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Cytokine mRNA profiling of peripheral blood mononuclear cells from trypanotolerant and trypanosusceptible cattle infected with Trypanosoma congoense

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1Animal Genomics Laboratory, School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin, Ireland; 2School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom; 3International Livestock Research Institute, Nairobi, Kenya; and 4The Institute of Genomics and Bioinformatics, University of New England, Armidale, Australia

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O’Gorman GM, Park SD, Hill EW, Meade KG, Mitchell LC, Agaba M, Gibson JP, Hanotte O, Naessens J, Kemp SJ, MacHugh DE. Cytokine mRNA profiling of peripheral blood mononuclear cells from trypanotolerant and trypanosusceptible cattle infected with Trypanosoma congoense. Physiol Genomics 28: 53–61, 2006. First published September 19, 2006; doi:10.1152/physiolgenomics.00100.2006.—To examine differences in cytokine profiles that may confer tolerance/susceptibility to bovine African trypanosomiasis, N’Dama (trypanotolerant, n = 8) and Boran (trypanosusceptible, n = 8) cattle were experimentally challenged with Trypanosoma congoense. Blood samples were collected over a 34-day period, and RNA was extracted from peripheral blood mononuclear cells. The expression levels of a panel of 14 cytokines were profiled over the course of infection and between breeds. Messenger RNA (mRNA) transcript levels for the IL2, IL8, and IL1RN genes were significantly downregulated across the course of infection in both breeds. There was an early increase in transcripts for genes encoding proinflammatory mediators (IFNG, IL1A, TNF, and IL12) in N’Dama by 14 days postinfection (dpi) compared with preinfection levels that was not detected in the susceptible Boran breed. By the time of peak parasitemia, a type 2 helper T cells preinfection levels that was not detected in the susceptible Boran and may explain why N’Dama control parasitemia more efficiently than Boran during the early stages of infection.

trypanosomiasis; gene expression; quantitative real-time RT-PCR

BOVINE AFRICAN TRYPANOSOMIASIS, or “nagana,” is a wasting disease that affects cattle populations across much of central Africa. The disease is caused by the protozoan parasites Trypanosoma congoense, T. vivax, and T. brucei brucei and is transmitted in the saliva of biting tsetse flies (Glossina spp.). When an infected tseste fly bites a mammalian host and deposits metacyclic forms of the parasite at the site of inoculation, a raised inflammatory swelling called a chancre develops within a few days that precedes parasitemia and other clinical signs (1). Thereafter, metacyclics differentiate into bloodstream forms, migrate to the bloodstream, and cause systemic infection (34). Parasitemia, detected in the peripheral blood, is usually apparent 1–3 wk later and may persist in waves for many months with fever occurring intermittently (14). From the period when parasitemia is first detected, there is a rapid decrease in hematological parameters. Major clinical signs in ruminants include anemia (6, 30), lymphoid enlargement, lethargy, and loss of condition with progression of the disease. Marked immunosuppression is usually observed with reduced host resistance to secondary infections (7). Typically, this disease is chronic, extending over many months, and fatal if untreated (14).

The scale of this problem is immense; indeed, the tsetse belt of sub-Saharan Africa, where the disease is endemic and a major constraint to livestock production, covers 37 countries and an area of ~10 million km². In many parts of Africa, cattle are a vital source of income, particularly for smallholder farmers, and the annual cost of bovine trypanosomiasis to African agriculture has been estimated at 1 billion US dollars per annum (21). However, over thousands of years, and presumably under high tsetse challenge, certain West African Bos taurus cattle populations have evolved tolerance to trypanosomiasis (31). One of the best-characterized trypanotolerant breeds is the N’Dama, and this cattle population represents both a unique genetic resource for African cattle agriculture and an important model to understand mechanisms of trypano-tolerance (29). In contrast, zebu populations (B. indicus) tend
to be susceptible and generally can only be maintained through the use of costly trypanocidal drugs. The well-characterized trypanosusceptible East African Boran breed has been used as a model of trypanosusceptibility for many previous studies (44).

