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# CYTOKINE PATTERNS IN A COMPARATIVE MODEL OF ARENAVIRUS INFECTION: IMPLICATIONS FOR VIRULENCE AND CONTROL OF VIRAL REPLICATION

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# CYTOKINE PATTERNS IN A COMPARATIVE MODEL OF ARENAVIRUS INFECTION: IMPLICATIONS FOR VIRULENCE AND CONTROL OF VIRAL REPLICATION

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For my husband Ben, my UTMB romance for whom I am eternally thankful

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# Cytokine patterns in a comparative model of arenavirus infection: Implications for virulence and control of viral replication

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Guinea pig infection with the arenavirus Pichinde provides an animal model for human Lassa fever, a disease that affects 300,000 to 500,000 people a year in western Africa. Low passage Pichinde virus (P2) induces a mild disease with low viremia, while high passage Pichinde (P18) induces a severe disease with high viremia, ending in terminal shock. We hypothesized that severe disease would be associated with a suppression of potentially antiviral cytokines early in infection, and high levels of potentially pathogenic pro-inflammatory cytokines late in infection. Cytokine responses to P2 and P18 infection were measured from primary guinea pig peritoneal macrophages (PM) *in vitro* when measured by real time RT-PCR. In general, neither P2 nor P18 infection altered cytokine production from unstimulated PM. P18 infected PM did have lower mRNA levels of IL-1β, IL-12p40, and MCP-1 after LPS addition when compared to P2 infected PM. During experimental guinea pig infection, P18 infection was associated with markedly increased IFN-γ and MCP-1 mRNA levels from the initial peritoneal target cells relative to P2. P18 infected peritoneal cells had slightly decreased TNF-α, IL-8, and IL-12p40 transcripts relative to mock infected peritoneal cells. Late in infection, P18 infected spleens and livers had similar cytokine patterns relative to P2, but P18 infected PBL had decreased TNF-α, IFN-γ, and RANTES transcripts. We also examined the ability of a decoy AP-1 thioaptamer, XBY-S2, to alter morbidity, mortality, and cytokine expression during P18 infection of guinea pigs. After two doses of XBY-S2, 50% (p=.024) of treated guinea pigs survived infection and had undetectable viremia. XBY-S2-treated P18 infected guinea pigs over time had overall increased cytokine mRNA expression of TNF-α, IL-8, IL-1β, and IL-10 compared to PBS-treated P18 infected guinea pigs. A suppression of PBL IL-1β and RANTES mRNA at day 12 of P18 infection was repeatedly observed. Conclusions from these experiments are 1) macrophage-derived cytokines do not explain the differential replication of P2 and P18 viruses, 2) high levels of IFN-γ and MCP-1 may contribute to virulence of P18 virus, 3) over-expression of pro-inflammatory cytokines in PBL, liver, or spleen is not associated with terminal shock, 4) boosting of pro-inflammatory cytokines by an AP-1 aptamer correlates with reduced viremia and survival of P18 infection.

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| | |
|---|---|
| **BMDM** | Bone marrow-derived macrophage |
| **GAPDH** | Glyceraldehyde phosphate dehydrogenase |
| **GRO** | Growth regulated gene |
| **IFN-γ** | Interferon-gamma |
| **IL-1β** | Interleukin-1 beta |
| **IL-8** | Interleukin-8 |
| **IL-10** | Interleukin-10 |
| **IL-12** | Interleukin-12 |
| **LAS** | Lassa virus |
| **LCMV** | Lymphocytic choriomeningitis virus |
| **LPS** | Lipopolysaccaride |
| **MCP-1** | Monocyte chemoattractant protein-1 |
| **PBL** | Peripheral blood leukocytes |
| **PIC** | Pichinde virus |
| **PM** | Peritoneal macrophage |
| **RANTES** | Regulated upon activation, normal T-cell expressed and secreted |
| **TNF-α** | Tumor necrosis factor-alpha |
| **TGF-β** | Transforming growth factor-beta |

# CHAPTER 1: INTRODUCTION

## ARENAVIRIDAE

Arenaviruses are members of the family *Arenaviridae* and are enveloped single-stranded RNA viruses. Ribosomal particles give these viruses a grainy appearance by electron microscopy from which arenaviruses derive their name (arenosus = sandy) (Murphy *et al*., 1970). The genome has two RNA segments, a small (S, 3.4 kb) segment encoding a nucleoprotein and glycoprotein precursor (GPC), and a large (L, 7.2 kb) segment encoding the viral polymerase and a Z protein (Harnish *et al*., 1983). Arenaviruses utilize an ambisense method of replication;(Auperin *et al*., 1984) the GPC and Z proteins are translated from subgenomic virus sense mRNA, while the nucleoproteins and polymerase are translated from subgenomic virus anti-sense mRNA. Both genomic and anti-genomic RNA can therefore be used as templates.

Arenaviruses have five proteins encoded in four genes. Nucleocapsid proteins (NP) are the most abundate viral protein found in host cells (Salvato *et al*., 1992). The NP associate with viral RNA to form nucleocapsids (Young *et al*., 1983). The L protein is an RNA-dependent RNA polymerase and is also a component of nucleocapsids (Salvato *et al*., 1989; López *et al*., 2001). In most arenaviruses, the GPC protein is processed in the endoplasmic reticulum into two glycoproteins, GP1 and GP2 by the host-cell protease SKI-1/S1P (Beyer *et al*., 2003; Lenz *et al*., 2001). The two glycoproteins both homotetramize, and then combine to form club-shaped spikes on the

outside of the virion (Vezza et al., 1977; Buchmeier et al., 2002). GP1 probably interacts with the receptor, since antibodies to GP1 blocked viral entry of LCMV *in vitro* (Burns *et al*., 1991). GP2 has a trans-membrane domain and has some properties of a fusion protein (Gallaher *et al*., 2001). The Z protein is very small, 11 kDa, and has a zinc-binding RING motif (Salvato *et al*., 1992). Mutational analysis of the Z protein in a LCMV reverse genetics system revealed this protein is important for viral budding (Perez *et al*., 2003). Further evidence for the role of the Z protein in budding was demonstrated when Vero cells transfected with a plasmid encoding the Z protein produced lipid enveloped Z-containing particles (Strecker *et al*., 2003). The Z protein of LCMV was also demonstrated to inhibit transcription and replication of LCVM in a reverse genetic system (Cornu *et al*., 2001). Recently, the Z protein has been demonstrated to interfere with viral polymerase activity for both LCMV and Tacaribe viruses (Cornu *et al*., 2004; Jácamo *et al*., 2003). Although the viral strategy for such a mechanism is not understood, a regulatory effect on transcription may contribute to the noncytopathic effect of arenaviruses (Cornu *et al*., 2004).

Two divisions of arenaviruses have been identified serologically and phylogenetically (Wulff *et al*., 1978; Bowen *et al*., 1997). The New World (North and South America) viruses include Junin, Machupo, Guanarito, and Pichinde among others, and the Old World (Africa, Asia, and Europe) viruses include Lassa, lymphocytic choriomeningitis (LCM), Mopeia, Ippy, and Mobala. New World viruses are further phylogenetically divided into clades A, B, and C (Bowen *et al*., 1996). Several of these

viruses are known human pathogens, the most common of which is LCMV. LCMV is the prototypical arenavirus. It has a world-wide distribution and causes aseptic meningitis. Other arenaviruses of medical importance include Lassa virus, an endemic/epidemic virus of West Africa and four New World arenaviruses, Guanirito, Machupo, Junin, and Sabia, all of which cause human hemorrhagic fever. The pathogenic mechanisms of these viruses have not been well studied and are poorly understood.

For the most part, the arenaviruses remain geographically localized within the regions inhabited by their rodent reservoirs. LCMV is distributed world-wide since the reservoir is the common *Mus musculus* (Salazar-Bravo *et al*., 2002). Humans are infected by eating food contaminated by rodents, aerosolization of rodent excreta, or preparing and eating infected animals (Monath *et al*., 1974; ter Meulen *et al*., 1996; McCormick *et al*., 1987b). Human to human transmission can occur, likely by direct contact with infected body fluids, but aerosol transmission has not been completely ruled out (Peters *et al*., 2002a; Charrel *et al*., 2003; Monath *et al*., 1973).

The receptor for Old World and New World clade C arenaviruses has been identified as α-dystroglycan (Smelt *et al*., 2001; Cao *et al*., 1998; Spiropoulou *et al*., 2002). Tropism and disease severity of various strains of LCMV correlated with their binding-affinities to α-dystroglycan (Smelt *et al*., 2001). Higher binding affinity to α-dystroglycan resulted in splenic white pulp tropism, immunosuppression, and viral clearance, while lower affinity binding resulted in splenic red pulp tropism and viral

clearance (Smelt *et al*., 2001). Some level of infection can occur without α-dystroglycan, and some arenaviruses do not bind this protein, indicating other receptors exist for arenaviruses (Cao *et al*., 1998; Kunz *et al*., 2005; Smelt *et al*., 2001; Spiropoulou *et al*., 2002).

## LASSA FEVER

Lassa virus causes an estimated 100,000-300,000 infections per year in western Africa (McCormick *et al*., 1987b). Mortality for Lassa fever was initially reported to be as high as 50% (Monath *et al*., 1973; Carey *et al*., 1972). It was later discovered that seroprevalence can be very high in some portions of west Africa; in Sierra Leone seroprevalence ranged from 8-52% in different villages and seroconversion of susceptible persons was as high as 22 per 100 people per year (McCormick *et al*., 1987b). Many of the febrile illnesses in these villages were attributed to Lassa (5-14%), but overall the mortality of Lassa virus infection seems to be much lower than originally described (1-2%) (McCormick *et al*., 1987b). A more recent survey of Guinea revealed 7% of hospitalized patients had confirmed Lassa fever, and analysis of IgG titers revealed 14% of patients had evidence of previous infection (Bausch *et al*., 2001). Fatality rates of hospitalized cases are still quite high, 16.5-18%, and are even higher for pregnant women, approximately 25-40% (McCormick *et al*., 1987a; Bausch *et al*., 2001; Johnson *et al*., 1987). These data establish the importance of Lassa fever to public health in western Africa, but Lassa virus can be imported to other countries as well. Imported

cases of fatal Lassa fever have been documented, with cases in several countries including Germany, the United Kingdom, and the US (Schmitz *et al*., 2002; Haas *et al*., 2003; Centers for Disease Control and Prevention (CDC) *et al*., 2004).

Typical of the arenaviruses, human infection with Lassa virus is often acquired by contact with rodent feces or urine of the reservoir, rodents of the genus *Mastomys* (Monath *et al*., 1974). Hunting and eating of rodents is also correlated with transmission (ter Meulen *et al*., 1996). *Mastomys* was found in higher concentrations in mining villages in forested areas as opposed to agricultural villages, but did not necessarily correlate with seroprevalence in humans (McCormick *et al*., 1987b). Studies in Guinea also found higher infection rates of Lassa in forested areas that parallel *Mastomys* infection (Demby *et al*., 2001). Human-to-human transmission can also occur by contact with body fluids from an infected person (Monath *et al*., 1973; Carey *et al*., 1972)

After an incubation period of up to 3 weeks (Mertens *et al*., 1973), Lassa fever presents as a non-specific febrile illness, with symptoms including fever, headache, malaise, sore-throat, severe lower back or abdominal pain, vomiting, diarrhea, conjunctivitis, and occasionally maculopapular rash. Other complications include facial edema, bleeding, acute respiratory distress syndrome, and neurological manifestations such as ataxia, tremors, and coma. Overt hemorrhage is not usually seen. Death is usually within 12 days after onset of symptoms, and is highly associated with the high levels of viremia and serum aspartate aminotransferase (AST) (Johnson *et al*., 1987). Those who recover have a high incidence (approximately 30%) of sensorineural hearing

loss; many of these patients retain some permanent loss of hearing function (Cummins *et al*., 1990).

## TREATMENT OF LASSA

Treatment for Lassa fever is mainly limited to supportive measures and ribavirin. The efficacy of ribavirin was studied in the hamster Pichinde virus model of Lassa fever, and in Lassa virus infected rhesus monkeys. In both models, ribavirin administered beginning at the time of viral inoculation increased survival to 100%, and significantly reduced viremia (Jahrling *et al*., 1980; Stephen *et al*., 1980). Delayed administration of ribavirin (4-5 days after inoculation), still resulted in 100% survival, but the disease was more severe (Jahrling et al., 1980). In Lassa infection of rhesus monkeys, ribavirin administration delayed until day 7 post-inoculation resulted in only 50% survival (Jahrling *et al*., 1984b). Animal studies of ribavirin efficacy were confirmed in a human study in Sierra Leone with patients who had high AST or viremia levels. When ribavirin was administered starting within the first six days after fever onset, mortality was significantly lower than for untreated patients (5-9% vs. 55-76%) (McCormick *et al*., 1986a). When ribavirin was administered starting more than seven days after fever onset, mortality was 26-47% (McCormick *et al*., 1986a). These results suggest that persons exposed to Lassa virus should be treated immediately with ribavirin. Treatment with immune plasma protected cynomolgus monkeys completely from Lassa when administered within 4 days after Lassa virus inoculation (Jahrling *et al*., 1984b). Lassa

virus infection of cynomolgus monkeys and guinea pigs indicate that immune plasma therapy can prevent death, but only when the concentration of neutralizing antibody is >2.0 LNI (log pfu neutralizing index) (Jahrling et al., 1984a; Jahrling et al., 1983). A combined treatment of ribavirin and immune plasma completely protected cynomolgus monkeys from Lassa fever, even when treatment was not administered until day 10 (Jahrling *et al*., 1984b). Detailed human studies on the efficacy of immune plasma treatment have not been documented.

## LASSA PATHOLOGY AND PATHOGENESIS

Lassa virus has an initial tropism for cells of reticuloendothelial origin (Walker *et al*., 1985a; Jahrling *et al*., 1982). During human infection, Lassa virus is pantropic, with virus replication detected in the spleen, liver, adrenal, heart, lung, and pancreas (Walker *et al*., 1982). Unlike the South American hemorrhagic arenaviruses, Lassa virus is only occasionally detected in the central nervous system (CNS), but clinical encephalitis is occasionally reported (Walker *et al*., 1982; Cummins *et al*., 1992; Gunther *et al*., 2001). Lesions are observed in the spleen, liver, adrenal, heart, lung and kidney (Table 1) (Walker *et al*., 1982). However, these lesions are typically not severe enough to attribute as the cause of death (Walker *et al*., 1982). The most consistent human lesion associated with Lassa is multifocal hepatocellular necrosis, although these lesions are usually not severe enough to induce hepatic failure (McCormick *et al*., 1986c; Walker *et al*., 1982). Additionally, analysis of liver function tests are not indicative of hepatic failure, although

modest elevations of AST (aspartate aminotransferase), particularly in combination with high viremia, are an indicator of poor prognosis (McCormick *et al*., 1987a; Johnson *et al*., 1987; McCormick *et al*., 1986a; McCormick *et al*., 1986c).

Coagulation tests, platelet turnover, and fibrinogen degradation products are not remarkably altered in either human or primate Lassa infection, indicating a lack of disseminated intravascular coagulation (Fisher-Hoch et al., 1988b; Fisher-Hoch et al., 1987). Only a mild thrombocytopenia is seen in Lassa infection, although platelet aggregation is greatly depressed in severe Lassa fever, and may be associated with hemorrhage (Fisher-Hoch et al., 1988b). Mild thrombocytopenia is accompanied by lymphopenia and neutrophilia during the course of Lassa fever (Fisher-Hoch et al., 1988b). An unknown component of serum from Lassa patients inhibits neutrophil and platelet activity *in vitro*.(Roberts *et al*., 1989)

Patients who succumb to Lassa fever usually die of shock, the cause of which is unknown but is not due to massive hemorrhage (McCormick *et al*., 1987a). As mentioned previously, significant pathological lesions are not seen, and the lack of cellular infiltrates suggests immunopathologic mechanisms are not involved (Walker *et al*., 1982). These findings have led many to suggest that the terminal shock is due to vascular dysfunction and collapse, possibly due to soluble mediators such as platelet activating factor, leukotrienes, or pro-inflammatory cytokines (Walker *et al*., 1987; Fisher-Hoch *et al*., 1987; Peters *et al*., 2002b). In humans, only cytokines have been measured to assess this theory, for which there is conflicting evidence. Fatal Lassa fever

was correlated with a suppression of serum IL-8, IP-10, IL-10, and IL-6 when compared to non-fatal Lassa fever (Mahanty *et al*., 2001). In contrast, an increase of serum TNF-α and IFN-γ was documented for one fatal case of Lassa fever (Schmitz *et al*., 2002). Further studies are needed to clarify the events of terminal shock in Lassa fever, and animal models provide a valuable tool for such studies.

## ANIMAL MODELS OF LASSA FEVER

Despite intensive efforts, a mouse model for Lassa fever has not been developed (Peters *et al*., 1987). The best characterized animal models for Lassa fever utilize non-human primates or guinea pigs. Comparison of Lassa infection of inbred guinea pigs and rhesus monkeys to human Lassa infection revealed similarities in 1) tropism, 2) humoral responses, 3) lack of immune cell infiltrates, and 4) pathological lesions (Jahrling *et al*., 1980; Jahrling *et al*., 1982; Callis *et al*., 1982). The rhesus model has been further characterized to reveal other similarities to human Lassa fever including lymphopenia, correlation between high viremia and AST levels with death, reduction of platelet aggregation, lack of disseminated intravascular coagulation, and death by terminal shock with the lack of overt hemorrhage (Fisher-Hoch *et al*., 1987). The use of Lassa virus in these two models is a drawback, since BSL-4 containment is required. Two other animal models for Lassa fever have been developed that use less pathogenic arenaviruses. A comparative model of Lassa fever was developed using intravenous (i.v.) or intragastric (i.g.) infection of LCMV WE strain in rhesus macaques. Intravenous infection results in

a severe disease similar to fatal Lassa fever, while intragastric inoculation results in an attenuated infection (Lukashevich *et al*., 2002; Peters *et al*., 1987). Another comparative model of Lassa fever utilizes Pichinde virus infection of guinea pigs. Pichinde virus is non-pathogenic to humans, but can be passage-adapted to produce severe or mild Lassa fever-like disease in guinea pigs (Jahrling *et al*., 1981; Aronson *et al*., 1994). Guinea pig and primate models have been very useful for studies of pathogenesis, immunity, and testing of antivirals and vaccines for Lassa fever (Lucia et al., 1989; Qian et al., 1994; Pushko et al., 2001; Morrison et al., 1989; Jahrling et al., 1983; Cosgriff et al., 1987; Aronson et al., 1995; Fisher-Hoch et al., 1987; McCormick et al., 1992; Jahrling et al., 1980).

## IMMUNOLOGY OF LASSA FEVER

Very little is known about the immunology of Lassa fever and the majority of the information comes from animal models. It is believed that humoral responses do not contribute greatly to control of infection. The evidence for this conclusion is: 1) the production of IgM and IgG do not correlate with clearance of virus; 2) antibody titers do not correlate with outcome; and 3) neutralizing antibodies do not appear until weeks after the virus is cleared, and even then titers are often very low (Jahrling *et al*., 1985; Johnson *et al*., 1987; McCormick *et al*., 1992). Passive immunization, however, can increase survival of Lassa infected cynomolgus monkeys and guinea pigs (Jahrling et al., 1985; Jahrling et al., 1984b; Jahrling et al., 1983). Efficacy of passive immunization is

dependent of the titer of neutralizing antibody in the serum (Jahrling et al., 1985; Jahrling et al., 1984b; Jahrling et al., 1983). Serum transfer from convalescent Z-158 (non-lethal Lassa strain) infected guinea pigs into Lassa Josiah inoculated guinea pigs was not protective until 55 days after Z-158 infection, when neutralizing antibody had developed (Peters *et al*., 1987).

Cell-mediated immunity appears to be important for clearance of Lassa virus, although studies have been very limited. Immune spleen cells from guinea pigs infected with a non-lethal Lassa strain (Z-158) exhibited a cytotoxic T lymphocyte response, and protected naïve guinea pigs from lethal challenge (Lassa Josiah strain) (Peters *et al*., 1987). A rhesus macaque intragastrically inoculated with LCMV-WE, survived a lethal challenge of intravenous LCMV-WE and had strong cytotoxic T lymphocyte (CTL) and lymphocyte proliferation responses to LCMV specific antigens (Rodas *et al*., 2004). Two other i.v. LCMV-WE challenged animals had low CTL responses, undetectable proliferation responses, and did not survive (Rodas *et al*., 2004). Recombinant virus vaccines containing Lassa proteins protect against lethal challenge in guinea pig and primate models, while a whole killed vaccine that ellicits only a humoral response is not protective (Fisher-Hoch *et al*., 2000; Pushko *et al*., 2001; Morrison *et al*., 1989; McCormick *et al*., 1992). Proliferation of CD4+ cells from Lassa antibody-positive individuals in response to recombinant Lassa nucleoprotein suggests a strong memory T-cell response (ter Meulen *et al*., 2000). Evidence that Lassa virus suppresses a cell-mediated response in severe disease is suggested by an inhibition of the lymphocyte

response to mitogen in Lassa infected primates (Fisher-Hoch *et al*., 1987). Together these results suggest cell-mediated immunity is more important for viral clearance than humoral immunity.

## LASSA VIRUS INNATE IMMUNE RESPONSES

IFN-α/β release is considered to be the prototypical innate, antiviral cytokine response, that inhibits the replication of many viruses (reviewed in (Guidotti *et al*., 2000a). In general, arenaviruses are not particularly sensitive to the effects of IFN-α/β, (Peters et al., 1989; Stephen et al., 1977a)(Marriott and Aronson, UTMB dissertation). Accordingly, Lassa virus titers are reduced only 1-2 logs in the presence of IFN-α/β during Vero or Huh-7 cell infection (Asper *et al*., 2004). *In vivo* characterization of IFN-α/β responses during Lassa infection have not been reported. Few other innate responses have been investigated for Lassa fever. Recent *in vitro* studies suggest Lassa virus does not induce the activation of macrophages and dendritic cells to produce cytokines or express cell surface molecules such as CD80, CD86, CD40, and HLA (Mahanty *et al*., 2003; Lukashevich *et al*., 1999; Baize *et al*., 2004). Other innate effector mechanisms such as the role of NK cells, NK T-cells, neutrophils, or complement have not been well-investigated in human Lassa fever.

## VACCINES FOR LASSA

The importance of cell-mediated immunity in Lassa virus clearance has mostly been elucidated through the investigation of vaccine efficacy. Although there is no commercially available vaccine for Lassa, many different experimental vaccines have been developed. A whole killed virus vaccine did not reduce viremia or provide any protection against Lassa challenge in non-human primates, although antibodies to the virus were detected (McCormick *et al*., 1992). In the search for an effective vaccine for Lassa, non-pathogenic arenaviruses were tested for their ability to protect against Lassa challenge. Mopeia, an Old World arenavirus that appears to be non-pathogenic in monkeys, protected rhesus monkeys from Lassa challenge, but the questionable safety of Mopeia in humans prevent its use (Fisher-Hoch *et al*., 1989). One of the first recombinant Lassa vaccines consisted of Lassa glycoproteins in a vaccinia vector, and was tested in both guinea pigs and rhesus monkeys (Fisher-Hoch *et al*., 1989; Morrison *et al*., 1989). Survival from Lassa challenge was 100% for all four monkeys vaccinated, and 79% of guinea pigs vaccinated compared to 0% and 14% of unvaccinated animals respectively (Morrison *et al*., 1989; Fisher-Hoch *et al*., 1989). Several combinations of vaccinia with GP1, GP2, GPC, or N (nucleoprotein) were tested. Further analysis of the vaccinia vaccines determined vaccine efficacy in rhesus monkeys as GPC+N>GPC>N>GP1 or GP2 (Fisher-Hoch *et al*., 2000). Replicon vaccines with a Venezuelan equine encephalitis vector and Lassa GPC protected guinea pigs 100% from a 160 $LD_{50}$ challenge of Lassa (Pushko *et al*., 2001). Recently, a recombinant replication-competent vaccine using Lassa virus GPC in a vesicular stomatitis virus vector (VSV-LAS) was

developed (Garbutt et al., 2004). The VSV-LAS vaccine protected 100% of cynomolgus macaques against morbidity and mortality when challenged with a high dose of Lassa (Geisbert et al., 2005). However, no vaccine to date provides complete sterilizing immunity; viremia is often detected in surviving animals from both primate and guinea pig studies (Fisher-Hoch et al., 2001; Pushko et al., 2001; Geisbert et al., 2005).

## PICHINDE MODEL OF LASSA FEVER

The studies described above establish the importance of animal models in arenavirus research. Development of a model for Lassa fever that could be studied outside of the BSL-4 environment was desired. A suitable model has been developed utilizing Pichinde (PIC), a New World arenavirus originally isolated from the rodent *Oryzomys albigularis* in Colombia (Trapido *et al*., 1971). In contrast to several of the arenaviruses, Pichinde is not known to be virulent to humans, although antibodies have been detected in lab workers (Buchmeier *et al*., 1974). A BSL-2 model of human Lassa fever utilizing Pichinde virus was originally developed in MHA (inbred) and LVG (outbred) hamsters. MHA hamsters were killed by 3,000-300,000 pfu (plaque forming units) of Pichinde, while LVG hamsters were resistant to disease (Buchmeier *et al*., 1977). A guinea pig model of Pichinde was also developed using guinea pig spleen passaged Pichinde. Virulence of Pichinde increased with serial spleen passage. Pichinde virus strain CoAn 4763 passed four or more times killed 100% of inbred strain 13 guinea pigs at a dose of 4.4 $\log_{10}$ pfu (Jahrling *et al*., 1981). Pichinde passaged 8 times (termed

"adapted Pichinde") had a $LD_{50}$ of 3 pfu (Jahrling *et al*., 1981) in strain 13 guinea pigs. Outbred guinea pigs were less susceptible to adapted Pichinde, with an $LD_{50}$ greater than 3000 pfu (Jahrling *et al*., 1981). Adapted Pichinde infection of inbred guinea pigs was further characterized. Clinical signs included fever, a dramatic decrease in food and water intake, profound weight loss, hyperventilation, ruffled fur, and excessive saliva production (Connolly *et al*., 1993). Death usually occurred within 2-3 weeks, with no overt signs of hemorrhage (Connolly *et al*., 1993).

A comparative Pichinde model was further developed for outbred Hartley guinea pigs, using high spleen-passaged virus (P18), and low spleen-passaged virus (P2) (Zhang *et al*., 2001; Zhang *et al*., 1999). P18 is highly lethal, inducing a severe disease with an $LD_{50}$ of 3 pfu (Zhang *et al*., 2001; Zhang *et al*., 1999). Guinea pigs infected intraperitoneally with P18 develop fever around day 3-4, and virus disseminates to the spleen by day 3 (Aronson *et al*., 1994; Zhang *et al*., 2001). Clinical signs of severe P18 infection are similar to adapted Pichinde infection of strain 13 guinea pigs, including fever, severe weight loss, rapid breathing, and ruffled fur (Connolly *et al*., 1993; Zhang *et al*., 1999). Death is attributed to shock and cardiovascular collapse beginning around day 12 (Qian *et al*., 1994; Aronson *et al*., 1994; Zhang *et al*., 2001; Katz *et al*., 1990). P2, on the other hand, is attenuated and induces a self-limited disease, with an $LD_{50}$ of >1000 pfu (Zhang *et al*., 2001; Zhang *et al*., 1999). Guinea pigs infected with P2 have a delayed onset of fever at approximately day 7-8 (compared to P18 infection), mild clinical disease and low organ virus titers (Zhang *et al*., 2001; Zhang *et al*., 1999). P2 induced fever

usually resolves by day 12 as the virus is cleared (Zhang *et al*., 2001). Through the use of reassortant viruses, the virulence of P18 has been mapped to the S-segment that encodes the GPC and nucleoprotein genes (Zhang *et al*., 2001).

### PICHINDE PATHOLOGY AND PATHOGENESIS: COMPARISON WITH LASSA FEVER

The pathology of adapted Pichinde infection in strain 13 guinea pigs is well-characterized. Infection of hamsters detailed the initial tropism for Pichinde as cells of the reticuloendothelium (Murphy *et al*., 1977). By immunohistochemistry, it was later confirmed that adapted Pichinde virus in guinea pigs is first detectable in splenic macrophages by day 5, followed by Kupffer cells and hepatocytes. Virus then disseminates to other tissues (Connolly *et al*., 1993). Virus is detectable by plaque assay, immunohistochemistry, and/or direct fluorescence assay in the plasma, spleen, liver, thymus, bone marrow, adrenal, pancreas, kidney, salivary gland, lung, and intestinal tract, revealing that tissue tropism and pathological lesions are very similar to human Lassa fever (Table 1) (Jahrling *et al*., 1981; Walker *et al*., 1982; Aronson *et al*., 1994; Connolly *et al*., 1993). The liver is a major site of viral replication in both Lassa and Pichinde infection (Jahrling *et al*., 1981; Connolly *et al*., 1993; Aronson *et al*., 1994; McCormick *et al*., 1986c). Elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) suggest hepatic and renal injury (Connolly *et al*., 1993). Hepatic steatosis and lesions have been reported, although these results are somewhat attributed to decreased food and water intake of sick guinea pigs,

rather than a direct virus effect (Connolly *et al*., 1993; Lucia *et al*., 1990). Immune cell infiltrates are not associated with Lassa fever or Pichinde infection, suggesting immunopathologic mechanisms are not responsible for tissue damage (Jahrling *et al*., 1981; Connolly *et al*., 1993; Walker *et al*., 1982). The non-cytopathic nature of Pichinde and Lassa, the lack of severe tissue pathology, and the lack of overt hemorrhage, do not provide for an obvious cause of death. Viremia has the most striking correlation with death in both Lassa fever and Pichinde (Aronson *et al*., 1994; Zhang *et al*., 2001; Johnson *et al*., 1987). The ability to control viral replication appears crucial for survival, but mechanisms for viral control and virus-induced terminal shock remain elusive.

TABLE 1 Fatal Lassa vs. Fatal Pichinde Pathology

| | Lassa (human) | Pichinde (guinea pig) |
|---|---|---|
| Liver | Focal hepatocellular necrosis | Focal hepatocellular necrosis, steatosis |
| Spleen | Necrosis of marginal zone | Necrosis of marginal zone |
| Lungs | Mild interstitial pneumonia | Mild interstitial pneumonia |
| Heart | Mild myocarditis | None |
| Adrenals | Necrosis, cytoplasmic inclusions | Necrosis, cytoplasmic inclusions |
| Kidney | Mild renal tubular injury | None |
| Brain | None | None |
| Pancreas | None | None |
| Intestine | Not described | Mild necrosis, villus atrophy, apoptosis |

Hemostatic processes were analyzed for evidence of disseminated intravascular coagulation (DIC) in PIC infected strain 13 guinea pigs. Fibrin-degradation products were elevated, but were not accompanied by an elevation in fibrinopeptide A, as would be expected for DIC (Chen *et al*., 1993). Additionally, fibrin thrombi were not found during necropsy of Pichinde infected guinea pigs (Cosgriff *et al*., 1987), therefore DIC is

not considered a hallmark of Pichinde infection. Terminal events in Pichinde infection include cardiovascular dysfunction characterized by reductions in cardiac output, heart rate, and blood pressure (Qian *et al*., 1994). Terminal pulmonary dysfunction is mainly limited to hyperventilation, but progresses to respiratory failure (Guo *et al*., 1991; Peters *et al*., 1989).

It is important to emphasize that Lassa and Pichinde are virtually non-cytopathic; extensive cellular destruction of target organs does not occur. This is in direct contrast to other viral hemorrhagic fever viruses like Rift Valley Fever, Ebola, and Marburg, which cause extensive cellular and organ damage (Peters *et al*., 1989; Connolly *et al*., 1999; Peters *et al*., 1999). Therefore, the physiologic dysfunctions associated with Lassa and Pichinde are likely not due to direct damage of parenchymal cells. There are several theories as to how Lassa infection may lead to terminal shock. One possibility is that Lassa virus inhibits the ability of infected cells to perform "luxury" actions required for normal host function, but without killing the cell. For example, LCMV has been demonstrated to inhibit growth hormone production from pituitary cells, resulting in retarded growth of some mouse strains (Oldstone *et al*., 1985). Therefore, alterations of the normal functions of heart or lung cells for example, during Lassa infection could result in cardiopulmonary failure. Another possibility is that viral infection induces the production of soluble mediators that can cause generalized vascular dysfunction. Leukotrienes were one of the first mediators investigated. Leukotrienes are lipid mediators produced by leukocytes, that have vasoactive, bronchoconstrictive, and pro-

inflammatory effects (Peters-Golden *et al*., 2004). By bioassay and radioimmunoassay, plasma leukotrienes ($LTE_4$) of infected guinea pigs reached peak concentrations at day 11 post-inoculation (PI) and disappeared by day 14 (Liu *et al*., 1986). An inhibitor of leukotrienes administered three times a day starting at day 3 PI, did not reduce mortality, but prolonged survival (Liu *et al*., 1986). Another lipid mediator, platelet activating factor (PAF), has been demonstrated to induce platelet and neutrophil aggregation, and can induce guinea pig cardiopulmonary dysfunction similar to that seen in PIC infection (Snyder *et al*., 1990; Qian *et al*., 1993). Qian *et al* reported that compared to control animals, Pichinde infected guinea pigs had elevated PAF in the blood, heart, and lungs by day 14 PI, but not in the liver and kidneys (Qian *et al*., 1992). PAF was also increased in the brain, along with dopamine and norepinephrine (Guo *et al*., 1993). The specificity of the above results for severe disease is unknown since comparisons with mild disease were not done. However, the results suggest that the cause of cardiopulmonary dysfunction and shock in severe Pichinde infection is complex. Limited studies have been done in a comparative model of Pichinde infection. In the P18 and P2 comparative model of severe vs. mild infection, complement activation does not appear to be responsible for terminal shock (A. Shurtleff, UTMB dissertation). Cytokines are additional soluble mediators that may contribute to terminal shock. Our lab has previously reported that severe Pichinde infection is associated with an increase in serum TNF in inbred, but not outbred, infected guinea pigs (Aronson *et al*., 1995). Animal studies on the pathophysiological effects of cytokines during terminal shock are very sparse. The results

reported in chapter 2 further characterize cytokine patterns during P18 and P2 infection of guinea pigs.

## PICHINDE IMMUNOLOGY

The immunological aspects of Pichinde infection in guinea pigs have not been well-studied because of the lack of commercial immunological reagents. In the original hamster model of Pichinde, susceptibility between adult MHA and LSH (inbred), and LVG (outbred), hamsters was characterized. By intraperitoneal infection, mortality was 80% for MHA hamsters with high viral titers, while mortality was 0% for LVG and LSH hamsters with low viral titers (Buchmeier *et al*., 1977). Susceptibility to Pichinde correlated with endogenous natural killer (NK) cell activity as well as increased infectious centers from NK cells (Gee et al., 1979). The authors suggested that NK cells might be a viral target for Pichinde, therefore, susceptibility was associated with greater numbers of available NK cells (Gee *et al*., 1979). Reduction of NK cells by anti-asialo GM1 serum in MHA hamsters reduced early splenic infectious centers, however, infectious centers were later increased and mortality was not reduced (Wright *et al*., 1987). These results suggest that cells with NK activity may be initial target cells of Pichinde virus, but are not ultimately important for the outcome of disease.

Further characterization of Pichinde responses in the hamster model revealed that compared to resistant LSH hamsters, susceptible MHA hamsters did not develop a delayed-type hypersensitivity response (swelling) at the site of footpad inoculation of

Pichinde, despite similar viral titers and antibody responses (Chan *et al*., 1983). In contrast, footpad swelling occurred in both hamster strains in response to vesicular stomatitis virus and vaccinia virus (Chan *et al*., 1983). MHA hamsters pre-treated with cyclophosphamide, a chemical known to inhibit suppressor cell populations, had footpad swelling similar to LSH hamsters (Chan *et al*., 1983). Furthermore, LSH footpad swelling was reduced in response to adoptive transfer of MHA splenic immune cells (Chan *et al*., 1983). These results suggest the suppression of footpad swelling by Pichinde was a result of a suppressor cell population. The only immunology data available for the guinea pig model is the observation that virulent Pichinde virus infection suppresses T-cell proliferatation responses to mitogen stimulation (Aronson, T. Jerrells, unpublished data). These limited data suggest that severe Pichinde infection suppresses cell-mediated immunity. Innate immunity has not been extensively studied in experimental models of Lassa fever, but is important for understanding potential mechanisms of host susceptibility or resistance.

