Cytokine patterns in a comparative model of arenavirus haemorrhagic fever in guinea pigs

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Arenaviruses such as Lassa virus cause a spectrum of disease in humans ranging from mild febrile illness to lethal haemorrhagic fever. The contributions of innate immunity to protection or pathogenesis are unknown. We compared patterns of expression of cytokines of innate immunity in mild versus severe arenavirus disease using an established guinea pig model based on the macrophage-tropic arenavirus Pichinde virus (PICV). Cytokine transcripts were measured by using real-time RT-PCR in target organs and blood during mild infection (caused by PICV, P2 variant) and lethal haemorrhagic fever (caused by PICV, P18 variant). In the initial peritoneal target cells, virulent P18 infection was associated with significantly increased gamma interferon (IFN-γ) and monocyte chemotactic protein-1 (MCP-1, CCL2) mRNA levels relative to P2 infection. Peritoneal cells from P18-infected animals had decreased tumour necrosis factor alpha (TNF-α), interleukin (IL)-8 (CXCL-8) and IL-12p40 transcripts relative to mock-infected animals. Late in infection, P18-infected peripheral blood leukocytes (PBL) had decreased TNF-α, IFN-γ; and regulated upon activation, normal T cell expressed and secreted (RANTES, CCL-5) cytokine transcripts relative to P2-infected PBL. We conclude that, in severe arenavirus disease, patterns of cytokine expression in the initially infected cells favour recruitment of additional target monocytes, while inhibiting some of their pro-inflammatory responses. Suppression rather than overexpression of pro-inflammatory cytokines accompanied the terminal shock in this model of arenavirus haemorrhagic fever.

INTRODUCTION

Arenaviruses such as Lassa virus, Junin virus, Machupovirus and Guanarito virus can cause haemorrhagic fever syndromes in humans, but clinical expression ranges widely between mild subclinical febrile disease and lethal haemorrhagic fever (Levis et al., 1985; McCormick et al., 1987). Host factors that determine disease severity, such as innate immune responses, have not been identified completely. Arenaviruses are generally considered to be poor interferon inducers, and weakly responsive to antiviral effects of type I interferons (Asper et al., 2004; Martinez-Sobrido et al., 2006; Stephen et al., 1977). However, the contributions of other cytokines of innate immunity to arenavirus disease or protection from disease have not been defined. We studied cytokines of innate immunity as possible correlates of disease protection or pathogenesis in a guinea pig model of arenavirus disease.

Arenaviruses are non-cytopathic viruses with a tropism for macrophages and dendritic cells (Aronson et al., 1994; Baize et al., 2004; Lukashevich et al., 1999; Mahanty et al., 2003b; Murphy et al., 1977). Upon activation by pathogens, including filovirus agents of viral haemorrhagic fever, macrophages typically secrete pro-inflammatory cytokines and chemokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL)-8 (Bosio et al., 2003; Feldmann et al., 1996). However, there is growing evidence that arenavirus infection of macrophages or dendritic cells decreases the capacity of those target cells to generate cytokine responses. For example, Lassa virus infection of both macrophages and dendritic cells fails to induce antiviral or pro-inflammatory cytokines, co-stimulatory molecules or other activation markers. By contrast, the related but benign arenavirus Mopeia virus, does induce macrophage and dendritic cell responses, including expression of TNF-α, and IL-12p35 (Baize et al., 2004; Lukashevich et al., 1999; Mahanty et al., 2003b; Pannetier et al., 2004). We have shown recently that a virulent arenavirus induces narrower and more restricted signalling responses in infected macrophages than does a benign arenavirus variant (Bowick et al., 2006, 2007). It is plausible that virulent arenaviruses interfere with the development of antiviral cytokine responses in their initial target cells of the monocyte/macrophage series. This has never been demonstrated in vivo.

