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Effects of transforming growth factor beta1 on Hantavirus cardiopulmonary syndrome

Stephanie Flynn James

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

EFFECTS OF TRANSFORMING GROWTH FACTOR BETA 1 ON HANTAVIRUS CARDIOPULMONARY SYNDROME

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Stephanie F. James

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Accepted by the Doctoral Committee

_____
William Schountz, Ph.D., Chair

_____
Patrick Burns, Ph.D., Committee Member

_____
Richard Jurin, Ph.D., Committee Member

_____
Steven Pulos, Ph.D., Faculty Representative

Date of Dissertation Defense_____

Accepted by the Graduate School

_____
Linda L. Black, Ed.D., LPC
Acting Dean of the Graduate School and International Admissions

ABSTRACT

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Hantavirus cardiopulmonary syndrome (HCPS) is characterized by fatigue, fever, and thrombocytopenia and results in pulmonary edema and shock. HCPS currently has a 36% mortality rate in the United States. The small animal model for studying HCPS was the Syrian golden hamster (*Mesocricetus auratus*), which develops a similar disease when infected with Andes (ANDV) or Maporal hantavirus (MAPV). We tested the use of anti-inflammatory cytokines, transforming growth factor-β1 (TGFβ1) or interleukin-10 (IL-10) as therapeutic agents for attenuating disease severity. Gene expression in both lung and spleen suggested an innate immune response with elevation of STAT 1 and MxA. The administration of TGFβ1appeared to suppress expression of several vasoactive cytokines, tumor necrosis factor (TNF), and interferon-γ (IFN-γ) in the lungs of infected animals and decreased lung congestion and pleural fluid volume; however, no significant attenuation of lesion severity was observed. Administration of IL-10 resulted in increased lesion score and no suppression of gene expression. This suggested that the noncognate functions of TGFβ1 may play a role in HCPS pathology and that IL-10 augments disease pathology.

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CHAPTER I

INTRODUCTION

Humans have long been subjected to various infectious diseases, many of which have had lasting impacts on society. A particularly significant example includes the bacterium *Yersinia pestis* that causes the plague. Between 1346 and 1350, this disease caused thousands of deaths, wiping out approximately one-fourth of the population of Europe. Spread through bites from infected rat fleas, this disease manifests itself with swollen, discolored lymph nodes, leading to the name many are familiar with--the Black Death. Currently, untreated plague has a mortality rate between 40-100% (Stenseth et al., 2008) and is still endemic in many rodent populations in the United States. Another example of a historically significant infectious disease is smallpox, a once common childhood infection, caused by *Variola* virus. This viral infection is highly contagious because it spreads by respiratory transmission. Although vaccination efforts of smallpox have eradicated the virus, it is considered a potential bioterrorism weapon (Parrino & Graham, 2006).

Many infectious diseases manifest as hemorrhagic fevers--diseases characterized by fever and bleeding diathesis. Also consistent with these severe illnesses are fatigue, edema, and hypotension (Center for Disease Control, 2011). Common examples are ebolaviruses and Marburgvirus, which are highly

virulent and result in high mortality rates. One of the earliest hemorrhagic fevers described is dengue hemorrhagic fever--an arborvirus transmitted by mosquitoes. Between 1927 and 1928, this virus caused the deaths of over 1500 people in Greece (Rosen, 1986). Worldwide, this disease infects a staggering amount of humans (estimated to be 50 million per year), predominantly in Africa, Asia, and South America (Gurugama, Garg, Perera, Wijewickrama, & Seneviratne, 2010). More recently, this virus has spread into North America for the first time, occurring in the Florida Keys (Center for Disease Control, 2010b).

In addition to conditions that give hemorrhagic fevers their names, many also have a renal component. One virus that causes such a disease is Seoul virus. Seoul virus is a member of the genus *Hantavirus*, family *Bunyaviridae.* However, it should be noted that there are many other viruses that cause hemorrhagic fevers with renal complications.

Although the hantavirus disease had occurred for centuries, hantaviruses were first noticed by Western scientists after they gained the interest of American military physicians during the Korean War in 1951. In his 1953 paper, Joseph Smadel described the symptoms of a hemorrhagic fever in American troops and noted that the suspected reservoir was a rodent-associated arthropod. However, it was later shown that it is transmitted directly by the rodents. In 1976, Lee, Lee, and Johnson (1978) isolated the etiologic agent of Korean hemorrhagic fever from the striped field mouse (*Apodemus agrarius coreae)*. His group named the agent Hantaan virus after the Hantaan river region where the mice were found. Later, the virus was classified as a member of the *Bunyaviridae* family. Since

this first identification, several other hantaviruses have been discovered including Puumala virus (host *Clethrionomys glareolus)* and Seoul virus (host *Rattus rattus)*, both of which also cause hantavirus fever with renal syndrome (HFRS).

Until the 1990s, hantaviruses had only been identified in Eurasian countries; those that were pathogenic resulted in diseases with a substantial renal component. In 1993, an outbreak of an unknown illness occurred among otherwise healthy young people in the Four Corners region of the United States. Known as the Colorado Plateau, this area has varied types of vegetation that sustain numerous types of wildlife including many rodent species.

Infected individuals were first presented at medical facilities in New Mexico with an abrupt onset of influenza-like symptoms including fever and headaches. These symptoms then rapidly progressed into respiratory distress and non-cardiogenic shock (Center for Disease Control, 1993). From December of 1992 through June 7, 1993, 24 victims were identified. Fourteen of these patients became ill during May of 1993. Of these initial 24 patients, 12 died resulting in a significant mortality rate of 50%. By November of 1993, new cases brought the mortality rate to above 75% (Nichol, 1993).

P*olymerase chain reaction* (PCR) analysis was conducted using tissue samples from victims to identify the agent responsible for the illnesses. The sequences obtained were homologous to other known hantavirus species with a difference of approximately 30%. Serology also suggested high cross-reactivity with Prospect Hill (PHV) and Puumala hantaviruses (PUUV; Nichol et al., 1993).

This led researchers to believe they were dealing with a previously undiscovered hantavirus most closely related with PHV and PUUV viruses.

Since all known hantaviruses were hosted by rodents, investigators began trapping and testing rodents in the region. The most commonly trapped rodent was the deer mouse (*Peromyscus maniculatus*). Trapped deer mice were tested for the presence of antibody to conserved epitopes on various other hantaviruses. Of all trapped deer mice, 30% had antibodies to hantavirus antigens. PCR results demonstrated that the virus sequenced from the deer mice was identical to those from human cases (Childs et al., 1994), suggesting the etiologic agent was a hantavirus whose reservoir was the deer mouse. This newly discovered hantavirus was eventually named Sin Nombre virus (SNV).

Since the 1993 outbreak, more *hantavirus* cardiopulmonary syndrome (HCPS) cases have been attributed to other newly discovered hantaviruses in North and South America; the hosts for most of these have also been identified. In South America, several species of hantaviruses have been identified: Andes, Juquitiba, Araraquara, Laguna Negra-like, Castelo dos sonhos, and Anajatuba viruses. Each of these has been associated with HCPS and has distinct rodent reservoirs (Oliveira et al., 2009). It is likely that as more animals are tested, new species of hantaviruses will be found.

While hantaviruses clearly cause dramatic health problems for infected humans, their reservoirs do not suffer ill effects. Due to their ability to mount a non-sterilizing antibody response, it has been suggested that hantaviruses and their rodent hosts have co-evolved. Recent PCR data suggest deer mice have

an increased expression of the cytokine-transforming growth factor beta (TGF-β1) and transcription factor fork head box P3 (FoxP3), which is suggestive of T regulatory cell involvement (Schountz et al., 2007). The increase in this cytokine may be important in the deer mice's lack of pathogenesis.

The goal of this project was to further elucidate the role of TGF-β1 in the resistance of disease. A better understanding of factors that aid in host resistance to disease may lead to therapeutics to treat infected patients.

Aim 1

Aim 1 was to characterize the cytokine gene expression in Syrian golden hamsters infected with Maporal hantavirus (MAPV).

H1 The pulmonary inflammation in the lungs of hamsters infected with Maporal virus is caused by inflammatory cytokines.

Hamsters were infected with MAPV at the University of Texas Medical Branch in Galveston under biosafety level four safety conditions. Messenger RNA was evaluated in lung, spleen, and kidney tissues of the infected animals.

Aim 2

Aim 2 was to characterize the effect of TGFβ1 or IL-10 treatment in Syrian golden hamsters infected with Maporal hantavirus.

H2 The pulmonary inflammation observed in the lungs of hamsters infected with Maporal virus will be reduced with the administration of a TGF-β therapy.

We infected hamsters with either MAPV or a saline control and then treated with active TGF-β, IL-10 or a saline control. Pleural fluid was recovered at necropsy and tissues were evaluated for immune cytokine mRNA.

Aim 3

Aim 3 was to determine the viral load in lung, spleen, and kidney of hamsters infected with MAPV.

H3 Viral replication occurs predominantly in the lung tissue of hamsters infected with Maporal virus.

RNA was extracted from lungs, spleens, and kidneys and reverse transcribed using primers specific to the nucleocapsid gene. Viral cDNA was quantified using multiplex PCR with a probe to either the (+) or (-) strand using GAPDH as a control.

CHAPTER II

REVIEW OF THE LITERATURE

Host Reservoirs

Deer mice are found throughout most of North America in a wide variety of environments and habitats. Their main diet consists primarily of seeds and green vegetation. Other hantaviruses causing HCPS exist in other rodent reservoirs including New York-1 virus (white footed mouse, *Peromyscus leucopus*) and Andes virus (long-tailed pygmy rice rat, *Oligoryzomys longicaudatus*). Because an increasing number of rodents is now associated with emerging zoonotic diseases, scientists have a greater appreciation of the importance of these associations. Where rodents are found, there is also the possibility of an associated hantavirus. Many of the identified viruses may have no or little pathogenicity in humans. There have been species identified that do not cause human disease, e.g., Prospect Hill, Tula, and Thottapalayam viruses. The degree of pathogenicity may be due to immune evasion strategies that have yet to be fully elucidated. Studies suggest that the difference in pathogenicity may be due to the innate interferon response. When Vero E6 cells (which are defective in the type I interferon pathway) were infected with pathogenic viruses (SNV, HTNV, Seoul) and non pathogenic viruses (PH, Tula, Thottapalayam), the replication rates were equivalent. However, when these viruses were used to

infect A549 lung cells, the non pathogenic strains induced a strong interferon response as measured by MxA and IFN-β. This response was not observed using pathogenic hantaviruses until later time points (Shim et al., 2011). When Tula and HTNV were used to infect human endothelial cells, it was observed that the HTNV had a faster replication rate. This correlated to a decreased expression of MxA as compared to Tula. It was also observed that HTNV had decreased expression of major histocompatibility complex I (MHC I) compared to the non pathogenic virus (Kraus et al., 2004). Appendix A lists known hantaviruses and their reservoirs.

It has been suggested that hantavirus developed co-evolutionary relationships with their rodent hosts (Yates et al., 2002). Phylogenetic analyses revealed that the more similar the rodent hosts, the more similar the infecting hantaviruses. HCPS is a New World disease found primarily in Sigmodontinae rodents; whereas, a less severe form of hantavirus disease with a renal component (HFRS) is seen in Eurasian continents. It has been speculated that the New World hantaviruses were carried to the Americas on ships and mutated as they spread across North and South America and crossed species (Morzunov et al. 1998; Plyusnin, 2002). This suggestion of genetic drift is supported by studies that demonstrated many hantavirus species differ by only a few point mutations, yet have distinct rodent hosts (Plyusnin, 2002). Several phylogenic studies have evaluated the relatedness of rodent hosts using trees prepared from rodent genetic sequences and sequences encoding the glycoprotein epitopes of hantaviruses. The trees were very comparable; however, there was some

evidence for host switching approximately two to four million years ago (Morzunov et al., 1998; Nemirov, Henttonen, Vaheri, & Plysnin, 2002; Vapalahti et al., 1999). Hence, it is still likely to see the evolution of new hantaviruses over time. While such studies support a hypothesis for co-evolution of virus host relationships, it should be noted that other host virus relationships have been observed, namely in arenaviruses with their rodent reservoirs. A capture study of rodents in central Venezuela suggested specific host relationships also existed between these viruses and a rodent host. Captured *Sigmodon alstoni* (cotton rats) were infected with Pirital arenavirus while *Zygodontomys brevicauda* (cane mice) were infected with Guanarito arenavirus, even though these two rodents occupied the same habitat (Bowen, Peters, & Nichol, 1997; Fulhorst et al., 1999).

SNV has likely been circulating in deer mouse populations for many years. After the 1993 outbreak, researchers evaluated tissue samples from deer mice collected from 1989-1993 at the Sevilleta Long Term Ecological Research Center (LTER) in New Mexico. Samples collected in 1989 showed that only small percentages of deer mice were infected with SNV. By 1991, the incidence of the virus in deer mice was distributed over 100,000 hectares, indicating it could spread easily between deer mice (Yates et al., 2002). In preserved human tissues kept from the 1970s, several individuals who died from respiratory disease were found to have been infected with SNV (Zaki et al., 1996), indicating the virus was present prior to the 1993 outbreak.

It has been postulated that the outbreak in 1993 was due to the 1992 El Niño weather oscillations. The deer mouse population near the Four Corners

area increased dramatically according to data collected at the LTER (Yates et al., 2002). Precipitation during this time increased more so than in previous years, providing increased vegetation and habitats for deer mice. This increase in the deer mouse population spilled over into human habitats such as sheds and woodpiles, thus providing an opportunity for human exposure.

After identification of the virus as a potential hantavirus (during the 1993 outbreak), scientists from the Centers of Disease Control and Prevention (CDC) in collaboration with researchers from Colorado State University and the University of New Mexico trapped over 1,000 rodents from various sites surrounding the Four Corners region. The majority of rodents trapped were from either the *Peromyscus* (mouse) or *Tamias* (chipmunk) genera. Of all trapped animals, 47.9% were *Peromyscus maniculatus*. Serological assays were used to evaluate if rodents had antibodies to known hantaviruses that caused HFRS. Deer mice had the highest rates of reactivity to PHV and SEOV. These animals also had virus in their tissues as evaluated using polymerase chain reaction (PCR; Childs et al., 1994).

Lung tissue was obtained from two infected deer mice for viral RNA sequencing. Primers were designed using aligned hantavirus sequences from Puumala (PUUV) and Prospect Hill viruses for viral gene amplification. The amplified PCR products were sequenced and compared to known hantaviruses using phylogenetic tree analysis, resulting in a novel hantavirus. The new virus was initially named Four Corners virus and had the most homology with PHV and

PUUV. Due to concerns from the local tourist industry, the name of the virus was later changed to Sin Nombre virus (Hjelle et al., 1994).

In 2000, a deer mouse colony was founded at the University of New Mexico using wild caught deer mice. When these mice were infected with SNV, researchers did not note a difference in the lung tissue between infected and uninfected controls (Botten et al., 2000). In this experiment, animal tissues were perfused with formalin fixed upon euthanization and paraffin embedded. They were evaluated using hemotoxylin and eosin staining; the results are the most reliable to date (Botten et al., 2003). A separate study prepared SNV specific antigen and used this to generate specific anti-SNV antibodies. These antibodies were then used to evaluate presence of viral antigen in experimentally infected deer mice tissues. Positive staining for virus was observed in cardiac and pulmonary (lung) tissues; however, no apparent pathology was noted in these tissues (Green et al., 1998).

In experimentally infected deer mice, viral RNA was predominantly in the lung, heart, and brown fat at days 14-35 post-infection (Botten et al., 2003). Levels peaked between day 60 and 120 days post-infection (dpi) when viral antigen was evaluated using immunohistochemistry (IHC) and then decreased; however, the virus was still readily detectable by PCR. Antibodies to the viral nucleocapsid were detected beginning at day 14 and continuing to the day 28 endpoint. These data suggest the virus may have developed persistent infection in these tissues.

The virus replicated in lung endothelial cells; however, little if any cytopathic effect was observed in these cells. Peak values of positive strand vRNA were observed between days 21 and 60 post-infection in the heart, lung, brown fat, and kidney. Values began to decrease after day 60; only the lung, heart, and brown adipose tissue (BAT) displayed appreciable levels of vRNA with the highest levels present in the lungs (Botten et al., 2003). Positive strand vRNA decreased dramatically after 60 days post-infection, indicating that the virus was not actively replicating and producing viral proteins. As the levels of viral and message RNA decreased, there was an increase in neutralizing antibody titers that peaked between days 120 and 180 post-infection (Botten et al., 2003).

One study evaluated the potential of SNV to replicate in T cells. Cells from infected deer mice were cultured and evaluated for the presence of vRNA. After 25 days of culture, vRNA was never detected in the T cells, suggesting that these cells were not capable of hosting replicating virus nor were they a likely location of persistent viral infection (Schountz et al., 2007).

Assessing the deer mouse immune response to SNV infection has been a challenging task, predominantly due to a lack of reagents. Many mRNA sequences and proteins of commonly used laboratory animals (mice, rats, etc.) do not share significant homology with the deer mouse. Progress has been made as several deer mouse immune genes have been sequenced; however, the corresponding proteins are not yet available (Oko et al., 2006). Progress is underway to sequence the entire deer mouse genome. In the future, these

sequences can be used to detect immune gene regulation using techniques such as in-situ hybridization and PCR. This will allow a better evaluation of the immune response to SNV in the deer mouse.

In studies from persistently infected animals, there appeared to be a development of an anti-inflammatory response (Mori et al., Schountz et al., 2007). One study evaluated the gene expression of available deer mouse genes. T cell proliferation from experimentally infected deer mice was assessed by culturing spleen cells from infected deer mice and challenged with SNV nucleocapsid antigen. T cells from infected deer mice had weaker proliferation than those from control animals. Additionally, the T cell cytokine profiling indicated increased expression of TGFβ1 in persistently infected deer mice, suggestive of a T regulatory cell response that may be mediating an inflammatory reaction to the virus. Interestingly, interleukin-10 (IL-10) expression was decreased in these animals along with Interferon gamma (IFN-γ). There was also a noticeable lack of tumor necrosis factor up regulation, which has been implicated in human pathology (Mori et al., Schountz et al., 2007). These findings are supported by a similar study (Easterbrook, Zink, & Klein, 2007) using Seoul virus (an HFRS-causing hantavirus) in the reservoirs of Norway rats (*Rattus norvegicus*). In addition to observing an increase in TGFβ1 in the lungs of infected animals when T regulatory cells were depleted, the expression of TGFβ1 significantly decreased. Levels of TNF were also notably higher in infected animals compared to infected animals with inactivated T regulatory cells (Easterbrook et al., 2007). It has been documented that T regulatory cells can

secrete TGFβ1, which may then decrease expression of TNF (Wei et al., 2008). Thus, it is not surprising that suppressing T regulatory cells may also attenuate TNF levels.

Pathology of Human Hantavirus Cardiopulmonary Disease

Sin Nombre virus infects humans principally by the respiratory route. The virus, excreted from the deer mice, is aerosolized and inadvertently inhaled. This is typically done during the sweeping and cleaning of areas mice inhabit, e.g., outdoor sheds and woodpiles. Upon entering the respiratory tract of an individual, the virus binds to endothelial cells. In a study (Yanagihara & Silverman, 1990) using Seoul, Hantaan, Puumala, and the non pathogenic Prospect Hill and Leaky hantaviruses, human vascular endothelial cells and human umbilical vein endothelial cells were infected with virus. Although these viruses cause HFRS, their transmission to humans is also through the respiratory route. Cells were observed at 3 days post-infection and 10 days post- infection. Approximately 20% of endothelial cells and 100% of the HUVECs had cytoplasmic staining for virus. However, no conspicuous cytopathic effects were observed in the infected cells study (Yanagihara & Silverman, 1990). This suggests that HFRS-causing viruses can bind to a receptor on these cells and enter into the cytoplasm, making them a potential target for infection.