## CYTOKINES AND ANTIVIRAL IMMUNITY

Cytokines that are elaborated during innate immune responses may exhibit indirect and direct antiviral effects. Chemokines contribute indirectly by recruiting immune cells. Pro-inflammatory cytokines activate immune cells to express MHC molecules, co-stimulatory molecules, and adhesion molecules, all of which contribute to virus-specific cell-mediated immunity. Another mechanism by which cytokines indirectly

control the spread of a viral infection is by inducing cytotoxic T-cell killing or apoptosis of infected cells (Beadling *et al*., 2005; Barber *et al*., 2001). In addition to indirect effects, some cytokines have been shown to have direct effects on viral replication. The best described anti-viral effects belong to the pro-inflammatory cytokines such as TNF-α, IFN's (α, β, and γ), IL-1β, and IL-12, among others. TNF-α and IFN-γ have well-known direct anti-viral effects (reviewed in (Guidotti *et al*., 2000a)). Guidotti *et al* have termed the inhibition of viral replication by cytokines "noncytopathic clearance" in the mouse hepatitis B model (Guidotti *et al*., 1994; Cavanaugh *et al*., 1997; Guidotti *et al*., 1999). Guidotti and Chisari have demonstrated that hepatitis B, and the arenavirus lymphocytic choriomeningitis, are cleared from infected hepatic macrophages through an IFN-γ and TNF-α dependent mechanism (Guidotti *et al*., 1994; Cavanaugh *et al*., 1997; Guidotti *et al*., 1999). TNF-α can inhibit viral replication of Sin Nombre virus, influenza, and vaccinia virus (Khaiboullina *et al*., 2000; Seo *et al*., 2002; Sambhi *et al*., 1991). Mechanisms of TNF-α antiviral activities are not well understood. It has been reported that the antiviral effects of TNF-α on respiratory syncytial virus are dependent on NF-κB activation, and independent of IFN-β (Bose *et al*., 2003). IFN-γ also inhibits replication of viruses such as measles, HIV, Sindbis, hepatitis C, ectromelia, and varicella zoster (Ryman *et al*., 2002; Patterson *et al*., 2002; Dhawan *et al*., 1994; Frese *et al*., 2002; Presti *et al*., 2001; Desloges *et al*., 2005; Karupiah *et al*., 1993). There are the three well-known IFN-α/β induced intracellular anti-viral pathways: Mx proteins, 2'5'-oligoadenylate synthetases, and RNA-activated protein kinase (PKR) (reviewed in

(Samuel *et al*., 2001). It is interesting to note that IFN-γ antiviral effects are usually independent of these three pathways (Presti *et al*., 2001; Desloges *et al*., 2005; Ryman *et al*., 2002). Alternative IFN-γ antiviral pathways are poorly understood, but the induction of nitric oxide (NO) is sometimes implicated (Karupiah *et al*., 1993; Guidotti *et al*., 2000b). Nitric oxide itself has antiviral properties (Croen *et al*., 1993; Karupiah *et al*., 1993; Sanders *et al*., 1998), and is responsible for at least a portion of the antiviral effects of IL-12, as well as IFN-γ. IL-12 has been demonstrated to inhibit viral replication of vesicular stomatis virus *in vitro* (Komatsu *et al*., 1999), and *in vivo* attenuated vaccinia, coxsackievirus, and hepatitis B infection of mice (Potvin *et al*., 2003; Cavanaugh *et al*., 1997; Gherardi *et al*., 1999). The antiviral effect of IL-12 is usually attributed to either the induction of IFN-γ or NO (Gherardi *et al*., 1999; Komatsu *et al*., 1998; Potvin *et al*., 2003; Cavanaugh *et al*., 1997). IL-1β is similar to IL-12, in that its antiviral functions are attributed to induction of other antiviral cytokines, particularly IFN-α/β (Van Damme *et al*., 1987; Van Damme *et al*., 1988). The direct antiviral effects of pro-inflammatory cytokines are a significant contribution to host defense.

In comparison to the pro-inflammatory cytokines, some chemokines have been demonstrated to increase viral replication. IL-8 (CXCL8) is principally a neutrophil chemoattractant and activator, but has been demonstrated to increase viral replication of cytomegalovirus, encephalomyocarditis virus, HIV, and poliovirus (Murayama *et al*., 1994; Khabar *et al*., 1997b; Lane *et al*., 2001a). Khabar *et al*. suggest that IL-8 increases

viral replication by suppressing IFN-α antiviral activity (Khabar *et al*., 1997a). GRO-α (CXCL1) is also a neutrophil chemoattractant, and increases HIV-1 replication in macrophages through an unknown mechanism (Lane *et al*., 2001b). MCP-1 (CCL2), is a monocyte chemoattractant protein that has recently been demonstrated to increase HIV-1 replication in macrophages at a late stage in the HIV-1 life cycle (Vicenzi *et al*., 2000; Fantuzzi *et al*., 2003). The function of chemokines in viral infections may be contradictory; controlling viral replication by recruiting immune cells, or enhancing viral replication.

Anti-inflammatory cytokines such as IL-10 and TGF-β sometimes contribute to increasing viral replication, mainly by down-regulating pro-inflammatory cytokines (Zielinska-Jenczylik *et al*., 1991; Benveniste *et al*., 1994; Marques *et al*., 2004). Such a mechanism has been described for respiratory syncytial virus infection of alveolar macrophages, in which pro-inflammatory cytokines were not upregulated, but IL-10 production was markedly increased (Panuska *et al*., 1995). Antibodies to IL-10 neutralized the RSV suppression of LPS-induced pro-inflammatory cytokines (Panuska *et al*., 1995). A balance of anti-inflammatory cytokines is needed to counter unchecked pro-inflammatory cytokine production, but without completely abrogating the protective pro-inflammatory response.

## PATHOGENIC EFFECTS OF CYTOKINES IN VIRAL HEMORRHAGIC FEVERS

Cytokines can be protective in viral infections, but can also contribute to pathology. Increased serum IL-8 and TNF-α are associated with dengue hemorrhagic fever when compared to less severe dengue infection (Raghupathy et al., 1998; Hober et al., 1993). Fatal human Ebola virus infection is also associated with increased IFN-γ, IFN-α, IL-2, IL-10, and TNF-α when compared to non-fatal Ebola virus infection (Villinger et al., 1999). The non-cytopathic nature of arenaviruses, and the lack of immunopathological mechanisms, has led many to speculate that the terminal events of Lassa fever are due to vascular dysfunction and permeability. Clinical signs of vascular dysfunction and leakiness in Lassa fever include facial edema, pulmonary edema, and hypovolemic shock (McCormick *et al*., 2002). Endothelial dysfunction during Lassa infection is indicated by reduced prostacyclin production in a primate model (Fisher-Hoch *et al*., 1987). Although endothelial cells can be infected by Lassa *in vitro* and *in vivo*, direct cellular damage is not observed (Shieh *et al*., 1997; Lukashevich *et al*., 1999). Vascular dysfunction may therefore be mediated by soluble factors such as pro-inflammatory cytokines. Cytokines implicated in vascular dysfunction and shock are produced by macrophages, important targets of arenaviruses (Murphy et al., 1977; Connolly et al., 1993). It is conceivable that the activation of macrophages during infection can result in large amounts of cytokines that can contribute to pathology. Figure 1 illustrates how cytokines may contribute to arenavirus pathogenesis and pathophysiology.

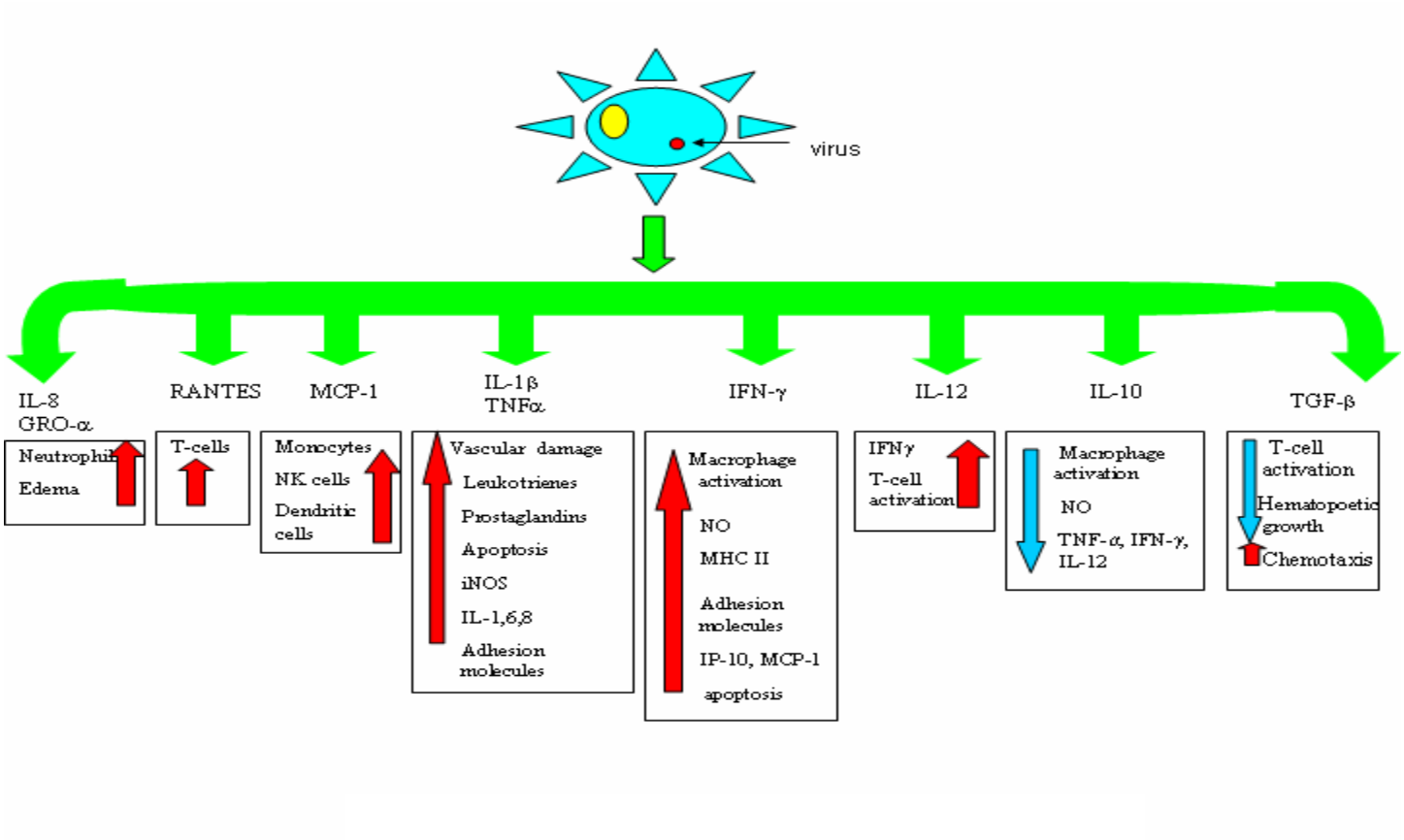


*Figure 1. Cytokine effects in relation to arenavirus pathology.*

Endothelium and the vascular wall are major targets of pro-inflammatory cytokines. Figure 2 shows just a few of the many potential effects of soluble macrophage mediators on endothelium, including permeability changes and upregulation of adhesion molecules. Cytokine-activated endothelial cells can also produce other soluble mediators like platelet activating factor and leukotrienes, both of which are elevated in the serum of Pichinde infected guinea pigs (Liu *et al*., 1986; Qian *et al*., 1992). The mechanisms by which Lassa induces shock are unclear, however, some hypotheses have been proposed based on studies of septic shock, for which there is abundant evidence that pro-inflammatory cytokines play a key role. TNF-α, IL-1α and β, and IL-6 have been implicated as the major inducers of septic shock (Butler *et al*., 1989; Hack *et al*., 1989; Tracey *et al*., 1987). TNFα and IL-1α also increase vascular permeability, one of the events leading to shock (Royall *et al*., 1989; Worrall *et al*., 1997). Other cytokines such as IL-10, TGF-β, IFN-γ, and IL-12 may play a role in shock by modulating or synergizing with TNF-α and IL-1 (van der Poll *et al*., 1999; Heinzel *et al*., 1994; Wysocka *et al*., 1995).

## LASSA AND CYTOKINES

Several studies suggest that Lassa virus inhibits cytokine production. The majority of cytokine data for Lassa is from *in vitro* studies of macrophages and dendritic cells. In a study done by Lukashevich *et al*., human macrophages and endothelial cells (HUVECS) infected with Lassa did not induce TNF-α and suppressed IL-8 compared to

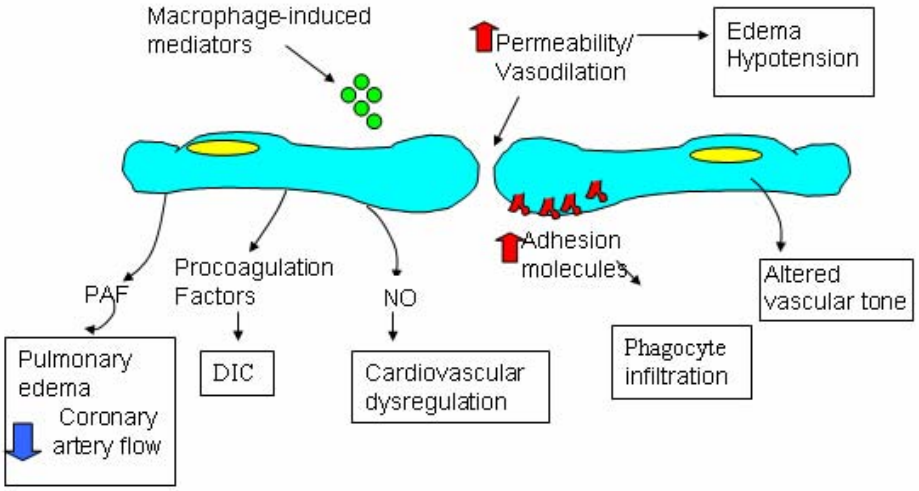


*Figure 2. Effect of macrophage-derived mediators on endothelium.*

mock infected cells (Lukashevich *et al*., 1999). Lassa also suppressed LPS-induced production of TNF-α and IL-8 by macrophages (Lukashevich *et al*., 1999). An additional *in vitro* study confirmed that Lassa virus did not activate human macrophages to induce cytokine mRNA, and actually suppressed IL-1β mRNA levels after LPS stimulation (Baize *et al*., 2004). Dendritic cells likewise are not activated to produce pro-inflammatory cytokines during Lassa infection, but an induction of IL-8, MIP-1α and

MIP-1β was reported (Mahanty *et al*., 2003; Baize *et al*., 2004). In contrast to Lassa virus, Mopeia, a non-pathogenic Old World arenavirus, can induce cytokine expression from macrophages. Mopeia infected macrophages had similar TNF-α and IL-8 expression compared to Lassa infected macrophages, but Mopeia induced expression of IL-8 in HUVEC's (Lukashevich *et al*., 1999). In another study, Mopeia infection of human macrophages, but not dendritic cells, up-regulated IFN-β, TNF-α, and IL-12p35 mRNA levels (Pannetier *et al*., 2004). From these *in vitro* reports, it appears that the pathogenicity of Lassa is associated with a suppression of early cytokine responses that may potentially inhibit viral replication.

High levels of pro-inflammatory cytokines have been suggested to induce terminal shock and vascular dysfunction in Lassa fever. However, the data on this subject has been contradictory, with both elevation and suppression of pro-inflammatory cytokines described. In two imported cases of fatal human Lassa fever, one patient had elevated IFN-γ and TNF-α within 2-days of death compared to earlier timepoints, while another patient had elevated IL-10 only (Schmitz *et al*., 2002). A primate LCMV infection model revealed animals with severe disease had elevated serum IFN-γ and IL-6, but undetectable TNF-α and IL-1β (Lukashevich *et al*., 2003). In addition, IL-1RA (receptor antagonist) protected mice from death after an intracerebral inoculation of Lassa, suggesting that IL-1 contributes to death in this model (Ignatyev *et al*., 2000).

In contrast, human studies revealed that patients with fatal Lassa infections had significantly lower serum levels of IL-8, IL-6, IL-10, and the chemokine IP-10 when compared to non-fatal Lassa fever (Mahanty *et al*., 2001). In addition, IFN-γ was undetectable in all fatal Lassa cases, and all but one fatal Lassa fever patient had undetectable levels of IL-1β and IL-12 compared to elevated levels in nonfatal Lassa and non-Lassa patients.(Mahanty *et al*., 2001) TNF-α was low to undetectable for both fatal and non-fatal Lassa fever (Mahanty *et al*., 2001). The apparent contradictions observed, especially in the human data, may be due to comparison of cytokines at different stages of infection, since blood samples were not continuously collected. Discrepancies in the literature may be further clarified by a complete timecourse animal experiment. In chapter 3, we report cytokine mRNA levels in the peripheral blood lymphocytes and tissues throughout the course of Pichinde virus infection of guinea pigs, an experimental model of Lassa fever.

## PICHINDE AND CYTOKINES

Very little has been documented on the role of cytokines in Pichinde. *In vitro*, infection of either primary guinea pigs macrophages or JH4 cells (a guinea pig fibroblast cell line) with P18 or P2 did not induce detectable IFN-α/β (K. Marriott, UTMB dissertation). Addition of recombinant murine IFN-α/β reduced JH4 titers of both virus variants by 1-2 logs, with no difference in the sensitivities of the two PIC variants (K. Marriott, UTMB dissertation). Limited studies of Pichinde in P388D1 cells (murine

macrophage cell line) indicated that both P2 and P18 induced small amounts of TNF-α and IL-6 protein, but levels were not significantly different (Fennewald *et al*., 2002). P18 infection did, however, suppress LPS-induced production of both TNF-α and IL-6 (Fennewald *et al*., 2002). In Chapter 2, we report the cytokine responses of primary guinea pig macrophages to Pichinde infection. To determine direct antiviral effects of certain cytokines on *in vitro* Pichinde infection, we measured viral replication after treatment with exogenous human cytokines. The results of these experiments are also reported in Chapter 2.

Cytokine data during guinea pig infection of Pichinde has been limited to IFN-α/β and TNF-α. Serum levels of IFN-α/β were not different between P18 and P2 infected animals (Marriott, K.A., UTMB dissertation), and treatment of guinea pigs with recombinant human IFN-α did not reduce mortality of high-passage Pichinde infection (Lucia *et al*., 1989). These results suggest that, similar to other arenaviruses, Pichinde virus is not particularly sensitive to the effects of IFN-α/β (Stephen et al., 1977a; Asper et al., 2004). In regards to cytokines in terminal shock, elevated levels of serum TNF have been reported late in severe Pichinde infection for inbred strain 13 guinea pigs, but were undetectable in outbred guinea pigs (Aronson *et al*., 1995). Because of the lack of data, few conclusions can be made for the role of cytokines during *in vivo* Pichinde infection. In chapter 3, we present cytokine expression patterns induced by P2 and P18 in guinea pigs over the course of infection.

## AP-1 REGULATION OF CYTOKINES

The regulation of cytokines is complex, but is often under the control of transcription factors such as NF-κB and AP-1. AP-1 is not an individual transcription factor, but is the name for the DNA binding activity of the sequence 5' TGAGTCA 3' (Wisdom *et al*., 1999). The AP-1 family of transcription factors is comprised of heterodimers and homodimers of Fos, Jun, and activating transcription factor (ATF) proteins that bind the AP-1 sequence. Some of the dimers are transcriptionally active such as c-Jun/c-Fos, but others containing Fra2 and JunB can be repressive (Suzuki *et al*., 1991; Rutberg *et al*., 1997). In response to certain stimuli such as growth factors, UV irradiation, cytokines, and viruses, cellular kinases interact with receptors that initiate a signaling cascade. The cascade initiates kinase-mediated phosphorylation of AP-1 proteins, which then bind to AP-1 sequences in the promoters of cytokines (Koj *et al*., 1996). Many cytokines including TNF-α, IL-1β, IL-8, IL-12p40, MCP-1, and RANTES have AP-1 binding sites in their promoters (Hiura TS. *et al*., 1999) (Martin *et al*., 1997; Ma *et al*., 1996; Roebuck *et al*., 1999; Tsukada *et al*., 1994; Angel *et al*., 1991). Thus, alteration of AP-1 functions could alter the transcription of several cytokines regulated by AP-1. In chapter 4 we report the ability of an AP-1 thioaptamer decoy to alter cytokine mRNA levels *in vitro* and *in vivo* and decrease mortality of P18 infected guinea pigs.

## RATIONALE FOR STUDIES

Cytokines are important to antiviral defense because of their direct antiviral properties, and their regulation of virus-specific cell-mediated immunity. Despite the importance of cytokines in viral infections, studies of cytokine responses and their activities during Pichinde infection are very limited. **In this study, we addressed the hypothesis that (1) PIC infection alters cytokine gene expression in target macrophages, (2) representative patterns of cytokine expression are associated with P2 vs. P18 infection of primary macrophages and (3) patterns of cytokine expression correlate with PIC virulence, viral replication, and viral inactivation.**

Experiments in chapter 2 were designed to investigate the hypothesis that attenuated P2 virus would up-regulate potential antiviral cytokines during infection of primary guinea pig macrophages, and virulent P18 virus would suppress expression of potential antiviral cytokines. Experiments in chapter 2 also investigated the effects of select cytokines on P2 and P18 viral replication. This study is the first report of cytokine responses to PIC infection in primary macrophages.

The experiments performed in chapter 3 were designed to meet two goals. The first goal was to identify a pattern of early cytokine expression at the initial site of infection that could explain the differential replication of P2 and P18 viruses. The second goal was to clarify the pattern of cytokines, particularly pro-inflammatory cytokines, that are associated with terminal shock in arenavirus hemorrhagic fever. This study is the first time cytokines have been systematically described in an animal model of arenavirus hemorrhagic fever.

In chapter 4, experiments were performed to evaluate the efficacy and mechanism of a potential treatment for arenavirus hemorrhagic fever. We hypothesized that an AP-1 thioaptamer would boost cytokine production and increase survival of guinea pigs infected with P18 virus. These experiments are the first time alteration of transcription factor proteins have been used for treatment of arenavirus hemorrhagic fevers.

# CHAPTER 2: CYTOKINE RESPONSES OF PRIMARY MACROPHAGES TO PICHINDE VIRUS *IN VITRO*

## INTRODUCTION

As mentioned in the rationale for studies, the main goal of experiments described in chapter 2 is to characterize the cytokine response of primary guinea pig macrophages to P2 and P18 infection. *In vitro* studies using macrophages are especially relevant since the primary infection of Pichinde (PIC) occurs in the macrophage, which has important implications for the innate immune response (Murphy *et al*., 1977). Direct infection of macrophages can inhibit the functions involved in both innate and adaptive immunity, allowing the virus to replicate uncontrolled. On the other hand, infection of macrophages can activate them to produce antiviral mediators like cytokines and nitric oxide, and to up-regulate antigen presentation of viral peptides, all of which may eventually lead to viral clearance.

One of the innate immune functions of macrophages is the production of cytokines. Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-12 can contribute to viral control by the activation and regulation of adaptive immune responses. Pro-inflammatory cytokines can also contribute to viral clearance through direct antiviral mechanisms. IFN-α/β, for example, has well-known antiviral effects. However arenaviruses typically have poor sensitivity to IFN-α/β (Lucia et al., 1989; Stephen et al., 1977a; Moskophidis et al., 1994). Previous studies in our lab indicate that P2 and P18 do

not differ in their abilities to induce IFN-α/β, and are not differentially sensitive to exogenous IFN-α/β (K. Marriott, UTMB dissertation). We were therefore interested in examining the potential role of other cytokines, particularly cytokines involved in innate immunity, in PIC pathogenesis. TNF-α, in particular, has been demonstrated to inhibit viral replication of Sin Nombre virus, influenza, vaccinia, and hepatitis B (Khaiboullina *et al*., 2000; Seo *et al*., 2002; Sambhi *et al*., 1991; Gilles *et al*., 1992). Other pro-inflammatory cytokines like IL-12 and IL-1β also have antiviral effects, often through the induction of IFN-γ or nitric oxide (Potvin *et al*., 2003; Van Damme *et al*., 1987; Cavanaugh *et al*., 1997; Komatsu *et al*., 1999). Pro-inflammatory cytokines are not the only cytokines that contribute to antiviral defense. Chemokines including MCP-1, RANTES, GRO-α, and IL-8 are important for recruiting monocytes, T cells, and neutrophils to the site of viral infection. On the other hand, chemokines can enhance replication of some viruses such as HIV, cytomegalovirus, and poliovirus (Murayama *et al*., 1994; Lane *et al*., 2001b; Lane *et al*., 2001a; Khabar *et al*., 1997b; Fantuzzi *et al*., 2003). In contrast to chemokines and pro-inflammatory cytokines, the anti-inflammatory cytokines IL-10 and TGF-β do not typically have direct effects on viral replication, but their induction may contribute to viral pathogenesis by inhibiting the antiviral response (Zielinska-Jenczylik *et al*., 1991; Panuska *et al*., 1995). Thus, macrophage-derived cytokines can have contrasting effects on viral replication, through both indirect and direct mechanisms. Although macrophage-derived cytokine responses have been characterized in many other viral systems, cytokine responses of primary macrophages to

PIC have not been previously reported. We therefore wanted to investigate the macrophage cytokine response to attenuated and virulent PIC. We hypothesized that P2 and P18 virus would induce differential macrophage responses that would alter viral replication.

## MATERIALS AND METHODS

### *Virus*

The Munchique strain CoAn 4763 of Pichinde virus was spleen-passaged twice in strain 13 guinea pigs to derive the P2 variant, and 18 times to derive the P18 variant (Zhang *et al*., 1999). Virus stocks used were prepared in P388D1 cells (murine macrophage-like cell line). Mock controls used were uninfected P388D1 supernatants prepared simultaneously with P2 and P18 stocks. P388D1 cell culture stock were used instead of spleen stocks because of the necessity of high titers for *in vitro* experiments. P388D1-prepared P2 viral stocks were $6.6x10^6$ pfu/ml, and P18 viral stocks were $4.2x10^7$ pfu/ml, compared to spleen stocks of $1.2x10^5$pfu/ml and $1.2x10^6$pfu/ml respectively. For some experiments, polyethyleneglycol (PEG) purified viral stocks were used. PEG stocks were prepared by first clarifying virus P388D1 cell supernatants by centrifugation at 3000xg for 10 minutes at 4$^o$C. Clarified supernatants were adjusted to final concentrations of 0.4M NaCl and 6% w/v PEG followed by precipitation for 2 hours at 4$^o$C. The precipitate was then pelleted by centrifugation at 10,000xg for 20 minutes, and the pellet resuspended in buffer containing 20mM Hepes pH 7.8, 200mM NaCl, and 2mM EDTA. The suspension was sonicated for 1 minute in a water bath, then

centrifuged at 3000xg for 10 minutes to remove particulate material (Young *et al*., 1983; Gschwender *et al*., 1975). Supernatants were aliquoted and stored at -80$^{o}$C until use. Titers of PEG viral stocks were determined by plaque assay described below.

## *Cells and Reagents*

Primary guinea pig peritoneal macrophages were collected from healthy male Hartley outbred guinea pigs obtained from Charles River. Following humane sacrifice by carbon dioxide asphyxia, guinea pig peritoneal macrophages were aseptically harvested by lavage in ice-cold phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (dPBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were centrifuged at 3,000xg for 10 minutes at 4$^{o}$C, and the pellet resuspended in RPMI-1640 media (Gibco) containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin. Macrophages were adherence purified by culture in 75cm$^2$ flasks at approximately 1x10$^6$ cells/ml overnight. Vero cells for the viral plaque assay, and L929 cells for the TNF bioassay, were cultured in minimal essential media (MEM) containing 5% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Vero cells less than passage 40 were used for plaque assay.

Bone marrow derived macrophages were obtained as described in Appendix A. Briefly, bone marrow was flushed from femurs of outbred guinea pigs and the red blood cells lysed. The remaining cells were incubated with 2mg/ml of soybean agglutinin to eliminate mature cells and enrich for hematopoeitic precursors. Non-agglutinated cells were cultured in tissure culture dishes with media containing 20% v/v supernatant from

the murine fibroblast L929 cell line as a source of macrophage-colony stimulating factor. After 5-6 days in culture, adherent bone marrow-derived macrophages (BMDM) were harvested and used for infection experiments.

For exogenous cytokine addition experiments, mineral oil elicited peritoneal exudate cells were used. 10 ml of sterile mineral oil was injected into the peritoneal cavity of an anesthetized Hartley guinea pig. Four days later, the peritoneal exudate cells were collected as described above, with the following modifications. After centrifugation at 3,000xg, the cell pellet was washed with PBS and centrifuged again. The cell pellet was resuspended in approximately 10mls of complete RPMI-1640 media (see above) to a concentration of $4x10^6$ macrophages/ml and added to tissue culture plates for the described exogenous cytokine addition experiments. The percentage of macrophages in the lavage was determined by DifQuik (Fisher Scientific) staining of a cytospin preparation.

Human recombinant cytokines IL-8, TNF-α, TGF-β, IL-1β, and GRO–α were obtained from R&D Systems (Minneapolis, MN). Recombinant human IL-8 and GRO-α were chosen since mice do not produce IL-8 or GRO. Guinea pig TNF-α, TGF-β, and IL-1b had either similar, or better, amino acid homology with human cytokines than murine cytokines, therefore, recombinant human cytokines were used (Scarozza et al., 1998; Yoshimura et al., 1999).

### *Macrophage infection by PIC variants*

Adherence purified guinea pig macrophages or bone marrow derived macrophages were collected by scraping in dPBS. Purified macrophages from multiple guinea pigs were pooled together, centrifuged, and the cell pellet suspended in approximately 500-1000 μl of dPBS. The cells were counted and divided into three separate microfuge tubes. Macrophages were infected in suspension by adding viral inoculum of P2 or P18 diluted in PBS + 1% v/v FBS, to one microfuge tube of cells each at an MOI=1. An equivalent volume of mock P388 cell supernatant, diluted in the same ratio as the P2 inoculum, was added to the third tube. Cell/virus suspensions were incubated at 37$^{o}$C for 40 minutes. $2.5x10^{5}$ cells of the cell/virus suspension were then added to individual wells of a 24 well plate in 500 μl of complete RPMI-1640 media. Final volume was approximately 650 μl/well. For experiments involving LPS stimulation, 100 μl of a 1ng/ml LPS *E. coli* 011:B4 solution (diluted in complete RPMI-1640 media), or media alone, was added two hours after infection to the appropriate wells for a total volume of approximately 750 μl. The final LPS concentration was 0.1 ng/ml LPS. At indicated timepoints after infection, supernatants and total cellular RNA were harvested and assayed for cytokines (by real-time RT-PCR, bioassay, and ELISA) and viral titers. RNA was collected from macrophage cell pellets using the Ambion RNA Aquaeous kit (Austin, TX) per manufacturer's instructions. RNA was eluted in 50ul of elution solution and treated with Ambion's Turbo DNA-free DNase treatment (Austin,

TX) per manufacturer's instructions. Supernatants and DNase-treated RNA were stored at -80$^{o}$C until use.

## *Addition of exogenous cytokines during macrophage infection*

In 24 well plates, 100 µl of peritoneal exudate macrophages were plated immediately after collection (without overnight adherence purification) for a total of $4x10^5$ macrophages/well. Cells were allowed to adhere for four hours, then the medium was removed and replaced with medium containing 0, 1, or 100 ng/ml of recombinant cytokines. Because guinea pig cytokines were not commercially available, human recombinant cytokines were used. Cells were incubated overnight, then the media was removed and the cells were infected by adding 40µl of P2 virus or 60µl of P18 virus (diluted in PBS with $Ca^{2+}$ and $Mg^{2+}$ containing 1% v/v FBS) directly in the well at an MOI=1 ($4x10^5$ pfu/well). For mock-infected cells, 40µl of uninfected P388D1 cell supernatant was applied. After a 40 minute incubation at 37$^{o}$C, 500 µl of media containing 0, 1, or 100 ng/ml of recombinant human cytokine was again added to the appropriate wells. Supernatants were collected 48 hours later and viral titers determined by plaque assay.

## *Real Time RT-PCR*

A two-step RT-PCR approach was used, with reverse transcription (RT) followed by real time PCR. First, DNase-treated RNA (unknown concentration), 8.3 nM random decamer primers (Ambion, Austin, TX), and nuclease-free water were heated for three minutes at 85$^{o}$ in a PCR thermocycler (Twin Block system, Easy Cycler Series, Ericomp,

San Diego, CA). A mix of dNTP's (0.4mM each per reaction, Fischer Sci.Fairlawn, NJ), enzyme buffer, M-MLV reverse transcriptase (100 U, Ambion, Austin, TX), and RNAse inhibitor (30 U, Amersham Biosciences, Piscataway, NJ) was added to each reaction for a total RT reaction volume of 20µl. Reverse transcription was performed in a PCR thermocycler (Twin Block system, Easy Cycler Series, Ericomp, San Diego, CA) for one hour at 42$^{o}$C followed by 10 minutes at 95$^{o}$C to inactivate the RT enzyme. 2µl of resulting cDNA was analyzed by real time PCR using the iQ Sybrgreen PCR mix (Bio Rad, Hercules, CA), nuclease-free water, and 0.4nM cytokine specific primers (0.2nM for GAPDH) per 25µl reaction. Primers were designed based on published sequences (Table 2)(Yoshimura *et al*., 1999; Scarozza *et al*., 1998; White *et al*., 1997; Yoshimura *et al*., 1993b; Jeevan *et al*., 2003; Yoshimura *et al*., 1993a; Shiratori *et al*., 2001) using Oligo primer analysis software (Wojciech Rychlik). Real time PCR was performed with

| | Forward 5' to 3' | Reverse 5' to 3' | Product size |
|---|---|---|---|
| IL-1β | CAGGCGTTTATGCTGTATGTC | TGAAGAAGAGCCCATCGT | 106 |
| IL-8 | TAGGGTGGCAGATTTAACTCA | TCAGGAATTGGCTTGCTAC | 121 |
| IL-10 | CCAAGGCACGAACACCCAG | TGTTTAACAGCACGTTGTCCAG | 166 |
| IL-12p40 | GCTGCCCTTGACACTGAACTT | TCCAATGGCAAGACCTTTCTGAA | 111 |
| TNF-α | CGGAATCGGCAAAGTCAAGGTA | AGCGCAAGCCCTGGTATGAA | 114 |
| TGF-β | CTGAAGCGAAAGCCCTCTAAT | CTGACCCCCAGTGATACC | 101 |
| GRO | GGGAGCCTCCGGGTCT | ACATCCAGAGCGTAGCGGTGA | 107 |
| RANTES | CGGTTCTTTCGGGTAACA | ACTCCTTGCTGCTTTGCCTAC | 119 |
| IFN-γ | CGTCATTGACCGAAATTTGAATCA | ATAGCATGAACACCATCAAGGAAC | 108 |
| MCP-1 | CCAGTTTGGCAATGTAGTCCT | CGGATGGAGTTAATACCC | 192 |
| GAPDH | ATCTCATCGTATTTGGCCGGT | AATGGGAAGCTCACAGGTATGG | 100 |
| GPC | TTGCCATCACAGTGTTATCA | CTTGGGACTTGAGTGACTCTA | 111 |

Table 2. Primer sequences of Pichinde GPC and guinea pig cytokines used for real time RT-PCR.

the Smart Cycler machine (Cepheid, Sunnyvale, CA). Cycling conditions included an initial 3 minute 95$^{o}$C denaturation, followed by 45 cycles of 95$^{o}$C 30 sec denaturation, 30 seconds annealing, 72$^{o}$C 30 seconds extension. A disassociation analysis was performed beginning at 60$^{o}$C to 95$^{o}$C at 0.2$^{o}$/sec to determine the melting temperature of the PCR product. A final fluorescence read step was performed at a temperature between the melting temperatures of the specific amplicon and that of the primer dimer or other non-specific products to ensure the fluorescence recorded was only due to Sybrgreen binding of the specific product. Primers for real time PCR were optimized on serial dilutions of plasmids containing an insert with the cytokine sequence of interest. Least-squares linear regression analysis of input DNA molecule number vs. cycle threshold had values of $r^2$>0.9. Resulting products were analyzed for correct product size by agarose gel electrophoresis.

RNA quantification was done by a relative approach rather than an absolute determination. Cycle threshold ($C_t$) values were normalized to GAPDH mRNA levels controlled for the amount of input RNA for each sample and resulted in a $\Delta C_t$ value. The formula for $\Delta C_t = C_t$ target - $C_t$ GAPDH. (Fold change vs. mock can be determined by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = (\Delta C_{t\ virus} - \Delta C_{t\ mock})$) (Livak *et al*., 2001). Samples which did not generate a $C_t$ value were given a default value of 45 (Kipar *et al*., 2001). $\Delta C_t$ values are inversely correlated with input RNA, therefore the axes of the graphs have been inverted to display comparative differences between the groups. Real time RT-PCR performed on serial dilutions of guinea pig spleen total RNA was used to determine the validity of the

$\Delta C_t$ method over various RNA concentrations. When $\Delta C_t$ was plotted vs input RNA concentration the resulting slope was usually < 0.1.

## *Analysis of cytokines from supernatant*

Commercial antibodies or ELISA's are not available for guinea pig cytokines, however the human IL-8 ELISA from R&D Systems has previously been shown to detect guinea pig IL-8, and was used for these experiments (Kuo *et al*., 1997; Lyons *et al*., 2002). A human TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN) has also been utilized to measure guinea pig TGF-β (Allen *et al*., 2003). TGF-β in supernatants was acid activated prior to analysis per manufacturer's instructions. TNF was measured by an L929 cytotoxicity bioassay as previously described (Aronson et al., 1995; Lasco et al., 2003).

## *Plaque assay*

Supernatants were analyzed for infectious virus by a Vero cell plaque assay. Cells less than passage 40 were used. Vero cells were plated in 6 well plates at $1x10^5$ cells/ml, 3 ml/well, and allowed to adhere overnight. Medium was removed from the plates and 100µl of serial 10-fold dilutions of supernatants (diluted in PBS w/ $Ca^{2+}$ and $Mg^{2+}$ + 1% v/v fetal bovine serum) were added to the wells in duplicate. The plates were then incubated at 37°C for 40 minutes in a humid incubator, followed by the addition of 3 ml/well of an overlay comprised of a 1:1 ratio of 2% methylcellulose and 2X MEM media (containing 2% v/v normal calf serum, 4mM L-glutamine, 200 U/ml penicillin, and 200 ug/ml streptomycin). Plates were incubated for four days at 37°C before staining

with an additional 3 ml overlay mixture (described above) containing 2% v/v neutral red (Sigma). After an overnight incubation, the overlay was removed and plaques counted to determine the plaque forming units/ml.

### *Indirect immunofluorescence assay*

An indirect immunofluorescence assay (IFA) was used to determine the percent of macrophages infected. At 48 hours post-infection, P2, P18, or mock-infected macrophages were scraped into PBS. Cytospin preparations of $1x10^4$ cells were prepared for each sample, fixed for 10 minutes in ice-cold methanol, and stored at -20$^o$C until use. The area containing cells was circled with a hydrophobic pen, then blocked against non-specific antibody binding in blocking solution (PBS, 1% v/v bovine serum albumin (BSA), 20% v/v normal horse serum (NHS)) for 30 minutes at room temperature.

Rabbit anti-PIC serum used as a source for anti-PIC primary antibody was incubated with P388D1 cytoplasmic cell lysate to reduce non-specific antibody reactivity. P388D1 cytoplasmic cell lysate was prepared as previously described (Dignam *et al*., 1983). Briefly, P388D1 cells were washed twice by centrifuging the cells at 2000xg at 4$^o$C for 15 minutes and resuspending with dPBS. After the second wash, the cell pellet was resuspended in dPBS, transferred to a microfuge tube, and re-pelleted. Cells were lysed by resuspending in 200µl of lysis buffer (10mM Hepes pH 7.9, 10mM KCl, 1mM EDTA, and 0.05%NP40). Following a 15 minute incubation on ice, the nuclei were pelleted by centrifugation at 2,500xg for 5 min. The supernatant was removed as cytoplasmic extract, a salt solution of 40mM Hepes, 150mM KCl, and 4mM MgCl2 was

added, and debris was spun out at 12,000xg for 20 min at 4°C. The supernatant was removed as cytoplasm, 30% v/v of glycerol was added, and the cytoplasmic extract was stored at -80°C until further use. Rabbit anti-PIC serum was absorbed with P388 cytoplasmic cell lysate at a ratio of 42:1 and incubated at room temperature for 30 minutes.

