Short communication: Lymphocyte proliferative responses in cattle naturally infected with digital dermatitis consist of CD8+ and $\gamma\delta$ -T cells but lack CD4+ T cells

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ABSTRACT

Digital dermatitis is an infectious disease of cattle and the leading cause of lameness. This disease is complicated by the reoccurrence of the lesions and the observation of lesions on more than one limb at different time points, indicating infection may not result in a protective immune response. The objective of this study was to characterize the peripheral blood cellular response in naturally infected and naïve cattle to bacterial antigens derived from pathogens associated with digital dermatitis lesions. Peripheral blood mononuclear cells were isolated from dairy cattle identified as having active or chronic lesions during routine hoof-trimming. Following bacterial antigen stimulation, cells were analyzed for proliferation and phenotype by flow cytometry, and culture supernatants were analyzed for IFN- γ secretion. Digital-dermatitis-infected animals had greater serum antibody titers to treponemal antigens, higher percentages of proliferating CD8+, $\gamma\delta$ -T cells, and B cells, and increased IFN- γ secretion in vitro when compared with responses of naïve animals. No increase in proliferation of CD4+ T cells was detected in infected or naïve cattle. Although CD8+ and $\gamma\delta$ -T cell responses may be antigen specific, the memory nature or long-lived response is yet unknown. The lack of responsiveness of CD4+ memory cells to treponemal antigens could explain the high rate of reoccurrence of digital dermatitis in infected animals.

Key words: digital dermatitis, cellular immunity, flow cytometry

Short Communication

Digital dermatitis (DD) is a leading cause of lameness in cattle worldwide and a significant cause of economic losses through decreased production, premature

culling, and cost of treatment/prevention. The disease can also affect welfare and well-being of food-producing animals (Losinger, 2006). Digital dermatitis is a polymicrobial infection causing ulcerative proliferative lesions between the digits, in the pastern area, and rarely, elsewhere on the leg and udder (Clegg et al., 2016). Although spirochetes from the genus *Treponema* are dominant isolates from lesions, other anaerobic and aerobic bacteria are present (Wilson-Welder et al., 2015). Lesions are hyperkeratinized, proliferative, necrotic, or ulcerative, may be painful to touch, and generally bleed easily (Plummer and Krull, 2017). An important contributor to the economic impact of DD is the chronicity of lesions and the frequency for recrudescence of lesions in previously affected animals. Affected animals develop a robust antibody response to *Treponema* antigens and to other bacteria thought to be involved in the lesions (Elliott and Alt, 2009; Moe et al., 2010). Trott et al. (2003) demonstrated a brief proliferative response by peripheral blood mononuclear cells (PBMC) to *Treponema phagedenis* antigens that declined with convalescence (Trott et al., 2003). Within lesions, small lymphocytes are occasionally observed, but their roles have not been characterized. The goal of this investigation was to characterize the cellular immune response [cluster of differentiation (CD) 4+, CD8+, gamma-delta T cell receptor ($\gamma\delta$ -TCR+), or B cell+] in cattle with previously diagnosed DD lesions and determine if activation of immunologic memory responses (i.e., CD4+ or CD8+ T cells) occurs. We hypothesized that there is a failure to generate immunological memory of one or more cell types, which may contribute to reoccurrence of DD lesions in some cattle.

Holstein dairy cattle (n = 6), 2 to 5 yr of age and lactating, were housed in production dairy-like facilities at the National Animal Disease Center (NADC), Ames, Iowa, and identified as having probable DD lesions. Lesions were observed during routine hoof-trimming and resulted from natural exposure (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13913>). Negative control cattle (n = 3) were Holstein-Angus crossbred

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female cattle, 4 yr of age, and were housed in a separate outdoor facility at NADC. Control animals had no lesions or exposure to animals with DD lesions during the 4 yr they had been at NADC. Visual inspection at postmortem indicated no scars or other signs of DD; however, no histology was performed to confirm this. Sample size was dictated by available animals on site that met definitions for infected and negative control groups. Presented data are the average of 2 independent samplings obtained at 4 and 5 wk after lesion identification in infected animals. All animal procedures were approved by National Animal Disease Center Institutional Animal Care and Use Committee (Animal Use Protocol Numbers ARS-2797, 2763, 2819, 2797, and 2500) in accordance with the standards established by the *Animal Welfare Act* (USDA, 2017) and the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010).

