HLA)-B27-associated AU. Hereby we wanted to enhance our knowledge about inflammatory processes in the cornea in AU and to further test the diagnostic usefulness of determining inflammatory cell densities in HAU.

MATERIALS AND METHODS
Consecutive patients were recruited from the Interdisciplinary Uveitis Centre Heidelberg,
Germany, a tertiary referral centre based in the university clinic.

The most important criterion for inclusion in this study was intraocular inflammation of the anterior segment of the eye with at least 0.5+ anterior chamber (AC) cells seen at the slit lamp. The clinical presentation of the HAU was evaluated according to the features documented in the literature: unilateral AU with sectorial atrophy of the iris and elevated intraocular pressure.12

Patients suffered either a first episode or a relapse of their disorder. Patients with keratouveitis were not included in this study. All of the patients met the clinical criteria. Diagnosis of HAU was suspected due to clinical presentation or confirmed either by AC puncture (2/10) or by response to acyclovir therapy in a previous episode (8/10).13 Most of the patients with HAU presented with a relapse (6/10) rather than a first episode (4/10). In four cases was herpes simplex virus assumed and in six cases varicella zoster virus. Treatment was initiated after confocal microscopy was performed with 5×800 mg oral acyclovir per day. The clinical response was judged as agreed by the SUN group.14 A reduction of AC cells down to a maximum of 0.5+ cells was expected. The herpetic treatment was continued following the results published in the literature.15 We examined in the HAU group 10 affected eyes and 6 healthy eyes that served as control.

All of the 19 patients with FUS were clinically diagnosed. Since FUS is a chronic unilateral AU that does not improve with any medical treatment,16 the Rostock Cornea Module (RCM) examination could be planned and 12 patients were called in for examination. Similar to the HAU group, we examined in the FUS group 19 affected eyes and 18 healthy control eyes.

Diagnosis of JIA-associated AU was made based on clinical features after joint affection was diagnosed.17 All HLA-B27AU patients showed a positivity for the HLA-B27 antigen as well as the typical clinical picture.17 In six patients, HLA-B27AU treatment was initiated after confocal microscopy, one was on topical and systemic treatment (inflimixab), two on systemic treatment (adalimumab and ciclosporin, respectively) and one on topical treatment. Four patients with JIA had no treatment, two patients with JIA were on topical and systemic treatment (adalimumab and methotrexate) and two were on systemic treatment with prednisone and methotrexate, respectively.

For confocal microscopy, an anaesthesia eye drop was first applied topically in each eye into the lower conjunctival fornix of the eye. Then the patients received a streak of lubricant ointment (Vidisic gel Bausch+Lomb, Dr. Mann Pharma, Berlin, Germany) to protect the cornea and to couple the microscope objective and the cornea. The microscope objective was then placed their forehead in the microscope support and to look straight ahead at the microscope-positioned white light in order to avoid eye movements and to standardise the examination condition. The entire examination time per eye took <5 min.

The in vivo confocal microscopy scanning was performed with the combination of Heidelberg Retina Tomograph II/III and RCM (HRT RCM) (Heidelberg Engineering, Germany) on each eye in the central region of the cornea. A 400 μm magnification lens was used for the examination (mag ×400/Achroplan ×63w/NA 0.95/AA 2.00 mm 670 nm/Zeiss), which resulted in a scan captured area of 400×400 μm (384×384 pixels) per image, with a transverse optical resolution of 2 μm and longitudinal optical resolution of 4 μm (Heidelberg Engineering, supplied information).

The area of interest consisted in the subbasal nerve fibre plexus, which appeared at a depth of 40–60 μm. The focus level was changed manually in axial direction. Sequential images were captured on this level from central areas of the cornea.

A total of three image sections were then selected and evaluated by two different observers (AK and SP), one of them blinded by the patients’ diagnosis, by manually counting the number of inflammatory cells in one visual field (250×250 μm) per section using a grid system with a 50 μm scale grid and the internal cell counting software. Cells touching image edges were only counted on two of four edges to avoid overestimation/underestimation of density. The final density of inflammatory cells was given as cells per square millimetre. Cells that presented a different localisation than that of the subbasal nerve fibre plexus region have not been taken into consideration.

The interobserver reliability for the determination of type 1 cells on the mean cells per mm² per patient for each observer was calculated with the Spearman test.

Table 1 and figure 1 show the grading of the inflammatory cells that assesses size and morphological form. Type 1 cells (T1) were defined as DCLCs with elongations and type 2 cells (T2) as round cells lacking dendrites. Dendrites of any kind were not observed in T2 cells in either deeper nor in higher localised adjacent layers.