Blocking solution was carefully removed and 40µl of the absorbed serum at a 1:40 dilution in dilution solution (PBS, 1% v/v BSA, 20% v/v NHS) was applied to the cells. Slides were incubated in a humid chamber for 30 minutes at room temperature. Slides were then washed with stirring twice, for 7 minutes each, in PBS. A 1:80 dilution of FITC-conjugated goat antibodies to rabbit IgG (Vector, Burlingame, CA) in dilution solution was applied to each well, and slides were incubated 30 minutes at room temperature in the dark. Following subsequent washing, a counterstain of Evan's Blue was applied (Sigma, St. Louis, MO). Negative and positive control preparations were assayed simultaneously with infected-macrophage preparations. Negative control was mock-infected macrophages, and positive control was Vero cells infected with P18 at an MOI=1 for 4 days. Slides were examined by fluorescent microscopy and the percent cells infected was determined by the number of positive cells containing fluorescent cytoplasmic granular staining per 100 cells (Connolly *et al*., 1993).

## *Statistics*

Differences between P18 and P2 viral titers were determined by Student's t-test. Differences in cytokine mRNA levels between the P2, P18, and mock infected cells were

determined by ANOVA with Bonferroni adjustment for multiple comparisons using Sigma Stat 3.0.1 (SPSS Inc.). Statistical significance is defined as p value<0.05.

RESULTS

To determine the effect of P2 and P18 infection on macrophage cytokine expression, two types of macrophages were used. Bone marrow-derived macrophages (BMDM) are a homogenous population with all the cells at the same stage of differentiation. Techniques for isolating guinea pig BMDM had not been previously reported for guinea pigs, therefore, we adapted murine BMDM techniques with the intention of developing large numbers of homogeneous primary macrophages for *in vitro* experiments. (For a detailed description of isolation and characterization of guinea pig BMDM, see Appendix A.) However, cell yields were lower than expected, therefore, the majority of experiments were done with peritoneal macrophages. Although a more heterogenous population than BMDM, peritoneal macrophages (PM) are the initial viral target of PIC after intraperitoneal inoculation of a guinea pig and, therefore, are useful for simulating early cytokine responses *in vivo*.

## *Pichinde replication in primary macrophages*

The ability of Pichinde virus to replicate in PM and BMDM was evaluated. P2 and P18 infection of PM showed peak titers of $1x10^6$pfu/ml and $2.5x10^6$pfu/ml respectively (Figure 3). BMDM titers for P2 and P18 peaked at $2.2x10^6$pfu/ml and $2.7x10^6$pfu/ml respectively (Figure 3). Analysis of infected cells at 48hr by indirect

immunofluorescence (IFA) showed a maximum of 4% of cells positive for viral antigen, although samples from many experiments had undetectable viral antigen for both P2 and P18 (data not shown). Previous experiments in our lab have shown that under similar conditions, resident PM can actively replicate virus as determined by infectious center assays, IFA, and viral titers (Shurleff, UTMB dissertation). Notably, unlike viral replication *in vivo, in vitro* titers were not significantly higher for P18 than P2 in either type of macrophages.

## *Cytokine mRNA levels from infected macrophages*

A possible explanation for similar P2 and P18 titers *in vitro* is that cytokines with antiviral effects may be induced during P18 infection, limiting P18 replication to similar levels as P2 virus. Conversly, cytokines that enhance viral replication may be induced by P2 virus. To test this possibility, PM and BMDM were infected with P2, P18, or mock supernatant and analyzed for cytokine mRNA production. At 48h post-infection of PM, there were no differences in cytokine mRNA levels between P18 and P2 infection. However, compared to mock infection, P18 infection elevated IL-8 mRNA 5-fold (Figure 4). At 72h, there were again no differences between the two virus variants, although both P2 and P18 infected cells had elevated levels of RANTES mRNA compared to mock (64-256 fold respectively). TGF-β mRNA was elevated by P2 infection at 72h compared to mock.

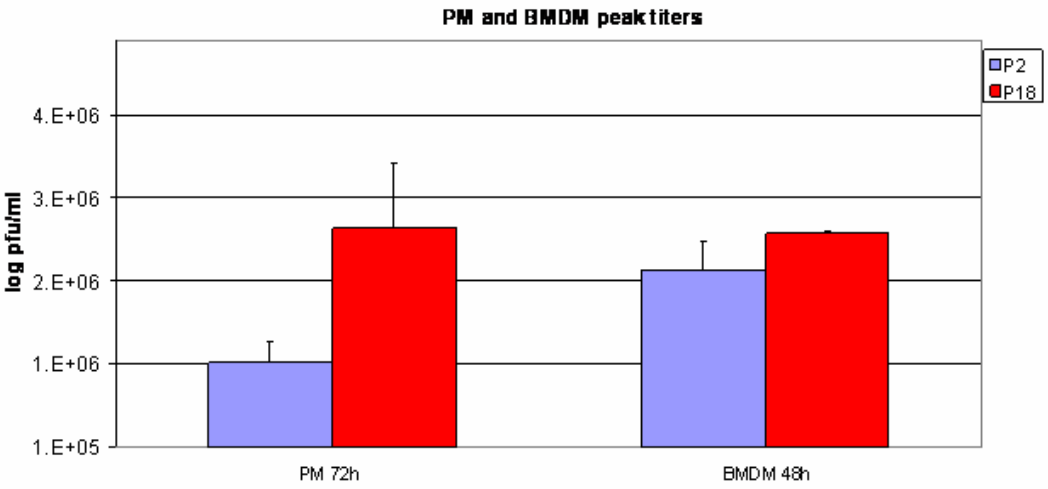


*Figure 3. Viral titers from supernatants of infected PM or BMDM.*
Cultures of $2.5x10^5$ BMDM or PM cells were infected in suspension with P2 or P18 at an MOI=1. Supernatants were harvested at the indicated timepoints and assayed by plaque assay. Points represent geometric mean ± SEM (n=2).

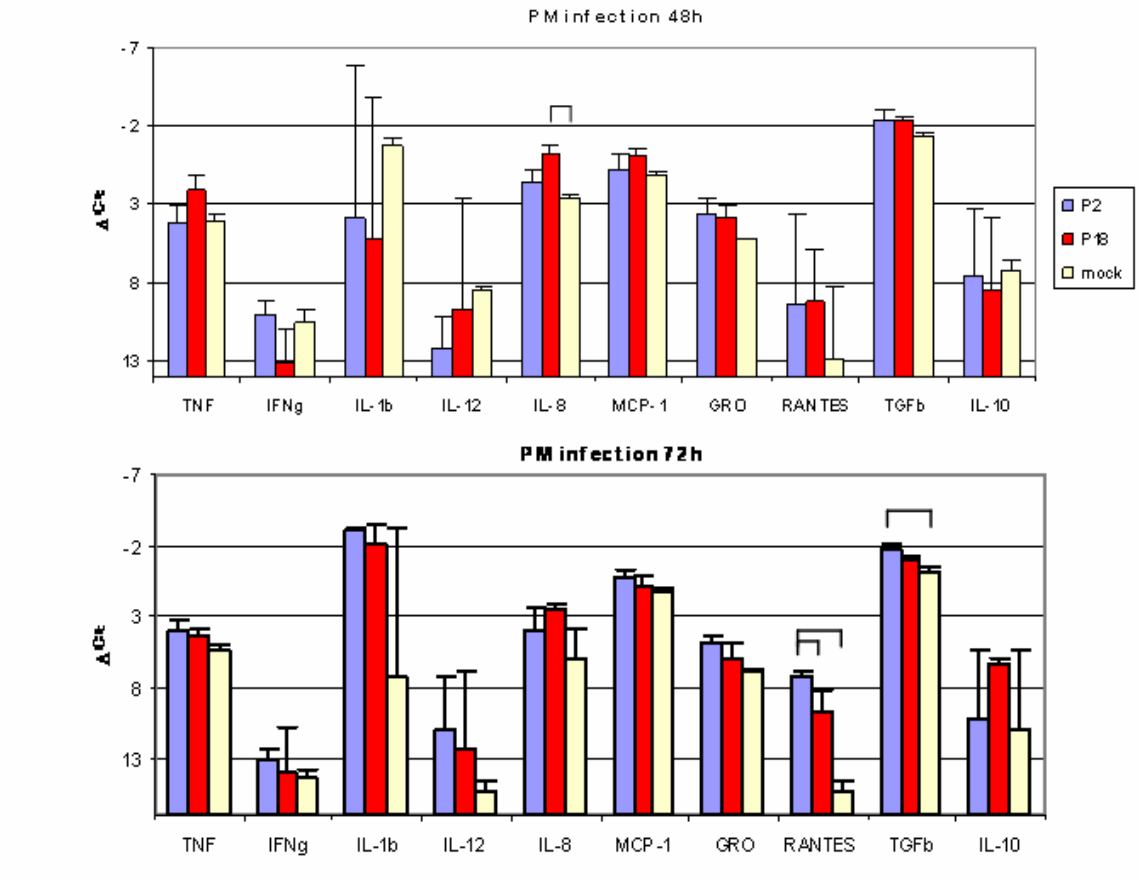


*Figure 4. Cytokine mRNA production from infected PM*

Peritoneal macrophages were infected in suspension at an MOI=1, with either P2, P18, or mock inoculum. At 48h and 72h post-infection, RNA was collected and analyzed for guinea pig cytokine mRNA by real time RT-PCR. Data is displayed as $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$, with standard deviations (n=2). Brackets indicate statistical significance between groups ($p < 0.05$). Statistics were done using one-way ANOVA. Results are representative of three independent experiments.

Similar to PM, BMDM at 48h revealed no differences between P18 and P2 infection. However, P2 infection elevated TGF-β mRNA 2-fold compared to mock. There were no differences detected at 72h (data not shown). At 96h, P18 infected cells had elevated levels of IL-12p40 mRNA compared to both P2 and mock. In contrast, P2 infected cells had elevated levels of IL-1β mRNA compared to both P18 and mock (Figure 5).

Overall, these data indicate that in PM, there is not a general activation of pro-inflammatory cytokines in virus infected macrophages; however, some alterations of the chemokines IL-8 and RANTES were observed. BMDM likewise did not respond with general activation of pro-inflammatory cytokines, although at a later timepoint, there was differential expression of IL-1β and IL-12p40 between P2 and P18 infected cells. TGFβ was slightly increased (2-fold) by P2 infection compared to mock in both cell types. In some viral systems, the induction of anti-inflammatory cytokines such as IL-10 and TGF-β down-regulates pro-inflammatory cytokine production (Panuska *et al*., 1995; Zielinska-Jenczylik *et al*., 1991). Whether the small upregulation of TGF-β contributed to the lack of macrophage activation by P2 is unknown. Aside from the similarities in TGF-β expression, PM and BMDM had different responses to P2 and P18 infection, although collectively these data suggest that neither P2 or P18 virus is a potent inducer of macrophage-derived cytokines.

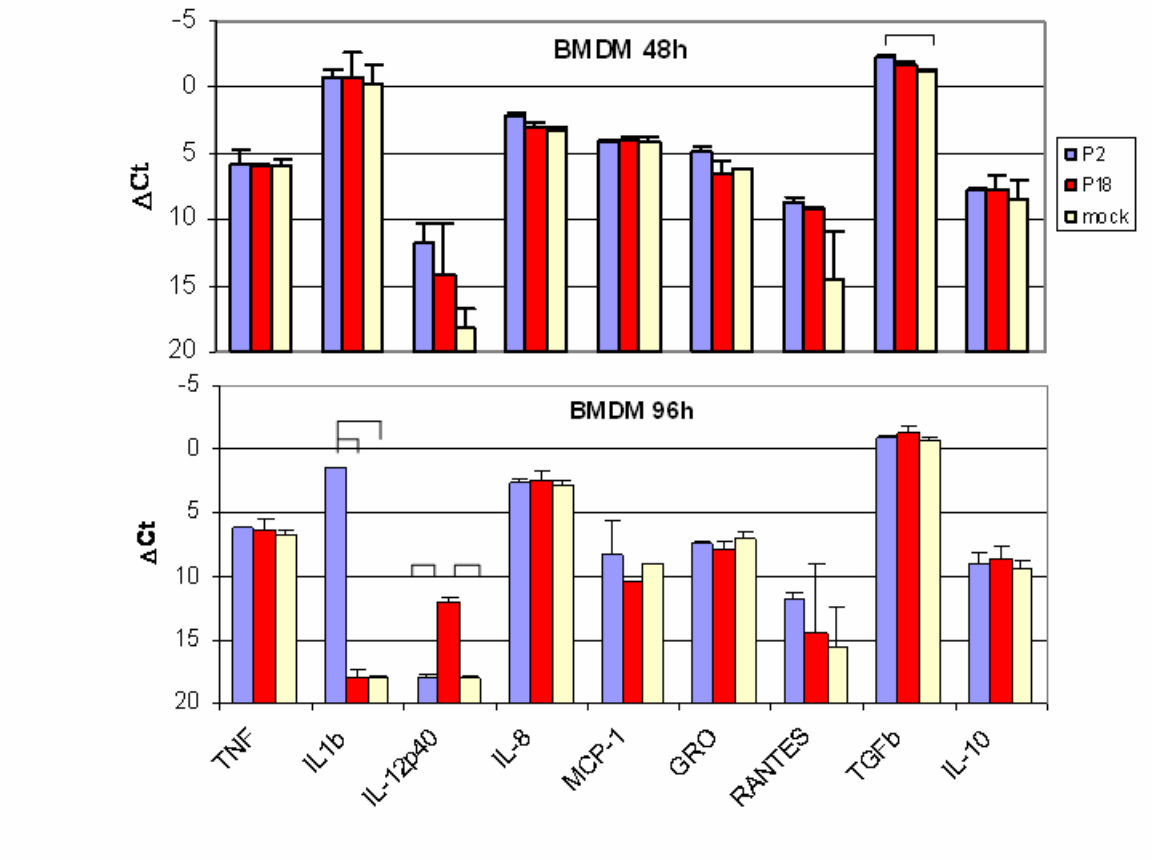


*Figure 5. Cytokine mRNA production from infected BMDM*

Bone marrow-derived macrophages were infected in suspension at an MOI=1, with either P2, P18, or mock inoculum. At 48h and 96h post-infection, RNA was collected and analyzed for guinea pig cytokine mRNA by real time RT-PCR. Data is displayed as $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$, with standard deviations (n=2). Brackets indicate statistical significance between groups ($p < 0.05$). Statistics were done using one-way ANOVA. Results are representative of three independent experiments.

Virus stocks used for this experiment were made in P388D1 cells. We were concerned that these macrophage-like cells might elaborate cytokines during production of viral stocks that would modulate primary macrophage cytokines responses to virus. To address this issue, viral stocks and mock P388-derived supernatants were diluted in media at least 3-fold after the initial 40 minute absorption. Furthermore, PM infection using polyethylene glycol purified stocks revealed that similar to experiments with P388D1 virus stock, there was no activation of pro-inflammatory cytokines or chemokines, and no differences between P2 and P18 infection were observed.

## *Cytokine mRNA levels from infected macrophages after LPS stimulation*

Although Pichinde virus did not activate pro-inflammatory cytokines, we wanted to determine if Pichinde virus was inhibiting the ability of macrophages to produce cytokines in response to another stimulus. mRNA levels of cytokines were analyzed after infected cells were stimulated with LPS. As expected, at 24hr, TNF and IL-8 protein for LPS-stimulated infected cells were 4000pg/ml - 400pg/ml respectively compared to undetectable levels from infected unstimulated cells (Figure 9). By 24h, there was no difference in mRNA levels between unstimulated and LPS-stimulated macrophages. A general pattern began to emerge of increased LPS-induced cytokine mRNA in P2 infected cells compared to both P18 and mock infected cells, particularly for IL-12p40, MCP-1, and IL-1β (Figure 6). At 24h, the increases in IL-12p40 and MCP-1 mRNA levels noted during P2 infection at 12h reached statistical significance above P18 and

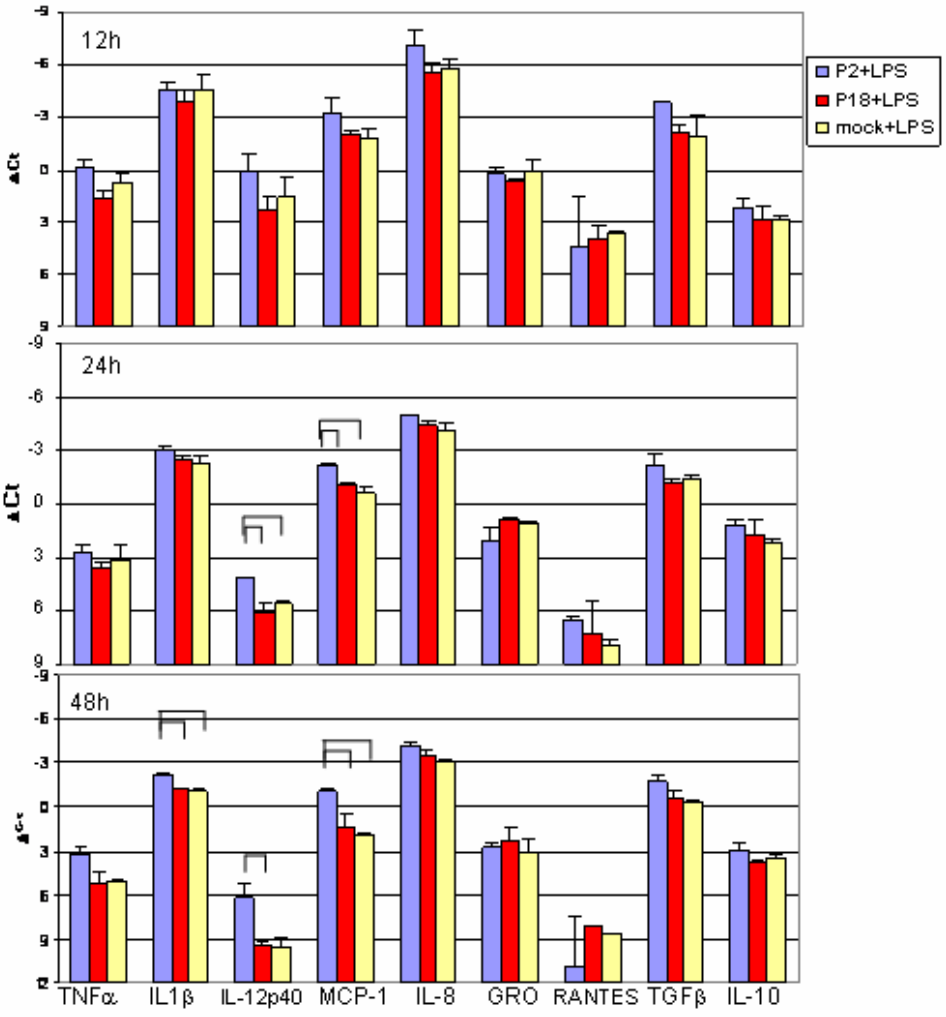


*Figure 6. Viral titers from supernatants of PM infected cells stimulated with LPS.*

PM were infected in suspension with virus at an MOI=1. LPS was added 2 hours after infection at a final concentration of 0.1ng/ml. Supernatants were harvested at the indicated timepoints and assayed by plaque assay. Points represent geometric mean ± SEM (n=2). Differences between P2 and P18 at each timepoint were determined by Student's t-test.

mock. At 48h, IL-12p40, MCP-1, and IL-1β mRNA levels were all elevated in P2 infected cells compared to P18 (Figure 6). This pattern of differential expression was not seen in LPS-stimulated BMDM. Infection with P2 and P18 viruses elevated MCP-1 and TNF-α at 48h post-infection compared to mock infected cells (Figure 7). These results indicate that unlike P2 infection, P18 infection did not boost LPS-induced mRNA levels of cytokines in PM.

## *Pichinde replication in LPS stimulated peritoneal macrophages*

LPS stimulation of macrophages induces pro-inflammatory cytokines with potentially antiviral effects. We were interested to see if LPS treatment of infected peritoneal macrophages would alter viral replication. P18 had a statistically higher titer than P2 at 24 hr (Figure 8). Peak titer for P18 infection was $4.7x10^5$ at 12h, and the peak titer for P2 was $1.97x10^5$ at 48h. BMDM titers after LPS stimulation have not been determined. These data indicate P18 virus replicates slightly better than P2 in LPS-stimulated PM.

## *IL-8, TGF-β and TNF levels from infected macrophage supernatant after LPS stimulation*

To correlate protein levels with mRNA expression, supernatants from infected PM were analyzed by ELISA (IL-8, TGF-β) and bioassay (TNF). No IL-8 or TNF could be detected from supernatants of P2, P18, or mock-infected, non-LPS stimulated macrophages. IL-8 levels from LPS stimulated macrophages revealed statistically lower IL-8 from P2 infected cells compared to mock at 48h. IL-8 from P18 infected cells had median values between P2 and mock (Figure 9). In contrast to protein levels, IL-8

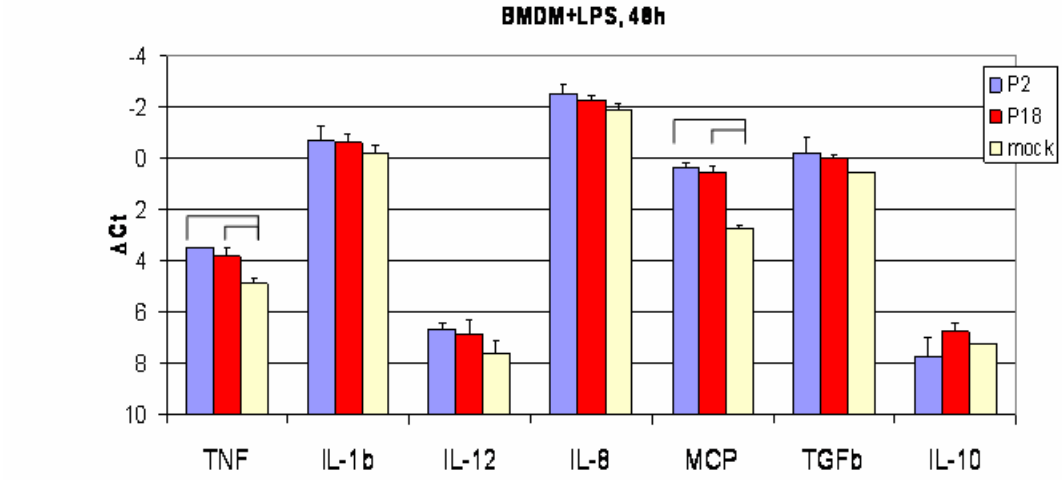


*Figure 7. Cytokine mRNA production from infected BMDM stimulated with LPS.*
Bone marrow-derived macrophages were infected in suspension at an MOI=1, with either P2, P18, or mock inoculum, and stimulated with LPS 2 hours after infection (final conc. 0.1ng/ml). At 48h post-infection, RNA was collected and analyzed for guinea pig cytokine mRNA by real time RT-PCR. Data is displayed as $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$, with standard deviations (n=2). Brackets indicate statistical significance between groups ($p < 0.05$). Statistics were done using one-way ANOVA.

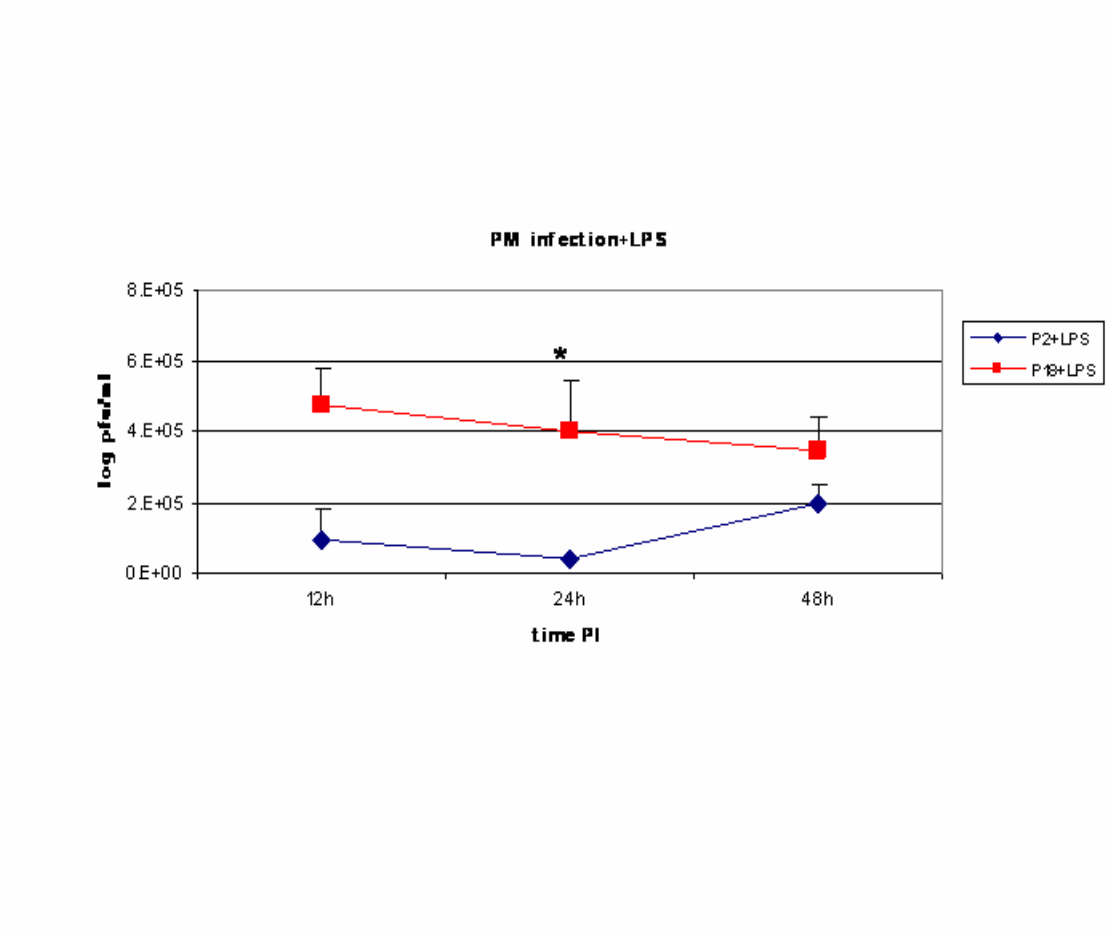


*Figure 8. Viral titers from supernatants of PM infected cells stimulated with LPS.*
PM were infected in suspension with virus at an MOI=1. LPS was added 2 hours after infection at a final concentration of 0.1ng/ml. Supernatants were harvested at the indicated timepoints and assayed by plaque assay. Points represent geometric means ± SEM (n=2).

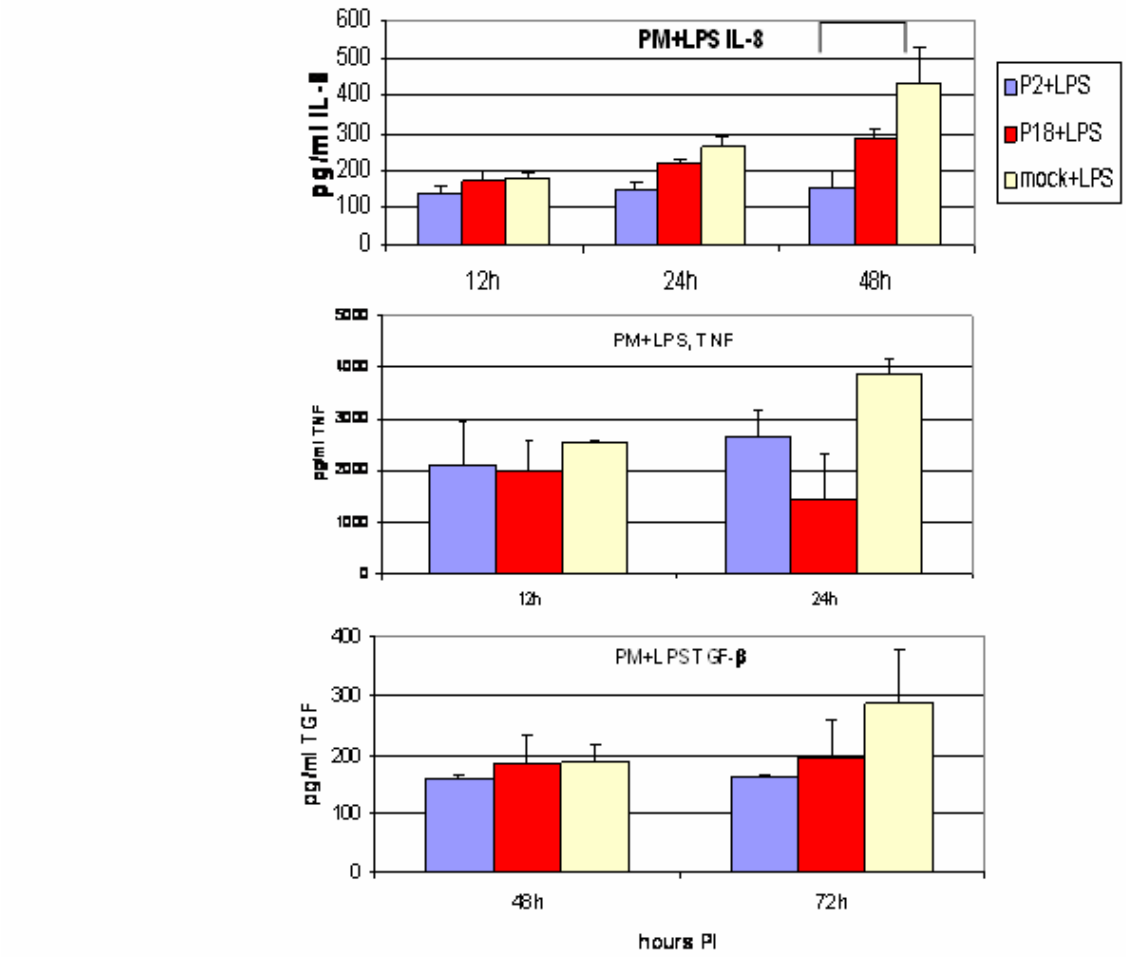


*Figure 9. IL-8, TGF-β and TNF protein production from infected PM stimulated with LPS.*

Peritoneal macrophages were infected in suspension at an MOI=1, with either P2, P18, or mock inoculum, and stimulated with LPS 2 hours after infection (final conc. 0.1ng/ml). At 12h, 24h, 48h, or 72h post-infection, supernatants were collected and analyzed for IL-8 and TGF-β by ELISA, and TNF by bioassay. Bars are the means ± standard deviations (n=2). Brackets indicate statistical significance between groups ($p < 0.05$). Statistics were done using one-way ANOVA. Results are representative of three independent experiments.

mRNA levels from the same experiment were not statistically different between groups. These differences may be attributed to post-transcriptional control, which has been described for IL-8 (Ma *et al*., 2004). There were no statistical differences between TNF levels from supernatants of P2, P18, or mock-infected, LPS-stimulated macrophages, although there was a suggestion of suppression by P18 ($p = 0.065$, 24h) (Figure 9). There were no statistical differences between TGF-β levels from supernatant of P2, P18, or mock-infected, LPS-stimulated PM (Figure 9). These results, in general, corroborate the mRNA data indicating that IL-8, TNF-α, and TGF-β are not induced by viral infection either with or without LPS stimulation.

### *Effects of exogenous cytokines on viral replication*

In results described in Chapter 3, we found that TNF-α, IL-8, and IL-1β were suppressed in total peritoneal cells explanted from PIC-infected guinea pigs early in infection (Figure 12). These cytokines have all been demonstrated to alter viral replication, and we were interested to determine if they could alter Pichinde replication in vitro (Khaiboullina et al., 2000; Seo et al., 2002; Gilles et al., 1992; Murayama et al., 1994; Van Damme et al., 1987; Lane et al., 2001b; Lane et al., 2001a). The effect of human GRO-α on PIC replication was also tested, since GRO-α has been demonstrated to increase HIV-1 replication in macrophages (Lane et al., 2001b). While TGF-β does not have antiviral effects, it does decrease expression of pro-inflammatory cytokines, and could contribute to an environment favoring viral replication, and the effects of TGF-β on PIC replication were tested as well. Guinea pig peritoneal exudate macrophages were

pre-treated with two doses (1ng/ml or 100ng/ml) of recombinant human cytokines overnight before infection, and treated again after infection. For all five cytokines tested, there were no significant changes in viral replication for either P18 or P2 at 48h post-infection (Figure 10).

Many groups have reported the use of heterologous cytokines for guinea pig studies since there are no commercially available cytokine reagents for guinea pigs (Lucia *et al*., 1989; Morris *et al*., 2002; Tjwa *et al*., 2003; Alloatti *et al*., 1999; Dai *et al*., 1999). However, we were concerned that perhaps there were no differences in viral replication because of the heterologous species difference of the recombinant cytokines. We know that TNF-α can induce IL-8, IL-1β can induce TNF and IL-8, and TGF-β can suppress cytokines (Dinarello et al., 2001; Flanders et al., 2001). We therefore decided to test the ability of human cytokines to stimulate IL-8 and TNF protein from guinea pig periteoneal exudate macrophages. Figure 11 demonstrates that IL-1β, TGF-β, GRO–α and IL-8 did not alter TNF production measured by bioassay. TNF-α, GRO–α, and IL-1β did not alter IL-8 production measured by ELISA, although TGF-β significantly suppressed IL-8 compared to peritoneal exudates cells treated with media alone (Figure 11). Without independent evidence of a guinea pig macrophage response to recombinant human TNF-α, IL-1β, and GRO-α, we cannot make any conclusions about the effect of these cytokines on PIC replication. However, the amino acid sequences of both TGF-β and IL-8 are highly conserved between guinea pigs and humans (Yoshimura et al., 1993b; Scarozza et al., 1998). In addition, human TGF-β has been demonstrated to affect

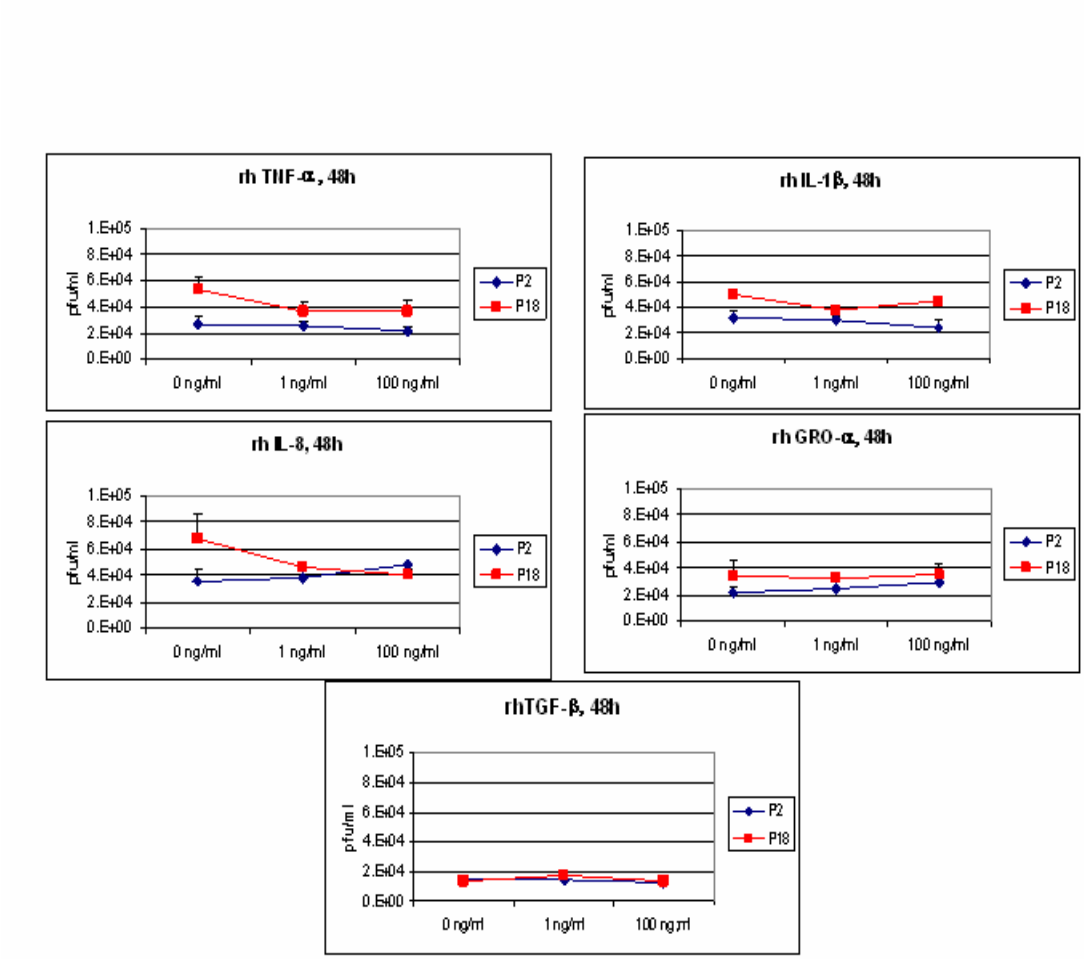


*Figure 10. Effect of exogenous human cytokines on viral replication in guinea pig peritoneal exudate macrophages.*

Peritoneal exudates macrophages were pretreated overnight with media containing 0, 1, or 100ng/ml of recombinant human cytokines. The medium was removed and cells were infected with P2, P18, or mock at an MOI=1, and further treated with medium alone, 1ng/ml, or 100ng/ml of the appropriate cytokine. 48h later, supernatants were harvested and assayed for viral titers by plaque assay. Titers are displayed as geometric means ± SEM, n=3. 1ng/ml and 100ng/ml titers were analyzed for statistical analysis by Student's t-test compared to medium alone titers (0ng/ml). Results are representative from two independent experiments.

