TITLE: Immunohistochemical characterization of feline lymphoplasmacytic anterior uveitis

AUTHORS: Lawrence Crossfield, Luca Fortuna, Rebekah Carling, Emma Scurrell, Marian Matas Riera, Yu-Mei Chang, Charlotte Dawson, Rachael Pittaway, Ian YL Yeung, Caroline Thaung, Simon L Priestnall, Oliver A Garden

JOURNAL: Veterinary Ophthalmology

PUBLISHER: Wiley

PUBLICATION DATE: 8 May 2018 (online)

DOI: https://doi.org/10.1111/vop.12569

Immunohistochemical characterization of feline lymphoplasmacytic anterior uveitis

Lawrence Crossfield[1] (first author), Luca Fortuna[1] BSc (first author), Rebekah Carling BSc (Hons) BVetMed MRCVS[1] (second author), Emma Scurrell[2] BVSc Dip ACVP MRCVS, Marian Matas Riera[1] DVM DipECVO MRCVS, Yu-Mei Chang[3] Phd CStat, Charlotte Dawson[1] BVetMed MVetMed DipECVO MRCVS, Rachael Pittaway BSc (Hons) BVSc MRCVS, Ian YL Yeung[5] MA FRCOphth, Caroline Thaung[5] FRCOphth FRCPath DPhil, Simon L Priestnall[4] BSc (Hons) BVSc PhD PGCert(VetEd) FHEA DipACVP FRCPath MRCVS (Senior author), Oliver A Garden[1] BSc BVetMed PhD FHEA FRSB FRSA FRCVS DipACVIM DipECVIM-CA (Senior author, corresponding author)

Small Animal Medicine and Surgery Group, The Royal Veterinary College, Royal College Street, London, NW1 0TU, United Kingdom, +44 (0)20 74685452

[1] Royal Veterinary College Department of Clinical Science and Services

[2] Cytopath

[3] Royal Veterinary College Research Support Office

[4] Royal Veterinary College Department of Pathobiology & Population Sciences

[5] Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom

Abstract

Objective

To characterise the immune cells present in different forms of feline anterior uveitis.

Samples

Eyes were obtained from 49 cats diagnosed with chronic idiopathic lympho-plasmacytic anterior uveitis, 7 cats with feline infectious peritonitis (FIP), and 9 cats euthanized for non-ocular disease.

Methods

H&E sections were scored on the level of infiltrate in the anterior uvea. Immunohistochemistry was performed for FoxP3, CD3 and IL-17A, and positive cells were quantified in multiple images of each sample. A generalised estimating equation tested for an association between level of inflammation and the prevalence of these cell types.

Results

Cells stained positive for IL-17A in idiopathic uveitis but not in FIP samples. We found significantly fewer FoxP3$^+$ and CD3$^+$ cells in low grade compared to high grade inflammation in idiopathic uveitis or FIP samples (p values all <0.005), but no difference between FIP and high grade samples.

Conclusions

Idiopathic, but not FIP-associated, uveitis appears to have Th17 cell involvement. The numbers of FoxP3$^+$ and CD3$^+$ T cells present appear directly correlated, thus severity of disease doesn't appear directly determined by regulatory cells numbers.

Introduction

Feline anterior uveitis is a clinically common condition, typically presenting bilaterally with no age or breed predispositions. Acutely, feline uveitis presents with signs of ocular pain and poorly-controlled chronic uveitis can lead to glaucoma, cataract formation and retinal detachment (1). Anterior uveitis (AU) can result from an underlying infection or arise secondary to trauma (2), but no inciting cause is identified in up to 70% of cases (3). Histopathological examination of affected eyes demonstrates lympho-plasmatic inflammation within the iris and ciliary body (4).

The recent identification of the pro-inflammatory Th17 subset of T cells has offered new grounds for investigation of the pathogenesis of anterior uveitis, and their marker IL-17 shows increased expression in human uveitis patients (5).

Previous investigations into functional defects of regulatory T cells (Tregs) in several inflammatory and autoimmune diseases have prompted research into associations between Tregs and AU (6). Tregs are a subset of T cell involved in modulation of the immune system and maintenance of self-tolerance, identified using the transcription factor Forkhead P3 (FoxP3) as a biomarker (7).