The profound susceptibility of many breeds of cattle to trypanosomiasis is easily understood when the complexity of the host-parasite relationship itself is considered. *T. congolense* is a unicellular, extracellular, motile parasite with a specific preference for the peripheral vasculature, where it is found either free swimming or attached via its anterior end to endothelial cells. The parasite is completely exposed to the host’s immune system and yet, in many instances, survives to proliferate, resulting in characteristic waves of parasitemia (for review, see Ref. 26). The array of host evasion mechanisms are complex (for review, see Ref. 9) and include the well-known phenomenon of antigenic variation of the variable surface glycoprotein (VSG) (2, 4, 8). In this regard, successful switching of the antigenic coat of the trypanosome plays a key role in avoidance of the host immune system. Despite the battery of tactics utilized by the parasite to evade detection and destruction, the superior response of N’Dama cattle has allowed these animals to survive and be productive in areas of tsetse challenge without the use of trypanocidal drugs (33). It is important, however, to acknowledge that N’Dama and Boran cattle are equally susceptible to the initial infection; however, once infected, the N’Dama have a superior capacity to control parasite proliferation, control anemia, and maintain body weight (43).

Although the mechanisms of trypanotolerance employed by trypanotolerant N’Dama cattle are still poorly understood, research efforts have focused on elucidating the enhanced control of parasitemia and anemia. For example, a hemopoietic chimera twin study reported that control of parasitemia was independent of the hemopoietic system, while control of anemia was dependent on the same system (36). In addition, T cell depletion studies suggested that both parasitemia and anemia were independent of T cells and antibody (38, 47). Overall, there appears to be two independent mechanisms, an innate control of parasite growth and control of anemia involving the hemopoietic system.

The cytokine environment and changes in the type 1/type 2 helper T cell (Th1/Th2) cytokine balance can influence disease progression in infected animals. Indeed, responses have been measured in an effort to understand their role in trypanotolerance and trypanosusceptibility (23, 27, 52). In particular, a study by Mertens et al. (27) examined the profiles of a number of cytokines in peripheral blood mononuclear cells (PBMC) from infected N’Dama and Boran cattle and suggested a possible protective role for the IL-4 protein. Due to the accessibility of mouse models and the prohibitive cost of bovine challenge experiments, a large number of these studies in recent years have been based on the murine immune response to trypanosome infection (20, 40, 50). It has been shown in the mouse model that survival requires a Th1 cytokine environment and classical macrophage activation in the early stage of infection, allowing the mice to control the first peak of parasitemia. Subsequently, a switch to the Th2 cytokine environment and alternatively activated macrophages during the chronic/late stages of the disease appears to be beneficial for survival, probably because the inflammatory parasite-control ling response is harmful to the host (2). However, caution should be exercised when extrapolating findings from these studies and applying them to the bovine model (34), where more work is needed to determine whether mechanisms similar to those in the murine model are at play. In terms of work carried out in cattle, some studies have focused specifically on lymph node cells (25). However, the immune response to bovine trypanosomiasis is highly compartmentalized, and the response in lymph node cells can be considerably different from that in PBMC. It is difficult to generalize cytokine profiles based on a small number of studies, considering the inherent variability in experimental design in terms of animals, parasite clone, and infection protocol including the dosage and time points examined. Consequently, the present study aims to improve our understanding of the bovine immune response, and in particular the cytokine response of PBMC, to *T. congolense* infection, building on previous transcriptional profiling work carried out with susceptible Boran (17). A panel of cytokines, chosen to characterize the presence of either a Th1 or Th2 type cytokine environment, was used for this purpose.

**MATERIALS AND METHODS**

**Animals and experimental challenge.** All the animal procedures were approved by the Institutional Animal Care and Use Committee of the International Livestock Research Institute (ILRI), in concordance with the Animals (Scientific Procedures) Act (United Kingdom) 1986. Animals were reared in a trypanosomiasis-free research farm at Kapiti Plains Estate, Kenya, and were therefore never infected with trypanosomes before the experimental challenge. Eight N’Dama and eight Boran (19- to 28-mo-old females) were experimentally infected with *T. congolense* clone IL1180 (15, 41) via the bite of eight infected tsetse flies (Glossina morsitans morsitans) per animal per (1, 10). Packed cell volume (PCV) was measured routinely throughout the course of infection (56), and animals with PCVs that dropped to 15% were removed from the experiment and treated with a trypanocide (Berenil, 7 mg/kg body wt). Parasitemia scores were also taken at the same time points as those used for the PCV measurements and monitored using the darkground/phase contrast buffy coat method (32, 45).