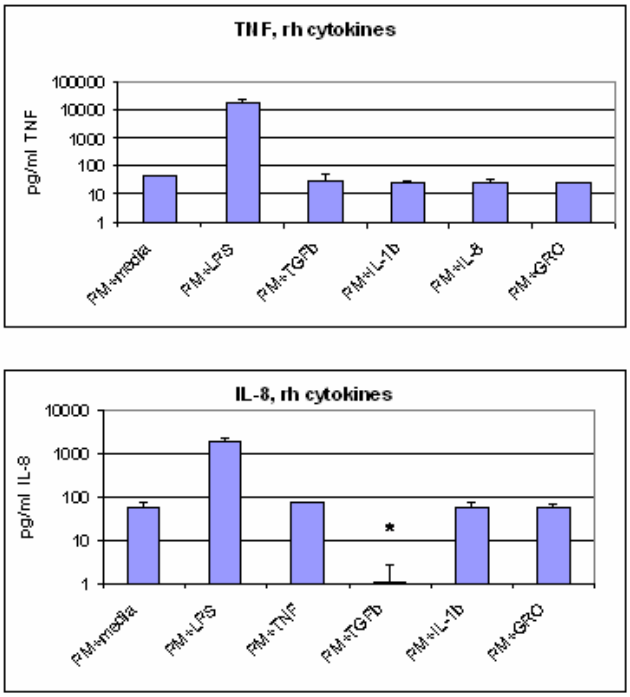


*Figure 11. TNF and IL-8 protein levels from guinea pig peritoneal exudate cell supernatants after treatment with recombinant human cytokines.*

Guinea pig peritoneal exudate cells were cultured at $4x10^5$ macrophages/well and treated with 100ng/ml of rh TNF-α, IL-1β, GRO-α, IL-8, or TGF-β for 18hrs. TNF production was measured from IL-1β, GRO-α, IL-8, and TGF-β treated cells by bioassay. IL-8 production from TNF-α, IL-1β, GRO-α, and TGF-β treated cells was measured by ELISA. Negative controls were media only treated wells and positive controls were LPS treated wells. Results are shown as means of triplicate wells ± standard deviations (n=2). * indicates $p<.05$ vs media control as determined by Student's t-test.

primary guinea pig lymphocytes (Dai et al., 1999). Previous experiments in our lab have demonstrated that human IL-8 caused an intracellular calcium influx in a guinea pig cell line (JH4 cells) (K. Marriott, UTMB dissertation). Therefore, although human TGF-β and IL-8 have been demonstrated to impact guinea pig cells, neither cytokine affects PIC replication under these conditions.

## DISCUSSION

In this study, we investigated the ability of attenuated (P2) and virulent (P18) Pichinde virus variants to alter cytokine mRNA production during infection of primary guinea pig macrophages. Infection studies of primary macrophages *in vitro* is highly relevant, since macrophages are the initial cell infected by Pichinde virus (Murphy *et al*., 1977). Macrophages also produce cytokines that have direct and indirect effects on viral replication and, therefore, contribute to the outcome of viral infection.

The activation of macrophages and dendritic cells and their production of cytokines like TNF-α and IL-12 are among the initial mechanisms of pathogen control. Many viruses, including Ebola, influenza A, hepatitis C, and equine infectious anemia virus, have been demonstrated to increase both pro-inflammatory cytokine and chemokine production from macrophages *in vitro* (Gupta *et al*., 2001; Lim *et al*., 2005; Hofmann *et al*., 1997; Lim *et al*., 2005). We hypothesized that P2 virus would stimulate production of pro-inflammatory cytokines that would contribute to the restriction of P2 replication. We also hypothesized that P18 would suppress pro-inflammatory cytokines,

possibly via the induction of anti-inflammatory cytokines, leading to unhindered viral replication. In this study of primary guinea pig macrophages, we found that neither P18 nor P2 virus induce pro-inflammatory cytokine mRNA expression of TNF-α, IL-1β, or IL-12p40 during PM infection. These results are similar to findings with Lassa virus. Lassa infection of macrophages and dendritic cells *in vitro* does not induce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, or IFN-γ (Mahanty *et al*., 2003; Lukashevich *et al*., 1999; Baize *et al*., 2004). In our study, chemokine and anti-inflammatory cytokines were also not particularly altered, with the exception of a 5-fold increase in IL-8 in P18 infected PM at 48h. IL-8 up-regulation was reported in Lassa-infected dendritic cells, but not macrophages (Baize *et al*., 2004).

Two different populations of primary macrophages were used in this study. The one similarity between PM and BMDM results was a 2-4 fold up-regulation of TGF-β during P2 infection, a result somewhat contrary to our expectations. TGF-β is primarily an anti-inflammatory cytokine, and is particularly known for suppressing T-cell activation . We would have expected P18 infection to have up-regulated TGF-β as a means of suppressing pro-inflammatory cytokines and cell-mediated immunity. TGF-β does, however, have other functions such as neutrophil and macrophage chemotaxis, as well as inhibiting endothelial cell growth and proliferation, therefore other functions of TGF-β may be involved in Pichinde infection . Besides TGF-β, PM and BMDM responded somewhat differently to Pichinde infection, although Pichinde virus did not appear to substantially up-regulate cytokines from either cell type. Variations in cytokine

expression between PM and BMDM are not entirely surprising. PM are a heterogenous population of cells taken from the peritoneal cavity shortly before use, while BMDM are a homogenous population derived *ex vivo* after 5-6 days of culture. Variations in cytokine mRNA between the two populations may also be due to the small number of replicates per group that resulted in unequal variance between the samples. There were no statistical differences between P2, P18, or mock groups at any time when data was analyzed using Kruskall-Wallis analysis of variance, a less sensitive non-parametric statistical test.

The lack of cytokine production during P2 infection was somewhat surprising. We had hypothesized that the attenuated P2 virus would up-regulate potentially antiviral pro-inflammatory cytokines that could limit replication of P2 *in vivo*. It was recently reported that Mopeia, another non-pathogenic arenavirus, increased mRNA levels of IFN-α/β, TNF-α, and IL-12p35 in macrophages, supporting this hypothesis (Pannetier *et al*., 2004). It was further illustrated that IFN-α/β reduced Mopeia replication by 2-logs, implying that Mopeia limits its own replication by inducing antiviral cytokines (Pannetier *et al*., 2004). In contrast, although Lassa virus replication can be moderately limited by IFN-α/β (1-log), it is not induced by Lassa infection (Asper *et al*., 2004; Pannetier *et al*., 2004). However, in our study, P2 did not up-regulate pro-inflammatory cytokines, suggesting mechanisms other than macrophage-derived cytokines are involved in the inhibition of P2 *in vivo*.

We also hypothesized that P18 infection would alter macrophage responses to stimulation with LPS. In our study, we did not detect a suppression of LPS-induced cytokine mRNA, although there was a suggestion of lower TNF-α protein compared to mock after P18 infection (p=0.066). We have previously reported a suppression of LPS-induced TNF-α and IL-6 from P388D1 cells infected with P18 (Fennewald *et al*., 2002). A suggested mechanism for this cytokine suppression was elucidated by electrophoretic mobility shift assay of *ex vivo* Pichinde infected PM. P18 infection was associated with an increase in transcriptionally repressive p50/p50 NF-κB homodimers, and a decrease in transcriptionally active relA/p50 NK-κB heterodimers (Fennewald *et al*., 2002). Viral alteration of transcription factors may play a key role in regulation of cytokine responses.

Interestingly, in this study we observed an increase in MCP-1, IL-12p40, and IL-1β in P2 infected cells stimulated with LPS. The nonpathogenic arenavirus Mopeia, when compared to LPS alone, also increased cytokine mRNA of IFN-β, TNF-α, and IL-12p35 after LPS stimulation (Pannetier *et al*., 2004). Both P2 and LPS induced transcriptionally active relA/50 heterodimers from peritoneal macrophages (Fennewald *et al*., 2002). Possibly P2, or a mediator associated with P2, is maintaining the transcriptional proteins and cytokines initially induced by LPS. Higher production of IL-12p40, MCP-1, and IL-1β LPS-induced cytokine mRNA during P2 infection was associated with lower viral titers when compared to P18. This suggests that although P2 infection does not increase macrophage-derived cytokines *in vitro*, exposure to IL-12p40, MCP-1, and IL-1β produced by other cells may inhibit P2 replication *in vivo*.

The inability of PIC to induce cytokines like TNF-α, IL-1β, and IL-12p40 from macrophages may be an indicator of viral-initiated macrophage dysfunction. Although we do not have enough evidence to support this speculation for PIC, several other viruses such as measles, parainfluenza type 3, and respiratory syncytial virus, can impair macrophage and dendritic cell activities (Schneider-Schaulies *et al*., 2002; Panuska *et al*., 1995; Plotnicky-Gilquin *et al*., 2001). These cells have impaired abilities to 1) produce IL-12, IL-1β, IL-8, and TNF-α 2) express cell-surface molecules, and 3) stimulate T-cell proliferation. Dysfunction of antigen presenting cells like PM may lead to immunosuppression *in vivo* characterized by inhibition of T-cell proliferation, lymphopenia, and suppression of macrophage-derived cytokines. Not coincidently, measles, parainfluenza type 3, and respiratory syncytial virus are all considered immunosuppressive (Schneider-Schaulies *et al*., 2002; Panuska *et al*., 1995; Plotnicky-Gilquin *et al*., 2001). Both fatal Lassa fever and P18 disease *in vivo* are also associated with immunosuppression characterized by a lack of pro-inflammatory cytokines ((Mahanty *et al*., 2001)(Figure 13), reduced T-cell response to mitogens, (Fisher-Hoch *et al*., 1987)(T. Jerrells, unpublished data), and lymphopenia (Fisher-Hoch et al., 1988b; Jahrling et al., 1981). The lack of macrophage-induced cytokines observed in this study corroborates the suggestion that immunosuppression is correlated with the inability of a virus to induce cytokines. Further studies are needed to determine the effects of PIC on macrophage antigen presentation and cell-surface molecule expression.

Although macrophage-derived cytokines were not produced during *in vitro* PIC infection, during *in vivo* infection PIC may be exposed to cytokines produced by other cells. Endothelial cells and T cells, for example, can produce a variety of cytokines including TNF-α, IL-1β, and IL-8 (Aggarwal et al., 2001; Iizasa et al., 2001). We therefore wanted to determine if cytokines known to affect viral replication could alter PIC replication. We opted to test TNF-α and IL-1β effects on PIC replication because of their known antiviral effects (Khaiboullina *et al*., 2000; Seo *et al*., 2002; Gilles *et al*., 1992; Van Damme *et al*., 1987). IL-8 was tested because in our experiment, IL-8 mRNA was increased by P18 during *in vitro* macrophage infection. IL-8 can also enhance viral replication of cytomegalovirus, poliovirus, and HIV (Murayama *et al*., 1994; Khabar *et al*., 1997b; Lane *et al*., 2001a; Poli *et al*., 1991). GRO-α and TGF-β were also chosen because of their ability to alter HIV infection of macrophages (Lane *et al*., 2001b; Poli *et al*., 1991). In our study, however, we did not observe any changes in either P2 or P18 replication after the addition of exogenous cytokines. A recent study in Vero and Huh-7 cells demonstrated TNF-α was not able to reduce Lassa replication (Asper *et al*., 2004), however studies in more pathologically relevant macrophages have not been done. Because of the probable lack of cross-reactivity between the human cytokines used in this study and the guinea pig cells, the results from experiments using human cytokines are inconclusive. It is possible that the cytokines tested do not have any direct antiviral effects on Pichinde replication, but we would like to pursue similar experiments with

recombinant guinea pig cytokines. Generation of recombinant guinea pig TNF-α is underway.

In summary, we have shown that, like Lassa infection of macrophages, Pichinde virus does not induce macrophages to produce pro- or anti-inflammatory cytokine or chemokine mRNA. This work further validates the use of Pichinde infection of guinea pigs as a model for Lassa fever. However, since there is no differential cytokine mRNA production from PM infected with P2 and P18 *in vitro*, studies of cytokine patterns during *in vivo* PIC infection would provide greater insight into potential contributions of cytokines to viral control.

# CHAPTER 3: CYTOKINE PATTERNS DURING COMPARATIVE PICHINDE INFECTION OF GUINEA PIGS.

## INTRODUCTION

Lassa is an Old World arenavirus which infects 100,000-300,000 people per year in western Africa (Johnson *et al*., 1987). Lassa virus causes a spectrum of disease from subclinical infection to fatal hemorrhagic fever (McCormick *et al*., 1987a). Clinical severity of disease closely parallels levels of viremia, but the mechanisms by which some patients control viral replication are unknown (Johnson *et al*., 1987). Clinical observation and experimental studies suggest that cell-mediated immunity plays a larger role in protection and viral clearance than does humoral immunity. (Johnson *et al*., 1987; Rodas *et al*., 2004; Fisher-Hoch *et al*., 1987; Jahrling *et al*., 1986; Peters *et al*., 1987; Fisher-Hoch *et al*., 2000). Innate immunity likely plays a role in control of early viral replication, but little is known about mechanisms of innate immunity in Lassa fever.

Arenaviruses are non-cytopathic viruses with a tropism for macrophages and dendritic cells (Mahanty *et al*., 2003; Lukashevich *et al*., 1999; Baize *et al*., 2004; Aronson *et al*., 1994), and macrophages are the initial target cell in experimental models of Lassa fever (Aronson *et al*., 1994; Murphy *et al*., 1977). Normally, upon activation by pathogens, macrophages secrete pro-inflammatory cytokines and chemokines such as TNF-α and IL-8, some of which are known to influence viral replication in various systems (Khaiboullina *et al*., 2000; Seo *et al*., 2002; Guidotti *et al*., 2000a; Murayama *et*

*al*., 1994; Cavanaugh *et al*., 1997). However, virulent arenaviruses appear to suppress the activation of macrophages. Human macrophages infected with Lassa virus fail to upregulate expression of IFN-α/β, TNF-α, and IL-12p35, whereas human macrophages infected with a related, avirulent arenavirus Mopeia do become activated to produce these cytokines (Pannetier *et al*., 2004; Lukashevich *et al*., 1999; Mahanty *et al*., 2003; Baize *et al*., 2004). It has also been reported that in human Lassa fever cases, lethal disease is associated with decreased serum levels of IL-8 and IP-10 compared to non-fatal cases. (Mahanty *et al*., 2001). It is plausible that virulent arenaviruses interfere with the development of antiviral cytokine responses in initial target cells of the monocyte/macrophage series.

Fatal Lassa fever is associated with a shock-like syndrome for which the pathogenesis is unknown. There is little evidence for direct viral-mediated cellular injury, nor do immunopathologic mechanisms seem to be responsible for the mild organ lesions (Walker *et al*., 1982). It has been suggested that over-production of pro-inflammatory cytokines, such as IL-1β and TNF-α, may contribute to vascular endothelial dysfunction and, consequently, shock (Peters *et al*., 2002b). The scanty existing data addressing this hypothesis for cytokines in Lassa fever terminal shock are contradictory. Elevated serum levels of TNF-α have been reported in a fatal Lassa case (Schmitz *et al*., 2002). Increased plasma levels of TNF, IFN-γ, and IL-6 have also been reported in experimental models of Lassa, and administration of IL-1 receptor antagonist (IL-1RA) provided protection against death from an intracerebral Lassa inoculation of

mice (Ignatyev *et al*., 2000; Aronson *et al*., 1995; Lukashevich *et al*., 2003). In contrast, there is also evidence to suggest that fatal Lassa fever is associated with a suppression of certain pro-inflammatory cytokines (Mahanty *et al*., 2001). To date, no comprehensive animal study has been performed to validate either of these hypotheses.

Animal models provide the ability to experimentally study various stages of infection in multiple tissue compartments. Animal studies using models for Lassa fever are greatly lacking in large part, because the most accepted models utilize costly non-human primates, or guinea pigs for which there are a lack of immunological reagents. Because of these limitations, macrophage-derived cytokine responses *in vivo* have not been systematically described in experimental models of Lassa fever. Therefore, we have developed real-time RT-PCR assays for guinea pig cytokines in order to study the cytokine response to Pichinde virus infection, which in guinea pigs provides an accepted animal model for Lassa fever (Jahrling *et al*., 1981; Connolly *et al*., 1993; Aronson *et al*., 1994).

Pichinde virus is a New World arenavirus that is non-pathogenic to humans, but causes severe Lassa fever-like disease after serial passage in guinea pigs (Jahrling *et al*., 1981). Pichinde virus can be used in a BSL-2 environment, as opposed to the BSL-4 environment for Lassa virus. We use two different variants of Pichinde virus to provide a comparative model simulating the spectrum of disease seen in Lassa fever. High-passage Pichinde virus (P18) causes severe disease in guinea pigs, with onset of fever during the first week of infection, progressive weight loss during the second week of infection, high

viremia, and death by days 14-21 post-infection due to partially compensated vascular leakage and myocardial suppression (Qian et al., 1994; Katz et al., 1990; Zhang et al., 2001; Zhang et al., 1999). The pathology of virulent P18 infected guinea pigs is similar to that of human Lassa fever with foci of splenic and liver necrosis, lack of cellular infiltrate, and lack of overt hemorrhage (Jahrling *et al*., 1981; Connolly *et al*., 1993; Aronson *et al*., 1994; Walker *et al*., 1982). Low-passage Pichinde virus (P2) causes a mild immunizing infection in guinea pigs, with onset of fever by day 6, low viremia, and resolution of clinical signs and viremia by approximately 2-3 weeks after infection (Zhang *et al*., 2001; Zhang *et al*., 1999; Jahrling *et al*., 1981). The model was originally described in strain 13 inbred guinea pigs (Jahrling *et al*., 1981). We have utilized outbred guinea pigs due to the current commercial unavailability of strain 13 guinea pigs, and the relevance of an outbred model to the human population. Using this comparative model, we studied cytokine mRNA responses to mild and severe arenavirus infection in various tissue compartments and several timepoints during experimental infection.

MATERIALS AND METHODS

## *Cells & Reagents*

Vero 76 cells for plaque assay were obtained from American Type Culture Collection (ATCC) and maintained in minimal essential media (MEM, Gibco) containing 5% v/v normal calf serum, 2mM L-glutamine, 100 U/ml penicillin, and 100μg/ml streptomycin.

## *Viruses*

P2 and P18 viruses were derived from serial spleen passages of Pichinde virus, Munchique strain (CoAn 4763) in inbred guinea pigs (Zhang *et al*., 1999). All virus stocks used were pooled spleen 10% w/v homogenates from inbred strain 13 guinea pigs infected with the indicated passage virus. For inoculations, spleen virus stocks was diluted in endotoxin free phosphate buffered saline (Gibco). Mock infected animals received an equivalent dilution of spleen homogenate from strain 13 uninfected, non-immune guinea pigs.

## *Animals*

National Institutes of Health guidelines for the humane treatment of animals were followed throughout the course of the experiment. Male Hartley outbred guinea pigs, 350-400g, were obtained from Charles River (colony K81). Guinea pigs were housed individually and acclimated for one week prior to virus infection. Animals were inoculated intraperitoneally with 1000 pfu of P2 or P18 in 1 ml, or with 1 ml of diluted mock-infected spleen cell homogenate. At days 1, 2, 6, and 12, seven guinea pigs from each group were sacrificed. Prior to sacrifice, blood was collected via cardiac puncture and stored in lithium heparin coated plasma separator tubes (Microtainer, Franklin Lakes, NJ). Plasma was reserved and RNA was isolated from the remaining cell pellet. Post-sacrifice, peritoneal cells were harvested by lavage with ice-cold PBS without Ca2+ and Mg2+ and stored on ice. Cells were centrifuged and RNA isolated from the cell pellet. Small portions of liver and spleen were harvested and stored in RNA Later (Ambion,

Austin TX) according to the manufacturer's instructions. Cytospin preparations of peritoneal cells and blood smears were stained using Leukostat (Fisher Scientific) for differential cell counts. At days 6 and 12, animals were considered outliers and excluded from cytokine analysis when their rectal temperature did not reach 39.8$^{o}$C, and viremia was undetectable (<10 pfu/ml) by plaque assay at the time of sacrifice. Because the disease outcome could not be known at days 1 or 2, all seven 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 using the RNA Ribo-Pure blood kit (Ambion, Austin TX) according to the manufacturer's instructions. Briefly, red blood cells were lysed, followed by a phenol/chloroform extraction and RNA purification through a glass fiber filter. Peritoneal cell RNA was isolated using the RNA Aquaeous kit from Ambion (Austin, TX) per manufacturer's instruction. To isolate RNA from the spleen and liver, 1-2 mm$^3$ pieces were removed from the larger pieces initially stored in RNAlater, and placed in tubes containing a 5mm stainless steel bead and Ultraspec RNA isolation reagent (Biotecx, Houston TX). Tissues were homogenized twice for two minutes each using a Mixer Mill machine (Qiagen, Valencia, CA). Following homogenization and a five minute incubation on ice, 200ul of phenol-chloroform were directly added to the tube. Samples were then vortexed and placed on ice for 10 minutes. The samples were then centrifuged at 16,100 x g for 1 minute and the aqueous layer

transferred to another tube. One volume of 75% ethanol in nuclease-free water was added to the aqueous layer and the solution was passed through a glass-fiber column from the RNA Aqueous kit. RNA was washed and eluted according the RNA Aqueous kit instructions. After elution of RNA, all RNA was DNAse treated using the DNA-free DNAse kit (Ambion, Austin TX) according the manufacturer's instructions. Following RNA extraction and DNAse treatment, RNA was analyzed for cytokines by real time RT-PCR as described in chapter 2.

### *Plaque assay*

Plasma samples collected at days 6 and 12 post-infection were analyzed for infectious virus by a Vero cell plaque assay, which was performed as described in chapter 2.

### *Indirect immunofluorescence assay*

Antigen dot slides for indirect immunofluorescence assay (IFA) were made by infecting Vero cells with P18 at a multiplicity of infection of 1. At day 4 post-infection, $10^3$ infected Vero cells were dried onto the wells of glass antigen dot slides (Erie Scientific, Portsmouth, NH), fixed for 10 minutes in ice-cold methanol, and stored at -20$^{o}$C until use. Wells were blocked against non-specific antibody binding in blocking solution (PBS, 1% v/v bovine serum albumin (BSA), 5% v/v normal horse serum (NHS)) for 30 minutes at room temperature. Blocking solution was carefully removed and serial 2-fold dilutions of serum in dilution solution (PBS, 1% v/v BSA, 1% v/v NHS) were added in duplicate to antigen wells. Dilutions ranged from 1:20 to 1:360 for each serum

sample. Slides were incubated in a humid chamber for 30 minutes at room temperature. Slides were then washed with stirring twice for 7 minutes each in PBS. A 1:80 dilution of rabbit anti-guinea pig FITC-conjugated antibody to Ig (Accurate Chemical, Westbury, NY) in dilution solution was applied to each well, and slides were incubated 30 minutes at room temperature in the dark. Following subsequent washing, a counterstain of Evan's Blue was applied (Sigma, St. Louis, MO). Each slide contained a positive control well of serum with known antibody titer and a negative control well of secondary antibody only. Slides were examined by fluorescent microscopy and final antibody titer was defined as the highest dilution of serum containing fluorescent cytoplasmic granular staining.

### *Measurement of plasma cytokines by bioassay and ELISA*

Commercial antibodies or ELISA's are not available for guinea pig cytokines, however, the human IL-8 ELISA from R&D Systems has previously been shown to cross-react with guinea pig, and was used for these experiments (Kuo et al., 1997; Lyons et al., 2002). TNF was measured by an L929 cytotoxicity bioassay as previously described (Aronson et al., 1995; Lasco et al., 2003).

### *Statistics*

Significant differences of cytokine mRNA ΔCt values between groups were analyzed by one-way analysis of variance with a Bonferroni adjustment for multiple comparisons. Correlations between cytokine mRNA and virus mRNA, or cytokine mRNA and differential cell counts, were analyzed using Pearson product moment

correlation analysis. All analyses were performed using Sigma Stat 3.0.1 (SPSS Inc.). Statistical significance is defined as p value<0.05.

RESULTS

After infection by the intraperitoneal route, peritoneal macrophages are the initial target of Pichinde virus (Aronson et al., 1994). Virus next becomes detectable in spleen, and subsequently in liver and other visceral, reticuloendothelial organs prior to the onset of viremia (Connolly *et al*., 1993; Jahrling *et al*., 1981; Aronson *et al*., 1994). For the purposes of this study, cytokine responses were studied at early time points (days 1-2) in the initial target cell (peritoneal cells) and mid-late time points in liver and spleen (days 6 and 12). Peripheral blood leukocyte cytokine expression was examined at all the aforementioned time points. Data from the mock-infected group is explicitly illustrated because we felt it important to demonstrate any cytokine changes resulting from stress, manipulation, or mock-treatment.

## *Disease phenotypes during Pichinde infection*

In the course of this study, we noted that there was not universal mortality for the guinea pigs challenged with virulent P18 virus. Whereas we have previously reported uniform lethality of P18 in outbred guinea pigs with an $LD_{50}$ of 3 plaque-forming units (Zhang *et al*., 2001; Zhang *et al*., 1999), over the last few years, using the same viral stock and dose, we have noticed a variable susceptibility of guinea pigs to Pichinde infection. In this study, by day 12 post-infection, four out of seven P18 infected guinea

pigs had developed disease with fever and other clinical signs, high serum viremia, high levels of viral RNA in the spleen, and seroconversion. Three out of the seven P18 infected guinea pigs remained clinically well and afebrile, did not have detectable viremia or antibody levels, and had very low viral RNA in spleens (Table 3). Similarly at day 6 post-infection, there were three P18 infected animals and one P2 infected animal that displayed no clinical signs or evidence of viral replication. We have labeled the guinea pigs that do not develop P18 disease "P18 disease resistant." P18 disease resistant animals were excluded from the cytokine analyses described below.

Table 3. Disease indicators of P2 and P18 virus "susceptible" vs. "resistant" guinea pigs at day 12.

| virus | Serum titer pfu/ml | IFA titer | fever? | weight change[a] | spleen virus ΔCt |
|---|---|---|---|---|---|
| P2 | Undetectable | >1:320 | Y | +37 | -1.7 |
| P2 | Undetectable | 1:320 | N | +88 | 5.4 |
| P2 | Undetectable | 1:320 | N | +51 | 2.9 |
| **P2** | **undetectable** | **neg** | **N** | **+110** | **6.9** |
| P2 | Undetectable | 1:320 | N | +68 | 7.5 |
| P2 | Undetectable | 1:320 | N | +37 | 1.7 |
| P2 | Undetectable | 1:320 | N | +33 | 0.9 |
| P18 | $2.05x10^5$ | 1:320 | Y | -73 g | -7.2 |
| P18 | $9.05x10^5$ | >1:320 | Y | -87 g | -6.4 |
| P18 | $4x10^3$ | 1:80 | Y | -3 g | -4.0 |
| P18 | $1.35x10^4$ | 1:160 | Y | -31 g | -4.2 |
| **P18** | **undetectable** | **neg** | **N** | **+102 g** | **8.7** |
| **P18** | **undetectable** | **neg** | **N** | **+110 g** | **4.3** |
| **P18** | **undetectable** | **neg** | **N** | **+146 g** | **14.8** |

[a] weight change from day 0 to day 12

### *Cytokine patterns from peritoneal cells and PBL early in infection.*

Examination of cell populations in the peritoneal cavity indicated a slight increase in granulocytes, predominantly neutrophils, in P18 infected animals (Table 4). Kurloff cells, the unique population in guinea pigs with reputed NK-like activity (Debout *et al*., 1993), were not observed in the peritoneal cavities. To determine cytokine patterns at the initial site of infection, peritoneal cells collected at days 1 and 2 post-infection were analyzed for cytokine mRNA levels. Peritoneal cells from P18 infected animals at day 2 had a striking increase in mRNA levels of IFN-γ and MCP-1 (CCL2) when compared to P2 and mock infected animals (Figure 12a-b). Of all ten cytokines examined, these were the only two that showed differential mRNA expression in infection with virulent vs. attenuated viruses. Notably, neither virus significantly induced mRNA expression of TNF-α, IL-1β, or IL-12p40 from peritoneal cells. However, P18 infection led to decreased peritoneal cell mRNA expression of pro-inflammatory cytokines TNF-α and IL-12p40, and the chemokine IL-8 (CXCL8) compared to mock infection (Figure 12a-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. However, peritoneal cells from P2 infected guinea pigs had suppressed mRNA expression of IL-1β compared to mock-infected cells (Figure 12a). Overall, infection with virulent P18 virus was associated with an early increase in IFN-γ and MCP-1 expression and suppression of certain pro-inflammatory cytokines in the initial target cells.

Table 4. Differential cell counts for peritoneal cells and peripheral blood of P2 and P18 infected guinea pigs

**Peritoneal cells**

| | %neutrophils | | | %lymphocytes | | | %macrophages | | |
|---|---|---|---|---|---|---|---|---|---|
| | P2 | P18 | mock | P2 | P18 | mock | P2 | P18 | mock |
| day 1 | 29.5 +/- 23.0 | 27.2 +/- 13.0 | 28.4 +/- 17.4 | 21.7 +/- 16.4 | 21.1 +/- 15.8 | 10.6 +/- 8.5 | 48.8 +/- 28.0 | 51.8 +/- 13.5 | 61.0 +/- 17.4 |
| day 2 | 32.6 +/- 11.7 | 37.1 +/- 14.3* | 16.1 +/- 7.7 | 12.2 +/- 8.4 | 7.8 +/- 5.4 | 9.7 +/- 11.1 | 55.2 +/- 12.2 | 55.1 +/- 13.8 | 74.3 +/- 14.3 |

**Peripheral blood leukocytes**

| | %neutrophils | | | %lymphocytes | | | %monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | P2 | P18 | mock | P2 | P18 | mock | P2 | P18 | mock |
| day 1 | 42.3 +/- 21.2 | 30.4 +/- 15.6 | 31.8 +/- 19.9 | 54.3 +/- 20.4 | 64.7 +/- 17.8 | 67.0 +/- 19.7 | 2.6 +/- 2.05 | 3.4 +/- 4.9 | 0.9 +/- 1.1 |
| day 2 | ND[a] | 31.7 +/- 11.5 | 23.8 +/- 5.1 | ND | 61.6 +/- 12.1 | 62.5 +/- 6.8 | ND | 4.21 +/- 0.5 | 9.4 +/- 4.1 |
| day 6 | 57.3 +/- 5.8 | 70.9 +/- 16.6 | 25.8 +/- 10.1 | 37.0 +/- 7.9 | 23.4 +/- 12.1* | 70.4 +/- 11.5 | 5.4 +/- 4.4 | 4.8 +/- 3.6 | 2.2 +/- 1.2 |
| day 12 | 35.2 +/- 13.7 | 62.2 +/- 4.7 | 37.6 +/- 20.2 | 57.5 +/- 15.0 | 25.2 +/- 1.9 | 57.9 +/- 21.0 | 6.4 +/- 3.6 | 12.5 +/- 5.7* | 4.1 +/- 3.2 |

*Indicates $p<0.05$ vs mock [a]Not determined

Cell types were distinguished by morphology as described in the methods

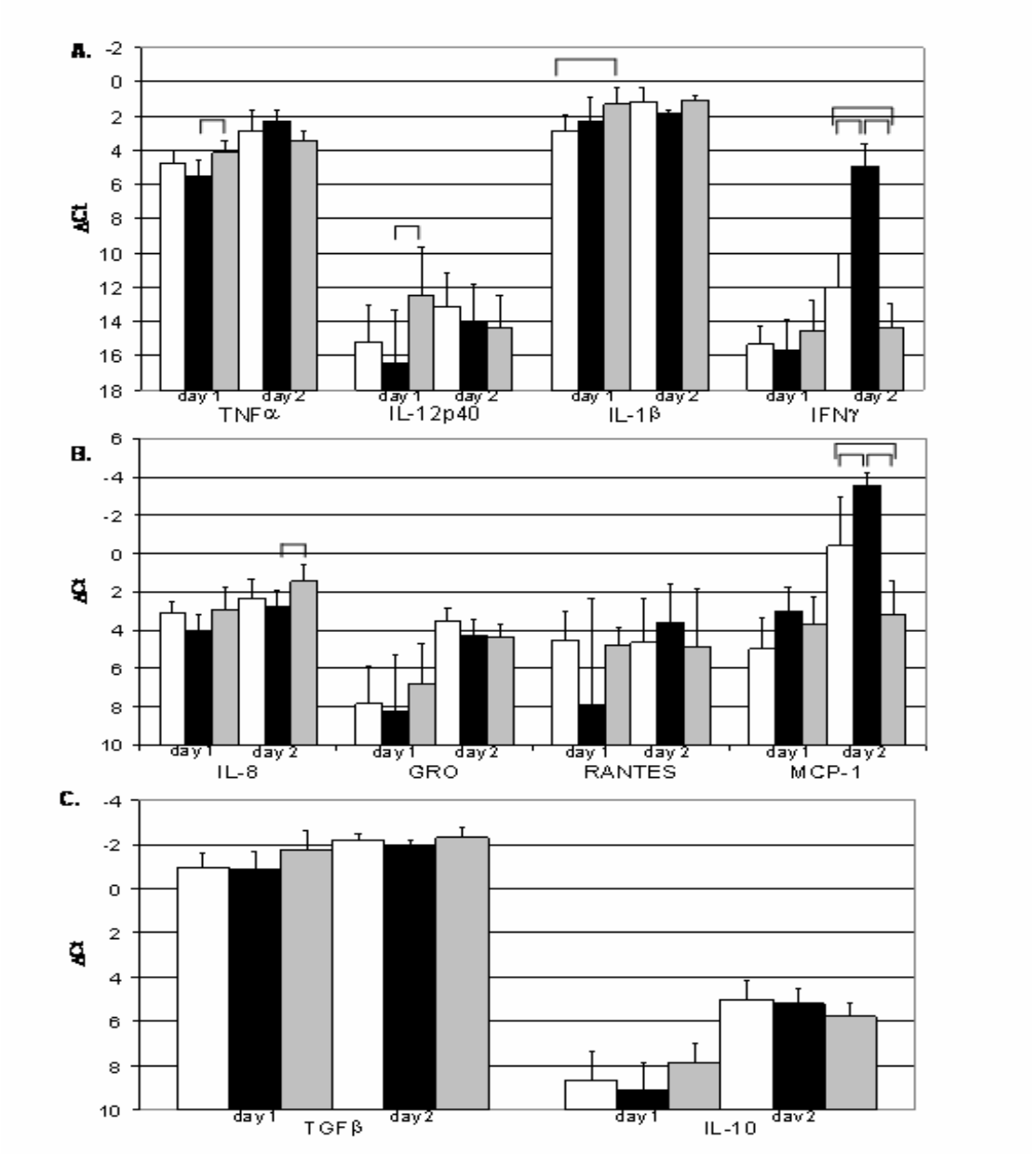


*Figure 12 Cytokine mRNA levels from peritoneal cells harvested day 1 and day 2 post-infection*. Mean ΔCt values with standard deviation are shown for seven animals each for P2-infected group (white bars), P18-infected-group (black bars) and mock-infected group (gray bars). $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$. **(A)** Pro-inflammatory cytokines **(B)** chemokines **(C)** anti-inflammatory/regulatory cytokines. Brackets indicate p value <.05 between groups by one-way ANOVA with Bonferroni adjustment for multiple comparisions.

Analysis of peripheral blood leukocyte (PBL) cytokine levels at days 1 and 2 did not mirror peritoneal cell mRNA levels, although a suppression of cytokines by P18 infection was again revealed. IL-8 and RANTES (CCL5) mRNA were suppressed in P18 infected PBL compared to P2 and mock (Figure 13). There were no other cytokine differences between groups at these timepoints. IL-1β and IL-10 mRNA's were undetectable from PBL at days 1 and 2. Differential cell counts of the blood at days 1 and 2 revealed no alterations (Table 4). Total peripheral white blood cell counts were not done in our study, but leukopenia during severe Pichinde disease has been reported by others (Cosgriff et al., 1987; Connolly et al., 1993). Similar to peritoneal cells, PBL from P18-infected animals showed a suppression of certain chemokine responses, and there was no upregulation of cytokine mRNA early in infection by either virus.