On the other hand, for several of the viral haemorrhagic fevers, it has been suggested that overproduction of pro-inflammatory cytokines such as IL-1β and TNF-α late in
the disease course may contribute to vascular endothelial dysfunction and consequent shock (Geisbert & Jahrling, 2004; Marta et al., 1999). The small number of reported human studies do not support this idea for arenavirus haemorrhagic fevers consistently (Mahanty et al., 2001; Schmitz et al., 2002). However, sampling limitations of human studies make it difficult to correlate cytokine levels with disease stage or outcome. Animal models provide the opportunity to study various stages of infection in multiple tissue compartments. In the present study, we sought to determine whether patterns of cytokine induction or suppression correlated with disease severity in a well characterized and accepted guinea pig model of arenavirus disease.

The model is based on the use of Pichinde virus (PICV), Munchique strain (Trapido & SanMartín, 1971), that was serially passaged in inbred guinea pigs and acquired virulence in that species. Infection with low-passage attenuated PICV variants, such as P2, results in onset of fever by day 6 and resolution of clinical signs and viraemia by approximately 2–3 weeks after infection (Jahrling et al., 1981; Zhang et al., 1999, 2001). Infection with high-passage virulent variants, such as P18, results in fever onset during the first week of infection (days 4–6), progressive weight loss during the second week of infection, and death by days 12–21 post-infection with accompanying hypothermia, metabolic acidosis, microvascular leak and myocardial suppression (Katz & Starr, 1990; Qian et al., 1994). After infection by the intraperitoneal route, peritoneal macrophages are the initial target, with virus becoming detectable next in spleen, and subsequently in liver and other visceral, reticuloendothelial organs prior to the onset of viraemia (Aranson et al., 1994; Connolly et al., 1993; Jahrling et al., 1981). We have used these two variants of PICV to provide a comparative model simulating the spectrum of clinical severity of human arenavirus disease. We compared cytokine mRNA responses to mild and severe arenavirus infection in several tissue compartments and time points during experimental infection, focusing on selected cytokines important in innate immunity.

**METHODS**

**Cells and reagents.** Vero 76 cells for plaque assay were obtained from the ATCC and maintained in minimal essential medium (Gibco) containing 5% (v/v) normal calf serum, 2 mM l-glutamine, 100 U penicillin ml\(^{-1}\), and 100 μg streptomycin ml\(^{-1}\).

**Viruses.** Attenuated P2 and virulent P18 viruses were derived from serial spleen passages of PICV, Munchique strain (CoAn 4763) in inbred guinea pigs (Zhang et al., 1999). All virus stocks used were pooled 10% (v/v) homogenates of spleen from the inbred strain 13 guinea pigs infected with the indicated passage virus. For inoculations, virus stock was diluted in endotoxin-free PBS (Gibco). Mock-infected animals received 1 ml of a 1:120 dilution of spleen homogenate from uninfected, non-immune strain 13 guinea pigs. This dilution was equivalent to the dilution of the lower titre P2 spleen stock.

**Animal experiments.** All animal experiments were conducted in accordance with the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals (National Academy of Sciences). All facilities in which animal experiments were conducted were accredited by The Association for Assessment and Accreditation of Laboratory Animal Care International. Male Hartley outbred guinea pigs, 350–400 g, were obtained from Charles River. Guinea pigs were housed individually and acclimatized for 1 week prior to virus infection. Animals received intraperitoneal inoculations of 1000 p.f.u. P2 or P18 in 1 ml or 1 ml of diluted mock-infected spleen cell homogenate. Final dilutions of spleen cell homogenates were 120-fold (for P2) and 1000-fold (for P18), and 120-fold for uninfected spleen homogenate. When applicable, weights and temperatures were determined 1 day prior to inoculation and at days 5, 6 and 12 post-infection. At days 1, 2, 6 and 12, guinea pigs from each group underwent cardiac puncture under xylazine/ketamine anaesthesia. Blood was collected and stored in lithium-heparin-coated plasma separator tubes (Microtainer; Becton Dickinson). Plasma was removed and RNA was isolated from the remaining cell pellet. After cardiac puncture, animals were killed humanely using carbon dioxide inhalation, and immediately after death peritoneal cells were harvested by lavage with ice-cold, Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS and stored on ice. Cells were centrifuged and RNA was isolated from the cell pellet. Small portions of liver and spleen were harvested and stored in RNA Later (Ambion) according to the manufacturer’s instructions. Cytospin preparations of peritoneal cells were stained by using Leukostat (Fisher Scientific) for differential cell counts.

