doi: 10.17221/7/2015-VETMED

Changes in regulatory T cells in dogs with B-cell lymphoma and association with clinical tumour stage

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ABSTRACT: Among several mechanisms that allow tumours to disarm the host immune system and thus to evade or suppress protective anti-tumour immunity, an important role for $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) has emerged. Numerous studies in humans have demonstrated increased Tregs in patients with carcinomas of the breast, lung, and pancreas, and this increased Treg has been correlated with poor prognosis. This study was performed (1) to investigate the percentage of Tregs in total lymphocytes of the peripheral blood in 12 canine patients with B cell lymphoma and (2) to investigate the change in the percentage of Tregs in canine lymphoma of different clinical tumour stages. On the flow cytometric analysis, the relative and absolute numbers of Tregs were significantly increased in 12 canine patients with B-cell lymphoma compared to five healthy beagles included in this study, and the greatest increases in the relative and absolute number of Tregs occurred in two dogs with more advanced World Health Organization clinical stages with bone marrow involvement compared to those in less advanced tumour stages without bone marrow involvement. This study provides basic information regarding the negative role of Treg recruitment in canine lymphoma patients and highlights the potential value of Treg levels as prognostic indicators in canine cancer patients.

Keywords: cancer; regulatory T cell; Treg; canine lymphoma; immune escape; WHO clinical tumour stages

List of abbreviations

APC = antigen presenting cells, **Tregs** = regulatory T cells

The field of cancer immunity has been the subject of intense research ever since the theory of cancer immunosurveillance, which posits that the immune system recognises and eradicates nascent transformed cells, was proposed in the late 1950s (Burnet 1971). Despite the evidence for the recognition and destruction of tumours by the immune system, there have been significant barriers to the generation of effective anti-tumour immunity. Among the prominent studies seeking answers for ineffective cancer immunity, immunoediting, which carries out dual roles in the control of malignancy, was proposed as a reasonable answer by Dunn and colleagues (Dunn et al. 2002). The importance of the concept of immunoediting is due to the fact that the immune system plays a critical

Supported by the Cooperative Research Program for Agriculture Science and Technology Development, project Functional feed material development and efficacy evaluation for detector dog's ability improvement (Project No. PJ011989), Rural Development Administration, Republic of Korea.

doi: 10.17221/7/2015-VETMED

role in sculpting the tumours, resulting in tumour variants with reduced immunogenicity or those that are better equipped to evade or suppress protective anti-tumour immunity (Koebel et al. 2007; Quezada et al. 2011).

Among several mechanisms that allow tumours to disarm the host immune system and thus to evade or suppress protective anti-tumour immunity, an important role for $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) has emerged. Tregs, previously termed suppressor T cells, are a distinct $CD4^+$ T cell subset defined in mice and humans by expression of the CD25 surface molecule, along with surface expression of CTLA-4, GITR, Lag3 and intracellular expression of the FoxP3 transcription factor (Sakaguchi 2000; Sakaguchi 2005). The primary function of Tregs is to suppress harmful autoimmune responses by inducing immunological tolerance. In the tumour microenvironment, however, the immunosuppressive function of Tregs plays a detrimental role by suppressing protective anti-tumour immune responses. Treg-mediated immunosuppression of natural killer cells, $CD8^+$ T cells and antigen presenting cells (APC) has been considered as the major mechanism which allows tumour cells escape from the host-protective immune system (Yang et al. 2004).

Increased numbers of Tregs have been documented in mice and humans with cancer, and numerous studies in humans have demonstrated increased numbers of Treg in blood, draining lymph nodes and tumour tissues in patients with breast, lung and pancreatic carcinoma (Mukherjee et al. 2001; Sasada et al. 2003). Moreover, it has been shown that an increased number of Tregs in cancer patients is correlated with poor prognosis (Curiel et al. 2004).

To the best of the authors' knowledge, however, only few studies have analysed the changes in Tregs in companion animals with cancer. Although there have been a few reports showing an increased number of Tregs in dogs with cancer compared with control dogs, the numbers of animals evaluated was relatively small and these studies were not designed to show any relationship between tumour stage and changes in Tregs (Biller et al. 2007; O'Neill et al. 2009). This study was performed to investigate the changes in the percentage of total lymphocytes represented by Tregs in the peripheral blood of canine patients with different clinical tumour stages of lymphoma, the most frequently occurring cancer in canine species. Thus, the goal of this study was (1) to investigate the changes in the percentage of total lymphocytes represented by Tregs in of the peripheral blood in 12 canine patients with lymphoma and (2) to investigate the change in the percentage of Tregs in different clinical tumour stages of canine lymphoma patients. We hypothesised that any increase in the number of Tregs would be more pronounced in canine patients with more advanced clinical tumour stages.

