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Research paper

Abomasal mucosal immune responses of cattle with limited or continuous exposure to pasture-borne gastrointestinal nematode parasite infection

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ABSTRACT

It has been well documented that cattle raised on pasture are slow in weight gain when compared to those fed with grain. Inflammation in the digestive system commonly caused by pasture-transmitted gastrointestinal (GI) nematode parasites that could negatively impact feed conversion has never been compared in cattle raised with no pasture exposure (NPE, uninfected), limited pasture exposure (LPE, exposure until weaning), or continuous pasture exposure (CPE, life time exposure). In the present study, the abomasal mucosal immune responses and inflammation of LPE and CPE cattle were investigated. Our results indicate that CPE cattle displayed inflamed abomasa with enlarged draining lymph nodes, the presence of *Ostertagia ostertagi* larvae and higher levels of *Ostertagia*-specific antibodies in circulation. The level of B cells was elevated in the abomasal mucosa in the presence (nodular) or absence (non-nodular) of *Ostertagia*-specific pathology, where B cells were 4-fold higher in the nodular mucosa. Foxp3$^+$ CD4T cells were also noticeably elevated in both the abomasal mucosa and blood, but were only slightly higher in non-nodular mucosa than in the nodular mucosa of CPE animals. In contrast, LPE animals presented no enlargement of abomasal draining lymph nodes and exhibited little to no immune cell infiltration in the abomasal mucosa. Further, CPE animals had higher numbers of mucosal mast cells when compared to LPE animals, though mucosal mast cells were high in all animals. Overall, CPE cattle displayed significantly higher levels of inflammation and pathology in their abomasa and may explain in part slowed weight gain relative to LPE animals. The results of this study emphasize the need for GI nematode parasite control in CPE animals and development and application of vaccines which are compatible with the organic cattle production system.

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1. Introduction

Prior to the era of confined, intensive livestock farming, cattle were raised on family farms where crop residues and pasture grass were the primary sources of animal feed (Rinehart, 2011). In the modern feedlot, however, cattle are either fed or finished with grain in order to achieve the desired market weights and meat grades with less time and cost. In recent years, sustainable livestock farming gradually gained popularity due to environmental factors (Herrero and Thornton, 2013), specific beneficial nutrients, and preferred flavors in the meat (Bjorklund et al., 2014; Daley et al., 2010; Kurve et al., 2015; Maughan et al., 2012; McAfee et al., 2011; Melton, 1990; Resconi et al., 2010; Van Elswyk and McNeill, 2014; Yang et al., 2002). In general, meats derived from pasture-raised animals, or from organically-raised animals which require access to pastures, are considered to have increased health benefits (Daley et al., 2010). However, natural methods of controlling infectious diseases, including pasture-transmitted gastrointestinal (GI) nematode parasites, are problematic given the restricted use of chemical intervention such as antibiotics and anthelmintics (Cabaret et al., 2002).

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One of the major differences between cattle raised with continuous pasture exposure (CPE) and limited pasture exposure (LPE) in temperate regions of the world is exposure of the abomasum to GI nematode parasites, including *Ostertagia ostertagi*, which remains the most pathogenic nematode parasite of cattle (Gasbarre et al., 2001a,b). *Ostertagia* has a direct life cycle with a prepatent period of 18–21 days; like other trichostrongyles, the infective 3rd stage larvae (L3) are transmitted to the host through grazing on pasture. The worm causes significant pathology in the abomasal mucosa upon entering and exiting the gastric glands. Upon exiting the gastric glands, the 4th stage larvae (L4) generate high levels of local inflammation, resulting in elevated pH in the lumen, compromised acid hydrolysis, and breakdown of microbials and dietary proteins. The parasite is most prevalent in young calves during the first grazing season (Fox, 1997). The cattle suffering from the infection normally exhibit poor feed conversion accompanied by weight loss (see review by Fox, 1997). Repeated exposure does not elicit significant immune protection until animals are much older. Over the past 50 years, control of the parasite has relied heavily on the use of anthelmintics; however, anthelmintics are prohibited in organically-raised cattle and not the preferred method of control in some of the pasture-raised practices.

Pasture-raised cattle have been studied mostly for meat quality, nutrient content, and biochemical/genetic changes (Baeza et al., 2013; Daley et al., 2010; Kurve et al., 2015; Li et al., 2015; Carrillo et al., 2016). However, the mucosal immune status in the abomasum of cattle maintained on pasture has never been compared to those raised with limited exposure to pasture or pasture-transmitted nematode parasites. The present study compared the pathology typical of ostertagiosis, systemic *Ostertagia*-specific antibody titers, and immune cell composition in the abomasum, the lymph nodes draining the abomasum, and in the peripheral blood between steers with no, limited, or continuous exposure to pastures. The goal of this research was to document and understand the systemic and local immune responses and inflammation elicited by pasture-transmitted abomasal pathogens such as *O. ostertagi*, and to provide basic information for controlling parasites of pastured cattle and aid in developing immune-based countermeasures including vaccines which are permitted in organic production systems.

