Innate Immune Response to Rift Valley Fever Virus in Goats

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Abstract

Rift Valley fever (RVF), a re-emerging mosquito-borne disease of ruminants and man, was endemic in Africa but spread to Saudi Arabia and Yemen, meaning it could spread even further. Little is known about innate and cell-mediated immunity to RVF virus (RVFV) in ruminants, which is knowledge required for adequate vaccine trials. We therefore studied these aspects in experimentally infected goats. We also compared RVFV grown in an insect cell-line and that grown in a mammalian cell-line for differences in the course of infection. Goats developed viremia one day post infection (DPI), which lasted three to four days and some goats had transient fever coinciding with peak viremia. Up to 4% of peripheral blood mononuclear cells (PBMCs) were positive for RVFV. Monocytes and dendritic cells in PBMCs declined possibly from being directly infected with virus as suggested by in vitro exposure. Infected goats produced serum IFN-α, IL-12 and other proinflammatory cytokines but not IFN-γ. Despite the lack of IFN-α, innate immunity via the IL-12 to IFN-γ circuit possibly contributed to early protection against RVFV since neutralising antibodies were detected after viremia had cleared. The course of infection with insect cell-derived RVFV (IN-RVFV) appeared to be different from mammalian cell-derived RVFV (MAM-RVFV), with the former attaining peak viremia faster, inducing fever and profoundly affecting specific immune cell subpopulations. This indicated possible differences in infections of ruminants acquired from mosquito bites relative to those due to contact with infectious material from other animals. These differences need to be considered when testing RVF vaccines in laboratory settings.

Introduction

Rift Valley fever (RVF) is a disease of ruminants and man caused by the mosquito transmitted Rift Valley fever virus (RVFV), genus Phelodovirus, family Bunyaviridae [1]. This spherical shaped, enveloped virus has a negative-sense single-stranded RNA genome made up of 3 segments. The large (L) segment encodes for the viral RNA-dependent RNA polymerase while the medium (M) segment encodes the external glycoproteins (Gn and Gc) and the non-structural protein (NSm). The small (S) segment is ambisense, coding for the nucleoprotein (N) in the antigenomic sense and the non-structural protein (NSs) in the genomic direction [2].

RVF outbreaks are frequently reported in Sub-Saharan African countries where the disease is endemic. These include Kenya, Tanzania, Somalia, South Africa, Sudan, Uganda, Madagascar and Senegal. However, outbreaks were also reported in Egypt, Yemen and Saudi Arabia indicating an expanding range for this disease [3]. RVFV is transmitted primarily by Aedes and Culex mosquitoes, with the latter serving as a magnifying host during outbreaks [2]. In addition to infectious mosquito bites, humans can also acquire RVF through contact with blood of diseased animals [4,5]. Outbreaks of RVF in endemic countries usually coincide with conditions such as periods of heavy rainfall and flooding, which favour heavy breeding of mosquito vectors [6,7].

RVF is characterized by large abortion storms and close to 100% mortality in newborn sheep, goats and cattle resulting in severe adverse socio-economic effects [8]. These animals carry high titres of virus (6 log10 to 8 log10 PFU/mL) in their blood resulting in fever, inappetence, nasal discharges and diarrhoea [3]. However, adult sheep, goats and cattle are more resistant to RVFV and experience lower mortality rates between 10–30% [3]. Human RVF usually manifests as a mild and self-limiting fever, but in some patients may progress to a haemorrhagic fever, neurological disorder or blindness [2,3].

Innate and adaptive immune responses contribute to the clearance of RVFV in infected animals [3,9]. Evidence for the role of innate immunity is mostly based on results from experimental models [9–12]. Interferon alpha (IFN-α) is believed to protect against RVFV because monkeys that secreted this cytokine within 12 h of being challenged with RVFV did not develop disease [11]. However, RVFV NSs protein inhibits IFN-α and IFN-β production/induction, thereby enabling early replication and viremia [12–14]. Anti-RVFV antibodies are detectable 4 to 8 days following infection [15–17]. Neutralising antibodies are believed to be crucial for the protection of infected animals [2,11].

Although ruminants have since been recognized as the primary animal hosts, there is little knowledge of the pathogenesis of RVFV in goats. In 2–3 months old goats experimentally infected

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Author Summary

Rift Valley fever (RVF) is a mosquito-transmitted disease of ruminants and man, which occurs in Africa, Saudi Arabia and Yemen but could spread to other areas. There isn’t much information on some aspects of the immune response to this disease and how it affects cells of the immune system in the natural animal hosts. To fill in some of this knowledge gap, we studied RVF in goats experimentally infected with the RVF virus. We also compared RVF virus grown in an insect cell-line and that grown in a mammalian cell-line for differences in the course of infection. Virus was present in the blood of the goats one day after infection. Some goats had fever coinciding with the time when the virus level in the blood was highest. Some cells in the blood dropped in number possibly as a direct effect of virus. Infected goats secreted cytokines (interferon gamma and interleukin-12), which possibly contributed to protection against RVF. Virus from an insect cell-line appeared to have more obvious effects in infected goats suggesting that differences may exist in infections of ruminants acquired from mosquito bites compared to those due to contact with infectious material from other animals.

with RVFV, viremia was detected 24 h post subcutaneous inoculation and lasted for 3 days [18]. These goats also had a mild transient increase in rectal temperature. Mild fever was equally observed in goats inoculated by inhalation and virus could be recovered from throat washes 2 days after inoculation [18]. In addition, virus was apparently transmitted to contact goats. Clinical signs varied in severity depending on the route of inoculation and included lethargy, diarrhoea and occlusion of the eyes. All the goats died between days 9 and 70 post inoculation possibly due to RVF but secondary infections could have also contributed to these deaths. Gross and histopathology lesions were observed in the liver, lungs, kidneys, spleen and brain of infected goats [18]. An attenuated live RVFV vaccine (Smithburn strain) has also been shown to cause abortion in vaccinated pregnant goats and pathology in the liver, kidney and other organs of vaccinated kids [19].