Animals were considered ‘disease resistant’ and analysed separately when they did not show signs of infection or disease, i.e. their rectal temperature did not exceed 39.8 °C, they did not seroconvert, and they did not display detectable viraemia at the time of killing (limit of detection 10 p.f.u. ml\(^{-1}\)). Because the disease outcome could not be known at days 1 or 2, all animals in each group were used in cytokine analyses at those early time points.

**RNA isolation.** Following the removal of plasma from the blood collection tube, RNA was isolated from the peripheral blood leukocytes (PBL) using the RNA Ribo-Pure blood kit (Ambion) according to the manufacturer’s instructions. Peritoneal cell RNA was isolated using the RNA Aqueous kit from Ambion. To isolate RNA from the spleen and liver, 1–2 mm\(^3\) pieces were placed in tubes containing a 5 mm stainless steel bead and Ultraspec RNA isolation reagent (Biotecx). Tissues were homogenized twice for 2 min each using a Mixer Mill machine (Qiagen), and RNA was isolated and purified using Biotecx reagent and RNA Aqueous kit, according to the manufacturer’s instructions (Biotecx and Ambion). After elution of RNA, all RNA was DNase-treated using the DNA-free DNase kit (Ambion).

**Real-time RT-PCR.** A two-step RT-PCR approach was used, with reverse transcription followed by real-time PCR. First, 2 μl DNase-treated RNA, 8.3 nM random decamer primers (Ambion) and nuclease-free water were heated for 3 min at 85 °C in a PCR thermocycler (Twin Block system, Easy Cycler Series; Eriomp). A mix of dNTPs (0.4 mM each per reaction; Fisher Scientific), enzyme buffer, M-MLV reverse transcriptase (100 U; Ambion) and RNase inhibitor (30 U; Amersham Biosciences) was added to each reaction for a total RT reaction volume of 20 μl. Reverse transcription was performed in a thermocycler (Twin Block system, Easy Cycler Serie; Eriomp) for 1 h at 42 °C followed by 10 min at 95 °C to inactivate the RT enzyme. A 2 μl volume of the resulting cDNA was analysed by real-time PCR using the IQ Sybгрgreen PCR mix (Bio-Rad), nuclease-free water and 0.4 nM cytokine-specific primers (0.2 nM for glyceraldehyde-3-phosphate dehydrogenase, GAPDH) per 25 μl reaction. Primers were designed based on published sequences (Table 1) (Jeovan et al., 2003; Scarozza et al., 1998; Shiratori et al., 1999).
concentrations. When

Plaque assay. Plasma samples collected at days 6 and 12 post-infection were analysed for infectious virus by a plaque assay. Vero cell monolayers were exposed to plasma samples, then overlaid with seven animals in these groups for the experiment shown above. Animals at days 6 or 12 that lacked any clinical signs of disease (fever, weight loss), serum antibody responses (by IFA) or viraemia were analysed separately, as described below (Table 2). Because the onset of clinical signs and viraemia typically does not occur until days 4–6 of infection, we were not able to predict which animals in the groups killed humanely at days 1 or 2 would have become ill, thus all seven animals in these groups for the experiment shown were included in the analysis.

