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# Cellular distribution of the prion protein in palatine tonsils of mule deer (*Odocoileus hemionus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*)

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**ABSTRACT.** Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects members of the Cervidae family, including deer (*Odocoileus* spp.), elk (*Cervus Canadensis* spp.), and moose (*Alces alces* spp.). While CWD is a neurodegenerative disease, lymphoid accumulation of the abnormal isoform of the prion protein (PrP<sup>Sc</sup>) is detectable early in the course of infection. It has been shown that a large portion of the PrP<sup>Sc</sup> lymphoid accumulation in infected mule deer takes place on the surface of follicular dendritic cells (FDCs). In mice, FDC expression of PrP<sup>C</sup> has been shown to be essential for PrP<sup>Sc</sup> accumulation. FDCs have been shown to normally express high levels of PrP<sup>C</sup> in mice and humans but this has not been examined in natural hosts for CWD. We used double immunofluorescent labeling and confocal microscopy to determine the PrP<sup>C</sup> expression characteristics of B and T lymphocytes as well as FDCs in palatine tonsils of CWD-negative mule deer and elk. We detected substantial PrP<sup>C</sup> colocalization with all cellular phenotypic markers used in this study, not just with FDC phenotypic markers.

**KEY WORDS:** chronic wasting disease, confocal microscopy, follicular dendritic cell, palatine tonsil, T lymphocyte

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Chronic wasting disease (CWD) is a fatal neurodegenerative disease that affects wild, as well as captive cervids including deer (*Odocoileus* spp.), elk (*Cervus canadensis* spp.), and moose (*Alces alces* spp.) [2, 42, 43]. Classified as a transmissible spongiform encephalopathy (TSE), CWD and other TSEs are commonly referred to as prion diseases to denote the accumulation of an abnormal isoform (PrP<sup>Sc</sup>) of the normal cellular prion protein (PrP<sup>C</sup>) [41]. Both PrP<sup>C</sup> and PrP<sup>Sc</sup> have the same primary structure and differ only in conformation. Details concerning the natural transmission of CWD are unknown, but horizontal transmission and oral assimilation of infectious PrP<sup>Sc</sup> are thought to be important in maintaining the disease in wild populations [28].

Early in the course of CWD infection, PrP<sup>Sc</sup> propagates and accumulates in lymphoid tissues prior to neuroinvasion [27]. This characteristic early lymphoid accumulation is seen in nearly all cases of CWD-positive mule deer and approximately 80% of CWD-positive elk [33, 34, 40]. The degree of early lymphoid involvement varies throughout the TSEs. Scrapie of sheep and goats, and variant Creutzfeldt-Jakob disease in humans all exhibit PrP<sup>Sc</sup> accumulation within secondary lymphoid tissues while there is a lack of appreciable lymphoid accumulation in cattle with bovine spongiform encephalopathy (BSE) [10, 39]. The results of a study by Jeffrey *et al.* [14] suggest that the extent of lymphoid involvement might be related to the species being inoculated rather than to the origin of the infectious prions since sheep inoculated with BSE material exhibit lymphoid accumulation of PrP<sup>Sc</sup>. In contrast, European red deer (*Cervus elaphus elaphus*) orally inoculated with infectious BSE material developed disease, but did not accumulate PrP<sup>Sc</sup> within lymphoid tissues whereas CWD inoculated animals of the same species exhibited intense PrP<sup>Sc</sup> immunohistochemical (IHC) staining in rectal lymphoid tissue and retropharyngeal lymph node [24].

Mule deer and elk with CWD display lymphoid PrP<sup>Sc</sup> accumulation that is focused on the germinal center within lymphoid follicles [34, 36]. Within the germinal centers of CWD-positive mule deer, the surfaces of follicular dendritic cells (FDCs) and B lymphocytes have been shown to accumulate the majority of the lymphoid PrP<sup>Sc</sup> [32]. The accumulation of PrP<sup>Sc</sup> on the surface of FDCs is not unique to CWD in mule deer. Both sheep and mice orally exposed to scrapie exhibit widespread PrP<sup>Sc</sup> accumulation on the surface of FDCs as well as within tingible body macrophages of secondary lymphoid follicles [13, 19, 25]. A similar

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accumulation of PrP<sup>Sc</sup> on FDCs is seen in mouse models and in humans with variant Creutzfeldt-Jakob disease (vCJD) [10, 16]. While Sigurdson *et al.* [32] did not examine lymphoid tissues of elk with CWD, similar staining characteristics reported in mule deer by Spraker *et al.* [35] and in elk by Spraker *et al.* [33] suggest that preferential FDC accumulation of PrP<sup>Sc</sup> also takes place in elk.

The reason for the preferential accumulation of PrP<sup>Sc</sup> on the surface of FDCs in cervids is not known although McCulloch *et al.* [26] have shown that FDC expression of PrP<sup>C</sup> is required for the accumulation of PrP<sup>Sc</sup> in the spleen of mice. FDCs are stromal cells that play a crucial role within the germinal center as antigen presenting cells [18]. FDCs present unprocessed antigens to B lymphocytes via complement and/or Fc receptors and turn-off apoptosis of those B lymphocytes that meet antigen affinity and specificity binding requirements [21, 22]. In persistent viral diseases such as HIV-1 and bovine viral diarrhea virus (BVDV) infection, FDCs have been shown to trap and express antigens on their surface for extended periods (up to 1 year) [7, 15]. A similar antigen trapping mechanism may be responsible for the accumulation of PrP<sup>Sc</sup> on the surface of FDCs in natural hosts of CWD. However, FDCs may simply express an increased number of PrP<sup>C</sup> on their surface, and simple *in situ* conversion of the isoform to PrP<sup>Sc</sup> would also explain the preferential accumulation of PrP<sup>Sc</sup> on the surface of FDCs.

