THEILERIA PARVA: IMMUNOPATHOLOGY, CORRELATES OF PROTECTION, AND VACCINE DEVELOPMENT

By

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East Coast Fever (ECF), caused by the apicomplexan parasite, *Theileria parva*, kills over a million cattle each year in sub-Saharan Africa. Cattle that develop ECF succumb to respiratory failure-induced pulmonary edema; however, the immunopathogenesis of these lesions is poorly understood. While Cape buffalo and cattle herds raised in *T. parva* endemic areas seem to develop innate resistance to disease and experience a low mortality rate, cattle herds newly introduced to regions with *T. parva* often experience up to a 90% mortality rate. These findings led us to hypothesize that ECF results from immune dysregulation during acute infection in naïve cattle. To test this hypothesis, we compared antemortem clinical pathology data, necropsy findings, and histopathology results from twenty African Boran calves and five Holstein calves infected with a lethal dose of *T. parva*. Infected cattle developed severe vasculitis of medium to large caliber vessels within the lungs and lymphoid organs. Immunohistochemical studies revealed that intrallesional macrophages were positive for the pro-inflammatory cytokine, IL-17, and expressed the marker of alternative activation,
CD163. These findings, coupled with the antemortem clinical pathology results, suggest that ECF is a form of macrophage activation syndrome (MAS), an often-fatal form of immune dysregulation observed in many infectious diseases and neoplasia. In ECF, parasite-driven lymphoproliferation likely leads to secondary systemic macrophage activation syndrome, vasculitis, pulmonary edema, respiratory failure and death. These findings are discussed in Chapter 1.

Following our discovery of the complexity of the immune response in acute *T. parva*, we began work to better characterize responding leukocyte populations and to define cellular phenotypes associated with both protection and harm in *T. parva*. Although cytotoxic CD8+ T-cell responses specific for *T. parva*-infected cells correlate well with protection from *T. parva* challenge, other features of protective and detrimental immune responses have not yet been elucidated. To fill this gap, we developed flow-cytometric assays to assess cytotoxicity and cytokine production in bovine leukocytes. These assays enable concurrent, multi-parameter assessment of responding leukocytes, and will greatly enhance our understanding of the immune response to *T. parva*. The development and optimization of these assays is described in Chapter 2.
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GENERAL INTRODUCTION

*Theileria parva* is an intralymphocytic apicomplexan parasite of Cape buffalo (*Syncerus caffer*) and cattle in sub-Saharan Africa. Although Cape buffalo do not develop disease when infected with *T. parva*, the parasite kills over a million cattle in the region each year, resulting in greater than $300 million dollars in annual losses [1, 2005]. Smallholder migratory farmers, who depend on livestock to feed and provide for their families, are the most heavily impacted by *T. parva* [2].

*T. parva* is primarily transmitted by the three-host tick, *Rhipicephalus appendiculatus*. Larval or nymphal ticks acquire the parasite while feeding on infected buffalo or cattle, and the parasite undergoes sexual reproduction in the tick midgut [1]. While the tick moults to its next instar, *T. parva* kinetes migrate to the tick salivary glands and mature into sporoblasts. During subsequent feeding, sporoblasts mature into the infective sporozoite stage, and are transmitted to their bovine or buffalo host [3]. Following transmission by infected ticks, sporozoites quickly enter lymphocytes and mature into schizonts [1].

Schizonts lie free within the host cell cytoplasm, and, through incompletely understood mechanisms, prevent apoptosis of infected cells and lead to cell transformation and lymphoproliferation [4-6]. Due to close association with the host cell spindle apparatus, schizonts divide along with their host cell [7]. In this, each daughter cell will contain a schizont, and exposure of the parasite to the extracellular environment is minimal. *T. parva* infected lymphocyte cultures maintained *in vitro* are considered immortal, and continue to divide until theileriacidal drugs are added to the medium [8].

In infected cattle, morbidity and mortality are the direct result of schizogony, and the constellation of resultant clinical signs is termed East Coast Fever (ECF). ECF is
characterized by prolonged, high fever and generalized peripheral lymphadenomegaly. In severe cases, animals develop terminal respiratory distress and die of respiratory failure[9, 10]. The pathogenesis of ECF is not fully understood; however, tissue infiltration by transformed lymphocytes likely plays a role in lesion development.

As the infection progresses, schizonts give rise to merozoites, which invade erythrocytes following lymphocytolysis. Unlike the related Apicomplexa *Theileria equi* and *Theileria annulata*, however, *T. parva* does not cause significant erythrocyte damage or anemia [11-13]. Mortality rates due to ECF are highest in cattle raised in non-*T. parva* endemic areas [14], but herd resistance can develop after several generations of consistent exposure to *T. parva*. Cattle that survive are often *T. parva* carriers [15] and have robust, protective immunity to similar strains of the parasite.

Due to rapid development of acaricide resistance and the infeasibility of consistent acaricide treatment in the field, prevention of ECF is largely based on the infection and treatment method (ITM), by which cattle are infected with live *T. parva* sporozoites and treated with long-acting oxytetracycline [16]. ITM is has been used effectively in Tanzania [1], and generates a strong protective immune response to similar strains of *T. parva*.

Protective immunity to *T. parva* is consistently achieved with sub-lethal natural infection and ITM, and is mediated primarily by CD8+ cytotoxic T cells specific for schizont-infected lymphocytes [13, 17]. Indeed, transfer of cytotoxic CD8+ T cells from a *T. parva*-immune calf to its *T. parva*-naïve, identical twin sibling prevented disease in the naïve sibling upon *T. parva* challenge [18]. Apart from CD8+ T cell killing of *T. parva* infected lymphocytes, to date there are no other known immune correlates of
protection in *T. parva*. Indeed, other common *in vitro* measures of T cell responsiveness, including the production of interferon gamma and cellular proliferation, provide no indication of protection from subsequent challenge [19].

Although quite effective, ITM is not an ideal vaccine for several reasons. First, production of the live sporozoite stabilate is incredibly labor-intensive and expensive, and requires over 600,000 ticks, 500 rabbits, and 130 cattle to produce a batch large enough to immunize one million cattle [1]. Second, as they are infected with live sporozoites, ITM vaccinees become *T. parva* carriers [20], and serve as a reservoir of infection. Third, to maintain infectivity, the live sporozoites must be stored in liquid nitrogen, which severely limits practical use of this vaccine in the field [1]. Finally, the current ITM formulation, known as the Muguga cocktail, is a mixture of three *T. parva* strains. Although this combination of strains elicits protection against many *T. parva* strains in Kenya and Tanzania, vaccine breakthroughs have been reported when cattle are co-grazed with Cape buffalo, or when the vaccine is used in regions outside of Kenya and Tanzania [1]. These limitations of ITM underscore the urgent need to develop an effective subunit vaccine for *T. parva*.

Although ITM is known to elicit protective, strain-specific CD4+ and CD8+ T-cell responses to *T. parva* in cattle, subunit vaccines utilizing known CD4+ and CD8+ *T. parva* T-cell antigens produce a protective response in only a small subset of cattle [19]. As in malaria, HIV, and tuberculosis, a means of generating CD8+ T-cell immunity with a subunit vaccine is the critical next frontier in *T. parva* vaccinology. While progress has been made in understanding *T. parva* immunity, significant gaps, including the role of immune dysregulation in ECF, as well as more complete characterization of immune
correlates of protection against *T. parva*, must be filled before a better vaccine can be developed.

We chose to first explore the role of immune dysregulation in ECF. Histologic evaluation of tissue from calves that succumbed to ECF led to the discovery that *T. parva* causes severe vasculitis of small to medium-caliber vessels within the lungs, lymphatic organs, and liver. Immunohistochemical studies on these tissues revealed that infiltrating leukocyte populations within vasculitis lesions were almost entirely comprised of macrophages and T cells, and that *T. parva* infected cells were not a significant component of most lesions [21].

Furthermore, infiltrating macrophages almost always expressed high levels of the pro-inflammatory cytokine, IL-17, and the marker of alternative activation, CD163. These findings, coupled with antemortem clinical signs and clinical pathology data, suggest that ECF is a form of macrophage activation syndrome (MAS). Not only can MAS cause severe tissue damage, it can alter T-cell responsiveness and functionality, thus rendering the cellular immune response ineffective. These data, described in detail in Chapter 1 of this dissertation, provide new insight into the role of the innate immune response in ECF pathogenesis and immunity, and provide a valuable first clue about barriers to the development of protective T-cell immunity in cattle [21].

Our recent discovery of the complexity of ECF immunopathology emphasizes the urgent need for complete characterization of responding leukocyte populations and a definition of cellular phenotypes associated with both protection and harm in *T. parva*. These definitions will allow more targeted vaccine development, in which adjuvants and delivery platforms can be selected or avoided based on the desired immune response,
rather than repeated cycles of trial and error using expensive bovine *T. parva* challenge studies. In addition, knowledge of necessary immune correlates of protection will enhance our ability to assess and compare new vaccine candidates across numerous research laboratories and experimental protocols, expediting collaborative vaccine development between laboratories in Africa, Europe, and the United States.

To this end, we developed a flow cytometric assay to measure cell killing by bovine leukocytes. Although similar assays are commonly used to assess human and murine immune responses [22, 23], these assays were not previously adapted to the bovine due to limited availability of bovine antibody reagents and the paucity of bovine models of successful cell-mediated immunity. Our herd of ITM-immunized cattle are solidly immune to lethal *T. parva* challenge, and serve as an excellent model of successful cell-mediated immunity. Additionally, we maintain *T. parva* infected, immortalized cell lines for each of these animals, which serve as a constant source of antigen for use in assay development. Thus, using immune lymphocytes from our cattle and autologous *T. parva* infected cell lines, as well as bovine reagents from the WSU Monoclonal Antibody Center, we developed and optimized the bovine flow cytometric killing assay. In addition, in order to begin to further expand our knowledge of cytokine production by memory T cells in *T. parva*, we optimized an ex-vivo, whole blood bovine intracellular interferon-gamma staining assay [24] for our *T. parva* system.