Pathogenic and nonpathogenic hantaviruses enter cells by using a Beta-integrin receptor. A study by Gavrilovskaya, Shepley, Shaw, Ginsberg, and Mackow (1998) showed that when Vero E6 cells and Chinese hamster ovary cells were pretreated with vitronectin (a ligand for β3 integrin), there was

approximately 70% inhibition of viral infection with New York hantavirus (a HPCS virus), indicating this virus used a cellular entry. Nonpathogenic Prospect Hill, Tula, and Thottalapom viruses were inhibited by fibronectin, indicating β1 integrins were required for their entry (Gavrilovskaya et al., 1998). In addition, when HUVEC cells were pretreated with an anti-β3 antibody, infection with pathogenic hantavirus was blocked. This was not observed using non-pathogenic virus, indicating that the cellular receptor used to gain viral entry may be significant to pathogenicity.

A study using Andes hantavirus demonstrated that infection of hamster trachea epithelial cells (TEC) can occur at both the apical and basolateral membranes (Rowe & Pekosz, 2006). However, the cells infected at the apical membrane supported viral replication more than the other (100 fold difference in viral RNA copy number). When the cells were double stained with markers for ciliated and non-ciliated cells, Andes infected the non-ciliated cells predominantly. Furthermore, the non-ciliated cells expressed β3 integrin more than ciliated cells (Rowe & Pekosz, 2006). Once virus has been released inside the host cell, it uses an RNA dependent RNA polymerase (RdRp) for viral replication and transcription of viral proteins. Hantaviruses are negative sense RNA viruses and synthesize a plus strand that functions as messenger RNA for translation or as a template for new viral RNA for viral replication. SNV contains an L segment (encodes the RdRp), M segment (encodes viral glycoproteins G_c and G_n), and an S segment (encodes the viral nucleocapsid.)

After transcription and translation of viral polypeptides, vRNA is synthesized and packaged into the nucleocapsid. The glycoproteins and nucleocapsid are co-localized in the Golgi complex, although the location of the viral packaging in the host cell is unknown (Li et al., 2010).

While it is accepted that hantavirus can infect and replicate within endothelial cells, little is known of how the virus spreads to other cells in the body. There are currently no published reports explaining viral shedding from endothelial cells. As it is an enveloped virus, it can be hypothesized that the virus buds out of infected endothelial cells.

Hantaviruses have been shown to infect monocytes/macrophages where the virus can also replicate, demonstrated by *in vitro* assays using HFRS that causes hantaviruses (Nagai et al., 1985; Temonen et al., 1993). However, this has yet to be shown in models using HCPS hantaviruses.

Other cells that may be subject to infection are dendritic cells. Human dendritic cells have been successfully infected with Hantaan virus *in vitro* (Raftery, Kraus, Ulrich, Kruger, & Schonrich, 2002). Dendritic cells were incubated with HTNV; after four days, nucleocapsid antigen could be identified in cells, which also correlated with a peak in viral titer in cell supernatant. Follicular dendritic cells in autopsy sections have also been observed to contain SNV antigen by immunohistochemistry, suggesting they too may be infected (Zaki et al., 1995). However, it cannot be discounted that these cells may simply be performing their designated function of phagocytosing virus and processing it for MHC presentation.

People infected with SNV typically have flu-like symptoms. During the 1993 outbreak, individuals infected with SNV proceeded to die an average of four days after the onset of symptoms (Zaki et al., 1995). The average incubation of exposure to symptoms is 9 to 33 days (Young et al., 2000).

After the incubation period, the disease follows a pattern that includes five distinct phases: Phase 1--fatigue, fever, vomiting; Phase 2--thrombocytopenia; and Phase 3--pulmonary edema. The fourth and fifth phases are signaled by dieresis and a slow recovery, respectively (Jonsson, Hooper, & Mertz, 2008; Maes, Clement, Gavrilovskaya, & Van Ransdt, 2004).

Treatment of HCPS is limited, in part, due to not being recognized until late in its progression. Antiviral therapies with ribavirin have been successful with HFRS (Chapman et al., 1999; Huggins et al., 1991). A recent report using the Andes hamster model demonstrates that ribavirin (an anti-viral medication commonly used to treat hepatitis) can prevent lethal HCPS disease when administered either intravenously or orally and decreases disease severity (Safronetz, Haddock, Feldmann, Ebihara, & Feldmann, 2011). Currently, the most successful treatment regimen used in humans is extracorporeal membrane oxygenation or ECMO (Dietl et al., 2008). This is also known as heart lung bypass--the blood is oxygenated in a machine outside the body and then returned. According to the Center for Disease Control (2010a), reports of mortality were 29% in 2010; overall, they were 35% from 1993-2009 (MacNeil, Ksiazek, & Rollin, 2011).

The Innate Immune Response

The human immune response includes both innate and adaptive mechanisms. Most viruses are typically engaged by the host innate responses, while an adaptive response develops and is more effective later in the course of disease. Innate molecules include pattern recognition receptors (PRR), cytokines, complement, phagocytic cells, and interferons.

In many human pathogens, viral RNA can cause an interferon response by binding to a PAMP such as TLR3 or RIG-I. These proteins activate immune gene transcription factors, namely, IFN regulatory factor 3 (IRF-3) and IFN regulatory factor 7 (IRF-7), both which play a role in the transcription of interferons α and β. These interferons then activate interferon stimulated genes (ISG) such as MxA and ISG56. SNV appears to be able to also turn on ISGs without using IRF-3 or IRF-7 (Prescott, Hall, Bondu-Hawkins, Ye, & Hjelle, 2007). When IRF-3 and IRF-7 were silenced in hepatoma cell line Huh3, there was still increased expression of ISG when exposed to irradiated SNV as compared to controls (Prescott et al., 2007). It is plausible that the G_c cytoplasmic tail could be functioning by interacting with transcription factor CREB for regulation of interferon expression.

The interactions of PRRs and interferon (IFN) regulation have been given particular attention in their response to hantavirus infection. In a study by Prescott, Ye, Sen, and Hjelle (2005), endothelial cells were infected with SNV and gene expression evaluated by microarray and quantitative PCR. These results were compared to endothelial cells treated with UV-inactivated SNV.

Both groups were studied at early time points (<24 hours) and later time points (three days). At 24 hours, there was no measurable difference in interferon stimulated genes (ISG) including MxA and ISG56 (both were elevated). This suggests that initially a particle of SNV may be used as a pathogen associated molecular pattern (PAMP) instead of double stranded RNA activating Toll-like receptors to trigger enhanced ISG expression. At day 3 post-infection, the cells treated with active (replicating) virus continued to induce ISG; whereas, the other group had reduced expression (Prescott et al., 2005).

However, a study by Alff et al. in 2006 showed that pathogenic hantavirus NY-1, an HCPS causing virus and HTNV, decreased interferon signaling at day 1 post-infection as measured by interferon response genes (ISG) MxA and ISG56. However, non-pathogenic PHV, which was used as a control, did not have this reduction in gene expression. By day 3 post-infection, all viruses had reduced MxA expression; however, it was still notably elevated in PHV as compared to NY-1. Additionally, NY-1 and HTNV had increased S segment gene expression, indicating they could replicate inside the endothelial cells. PHV had very little S segment gene expression.

This study also demonstrated that cells treated with the G_c cytoplasmic protein of pathogenic hantavirus reduced RIG-I (retinoic acid inducible gene) function. Cells were transfected with a RIG-I and interferon stimulated response element (IRSE) driven luciferase gene; decreased activity was observed when cells were also co-transfected with the G_c portion of NY-1 as compared to the N protein or PHV G_c. RIG-I activates IFN signaling pathways; thus, it is possible

that the G_c portion of pathogenic hantavirus may function as an inhibitory molecule of RIG-I and limit the downstream innate type I IFN response (Alff et al., 2006).

A subsequent study (Shim et al., 2011) used A549 and Vero E6 cells to study the differences of innate immune responses to pathogenic and non pathogenic hantaviruses. Both sets of viruses could replicate easily in Vero E6 cells; however, in A549 cells only, the pathogenic viruses had replication equivalent to rates seen in Vero E6 cells. This also correlated with a delayed increase in MxA and IFN-β (Shim et al., 2011).

Collectively, results of such studies indicate that pathogenic hantaviruses are delaying or subverting the type I interferon response to allow for increased viral replication and dissemination.

Cell Mediated Host Response

In addition to innate responses, an adaptive immune response also appeared relevant during hantavirus infection. A hallmark study by Zaki et al. in 1995 showed the pulmonary infiltrate of infected individuals contained both $CD4^+$ and $CD8^+$ T cells along with macrophages. The most common T cell subtype was the $CD8^+$ phenotype. This cell type binds to cells displaying antigen in a MHC I. In a study infecting endothelial cells with HTNV (Kraus et al., 2004), there was a delayed increase in MHC I production (peak levels day 4-5- post-infection) as to cells infected with a non pathogenic Tula virus, which had a peak production after two-three days post-infection.

As T cells and macrophages are well known producers of inflammatory cytokines, researchers have begun to evaluate the presence of cytokines in lungs and spleens of infected individuals. Cytokine presence has been evaluated by immune staining in both the lung and spleen of infected individuals. T cell cytokines IFNγ, IL-2, IL-4, lymphotoxin (LT), and TNF were increased in lung and spleen tissues, as well as monocyte derived IL-1α, IL-1β, and IL-6 (Mori et al., 1998). The presence of cytokine producing T cells is suggestive of an immune response to HPS infection that may mediate capillary leak, causing the pulmonary edema seen in these patients.

In blood samples from infected individuals, $CD8^+$ T cells have been isolated that immunoprecipitate with specific regions of the SNV nucleocapsid protein. When amino acid substitutions were made in the nucleocapsid region, they were no longer recognized. Cell death was averted when anti-MHC-I antibody was added to the experiment. From these data, it could be hypothesized that the alveolar monocytic cells were presenting SNV nucleocapsid antigen in the MHC-I complex and activating $CD8^+$ T cells (Ennis et al., 1997; Zaki et al., 1995). Memory $CD8^+$ T cells have been isolated from patients who fully recovered from Hantaan infections. One sample recognized the G_c portion and two others recognized a region of the nucleocapsid. When these cells were exposed to SNV peptides, they only recognized the C terminal region of the nucleocapsid, which tends to be conserved across all hantaviruses (Van Epps, Schmaljohn, & Ennis, 1999). A subsequent study (of 78 convalescent people supported this finding. Patients monitored up to 13 years

post infection with Andes hantavirus continued to have $CD8^+$ T cells that were specific for the C terminal region of the nucleocapsid (Manigold et al., 2010). Two patients infected with SNV also had $CD8^+$ T cell responses measured by SNV nucleocapsid antigen T cell recall assays (Ennis et al., 1997). Collectively, these studies provide evidence that upon infection with SNV, patients develop a $CD8^+$ T cell response. Hence, the virus must be processed using the MHC-I pathway. It is well known that SNV has tropism for endothelial cells; however, no cytopathic effects were observed. Some studies have suggested that the HPS hantavirus can also infect alveolar macrophages and dendritic cells. It could possibly be these macrophages display the nucleocapsid antigen for $CD8^+$ T cell recognition (Nagai et al., 1985, Raftery et al., 2002; Temonen et al., 1993; Zaki et al., 1995).

Although less has been studied regarding $CD4^+$ T cells, such a response must occur because IgG antibodies have been detected during acute and convalescent patients. In acute patients there is a high titer of IgM and IgG antibodies, indicating a $CD4^+$ T cell and B cell interaction. IgA titers are also observed, which most likely come from the epithelial mucosa where primary infection occurs (Bostik et al., 2000).

It must also be considered that HCPS may not be a T cell mediated disease, at least in the traditional sense. A study by Hammerbeck and Hooper (2011) evaluated T cell responses in hamsters infected with ANDV. Their study used cyclophosphamide to inactivate T cells and anti-CD8 antibody to specifically deplete $CD8^+$ T cells. The administration of these agents and subsequent

infection with ANDV did not alter disease pathology (Hammerbeck & Hooper, 2011).

Animal Model of Disease

Until recently, study of hantavirus pathogenesis has been slowed by the lack of an animal model. The usual laboratory mice and rat strains do not develop a clinical response to viral infection (Wahl-Jensen et al., 2007). In 2002, it was discovered that Syrian golden hamsters could become infected with Andes virus (a South American HCPS virus) and suffer a clinical course similar to humans with HCPS. Hamsters infected with Andes virus were asymptomatic for the first 10 days. At day 11, post-infection hamsters became moribund, developing respiratory distress and dying within 24 hours of onset of symptoms. Similar to human disease, the animals had pleural edema and an increase in lymphocyte infiltration in the lungs. Spleens were enlarged and had large, possibly apoptotic cells in the red pulp. Viral antigen was observed in vital organs--liver, kidney, lung, spleen, and heart. It should be noted that no infectious virus was found in brain tissue. Although the virus is highly lethal in hamsters, those that survived after 12 days developed neutralizing antibodies to the virus. When subsequently challenged again, they did not develop the disease (Hooper, Larsen, Custer, & Schmaljohn, 2001).

Hamsters infected with MAPV became symptomatic beginning day 8 post-infection (lethargic, loss of appetite). Beginning of day 9 post infection, animals become moribund. As with the Andes model, surviving animals became clinically normal with increasing IgG antibody titer, specific to the MAPV virus. Infected

animals had reddened lungs that had also developed fibrin deposits, congestion, and edema. As in humans and Andes models, there were also macrophages and lymphocytes in increased numbers compared to control animals. Viral antigen was commonly found in the lungs, lymph nodes, spleen, and kidney (Milazzo, Eyzaguirre, Molina, & Fulhorst, 2002).

Transforming Growth Factor Beta-1

Transforming Growth Factor-Beta-1 (TGFβ1) has been implicated as a potential factor in the persistent infections of hantavirus in both the SNV and Seoul reservoirs (Easterbrook et al., 2007; Schountz et al., 2007). It may be that TGFβ1 drives the development of T regulatory cells that suppress inflammation while an adaptive immune response builds. Although the adaptive response is not sterilizing, it most likely serves to inhibit any pathology.

TGFβ1 is a multifunctional cytokine that consists of three different isoforms (TGFβ-1, 2, 3) found on three separate genes. All exhibit similar biological effects such as cell growth, embryogenesis, tissue remodeling, and repair. They are not secreted by one cell type or organ; rather, they are found in all systems in the body. All forms have a molecular weight of 25kDa and consist of two identical monomers, each 112 amino acids in length and held together by disulfide bonds (Koppa, 1994).

Although their functions are similar, the magnitude of their action depends on the isoform. TGFβ1 is of particular interest as it is essential in normal (tissue) repair processes. TGFβ1 induces the production of extracellular matrix proteins and of protease inhibitors. These protease inhibitors prevent the degradation of

the accumulating matrix, resulting in buildup of fibrotic scar tissue (Border & Noble, 1993). This sclerotic process is similar to scar formation when cut skin is healed. The scar is a meshwork of extracellular matrix proteins that close up the wound.

TGFβ1 is initially secreted from cells in an inactive form and is non-covalently bound to a latency associated peptide (LAP) at the N-terminus of TGF (Crawford et al., 1998). The LAP-TGFβ1 complex is non-covalently bound to another molecule--Latent TGFβ1 Binding Protein (LTBP.) It has been suggested that the LTBP may increase TGFβ1's affinity for cellular receptors. This LAP-TGFβ1-LTBP complex may enhance the stability of TGFβ1 in the extracellular matrix ready for activation (Crawford et al., 1998; Munger et al., 1997).

The LAP portion of the pro TGFβ1 is cleaved from the TGFβ1-LAP complex to activate TGFβ1. TGFβ1 has been found to signal cells via serine/threonine kinases (Souchelnitskiy, Chambax, & Feige, 1995). Active TGFβ1 binds to a kinase receptor called TGF Receptor-II (TGR-II) that recruits and forms a complex with TGF Receptor-I (TGR-I). TGR-I has a phosphate on the intracellular domain and TGFβ1 binding is followed by a TGF receptor phosphorylation reaction and propagation of a signal using the SMAD pathway (Bottinger, Letterio, & Robers, 1997).

TGF β1 is a pleiotropic cytokine. In addition to growth and healing, it is also capable of anti-inflammatory functions. Macrophages that phagocytose apoptotic cells as a function of the immune pathway secrete increased levels of TGFβ1 as compared to other cytokines, namely IL-10. The TGFβ1 can then

function to down regulate the inflammatory cytokine tumor necrosis factor (TNF) produced by macrophages (Fadok et al., 1998). TGFβ1 is also released from T cells upon apoptosis; this TGFβ1 can further suppress inflammatory cytokine production from macrophages (Chen, Frank, Jin, & Wahl, 2001).

The expression of TGFβ1 can be induced by cross linking of the CTLA-4 in $CD4^+$ T cells, which then inhibits T cell proliferation (Chen, Jin, & Wahl, 1998). Interestingly, recent studies have demonstrated a role of TGFβ1 in the induction and proliferation of peripheral T regulatory cells. When $CD4^+CD25^+$T cells are stimulated with TGFβ1 and IL-2 they become inducible T regulatory cells (iTregs) that are positive for Forkhead Box p3 transcription factor (Zheng, 2008). When iTregs cells are transplanted into a murine hepatitis model, pathology is decreased. This effect is blocked when anti- TGFβ1 antibodies are added(Wei et al., 2008). This suggests that the iTreg response controlling liver inflammation is mediated by TGFβ1. Another study also evaluated iTregs ability to control endothelial cell activation by measuring the level of E-selectin present on these cells. Cells were induced to become iTregs by TGFβ1 and the supernatant used to measure the migration ability of Th1 cells through heart muscle endothelial cells. The use of the iTreg supernatant significantly limited the ability of Th1 migration. It was also shown that levels of E-selection were decreased after exposure to the iTreg supernatant (Maganto-Garcia et al., 2011). This suggests that TGFβ1 induced Tregs can decrease tissue inflammation by restricting the migration of Th1 cells across the endothelial cell barrier.

Evidence for Cytokine Therapy

Although cytokines may appear to be attractive modalities for a variety of diseases, very few have been utilized in viral infections. For the most part, strategies to fight viruses are immunization (when available) and anti-viral medications, such as ribavirin, that block viral replication.

One of the first cytokines that was evaluated for efficacy in viral infection was a mixture of Interferon-α (IFNα) and Interferon-β (IFNβ). During initial experiments, it was discovered these molecules have anti-tumor activity and focus shifted toward using them for this purpose. However, IFN-β in particular has found use for treatment of multiple sclerosis (trade names Rebif, Avonex), which is thought to be triggered by a still unknown virus. Clinical studies have shown that IFN-β treatments decrease relapse rates of Multiple Sclerosis by approximately 30% and new lesion activity by about 65% (Rudick & Goelz, 2011). IFN-α is one of the most popular treatments for hepatitis C, a chronic liver infection caused by the hepatitis C virus. The success of IFN-α treatment depends on the patient genotype but complete remission has been observed. Due to the ability of the virus to mutate rapidly, the use of a cytokine therapy has shown more promise than anti-viral medications (Aghemo, Rumi, & Colombo, 2009).

CHAPTER III

MATERIALS AND METHODS

Aim 1

Animals

For the first set of experiments evaluating gene expression, 12 four-week-old out bred female Syrian golden hamsters (Harlan Sprague Dawley) were used for experiments and divided into three groups: (a) uninfected controls, (b) MAPV infected and euthanized at day 8 post-infection, and (c) MAPV infected and euthanized at day 10 post-infection.

Animals were infected by intramuscular injection into the right hind leg with a 0.2 mL suspension containing 3.1 $\log_{10}$ median cell culture infectious doses in PBS of the MAPV strain 97021050 (Milazzo et al., 2002). Animals were housed under BSL-4 safety conditions at the University of Texas Galveston medical branch (UTMB) and treated in accordance with UTMB animal handling guidelines and approved protocol.