There is still a remarkable paucity of data on RVFV innate and cell mediated immune responses in sheep, goats and cattle. Knowledge of the pathogenesis and immune response to RVFV in these domestic ruminants is crucial for rational design of new vaccines and/or evaluation of existing vaccines for veterinary and human use. Therefore, to better understand RVF in small ruminants, we performed experimental infection of goats with RVFV.

The C-type lectins, DC-SIGN and L-SIGN have been identified as probably receptors for arthropod borne viruses (arboviruses) [20]. Similarly, DC-SIGN has recently been identified as a receptor for Phlebovirus including RVFV [21]. Furthermore, insect cell-derived arboviruses belonging to the Alphavirus genus were more infectious to monocyte-derived dendritic cells (MoDCs) compared to mammalian cell-derived virus [20,22] possibly due to their stronger recognition and binding to the C-type lectin receptors. In addition, insect cell-derived Alphavirus was poor at inducing type 1 interferon responses in MoDCs, further enhancing its ability to replicate in these cells [22]. These data collectively suggest that there might be differences between insect cell-derived and mammalian cell-derived arbovirus in in vivo infectivity and disease pathogenesis in susceptible animals. To investigate this, we inoculated goats with insect cell-derived and mammalian cell-derived RVFV and monitored in vivo differences in the course of infection. In addition, we evaluated RVFV from these 2 sources for differences in in vitro infectivity of MoDCs.

Materials and Methods

Ethics statement

All animal experiments were carried out in enhanced biosafety level 3 (BSL3+) at the National Centre for Foreign Animal Disease (NCFAD), Winnipeg, Manitoba. All protocols for animal use, under animal use document (AUD) number C-09-004, were approved by the Canadian Science Center for Human and Animal Health, Winnipeg, Manitoba, Canada Animal Care Committee. Only the NCFAD veterinarian and trained animal care personnel were allowed access to the animals. Care was taken to minimise animal suffering, respecting the Canadian Council on Animal Care guidelines for animal manipulations.

Virus production and titration

RVFV strain ZH501 [23] was kindly provided by Dr Heinz Feldmann, National Microbiology Laboratory, Winnipeg, Canada.

The mammalian cell-derived RVF virus (MAM-RVFV) was propagated on Vero E6 cells (American Tissue Culture Collection, ATCC, Manassas, VA, USA). Infection of Vero E6 cells with RVFV was done in dulbecco’s modification eagle’s medium (DMEM) supplemented with 0.3% bovine serum albumin (BSA, Wisent, QC, Canada) at an MOI of 0.1 and the cultures maintained in DMEM with 0.3% BSA at 37°C, 5% CO2 and 95% relative humidity. The virus eventually used as inoculum for goats was from the 4th passage in Vero E6 cells.

Insect cell-derived RVFV (IN-RVFV) was obtained by propagating the passage 3 RVFV from Vero E6 cells above in a mosquito cell line (C6/36, ATCC). C6/36 cells were infected at an MOI of 0.1 and maintained at 28°C in a 1:1 mixture of EMEM (Wisent) and ESF-921 (Expression Systems, Woodland, CA, USA) supplemented with 2.5% FBS, 25 mM HEPES and 1 mM sodium pyruvate. The IN-RVFV eventually used as inoculum for goats was from the 2nd passage in C6/36 cells.

The sequences for the M and S segments of MAM-RVFV and IN-RVFV were compared for any differences that might result from propagation in the different cell lines. The M segment was selected for sequencing because it was recently shown that a single nucleotide substitution in the glycoprotein (Gn) can have a significant effect on the virulence of RVFV [24]. In addition, the NSs protein encoded by the S segment is also of importance in the virulence of RVFV [13,14,25]. For sequencing, viral RNA was isolated as previously described [26] and RT-PCR performed using published primers and protocol [27]. The RT-PCR products were purified and then cloned using the cloneJet2.1/blunt vector and the CloneJet PCR cloning kit (Fermentas, Canada). Three positive clones per gene segment were identified and sequenced and a consensus sequence obtained as previously described [28].

Vero E6 cells were used to determine the titres of RVFV derived from both cell lines. Briefly, serial 10 fold dilutions of virus in 200 µL DMEM were transferred onto a 24-well plate containing confluent Vero E6 cell monolayer. After 1 h, at 37°C, 5% CO2 and 95% relative humidity, an overlay of 1.75% carboxymethylcellulose in DMEM containing 0.3% BSA (CMC overlay) was added to all wells and plates incubated as above. After 4–5 days cells were fixed with 10% formalin, stained with 0.5% crystal violet and plaques counted.
Goat inoculation

Healthy 4 month old Boer-cross goats were obtained from breeders in Manitoba, Canada and allowed 10 days to acclimatize to BSL3+ containment at NCFAD, during which they were monitored daily for any signs of disease. After acclimatization, the goats were divided into 2 groups (4 per group) and housed in separate cubicles. One group was inoculated with 5 log_{10} PFU of IN-RVFV and the 2nd group with 5 log_{10} PFU of MAM-RVFV per animal by the subcutaneous route. Daily monitoring was continued and rectal temperatures recorded. Blood for serum samples and for peripheral blood mononuclear cells (PBMCs) isolation was collected prior to and daily for the first 7 days post infection with RVFV. Additional sampling was done at 14, 21 and 30 DPI. Serum samples were stored at −70°C.

Peripheral blood mononuclear cells (PBMCs) isolation and flow cytometry

Blood for PBMCs isolation was collected in EDTA-treated vacutainers prior to and daily for the first 7 days post infection (DPI) of goats with RVFV. PBMCs were purified from this blood using Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with minor modifications to manufacturer’s protocol. Briefly, blood was mixed with an equal volume of sterile phosphate buffered saline (PBS, pH 7.2, Sigma), layered over Ficoll-Paque Plus and centrifuged at 300 g for 30 min with the centrifuge brake off. The PBMC layer was collected and washed twice with PBS.