MATERIAL AND METHODS

Study population. To investigate the percentage of Tregs in the peripheral blood of healthy dogs, five healthy beagles were used in this experiment. For analysis of Tregs in dogs with lymphoma, blood was collected from 12 dogs who had just been diagnosed with canine lymphoma at the Chonbuk National University. For the analysis of changes in Tregs in different clinical stages of malignancies, 12 canine patients with lymphoma were classified based on the World Health Organization (WHO) clinical staging system for lymphosarcoma in domestic animals. The study protocol was approved by the institutional Animal Care and Use Committee of Chonbuk National University.

Flow cytometric analysis. To evaluate the *ex vivo* expression of cell surface markers on peripheral blood mononuclear cells of canine lymphoma patients, the following monoclonal antibodies were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-canine CD4 (AbD Serotec, Oxford, UK), PE-conjugated anti-canine monoclonal CD25 (eBioscience, San Diego, USA) and APC-conjugated anti-mouse/rat FoxP3 (eBioscience, San Diego, USA). Three millilitres of blood were drawn into an ethylenediaminetetraacetic acid tube (Becton Dickinson, Franklin Lakes, USA) for flow cytometric analysis. Peripheral blood mononuclear cells were immediately separated by centrifugation at 700 × *g* for 30 min at room temperature using Histopaque 1077 (Sigma-Aldrich, St Louis, USA). Peripheral blood mononuclear cells were washed twice with phosphate buffered saline and incubated with antibodies to the extracellular markers of Treg, FITC-conjugated anti-canine CD4 and PE-conjugated anti-canine CD25, in fluorescence-activated cell sorting (FACS) buffer (1% BSA in phosphate buffered saline) for 30 min at room tem-

doi: 10.17221/7/2015-VETMED

perature. Samples were washed using a fixation/permeabilisation buffer (eBioscience, San Diego, USA) and incubated at 4 °C for 12 hours. APC-conjugated anti-mouse/rat FoxP3 antibody was used to stain intracellular markers of Tregs, with APC-conjugated Rat IgG2a (eBioscience, San Diego, USA) antibody as the isotype control. After staining for the intracellular marker FoxP3, samples were incubated on ice for 30 minutes and washed using permeabilisation buffer. Flow cytometry was used to detect the percentage of CD4 (+)/CD25 (+)/FoxP3 (+) Tregs in the total population of peripheral blood lymphocytes. Lymphocytes were gated by a combination of light scatter and forward scatter. The percentage of CD4 (+)/CD25 (+)/FoxP3 (+) cells were calculated from these gated lymphocytes and compared with CD4 (+)/CD25/isotype control (+) cells. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, USA) equipped with a 15-mW air-cooled argon laser. Data were acquired from a minimum of 10,000 events defined by light scatter and forward scatter gate to include lymphocytes. Data analyses including spectral compensation were performed using FCS Express (De Novo Software, Los Angeles, USA).

Calculation of cell numbers. The total number of lymphocytes in blood samples was determined for each dog by routine CBC and differential counting on the blood smear test. The total lymphocyte number was then used to calculate the number of Tregs.

Phenotyping of lymphoma. Polymerase chain reaction (PCR) assays were utilised to determine phenotypes from lymph node cytological smears and peripheral blood.

Statistical Analyses. Statistical analysis was performed using Graphpad Prism5 (GraphPad Software, Inc., USA). Differences between two groups were compared using the Mann-Whitney *U*-test, which assumes non-Gaussian distribution of data. For all comparisons, a $P \leq 0.05$ was considered significant.

RESULTS

Study patients

Twelve dogs diagnosed with canine B-cell lymphoma were included in the study, and all of the animals were classified with the WHO clinical staging system. Ten dogs were classified as WHO stage IV which implies liver and/or spleen involvement, and two dogs with lymphoma were classified as stage V indicating the involvement of bone marrow (Table 1).