### *Cytokine mRNA levels in the spleen at mid-late stage infection*

The spleen is an important site of viral infection and pathology during infection with Lassa virus and its surrogate Pichinde (Aronson *et al*., 1994; Walker *et al*., 1982). In this experimental model, virus spreads quickly from the peritoneal cavity to the spleen, and virus titers in the spleen for P18 exceed those for P2 (Jahrling *et al*., 1981; Connolly *et al*., 1993; Aronson *et al*., 1994; Zhang *et al*., 2001). We measured cytokines in the spleen to determine whether patterns of expression correlated with disease outcome or viral load. At day 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 were

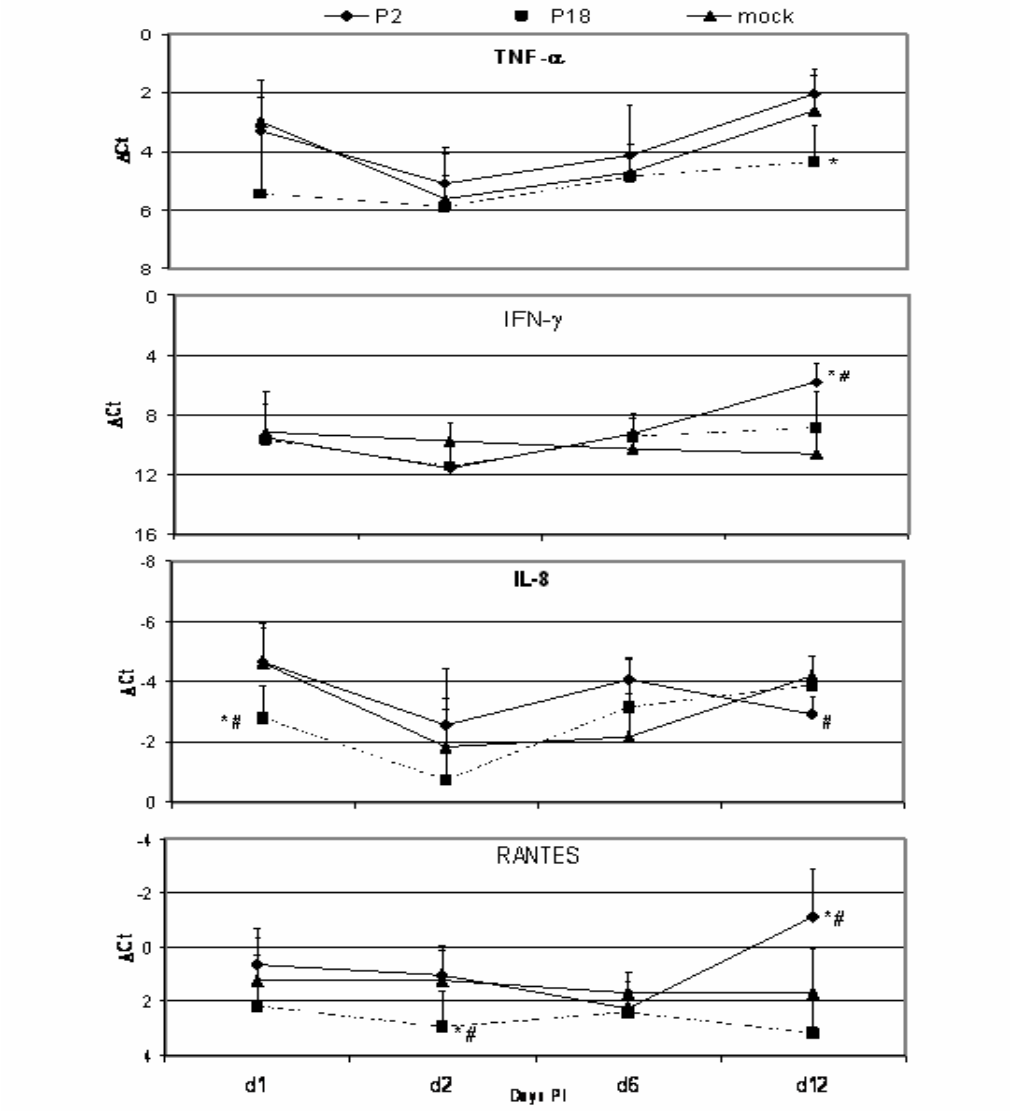


*Figure 13. Cytokine mRNA levels from peripheral blood leukocytes over time.* Mean ΔCt values and standard deviations are shown for indicated cytokines, comparing P18-infected animals (square symbol-dashed line), P2-infected animals (diamond symbol) and mock-infected animals (triangle symbol). For day 6 and 12 analyses, only animals with clinical signs of disease and measurable viremia are included. Group sizes are as follows: all groups n=7 for days 1 and 2; Days 6 and 12, P18 n=4, P2 n=6, mock n=7. Statistical significance is defined as p value <.05 by one-way ANOVA with Bonferroni adjustment for multiple comparisions. Error bars indicate standard deviation of the mean. * indicates significant difference compared to the other virus variant, # indicates significant difference compared to mock only.

elevated in both P2 and P18 infected spleens, and IL-1β and IL-12p40 were suppressed in both P2 and P18 infected spleens when compared to mock (Figure 14a). Chemokines IL-8, GRO, and MCP-1 showed increased relative mRNA levels in P18 infected spleens compared to P2 or mock-infected spleens. By contrast, RANTES mRNA expression was decreased in P18-infected spleens at day 12 (Figure 14b), echoing the lower RANTES levels in PBL from P18 infected animals at day 2. For the regulatory cytokines, P18 infection suppressed TGF-β mRNA levels in the spleen compared to mock-infected animals at both days 6 and 12. At day 12, there was a slight increase in IL-10 expression in P18-infected spleens compared to P2-infected spleens, in which IL-10 levels were down-regulated relative to mock-infected spleens (Figure 14c). Overall, the results suggest that higher levels of chemokine expression correlate with higher virus titers and splenic pathology associated with P18 infection, and that over-expression of pro-inflammatory cytokines in the spleen are not responsible for terminal shock of P18 disease.

### *Cytokine mRNA levels in the liver at mid-late stage infection*

The liver is also an important target organ in Lassa fever and Pichinde infection (Walker *et al*., 1982; McCormick *et al*., 1986b; Callis *et al*., 1982; Connolly *et al*., 1993; Aronson *et al*., 1994). Similar to the spleens, livers in P18 infected animals have moderate pathological changes with high virus titers, compared to P2 infected livers which have minimal pathological changes and low virus titers at day 12 (Aronson,

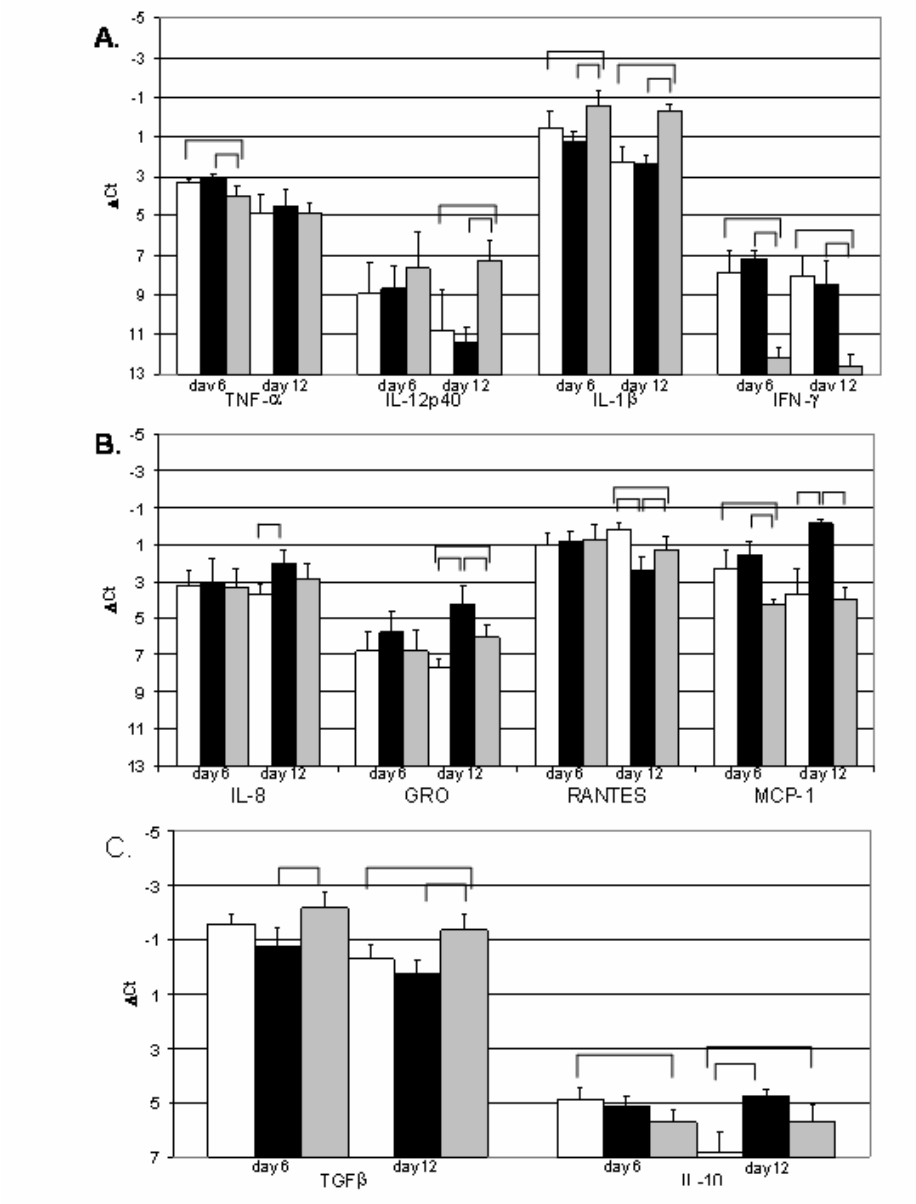


*Figure 14. Cytokine mRNA levels from spleens harvested day 6 and day 12 post-infection.* Mean ΔCt values and standard deviations are shown for P2-infected group (white bars, n=6), P18 infected group (black bars, n=4) and mock-infected group (gray bars, n=7). ΔCt = Ct cytokine- Ct GAPDH . Only animals showing clinical signs of illness and viral replication were included in the analysis. (A). pro-inflammatory cytokines (B). chemokines (C). anti-inflammatory cytokines. Brackets indicate p value <.05 between groups by one-way ANOVA with Bonferroni adjustment for multiple comparisons.

unpublished data). In terms of hepatic cytokine mRNA levels, there were few differences between P18 and P2 infection. P18 infected livers had elevated IL-8 at day 6, and suppressed IL-1β at day 12, compared to P2 infected livers (Figure 15a-b). Livers from both P18 and P2 infected animals had generally increased levels of the pro-inflammatory cytokines TNF-α and IFN-γ, when compared to mock infected livers. Similar to the spleens, the livers did not show differential elevation of pro-inflammatory cytokine mRNA's in P18 infection compared to P2 infection. Both P2 and P18 infected livers also showed increased mRNA levels for IL-8, GRO, MCP-1, RANTES, and TGF-β compared to mock infected livers (Figure 15a-c). Overall the results show parallel increases in liver cytokine and chemokine mRNA levels in both Pichinde infections, suggesting that liver over-expression of pro-inflammatory cytokines does not account for terminal shock and vascular collapse characteristic of P18 infection.

### *Cytokine mRNA levels in the PBL at mid-late stage infection*

Increased levels of pro-inflammatory cytokines in the serum have been suggested as the cause of terminal shock for Lassa fever. Contrary to this hypothesis, a pattern of suppression of PBL cytokines emerged. At day 12 post-infection, PBL from P18 infected animals had lower levels of TNF-α and IFN-γ mRNA compared to P2 infected PBL (Figure 13). Also of note, RANTES expression was lower for P18 infected PBL compared to P2-infected PBL at day 12, paralleling the relative suppression of this cytokine also seen in P18-infected spleens at day 6 (Figure 13). There were no differences between P2 and P18 mRNA levels for other PBL cytokines analyzed.

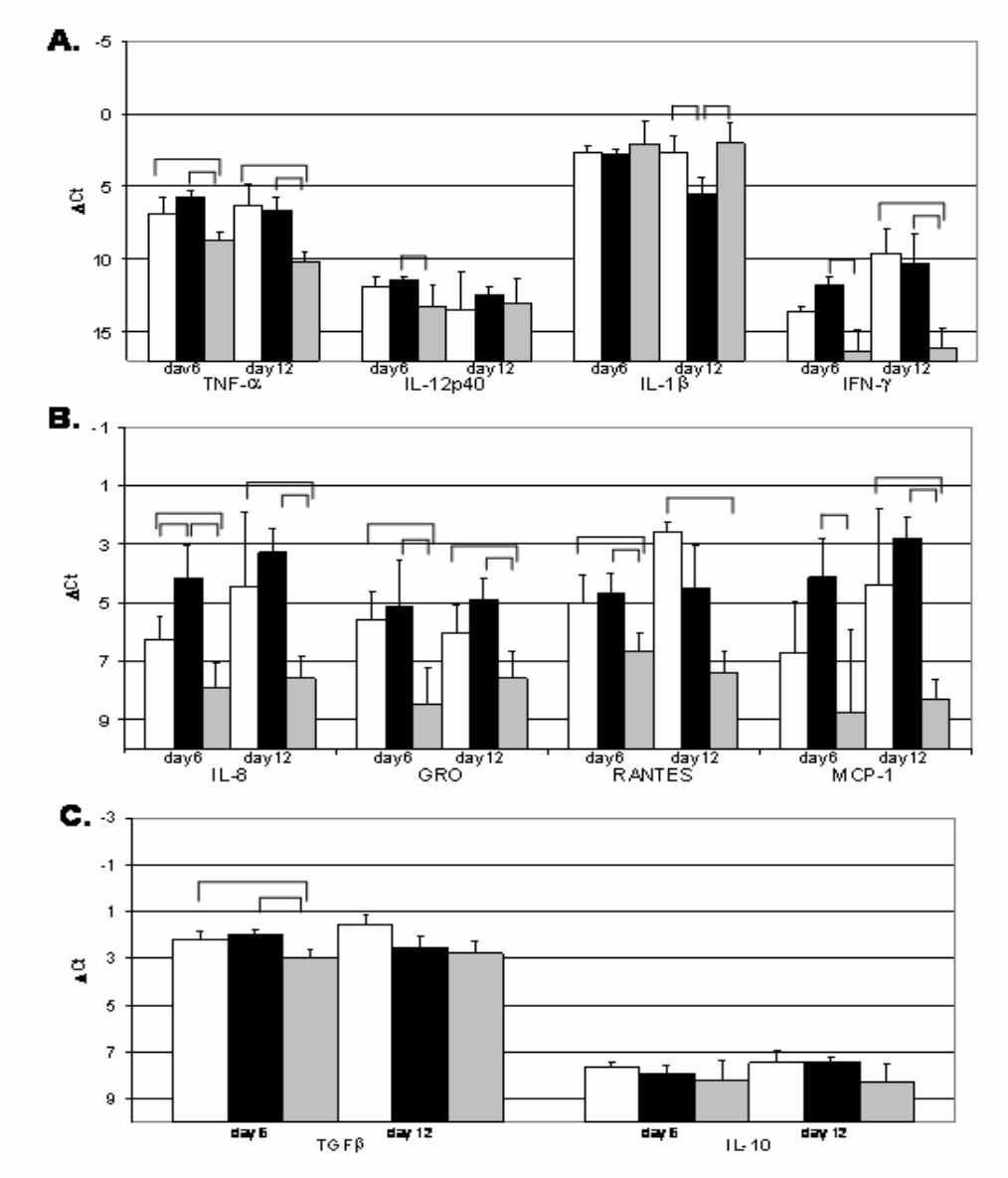


*Figure 15. Cytokine mRNA levels from livers harvested day 6 and 12 post-infection.*

Mean ΔCt values and standard deviations are shown for P2-infected group (white bars, n=6), P18 infected group (black bars, n=4) and mock-infected group (gray bars, n=7). ΔCt = Ct cytokine- Ct GAPDH . Only animals showing clinical signs of illness and viral replication were included in the analysis. (A). pro-inflammatory cytokines (B). chemokines (C). anti-inflammatory cytokines. Brackets indicate p value <.05 between groups by one-way ANOVA with Bonferroni adjustment for multiple comparisons.

However, P2 infection resulted in increased mRNA levels of IL-10 (day 6), IFN-γ, and RANTES (day 12) compared to mock infected PBL (Figure 13). Taken together, these results suggest that a suppression of cytokines, such as TNF-α, IFN-γ, and RANTES, is associated with a more severe infection. We did not find increased expression of pro-inflammatory cytokines to be associated with the lethal shock-like disease. Differential cell counts from the PBL confirm earlier reports of lymphopenia at day 6 (Jahrling *et al*., 1981; Cosgriff *et al*., 1987) and increased monocytes at day 12 in P18 infected guinea pigs compared to mock (Table 4).

## *Plasma cytokine levels by bioassay and ELISA*

Measurement of cytokine protein expression levels in the guinea pig is hampered by the paucity of immunological reagents for that species. Using available bioassays for TNF, and immunoassays for IL-8 and TGF-β that have been shown to cross-react with guinea pig cytokines, we did attempt to correlate mRNA and protein levels for those cytokines (Aronson *et al*., 1995; Kuo *et al*., 1997). No bioactive TNF could be detected in serum samples from any animal at day 12 post infection (limit of detection 32 pg/ml). IL-8 could not be detected in any serum sample from all groups at all time points (limit of detection 10 pg/ml). TGF-β was detectable and ranged from 200-500 pg/ml in peripheral blood, but in accordance with TGF-β PBL mRNA levels, there were no differences in levels between groups at any timepoint (data not shown).

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

Despite the fact that approximately 40% of P18 inoculated guinea pigs ultimately did not develop signs of disease or seroconversion, viral RNA levels in peritoneal cells during the first 2 days of infection with P18 were tightly clustered, showing no significant outliers (Figure 16). Quantitative real-time RT-PCR assay for viral RNA indicated that there was some degree of viral replication in peritoneal cells in all seven P18 infected animals at day 1 and day 2 post-infection (Figure 16). This assessment was based on two observations: 1) all animals inoculated with P18 displayed higher levels of viral RNA than P2-infected animals in the peritoneal and splenic compartments within the first two days of infection and 2) there were increased viral RNA levels at day 2 compared to day 1. This suggested to us that "disease resistance" was not simply the result of refractoriness of peritoneal cells of some animals to initial infection with P18.

We were particularly interested to determine whether differences in the cytokine patterns would correlate with virus levels early in infection, because of the strong correlation between virus replication and disease severity. When all P2 and P18 infected animals were considered together, Pearson product-moment correlation analysis demonstrated a positive correlation between peritoneal cell viral RNA level and MCP-1 mRNA at days 1 and 2. At day 2 in peritoneal cells, there was also a positive correlation between TNF-$\alpha$ and IFN-$\gamma$ mRNA levels and viral RNA levels (Figure 16). There were no cytokines for which a negative correlation was found.

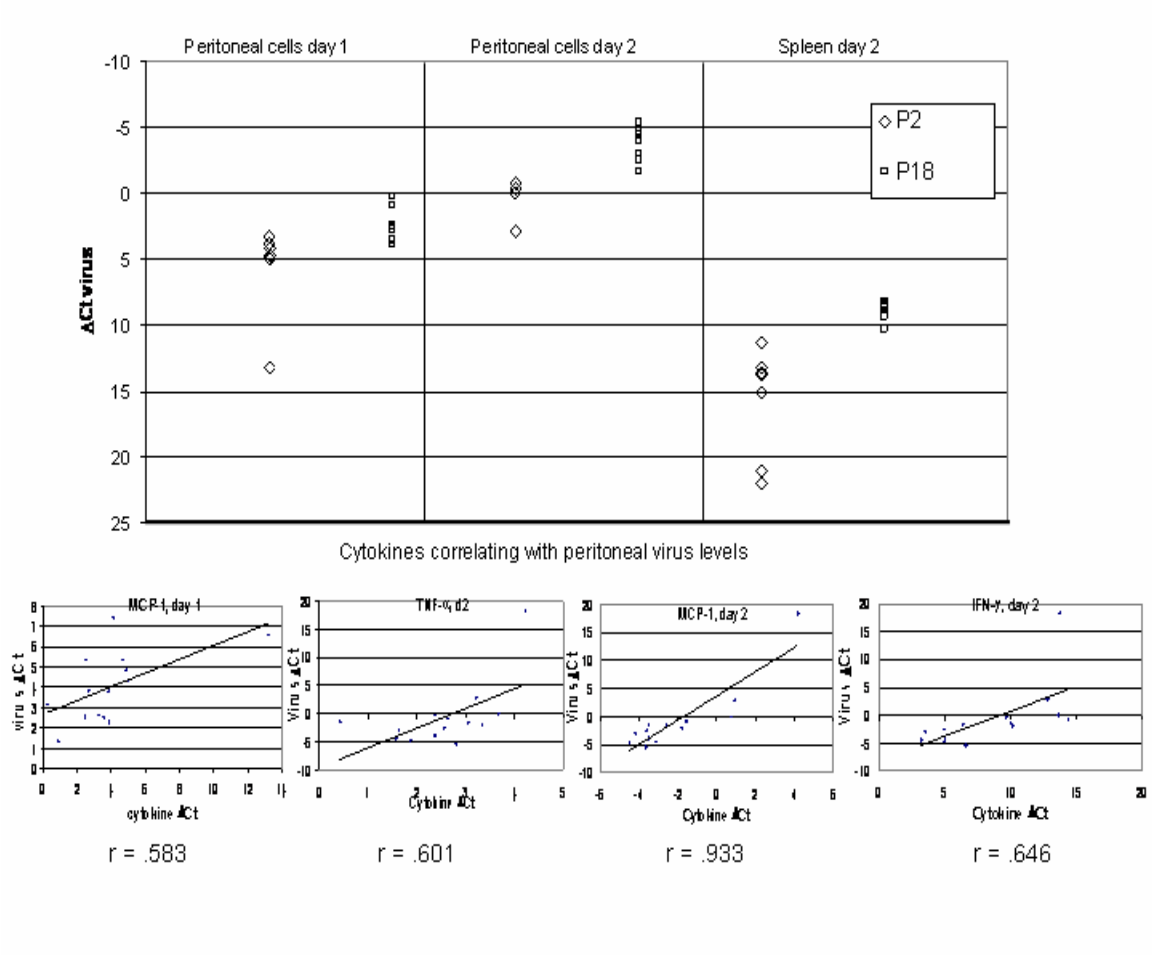


*Figure 16. Virus mRNA levels in peritoneal cells and spleen at days 1 and 2.* PIC viral RNA levels are shown in peritoneal cells or spleen tissue from P2 (diamond) or P18-infected (square) guinea pigs at days 1 and 2 post-infection. RNA was isolated from the peritoneal cells or spleen and analyzed by real-time RT-PCR with Pichinde GPC specific virus primers (table 2). Results are expressed in ΔCt; $\Delta Ct = Ct_{virus} - Ct_{GAPDH}$. Cytokines that showed a statistically significant correlation ($p<0.05$, Pearson product moment correlation analysis) between cytokine mRNA level and viral RNA level are indicated beneath the appropriate time point.

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 (cytokine data shown in figures 12-15). When analyzed separately, "disease resistant" P18 animals at days 6 and 12 showed distinct patterns compared to clinically ill, viremic animals. "Disease resistant" guinea pigs had cytokine patterns similar to those of mock infected animals in most compartments at most time points, with a few exceptions. Disease resistant guinea pigs showed alterations in GRO expression compared to mock infected animals, with increase in liver at day 6, and decrease in spleen and PBL at day 12. 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 to mock-infected animals.

### *PBL cytokine patterns associated with disease outcome*

In addition to the experiment discussed above (experiment #1), we were also interested to determine if we could find early and late PBL cytokine markers that correlated with outcome. For this experiment, PBL cytokine mRNA was measured in the same individuals throughout the course infection (experiment #2). Therefore we had early PBL samples for guinea pigs in which the outcome was known. During experiment #2, blood was collected from the guinea pigs by clipping a toenail, in contrast, blood was collected via cardiac puncture from experiment #1. When comparing P2 and P18 infected guinea pigs, there were no obvious PBL cytokine markers indicative of outcome from either experiment #1 or #2 (data not shown). However, there were two similarities

between the two experiments at day 12. First, both experiments demonstrated a sharp elevation (8-32 fold) of RANTES expression in P2 infected animals compared to both P18 and mock (Figure 13 and 17). Secondly, a distinct suppression of IL-1β was noted in P18 infected guinea pigs compared to P2 and mock in experiment #2 (Figure 17). A similar pattern of IL-1β suppression in P18 infected guinea pigs was seen in experiment #1, but did not reach statistical significance ($p=0.067$) (data not shown). These data indicate that the suppression of RANTES and IL-1β mRNA seen late in P18 infection compared to P2, is reproducible despite different methods of blood collection.

PBL cytokine patterns were also analyzed in order to detect markers of susceptibility between P18 susceptible and P18 resistant guinea pigs. From two separate experiments (experiment #2 and #3), we were unable to correlate day 1 and day 2 cytokine patterns with known outcome due to small samples sizes (n=1) of resistant guinea pigs. However, at day 12, P18 resistant animals from experiment #1 (n=3) and experiment #2 (n=1) had a pattern of suppressed GRO expression at day 12 compared to P18 susceptible, P2, and mock (Figure 18). These data emphasize that P18 resistant animals develop a unique cytokine response to P18 virus.

## DISCUSSION

Many factors including virus and host genetics may be involved in the spectrum of disease associated with Lassa fever, and there are likely immune mechanisms that control viral replication in mild and subclinical infections that have yet to be defined.

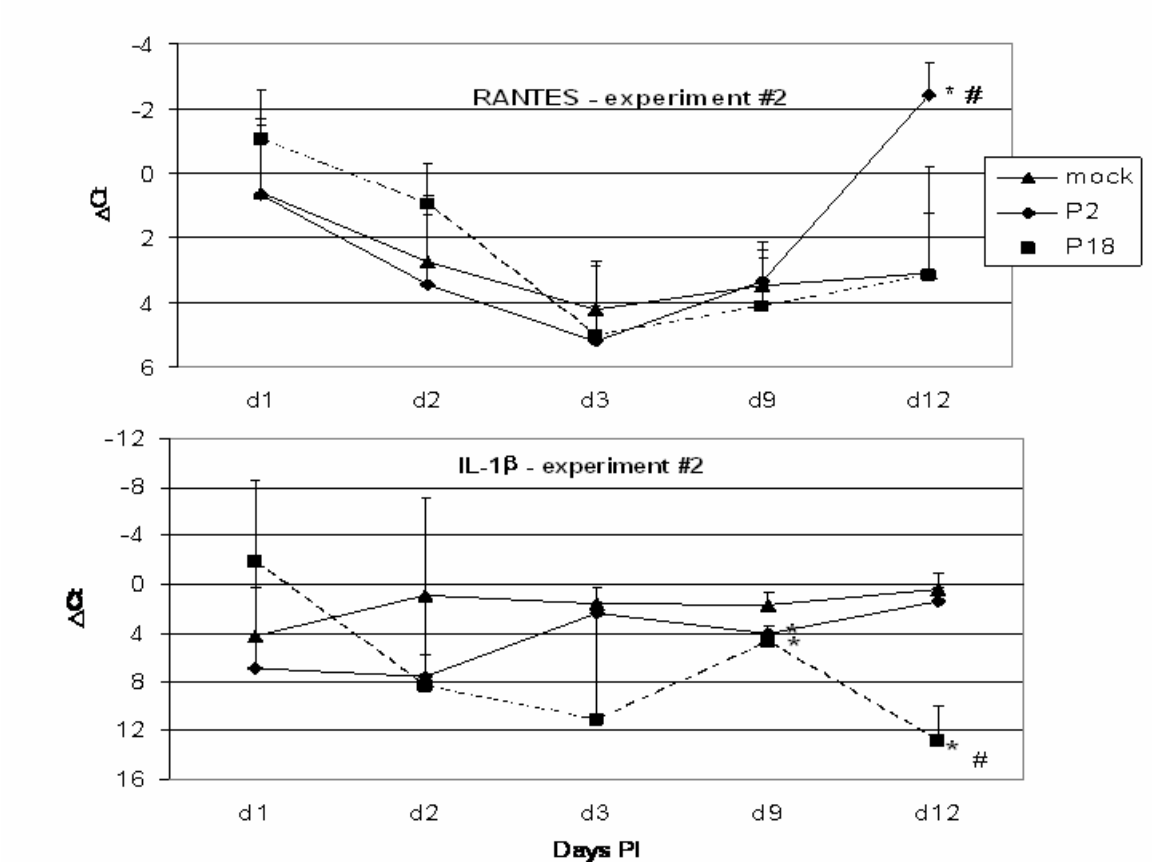


*Figure 17. RANTES and IL-1β mRNA from infected guinea pig PBL.* Guinea pigs were inoculated intraperitoneally with 1000 pfu of P2 (diamond), P18 (square, dashed line), or mock supernatant (triangle).Blood was collected at indicated timepoints by clipping a toenail. RNA was extracted from the PBL and analyzed by real-time RT-PCR for cytokines. Mock and P2, n=5. P18, n=4. $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$. Statistical significance is defined as p value <.05 by one-way ANOVA with Bonferroni adjustment for multiple comparisions. Error bars indicate the standard deviation. * indicates significant difference compared to the other virus variant, # indicates significant difference compared to mock only.

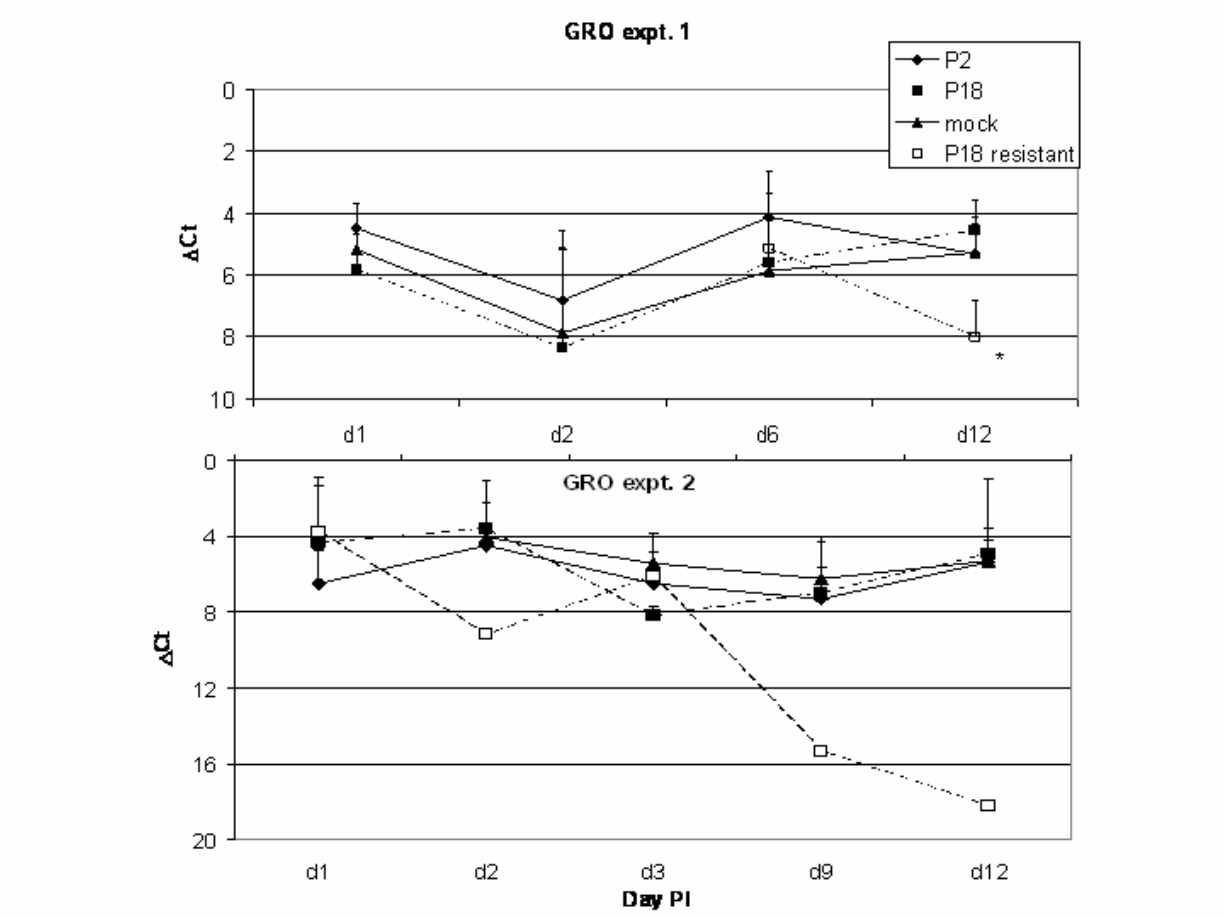


*Figure 18. Infected guinea pig PBL GRO mRNA from two independent experiments.* Guinea pigs were inoculated intraperitoneally with 1000 pfu of P2 (diamond), P18 (square, dashed line), or mock supernatant (triangle). Blood was collected at indicated timepoints by clipping a toenail (expt. 2), or by cardiac puncture (expt. 1). RNA was extracted from the PBL and analyzed by real-time RT-PCR for cytokines. Expt. #1, n=7 at days 1-2. Days 6 and 12, mock n=7, P2 n=6, P18, n=4, P18 resistant, n=3. Expt. #1, mock n=5, P2 n=5, P18 n=4, P18 resistant n=1. $\Delta Ct = Ct_{cytokine} - Ct_{GAPDH}$. Statistical significance is defined as p value <.05 by one-way ANOVA with Bonferroni adjustment for multiple comparisions. Error bars indicate standard deviation of the mean. * indicates significant difference compared to the other virus variant, # indicates significant difference compared to mock only.

The goal of this study was to characterize cytokine patterns in a Pichinde virus comparative model of Lassa fever, with a focus on cytokines important for innate immunity. This is the first detailed study of cytokine responses in a guinea pig model of arenavirus hemorrhagic fever. Our approach was two-fold: (1) to study very early cytokine responses in the initial target cells, testing the hypothesis that the virulent virus suppresses macrophage pro-inflammatory cytokine responses and (2) to study cytokine responses in reticuloendothelial target organs during later phases of infection, to test the hypothesis that over-expression of pro-inflammatory cytokines correlates with lethal shock and vascular collapse.

With regard to early cytokine responses, the most dramatic finding of our study was increased expression of IFN-γ and MCP-1 at day 2 in initial target cells of the virulent virus, P18. NK cells, γδ 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 *et al*., 2001; Schaible *et al*., 2000). Granulocytes can also be induced to produce IFN-γ (Ethuin *et al*., 2004). The cellular source of the IFN-γ we observed is unknown at this time. Kurloff cells, guinea pig NK cells, were not identified morphologically in peritoneal cavities, but an increase in granulocytes was noted. The increase in IFN-γ in P18 infection was somewhat unexpected, since IFN-γ has important antiviral and macrophage-activating effects in many systems. However, it has been previously shown that IFN-γ has minimal antiviral activity against Lassa virus in human cells (Asper *et al*., 2004). IFN-γ is a potent inducer of nitric oxide (NO) production from macrophages.

Interestingly, although nitric oxide can have antiviral effects, it can also exert pathogenic effects, possibly as a result of NO's ability to suppress T-cell responses (Akaike *et al*., 2001). During infection with virulent Pichinde variants, a marked suppression of T cell mitogen responsiveness has been observed (TR Jerrells, personal communication, unpublished results). An early suppression of T-cell proliferation by NO could explain this phenomenon and may contribute to lack of immune control of virus replication. Further studies are needed to determine the role of IFN-γ and nitric oxide in arenavirus pathogenesis

Paralleling the early increase in IFN-γ expression in P18 infected peritoneal cells was an increase in MCP-1 mRNA at day 2. MCP-1 (CCL2) 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 (Rollins et al., 2001). MCP-1 functions to recruit monocytes, but also T cells and NK cells to inflammatory sites . Elevated MCP-1 levels have been reported in a simian model of disseminated mycobacterial infection in SIV-infected macaques. These authors hypothesized that disease progression relates to an increased pool of susceptible target cells recruited by MCP-1 (Hendricks *et al*., 2004). It is plausible that MCP-1 could accelerate/enhance Pichinde infection by recruiting monocyte/macrophage target cells to the peritoneal cavity. In our study, we did not observe an increase in peritoneal macrophages in P18 infection at days 1 or 2, but later time points were not examined. We were interested to note that P18 infection was associated with increased MCP-1 mRNA levels in several

compartments at several time points. This suggested that MCP-1 could be reducing macrophage responsiveness to virulent virus. An analogous observation was that transgenic mice over-expressing MCP-1 had increased sensitivity to bacterial pathogens that grow within monocyte/macrophages (Rutledge *et al*., 1995). MCP-1 has also been found to directly enhance HIV-1 replication in human monocyte-derived macrophages, via a post-entry mechanism (Fantuzzi *et al*., 2003). There have been no studies on the effects of endogenous or exogenous MCP-1 on arenavirus replication to our knowledge.

Another important observation in the present study was a 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 was a slight decrease in TNF-α, IL-8, and IL-12p40 mRNA in peritoneal cells from P18 infected guinea pigs compared to mock-infected guinea pigs. These 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 nonpathogenic Mopeia virus (Lukashevich *et al*., 1999; Pannetier *et al*., 2004; Baize *et al*., 2004). We also observed a slight decrease in PBL IL-8 mRNA expression during the first days of P18 infection. This observation corroborates previous reports that decreased serum IL-8 levels are associated with lethal outcome in Lassa fever (Mahanty *et al*., 2001), and that Lassa inhibits IL-8 expression from human monocyte-derived macrophages and endothelial cells (Lukashevich *et al*., 1999). The mechanism by which Lassa and Pichinde 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 TGFβ. Another means of suppressing cytokine expression is through the inhibition of transcriptional activators like NF-κB. Indeed, we have previously shown that compared to P2, P18 infection of primary guinea pig macrophages fails to activate NF-kB RelA/p50 heterodimers, while increasing nuclear translocation of repressive NF-κB p50/p50 homodimers (Fennewald *et al*., 2002). The differential suppression of TNF-α responses suggests the possibility that TNF-α may exert a direct or indirect antiviral effect on Pichinde infected macrophages, as has been observed for other viruses (Mestan *et al*., 1986; Khaiboullina *et al*., 2000; Seo *et al*., 2002). However, in a previous report, TNF-α did not inhibit Lassa replication in human or primate liver or fibroblast cells (Asper *et al*., 2004). We have also found that neither exogenous human TNF-α nor IL-8 inhibits Pichinde replication in guinea pig macrophages (Figure 10). It is likely that TNF-α, IL-12, and IL-8 function by influencing recruitment, activation, and maturation of inflammatory and immune cells, rather than by directly inhibiting viral replication in target cells.

The second question addressed by this study involves the role of cytokines in the terminal shock associated with severe arenavirus disease. High levels of pro-inflammatory cytokines have been associated with disease severity in other hemorrhagic fevers including those caused by Ebola and Marburg viruses, hantavirus, and the South American arenavirus Junin (Peters *et al*., 2002b; Schnittler *et al*., 2003; Marta *et al*., 1999). However, in this comparative Pichinde model, we did not find differentially

increased pro-inflammatory cytokine transcripts in blood or organs during infection with the virulent, hemorrhagic fever-inducing P18 variant; TNF and IL-8 were not detected in serum of P18 infected animals at late time points. Instead, severe disease was associated with suppression of IFN-γ and TNF-α transcripts in PBL. Previous studies of cytokines in the terminal shock of Lassa fever have reported disparate results. One anecdotal clinical case report of Lassa fever noted terminal elevation in serum TNF-α and IFN-γ (Schmitz *et al*., 2002). Additional support for cytokine-mediated terminal shock comes from an animal study in which IL-1RA protected mice from death after intracerebral inoculation of Lassa, although viremia was not reduced (Ignatyev *et al*., 2000). This model has not been well-characterized, however, and may have significant pathological differences than human Lassa fever. Our lab has previously reported terminal increase in TNF bioactivity in sera from inbred strain 2 guinea pigs during Pichinde infection (Aronson *et al*., 1995). On the other hand, other reports have indicated suppression of pro-inflammatory cytokines associated with lethality. In an LCMV/primate model of Lassa, fatally infected rhesus macaques had undetectable serum TNF-α and IL-1β, with a moderate elevation of IFN-γ (Lukashevich *et al*., 2003). In humans, Mahanty *et al* reported a suppression of IL-8 and interferon inducible protein (IP-10) was associated with fatal Lassa fever compared to non-fatal Lassa (Mahanty *et al*., 2001). IFN-γ, IL-1β, and IL-12 were undetectable in five of six fatal Lassa cases. In addition, TNF-α was not detected in the serum from either group (Mahanty *et al*., 2001). Our lab has previously reported undetectable levels of TNF in virulent Pichinde infected outbred guinea pigs at

day 12 (Aronson *et al*., 1995). The present results reiterate that, contrary to other hemorrhagic fevers, Pichinde virus infection with terminal shock is not associated with high levels of pro-inflammatory cytokine expression.

