J. Comp. Path. 2017, Vol. 157, 15–22

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

www.elsevier.com/locate/jcpa

NEOPLASTIC DISEASE

Prevalence of FoxP3$^+$ Cells Does Not Correlate With Ki67 Expression in Canine Diffuse Large B-cell Lymphoma

C. F. Muir[*,†], S. L. Priestnall[†], A. Hibbert[‡], C. Brown[*], O. A. Garden[§,‖] and T. Scase[*,‖]

** Bridge Pathology Ltd, 637 Gloucester Rd, Bristol, † Pathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, Hatfield, Hertfordshire, ‡ RVC Imaging Suite, Royal Veterinary College, Royal College Street and § Immune Regulation Laboratory, Comparative Physiology and Medicine Research Group, Department of Clinical Sciences and Services, Royal Veterinary College, London, UK*

Summary

Diffuse large B-cell lymphoma (DLBCL) is the most common type of canine lymphoma and survival times are currently <1 year. Manipulation of the tumour microenvironment, of which the regulatory T cell (Treg) is a principal player, represents a potentially exciting way to curb the rapid proliferation of neoplastic cells. Tregs, characterized by the stable expression of the transcription factor FoxP3, suppress innate and adaptive arms of the immune response and represent a potential therapeutic target within neoplastic lymph nodes. This retrospective study explored the hypothesis that Tregs promote the proliferation of neoplastic large B cells, employing immunohistochemistry to assess both FoxP3 and Ki67 expression within canine lymph nodes. Fifty-seven biopsy samples of canine nodal DLBCL were examined. There were significantly fewer FoxP3$^+$ cells in lymph nodes effaced by DLBCL than in reactive lymph nodes (27 versus 369 cells/mm^2; Mann–Whitney $U = 16$, $P = 0.011$). There was no relationship between the number of intratumoural FoxP3$^+$ cells and neoplastic cell proliferation (Spearman's rank $r = 0.058$, $P = 0.670$, 95% confidence interval). The results of this study show that FoxP3$^+$ cells are reduced in lymph nodes effaced by DLBCL and that this change is unrelated to the expression of Ki67. This study also describes a robust digital method to standardize cell counts and facilitate future comparative studies.

Keywords: B-cell lymphoma; dog; FoxP3; regulatory T cell

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of canine lymphoma (Valli *et al.*, 2011) and survival times in dogs are currently <1 year (Valli *et al.*, 2013; Aresu *et al.*, 2015). DLBCL is characterized by a high mitotic index, with complete effacement of nodal architecture; this strong drive for rapid cell division is mediated by loss of proteins controlling the cell cycle and apoptotic pathways, such as nuclear factor (NF)-κB (Richards *et al.*, 2013). The molecular pathways underlying the development of canine DLBCL are similar to that of human DLBCL and canine DLBCL can offer a potentially robust model of this common human malignancy (Richards *et al.*, 2011).

To curb the rapid proliferation of neoplastic cells, possible ways of manipulating the immune system have been examined and one potential candidate is the regulatory T cell (Treg) (Tanaka and Sakaguchi, 2017). Tregs, characterized by the

Correspondence to: C. F. Muir (e-mail: cmuir@rvc.ac.uk).
‖ Both authors contributed equally to this study.

0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2017.03.010

expression of the transcription factor Forkhead box P3 (FoxP3) (Zheng and Rudensky, 2007), suppress innate and adaptive arms of the immune system. This is achieved in part by the release of inhibitory cytokines, consumption of interleukin (IL)-2 and expression of co-inhibitory receptors that block $CD4^+$ T-cell activation and induce $CD4^+$ T-cell apoptosis (Read *et al.*, 2006; Pandiyan *et al.*, 2007; Rubtsov *et al.*, 2008). Together these proteins suppress immune-mediated destruction of neoplastic cells and are potential targets for cancer immunotherapy (Smyth *et al.*, 2016). Antibodies against cytotoxic T lymphocyte antigen 4 (CTLA-4), a protein that controls Treg differentiation and function (Wing *et al.*, 2008), are currently being developed as human cancer treatments and antagonism of CTLA-4 has been shown to decrease the number of tumour infiltrating Tregs (Simpson *et al.*, 2013).