2. Materials and methods

2.1. Cattle

The Wye Angus has been maintained as a closed herd since 1958 by the Wye Research and Education Center, University of Maryland Experimental Station (Queenstown, MD). Experiments were conducted over a 2.5-year period; specifically, CPE steers were studied in 2013–2014, and steers with no pasture exposure (NPE) and LPE steers were studied in 2014–2015. All calves were born between January and April and weaned when they were approximately 6 months old and all calves had access to pastures prior to weaning. Thus, all LPE and CPE animals were considered exposed to various pasture-borne GI nematode parasites including *O. ostertagia* during their lifetime. The CPE and LPE calves were dewormed once with 1 mg/10 kg body weight of Dectomax Pour-On Solution (5 mg/ml, Zoetis, Inc., Florham Park, NJ) at weaning. After weaning, the calves were randomly assigned as follows, LPE (n = 10), CPE (n = 10), or NPE (n = 8). The LPE group had no additional access to pasture after weaning and received a mixed diet consisting of corn silage, shelled corn, and soybean supplemented with trace elements. The CPE group was permitted to forage on pasture consisting primarily of alfalfa during grazing season and bailage during the winter. The NPE steers were raised in confinement from birth and used in 2015 as uninfected controls for blood immune cell phenotyping and serum antibody titers. LPE steers reached market weight at approximately 14 months of age, whereas CPE steers reached market weight at 20 months of age (Li et al., 2015). All animals were euthanized at a commercial facility (Old Line Custom Meat Company, Baltimore, MD). Due to the large work load particularly in cell isolation and phenotyping using numerous antibodies in multiple combinations, not all animals were processed for cell isolation and staining. Jugular venous blood was obtained for PBMC and serum preparation. An Animal Care and Use Protocol was approved by the Beltsville Institutional Animal Care and Use Committee.

2.2. Abomasal pathology assessment

Presence (+) or absence (−) of erythema, enlarged (visible, +) abomasal draining lymph nodes, nodular pathology (nodules on abomasal surface), and the average number of nodules present per cm^2 (nodules from 5 random areas were counted) were used as criteria for gross lesion assessments of the abomasa. The distribution of nodules is classified in 4 categories, none (−), sporadic (+), 0.5–2 nodules per cm^2 (++), and greater than 2 nodules per cm^2 (+++). For microscopic lesions, levels of inflammatory cell infiltration and superficial ulceration of the mucosa were determined. Ulceration is defined as localized denuded mucosal surface where massive inflammatory cell infiltration is present. The different levels of cell infiltration are divided into 4 categories, representing low (+), intermediate (++), high (+++), or very high (++++) infiltrating cells.

2.3. Tissue and blood collection, tissue digestion and cell preparation

Tissue samples were collected from steers at the time of euthanasia. As soon as the GI tract was exposed, the abomasal tissues and the draining lymph nodes were collected and fixed in 10% neutral formalin (pH 7.2), in zinc-based fixative (0.05% calcium acetate 0.5% zinc acetate 0.5% zinc chloride in 0.1 M Tris buffer, pH 7.4) (Rieger et al., 2013), or immersed in ice-cold RPMI 1640 until processed. After processing, formalin- and zinc-fixed tissues were paraffin-embedded, sectioned and H&E-stained by Histoserv, Inc. (Rockville, MD).

Abomasal fundic mucosa with no visible nodular pathology was collected as potentially infected, but non-nodular mucosa (NNM), and the mucosa with nodular pathology (visible nodules on the mucosa) (Fig. 1A) were collected as infected nodular mucosa (NM). Individually collected nodules were pooled to obtain a single cell suspension for subsequent phenotypic analysis (Liyanage et al., 2002). Mucosa free of connective tissue and muscle were cut into 2–3 mm^3 pieces and digested at 37 °C for 2 h in 5 ml of RPMI 1640 medium containing 10% fetal bovine serum (FBS) (complete medium), 400 U/ml collagenase V, 0.1 mg/ml DNase, and 2.5 U/ml hyaluronidase (Garcia et al., 2014; Li et al., 2011; Liyanage et al., 2002). Cells were further washed 3 times with Hank's balanced salt solution (HBSS) and suspended in complete medium. Due to high numbers of total cells recovered from the abomasal tissue digestions, phenotypically defined immune cells from the pasture-raised animals (n = 9) were calculated by multiplying the total volume of cell suspension by the percent phenotypically defined live cells determined by flow cytometry. Low numbers of total cells from the LPE animals (n = 5) were recovered from the abomasal tissue digestion; therefore, total phenotype-specific cells in 0.6 g of tissue digestion were entirely enumerated by flow cytometry.

Peripheral blood from steers was collected either postmortem (LPE and CPE steers) or from the jugular vein with a vacutainer using EDTA as anticoagulant (uninfected controls). Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte sepa-