Table 1. Relative and absolute numbers of regulatory T cells in peripheral blood in 12 canine patients with B-cell lymphoma; clinical tumour stages are based on the World Health Organisation (WHO) clinical staging system

Patients	WHO stages	Phenotypes	%	Absolute number ($\times 10^3$/μl)
1	IV	B	16.92	970
2	IV	B	12.84	271
3	IV	B	10.38	1797
4	IV	B	20.48	713
5	IV	B	11	540
6	IV	B	16.37	889
7	IV	B	13.97	556
8	IV	B	10.38	554
9	IV	B	8.93	234
10	IV	B	21.28	307
11	V	B	35.77	3469
12	V	B	57.19	5809

Regulatory T cells in dogs with B-cell lymphoma

Blood samples from canine patients with B-cell lymphoma were evaluated using flow cytometry for quantification of the percentage of Tregs in peripheral blood. A typical graph with dot plots for Treg analysis is shown in Figure 1. For this analysis, the percentage of Tregs was calculated by dividing the $CD4^+CD25^+FoxP3^+$ T cells by the total number of $CD4^+$ T cells.

We found that all 12 canine patients with B-cell lymphoma included in this study showed a significant increase in the percentage of Tregs in peripheral blood compared to the average percentage of Tregs in five healthy beagles (5.15%). The mean percentage of Tregs in the 12 canine patients diagnosed with B-cell lymphoma was 19.62%. The absolute numbers of Tregs in the peripheral blood of dogs with B-cell lymphoma were also significantly increased (mean of 1342×10^3 cells/μl blood versus 51×10^3 cells/μl blood in five healthy beagles.