Unlike previous assays that allow measurement of a single immune response outcome (e.g. proliferation or cytotoxicity) but provide no data on the phenotype of cells responsible for the outcome, our assays allow concurrent, quantitative measurement of multiple parameters, including cytokine production, cell killing, and determination of the
detailed surface phenotype of responding leukocyte populations. These assays will significantly expand our understanding of the attributes that comprise immune protection in ITM immunized animals and *T. parva* survivors, providing a target for future vaccine development efforts. These assays are described in detail in Chapter 2.
REFERENCES


CHAPTER ONE

East Coast Fever Caused by Theileria parva is Characterized by Macrophage Activation Associated with Vasculitis and Respiratory Failure

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ABSTRACT

Respiratory failure and death in East Coast Fever (ECF), a clinical syndrome of African cattle caused by the apicomplexan parasite *Theileria parva*, has historically been attributed to pulmonary infiltration by infected lymphocytes. However, immunohistochemical staining of tissue from *T. parva* infected cattle revealed large numbers of CD3- and CD20-negative intralesional mononuclear cells. Due to this finding, we hypothesized that macrophages play an important role in *Theileria parva* disease pathogenesis. Data presented here demonstrates that terminal ECF in both Holstein and Boran cattle is largely due to multisystemic histiocytic responses and resultant tissue damage. Furthermore, the combination of these histologic changes with the clinical findings, including lymphadenopathy, prolonged pyrexia, multi-lineage leukopenia, and thrombocytopenia is consistent with macrophage activation syndrome. All animals that succumbed to infection exhibited lymphohistiocytic vasculitis of small to medium caliber blood and lymphatic vessels. In pulmonary, lymphoid, splenic and hepatic tissues from Holstein cattle, the majority of intralesional macrophages were positive for CD163, and often expressed large amounts of IL-17. These data define a terminal ECF pathogenesis in which parasite-driven lymphoproliferation leads to secondary systemic macrophage activation syndrome, mononuclear vasculitis, pulmonary edema, respiratory failure and death. The accompanying macrophage phenotype defined by CD163 and IL-17 is presented in the context of this pathogenesis.
INTRODUCTION

Theileria parva is an intracellular apicomplexan parasite of Cape buffalo (Syncerus caffer) and Bos indicus and Bos taurus cattle in sub-Saharan Africa. Mortality rates are high in most imported Bos taurus breeds and indigenous breeds raised in non-endemic areas [1]. T. parva kills over one million cattle each year in sub-Saharan Africa, resulting in severe economic disadvantage for pastoral farmers. Like the closely related protozoan pathogens Plasmodium and Babesia, and the distantly related protozoan pathogens Trypanosoma and Leishmania, which cause disease in animals and humans, T. parva is arthropod-borne.

T. parva is primarily transmitted by the three-host tick Rhipicephalus appendiculatus. Following transmission to cattle by infected ticks, sporozoites enter lymphocytes and mature into multinucleated schizonts. Schizonts induce constitutive activation of multiple host-cell signal transduction pathways, preventing apoptosis and causing cell transformation and marked lymphoproliferation [2-5]. Association of the schizont with the host cell mitotic spindle apparatus allows the schizont to divide in concert with infected host cells, and also minimizes schizont exposure to extracellular clearance mechanisms. Prior to data presented here, clinical disease, including terminal ECF, was thought to be driven solely by lymphoproliferation and resultant organ infiltration by infected, transformed lymphocytes.

Clinical disease (East Coast Fever) is characterized by marked peripheral lymphadenopathy, fever, anorexia and respiratory distress. Small numbers of infected cattle develop neurologic signs (turning sickness), due to blockage of cerebral vessels by clusters of parasitized cells. Clinicopathologic changes described in end-stage
disease include severe leukopenia, thrombocytopenia, lymphocytolysis in lymphoid tissues and hemorrhage [6, 7]. The causes of leukopenia, which involves both lymphoid and myeloid cells, are unclear but likely include rupture of infected lymphocytes during merozoite production, and lysis of uninfected cells in lymphoid tissues. Treatment with antiparasitic drugs, such as buparvaquone early in the course of infection, can lessen disease severity, but does not always eliminate the parasite [8]. Immunization of cattle by concurrent infection of cattle with live \textit{T. parva} sporozoites and treatment with long-acting oxytetracycline, known as the infection and treatment method (ITM) [9], results in transient clinical reactions, apparently due to the impact of oxytetracycline on parasite replication. At least one \textit{T. parva} strain included in the ITM cocktail results in long-term \textit{T. parva} infection [10].

Cattle that survive natural infections or are immunized using ITM develop solid immunity to similar strains. In these animals, the protective immune response is largely mediated by major histocompatibility complex (MHC) class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) specific for \textit{T. parva} schizont-infected lymphocytes [11] and is often strain-specific [12]. Cattle immunized using ITM also generate MHC class II-restricted CD4$^+$ T cells specific for \textit{T. parva} schizont-infected lymphocytes [12, 13]. Like parasite-specific CTLs, parasite-specific helper T cells are sometimes strain-specific [12].

Unfortunately, animals infected with \textit{T. parva}, even with concurrent treatment, often fail to control the parasite and succumb to severe ECF within three weeks of infection. Severely affected cattle usually succumb to pulmonary edema [6]. Widespread tissue infiltration by transformed lymphocytes, including infiltration of alveolar septae, has been documented by histopathologic evaluation [6, 14-16]. These
data led to the understanding that pulmonary infiltration by transformed lymphocytes was associated with respiratory failure.

Two immunohistochemical (IHC) studies on tissue from infected cattle have been published [17, 18], and support previous histologic data that large numbers of T cells infiltrate the lungs of cattle with ECF. While our initial IHC analyses also demonstrate abundant T cells within the lungs of cattle, we also documented a large population of CD3- and CD20-negative mononuclear cells within tissues of affected cattle. Based on these studies, we hypothesized that histiocytic cells (cells of the monocyte/macrophage lineage) play a role in the development of acute ECF in cattle. Histology, IHC, and morphometry were used to garner an understanding of the role macrophages play in the development of acute ECF.

Although some of the components of the immune response to *T. parva* are well-understood, the pathogenesis of lethal disease, including the possible role of aberrant immune responses, was hitherto largely unexplored. Since macrophages can serve both immunostimulatory and immunosuppressive roles, and thus alter the efficacy and character of the adaptive immune response, we also sought to provide initial characterization of the histiocytic response in *T. parva*. CD163 is a macrophage scavenger receptor that is generally up-regulated in anti-inflammatory, immunosuppressive states, and also in response to severe tissue injury ([19, 20]). In contrast, IL-17 is a pro-inflammatory cytokine produced by many leukocytes (including macrophages) that is often associated with severe immunopathology ([21-23]). We used immunohistochemical labeling of CD163 and IL-17 to provide basic phenotypic information about the macrophage response in *T. parva*. These data were coupled
with clinical data to generate a more detailed understanding of the immunopathogenesis of acute ECF.

Data presented here expand ECF pathogenesis to include a macrophage-centric inflammatory response. While a robust innate immune response is likely required to trigger a disease-limiting adaptive immune response to *T. parva*, excessive macrophage activation and resultant tissue destruction likely trigger ECF. Importantly, infection with lethal doses of *T. parva* was found to cause severe lymphohistiocytic vasculitis of the lungs, lymph nodes, spleen and liver, and these organs were shown to contain large numbers of CD163+ and IL-17+ macrophages. We propose that pulmonary edema and respiratory failure during ECF are due to the development of pulmonary vasculitis, and that the induction of a multisystemic histiocytic response contributes significantly to clinical disease in ECF.

**MATERIALS AND METHODS**

**Holstein cattle (*Bos taurus)*.** All animal experiments were approved by the Washington State University and University of Idaho Institutional Animal Care and Use Committees, protocol numbers 2013-66 (U of I) and 04515-002 (WSU). All therapeutic drugs were administered according to the manufacturer’s dosing instructions. This study utilized five, three month-old Holstein steers from dairies in northern Idaho and central Washington. Cattle were quarantined at the USDA-ARS-ADRU vector disease research barns for two weeks before the onset of the study, and received regular health checks from a licensed veterinarian at this time. Pre-infection complete blood counts (CBCs) and serum chemistry panels were normal. After quarantine, calves were
housed in pairs in box stalls in the USDA-ARS-ADRU vector disease research barn. Stalls were bedded with wood shavings. Animals had continuous access to fresh water and were fed grass and alfalfa hay and a small amount of Calf Manna (MannaPro®, USA) twice daily. Mechanical heating and cooling units, coupled with open-air ventilation were used to maintain the barn at approximately 16º C. Calves were exposed to overhead lighting from 7 a.m. to 5 p.m. daily, and were closely monitored by animal care staff and veterinarians.

To infect animals with *T. parva*, 0.2-0.5 mL of cryopreserved *T. parva* Muguga sporozoite stabilitate Ed80 was injected subcutaneously in the left parotid region. Following infection, complete physical examination, including rectal temperature, palpation of peripheral lymph nodes and thoracic auscultation, was performed on each animal at least once per day. At the onset of pyrexia (rectal temperature ≥ 39.4º C), CBCs were performed regularly to monitor leukocyte, erythrocyte, and platelet counts. As soon as peripheral lymph node enlargement was detected, needle aspirates were collected from affected nodes once daily, and Giemsa-stained smears of aspirates examined for schizont-infected lymphocytes.