Treponema phagedenis, strains 4A and 5B, *Treponema denticola* [strain: CIP 103919, ATCC #35405, American Type Culture Collection (ATCC), Manassas, VA], *Treponema vincentii* (strain: Smibert, ATCC #35580), and *Treponema pedis* (strain T3552B; DSMZ culture #18691, Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were grown in Oral Treponeme Enrichment Broth (OTEB, Anaerobe Systems, Morgan Hill, CA) to confluence, harvested and washed by centrifugation, and antigen prepared from whole-cell sonicates as described previously (Jergens et al., 2007; Wilson-Welder et al., 2013). Briefly, after washing by centrifugation, cells were suspended in minimal amount of sterile distilled water, frozen at -80°C overnight, and lyophilized (FreeZone 2.5, Labconco, Kansas City, MO). Dried powdered cells were stored at -20°C until used to create 10 mg/mL suspensions in sterile PBS (pH 7.4). Rehydrated suspensions were sonicated for 1 min in 15-s pulsed bursts while stored on ice. Sonicated solutions were sterilized using a UV-Stratalinker, aliquoted, and stored at -20°C until use.

Blood was collected 4 wk following hoof-trimmer visit via jugular venipuncture. Serum was separated by centrifugation and stored at -20°C . Whole cell sonicates of *Treponema* antigens (10–20 $\mu\text{g}/\text{mL}$ in PBS) were bound overnight to wells in 96-well plates (Nunc Maxisorp, Thermo Fisher, Rochester, NY). Serum was diluted 1:50 and serially diluted by factor of 2 and 100 μL added to individual wells, and incubated at 37°C for 1 h and 4°C overnight. After 3 washes with PBS containing 0.05% (vol/vol) Tween 20 (Sigma, St. Louis, MO), 100 μL of 1:1,000 dilution horseradish peroxidase-conjugated sheep anti-bovine IgG (γ -chain; KPL, Gaithersburg, MD) was added and plates incubated for 1 h at 37°C . Plates were washed 5 times with PBS-

Tween, and 100 μL of 1-step SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL) was added to all wells and the reaction stopped using 100 μL of TMB SureBlue Stop Solution (R & D systems, Minneapolis, MN). Plates were read on a microtiter plate reader at 650 nm. The ELISA data for each animal are reported as the lowest dilution of serum with an optical density that was greater than 2 standard deviations above the optical density mean for wells containing PBS only. The assay was performed in duplicate with samples blinded. The ELISA data were log-transformed and analyzed using a 2-way ANOVA with repeated measures and means separated by Tukey's multiple comparison test (GraphPad Prism 7, GraphPad Software Inc., La Jolla, CA).

Blood was collected by jugular vein into 10 mL of $2\times$ Acid-Dextran-Citrate purified on a density gradient (Histopaque, density 1077, Sigma). Isolated PBMC (5×10^6 cells) were labeled with 10 nM Cell Trace Violet (Invitrogen, Carlsbad, CA) and incubated in duplicate to wells of a 96-well microtiter plate containing 25 $\mu\text{g}/\text{mL}$ of bacterial whole cell sonicates, 10 $\mu\text{g}/\text{mL}$ of pokeweed mitogen or medium alone (no stimulation) in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% Pen-Strep (10,000 U/mL, Gibco, Waltham, MA), 25 mM HEPES buffer, 1% NEAA, 1% EAA, 1% sodium pyruvate, 50 μM 2- β -mercaptoethanol, and 100 $\mu\text{g}/\text{mL}$ of gentamicin sulfate with sodium bicarbonate solution added to restore the pH to approximately 7. Plates were incubated at 37°C , 5% CO_2 for 5 d. A duplicate plate was inoculated with unlabeled cells from which 150 μL of cell culture supernatants were collected at 48 h after stimulation. Supernatants were frozen at -20°C until assayed for cytokine concentrations.