The purpose of this study was to test the hypothesis that feline lympho-plasmacytic anterior uveitis is characterised by an influx or induction *in situ* of pro-inflammatory T cells associated with a perturbation of the balance of conventional and regulatory T cells in the uveal tract.

Materials and methods

Clinical cases and controls

Formalin-fixed, paraffin-embedded eyes from 49 cats with a clinical and histopathological diagnosis of idiopathic lympho-plasmacytic anterior uveitis were obtained from a board-certified pathologist at CytoPath, Herefordshire, UK (EJS). Samples were previously enucleated by licensed veterinarians for animal welfare reasons and diagnostic purposes under The Veterinary Surgeons Act (1966) following written informed consent by owners of the animals. Eyes were obtained from seven cats diagnosed with microscopic lesions consistent with, and immunohistochemically confirmed as, feline infectious peritonitis (FIP). These cases were provided by CytoPath or the RVC Pathology Service. Nine eyes were obtained through the RVC Pathology Service from cats that were euthanized for reasons other than ocular disease for use as disease free controls. Reactive feline lymph nodes from the RVC Pathology Service were obtained as positive controls for immunohistochemical labelling.

Scoring system

Uveitic and control eyes were stained with haematoxylin and eosin (H&E). The iris and ciliary body in each section were scored for the severity of the inflammatory infiltrate. A score of 0 represented no inflammatory cell infiltration, 1 mild infiltration (<25% of the structure/s infiltrated), 2 moderate infiltration (25-75% of the structure/s infiltrated), and 3 marked infiltration (>75% of the structure infiltrated). Sagittal sectioning of the globe created two areas of iris and ciliary body on each section, which were scored separately; the final sample score

was the sum of the two halves. Samples with a total score of 0 were classed as having no inflammation, those with scores of 1-4 as having low-grade inflammation, and those with scores of 5+ as having high-grade inflammation.

Immunohistochemistry (IHC)

4μm thick tissue sections were mounted onto positively-charged slides (Leica) for immunohistochemical labelling. Tissue were deparaffinised and hydrated, and heat-induced antigen retrieval was performed with 10mM citrate-buffered saline at pH 6.0 with 0.05% v/v Tween®-20 in a water bath at 90°C. A peroxidase block was performed for 20 minutes to quench endogenous peroxidase activity, and a serum block was performed for 30 minutes to prevent non-specific antibody binding. Immunolabelling with primary antibodies was performed at 4°C overnight. Slides were covered with appropriate secondary antibody and incubated for 30 minutes at room temperature. Slides were rinsed and a DAB+ chromogen solution (Dako) was added for 5 minutes at room temperature. A counter-stain of haematoxylin was applied; samples were then dehydrated and mounted with DPX (Fisher-Scientific). Details of antibodies and solutions are found in Table 1.

Image analysis

Any section that had suffered damage or yielded a negative positive control was discounted. A photomicrograph was taken of each iris and ciliary body in every section (4 images in total in each eye) at 2.5x magnification using a Leica DFC300 FX camera (Leica Microsystems UK LTD). Each image was overlain with a standardised digital grid, the coordinates of boxes incorporating at least 50% tissue noted, and half of these coordinates were randomly selected using random.org for each slide.

These randomly selected coordinates were used as a centre point to image at a higher magnification (20x) to obtain regions of interest (ROI) for use in quantification analysis. In some imaging fields different ROIs over-lapped; photomicrographs were therefore processed using Photoshop CS4 version 11.0 (Adobe Systems; Mountain View, CA) to merge overlapping images to prevent over-counting of cells. Areas of pigmentation, dead space, fractures or folding of the tissues, and tissue that was neither ciliary body nor iris, were excluded with Adobe Photoshop.

Each image was quantified for expression of the labelling antibody. For CD3 and FoxP3 labelling, positive cells were counted manually, with help from the counter function in Photoshop. The area of non-excluded tissue in each image was measured using a macro in Volocity® software (PerkinElmer Inc, Massachusetts, USA), and this was used to normalise the cell counts for each image to standardised areas. For CD3 this was expressed as the number of cells per $100,000\mu m^2$, and for FoxP3 this was expressed as number of cells per $250,000\mu m^2$.

As the less distinct labelling for IL-17A meant that accurately counting individual cells was impractical, images were instead simply noted whether labelling was present or not.

