CD4- and CD8-Expressing Cells in the Chambers of Normal, Cataract and Uveitic Eyes: A Comparative Study in Dogs

Tomasz Maślanka (✉ tomasz.maslanka@uwm.edu.pl)

University of Warmia and Mazury in Olsztyn <https://orcid.org/0000-0002-3807-0149>

Hubert Ziółkowski

Uniwersytet Warmińsko-Mazurski Wydział Medycyny Weterynaryjnej

Jacek Garncarz

Klinikum rechts der Isar der Technischen Universität München Klinik und Poliklinik für Augenheilkunde

Natalia Ziółkowska

Uniwersytet Warmińsko-Mazurski Wydział Medycyny Weterynaryjnej

Research article

Keywords: Aqueous humor, Eye chambers, CD4, CD8, Cataract, Uveitis, Dog

Posted Date: May 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-24926/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Research in Veterinary Science on October 1st, 2020. See the published version at <https://doi.org/10.1016/j.rvsc.2020.07.021>.

Abstract

Background The eye chambers constitute an immunologically privileged compartment. The presence of CD4⁺ and CD8⁺ cells in the chambers of normal and cataract eyes is a research problem that so far has been explored only fragmentarily. The first aim of our research was to determine whether CD4⁺ and CD8⁺ cells are present in the normal chambers of the eye in dogs, and if so, what the relative and absolute counts of these cells are. Another objective was to verify the hypothesis that uncomplicated cataract may be associated with the local recruitment of CD4⁺ and CD8⁺ cells.

Results The presence of CD4⁺ and CD8⁺ cells was detected in aqueous humor (AH) samples of normal and cataract eyes. However, on average only 55 and 236 events for combined subsets of CD4⁺ and CD8⁺ cells were collected for the entire volume of AH from normal and cataract eyes, respectively. The statistical analysis did not reveal significant differences in the percentage and absolute number of CD4⁺ cells between normal and cataract eyes. However, the values of these parameters in AH samples from cataract eyes were approximately 2- and 3-fold higher than in normal eyes, respectively. The mean percentage and absolute count of CD8⁺ cells increased approximately by 2.7- and 6-fold, respectively, in AH samples from cataract eyes compared to normal ones. The absolute count, but not the percentage, of CD4⁺ and CD8⁺ cells in AH of uveitic eyes was approximately 5- and 3-fold higher than in cataract eyes.

Conclusions The results indicate that CD4⁺ and CD8⁺ cells occur constitutively in the normal chambers of the eye in dogs. However, considering the extremely low abundance of these cells, it needs to be concluded that both of these cell populations appeared in trace amounts. The development of uncomplicated cataract in dogs may not be immunologically neutral in terms of the local immune response, but it may be associated with the recruitment of CD8⁺ cells into the eye chambers. This event does not seem to be of an inflammatory nature because it appears on a scale a few times smaller than in the course of uveitis.

Background

This study is the continuation of our previous investigations [1, 2], aiming to assess the presence of CD4⁺ and CD8⁺ T cells in the chambers of the normal eye. The eye is an immune privileged site, where some immune responses are down-regulated or completely abolished to protect the delicate internal structures of the eye from the damage and permanent injury that could be consequences of strong inflammatory responses. Blood-aqueous barriers are the fundamental structure restricting the ingress of blood cells into the anterior chamber of the eye. Taking into consideration this unique immune privilege status of the eye, we were intrigued whether eye chambers are completely devoid of CD4⁺ and CD8⁺ T cells or whether these cells are present at this site. CD4⁺ and CD8⁺ T cells occur not only in lymphoid tissues and inflamed non-lymphoid tissues, but also reside in uninfamed non-lymphoid tissues. Relatively much is known about tissue-resident memory T (Trm) CD4⁺ and CD8⁺ T cells which occupy peripheral tissues and organs without recirculating [3]. Another fact that should be taken into consideration is that eye chambers may

constitute part of the normal migratory pathway of naïve CD4⁺ and CD8⁺ T cells. It was once thought that only activated and effector memory T cells were able to access non-lymphoid tissues, while naïve T cells were assumed to recirculate exclusively between secondary lymphoid tissue. However, the research results obtained over the last 20 years indicate that naïve T cells routinely traffic through non-lymphoid organs in a manner similar to that of memory T cells [4–9]. In the light of these findings, it can be hypothesized that naïve CD4⁺ and CD8⁺ T cells may circulate through non-lymphoid tissues, such as the eye, as part of their normal migratory pathway.