Two animals were co-treated with Liquamycin® (Zoetis, USA), a long-acting form of oxytetracycline (LA OTC) at the time of infection. In the three remaining calves, Terramycin® (Zoetis, USA), a short-acting oxytetracycline (SA OTC) was administered intramuscularly every 24 hours after the onset of pyrexia in an attempt to curtail schizont parasitemia. In all calves, pyrexia was controlled via parenteral administration of flunixin meglumine (Pfizer Animal Health, USA), and anorectic calves were given Resorb® (Zoetis, USA) oral electrolyte solution three times a day. If animals developed
dyspnea or tachypnea, furosemide (Bayer Healthcare, USA) was administered via
intramuscular injection to decrease pulmonary edema. Buparvaquone (Bimeda,
Ireland) was administered via intramuscular injection every 48 hours to aid in controlling
disease in two calves.

Three inoculated calves developed severe dyspnea that failed to respond to the
aforementioned treatment protocols and were euthanized via intravenous injection of
Fatal Plus (Vortech Pharmaceuticals, USA), and one calf died suddenly of respiratory
failure just before euthanasia could be administered. In each case, a complete
necropsy was performed within twelve hours of death. Three uninfected, age and
breed-matched calves were sacrificed and used as negative controls for necropsy,
histopathology, IHC, and morphometry. Table 1 summarizes information on each
infected Holstein calf included in this study.

<table>
<thead>
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<th>Animal Number</th>
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<th>Antibiotic Treatment</th>
<th>Buparvaquone</th>
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<tr>
<td>1412</td>
<td>0.5</td>
<td>LA OTC, ITM</td>
<td>Yes</td>
<td>Survived</td>
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<tr>
<td>1415</td>
<td>0.3</td>
<td>LA OTC, ITM</td>
<td>Yes</td>
<td>Euthanized</td>
</tr>
<tr>
<td>1419</td>
<td>0.2</td>
<td>SA OTC</td>
<td>No</td>
<td>Euthanized</td>
</tr>
<tr>
<td>1420</td>
<td>0.2</td>
<td>SA OTC</td>
<td>No</td>
<td>Died</td>
</tr>
<tr>
<td>1435</td>
<td>0.2</td>
<td>SA OTC</td>
<td>No</td>
<td>Euthanized</td>
</tr>
</tbody>
</table>
**Histopathology and immunohistochemistry, Holstein cattle.** Tissue sections were fixed in 10% neutral buffered formalin for at least seven days. For basic histopathology, formalin-fixed sections of lung, lymph node, heart, liver, spleen, kidney, adrenal gland, fore stomachs, intestines, brain and pituitary gland were trimmed, routinely processed, stained with hematoxylin and eosin (H&E), and examined with a light microscope. Immunophenotyping of the leukocyte infiltrates was performed using immunohistochemical (IHC) detection of CD3 (T cell marker [24]), CD20 (B cell marker [25]), IBA-1 (pan-histiocytic (monocyte/macrophage/dendritic cell) marker [26, 27]), CD163 (macrophage marker [28]), and, due to recent studies on its importance in lesion development in several protozoal infections, interleukin-17 [21-23, 29]. Detection of *T. parva* schizonts was performed using a monoclonal antibody to the *T. parva* schizont surface-bound polymorphic immunodominant molecule (PIM) antigen [18, 30]. The species of origin, isotype, source, concentration, and Antibody Registry numbers (where applicable) of the primary antibodies used in this study are provided in Table 2.
Table 2. Antibodies used for IHC and Fluorescent Co-Labeling IHC

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
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For all IHC, 2-3 µm thick serial sections of formalin-fixed, paraffin-embedded tissues were placed onto positively charged glass slides (Superfrost®/Plus, Fisher Scientific, Pittsburg, PA). An automated processor (Discovery XT, Ventana Medical Systems, Tucson, AZ) was used for deparaffinization, antigen retrieval and immunolabeling. For single immunolabeling, sections were incubated with the primary antibody for two hours, followed by the Ventana universal secondary antibody and detection with the Discovery DAB® (3,3’-diaminobenzidine chromagen) Map Kit (Ventana). For double-labeling studies, sections were then incubated with the second primary antibody for three hours, followed by the Ventana universal secondary antibody and detection with the Discovery® RedMap (Red/Napthol chromagen) Kit (Ventana). All slides were then counterstained with hematoxylin, and evaluated using a light microscope. Immunostaining of tissues from an uninfected calf, as well as incubation
of tissues with irrelevant, isotype-matched primary antibodies or substitution of primary
and secondary antibodies with antibody diluent were used as controls in all reactions.

**Fluorescent Immunohistochemical Co-Labeling.** Thin section
deparaffinization, antigen retrieval and fluorescence immunohistochemistry were
performed using the automated slide staining platform, Discovery XT (Ventana Medical
Systems). Following extended deparaffinization (75 °C), epitope retrieval was achieved
using a tris-based buffer, pH 8.5, (Cell Conditioning Solution CC1, Ventana Medical
Systems) held at 95 °C for ~60 minutes ("standard cell conditioning" protocol).
Thereafter, the sequence of automated protocol steps for dual immunofluorescence
labeling were: antibody blocking, application of 1<sup>st</sup> primary antibody and detection steps
using tyramide-FITC signal amplification (RUO anti-Rabbit or anti-Mouse IgG FITC,
Ventana Medical Systems), denaturation (32 minutes at 95 °C), application of 2<sup>nd</sup>
primary antibody and detection steps using Goat anti-Rabbit or anti-Mouse IgG
conjugated to AlexaFluor 647 (Invitrogen).

Dual labeling was performed to detect cells co-expressing IL-17 (1<sup>st</sup> primary
antibody, 4 hours incubation, Table 2) and each of the cell lineage markers CD3, CD163,
and IBA-1 (2<sup>nd</sup> primary antibodies, 12 hours incubation, Table 2). Control reactions
included substitution of the 1<sup>st</sup> primary with 10 µg/mL Rabbit non-immune serum (Dako),
the 2<sup>nd</sup> primary with 6 µg/mL Rabbit non-immune serum or 2.5 µg/mL of a Mouse
monoclonal IgG1 irrelevant epitope antibody. No fluorescence bleed through or cross-
reactivity were observed in these control reactions.

Dual labeling of CD163 and IBA-1 was conducted as above for IBA-1 (2 µg/mL) as
the 2<sup>nd</sup> primary antibody, but substituting MCA1853 (1 µg/mL, 2 hour incubation) as the
1st primary antibody. No fluorescence bleed through or cross-reactivity were observed when substituting 1 µg/mL of a Mouse monoclonal IgG1 irrelevant epitope antibody as the 1st primary antibody or 2 µg/mL Rabbit non-immune serum as the 2nd primary antibody.

Epifluorescence microscopy was conducted using an Axio Imager.M1 microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with an X-Cite 120 Fl Illuminating system (EXFO Photonic Solutions, Mississauga, Ontario, Canada), an AxioCam MRm digital camera, and a computer workstation running AxioVision 4.8.1 imaging software. Fluorescence images were captured as a z-stack (step increment 275 nm) using an EC Plan-Neofluar 40x/1.3 oil M27 objective. Maximum intensity projections of z-stack images were processed using Fiji (an ImageJ-based open source image processing package) and figures organized using Photoshop Elements software.

**Boran Cattle (Bos indicus).** H&E stained sections of the left pre-scapular lymph nodes from twenty African Boran calves were evaluated. All cattle were used in an ECF infection kinetics study at the International Livestock Research Institute in Nairobi, Kenya in 1981 [31]. In the study, 17/20 cattle were infected with a lethal dose of *T. parva* Muguga, and three calves were used as negative controls. Following infection, pairs of cattle were euthanized on days 3, 6, 8, 10, 12, 14, and 16 days post infection. Of the remaining three infected animals, one was euthanized on day 18, one on day 19, and one animal succumbed to ECF on day 19. The negative control animals were euthanized at the end of the experiment. Lymph nodes were collected during postmortem examination, fixed in 10% neutral buffered formalin, and routinely
processed for histopathology. Slides were stored until 2014 by Dr. Ivan Morrison, and were shipped to Pullman, WA for histologic evaluation.

**Morphometric Measurements of Lungs.** To provide quantitative measurements of CD163, IBA-1 and IL-17 positive staining in lung tissue, whole slide digital images were collected and automated image analysis performed. Briefly, sections of lung from infected and control animals immunohistochemically labelled for IBA-1, CD163, and IL-17 as described above were scanned in bright field with a 20X objective using a Nanozoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, New Jersey). The whole slide digital images were then imported into Visiopharm software (Hoersholm, Denmark) for analysis. The Visiopharm Image Analysis module automatically detected tissue on slides, from which sections of lung were selected. For each marker, 184-234 mm$^2$ of control calf lung tissue was analyzed and 497-625 mm$^2$ of infected calf lung tissue was analyzed. 100% of each lung section was analyzed. Digital images were converted to gray scale values using RGB-R and RGB-B filters with a mean size of 5 pixels by 5 pixels. Visiopharm was trained to label positively stained cells for CD163, IBA-1 and IL17, and to detect background tissue counter stain (Hematoxylin) using an analyte-specific configuration based on a threshold of pixel values. Images were processed in batch mode using the configuration developed for each analyte to generate the desired outputs. For each analyte, the following were calculated: A. Area of positive immunolabeling, B. Area of hematoxylin stained tissue, C. Total area of tissue (A+B), and D. Ratio of positive analyte staining to total tissue area (A/C). Any non-specific staining (e.g., staining along the edge of tissues) on tissue sections or any tissue that showed substantial damage
from processing was excluded from analysis. Based on output for each section analyzed, the mean value for each analyte was calculated by averaging the results obtained from each animal and then averaging the results for the control cattle and cattle infected with *T. parva*. Mean values for control and infected groups were compared using a one-tailed student’s t-test (α<0.05).