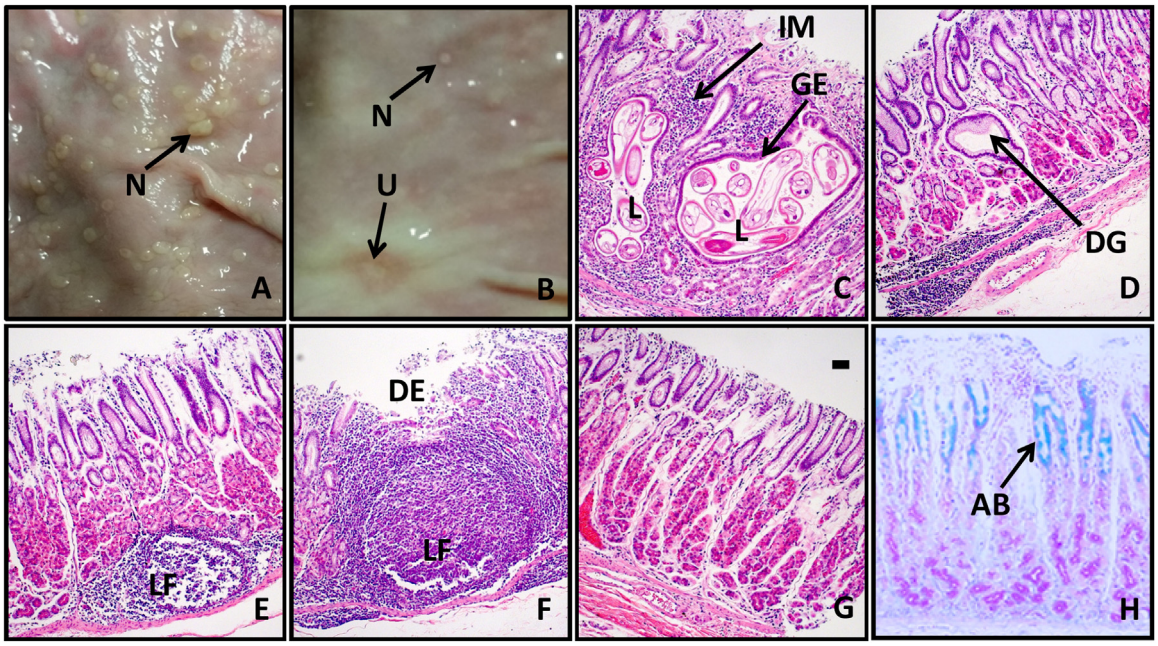

Fig. 1. Representative gross (A and B) and microscopic (C through F) pathology of the abomasum, typical of *Ostertagia ostertagi* infection, in cattle maintained on pasture; A) typical nodular pathology (N) as a result of cellular hyperplasia and inflammatory cell infiltration caused by *O. ostertagi* invasion into the gastric glands; B) abomasal pathology showing primarily the receding nodules (N) and mucosal ulcerations (U); C) Cross section of the abomasum infected with *O. ostertagi* larvae (L) that were surrounded by intact glandular epithelial cells (GE) which separated the larvae from the infiltrating immune cells (IM); D) dilated abomasal gland (DG) caused by *Ostertagia* larvae and typical immune cell infiltration at the base of the glands; E) and F) massive infiltration of immune cells (lymphoid follicle-like, LF) in place of *O. ostertagi* larvae-infected abomasal glands; G) uninfected abomasal mucosa; and H) Alcian blue (AB) staining of the abomasal mucosubstances. The *Ostertagia*-caused nodules had a size of 1–2 mm in diameter (A and B). Magnification, 400× for C) to G) and 100× for H). Scale bar (Panel G) for panels C to G, 30 μm.

ration medium (Mediatech, Manasas, VA), suspended in complete medium and then counted prior to use.

2.4. Flow cytometry

Bovine-specific antibodies were obtained from the Monoclonal Antibody Center of Washington State University (Pullman, WA). Antibodies to the following surface markers were used: (1) B-B2 (clone BAQ44A) which defines all B cells; (2) CD4 (clone CACT83B) and CD8 (clone CACT130A) which define all αβ TCR^+ T cells; and (3) δ chain of γδ TCR (clone GB21A) which defines all γδ TCR^+ T cells. Fluorescently labeled, secondary antibodies were purchased from Biolegend (San Diego, CA). Intracellular staining was performed for bovine Foxp3, using the mouse anti-bovine Foxp3 antibody (FOX5A) as described previously (Seo et al., 2009). Phenotypic analysis was performed using a FACSCalibur™ flow cytometer and CELLQuest™ software (BD Biosciences). Flowjo software (Tree Star Inc.) was used for data analysis.

2.5. Serum total IgG ELISA

Serum total antigen-specific IgG, IgG1 or IgG2 was determined as described elsewhere (Yin et al., 2012). Briefly, 96-well plates were coated with crude adult *O. ostertagi* lysate at 10 ug/ml at 37 °C for 1 h then 4 °C overnight. After washing with PBS-T (PBS, pH 7.2, 0.05% Tween-20), the plates were blocked with 25% horse serum in PBS for 1 h at 37 °C. Sera diluted 1:500 (100 ul/well) were then added in duplicate and incubated for 1 h at room temperature followed by the addition of 100 ul per well of horse radish peroxidase-labeled, goat anti-bovine IgG (H + L) (HRP, 1:2500) (KPL, Gaithersburg, MD), sheep anti-bovine IgG1-HRP or sheep anti-bovine IgG2-HRP (1:5000) (Novus Biologicals USA, Littleton, CO). TMB (Thermo Fisher) was used as substrate and the plates were read at 450 and 570 nm.

2.6. Histology and histological staining

H&E staining of formalin-fixed sections was done by Histoserv (Rockville, MD). Mast cell staining was performed on tissue sections fixed with zinc-based fixative using the toluidine blue method (Rieger et al., 2013). Immunostaining for *O. ostertagi* was conducted using a sheep antibody against the *O. ostertagi* macrophage migration inhibitory factor (Oos-MIF) (Qu et al., 2014). Briefly, tissue sections were rehydrated through xylene and ethanol (100–50%) to water, and incubated in pre-warmed antigen-retrieval solution (0.4% pepsin in 0.01 N HCL) for 15 min followed by incubation in blocking buffer (PBS, pH7.2, containing 0.5% Na caseinate, 0.75% BRIJ-35) for 10 min. The sections were then incubated for 30 min at 37 °C with sheep antibody to Oos-MIF (1:500) diluted in blocking buffer, followed by rabbit anti-sheep IgG-HRP (1:2500; KPL, Gaithersburg, MD) for 30 min at 37 °C. The HistoMark TrueBlue (KPL, Gaithersburg, MD) was used as substrate and orcein was used as the counterstain. Micrograph imaging of the stained tissue sections and toluidine blue-stained mast cell counting were conducted using the Axioskope system and the software AxioVision (Zeiss MicroImaging, Inc. Thornwood, NY).