Results were evaluated for statistical significance with one-way analysis of variance (ANOVA) test, Tukey’s post hoc test, Kolmogorov–Smirnov test and Mann–Whitney U test for affected eye and contralateral eye pair comparing in each of the four groups. The interobserver reliability was calculated with Spearman test performed on the mean cells per mm² per patient by each observer. Graphics and statistics were generated with GraphPad Prism V6.0 and SPSS V20.0 software.

RESULTS

A total of 48 patients were examined (21 females and 27 males) in this study with analysis of a total of 89 eyes. Demographic data and numbers of type 1 and 2 cells seen are listed in table 2.

Comparison of frequency of type 1 in all four groups

Interobserver correlation of type 1 (T1) cells counts was excellent (Spearman r=0.9622, p<0.0001). The difference between means of T1 cells density of affected eyes in all four groups was significant (one-way ANOVA p=0.039) (figure 2). In the subsequent post test, means of T1 cell densities of affected eyes in patients with HAU and that of patients with FUS was significant

<table>
<thead>
<tr>
<th>grading morphology</th>
<th>size (approximately) (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>type 1</td>
<td></td>
</tr>
<tr>
<td>a. dendritic-like small</td>
<td>25</td>
</tr>
<tr>
<td>b. dendritic-like medium</td>
<td>40</td>
</tr>
<tr>
<td>c. dendritic-like large</td>
<td>55</td>
</tr>
<tr>
<td>type 2</td>
<td></td>
</tr>
<tr>
<td>a. cell bodies lacking dendrites small</td>
<td>10</td>
</tr>
<tr>
<td>b. cell bodies lacking dendrites medium</td>
<td>20</td>
</tr>
<tr>
<td>c. cell bodies lacking dendrites large</td>
<td>40</td>
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</tbody>
</table>

Type 1 includes bright, branched structures while type 2 describes hyperreflective cell bodies lacking elongations.
(Tukey’s post hoc p=0.025) (figure 3). There was no significant difference when patients with HAU were compared with JIA and HLA-B27AU (Tukey’s post hoc; p=0.181 and 0.300).

**Comparison of frequency of type 2 in all four groups**

Type 2 (T2) cells were less frequently seen (see table 2). Evaluation of T2 cells showed no difference between means of affected eyes in all four groups (one-way ANOVA p=0.185; Tukey’s post hoc; HAU vs FUS p=0.502, HAU vs JIA p=0.877 and HAU vs B27AU p=0.739) (figures 4 and 5).

**Ratio type 1 cells:type 2 cells**

Comparing the ratios between T1 vs T2 cells in affected eyes in HAU, FUS and B27-AU, these were approximately 2:1, whereas the ratio in the JIA group was 1:1 (see table 2). On the other hand, the ratios between T1 vs T2 in unaffected eyes showed a result of approximately 3:1 in HAU and FUS, and a ratio of 1.6:1 in B27-AU and a ratio of around 1:1 in JIA.

Nearly all patients showed T1 cells in their affected eyes, but only up to 50% T2 cells. Especially patients with FUS and JIA showed T2 cells only in two cases. This was significant comparing FUS and HAU in Fisher’s exact test (p=0.0302), but not for...
We further distinguished the different cell types into type 1a–c and type 2a–c regarding size and presence of dendrites or not (see Table 1). The pattern of type 1b cells was present in the majority of all four patient groups: patients with HAU presented type 1b cells (60% of the cases), type 1c, 2a and 2b (each 30%) and type 1a (20%). Patients with FUS showed type 1b cells (57.9%), 1a (31.6%), 2a and 2c (5.3%). In patients with JIA, we found the following cell types: 1b (66.7%), 2a (22.2%), 1a (11.1%) and in HLA-B27 patients type 1b (50%), 1a (40%), 2a (30%), 1c and 2b (each 20%).

Validity of the method

The validity of the diagnostic method was analysed by generating an receiver operating characteristics (ROC) curve and by determining the value of the area under the curve (AUC) =0.769, suggesting a high reliability of the method. Furthermore, we determined a cut-off point of 61 type 1 cells/mm² with a Youden index of 0.558 and a sensitivity of 0.900 and a specificity of 0.342. Above this value, the diagnosis of HAU is more probable.

Correlation between AC cells and DLC count

Slit-lamp biomicroscopy revealed no correlation between amount of AC cells and density of the DLC in the central cornea. The amount of AC cells ranged between 0.5+ and 2+ (2+ only in one patient) in the HAU group, between 0.5+ and 1+ in the FUS group, between 0.5+ and 4+ (4+ only in one patient) in the JIA group and between 0.5+ and 2+ in the HLA-B27 group.