The cells were then resuspended in FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide) and stained for flow cytometry using antibodies known to cross react with goat cell surface markers [29]. Approximately 10^6 PBMCs/tube were each stained with mouse anti bovine CD5-FITC (clone CC17), mouse anti sheep CD6:RPE (clone 38.65), mouse anti bovine CD21:RPE (clone CC21) all from AbD Serotec (Oxford, UK) or mouse anti bovine CD172a (SWC3, clone DH59B) from VMRD (Pullman, WA, USA) on ice for 30 min. Isotype control antibodies were included to check for non-specific binding. Cells were washed twice with FACS buffer and for CD172a (unlabelled primary antibody), rat anti mouse IgG1:FITC (Abd Serotec) was added for another 30 min on ice.

For RVFV detection, PBMCs from infected goats were permeabilized using BD cytometric perm reagent (BD Biosciences, San Diego, CA, USA) according to manufacturer’s protocol. An optimal amount of rabbit polyclonal anti RVFV NSm1 antibody was then added to the cells and incubated on ice for 30 min. The rabbit polyclonal anti RVFV NSm1 antibody (R1105) was produced by the EvoQuest Team, Invitrogen (Carlsbad, California, USA) using a synthetic NSm1 polypeptide. Antibody from a naive rabbit was used as isotype control. Cells were washed twice with BD perm/wash buffer, then stained with Alexa Fluor 594 donkey anti rabbit IgG (Invitrogen, Ore gum, USA) for 30 min on ice, followed by 2 more washes with BD perm/wash buffer.

After the final wash in all staining protocols, cells were fixed overnight in 10% phosphate-buffered formalin before running on the FC500 two laser flow cytometer (Beckman Coulter). At least 25,000 events were acquired per sample and data analysed with the CXP analysis software (Beckman Coulter).

Generation and infection of goat monocyte-derived dendritic cells

Peripheral blood mononuclear cells isolated from naive goats as described above were resuspended in RPMI supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 200 µM/mL glutamx, 10 mM HEPEs and 0.5 µM 2-mercaptoethanol (complete medium) and incubated in cell culture flasks at 37°C overnight for monocytes to attach. Non-adherent cells were removed, adherent monocytes washed twice with sterile PBS and then incubated at 37°C, 5% CO2 and 95% relative humidity in complete medium containing 1 in 10 dilution of recombinant bovine GM-CSF and 0.1 µg/mL recombinant bovine IL-4 (both from Serotec). These conditions have been shown to differentiate bovine monocytes into MoDCs [30]. As controls, adherent monocytes were also cultured in complete medium only (Mo). Medium was supplemented after 3 days and MoDCs and MΦ harvested after 7 days. Flow cytometry for CD14, CD172a and CD11c surface markers was performed as described above.

For RVFV infection, approximately 5×10^5 MoDCs were exposed to either IN-RVFV or MAM-RVFV at 0.1 MOI in RPMI without FBS for 1 h at 37°C, 5% CO2 and 95% relative humidity. The cells were then washed, resuspended in complete medium and incubated at 37°C for 24 h after which supernatants were harvested. The amount of virus in culture supernatants was measured by plaque assay on Vero E6 cells as described earlier.

Determination of adaptive cell-mediated immunity to RVFV

Recall cell-mediated immunity (CMI) was determined by measuring RVFV-specific IFN-γ response [31] in PBMCs from goats at DPI 21. Antigen-specific induction of IFN-γ is one of the accepted methods in immunology for detecting CMI. PBMCs were isolated as described above and resuspended in complete medium. PBMCs were adjusted to 10^6/mL, 100 µL added per well of a 96-well plate and duplicate wells stimulated with IN-RVFV or MAM-RVFV at 0.1 MOI to a final volume of 200 µL well. Complete medium was added to negative control wells while ConA was used as positive control. Plates were incubated at 37°C, 5% CO2 and 95% relative humidity for 48 h, supernatants harvested and stored at −70°C for subsequent IFN-γ ELISA as described below.

RNA extraction and qRT-PCR

RVFV RNA was extracted from serum using the TriPure Isolation reagent (Roche) according to the manufacturer’s protocol. The purified RNA was stored at −70°C. Primers (Invitrogen) and probe (Applied Biosystems) designed to target nucleotides 2912 to 2981 of the RVFV L gene segment [26] were used for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) as previously described [26].

Cytokine ELISA

Antibody pairs known to cross-react with goat IL-12 and IFN-γ were obtained from AbD Serotec and used as previously described [29]. Based on the cross-reactivity of other bovine and ovine antibodies with related targets in goats [29,33], there was a high probability that other sheep and bovine cytokine ELISA antibodies will also cross-react with goat. We therefore obtained cytokine ELISA kits for sheep TNF-α, IL-6 and IL-1β from TSZ ELISA (Framingham, MA, USA) and bovine IFN-γ from USCN Life Science Inc. (Wuhan, China) and used them with goat serum according to the manufacturers’ instructions.

Interferon gamma antiviral assay

To test the in vitro antiviral effect of ruminant IFN-γ against RVFV, recombinant bovine IFN-γ (RB- IFN-γ, Thermo Scientific) and Mardin-Darby bovine kidney (MDBK) cells were used.
Approximately 2 × 10^5 cells/well in 250 μL AMEM were added to a 24-well plate and an equal volume of various concentrations of RB-IFN-γ added in quadruplicates. Medium only was added to cells in the control wells. Plates were incubated at 37°C, 5% CO2 and 95% relative humidity for 24 h and checked for confluence. Well contents were emptied and 100 PFU of RVFV in 200 μL added to all wells except the control controls. After 1 h at 37°C, CMC overlay was added to all wells and plates incubated at 37°C, 5% CO2 and 95% relative humidity. On day 3 after addition of virus, cells were fixed with 10% formalin and stained with 0.5% crystal violet. Plaques were counted in all wells and the percent plaque inhibition calculated.