A perusal of the literature has revealed complete lack of data on the occurrence of T cells in the eye chambers of healthy animals. There have been only few reports evaluating the CD4/CD8 ratio in aqueous humor (AH) of human patients with uveitis. AH samples collected from patients who had been operated on for uncomplicated cataracts served as control in these investigations. Thus, the underlying assumption was that AH from such patients was representative of the condition of a completely healthy eye with respect to the presence of CD4⁺ and CD8⁺ T cells. It is worth adding that results of the cited studies are not concordant in this regard. In some studies, these cells were not detected in AH samples [10–13]. In turn, Avundruk et al. [14, 15] determined the CD4/CD8 ratio in AH samples which were taken from patients operated on due to senile cataract. However, their study did not reveal what percentage within the lymphocyte population was composed of CD4⁺ and CD8⁺ T cells. Also, Martin et al. [16] found these cells in AH samples from patients operated on for uncomplicated cataracts; their study was the only one providing information about the percentages of CD4⁺ and CD8⁺ T cells in AH. Noteworthy the results of earlier studies indicated that CD4⁺ and CD8⁺ T cells were not present on normal rodent and human corneas [17–21]. However, more in-depth research demonstrated the presence of both types of cells on the corneas of naïve mice [22].

To the best of our knowledge, there were no research findings concerning the presence of CD4⁺ and CD8⁺ T cells in the eye chambers beside the aforementioned. In our opinion, no studies have been conducted thus far with the focus on establishing whether these cells occur in the chambers of healthy eyes, which is a serious gap in knowledge. We therefore undertook to perform broader investigations into this problem. In our previous papers, we presented results on the presence of CD4⁺ and CD8⁺ cells in the normal eye chambers of mouse [1], cattle and swine [2]. The results implicate very big interspecies differences in this respect. Moreover, interesting differences emerged in the proportion of CD4⁺ and CD8⁺ cells between peripheral blood (PB) and AH. The current study is the continuation of the above investigations, which broadens the research scope by including another animal species. Thus, the first aim was to determine whether CD4⁺ and CD8⁺ cells were present in the normal eye chambers of a dog, and if so, what the proportions of these subsets in the total lymphocyte population were, and whether there were any fundamental differences from the corresponding results obtained from PB samples.

However, the research had another underlying objective. Uncomplicated cataract [which first and foremost can be equated with age-related cataract (ACR)] is traditionally regarded as a “non-immunological” ocular disease. This view is predominantly anchored on the fact that the anterior

chamber is an immune-privileged compartment secluded from the immune system. Moreover, no inflammation was detected in ocular tissues of patients with ARC [23]. Therefore, in the studies quoted earlier, the AH samples collected from patients undergoing uncomplicated cataract surgery were treated as sample material representing the AH of a healthy eye. However, several reports implicate the involvement of immune mechanisms in the pathology of uncomplicated cataract [23–25]. Recently, it has been demonstrated that increased total IgE levels were significantly associated with ARC [26]. Circulating auto-antibodies against lens antigens are prevalent in patients with ARC [25]. As early as in 1979, Brinkman and Broekhuysse [24] indicated that leakage of lenticular proteins into AH (occurring during cataract maturation) may trigger the primary immune response leading to a population of sensitized lymphocytes. Considering all the above, we hypothesized that, independently from systemic immune responses, uncomplicated cataract may be associated with the local recruitment of CD4⁺ and CD8⁺ cells. Hence, another objective of our investigations was to verify this hypothesis by comparing the percentage and absolute counts of CD4⁺ and CD8⁺ cells in the eye chambers between dogs with normal and cataract eyes. Furthermore, we decided it was reasonable to confront and compare these results with the corresponding data in dogs with uveitis. Presentation of values of the evaluated parameters obtained in normal and cataract eyes in the context of the inflammation-induced influx of CD4⁺ and CD8⁺ cells into the eye chambers ensures a broader perspective, thus enabling us to see the proper scale of the results.

Results

The presence of CD4⁺ and CD8⁺ (i.e. CD4⁺CD8⁻ and CD4⁻CD8⁺ cells) cells was detected in AH samples of normal and cataract eyes. However, it should be emphasized that the absolute number of these cells was extremely low; on average, only $55 \pm (\text{SD}) 48$ and 236 ± 65 events for combined subsets of CD4⁺ and CD8⁺ cells were collected for the entire volume of AH obtained from normal and cataract eyes, respectively. This outcome means that the amount of CD4⁺ and CD8⁺ cells in AH samples from normal eyes was only slightly above the limit of detection by flow cytometry.

The statistical analysis did not reveal any significant difference in the values of percentage (Fig. 1a, b; Fig. 2) and absolute number (Fig. 1a', b'; Fig. 2) of CD4⁺ cells between normal and cataract eyes. However, it should be noted that the mean percentage and absolute count of CD4⁺ cells in AH samples from cataract eyes were almost 2- and 3-fold greater than in normal eyes, respectively. The percentage (Fig. 1c, d; Fig. 2) and absolute count (Fig. 1c', d'; Fig. 2) of CD8⁺ T cells in AH samples from cataract eyes were significantly ($p < 0.001$ and $p < 0.05$, respectively) higher than the values of corresponding parameters in normal eyes. The mean percentage and absolute count of CD8⁺ cells were increased by approximately 2.7- and 6-fold, respectively, in AH samples derived from cataract eyes.