As assessed by flow cytometric analysis of affected tissue, canine cases of DLBCL survive for longer when fewer Tregs are present (O'Neill *et al.*, 2009; Pinheiro *et al.*, 2014), but the converse has been found in human immunohistochemical studies (Lee *et al.*, 2008; Tzankov *et al.*, 2008; Ahearne *et al.*, 2014; Coutinho *et al.*, 2015). The present study aims to build on this work by using immunohistochemistry (IHC) to characterize the number of Tregs in canine DLBCL tissues, with the overarching hypothesis that Tregs promote the proliferation of neoplastic large B cells. The results of this study may offer preliminary data for studies investigating whether Treg suppression (using drugs such as Ipilimumab™) could be used as treatment for canine DLBCL.

In veterinary medicine, studies of prognosis are confounded by humane destruction of animals prior to natural death, a plethora of different treatment protocols and difficulties obtaining robust follow-up data (Chun, 2009; Valli *et al.*, 2013). This study examines a marker of cell division, Ki67, as an indirect measure of the rate at which DLBCL would be expected to progress without treatment. Without treatment, tumours with a high rate of cell division would be expected to develop more quickly, leading to a shorter survival time than tumours with a low mitotic index (Valli *et al.*, 2013).

Ki67 is a nuclear protein that is expressed at all stages of the cell cycle except G0 and therefore offers a more robust measure of the growth fraction than visual assessment of the cell spindle, as assessed by a count of the number of mitotic figures (Huang *et al.*, 1994; Phillips *et al.*, 2000; Scholzen and Gerdes, 2000).

This study offers a robust digital measurement of the number of $FoxP3^+$ cells in canine DLBCL tissue samples and examines the correlation between $FoxP3^+$ cells and Ki67 expression.

Materials and Methods

Study Population

Sixty-five incisional or excisional biopsy samples of nodal DLBCL submitted to Bridge Pathology Ltd., Bristol, between June 1st, 2009 and May 1st, 2015, were selected for inclusion in this study. In all cases, the tumour cells expressed CD79a and did not express CD3. Dogs with T-cell-rich large B-cell lymphoma were not included and cases in which the lymph node site was unknown or in which the lymph node was fragmented (with <2 mm of tissue available) were also excluded. Diagnosis was made by board certified veterinary pathologists. Dogs had not received any prior treatment for lymphoma and had no previous history of neoplasia. Owner consent was obtained indirectly as an 'opt out' option on the submission form. Case details are included in Supplementary Table 1.

Case Selection

Samples were fixed in 10% neutral buffered formalin for approximately 24 h, processed routinely and embedded in paraffin wax. Sections were stained with haematoxylin and eosin (HE). A diagnosis of DLBCL was based on the presence of a diffuse infiltrate of neoplastic round cells that completely effaced the architecture of the lymph node. Cells measured >2 times the width of an erythrocyte, contained minimal amounts of cytoplasm and possessed round to oval, frequently cleaved nuclei. Mitoses were identified in all high-power fields (×400). Four control lymph nodes were selected from adult dogs with no history of disease or receipt of medication.

Preparation of Samples

Paraffin wax blocks from each case were overlaid by a stainless steel cassette lid (Cell Path EAX-0114-08A; Newtown, UK). The holes within the grid were designated as a number within the range 1−30. A sample hole was selected using a random number generator (https://www.random.org/) and tissue cores were punched from each block, through the respective grid hole, using a 2 mm^2 core biopsy needle (Kai biopsy punch; pfmmedical, Poynton, UK). Large areas of necrosis were avoided by marking with a pen affected areas on the HE-stained slide (Parsons and Grabsch, 2009). Two cores were made from each wax block and the cores were arrayed into the recipient block with a maximum of 30 cores included in a single block. In each block, a single core of splenic tissue was included to aid orientation and a single core of a lymph node was included as a positive control.