**Blood collection, PBMC isolation, and RNA extraction and purification.** Peripheral blood (200 ml) was collected before infection and at 14, 21, 25, 29, and 34 days postinfection (dpi) in heparanized syringes. These time points were chosen to coincide with the first wave of parasitemia. PBMC were isolated, using Percoll gradients (GE Healthcare, Buckinghamshire, UK) and previously described methods (53). The isolated PBMC were washed with sterile PBS and stored immediately at −80°C in Tri-reagent (Medical Research Center, Cincinnati, OH) for further processing. Total RNA was extracted with chloroform and precipitated with isopropanol. Each sample was further DNase treated with RQI RNase-free DNase (Promega, Southhampton, UK) and purified using the RNaseasy mini kit (Qiagen, Crawley, UK). RNA quality and quantity were subsequently assessed using the 18S/28S ratio and RNA integrity number (RIN) on an Agilent Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Dublin, Ireland).

**cDNA synthesis and real-time quantitative RT-PCR.** Four micrograms of total RNA from each sample were reverse transcribed into cDNA with oligo(dT) primers, using a SuperScript III first-strand synthesis SuperMix kit according to the manufacturer’s instructions (Invitrogen, Paisley, UK). The converted cDNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), diluted to 20 ng/µl working stocks, and stored at −20°C for subsequent analyses. Primers for real-time quantitative
RT-PCR (qRT-PCR) were designed, where possible, to span two exons, using the Vector NTI Advance software package (Invitrogen), and commercially synthesized (Invitrogen). Details for these primer sets are provided in Table 1. Each reaction was carried out in a total volume of 25 μl with 2 μl of cDNA (20 ng/μl), 12.5 μl of 2× PCR master mix (BioGene, Cambridgeshire, UK), 1.25 μl of SYBR Green 1 (1/40,000 dilution with DMSO; BioGene, Cambridgeshire, UK), and 9.25 μl of primer-H2O. Optimal primer concentrations were determined by titrating 100, 300, and 900 nM final concentrations, and dissociation curves were examined for the presence of a single product. Real-time qRT-PCR was performed using an MX3000P quantitative PCR system (Stratagene, La Jolla, CA) with the following cycling parameters: 95°C for 10 min (PCR hot start), followed by 45 cycles of 95°C for 15 s and 60°C for 1 min, followed by amplicon dissociation (95°C for 1 min, 55°C for 30 s, increasing 0.5°C/cycle for 81 cycles).

Flow cytometry of PBMC samples. To quantify any changes in the percentages of different cell subsets in the PBMC samples across the time course, cell surface markers for CD4+ and CD8+ T cells, γδ T cells, and B cells were examined using flow cytometry. These analyses were carried out using the PBMC samples from each animal before infection and at 14, 21, 25, 29, and 34 dpi. Briefly, 1 × 10^6 PBMC in PBS were added to 96-well microtiter plates, primary monoclonal antibody was added, and these samples were incubated on ice for 30 min. Cells were then washed three times in fluorescence-activated cell sorting (FACS) medium [RPMI 1640, no HEPES, 5% horse serum (Sigma-Aldrich), and 0.01% sodium azide]. Secondary FITC-conjugated antibody was then added, and cells were incubated on ice in the dark for 30 min and fixed with 2% formaldehyde. The monoclonal antibodies used were as follows: IL4 [bovine CD4+ T cells (5)], CC15 [bovine B cells (11, 49)], ILA11 [bovine CD8+ T cells (3)], ILA51 [bovine CD8+ T cells (11, 49)], CC15 [bovine γδ T cells (5)], and ILA30 [bovine B cells, IgM (37) (from J. Naessens, ILRI)]. Data were collected using a BD FACScan instrument and analyzed with the CellQuest version 3.3 software package (BD Biosciences, San Jose, CA).