Expectedly, the present study demonstrated a significant increase in the percentage and absolute number of CD4⁺ (Fig. 1a, a', b, b'; Fig. 2; $p < 0.05$ and $p < 0.001$, respectively) and CD8⁺ (Fig. 1c, c', d, d'; Fig. 2; $p < 0.01$ and $p < 0.001$, respectively) T cells in AH samples derived from uveitic eyes compared to normal ones. The mean percentage and absolute count of CD4⁺ cells were increased by approximately 3- and 15-

fold, respectively, in uveitic eyes. The values of corresponding parameters for CD8⁺ cells were about 3- and 18-fold greater in uveitic eyes compared with normal ones. The absolute count, but not the percentage, of CD4⁺ (Fig. 1a', b') and CD8⁺ (Fig. 1c', d') cells in AH samples from uveitic eyes was also considerably ($p < 0.001$) higher compared to cataract eyes; the mean values of these parameters were increased by approximately 5- and 3-fold, respectively. No differences were found in the CD4/CD8 ratio between any groups (Fig. 3).

Huge differences were seen at first glance in the percentages of the analyzed cells between AH and PB samples, which urged us to visualize these data graphically alongside the statistical analysis applied (Fig. 4). The percentage of CD4⁺ cells within the total lymphocyte population of PB in dogs with normal, cataract and uveitic eyes was approximately 10-, 7- and 4-fold higher ($p < 0.001$), respectively, than the corresponding values determined in AH (Fig. 4a). Similarly, the percentage of CD8⁺ cells was by 8- and 2.5-fold greater ($p < 0.001$) in PB of dogs with normal and cataract eyes, respectively, than the respective values obtained from AH samples (Fig. 4b). Although the frequency of CD8⁺ cells in PB of dogs with uveitis was by 2.4-fold higher than in AH, this difference was not significant (Fig. 4b). No differences were found in the CD4/CD8 ratio between PB and AH samples in both dogs with normal eyes and dogs with uveitis (Fig. 4c). In turn, the CD4/CD8 ratio was significantly lower ($p < 0.05$) in AH than in PB in dogs with cataract (Fig. 4c).

In addition, a case of a dog who choked on a bone was brought to attention in the context of the evaluated parameters. In contrast to normal, cataract, and even uveitic, eyes, the percentage, shape and location of CD4⁺ and CD8⁺ cell subsets in AH of this dog were almost the same as those determined in PB (Fig. 5).

Discussion

CD4⁺ and CD8⁺ cells were detectable in AH samples from normal eyes, hence these results strongly suggest that CD4⁺ and CD8⁺ cells occur constitutively in the eye chambers of the normal canine eye. Presumably, they belong to the pool of Trm cells or represent migratory naïve T cells, or both. However, amounts of the analyzed cells were only slightly above the limit of detection of flow cytometry. Therefore, it should be concluded that in terms of their absolute count, CD4⁺ and CD8⁺ cells represent only trace populations of cells in the eye chambers of the normal canine eye. This conclusion is relatively concordant with the outcome of our earlier research, in which we were able to demonstrate the presence of these cells in normal bovine and porcine chambers of the eye [2]. In both of these species, the said cells appeared in very low amounts, although their absolute count was distinctly higher than in dogs (which to some extent can be explained by the fact that the ocular globe of pigs and cows is much bigger than in dogs).

With respect to the percentage of CD4⁺ cells, much similarity to the results obtained in mice [1] and pigs but not in cattle [2] was achieved in the current study, as in the two former species the single positive

CD4⁺ cells constituted only a trace subset among the lymphocyte population of eye chambers. However, as regards the proportion of CD8⁺ in the lymphocyte population of the eye chambers, dogs are distinctly different from the other species. The value of this parameter in dogs was by 9-, 4- and 12-fold lower than in mice [1], cattle and pigs [2], respectively. These results are in agreement with our previous conclusion that the proportion of CD4⁺ and CD8⁺ subsets in the lymphocyte population of eye chambers can differ considerably between animal species.