After 5 d culture, labeled PBMC were harvested by centrifugation and labeled with live/dead discriminator dye (Zombie Yellow, BioLegend, San Diego, CA), then labeled with antibodies to cell surface markers for CD4, CD8, $\gamma\delta$ -TCR, and B cells. Primary antibodies, secondary antibodies, dilutions, and suppliers are given in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13913>). After labeling with secondary antibodies cells were then fixed and analyzed on a BD LSR II Flow cytometer and data analyzed using FlowJo software with a minimum of 2,000 cells within the live gate required for analysis. Lymphocyte populations were plotted against fluorescent intensity of each antibody using: CD4 versus B cell markers and CD8 versus $\gamma\delta$ -TCR with fluorescence-minus-one for gate restrictions similar to previously published reports (Krueger et al., 2016). The percentage of each identified subset was analyzed for decrease in fluorescence intensity of cell membrane proliferation dye as compared with background or no

stimulation wells. Data were further analyzed using GraphPad Prism 7 software fitting 2 way-ANOVA with Sidak's multiple comparisons post-test, comparing within groups (negative or DD+) the effect of well stimulation as compared with background or no stimulation wells and Dunnett's multiple comparisons post-test for within well stimulation between groups with groups being the cattle DD status (DD+, negative) and treatments being well stimulation (bacterial antigen, $\pm$ controls). Means were significant if $P \leq 0.05$.

Concentrations of IFN γ in cell culture supernatants collected at 48 h post-stimulation were determined using an AlphaLisa kit (PerkinElmer) following the manufacturer's suggested protocol. Data were analyzed using a 2-way ANOVA with Sidak's multiple comparisons post-test for comparing within group effect of well stimulation as compared with no stimulation wells and Dunnett's multiple comparisons for comparison between treatment groups (DD+ vs. negative) and between bacterial antigens. Means were significant if $P \leq 0.05$.

Serum antibody, cellular proliferation, and cellular secretion of IFN γ was measured in response to 4 *Treponema* species and 5 strains associated with DD. No significant difference was observed in responses of infected or negative cattle; thus, data from individual *Treponema* species and strains are presented as one group.

In the current study, cattle with DD lesions had greater ($P < 0.0001$) antibody responses to *Treponema* antigens, regardless of *Treponema* strain when compared with control cattle (Figure 1). This increase in response to treponemal antigens was also reflected in total PBMC proliferation (Figure 2A). Examining the phenotypes of cells proliferating in response to treponemal antigens, there was no difference between DD+ and negative groups in percentages of CD4+ cells (Figure 2B). However, there was a small but significant difference ($P = 0.003$) in percentage of proliferating CD8+ cells (Figure 2C). The CD8+ population did not include CD8+ $\gamma\delta$ -TCR+ cells. Significant differences between negative and DD+ groups were observed in percentage of proliferating $\gamma\delta$ -TCR+ cells (Figure 2D) and percentage of proliferating B cells (Figure 2E). No differences was observed ($P > 0.05$) between negative and DD+ cattle in proliferation of unstimulated cells or cells stimulated with mitogen (data not shown). After stimulation for 48 h with *Treponema* antigens, wells with PBMC from DD+ animals had greater ($P < 0.05$) concentrations of IFN γ in supernatants when compared with responses from negative animals (Figure 3).