### Data analysis.
Analyses of putative reference, or “housekeeping,” genes were carried out using the geNorm version 3.4 Microsoft Excel add-in (54). Briefly, geNorm calculates a gene expression stability measure “M” for a control gene as the pairwise variation for that gene with all other tested control genes. The

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Amplicon Size, bp</th>
<th>Primer Concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>Actin, beta</td>
<td>F: CAGCAAGATGGGATGACCAAGG&lt;br&gt;R: AAGCCAGCTAACATCCTGGC</td>
<td>91</td>
<td>300</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>F: TTTCTAGGCGGCTGCAGAAGG&lt;br&gt;R: GATCCGAAAACAGACGCTGGG</td>
<td>107</td>
<td>300</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide</td>
<td>F: GCATCCCAAGAGACTAATCCTC&lt;br&gt;R: GCAAGAACGAAATGACGACCA</td>
<td>120</td>
<td>300</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase-1</td>
<td>F: TGCATGAGATTGGAAGAGG&lt;br&gt;R: CAAGAGGGTGGCAAAAGCT</td>
<td>154</td>
<td>300</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein</td>
<td>F: GAGAAGAGCTAGATTTGTTG&lt;br&gt;R: CGTAGGAGGGCTGTGCTT</td>
<td>185</td>
<td>300</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
<td>F: CATACAGGTCCTGGCTTCTG&lt;br&gt;R: CACGTCGTTTCGCTCCACAC</td>
<td>108</td>
<td>300</td>
</tr>
<tr>
<td>IFNG</td>
<td>Interferon, gamma</td>
<td>F: TCAAATCCGGGTTAGATGCTG&lt;br&gt;R: GACGATACGGTGAGCTTCTGG</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td>LTA</td>
<td>Lymphotoxin alpha (TNF superfamily, member 1)</td>
<td>F: AGCCTCTCTCTCTCTCTG&lt;br&gt;R: CCGAGGAGCGCTAAGAATC</td>
<td>111</td>
<td>900</td>
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<tr>
<td>IL1RN</td>
<td>Interleukin-1 receptor antagonist</td>
<td>F: CGAACCGCCATACATGCTCC&lt;br&gt;R: AACTCTGATGTCCTTCCGC</td>
<td>111</td>
<td>300</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>F: GCCTGAAACTTGCCTCTG&lt;br&gt;R: CTGGTGAATGATAGCAGG</td>
<td>147</td>
<td>300</td>
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<td>IL1A</td>
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<td>116</td>
<td>300</td>
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<tr>
<td>IL2</td>
<td>Interleukin-2</td>
<td>F: CAGAGAGATCGAATATGCTT&lt;br&gt;R: CAGGTTTCTACTGCTGATCAG</td>
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<td>300</td>
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<td>IL4</td>
<td>Interleukin-4</td>
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<td>900</td>
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<tr>
<td>IL6</td>
<td>Interleukin-6</td>
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<td>100</td>
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<tr>
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<td>Interleukin-8</td>
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<td>300</td>
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<tr>
<td>IL10</td>
<td>Interleukin-10</td>
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<td>110</td>
<td>100</td>
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<tr>
<td>IL12</td>
<td>Interleukin-12</td>
<td>F: AGATATAAAACAGGACAGG&lt;br&gt;R: GATACTCTAAGGCAAGG&lt;br&gt;</td>
<td>154</td>
<td>100</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor (TNF superfamily, member 2)</td>
<td>F: GCCTGAGAAGGCTTCTGC&lt;br&gt;R: AACCAGAGGCTGCTTTG&lt;br&gt;</td>
<td>149</td>
<td>900</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor, beta 1</td>
<td>F: TGCTGACGCTCCAGAAAGAA&lt;br&gt;R: AGGCACAAATTGGCCTTG&lt;br&gt;</td>
<td>116</td>
<td>300</td>
</tr>
</tbody>
</table>

qRT-PCR, quantitative RT-PCR; F, forward; R, reverse.
tested genes are then ranked in order of decreasing $M$ values or increasing mRNA expression stability. The optimal number of reference genes can also be estimated by geNorm by examining the pairwise variation “$V$” between two sequential normalization factors. It is recommended that the inclusion of additional reference genes is not required below $V$ values of 0.15 (54). The $2^{-\Delta\Delta CT}$ method (where CT is cycle threshold) was used to determine mean fold changes in gene expression between breeds at a particular time point and between time points for each breed (22). On the basis of the geNorm analyses, the peptidylprolyl isomerase A gene ($PPIA$) alone was used as the reference gene (additional reference genes were not required based on geNorm criteria), and preinfection values were used as calibrators to generate the graphs shown in Fig. 1. Student’s $t$-test was used to identify significant differences in gene expression between breeds and time points.