In this study, all parameters assayed in AH versus their values in PB were compared. Such comparisons might be considered as redundant because considerable differences in the composition of the lymphocyte population between PB and the effector sites of immune response (and the eye chambers should be counted as a site of this type) are a natural event. However, such comparisons make it more evident that the proportion of CD4⁺ and CD8⁺ cells in the lymphocyte population of the eye chambers of normal eyes is very small; the mean values of both parameters equalled mere 3.66 and 3.28%, respectively, which means that they were by 10- and 8-fold lower, respectively, than the analogous percentages in PB. It can be suspected that such low and specific homing of CD4⁺ and CD8⁺ cells to the eye chambers is involved in the induction and maintenance of ocular immune privilege. Let us not ignore the finding arising from our research, i.e. that the combined proportion of CD4⁺ and CD8⁺ cells constitutes no more than 7% of the total lymphocyte population of the eye chambers. Determination of what type of other immunocompetent cells hides within this population requires further tests including a wider antibody panel.

Another purpose of confronting the data obtained from AH samples with the corresponding data achieved in PB was to confirm that the small percentage of CD4- and CD8-expressing cells in AH samples was not caused by erroneous labeling of cells, or that the presence of lymphocytes in AH samples was not due to their contamination with blood. The typical distribution of CD4- and CD8-expressing cells in PB samples as well as the fact that this distribution was distinctly different from the one determined in AH samples prove that this aim was achieved. Some doubt may arise whether the presence of lymphocytes in eye chambers might not have been a consequence of their postmortem extravasation from the systemic circulation into eye chambers. However, if that had been the case, the percentage of the analyzed cells would have been very close to the one identified in PB. This line of reasoning is supported by the results concerning the distribution of CD4⁺ and CD8⁺ cells in AH sample from a dog which choked on a bone. The percentage, shape and location of both cell subsets in AH were almost the same as those in PB. Furthermore, the number of events collected for combined subsets of CD4⁺ and CD8⁺ cells in AH sample of this dog was approximately 12-fold higher (data not shown) than in AH samples from normal eyes. It is common knowledge that strangulation may cause capillary rupture in the conjunctiva and sclera. Therefore, these results strongly suggest that choking may result in passive leakage of lymphocytes across damaged blood vessels into the eye chambers. To the best of our knowledge, this is the first report on this subject.

The mean percentage and absolute count of CD4⁺ cells in AH samples from cataract eyes were almost 2- and 3-fold greater than in normal eyes, respectively. Nevertheless, these differences were not statistically

significant. Hence, there is only enough evidence to claim that there was a certain trend towards an increasing number of CD4⁺ cells in cataract eyes. The situation looks different in the case of CD8⁺ cells. The percentage and absolute counts of these cells in the eye chambers of the cataract eyes were increased by about 2.7- and 6-fold, respectively, compared to normal ones, and the differences were significant. These results strongly suggest that in terms of the local immune response, the development of uncomplicated cataract in dogs may not be immunologically neutral, but rather it may be associated with the recruitment of CD8⁺ cells into the eye chambers. This conclusion implicates that in terms of the presence of CD8⁺ cells in the eye chambers, the cataract eye may not represent faithfully the condition of a healthy eye, as it is suspected. For reasons discussed in the section “A major limitation of the study”, the currently available research capabilities preclude any deeper interpretation of the cataract-related local CD8⁺ cell recruitment or the trend demonstrated by CD4⁺ cells towards this direction, as such phenotypes might hide different types of cells (e.g. naïve, memory, effector or regulatory T cells or even non T cells).

The mean percentage and absolute count of CD4⁺ cells were increased approximately 3- and 15-fold in uveitic eyes, respectively, compared to normal eyes. The values of corresponding parameters for CD8⁺ cells were about 3- and 18-fold greater in uveitic eyes than in normal ones. These results indicate that uveitis was associated with the infiltration of the eye chambers by CD8⁺ and CD4⁺ T cells. To our knowledge, this is the first report on the evaluation of the numbers of these cells in the eye chambers of dogs with uveitis. However, this is not an original finding, because it has been widely accepted that T cells play a predominant role in uveitis [12, 16, 27, 28]. The relatively selective recruitment of CD8⁺ and CD4⁺ T cells into eye chambers was reported in patients with Fuchs’ heterochromic iridocyclitis and idiopathic anterior uveitis [27], respectively. Considering the literature data [28, 29], it can be assumed that CD8⁺ and CD4⁺ cells infiltrating the chambers of uveitic eyes constitute largely proinflammatory T cells.

It should be highlighted that the assessment of the abundance of the analyzed cells in the eye chambers of uveitic eyes was not a research problem *per se* as set in this study. A group of dogs with uveitis was included so as to confront the scale of cataract- and uveitis-induced changes in evaluated parameters, hence it served as a reference. The mean absolute counts of CD8⁺ and CD4⁺ cells in the eye chambers of uveitic eyes were approximately 3- and 5-fold higher, respectively, compared to cataract eyes. Thus, the demonstrated increase in the number of CD8⁺ cells in the eye chambers of cataract eyes was much smaller than the one occurring in uveitic eyes. The study did not show differences in the CD4/CD8 ratio between any of the experimental groups, which means that CD8⁺ cells increased proportionally to CD4⁺ cells. This conclusion confirms our suspicion that in the course of canine cataract there is a tendency towards an increasing number of CD4⁺ cells in the eye chambers. However, it needs to be emphasized strongly that the scale of this trend was minimal when compared to the impressive increase in the CD4⁺ cell counts in uveitic eyes.