The objective of this study was to characterize cellular responses in cattle naturally infected with DD. Others have demonstrated that cattle with DD have

robust antibody responses to treponemal antigens and other types of bacteria within lesions (Walker et al., 1997; Demirkan et al., 1999; Trott et al., 2003; Elliott and Alt, 2009; Vink et al., 2009; Moe et al., 2010). Therefore, it was not surprising that we observed B cells from cattle with DD lesions proliferated in response to *Treponema* antigens in significantly greater percentages than from naive cattle. Whole cell sonicate preparations may contain crosslinking antigens that can induce proliferation of cells and secretion of antibody in the absence of any T cell help (Murphy et al., 2008). Elliot and Alt (2009) indicated that antibody to *T. phagedenis* antigens was biased toward IgG subclass 1, indicating a T-helper type-2 response; however, presence of large amounts of IgG subclass 3, a subclass typically generated by short-lived memory B cells to polysaccharide and LPS-like antigens in T-independent pathways, could not be ruled out (Elliott and Alt, 2009). Regardless, others have observed that antibody responses fade quickly after lesion resolution and anecdotal evidence from producers would seem to indicate that an antibody response does not help resolve lesions or protect from reinfection (Walker et al., 1997; Trott et al., 2003; Gomez et al., 2014).

We observed that $\gamma\delta$ -T cells of cattle with DD proliferate in response to stimulation with bacterial antigens. Other investigations report antigen-specific $\gamma\delta$ -T cells proliferating and producing IFN γ isolated vaccinated or experimentally infected animals to other pathogens (Naiman et al., 2001; Krueger et al., 2016). Bovine $\gamma\delta$ -T cells have been described as innate-like immune cells with the ability to recognize diverse ligands including some pathogen-associated molecular patterns

Figure 1. Serum antibody titers to treponemal antigen as measured by dilution ELISA. Control animals (negative) were never exposed to digital dermatitis; DD+ are animals with digital dermatitis lesions observed during routine foot-trimming 4 wk before blood collection. Group means and SEM are shown. * indicates a statistically significant difference ($P < 0.0001$) between negative and DD+.

Figure 2. Proliferative responses and cellular phenotypes of peripheral blood mononuclear cells isolated from the blood of cattle negative (no exposure) to digital dermatitis or with lesions detected during routine foot-trimming 4 wk before blood collection (DD+). Peripheral blood mononuclear cells were stimulated for 5 d with treponemal antigens. Group mean and SEM shown. * indicates a statistically significant difference between negative and DD+ groups. (A) Percentage of proliferating total lymphocytes, $P = 0.0004$. (B) Percentage of proliferating cluster of differentiation (CD) 4+ cells, no significant difference. (C) Percentage of proliferating CD8+ cells, $P = 0.0003$. (D) Percentage of proliferating cells expressing $\gamma\delta$ -T cell receptor (gdTCR+), $P < 0.0001$. (E) Percentage of cells expressing surface IgM (B cells), $P < 0.0001$.

(Hein and Dudler, 1997; Guzman et al., 2014). The highly diverse receptor repertoire of bovine $\gamma\delta$ -T cells is one of many differences between ruminants, mice, and humans, along with the relatively high numbers of these cells in ruminant tissues, including the skin and peripheral blood circulation (Guzman et al., 2014). The WC1 receptor on bovine $\gamma\delta$ -T cells appears to have affinity for protein and nonprotein antigens from *Lep-tospira* and *Mycobacteria*, indicating a possible role in detecting bacterial antigens in non-MHC presentation (Baldwin et al., 2014). In this limited study, the role of WC1+ specificity to *Treponema* antigens was not investigated. Much like CD4+ or CD8+ T cells, $\gamma\delta$ -T cells can have memory-like phenotypes, proliferating and releasing IFN γ upon recognition of their cognizant antigen. Interferon γ , a potent pro-inflammatory cytokine, has multiple functions activating cells of innate and adaptive immunity for long-lived memory and pathogen clearance, and is generally associated with T-helper type 1 responses (Murphy et al., 2008). We observed increase in IFN γ release from stimulated cells from cattle with DD over DD-negative cattle; however, more study is needed to discern the phenotype of cells secreting IFN γ .