**RESULTS**

**Clinical disease kinetics of ECF in Holstein calves.** Left parotid lymphadenopathy was noted 4-7 days post infection, and enlargement of contralateral and other regional lymph nodes developed by day 9. Schizonts were detected in Giemsa-stained aspirates of the left parotid lymph node within 24 hours of the development of lymphadenopathy, and the percentage of schizont-positive, blasting lymphocytes ranged from 9%-34% during acute disease. Large numbers of uninfected lymphoblasts were also detected in all aspirates. All cattle exhibited severe, sustained pyrexia (39.5-41.6 °C) from 6-9 days post infection onward. Resting respiratory rates in all calves began to increase between days 6 and 11 post-infection, and cattle exhibited terminal dyspnea characterized by neck extension, rapid, shallow inspiration and marked expiratory effort consistent with restrictive pulmonary disease. During this time, crackles were auscultated over all lung fields. Between days 10 and 15, four of five animals developed petechiae over the gingival and scleral mucosae. Over the course of acute disease, the body weight of all animals decreased 25%-33%, and calves exhibited decreased skeletal muscle mass and increased skeletal prominence. Similar clinical reactions were observed in the Boran cattle used in the ECF infection kinetics study at ILRI in 1981 (Morrison, et al., 1981).
From day 8-11 post infection, total leukocyte counts dropped significantly, with nadirs of 300-3000 leukocytes/µL of blood (normal 4,000-12,000/µL). Both neutrophil and lymphocyte subsets were affected; however, neutropenia was far more severe than lymphopenia in all animals. The Boran cattle infected at ILRI in 1981 also developed severe terminal leukopenia [31]. In our study, four calves developed severe thrombocytopenia, with nadirs of 42,000-59,000 platelets/µL of blood (normal 100,000-700,000/µL). The development of thrombocytopenia corresponded to the development of mucosal petechiation. Terminally, three calves (1415, 1419, and 1435) developed mild anemia (PCV 21%-23%, pre-infection PCV for these calves was 29%-31%), and all animals developed moderate hypoproteinemia. Beginning seven days post infection, schizont-infected lymphoblasts were consistently detected on Giemsa-stained blood smears; however, merozoite infected erythrocytes were rarely detected. Sustained, moderate liver enzyme elevations were noted in two of five (1415 and 1412) animals from day seven post infection onward.

**Gross Lesions of ECF in Holstein calves.** Necropsy findings were similar in all animals. The pleural, pericardial, and peritoneal cavities contained abundant free serosanguinous fluid (Supplementary Fig 1), and petechiae and ecchymoses were disseminated across the epicardium, tracheal mucosa, visceral pleura, and serosa of the forestomachs, and intestines. All animals had severe interstitial pneumonia affecting all lung lobes, as well as severe pulmonary edema, with expansion of interlobular septae and abundant foam within the airways (Supplementary Fig 2). Bilaterally, the retropharyngeal, parotid, cervical, prescapular, tracheobronchial, pre-hepatic and sublumbar lymph nodes were enlarged to 2-20 times normal size. On cut
surface, enlarged nodes were edematous, and contained large foci of hemorrhage and necrosis. Two animals (1415 and 1420) had severe, subcutaneous intermandibular edema. Calf 1415 also had severe fibrinosuppurative tracheitis and bronchopneumonia (secondary bacterial infection).

**Histopathologic lesions of ECF in Boran and Holstein calves.** In order to provide a more comprehensive understanding of ECF pathogenesis, histologic evaluation of the lungs, lymph nodes, spleen, liver, heart, brain, and intestines of Holstein calves with terminal ECF was performed. In multiple foci throughout the lungs of all calves, small to medium caliber blood and lymphatic vessel walls were severely disrupted by fibrinoid degeneration, necrotic cellular debris, edema, and macrophages and lymphocytes (mononuclear vasculitis). Affected vessels were lined by reactive endothelial cells, and clusters of monocytes were frequently adhered to the luminal aspect of the endothelium. In general, thin-walled veins and lymphatic vessels were more severely affected than arterioles. Alveoli and interlobular septae were filled with and widened by edema, fibrin, hemorrhage, and alveolar macrophages, and alveolar septae were markedly widened by fibrin mats and infiltrating macrophages and lymphocytes (Fig 1).

Within lymph nodes, corticomedullary distinction was largely lost, and normal architecture replaced by sheets of macrophages and lymphoblasts separated by large aggregates of necrotic cells, fibrin and hemorrhage. As in the lungs, small to medium caliber blood and lymphatic vessels within lymph nodes were often disrupted by vasculitis. Lymph nodes draining the site of infection were most severely affected (Fig 1).
Hepatic portal triads were rimmed by moderate to large numbers of macrophages and lymphoblasts, and portal vessels were multifocally, mildly disrupted by vasculitis.

Within the spleen, the distinction between the red and white pulp was often unapparent. In some foci, periarteriolar lymphatic sheaths were often greatly expanded by monomorphis populations of lymphoblasts or replaced by sheets of macrophages. Numerous macrophages were filled with hemosiderin, suggesting increased erythrocyte turnover and erythrophagocytosis.

In order to establish a kinetic framework for the development of histologic lesions in ECF, sections of lymph nodes collected from Boran cattle at regular intervals following T. parva infection were examined and compared. Within these sections, mild vasculitic lesions were evident as early as three days post infection. From day 10 to 19, severe lymphoid necrosis, edema and hemorrhage developed within affected nodes. During this time period, vasculitis lesions became more prevalent and more severe, with marked fibrinoid degeneration, endothelial disruption, and fibrin thrombus formation observed in the majority of vessels by day 19. Representative histologic lesions are shown in Fig 1.

**Tissue distribution of T. parva infected cells.** In all infected animals, large numbers of schizont-infected cells were detected within the lungs, lymph nodes, spleen, liver, and bone marrow, and rare infected cells were detected within the brain, adrenal glands, thyroid gland, heart, and tongue. Schizont-infected cells were most prevalent in the spleen (Fig 2), and were widely dispersed throughout the red and white pulp. Within the lungs, infected cells infiltrated interlobular and alveolar septae, and were
occasionally within perivascular connective tissue and vessel walls. Infected cells were within the cortex and medulla of lymph nodes, and often surrounded portal vessels within the liver. No staining with the anti-schizont antibody was detected in tissues from an uninfected control calf.

\section*{Immunohistochemical characterization of mononuclear cell tissue infiltrates.} In all animals, the lungs, lymph nodes, spleen, and liver contained markedly increased numbers of T cells (CD3$^+$ cells); however, B cells (CD20$^+$ cells) were rarely detected within the lungs and liver, and were greatly decreased within the spleen and lymph nodes (Supplementary Fig 3). Within the lungs, T cells were found within interlobular and alveolar septae, within perivascular connective tissue, and within vessel walls and lumina (Fig 3). Large sheets of blastic T cells expanded the red and white pulp of the spleen and the cortex and medulla of lymph nodes, and were often detected within vessel walls of both organs. T-cells expanded the periportal space within the liver.

The lungs, lymph nodes, spleen, and liver of all infected animals contained large numbers of IBA-1 positive (histiocytic) cells. Within the lungs of infected animals, large numbers of macrophages infiltrated vessel walls, alveolar septae, alveoli, and interlobular septae, and large clusters of macrophages were often adhered to the endothelium of small to medium caliber vessels. In lung sections from the control animals, normal alveolar macrophages were observed in the alveolar septae. In the lymph nodes of infected calves, large sheets of macrophages obscured normal corticomedullary architecture, disrupted vessel walls, and partially occluded vessels. In lymph nodes from the infected calf, macrophages were confined to the subcapsular and medullary sinuses. Within infected animals, but not uninfected controls, sheets of
macrophages multifocally disrupted the red and white pulp of the spleen, and small to moderate numbers of macrophages were mixed with lymphocytes in periportal areas of the liver. Further sub-typing of macrophage populations using CD163 labeling revealed that the vast majority of tissue-associated macrophages within infected animals were CD163+ (Fig 3). The mean percent sectional area positive for CD163 and IBA-1 in lung sections from *T. parva* infected and control calves is listed in Table 3. The mean percent sectional area positive for CD163 was significantly greater in the *T. parva* infected calves than in the control calves (p=0.005). This finding supports the histologic impression that the lungs of infected calves contain larger numbers of CD163+ macrophages than those of the control calves. Although the CD163+ macrophage subset is increased in the lung during acute ECF, there was no significant difference in total histiocytic cell (IBA-1 positive) sectional area in *T. parva* infected animals and control animals (p=0.28).

<table>
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Expression of IL-17 by macrophages. In all infected animals, the lungs, lymph nodes, spleen, and liver contained large numbers of IL-17-positive mononuclear leukocytes. Within the lungs, IL-17-positive cells were found within alveolar and interlobular septae, alveoli, and within vessel walls and vasculitic lesions (Fig 3). Sheets of IL-17-positive cells were most abundant within the subcapsular and medullary sinuses of lymph nodes, and small numbers of IL-17-positive cells were found within the superficial cortex. IL-17-positive cells were often found within periportal regions of the liver, and scattered throughout sheets of macrophages within the spleen. Importantly, vascular endothelial cells were frequently strongly positive for IL-17 (Fig 3). Non-cell associated IL-17 staining was frequently observed within mats of fibrin and within connective tissue of vessel walls. The mean percent sectional area positive for IL-17 in lung sections from *T. parva* infected and control calves is listed in Table 3. The mean sectional area positive for IL-17 within the lungs of *T. parva* infected cattle was significantly greater than that in the control cattle (p=0.01).