Conclusions

The results indicate that CD4⁺ and CD8⁺-expressing cells occur constitutively in the eye chambers of the normal canine eye. However, considering the extremely low abundance of these cells, it needs to be concluded that the presence of both populations in the chambers of normal canine eye is within trace amounts. The development of uncomplicated cataract in dogs may not be immunologically neutral in the aspect of the local immune response, but rather it may be associated with the recruitment of CD8⁺ cells into the eye chambers. This phenomenon does not seem to be of an inflammatory nature, because its scale is a few times smaller than in the course of uveitis.

A Major Limitation Of The Study

Traditionally, the CD4⁺ and CD8⁺ phenotypes have been considered as helper and cytotoxic/suppressor T lymphocytes, respectively. However, currently it has emerged from research on human and murine models that CD4⁺ and CD8⁺ T cells include various effector and regulatory subsets. Furthermore, although CD4 and CD8 are predominantly expressed on T lymphocytes, these molecules are also present, but to a very limited extent, on dendritic and certain other cells. In our previous study [1] conducted on mice, we were able to subdivide CD8⁺ T cells into some subsets because AH withdrawn from globes of several mice was pooled into one sample. Moreover, it was interesting to discover that the cell yield was disproportionally larger than in pigs, cattle [2] or dogs. Unfortunately, in the current study it was impossible to distinguish particular subsets within the two analyzed populations for two reasons. Firstly, the lymphocyte yield from AH of normal or cataract eyes of single dogs was obtained only in trace amounts, making it unfeasible to subdivide the CD4⁺ and CD8⁺ T cell subset into yet smaller subsets. And secondly, antibodies to perform flow cytometric differentiation of canine CD4⁺ and CD8⁺ T cells into particular subtypes are almost nonexistent. In view of these facts, in order to exercise due caution, we refrained from performing deeper immunological interpretation of the results with regard to the presence of CD4⁺ and CD8⁺ cells in the chambers of normal eye, or the meaning of the presence as well as changes in the number of these cells in relation to the development of cataract. The current state of knowledge makes it obvious that the CD4⁺ and CD8⁺ phenotypes can hide different types of cells. However, their identification was impossible, and hence any interpretation would be purely speculative.

Methods

Animals

The study presented in this article was neither experimental nor clinical in its essence. The dogs involved were not submitted to any procedure to obtain the results discussed in this article. We were able to gather the experimental material owing to the kind help of both the owners of the dogs from which the material was obtained and the veterinary doctors who acted as intermediaries in collecting this material. In most cases, AH was obtained during a surgical procedure on dogs due to uncomplicated or senile cataracts (dogs no 10–16) or lens luxation (dogs no 19–22). Such surgeries require the emptying of eye chambers

of AH. It is therefore useful to stress that the collection of AH was not associated with any loss incurred to a canine patient. In some cases, AH was collected directly after the death of dogs which had to be euthanized for verifiable reasons. These dogs either had no symptoms of ocular disease (dogs no 1–9), or were diagnosed as having cataract (dogs no 17 and 18) not accompanied by any other ophthalmological disorder. Characteristics of dogs and the number assigned to each dog are shown in Table 1. Blood samples were taken while making injections to administer an agent for anaesthesia or euthanasia. In each case, the owners expressed consent for intraoperative or postmortem collection of AH and PB samples for scientific research. AH and PB were sampled by the veterinary doctors performing an eye surgery or euthanasia of the animals. The material was obtained from small animal clinics in Biskupiec, Olsztyn and Warsaw, in 2016–2019. The acquisition of biological material for research under the circumstances as described above (i.e. acquisition of fluids or tissues lost by the patient during a surgical treatment or from animals that were euthanized for reasons unrelated with the study) does not require an approval of the Ethics Commission in Poland.