It is notable that expression of the chemokine RANTES (CCL5) was often suppressed by P18 infection, both in the organs and PBL. We have repeatedly observed that severe Pichinde disease is associated with decreased PBL expression of RANTES mRNA (Scott and Aronson, unpublished data). A low level of RANTES is associated with poor outcome in septic shock, a syndrome many have considered similar to the terminal shock seen in hemorrhagic fevers (Cavaillon *et al*., 2003). RANTES is typically produced by T-cells approximately 3-5 days after activation (Song *et al*., 2000) and has chemoattractant activity towards T cells, monocytes, and eosinophils (Schall *et al*., 2001). Relative decrease in RANTES expression during P18 infection may suggest diminished T cell activation and suppression of cell-mediated immune responses. Cell-mediated immunity has been suggested to be vital for the clearance of arenaviruses, and a suppression of this response likely allows these viruses to replicate virtually unchallenged (Fisher-Hoch *et al*., 2000; Fisher-Hoch *et al*., 1987).

During the course of this study, we observed a variable resistance to Pichinde disease in outbred guinea pigs, as had been reported previously (Jahrling *et al*., 1981). Although 40% of P18-inoculated guinea pigs ultimately did not develop viremia, antibodies, or clinical signs, all animals inoculated with the virulent P18 showed similar levels of viral replication within the first two days of infection. Events important in

clearance for the P18 "resistant" animals likely occurred between days 2 and 6 of infection. With the design of this study, we were unable to determine whether induction of specific cytokine patterns in initial target cells could account for differences in disease expression. Additionally, cytokine expression patterns in animals infected with the attenuated P2 virus differed from those animals which appeared "resistant" to P18 disease. This observation suggests that putative cytokine mechanisms of viral clearance differ for animals infected with P2 and animals capable of halting P18 infection at an early stage. 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.

In summary, we report the first detailed study of cytokine responses in a guinea pig model of Lassa fever. Acknowledging the limitations inherent in the guinea pig model that allowed us to measure transcripts only, we conclude that: 1) severe arenavirus hemorrhagic fever causes a suppression of selected pro-inflammatory cytokines very early in infection in initial target cells, 2) early induction of MCP-1 and IFN-γ in initial target cells is associated with progression of disease, and 3) lethal hemorrhagic fever is not associated with over-expression of pro-inflammatory cytokines. Future studies should investigate the role of each of these cytokines on arenavirus replication, pathogenesis, and immunity.

# CHAPTER 4: TREATMENT OF P18-INDUCED DISEASE WITH AN AP-1 THIOAPTAMER

## INTRODUCTION

Lassa fever, caused by the arenavirus Lassa, is endemic in areas of western Africa where there are an estimated 100,000-300,000 cases per year (McCormick *et al*., 1987b). Lassa virus has been classified a category A bioterrorism threat. Lassa virus infection of humans causes a wide-spectrum of manifestations, from asymptomatic to fatal hemorrhagic fever (McCormick *et al*., 1987b). Those patients who succumb to Lassa fever die of a shock-like syndrome, the pathogenesis of which has been particularly elusive since Lassa is non-cytopathic and no specific organ lesion seems to be responsible for death (Walker *et al*., 1982). In addition, there is little evidence of immune cell infiltrates, suggesting immunopathology is not a significant pathogenic mechanism (Walker *et al*., 1982). A hallmark of Lassa infection is the correlation between the level of serum viremia and mortality, but the mechanisms by which viral replication is limited in mild cases are unknown (Johnson *et al*., 1987). Neutralizing antibody does not appear to be primarily responsible for clearing virus during natural or experimental infection (Johnson *et al*., 1987; Fisher-Hoch *et al*., 1987). Previous studies suggest that cell-mediated immune responses are likely important for the resolution of infection (Fisher-Hoch *et al*., 1987), (TJ Jerrells, unpublished data). However, this study explores potential innate immune contributions to limiting viral replication in a model of Lassa fever.

Lassa infection of target cells inhibits expression of certain cytokines involved in innate immunity, such as TNFα, IL-1β, and IL-8 (Baize *et al*., 2004; Lukashevich *et al*., 1999; Mahanty *et al*., 2001; Mahanty *et al*., 2003). Proinflammatory cytokines in particular regulate innate responses such as induction of nitric oxide synthase and apoptosis, and contribute to adaptive responses by activating immune cells (Barber *et al*., 2001; Guidotti *et al*., 1994; Karupiah *et al*., 1993; Komatsu *et al*., 1999). Additionally, cytokines such as TNFα and type I and II interferons are well known to have direct antiviral effects (Guidotti *et al*., 2000a). Existing data suggest boosting an early pro-inflammatory response during arenavirus infection is a potential therapeutic strategy. To this end, we have targeted transcription factors that control cytokine expression in order to modulate cytokine expression and disease outcome. Our goal was to determine if a modified DNA oligonucleotide targeting the AP-1 family of transcription factors could modulate cytokine expression *in vivo*, and alter morbidity and mortality in a guinea model of arenavirus hemorrhagic fever.

DNA and RNA oligonucleotides can be designed to directly bind and inhibit transcription factors such as NFκB and AP-1 (Horishita *et al*., 1997; Lenert *et al*., 2003; Bielinska *et al*., 1990). These oligonucleotides, or "aptamers", have therapeutic potential by inhibiting transcription factors that regulate expression of proteins that are relevant in the pathogenesis of a disease. This concept has already been shown effective in that administration of NF-κB aptamers ameliorate rat myocardial infarction and experimental colitis in mice (Horishita *et al*., 1997; Neurath *et al*., 1996). Modification of aptamers to

include dithiophosphates enhances protein binding, stability, and the ability to enter cells (King *et al*., 2002; Marshall *et al*., 1993; Yang *et al*., 2002a).

The AP-1 transcription factor family consists of dimers of Jun, Fos, Maf, ATF, and CREB sub-family of proteins (reviewed in (Karin *et al*., 1997)). These dimers have a variety of functions including the regulation of cell proliferation, cell death, inflammation, and cytokine expression (Angel *et al*., 1991; Koj *et al*., 1996; Liebermann *et al*., 1998). Our group has shown that the AP-1 dithioaptamer "XBY-S2" binds mainly to complexes containing Fra 2 and JunB proteins in P388D1 murine macrophage-like cells (Fennewald et al, submitted for publication), proteins which have known repressive effects on AP-1 activity (Rutberg *et al*., 1997; Suzuki *et al*., 1991). Treatment of cells with XBY-S2 increased TNFα, IL-6, and IL-12 expression in poly I:C treated P388D1 cells (murine monocyte cell line); these observations are compatible with the proposed action of XBY-S2 as a decoy "inhibitor" of a repressive AP-1 dimer(s) (Fennewald,el al, submitted for publication). We sought to determine the potential of XBY-S2 to upregulate cytokines *in vivo* during experimental arenavirus infection.

To test the aptamer, XBY-S2, we utilized the Pichinde virus model of arenavirus hemorrhagic fever in guinea pigs. A high passaged variant of Pichinde CoAn 4763 (P18), while avirulent in humans, leads to death in the guinea pigs by a shock-like syndrome in approximately two weeks, with pathology similar to human Lassa fever (Aronson *et al*., 1994; Connolly *et al*., 1993; Jahrling *et al*., 1981; Liu *et al*., 1982; Zhang *et al*., 2001). P18 infection of P388D1 cells induced only moderate levels of TNF-α and

IL-6 compared to that of an attenuated variant of Pichinde (P2) (Fennewald *et al*., 2002). P18 infection also caused a suppression of TNF-α and IL-6 in LPS-stimulated P388D1 cells, and induced large amounts of the inhibitory p50/p50 NF-kB dimer in guinea pig peritoneal macrophages (Fennewald *et al*., 2002). These results suggest P18 is inhibiting the activation of macrophages, thereby preventing an adequate cytokine response that could potentially inhibit viral replication, similar to Lassa virus. We hypothesize that treatment of P18 infected guinea pigs with AP-1 thioaptamers would increase cytokine expression *in vivo*, reducing viremia and increasing survival.

MATERIALS AND METHODS

## *Thioaptamer synthesis*

XBY-S2 was synthesized as previously described for phosphodithoiated aptamers (Yang *et al*., 1999). Following oxidation of the thiophosphite triesters with a sulfurization reagent (EDITH), the crude oligonucleotides were purified by FPLC ion exchange with a Mono Q HR 10/10 column (Pharmacia) (Yang *et al*., 2002b; Xu *et al*., 1996). After purification, the oligonucleotides were desalted and concentrated with Centricon-3 centrifugal concentrators (Amicon). Complementary strands were mixed in equal molar concentrations, annealed at 90$^{o}$C, and cooled slowly for duplex formation.

| XBY-S2 | XBY-S1 |
|---|---|
| 5’ CC AGTsG ACTsC AGTsG 3’ | 5’ TsTsGCGCGC A AC ATsG 3’ |
| 3’ GGs TCA CsTGA GsTCA C 5’ | 3’ A A CGCGCGsTsTGsTA C 5’ |

## *Virus and aptamer/liposome preparation*

For XBY-S2 treatment, Tfx-50 liposomes (Promega, Madison, WI) were used as a delivery vehicle. Liposomes were prepared exactly according to manufacturers instructions. Thirty minutes prior to guinea pig inoculation, 200ug of aptamer in 40ul nuclease free water was added to one vial of prepared lipid (400ul, 1mM lipid, DNA:lipid charge ratio = 1.3:1)(Ono *et al*., 1998) and incubated at room temperature for thirty minutes Immediately prior to intraperitoneal injection, liposomal aptamer was diluted 10-fold in endotoxin-free PBS containing $Ca^{2+}$ and $Mg^{2+}$ for a final concentration of 50μg aptamer/ml to reduce toxicity.

Pooled guinea pig spleen stock of Pichinde variant P18 virus derived from Pichinde Munchique strain (CoAn 4763) (Zhang *et al*., 1999) was diluted to 1000 plaque forming units/ml in endotoxin-free PBS containing $Ca^{2+}$ and $Mg^{2+}$, and used for *in vivo* experiments.

## *Thioaptamer treatment of primary guinea pig macrophages*

Peritoneal macrophages from 3 male outbred Charles River guinea pigs (350g) were harvested by aseptic peritoneal lavage with 100 ml of sterile PBS (Ca and Mg-free). The cells were collected by centrifugation and the cells were resuspended in 2.5 ml ACK red cell lysis buffer (0.15M $NH_4Cl$, 0.1mM $Na_2$ EDTA, 1.0mM $KHCO_3$, pH 7.3) and placed in a 37$^{o}$C incubator for five minutes with occasional mixing. We then added 2.5 ml of 1X RPMI 1640 supplemented with 10% v/v FBS, 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine and plated cells into 96 well plates at a concentration

of $1x10^5$ cells/well. Cells in various wells were then treated with 40, 20, 10, 5, or 2μg/ml of XBY-S2 in PBS for 24 hours. Some of the wells were then treated with *E. coli* 011:B4 LPS in RPMI (for a final concentration of 0.5μg/ml) and incubated for an additional 24 hours. The supernatants were collected and analyzed for TNF by bioassay and IL-8 using a human IL-8 ELISA (R&D Systems, Minneapolis, MN) previously shown to be applicable for measuring guinea pig IL-8 (Kuo *et al*., 1997).

## *Animals*

Guinea pigs were handled in accordance with guidelines for the humane use of laboratory animals established by the National Institutes of Health. Male Hartley outbred guinea pigs, approximately six weeks old, were obtained from Charles River Laboratories (Wilmington, MA, colony K81). The guinea pigs were acclimated for one week prior to treatment.

## *XBY-S2 treatment experiments*

Guinea pigs were treated two hours prior to virus infection with a 1ml intraperitoneal (ip) injection of XBY-S2 in liposomes (50μg aptamer/ml). A mock-treated control group received an injection of endotoxin-free PBS only. Two hours after the first XBY-S2/liposome injection, 1000 pfu of P18 was inoculated ip into all guinea pigs. Two days post-virus infection, guinea pigs received a second injection of aptamer/liposomes or PBS at the same dose as day 0. Guinea pigs weights and rectal temperatures were monitored daily and when weight dropped 25% below pre-infection

weight or temperature dropped below 38.5°C, guinea pigs were humanely euthanized, per previously described surrogate criteria for death (Aronson *et al*., 1994).

## *Blood collection*

Serial blood samples from each guinea pig were acquired at days –2, 1, 2, 3, 6, 9, and 12 by clipping a hindleg toenail. 300-600µl of blood was collected in lithium heparin serum separator gel tubes (Microtainer, Franklin Lakes, NJ). After collection, the tubes were centrifuged at 7,000xg for one minute and the plasma was collected and stored at –80°C.

## *RNA isolation from PBL*

After plasma removal, the gel separator was removed with a pipet and the cells transferred into a microfuge tube containing 800µl of lysis solution from Ambion's (Austin, TX) RiboPure blood kit for RNA extraction. RNA from cells was isolated according to the manufacturer's instructions. RNA was DNase treated for 30 minutes at 37°C using DNAfree (Ambion, Austin, TX) according to the manufacturer's instructions. Due to the small amount isolated, RNA was not quantitated. RNA was tested for genomic DNA contamination by real time RT-PCR with heat-inactivated M-MLV reverse transcriptase (Ambion, Austin, TX) using GAPDH specific primers for both the reverse transcriptase (1nM reverse primer) and real time PCR (0.2nM primers).

## *Real time RT-PCR, Plaque Assay, and Indirect immunofluorescence assay*

Real time RT-PCR, plaque assay, and indirect immunofluorescence assay were all performed as described in Chapters 2 and 3.

## *Statistics*

Statistical differences of TNF and IL-8 production between aptamer treated cells and untreated cells were determined using Student's t-test. For comparison of survival curves between treated and mock treated animals, statistical significance was assessed using Kaplan-Meier survival analysis. Fisher's least significant difference procedure was used for multiple comparisons with Bonferroni adjustment for a number of comparisons between XBY-S2 treated survivors, and XBY-S2 treated non-survivors and PBS control. Analysis of variance is computed using PROC MIXED in SAS®, Release 8.2. Main effects and interaction are assessed at the 0.05 level of significance. Student's t-test was used to compared viremia levels and $\Delta$Ct values of XBY-S2 treated vs. PBS control.

RESULTS

## *XBY-S2 pertubation of cytokine expression in primary guinea pig macrophages*

Cytokine dysregulation likely contributes to pathogenesis in arenavirus infection of humans and experimental infection of guinea pigs (Mahanty *et al*., 2003; Lukashevich *et al*., 1999; Baize *et al*., 2004; Mahanty *et al*., 2001). XBY-S2 is a thioaptamer that binds to the AP-1 family of transcription factor proteins, well-known regulators of

cytokine expression. Previous studies have shown that XBY-S2 binds putative negative regulators of transcription in this family (Fennewald et al, submitted). To determine if XBY-S2 can influence cytokine gene expression in primary cells, we examined the cytokine production from primary peritoneal guinea pig macrophages in response to *ex vivo* LPS stimulation following treatment with the XBY-S2 thioaptamer. The supernatants of cultured primary guinea pig peritoneal macrophages were assayed for TNF by standard bioassay and IL-8 was measured by ELISA based on human IL-8. These two cytokines were chosen because they are among the few guinea pig macrophage-derived cytokines for which available standardized assays exist, and because AP-1 is known to be important in regulation of their transcription (Hoffmann *et al*., 2002a). As can be seen in Figure 19, XBY-S2 increased the expression of IL-8 in a dose dependent fashion over the levels expressed in unstimulated macrophages. At doses below 20μg/ml, XBY-S2 appeared to increase basal level of TNF expression but there was no clear dose-dependence at higher doses (Figure 19). XBY-S2 showed a tendency toward decreasing LPS-inducible TNF-α from macrophages at higher doses (10-40 μg/ml) (data not shown). However, in general, XBY-S2 treatment does not appear to significantly influence cytokine gene expression in macrophages stimulated with LPS. Therefore, these data, in part, confirm the results seen in the P388D1 cell line that indicate that XBY-S2 targets AP-1 proteins that repress the transcription of these cytokines, or AP-1 regulates the expression of another protein that serves as a repressor. XBY-S2 does not block the activities of transcription factors that activate the expression

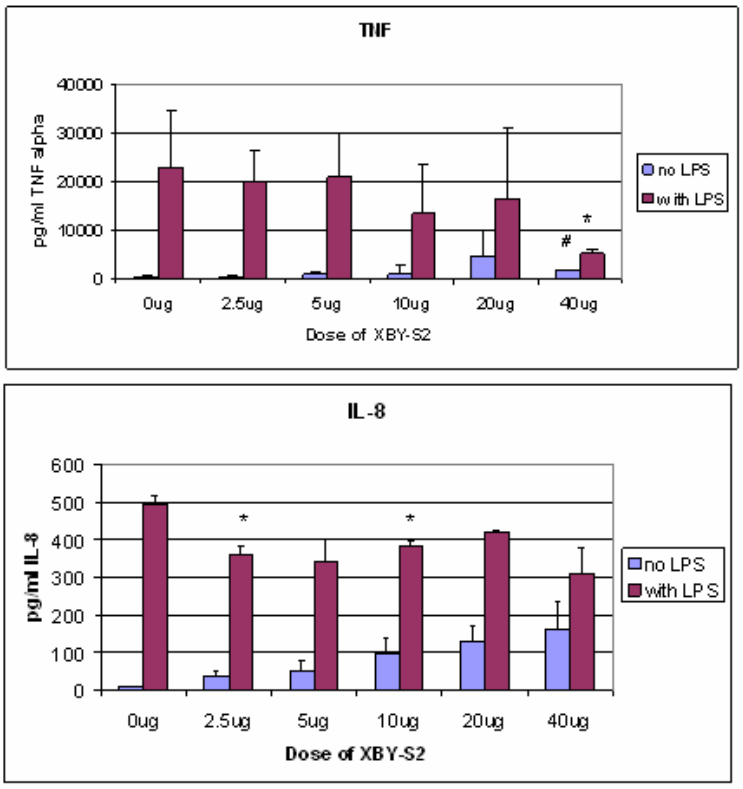


*Figure 19. Influence of XBY-S2 TNF and IL-8 protein levels in primary guinea pig macrophages.* Primary peritoneal macrophages were harvested by lavage from guinea pigs and treated with varying amounts of XBY-S2 for 24hr. Some of the cells were then stimulated with LPS (0.5µg/ml final concentration) and culture supernatants were harvested after an additional 24 hr. TNF and IL-8 were measured by bioassay and ELISA respectively. Bars indicate the average of triplicate wells ± standard deviation. * indicates $p < 0.05$ vs. 0ug with LPS. # indicates $p < 0.05$ vs. 0ug without LPS.

of these cytokines. However, XBY-S2 increases the basal level of expression of pro-inflammatory cytokines that could influence innate immunity induced in response to viral challenge.

### *XBY-S2 aptamer effects on guinea pig morbidity, mortality, and viremia*

The aptamer XBY-S2 was tested for its ability to influence cytokine expression and disease outcome in experimental guinea pig infection with Pichinde virus variant, P18. A survival curve for one of two experiments is shown in Figure 20. In order to reduce aptamer dose and target macrophages, aptamers were given in a cationic liposome vehicle as described in the methods section. When administered intraperitoneally at days 0 and 2 of infection, liposomal XBY-S2 increased survival of P18 infected guinea pigs compared to mock-treated guinea pigs by 37.5-50% in separate experiments ($p=.024$) (Figure 20). Four out of five of the treated surviving guinea pigs showed no characteristic clinical signs of morbidity such as fever, ruffled fur, or loss of body weight (Zhang *et al*., 2001). One aptamer treated survivor developed fever, ruffled fur, and weight loss by day 12, but eventually recovered. None of the mock-treated control guinea pigs survived infection. In previous experiments, a scrambled aptamer (XBY-S1) that lacked an AP-1 binding sequence did not ameliorate morbidity or mortality in P18 infected guinea pigs, compared to liposome vehicle treated or PBS treated animals alone (data not shown).

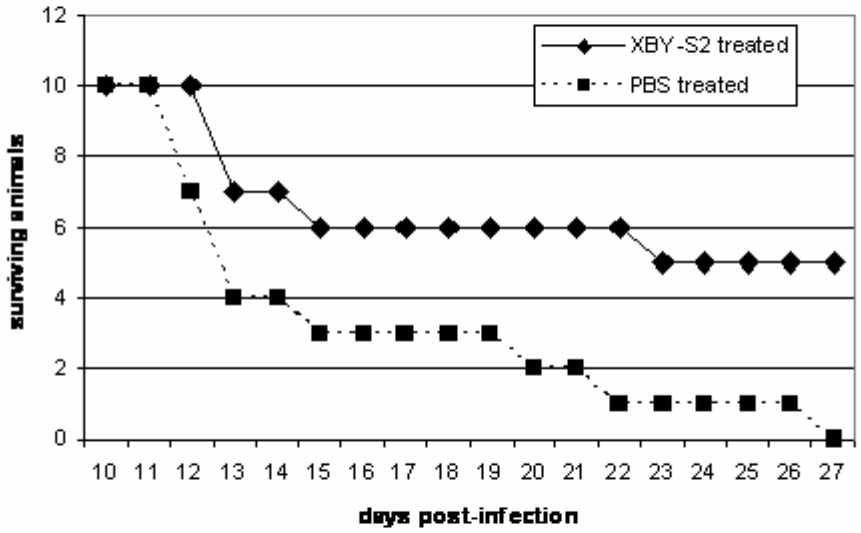


*Figure 20. Survival of guinea pigs infected with P18 virus after XBY-S2 treatment.* Survival of guinea pigs infected intraperitoneally with 1000 pfu of high passage Pichinde virus (P18) after treatment with an AP-1 thioaptamer (XBY-S2) or PBS alone. Outbred guinea pigs were given two doses of XBY-S2 in liposomes; one dose 2 hours prior to infection and a second dose 2 days post-infection. Mock guinea pigs were given PBS only. Diamond with solid line indicates XBY-S2 treated guinea pigs (n=10). Square with dashed line indicates PBS treated guinea pigs (n=10). Survival of XBY-S2 treated guinea pigs was statistically different than PBS treated (Kaplan-Meier survival analysis, p=.024).

The decreased disease severity in XBY-S2 treated guinea pigs was accompanied by decreased viremia titers (Figure 21). XBY-S2 treated survivors that developed no clinical signs of illness had no detectable virus in the serum at any timepoint and did not seroconvert by day 28 post-infection. XBY-S2 treated animals that developed clinical illness had approximately 10-fold lower viremia than mock-treated control animals throughout the course of infection (Figure 21). All animals with clinical disease (irrespective of treatment group) developed antibody titers of at least 1:160 with no differences between the treated and mock-treated groups. Pre-infection serum samples from all guinea pigs were negative for cross-reactive antibody by immunofluorescence assay.

Histopathological evaluation of liver and spleen of lethally infected animals showed no differences between XBY-S2 treated and mock treated animals in terms of liver inflammation, necrosis, steatosis, or splenic necrosis (data not shown).

## *XBY-S2 effect on cytokine expression in peripheral blood leukocytes during PIC infection*

*In vitro* experiments on primary guinea pig peritoneal macrophages showed that XBY-S2 increased basal levels of IL-8 and TNF proteins (Figure 22). In order to determine *in vivo* effects of XBY-S2 on cytokines, serial blood samples were collected two days prior to infection to establish baseline levels, and at days 1, 2, 3, 6, 9, and 12 post-infection to measure cytokine mRNA expression from peripheral blood leukocytes

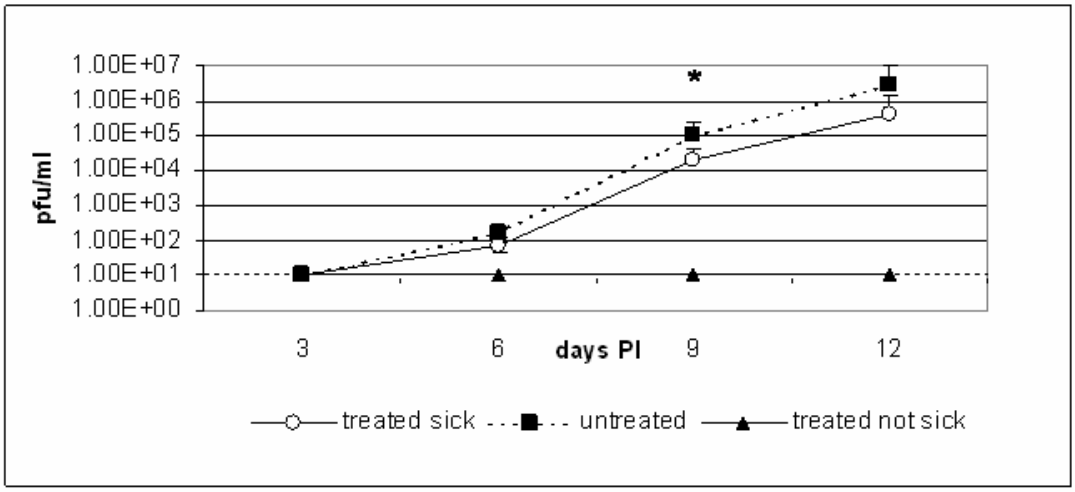


*Figure 21. Viremia of guinea pigs infected with P18 virus after XBY-S2 treatment.* Viremia of guinea pigs infected intraperitoneally with 1000 pfu of P18 virus. Guinea pigs were either treated with two doses of an AP-1 thioaptamer (XBY-S2) in liposomes or PBS alone. Viremia was measured by plaque assay from serum collected at days 3, 6, 9, and 12 post-infection. Three groups are represented, XBY-S2 treated guinea pigs that did not develop illness (triangle solid line, n=4), XBY-S2 treated guinea pigs that did develop illness (open circle solid line, n=6), and PBS alone guinea pigs (all became ill)(square dashed line, n=10). Points represent geometric means ± SEM. * denotes $p < .05$ by a t-test.

(PBL) by real time RT-PCR. Primers for real time RT-PCR were designed and optimized as described in the methods section of Chapter 2 (Table 1).

Figures 22-23 show the comparison of average $\Delta C_t$ ($C_{t\ cytokine} - C_{t\ GAPDH}$) values from PBL of XBY-S2 treated and mock- treated groups over time. There was a pattern of higher TNFα, IL-8, IL-1β, and IL-10 expression over time in XBY-S2 treated animals compared to mock treated animals, suggesting XBY-S2 has similar effects *in vivo* as *in vitro* (Figures 22-23). In contrast, there was a trend toward lower IFNγ mRNA expression over time in treated animals compared to control animals. Both XBY-S2 treated and mock treated groups had large (256 - 8,000 fold) increases in GRO, TGFβ, and RANTES mRNA expression by day 2 post infection when compared to prebleed values, which remained elevated throughout the course of infection (Figure 23). These data may reflect either a P18 cytokine response unaltered by XBY-S2 treatment, or a stress/inflammation response due to repeated blood collection.

Analysis of individual timepoints revealed that early in infection (days 1-3), PBL from XBY-S2 treated animals had increased expression of the pro-inflammatory cytokines TNFα, IL-12p40, and IL-1β compared to mock treated animals (Figure 22). The chemokines RANTES and IL-8 were also upregulated. Few differences were seen during mid-stage infection (days 6 and 9), however by the late stage of disease (day 12) IL-1β and IL-8 expression was again higher in XBY-S2 treated guinea pigs compared to mock treated guinea pigs, while GRO expression was lower (Figures 22-23).

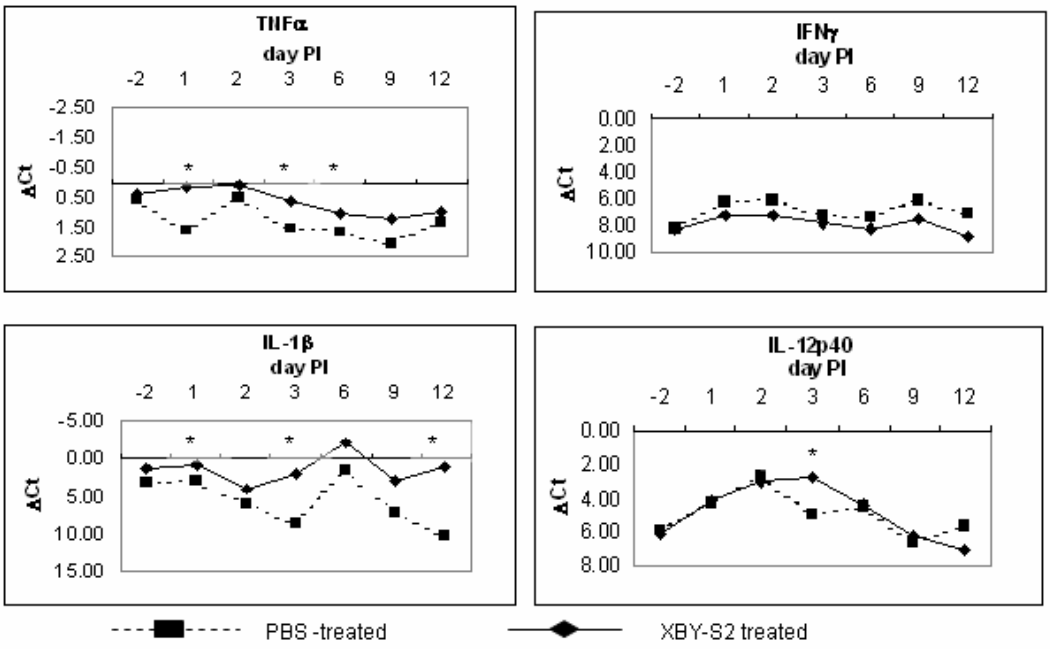


*Figure 22. Pro-inflammatory cytokine mRNA expression from peripheral blood lymphocytes (PBL) of XBY-S2 thioaptamer treated or PBS treated P18 infected guinea pigs, independent of survival.* Blood from guinea pigs was collected 2 days prior to infection, and days 1, 2, 3, 6, 9, and 12 post-infection. RNA isolated from PBL was analyzed by real time RT-PCR. Graphs represent the $\Delta C_t$ values from each cytokine over time ($\Delta C_t = Ct_{\text{cytokine}} - Ct_{\text{GAPDH}}$). The data points represent the mean $\Delta C_t$'s from either XBY-S2 treated animals (diamond solid line, n=10) or PBS alone treated guinea pigs (square dashed line, n=10). * denotes statistical significance between the two groups ($p \leq .05$, by Students t-test).

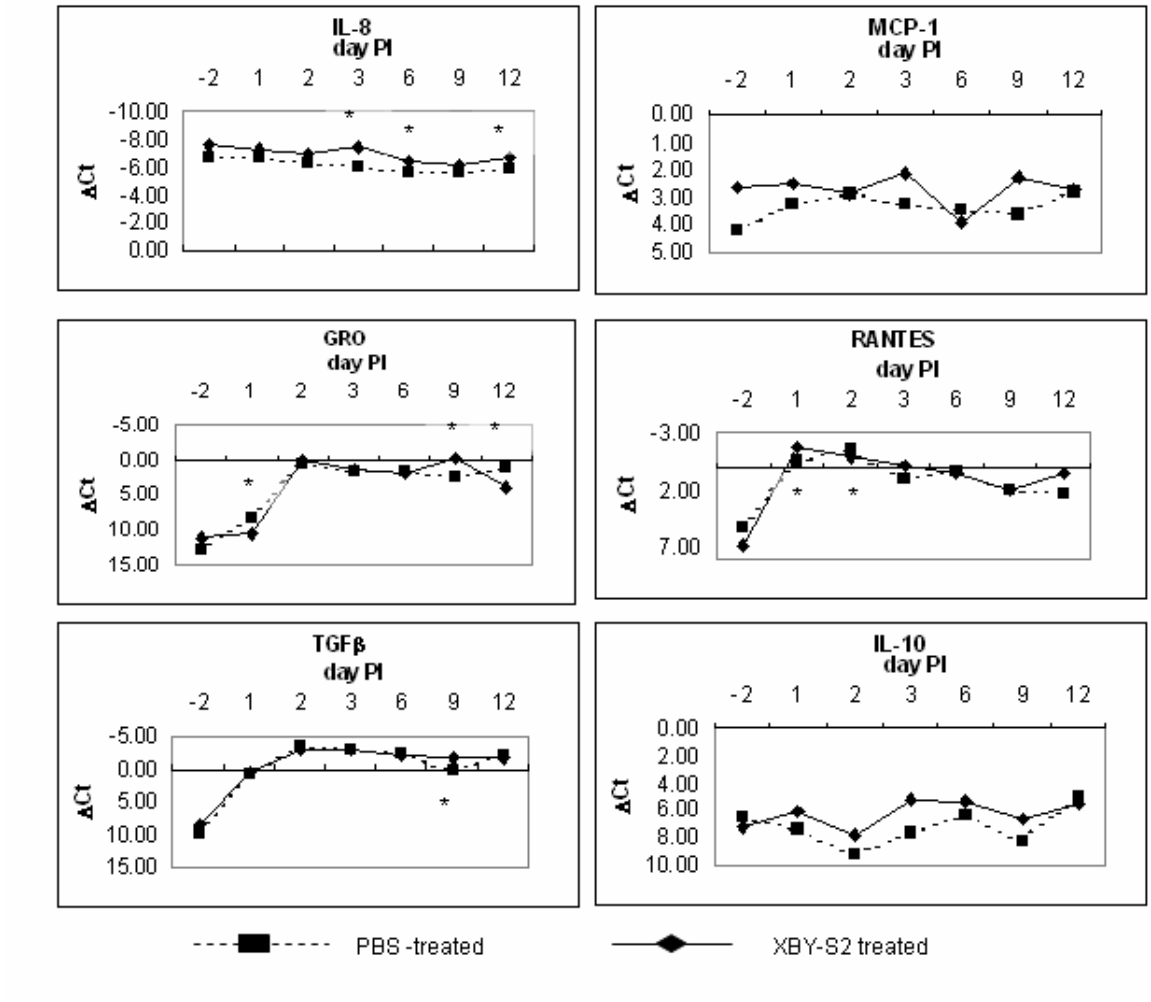


*Figure 23. Chemokine and anti-inflammatory cytokine mRNA expression from peripheral blood lymphocytes (PBL) of XBY-S2 thioaptamer treated or PBS treated P18 infected guinea pigs, independent of survival.* Blood from guinea pigs was collected 2 days prior to infection, and days 1, 2, 3, 6, 9, and 12 post-infection. RNA isolated from PBL was analyzed by real time RT-PCR. Graphs represent the $\Delta C_t$ values from each cytokine over time ($\Delta C_t = Ct_{\text{cytokine}} - Ct_{\text{GAPDH}}$). The data points represent the mean $\Delta C_t$'s from either XBY-S2 treated animals (diamond solid line, n=10) or PBS alone treated guinea pigs (square dashed line, n=10). * denotes statistical significance between the two groups ($p \leq .05$, by Students t-test).

To determine if any cytokine patterns correlated with survival, we also compared PBL cytokine expression between survivors and non-survivors within the aptamer treated group. Although the TNF-α and IL-1β expression was significantly elevated in the treated group at day 1 post infection (Figure 22), there was no difference in expression of either of these cytokines between treated survivors and treated non-survivors (Figure 24). No cytokine expression patterns predictive of survival were identified in the first 6 days of infection. Survivors did have higher IL-1β expression at days 9 and 12, and higher RANTES expression at day 12 compared to non-survivors (Figure 24).

Because of limited availability of immunoassays for guinea pig cytokines, only serum TNF and IL-8 were measured by bioassay and ELISA (R&D Systems, Minneapolis, MN) (Aronson *et al*., 1995; Kuo *et al*., 1997). At day 9, serum levels of TNF measured by bioassay were significantly higher for XBY-S2 treated animals compared to mock treated (Figure 25). No significant differences were observed in TNF between XBY-S2 treated survivors and non-survivors (data not shown). Serum samples were below the limit of detection for TNF at days 1, 2, 3, and 6, and for IL-8 at all timepoints.