PCR for Gene Expression

Tissues were homogenized in Trizol reagent (Invitrogen) by collaborators in Texas and shipped to the University of Northern Colorado in 1 mL aliquots. Two hundred microliters of chloroform were added to each sample and incubated at room temperature for 2.5 minutes. Samples were then spun at 12,000 x g for

15 minutes at 4° C. The aqueous upper layer was removed and transferred to a fresh tube with an equal volume of 70% ethanol and mixed. RNA was purified over RNAeasy columns according to kit instructions (Qiagen). Samples were bound to spin columns for 15 seconds at 8,000 x g at room temperature and then washed using kit buffers. RNA was then eluted from columns using sterile Millipore water by spinning for one minute at 8,000 x g. All RNA samples were stored at -80° C.

Reverse transcription PCR (RT-PCR) was performed using random hexamers and a qScript kit (Bio-Rad). Three microliters of RNA was added to 4 uL of reaction buffer, 1 uL reverse transcriptase, and 10 uL of RNAse free water (all provided by the kit manufacturer.) Samples were then reverse transcribed into cDNA using a Bio-Rad thermocycler under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Samples were kept frozen at -20°C until used.

The cDNA generated was used for quantitative real time PCR using primers established by Zivcec, Safronetz, Haddock, Feldmann, and Ebihara (2011; see Appendix C). The protocol for the quantitative PCR assay was adapted from a previously established protocol (Oko et al., 2006). Thirty-nine microliters of the cDNA were mixed with 487.5 uL of Millipore water and 487.5 SYBR Green reaction mix. Aliquots of 45 uL were added to real time plates and 5uL of primers were added. Experiments were performed using the iQ SYBR Green kit for 40 cycles (45 seconds at 94°C, 50 seconds at 54°C, and 60 seconds at 72°C) in a MyiQ real time thermal cycler (Bio-Rad). Cycle threshold

values were calculated by averaging the relative fluorescence unit from cycles 10-14, +3 standard deviations. Values were considered positive when they reached this value and were continuously 20% over previous values. Fold expression was calculated using the delta delta Ct method.

Aim 2

Animals

Forty-two 4-week-old out bred Syrian golden hamsters (female) were used for the experiments. Animals were divided into the following groups: (a) MAPV infected and treated with TGFβ1, (b) MAPV infected and treated with IL-10, (c) MAPV infected and treated with 1x PBS, (d) MAPV infected only, (e) TGFβ1 treatment only, (f) IL-10 treatment only, and (g) 1x PBS only. One day prior to infection, hamsters were anesthetized and surgically implanted subcutaneously between the scapulae with ALZET osmotic pumps (Model 2001). Incisions were closed using 3M Precise disposable skin staplers. Pumps contained either 200 ng active human rTGFβ1 or interleukin-10 (R&D Systems) in 200 µL of PBS or PBS only. These pumps are designed to release 1 uL/hour of solution so that animals received a continual dose of 1 ng/hour of the treatment cytokine.

Animals were infected by intramuscular injection into the right hind leg with a 0.2 mL suspension containing 3.1 $\log_{10}$ median cell culture infectious doses in PBS of the MAPV strain 97021050 (Milazzo et al., 2002). Animals were housed under BSL-4 safety conditions at the University of Texas Galveston medical branch and treated in accordance with UTMB animal handling guidelines and approved protocol.

TGFβ1 Dosing Study

To evaluate appropriate dosage of TGFβ1 into Syrian golden hamsters, animals were assigned to one of five groups: A, B, C, D, or E. Group A served as a control group and received infusions of saline. Groups B, C, D, and E received infusions of recombinant TGFβ1 (R&D Systems). Groups were dosed at concentrations of 20 ng, 200 ng, 2,000 ng, and 20,000 per mL, respectively. Each group was made up of four hamsters for a total of 20 animals.

TGFβ1 was administered using ALZET osmotic pumps for seven days. Hamsters were anesthetized with 3% isoflourane and fur plucked from incision site. Surgical sites were cleaned using betadine wipes and 70% ethanol. Incisions were made in the subscapsular area using surgical scissors and pumps inserted with hemostats. Incisions were then closed using 3M Precise surgical staples. Polysporin was administered to the wound to prevent infection. Animals were then warmed and observed during recovery. Hamsters were observed daily for biological effects by appearance, food and water consumption, behavior, and weight. Observations were recorded and scored in a table (see Appendix C.) After seven days, animals were euthanized using respiratory hyper anesthesia with isoflourane and compromise of the pleural sac. Organs were removed for histological evaluation of pathology compared to the control group.

Pathology

Pleural fluid was collected from each animal upon death by aspiration with a syringe and stored at -80 C.

Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Five μm thick sections were prepared, stained with hematoxylin-eosin, and evaluated by light microscopy. Histopathologic lesions were scored based on the severity of lesions utilizing the following grading scheme. Histologic sections of lung were scored on a 0-9 (0--*no discernible lesions*, and 9--*most severely affected*) scale based on the severity of vascular lesions (0-3), alveolar lesions (0-3), and lesions of larger airways (0-3). Phosphotungstic acid haematoxylin (PTH) staining was performed on lung sections to assess fibrin deposition.

Quantitative Polymerase Chain Reaction for Gene Expression

Tissues were homogenized in Trizol reagent (Invitrogen) by collaborators in Texas and shipped to the University of Northern Colorado in 1 mL aliquots. Two hundred microliters of chloroform was added to each sample and incubated at room temperature for 2.5 minutes. Samples were then spun at 12,000 x g for 15 minutes at 4° C. The aqueous upper layer was removed and transferred to a fresh tube with an equal volume of 70% ethanol and mixed. RNA purified over RNAeasy columns according to kit instructions (Qiagen). Samples were bound to spin columns for 15 seconds at 8,000 x g at room temperature and then washed using kit buffers. RNA was then eluted from columns using sterile Millipore water by spinning for 1 minute at 8,000 x g. All RNA samples were stored at -80° C.

Reverse transcription PCR (RT-PCR) was performed using random hexamers and a qScript kit (Bio-Rad). Three microliters of RNA was added to 4

uL of reaction buffer, 1 uL reverse transcriptase, and 10 uL of RNAse free water (all provided by the kit manufacturer.) Samples were then reverse transcribed into cDNA using a Bio-Rad thermocycler using the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Samples were kept frozen at -20°C until used.

The cDNA generated was used for quantitative real time PCR using primers listed in Appendix D. The protocol for the quantitative PCR assay was adapted from a previously established protocol (Oko et al., 2006). Thirty-nine microliters of the cDNA was mixed with 487.5 uL of Millipore water and 487.5 SYBR Green reaction mix. Aliquots of 78 uL were added to deep well dishes and 3 uL of primer was added. Twenty-five microliters of mixture were then transferred into real time plates in triplicate. Experiments were performed using the iQ SYBR Green kit for 50 cycles (45 seconds at 94°C, 50 seconds at 54°C, and 60 seconds at 72°C) in a MyiQ real time thermal cycler (Bio-Rad). Cycle threshold values were calculated by averaging the relative fluorescence unit from cycles 10-14, +3 standard deviations. Values were considered positive when they reached this value and were continuously 20% over previous values. Fold expression was calculated using the delta delta Ct method.

Aim 3

Reverse Transcription Quantitative Polymerase Chain Reaction to Evaluate Viral Copy Number

Tissues were homogenized in Trizol reagent (Invitrogen) by collaborators in Texas and shipped to the University of Northern Colorado in 1 mL aliquots.

Two hundred microliters of chloroform was added to each sample and incubated at room temperature for 2.5 minutes. Samples were then spun at 12,000 x g for 15 minutes at 4° C. The aqueous upper layer was removed and transferred to a fresh tube with an equal volume of 70% ethanol and mixed. RNA purified over RNAeasy columns according to kit instructions (Qiagen). Samples were bound to spin columns for 15 seconds at 8,000 x g at room temperature and then washed using kit buffers. RNA was then eluted from columns using sterile Millipore water by spinning for 1 minute at 8,000 x g. All RNA samples were stored at -80° C.

Samples were quantified using a Nanodrop 2000 spectrophotometer and 500 ng of RNA was used for reverse transcription. Samples were reverse transcribed using a qScript kit with random hexamers and a single strand primer for either the negative (5'- TGTTATCCACAAGAGGGAGACAGAC -3') or plus (5'-CCTATCCATCCAGTCCTTCACAAAG-3') strand of MAPV. Five microliters of cDNA was removed and used in real-time PCR with the iQ SYBR green kit under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 50°C for 10 s, and 72° C for 30 s. A standard curve was generated using 100-fold dilutions of MAPV from 10^7 to 70 copies.

Statistical Analysis

Gene expression was evaluated using Prism GraphPad software. Significance was calculated using one way ANOVA followed by a Tukey post test. A p-value < 0.05 was considered significant. Data tables may be found in Appendices E and F.

CHAPTER IV

RESULTS

Aim 1: Infected and Uninfected Control Animals

The spleens of infected animals had significant increases ($p<0.05$) in interferon related genes Stat1 and Mx2; however, some were significant on day 8 but not on 10 and vice versa (see Figure 1). Pro-inflammatory gene IL-6 was also increased as were chemokine CXCL10, cyclin kinase inhibitor p27, and eukaryotic translation initiation factor (Eif2ak2). Other pro-inflammatory genes were also increased over controls but *p* values did not achieve significance.

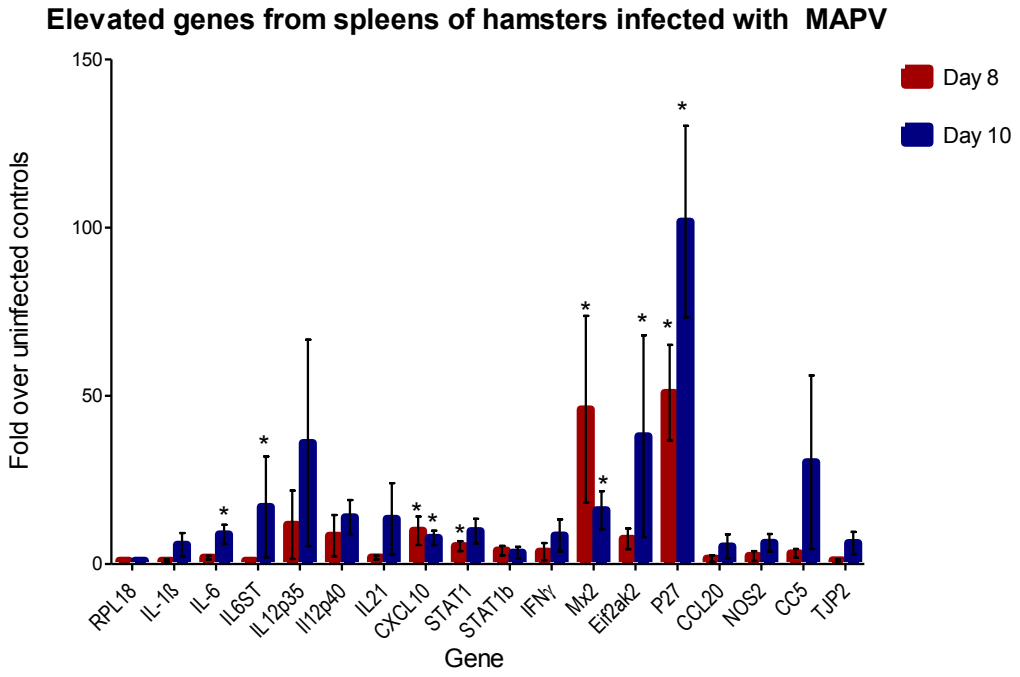

Figure 1. Gene expression from spleens of hamsters infected with MAPV. Several pro-inflammatory genes were increased in infected animals compared to controls. Significance is noted by an asterisk (*).

Lung tissue from infected animals had significant differences in CXCL10. Stat1 and Stat1b were all elevated compared to controls. Many pro-inflammatory genes were elevated, although not significantly (see Figure 2).

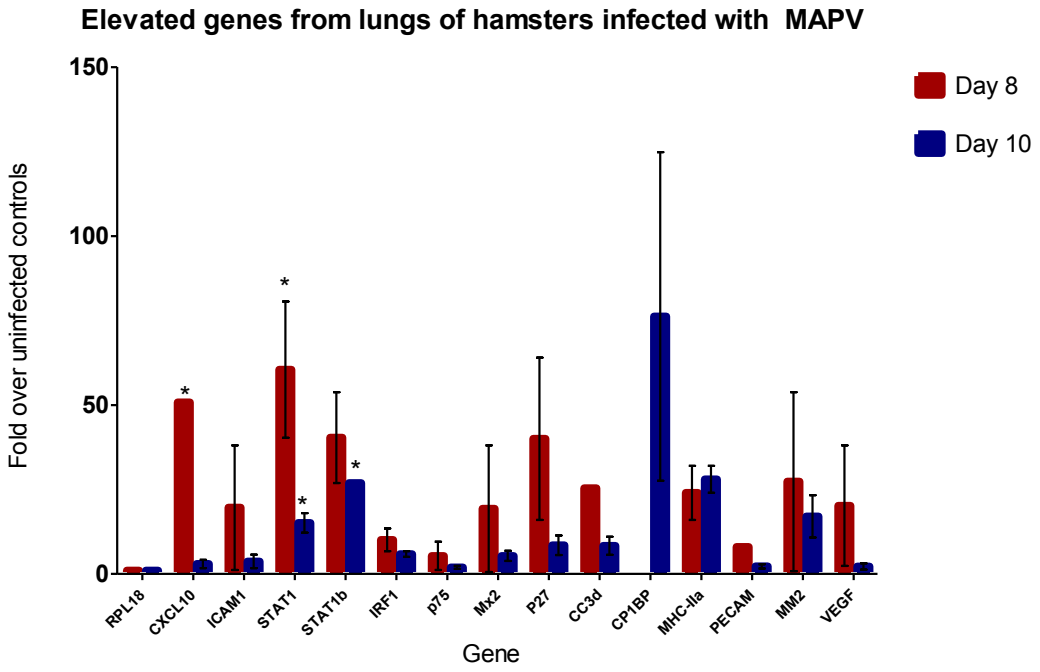

Figure 2. Gene expression from lungs of hamsters infected with MAPV. Several pro-inflammatory genes were increased in infected animals, although none were considered statistically significant.

Aim 2: Pathology

There was no difference in survival between animals treated with TGFβ1 or IL-10 versus controls (see Figure 3). There was a difference in lung congestion between treated and control animals. The lungs from hamsters treated with TGFβ1 were thin, pink, and had less lung congestion; the volume of pleural fluid recovered from thoracic cavities was significantly less than controls (p=0.05; see Figure 4).

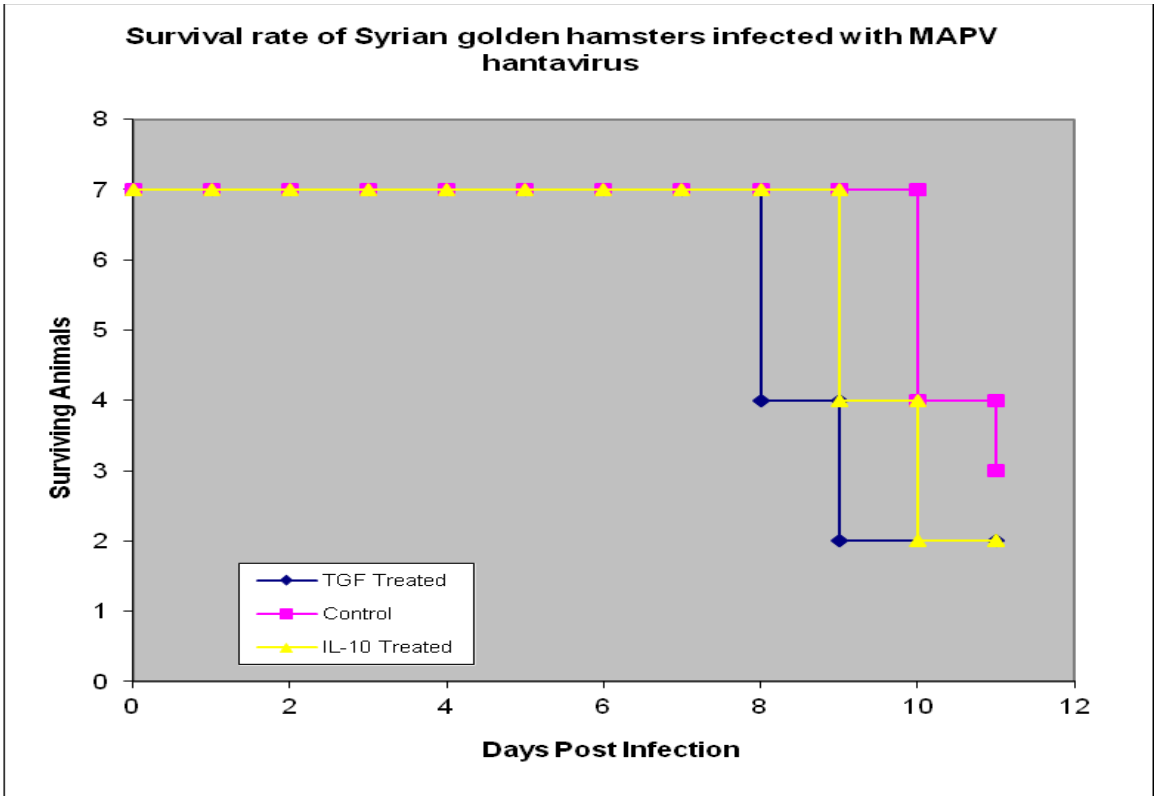

Figure 3. Survival curve of hamsters infected with MAPV and treated with TGFβ1 or IL-10. Animals infected with MAPV and treated with either TGFβ1 or IL-10 did not have a significant difference in survival time compared to animals treated with PBS.

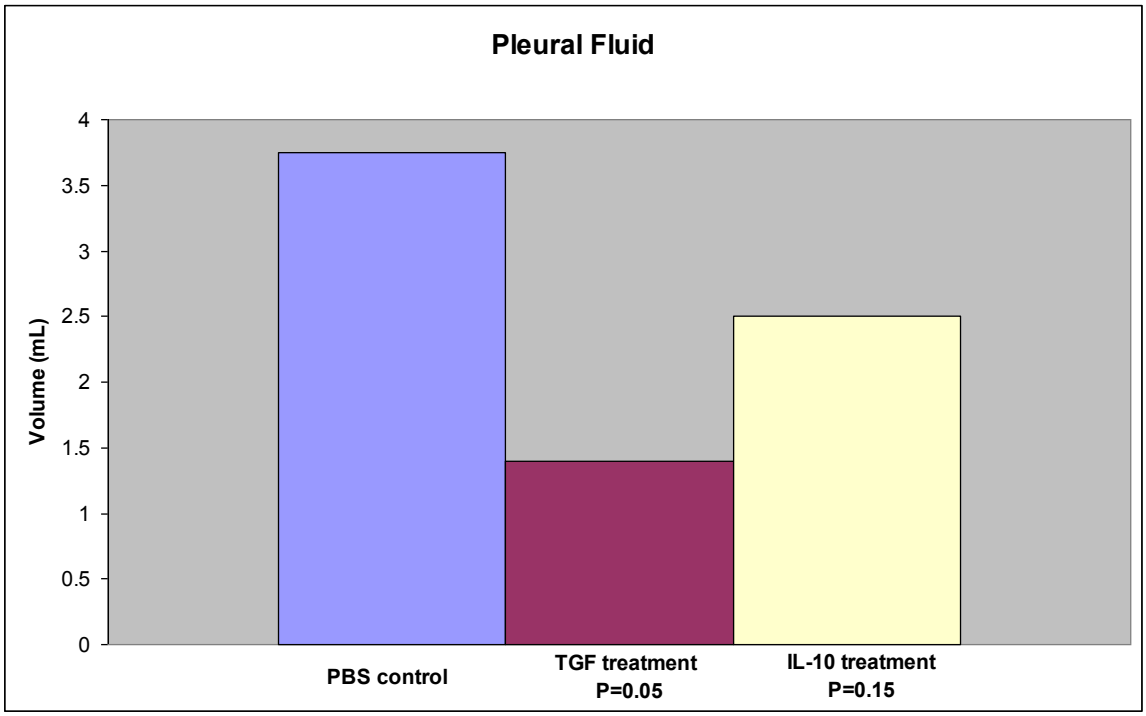

Figure 4. Average pleural fluid from lungs hamsters infected with MAPV and treated with TGFβ1 or IL-10. The average amount of pleural fluid recovered from the pleural cavity of animals infected with MAPV and treated with a PBS control was 3.75mL. In animals treated with TGFβ1, there was a significant decrease in the fluid recovered (1.4mL). In animals treated with IL-10, there was no significant decrease in pleural fluid recovered.