In both humans and mice, FDCs have been shown to express high levels of PrP<sup>C</sup> relative to other lymphoid cell types [3, 37]. In the same mouse model, PrP<sup>C</sup> expression by FDCs was shown to be a requirement for lymphoid accumulation of PrP<sup>Sc</sup> and eventual neuroinvasion [3, 4]. While these studies portray the PrP<sup>C</sup> characteristics of FDCs as important in the pathogenesis of other prion disease models, the PrP<sup>C</sup> association with FDCs has previously not been examined in cervids. Therefore we studied the PrP<sup>C</sup> distribution relative to cell phenotype markers within palatine tonsils of uninfected mule deer and elk using double immunofluorescent labeling and laser scanning confocal microscopy. We set out to determine if the high association of PrP<sup>Sc</sup> with FDCs that was seen by Sigurdson *et al.* [32] can be explained by a high relative expression of PrP<sup>C</sup> on FDC surfaces prior to infection. We chose to examine palatine tonsils because the anatomic location in the oropharynx and close proximity to orally acquired potential antigens suggests they may serve as initial portals of entry for naturally acquired infectious prions.

## MATERIALS AND METHODS

### Collection of deer and elk tonsil tissues

Palatine tonsils were collected from carcass heads of hunter-killed mule deer and elk brought to the Wyoming State Veterinary Laboratory (WSVL) for CWD diagnostic testing. Tonsils were placed in 10% neutral buffered formalin before being processed and embedded in paraffin wax. Paraffin embedded blocks of tonsils from eight mule deer and eight elk that had been diagnosed as negative for CWD via IHC of retropharyngeal lymph node were selected for this study. The age of mule deer and elk used for this study ranged from approximately 1–6 years.

Formalin-fixed, paraffin embedded tonsil tissues from CWD positive mule deer and elk were archived samples from previous CWD research projects in the Department of Veterinary Sciences at the University of Wyoming. Prior to the initial trimming, these CWD positive tissues had been allowed to fix in neutral buffered formalin, immersed in 98% formic acid for 1 hr and rinsed extensively in running tap water before being processed and embedded in paraffin wax.

### Antibodies

Primary antibodies used in this study are summarized in Table 1. To label PrP<sup>C</sup> and PrP<sup>Sc</sup>, mAb F99/97.6.1 was used that is specific for amino acids 220–225 near the C-terminus of the mule deer and elk prion protein [29]. Validated diagnostic tests utilize mAb F99/97.6.1 and the staining characteristics have been previously described [35]. Secondary antibodies used for confocal microscopy were species or isotype specific and conjugated to synthetic fluorescent tags DyLight 488 or DyLight 549.

### Validation of antibody reactivity to cervid tissues

Due to a lack of commercially available antibodies raised against cervid antigens, we tested potential commercial antibodies in both cervid tissues and the species of antigen origin to assure adequate cross reactivity to cervid tissues. For the cross validation

**Table 1.** Summary of primary antibodies used in this study

Antibody	Cell phenotype	Specificity	Isotype	Source
pAb A 0452	T cells	Human CD3	Rabbit immune serum	DAKO <sup>d)</sup>
mAb BAQ15A	B cells, FDCs	Bovine CD21	IgM	VMRD <sup>e)</sup>
mAb BAQ44A	B cells	Undefined <sup>a)</sup>	IgM	VMRD <sup>e)</sup>
mAb CNA.42	FDCs	120-kd FDC specific glycosylated antigen [30]	IgM	DAKO <sup>d)</sup>
mAb 2–137	FDCs	Undefined <sup>b)</sup>	IgM	A. Young <sup>c)</sup>
mAb F99/97.6.1	N/A	PrP	IgG1	VMRD <sup>e)</sup>

a) Exact epitope undefined. Antibody developed using bovine PBMCs as antigen. Staining characteristic determined to specifically label a portion of B cells in peripheral blood by FACS profile available at www.vmr.com. b) Exact epitope undefined. Antibody developed using membrane suspension of sheep ileal Peyer's patches as antigen [7]. Specifically labels the surface and cytoplasm of cells within the GC that are morphologically consistent with follicular dendritic cells (FDCs). c) Antibody generously gifted by Dr. Alan Young, South Dakota State University. d) Dako, Glostrup, Denmark. e) VMRD, Pullman, WA, U.S.A.

of antibodies pAb A 0452 and mAb CNA.42, human tonsils were obtained from Ivinson Memorial Hospital in Laramie, Wyoming from routine tonsillectomy procedures. A proposal submitted to the University of Wyoming Institutional Review Board for the use of human tissues was reviewed and given exempt status. All antibodies used in this study showed adequate cross reactivity as they were found to bind to cells with the same morphology and distribution, regardless of the species.

### *PrP<sup>C</sup> labelling*

Because the validated diagnostic PrP<sup>Sc</sup> IHC detection protocol eliminates the staining of PrP<sup>C</sup> in cervid tissues, we developed a tissue preparation protocol that produced optimal staining of PrP<sup>C</sup> using mAb F99. To accomplish this, we omitted the one hour formic acid treatment used for CWD diagnostic testing, as well as the 5 min formic acid treatment of tissue sections, and lowered the target retrieval solution temperature from 120°C to 93°C. This allowed for adequate epitope retrieval and binding of PrP<sup>C</sup> without complete denaturation of the epitope.

### *Immunohistochemistry*

Unless otherwise noted, all staining reagents and supplies were purchased from DAKO, Glostrup, Denmark. Five micron thick sections of CWD-negative tissue were cut and mounted onto positively charged slides and air dried for a minimum of 12 hr. Slides were then deparaffinized, rehydrated and immersed in target retrieval solution for 20 min at 93°C for PrP<sup>C</sup> labeling or 120°C for cell phenotype markers. Slides were cooled for 10 min at room temperature, rinsed in phosphate buffered saline (PBS), incubated with dual endogenous enzyme blocking reagent to quench endogenous peroxidase for 5 min at room temperature, and incubated 5 min in serum free protein block. Slides were incubated with primary antibodies for 1 hr at room temperature followed by host and isotype specific secondary antibodies conjugated to horse radish peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA, U.S.A.) for an additional 20 min at room temperature. Slides were then incubated with 3,3'-Diaminobenzidine (DAB) chromogen solution for 10 min before counterstaining with hematoxylin and coverslipping. Negative controls consisted of either mouse isotype matched antibody or normal rabbit serum at equal protein concentrations.

CWD positive tissue sections were cut and deparaffinized as above. Prior to immersion in target retrieval solution for 20 min at 120°C, slides were pretreated with 98% formic acid for 5 min and washed 3X in tris buffered saline (TBS) for 2 min. Incubation with primary antibody (F99 1:100 dilution) was for 10 min at room temperature to probe for PrP<sup>Sc</sup>. Secondary antibody, DAB chromogen and counterstaining was as described above for staining PrP<sup>C</sup> and cell markers.

