Original article

CD4- and CD8-expressing cells found in the bovine and porcine anterior chamber of the eye

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Abstract

The aim of the present study was to investigate whether the anterior chamber constitutes part of the normal migratory pathway of $CD4^+$ and $CD8^+$ lymphocytes in cattle and swine. The cells obtained from aqueous humor of cows and pigs were stained for CD4 and CD8 receptors, and subsequently analyzed with flow cytometry. The mean percentage of $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^+CD8^+$ cells within the total lymphocyte population of the bovine anterior chamber was, respectively, 17.88, 12.64 and 27.26%. In turn, the mean values of these parameters in pigs were 1.77, 38.48 and 17.45, respectively. Among bovine and porcine $CD4^+CD8^+$ cells prevalent were those displaying $CD4^{low}CD8^{low}$ and $CD4^{low}CD8^{high}$ phenotypes, respectively. The results suggest that the anterior chamber in cattle and swine is an element in the normal migratory pathway of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ cells. Furthermore, the contribution of these subsets in the anterior chamber lymphocyte population can differ considerably between animal species.

Key words: anterior chamber, $CD4^+$ cells, $CD8^+$ cells, DP cells, cattle, pig

Introduction

It was commonly held that only activated and effector memory T cells migrate/home to non-lymphoid tissues. In contrast, naive T cells were thought to recirculate exclusively between secondary lymphoid tissue via the blood and lymphatic systems. Evidence is now emerging that this view may be too simplistic and that naive T cells routinely traffic through non-lymphoid organs in a manner similar to that of memory T cells

(Cose 2007). A series of studies performed over the past 15 years clearly indicates that naive T cells may circulate through non-lymphoid tissues as part of their normal migratory pathway (Westermann et al. 2001, Cose et al. 2006, Luettig et al. 2007). Taking into consideration this fundamental shift in the current paradigm of T cells migration through different types of tissue, the purpose of the present study was to investigate whether the anterior chamber constitutes part of the normal migratory pathway of CD4$^+$ and CD8$^+$ lymphocytes in cattle and swine. It is worth mentioning that apart from single reports (Avundruk et al. 1997, 1998, Maślanka et al. 2017) concerning humans and mice, the available literature lacks data regarding the presence of CD4$^+$ and CD8$^+$ cells in the anterior chamber of healthy humans and animals.

Materials and Methods

Animals and sample collection

Globes and peripheral blood (PB) were obtained from 5-year-old cows (n=20) and 5- to 6-month-old pigs (n=16) slaughtered in abattoirs in north-eastern Poland. Law in Poland (Act of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes) does not require a permit from an ethics commission to conduct experiments in which samples for research are obtained *post mortem* from animals not submitted to any procedure while alive. Globes were collected on average 1 h after slaughter and transported on ice within 2 to 3 h to the laboratory. They did not show any abnormalities or signs of disease. Immediately afterwards, the cornea was incised with a 2.75-mm corneal knife (Kai Medical, Gifu, Japan) and then aqueous humor (AH) from both globes of the same animal was poured into a tube containing fluorescence-activated cell-sorting buffer [FB; 1 x Dulbecco's PBS devoid of Ca^{2+} and Mg^{2+} with 2% (v/v) heat-inactivated FBS] (both from Sigma-Aldrich, Schnelldorf, Germany).

Staining for flow cytometry analysis

Samples of PB were prepared as it was previously described (Maślanka 2014). The cells were re-suspended in FB and stained for surface antigens with fluorochrome conjugated monoclonal antibodies (1:20): (a) cattle: AF647 mouse anti-bovine CD4 (clone CC8) and FITC mouse anti-bovine CD8 (clone CC63); (b) pigs: PE mouse anti-porcine CD4 (clone MIL17) and FITC mouse anti-porcine CD8 alpha (clone MIL12) (all from Serotec, Oxford, UK).

FACS acquisition and analysis

Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA). The data were acquired by FACS*Diva* version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, CA, USA). Unstained and single fluorochrome-stained samples were used to set fluorochrome compensation levels. The entire volume of each sample was always acquired. As the first step, the lymphocyte population in peripheral blood was gated on the basis of forward and side scatter properties. The location of this gate served as a point of reference to set the lymphocyte gate for AH samples. CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^+$CD8$^+$ cell subsets were defined according to the expression of CD4 and CD8 within the gated lymphocyte subpopulation. Fluorescence minus one (FMO) controls were used to establish the gating strategy for identifying CD4- and CD8-expressing cells.