## DISCUSSION

Fatal Lassa fever is strongly correlated with viremia; survivors of Lassa are able to control viral replication through unknown mechanisms.(Johnson *et al*., 1987) Pro-inflammatory cytokines have direct antiviral effects as well as activating innate immune

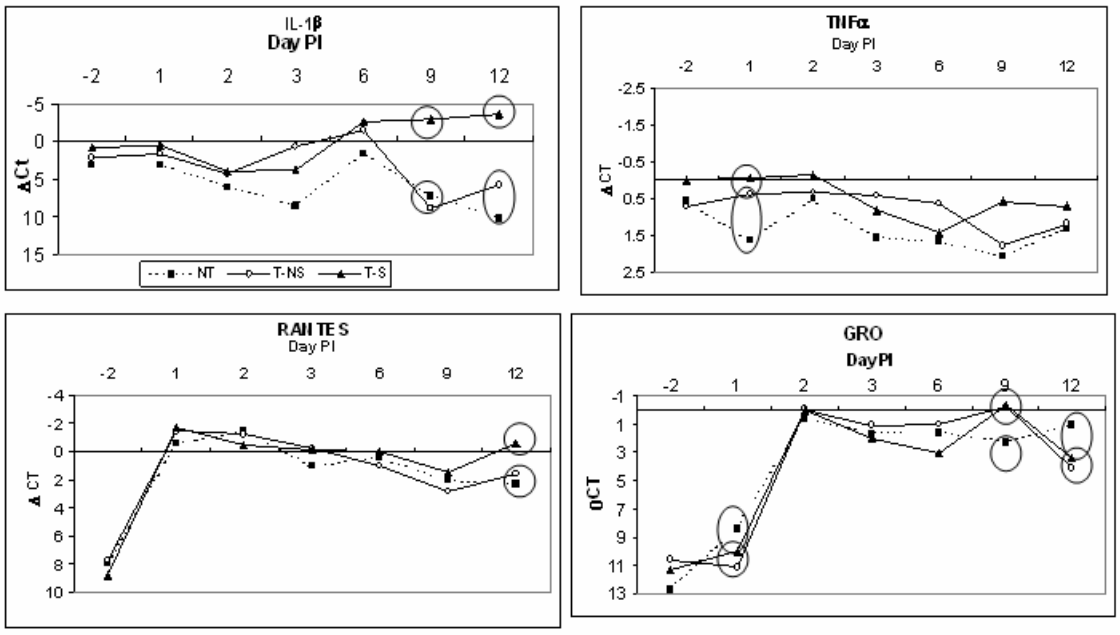


*Figure 24. Cytokine mRNA expression from PBL of P18 infected guinea pigs, comparison of XBY-S2 treated virus survivors, XBY-S2 treated non-survivors, or PBS treated groups.* PBL RNA was isolated from blood collected two days prior, days 1, 2, 3, 6, 9, and 12 post-infection. RNA was analyzed by real-time RT-PCR. Data points are the mean $\Delta C_t$'s from each group ($\Delta C_t = Ct_{\text{ cytokine}} - Ct_{\text{ GAPDH}}$). Groups represented are XBY-S2 treated guinea pigs that survived (triangle solid line), XBY-S2 treated guinea pigs that did not survive (open circle solid line), and PBS alone treated guinea pigs, none of which survived (square dashed line). Two data points within an ellipse indicates they are not statistically different from each other. One data point alone in an ellipse indicates it is statistically different than the other two groups. Statistical significance is $p < .05$ by Fisher least significant difference test for multiple comparisons.

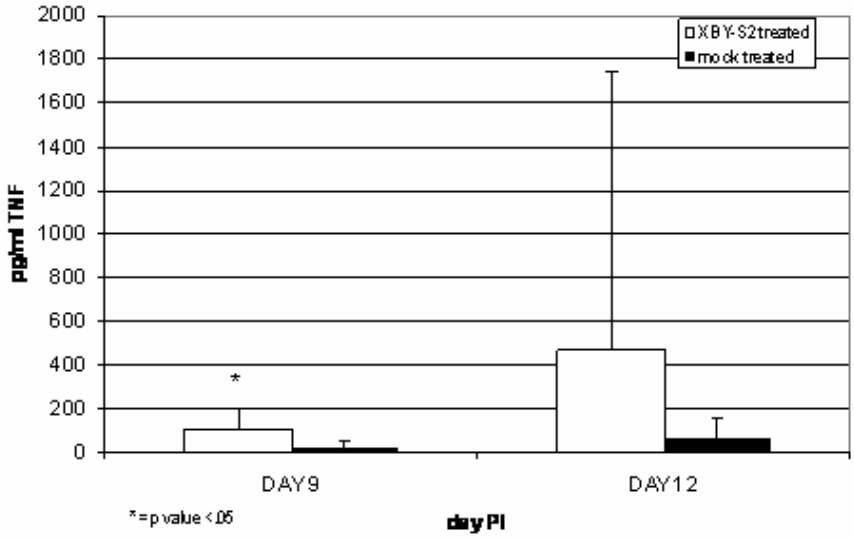


*Figure 25. Serum TNF levels from P18 virus infected XBY-S2 treated or mock treated animals.* Serum TNF was measured by bioassay for days 9 and 12 post-infection. * indicates a p-value of <.05 compared to mock-treated group by Students t-test.

cells.(Guidotti *et al*., 2000a) Suppression of these cytokines could enable a virus to evade the cytokine-mediated viral control mechanisms and allow the virus to replicate unchallenged. Lassa virus has been shown to suppress macrophage and dendritic cell production of pro-inflammatory cytokines,(Mahanty *et al*., 2003; Lukashevich *et al*., 1999; Baize *et al*., 2004) and have the ability to suppress LPS-induced cytokines (Baize *et al*., 2004; Lukashevich *et al*., 1999; Mahanty *et al*., 2003; Fennewald *et al*., 2002). In addition, an overall lack of pro-inflammatory cytokine induction is associated with fatal Lassa fever (Mahanty *et al*., 2001). An early innate immune response, such as induction of pro-inflammatory cytokines, could potentially inhibit virus replication and spread. Therefore, boosting of early pro-inflammatory cytokines is a potential therapeutic approach for hemorrhagic fever viruses.

In order to induce pro-inflammatory cytokines, we have targeted the AP-1 family of transcription factors known to regulate cytokine expression. A double stranded DNA thioaptamer (XBY-S2) was shown to bind dimers containing Fra-2, which can have suppressive effects on AP-1 activation (Rutberg *et al*., 1997; Suzuki *et al*., 1991). XBY-S2 acts as a decoy for a transcriptional repressor (hence functions as a "repressor of the repressor") and upregulates TNF and IL-8 in primary guinea pig peritoneal macrophages. We hypothesized that this thioaptamer (XBY-S2) would ameliorate disease by upregulating early pro-inflammatory cytokine expression in a guinea pig model of arenavirus hemorrhagic fever.

We have demonstrated a 50% decrease in mortality and a 40% reduction in morbidity in XBY-S2 treated, Pichinde virus infected guinea pigs compared to mock treated infected guinea pigs. Four of the ten aptamer- treated guinea pigs not only survived viral challenge, but also showed no signs of clinical illness, did not develop detectable viremia, and did not seroconvert. The treated group on average had lower serum viremia than the untreated group; when considering only the animals that developed clinical signs, the treated group still had a nearly 10-fold lower viremia than the untreated group. These beneficial effects were seen under stringent challenge conditions in the absence of specific antiviral therapy. The ability of XBY-S2 treatment to reduce viremia is important and relevant due to the correlation of viremia and severity of disease in both Lassa fever and the Pichinde virus infection model of Lassa fever (Johnson *et al*., 1987; Zhang *et al*., 2001).

The mechanism by which XBY-S2 decreases PIC replication *in vivo* is unknown. We considered the possibility that XBY-S2 directly inhibits virus binding or entry into host cells. However, pilot studies of pre-treatment of guinea pig peritoneal macrophages with XBY-S2 in culture did not affect viral titers generated in those cultures, suggesting that aptamer did not interfere with virus binding or subsequent infection in isolated cells (Figure 26).

XBY-S2 treatment did not result in increased serum anti-PIC antibody titers, suggesting that the beneficial effect of aptamer was not attributable to a boost in humoral immune response. We have not ruled out the possibility that cell-mediated immune

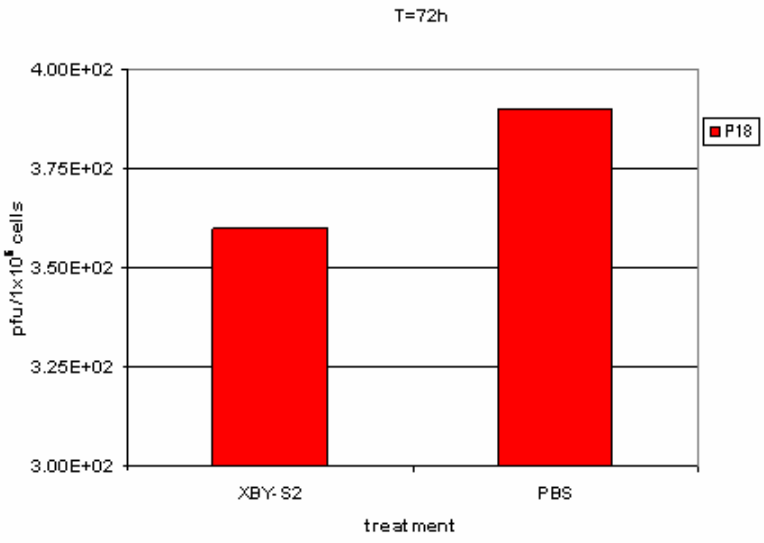


*Figure 26. Influence of XBY-S2 on P18 replication in primary guinea pig macrophages.* Primary peritoneal macrophages were harvested by lavage from guinea pigs and plated at $1x10^5$ macrophages per well in 100µl of RPMI-1640 medium. Macrophages were immediately treated with PBS, or XBY-S2 (diluted in PBS) at a final concentration of 2µg/ml, overnight. Medium containing XBY-S2 was removed, and cells were infected with P18 virus (MOI=1). Fresh medium without XBY-S2 was then added to each well (n=1). Culture supernatants were harvested 72h after viral infection and assayed for infectious virus by plaque assay.

responses are enhanced in XBY-S2 treated animals. However, because most of the protected animals failed to develop viremia, clinical disease, or antibody response, we believe that XBY-S2's beneficial effect is exerted very early in infection, possibly via induction of some macrophage-specific innate effector mechanisms such as AP-1 regulated cytokine expression.

In order to test this possibility, we developed real-time RT-PCR for ten guinea pig cytokines including pro-inflammatory and regulatory cytokines, as well as selected chemokines. Many of the cytokines analyzed are predominantly expressed in macrophages, important target cells for Pichinde (Aronson *et al*., 1994). Real time RT-PCR was used to measure mRNA expression in peripheral blood leukocytes. Many cytokine genes have AP-1 sequences in their promoters including IL-1β, IL-8, IL-12p40, IL-10, TNFα, TGFβ, RANTES, MCP-1, and GRO (Angel et al., 1991; Ma et al., 1996; de Waal Malefyt et al., 2001; Hiura TS. et al., 1999; Martin et al., 1997). AP-1 response elements have been shown to be required for transcription of TNF-α, IL-1β, TGF-β, RANTES, IL-8, and MCP-1 (Birchenall-Roberts *et al*., 1990; Martin *et al*., 1997; Hiura TS. *et al*., 1999; Hoffmann *et al*., 2002b; Roebuck *et al*., 1999). Over the course of infection, we found overall increased mRNA expression in several of these cytokines in XBY-S2 treated guinea pigs compared to mock-treated guinea pigs, supporting our hypothesis that induction of cytokines is beneficial in the outcome of disease.

An important finding of this study was that TNF-α was up-regulated very early in infection in XBY-S2 treated animals compared to mock-treated animals. Other studies

from our laboratory indicate that PIC infection suppresses TNF production from primary target cells (Figure 12) (Fennewald *et al*., 2002). The aptamer XBY-S2 apparently counteracts the virus's suppressive effects on TNF-α expression, restoring macrophage responsiveness and ameliorating disease. TNF-α has been shown to have antiviral effects in a variety of viral systems, such as respiratory syncytial virus, Sin Nombre virus, influenza virus, vesicular stomatitis virus and others (Bose *et al*., 2003; Khaiboullina *et al*., 2000; Seo *et al*., 2002; Mestan *et al*., 1986). Asper and colleagues have shown that TNF-α does not affect growth of the Old World arenaviruses Lassa and LCMV in Vero or Huh-7 cells (Asper *et al*., 2004). Human TNF-α likewise did not inhibit Pichinde replication in guinea pig macrophages (Figure 10), however, the cross-reactivity of human TNF-α on guinea pig cells is in question (Figure 11). The effects of guinea pig TNF-α on arenavirus replication in macrophages, which are more relevant target cells, are unknown at this time. TNFα also activates many facets of the immune response by activating macrophages, NK cells, and dendritic cells, as well as inducing apoptosis and iNOS production . XBY-S2 treated guinea pigs also had increased expression of IL-1β, a pro-inflammatory cytokine which has similar functions as TNFα, but has not been shown to have any direct anti-viral effects. It is currently unknown whether these prototypical pro-inflammatory cytokines exert a beneficial effect by direct stimulation of anti-viral responses in target macrophages, or by fostering inflammation and innate immune responses.

Interferons are the prototypical anti-viral cytokines. Sequences for guinea pig IFNα/B are not currently available, and we have not developed RT-PCR assays for these genes. However, we have previously shown that attenuated and virulent variants of Pichinde do not differ in terms of interferon induction *in vitro* or *in vivo* as measured by bioassay (K. Marriott, UTMB dissertation). In this study, we identified no statistically significant differences in IFN-γ mRNA expression between treated and mock-treated groups at any timepoint. These data corroborate previous observations that hemorrhagic-fever causing arenaviruses in general are poor interferon inducers, only moderately sensitive to the antiviral effects of interferons, and sensitivity to interferons does not correlate with disease severity (Asper et al., 2004; Peters et al., 1989; Levis et al., 1984; Stephen et al., 1977b; Moskophidis et al., 1994). It is likely the reduction of viremia we observe in XBY-S2 treated guinea pigs is not due to the antiviral properties of interferons.

The CXC chemokines GRO and IL-8 (CXCL8) had altered expression in the aptamer treated animals compared to mock-treated animals. There was higher IL-8 mRNA expression in PBL of the treated animals compared to mock-treated animals over time. This result parallels our finding that XBY-S2 increased IL-8 expression in cultured primary guinea pig macrophages (Figure 19). Interestingly, a correlation between elevated serum IL-8 levels and improved survival has been described in human Lassa fever cases (Mahanty *et al*., 2001). Also, Lassa virus suppresses IL-8 expression in human monocytes whereas an avirulent, related arenavirus, Mopeia, does not

(Lukashevich *et al*., 1999). Taken together, these results again suggest that treatments for Lassa fever that restore IL-8 responses of infected macrophages could be beneficial.

IL-8 and GRO are chemoattractants for neutrophils. The role of chemokines and neutrophils are virtually unknown in viral hemorrhagic fevers. Neutrophilia is often noted late in the course of severe arenavirus hemorrhagic fever cases (Fisher-Hoch et al., 1988a; Jahrling et al., 1981; Fisher-Hoch et al., 1985). High levels of neutrophils may be contributing to the dysfunction of vascular endothelium in viral hemorrhagic fever by directly damaging the endothelium (Wyman *et al*., 2002; Rabinovici *et al*., 1993), therefore, the correlation between high IL-8 expression and survival is surprising. However, Gimbrone *et al* have shown that high levels of IL-8 actually reduce neutrophil adhesion and subsequent damage to vascular endothelium suggesting a possible beneficial role for IL-8 (Gimbrone *et al*., 1989). The role of neutrophils and IL-8 in viral hemorrhagic fever needs to be further examined.

Late in the course of Pichinde infection in the studies reported here, a marked decrease in PBL expression of RANTES (CCL5) was observed in lethally infected animals when compared to survivors, irrespective of treatment group. The early immunosuppression of macrophage and dendritic cell activation during Lassa infection (Mahanty *et al*., 2003; Baize *et al*., 2004) likely inhibits the ability of these cells to activate T-cells, resulting in an immunosuppressive cascade leading to uncontrolled viremia and death. RANTES is produced primarily by CD8+ T-cells and is expressed 3-5 days after T-cells become activated (Song *et al*., 2000). Therefore the difference in

RANTES expression between survivors and non-survivors may be a reflection of impaired T-cell activation and generalized immunosuppression. Alternately, Pichinde may be directly infecting T-cells and inhibiting their functions, in light of observations of profound suppression of T-cell unresponsiveness to mitogens in experimental models of Lassa fever (Fisher-Hoch *et al*., 1987)(Jerrells, TR, unpublished data). Suppression of the pro-inflammatory IL-1β was also seen in lethally infected animals; a similar mechanism of suppression in monocytes may be responsible.

The data we present must be interpreted in light of several caveats. First, mRNA levels do not necessarily correlate with bioactive protein levels for a given cytokine. Due to the limitations of the guinea pig system, it is not possible to measure serum levels of most of these cytokines. For the two cytokines that we could measure by ELISA or bioassay, IL-8 and TNF, serum differences were only detected for TNF at days 9 and 12, and only at day 9 in the treated group. The lack of detectable serum TNF in treated animals despite two fold increase in mRNA levels compared to mock treated animals, could represent either relative insensitivity of the bioassay, or post-transcriptional regulation of TNF synthesis. Mahanty *et al* were also unable to detect serum TNF in either fatal or non-fatal Lassa patients (Mahanty *et al*., 2001). The second caveat is that although input RNA was normalized based on GAPDH mRNA, we have not corrected for any potential shifts in populations of circulating leukocytes. In this experiment, no peripheral blood smears or differential counts were done. However, previous reports have shown no significant alterations in PBL differential counts within the first six days

of infection (Jahrling *et al*., 1981). It appears unlikely to us that early changes in PBL cytokine mRNA's could reflect simply dramatic shifts in cell populations. However, late in infection, we would expect increased neutrophil counts (relative neutrophilia) in lethally infected animals (Jahrling *et al*., 1981); it is possible at the late time points that shifts in cell types circulating in the blood could explain cytokine mRNA changes.

In summary, we have reported the first therapeutic study of an AP-1 decoy thioaptamer in a viral hemorrhagic fever model. Treatment with the candidate aptamer enhanced survival and decreased viremia in arenavirus challenged animals. Improved outcome of treated animals was also associated with a general pattern of early TNF-$\alpha$ expression, and sustained increases in pro-inflammatory cytokine and CXC chemokine expression throughout the two-week course of infection. These findings suggest that an early inflammatory environment may limit establishment of viral infection in this system. Current studies are investigating potential antiviral effects of pro-inflammatory cytokines. Detailed studies of cytokine expression in mild and severe PIC infections in various tissue compartments are ongoing in the laboratory; results from such studies will further refine therapeutic strategies.

# CHAPTER 5: SUMMARY

During the initial host response to viral infection, cytokines play a vital role in controlling viral replication through direct antiviral effects, or by activating and recruiting immune cells. Pro-inflammatory cytokines activate macrophages, dendritic cells, T cells, neutrophils, and other immune cells. The activation of immune cells by TNF-α, IL-1β, IFN-γ, and IL-12 is important for initiating innate defenses like phagocytosis, nitric oxide production, and the secretion of more cytokines. Furthermore, antigen presentation through pro-inflammatory cytokine-induced MHC II molecules is critical for virus-specific adaptive immunity. Chemokines like IL-8 and RANTES have the responsibility of recruiting the immune cells to the site of infection so they can become activated. Meanwhile, anti-inflammatory cytokines like IL-10 and TGF-β regulate the pro-inflammatory response to prevent immunopathologic damage to the host. Often times, this complex system is enough to control a viral infection. But some viruses have adapted methods to avoid the immune system, and replicate uncontrolled. P18 virus appears to be one of those viruses.

In the guinea pig, P18 virus replication is not controlled, leading to severe disease and death. P2 virus replication on the other hand, is controlled, resulting in an attenuated disease. Early events during Pichinde infection may explain the differential levels of viral replication. We hypothesize that innate responses during the initial infection of target macrophages are key to controlling P2 replication. In contrast, we hypothesize P18 virus

inhibits or evades the mechanisms that control P2. Since cytokines are vitally important to immune cell activation and are also known to have direct antiviral effects, we proposed to investigate the pattern of cytokine responses from the initial target cell, the macrophage.

In chapter 2, we reported cytokine mRNA levels from peritoneal and bone-marrow derived macrophages infected *in vitro*. P2 and P18 virus replicated to similar titers in both types of macrophages. We had hypothesized that P2 infection would up-regulate potentially antiviral cytokines such as TNF-α and IL-1β, and P18 infection would suppress cytokines with antiviral abilities. Surprisingly, there was little cytokine alteration in response to either P2 or P18 virus in either cell type. However, Lassa virus also did not induce cytokine production for macrophages (Baize *et al*., 2004; Lukashevich *et al*., 1999). The fact that macrophages do not produce cytokines in response to either PIC or Lassa infection further validates the use of PIC as a model for Lassa.

The lack of macrophage cytokine induction in response to P2 infection is contrary to the macrophage induction of TNF-α and IL-12p35 mRNA in response to another attenuated arenavirus, Mopeia (Pannetier *et al*., 2004). However, both Mopeia and P2 synergized with LPS to increase MCP-1, IL-1β, and IL-12p40 mRNA in macrophages (Pannetier *et al*., 2004). We speculate that the cytokines up-regulated by P2 infection in response to LPS-stimulation may have had an antiviral effect on P2, resulting in lower

titers for P2 virus compared to P18 virus after LPS stimulation. We were interested to determine if cytokines known to alter the replication of other viruses could affect P2 and P18 viral replication. However, neither P2 nor P18 replication was altered by recombinant TNF-α, IL-1β, IL-8, GRO-α, or TGF-β. Although the cross-reactivity of these human cytokines with guinea pig cells is in question, the lack of PIC sensitivity to TNF-α could be another validation of the use of PIC to model Lassa fever. It has recently been reported that Lassa virus is not sensitive to the antiviral effects of TNF-α (Asper *et al*., 2004).

The lack of differential cytokine production in response to P2 and P18 infection *in vitro* further necessitated experiments describing cytokine responses *in vivo*. In chapter 3, we report the *in vivo* cytokine responses to P2 and P18 infection. We first examined the peritoneal cell population after intraperitoneal inoculation. There were several striking differences between *in vitro* and *in vivo* infections. First, unlike *in vitro* infection, P2 did not replicate as well as P18 *in vivo*, with lower viral RNA levels in both the peritoneal cells and spleen at day 2. Secondly, P18 infection markedly elevated levels of IFN-γ and MCP-1, whereas no elevation was observed *in vitro*. Since cytokines were not up-regulated in peritoneal macrophages *in vitro*, the source of IFN-γ and MCP-1 is likely from other cells in the peritoneal population, such as NK cells, T cells, or neutrophils. Indeed, neutrophils were significantly increased at day 2 in P18 infection compared to

mock, and can be induced to produce both MCP-1 and IFN-γ (Ethuin *et al*., 2004; Yamashiro *et al*., 2000).

Induction of IFN-γ during P18 infection was surprising, considering IFN-γ is typically considered an antiviral cytokine. However, some viruses are not sensitive to the effects of IFN-γ, and instead, IFN-γ induces the differentiation of macrophages to make them more permissive to infection. A study of cytomegalovirus (CMV) showed that CD8+ T-cell produced IFN-γ and TNF-α increased production of CMV without antiviral effects (Söderberg-Nauclír *et al*., 1997). There may be an IFN-γ-dependent effect on macrophages that makes them more permissive to P18 infection *in vivo*, that is not reproducible *in vitro* because of the low amounts of IFN-γ produced by macrophages. Previous studies with adapted Pichinde (8-serial passages) revealed that differentiation of a monocytic cell line by phorbol ester (PMA) was required to support replication (Polyak *et al*., 1991). From the data reported here, we hypothesize that P18 induces high levels of IFN-γ, which then differentiates cells to become permissive to infection. At the same time, this hypothesis would suggest that restriction of P2 replication is due to non-permissiveness of target cells, as opposed to a direct antiviral effect of cytokines. P2 infection does induce IFN-γ 5-fold *in vivo* compared to mock, but the amount is significantly lower (128-fold) than IFN-γ induced during P18 infection. Therefore both viruses induce IFN-γ, but the higher amount of IFN-γ during P18 infection could also result in a higher number of permissive cells, and higher virus levels. A simple

experiment to test this hypothesis would be to add a source of IFN-γ, such as Concanavalin A-stimulated splenocyte supernatants, to macrophage cultures before infection and measure replication.

Both severe Lassa and Pichinde are associated with a suppression in cell-mediated immunity, characterized by a lack of T cell proliferation in response to mitogens, and limited cytotoxic T cell activity (Fisher-Hoch *et al*., 1987)(T. Jerrells, unpublished data). Immunosupression can be a result of many factors. In this study, we reported that P18 suppressed TNF-α, IL-12p40, and IL-8 early in infection *in vivo*. Without appropriate macrophage and T-cell activation by pro-inflammatory cytokines, an effective cell-mediated response will not develop. Additionally, the elevated levels of IFN-γ induced during P18 infection could lead to the production of nitric oxide (NO). NO is a strong suppressor of T-cell proliferation and T-cell-mediated immunity (van der Veen *et al*., 2001). Suppression of pro-inflammatory cytokines, and induction of NO could be mechanisms by which Lassa and Pichinde cause immunosuppression. Future studies on the role of IFN-γ and NO could provide important information on Pichinde pathogenesis.

The characteristic immunosuppression during Lassa and Pichinde infection results in uncontrolled viremia and terminal shock. The events leading to terminal shock are not clear for either Lassa or Pichinde infection, but many investigators have hypothesized over-expression of TNF-α and IL-1β contributes to vascular dysfunction, which then leads to shock. In chapter 3, we detail the cytokine responses during P2 and P18

infection to clarify whether elevated pro-inflammatory cytokines are associated with terminal shock. In contrast to the popular hypothesis, the most thorough human study of cytokines in Lassa fever indicates that fatal Lassa is not associated with elevated pro-inflammatory cytokines (Mahanty *et al*., 2001). Our investigation corroborates the human data, indicating severe Pichinde is not associated with elevated pro-inflammatory cytokine mRNA, even in the presence of high levels of virus. In fact, compared to attenuated disease, guinea pigs with severe Pichinde disease had significantly lower amounts of TNF-α and IFN-γ mRNA in PBL late in infection. In addition, there were no differences in P2 and P18 levels of TNF-α, IL-1β, IL-12p40, or IFN-γ in the spleen or liver, where both viruses up-regulated pro-inflammatory cytokines relative to mock. In general, there was a pattern of suppressed RANTES and IL-1β associated with P18 infection. It is clear that IL-1β from the PBL is therefore not a major contributor to vascular dysfunction. Aside from indicating immunosuppression, the implications of decreased RANTES and IL-1β during severe Pichinde infection are unclear at this time. The only cytokine differences between P2 and P18 in target organs were increases in the chemokines IL-8, MCP-1, and GRO-α in P18 infected spleens compared to P2 at day 12. An increase in chemokines is typically associated with immunopathological damage during viral infections. In the absence of splenic inflammatory infiltrates during P18 infection, the contribution of chemokines to pathology is unknown.

We have demonstrated that both early and terminal events during virulent P18 infection are associated with down-regulation of pro-inflammatory cytokines. We also hypothesized that up-regulating pro-inflammatory cytokines would be therefore be protective. Indeed, in Chapter 4 we report that an AP-1 thioaptamer decoy increased PBL TNF-α, IL-1β, and IL-8 mRNA levels throughout the course of infection, and correspondingly decreased P18 mortality by 50%. The increase in survival was accompanied by an absence of detectable viremia. *In vitro* experiments suggest that the aptamer does not have direct antiviral effects, therefore we do not know the mechanism by which viral replication was inhibited. We suspect the mechanism is different than the mechanism which inhibits P2 replication.

In summary, neither P18 or P2 activates macrophages to produce cytokines *in vitro*, however, *in vivo* P18 infection is associated with increased IFN-γ and MCP-1 at the site of initial infection. P18 infection is also associated with an early suppression of peritoneal cell TNF-α and IL-8. Counter-action of P18-induced cytokine suppression by an AP-1 aptamer up-regulated PBL TNF-α and IL-8 and increased survival. A late suppression of pro-inflammatory cytokines in the PBL is also characteristic of severe disease; elevated levels of pro-inflammatory cytokine mRNA in the spleen, liver, or PBL do not appear to be responsible for terminal shock. In regards to P2 virus, control of replication does not appear to be cytokine mediated, since antiviral cytokines are not upregulated either *in vitro* or *in vivo*. Figure 26 summarizes the potential cytokine-mediated events during Pichinde infection.

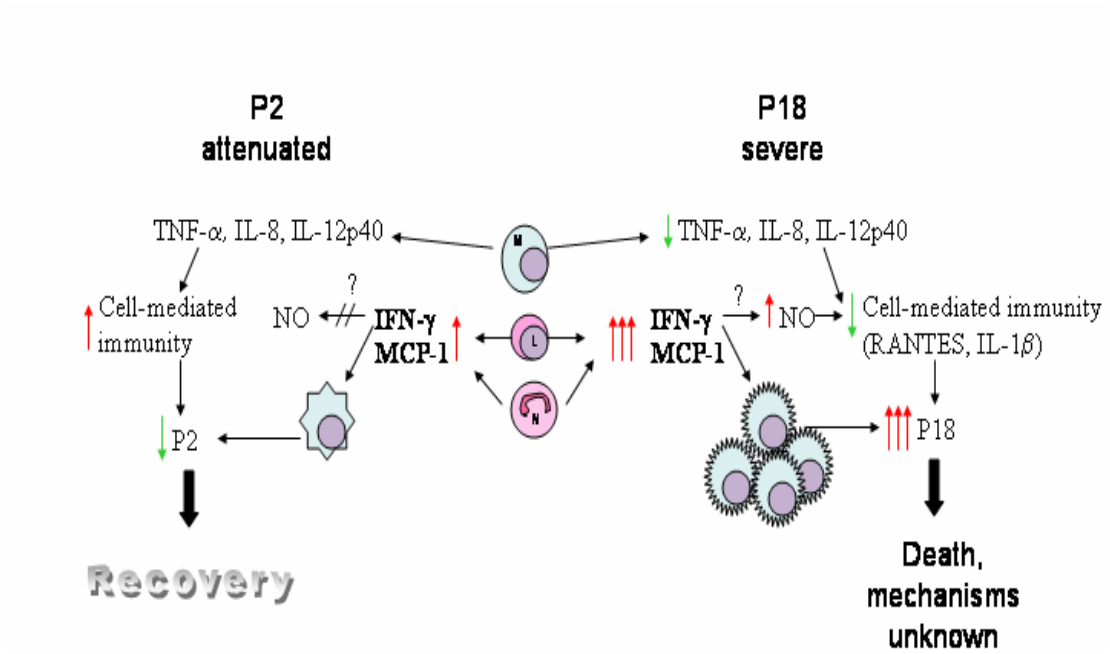


*Figure 27. Hypothesis of cytokine involvement in P2 disease vs. P18 disease.* M=macrophage, L=lymphocyte, N=neutrophil. During early P18 infection, large amounts of IFN-γ and MCP-1 activate macrophages resulting in a large number of permissive cells, as well as stimulating nitric oxide production (NO). Suppressed production of TNF-α, IL-8, and IL-12p40, in conjunction with NO, results in a lack of cell-mediated immunity. High levels of P18 virus, produced from permissive cells and uncontrolled by cell-mediated immunity, results in death due to unknown mechanisms. During P2 infection, small amounts of IFN-γ and MCP-1 activate some macrophages resulting in a small number of permissive cells. NO is not produced in significant amounts and cell-mediated immunity is not suppressed. Cell-mediated immunity is able to clear the low levels of P2 virus produced by the small number of permissive cells, resulting in recovery.

# APPENDIX A

## CULTURE AND CHARACTERIZATION OF GUINEA PIG BONE MARROW-DERIVED MACROPHAGES

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ABSTRACT

The guinea pig is an important model for various diseases in which macrophages play a key role in pathogenesis. Our goal was to develop a technique that yields a pure population of guinea pig bone marrow derived macrophages (BMDM). Adapting culture techniques for mouse BMDM to the guinea pig resulted in extensive fibroblast contamination. Utilizing the plant lectin soybean agglutinin to remove fibroblasts from the bone marrow, we obtained a pure macrophage population. BMDM had characteristic macrophage morphology by light and electron microscopy, and exhibited non-specific esterase and phagocytic activity. BMDM did not react with the guinea pig tissue-macrophage antibody MR-1. BMDM could be activated to express MHC class II expression in response to IFNγ, and produce IL-8 and TNFα in response to LPS stimulation. Cultures of homogenous primary guinea pig macrophages will be useful for the study of a variety of infectious and allergic diseases modeled in guinea pigs.

## 1. Introduction

Guinea pigs are used as a model for numerous diseases including viral hemorrhagic fevers, tuberculosis, herpes simplex infection, and allergic reactions (Amminikutty et al., 2003; Aronson et al., 1995; Ho et al., 1991; White et al., 1997; Connolly et al., 1999; Oubina et al., 1984). For many of these processes macrophages play a key role in pathogenesis and immunity. Homogenous primary macrophage culture systems are desirable to study specific effects of various pathogens and stimulants on gene expression, activation, and proliferation without the influence of previous in vivo activation or other cell types. Peripheral blood monocytes and peritoneal macrophages have been used for primary macrophage studies in animal models, but cell number and longevity are limiting due to lack of further replication. In addition, these cells are in various stages of differentiation and activation. Alternatively, in several mammalian systems, culturing of bone marrow produces a homogenous population of bone marrow-derived macrophages (BMDM) which can be sequentially passaged (Adler et al., 1994; Hume et al., 1985; Stewart et al., 1981; Boltz-Nitulescu et al., 1987; Daniel et al., 1993; Francey et al., 1992; Tipold et al., 1998). Methods for establishing a pure culture of guinea pig BMDM have not previously been reported, although other groups have used mature guinea pig bone marrow macrophages (Dreher et al., 1981; Phillips et al., 1979) and mixed bone marrow cultures (Gonzalez-Serva et al., 1978). BMDM from many species have been cultured using conditioned media from the murine L929 cell-line as a

source of macrophage colony stimulating factor (M-CSF) (Boltz-Nitulescu et al., 1987). Our goal was to develop a technique for the culture of guinea pig BMDM and assess the basic morphological and functional characteristics of these cells.

## 2. Materials and Methods

### *2.1. Animals*

Animals were handled in accordance with National Institutes of Health guidelines for the humane use of laboratory animals. Typically, 250-400gm male outbred Hartley guinea pigs with an average age of six weeks were used (Charles River Laboratories, Wilmington, MA)

### *2.2 Reagents and Media*

The supernatant from the murine fibroblast L929 cell line is a known source of macrophage-colony stimulating factor (M-CSF) . To obtain M-CSF rich conditioned supernatant, L929 cells (gift of Dr. T. Jerrells, University of Nebraska Medical Center, Omaha, NE) were grown in Cellgro high-glucose DMEM media (Mediatech Inc., Herndon, VA) containing 5% fetal bovine serum (FBS, Invitrogen-Gibco Corp., Grand Island, N.Y.), 2mM L-glutamine, and 0.5mg/ml gentamicin. Cells were passaged approximately twice per week and the supernatant was collected after the cells reached confluency. Supernatant was filtered with a 0.22μm filter (Millicore Corp., Bedford, MA) and stored at $-20^{o}$C until use.

Media for bone marrow-derived macrophages (BMDM) consisted of 60% v/v high-glucose DMEM (containing 5% FBS, 2mM L-glutamine, 0.5 mg/ml gentamicin), 20% v/v heat-inactivated FBS and 20% v/v conditioned L929 cell supernatant. Media was filtered with a 0.22μm filter before use. In our hands, 20% L929 cell supernatant was found to be the optimum concentration, with 50% L929 cell supernatant inhibiting cell growth. A source of guinea pig IFNγ for stimulating BMDM was obtained from concanavalin A-stimulated guinea pig spleen cell supernatants (CAS) (Amminikutty *et al*., 2003). To prepare CAS, spleens were aseptically removed, placed in 10 mls of ice-cold PBS w/o $Ca^{2+}Mg^{2+}$ containing penicillin (100 IU/ml) and streptomycin (100 μg/ml) and triturated into a single-cell suspension in a tissue culture dish (Nunc, Rochester, NY). Nucleated cell counts were adjusted to $10^7$ cells/ml of phosphate buffered saline (PBS) and 6 mls of cell suspension carefully layered onto 3 ml of Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were centrifuged at 450xg for 30 minutes at room temperature and the buffy coat transferred to a polypropylene tube. After two washings in PBS, mononuclear cells were resuspended at 1 x $10^6$ cells/ml in complete RPMI-1640 media (Invitrogen-Gibco, Grand Island, NY) containing 10% v/v FBS, 2mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Five mls of cell suspension was cultured in a 25 $cm^2$ flask (Corning Inc., Corning, NY), containing concanavalin A (Sigma, St. Louis, MO) to produce a final concentration of 5μg/ml. A second flask of cell suspension prepared without concanavalin A was the source of "control supernatant". After a 96 h incubation at 37$^o$C in a 5% $CO_2$, humid atmosphere,

supernatants were collected, centrifuged to remove cells, filtered with a 0.22μm filter, and stored at –80°C.

Antibodies used include: mouse monoclonal antibody specific for guinea pig MHC II (clone number Cl.13.I) (Accurate Chemical, Westbury, NY) (Tan *et al*., 1985), monoclonal mouse anti-guinea pig macrophage antibody (MR-1) (Serotec, Kidlington, England )(Kraal *et al*., 1988), FITC-conjugated sheep anti-mouse $F(ab)_2$ IgG (Boehringer Mannheim Biochemica, Mannheim, Germany), and horse anti-mouse IgG Texas Red conjugate (Vector Laboratories, Burlingame, CA). *E. coli* 011:B4 lipopolysaccaride for BMDM stimulation was obtained from Sigma, St. Louis, MO.

*2.3. Collection of Bone marrow-derived macrophages (BMDM)*

Complete femurs were removed from six week old, male, Hartley outbred guinea pigs (350-400 gms) purchased from Charles River Laboratories. When larger guinea pigs were used there was a decrease in BMDM numbers. Extraneous tissue was scraped off the femur and the ends were clipped. Bone marrow was flushed from the femur with a needle and syringe containing 8 mls bone marrow media and collected in a 50 ml conical tube. Cells from both femurs from one guinea pig were collected in one conical tube.