doi: 10.17221/7/2015-VETMED

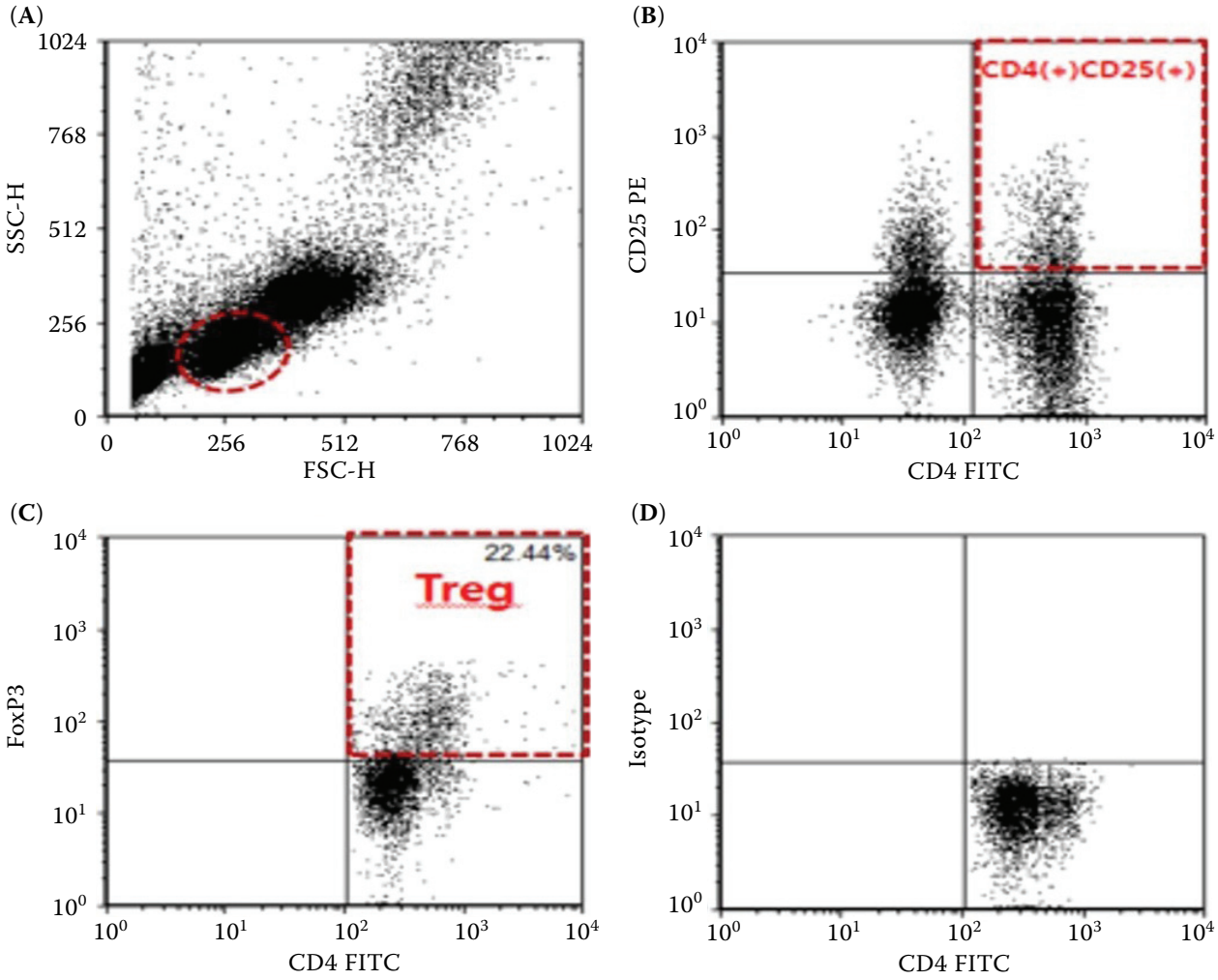

Figure 1. Flow cytometric analysis of regulatory T cells (Tregs) in the blood of dogs
FITC = fluorescein isothiocyanate

Treg changes in dogs with B-cell lymphoma and association with clinical tumour stage

We also found that dogs in more advanced WHO clinical stages showed a much higher increase in the numbers of Tregs in peripheral blood compared to those in less advanced WHO clinical stages (Figure 2A). Among 12 dogs included in this study, 10 dogs with B-cell lymphoma were classified as WHO clinical stage IV, which indicates the involvement of liver or spleen, and two of the canine patients with B-cell lymphoma in this study were classified into WHO clinical stage V, implying bone marrow involvement. Although the number of dogs with more advanced clinical stages included in this study is relatively small, both the relative and absolute numbers of Tregs in two dogs with WHO clinical stage V showed a marked increase compared to the mean percentage (57.19% vs 16.21%; Figure 2A) and mean absolute numbers (5809×10^3/μl vs 936×10^3/μl; Figure 2) of Tregs in the remainder of dogs with WHO clinical stage IV included in this study.

DISCUSSION

The major findings to emerge from this study were that the relative and absolute numbers of Tregs were significantly increased in dogs with lymphoma and that the greatest increases in the relative and absolute numbers of Tregs occurred in dogs in more advanced WHO clinical stages with bone marrow involvement.

Tregs are a distinct lineage of T lymphocytes that comprise approximately 5–10% of total T cells in

doi: 10.17221/7/2015-VETMED

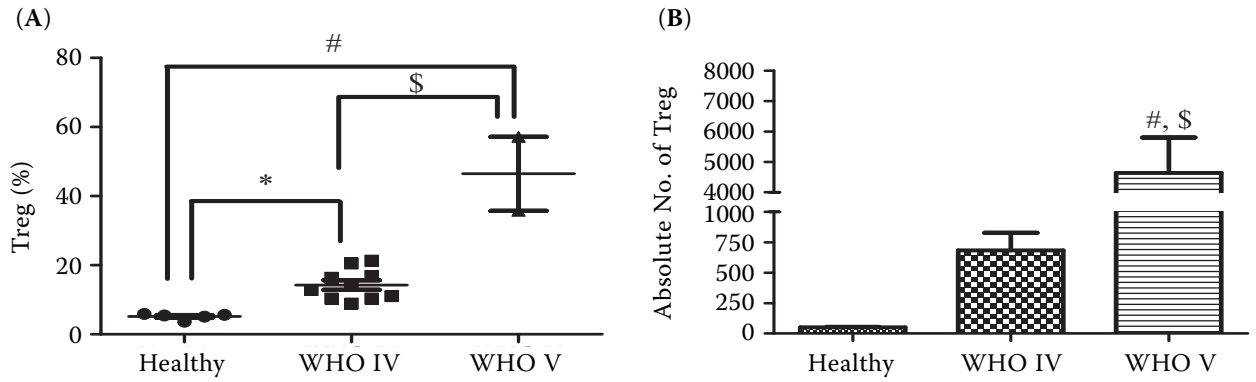

Figure 2. Percentages and absolute numbers (103/μl) of regulatory T cells (Tregs) in 12 canine patients with B-cell lymphoma classified according to the World Health Organisation (WHO) clinical tumour staging system compared to those of five healthy beagles

rodents, cats and humans (Itoh et al. 1999; Shevach 2002). Several studies have described the different subsets of Tregs, which include natural Tregs derived from the thymus and inducible Tregs which are induced and activated by exposure to antigens under certain circumstances (Beyer and Schultze 2006). Despite these functional and phenotypic differences between Treg subsets, the Treg population as a whole normally plays a critical role in maintaining the peripheral tolerance and in preventing the occurrence of a variety of autoimmune diseases (Sakaguchi et al. 1995).