Table 1
Characteristics of dogs and the number assigned to each dog

Normal eyes			Cataract eyes			Uveitic eyes		
Dog no.	Age (approx. years) / Sex	Breed	Dog no.	Age (approx. years) / Sex	Breed	Dog no.	Age (approx. years) / Sex	Breed
1	3/♀	German shepherd	10	3/♀	Mixed breed	19	4/♀	Fox Terrier
2	8/♀	Mixed breed	11	5/♀	Jack Russell Terrier	20	6/♀	British bulldog
3	10/♀	Golden Retriever	12	6/♀	Polish Lowland Sheepdog	21	7/♀	Mixed breed
4	10/♀	Mixed breed	13	7/♀	Yorkshire Terrier	22	11/♀	Cocker Spaniel
5	11/♀	Boxer	14	8/♀	Mixed breed			
6	13/♀	Dalmatian	15	9/♀	Greater Swiss Mountain Dog			
7	13/♀	Mixed breed	16	10/♀	Mixed breed			
8	15/♀	Mixed breed	17	14/♀	Mixed breed			
9	16/♀	Mixed breed	18	15/♀	Mixed breed			

Staining For Flow Cytometry

AH was collected during surgery. In the remaining cases, immediately after euthanasia, aqueous humor was taken from the anterior chamber of the eye by aspiration with a syringe. AH was collected into tubes containing fluorescence-activated cell sorting (FACS) buffer [FB; Dulbecco's PBS devoid of Ca^{2+} and Mg^{2+} with 2% (v/v) heat-inactivated FBS (both from Sigma-Aldrich)] and centrifuged. PB was obtained aseptically, being drawn from the cephalic vein into heparinized sterile vacutainer tubes [Becton Dickinson (BD) Biosciences, San Jose, CA, USA]. Red blood cells were removed by lysis in BD Pharm Lyse buffer (BD Biosciences) according to the manufacturer's protocol. AH and PB samples were washed in 2 mL FB, and subsequently, cells were resuspended in FB and stained with fluorochrome-conjugated monoclonal antibodies (mAbs): anti-canine CD4 FITC (1:40; clone YKIX302.9) and anti-canine CD8a APC

(1:40; clone YCATE55.9; both from eBioscience, San Diego, CA, USA). After 45 min incubation (on ice and in the dark), the cells were washed in 2 mL FB and analyzed by flow cytometry.

Facs Acquisition And Analysis

Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences). The data were acquired by FACSDiva version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, USA). The cytometry setup and tracking beads (BD Biosciences) were used to initialize PMT settings. Unstained and single fluorochrome-stained PB samples were used to set fluorochrome compensation levels. In order to maximise cell yield, AH samples were diluted to 500 μ L with FB and acquired at a low flow rate. The entire volume of each AH sample was always acquired. Apart from assessing the percentage of CD4⁺ and CD8⁺ cells, the semi-absolute quantification of these cells was performed (for simplification, referred to as “absolute count/number”). Since the flow cytometer flow-rate is constant and samples were resuspended in the same volume, the number of events acquired for CD4⁺ and CD8⁺ cells can be compared. This method was successfully used to estimate the number of cells derived from the conjunctiva of dry eye patients [30]. FlowJo software provides directly the number of cell events per sample. Thus, the absolute number of CD4⁺ and CD8⁺ cells represents the number of cells collected from the entire volume of AH samples. To ensure mutual comparability of this parameter, the number of CD4⁺ and CD8⁺ cells collected from AH samples derived from one eye (dogs no 10–16 and 19–22) was multiplied by 2 (in the remaining dogs AH was collected from both eyes). The lymphocyte gate usually could not be defined in AH samples as such because of extremely low cell yield. Therefore, the lymphocyte population was gated - on the basis of forward and side scatter properties - in PB samples. The positioning of this gate served as an approximate reference for setting the lymphocyte gate in AH samples.

Statistical Analyses

Data were expressed as the mean ($\pm$ SD) for 9 (normal and cataract eyes) or 4 (uveitic eyes) dogs per group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post-hoc* test or unpaired Student's t-test. Differences were deemed significant when the *p* values were < 0.05.

Abbreviations

AH

Aqueous humor; APC:Allophycocyanin; FB:Fluorescence-activated cell sorting buffer; FITC:Fluorescein isothiocyanate; PB:peripheral blood; SD:Standard deviation; Trm:tissue-resident memory T cells

Declarations

Ethics approval and consent to participate

The acquisition of biological material for research under the circumstances as described in this study (i.e. acquisition of fluids or tissues lost by the patient during a surgical treatment or from animals that were euthanized for reasons unrelated with the study) does not require an approval of the Ethics Commission in Poland.

Consent for publication

Not applicable

Availability of data and materials

Data are available on request from the authors.

Competing interests

The authors declare that they have no conflict of interests.

Funding

The project financially supported by Minister of Science and Higher Education in the range of the program entitled "Regional Initiative of Excellence" for the years 2019-2022, Project No. 010/RID/2018/19, amount of funding 12.000.000 PLN.

The project was co-supported by a grant (No. 15.610.008-300) from the University of Warmia and Mazury in Olsztyn.