**RESULTS**

**Parasitemia and PCV.** Trypanosomes were detected in the peripheral blood of all animals experimentally infected in this study by 12–15 dpi, with peaks of the first wave of parasitemia occurring at 15–22 dpi. The mean PCVs for both the N’Dama and Boran groups fell from 38.1 and 37.8%, respectively, at 8 dpi to 28.7 and 28.4%, respectively, at 22 dpi with a marked reduction from 12 dpi onward, when trypanosomes first appeared in peripheral blood. Between 12 and 22 dpi, there were similar rates of PCV decline in both breeds. In the period between 22 and 35 dpi, the rate of PCV decline in N’Dama was slower than for Boran, with a mean minimum PCV measure-
Table 2. Significant mean fold differences in cytokine mRNA expression between N’Dama and Boran animals at each time point

<table>
<thead>
<tr>
<th>Gene</th>
<th>0 dpi</th>
<th>14 dpi</th>
<th>21 dpi</th>
<th>25 dpi</th>
<th>29 dpi</th>
<th>34 dpi</th>
</tr>
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<tbody>
<tr>
<td>IL4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>ND</td>
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<td>IL10</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>IL2</td>
<td>ND</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>IL8</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>IL4</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>GFAP</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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</tr>
<tr>
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<td>IL4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tr>
</tbody>
</table>

ND, N’Dama; BN, Boran; †, increase; NS, not significant at \( P \leq 0.05 \); dpi, days postinfection.

ment of 24.8 ± 3.2% at 29 dpi in N’Dama and 19.9 ± 4% at 32 dpi in Boran.

**PBMC cell subset analyses.** The fluctuations in cell subsets that were measured within breeds used preinfection values (and not uninfected controls) to determine “unchallenged variation,” and this must be taken into account when assessing the significance of the variation measured after infection. Overall, there was a decrease in the percentage of CD8⁺ T cells in both groups across the time course with the exception of a slight increase in CD8⁺ and γδ T cells in Boran at 25 dpi. The percentage of CD4⁺ T cells in N’Dama decreased until 25 dpi, after which the numbers stabilized. There was no significant decrease in the percentage of CD4⁺ T cells in Boran over the time course. The percentage of B cells fell in both N’Dama and Boran from 0 to 14 dpi and then increased until the end of the time course. There was no significant difference between the two groups in CD4⁺ T cells at 0, 14, 29, and 34 dpi. There were modest differences in CD4⁺ T cells in Boran compared with N’Dama at 21 and 25 dpi (\( P = 0.045 \) and \( P = 0.035 \), respectively; higher in Boran). There were no significant differences in CD8⁺ T cells between the two groups at 0, 14, and 29 dpi; however, there were significant differences at 21, 25, and 34 dpi (\( P = 0.015, P = 0.024, \) and \( P = 0.028 \), respectively; higher in Boran). There were no significant differences in either γδ T cells or B cells between the two groups at any of the time points.

**qRT-PCR reference gene analyses.** Because the effect of various experimental treatments on the expression of traditional qRT-PCR reference, or housekeeping, genes is largely unknown, it was important to identify a stable reference gene for the particular physiological conditions inherent in the present study. Six genes were chosen to represent a panel of potential qRT-PCR reference genes for this study [β-actin (ACTB), GAPDH, tyrosine-3-monoxygenase/tryptophan-5-monoxygenase activation protein, ζ-polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase-1 (HPRT1), succinate dehydrogenase complex, subunit A, flavoprotein (SDHA), and PPIA]. Real-time qRT-PCR was carried out with primers for all six genes on all animals over the entire time course (0–34 dpi). The data were then analyzed using the geNorm package, and \( M \) stability values were generated for specific comparisons between the N’Dama and Boran groups at each time point and also across the time course for each breed (i.e., 0 vs. 14 dpi, 0 vs. 21 dpi, 0 vs. 25 dpi, etc.). The recommended \( M \) value cutoff is 0.5, with higher \( M \) values representing more variable gene expression. The gene that exhibited the greatest stability throughout the time course was PPIA, with \( M \) values ranging from 0.13 to 0.28. In contrast to this, the most variable of the putative qRT-PCR reference genes was SDHA, with \( M \) values ranging from 0.51 to 0.88. In addition, geNorm was used to determine the optimal number of reference genes for normalization, based on a recommended cutoff \( V \) value of 0.15; additional reference genes would not have contributed to a more accurate normalization factor. Therefore, PPIA was used as a single standard reference gene for these experiments.