DISCUSSION

In this study, we describe the presence of DLC in the cornea in patients with different subsets of AU. The finding of these cells of possible inflammatory character in this tissue is surprising as the cornea is not thought to be involved in AU. Zhivov et al reported a moderate density (34±3 cells/mm²) of LCs, which we find similar to that we described in our study as DLC, in central corneal epithelium of healthy volunteers.17 This could account for the numbers of DLC seen in fellow eyes of FUS, JIA and B27AU, but not for those seen in affected eyes of all groups nor fellow HAU eyes.

It is, therefore, imaginable that an inflammatory response in the cornea is part of the uveitic picture. The alternative is that it is a reactive response of the AC inflammation. We found no correlation between amount of inflammatory cells in the AC and numbers of DLCs (the four-step grading system may be too rough to detect a correlation). Mocan et al reported that the eight patients with keratouveitis had ≥2 cells in the AC, but do not show mean or maximum AC cell readings and no correlation with presence and numbers of inflammatory cells in the cornea.

Note has to be taken of the high variation of frequencies of DLC seen in our patients and the relatively small number included due to the rarity of the disease. To our knowledge, so far this observation has not been published in non-corneal disease such as AU, so we have to compare our findings with those published in corneal diseases.

DLC in the literature

Mocan et al found dendritiform and small round cells by in vivo confocal microscopy in 52% of patients with non-epithelial herpes keratitis. This included eight patients with keratouveitis,
of the cornea in AU. Mayer et al\textsuperscript{31} found slightly lower numbers in patients post herpes keratitis (53.8 cells/mm\textsuperscript{2}).

**Frequency of DLC in AU**

Our study confirmed the finding of our previous study in more patients that the density of the DLCs in affected eyes in patients with HAU is significantly higher in the central subbasal nerve plexus than in patients with FUS, but not in patients with JIA and HLA-B27AU.

Still, the non-invasive in vivo confocal microscopy may support the diagnosis of HAU in clinical practice when >61 cells/mm\textsuperscript{2} are seen. As results of herpetic testing as AC taps or response to treatment take some time, confocal microscopy gives a fast information but may facilitate a diagnostic and treatment decision. Based on the ROC curve and on the value of AUC, the diagnostic reliability seems good, meaning that the confocal imaging holds a high sensitivity. We chose the analysis of the interobserver correlation for T1 cells since the T1 cells represent the main result of the study and these cells are the one that appear more often. Since the interobserver correlation for T1 cells resulted to be consistently strong, we presumed that the interobserver agreement for T2 cells would be similar.

If one looks at the distribution of frequencies in the different AU groups, a clustering around the mean is seen except for B27AU, where five patients have high numbers of T1 cells and five very low numbers (see figure 2). We went back to the clinical charts of these patients to see whether there was a possible explanation for this. The patients did not show differences in time since onset of flare, limited or chronic disease, age, anti-nuclear antibody (ANA) status or medication versus no medication (data not shown). So we cannot explain the observation by clinical features.

It may be that in a larger sample size the trend seen would also become significant for the other AU subtypes. The small sample size is a limitation of our study but is a limitation we see it is difficult to get a larger sample size in a reasonable amount of time with clear-cut phenotypes. Our study already took 3 years to get to the sample size presented.

Another limitation is introduced by different length of disease or flare, age and treated and untreated patients. Still, it did not seem that these parameters influenced type and number of DLC seen. Some differences (as the presence of round T2 cells) could be explained by a more acute type of uveitis (as HAU and B27AU) versus chronic uveitis (JIA and FUS).

**DLCs’ morphological characteristics**

We further described the pattern and the morphological characteristics of DLC by assessing and establishing a standardised grading (table 1). Size of cell body, dendrites and morphology was taken into consideration. We identified DLCs with long-branched dendrites (type 1) and cell bodies lacking dendrites (type 2) at a depth of 40–60 μm in the subbasal nerve plexus of the central cornea. The interobserver correlation was good, which we think to be a valid method to distinguish these cells. The T2 cells might be inflammatory cells like lymphocytes, neutrophils, monocytes or very early infiltrating DLCs evolving into mature DLCs. Other authors also describe resident cell population in the central cornea that are being attracted by different chemokines during an acute inflammation.\textsuperscript{7} Furthermore, we also found DLCs forming local networks assisted by long branches, similar to the ‘wire netting’ described by de Jong et al\textsuperscript{22} concerning epidermal LCs.