Lung sections were examined and scored for lesion severity (see Figure 5). Histopathologic changes in the lungs of MAPV infected animals were characterized by diffuse, mild expansion of alveolar septa by infiltrating inflammatory cells consisting of lymphocytes, macrophages, and fewer neutrophils (see Figure 5). Alveoli contained increased numbers of alveolar macrophages with abundant foamy cytoplasm, occasional neutrophils, and areas of edema and hemorrhage. Alveolar walls appeared largely intact and hyaline membranes were rarely identified, with the exception of two animals who

received IL-10, where they were more abundant and frequently positive for Phosphotungstic acid haematoxylin (PTH). There was prominent perivascular edema with large accumulations of lymphocytes and macrophages with fewer neutrophils surrounding congested, small, medium, and large pulmonary vessels. Vessels frequently contained large number of neutrophils pavemented on the luminal surfaces. Occasionally, medium sized vessel walls were expanded and disrupted by inflammatory cells and nuclear debris, but none contained PTH positive material within the walls. There was regional mesothelial cell hypertrophy on the pleural surface (see Figure 6).

		Spleen	Lung	Other	Total
	K006026	2	2	2	6
	K006030	2	2	1	5
TGFβ1	K006004	1	1	0	2
	K006007	3	2	2	7
	K006009	1	1	1	3
IL-10	K006013	2	2	1	5
	K006016	3	3	3	9

Figure 5. Histologic scores from tissues of MAPV infected, TGFβ1, and IL-10 treated hamsters.

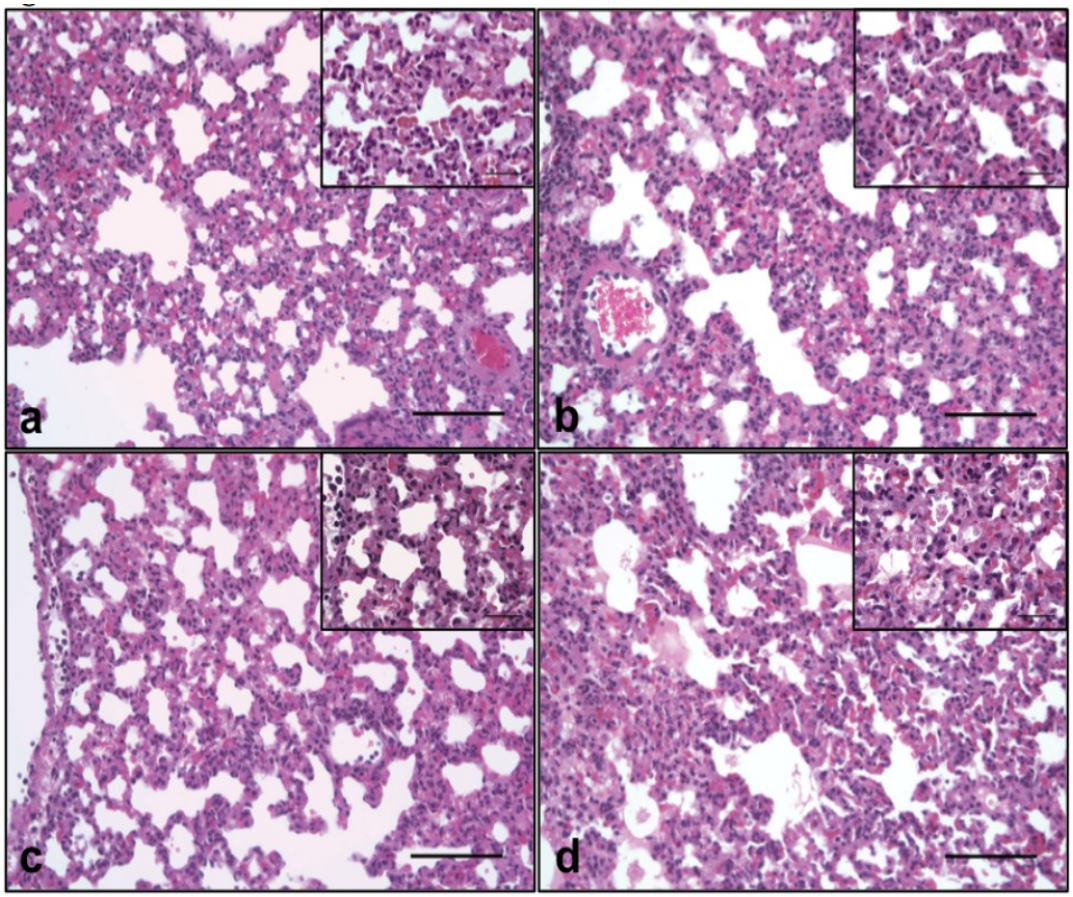

Figure 6. Histologic lung sections from hamsters infected with MAPV. Histologic lung sections are from Syrian hamsters on day 12 post- inoculation: **(a)** Normal Lung. Uninfected, untreated control animal. **(b)** There is interstitial and perivascular infiltration of lymphocytes, histiocytes and fewer neutrophils with minimal accumulations of edema and inflammatory cells within alveoli. MAPV infected, untreated animal. **(c)** Histopathologic features are similar to (b). MAPV infected, TGF-β treated animal. **(d)** MAPV infected, IL-10 treated animal. Alveolar accumulations of edema, fibrin and inflammatory cells, mainly consisting of alveolar histiocytes and neutrophils, are greater when compared to (b) and (c). MAPV infected, IL-10 treated animal. (Hematoxylin and Eosin, Bar (a-d) = 150μm, Bar (insets) = 50μm)

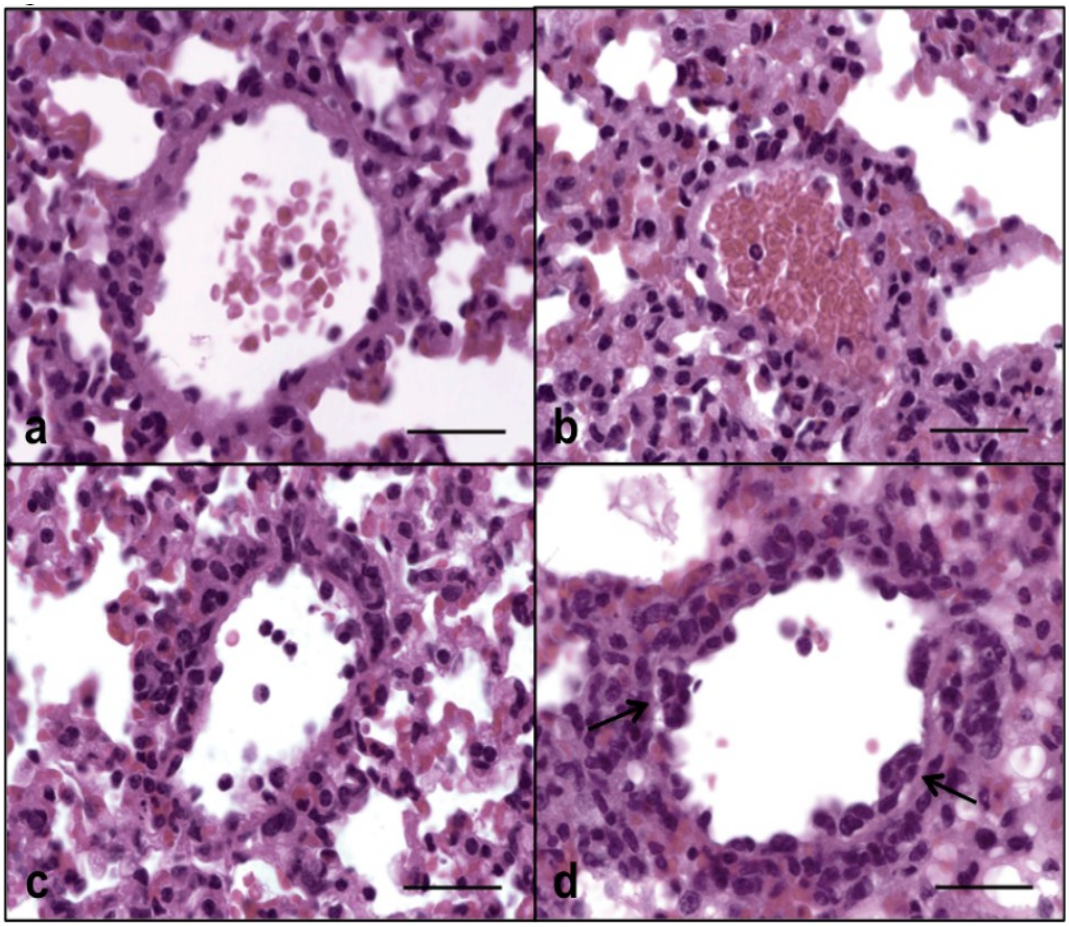

Figure 7. Histologic sections of pulmonary vessels from hamsters infected with MAPV. Histologic sections of medium-sized pulmonary vessels are from Syrian hamsters on day 12 post-inoculation. **(a)** Normal pulmonary vein. Uninfected, untreated control animal. **(b)** Infiltrating lymphocytes, histiocytes and fewer neutrophils are restricted to the tunic adventicia and adjacent perivascular tissue. MAPV infected, untreated control animal. **(c)** The Perivascular inflammation is similar to (b). MAPV infected, TGF-β treated animal. **(d)** Large numbers of inflammatory cells are present both within the inner tunics of the vessel wall (arrows) and surrounding it. MAPV infected, IL-10 treated animal. (Hematoxylin and Eosin, Bar (a-d) = 50μm)

Collectively, there was considerable variation in lesion severity amongst individual animals within groups. Two of the three IL-10 treated hamsters had the most severe histologic changes. MAPV-infected, TGFβ1 treated animals did not differ in lesion severity from those untreated MAPV-infected animals. There were no appreciable histologic differences between cytokine treated uninfected animals when compared to uninfected controls. However, one of the IL-10 treated animals closely resembled controls; it may be questioned whether the pump functioned properly. The lungs were only slightly congested and there was no pleural fluid.

Histopathologic changes in the spleen were mainly confined to the periartiolar lymphoid sheaths (PALs) in which there were mildly increased cellularity and prominence of follicular germinal centers in MAPV-infected animals (see Figure 8). Two of the animals from the TGFβ1 group had spleens 1.5-2 times the normal size and both of these animals survived. Within affected follicles, there were increased numbers of large lymphocytes with frequent mitotic figures, increased numbers of tingible body macrophages, and lymphocytes undergoing apoptosis. No significant changes were observed in the marginal zone or sinus. Changes were present within the red pulp, namely lymphocyte apoptosis, but this was also present in the uninfected controls.

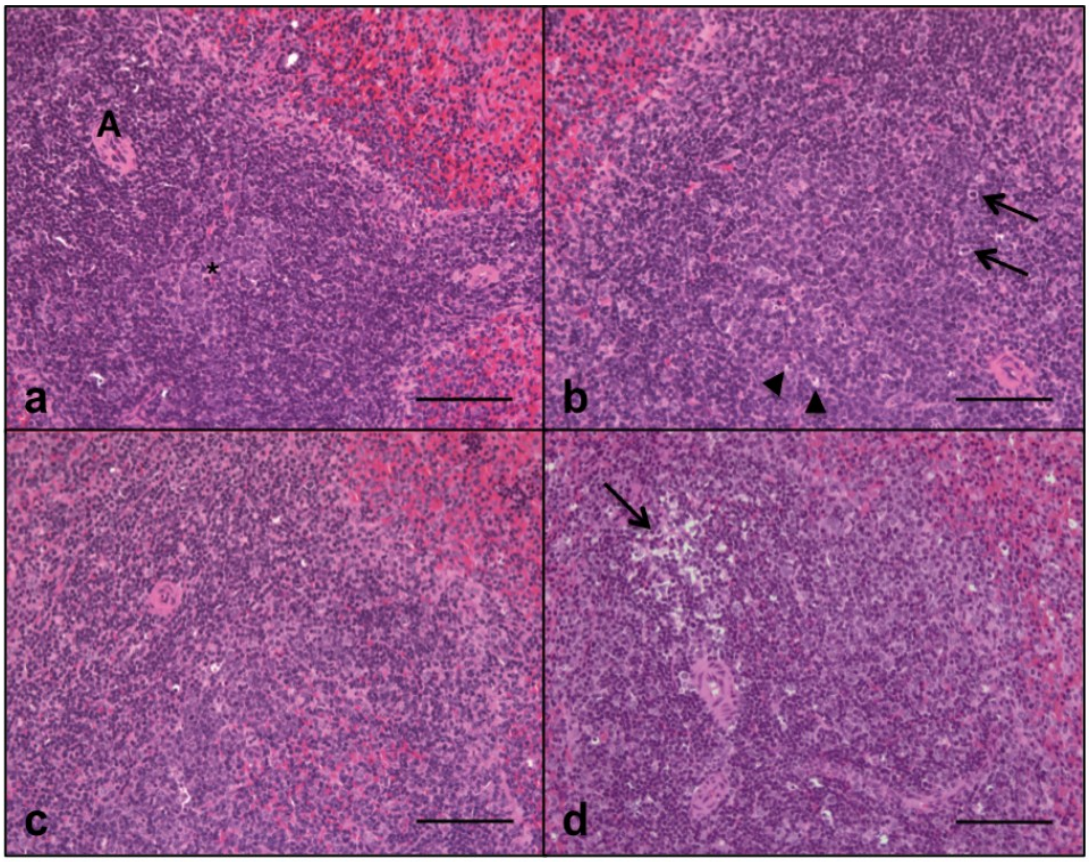

Figure 8. Histologic sections of spleen from hamsters infected with MAPV. Histologic sections of spleen are from Syrian hamsters on day 12 post-inoculation. **(a)** Normal periartiolar lymphoid sheath and follicle (germinal center denoted by asterisk) surrounding artery (A), Uninfected, untreated control animal. **(b)** There is expansion of the germinal center by proliferating large lymphocytes with multiple mitotic figures (arrows) as well as lymphocytes undergoing apoptosis (arrowheads). MAPV infected, untreated control animal. **(c)** The section contains features similar to (b). MAPV infected, TGF-β treated animal. **(d)** Note the increased numbers of lymphocytes undergoing apoptosis (arrow) and the less well-defined germinal center when compared to (b or c). MAPV infected, IL-10 treated animal. (Hematoxylin and Eosin, Bar (a-d) = 150μm)

There were mildly increased numbers of hematopoietic cells within the red pulp of infected animals. Spleens from cytokine treated uninfected animals resembled those from control animals. There were no appreciable differences between MAPV infected TGFβ1 treated and untreated MAPV infected animals. Two of three MAPV-infected, IL-10 treated animals had proportionally greater numbers of apoptotic cells within the PALs.

Quantitative Polymerase Chain Reaction for Gene Expression

The spleens of TGFβ1 treated animals had a significant difference in IL-13 expression level compared to controls which was increased. There were no significant differences in gene expression in the lungs. The IL-10 treatment group had elevated expression of several genes in the spleen as well as in the lung. However, only IL-13 was statistically significant (see Figures 9 and 10). The administration of cytokines in uninfected animals did not appear to adversely affect animals.

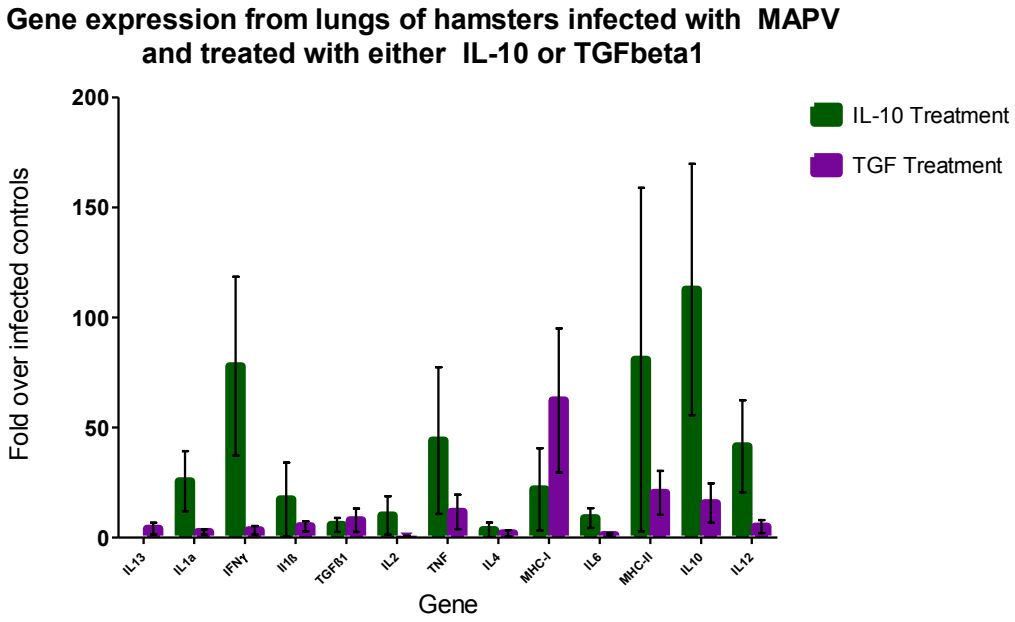

Figure 9. Gene expression from lungs of hamsters infected with MAPV. Although there were wide increases in gene expression for immune cytokines from lungs of infected and treated hamsters, none were significant by statistical analysis.

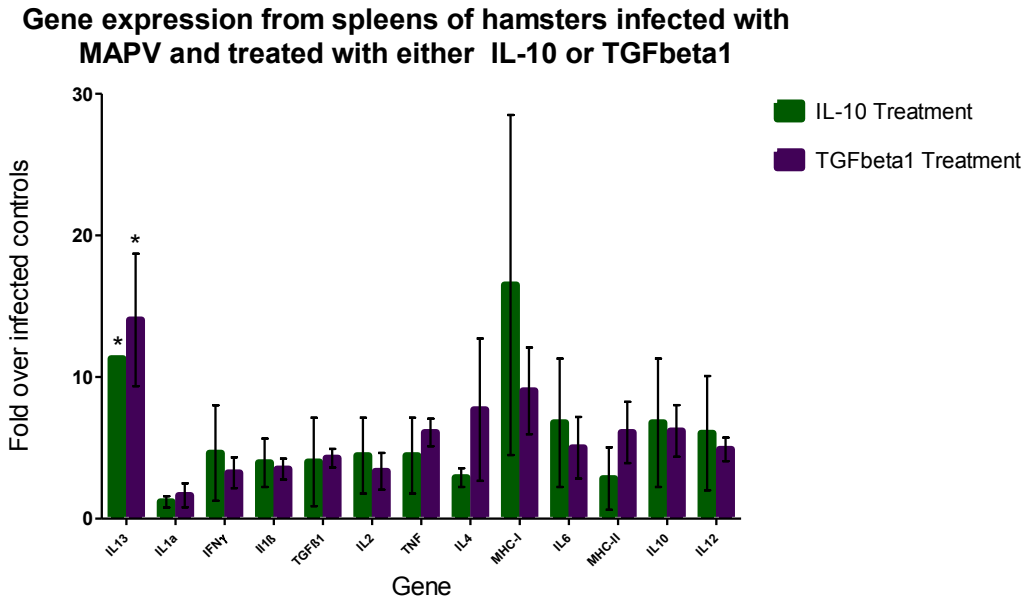

Figure 10. Gene expression from spleens of hamsters infected with MAPV. Several immune genes from spleens of infected hamsters treated with TGFβ1 were significantly different from infected, untreated control animals. None were statistically significant in the IL-10 treatment group.