Authors' contributions

TM conceived and designed the study, performed the laboratory work, analyzed and interpreted the data, and wrote the manuscript. HZ performed statistical analyses, participated in the laboratory assays and in writing of the manuscript. JG collected samples and participated in data interpretation. NZ made substantial contributions to the design of the project, collected samples, participated in data interpretation and was involved in drafting the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The authors would like to thank the following individuals for providing the research material: Sylwia Lew, DVM, PhD, Elżbieta Mikulska-Skupień, DVM, PhD and Piotr Socha, DVM, PhD.

References

1. Maślanka T, Ziółkowska N, Ziółkowski H, Małaczewska J. CD25⁺CD127⁺Foxp3⁻ cells represent a major subpopulation of CD8⁺ T cells in the eye chambers of normal mice. *PLoS One*. 2017;12:e0170021.
2. Maślanka T, Socha P, Ziółkowska N, Dąbrowski M, Małaczewska J, Ziółkowski H. CD4- and CD8- expressing cells found in bovine and porcine anterior chamber. *Pol J Vet Sci*. 2018;21:293–8.
3. Schenkel JM, Masopust D. *Tissue-resident memory T cells*. *Immunity*. 2014;41:886 – 97.
4. Luettig B, Pape L, Bode U, Bell EB, Sparshott SM, Wagner S, Westermann J. Naive and memory T lymphocytes migrate in comparable numbers through normal rat liver: activated T cells accumulate in the periportal field. *J Immunol*. 1999;163:4300–7.
5. Luettig B, Kaiser M, Bode U, Bell EB, Sparshott SM, Bette M, Westermann J. Naive and memory T cells migrate in comparable numbers through the normal rat lung: only effector T cells accumulate and proliferate in the lamina propria of the bronchi. *Am J Respir Cell Mol Biol*. 2001;25:69–77.
6. Westermann J, Ehlers EM, Exton MS, Kaiser M, Bode U. Migration of naive, effector and memory T cells: implications for the regulation of immune responses. *Immunol Rev*. 2001;184:20–37.
7. Cose S, Brammer C, Khanna KM, Masopust D, Lefrancois L. Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. *Eur J Immunol*. 2006;36:1423–33.
8. Cose S. T-cell migration: a naive paradigm? *Immunology*. 2007;120:1–7.
9. Lewis M, Tarlton JF, Cose S. Memory versus naive T-cell migration. *Immunol Cell Biol*. 2008;86:226–31.
10. Flynn TH, Mitchison NA, Ono SJ, Larkin DF. *Aqueous humor alloreactive cell phenotypes, cytokines and chemokines in corneal allograft rejection*. *Am J Transplant*. 2008;8:1537-43.
11. Kim TW, Chung H, Yu HG. Analysis of Intraocular Chemokine and Chemokine Receptor in Patients with HLA-B27-associated Anterior Uveitis. *J Korean Ophthalmol Soc*. 2008;49:1475–82.
12. Sanz-Marco E, Garces M, Sempere A, Diaz-Llopis M. *CD4/CD8 ratio in aqueous humor in Uveitis*. *Ocul Immunol Inflamm*. 2013;21:408-9.
13. Dave N, Chevour P, Mahendradas P, Venkatesh A, Kawali A, Shetty R, et al. *Increased Aqueous Humor CD4+/CD8 + Lymphocyte Ratio in Sarcoid Uveitis*. *Ocul Immunol Inflamm*. 2019;27:1033-40.
14. Avunduk AM, Cetinkaya K, Kapicioğlu Z. Comparison of T helper/T suppressor ratios in aqueous humor and blood samples by flow cytometric analysis. *Ophthalmologica*. 1997;211:84–6.
15. Avunduk AM, Avunduk MC, Tekelioğlu Y, Kapicioğlu Z. CD4⁺ T cell/CD8⁺ T cell ratio in the anterior chamber of the eye after penetrating injury and its comparison with normal aqueous samples. *Jpn J Ophthalmol*. 1998;42:204–7.
16. Martin CM, Santos Lacombe M, Serrano Molina CI, Ramirez Chamond R, Collantes Estevez E. *ICAM-1 expression on the surface of T lymphocytes in patients with uveitis: a comparative study between the eye and peripheral blood*. *Ophthalmologica*. 2002;216:203-8.