2.7. Statistics

Ostertagia-specific ELISA antibody titers, immune cells (phenotypes/g of tissue), mast cells/mm^2 of tissue, and percent $B\text{-}B2^+$ B cells, $CD4^+$, $CD8^+$ and γδ TRC^+ T cells were all analyzed by one-way ANOVA with the Tukey-Kramer multiple comparisons test (GraphPad InStat Software, Inc., La Jolla, CA). Data are expressed as mean ± SEM. A probability of $P < 0.05$ was considered statistically significant.

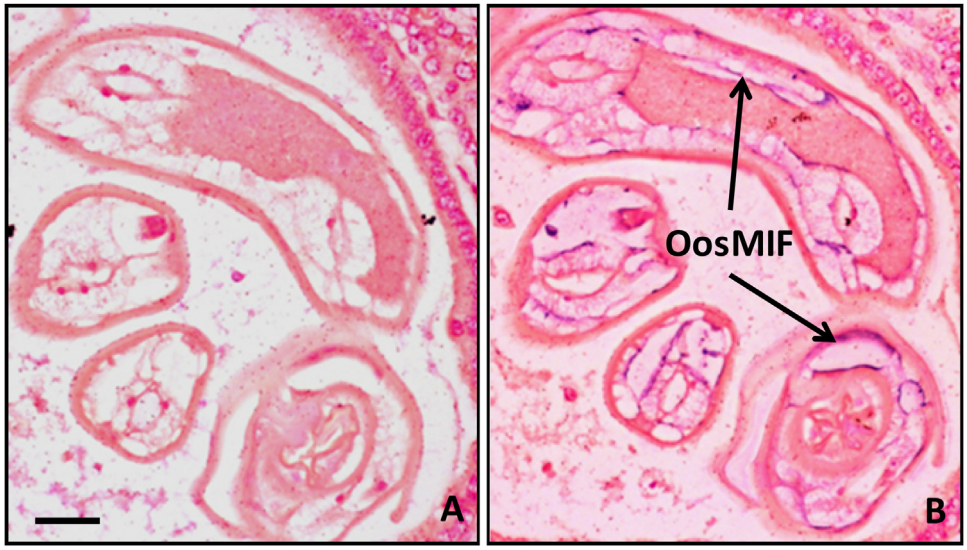

Fig. 2. Representative micrographs of immunohistochemically stained *Ostertagia* larvae in abomasal glands. (A) Control staining with pre-immune sheep sera (1:500); (B) staining with sheep anti-sera (1:500) against *Ostertagia ostertagi* macrophage migration inhibitory factor (Oos-MIF). Immunohistochemistry was conducted using the TrueBlue substrate, where the blue color represents specific staining for Oos-MIF, and the red color represents counter staining with orcein. Magnification, 400×. Scale bar (Panel A), 30 μm.

Table 1
Lesions in the abomasal fundus of steers with continuous (CPE) or limited (LPE) exposure to pasture-borne gastrointestinal nematode parasites.

	CPE steers						LPE steers				
	Lesions						Lesions				
	Gross[1]			Microscopic			Gross			Microscopic	
ET#[2]	NOD	ERY	dLN	INF	ULC	ET#	NOD	ERY	dLN	INF	ULC
359	+	+	+	++	–	562	–	–	–	+	–
393	+	+	+	++	–	565	–	–	–	+	–
400	++	+	+	++++	+	613	–	–	–	+	–
450	++	+	+	+++	+	618	–	–	–	+	–
467	+	+	+	++	–	624	–	–	–	+	–
476	++	+	+	++	–	628	–	–	–	+	–
482*	+++	+	+	+++	+	644	–	+	–	+	–
484*	+++	+	+	++++	+	647	–	+	–	+	–
489	+++	+	+	+++	+	649	–	+	–	++	–
490	+++	+	+	+++	+	650	–	+	–	++	–

[1] Gross lesions were assessed by visual inspection at slaughter and later on formalin-fixed tissues, and microscopic lesions were determined with formalin-fixed, paraffin-embedded, H&E stained tissue cross sections.

[2] ET#, animal ear tag number; NOD, nodular pathology or nodules on the abomasal surface; ERY, erythema or elevated redness of the mucosal surface; dLN, lymph node draining the entire abomasum; INF, inflammatory cell infiltration; ULC, localized ulcerations on mucosa where the mucosal surface was denuded. The distribution of nodules is classified in 4 categories, none (−), sporadic (+), 0.5-2 nodules per cm^2 (++), or greater than 2 nodules per cm^2 (+++). *, *Ostertagia ostertagi* larvae were present in the abomasal mucosa of these animals.

3. Results

3.1. Gross and histological lesions and mucosal and O. ostertagi staining

Gross lesions were absent from the abomasal surface of LPE steers while the CPE steers had apparent inflammation (erythema, as elevated redness) and nodular pathology characteristic of *O. ostertagia* infections (Fig. 1A and B; Table 1). In the CPE steers, 3 had sporadic distribution of nodules, 3 had intermediate numbers of nodules (<2 nodule/cm^2), and 4 had high numbers of nodules (>2 nodules/cm^2) (Table 1). Two steers with the most nodules on the abomasum also had *O. ostertagi* L4 in the gastric glands (Fig. 1C). Most CPE steers had high levels of immune cell infiltration with either diffuse or localized patterns in areas where previous infections may have existed (Fig. 1C through F). Some of the CPE animals showed localized surface mucosal denudation, or ulceration in the abomasum (Fig. 1F). Below the surface epithelial cell layer, massive inflammatory cell infiltration was observed that was similar to a lymphoid follicle (Fig. 1F). Furthermore, the abomasal draining lymph nodes were enlarged and numerous in CPE animals. The LPE animals had low or undetectable levels of inflammation in the abomasum, as indicated by occasional low levels of infiltration at the base of the lamina propria (data not shown) and exhibited no changes in the size and numbers of draining lymph nodes (Table 1).