Aim 3: Viral Copy Number

Viral copy number was evaluated in tissues from control animals and those treated with TGFβ1 or IL-10. Both positive and negative strand viral RNA were detected in the lungs of three of the five animals infected with MAPV and treated with TGFβ1; no significance difference was noted between the positive and negative strands (see Figure 11). Viral copy numbers were similar in animals treated with IL-10 (two of three animals examined had detectable viral RNA) and no significance difference was observed. All standard curves produced a 0.89 correlations or better between the relative fluorescence value and copy number.

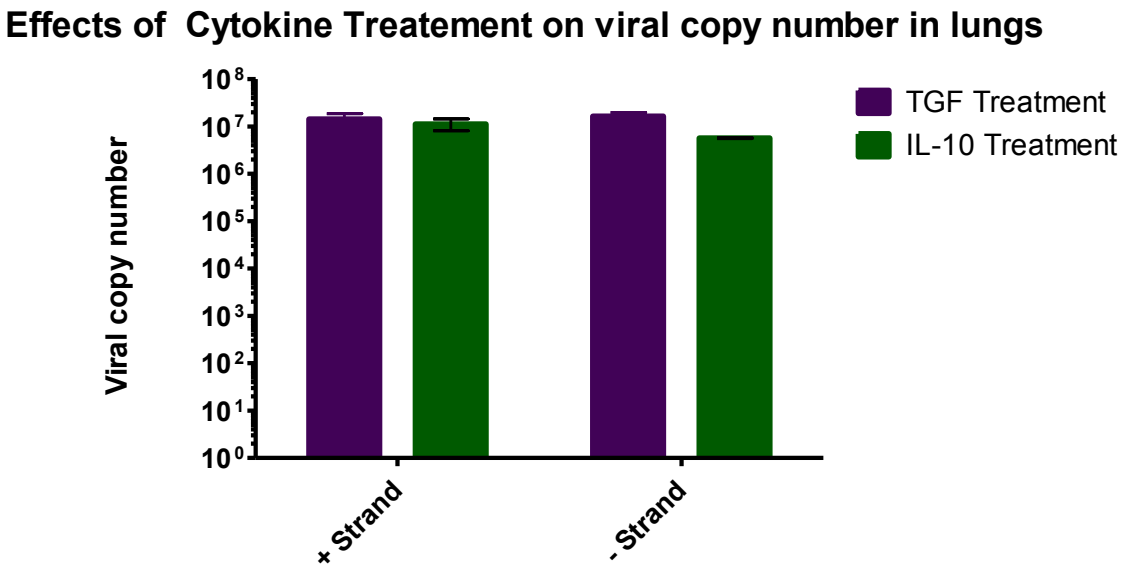

Figure 11. Viral copy number in lungs of infected hamsters. Viral copy number was evaluated in three hamsters infected with MAPV. There was no significant difference in copy number between positive and negative strand. There was also no difference in copy numbers between the TGFβ1 and IL-10 treated groups (not shown). RPL18 was used as a negative control.

CHAPTER V

DISCUSSION

Aim 1

This is the first time gene expression has been evaluated in the hamster HCPS model. Unfortunately, there are no hamster specific reagents that can evaluate protein expression. Instead, we utilized available sequences to measure gene expression using real-time PCR. Results presented here suggest that HCPS is not mediated by pro-inflammatory cytokines in the hamster model, dissimilar to human disease.

Gene expression studies demonstrated an obvious lack of elevation in inflammatory cytokines such as TNF and IFNγ. Data of gene expression from infected human tissues are limited. Mori et al. (1998) used immunohistochemistry to evaluate cytokine levels in human autopsy sections and described significant increases in TNF and IFNγ staining in lungs. Our panel of genes investigated in the hamster model was larger than that evaluated with IHC; we observed increases in other cytokines that implicated innate mechanisms rather than T cell mediated disease, e.g., transcription factors STAT1 and STAT1b, which are part of the interferon signaling system. We also observed an increase in CXCL10, a chemokine known to be induced by IFNγ. It is therefore

plausible that IFNγ is elevated at earlier time points than evaluated here and functions to increase expression of CXCL10.

Our studies support those of Hammerbeck and Hooper (2011) that HCPS may not be a T cell mediated disease in the hamster model. In their study using ANDV in the hamster model, T cells were activated at disease onset; however, when T cell function was blocked by cyclophosamide, there was similar disease pathology. This may indicate that the (T cell specific) cytokines observed in HCPS may not be the sole cause of pathology. The T cell responses that did occur appeared to be predominately CD8+ T cells (Lindgren et al., 2011).

Aim 2

Deer mice with persistent infection of SNV develop increased gene expression of TGFβ1 as do Norway rats infected with Seoul hantavirus (Easterbrook et al., 2007; Schountz et al., 2007). Because of this, we postulated the anti-inflammatory activity of TGFβ1 might be essential in the reservoir not developing pathology. Because IL-10 and TGFβ1 are both potent anti-inflammatory molecules that may be secreted from T regulatory cells, IL-10 was also investigated. Here we demonstrated that TGFβ1 might benefit hamsters infected with Maporal hantavirus while IL-10 augmented disease pathology. TGFβ1 treated hamsters had decreased pleural fluid and congestion, although lesion severity scores closely resembled infected, untreated control animals. The lungs of IL-10 treated animals had severe histological changes, including edema and more inflammatory cells, compared to TGFβ1 and control groups. This coincided with an increase in pro-inflammatory gene expression. Although not

statistically significant, the authors believe they were still meaningful due to the unpredictable natures of using whole animals in experiments rather than *in vitro* work with cells. A study by Maganto-Garcia et al. (2011) demonstrated the ability of TGFβ1 to suppress endothelial cell activation in heart tissue; however, IL-10 was not included in this study.

The increase in IL-13 in the spleens of infected and treated animals was suggestive of a Th2 involvement. IL-13 and IL-4 are usually observed influencing T cells to differentiate into a Th2 response and may be secreted by tissue macrophages during inflammatory responses. In TGFβ1 treated animals, there were no significant changes observed in the marginal zone or sinus. Changes were present within the red pulp, namely lymphocyte apoptosis, but this was also present in the uninfected controls; in all likelihood, this represented a stress response that may have been simply exacerbated by infection in some animals. In contrast, IL-10 treated animals had an increase in the number of cells undergoing apoptosis in a less well defined germinal center, which may be indicative of a more severe disease. This group also had a significant increase in lesion scores in the spleen compared to infected control animals.

TGFβ1 is a pleiotropic cytokine that can have both pro-fibrotic and immunosuppressive actions. Several therapeutics involving TGFβ1 were formulated to limit its action and thus ameliorated fibrotic diseases such as glomerulosclerosis. Because of TGFβ1 adverse effects, it has not been actively pursued as a potential pharmacologic agent (Prud'homme, 2007).

However, in studies with viral infections, TGFβ1 administration may have some therapeutic benefit. In cases of DHF, high levels of TGFβ1 have been associated with less severe pathology (Perez et al., 2010; Sierra et al., 2010). However, the level of IL-10 in these studies was associated with more severe DHF; this severity correlated with specific polymorphisms in the IL-10 gene (Perez et al., 2010; Sierra et al., 2010). Patients infected with Dobrava hantavirus (which causes HFRS) also had significantly elevated levels of IL-10 as measured by ELISA. This level of IL-10 correlated with disease severity (higher levels of IL-10 were associated with more severe disease; Saksida, Wraber, & Avsic-Zupancic, 2011).

Human lung fibroblasts pretreated with TGFβ1 were more susceptible to rhinovirus infection and replication; myofibroblasts have deceased type I interferon responses as measured by ISGs RANTES and IP-10 (Thomas et al., 2009). Hence, TGFβ1 may be helping cells to subvert the interferon response. This, in turn, allows for increased viral replication while also delaying an inflammatory response, which has also been the suggested role in deer mice infected with SNV.

The interferon response to pathogenic hantavirus in humans appears to be disrupted, perhaps by a part of the nucleocapsid or glycoprotein (Alff et al., 2006; Prescott et al., 2005). Endothelial cells infected with HCPS-causing viruses have augmented ISG expression compared to cells with non-pathogenic hantavirus (Alff et al., 2006; Prescott et al., 2005). Because IL-10 also uses the IFN receptor and associated Jak/Stat pathway, it may be that this cytokine is

somehow interfering with the Jak/Stat signaling and leading to disease attenuation. In a study with Dengue virus (Ubol, Phuklia, Kalayanarooj, & Modhiran, 2010), it was observed that increased levels of IL-10 activated expression of suppressor of cytokine signal 3, limiting the Jak/Stat pathway. Pathogenic virus may then take advantage of this suppression to promote their own survival. Our results correlated with this as we did not observe a significant increase in interferon α or β. Interestingly, it was impossible to obtain any statistical significance with either IL-10 or TGFβ1 treatments. Both groups had similar amounts of genomic and anti-genomic viral copies in the lungs of infected animals, indicating that the type of treatment did not affect viral burden.

This study demonstrated that TGFβ1 might improve the outcome for hamsters infected with MAPV. Because only little improvement was noted, future directions might include a higher dosage of TGFβ1. It appeared clear that IL-10 would be of no therapeutic benefit. Because of the use of Andes as a model of HCPS, it might also be suggested to study TGFβ1 in hamsters infected with this virus. This study used only the few cytokine sequences that were available. Since then, many more hamster sequences have become available and should be included in further studies.

Statistical Analysis

To evaluate significance of gene expression studies and lesion scores, one way ANOVA statistical tests were conducted for each gene. This enabled the means to be compared between each of the groups and reduced the risk of observing a significant *P* value by chance; it was the most appropriate method to

evaluate gene expression studies. One potential problem with this study was the limited amount of animals. This might be a reason for *R* values lower than desired in our results. However, due to costs and time constraints, the author was limited in the number of animals that could be used and could not perform repeated studies.

A Tukey-Kramer post hoc test was also utilized to compare each mean with the others. Using this test helped decrease the likelihood Type I and II errors were made.

Such tests also assumed that the population of animals used in these studies was a random representation of Syrian golden hamsters. In fact, this might not be true. Colonies of hamsters were developed using only a few wild caught animals. Hence, these animals might be more heavily inbred and not a true representation.

Future Directions

This study sheds new light on a potential mechanism for hantavirus cardiopulmonary pathogenesis. It was hypothesized that the virus entered via the respiratory route and infected endothelial cells. The virus then modulated the interferon response while it replicated itself and was released into the intra-alveolar space, where it might infect or be engulfed by alveolar macrophages. This allowed the virus to be disseminated to other organs of the body, namely the spleen. It is plausible that there was an interaction between macrophages and T cells that induced the production of IFNγ, which then acted to increase the expression of CXCL10 on endothelial cells.

In vitro studies suggested that after three days post-infection, cells were able to develop a more robust type I interferon response (Prescott et al., 2005), while earlier time points had decreased levels of interferons and interferon response genes compared to non-pathogenic hantaviruses (Spiropoulou, Albarino, Ksiazek, & Rollin, 2007). Hence, we hypothesized that initially the virus suppressed the interferon response and disseminated throughout the body. After a few days, infected cells overcame the virus but developed a modified interferon response. After the virus infected the macrophages, they traveled to the spleen, replicated further, and presented antigen in the MHC-I complex to $CD4^+$ and $CD8^+$ T cells.

A recent study suggests a role for vascular endothelial growth factor (VEGF) and cadherin. Human pulmonary endothelial cells infected with Andes hantavirus increased expression of VEGF that coincided with a decrease in cadherin. This protein is known to play a major role in the adherens junctions of endothelial membranes. Endothelial cells infected with Andes also had disrupted cell membranes and evidence of increased permeability. Due to the lack of observed TNF in these studies, it was also hypothesized that the pulmonary edema seen in HCPS victims was due to deregulation of VEGF and cadherin rather than a cytokine storm as originally hypothesized.

A major pitfall of the hamster model was there are no antibody reagents that are cross-reactive in hamsters; thus, it is unknown if some of the cytokines expressed were from macrophage, NK cell, T cell, or endothelial cell origins.

To study any disease, it is necessary to use an animal model that presents with a similar disease seen in humans. Pathologically, the hamster appears to be a useful model. However, few reagents are cross reactive with hamster proteins. Antibodies that are useful in rat and mouse models are not cross reactive in the hamster or the deer mouse. This limits scientists to gene expression studies. The entire genome has not been sequenced in both animals so many genes are still unavailable.

One of the most useful tools would be the development of antibodies against T cell subsets, macrophages, and endothelial cells. With these tools, immunohistochemistry could be performed evaluating not only organ lesions but the cellular component of these lesions would also be known. This could give researchers insight into the immune mechanisms mediating the disease. Although such antibodies might also be used in flow cytometry, the use of a BSL-4 virus in such a machine might not be practical for many labs.

In addition to better and a wider variety of reagents for use in the hamster model, it was also evident from the studies conducted here that gene expression should also be monitored at earlier time points. This has been easily accomplished in cell culture systems but not in an animal model. Our results supported evidence that many genes might have been expressed at earlier time points and were back to relatively normal levels by day 8. This might explain why we did not observe an increase in TNF and/or IFNγ.

Although the deer mice had increased expression of TGFβ1 and $Foxp3^{+}$, which is suggestive of a Treg response, this observation has not been made in

animal models. To better evaluate if this is indeed attenuating pathology in the host, the blocking the development of T regulatory cells with a drug such as tacrolimus might be useful in experimentally infected deer mice.

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APPENDIX A

LIST OF SELECTED HANTAVIRUSES AND THEIR HOSTS

List of Selected Hantaviruses and
Known Rodent Reservoirs

Hantaan virus (HTNV)*	Apodemus agrarius
Seoul virus (SEOV)*	Rattus norvegicus
Dobrava-Belgrade virus (DOBV)*	A.flavicollis
Puumala virus (PUUV)*	Myodes glareolus
Sin Nombre virus (SNV)**	Peromyscus maniculatus
New York virus (NYV)**	Peromyscus leucopus
Bayou virus (BAYV)**	Oryzomys palustris
Andes virus (ANDV)**	Oligoryzomys longicaudatus
Maporal virus (MAPV)	Oligoryzomys fulvescens
Prospect Hill virus (PHV)	Microtus pennsylvanicus
Thottapalayam virus (TPMV)	Suncus murinus
Tula virus (TULV)	Microtus arvalis

* indicates known HFRS causing virus
** indicates known HCPS causing virus. Others are of unknown pathology.

Source: Yates et al., 2002.

APPENDIX B

EUTHANASIA TABLE USED TO EVALUATE INFECTED HAMSTERS

Euthanasia Table Used to Evaluate Infected Hamsters on a Daily Basis

Euthanasia Scoring Evaluation

Animal: _____ Date: _____ Time: _____

Animals will be evaluated twice daily. If a score of 1 or more is noted at any evaluation, animals will be observed thrice daily for disease progression.

Eating/Drinking	Normal amount of feces Moderate amount of feces Scant/no feces	0 1 2
Behavior	Normal nesting Moderate amount of nesting No nesting	0 1 2
Movement*	Normal when touched Reluctant to move when touched No movement when touched	0 1 2
Hair Coat*	Normal Slightly rough Very rough	0 1 2
Posture*	Normal Moderately hunched Very hunched	0 1 2
Total		

Criteria for immediate euthanasia:
A score of 5 or more in an evaluation
Animals that are moribund or suffer 10% or more weight loss in an evaluation
Visible hemorrhaging in an evaluation
Any animal in groups denoted with an '*' with a score of 2 in two consecutive evaluations
Any animal that LAR veterinarian deem suffering