Results

Distribution of CD4$^+$ and CD8$^+$ single positive cells and CD4$^+$CD8$^+$ double positive cells in the bovine anterior chamber of the eye

It should be underlined that the number of lymphocytes in the bovine anterior chamber was extremely low; on average, only 2054 ± (SD) 958 events in the lymphocyte gate were collected for the entire volume of AH samples. In the analyzed samples of bovine AH, the presence of lymphocytes with the phenotypes CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ as well as CD4$^+$CD8$^+$ [further referred to as CD4$^+$, CD8$^+$ and DP (double positive) cells, respectively] was determined. The mean percentage of AH CD4$^+$ lymphocytes was 17.88 ± 6.73, and did not differ significantly from the value of this parameter obtained in PB (Fig. 1A). The percentage of CD4$^{high (hi)}$ and CD4$^{low (lo)}$ cells was not observed to differ considerably between AH and PB (Fig. 1B and B'). The mean percentage of AH CD8$^+$ cells was 12.64 ± 8.65, thus being significantly lower (p<0.001) than the value of this parameter for PB (Fig. 1A). It was observed that percentages of CD8^{hi} and CD8^{lo} cells within the total lymphocyte population were considerably lower (p<0.001) in AH than in PB (Fig. 1C). On the other hand, it was not demonstrated that the percentage of these cells within CD8$^+$ cell subset was considerably different between AH and PB (Fig. 1C'). Thus, with respect to shares of cells with high and low expression of CD8 molecule, bovine AH CD8$^+$ cells do not differ from their counterpart in PB. Quite unexpectedly, it was found than DP cells prevailed in their number over
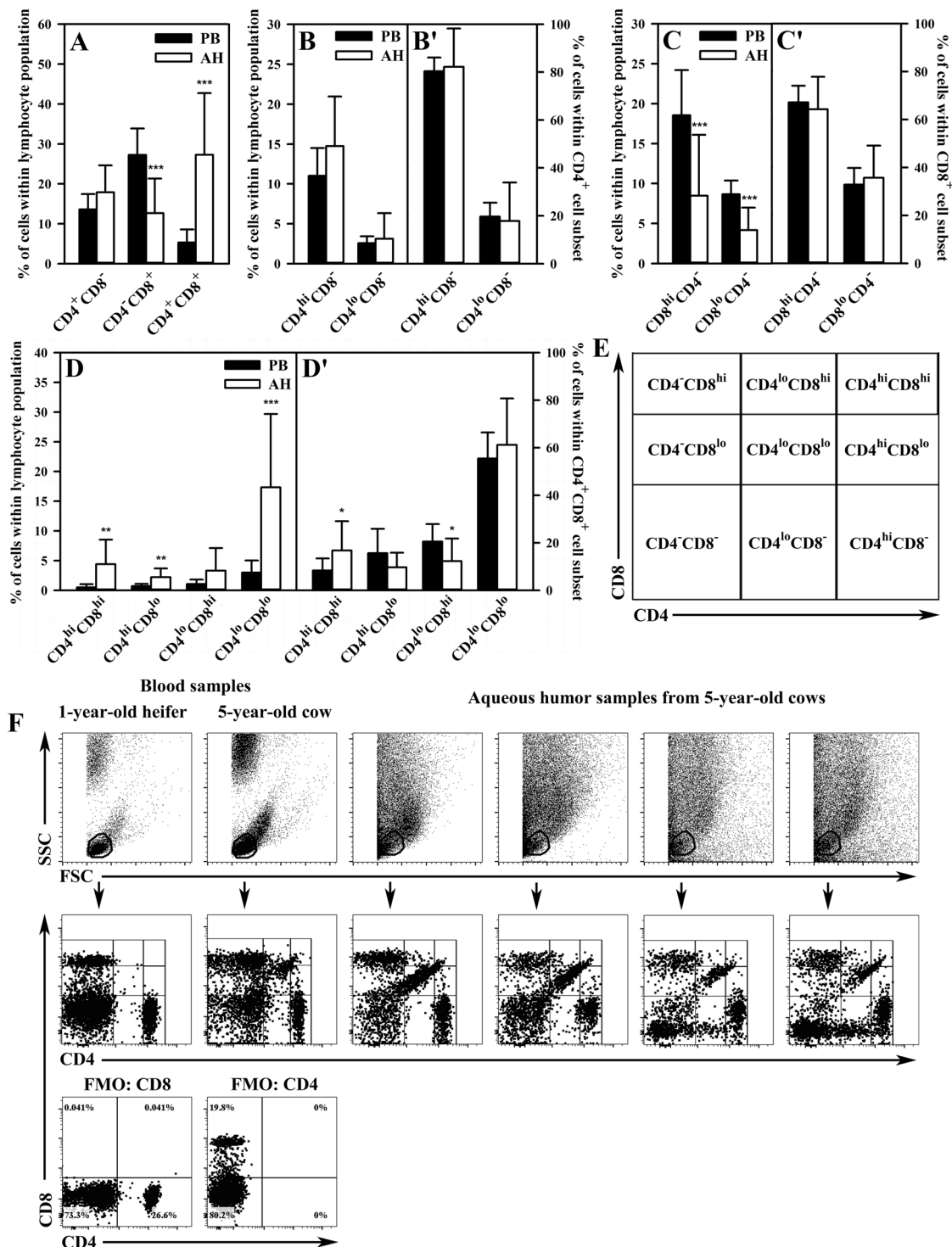