Table 1. Guinea pig cytokine and viral primers for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5′–3′</th>
<th>Reverse 5′–3′</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>CAGGCGTTATTTATGCTATGTC</td>
<td>TGAAGAAGGCCATCGT</td>
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</tr>
<tr>
<td>IL-8</td>
<td>TACGGTTGAGCAGTAAAACCTCA</td>
<td>TCAGGAATTGCTGCTAC</td>
<td>121</td>
</tr>
<tr>
<td>IL-10</td>
<td>CAAAGGCAACGACACCCAG</td>
<td>TTGTAAACACGCTGTCAGCAG</td>
<td>166</td>
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<tr>
<td>IL-12p40</td>
<td>GCCGGCTGTGACACTGAACTTT</td>
<td>TCCAAAGCAGGTACCCCTGAA</td>
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<tr>
<td>TNF-α</td>
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</tr>
<tr>
<td>TGF-β</td>
<td>CTGAACGGCAAGGCGCTCTAAT</td>
<td>CGTGGCGCCATGATACC</td>
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</tr>
<tr>
<td>GRO</td>
<td>GGGAGCCTCAGGGGTCT</td>
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</tr>
<tr>
<td>RANTES</td>
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<td>ACTCCTTGCTGTTGCTGCTAC</td>
<td>119</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CGTTCATGTGCAAGATTTGAACTCA</td>
<td>ATAGGATAAGCAGGACTGAGAAGAC</td>
<td>108</td>
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<tr>
<td>MCP-1</td>
<td>CAAGCGCGCATGCTGTTACCTC</td>
<td>CGGTGAGGATACGCC</td>
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<tr>
<td>GAPDH</td>
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<td>AATCGGAAACTGCGATGTTG</td>
<td>100</td>
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<tr>
<td>PICV GPC</td>
<td>TTGCCATCAGCTGTTATTA</td>
<td>CTTGGGACCTGATGACTCTA</td>
<td>111</td>
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</tbody>
</table>
Cytokine patterns from peritoneal cells early in infection

We selected target cells or tissues from which to measure cytokines based on the pattern of virus spread in the host, described in the Introduction. We chose early time points (days 1 and 2) to measure cytokine responses in initial target cells (peritoneal cells) to determine whether there are cytokine/chemokine responses that could predict benign disease course. Peritoneal cells from virulent P18-infected animals at day 2 had a striking increase in mRNA levels of gamma interferon (IFN-γ) (128–500-fold) and monocyte chemoattractant protein-1 (MCP-1, CCL2) (8–250-fold) when compared with attenuated P2-infected and mock-infected animals (Fig. 1a and b). Among the 10 cytokines examined from peritoneal cells, MCP-1 and IFN-γ were the only two that showed differential mRNA expression in infection with virulent (P18) versus attenuated (P2) viruses. Notably, neither virus significantly induced increased mRNA levels of TNF-α, IL-1β or IL-12p40 from peritoneal cells (Fig. 1a). However, P18 infection was associated with decreased peritoneal cell mRNA expression of pro-inflammatory cytokines TNF-α (fourfold at day 1) and IL-12p40 (16-fold at day 1), and the chemokine IL-8 (CXCL8) (2.5-fold at day 2) compared with mock infection (Fig. 1a and b). Mean mRNA levels for these cytokines in P2 infection were intermediate between P18 and mock levels, and were not significantly different from either group. In contrast, peritoneal cells from P2-infected guinea pigs had slightly suppressed mRNA expression of IL-1β (approximately threefold) compared with mock-infected cells (Fig. 1a).

To determine whether differences in cytokine transcript levels between groups reflect significant differences in peritoneal cell populations, differential cell counts were done. Peritoneal cell populations at days 1 and 2 post-infection consisted of 49–61% macrophages, 10–21% lymphocytes and 17–37% neutrophils. The only statistically significant difference between groups was an increased proportion of neutrophils at day 2 for P18-infected guinea pigs compared with mock-infected guinea pigs (P2=32.6±11.7%; P18=37.1±14.3%; mock=16.1±7.7%). We did not identify any cells with the characteristic morphology of the NK-like Kurloff cells in peritoneal cell populations from any group (Debout et al., 1993).