Staining with Alcian blue (pH 2.5), which stains for acidic sulfated mucosubstances and sialomucins, revealed no significant difference in mucosubstances between the abomasal mucosa of LPE and CPE steers (Fig. 1H). Nematode larvae which were present in 2 of the CPE steers were specifically detected using an antiserum against Oos-MIF (Fig. 2B), but not by the pre-immune serum (Fig. 5A) as visualized by immunohistochemistry (Qu et al., 2014).

3.2. Ostertagi adult worm antigen-specific antibody titers and phenotypically defined immune cells in abomasal mucosa

Eight 5-month old calves raised indoors on concrete slabs were used as *O. ostertagia*-uninfected controls, and these calves had undetectable levels of anti-*O. ostertagi* antibodies (Fig. 3). The 20 steers raised on the Wye Farm, MD all had significant levels of antibodies reactive to the crude adult *O. ostertagi* antigen. By the end of the study, the total IgG titers were higher in CPE than the LPE steers ($P < 0.001$) (Fig. 3A). In addition, total antigen-specific IgG1 in CPE animals was higher ($P < 0.001$) than that in the LPE or NPE animals and higher ($P < 0.001$) than total IgG2 in CPE and LPE animals (Fig. 3B).

In CPE steers, B cells isolated from NM or NNM were more abundant than other immune cell phenotypes ($P < 0.05$), with NM B cells being 4-fold higher than those in NNM ($P < 0.001$) (Fig. 4). The percent B cells in abomasal draining lymph nodes and in NM was similar ($P > 0.05$). In addition to B cells, the percentage of $CD4^+$ T cells in abomasal draining lymph nodes was also high and more abundant than $CD8^+$ and $\gamma\delta^+$ T cells ($P < 0.001$). CD4, CD8, or γδ T

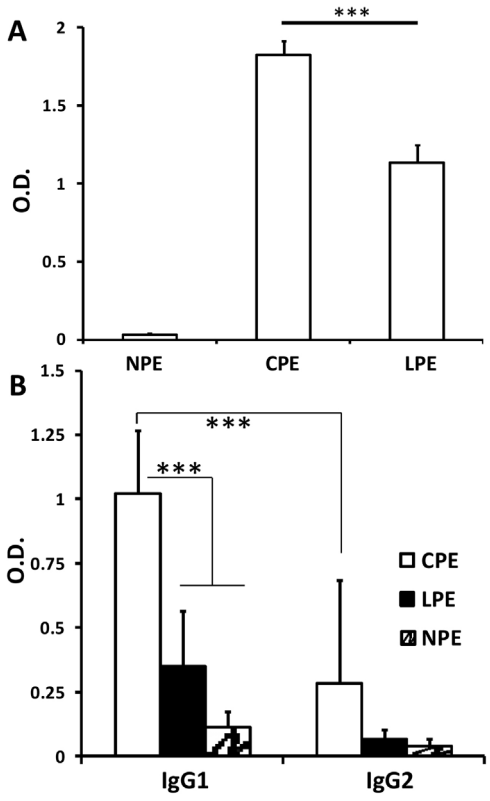

Fig. 3. *Ostertagia ostertagi* adult antigen-specific total IgG (A) and IgG1 and IgG2 (B) in sera of steers with no pasture exposure (NPE), limited pasture exposure (LPE), and continuous pasture exposure (CPE). All sera were diluted at 1:500. *Ostertagia*-specific antibodies were not detectable in uninfected steers, but readily detectable in sera from either CPE or LPE animals. ***, P < 0.001.

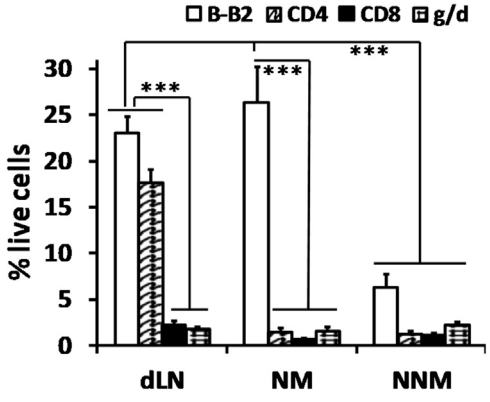

Fig. 4. Phenotypically defined immune cells isolated from tissues of steer with limited pasture exposure (LPE) (n = 5) or continuous pasture exposure (CPE) (n = 9). Single cell suspension was stained with antibodies against markers of T and B lymphocytes and the percentage of each population was determined based on live cell gate. The data represent mean cells/g of tissue + SEM. dLN, draining lymph node; NNM, non-nodular mucosa; NM, nodular mucosa. ***, P < 0.001.

cells in NM or NNM were approximately 2% or lower in CPE animals (Fig. 4).