Fig. 1. Comparative analysis of the percentage of CD4+CD8-, CD4-CD8+ and CD4+CD8+ cells and their selected subsets in peripheral blood (PB) and aqueous humor (AH) of cattle. The results are expressed as a percentage of: (i) CD4+CD8-, CD4-CD8+ and CD4+CD8+ cells within the total lymphocyte population (A); (ii) CD4$^{high(hi)}$CD8-, CD4$^{low(lo)}$CD8- (B), CD4-CD8^{hi}, CD4-CD8^{lo} (C), CD4^{hi}CD8^{hi}, CD4^{hi}CD8^{lo}, CD4^{lo}CD8^{hi} and CD4^{lo}CD8^{lo} cells (D) within the total lymphocyte population; (iii) CD4^{hi}CD8-, CD4^{lo}CD8- (B'), CD4-CD8^{hi}, CD4-CD8^{lo} (C'), CD4^{hi}CD8^{hi}, CD4^{hi}CD8^{lo}, CD4^{lo}CD8^{hi} and CD4^{lo}CD8^{lo} cells (D') within CD4+CD8-, CD4-CD8+ and CD4+CD8+ cell subsets, respectively. Results are the mean (± SD) of two independent experiments with 10 animals per experiment (n=20, *p<0.05, **p<0.01, ***p<0.001, unpaired Student's t-test). Examples of cytograms of selected samples of PB and AH (F). As the first step, the lymphocyte population in PB was gated on the basis of forward and side scatter (FSC and SSC, respectively; panel 1). The location of this gate served as a point of reference to set the lymphocyte gate for aqueous humor samples. CD4+CD8-, CD4-CD8+ and CD4+CD8+ T cell subsets were defined according to the expression of CD4 and CD8 within the gated lymphocyte subpopulation. Relative to the intensity of CD4 and CD8 expression, the CD4+CD8-, CD4-CD8+ and CD4+CD8+ cell populations were subdivided into CD4^{hi}CD8-, CD4^{lo}CD8-, CD4-CD8^{hi}, CD4-CD8^{lo}, CD4^{hi}CD8^{hi}, CD4^{hi}CD8^{lo}, CD4^{lo}CD8^{hi} and CD4^{lo}CD8^{lo} subsets (E and F, panel 2). Fluorescence minus one (FMO) controls were used to establish the gating strategy for identifying CD4- and CD8-expressing cells (F, panel 3).