### *Immunofluorescent staining*

CWD negative tissue sections were cut (5 µm) and mounted onto positively charged slides and air dried for a minimum of 12 hr. Following deparaffinization, rehydration and immersion in target retrieval solution (20 min, 93°C, 10 min cooling), slides were rinsed twice in PBS and incubated with serum free protein block for 5 min. All antibody incubation steps were separated with two rinses in PBS and one rinse in wash buffer. Sections were first exposed to F99 (1:100 dilution) anti-PrP mAb overnight at 4°C followed by 60 min incubation at 37°C with a mouse IgG1 specific secondary antibody conjugated to the synthetic tag DyLight 488 (Jackson ImmunoResearch). Slides were then incubated with cell marker antibodies for 10–12 hr at 4°C followed by incubation with an IgM or rabbit immunoglobulin specific secondary antibody conjugated to the synthetic tag DyLight 549 (Jackson ImmunoResearch) for 60 min at 37°C. Slides were coverslipped using Permount anti-fading medium (Fisher Healthcare, Waltham, MA, U.S.A.) and kept in the dark at 4°C overnight before being examined by confocal microscopy. Negative control protocols included either isotype matched mouse immunoglobulin or normal rabbit serum at the appropriate protein concentration.

For fluorescent labeling of PrP<sup>Sc</sup> within CWD positive tissue samples, slides were prepared as described above for CWD positive chromogenic labeling. Primary antibody was incubated overnight at 4°C followed by secondary anti-mouse IgG1 DyLight 488 antibody incubation for 60 min at 37°C.

### *Experimental treatments*

To examine the degree of overlap of PrP<sup>C</sup> with the cell phenotype markers, each antibody pair treatment consisted of antibodies to PrP<sup>C</sup> and the respective cell marker. For each antibody pair treatment, 8 sections from each species were stained. With 5 cell phenotypic markers used, the result was 40 sections examined for mule deer and 40 for elk. For all 80 sections used in this study, images of 2 follicles were captured to be analyzed for colocalization. With two channels for each follicle, one for the 488 nm Argon laser that displayed PrP<sup>C</sup> staining and one for the 543 nm Helium-neon (HeNe) laser that displayed the cell phenotype marker staining, the study encompassed 320 lymphoid follicle images.

### *Confocal microscopy*

A Leica TCS SP2 laser scanning confocal microscope and Argon 488 nm and HeNe 543 nm lasers were used to excite tissues within the respective channels sequentially and images were captured using 8-bit resolution. Two follicles were examined from each animal and images were captured at 200X total magnification. All images were captured using a line scanning average of 8.

### *Colocalization analysis*

Digital data from PrP<sup>C</sup> and cell phenotype marker images were analyzed for colocalization using ImageJ software [30]. Colocalization was calculated using two methods. First, the Colocalisation Threshold program within ImageJ was used to obtain a Pearson's correlation coefficient (Rcoloc) value for each 488 nm (PrP<sup>C</sup>) and 543 nm (cell marker) pair of images using pixels

of intensity equal to or greater than the threshold determined by the software. Pearson's correlation coefficient is considered a standard measure of pattern recognition describing intensity distribution correlation between images. Rcoloc values range from -1.0 to 1.0 where -1.0 indicates complete negative correlation, 0 indicates no significant correlation and 1.0 indicates complete positive correlation [44]. For each antibody pair, Rcoloc values from the two follicles of each animal were averaged. These mean Rcoloc values were analyzed for significance using a one-way analysis of variance (ANOVA) with an alpha of 0.05 and Tukey's least significant difference (LSD).

The second method of analysis also used the Colocalisation program within ImageJ and thresholds were determined as above. An image of above-threshold colocalized pixels was obtained and the calculated percent area of colocalized pixels was divided by the percent area of each respective cell marker. The result is the percent area of each cell marker image that is both above threshold, and colocalized with PrP<sup>C</sup>. For data analysis, a one-way ANOVA with an alpha of 0.05 and Tukey's LSD as above was used to determine significant differences of percent colocalization among the cell phenotypes.

## RESULTS

### *B lymphocytes express PrP<sup>C</sup>*

BAQ44A staining with fluorescently tagged secondary antibodies was relatively uniform throughout the tonsil with a slight increase in cellular density in and around the germinal centers (Fig. 1a and 1d). Both mule deer and elk tonsils displayed colocalization of PrP<sup>C</sup> and B lymphocytes shown as yellow pixels when PrP<sup>C</sup> and B lymphocyte images of the same field of view are merged (Fig. 1c and 1f). Isotype matched negative control sections exhibited no detectable staining (Fig. 1g-i). When all the fluorescent PrP<sup>C</sup>+B cell image pairs were analyzed, mAb BAQ44A and mAb F99 had a mean Rcoloc of 0.8161 for mule deer and 0.8886 for elk indicating positive colocalization in both species. Also, the average percent of mAb BAQ44A found to colocalize with mAb F99 was 50.95% for mule deer and 56.54% for elk. Since mAb BAQ44A labels mainly the cell surface of B lymphocytes, these results suggest that tonsillar B lymphocytes in deer and elk express surface PrP<sup>C</sup> (Fig. 1j-l).