Statistical analysis

Statistical analysis was conducted with the use of SPSS® software (IBM). A generalised estimating equation with ordinal logistic link function was used to assess associations between the expression of immunolabelling between experimental groups and between iris and ciliary body. To account for variations in the numbers of ROIs, an exchangeable correlation matrix was utilised. Graphs were made using R Software (R Foundation for Statistical Computing)

Results

Immunohistochemistry

Preliminary immunohistochemistry (IHC) on feline reactive lymph nodes showed moderate cytoplasmic and strong membranous anti-CD3 labelling of a population of lymphocytes, and strong nuclear anti-FoxP3 labelling of a smaller population of cells, while the no-primary negative controls showed no non-specific staining (Fig. 1)

Sections from all feline eyes with the diagnosis of lymphoplasmacytic anterior uveitis and FIP exhibited compelling positive immunolabelling for CD3 on the membranes of mononuclear inflammatory cells infiltrating the iris and ciliary body stroma (Fig. 2a,c,e). Nuclear labelling for FoxP3$^+$ cells was identified in all FIP and all but one uveitic eye, with varying degrees of intensity (Fig. 2b,d,f). For both antibodies immunolabelling occurred in the same anatomical regions, with FoxP3$^+$ cells present in far lower numbers. Labelling was strongest for in lymphoid follicles or clusters of infiltrating inflammatory cells. The base of the iris in uveitic eyes was a region consistently containing strong CD3 membranous labelling. Disease free controls showed no compelling positive labelling for either antibody, although weak non-specific FoxP3 labelling was seen in fibrous connective tissue.

In uveitic eyes, IL-17 labelling was seen in varying degrees in all samples, present as a weak cytoplasmic, nuclear and extracellular labelling found almost entirely in lymphoid follicles. Labelling was absent from FIP and non-disease control eyes (Fig. 3).

Statistical analysis showed that low grade uveitis had a lower CD3 cell count (mean=10.54 cells per 100,000μm^2) than high grade (mean =62.22 cells per 100,000μm^2, p<0.0001) and FIP (mean=59.49 cells per 100,000μm^2, p<0.0001), while FIP and high grade uveitis could not be differentiated (Fig. 4a). A similar result was found with FoxP3, with low grade uveitis (mean=0.59 cells per 250,000μm2) having significantly lower counts than high grade uveitis

(mean =5.05 cells per 250,000μm^2, p<0.0001) and FIP (mean=4.78 cells per 250,000μm^2, p<0.0001), while again no difference was found between FIP and high grade uveitis (Fig. 4b).

Discussion

Our work set out to determine whether feline lymphoplasmacytic anterior uveitis is characterised by an induction of pro-inflammatory T cell mediators associated with a disturbance of the balance of Tregs in the anterior uveal tract. Our data demonstrated no significant difference in FoxP3-labelling in the anterior uvea between the uveitis and FIP groups, which would appear to indicate that a reduction in the frequency of Tregs is not a contributing factor for the idiopathic form of the disease. Similarly, the number of FoxP3^{+} cells appeared to be directly proportional to the number of CD3^{+} cells in a sample, with low grade samples not having proportionally higher numbers of Tregs, suggesting that Treg number does not have a protective effect. This corresponds with the results of a study investigating the frequency of Tregs in human idiopathic uveitis, in which inflammation persisted despite the presence of Tregs (5). However, this does not rule out a functional deficit in the Tregs that are present.

Sun *et. al*. documented a correlation between the increased frequency of Tregs and increased chronicity of the disease in an experimental model of anterior uveitis, the highest expression coinciding with the peak stage of intraocular inflammation, preceding remission (8). In the current study, all the uveitic samples were obtained from cats with chronic manifestations of the disease. The high expression of FoxP3 found in this study could be attributed to the stage of the disease when the eyes were enucleated – our results may not reflect the initial phase of the disease. Further investigations using globes at different stages of the disease would help to define the role that Treg frequency plays in the development of uveitis in cats.

The expression of IL-17 was significantly higher in the uveitic eyes compared to the FIP samples. We speculate that Th17 cells are crucial to the pathogenesis of non-infectious uveitis, which has previously been documented in humans (6). Th17 cells could therefore potentially be a therapeutic target for feline idiopathic uveitis, and their suppression has already been shown to yield therapeutic benefit in uveitis in Behçet's disease in humans (9).