**Antibody detection**

Neutralising antibody response to RVFV was determined by plaque reduction neutralization test (PRNT) modified from a previously described protocol [34]. Serial 2-fold dilutions of serum in DMEM were made starting from 1 in 20 to obtain triplicates of 100 μL/well for each serum sample. 100 μL of DMEM containing 100 PFU of RVFV was added to each serum dilution, mixed and incubated at 37°C, 5% CO2 and 95% relative humidity for 1 h. 200 μL of the virus/serum mixture was then transferred onto a 24-well plate containing confluent Vero E6 cell monolayer and incubated for another 1 h. CMC overlay was then added to all wells and plates incubated at 37°C, 5% CO2 and 95% relative humidity. Assay of negative and positive control sera as well as the test sera. After 5 days the cells were fixed with 10% formalin, stained with 0.5% crystal violet and plaques counted. The reciprocal of the highest serum dilution that prevented at least 70% CPE was taken as the PRNT_70 titre for that sample.

**Statistical analysis**

Data from multiple time points was analyzed by ANOVA with the Dunnett multiple comparisons test using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA). Differences between groups for data collected at a single time point were analysed using the Student t-test. A p ≤ 0.05 was considered statistically significant.

**Results**

**Sequence identity of the Rift Valley fever virus**

The M gene sequences of MAM-RVFV and IN-RVFV were identical to each other (data not shown) as well as to a published sequence for the M segment (GenBank accession number DQ380200) of RVFV ZH501 strain [27]. Similarly, the gene sequences for the S segment of MAM-RVFV, IN-RVFV and a GenBank publication (accession number DQ380149) [27] were identical to each other.

**Viremia and clinical signs**

All the goats infected with RVFV developed viremia starting at DPI 1. In IN-RVFV-infected goats, peak viremia was attained at DPI 1–2 and by DPI 4 all goats were afebrile (Figure 1A). On the other hand, MAM-RVFV-infected goats had peak viremia at DPI 3 and at DPI 4, 50% of the goats were still viremic (figure 1A). Indeed, on DPI 1 and 3 the difference in viremia between the 2 groups reached statistical significance (p<0.02). Peak viremia in MAM-RVFV-infected goats was higher than for IN-RVFV-infected goats but this difference was not statistically significant. By DPI 5, no virus could be detected in the blood of all the goats. The only clinical sign observed was a slight increase in rectal temperature in IN-RVFV infected goats. Following infection with IN-RVFV, rectal temperatures rose to 39.9–40.3°C, with maximum temperatures corresponding to peaks of viremia at DPI 1–2 (Figure 1B). In MAM-RVFV-infected goats, the increase in rectal temperature was barely noticeable, with a maximum of 39.9°C in 1 goat at DPI 2, and not exceeding 39.5°C in the rest of the goats (Figure 1B). However, there was no significant difference in rectal temperatures between the 2 groups. All goats survived through out the duration of the experiment.

**Changes in frequencies of cell types in PBMCs of RVFV-infected goats**

Monocyte/DC, T lymphocytes, cytotoxic T cells and B cells were identified with antibodies against CD172a, CD5, CD8 and CD21 surface markers respectively. Frequencies of these cells in naïve goats ranged from 11.1–19.3% (CD172a+ monocytes/DC), 15.8–35.4% (CD5+ T cells), 5.7–20.1% (CD8+ T cells) and 8.1–20.9% (CD21+B cells). Following infection there was a drop in CD172a+ monocytes/DC starting on DPI 2 in both IN-RVFV and MAM-RVFV infected goats (Figure 2). However, this decline in CD172a+ monocytes/DC, expressed as a percentage of baseline frequencies, was more pronounced and statistically significant (p<0.01 on DPI 2 and 4, p<0.05 on DPI 3) in IN-RVFV-infected goats compared to MAM-RVFV-infected goats in which the drop never attained statistical significance (figure 2). On the other hand, CD5+ T cells and CD8+ T cells reduction was less than 20% in goats infected with MAM-RVFV while goats infected with IN-RVFV suffered approximately 40% drop (Figure 2). Conversely, in MAM-RVFV-infected goats, CD21+B cell frequencies increased by approximately 2 fold on DPI 1 and 2 (p<0.05 and p<0.01 respectively), returning to within baseline values on DPI 3 but never dropping below baseline frequencies (Figure 2). On the contrary, in IN-RVFV-infected goats, the changes in CD21+B cell frequencies were not statistically significant, only increasing slightly on DPI 3 but declining to 30% below baseline frequencies on DPI 4 and 5 (Figure 2). However, by DPI 14, CD21+B cell frequencies were above baseline values in both groups.

**Ex vivo infectivity of goat PBMC by RVFV**

To investigate whether the decrease in frequencies of identified PBMC subsets was as a result of permissiveness to RVFV, PBMC from infected goats were examined for the presence of virus by intracellular staining for the non-structural protein (NSm1) and flow cytometry. At DPI 1, 1.4 to 2% of PBMCs in MAM-RVFV and IN-RVFV-infected goats were positive for RVFV which increased to 3 to 4% on DPI 3 (Figure 3). More PBMCs stained for NSm1 in IN-RVFV-infected than in MAM-RVFV-infected goats, reaching statistical significance at DPI 1 (p ≤ 0.05). However, this difference was not statistically significant at DPI 3.

**In vitro infectivity of goat MoDC by RVFV**

Since goat MoDCs have not been previously described, we first confirmed that the cells derived from goat monocytes with bovine GM-CSF and IL-4 had the phenotype of related bovine MoDCs [30,35]. These cells had the morphology of DC and were CD14 negative, CD11c and CD172a low as opposed to MoDCs that were CD14+, CD11c and CD172a high (supplementary figure 1). When these cells were infected with RVFV at MOI 0.1, approximately 1 log10 PFU/mL more virus (p<0.05) was obtained from IN-RVFV-infected MoDCs compared to MAM-RVFV at 24 h post infection (Figure 4).