Fig. 1. Cytokine mRNA levels from peritoneal cells harvested on days 1 and 2 post-infection. Mean ΔCt values with standard errors are shown for seven animals each from the P2-infected group (white bars, n=7), P18-infected group (black bars, n=7) and mock-infected group (grey bars, n=7). ΔCt=Ct cytokine−Ct GAPDH. Brackets indicate P<0.05 between groups by one-way ANOVA with Bonferroni adjustment for multiple comparisons. (a) Pro-inflammatory cytokines. (b) Chemokines. (c) Regulatory cytokines.

Table 2. Features of PICV P18 infection in disease ‘susceptible’ and disease ‘resistant’ guinea pigs

Indicated signs or measurements were noted on day 12 post-infection with 1000 p.f.u. P18. Y, Yes; N, no.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Fever change from baseline (g)</th>
<th>Virus titre in plasma (p.f.u. ml⁻¹)</th>
<th>IFA reciprocal titre</th>
<th>Ratio viral RNA/ GAPDH RNA in spleen</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Y</td>
<td>-73</td>
<td>2.0 x 10⁵</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>Y</td>
<td>-87</td>
<td>9.0 x 10⁵</td>
<td>&gt;320</td>
</tr>
<tr>
<td>3</td>
<td>Y</td>
<td>-3</td>
<td>4.0 x 10⁵</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>-31</td>
<td>1.4 x 10⁶</td>
<td>160</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td>+102</td>
<td>&lt;10</td>
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<td>7</td>
<td>N</td>
<td>+146</td>
<td>&lt;10</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>
Cytokine mRNA levels in the spleen and liver at mid–late stage infection

We selected the time points days 6 and 12 to determine whether there are subsequent waves of cytokine responses, particularly pro-inflammatory cytokines, that are differentially expressed in severe versus mild infection. At days 6 and 12 post-infection, there were no differences between P18- and P2-induced pro-inflammatory cytokine mRNA levels in the spleen. TNF-α and IFN-γ transcripts were increased in spleens of guinea pigs infected with either PICV variant compared with mock-infected animals (2-32-fold, Fig. 2a), whereas IL-1β and IL-12p40 transcripts were decreased (Fig. 2a). Chemokines, IL-8, growth-related oncogene (GRO) and MCP-1 showed increased relative mRNA levels in P18-infected spleens compared with P2- or mock-infected spleens (Fig. 2b). By contrast, RANTES...
mRNA expression was decreased in P18-infected spleens at day 12 (Fig. 2b).

In terms of hepatic cytokine mRNA levels, there were few differences between P18 and P2 infection. P18-infected livers had elevated IL-8 (fourfold) at day 6, and suppressed IL-1β (eightfold) at day 12, compared with P2-infected livers (Fig. 3a and b). Both P2- and P18-infected livers showed increased mRNA levels for TNF-α, IFN-γ, GRO, MCP-1, RANTES and transforming growth factor beta (TGF-β) compared with mock-infected livers, but did not have differential levels of these chemokines compared with each other (Fig. 3).

**Cytokine transcript levels in PBL**

PBL cytokine transcript levels were examined at all the aforementioned time points. Although PBL cytokine transcript levels at days 1 and 2 did not mirror peritoneal cell mRNA levels completely, suppression of certain cytokines in P18 infection was again revealed. IL-8 and RANTES (CCL5) mRNA were decreased approximately fourfold in P18-infected PBL compared with P2- and mock-infected PBL at early time points (Fig. 4). There were no other cytokine differences between groups at these time points. In particular, there was no upregulation of PBL cytokine mRNA early in infection by either virus.

We observed a pattern of suppression of PBL cytokines mRNAs late in infection. At day 12 post-infection, PBL from P18-infected animals had approximately fourfold lower levels of TNF-α and IFN-γ mRNA compared with P2-infected PBL (Fig. 4). Also of note, RANTES expression was lower for P18-infected PBL compared with P2-infected PBL at day 12, paralleling the relative suppression of this cytokine also seen in P18-infected spleens at day 6 (Figs 2b and 4). There were no differences between P2 and P18 mRNA levels for other PBL cytokines analysed. However, P2 infection resulted in increased mRNA levels of IL-10 (day 6, data not shown), IFN-γ and RANTES (day 12) compared with mock-infected PBL (Fig. 4). Taken together, these results suggest that a relative suppression of cytokines, such as TNF-α, IFN-γ and RANTES, in PBL is associated with a more severe PICV disease. We did not find increased expression of pro-inflammatory cytokines to be associated with the lethal-shock-like disease. The day 12 time point correlates with the presence of vascular leakiness in PICV-infected guinea pigs (Katz & Starr, 1990).

