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Short communication: Lymphocyte proliferative responses in cattle naturally infected with digital dermatitis consist of CD8+ and γδ-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+, γδ-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 γδ-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 (**γδ-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, Leibinz-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 μg/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× Acid-Dextran-Citrate purified on a density gradient (Histopaque, density 1077, Sigma). Isolated PBMC (5×10^6 cells) were labeled with 10 n*M* Cell Trace Violet (Invitrogen, Carlsbad, CA) and incubated in duplicate to wells of a 96-well microtiter plate containing 25 μg/mL of bacterial whole cell sonicates, 10 μg/mL of pokeweed mitogen or medium alone (no stimulation) in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 1% Pen-Strep (10,000 U/mL, Gibco, Waltham, MA), 25 m*M* HEPES buffer, 1% NEAA, 1% EAA, 1% sodium pyruvate, 50 μ*M* 2-β-mercaptoethanol, and 100 μg/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, γδ-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 LSRII 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 γδ-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