**Serum cytokine response to RVFV infection in goats**

RVFV infection in goats was characterized by 2 cytokine response patterns. Serum levels of IL-12 and IFN-γ peaked early
post-infection while TNF-α, IL-6 and IL-1β levels peaked later. Serum IL-12 levels peaked at DPI 1 in both IN-RVFV-infected and MAM-RVFV-infected goats (Figure 5). The increase in IL-12 response for IN-RVFV-infected goats reached statistical significance on DPI 1 and 2 compared to baseline (p<0.01). On the contrary, the increase in IL-12 response for MAM-RVFV-infected goats did not reach statistical significance. The peak IL-12 response was significantly different between IN-RVFV-infected and MAM-RVFV-infected goats (p = 0.03). However, when all infected goats were analysed together, the IL-12 response was significant at DPI 1 (p<0.01) and DPI 2 (p<0.05). Maximum levels of serum IFN-γ was reached in IN-RVFV-infected goats at DPI 2 but this was delayed until DPI 4 in MAM-RVFV-infected goats (Figure 5). The IFN-γ response reached statistical significance on DPI 2 in IN-RVFV-infected goats (p<0.05) and DPI 4 in MAM-RVFV-infected goats (p<0.05) compared to baseline. There was no significant difference in peak IFN-γ response between the 2 groups. Serum TNF-α, IL-6 and IL-1β levels rose slightly at DPI 1 followed by a significant increase (p<0.05) at DPI 6 (Figure 5). There were no significant differences between IN-RVFV-infected and MAM-RVFV-infected goats with regards to TNF-α, IL-6 and IL-1β response. Minute levels of IFN-α (≤5 pg/ml) were detected in serum from some naive goats but these levels did not increase early post infection (Figure 5).

**In vitro antiviral effect of interferon gamma against RVFV**

Due to the high serum IFN-γ response in RVFV-infected goats, direct antiviral effect of this cytokine on RVFV was tested in vitro...
Figure 2. Changes in cell population frequencies in peripheral blood mononuclear cells in RVFV-infected goats. Column A: PBMCs from IN-RVFV-infected goats; Column B: PBMCs from MAM-RVFV-infected goats; Column C: Cell frequencies expressed as a percentage of pre-infection value for — ■ — IN-RVFV and — • — MAM RVFV-infected goats (n = 4 goats each). Data points in column A and B represent individual animals and the line represents the means. In column C data points represent means ± standard deviation (error bars). MAM-RVFV = RVFV produced in the mammalian cell line Vero E6, IN-RVFV = RVFV produced in the insect cell line C6/36.

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unstimulated cells secreted significantly less IFN-c the non-specific mitogen, ConA, was similar in both groups.

The cells exposed to either virus (p maximum at DPI 21–30 in all goats. However, PRNT 70 titres for antibodies rose to a lower (p 0.05) compared to MAM-RVFV infected goats (Table 1). There was no significant difference in antibody response between the IN-RVFV-infected and MAM-RVFV-infected goats by the filled histograms. Error bars represent standard deviation of means (n = 3 goats each).

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Adaptive cell-mediated immune response to RVFV

In vitro RVFV-induced IFN-c secretion by PBMCs from convalescent goats was used to determine specific cell-mediated immunity (CMI) [31]. PBMCs harvested from convalescent goats at DPI 21 secreted high levels of IFN-c in response to RVFV re-exposure (Figure 6). This IFN-c response was almost identical between the IN-RVFV-infected and MAM-RVFV-infected goats irrespective of which virus (IN-RVFV or MAM-RVFV) was used for in vitro re-stimulation of PBMCs. In addition, the response to the non-specific mitogen, ConA, was similar in both groups. Unstimulated cells secreted significantly less IFN-c compared to the cells exposed to either virus (p<0.02).

Antibody response in RVFV-infected goats

Antibody response, based on PRNT 70, commenced on DPI 5 with low titres in most of the goats. Antibody titres rose to a maximum at DPI 21–30 in all goats. However, PRNT 70 titres for IN-RVFV infected goats at DPI 7, 14 and 21 were significantly lower (p<0.05) compared to MAM-RVFV infected goats (Table 1). There was no significant difference in antibody response between the 2 groups at DPI 30.

Discussion

Cattle, sheep and goats have long been recognized as the natural hosts of RVFV. The clinical manifestation and pathology of natural and experimental RVF in cattle and sheep have been reported [8,31,36–38]. To the best of our knowledge, only one report of experimental infection in goats is published, and it did not address the innate immune response to the virus in goats [18]. Pathology following vaccination of goats with an attenuated strain of RVFV has been studied. However reports from other models reveal that this is not the same as infection with the virulent strain [19]. In this report we attempted to address some aspects of the innate and adaptive immune response to RVFV in goats. In addition, we compared these parameters between insect cell-derived and mammalian cell-derived RVFV. As in the previous report [18], the incubation period for RVFV in goats was 24 h. A similar incubation period is recorded for sheep, cattle, non-human primates and humans [3]. Peak viremia at DPI 1–3 was similarly reported in goats [18] and other susceptible species [3,11,31]. There were no mortalities and the only clinical sign we observed in these RVFV-infected goats was a mild fever in a subset of animals. Therefore, experimental infection of goats with RVFV produces a fairly typical disease course similar to what has been observed in other ruminants of a similar age group [3,18,31].