**Relationship between disease severity and cytokine mRNA levels**

To determine whether viral replication in the initial target population could predict which animals would not develop
disease, viral RNA levels were determined by real-time RT-PCR in peritoneal cells of all animals at days 1 and 2 post-inoculation. Although 40% of P18-inoculated guinea pigs did not develop signs of disease or seroconversion ultimately, viral RNA levels in peritoneal cells during the first 2 days of infection with P18 were tightly clustered with no outliers (Fig. 5). Quantitative real-time RT-PCR assay for viral RNA indicated that there was some degree of viral replication in peritoneal cells in all P18-infected animals at days 1 and 2 post-infection (Fig. 5). This assessment was based on the observations that: (i) animals infected with P18 displayed generally higher levels of viral RNA than P2-infected animals in the peritoneal compartments within the first 2 days of infection and (ii) there were increased viral RNA levels at day 2 compared with day 1. These findings suggested to us that ‘disease resistance’ was not due to peritoneal cells of some animals being refractory to infection with P18.

We were particularly interested in identifying any cytokines whose expression was inversely correlated with viral RNA levels early in infection, to indicate a possible protective cytokine or biomarker for less severe disease. When all P2- and P18-infected animals were considered together, Pearson’s product moment correlation analysis demonstrated a positive correlation between peritoneal cell viral RNA level and MCP-1 mRNA at days 1 and 2 (r=0.583 and 0.933, respectively, data not shown). At day 2 in peritoneal cells, there was also a positive correlation between TNF-α and IFN-γ mRNA levels and viral RNA levels (r=0.601 and 0.646, respectively, data not shown). There were no cytokines for which a negative correlation was found.

We then compared cytokine responses in the P18 ‘disease resistant’ group (three of seven P18-infected animals each for days 6 and 12) with those of the P18-infected animals with typical disease features. When analysed separately, ‘disease resistant’ P18 animals at days 6 and 12 showed distinct patterns compared with clinically ill, viraemic animals. Their cytokine patterns were more similar to those of mock-infected animals than to those of P2-infected animals in most compartments at most time points, with a few exceptions. Disease-resistant guinea pigs showed alterations in GRO expression compared with mock-infected animals, with an increase in liver at day 6, and a decrease in spleen and PBL at day 12 (data not shown). Resistant animals also had decreased mRNA levels for IFN-γ (day 12, liver), TNF-α (day 12, PBL) and IL-8 (day 12, PBL) compared with mock-infected animals (data not shown).

**DISCUSSION**

The goal of this study was to characterize cytokine patterns in guinea pig infections with virulent and attenuated strains of PICV, with a focus on cytokines important for innate immunity and inflammation. We report the first study of cytokine responses in a guinea pig model of arenavirus haemorrhagic fever.