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**Figure 4** Distribution of type 2 cells (mean with SEM) in subjects with herpetic anterior uveitis (HAU), Fuchs’ uveitis syndrome (FUS), juvenile idiopathic arthritis (JIA) and HLA-B27, both eyes. Affected eyes of all four patient groups reveal the following cell densities: HAU 44.9 ±22.6 cells/mm\textsuperscript{2}, FUS 22.6 ±11.0 cells/mm\textsuperscript{2}, JIA 43.3 ±22.6 cells/mm\textsuperscript{2}, FUS 22.6 ±11.0 cells/mm\textsuperscript{2}, JIA 56.0 ±18.3 cells/mm\textsuperscript{2} and HLA-B27 36.1 ±24.1 cells/mm\textsuperscript{2}. One-way analysis of variance test for affected eyes p = 0.185. The fellow eyes showed the following cell counts: HAU 56.5 ±64.0 cells/mm\textsuperscript{2}, FUS 22.6 ±26.1 cells/mm\textsuperscript{2}, JIA 43.3 ±32.0 cells/mm\textsuperscript{2} and HLA-B27 25.0 ±23.1 cells/mm\textsuperscript{2}.

**Figure 5** Amount of corneal type 2 cells/mm\textsuperscript{2} (mean with SEM) in affected eyes of patients with herpetic anterior uveitis (HAU) (44.9 ±22.6 cells/mm\textsuperscript{2}, n=5) versus patients with Fuchs’ uveitis syndrome (FUS) (20.0 ±11.0 cells/mm\textsuperscript{2}, n=2). *p = 0.302.
Clinical science

Previous studies reported that branched DLCs are more likely immunologically mature and round ones, lacking branches, may be immunologically immature DLCs. Along with the process of maturation, the DLCs migrate from the periphery to the central region of the cornea most probably stimulated by cell-derived cytokines and chemokines due to an injury trigger. This migration led Thoft and Friend to establishing the so-called X, Y, Z hypothesis that provides a possible explanation concerning the migration of the LCs. The premise consists in the fact that LCs migrate from the periphery to the central cornea. In accordance to this hypothesis, LCs not only pass from periphery to central but they also migrate from deep layers beneath the surface, explaining, therefore, the presence of immature LCs in dermidents in the central cornea. This hypothesis accords with our observations and could describe T2 cells in our study. Some authors believe that T2 cells could be resident macrophages; however, their presence is rather rare and mostly limited to the lower stromal region of the cornea. From the cell size we measured, type 2a cells could correspond to lymphocytes, type 2b to monocytes and type 2c to macrophages, which histologically measure 4–15 μm, 20 μm and 20–50 μm, respectively. Mayer et al published a study comparing confocal microscopy to histology in patients that underwent keratoplasty the day after confocal microscopy had been performed. They found that the dendritiform cells stained for major histocompatibility complex (MHC) class II, Langerin and DC sign, concluding that these are different antigen-presenting cells. They did not apply markers for other inflammatory cells as, for example, CD4. We conclude that a complex non-homogeneous cell population with different phenotype and probably diverse functions can be seen in the subbasal nerve plexus of the central cornea in AU and may clinically be relevant to distinguish HAU from other types of AU. Still these preliminary findings have to be validated in a larger patient group, ideally early on in the inflammatory process and before treatment is started. Another very interesting aspect would be longitudinal data, which so far we could not collect due to patients’ reluctance to return to clinic only for confocal microscopy.

Contributors ASP made substantial contribution to acquisition of data, analysis and interpretation of data and drafted the manuscript. ABK made substantial contribution to acquisition of data, participated in its coordination and helped to draft the manuscript. GUA had been involved in the design and coordination, revised the manuscript critically for important intellectual content, and helped to draft the manuscript. FM was involved in its design and coordination, revised the manuscript and helped to draft the manuscript. GUA had been involved in the design and coordination of the study. AK has received a Travel Grant by Heidelberg Engineering. We have loaned the Heidelberg Retina Tomograph III and the Rostock Cornea Module Engineering, AK has received a Travel Grant by Heidelberg Engineering. We have been loaned the Heidelberg Retina Tomograph III and the Rostock Cornea Module Engineering. We have been loaned the Heidelberg Retina Tomograph III and the Rostock Cornea Module Engineering.

Patient consent Obtained.

Ethics approval The study protocol was approved by the Institution’s Ethical Review Commission. The tenants of the Declaration of Helsinki were followed.

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