*2.4. Soybean Agglutinin purification*

Figure 1 is a schematic representation of the procedures which yielded maximum purity of guinea pig BMDM. Bone marrow cells were treated with soybean agglutinin (SBA) to enrich for hematopoeitic precursors and eliminate mature cells by the following

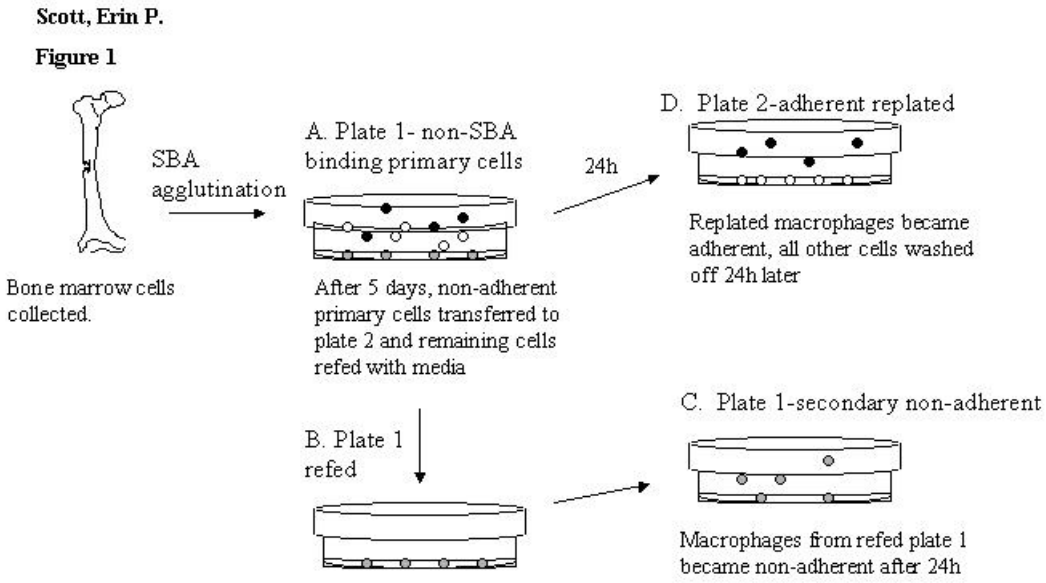



**Figure 1: Schematic for derivation of BMDM**.

After SBA enrichment, cells were plated in Nunclon dishes (plate 1). Five days later, non-adherent primary cells from plate 1 were transferred to another Nunclon dish (plate 2, D) where the macrophages become adherent. Plate 1 was washed and refed (B) with fresh media after the transfer of non-adherent cells to plate 2. The remaining cells from plate 1 became non-adherent over time (C).

procedure (Ebell *et al*., 1985; Reisner *et al*., 1980). Bone marrow cells were centrifuged at 200xg for 5 minutes, resuspended in ACK lysis buffer, (0.15M $NH_4Cl$, 0.1mM $Na_2$ EDTA, 1.0mM $KHCO_3$, pH 7.3) and incubated at $37^o$ for 5 minutes to lyse the red blood cells. In a 12 x 75mm polystyrene tube, approximately 1 x $10^8$ nucleated cells were then added to one volume SBA solution (2mg/ml in PBS) (Vector Labs, Burlingame, CA) and incubated at room temp for 5 minutes. The solution was then layered over eight volumes of 5% bovine serum albumin (Sigma-Aldrich Co., Dallas, TX) in $dH_20$. After a 10 minute incubation at room temperature, the non-agglutinated cells at the interface were transferred to a conical tube and the cells were washed once with PBS. Cells were resuspended in 8 mls of BMDM media and all 8 mls placed on one Nunclon 100 x 30 mm polystyrene tissue culture dishes (Nunc, Rochester, NY) at a density of approximately $1x10^6$ cells/ml (Figure 1A). Nunclon (Nunc) cell culture dishes produced more macrophage growth than untreated petri dishes. Cells from individual guinea pigs were cultured separately. Cells were cultured at $37^o$ in 5% $CO_2$. 48-72 hours after collection, fresh media (25% of the original collection volume) was added. Five days post-collection, non-adherent primary cells were transferred to another Nunclon plate and the original plates were refed with fresh media (Figure 1B). Within 24 hours, two macrophage populations were derived: "adherent replated" (Figure 1D) and "secondary non-adherent" (Figure 1C) macrophages.

*2.5. Electron Microscopy*

Secondary non-adherent BMDM were harvested at day 10 and centrifuged in a microcentrifuge at 1,200xg for 5 minutes. The pellet was then fixed in a mixture of 2% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% $OsO_4$ in the same buffer, stained *en bloc* in 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in epoxy resin Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips 201 electron microscope at 60 kV.

*2.6. BMDM stimulation/MHC class II detection*

BMDM cells harvested as described above on day 7 were centrifuged, resuspended in BMDM media at a density of 1 x $10^6$ cells/ml in 60 x 15 mm Falcon tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight. Each BMDM plate was stimulated with CAS (described above) diluted 1:5, 1:10, or 1:20 in BMDM media. BMDM cells exposed to control spleen supernatant at a 1:5 dilution was used as a control. After incubation for 18 hours to stimulate MHC II, cells were washed with cold PBS and stained with 20 µl of mouse anti-guinea pig MHC II (1:40 dilution). Following a 0.5 hour incubation, cells were washed with PBS and 20 µl of FITC-conjugated sheep anti-mouse $F(ab)_2$ IgG antibody (1:20 dilution) was added for 0.5 hours. After subsequent washing, the cells were resuspended in 500 µl of 4% paraformaldehyde dissolved in PBS. Cells were analyzed by flow cytometry with immunoglobulin (IgG) matched isotype control antibodies and cells incubated only with secondary antibodies were used to set baseline values for positive staining quantitation. Forward versus side

light scatter was used for isolation of cell populations based on size. Acquisition and analysis of the data was performed using Cellquest software (Becton Dickinson, Mountainview, CA).

*2.7. Histochemical techniques*

BMDM at days 0, 7, and 17 post-collection were harvested and cytocentrifuge preparations of 1 x $10^4$ cells in PBS centrifuged onto uncoated glass slides using a Cyto-tek cytocentrifuge (Miles Scientific, Mishawaka, IN). Slides were either used immediately or air-dried and stored at –20$^o$C. Non-specific esterase staining was done using a commercial non-specific esterase kit according to manufacturer's instructions (Sigma, St. Louis, MO) based on the method previously described (Li et al., 1973). Cell morphology was characterized by Diff-Quik stain (Merz & Dade, Switzerland).

*2.8. Immunohistochemistry*

MR-1 is a monoclonal antibody that detects a component of the cytoplasm in guinea pig tissue macrophages (Kraal et al., 1988). For MR-1 staining, cytocentrifuge preparations of day 7 and day 17 adherent replated and secondary non-adherent cells were fixed for 10 minutes in acetone at –20$^o$C. Following PBS washes, samples were blocked with buffer containing 1xPBS/1% BSA/5% normal horse serum/0.02% v/v sodium azide, for one hour at room temperature. After blocking, MR-1 was added to the cells (1:10 in dilution buffer 1xPBS/1% BSA/1% NHS/0.02% v/v sodium azide) and the slide was incubated for 45 minutes at room temperature. Slides were rinsed twice for 7 minutes each with

gentle stirring, followed by the addition of Texas-Red conjugated anti-mouse Ig (1:150 in dilution buffer) secondary antibody and incubation at room temp for 30 minutes in the dark. After additional washes with PBS, slides were coverslipped with GelMount (Biomeda, Foster City, CA) and viewed with a fluorescent microscope (Optiphot, Nikon Inc. Instrument Group, Garden City, NY). Control slides were incubated with diluent in lieu of primary antibody. Frozen sections of normal guinea pig liver containing MR-1 positive Kupffer cells served as positive controls.

*2.9. Phagocytosis Assay*

BMDM were plated in 48 well plates at 1 x $10^5$ cells/well and incubated overnight at $37^0$C to allow cells to adhere. *Saccharomyces cerevisae* (yeast) in PBS was inactivated by a 30 minute incubation at 60$^o$C. Yeast particles (1 x $10^8$ particles/ml) were incubated at a 1:1 dilution with normal guinea pig serum for one hour at 37$^o$C for opsonization and then diluted in PBS to 7.5 x $10^6$ cells/ml. Opsonized yeast suspension (100μl) was added to each well, and cells were incubated for 2 hours at 37$^o$C. Parallel plates were incubated with yeast suspension at 4$^o$C as a negative control. Cells were washed twice with PBS, resuspended in PBS and prepared on slides by cytocentrifugation. A Wright stain (Diff-Quik, Merz & Dade, Switzerland) was applied and cells counted by light microscopy. A minimum of 200 cells per slide was counted and cells containing more than three yeast particles were considered positive.

*2.10. Cytokine assays*

BMDM were plated at 1 x $10^5$ cells in a 48 well plate and allowed to adhere overnight. LPS (100μl) was added at a concentration of 1μg/ml for 24h and supernatants collected and analyzed for cytokine production. IL-8 ELISA was performed using a kit for human IL-8 (R&D Systems, Minneapolis, MN) validated previously for guinea pig (Kuo et al., 1997). The protocol provided by the manufacturer was used. TNF production was determined using a L929 cell cytotoxicity bioassay described previously (Aronson et al., 1995).

## 3. RESULTS

### *3.1. BMDM culture*

Initially, techniques of BMDM isolation described for murine systems were adapted for the guinea pig, including the use of murine L929 cell supernatant as the source for M-CSF . However, the cultivation of guinea pig BMDM using these techniques produced a mixed population of macrophages and fibroblast-like cells. By approximately day 10 of culture, fibroblast-like cells were predominant with less than half of the cells displaying macrophage properties of non-specific esterase and phagocytic activity (Table 1, non-SBA column). Electron microscopy also showed a fibroblast-like morphology (not shown). In order to enrich for hematopoetic precursors, a negative selection was developed utilizing soybean agglutinin (SBA), a plant lectin that binds and agglutinates mature cells including fibroblasts (Ebell *et al*., 1985; Reisner *et al*., 1980). Figure 1 summarizes the procedures for deriving pure BMDM. When viewed

by light microscopy, after five-days of culture SBA purified bone marrow cultures showed substantially fewer fibroblast colonies than non-SBA treated cultures. Many non-adherent cells were present, and Wright stain analysis showed that the non-adherent "primary" cells were a mixed population including macrophages, neutrophils, and other cells (not shown). In order to isolate the macrophages from these non-adherent primary cells, all the cells were removed from the original plate at day 5 (Figure 1A) and replated into a second Nunclon plate for adherence purification (Figure 1D). Some of the replated primary cells became adherent and were designated "adherent replated". These cells were determined to be macrophages by light microscopy (Figure 2A, B). Electron microscopy confirmed macrophage morphology (Figure 2C). After washing with PBS and refeeding with BMDM media, the original plates yielded more non-adherent cells, designated "secondary non-adherent cells" (Figure 1C). At any time point when these secondary non-adherent cells were replated, they became adherent. The timing of non-adherent primary cell transfer from the original plate (Figure 1, A to D) was critical. When non-adherent primary cells were removed one day post bone marrow collection, there were very few adherent cells left to produce secondary non-adherent cells. When non-adherent primary cells were replated at day 3 post collection, numerous "adherent replated" cells were observed, while the secondary non-adherent cell population was decreased and very few cells remained in the original plate. In contrast, when non-adherent primary cells were replated on day 5, fewer numbers of "adherent replated" cells were observed, with greater numbers of secondary non-adherent cells. These results

indicate that at day 3, many of the precursors were non-adherent and matured once replated, leaving few cells in the original plate to mature and produce secondary non-adherent cells. When we waited until day 5 to replate the non-adherent primary cells, many of the precursor cells had become adherent, leaving more cells in the original plate to produce secondary non-adherent cells. The best BMDM yields overall were obtained by replating at day 5.

| **Day of culture** | **Test** | **Non-SBA treated** | **SBA treated** | |
|---|---|---|---|---|
| | | | **$2^o$ non-adherent** | **Adherent replated** |
| 0 | NSE | 16 | NA | NA |
| 6-7 | NSE | 26 | 57 | 75 |
| | Phagocytosis | 55 | 49 | 86 |
| 17-20 | NSE | 18 | 91 | 95 |
| | Phagocytosis | 35 | 69 | 71 |

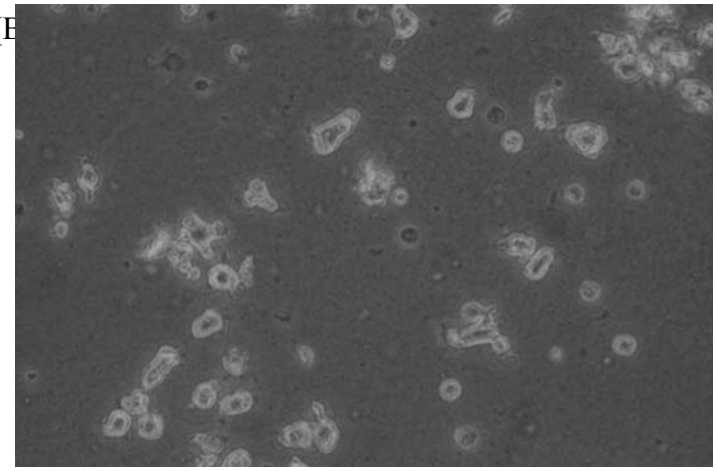
Table 1: Comparison of soybean agglutinin (SBA) treated vs non-treated BMDM cultures. Bone marrow cells were either untreated (non-SBA), or treated with SBA prior to culture (SBA treated). At the indicated day of culture, BMDM were assayed for non-specific esterase (NSE) activity and the ability to phagocytose yeast particles. Data reported is the percentage of BMDM cells which showed NSE or phagocytic activity out of 200 cells counted. Results are representative of multiple experiments. For a description of Secondary non-adherent and adherent replated cells, see Figure 1.

**Figure 2: Morphology of SBA purified BMDM.**

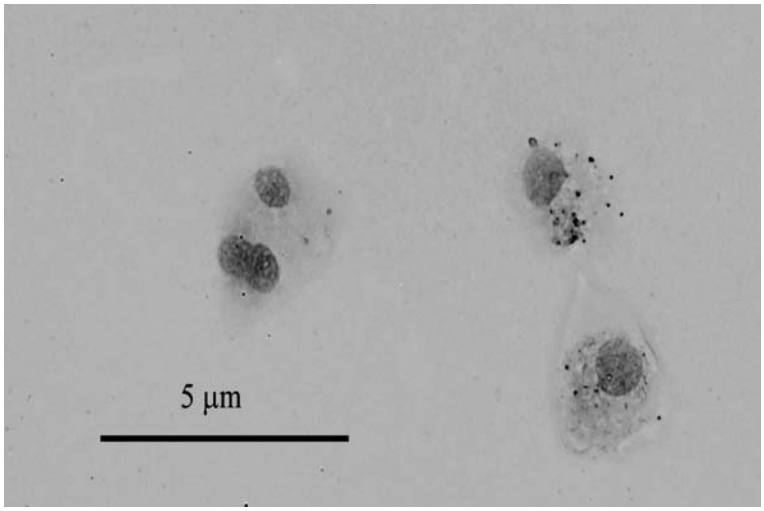
(A) Inverted phase contrast microscopy of day 11 adherent replated cells (20X). (B) Wright stain of day 17 adherent replated cells (40X). (C) Electron microscopy of day 10 non-adherent BMDM. Arrow points to probable phagocytosed particles indicative of macrophages (14,100X).

A.

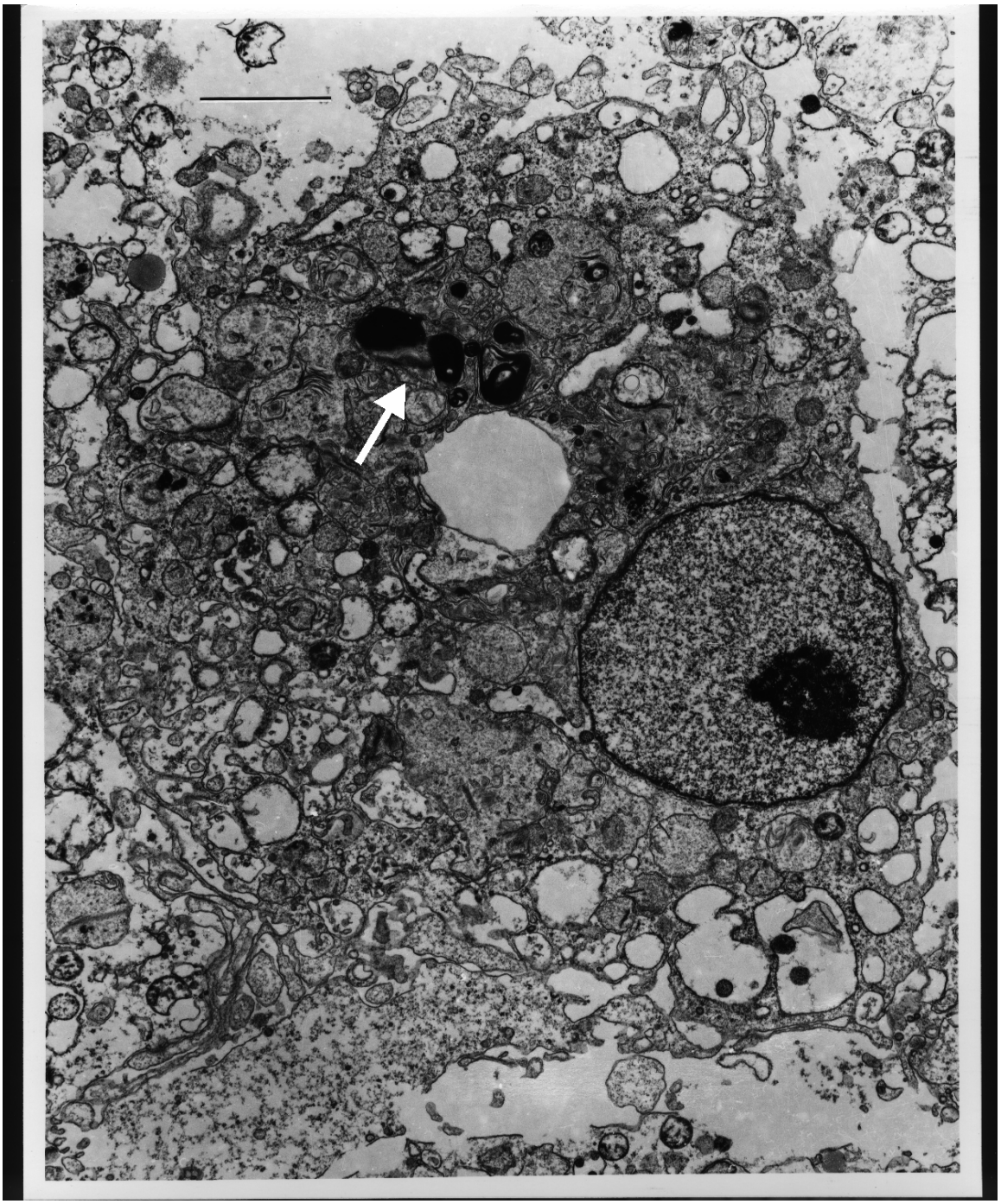
(B



B.



C.



Typical cultures of murine BMDM result in a relatively pure population with approximate yields of 1 x $10^7$ BMDM per femur by day 7 . In contrast, typical yields for our procedure were approximately 1 x $10^6$ cells per guinea pig at day 7, representing 90% secondary non-adherent and 10% adherent replated. We speculated that lack of guinea pig BMDM proliferation was due to poor cross-reactivity of murine M-CSF with guinea pig monocyte precursors. However, there was no increase in BMDM yields when L929 conditioned media was substituted with conditioned media from non-SBA purified bone marrow cultures as a source for guinea pig growth factors.

## 3.2. BMDM characterization

*3.2a.Histochemical analysis*

Cells contain different isoenzymes of esterase which can be used histochemically to differentiate cells of monocytic and granulocytic lineages (Li *et al*., 1973). For example, hydrolysis of α-naphyl acetate by nonspecific esterase (NSE) identifies cells of the monocytic lineage (Li *et al*., 1973). Cytopreparations of guinea pig BMDM cells were collected and analyzed for NSE activity. Cells showed a range of intensity of histochemical staining for NSE, with some cells having distinct black cytoplasmic granulation, and other having a more diffuse dark gray cytoplasmic staining compared to the light blue cytoplasm of negative cells (Figure 3A). A low proportion of non-SBA treated cells had NSE activity, ranging from 33% on day 6 to 18% cells positive on day 20. A higher proportion of SBA treated cells had NSE activity, with 57% of non-

adherent secondary cells positive on day 7 and 91% positive on day 17, while adherent replated were 75% positive on day 7 and 95% positive on day 17 (Table 1).

*3.2b. Phagocytosis assay*

We compared the ability of non-SBA treated BMDM cells, SBA non-adherent, and SBA adherent replated cells to phagocytose opsonized yeast particles. The proportion of non-SBA treated cells with phagocytosis activity was 55% on day 7, but decreased to 35% by day 20. In comparison, SBA treated cultures showed an increase in phagocytic activity during the same time period, with up to 70% positive cells by day 17. In addition, the adherent replate BMDM had higher levels of phagocytic activity than secondary non-adherent BMDM at day 7, however, by day 17 both populations had a similar proportion of phagocytic capability (Table 1). The lower levels of NSE and phagocytosis activity at the earlier timepoint for the secondary non-adherent cells compared to the adherent replated cells, likely indicates a different level of maturation since the activity of both cell populations becomes nearly equal by day 17.

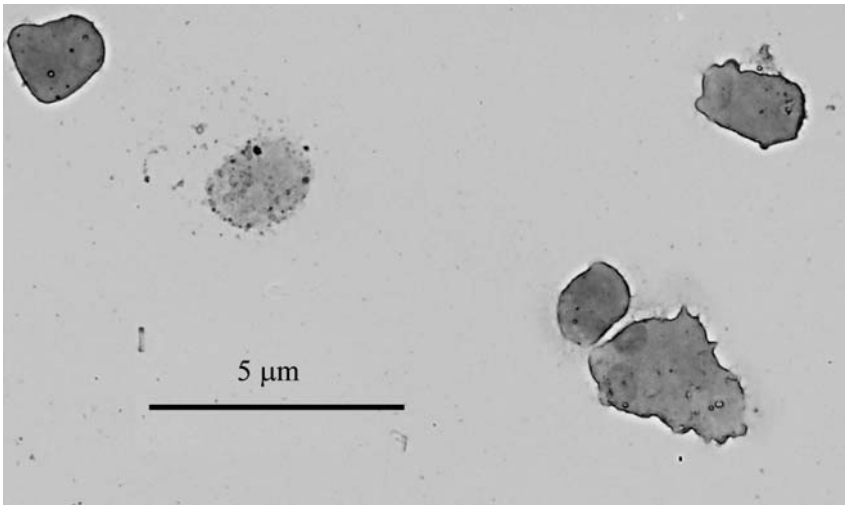
*3.2c. Immunohistochemistry*

MR-1 is a guinea pig macrophage monoclonal antibody that targets a cytoplasmic component of many tissue macrophages (Kraal *et al*., 1988). Secondary non-adherent and adherent replated BMDM did not stain well with MR-1, with weak to absent staining

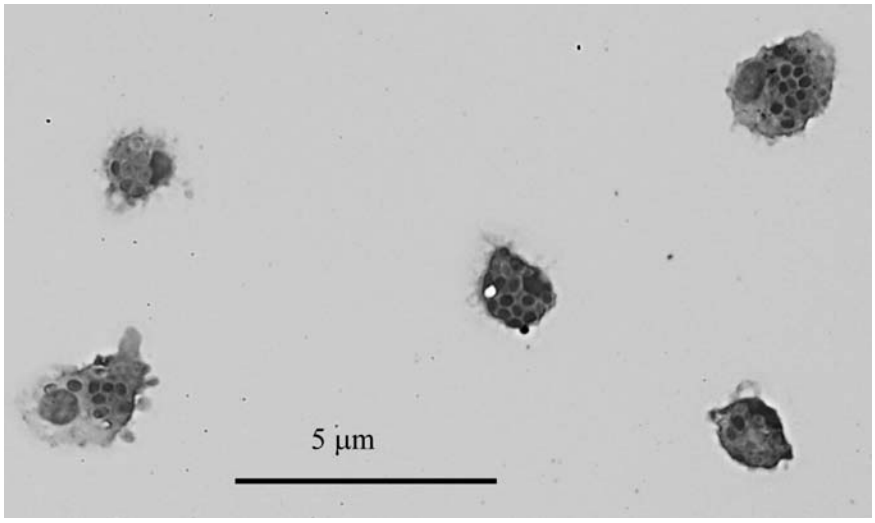
**Figure 3**: **Micrographs of NSE positive and phagocytic SBA purified BMDM**.

SBA-purified BMDM were harvested at days 7 and 17 and assayed for (A) non-specific esterase staining and (B) phagocytosis activity. Micrographs are representative of positive cells.

A.




B.



compared to secondary antibody only treated controls, while control liver sections exhibited very bright positive staining of Kupffer cells. A similar finding has been reported for peritoneal macrophages, which exhibit less intense staining levels than tissue macrophages (Kraal *et al*., 1988). Few commercially available guinea pig macrophage-specific immunologic markers are available, which limited our ability to further characterize the BMDM by this method.

### 3.2d. MHC class II analysis

As part of our characterization of these BMDM, we were interested in determining whether IFNγ could induce MHC II expression. One of the few commercially available anti-guinea pig antibodies recognizes MHC class II (Tan *et al*., 1985). To determine whether MHC II expression by BMDM could be induced, BMDM were treated with varying dilutions of CAS as a source of IFNγ. Using forward and side scatter analysis, the cells were consistently homogenous in multiple experiments (Figure 4A). expression (7.11% positive cells). However, a dose-dependent increase in MHC II expression was detected in CAS stimulated BMDM, with up to a three-fold induction of MHC II (Table 2). Figure 4B demonstrates a representative shift of positive staining cells compared to secondary antibody alone controls.

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Figure 4

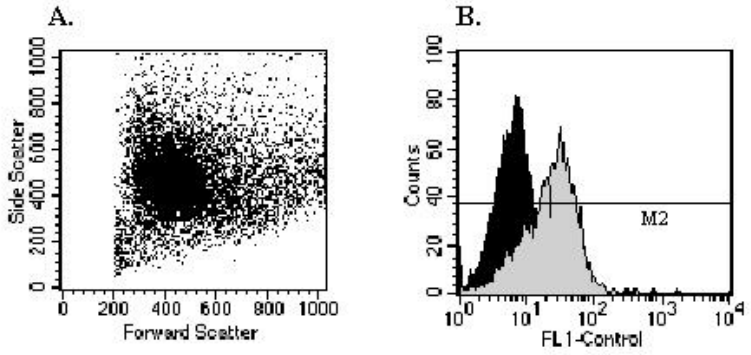


**Figure 4**: **BMDM MHC class II expression.**

SBA-purified BMDM were stimulated for 18 hours with either CAS or control supernatant. Cells were stained with a primary anti-guinea pig MHC class II antibody and secondary FITC antibody then analyzed by flow cytometry. (A) Forward and side scatter indicates a homogenous population. (B) A representative histogram shows an overlay of MHC II expression (gray) and secondary antibody only control (black).

| Supernatant | Dilution | % positive |
|---|---|---|
| CAS | 1: 5 | 21.73 |
| CAS | 1:10 | 17.18 |
| CAS | 1:20 | 10.65 |
| Control | 1:5 | 7.11 |

Table 2: Detection of MHC class II on stimulated BMDM.
BMDM were stimulated for 18 hours with the indicated dilution of supernatant from either 96 hour concanavalin A stimulated guinea pig spleen cells (5μg/ml) or control supernatant. BMDM were then analyzed by flow cytometric analysis for MHC class II expression. Data was collected from 10,000 cells for each sample. Results are percent positive MHC II cells from a representative experiment. Control levels for secondary antibody only stained cells were 2.9-4.5%.

### 3.2e. Cytokine assays

To determine whether BMDM were capable of cytokine induction, 1 x $10^5$ BMDM were stimulated with 1 g/ml of LPS overnight. The supernatant was assayed for TNFα by bioassay, and IL-8 by ELISA for detection of human IL-8, previously validated for use for guinea pig IL-8 (Kuo et al., 1997). A 1000-fold increase in TNFα and a 4-10 fold increase in IL-8 production were seen in LPS-stimulated BMDM (Figure 5).

## *4. DISCUSSION*

Culture of a pure bone marrow-derived macrophage population has been described for many species including, but not limited to, mouse, human, rat, cat, dog, cow, and sheep (Boltz-Nitulescu et al., 1987; Adler et al., 1994; Daniel et al., 1993; Francey et al., 1992; Hume et al., 1985; Tipold et al., 1998). To date however, this list does not include guinea pig, an important animal model for the study of a variety of

infectious and allergic diseases. This investigation provides the first report of the generation and characterization of guinea pig bone marrow-derived macrophages.

For culture of murine and rat BMDM, bone marrow cells are simply flushed from the femurs and cultured in media containing a source of murine M-CSF (; Boltz-Nitulescu et al., 1987). By approximately day 17, high numbers of macrophages have been reportedly obtained with little evidence of contaminating cells (Boltz-Nitulescu et al., 1987; Hume et al., 1985; Tipold et al., 1998). However, when we used a similar technique for guinea pigs, there were large colonies of contaminating fibroblast-like cells by day 10 of culture. These results were not surprising, as bone marrow normally contains large numbers of specialized fibroblasts. Hsiung and Gonzalez-Serva also noted a large population of fibroblasts by day 10 in cultures of guinea pig bone marrow (Gonzalez-Serva *et al*., 1978). These investigators did not utilize any source of M-CSF in their cultures, and no further attempts to purify the macrophages were made. We adapted a strategy to eliminate the contaminating cells from guinea pig BMDM cultures. This strategy was based on the reported purification of hematopoietic precursors for bone marrow transplant purposes by using the plant lectin soybean agglutinin (SBA) that binds to and agglutinates fibroblasts and mature hematopoietic cells (Ebell *et al*., 1985; Reisner *et al*., 1978; Reisner *et al*., 1980). When SBA was used to deplete bone marrow of fibroblasts and enrich for hematopoietic precursor cells, including monocyte precursors, we observed a marked reduction of fibroblast colonies.

**Figure 5**: **Effect of LPS on BMDM cytokine production.**

Non-purified or SBA purified BMDM ($1x10^5$ cells) were stimulated for 18 hours with media alone or 1μg/ml of LPS. Supernatants were collected and assayed for (A) TNFα by L929 cytotoxicity assay and (B) IL-8 by human ELISA.

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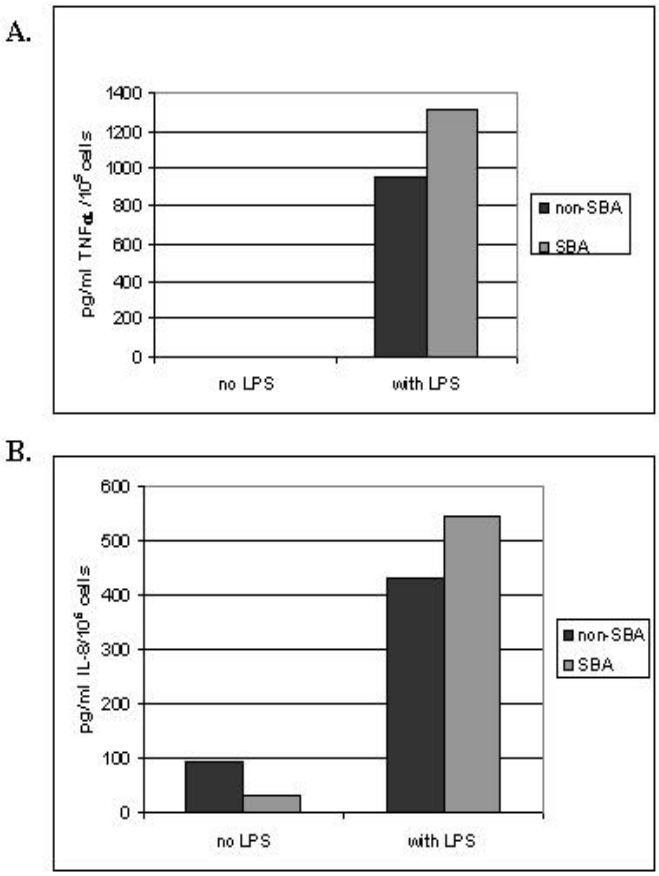
**Figure 5**




After elimination of the fibroblasts by SBA agglutination, we observed both adherent and non-adherent populations from the initial culture of primary cells. Replating the primary non-adherent cells in a fresh dish produced a population of

"adherent replated" cells. A "secondary non-adherent" cell population arose in the original plate; this can be attributed to either loss of adherence or proliferation of the original adherent primary cells. Analysis of the secondary non-adherent cells and the adherent replated cells revealed different degrees of NSE and phagocytic activity. We speculate that the non-adherent cells become more mature after replating as evidenced by the development of adherence and increased incidence of phagocytic and NSE activity (Table 1). Other investigators have found similar results. For example, human BMDM non-adherent cells at day 7 were found to be NSE negative, but had a mononuclear cell morphology and became adherent and NSE positive when replated on serum coated flasks (Hume *et al*., 1985). Murine BMDM cultures have non-adherent and adherent populations that display different monoclonal antibody staining properties. The non-adherent cells will also produce adherent macrophage colonies after replating (Walker *et al*., 1985b). The maturity of non-adherent cells after replating may be due simply to cell maturation over time, as evidenced by the increase in phagocytic and NSE activity by non-adherent cells without replating (Table 1). Alternatively, considering the sensitivity of cells to any environmental change, the stress of being replated and refed with fresh media may cause the cells to mature. We consider both the adherent replated and non-adherent secondary cells to be BMDM, which can be pooled together for use in functional studies at days 17-20 of culture.

The major limitation of our method for producing guinea pig BMDM is the lack of large cells numbers. The yield was approximately ten-fold less cells than for other

BMDM systems (Daniel et al., 1993; Francey et al., 1992). In some systems, high levels of serum in the media are sufficient to produce large numbers of macrophages (Adler *et al*., 1994; Francey *et al*., 1992) while other systems need a source of M-CSF (Boltz-Nitulescu et al., 1987; Hume et al., 1985). Murine L929 cell supernatant, a crude source for M-CSF, was used for the production of BMDM from rat (Boltz-Nitulescu *et al*., 1987). Utilizing a media combination of high serum concentrations and L929 supernatant, we obtained an increase in macrophages by day 7, however subsequent cell numbers did not significantly increase. This result is similar to bovine and ovine BMDM for which yields are not enhanced by L929 supernatant (Adler *et al*., 1994; Francey *et al*., 1992). A possible explanation is that murine M-CSF does not cross-react well with bovine, ovine, and guinea pig cells. An experiment using crude supernatant from unpurified guinea pig bone marrow culture as a potential source of guinea pig M-CSF did not increase yields. We did observe that in the unpurified bone marrow cultures, the macrophages congregated near the fibroblast colonies. This could be due to a growth factor or an extracellular matrix component released by the fibroblasts that the macrophages favor. Experiments using plates coated with different types of matrix proteins are ongoing.

Few immunophenotyping markers are available for guinea pig macrophages. Little if any immunoreactivity to MR-1 was observed in BMDM. MR-1 is a commercially available monoclonal antibody which recognizes a cytoplasmic component primarily in guinea pig tissue macrophages and is reported to be strongly reactive to

Kupffer cells, spleen macrophages, alveolar macrophages; moderately reactive to peritoneal exudate cells and monocytes; and non-reactive to Langerhans and microglial cells (Kraal *et al*., 1988). In contrast, most mature macrophages isolated directly from the bone marrow are MR-1 positive (Kraal *et al*., 1988). Considering monocytes only stain moderately well with MR-1, it is not surprising that BMDM showed poor immunostaining with this antibody. MR-1 likely recognizes a marker of maturation attained by tissue macrophages, but not yet attained by monocytes and BMDM.

BMDM did show a moderate ability to upregulate MHC class II molecules in response to CAS, a putative source of IFNγ. At 24h, unstimulated cells expressed low levels of MHC II, and a three-fold induction was seen in cells exposed to the highest concentration of CAS. These results are similar to mouse BMDM, where recombinant mouse IFNγ induced a three-fold induction of MHC class II after 24 hours (Cullell-Young *et al*., 2001). Guinea pig BMDM were responsive to LPS treatment. Large increases in TNFα and IL-8 were detectable 24 hours after LPS stimulation, with slightly higher levels for the SBA purified macrophages. This is consistent with reports of BMDM from other species in which LPS upregulates cytokines (Adler *et al*., 1994; Ogle *et al*., 1997; Tipold *et al*., 1998; Daniel *et al*., 1993).

In summary, we have developed a method to produce reasonably pure guinea pig bone marrow-derived macrophages. These BMDM exhibit functionally characteristic macrophage-like activity by NSE and phagocytosis assays and can be activated to express

MHC class II molecules and cytokines. These cells will be useful for the study of macrophage function, activation and differentiation in a variety of disease processes.

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Vita

Erin Scott was born on June 17, 1977 to Steve and Sharon Webb. Erin is married to Dr. Benjamin Scott. During her undergraduate career at the University of Kansas, Erin gained laboratory experience in the department of Pharmaceutical Chemistry, and completed a summer internship at the United States Army Military Research Institute of Infectious Diseases. While in graduate school, Erin has presented both oral and poster presentations at several local, national, and international meetings. Erin received two awards for her presentations in the annual Experimental Pathology poster session. In addition, Erin served on multiple UTMB and departmental committees, and was a co-founder of the Experimental Pathology Graduate Student Organization.

Erin's teaching experience included assistant teaching in several University of Texas Medical Branch medical school courses. She was also a tutor for the Graduate School of Biological Sciences Molecular Biology and Genetics course.

Erin has recently accepted a post-doctoral fellowship at the University of Pennsylvania under Dr. Susan Weiss. She will be researching the neuroimmunological aspects of murine hepatitis virus.

Education

B.S., May 1999, The University of Kansas, Lawrence, KS

Publications

Youngbo, H.; Webb, E.; Singh, J.; Morgan, B.A.; Gainor, J.A.; Gordon, T.D.; Siahaan, T.J. (2002) Rapid determination of substrate specificity of *Clostridium histolycticum* B-collagenase using an immobilized peptide library. J Bio Chem 277:8366-8371

Scott, E.P., Marriot, K.A., Aronson, J.F. "Cytokine patterns in a guinea pig model of Lassa fever: Comparison of mild and severe disease." Submitted to Virology on June 1st, 2005.

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Scott, E.P.; Yang, X.B.; Gorenstein, D.G.; Aronson, J.F. "An AP-1 thioaptamer increases survival and alters cytokine expression in a guinea pig model of arenavirus hemorrhagic fever." In preparation.

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## Selected Abstracts

"Genomic–wide expression of *Saccaromyces cerevisiae* in response to voriconazole"

Presented at the Structural Biology Symposium – UTMB-2001
Presented at ICAAC – Chicago, IL – 2001

"The effect of transcription factor aptamers on TNF alpha and IL-8 expression in primary guinea pig macrophages: therapeutic implications in a guinea pig model of arenavirus disease."

Presented at Negative Strand Virus – Pisa, Italy – 2003

"Cytokine patterns in guinea pig arenavirus infection."

Oral presentation at American Society for Virology – Montreal, Canada – 2004

"Early and late cytokine responses in a guinea pig model of arenavirus hemorrhagic fever."

Presented at the American Society for Virology – State College, Pennsylvania- 2005