With regard to early cytokine responses, a striking finding of our study was a differentially increased expression of MCP-1 (CCL2) mRNA at day 2 in initial target cells of the virulent virus, P18. MCP-1 is known to be induced by IFN-γ (Bauermeister et al., 1998; Inagaki et al., 2002), and can be produced by a variety of cells, including macrophages, T cells and granulocytes (Mantovani et al., 2003). MCP-1 functions to recruit monocytes, T cells and NK cells to inflammatory sites (Mantovani et al., 2003). MCP-1 expression in the peritoneal cavity could accelerate or enhance PICV infection by recruiting monocyte/macrophage target cells to the peritoneal cavity (Gupta et al., 2001; Hendricks et al., 2004). In addition to the early local increase in MCP-1 in P18-infected peritoneal cavities, we also observed increased MCP-1 mRNA levels in multiple compartments at different time points during P18 infection. MCP-1 is a marker of severe disease in murine and primate models of Ebola haemorrhagic fever (Geisbert et al., 2003; Hutchinson et al., 2001; Mahanty et al., 2003a). Recently, investigators have identified differentially upregulated expression of CCR2, the chief receptor for MCP-1, in blood cells of primates infected with a virulent strain of the arenavirus lymphocytic choriomeningitis virus.
mechanism (Fantuzzi et al., 2003; Rutledge et al., 1995). For example, MCP-1 enhances HIV-1 replication directly in human monocyte-derived macrophages, via a post-entry mechanism (Fantuzzi et al., 2003). There have been no studies on the effects of MCP-1 on arenavirus replication to our knowledge.

Paralleling the early increase in MCP-1 mRNA expression in P18-infected peritoneal cells was an increase in IFN-γ mRNA at day 2. The cellular source of the IFN-γ we observed is unknown at this time. NK cells, γ/δ T cells and NK T cells are known to produce IFN-γ during innate immune responses, particularly after stimulation by IL-12 and IL-18 (Billiau & Vandenbroeck, 2001; Schaible & Kauffman, 2000). Granulocytes can also be induced to produce IFN-γ (Ethuin et al., 2004). In our studies, Kurloff cells (guinea pig NK cells) were not identified morphologically in peritoneal cavities, but an increase in granulocytes was noted, suggesting the possibility that the IFN-γ was neutrophil derived. NK T cells or γ/δ T cells have not been well characterized in guinea pigs. Although IFN-γ has important antiviral and macrophage-activating effects in many systems, it has been shown to have minimal antiviral activity against Lassa virus in human cells (Asper et al., 2004). IFN-γ-stimulated macrophages produce nitric oxide (NO), which can have antiviral effects (Guidotti et al., 2000), but at high concentrations can also suppress T-cell proliferation and influence T helper (Th) cell polarization towards Th2 (Akaike, 2001; Butz et al., 1994). We speculate that early IFN-γ-induced NO production by target peritoneal macrophages leads to the inhibition of T-cell proliferation and Th1 differentiation, impairing subsequent cell-mediated immune control of virus replication (Fisher-Hoch et al., 1987, 2000). Relevant to this hypothesis, we have observed repeatedly that expression of RANTES (CCL5), a chemokine that recruits, activates and is produced by T cells (Song et al., 2000), was suppressed in organs and PBL during P18 infection, suggesting diminished T-cell activation. In addition, marked suppression of T-cell proliferative responses to mitogen late in infection with adapted virulent PICV strains has been observed (T. R. Jerrells, unpublished data). Further studies are needed to determine the role of IFN-γ and NO in arenavirus haemorrhagic fever pathogenesis.

Another finding in the present study was the differential suppression of certain other pro-inflammatory cytokine/chemokine responses in P18-infected peritoneal cells or PBL early in the course of infection. In particular, there were slight decreases in TNF-α, IL-8 and IL-12p40 mRNA in peritoneal cells from P18-infected guinea pigs compared with mock-infected guinea pigs. We have reported elevated serum TNF bioactivity and TNF-α mRNA levels in spleen during the late phases of virulent PICV infection previously (Aronson et al., 1995). However, in that study, different PICV strains and different guinea pig strains were utilized compared with the current report. We had observed elevated serum TNF previously only in sera of inbred strain 2 guinea pigs and not in outbred guinea pigs, as used here. The current results are in accordance with in vitro studies in which Lassa virus infection inhibited or failed to induce TNF-α, IL-12 and other cytokine responses from human macrophages, in contrast to the non-pathogenic Mopeia virus (Baize et al., 2004; Lukashevich et al., 1999; Pannetier et al., 2004). We also observed a slight decrease in PBL IL-8 mRNA expression during the first days of P18 infection. This observation parallels previous reports that decreased serum IL-8 levels are associated with lethal outcome in Lassa fever (Mahanty et al., 2001), and that Lassa virus inhibits IL-8 expression from human monocyte-derived macrophages and endothelial cells (Lukashevich et al., 1999). The mechanism by which Lassa virus and PICV suppress macrophage activation is unknown. In our study, induction of anti-inflammatory cytokines did not seem to be responsible, since no early differences were observed for IL-10 or TGF-β (data not shown).