### *PrP<sup>C</sup> expression by FDCs*

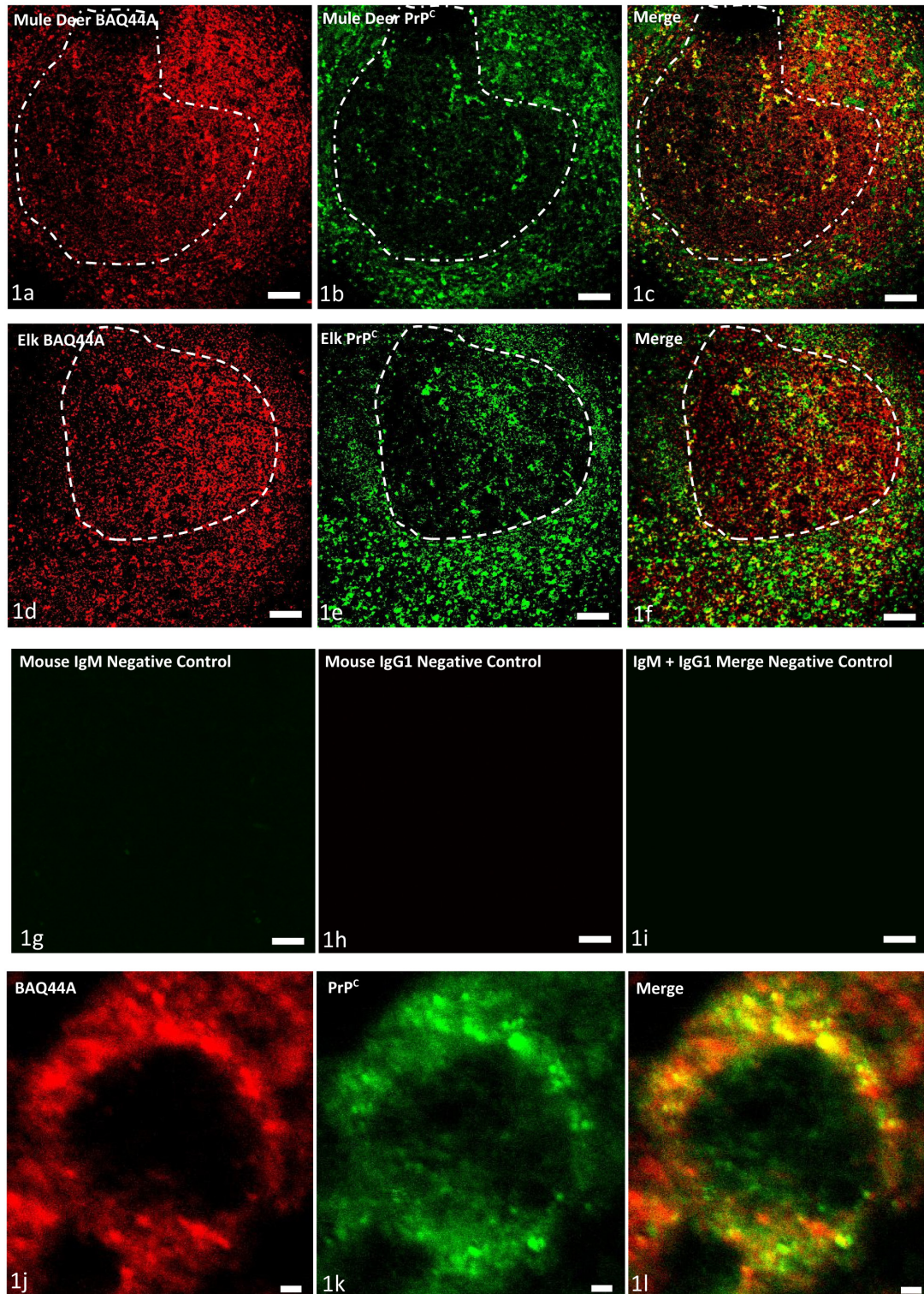
To investigate the PrP<sup>C</sup> expression level of FDCs we utilized anti-FDC mAbs CNA.42 and 2-137 (Table 1). Both antibodies label surface and cytoplasmic epitopes of FDCs on sections of mule deer and elk (Fig. 2). Also mAb 2-137 stained a small number of cells outside the germinal centers that are not morphologically consistent with FDCs (Fig. 3d). Since the number of positively stained perifollicular non-FDC cells was very small, their contribution to results concerning colocalization was considered minimal. Within the follicular zone, each antibody labels cells that are morphologically typical of FDCs. Both anti-FDC antibodies colocalize with mAb F99 as evident by yellow pixels in merged images (Fig. 3c and 3f). When individual FDCs were viewed at higher magnification, colocalization was seen both on the surface as well as within the cytoplasm of FDCs (Fig. 4). Colocalization analysis of mAb CNA.42 resulted in mean Rcoloc values of 0.8347 for mule deer and 0.8337 for elk samples. Mean percent colocalization results for mAb CNA.42 were 51.00% for mule deer and 48.02% for elk. When mAb 2-137 images were analyzed, the mean Rcoloc and percent colocalization values were 0.9280 and 57.06% respectively for mule deer whereas elk values were 0.9430 and 54.52% respectively. Rcoloc values and percent colocalization of the images for both anti-FDC antibodies indicate expression of PrP<sup>C</sup> by FDCs within palatine tonsil germinal centers of both species.

### *Colocalization of CD21 and PrP<sup>C</sup>*

As a supplemental cell marker for B lymphocytes and FDCs, we analyzed the colocalization of PrP<sup>C</sup> with mAb BAQ15A that is specific for CD21 (Fig. 5). Since CD21 is expressed by both B lymphocytes and FDCs [38], BAQ15A colocalization characteristics could assist in validating the results mentioned above for mAbs BAQ44A, CNA.42 and 2-137. Upon image analysis, the Rcoloc values for BAQ15A concerning PrP<sup>C</sup> colocalization were 0.9017 and 0.9060 for mule deer and elk respectively. The BAQ15A percent colocalization was determined to be 35.99% for mule deer and 38.53% for elk. While the Rcoloc values for BAQ15A in both species (mule deer-0.9017, elk-0.9060) falls between the Rcoloc values for mAbs BAQ44A (mule deer-0.8161, elk-0.8886) and 2-137 (mule deer-0.9280, elk-0.9430), the percent colocalization is substantially lower than all other cell types. This result may be explained by CD21 expression levels being lower than expression levels of the epitopes for mAbs BAQ44A, CNA.42 and 2-137. Another possible factor that may have influenced this result is the fact that mAb BAQ15A was raised against bovine CD21 and may have a slightly decreased affinity for cervid CD21. While the affinity of mAb BAQ15A may have influenced our percent colocalization values, there is no evidence to suggest that mAb BAQ15A is not specific for cervid CD21, and thus the Rcoloc and percent colocalization results indicate that CD21 colocalizes with PrP<sup>C</sup>. This BAQ15A colocalization data supports our previous conclusions that PrP<sup>C</sup> is expressed by tonsillar B lymphocytes and FDCs.