Total numbers of each phenotypically defined immune cell type per g of abomasal mucosa were dramatically higher in CPE animals relative to LPE animals (88-fold in $CD4^+$, 18-fold in $CD8^+$, 27-fold in γδ TCR^+ T cells, or 207-fold in B cells) (P < 0.001) (Fig. 5). For immune cells derived from the abomasa of LPE steers, the γδ TCR^+ T cells were more abundant than $CD4^+$ T cells (P < 0.05), but not significantly different from $CD8^+$ T cells or B cells (P > 0.05). In CPE steers, the total numbers of B cells per g of tissue were higher than

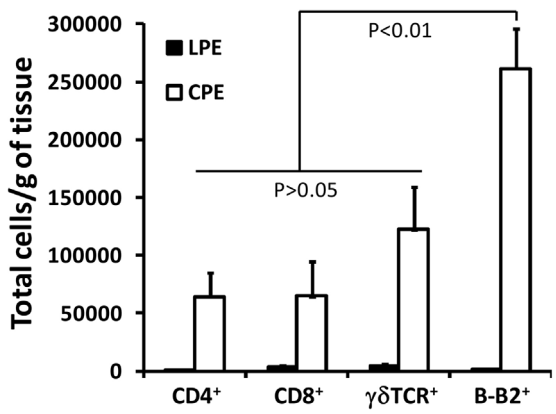

Fig. 5. Total number of phenotypically defined $CD4^+$, $CD8^+$, and γδTCR^+ T cells and B-$2B^+$ B cells from tissues of steer with limited pasture exposure (LPE) (n = 5) or continuous pasture exposure (CPE) (n = 9). Single cell suspension was stained with antibodies against markers of T and B lymphocytes and the percentage of each population and total number of cells was determined based on live cell gate. The data represent mean cells/g of tissue + SEM. LN, lymph node; NNM, non-nodular mucosa; NM, nodular mucosa; g/d, gamma/delta T cells. **, P < 0.01.

those of $CD4^+$, $CD8^+$, or γδ TCR^+ T cells in the abomasa (P < 0.001) (Fig. 5). The abomasal draining lymph nodes were not visible or not obvious in LPE steers, thus, the immune cell analysis was not performed on these lymph nodes.

Peripheral blood $CD4^+$ $Foxp3^+$ T cells of CPE steers were higher (15%) (P < 0.001) than those of the NPE uninfected control (6%) or the LPE animals (<1%) (Fig. 6D). When compared to NNM of LPE (<1%) or NM of CPE (<1%) animals, NNM of CPE (3%) animals had the highest percentage of $CD4^+$ $Foxp3^+$ T cells (Fig. 6D). In general, LPE steers had the lowest and CPE steers had the highest $CD4^+$ $Foxp3^+$ T cells in peripheral blood and abomasal mucosa.

3.3. Mast cell counts in abomasal tissues

For both LPE and CPE animals, mast cell distribution patterns in different regions of the abomasum were similar (Fig. 7). Mast cells in all abomasal mucosal regions examined were higher (P < 0.05) in CPE than in LPE animals, except for those of the fundic submucosa which did not differ (P > 0.05) between the 2 groups. The fundic body mucosa had the highest (P < 0.001) level of mast cells when compared to all other locations, including the tip of the fundic fold, pyloric mucosa, and fundic submucosa (Fig. 7), while the fundic submucosa had the lowest levels of mast cells (P < 0.001). Mast cells were similar (P > 0.05) in the fundic tip region, fundic lamina propria, and the pyloric mucosal region. Within the fundic body, mast cells were significantly higher (P < 0.001) in the fundic lamina propria than in the epithelial region.

4. Discussion

Due to the endemic nature of the GI nematode parasite infections under temperate climates, clinical and subclinical diseases caused by these parasites constitute one of the major constraints on ruminant livestock production. The overall annual cost to the industries may well exceed $2 billion in the US alone (Gasbarre, 1997). GI nematode parasite control is particularly problematic in organic production systems where anthelmintic use is prohibited. Even under pasture-raised conditions, some of the farms also prefer to have the animals free of chemical drugs and attempt to manage the infection by increased pasture rotation and lower stocking densities. In addition, reduced and/or strategic use of anthelmintics is also preferred given the emergence of drug resistance(Kaplan and Vidyashankar, 2012).

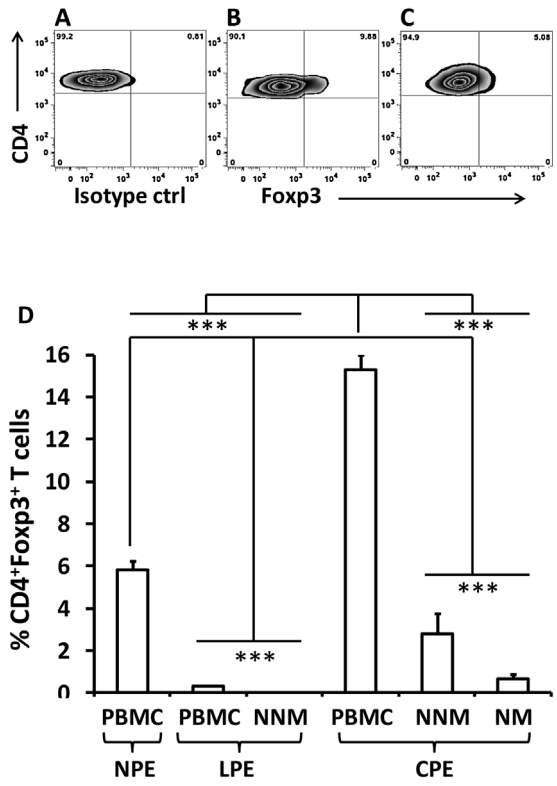

Fig. 6. $CD4^+$ $Foxp3^+$ T cells in peripheral blood and abomasal mucosa of steer with continuous pasture exposure (CPE) and limited pasture exposure (LPE). A, B and C are representative flow cytometric analyses of $Foxp3^+$ $CD4^+$ T cells of pasture-raised (A and B) and indoor-raised uninfected (C) animals; A) cells stained with anti-CD4 antibody and an isotype control for anti-bovine Foxp3 antibody; B) and C) Peripheral blood mononuclear cells (PBMCs) stained with anti-CD4 and anti-Foxp3 antibodies; D) % $CD4^+$ $Fop3^+$ T cells in peripheral blood and abomasal mucosal cells. The data represent mean% $CD4^+$ $Fop3^+$ T cells + SEM. Fluorescent intensity in log scale is indicated on the axis (A, B, and C). **, $P < 0.01$; ***, $P < 0.001$.