17. Planck SR, Rich LF, Ansel JC, Huang XN, Rosenbaum JT. Trauma and alkali burns induce distinct patterns of cytokine gene expression in the rat cornea. *Ocul Immunol Inflamm.* 1997;5:95–100.
18. Ghiasi H, Wechsler SL, Cai S, Nesburn AB, Hofman FM. The role of neutralizing antibody and T-helper subtypes in protection and pathogenesis of vaccinated mice following ocular HSV-1 challenge. *Immunology.* 1998;95:352–9.
19. Kuffova L, Holan V, Lumsden L, Forrester JV, Filipec M. Cell subpopulations in failed human corneal grafts. *Br J Ophthalmol.* 1999;83:1364–9.
20. Hazlett LD, McClellan SA, Rudner XL, Barrett RP. The role of Langerhans cells in *Pseudomonas aeruginosa* infection. *Invest Ophthalmol Vis Sci.* 2002;43:189–97.
21. Streilein JW. Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. *J Leukoc Biol.* 2003;74:179–85.
22. Mott KR, Osorio Y, Brown DJ, Morishige N, Wahlert A, Jester JV, Ghiasi H. The corneas of naive mice contain both CD4⁺ and CD8⁺ T cells. *Mol Vis.* 2007;28;13:1802-12.
23. Ibaraki N, Lin LR, Dang L, Reddy VN, Singh DP, Sueno T, Chylack LT Jr, Shinohara T. Anti-beta-crystallin antibodies (mouse) or sera from humans with age-related cataract are cytotoxic for lens epithelial cells in culture. *Exp Eye Res.* 1997;64:229–38.
24. Brinkman CJ, Broekhuysen RM. Cell mediated immunity in relation to cataract and cataract surgery. *Br J Ophthalmol.* 1979;63:301–5.
25. Shinohara T, Singh DP, Chylack LT Jr. Review. Age-related cataract: immunity and lens epithelium-derived growth factor (LEDGF). *J Ocul Pharmacol Ther.* 2000;16:181 – 91.
26. Yoo TK, Kim SW, Seo KY. Age-Related Cataract Is Associated with Elevated Serum Immunoglobulin E Levels in the South Korean Population: A Cross-Sectional Study. *PLoS One.* 2016;11:e0166331.
27. Muhaya M, Calder V, Towler HM, Shaer B, McLauchlan M, Lightman S. Characterization of T cells and cytokines in the aqueous humour (AH) in patients with Fuchs' heterochromic cyclitis (FHC) and idiopathic anterior uveitis (IAU). *Clin Exp Immunol.* 1998;111:123–8.
28. Muhaya M, Calder VL, Towler HM, Jolly G, McLauchlan M, Lightman S. Characterization of phenotype and cytokine profiles of T cell lines derived from vitreous humour in ocular inflammation in man. *Clin Exp Immunol.* 1999;116:410–4.
29. Sharma RK, Gupta V, Bansal R, Sharma K, Gupta A, Sachdeva N. Immune Profiling of T Cells Infiltrating Vitreous Humor in Tubercular Uveitis. *Immunol Invest.* 2018;47:615–31.
30. Barabino S, Montaldo E, Solignani F, Valente C, Mingari MC, Rolando M. Immune response in the conjunctival epithelium of patients with dry eye. *Exp Eye Res.* 2010;91:524-9.

Figures

Figure 1

The number of CD4⁺ and CD8⁺ cells in the aqueous humor of normal, cataract and uveitic canine eyes. The bars depict group mean ($\pm$ SD) (a, a', c, c') and the dots represent individual dogs (b, b', c, c'). (a, b, c, d) The relative count is expressed as a percentage of CD4- and CD8-expressing T cells within the total lymphocyte population. (a', b', c', d') The semi-absolute quantification represents the total number of CD4⁺ or CD8⁺ events acquired by flow cytometry for the entire volume of sample. Statistical differences

were evaluated by one-way ANOVA followed by Student-Newman-Keuls post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Selected cytograms showing the distribution of CD4+ and CD8+ cells in aqueous humor (AH) and peripheral blood (PB) of dogs with normal, cataract and uveitic eyes. The results are expressed as percentages of CD4+CD8- (lower right quadrant) and CD4-CD8+ (upper left quadrant) cells within the lymphocyte gate. PB samples served, among other things, to verify the accuracy of staining method and gate placement. Furthermore, the existence of the enormous differences in the percentages of CD4+ and CD8+ cells between PB and AH samples excludes the possibility of contamination of AH samples by lymphocytes from PB.

Figure 3

CD4/CD8 ratio in the aqueous humor of normal, cataract and uveitic canine eyes. The bars depict group mean ($\pm$ SD) (a) and the dots represent individual dogs (a').

Figure 4

The percentage of CD4⁺ and CD8⁺ cells and their ratio in the aqueous humor (AH) and peripheral blood (PB) of dogs with normal, cataract and uveitic eyes. (a, b) The results are expressed as a percentage of CD4- and CD8-expressing cells within the total lymphocyte population. Differences between PB with AH within individual groups were evaluated by unpaired Student's t-test; *p < 0.01, **p < 0.001. Additionally, PB parameters were compared among all groups by one-way ANOVA, but this analysis did not reveal significant differences.

Figure 5

Distribution of CD4+ and CD8+ cells in the aqueous humor and peripheral blood in a dog which choked on a bone.