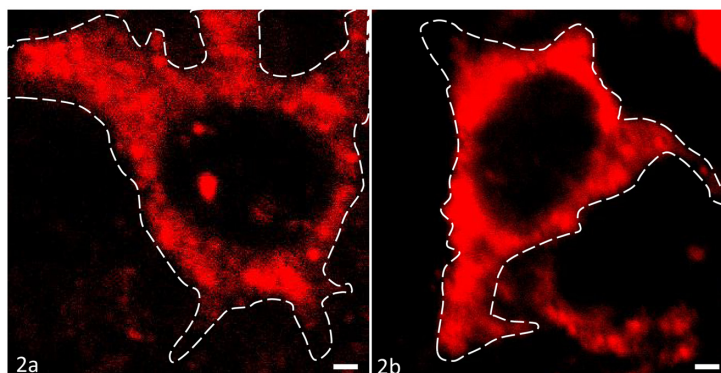
### *T lymphocytes express PrP<sup>C</sup>*

To label T lymphocytes we used pAb A 0452 that is specific for the human CD3 molecule on the surface of T lymphocytes. PAb A 0452 staining labeled many cells lining the outside of the germinal center in the "T cell zone" with follicular T cells lightly dispersed within the germinal center. The general location of positively stained cells in and around the germinal center was consistent with human tonsil sections chromogenically stained, with an apparent slight decrease in affinity for the cervid CD3. The fluorescent signal from pAb A 0452 staining originated from on or very near the cell surface of T lymphocytes (Fig. 6). On sections examined for both mule deer and elk, pAb A 0452 colocalized with PrP<sup>C</sup> in merged images (Fig. 7c and 7f). The majority

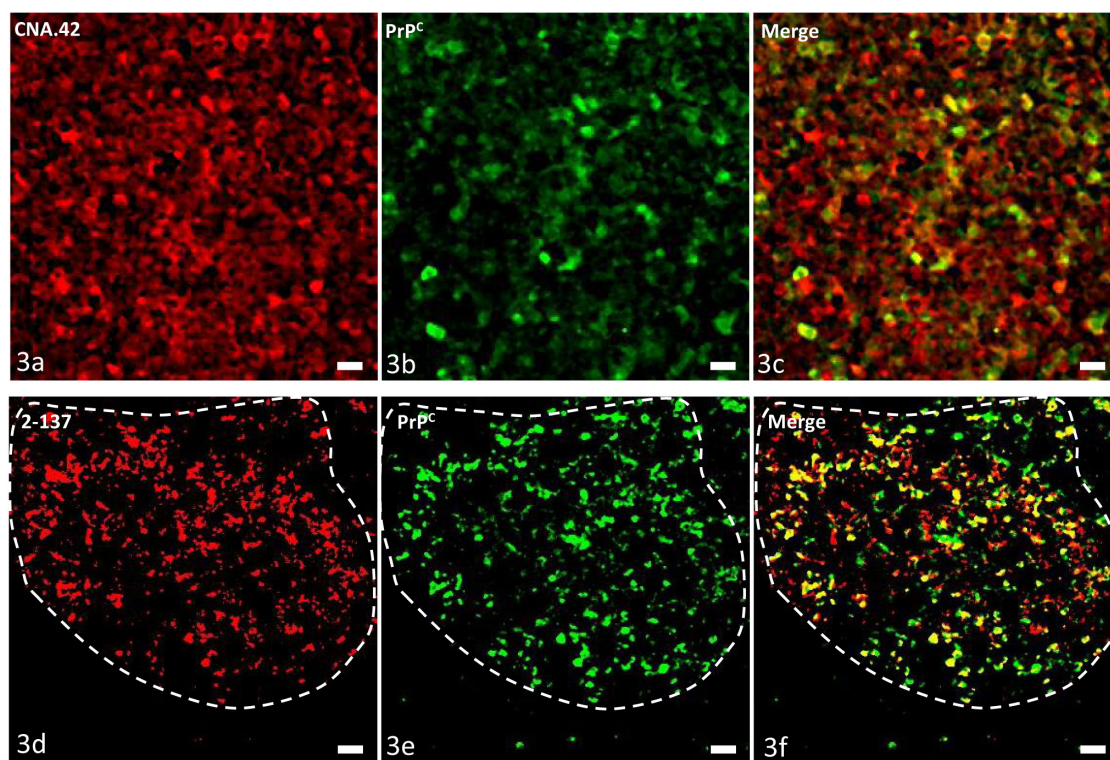


**Fig. 1.** BAQ44A (red) and PrP<sup>C</sup> (green) images in the same field of view of mule deer (panels a, b) and elk (panels d, e) tonsils colocalized as yellow in merged images (panels c, f). Isotype matched negative controls (panels g–i). Bars, 80 μm. High magnification images showing surface colocalization of mAb BAQ44A and PrP<sup>C</sup> (panels j–l). Bars 1 μm. Dashed line represents approximate germinal center borders.

of colocalization for pAb A 0452 and PrP<sup>C</sup> occurred outside the germinal center within the “T cell zone”. The number of follicular T cells varied greatly within follicles of each species. Mean Rcoloc values for pAb A 0452 colocalization were 0.8974 and 0.8776 for mule deer and elk respectively. The percent colocalization of pAb A 0452 was 48.75% for mule deer while the elk images had a 59.05% colocalization. We interpret these results as indicating expression of PrP<sup>C</sup> on the surface of CD3<sup>+</sup> T lymphocytes. Rcoloc



**Fig. 2.** Fluorescent images of follicular dendritic cells. (a) Mule deer section stained using mAb CNA.42. (b) Mule deer section stained using mAb 2-137. Note characteristic dendritic processes as well as surface and cytoplasmic staining. Sections of elk tonsil displayed the same staining characteristics (data not shown). Bars, 2  $\mu$ m. White dashed lines outline the cellular surface.