In contrast to their original purpose in maintaining the immunotolerance of self-antigens, Tregs can also confer an immunological tolerance to tumour antigens, hampering a wide range of beneficial anti-tumour immune responses involving $CD4^+$ helper T cells, $CD8^+$ T cells, natural killer cells and natural killer T cells (Shevach 2002). The recruitment of Tregs has recently emerged as a major mechanism that tumour cells can use not only to evade the immune surveillance of the body, but also to promote conditions that favour its progression.

Numerous studies in humans have described increased numbers of Tregs in patients with carcinoma of the breast, lung, and pancreas, and this increased Treg expression has been correlated with poor prognosis (Woo et al. 2001; Sasada et al. 2003; Wolf et al. 2003; Curiel et al. 2004). Moreover, Tregs are known to be overexpressed and comprise a major portion of tumour-infiltrating lymphocytes (Viguier et al. 2004), implying a potential role in the development and progression of cancer. The markedly increased percentages and absolute numbers of Tregs in canine patients with lymphoma in this study offers further support for a possible correlation between an increased number of Tregs and lymphoma development and progression.

The fact that Tregs can dampen anti-tumour immunity, becoming a major obstacle for effective immune surveillance of body, is also a promising therapeutic clue for the field of immunotherapy. Immunotherapy describes the concept of harnessing the body's own immune system for cancer surveillance and annihilation. To make immunotherapy more effective in treating cancer, it is necessary to understand the exact mechanisms of both how our body's own immune system works to recognise and eliminate the tumour antigens from the body and how tumour cells, on the other hand, can escape and counteract this anti-tumour immunity. Although the recruitment of Tregs is not the only mechanism that helps tumour cells evade anti-tumour immunity, it has emerged as one of the major obstacles to effective immunotherapy. Moreover, the fact that Tregs are apparently capable of suppressing almost every aspect of anti-tumour immune responses including $CD4^+$ helper T cells, $CD8^+$ T cells, natural killer cells and natural killer T cells highlights the importance of controlling the activity of Tregs as well as reinforcing the components of anti-tumour immunity when applying immunotherapy. For example, several clinical trials based on natural killer cell immunotherapy alone performed at the end of 1990s were unsuccessful because of a failure to control the expansion of Tregs, which directly inhibited the natural killer cell killing effects (Terme et al. 2008).

Although further studies are needed with larger study populations, our finding that the greatest increases in the percentages and absolute numbers of

doi: 10.17221/7/2015-VETMED

Tregs occurred in canine lymphoma patients with bone marrow involvement supports a possible relationship between Treg recruitment and tumour progression. The percentages of Tregs in two dogs diagnosed with B-cell lymphoma with bone marrow involvement were 2.5–4 times higher in the remainder of the dogs (B-cell lymphoma) without bone marrow involvement. Moreover, the absolute numbers of Treg in dogs with bone marrow involvement were 5–8.5 times higher than those in dogs without bone marrow involvement. We suggest that this finding is indicative of a potential role for Treg changes as a prognostic marker in canine lymphoma patients. It also necessitates future studies focusing on subgrouping canine lymphoma patients using a variety of criteria such as WHO clinical stage system, immunophenotyping or histologic findings, and establishing a reasonable prognostic correlation between the Treg changes and different classification criteria.

In conclusion, canine patients with B-cell lymphoma had significantly increased percentages and absolute numbers of Tregs in their peripheral blood compared to healthy Beagles. Additionally, canine B-cell lymphoma patients with bone marrow involvement exhibited greater increases in both mean percentages and absolute numbers of Tregs in peripheral blood compared to those with less advanced WHO clinical stages as well as healthy dogs. The findings from this study suggest a number of follow-up studies. For example, does the Treg percentage actually correlate with clinical tumour stage or any other classifying criteria in canine B-cell lymphoma patients? Can blocking the recruitment of Tregs actually reinforce other modalities of immunotherapy such as natural killer cell-based immunotherapy? Such a finding would suggest a direct association between the lymphoma and induction of Treg recruitment. And finally, it may be possible in the future to develop an effective immunotherapy which could control the recruitment of Tregs, reinforcing the efficacy of other modalities of immunotherapy as well as establishing a reliable prognostic marker in canine lymphoma patients based on the systematic analysis of Treg changes.

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Received: January 8, 2015
Accepted after corrections: September 9, 2017