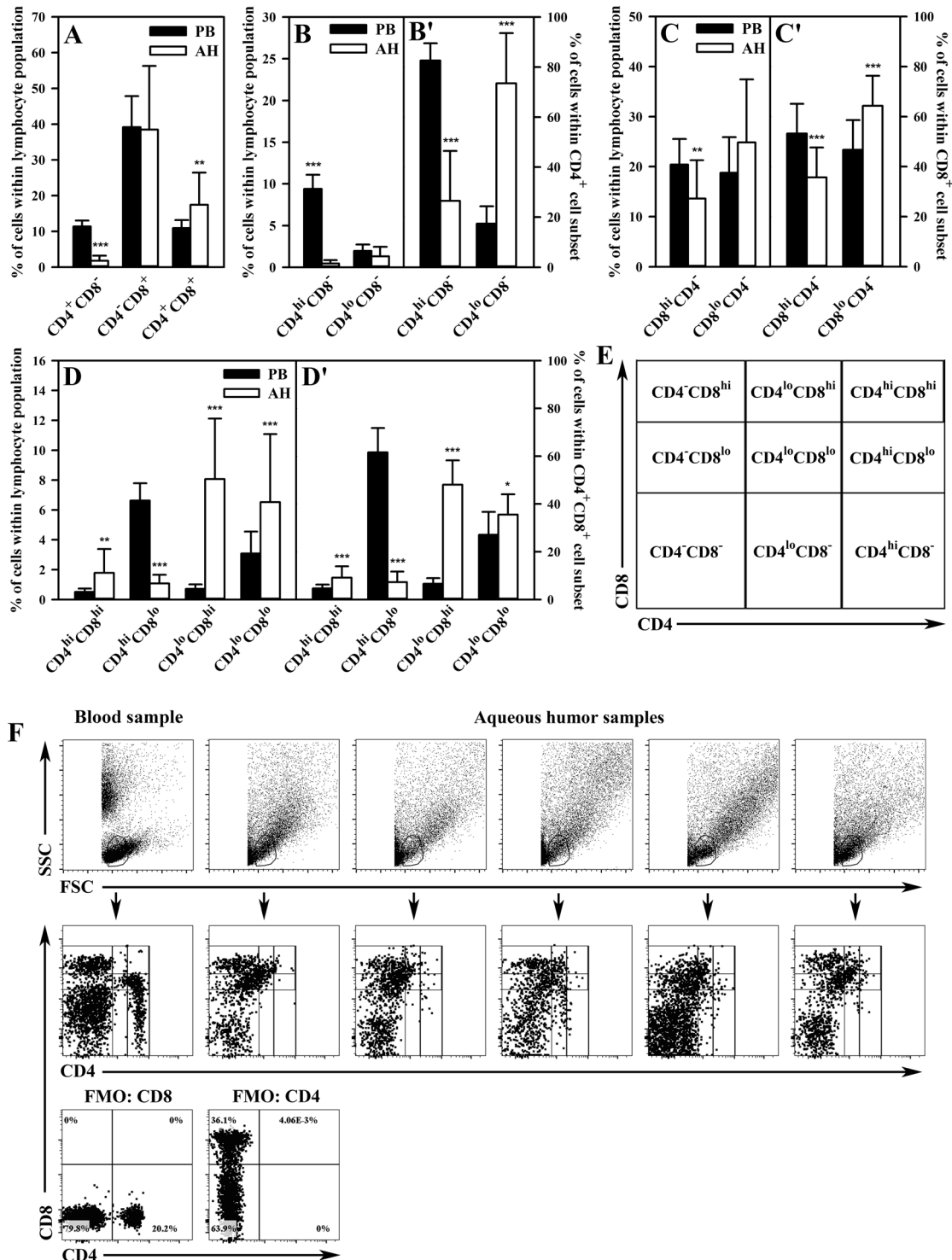
Fig. 2. Comparative analysis of the percentage of CD4+CD8-, CD4-CD8+ and CD4+CD8+ cells and their selected subsets in peripheral blood (PB) and aqueous humor (AH) of pigs. The results are expressed as a percentage of: (i) CD4+CD8-, CD4-CD8+ and CD4+CD8+ cells within the total lymphocyte population (A); (ii) CD4high(hi)CD8-, CD4low(lo)CD8- (B), CD4-CD8hi, CD4-CD8lo (C), CD4hiCD8hi, CD4hiCD8lo, CD4loCD8hi and CD4loCD8lo cells (D) within the total lymphocyte population; (iii) CD4hiCD8-, CD4loCD8- (B'), CD4-CD8hi, CD4-CD8lo (C'), CD4hiCD8hi, CD4hiCD8lo, CD4loCD8hi and CD4loCD8lo cells (D') within CD4+CD8-, CD4-CD8+ and CD4+CD8+ cell subsets, respectively. Results are the mean (± SD) of two independent experiments with 8 animals per experiment (n=16, *p<0.05, **p<0.01, ***p<0.001, unpaired Student's t-test). Examples of cytograms of selected samples of PB and AH (F). As the first step, the lymphocyte population in PB was gated on the basis of forward and side scatter (FSC and SSC, respectively; panel 1). The location of this gate served as a point of reference to set the lymphocyte gate for aqueous humor samples. CD4+CD8-, CD4-CD8+ and CD4+CD8+ T cell subsets were defined according to the expression of CD4 and CD8 within the gated lymphocyte subpopulation. Relative to the intensity of CD4 and CD8 expression, the CD4+CD8-, CD4-CD8+ and CD4+CD8+ cell populations were subdivided into CD4hiCD8-, CD4loCD8-, CD4-CD8hi, CD4-CD8lo, CD4hiCD8hi, CD4hiCD8lo, CD4loCD8hi and CD4loCD8lo subsets (E and F, panel 2). Fluorescence minus one (FMO) controls were used to establish the gating strategy for identifying CD4- and CD8-expressing cells (F, panel 3).