Within the lymph nodes and lungs of both the infected and control calves, almost all IL-17-positive mononuclear cells were co-positive for IBA-1, and only rarely co-positive for CD3, suggesting that, in acute *T. parva* infection, IL-17 is produced predominantly by macrophages (Fig 4). Interestingly, in all calves, the majority of IL-17-positive macrophages co-expressed CD163 (Fig 4). The number of IL-17-positive cells was significantly greater in the infected calves than in the control cattle. Since CD163 is only expressed on a subset of macrophages, and is up-regulated in actively phagocytic cells, we performed co-labeling studies using IBA-1 and CD163. As expected, all CD163-positive cells were also positive for IBA-1, but only a subset of IBA-1 positive
cells were also positive for CD163 (Fig 5). In the infected cattle, a subjectively larger proportion of IBA-1-positive (histiocytic) cells co-expressed CD163 than in the control cattle.

**DISCUSSION**

The results of this study provide new insights into the immunopathogenesis of acute, fatal ECF. Protozoa -- including *Plasmodium* sp., *Trypanosoma* sp., and *Toxoplasma* sp., are adept at immune system subversion, and numerous pathogen-mediated forms of immune evasion -- such as antigenic variation, limited antigen production, persistence in immune-privileged sites, and altered antigen presentation have been described in protozoal infections [32-35]. Protozoa have also been shown to subvert T-cell responses by triggering anergy, exhaustion, or apoptosis [36]. Another mechanism of T-cell inhibition in protozoal disease is the induction of alternatively activated, or suppressor, macrophages [37]. Although definitive phenotypic characterization of macrophage subsets *in situ* is difficult because gene expression in macrophages is highly plastic and changes with fluctuating environmental cues [38], alternatively activated macrophages in humans and mice are often characterized by high expression of CD163 [37].

CD163 is a membrane-bound, hemoglobin/haptoglobin scavenger receptor protein in the scavenger receptor cysteine-rich superfamily group B [39]. Hemoglobin is released from damaged erythrocytes, and can cause oxidative damage to cells. After binding to CD163, hemoglobin is degraded within the macrophage, and several anti-inflammatory substances are released [40, 41]. In addition to its scavenger receptor role, CD163 can also bind bacteria and viruses, and its expression is up-regulated on
mature, actively phagocytic tissue macrophages [19]. In general, CD163 expression is up-regulated by glucocorticoids and IL-10, and down-regulated by pro-inflammatory cytokines, including IFN-γ [20].

In infectious disease, alternatively activated macrophages have been shown to suppress T-cell activation. The presence of CD163+ macrophages is associated with increased tissue invasion, decreased T-cell activity, and resultant poor prognosis in many forms of human cancer [42, 43], and alternatively activated macrophages are associated with altered T-cell function and parasite persistence in trypanosomiasis [44-46] and toxoplasmosis [47]. It is possible that lysis of T. parva infected lymphocytes and erythrocytes, and the resulting free iron and pro-inflammatory milieu of necrosis stimulates a robust CD163+ macrophage response in acute T. parva. The presence of large numbers of CD163+ macrophages, could, in turn, inhibit necessary proliferative and cytotoxic T-cell responses. Alternatively, since T. parva infected lymphocytes have been shown to produce significant amounts of IL-10 [48], it is possible that schizont-infected lymphocytes themselves stimulate a CD163 macrophage response in acute ECF.

Robust CD163+ macrophage responses are also observed in macrophage activation syndrome (MAS, also referred to as hemophagocytic lymphohistiocytosis), an exaggerated systemic macrophage response observed in numerous neoplastic, autoimmune, and infectious diseases [49], including trypanosomiasis in cattle [50]. Clinical components of MAS include prolonged fever, lymphadenomegaly, splenomegaly, hemorrhage, cytopenias that affect at least two leukocyte lineages, thrombocytopenia, hypoproteinemia, elevated liver enzymes, hemophagocytosis, tissue
infiltration by activated macrophages, hyperferritinemia, and elevated soluble CD163 (sCD163) [51]. CD163 is cleaved from the membrane of macrophages in response to severe inflammation, exposure to LPS, and cross linkage of FC receptors [19]. In humans and mice, serum levels of sCD163 increase markedly during acute inflammation, and serve as a means of monitoring macrophage activation [52, 53].

The cattle in this study demonstrated several of the hallmark criteria of MAS, including fever, lymphadenomegaly, hemorrhage, panleukopenia, thrombocytopenia, hypoproteinemia, elevated liver enzymes, and infiltration of numerous tissues by macrophages. Consistent with MAS, the spleens of these cattle contained increased numbers of macrophages, the majority of which were CD163+. Many macrophages contained hemosiderin pigment, which is consistent with hemophagocytosis and increased erythrocyte turnover. Large numbers of schizont-infected lymphocytes were present in the spleens of the animals. Elucidation of the direct effect of schizont-infected lymphocytes on macrophage induction in acute ECF requires further study. As assays do not yet exist to quantify soluble CD163 in the serum of cattle, we were unable to measure sCD163 in this study.

Interestingly, many of the CD163+ macrophages also exhibited positive immunolabeling for IL-17, a pro-inflammatory cytokine. CD163 is considered a multifunctional receptor, and thus, CD163+ macrophages often serve multiple, disparate functions [19]. Monoclonal antibody binding to CD163 caused production of pro-inflammatory cytokines in rats [54], and engagement of CD163 by bacteria also led to pro-inflammatory cytokine production [55]. Thus, CD163+ cells may play myriad immunomodulatory roles throughout the disease course in ECF.
It is likely that the marked macrophage-mediated IL-17 response contributes significantly to the development of severe lesions in fatal ECF. In many protozoal infections, including *Leishmania major* [21], *Toxoplasma gondii* [23] and *Eimeria tenella* [22], severe tissue damage is associated with an overly robust IL-17 response, and can often be abrogated by administration of anti-IL-17 antibodies [22].

IL-17 can be produced by many leukocyte subsets, including Th17 CD4+ cells, CD8+ T cells, γδ T cells, macrophages, dendritic cells and NK cells [56], and acts on many cell types, including leukocytes, endothelial cells, epithelial cells, chondrocytes, osteoclasts, and fibroblasts. Binding of IL-17 to the IL-17 receptor leads to activation of MAP-kinase, NF-κB, and C/EBPβ pathways, leading to production of numerous chemokines, cell adhesion molecules, matrix metalloproteinases, and antimicrobial substances [57]. In psoriasis, activation of ERK and MAP kinases after IL-17 binding to endothelial cells leads to increased cell adhesion molecule and chemokine expression and vascular inflammation [58]. IL-17 production is also a component of giant cell arteritis, and is believed to exert its effects on endothelial cells and fibroblasts within vessels, leading to endothelial cell activation, increased expression of adhesion molecules and cytokines, recruitment of mononuclear cells, and vascular damage [59, 60]. In this study, endothelial cells were strongly positive for IL-17, suggesting that IL-17 produced by leukocytes in the affected tissues was binding to endothelial cells. Downstream effects of IL-17 binding to endothelial cells likely gave rise to the severe vasculitis noted in many tissues in these calves.

Significantly, the most severe vasculitic lesions were present within the lungs of both Boran and Holstein calves, suggesting that the pathogenesis of ECF is the same in
both *Bos indicus* and *Bos taurus* cattle. Inflammation of pulmonary blood and lymphatic vessels is the cause of terminal pulmonary edema in ECF. In this, vasculitis leads to leakage of protein-rich fluid into the airways and pleural space, causing severe restrictive and obstructive respiratory distress, hypoxia, and death. The histological and immunohistochemical findings of this study further suggest *Theileria parva* infection triggers macrophage activation syndrome and IL-17 production leading to the development of vasculitis.