During the course of this study, we observed a variable ‘resistance’ to PICV disease in outbred guinea pigs, as had been reported previously (Jahrling et al., 1981). Since our previous description of PICV infection of outbred guinea pigs (Zhang et al., 1999, 2001), we have noticed repeatedly only a 30–50% mortality among outbred guinea pigs infected with 1000 p.f.u. P18. We do not know the reason for this difference in disease profiles for these outbred animals in our hands, but suspect host genetic factors. In the current study, we showed that, although 40 % of P18-inoculated guinea pigs did not develop viraemia, antibodies or clinical signs ultimately, all animals inoculated with the virulent P18 showed similar levels of viral replication within the first 2 days of infection. We do not have an explanation for the fact that disease-resistant animals did not develop antibodies by day 12, despite evidence of viral replication in peritoneal cells and spleen at early time points. It is possible that seroconversion in these animals was delayed and would have been detected at later time points (Jahrling et al., 1981). Further study of disease resistance to P18 would be valuable in identifying biomarkers of resistance or prognostic indicators. This ‘resistance’ phenomenon may be particularly relevant in comparison with the human population, and requires further evaluation.

The studies reported here do not address type I interferons. This is primarily due to the lack of sequence information for guinea pig non-immune interferons during the period of funding for this work. In our hands, P2 and P18 PICV variants do not differ in terms of interferon induction in vivo or in vitro, or sensitivity to purified murine interferon-α/β (K. Marriott & J. F. Aronson, unpublished data). Administration of exogenous consensus human interferon (interferon alfacon-1) does have a beneficial effect in PICV disease in the hamster model (Gowen et al., 2005). Modulation of the interferon response may be of key importance in innate immune evasion by arenaviruses, as the nucleoprotein (NP) of the prototype arenavirus,
LCMV, inhibits interferon signalling by preventing interferon regulatory factor 3 activation (Martinez-Sobrido et al., 2006). It would be of particular interest to determine whether PICV NP has similar interferon antagonistic activity, and whether P2 and P18 NP differ in this regard. We have shown previously that P2 infection of macrophages is associated with increased evidence of JAK/STAT pathway signalling compared with P18 infection (Bowick et al., 2007). The significance of this finding for interferon responses in this system is not known at present. Another limitation of the current study is the lack of correlation of transcript levels and protein levels of cytokines. Immunological methods for the detection of guinea pig cytokines were not available at the time of this study, and in our experience, immunological reagents directed against murine or human cytokines are not reliably cross-reactive with guinea pig cytokines. The completion of the guinea pig genome sequence, reported after these studies were done, will enable future expanded studies of innate immune responses in guinea pig-dependent disease models, such as arenavirus haemorrhagic fevers.

In summary, we report the first study, to our knowledge, of cytokine responses in a guinea pig model of arenavirus haemorrhagic fever. We were not able to demonstrate increased expression of pro-inflammatory cytokine transcripts in peripheral blood or hepatosplenic compartments during the terminal phases of lethal disease, suggesting that these cytokines may not be associated uniquely with terminal microvascular insufficiency characteristic of arenavirus haemorrhagic fevers. We found that early induction of MCP-1 and IFN-γ in initial target cells correlated with disease severity and that severe arenavirus haemorrhagic fever was associated with an early suppression of selected pro-inflammatory cytokine expression in initial target cells in vivo. The results reported here will set the groundwork for future investigations of the pathogenesis of arenavirus haemorrhagic fever, which will be facilitated by the increasing characterization of the guinea pig genome and immune system.

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