**Cytokine mRNA profiles of PBMC from N’Dama and Boran.** The mRNA profiles of 14 cytokine genes were examined by real-time qRT-PCR. The graphs in Fig. 1 represent mean fold changes relative to preinfection values for both the N’Dama and Boran groups. Because of differences in expression before infection, these graphs represent changes within a particular breed over time, while significant differences between the breeds are represented in Table 2. The list of cytokines represented in Table 2 excludes cytokines with no significant difference between the breeds at any time point measured. With the 2⁻ΔΔCT method of data analysis, the mean fold change at time 0 should be very close to 1 (since \( 2^0 = 1 \)) (22). Minor deviations from a value of 1 can be attributed to experimental variation. An advantage of the 2⁻ΔΔCT method is that verification of the time 0 fold change is a convenient way to check for variation among samples. Importantly, in the present experiment, only minor deviations were observed.

By 14 dpi, when parasites were detected in the peripheral blood of all animals, the mRNA expression of a number of
proinflammatory mediators increased in N’Dama but not Boran compared with preinfection levels. The expression of the IFNG gene increased by 4.3-fold ($P = 0.037$), that of ILIA by 5.5-fold ($P = 0.011$), that of IL12 by 2.0-fold ($P = 0.041$), that of NFKB1 by 1.6-fold ($P = 0.021$), and that of TNF by 2.1-fold ($P = 0.009$). There was a 1.5-fold higher expression level ($P = 0.047$) of the TNF gene in N’Dama compared with Boran at 14 dpi. At the same time, the expression of the IL2 gene was downregulated in Boran by 3.1-fold ($P = 0.000$).

At 21 dpi, there was a significant 4.3-fold higher level of IL10 gene expression in Boran compared with N’Dama ($P = 0.001$). A number of proinflammatory mediators remained higher than preinfection levels in N’Dama including the IL1A gene (4.2-fold, $P = 0.034$) and TNF (2.7-fold, $P = 0.025$), whereas IL2 [6.3-fold in N’Dama ($P = 0.000$) and 9.0-fold in Boran ($P = 0.000$)], IL8 [5.0-fold in N’Dama ($P = 0.009$) and 3.0-fold in Boran ($P = 0.014$)], and ILIRN [3.0-fold in N’Dama ($P = 0.000$) and 2.0-fold in Boran ($P = 0.000$)] were significantly downregulated at this time compared with preinfection.

A continued trend of IL2, IL8, and ILIRN downregulation at 25 dpi compared with preinfection values was observed. There were significant differences between N’Dama and Boran at 25 dpi in the expression levels of the LTA, IL10, and TGFBI genes. LTA was 4.5-fold higher in N’Dama compared with Boran ($P = 0.006$), and TGFBI was also higher in N’Dama compared with Boran (5.9-fold, $P = 0.037$), while Boran had higher levels of the IL10 transcript (2.1-fold, $P = 0.008$).

Differences in the expression levels of the IL10, IL6, TGFBI, and IL4 genes were detected at 29 dpi between N’Dama and Boran. In each case, the levels of expression were higher in the susceptible Boran [IL10, 1.7-fold, $P = 0.047$; IL6, 5.4-fold, $P = 0.030$; TGFBI, 2.1-fold, $P = 0.056$; and IL4, 3.6-fold, $P = 0.020$]. Again, there was a continued trend of downregulation for the ILIRN, IL2, and IL8 genes that continued through 34 dpi. The largest significant difference between N’Dama and Boran at 34 dpi was in IL6 gene expression, with a 7.1-fold higher level in Boran compared with N’Dama ($P = 0.010$).