Future work will be undertaken to determine the cellular mechanisms of macrophage activation and the effect of CD163+ macrophages on T-cell function. This enhanced understanding of the role of the innate immune response in *T. parva*, and its subsequent effect on the trajectory of the adaptive immune response, will aid in the development of a vaccine that elicits an efficacious cytotoxic T-cell response without causing severe immunopathology. Furthermore, the results of this study suggest that immunosuppressive drug therapy or anti-IL-17 therapy may provide clinical benefit to cattle with ECF.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LMF, DAS, CWF, WIM DPK. Analyzed data: LMF, DAS, CWF, WIM, DDN, DPK. Performed experiments: LMF, DAS, CWF, WIM. Contributed reagents/materials/analysis tools: CWF, WIM. Wrote the paper: LMF.
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Fig 1. Histologic Lesions in Holstein and Boran cattle. Representative photomicrographs of H&E stained sections of lymph node medulla in uninfected control and infected Boran (day 12) and Holstein calves, and lung from uninfected control and infected Holstein calves. Note: Severe disruption of vessel walls by mononuclear cells and fibrinoid degeneration (lung and lymph node), and lymphohistiocytic interstitial pneumonia with edema (lung). Scale bar: 200 µm.
Fig 2. Splenic PIM Immunoreactivity. Shown are the results of immunohistochemical labeling of the *T. parva* antigen, PIM, in the spleen. A. The spleen of the control calf lacks PIM immunoreactivity. B. In a representative infected Holstein calf, there is abundant PIM immunoreactivity within lymphocytes of the red and white pulp. Scale bar: 200 µm.
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Fig 3. Immunohistochemical Labeling of Leukocytes in the Lungs. Shown are the results of IHC labeling for CD3-like immunoreactivity (CD3-li), CD163-li, IBA-1-li, and IL-17-li in the lung of uninfected control and representative infected Holstein calves. Note: CD3-li, CD163-li, and IL-17-li is significantly increased in the lungs of the infected calf. Furthermore, CD3+, CD163+, IBA-1+ and IL-17+ mononuclear cells are components of vasculitis lesions. Vascular endothelial cells consistently exhibit pronounced IL-17-li in the infected calf, but endothelial IL-17-li is rare in the control calf. Scale bar: 200 µm.
Fig 4. Dual Fluorescence Labeling of Leukocytes in Lymph Nodes. Shown are the results of dual fluorescence labeling for IL-17-li (pseudocolored cyan) and either CD3-li, IBA-1-li, or CD163-li (pseudocolored magenta) in the medulla of a lymph node from a control calf and representative infected calves. Note: IL-17-li was infrequent in the medulla of control calf lymph node but was widespread throughout the medulla of infected calves. Where it occurred within the non-vascular elements, IL-17-li was generally punctate and appeared intracellular. The cell-associated, punctate IL-17-li was considerably weaker in intensity and less dense as compared to the infected calves. In both the infected calves and, where present, in the control calf, intracellular punctate IL-17-li was most frequently co-localized with IBA-1-li cells and CD163-li cells but not with CD3-li cells. Scale bar: 20 µm.
Fig 5. CD163 and IBA-1 Dual Fluorescence Labeling. Shown are the results of dual fluorescence labeling for CD163-li (pseudocolored cyan) and IBA-1-li (pseudocolored magenta) in a lymph node cortical follicle and medulla from the control calf and representative infected calves. In both calves, most CD163-li cells were co-labeled by IBA-1-li but not all IBA-li cells were co-labeled with CD163-li. IBA-1-li cells without definitive CD163-li were most frequent in the control calf lymph parafollicular regions. Scale bar: 20 µm.
S1 Fig. **Severe Pleural and Peritoneal Effusion, Calf 1435.** In all deceased calves, large amounts of free pleural and peritoneal fluid was noted during the gross exam.
S2 Fig. Severe Interstitial Pneumonia and Pulmonary Edema, Calf 1435. In all deceased calves, lungs were reddened, wet, and heavy, and there was marked expansion of interlobular septae by edema.
S3 Fig. CD20 Immunohistochemical Labeling in the Lungs. In all cattle, CD20-positive cells (B lymphocytes) were rare in the lungs. Depicted is part of a normal bronchiolar associated lymphoid follicle. Note the lack of B lymphocytes within alveolar septae, vessel walls, and vessel lumina. Scale bar = 200 µm.
CHAPTER TWO

Development of flow cytometric assays to measure bovine Leukocyte responses to *Theileria parva*.

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ABSTRACT

The intra-lymphocytic, apicomplexan parasite, *Theileria parva*, is a leading cause of death in African cattle, resulting in severe economic hardship for pastoralist farmers. Although the development of a sustainable, next-generation vaccine to protect cattle from *T. parva* is a major focus of international aid organizations, vaccine development is hindered by the lack of multi-dimensional bovine immune assays essential for elucidating the mechanisms associated with development of protective immunity against *T. parva*. The objectives of the present studies were to develop and optimize flow-cytometric assays to assess cytotoxic effector function and cytokine production in bovine leukocytes. These assays were developed with cells from *T. parva*-immune cattle. In the future, these assays can be used to provide a more complete picture of the cellular phenotypes associated with immune protection from *T. parva*, and to thoroughly evaluate multiple aspects of the cellular immune response elicited by candidate *T. parva* vaccines. In addition, these assays can also be used to answer similar questions in other bovine diseases, such as *Mycobacterium* sp., in which T-cell responses are required for control. Finally, since cattle are outbred, insights gained from the present studies may expand approaches available for the study of other additional in humans such as tuberculosis, malaria and AIDS.
INTRODUCTION

The tick-borne, apicomplexan parasite *Theileria parva* kills over a million cattle annually in sub-Saharan Africa [1]. Mortality rates are highest in European cattle breeds imported for higher meat and milk yields, and most losses are incurred by pastoralist farmers [2]. Indeed, livestock morbidity and mortality due to *T. parva* are among the leading causes of poverty in the region. Improved control of *T. parva* via subunit vaccine development is a critical aspect of international aid programs to combat poverty in sub-Saharan Africa. Immunity to *T. parva*, like malaria, tuberculosis, and HIV, is centered on development of a cytotoxic T-lymphocyte (CTL) response [3]. Unfortunately, the ability to generate CTL immunity using a subunit vaccine remains elusive.

The key component of *T. parva* immunity is CD8+ CTLs specific for schizont-infected lymphocytes [3, 4]. A protective CTL response is elicited by natural infection and the infection and treatment method (ITM), in which cattle are infected with *T. parva* and co-treated with oxytetracycline [5]. ITM-immunized cattle develop mild disease, and become immune to similar strains of *T. parva* [6]. Although effective, logistical requirements and regional strain variation preclude the sustainability of ITM as a vaccine, and necessitate next-generation vaccine development [1].

To date, a number *T. parva* CTL antigens have been tested in cattle using multiple delivery platforms [7]. Although T cells from immunized cattle consistently exhibited antigen-specific interferon gamma responses, only one third of the animals developed CTL responses to infected cells, and many developed severe disease during subsequent challenge [7]. Although the requirement for a CTL response in *T. parva* is
clear, attempted exploitation of this single immune dimension is clearly insufficient to generate a protective vaccine. As in malaria, tuberculosis, and HIV, the complete profile of a successful *T. parva* immune response is lacking.

Since cattle are outbred, an understanding of the bovine cellular immune response to *T. parva* is an ideal model for human cellular immune responses to malaria, tuberculosis, and HIV. In contrast to these diseases, ECF can be studied directly in the natural host. ITM-immunized cattle can serve as a constant source of effector cells mediating a protective immune response. Sufficient progress has been made in the development immune reagents to capitalize on this opportunity to develop multi-dimensional bovine immune assays that facilitate detailed analysis of the phenotype and function of leukocyte subsets mediating protective cellular immunity. With this in mind, we developed a flow-cytometric based bovine cytotoxicity assay, and tested it in ITM-immunized cattle. We also optimized an *ex vivo* bovine intracellular cytokine staining assay for use in the *T. parva* system.

**MATERIALS AND METHODS**

**Animals:** Eight MHC class I-matched Holstein steers from central Washington were used in the present study. MHC class I and class II typing was performed as previously described [8]. Cattle were maintained as described in previous studies [9]. Four steers were ITM-immunized with 0.15 mL of *T. parva* sporozoite stabilate Ed80 and oxytetracycline. Infection was confirmed, and clinical disease managed as previously described [9]. All cattle recovered. Twenty months after initial infection, cattle
were challenged with 1 mL of *T. parva* sporozoite stabilate P2015/1 (homologous to Ed80). Due to the lack of overt clinical disease, increases in serum antibody titers to *T. parva*, measured via indirect ELISA [10], were used to confirm successful challenge. Four uninfected animals were maintained as negative controls. Blood was collected from cattle following challenge for use in assay development. Experiments were approved by the Washington State University Institutional Animal Care and Use Committee, protocol number 04515-002.

**Theileria parva infected cell lines:** *T. parva* infected lymphocyte cell lines were established and maintained using standard methods [11], with slight modifications. Briefly, 4 x 10⁷ peripheral blood mononuclear cells (PBMC) were isolated using density centrifugation and incubated with 300 µL *T. parva* Muguga sporozoite stabilate P2015/1 for 90 minutes at 37°C/5% CO₂ in air. During incubation, cells were gently mixed every 15 minutes. Following incubation, cells were washed in 15 mL of fresh RPMI (Thermo-Fisher, USA), and then plated at 2 x 10⁶ cells/well in 24-well flat-bottom culture plates. Cells were cultured in complete RPMI [11] at 37°C/5% CO₂ in air. Infection was verified using Giemsa-stained cytospin smears 7-10 days post infection. Once established, cell lines were maintained at 37°C/5% CO₂ in air and subcultured every 2-4 days as needed. Aliquots of each culture were cryopreserved to avoid loss of the line.

**Generation of concanavalin-A blasts:** Autologous concanavalin-A (conA) stimulated PBMC were generated for use as un-infected target cells in the cytotoxicity assay. PBMC were plated at 2 x 10⁶ cells/well in 24-well culture plates in complete RPMI supplemented with 5 µg/mL conA (Sigma) for 48 hours, and then used in the cytotoxicity assays.
**Generation of *T. parva* specific T-lymphocyte lines:** *T. parva* specific T-lymphocyte lines were generated as previously described [11, 12], with slight modifications. Briefly, blood from immunized animals was collected in anticoagulant citrate dextrose (ACD) and PBMCs isolated by density gradient centrifugation. PBMCs were resuspended in RPMI and stimulated by coculture with autologous, γ-irradiated (5,000 rads) *T. parva*-infected cells at a ratio 20:1 for six days. After the first stimulation, cells were collected and subjected to density gradient centrifugation to remove dead cells. Harvested cells were phenotyped and depleted of NK and γδ cells using anti-NK (CD335) and δ T-cell receptor chain (GB21A) monoclonal antibodies (mAbs) (1 µg/10^6 cells each, Table 1) and rat anti-mouse IgG2a+b magnetic microbeads (Miltenyi Biotec, USA) according manufacturer protocols. The depleted cells, comprised of CD4+ and CD8+ T cells, were resuspended in RPMI and subjected to a second 5-6 day stimulation with autologous, γ-irradiated (5,000 rads) *T. parva*-infected cells.