The method of immunocharacterising the ocular lesions relied on the expression of biomarkers associated with the respective cells of interest. There are some confounding factors accompanying this technique. FoxP3 can be transiently expressed by conventional, non-regulatory T-cells: therefore, positive labelling does not necessarily correspond to Tregs specifically (10). However, despite this caveat, previous reports have demonstrated cross-reactivity with this particular anti-human FoxP3 clone with feline tissue (11). Other types of immune cells are known to express FoxP3 in the cytoplasm, but the low level of background staining we found in the cytoplasm of most cells with this antibody unfortunately made these impossible to reliably identify. IL-17 was used as a biomarker for Th17 cells; however, it is important to note that a variety of innate immune cells also secrete this cytokine (12). Furthermore, no comparisons of primary structure or cross-reactivity data regarding this antibody for use in feline tissue were available. Therefore, Western blots or immunoprecipitation will need to be carried out to confirm cross-reactivity. In addition, other associated cytokines such as IL-6 and IL- 23 could be used in conjunction with IL-17A in order to help identify TH17 cells.

CD3 is a commonly used biomarker for T-cells and the anti-human antibody has been shown to be cross-reactive. However there are many subtypes of CD3$^+$ cells, and these may have different levels of involvement in the disease. Work with additional markers would be needed in further work to characterise the CD3$^+$ population described in this study.

Another limitation was the lack of patient data on treatment prior to enucleation, as it is likely that medications could have influenced the cell populations in the eye. This means our results may not be representative of the natural progression of the disease.

In conclusion, we demonstrated that IL-17 appears to be expressed in the idiopathic form of the disease, in contrast to that caused by FIP, but the sensitivity and cross-reactivity of this antibody have yet to be established and so a role for IL-17 in FIP-induced uveitis cannot be ruled out. The frequency of FoxP3$^+$ cells present in the uveitic eye appears to be directly proportional to the general level of inflammation and so does not appear to have a role in pathogenesis, although functional studies would be warranted to examine the potential role that differential activity of Tregs may play in the disease.

References

1. Gould D, McLellan G. BSAVA manual of canine and feline ophthalmology. 3rd ed. British Small Animal Veterinary Association: British Small Animal Veterinary Association; 2014.

2. Powell CC. Chapter 39 - Uveitis. In: Wingfield WE, editor. Veterinary Emergency Medicine Secrets (Second Edition) [Internet]. Philadelphia: Hanley & Belfus; 2001 [cited 2016 Jan 4]. p. 159–63.

3. Chavkin M, Lappin M, Powell, et al. Seroepidemiological and clinical observation of 93 cases of uveitis in cats. Prog Vet Comp Opthalmol 1992;2:29-36

4. Peiffer RL, Wilcock BP. Histopathologic study of uveitis in cats: 139 cases (1978-1988). J Am Vet Med Assoc. 1991 Jan 1;198(1):135–8.

5. Kottoor SH. Regulatory T cells in human uveitis. University of Birmingham; 2011.

6. Amadi-Obi A, Yu C-R, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, et al. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat Med. 2007 Jun;13(6):711–8.

7. Tang Q, Bluestone J. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol. 2008; 9 (3) pp: 239-244

8. Sun M, Yang P, Du L, Zhou H, Ren X, Kijlstra A. Contribution of CD4+CD25+ T cells to the regression phase of experimental autoimmune uveoretinitis. Invest Ophthalmol Vis Sci. 2010 Jan;51(1):383–9.

9. Sugita S, Kawazoe Y, Imai A, Yamada Y, Horie S, Mochizuki M, et al. Inhibition of Th17 differentiation by anti-TNF-alpha therapy in uveitis patients with Behçet's disease. Arthritis Res Ther. BioMed Central; 2012;14(3):R99.

10. Wang J, Ioan-Facsinay A, van der Voort EIH, Huizinga TWJ, Toes REM. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol. 2007 Jan;37(1):129–38.

11. Banham AH, Lyne L, Scase TJ, Blacklaws BA. Monoclonal antibodies raised to the human FOXP3 protein can be used effectively for detecting Foxp3(+) T cells in other mammalian species. Vet Immunol Immunopathol. 2009 Feb;127(3–4):376–81.

12. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol. Nature Publishing Group; 2010 Jul 18;10(7):479–89