We observed a significant decline in CD172a+ cells (monocytes and dendritic cells) in RVFV-infected goats. There was also a pronounced decline in T cells (CD5+) and a transient decline in cytotoxic lymphocytes (CD8+) in IN-RVFV infected goats. Only a slight decline in the CD5 population was observed for MAM-RVFV infected goats. It has been suggested that RVFV can directly cause necrosis in infected cells as part of the disease pathogenesis [25,39] and RVFV has been isolated from human PBMCs in a natural outbreak [40]. RVFV has also been shown to infect human monocytes/macrophages [25,41]. Furthermore, RVFV was detected in Kupffer cells (resident liver macrophages) [39]. The differential effect of IN-RVFV and MAM-RVFV on PBMCs could be due to their differential ability to infect BMC subsets. Indeed, PBMCs from IN-RVFV-infected goats had significantly higher percentage of RVFV NSm1 positive cells than in their MAM-RVFV-infected counterparts at DPI 1 which might be linked to the observation of a more profound decline in CD172a+, CD5+ and CD8+ cells in IN-RVFV infected goats. This is further supported by our in vitro data which shows that IN-RVFV infects MoDCs more readily than does MAM-RVFV. Furthermore, RVFV has previously been shown to infect MoDCs [21]. In addition, in arboviruses, insect cell-derived alphaviruses infect MoDCs more efficiently than mammalian cell-derived ones. The presence of high mannose carbohydrates in the viral glycoproteins is thought to enable the former to readily bind receptors on target cells [20,22]. Contrary to the other cell subsets, CD21+ B cell frequencies increased post infection and never dropped below baseline in MAM-RVFV infected goats, while the slight increase in CD21+ B cell frequencies in IN-RVFV infected goats was followed by a decline below baseline frequencies. The amplification of B cells probably prepared the immune system for the more robust antibody production in MAM-RVFV infected goats as opposed to in IN-RVFV infected ones.
To the best of our knowledge, cytokine response to RVFV in ruminants has not been investigated. Here we report the detection of IL-12, IFN-γ, TNF-α, IL-6 and IL-1β in serum of RVFV-infected goats. Also of significance, is the absence of detectable IFN-α, one of the most potent antiviral cytokines. Experimental models have demonstrated a role for IFN-α in RVFV clearance [11] and the virus has developed mechanisms, via the NSs protein, to inhibit IFN-α response in infected cells [12–14,25]. The presence of other cytokines but not IFN-α in RVFV-infected goats suggests that the virus may have specifically blocked its production/induction. This would create a window for high viremia to be attained which usually occurs within 24 h of infection. On the other hand, IL-12 and IFN-γ peaked at DPI 2–4 suggesting an otherwise functional innate immune response to RVFV in goats. In previous reports in sheep, RVFV was cleared from blood several days before the detection of neutralising antibodies indicating that innate immunity was likely responsible for this early protection [31]. IL-12 is known to activate bovine
and ovine NK cells to secrete IFN-γ [42] which in turn activates NK cells to better cytotoxicity [43]. The response pattern in the current report suggests that IL-12 might have promoted the IFN-γ response, possibly from NK cells though other cells including macrophages and DC also secrete IFN-γ [44]. In previous studies [10], monkeys were protected from RVF when human IFN-γ was administered 24 h prior to infection. In addition to promoting NK cell cytotoxicity and downstream adaptive immune responses, IFN-γ is known to activate pathways that can directly inhibit virus [43]. However, using recombinant bovine IFN-γ, we did not detect any significant direct antiviral effect on RVFV replication in MDBK cells. Indeed, there was no antiviral effect at titres equivalent to the maximum serum IFN-γ response in RVFV-infected goats. Furthermore, it has been demonstrated that human IFN-γ has minimal in vitro antiviral effect against RVFV [45]. It is therefore, possible that IFN-γ and IL-12 may have played a role in the rapid clearance of viremia in RVFV-infected goats by activating NK cells, even though a direct antiviral effect of these cytokines cannot be ruled out. This will be investigated in subsequent experiments. The other pro-inflammatory cytokines may have also played a role in RVFV clearance despite reaching peak levels on DPI 6–7. Recent data from humans suggests that a strong pro-inflammatory response is linked to survival of RVF [25].

The detection of neutralising antibodies starting at DPI 5 reported here has been similarly observed in natural and experimental infections in other animal models and humans [2,15–17]. Neutralising antibodies are believed to be crucial for the early protection against RVFV [2]. Based on our observations in goats, the initial protection could be primarily due to innate immunity (mediated by cytokines and possibly NK cells). Nevertheless, neutralising antibodies are responsible for long term protection from subsequent challenge [2]. Adaptive cell mediated immunity may also be involved in long term protection from

**Table 1. Antibody response in RVFV-infected goats.**

| PRNT70 titre | Group | Goat # | DPI 0 | DPI 4 | DPI 5 | DPI 6 | DPI 7 | DPI 14 | DPI 21 | DPI 30 |
|--------------|-------|--------|-------|-------|-------|-------|-------|--------|--------|--------|
| MAM-RVFV     | 4     | <20    | <20   | 20    | 40    | 40    | 320   | 640    | 640    |        |
|              | 27    | <20    | <20   | 20    | 40    | 80    | 320   | 640    | 640    |        |
|              | 28    | <20    | <20   | 20    | 20    | 80    | 320   | 1280   | 5120   |        |
|              | 44    | <20    | <20   | 20    | 40    | 40    | 320   | 1280   | 640    |        |
| IN-RVFV      | 29    | <20    | <20   | 20    | 20    | 20    | 80    | 320    | 320    |        |
|              | 38    | <20    | <20   | 20    | 20    | 40    | 40    | 320    | 640    |        |
|              | 39    | <20    | <20   | 20    | 20    | 20    | 40    | 320    | 640    |        |
|              | 48    | <20    | <20   | 40    | 40    | 40    | 320   | 640    |        |        |

Antibody response to RVFV was measured by plaque reduction neutralisation test (PRNT). The reciprocal of the serum dilution giving at least 70% plaque inhibition relative to the virus control was taken as the PRNT70 titre for that sample. MAM-RVFV = mammalian cell-derived RVFV, IN-RVFV = insect cell-derived RVFV. doi:10.1371/journal.pntd.0001623.t001
RVFV as suggested by the high IFN-γ response following restimulation of cells from convalescent goats (this report) and sheep [31]. Experimental trials in mice have also suggested that cell mediated immunity is important for post-vaccinal protection against RVFV [46].