After the second stimulation, cells were collected and subjected to density gradient centrifugation to remove dead cells. Harvested cells were phenotyped and then used to generate subcultures of CD4+ and CD8+ T cells for use in the development of a flow cytometric cytotoxicity assay. CD8+ T cells were positively selected using a CD8-specific mAb (Table 1, 1 µg/10^6 cells) and rat anti-mouse IgG2a+b magnetic microbeads as previously described. The separated populations were phenotyped to verify purity. The cultures were expanded in RPMI containing bovine 10 ng/mL IL-2 (Kingfisher, USA) for 2-3 days and then used in the cytotoxicity assay.
Combinations of mAbs specific for CD4, CD8, CD335, TCR δ-chain, CD45RO, and CCR7 were used to determine the composition of lymphocyte cultures after each round of stimulation, after depletion of NK and γδ T cells, after positive selection of CD8+ T cells, and before setting up the cytotoxicity assay coculture (Table 1). Staining of cells was done as previously described [13] using fluorochrome-conjugated goat anti-mouse isotype-specific second step antibodies. (Table 2). Labelled cells were analyzed with a Becton Dickinson FACSCalibur flow cytometer and Cell Quest software (BD Immunocytometry Systems San Jose, CA).

<table>
<thead>
<tr>
<th>mAb clone</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILA11A</td>
<td>CD4</td>
<td>G2a</td>
<td>WSU Monoclonal Antibody Center, Pullman, WA, USA</td>
</tr>
<tr>
<td>7C2B</td>
<td>CD8</td>
<td>G2a</td>
<td>WSU Monoclonal Antibody Center, Pullman, WA, USA</td>
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<td>CACT80C</td>
<td>CD8</td>
<td>G1</td>
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<tr>
<td>ILA116A</td>
<td>CD45RO</td>
<td>G3</td>
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<td>G1</td>
<td>WSU Monoclonal Antibody Center, Pullman, WA, USA</td>
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<tr>
<td>CC302</td>
<td>IFN-γ</td>
<td>G1</td>
<td>AbD Serotec, USA</td>
</tr>
<tr>
<td>3D12</td>
<td>CCR7</td>
<td>G2a</td>
<td>BD Pharmingen, USA</td>
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<td>TE1A</td>
<td>CD28</td>
<td>IgM</td>
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<td>FW3-218</td>
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<td>G1</td>
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<td>AKS1</td>
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<td>G1</td>
<td>Norwegian University of Life Sciences [14]</td>
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<tr>
<td>AKS8</td>
<td>CD335</td>
<td>G2a</td>
<td>Norwegian University of Life Sciences [15]</td>
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</table>
Flow cytometric cytotoxicity assay: The assay was modified from [16] and optimized for use in cattle. A three-color flow cytometric assay was used to monitor killing of autologous *T. parva*-infected target cells by the *T. parva*-specific CD8+ T and CD4+ T-cell lines and the unseparated parent cell line.

Target cells were prepared at 0, 6, 24, and 48 hours just before use. *T. parva*-infected cells or ConA blasts were harvested, subjected to density gradient centrifugation to remove dead cells, and resuspended at $2 \times 10^6$ cells/mL in cRPMI with Cell Tracker Deep Red (Thermo-Fisher, USA) at 1:1000 for 30 minutes at room temperature. Cells were then washed and resuspended.

Effector cells (*T. parva*-specific parent unseparated T-cell line, CD8+ or CD4+ T-cell lines) were subjected to density gradient centrifugation to remove dead cells and resuspended in RPMI. Effector cells were cocultured with target cells for 24 and 48 hours at an effector:target (E:T) cell ratio 40:1 (for parent and CD4+ T-cell lines) and 20:1 (for CD8+ T-cell lines). One well containing only labelled target cells was included as a control for spontaneous target cell death.

At 0, 6, 24 or 48 hours of incubation, cells were collected and incubated with annexin V PE (BD Bioscience, USA) and 7-AAD (BD Bioscience, USA). In brief, cells were pelleted and resuspended in 1X annexin V binding buffer (BD Bioscience, USA).

<table>
<thead>
<tr>
<th>mAb</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Goat anti-mouse IgG2a PE.CY5.5</td>
<td>Thermo-Fisher, USA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG1 Alexa Fluor 647</td>
<td>Thermo-Fisher, USA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG1 FITC</td>
<td>Thermo-Fisher, USA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG3 FITC</td>
<td>Southern Biotech, USA</td>
</tr>
<tr>
<td>Goat anti-rat IgG FITC</td>
<td>Southern Biotech, USA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG2b PE</td>
<td>Southern Biotech, USA</td>
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Table 2. Secondary Antibodies Used in Flow Cytometric Assays
and mixed with Annexin V PE and 7-AAD (20 µL). Cell preparations were then incubated for 15 minutes. Labelled cells were analyzed by flow cytometry within one hour of staining according the recommended company protocol. 5x10^4 events were collected on each sample, with an electronic gate placed on the Cell Tracker Deep Red labelled target cells.

**γ interferon cytokine flow cytometric assay (CFC):** The protocol for CFC was carried out as previously described [17, 18] with slight modifications. Briefly, heparinized blood samples were aliquoted into six-well plates (5 ml/well). Anti-CD28 and -CD49d monoclonal antibodies (mAb) (Table 1) were added (1 µg/ml) as costimulatory molecules. One well was stimulated with 10^6 autologous, γ-irradiated (5,000 rads) *T. parva*-infected cells and one well was used as a negative control. Plates were incubated at 37°C/5% CO2 in air. After six hours, brefeldin A (BD Biosciences, USA) was added to each well (10 µg/ml), and plates were incubated for an additional ten hours. At the end of the incubation period, PBMCs were harvested by density gradient centrifugation, washed twice in PBS, re-suspended in first wash buffer (PBS-ACD-horse serum 0.02%-sodium azide 0.002%) and processed for cell labelling.

For surface labelling of cells, 10^6 cells were incubated with a mixture of anti-CD4, (or -CD8), -CD45R0, -CCR7 and -CD69 mAbs (0.75 µg each, Table 1) for 30 minutes on ice, washed twice in first wash buffer (700 x g for 2 minutes), resuspended in goat anti-mouse fluorochrome-conjugated isotype-specific secondary antibodies (Table 2) and incubated in the dark for 30 minutes on ice. Cells were then washed twice in second wash buffer (PBS-ACD-sodium azide 0.002%) and resuspended in 100 µl of Fixation/Permeabilization buffer (Cytofix/Cytoperm Fixation Permeabilization kit, BD


Biosciences, USA) for 20 minutes in the dark on ice. For intracellular staining, cells were subjected to two washes (1500 x g for 2 minutes) in Perm/Wash buffer (Cytofix/Cytoperm Fixation Permeabilization kit, BD Biosciences, USA), re-suspended in Perm/Wash buffer containing 0.1 µg of anti-bovine IFN-γ FITC (Table 1) and incubated for 30 minutes in the dark on ice. Cells were subjected to two final washes in Perm/Wash buffer, re-suspended in PBS-buffered formaldehyde (2%), and stored at 4°C in the dark until examined. A BD FACSCalibur flow cytometer equipped with Cell Quest software was used to collect the cells. Cell preparations were visualized in forward (FSC) and side scatter (SSC) to distinguish granulocytes from mononuclear cells. Electronic gates were used to distinguish small lymphocytes, gate 1 (R1) from monocytes and large lymphocytes, gate 2 (R2) and isolate the populations for analysis. 5x10^5 events were collected for analysis of each sample.

**Data analysis:** FCS Express software (De Novo software, Los Angeles, CA) was used to analyze all FC data. For the cytotoxicity assay, the percent cytotoxicity was determined by subtracting the percent of dead cells in wells with only *T. parva*-infected target cells from wells containing both effector and target cells for each specified time point. Mean values for percent cytotoxicity between 0 and 6, 24, or 48 hours incubation were compared using a one-tailed student’s t-test (α<0.05). For the *ex vivo* intracellular IFN-γ assay, mean IFN-γ-positive cell frequencies between ITM-immunized and control cattle were compared using a one-tailed student’s t-test, (α<0.05).
RESULTS AND DISCUSSION

The chromium release assay has been the primary method used to study cytotoxic T-cell activity against *T. parva*-infected target cells [3, 7]. Although useful, we were interested in finding ways to study cytotoxic T-cell activity in greater detail. We were especially interested in assays that could be used to phenotype cytotoxic T cells before and during interaction with target cells. The flow cytometric cytotoxic T-cell assay was adapted for use in cattle to achieve the first objectives. We also adapted cytokine flow cytometric assay for use in the studies starting with monitoring expression of IFN-γ.

Assessment of T-cell mediated killing of autologous, *T. parva*-infected lymphocytes using a flow-cytometric cytotoxicity assay: T-cell mediated cytotoxicity was assessed four months post-challenge. Cytotoxicity was measured in parent effector cell cultures at T0 and after 24 and 48 hours incubation with autologous *T. parva* infected cells or uninfected, autologous ConA blasts, and after 0 and 24 hours incubation of CD4+ and CD8+ T-cell lines with autologous *T. parva* infected target cells (Fig 1 and Table 3). Statistically significant cytotoxicity was detected at all time points when parent T-cell lines or CD8+ T-cell lines were used as effector cells, consistent with previous observations using the chromium release assay [19]. The lack of significant cytotoxicity when effector cells were incubated with uninfected, autologous con-A blasts or MHC-class I mismatched infected cells (data not shown) supports the premise that killing was MHC class I restricted and *T. parva*-specific.
Since significant *T. parva*-specific CD8+ T-cell mediated cytotoxicity is usually observed after a shorter incubation period with target cells using the chromium release assay [11], we repeated the assay with the effector parent T-cell lines incubated with autologous infected cells for only six hours (Fig 2, Table 4). Even with the incubation period reduced by 75%, statistically significant cytotoxicity was observed with cells from all animals used in the study.