APPENDIX C

PRIMERS USED FOR REAL TIME PCR--RPL18

Primers Used for Real Time PCR--RPL18

B-cell lymphoma 2 protein (bcl-2) (AJ582074)	Bcl2 F	CTTCGCAGAGATGTCCAGTC
	Bcl2 R	CATCTCCCTGTTGACGCTC
	Bcl2 TM	6FAM-TGACGCCCTTCACCGCGA-BBQ
Bcl-2 associated protein (AJ582075)	Bax F	GGCAACTTCAACTGGGG
	Bax R	CCACCCTGGTCTTGGATC
	Bax TM	6FAM-CCAGCCCATGATGGTTCTGATTAGC-BBQ
CD83 protein DQ094177	CD83 F	AACCTGGTACGGAACAAGCT
	CD83 R	CAAAGGAAGGTTGCCGTC
	CD83 TM	6FAM-TCCAGGCAGCATTCAGGTACACTGA-BBQ
Chemokine (C-X-C motif) ligand 10 (IP-10) (AY007988)	IP-10F	GCCATTCATCCACAGTTGACA
	IP-10 R	CATGGTGCTGACAGTGGAGTCT
	IP-10 TM	6FAM-CGTCCCGAGCCAGCCAACGA-BBQ
Chemokine CCL20/MIP-3 α (AY924377)	CCL20 F	AGTCAGTCAGAAGCAAGCAACT
	CCL20 R	TGAAGCGGTGCATGATCC
	CCL20 TM	6FAM-CACAAGGAGCACTATCCCACCCAGA-BBQ
Chemokine ligand 17 (FJ664143)	CL17 F	CGAGTGCTGCCTGGAGATC
	CL17 R	TGATGGCCTTCTTCACATGC
	CL17 TM	6FAM-TGGACCTGCCCTGGACAGTCACA-BBQ
Chemokine ligand 22 (FJ664144)	CL22 F	CGCGTAGTGAAGGAGTTCTTC
	CL22 R	TCTTCACCAGGCCAGCTTA
	CL22 TM	6FAM-ACCTCAAAGTCCTGCCGCAAGCC-BBQ
Claudulin-1 (EU856105)	ham cld1 F2	GCCACAGCATGGTATGGAA
	ham cld1 R1	GCAAGAAAGTAGGGCACCTC
	ham cld1 TM	6FAM- CCCGTCAATGCCAGGTATGAATT-BBQ
Complement C3 (complement C3d region) (AB024425)	CC3d F	GGAGCCTTACCTCAGCAAGT
	CC3d R	TAGCCGCCTCCGTAGTATCT
	CC3d TM	6FAM-CAGAAGCTCTACAATGTGGAGGCCA-BBQ
Complement component 5 (DQ369042)	CC5 F	GTAGTTCCCGATGCTGAAGTG
	CC5 R	TGATTAACTCCATTGACCAACG
	CC5 TM	6FAM-TGTGACTTGCATCGCTTTCGGC-BBQ
Complement protein C1qBP (DQ367730)	CP1qBP F	CAGAGGATGAGGTTGGACAA
	CP1qBP R	CCATTAGGTGGTCATACAAGGC
	CP1qBP TM	6FAM-TCCATTCAGAGTCACCAGTGGTCTGGA-BBQ
E-cadherin (DQ237892)	Ecad F	GTTAAGGTTCTGGAGATGAGATTGG
	Ecad R	CATCTTTCCCCTCCGAGACA
	Ecad TM	6FAM-TTATGTAGATGACCATGACTTTAATGACAA-BBQ
Hypoxanthine phosphoribosyltransferase (AF047041)	HPRT F	TGCGGATGATATCTCAACTTTAACTG
	HPRT R	AAAGGAAAGCAAAGTTTGTATTGTCA
	HPRT TM	6FAM-AAAGAATGTCTTGATTGTTGAAGGTAAAACTGACATTGG-BBQ
Inducible nitric oxide synthase-2 (AY297461)	iNOS F	TGGCAGGATGGGAAACTGA
	iNOS R	GCACCGCTTTCACCAAGACT
	iNOS TM	6FAM-CCCAGGAGGAGAGAGATCCGGCTC-BBQ
Intracellular adhesion molecule-1 (DQ093373)	ICAM1 F	TGCAGCCGGAGAACAGATG
	ICAM1 R	ATCTCCCGTGTGACAGTCTTCA
	ICAM1 TM	6FAM-AGCCCTGCTGCCCATCGGG-BBQ
Interferon-α inducible protein (p27-h) (AF212039)	p27 F	TCGTTGCTGCTCCCGTAGTC
	p27 R	ATGGATCCCGCTGCAATTC
	p27 TM	6FAM-TGGGTGCTGTGGGCTTCACTGG-BBQ
Interferon-γ (AF034482)	IFNg F	GGCCATCCAGAGGAGCATAG
	IFNg R	TTTCTCCATGCTGCTGTTGAA
	IFNg TM	6FAM-CACCATCAAGGCAGACCTGTTTGCTAACTT-BBQ
Interferon regulatory factor-1 (DQ092344)	IRF1 F	GGCATACAACATGTCTTCACG
	IRF1 R	GCTATGCTTTGCCATGTCAA
	IRF1 TM	6FAM-CACAATGACGCCAGACCTTGCTCA-BBQ
Interferon regulatory factor-2 (AY714581)	IRF2 F	AATGCCTTCAGAGTGTACCG
	IRF2 R	TGTTCACCGTACTATCCACTTCAT
	IRF2 TM	6FAM-CTGAAGTCAGGACCGCATACTCAGGA-BBQ
Interleukin-1β (AB028497)	IL-1b F	GGCTGATGCTCCCATTCG
	IL-1b R	CACGAGGCATTTCTGTTGTTCA
	IL-1b TM	6FAM-CAGCTGCACTGCAGGCTCCGAG-BBQ
Interleukin-2 (EU729351)	IL-2F	GTGCACCCACTTCAAGCTCTAA
	IL-2 R	AAGCTCCTGTAAGTCCAGCAGTAAC
	IL-2 TM	6FAM-AGGAAACCCAGCAGCACCTCGAGC-BBQ
Interleukin-4 (AF046213)	IL-4F	CCACGGAGAAAGACCTCATCTG
	IL-4 R	GGGTCACCTCATGTTGGAAATAAA
	IL-4 TM	6FAM-CAGGGCTTCCCAGGTGCTTCGCAAGT-BBQ
Interleukin-6 (AB028635)	IL-6F	CCTGAAAGCACTTGAAGAATTCC
	IL-6 R	GGTATGCTAAGGCACAGCACACT
	IL-6 TM	6FAM-AGAAGTCACCATGAGGTCTACTCGGCAAAA-BBQ
Interleukin-10 (AF046210)	IL-10F	GTTGCCAAACCTTATCAGAAATGA
	IL-10 R	TTCTGGCCCGTGGTTCTCT
	IL-10 TM	6FAM-CAGTTTTACCTGGTAGAAGTGATGCCCCAGG-BBQ
Interleukin-12 p40 subunit (AB085792)	IL12p40 F	TGGTTACCTCCTTAGCAGTCC
	IL12p40 R	TCAGCCTGATGATGAACCTGA
	IL12p40 TM	6FAM-TCCAGAGTGCCATAATAGCCACACAAA-BBQ
Interleukin-21 (FJ664142)	IL21 F	TCAACTGATGTGAAAGGAGC
	IL21 R	ATCTTGTGGAGCTGGCAG
	IL21 TM	6FAM-TCAGGGTCCTAGCCAAAAGAGAATC-BBQ
Interleukin-2 receptor-α (DQ093372)	IL2Ra F	AAAGCAAGCTACACCTAACCC
	IL2Ra R	GCCTTGTATCCTTGAATGCG
	IL2Ra TM	6FAM-CAGAAATCAGCACAGTCTGTGCACCA-BBQ
Interleukin-6 signal transducer (EF442778)	IL6ST F	TGAAGATACAGCATCTTCCCG
	IL6ST R	TGAAGATACAGCATCTTCCCG
	IL6ST TM	6FAM-TCACTCCAGTAGCCTTTGCCATCCT-BBQ
Junction adhesion molecule (EU856104)	ham jam F1	CGTCCAAGTTCCCGAGAGTA
	ham jam R1	CGTGATCTGGCTGTTATAGCA
	ham jam TM	6FAM-TAGTGCCACCCTGGACGAACTTC-BBQ
Matrix metalloproteinase-2 (AF260254)	MM2 F	GATGCTGCCTTTAACTGGAGT
	MM2 R	GAGCTTAGGGAAACCAGGAT
	MM2 TM	6FAM-CATACATCTTCGCTGGAGACAAGTTC-BBQ
MHC class II antigen alpha chain (DQ092501)	MHCAAC F1	CAGGGAGGACTGCAAGCTATA
	MHCAAC R	TGTCCACGAAGCAGATGAG
	MHCAAC TM	6FAM-TGCAGCAAAGCAGAACTTGGACATC-BBQ
Myxovirus resistance protein-2 (EU616539)	Mx2 F	CCAGTAATGTGGACATTGCC
	Mx2 R	CATCAACGACCTTGTCTTCAGTA
	Mx2 TM	6FAM-TGTCCACCAGATCAGGCTTGGTCA-BBQ
Nitric oxide synthase-2 (DQ355357)	NOS2 F	TGCCTTGCATCCTCATTGG
	NOS2 R	GTCGCTGTTGCCAGAAACTG
	NOS2 TM	6FAM-CCTGGCACGGGCATCGCTC-BBQ
Occuldin (EU856106)	ham occ F1	CTATTCTGGGCATCCTGGT
	ham occ R1	TTGCACATGGCATAGATCTG
	ham occ TM	6FAM- AGTCAACCCAACTGCCCAGGCT-BBQ
p75 tumor necrosis factor membrane receptor (AF315291)	p75 F	CCCCAGGCCACAGTCAC
	p75 R	GCCGTGGGAGGAATCTGAA
	p75 TM	6FAM-CTGCACAGGCCTCCTGAGACCCT-BBQ
Platelet endothelial adhesion molecule (AF508040)	PECAMF	CAGGATCAGAACTTCAGCAAGAT
	PECAMR	GCAGCTGATGGTTATAGCATGT
	PECAM TM	6FAM-TGTACCGCAGGCATCGGCAGA-BBQ
Protein kinase R (DQ645944)	Eif2ak2 F	ACGGACCTAAGAGATGGCAT
	Eif2ak2 R	AGGTAACTAAAGCGGAGTGC
	Eif2ak2 TM	6FAM-CCACGGATCGACCTAGTGCTTCTGA-BBQ
Ribosomal Protein L 18 (DQ403027)	RPL18 F	GTTTATGAGTCGCACTAACCG
	RPL18 R	TGTTCTCTCGGCCAGGAA
	RPL18 TM	YAK-TCTGTCCCTGTCCCGGATGATC-BBQ
Signal transducer and activator of transcription-1 β (AB177397)	STAT1b F	AGGTCCGTCAGCAGCTTAA
	STAT1b R	GCCGTTCCACCACAAAT
	STAT1b TM	6FAM-TCTGAATGAGCTGCTGGAAGAGGACA-BBQ
Signal transducer and activator of transcription-2 (AB177399)	STAT2 F	AATGCCTTCAGAGTGTACCG
	STAT2 R	TGTTCACCGTACTATCCACTTCAT
	STAT2 TM	6FAM-CTGAAGTCAGGACCGCATACTCAGGA-BBQ
Tight junction protein 2 (EU856099)	ham tjp2 F1	CTACACTGACAATGAGCTGGA
	ham tjp2 R1	CTCTGGGCTGGATTTCCTTA
	ham tjp2 TM	6FAM-TCATGCTGCACCGGCTCCGA-BBQ
Tissue inhibitor of matrix metalloproteinase-2 (AF260255)	TIMM2 F	AGAGCCTGAACCACAGGT
	TIMM2 R	CGGGTCCTCGATGTCAA
	TIMM2 TM	6FAM-CGAGTGCAAGATCACACGCTGCC-BBQ
Transforming growth factor-β1 (AF046214)	TGFb F	TGTGTGCGGCAGCTGTACA
	TGFb R	TGGGCTCGTGAATCCACTTC
	TGFb TM	6FAM-CGACTTTCGCAAGGACCTGGGCT-BBQ
Transforming growth factor 2 (AY007214)	TGFb2 F	TGCTGCCCTCCTACAGACT
	TGFb2 R	GCACAGAAGTTGGCATTATACC
	TGFb2 TM	6FAM-CACAACAGTCCAATCGGCGGA-BBQ
Transforming growth factor-β 3 (AF298188)	TGFb3 F	CAAGCTCAGGCTCACCAGT
	TGFb3 R	CCGACTCTGTGTTCTCCTGAG
	TGFb3 TM	6FAM-AGCCATCGGTGATGACCCACGT-BBQ
Transforming growth factor-β type I receptor (AF298187)	TGFbTIR F	ATCAAACTTGCTCTGTCTACGG
	TGFbTIR R	TGTCTGTGGCAGAATCATGC
	TGFbTIR TM	6FAM-ACAGCCAGTCCCAAGTCTGCAATAC-BBQ
Tumor Necrosis Factor-α (AF315292)	TNFa F	GGAGTGGCTGAGCCATCGT
	TNFa R	AGCTGGTTGTCTTTGAGAGACATG
	TNFa TM	6FAM-CCAATGCCCTCCTGGCCAACG-BBQ
Vascular endothelial growth factor (AF297627)	VEGF F	CAGGAGTACCCCGATGAGATAGA
	VEGF R	CCCCCACACCGCATCA
	VEGF TM	6FAM-TCTTCAAGCCGTCCTGTGTGCCC-BBQ

RPL18 was used as a negative control in gene expression experiments. Primers developed by Zivcec et al. (2011).

APPENDIX D

PRIMERS USED FOR REAL TIME PCR--GAPDH

Primers Used For Real Time PCR--GAPDH

Gene	Forward	Reverse
IL-1β	5'-CCAACAAGTGGTGTTCTCCATGAG -3'	5'- CAGGTACAGGTTCTTTCCCTTTAGG -3'
IL-2	5'- AATCCGAAACTCCCCATGATG -3'	5'- CTCCAAGTTCTTCTTCCAGGCAC -3'
IL-6	5'-AGCTGGAGTCACAAAAGGAGTGG -3'	5'- TTTGCCGAGTAGACCTCATGGTG -3'
IL-12p35	5'-TGAACTCCTCCTCCTGAAAAGCTC -3'	5'- GCCCACCTCTTGTTAAAATGCC -3'
TGFβ1	5'- AAGTGGATTCACGAGCCCAAG -3'	5'- GGCAAGGACCTTACTGTACTGTGTG -3'
MHC-I	5'-GTC GGC TAC GTG GAC GAC AC-3'	5'-CGT TCA GGG CGA TGT AAT C-3'
GAPDH	5'- AGCTTGTCATCAACGGGAAGG -3'	5'- GACGCCAGTAGACTCCACAACATAC -3'

GAPDH was used as a negative control in gene expression experiments.

APPENDIX E

ANOVA TABLES FOR INFECTED VERSUS UNINFECTED HAMSTERS

Study K006 Lung Tissue ANOVA Tables from qPCR Experiments

Parameter

Table Analyzed IFNgamma

One-way analysis of variance

P value	0.0447
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	5.007
R square	0.5886

ANOVA Table	SS	df	MS
Treatment (between columns)	124.6	2	62.31
Residual (within columns)	87.11	7	12.44
Total	211.7	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IFNg vs IL10	7.333	3.601	No	ns	-1.149 to 15.82
IFNg vs TGF	-0.6125	0.3215	No	ns	-8.547 to 7.322
IL10 vs TGF	-7.946	4.171	Yes	*	-15.88 to -0.01106

Parameter

Table Analyzed IL1alpha

One-way analysis of variance

P value	0.5197
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.7197
R square	0.1706

ANOVA Table	SS	df	MS
Treatment (between columns)	14.38	2	7.191
Residual (within columns)	69.94	7	9.991
Total	84.32	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL1a vs IL10	2.777	1.522	No	ns	-4.824 to 10.38
IL1a vs TGF	0.3050	0.1787	No	ns	-6.805 to 7.415
IL10 vs TGF	-2.472	1.448	No	ns	-9.582 to 4.638

Parameter

Table Analyzed IL1beta

One-way analysis of variance

P value	0.8383
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.1809
R square	0.04916

ANOVA Table	SS	df	MS
Treatment (between columns)	3.476	2	1.738
Residual (within columns)	67.24	7	9.606
Total	70.72	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Il1ß vs IL10	1.333	0.7451	No	ns	-6.120 to 8.786
Il1ß vs TGF	1.248	0.7453	No	ns	-5.724 to 8.219
IL10 vs TGF	-0.08583	0.05128	No	ns	-7.057 to 6.886

Parameter

Table Analyzed IL2

One-way analysis of variance

P value	0.5633
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.6238
R square	0.1513

ANOVA Table	SS	df	MS
Treatment (between columns)	13.34	2	6.669
Residual (within columns)	74.84	7	10.69
Total	88.18	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL2 vs IL10	0.8933	0.4732	No	ns	-6.969 to 8.756
IL2 vs TGF	-1.803	1.021	No	ns	-9.157 to 5.552
IL10 vs TGF	-2.696	1.527	No	ns	-10.05 to 4.659

Parameter

Table Analyzed IL4

One-way analysis of variance

P value 0.5638

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.6225

R square 0.1510

ANOVA Table	SS	df	MS
Treatment (between columns)	24.93	2	12.47
Residual (within columns)	140.2	7	20.02
Total	165.1	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL4 vs IL10	3.000	1.161	No	ns	-7.760 to 13.76
IL4 vs TGF	-0.6825	0.2824	No	ns	-10.75 to 9.383
IL10 vs TGF	-3.682	1.524	No	ns	-13.75 to 6.383

Parameter

Table Analyzed IL-6

One-way analysis of variance

P value 0.7143

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.3531

R square 0.09164

ANOVA Table	SS	df	MS
Treatment (between columns)	11.47	2	5.734
Residual (within columns)	113.7	7	16.24
Total	125.1	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL6 vs IL10	1.113	0.4785	No	ns	-8.577 to 10.80
IL6 vs TGF	-1.444	0.6636	No	ns	-10.51 to 7.620
IL10 vs TGF	-2.558	1.175	No	ns	-11.62 to 6.507

Parameter

Table Analyzed IL10

One-way analysis of variance

P value 0.3812

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1.110

R square 0.2408

ANOVA Table	SS	df	MS
Treatment (between columns)	35.34	2	17.67
Residual (within columns)	111.4	7	15.91
Total	146.7	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL10 vs IL10	4.780	2.075	No	ns	-4.813 to 14.37
IL10 vs TGF	1.724	0.8003	No	ns	-7.249 to 10.70
IL10 vs TGF	-3.056	1.418	No	ns	-12.03 to 5.917

Parameter

Table Analyzed IL12

One-way analysis of variance

P value 0.3516

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1.218

R square 0.2582

ANOVA Table	SS	df	MS
Treatment (between columns)	21.19	2	10.59
Residual (within columns)	60.89	7	8.698
Total	82.08	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL12 vs IL10	3.670	2.155	No	ns	-3.422 to 10.76
IL12 vs TGF	1.194	0.7497	No	ns	-5.440 to 7.828
IL10 vs TGF	-2.476	1.554	No	ns	-9.110 to 4.158

Parameter

Table Analyzed IL13

One-way analysis of variance

P value	0.3585
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.192
R square	0.2541

ANOVA Table	SS	df	MS
Treatment (between columns)	59.51	2	29.75
Residual (within columns)	174.7	7	24.96
Total	234.2	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL13 vs IL10	5.447	1.888	No	ns	-6.567 to 17.46
IL13 vs TGF	0.2225	0.08247	No	ns	-11.02 to 11.46
IL10 vs TGF	-5.224	1.936	No	ns	-16.46 to 6.013

Parameter

Table Analyzed MHC-I

One-way analysis of variance

P value 0.1480

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 2.541

R square 0.4207

ANOVA Table	SS	df	MS
Treatment (between columns)	38.22	2	19.11
Residual (within columns)	52.64	7	7.520
Total	90.86	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MHC-I vs IL10	2.553	1.613	No	ns	-4.041 to 9.147
MHC-I vs TGF	4.719	3.187	No	ns	-1.449 to 10.89
IL10 vs TGF	2.166	1.462	No	ns	-4.002 to 8.334

Parameter

Table Analyzed MHC-II

One-way analysis of variance

P value	0.2569
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.660
R square	0.3218

ANOVA Table	SS	df	MS
Treatment (between columns)	24.46	2	12.23
Residual (within columns)	51.56	7	7.365
Total	76.02	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MHC-II vs IL10	3.330	2.125	No	ns	-3.196 to 9.856
MHC-II vs TGF	3.471	2.368	No	ns	-2.634 to 9.575
IL10 vs TGF	0.1408	0.09609	No	ns	-5.964 to 6.245

Parameter

Table Analyzed TGF

One-way analysis of variance

P value	0.5684
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.6132
R square	0.1491

ANOVA Table	SS	df	MS
Treatment (between columns)	6.526	2	3.263
Residual (within columns)	37.25	7	5.321
Total	43.78	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFß1 vs IL10	1.890	1.419	No	ns	-3.657 to 7.437
TGFß1 vs TGF	1.643	1.318	No	ns	-3.546 to 6.831
IL10 vs TGF	-0.2475	0.1987	No	ns	-5.436 to 4.941

Parameter

Table Analyzed TNF

One-way analysis of variance

P value	0.4032
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.037
R square	0.2286

ANOVA Table	SS	df	MS
Treatment (between columns)	20.17	2	10.08
Residual (within columns)	68.06	7	9.723
Total	88.23	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TNF vs IL10	3.667	2.037	No	ns	-3.832 to 11.16
TNF vs TGF	1.864	1.107	No	ns	-5.150 to 8.878
IL10 vs TGF	-1.803	1.070	No	ns	-8.816 to 5.211

Study K006 Spleen Tissue Samples from qPCR

Parameter

Table Analyzed IFNgamma

One-way analysis of variance

P value	0.3360
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.367
R square	0.3536

ANOVA Table	SS	df	MS
Treatment (between columns)	3.553	2	1.777
Residual (within columns)	6.497	5	1.299
Total	10.05	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IFNg vs IL10	1.665	2.066	No	ns	-2.044 to 5.374
IFNg vs TGF	1.458	2.088	No	ns	-1.755 to 4.670
IL10 vs TGF	-0.2075	0.2973	No	ns	-3.420 to 3.005

Parameter

Table Analyzed IL1alpha

One-way analysis of variance

P value 0.9967

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.003329

R square 0.001330

ANOVA Table	SS	df	MS
Treatment (between columns)	0.03084	2	0.01542
Residual (within columns)	23.16	5	4.632
Total	23.19	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL1a vs IL10	0.1650	0.1084	No	ns	-6.838 to 7.168
IL1a vs TGF	0.04000	0.03035	No	ns	-6.025 to 6.105
IL10 vs TGF	-0.1250	0.09485	No	ns	-6.190 to 5.940

Parameter

Table Analyzed IL1beta

One-way analysis of variance

P value	0.0781
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	4.431
R square	0.6393

ANOVA Table	SS	df	MS
Treatment (between columns)	4.589	2	2.295
Residual (within columns)	2.589	5	0.5179
Total	7.179	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Il1ß vs IL10	1.830	3.596	No	ns	-0.5118 to 4.172
Il1ß vs TGF	1.703	3.863	No	ns	-0.3256 to 3.731
IL10 vs TGF	-0.1275	0.2893	No	ns	-2.156 to 1.901

Parameter

Table Analyzed IL2

One-way analysis of variance

P value 0.3580

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1.270

R square 0.3369

ANOVA Table	SS	df	MS
Treatment (between columns)	3.938	2	1.969
Residual (within columns)	7.749	5	1.550
Total	11.69	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL2 vs IL10	1.830	2.079	No	ns	-2.221 to 5.881
IL2 vs TGF	1.458	1.912	No	ns	-2.051 to 4.966
IL10 vs TGF	-0.3725	0.4886	No	ns	-3.881 to 3.136