Additionally, we observed an increase in CD8+ cell proliferation in antigen stimulated cells from cattle with DD lesions over DD-negative cattle. The CD8+ memory cells are linked to many dermal inflammatory conditions, such as atopic dermatitis, experimentally induced psoriasis, and viral infections (Gunderson et

al., 2013; Hijnen et al., 2013; Carbone and Gebhardt, 2014). Usually localized in tissues, these resident memory cells actively promote lymphocyte recruitment to the site of infection/inflammation, are capable of Th1/Th2 cytokine biases typically associated with CD4+ T cells. Additional study is needed to determine if the proliferating CD8+ cells observed from cattle with DD lesions are resident memory cells, expressing markers of memory cells such as CD45RO and skin-specific homing signals such as CCR8 (Denis et al., 2011; McCully et al., 2012).

Alternatively, the proliferating CD8+ cells observed could also be NK-like or NK-T cells. The NK-T cells in ruminants are capable of responding specifically to bacterial antigens in recall reactions, including proliferation and the production of IFN γ (Hope et al., 2002; Zuerner et al., 2007; Park et al., 2015). Like $\gamma\delta$ -T cells, NKT cells may respond to lipid antigens presented by CD1 rather than MHC (Hope et al., 2002). The NKT cells can comprise a significant proportion of CD8+ fraction in ruminants, and as in this study can be easily overlooked by utilizing too few cellular markers to fully identify cellular populations (Park et al., 2015).

Of particular note is the lack of proliferation in the CD4+ cell population. The cell type most often associated with helper T cell functions (i.e., cytokine secretion for B cell class switching, cytotoxic T cell activation, and macrophage activation) and long-lived memory cell responses (Murphy et al., 2008). It is unclear how long-lived memory $\gamma\delta$, NKT, or CD8+ T cell responses may be, or how the lack of CD4+ responses affect the effector functions of the cellular responses. The time point of several weeks after the hoof-trimmer visit was deliberately chosen to allow for any immune responses created in a new acute lesion to develop fully. As with the antibody response, it would appear that the cellular response induced by natural exposure or infection is not protective, and being slightly inflammatory (Th1 bias, increase in IFN γ), may contribute to lesion development.

It is not in the pathogen's best interest to generate immune responses that facilitate pathogen clearance, tissue healing, or other means of the pathogen's destruction or limitation to metabolites. The use of different adjuvants, immune modulators, or inoculation methodology can tweak the immune response into eliciting a differently biased immune response to that same pathogen (Sallusto et al., 2010). Heifers may be exposed to DD before they ever enter the milking herd; thus, vaccination is likely to occur in the face of pre-existing immune response (Gomez et al., 2015). It is critical to understand the pre-existing immune response to DD, even if it is not effective, so that vaccines can be generated that not only create the right type of im-

Figure 3. Interferon γ cytokine release from peripheral blood mononuclear cells isolated from negative (no exposure) cattle or cattle with digital dermatitis lesions detected during routine foot-trimming 4 wk before blood collection (DD+). Peripheral blood mononuclear cells were stimulated for 48 h with treponemal antigen lesions before supernatants were collected. Group mean and SEM shown. * indicates a statistically significant difference between negative and DD+, $P < 0.0001$.

mune response (e.g., humoral, cellular, Th1, Th17, and so on) but can do so with pre-existing responses.

Further study is needed to follow greater numbers of cattle for longer periods of time, including examining cellular responses in acute versus chronic and fully healed lesions over time. The limitations of this study show the need to take full advantage of the ever-expanding immunological toolbox available for bovines to conduct complex analysis into cellular phenotype and function.

In conclusion, we observed an antigen-specific proliferative response in cattle with DD lesions consisting of B cells, CD8+, and $\gamma\delta$ -TCR+ T cells. Further studies are needed to elucidate the further phenotype and memory properties of these cells. The immune response elicited by natural infection consisting mostly of antibody, CD8+, and $\gamma\delta$ -T cells is not effective at overcoming existing immune responses, effective or not, has implications for vaccine design.

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