<table>
<thead>
<tr>
<th>Effector Cell Type</th>
<th>0 Hours Incubation</th>
<th>24 Hours Incubation</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent T-cell Lines</td>
<td>2.4%</td>
<td>51.4%</td>
<td>0.00002</td>
</tr>
<tr>
<td>CD8+ T-cell Lines</td>
<td>2.2%</td>
<td>53.1%</td>
<td>0.00007</td>
</tr>
<tr>
<td>CD4+ T-cell Lines</td>
<td>1.4%</td>
<td>7.1%</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Although the percent cytotoxicity mediated by effector CD8+ T cells was significantly greater than that mediated by effector CD4+ T cells (Table 5) at 24 hours, the percent cytotoxicity mediated by CD4+ T cells was statistically significant (Table 3). The absence of significant cytotoxicity when effector and target cells were MHC class II mismatched and when effector cells were mixed with uninfected, autologous con-A blasts supports the assumption that the observed, CD4+ T-cell mediated cytotoxicity is
MHC class II restricted and *T. parva* specific (data not shown). To the author’s knowledge, this is the first documented evidence of cytotoxic CD4+ T-cell activity against *T. parva*. Although observed less commonly than cytotoxic CD8+ T-cell responses, cytotoxic CD4+ T-cell responses have been documented in cattle infected with *Neospora caninum* [20, 21] and in humans infected with *Toxoplasma gondii* [22, 23]. The importance of cytotoxic CD4+ T cells in immune control of *T. parva* is unclear. Previous studies suggest that transfer of CD8+ CTL, depleted of CD4+ T cells, between immune and naïve twin calves is sufficient to confer protection from *T. parva* [3]. However, whether CD4+ CTL significantly augments protection *in vivo* remains to be demonstrated.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>CD4+ T cells</th>
<th>CD8+ T cells</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>7.1%</td>
<td>53.1</td>
<td>0.00002</td>
</tr>
</tbody>
</table>

This assay, like flow-cytometric cytotoxicity assays used in humans and mice [16, 24], shows general agreement with historically reported cytotoxicity data obtained from *T. parva*-infected cattle using chromium-release assays. Unlike chromium release assays, this assay does not require use of radioactive material, and also allows quantitation of two different phases of cytotoxicity over time: Early induction of apoptosis (annexin-V binding) and late increases in cell-membrane permeability (7-AAD binding) [25]. Since chromium release only occurs with increased membrane permeability, its measurements exclude early apoptotic cells, and are thus a less-sensitive indicator of cytotoxicity. Furthermore, although effector cell phenotyping was
limited to CD4+ and CD8+ labeling in this study, other surface labels could be added to
further characterize effector cell populations [25, 26].

IFN-γ-producing memory CD4+ T cells, but not CD8+ T cells, from T. parva-
immune cattle, are detected at four months post-challenge: Ex vivo T-cell IFN-γ
production was assessed at one and four months post-challenge. In all experiments,
gating was performed so that CD45R0+, IFN-γ+ cells were selected first. Next, CD4+ or
CD8+ subsets of the CD45R0+/IFN-γ+ population were selected. This strategy showed
that the majority (>90%) of IFN-γ producing cells were CD4+ T cells. Thus, we
restricted data analysis to CD45R0+/IFN-γ/CD4+ T cells for the remaining analyses.
Finally, CD45R0+/IFN-γ/CD4+ T cells were examined for expression of CCR7 and
CD69 (Fig 3). At one month post-challenge, the average frequency of CD4+ T cells
producing IFN-γ in response to autologous, T. parva-infected cells ranged was 2.71%,
and decreased to 0.25% by four months post-challenge (Fig 4 and Table 6). At both
time points, the frequency of IFN-γ-producing cells was significantly greater than the
frequency in cells from control cattle (Table 6). In all cases, IFN-γ-producing cells were
positive for the memory T-cell marker, CD45R0, and negative for the marker of central
memory, CCR7. The IFN-γ producing cells were positive for the activation marker
CD69 consistent with expression in recent cell activation [27].

<table>
<thead>
<tr>
<th>Time Post Challenge</th>
<th>Immune (n=4)</th>
<th>Control (n=4)</th>
<th>P Value</th>
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<tr>
<td>One Month</td>
<td>2.71</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Four Months</td>
<td>0.25</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>
The fact that CD45RO+/IFN-γ+/CD8+ T cells were difficult to detect ex vivo in our assay is somewhat unexpected, since ex vivo CD8+ T-cell interferon-γ responses have been detected in ELISPOT assays between 5 and 13 days post-challenge [7, 12]. Studies were not conducted, in these investigations, however, to determine whether cells detected by ELISPOT had cytotoxic T-cell activity. It is likely that the number of circulating memory CD8+ T cells specific for *T. parva*-infected cells had dwindled substantially by one month post-challenge, to the point that they were below the limits of detection for our assay. Further studies are needed to reconcile our findings with earlier studies using this assay at the same earlier time points.

The strength of the memory CD4+ T-cell response is interesting, given the strong evidence that robust CD8+ T-cell responses, but not CD4+ T-cell responses, are required to protect cattle from ECF. It is likely that this CD4+ T-cell population is valuable in the development, maintenance, and reactivation of long-term memory CD8+ T cells against *T. parva*. Indeed, in other infections and immunization models, CD4+ T cells have been shown to enhance survival of memory CD8+ T cells via suppression of pro-apoptotic factors like TNF-related apoptosis inducing ligand (TRAIL) [28] or BCL-2 associated x-protein (BAX) [29]. This suppression can be achieved through interactions between CD4+ and CD8+ T cells during both the priming and secondary immune response phases [30, 31] and is largely depended on expression of certain cytokines, including IL-2 and IL-15 [29, 32]. In addition, CD4+ regulatory T cells play an important role in modulating memory CD8+ T-cell responses [33].

Although CD4+ T cell responses have been documented in *T. parva*, [34, 35] the role of memory CD4+ T cells in immunity *T. parva* has been largely unexplored. Since
this assay allows for simultaneous evaluation of multiple cellular parameters, it can be adapted to assess lymphocyte expression of pro-apoptotic proteins, other cytokines, and regulatory T-cell markers in future experiments, thus providing important insight into CD4+ T-cell memory and function in development of immunity against *T. parva*.

Finally, as new candidate *T. parva* vaccine antigens are discovered, MHC class I and class II tetramers can be used in conjunction with intracellular cytokine staining and extracellular phenotypic marker staining to better understand antigen-specific T-cell responses after immunization and challenge.

Continued use, expansion, and improvement of these assays in *T. parva* will expand data on protective T-cell populations in immune individuals, will aid in determining more accurate immune correlates of protection in *T. parva*, and will facilitate next-generation vaccine development. These assays will also be useful in addressing similar challenges in vaccine development in other bovine diseases, such as *Mycobacterium* sp., in which T-cell responses are required for control. Finally, since cattle are outbred, it is possible that comprehensive bovine T-cell response data may provide insights into how to elicit stronger cell-mediated immunity against diseases in humans such as HIV, malaria, and tuberculosis.
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cytotoxic T-cell clones react with a schizont-derived antigen associated with the surface  
Fig 1. Percent living and dead cells after 0, 24, and 48 hours incubation with autologous, *T. parva* infected cells in a representative ITM-immunized animal. Parent T-cell line (A), CD8+ T-cell line (B), CD4+ T-cell line (C), and *T. parva* infected target cells alone (D).
Fig 2. Percent living and dead cells after 0 and 6 hours incubation with autologous, *T. parva* infected cells in a representative ITM-immunized animal. Incubation with Parent T-cell effector line (A), vs. *T. parva* infected target cells alone (B).
Fig 3. **Gating strategy and phenotype of IFN-γ producing cells.** All cells positive for both IFN-γ and CD45RO were selected (A). From this population, CD4+ (or CD8+)/CD45RO+ cells were selected (B), and IFN-γ+/CD45RO+/CD4+ (or CD8+) cell populations were assessed for expression of CCR7 (C) and CD69 (D). Note that almost all CD45RO+/IFN-γ+ cells are CD4+ T cells, and that CD45RO+/IFN-γ+/CD4+ T cells are negative for CCR7.

Fig 4. **IFN-γ producing CD4+ T cells in representative ITM-immunized and control cattle, 4 months post-challenge.** In all cases, the frequency of IFN-γ producing T cells was higher in ITM immunized animals (A) than in naïve control animals (B) after incubation with autologous, *T. parva* infected cells.
CONCLUSION

*Theileria parva*, the causative agent of East Coast Fever, kills millions of cattle each year in sub-Saharan Africa, resulting in severe poverty and malnutrition of pastoralist farmers of the region. The development of a next-generation vaccine to prevent ECF is urgently needed; however, to date, all candidate *T. parva* vaccines have failed. This failure is due in large part to an incomplete understanding of ECF immunopathogenesis, and thus of the complete profile of successful and unsuccessful immune responses to *T. parva* in cattle. Our work revealed that immune dysregulation, in the form of systemic macrophage activation syndrome, leads to vasculitis, respiratory failure, and death in acute ECF. This work highlights the previously unrecognized complexity of the *T. parva* immune response, and begins to shed light on how early, innate events shape the outcome of *T. parva* infection and the cell-mediated immune response. Future work will strive to elucidate the mechanism of macrophage activation in *T. parva*, and to understand the interplay of these alternatively activated macrophages with the CD4+ and CD8+ T cell response to *T. parva*. This information, coupled with that gained from multidimensional assessment of the bovine immune response to *T. parva* using our new flow-cytometric assays, will provide much-needed information regarding immune correlates of protection and disease in *T. parva*, thereby expediting the development of a protective subunit vaccine.