**DISCUSSION**

The study of the bovine immune response to *T. congolense* infection is more meaningfully described in the context of the clinical data collected during the infection time course. The parasitemia observations are largely in agreement with a previous study that reported similar prepant times and time to first peak of parasitemia (43). In addition to becoming parastemic, both the N’Dama and Boran animals developed anemia, and the kinetics of this anemia, as measured by PCV, were also comparable with the study by Paling et al. (43). Cumulatively, the clinical data suggest that the animals in this experimental challenge exhibited typical phenotypic responses to *T. congolense* experimental infection.

Normal responses to infection in the N’Dama and Boran animals also include well-documented fluctuations in circulating leukocyte populations (12, 39, 55). The development of bovine monoclonal antibodies has facilitated a more accurate description of these changes in cell subset populations in both tolerant and susceptible breeds (for review, see Ref. 35). An early leukopenia has been well documented during experimental trypanosomiasis infection (12, 36, 42). Also, after parasites are detected in the blood, the proportion of B cells increases, mainly because of the expansion of the CD5+ B cell subpopulation (39). An expansion of B cells was also observed in this study after 14 dpi, but there were no significant differences between the N’Dama and Boran animals in the proportion of circulating B cells. There were also no significant differences in the proportions of γδ T cells, marginal differences in CD4+ T cell proportions, and only ~6% higher CD8+ T cell proportion in Boran at three time points. Fluctuations detected during the course of infection in this study would therefore not be expected to mask accurate interpretation of cytokine expression profiles between the N’Dama and Boran to any great extent.

The majority of studies in recent years that have profiled the host cytokine response to trypanosome infection have been based on the mouse model of the disease (2, 19, 20, 40, 50). Murine models have been used to a greater extent primarily because of the large expense and difficulty associated with bovine trypanosome challenge experiments. Indeed, murine models of tolerance and susceptibility to trypanosomiasis are well developed and offer an accessible means by which to study the underlying mechanisms involved. A recent review of the role of mouse models in African trypanosomiasis discusses their great potential but also highlights that there are clear differences between the bovine and murine systems (13). Furthermore, many of the bovine studies published to date have focused on the immune response in specific cell types, primarily lymph node cells, spleen cells, and isolated macrophages, or in vitro stimulation experiments (24, 25, 46, 48, 52). Overall, there are relatively few studies where profiling of cytokines in PBMC has been carried out in cattle (27, 52). Therefore, the aim of the present study was to enhance the understanding of cytokine responses in the bovine model of *T. congolense* infection and shed light on differences in cytokine profiles between N’Dama and Boran cattle that may be due to the mechanisms underlying trypanotolerance and trypanosusceptibility.

The relative mRNA expression levels for 14 cytokine genes were determined for the trypanosusceptible Boran and trypanotolerant N’Dama before infection and at 14, 21, 25, 29, and 34 dpi. By the time trypanosomes were observed circulating in blood, at 14 dpi, N’Dama were producing higher levels of proinflammatory and Th1 type cytokines than Boran, including the products of IFNG, IL12, TNF, and IL1A genes. Additionally, NFKB1 gene expression increased consistently over time, with greater increases in N’Dama. In the murine model, mice deficient in TNF protein have been found to be highly susceptible to *T. congolense* infection, and the TNF gene is thought to play a major role in resistance to infection in mice (18). However, the role of TNF in host tolerance/susceptibility to bovine trypanosomiasis is not fully understood, and in this study, the TNF transcript generally increased in both breeds up to a maximum at 25 dpi, with N’Dama showing significant increases at 14, 21, and 25 dpi compared with preinfection; it is therefore possible that TNF plays a role in disease progression in both N’Dama and Boran. Overall, these data suggest that, during the very early stages of systemic infection, N’Dama cattle are better able to induce classically activated macrophages and a Th1 type response. Classically activated macrophages are effective in combatting parasites by secreting...
nitric oxide (NO) and oxidative radicals; therefore, this could explain why N’Dama are able to control trypanosome numbers more efficiently during the first peak of parasitemia (36). The difference in peak parasitemia between N’Dama and Boran may have important consequences: lowering the amount of available trypanosomes may allow N’Dama to keep pathology under control and to maintain effective responses. Indeed, the highly polarized T_{H1} type response that is observed when mice are infected with African trypanosomes has recently been reviewed (26, 50), with IFN-γ secretion regulating key aspects of immunity (16).