Parameter

Table Analyzed IL4

One-way analysis of variance

P value	0.4476
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.9483
R square	0.2750

ANOVA Table	SS	df	MS
Treatment (between columns)	5.606	2	2.803
Residual (within columns)	14.78	5	2.956
Total	20.39	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL4 vs IL10	1.505	1.238	No	ns	-4.090 to 7.100
IL4 vs TGF	2.045	1.942	No	ns	-2.800 to 6.890
IL10 vs TGF	0.5400	0.5129	No	ns	-4.305 to 5.385

Parameter

Table Analyzed IL6

One-way analysis of variance

P value 0.1920

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 2.337

R square 0.4832

ANOVA Table	SS	df	MS
Treatment (between columns)	6.708	2	3.354
Residual (within columns)	7.176	5	1.435
Total	13.88	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL6 vs IL10	2.335	2.756	No	ns	-1.563 to 6.233
IL6 vs TGF	1.960	2.672	No	ns	-1.416 to 5.336
IL10 vs TGF	-0.3750	0.5112	No	ns	-3.751 to 3.001

Parameter

Table Analyzed IL10

One-way analysis of variance

P value 0.0817

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 4.309

R square 0.6329

ANOVA Table	SS	df	MS
Treatment (between columns)	8.806	2	4.403
Residual (within columns)	5.109	5	1.022
Total	13.92	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL10 vs IL10	2.335	3.267	No	ns	-0.9544 to 5.624
IL10 vs TGF	2.463	3.978	No	ns	-0.3862 to 5.311
IL10 vs TGF	0.1275	0.2060	No	ns	-2.721 to 2.976

Parameter

Table Analyzed IL12

One-way analysis of variance

P value 0.1324

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 3.112

R square 0.5545

ANOVA Table	SS	df	MS
Treatment (between columns)	7.207	2	3.604
Residual (within columns)	5.790	5	1.158
Total	13.00	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL12 vs IL10	2.165	2.845	No	ns	-1.337 to 5.667
IL12 vs TGF	2.205	3.346	No	ns	-0.8275 to 5.238
IL10 vs TGF	0.04000	0.06070	No	ns	-2.993 to 3.073

Parameter

Table Analyzed IL13

One-way analysis of variance

P value 0.0175

P value summary *

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 10.12

R square 0.8019

ANOVA Table	SS	df	MS
Treatment (between columns)	18.69	2	9.347
Residual (within columns)	4.619	5	0.9238
Total	23.31	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL13 vs IL10	3.505	5.157	Yes	*	0.3774 to 6.633
IL13 vs TGF	3.543	6.019	Yes	*	0.8339 to 6.251
IL10 vs TGF	0.03750	0.06371	No	ns	-2.671 to 2.746

Parameter

Table Analyzed MHC-I

One-way analysis of variance

P value	0.0597
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	5.218
R square	0.6761

ANOVA Table	SS	df	MS
Treatment (between columns)	15.15	2	7.577
Residual (within columns)	7.260	5	1.452
Total	22.41	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MHC-I vs IL10	3.500	4.108	No	ns	-0.4212 to 7.421
MHC-I vs TGF	2.955	4.005	No	ns	-0.4409 to 6.351
IL10 vs TGF	-0.5450	0.7386	No	ns	-3.941 to 2.851

Parameter

Table Analyzed MHC-II

One-way analysis of variance

P value	0.2182
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.096
R square	0.4560

ANOVA Table	SS	df	MS
Treatment (between columns)	7.720	2	3.860
Residual (within columns)	9.209	5	1.842
Total	16.93	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MHC-II vs IL10	0.8300	0.8649	No	ns	-3.586 to 5.246
MHC-II vs TGF	2.290	2.755	No	ns	-1.535 to 6.115
IL10 vs TGF	1.460	1.757	No	ns	-2.365 to 5.285

Parameter

Table Analyzed TGF

One-way analysis of variance

P value	0.1800
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.464
R square	0.4964

ANOVA Table	SS	df	MS
Treatment (between columns)	5.550	2	2.775
Residual (within columns)	5.631	5	1.126
Total	11.18	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFß1 vs IL10	1.330	1.772	No	ns	-2.123 to 4.783
TGFß1 vs TGF	2.040	3.139	No	ns	-0.9508 to 5.031
IL10 vs TGF	0.7100	1.092	No	ns	-2.281 to 3.701

Parameter

Table Analyzed TNF

One-way analysis of variance

P value	0.0608
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	5.163
R square	0.6737

ANOVA Table	SS	df	MS
Treatment (between columns)	8.665	2	4.332
Residual (within columns)	4.196	5	0.8392
Total	12.86	7	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TNF vs IL10	1.835	2.833	No	ns	-1.146 to 4.816
TNF vs TGF	2.545	4.537	No	ns	-0.03659 to 5.127
IL10 vs TGF	0.7100	1.266	No	ns	-1.872 to 3.292

APPENDIX F

ANOVA TABLES FOR TREATED VERSUS UNTREATED HAMSTERS

Study L005 Lung ANOVA tables from qPCR

Parameter

Table Analyzed ANOVA IL1b

One-way analysis of variance

P value 0.6129

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.5546

R square 0.2171

ANOVA Table	SS	df	MS
Treatment (between columns)	2.357	2	1.179
Residual (within columns)	8.500	4	2.125
Total	10.86	6	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	1.500	1.455	No	ns	-3.695 to 6.695
Control vs Day 10	0.5000	0.5314	No	ns	-4.242 to 5.242
Day 8 vs Day 10	-1.000	1.063	No	ns	-5.742 to 3.742

Parameter

Table Analyzed Bax

One-way analysis of variance

P value	0.2397
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.764
R square	0.3351

ANOVA Table	SS	df	MS
Treatment (between columns)	12.60	2	6.300
Residual (within columns)	25.00	7	3.571
Total	37.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-1.500	1.296	No	ns	-6.320 to 3.320
Control vs Day 10	-2.500	2.646	No	ns	-6.436 to 1.436
Day 8 vs Day 10	-1.000	0.8641	No	ns	-5.820 to 3.820

Parameter

Table Analyzed Bcl-2

One-way analysis of variance

P value 0.0628

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 4.219

R square 0.5466

ANOVA Table	SS	df	MS
Treatment (between columns)	55.75	2	27.88
Residual (within columns)	46.25	7	6.607
Total	102.0	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-3.250	2.065	No	ns	-9.806 to 3.306
Control vs Day 10	-5.250	4.085	No	ns	-10.60 to 0.1029
Day 8 vs Day 10	-2.000	1.271	No	ns	-8.556 to 4.556

Parameter

Table Analyzed CC3d

One-way analysis of variance

P value	0.1729
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.385
R square	0.4429

ANOVA Table	SS	df	MS
Treatment (between columns)	27.56	2	13.78
Residual (within columns)	34.67	6	5.778
Total	62.22	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	4.667	3.008	No	ns	-2.066 to 11.40
Control vs Day 10	2.667	2.054	No	ns	-2.966 to 8.299
Day 8 vs Day 10	-2.000	1.359	No	ns	-8.387 to 4.387

Parameter

Table Analyzed CC5

One-way analysis of variance

P value	0.2808
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.581
R square	0.3452

ANOVA Table	SS	df	MS
Treatment (between columns)	25.39	2	12.69
Residual (within columns)	48.17	6	8.028
Total	73.56	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-1.833	1.002	No	ns	-9.769 to 6.102
Control vs Day 10	-3.833	2.505	No	ns	-10.47 to 2.806
Day 8 vs Day 10	-2.000	1.153	No	ns	-9.528 to 5.528

Parameter

Table Analyzed CCL17

One-way analysis of variance

P value 0.3189

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1.352

R square 0.2786

ANOVA Table	SS	df	MS
Treatment (between columns)	43.35	2	21.68
Residual (within columns)	112.3	7	16.04
Total	155.6	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-4.250	1.733	No	ns	-14.46 to 5.964
Control vs Day 10	-4.250	2.123	No	ns	-12.59 to 4.089
Day 8 vs Day 10	0.0	0.0	No	ns	-10.21 to 10.21

Parameter

Table Analyzed CCL22

One-way analysis of variance

P value	0.1183
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.940
R square	0.4565

ANOVA Table	SS	df	MS
Treatment (between columns)	87.15	2	43.58
Residual (within columns)	103.8	7	14.82
Total	190.9	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-5.500	2.333	No	ns	-15.32 to 4.319
Control vs Day 10	-6.250	3.247	No	ns	-14.27 to 1.767
Day 8 vs Day 10	-0.7500	0.3181	No	ns	-10.57 to 9.069

Parameter

Table Analyzed CD83

One-way analysis of variance

P value	0.0022
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	16.70
R square	0.8267

ANOVA Table	SS	df	MS
Treatment (between columns)	75.15	2	37.58
Residual (within columns)	15.75	7	2.250
Total	90.90	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-5.250	5.715	Yes	*	-9.076 to -1.424
Control vs Day 10	-5.750	7.667	Yes	**	-8.874 to -2.626
Day 8 vs Day 10	-0.5000	0.5443	No	ns	-4.326 to 3.326

Parameter

Table Analyzed CXCL10

One-way analysis of variance

P value	0.0154
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	9.049
R square	0.7510

ANOVA Table	SS	df	MS
Treatment (between columns)	41.22	2	20.61
Residual (within columns)	13.67	6	2.278
Total	54.89	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	5.667	5.817	Yes	*	1.440 to 9.894
Control vs Day 10	1.167	1.431	No	ns	-2.370 to 4.703
Day 8 vs Day 10	-4.500	4.869	Yes	*	-8.510 to -0.4898

Parameter

Table Analyzed Ecad

One-way analysis of variance

P value	0.4489
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.9000
R square	0.2045

ANOVA Table	SS	df	MS
Treatment (between columns)	18.90	2	9.450
Residual (within columns)	73.50	7	10.50
Total	92.40	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.250	1.134	No	ns	-10.51 to 6.015
Control vs Day 10	-3.000	1.852	No	ns	-9.748 to 3.748
Day 8 vs Day 10	-0.7500	0.3780	No	ns	-9.015 to 7.515

Parameter

Table Analyzed Foxp3

One-way analysis of variance

P value	0.0128
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	8.669
R square	0.7124

ANOVA Table	SS	df	MS
Treatment (between columns)	96.60	2	48.30
Residual (within columns)	39.00	7	5.571
Total	135.6	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-6.000	4.151	No	ns	-12.02 to 0.02024
Control vs Day 10	-6.500	5.508	Yes	*	-11.42 to -1.584
Day 8 vs Day 10	-0.5000	0.3459	No	ns	-6.520 to 5.520

Parameter

Table Analyzed ICAM1

One-way analysis of variance

P value	0.5692
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.6114
R square	0.1487

ANOVA Table	SS	df	MS
Treatment (between columns)	10.35	2	5.175
Residual (within columns)	59.25	7	8.464
Total	69.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	2.750	1.544	No	ns	-4.670 to 10.17
Control vs Day 10	1.250	0.8593	No	ns	-4.809 to 7.309
Day 8 vs Day 10	-1.500	0.8419	No	ns	-8.920 to 5.920

Parameter

Table Analyzed IFNg

One-way analysis of variance

P value	0.4978
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.7853
R square	0.2075

ANOVA Table	SS	df	MS
Treatment (between columns)	10.97	2	5.486
Residual (within columns)	41.92	6	6.986
Total	52.89	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.167	1.270	No	ns	-9.570 to 5.236
Control vs Day 10	-2.417	1.693	No	ns	-8.610 to 3.777
Day 8 vs Day 10	-0.2500	0.1545	No	ns	-7.273 to 6.773

Parameter

Table Analyzed IL2

One-way analysis of variance

P value	0.0065
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	13.05
R square	0.8131

ANOVA Table	SS	df	MS
Treatment (between columns)	365.0	2	182.5
Residual (within columns)	83.92	6	13.99
Total	448.9	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-7.833	3.245	No	ns	-18.31 to 2.641
Control vs Day 10	-14.58	7.220	Yes	**	-23.35 to -5.820
Day 8 vs Day 10	-6.750	2.947	No	ns	-16.69 to 3.187

Parameter

Table Analyzed IL2Ra

One-way analysis of variance

P value	0.0026
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	15.65
R square	0.8172

ANOVA Table	SS	df	MS
Treatment (between columns)	115.2	2	57.58
Residual (within columns)	25.75	7	3.679
Total	140.9	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-7.250	6.173	Yes	**	-12.14 to -2.358
Control vs Day 10	-6.750	7.039	Yes	**	-10.74 to -2.756
Day 8 vs Day 10	0.5000	0.4257	No	ns	-4.392 to 5.392

Parameter

Table Analyzed IL-4

One-way analysis of variance

P value	0.2121
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.951
R square	0.3579

ANOVA Table	SS	df	MS
Treatment (between columns)	30.10	2	15.05
Residual (within columns)	54.00	7	7.714
Total	84.10	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-4.250	2.499	No	ns	-11.33 to 2.834
Control vs Day 10	-3.000	2.160	No	ns	-8.784 to 2.784
Day 8 vs Day 10	1.250	0.7349	No	ns	-5.834 to 8.334

Parameter

Table Analyzed IL6

One-way analysis of variance

P value 0.5173

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.7372

R square 0.1973

ANOVA Table	SS	df	MS
Treatment (between columns)	2.806	2	1.403
Residual (within columns)	11.42	6	1.903
Total	14.22	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-1.333	1.497	No	ns	-5.197 to 2.530
Control vs Day 10	-1.083	1.454	No	ns	-4.316 to 2.149
Day 8 vs Day 10	0.2500	0.2960	No	ns	-3.415 to 3.915

Parameter

Table Analyzed IL-10

One-way analysis of variance

P value 0.9725

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.0280

R square 0.007937

ANOVA Table	SS	df	MS
Treatment (between columns)	0.1500	2	0.0750
Residual (within columns)	18.75	7	2.679
Total	18.90	9	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs Day 10	-0.2500	0.3055	No	ns
Control vs Day 8	0.0	---	No	ns
Day 8 vs Day 10	-0.2500	---	No	ns

Parameter

Table Analyzed IL12p40

One-way analysis of variance

P value 0.1128

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 3.029

R square 0.4639

ANOVA Table	SS	df	MS
Treatment (between columns)	70.75	2	35.38
Residual (within columns)	81.75	7	11.68
Total	152.5	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-5.750	2.748	No	ns	-14.47 to 2.966
Control vs Day 10	-5.250	3.073	No	ns	-12.37 to 1.867
Day 8 vs Day 10	0.5000	0.2389	No	ns	-8.216 to 9.216

Parameter

Table Analyzed IL12p40

One-way analysis of variance

P value 0.1128

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 3.029

R square 0.4639

ANOVA Table	SS	df	MS
Treatment (between columns)	70.75	2	35.38
Residual (within columns)	81.75	7	11.68
Total	152.5	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-5.750	2.748	No	ns	-14.47 to 2.966
Control vs Day 10	-5.250	3.073	No	ns	-12.37 to 1.867
Day 8 vs Day 10	0.5000	0.2389	No	ns	-8.216 to 9.216

Parameter

Table Analyzed IL21

One-way analysis of variance

P value 0.0125

P value summary *

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 8.742

R square 0.7141

ANOVA Table	SS	df	MS
Treatment (between columns)	141.8	2	70.88
Residual (within columns)	56.75	7	8.107
Total	198.5	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-6.000	3.441	No	ns	-13.26 to 1.262
Control vs Day 10	-8.250	5.795	Yes	*	-14.18 to -2.320
Day 8 vs Day 10	-2.250	1.290	No	ns	-9.512 to 5.012

Parameter

Table Analyzed iNOS

One-way analysis of variance

P value	0.7531
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.2953
R square	0.07782

ANOVA Table	SS	df	MS
Treatment (between columns)	6.350	2	3.175
Residual (within columns)	75.25	7	10.75
Total	81.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-0.5000	0.2490	No	ns	-8.862 to 7.862
Control vs Day 10	-1.750	1.067	No	ns	-8.578 to 5.078
Day 8 vs Day 10	-1.250	0.6226	No	ns	-9.612 to 7.112

Parameter

Table Analyzed IRF1

One-way analysis of variance

P value	0.1644
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.363
R square	0.4030

ANOVA Table	SS	df	MS
Treatment (between columns)	18.90	2	9.450
Residual (within columns)	28.00	7	4.000
Total	46.90	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	3.250	2.654	No	ns	-1.851 to 8.351
Control vs Day 10	2.500	2.500	No	ns	-1.665 to 6.665
Day 8 vs Day 10	-0.7500	0.6124	No	ns	-5.851 to 4.351

Parameter

Table Analyzed IRF2

One-way analysis of variance

P value	0.0003
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	42.54
R square	0.9341

ANOVA Table	SS	df	MS
Treatment (between columns)	30.72	2	15.36
Residual (within columns)	2.167	6	0.3611
Total	32.89	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-3.167	8.164	Yes	**	-4.850 to -1.484
Control vs Day 10	-4.167	12.84	Yes	***	-5.575 to -2.759
Day 8 vs Day 10	-1.000	2.717	No	ns	-2.597 to 0.5967

Parameter

Table Analyzed JAM

One-way analysis of variance

P value	0.0080
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	10.38
R square	0.7479

ANOVA Table	SS	df	MS
Treatment (between columns)	35.60	2	17.80
Residual (within columns)	12.00	7	1.714
Total	47.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-3.500	4.365	Yes	*	-6.839 to -0.1606
Control vs Day 10	-4.000	6.110	Yes	**	-6.727 to -1.273
Day 8 vs Day 10	-0.5000	0.6236	No	ns	-3.839 to 2.839

Parameter

Table Analyzed MHC-IIa

One-way analysis of variance

P value	0.0970
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.316
R square	0.4865

ANOVA Table	SS	df	MS
Treatment (between columns)	52.35	2	26.18
Residual (within columns)	55.25	7	7.893
Total	107.6	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	4.500	2.616	No	ns	-2.666 to 11.67
Control vs Day 10	4.750	3.381	No	ns	-1.101 to 10.60
Day 8 vs Day 10	0.2500	0.1453	No	ns	-6.916 to 7.416

Parameter

Table Analyzed MM2

One-way analysis of variance

P value	0.2006
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.038
R square	0.3680

ANOVA Table	SS	df	MS
Treatment (between columns)	23.15	2	11.58
Residual (within columns)	39.75	7	5.679
Total	62.90	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	2.750	1.885	No	ns	-3.328 to 8.828
Control vs Day 10	3.250	2.728	No	ns	-1.713 to 8.213
Day 8 vs Day 10	0.5000	0.3426	No	ns	-5.578 to 6.578

Parameter

Table Analyzed Mx2

One-way analysis of variance

P value 0.5382

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.6777

R square 0.1622

ANOVA Table	SS	df	MS
Treatment (between columns)	12.15	2	6.075
Residual (within columns)	62.75	7	8.964
Total	74.90	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	2.250	1.227	No	ns	-5.386 to 9.886
Control vs Day 10	2.250	1.503	No	ns	-3.985 to 8.485
Day 8 vs Day 10	0.0	0.0	No	ns	-7.636 to 7.636

Parameter

Table Analyzed NOS2

One-way analysis of variance

P value	0.3525
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.247
R square	0.2936

ANOVA Table	SS	df	MS
Treatment (between columns)	23.56	2	11.78
Residual (within columns)	56.67	6	9.444
Total	80.22	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.667	1.344	No	ns	-11.27 to 5.941
Control vs Day 10	-3.667	2.209	No	ns	-10.87 to 3.535
Day 8 vs Day 10	-1.000	0.5314	No	ns	-9.166 to 7.166

Parameter

Table Analyzed p27

One-way analysis of variance

P value	0.2879
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.543
R square	0.3397

ANOVA Table	SS	df	MS
Treatment (between columns)	31.25	2	15.63
Residual (within columns)	60.75	6	10.13
Total	92.00	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	5.000	2.434	No	ns	-3.912 to 13.91
Control vs Day 10	2.750	1.600	No	ns	-4.706 to 10.21
Day 8 vs Day 10	-2.250	1.155	No	ns	-10.70 to 6.205