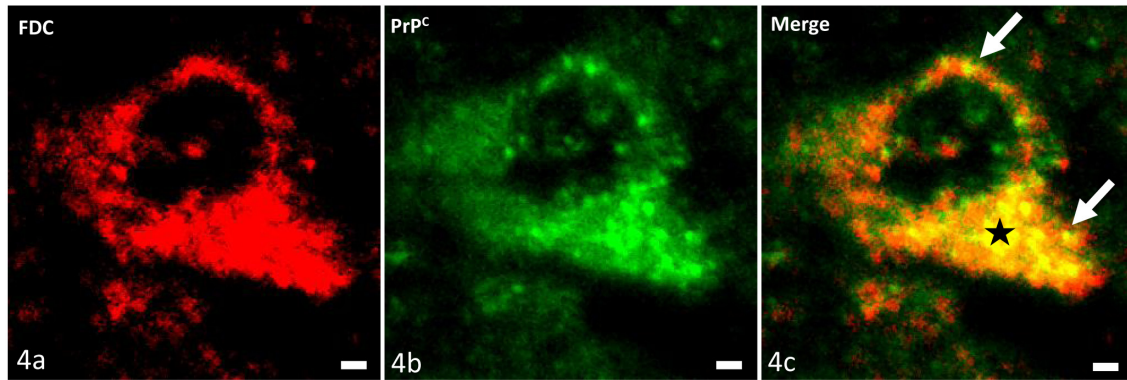


**Fig. 3.** Sections of elk tonsil. PrP<sup>C</sup> (green) colocalization with follicular dendritic cell markers (red) using mAb CNA.42 (top row) and mAb 2-137 (bottom row). Note the oval outline of the central follicular zone in the lower magnification images of panels d–f. Bars, 15  $\mu$ m (panels a–c), 40  $\mu$ m (panels d–f). Dashed line represents approximate germinal center borders.

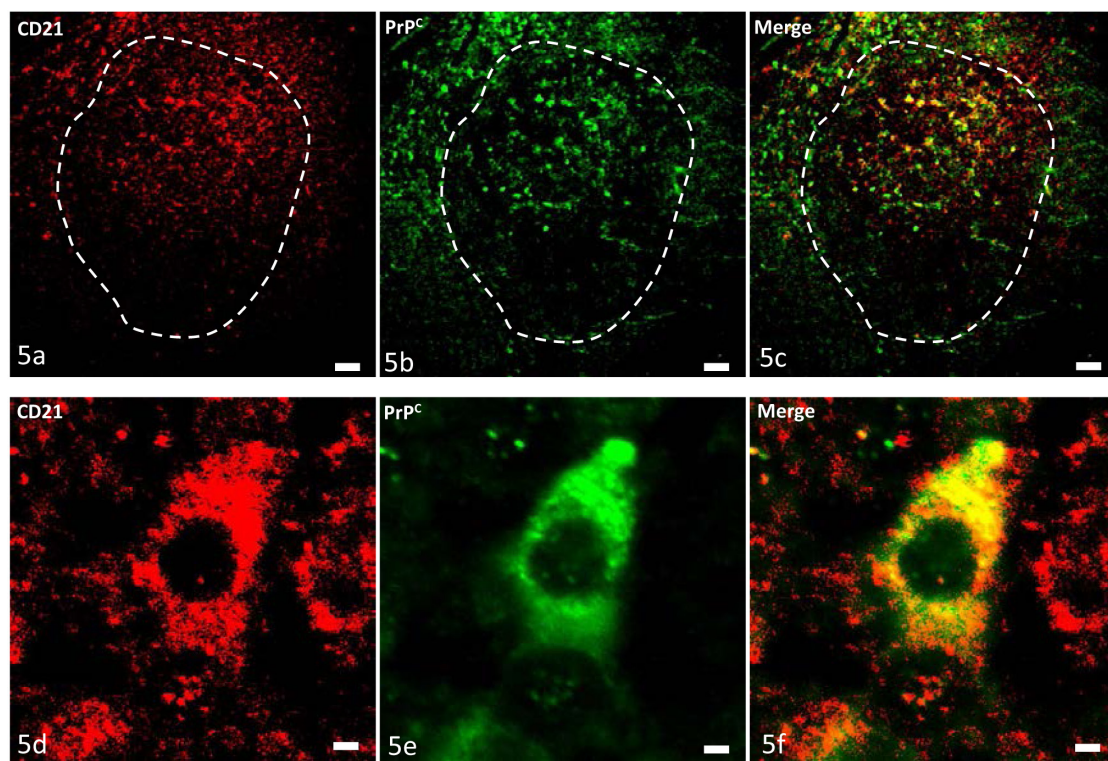
and percent colocalization data means for all antibody pair treatments used in this study are summarized in [Table 2](#).

#### *Comparing the PrP distribution in tonsils of CWD positive and CWD negative animals*

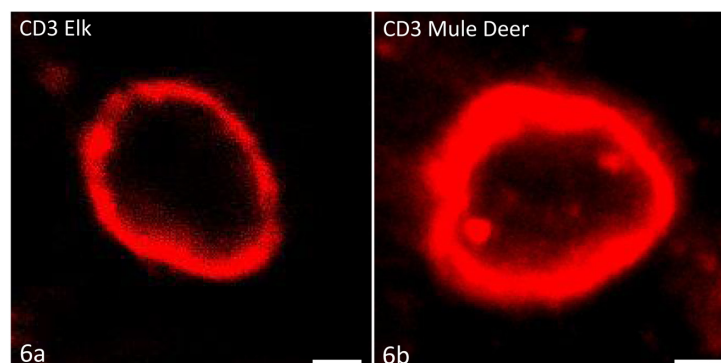
Using our developed PrP<sup>C</sup> tissue preparation protocol and the normal diagnostic PrP<sup>Sc</sup> detection protocol, we investigated the anatomical distribution of PrP<sup>C</sup> and PrP<sup>Sc</sup> within tonsils of CWD infected and uninfected mule deer and elk. Tonsils stained from infected mule deer exhibited a characteristic intense IHC staining of PrP<sup>Sc</sup> within the germinal centers of secondary follicles ([Fig. 8a](#)). However, the PrP<sup>C</sup> distribution within tonsils of uninfected mule deer appeared as widespread staining not defined by the apparent boundaries of the germinal centers ([Fig. 8b](#)). The same differential staining characteristics were seen when PrP of both isoforms was labeled with immunofluorescent secondary antibodies ([Fig. 9](#)). The same distributions were seen for PrP<sup>C</sup> and PrP<sup>Sc</sup> in tonsils of CWD negative and positive elk labeled for IHC and with immunofluorescent antibodies (data not shown). The PrP<sup>Sc</sup> staining confined to the germinal centers of CWD positive mule deer and elk seen here can be attributed to the surface



**Fig. 4.** Elk tonsil. Colocalization of PrP<sup>c</sup> (green) with mAb 2–137 (red). Bars, 1  $\mu$ m. White arrow highlighting surface colocalization. Black star highlighting intracytoplasmic colocalization.