In conclusion, experimental RVF in goats closely resembles natural and experimental infection in other ruminant hosts. Apparently, the virus infects DCs and monocytes and inhibits IFN-α response thereby allowing rapid replication. However other arms of innate and possibly adaptive immunity combine to protect animals from RVFV shortly after infection. The source of virus appears to influence events during infection, with IN-RVFV achieving peak viremia more rapidly, infecting more PBMCs, inducing slight fever and higher levels of early cytokines but lower levels of neutralising antibodies at onset of seroconversion. These findings seem to suggest that infections acquired from mosquito bites could differ somewhat from those due to contact with infectious material. However, this is far from conclusive considering the small sample size of 4 goats per group and the fact that in a natural setting things are much more complex, with other factors such as dose of infection, age and immune status likely to influence the course of disease. In addition, considering that these are outbred animals, genetic factors could also have contributed to the observed differences. Nevertheless, all 8 goats responded to RVFV by secreting cytokines irrespective of the source of virus. More work is required in goats and other ruminants to check if this results can be similarly observed in these species.

References
1. Bishop DH, Calisher CH, Casals J, Chumakov MP, Gaidamovich SY, et al. (1980) Bunyaviridae. Intervirology 14: 125–145.
2. Pepe M, Boulou M, Bird BH, Kemp A, Pawska J (2010) Rift Valley fever virus (Bunyaviridae: Phleboviridae): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. Vet Res 41: 61.
3. Bird BH, Ksiazek TG, Nichol ST, Macchialini NJ (2009) Rift Valley fever virus. J Am Vet Med Assoc 234: 883–893.
4. Archer BN, Weyer J, Pawska J, Nkosi D, Leman P, et al. (2011) Outbreak of Rift Valley fever affecting veterinarians and farmers in South Africa. 2008. S Afr Med J 101: 283–286.
5. Beaudoin AD, Ochiai Y, Peters CJ, Muchiri EM, King CH (2007) Spectrum of Rift Valley fever virus transmission in Kenya: insights from three distinct regions. Am J Trop Med Hyg 76: 795–800.
6. Anyamba A, Chretien JP, Small J, Tucker CJ, Formenty PB, et al. (2009) Prediction of a Rift Valley fever outbreak. Proc Natl Acad Sci U S A 106: 955–959.
7. Davies FG, Lanthicum KJ, James AD (1985) Rainfall and epizootic Rift Valley fever in the Western Highlands of Papua New Guinea. Bull World Health Organ 63: 941–943.
8. Coetzer JA (1982) The pathology of Rift Valley fever. II. Lesions occurring in field cases in adult cattle, calves and aborted foetuses. Onderstepoort J Vet Res 49: 11–17.
9. do Valle TZ, Billecocq A, Girault L, Alberts R, Gommet C, et al. (2010) A new mouse model reveals a critical role for host innate immunity in resistance to Rift Valley fever. J Immunol 185: 6146–6156.
10. Morrill JC, Czarnecki CW, Peters CJ (1991) Recombiant human interferon-gamma modulates Rift Valley fever virus infection in the rhesus monkey. J Interferon Res 11: 297–304.
11. Morrill JC, Jennings GB, Johnson AJ, Cougiff TM, Gibbs PH, et al. (1990) Pathogenesis of Rift Valley fever in rhesus monkeys: role of interferon response. Arch Virol 110: 195–212.
12. Bouley M, Jurenka J, Vialat P, Kuhn H, Pavlovic J, et al. (2007) Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein Ns. J Virol 71: 1371–1377.
13. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, et al. (2009) Dual functions of Rift Valley fever virus Ns0 protein: inhibition of host mRNA transcription and post-transcriptional downregulation of protein kinase PKR. Ann N Y Acad Sci 1171 Suppl 1: E75–E85.
14. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, et al. (2009) Dual functions of Rift Valley fever virus Ns protein: inhibition of host mRNA transcription and post-transcriptional downregulation of protein kinase PKR. Ann N Y Acad Sci 1171 Suppl 1: E75–E85.
15. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, et al. (2009) Rift Valley fever virus Ns protein promotes post-transcriptional downregulation of protein kinase PKR and inhibits eIF2alpha phosphorylation. PLoS Pathog 5: e1000257.
16. Pawska JT, Burt EF, Swanepoel R (2005) Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. J Virol Methods 128: 173–181.
17. Williams R, Ellis CE, Smith SJ, Potgieter CA, Wallace D, et al. (2011) Validation of an IgM antibody capture ELISA based on a recombinant nucleoprotein for identification of domestic ruminants infected with Rift Valley fever virus. J Virol Methods 177: 140–146.
18. Imam ZN, de-Karaiangany R, Kasem S (1978) Studies on goats experimentally infected with RVF virus. J Egypt Public Health Assoc 53: 273–280.
19. Kamal SA (2009) Pathological studies on postvaccinal reactions of Rift Valley fever in goats. Virol J 6: 94.
20. Klimstra WB, Nanglo EM, Smith MS, Yurochko AD, Ryman KD (2005) DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived alphaviruses. J Virol 77: 12902–12903.
21. Lozach PY, Kuhbacher A, Meier R, Mancini R, Ritto D, et al. (2011) DC-SIGN as a receptor for phleboviruses. Cell Host Microbe 10: 75–88.
22. Shabman RS, Morrison TE, Moore C, White L, Suthar MS, et al. (2007) Differential induction of type I interferon responses in myeloid dendritic cells by mosquito and mammalian-cell-derived alphaviruses. J Virol 81: 1937–1947.
23. Warabi KS, el-Baz LAM, el-Tayeb EM, Omar II, Osman MA, et al. (1978) Virus isolation and identification from cases of Rift Valley fever virus infection in Egypt. J Egypt Public Health Assoc 53: 201–203.
24. Morrill JC, Ikegami T, Yoshikawa-Iwata N, Lokugamage N, Won S, et al. (2010) Rapid accumulation of virulent rift valley Fever virus in mice from an attenuated virus carrying a single nucleotide substitution in the m RNA. PLoS One 5: e9986.
25. McIntyre AK, Nichol ST (2011) Rift Valley fever virus inhibits a pro-inflammatory response in experimentally infected human monocyte derived macrophages and a pro-inflammatory cytokine response may be associated with patient survival during natural infection. Virology.
26. Drolet BS, Weingartl HM, Jiang J, Neufeld J, Marszal P, et al. (2011) Development and evaluation of one-step RT-PCR and immunohistochemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. J Virol Methods.
27. Bird BH, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST (2007) Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. J Virol 81: 2805–2816.
28. Weingartl HM, Berhane Y, Hisanaga T, Neufeld J, Kehler H, et al. (2010) Genetic anne expression of genomic RNA from recombinant virus carrying a single nucleotide substitution in the m mRNA. Cell Host Microbe 10: 75–88.
29. Pawska JT, Burt EF, Swanepoel R (2005) Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. J Virol Methods 128: 173–181.
30. Denis M, Buddle BM (2008) Bovine dendritic cells are more permissive for Mycobacterium bovis replication than macrophages, but release more IL-12 and induce better immune T-cell proliferation. Immunol Cell Biol 86: 185–191.