single positive CD4⁺ and CD8⁺ cells. The mean percentage of DP cells in total AH lymphocyte population was as high as 27.26 ± 15.49, whereas in blood it only reached 5.30 ± 3.25; this difference was significant ($p < 0.001$) (Fig. 1A). However, it should be added that the results of our earlier studies suggest that the mean percentage of DP cells in PB of one-year-old heifers was very low (Fig. 1F), i.e it amounted to 0.69 ± 0.33 ($n = 28$; unpublished data). Therefore, in cattle there is an increase in the percentage of DP cells in PB with age. With respect to the density of CD4 and CD8 molecules, DP cells did not constitute a homogenous population. Instead, four subpopulations were distinguishable (Fig. 1E and F), among which the subpopulation with low expression of both molecules was evidently the dominant one. Lymphocytes of this phenotype (i.e. $CD4^{lo}CD8^{lo}$) represented as much as $17.66\% \pm 12.35$ of the total of lymphocytes, whereas the values of this parameter for the subpopulations with the phenotypes $CD4^{hi}CD8^{hi}$, $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ were $4.40\% \pm 4.12$, $2.21\% \pm 1.49$ and $3.32\% \pm 3.80$, respectively (Fig. 1D). Except for the last subpopulation, these values were much higher ($p < 0.01$) than those for PB. Analysis of the above subpopulations in regard of their counts within the DP cell subset showed that as much as $61.25\% \pm 19.47$ of these cells were composed of lymphocytes $CD4^{lo}CD8^{lo}$, while the contribution of $CD4^{hi}CD8^{hi}$, $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ cells was $16.78\% \pm 12.31$, $9.68\% \pm 6.15$ and $12.29\% \pm 9.49$, respectively (Fig. 1D'). The percentage of $CD4^{hi}CD8^{hi}$ cells in the AH DP cell subset was significantly higher ($p < 0.05$) than the value of this parameter in PB. In turn, the percentage of $CD4^{lo}CD8^{hi}$ cells in AH DP cell subset was lower ($p < 0.05$) than in PB (Fig. 1D').