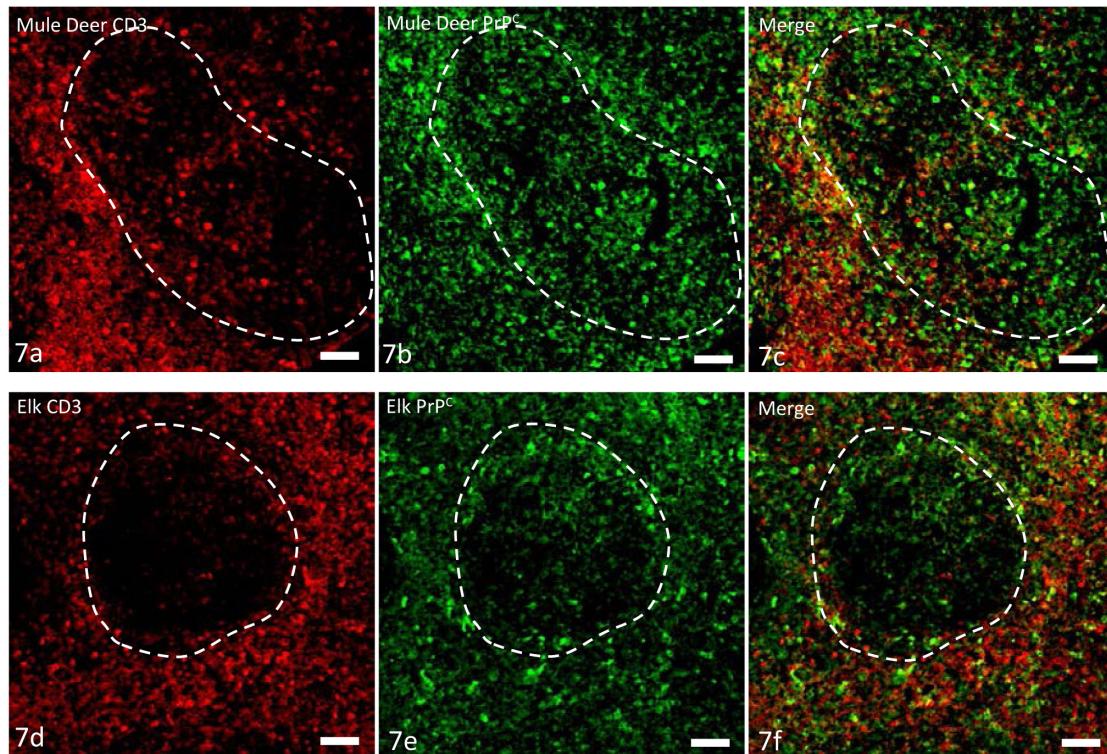


**Fig. 5.** Sections stained for CD21 (red) and PrP<sup>c</sup> (green). Colocalization is seen both within the follicular dendritic cell (FDC) network and outside the follicle on B lymphocytes (panels a, b, and f). Image showing the colocalization of CD21 and PrP<sup>c</sup> within the cytoplasm of a FDC (panels d–f). Bars, 50  $\mu$ m (panels a–c), 1.5  $\mu$ m (panels d–f). Dashed line represents approximate germinal center borders.



**Fig. 6.** T lymphocytes stained with mAb A 0452 on sections of elk (a) and mule deer (b). Bars, 1.5  $\mu$ m.





**Fig. 7.** PrP<sup>C</sup> (green) colocalization with CD3 (red) on tonsil sections from mule deer (top row) and elk (bottom row). Negative control sections exhibited no detectable staining (data not shown). Bars, 50  $\mu$ m. Dashed line represents approximate germinal center borders.

**Table 2.** Rcoloc and percent colocalization with mAb F99 means for all antibody pair treatments used in this study (n=8)

Antibody	Rcoloc (Mule deer)	Rcoloc (Elk)	% Colocalization (Mule deer)	% Colocalization (Elk)
mAb BAQ44A (B lymphocytes)	0.8161	0.8886	50.95	56.54
mAb BAQ15A (B lymphocytes & FDCs)	0.9016	0.9060	35.99	38.53
pAb A 0452 (T lymphocytes)	0.8974	0.8776	48.75	59.05
mAb CNA.42 (FDCs) <sup>a)</sup>	0.8347	0.8337	51.00	48.02
mAb 2-137 (FDCs)	0.9280	0.9430	57.06	54.52

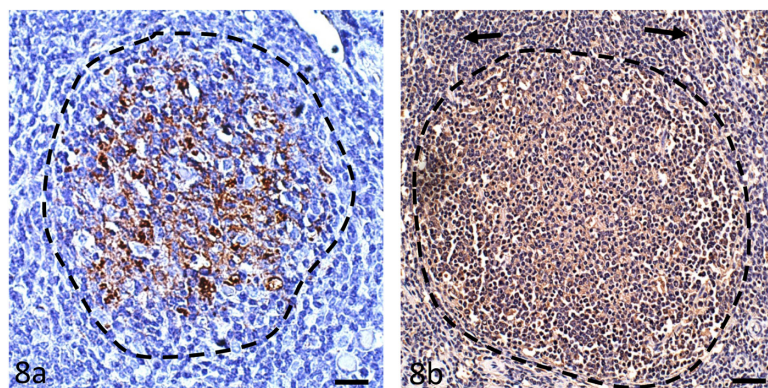
a) FDCs, follicular dendritic cells.

accumulation by FDCs and B lymphocytes as described by Sigurdson *et al.* [32]. Also, the widespread distribution of PrP<sup>C</sup> supplements the data described here concerning the colocalization of PrP<sup>C</sup> and cell markers by staining within the FDC network and also staining outside the germinal center in areas including the “T cell zone”.