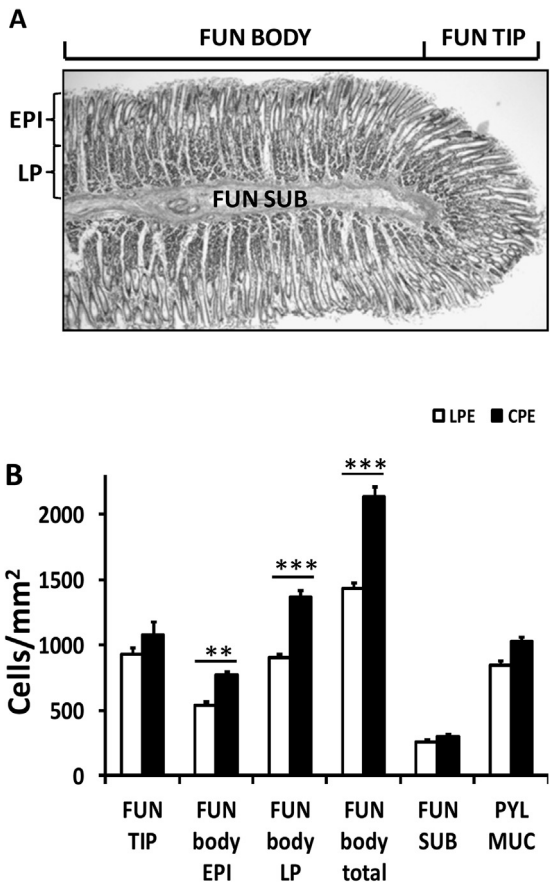

Fig. 7. Mast cell counts per mm^2 in abomasal mucosal tissues. A) a photomicrograph of the abomasal fundic fold showing sub-divisions; B) number of mast cells/mm^2 in tissue section of 5 μm in thickness. Tissues were fixed with zinc-based fixative and stained with the toluidine blue method. Stained mast cells in each piece of fundic fold or pyloric mucosa of 2–3 mm × 10–12 mm in size (3–4 pieces of each tissue were placed in each block) were counted in 3–4 random locations of each of the sub-sections as designated in the diagram (A) under a microscope with a magnification of 400×. Data are expressed as mean + SEM of 10 animals in each group. FUN, fundic mucosal region; FUN TIP, fundic mucosa at the distal end of the fundic fold; FUN body, fundic mucosa without the fundic tip; EPI, epithelial (luminal) portion of the fundic mucosa; LP, fundic mucosal lamina propria; FUN SUB, fundic submucosa; PYL MUC, pyloric mucosa. **, $P < 0.01$; ***, $P < 0.001$.

Overall exposure to various pathogens by cattle raised under either pastured or pasture-free conditions may be difficult to pinpoint; however, identification of abomasal infection by *O. ostertagi* is relatively simple and direct. There are only a few economically important, pathogenic nematode parasites that can infect the bovine abomasum, including *O. ostertagi*, *Haemonchus placei or H. contortus*, and *Trichostrongylus axei*. Among these, *O. ostertagi* is the only one that burrows into and resides in the gastric glands in the lower region of lamina propria during development until they emerge to the lumen as young adults. *H. placei* does not invade into the tissue and *T. axei* may only reside in the crypt of the abomasal surface mucosa. The pathology in bovine ostertagiosis, characterized by slightly raised, pale nodules on the abomasal surface as a result of cellular hyperplasia and inflammatory cell infiltration, is easy to identify. Mixed infections do occur in cattle, but *O. ostertagi* is considered the most pathogenic in temperate regions (Gasbarre et al., 2001a, 2001b).

Earlier studies indicate that cattle can be sensitized by exposure to *O. ostatertagi* L3 which results in an increase in globule leukocytes and mast cells (Wiggin and Gibbs, 1990). Immunoprotection against re-infection by *O. ostertagi* develops slowly and protection is not acquired until the animals reach adulthood. Antibody and cytokine responses to infection can be easily shown; however, T cell responses are difficult to demonstrate (Gasbarre, 1997; Klesius, 1993). In general, non-specific immunosuppression is noted in cattle exposed to *O. ostertagi* (Wiggin and Gibbs, 1989; Yang et al., 1993). The mechanisms for such a slow development of protective immunity against *O. ostertagi* remain unknown, despite several hypotheses (Klesius, 1993; Gasbarre, 1997).