Distribution of CD4⁺ and CD8⁺ single positive cells and CD4⁺CD8⁺ double positive cells in porcine anterior chamber of the eye

Similarly to cattle, the anterior chamber in pigs contained a very small number of lymphocytes; on average, only 4764 ± 4091 events in the lymphocyte gate were collected for the entire volume of AH samples. The mean percentage of CD4⁺ cells in the lymphocyte population of the anterior chamber in pigs was just 1.77 ± 1.43 (thus, being 6.42-fold lower than in PB), while the percentage of CD8⁺ cells within the same population was 38.48 ± 17.81 on average, not being considerably different from the value of this parameter in PB (Fig. 2A). However, it should be noted that the percentage of $CD8^{hi}$ cells within AH CD8⁺ cells was much smaller ($p < 0.001$) than in PB (Fig. 2C'). It is well known that the lymphocyte population in pigs, unlike in other animal species or in humans, contains a large

share of DP cells (Zuckermann 1999, Käser et al. 2008). Likewise, a large contribution of these cells was found in the anterior chamber of pigs, namely their percentage was 17.45 ± 8.97, and moreover it was significantly higher ($p < 0.01$) than that in blood (Fig. 2A). Further analysis demonstrated that AH DP cells differed drastically from PB DP cells with respect to the density of CD4 and CD8 molecules. The shares of individual subsets of DP cells differed with respect to both the total PB lymphocyte population and the PB DP cell subset (Fig. 2D and D'). The percentage of $CD4^{lo}CD8^{hi}$ cells in the total AH lymphocyte population and in the AH DP cell subset was 8.06 ± 4.06 and 48.01 ± 10.28, respectively, and these values were 11.19- and 7.24-fold higher, respectively, compared to those found for PB DP cells ($p < 0.001$). In turn, the percentage of $CD4^{hi}CD8^{lo}$ cells in the total AH lymphocyte population and in the AH DP cell subset was 1.08 ± 0.57 and 7.31 ± 4.40, respectively, whereas the values of these parameters for PB DP cells were 6.14- and 8.43-fold higher, respectively, and these differences were significant ($p < 0.001$).

Discussion

The results obtained suggest that CD4⁺, CD8⁺ and DP lymphocytes reside within the normal/noninflamed bovine and porcine anterior chamber as a consequence of constitutive trafficking. However, it should be noted that only about 2000 and 5000 events were collected in the lymphocyte gate for the entire sample of bovine and porcine AH, respectively. Although these data do not reflect the real number of lymphocytes, they suggest that the constitutive lymphocyte trafficking into the anterior chamber of the eye is relatively poor.

The results suggest that in regard of the presence of CD4⁺ cells, the lymphocyte population of the bovine anterior chamber does not differ significantly from that in PB. In turn, the share of CD8⁺ cells in the lymphocyte population of the anterior chamber was lower compared to that in PB. With respect to the contribution of cells with high and low expression of CD4 and CD8 molecules, bovine AH CD4⁺ and CD8⁺ cells do not differ from PB CD4⁺ and CD8⁺ cells. The bovine anterior chamber is unique in terms of the presence of DP cells, as the share of these cells in the lymphocyte population found there is about five-fold higher than in blood, with about 60% of these cells showing low expression of both CD4 and CD8 molecules. The results are completely different for pigs, where CD4⁺ cells in the anterior chamber constitute a very small subpopulation. In turn, regarding the share of CD8⁺ cells in the lymphocyte population that found in the porcine anterior chamber does not differ from that found in PB.

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However, the CD8^{+} cell subset present in the anterior chamber contained a considerably larger share of cells with low expression of CD8 compared to the PB CD8^{+} cell subset. Similarly to PB, the anterior chamber in pigs contains a large population of DP cells, although quite different from the former in respect of the density of molecules CD4 and CD8, namely DP cells with high expression of CD4 dominate in PB, while the dominant DP cells in the anterior chamber are those with high expression of CD8.

It should be underlined that the perusal of available literature has shown an almost complete absence of data on the occurrence of lymphocytes in the anterior chamber of healthy humans and animals. Avundruk et al. (1997, 1998) determined the CD4/CD8 ratio in AH samples which were taken from patients who were operated because of senile cataract. These investigations clearly demonstrate that counts of CD8^{+} cells in human are higher than those of CD4^{+} cells (CD4/CD8 cell ratio: 0.79 and 0.77) (Avundruk et al. 1997, 1998). Our recent studies have revealed only a trace presence of CD4^{+} and DP cells in the anterior chamber of normal mice, as the mean percentages of these cells in the total lymphocyte population were only 0.56% and 0.38%, respectively (Maślanka et al. 2017). On the contrary, the mean percentage of CD8^{+} cells in the total AH lymphocyte population was as high as 28.69. Thus, these data and the present results show that single positive CD8^{+} cells constitute a large subset of the lymphocyte population in the anterior chamber of humans, mice, cows and pigs. In turn, the share of single positive CD4^{+} cells in the lymphocyte population of the human and bovine anterior chamber is quite high, while these cells represent a very small subset in the anterior chamber of mice and pigs. Moreover, DP cells constitute a relatively large proportion of lymphocytes found in cattle and pig but not in the mouse anterior chamber.

In conclusion, the results suggest that the anterior chamber of the eye in normal cattle and swine is an element in the normal migratory pathway of CD4^{+}, CD8^{+} and DP cells. However, the trafficking of these cells into the chamber seems to be relatively poor. Furthermore, the contribution of CD4^{+}, CD8^{+} and DP subsets in the anterior chamber lymphocyte population can differ considerably between animal species.

Acknowledgements

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