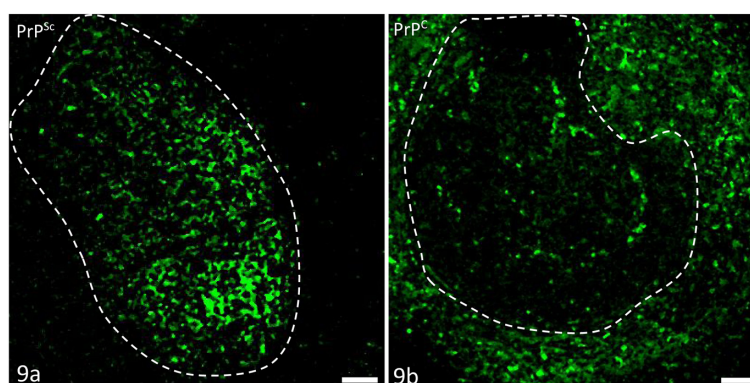
## DISCUSSION

Here we have demonstrated the distribution of PrP<sup>C</sup> among three major cell phenotypes in lymphoid follicles of two naturally occurring hosts of CWD. We used double immunolabeling and laser scanning confocal microscopy to show that PrP<sup>C</sup> is expressed by B and T lymphocytes as well as FDCs in mule deer and elk tonsils. While these three cell phenotypes express PrP<sup>C</sup>, other cells within lymphoid follicles, namely macrophages and mobile dendritic cells were not examined and might also express PrP<sup>C</sup>.

The only statistical difference in Rcoloc values of elk were those of mAbs 2–137 and CNA.42 which both label FDCs. Mule deer had an Rcoloc difference between mAbs 2–137 and CNA.42 as well as BAQ44A. The other cell markers' Rcoloc values were the same statistically both to each other, and to each of the anti-FDC mAbs. Since each anti-FDC antibody clearly labeled FDCs, this result could be due to a difference in the level of expression of their respective epitopes by FDCs. Having a high correlation value exist between cell markers for B lymphocytes and FDCs with PrP<sup>C</sup> is in agreement with the distribution seen among these cell types concerning their association with PrP<sup>Sc</sup> in infected animals [32]. An interesting result of this study was the relatively high Rcoloc values for CD3 and PrP<sup>C</sup> on T lymphocytes of both species. The expression of PrP<sup>C</sup> by T lymphocytes has been previously described in humans, goats, and mice [5, 12, 17, 20, 23] but not yet in cervids as far as the authors are aware. This is in contrast



**Fig. 8.** Immunohistochemistry staining for PrP in palatine tonsils of mule deer. (a) Tonsil follicle stained for PrP<sup>Sc</sup> in a chronic wasting disease (CWD) positive mule deer. (b) Tonsil follicle of CWD negative mule deer stained for PrP<sup>C</sup>. Note that the staining of PrP<sup>C</sup> appears much more widespread and substantial amounts of PrP<sup>C</sup> appear outside the central follicular zone (arrows). Bars a) 35  $\mu$ m, b) 70  $\mu$ m. Dashed line represents approximate germinal center borders.



**Fig. 9.** Confocal images of PrP stained tonsil follicles in mule deer. (a) Tonsil follicle stained for PrP<sup>Sc</sup> from a CWD positive mule deer. Notice staining is confined largely to the germinal center. (b) Tonsil follicle stained for PrP<sup>C</sup> from a CWD negative mule deer. Note much more dispersed staining outside the germinal center when compared to panel (a). [Figure 9b](#) is identical to [Fig. 1b](#). Bars, 70  $\mu$ m. Dashed line represents approximate germinal center borders.

to the very low level of PrP<sup>Sc</sup> seen in association with T lymphocytes in CWD infected animals [32]. This result could explain the apparent difference in distribution of PrP<sup>C</sup> since the more widespread distribution of PrP<sup>C</sup> on uninfected tissues occurs where a large portion of T lymphocytes are present (Figs. 8 and 9). This implies that the accumulation of PrP<sup>Sc</sup> is not solely due to the presence of PrP<sup>C</sup> and that there is a pathological characteristic of CWD that promotes the preferential accumulation of PrP<sup>Sc</sup> on the surfaces of FDCs and B lymphocytes, but not T lymphocytes.

The only difference in percent colocalization seen among the phenotypic markers for this study for both mule deer and elk is that of mAb BAQ15A (Table 2). Since CD21 is expressed by both B lymphocytes and FDCs [38], one would expect the percent colocalization of BAQ15A with PrP<sup>C</sup> to be much closer to the percent colocalization of cell marker mAbs BAQ44A, CNA.42 and 2–137. Yet as suggested above, mAb BAQ15A may have lower cross reactivity to cervid antigens compared to that of mAbs BAQ44A, CNA.42 and 2–137. While percent colocalization provides supplemental data to support the expression of PrP<sup>C</sup> in addition to Rcoloc values, percent colocalization cannot be used to determine the percentage of cells that express the protein of interest. This is because the cell phenotypic markers used for each cell type may be expressed at variable rates among the cell types.

When comparing the percent colocalization data for this study to that of PrP<sup>Sc</sup> determined by Sigurdson *et al.* [32] several differences are apparent. First is the increase in colocalization of mAb CNA.42 with PrP<sup>C</sup>. Since CNA.42 exhibited extensive intracellular labeling, its colocalization with PrP<sup>C</sup> suggests that FDCs actively produce PrP<sup>C</sup> intracellularly. Perhaps the most intriguing result of this study however is the percent colocalization of CD3 and PrP<sup>C</sup> seen in mule deer (48.75%) and elk (59.05%) compared to virtually no colocalization between the same in CWD infected animals with respect to PrP<sup>Sc</sup>.

These differences with respect to T lymphocyte PrP<sup>C</sup> vs PrP<sup>Sc</sup> characteristics would appear to support the results of Davenport *et al.* [6] that determined PrP<sup>C</sup> expression levels was not the sole driver of PrP<sup>Sc</sup> accumulation. It may be that T lymphocytes are not involved in PrP<sup>Sc</sup> propagation and they merely express PrP<sup>C</sup> prior to infection. They may not be susceptible to isoform conversion or are potentially not directly exposed to PrP<sup>Sc</sup> during infection.

This study provides data previously unknown for the expression levels of PrP<sup>C</sup> in natural hosts of CWD. The most common cell type implicated in prion disease lymphoid pathogenesis research seems to be the FDC [1, 3, 4, 8, 9, 11, 15, 18, 25, 31], and our results support that by showing FDC expression of PrP<sup>C</sup> in natural hosts for CWD. Future work that examines the distribution of PrP<sup>C</sup> utilizing anti-PrP antibodies with epitopes distinct from mAb F99/97.6.1 may be beneficial to determine if the protein is expressed in the same fashion across the cell types. Any differences in the expression characteristics may shed light on potential isoform conversion resistance mechanisms among the cell types.

CONFLICTS OF INTEREST. The authors declare that there are no conflicts of interest.

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