Supporting Information

Figure S1 Phenotype of goat monocyte-derived dendritic cells. MoDCs were differentiated from adherent blood monocytes using recombinant bovine GM-CSF and IL-4. For controls, adherent monocytes were cultured in culture medium only (Mφ). Pictures were taken on days 0, 3 and 6. On day 7, MoDCs and Mφ were harvested and analyzed by flow cytometry. 1A. shows progression from monocytes to MoDCs with characteristic dendrites. 1B. shows MoDCs (broken line) as CD14 negative, CD172a and CD11c low as opposed to Mφ (solid line) that were CD14+, CD172a and CD11c high. Filled histograms represent cells stained with isotype control antibody. (TIF)

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Author Contributions

Conceived and designed the experiments: CKN PM HMW. Performed the experiments: CKN PM SZ HMW. Analyzed the data: CKN SZ HMW. Wrote the paper: CKN HWM.
31. Busquets N, Xavier F, Martin-Folgar R, Lorenzo G, Galindo-Cardiel I, et al. (2010) Experimental infection of young adult European breed sheep with Rift Valley fever virus field isolates. Vector Borne Zoonotic Dis 10: 689–696.

32. Bird BH, Basioe DA, Ksiazek TG, Shoemaker TR, Nichol ST (2007) Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. J Clin Microbiol 45: 3506–3513.

33. Caro MR, Gallego MC, Buendia AJ, Navarro E, Navarro JA (1998) Postnatal evolution of lymphocyte subpopulations in peripheral blood and lymphoid organs in the goat. Res Vet Sci 65: 145–148.

34. Weringart HM, Drebot MA, Hubalek Z, Halouzka J, Andonova M, et al. (2003) Comparison of assays for the detection of West Nile virus antibodies in chicken serum. Can J Vet Res 67: 120–132.

35. Lei L, Hostetter JM (2007) Limited phenotypic and functional maturation of bovine monocyte-derived dendritic cells following Mycobacterium avium subspecies paratuberculosis infection in vitro. Vet Immunol Immunopathol 120: 177–186.

36. Coetzer JA (1977) The pathology of Rift Valley fever. I. Lesions occurring in natural cases in new-born lambs. Onderstepoort J Vet Res 44: 205–211.

37. Olaleye OD, Tomori O, Fajimi JL, Schmitz H (1996) Experimental infection of three Nigerian breeds of sheep with the Zinga strain of the Rift Valley Fever virus. Rev Elev Med Vet Pays Trop 49: 6–16.

38. Olaleye OD, Tomori O, Schmitz H (1996) Rift Valley fever in Nigeria: infections in domestic animals. Rev Sci Tech 15: 937–946.

39. Shieh WJ, Paddock CD, Lederman E, Rao CY, Gould LH, et al. (2010) Pathologic studies on suspect animal and human cases of Rift Valley fever from an outbreak in Eastern Africa, 2006–2007. Am J Trop Med Hyg 83: 38–42.

40. Saribou JL, Jousan A, Le Guennou B, Philippe B, Rieu O, et al. (1989) Isolation of Rift Valley fever virus from human peripheral blood mononuclear cells: Mauritanian epidemic. Res Virol 140: 263–270.

41. Lewis RM, Cosgriff TM, Peters CJ, Morrill JC (1987) Differentiation of a human monocytic cell line associated with increased production of Rift Valley fever virus by infected cells. J Med Virol 23: 207–215.

42. Elhmouzi Younes J, Boyen P, Pende D, Storset AK, Le Vern Y, et al. (2010) Oxine CD16+/CD14− blood lymphocytes present all the major characteristics of natural killer cells. Vet Res 41: 4.

43. Biron CA, Brossay L (2001) NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 13: 458–464.

44. Ansari AA, Mayne AE, Sundstrom JB, Bostik P, Grimm B, et al. (2002) Administration of recombinant rhesus interleukin-12 during acute simian immunodeficiency virus (SIV) infection leads to decreased viral loads associated with prolonged survival in SIVmac251-infected rhesus macaques. J Virol 76: 1731–1743.

45. Habjan M, Pichlmair A, Elliott RM, Overby AK, Glatter T, et al. (2009) NSs protein of rift valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. J Virol 83: 4365–4373.

46. Bodha H, Lorenzo G, Rodriguez F, Buen A (2011) A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR(−/−) mice upon lethal virus challenge. Vaccine 29: 4469–4475.