The expression levels of the IL2, IL4, IL8, and IL1RN genes were all significantly downregulated over the time course, with decreases in expression particularly noticeable after 14 dpi (when parasites are detectable in the bloodstream). Suppression of cellular immune responses is a feature of trypanosomiasis in the bovine and murine host. The decreased synthesis of the IL2 transcript and a depressed T cell proliferative ability (based on in vitro stimulation of cells from infected animals) have previously been documented in mice and bovine studies using lymph node cells, spleen cells, and mononuclear blood cells (24, 27, 28, 46, 48, 51). From real-time qRT-PCR data on PBMC of T. congolense-infected N’Dama and Boran animals, a decrease in the IL2 transcript, particularly at 21 dpi, was reported in both groups, and no significant difference was detected between the breeds (27). These data are in agreement with our observations in the present study, although we observed a slightly larger decrease in the Boran group. However, the same study also reported an increase in IL4 production in N’Dama at 32 dpi, and it was hypothesized that this might play a protective role in infection (27). In the present study, we initially observed a decrease in IL4 gene expression at 21 dpi in both breeds, which is in concordance with the previous work; however, we observed no increase in IL4 expression in either breed at 29 or 34 dpi. This may be due to subtle differences in the experimental setup or may even reflect slightly different time courses, such that we may have observed an upregulation of IL4 gene expression if PBMC samples had been collected after 34 dpi. The decrease in IL8 gene expression represented the most profound change in gene expression of any of the cytokines in this study and was similar in both breeds until 34 dpi. The decrease may reflect a switch to a more predominant T_{H2} type response or have a more specific undefined role (to the best of our knowledge, there are no published reports of the suppression of IL-8 production in trypanosome-infected cattle).

The gene expression profiles of the IL6 and IL10 genes showed a pattern of upregulation relative to preinfection between 14 and 21 dpi, followed thereafter by a decrease in expression. Maximal levels of IL6 transcript were reached at 21 dpi in both breeds, after which levels decreased but still remained high relative to preinfection levels. Increased expression of the IL6 transcript from PBMC of infected cattle has been reported for both N’Dama and Boran animals (27). In that study, a disease-promoting role for IL-6 was suggested, and in the present study, the significantly higher levels of IL6 transcript in Boran compared with N’Dama at 29 and 34 dpi would support this hypothesis. Elevated levels of IL10 mRNA have been reported in susceptible Boran and tolerant N’Dama (51, 52), coinciding with the first wave of parasitemia (52). The same study reported that the NO response of monocytes to IFN-γ was suppressed during infection and suggested a possible association with the upregulation of IL-10. Because immunosuppression is a feature of infection in both N’Dama and Boran, and elevated levels of IL-10 were detected in both breeds, the study suggested that IL10 gene expression was not associated with trypanotolerance. In our study, we have also observed a parallel increase in IL10 mRNA transcription for both the N’Dama and Boran animals; however, there are differences, both quantitatively and temporally, between the two breeds. After 14 dpi, the increase in IL10 gene expression in Boran, reaching maximal levels at 21 dpi (4.3-fold higher in Boran), was greater than that observed for the N’Dama animals.

By day 21, the time of peak parasitemia, we detected for both breeds a trend toward T_{H2} responses, but this was much more pronounced for the Boran, with increased levels of IL6 and IL10 transcripts. This trend toward alternative macrophage activation is not expected to facilitate clearance of the trypanosomes in the Boran animals, particularly at a time when parasite numbers reach a peak. Boran cattle are more dependent on antibody responses to control the first peak of parasitemia than N’Dama cattle (35). It has been shown in a mouse model that classically activated macrophages may be beneficial for parasite clearance; however, at the same time, it was also shown that they could contribute to pathology (2). A switch to alternatively activated macrophages during chronic stages of the disease appears to be beneficial for survival, probably because the inflammatory parasite-controlling response is harmful to the host (2). Further studies on isolated cell populations and knowledge of the parasite triggers and genetic components that lead to particular activation patterns in macrophages and T cells would allow us to understand, and perhaps interfere with, an ineffective response in susceptible animals.

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REFERENCES

BOVINE CYTOKINE PROFILES AFTER TRYPANOSOME CHALLENGE