The present study demonstrated that cattle raised on pasture, but not steers with limited access to pastures (only until weaning), had increased abomasal inflammation (redness and elevated numbers of mast cells), abomasal pathology and enlarged abomasal draining lymph nodes typical of ostertagiosis. The worm burden was not estimated in the abomasal contents of steers used in the present study. However, the presence of *O. ostertagi* L4 in 2 of the CPE steers was confirmed by the lamina propria location, typical nematode morphology and specific localization of Oos-MIF in the parasite (Fox, 1997; Qu et al., 2014). Nodular pathology typifies *O. ostertagi* infections, while the mucosal surface ulceration in some animals may be caused by *O. ostertagi* as well as other lumen dwelling nematodes. Increases in mast cells in abomasal tissues further indicate that the inflammation in CPE animal is helminth driven (Collington et al., 2011). Both CPE and LPE steers had detectable levels of *Ostertagia* antigen-specific antibodies at slaughter, suggesting that both groups were exposed to *O. ostertagia* infection while on pasture prior to weaning. The CPE animals had higher levels of *Ostertagia*-specific antibodies because they had a protracted period of pasture-borne parasite exposure. Higher

IgG1 antibody levels in CPE steers may be consistent with a biased Th2 type immune response elicited by the nematode parasites (Gasbarre et al., 2001a). Interestingly, the LPE animals had abomasal draining lymph nodes that were undetectable to the eye, and very low levels of total B and T cells in the abomasal mucosa. On the other hand, the CPE animals had high numbers of immune cells in both draining lymph nodes and mucosa, with a high percentage of B cells and CD4T cells in the lymph nodes, and dominant percentage of B cells in the mucosa. Furthermore, the abomasal mucosa with nodules caused by *O. ostertagi* infection harbored much higher levels of B cells than the mucosa without nodules. These data suggest that the dominant B cells that accumulate around the nodules may be attracted to the infection site and/or may proliferate locally at the infection site in response to the presence of the parasite larvae or their secretory products. By using celiac trunk lymphatic cannulation, it was previously demonstrated that B cells, but not T cells or monocytes, in abomasal lymphatic lymph were significantly elevated when the calves were pastured for either 1 month or in excess of 5 months, and that infected calves had higher numbers of total B cells than the uninfected controls (Baker et al., 1993). These data are consistent with our findings that the abomasa of CPE steers had higher numbers of B cells. Increased B cells in the lymph as shown by Baker et al. (1993) may be explained by increased overall B cells in the abomasa as demonstrated in the present study. Intriguingly, Baker et al. (1993) also showed that calves experimentally-infected with a large bolus of 200,000 *Ostertagia* L3 did not have elevated B cells in the lymph, suggesting that the accumulation of B cells in abomasal mucosa may be dose dependent (e.g. trickle infection which in part mimics natural exposure) or related to additional pasture-associated factors. Alternatively, the lymph may not represent all the cell populations seen in tissues. Indeed, at the transcriptional level abomasal mucosal immune responses induced by a large single dose or by multiple trickle infection doses are different (Claerebout et al., 2005; Mihi et al., 2014). Increases in IgM^+ B cells in the abomasal draining lymph nodes as shown by Baker et al. (1993) were later confirmed (Almeria et al., 1997). In contrast to the enzymatic digestion method used herein, however, Almeria et al. (1997) used a different method involving EDTA and Percoll gradient centrifugation to isolate abomasal mucosal cells which were further partitioned into intraepithelial lymphocytes and lamina propria lymphocytes. The procedural differences could in part explain why the uninfected control animals also had high levels of B cells in the mucosa when compared to the results of the present study (Almeria et al., 1997).

The present study also demonstrated that high levels of $CD4^+$ $Foxp3^+$ T cells were present in peripheral blood and abomasal NNM of CPE animals, suggesting that this phenotype may be associated with pasture/grass-transmitted pathogens including *O. ostertagi*. A subset of the $CD4^+$ $Foxp3^+$ T cells may represent the Treg phenotype in cattle (Seo et al., 2009), which has never been studied in cattle infected with *O. ostertagi*. The function of these $CD4^+$ $Foxp3^+$ T cells in the abomasum of CPE cattle remains to be determined. Consistent with the present study, recent research has shown that gene transcripts of bovine *Foxp3*, as well as those of the immunoregulatory cytokines transforming growth factor beta (*TGF-beta*),interleukin-10 (*IL-10*) and arginase 1 (*ARG1*) were upregulated upon infection by *O. ostertagi* (Mihi et al., 2014). Recent studies also showed that sheep (DRB1*1101 allele carrier) infected with *Teladorsagia circumcincta*, a sister parasite of *O. ostertagi*, exhibited early up-regulation of Treg-related cytokine genes and *Foxp3* (Hassan et al., 2011). More recently, increased $Foxp3^+$ T cells were demonstrated in *T. circumcincta*-infected sheep, with a concomitant upregulation of IL-10 which may be associated with suppression of T cell proliferation (McNeilly et al., 2013). It is intriguing that the NPE steers investigated in the present study had slightly higher levels of Foxp3+ CD4T cells in PBMC when compared to the LPE animals. At the time of the study, these steers were 5 months old and maintained indoors since birth. Whether elevation of those cells is due to the housing/feeding conditions or young age remains unknown. Indeed, the increase in the number of Treg cells in cattle could be explained in part by differences in environmental conditions under which they were raised, in addition to their exposure to GI nematode parasites (Chang et al., 2005; Seo et al., 2009). In other animals, bacterial strains in the GI tract can bias the accumulation of Tregs through short-chain fatty acid, bacterial fermentation products of dietary fibers produced by numerous bacteria (Benedé et al., 2016; Faith et al., 2014; Smith et al., 2013).

In conclusion, our studies demonstrate a major difference in animals with or without exposure to pasture-transmitted parasites. The inflammation seen in pasture-raised animals may be largely caused by chronic infection with *O. ostertagi* and the constant presence of inflammation in the stomach which likely leads to reduced feed conversion and weight gain. This in turn may explain in part why these animals were nearly 200 days delayed in achieving market weight (Li et al., 2015). Further research towards better understanding the functions of elevated B cells at the site of infection, and Tregs in circulation and in the abomasal mucosa will facilitate the development of immunologic countermeasures to control GI nematode parasite infections in cattle.

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