Parameter

Table Analyzed p75

One-way analysis of variance

P value	0.8483
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.1684
R square	0.04591

ANOVA Table	SS	df	MS
Treatment (between columns)	4.150	2	2.075
Residual (within columns)	86.25	7	12.32
Total	90.40	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	1.750	0.8141	No	ns	-7.203 to 10.70
Control vs Day 10	0.7500	0.4273	No	ns	-6.560 to 8.060
Day 8 vs Day 10	-1.000	0.4652	No	ns	-9.953 to 7.953

Parameter

Table Analyzed PECAM

One-way analysis of variance

P value	0.2419
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.750
R square	0.3333

ANOVA Table	SS	df	MS
Treatment (between columns)	12.00	2	6.000
Residual (within columns)	24.00	7	3.429
Total	36.00	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	3.000	2.646	No	ns	-1.723 to 7.723
Control vs Day 10	1.000	1.080	No	ns	-2.856 to 4.856
Day 8 vs Day 10	-2.000	1.764	No	ns	-6.723 to 2.723

Parameter

Table Analyzed Stat1

One-way analysis of variance

P value	0.0060
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	13.52
R square	0.8185

ANOVA Table	SS	df	MS
Treatment (between columns)	45.83	2	22.92
Residual (within columns)	10.17	6	1.694
Total	56.00	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	5.833	6.942	Yes	**	2.187 to 9.479
Control vs Day 10	3.833	5.453	Yes	*	0.7830 to 6.884
Day 8 vs Day 10	-2.000	2.509	No	ns	-5.459 to 1.459

Parameter

Table Analyzed Stat1b

One-way analysis of variance

P value	0.0288
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.142
R square	0.6370

ANOVA Table	SS	df	MS
Treatment (between columns)	58.35	2	29.18
Residual (within columns)	33.25	7	4.750
Total	91.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	5.250	3.934	No	ns	-0.3088 to 10.81
Control vs Day 10	4.750	4.359	Yes	*	0.2113 to 9.289
Day 8 vs Day 10	-0.5000	0.3746	No	ns	-6.059 to 5.059

Parameter

Table Analyzed Stat2

One-way analysis of variance

P value	0.8121
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.2156
R square	0.06704

ANOVA Table	SS	df	MS
Treatment (between columns)	4.306	2	2.153
Residual (within columns)	59.92	6	9.986
Total	64.22	8	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	0.8333	0.4085	No	ns	-8.017 to 9.684
Control vs Day 10	-0.9167	0.5371	No	ns	-8.322 to 6.488
Day 8 vs Day 10	-1.750	0.9043	No	ns	-10.15 to 6.647

Parameter

Table Analyzed TGFbeta

One-way analysis of variance

P value	0.8814
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.1286
R square	0.03543

ANOVA Table	SS	df	MS
Treatment (between columns)	3.150	2	1.575
Residual (within columns)	85.75	7	12.25
Total	88.90	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-0.7500	0.3499	No	ns	-9.677 to 8.177
Control vs Day 10	-1.250	0.7143	No	ns	-8.539 to 6.039
Day 8 vs Day 10	-0.5000	0.2333	No	ns	-9.427 to 8.427

Parameter

Table Analyzed TGFbeta2

One-way analysis of variance

P value	0.0046
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	15.00
R square	0.8333

ANOVA Table	SS	df	MS
Treatment (between columns)	33.33	2	16.67
Residual (within columns)	6.667	6	1.111
Total	40.00	8	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Control vs Day 10	-4.333	7.612	Yes	**
Control vs Day 8	-3.333	4.899	Yes	*
Day 8 vs Day 10	-1.000	1.549	No	ns

Parameter

Table Analyzed TGFbeta3

One-way analysis of variance

P value	0.3481
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.232
R square	0.2603

ANOVA Table	SS	df	MS
Treatment (between columns)	11.35	2	5.675
Residual (within columns)	32.25	7	4.607
Total	43.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.000	1.522	No	ns	-7.475 to 3.475
Control vs Day 10	-2.250	2.097	No	ns	-6.720 to 2.220
Day 8 vs Day 10	-0.2500	0.1902	No	ns	-5.725 to 5.225

Parameter

Table Analyzed TGFbetaR1

One-way analysis of variance

P value	0.3481
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.232
R square	0.2603

ANOVA Table	SS	df	MS
Treatment (between columns)	11.35	2	5.675
Residual (within columns)	32.25	7	4.607
Total	43.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.000	1.522	No	ns	-7.475 to 3.475
Control vs Day 10	-2.250	2.097	No	ns	-6.720 to 2.220
Day 8 vs Day 10	-0.2500	0.1902	No	ns	-5.725 to 5.225

Parameter

Table Analyzed TIMM2

One-way analysis of variance

P value	0.9128
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.09245
R square	0.02574

ANOVA Table	SS	df	MS
Treatment (between columns)	1.400	2	0.7000
Residual (within columns)	53.00	7	7.571
Total	54.40	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	1.000	0.5935	No	ns	-6.018 to 8.018
Control vs Day 10	0.5000	0.3634	No	ns	-5.230 to 6.230
Day 8 vs Day 10	-0.5000	0.2967	No	ns	-7.518 to 6.518

Parameter

Table Analyzed TJP2

One-way analysis of variance

P value	0.4352
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.9392
R square	0.2116

ANOVA Table	SS	df	MS
Treatment (between columns)	16.10	2	8.050
Residual (within columns)	60.00	7	8.571
Total	76.10	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	1.250	0.6972	No	ns	-6.217 to 8.717
Control vs Day 10	-2.000	1.366	No	ns	-8.097 to 4.097
Day 8 vs Day 10	-3.250	1.813	No	ns	-10.72 to 4.217

Parameter

Table Analyzed TNF

One-way analysis of variance

P value 0.5151

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.7304

R square 0.1727

ANOVA Table	SS	df	MS
Treatment (between columns)	9.600	2	4.800
Residual (within columns)	46.00	7	6.571
Total	55.60	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	-2.000	1.274	No	ns	-8.538 to 4.538
Control vs Day 10	-2.000	1.560	No	ns	-7.338 to 3.338
Day 8 vs Day 10	0.0	0.0	No	ns	-6.538 to 6.538

Parameter

Table Analyzed VEGF

One-way analysis of variance

P value	0.5195
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.7201
R square	0.1706

ANOVA Table	SS	df	MS
Treatment (between columns)	14.35	2	7.175
Residual (within columns)	69.75	7	9.964
Total	84.10	9	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs Day 8	3.250	1.681	No	ns	-4.801 to 11.30
Control vs Day 10	0.7500	0.4752	No	ns	-5.824 to 7.324
Day 8 vs Day 10	-2.500	1.293	No	ns	-10.55 to 5.551

Study L005 Spleen ANOVA Tables from qPCR

Parameter

Table Analyzed Bax

One-way analysis of variance

P value 0.9432

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.05882

R square 0.01290

ANOVA Table	SS	df	MS
Treatment (between columns)	0.1667	2	0.08333
Residual (within columns)	12.75	9	1.417
Total	12.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Bax vs Column B	0.0	0.0	No	ns	-2.350 to 2.350
Bax vs Column C	0.2500	0.4201	No	ns	-2.100 to 2.600
Column B vs Column C	0.2500	0.4201	No	ns	-2.100 to 2.600

Parameter

Table Analyzed Bcl2

One-way analysis of variance

P value	0.3452
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.200
R square	0.2105

ANOVA Table	SS	df	MS
Treatment (between columns)	2.667	2	1.333
Residual (within columns)	10.00	9	1.111
Total	12.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Bcl2 vs Column B	0.0	0.0	No	ns	-2.081 to 2.081
Bcl2 vs Column C	1.000	1.897	No	ns	-1.081 to 3.081
Column B vs Column C	1.000	1.897	No	ns	-1.081 to 3.081

Parameter

Table Analyzed CC3d

One-way analysis of variance

P value 0.8446

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.1721

R square 0.03684

ANOVA Table	SS	df	MS
Treatment (between columns)	1.167	2	0.5833
Residual (within columns)	30.50	9	3.389
Total	31.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CC3d vs 8	-0.2500	0.2716	No	ns	-3.885 to 3.385
CC3d vs Column C	0.5000	0.5432	No	ns	-3.135 to 4.135
8 vs Column C	0.7500	0.8148	No	ns	-2.885 to 4.385

Parameter

Table Analyzed CC5

One-way analysis of variance

P value	0.0703
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.617
R square	0.4456

ANOVA Table	SS	df	MS
Treatment (between columns)	21.50	2	10.75
Residual (within columns)	26.75	9	2.972
Total	48.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CC5 vs Column B	1.250	1.450	No	ns	-2.154 to 4.654
CC5 vs Column C	3.250	3.770	No	ns	-0.1541 to 6.654
Column B vs Column C	2.000	2.320	No	ns	-1.404 to 5.404

Parameter

Table Analyzed CCL17

One-way analysis of variance

P value	0.2571
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.586
R square	0.2606

ANOVA Table	SS	df	MS
Treatment (between columns)	6.167	2	3.083
Residual (within columns)	17.50	9	1.944
Total	23.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CCL17 vs Column B	0.7500	1.076	No	ns	-2.003 to 3.503
CCL17 vs Column C	1.750	2.510	No	ns	-1.003 to 4.503
Column B vs Column C	1.000	1.434	No	ns	-1.753 to 3.753

Parameter

Table Analyzed CCL20

One-way analysis of variance

P value	0.3798
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.080
R square	0.1935

ANOVA Table	SS	df	MS
Treatment (between columns)	10.50	2	5.250
Residual (within columns)	43.75	9	4.861
Total	54.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CCL20 vs Column B	-0.7500	0.6803	No	ns	-5.103 to 3.603
CCL20 vs Column C	1.500	1.361	No	ns	-2.853 to 5.853
Column B vs Column C	2.250	2.041	No	ns	-2.103 to 6.603

Parameter

Table Analyzed CCL22

One-way analysis of variance

P value 0.3227

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1.286

R square 0.2222

ANOVA Table	SS	df	MS
Treatment (between columns)	4.500	2	2.250
Residual (within columns)	15.75	9	1.750
Total	20.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CCL22 vs 8	0.7500	1.134	No	ns	-1.862 to 3.362
CCL22 vs Column C	1.500	2.268	No	ns	-1.112 to 4.112
8 vs Column C	0.7500	1.134	No	ns	-1.862 to 3.362

Parameter

Table Analyzed CD83

One-way analysis of variance

P value	0.0262
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	5.609
R square	0.5548

ANOVA Table	SS	df	MS
Treatment (between columns)	7.167	2	3.583
Residual (within columns)	5.750	9	0.6389
Total	12.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CD83 vs 8	0.2500	0.6255	No	ns	-1.328 to 1.828
CD83 vs Column C	1.750	4.379	Yes	*	0.1718 to 3.328
8 vs Column C	1.500	3.753	No	ns	-0.07823 to 3.078

Parameter

Table Analyzed CXCL10

One-way analysis of variance

P value	0.0161
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.763
R square	0.6005

ANOVA Table	SS	df	MS
Treatment (between columns)	22.17	2	11.08
Residual (within columns)	14.75	9	1.639
Total	36.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CXCL10 vs 8	3.000	4.687	Yes	*	0.4723 to 5.528
CXCL10 vs Column C	2.750	4.296	Yes	*	0.2223 to 5.278
8 vs Column C	-0.2500	0.3906	No	ns	-2.778 to 2.278

Parameter

Table Analyzed Ecad

One-way analysis of variance

P value	0.4053
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.000
R square	0.1818

ANOVA Table	SS	df	MS
Treatment (between columns)	4.167	2	2.083
Residual (within columns)	18.75	9	2.083
Total	22.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Ecad vs Column B	-1.250	1.732	No	ns	-4.100 to 1.600
Ecad vs Column C	0.0	0.0	No	ns	-2.850 to 2.850
Column B vs Column C	1.250	1.732	No	ns	-1.600 to 4.100

Parameter

Table Analyzed Eif2ak2

One-way analysis of variance

P value	0.0111
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	7.737
R square	0.6323

ANOVA Table	SS	df	MS
Treatment (between columns)	32.67	2	16.33
Residual (within columns)	19.00	9	2.111
Total	51.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Eif2ak2 vs 8	2.500	3.441	No	ns	-0.3689 to 5.369
Eif2ak2 vs Column C	4.000	5.506	Yes	**	1.131 to 6.869
8 vs Column C	1.500	2.065	No	ns	-1.369 to 4.369

Parameter

Table Analyzed Foxp3

One-way analysis of variance

P value 0.1850

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 2.048

R square 0.3127

ANOVA Table	SS	df	MS
Treatment (between columns)	7.167	2	3.583
Residual (within columns)	15.75	9	1.750
Total	22.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Foxp3 vs Column D	1.750	2.646	No	ns	-0.8620 to 4.362
Foxp3 vs Column E	1.500	2.268	No	ns	-1.112 to 4.112
Column D vs Column E	-0.2500	0.3780	No	ns	-2.862 to 2.362

Parameter

Table Analyzed ICAM1

One-way analysis of variance

P value	0.3680
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.119
R square	0.1992

ANOVA Table	SS	df	MS
Treatment (between columns)	4.167	2	2.083
Residual (within columns)	16.75	9	1.861
Total	20.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
ICAM1 vs 8	0.0	0.0	No	ns	-2.694 to 2.694
ICAM1 vs Column C	1.250	1.833	No	ns	-1.444 to 3.944
8 vs Column C	1.250	1.833	No	ns	-1.444 to 3.944

Parameter

Table Analyzed IFNgamma

One-way analysis of variance

P value	0.0909
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.167
R square	0.4130

ANOVA Table	SS	df	MS
Treatment (between columns)	12.67	2	6.333
Residual (within columns)	18.00	9	2.000
Total	30.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IFNg vs 8	1.000	1.414	No	ns	-1.792 to 3.792
IFNg vs Column C	2.500	3.536	No	ns	-0.2924 to 5.292
8 vs Column C	1.500	2.121	No	ns	-1.292 to 4.292

Parameter

Table Analyzed IL1b

One-way analysis of variance

P value	0.0909
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.167
R square	0.4130

ANOVA Table	SS	df	MS
Treatment (between columns)	9.500	2	4.750
Residual (within columns)	13.50	9	1.500
Total	23.00	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL-1ß vs Column B	-0.2500	0.4082	No	ns	-2.668 to 2.168
IL-1ß vs Column C	1.750	2.858	No	ns	-0.6683 to 4.168
Column B vs Column C	2.000	3.266	No	ns	-0.4183 to 4.418

Parameter

Table Analyzed IL-2

One-way analysis of variance

P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	110.0
R square	0.9607

ANOVA Table	SS	df	MS
Treatment (between columns)	287.2	2	143.6
Residual (within columns)	11.75	9	1.306
Total	298.9	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL-2 vs Column C	0.2500	0.4376	No	ns	-2.006 to 2.506
IL-2 vs Column D	10.50	18.38	Yes	***	8.244 to 12.76
Column C vs Column D	10.25	17.94	Yes	***	7.994 to 12.51

Parameter

Table Analyzed IL-2a

One-way analysis of variance

P value	0.0214
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.077
R square	0.5745

ANOVA Table	SS	df	MS
Treatment (between columns)	13.17	2	6.583
Residual (within columns)	9.750	9	1.083
Total	22.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL2Ra vs Column D	0.7500	1.441	No	ns	-1.305 to 2.805
IL2Ra vs Column E	2.500	4.804	Yes	*	0.4449 to 4.555
Column D vs Column E	1.750	3.363	No	ns	-0.3051 to 3.805

Parameter

Table Analyzed IL-6

One-way analysis of variance

P value	0.0090
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	8.314
R square	0.6488

ANOVA Table	SS	df	MS
Treatment (between columns)	16.17	2	8.083
Residual (within columns)	8.750	9	0.9722
Total	24.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL-6 vs Column B	0.7500	1.521	No	ns	-1.197 to 2.697
IL-6 vs Column C	2.750	5.578	Yes	**	0.8031 to 4.697
Column B vs Column C	2.000	4.057	Yes	*	0.05312 to 3.947

Parameter

Table Analyzed IL-6ST

One-way analysis of variance

P value	0.3065
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.800
R square	0.5455

ANOVA Table	SS	df	MS
Treatment (between columns)	12.00	2	6.000
Residual (within columns)	10.00	3	3.333
Total	22.00	5	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL6ST vs Column B	0.0	0.0	No	ns	-7.630 to 7.630
IL6ST vs Column C	3.000	2.324	No	ns	-4.630 to 10.63
Column B vs Column C	3.000	2.324	No	ns	-4.630 to 10.63

Parameter

Table Analyzed IL-10

One-way analysis of variance

P value 0.0797

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 3.395

R square 0.4300

ANOVA Table	SS	df	MS
Treatment (between columns)	7.167	2	3.583
Residual (within columns)	9.500	9	1.056
Total	16.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL10 vs 8	1.750	3.407	No	ns	-0.2786 to 3.779
IL10 vs Column C	1.500	2.920	No	ns	-0.5286 to 3.529
8 vs Column C	-0.2500	0.4867	No	ns	-2.279 to 1.779

Parameter

Table Analyzed IL-12p40

One-way analysis of variance

P value	0.0053
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	9.916
R square	0.6878

ANOVA Table	SS	df	MS
Treatment (between columns)	72.17	2	36.08
Residual (within columns)	32.75	9	3.639
Total	104.9	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Il12p40 vs Column B	-2.750	2.883	No	ns	-6.517 to 1.017
Il12p40 vs Column C	3.250	3.407	No	ns	-0.5165 to 7.017
Column B vs Column C	6.000	6.291	Yes	**	2.233 to 9.767

Parameter

Table Analyzed IL-21

One-way analysis of variance

P value 0.1004

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 3.000

R square 0.4000

ANOVA Table	SS	df	MS
Treatment (between columns)	13.17	2	6.583
Residual (within columns)	19.75	9	2.194
Total	32.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IL21 vs Column B	0.7500	1.013	No	ns	-2.175 to 3.675
IL21 vs Column C	2.500	3.375	No	ns	-0.4250 to 5.425
Column B vs Column C	1.750	2.363	No	ns	-1.175 to 4.675

Parameter

Table Analyzed iNOS

One-way analysis of variance

P value 0.6024

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.5364

R square 0.1065

ANOVA Table	SS	df	MS
Treatment (between columns)	4.500	2	2.250
Residual (within columns)	37.75	9	4.194
Total	42.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
iNOS vs 8	-0.7500	0.7324	No	ns	-4.794 to 3.294
iNOS vs Column C	0.7500	0.7324	No	ns	-3.294 to 4.794
8 vs Column C	1.500	1.465	No	ns	-2.544 to 5.544

Parameter

Table Analyzed IRF1

One-way analysis of variance

P value	0.6263
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.4932
R square	0.09877

ANOVA Table	SS	df	MS
Treatment (between columns)	2.000	2	1.000
Residual (within columns)	18.25	9	2.028
Total	20.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IRF1 vs 8	0.5000	0.7022	No	ns	-2.312 to 3.312
IRF1 vs Column C	-0.5000	0.7022	No	ns	-3.312 to 2.312
8 vs Column C	-1.000	1.404	No	ns	-3.812 to 1.812

Parameter

Table Analyzed IRF2

One-way analysis of variance

P value	0.4907
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.7714
R square	0.1463

ANOVA Table	SS	df	MS
Treatment (between columns)	1.500	2	0.7500
Residual (within columns)	8.750	9	0.9722
Total	10.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
IRF2 vs 8	0.0	0.0	No	ns	-1.947 to 1.947
IRF2 vs Column C	0.7500	1.521	No	ns	-1.197 to 2.697
8 vs Column C	0.7500	1.521	No	ns	-1.197 to 2.697

Parameter

Table Analyzed JAM

One-way analysis of variance

P value	0.4385
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.9048
R square	0.1674

ANOVA Table	SS	df	MS
Treatment (between columns)	3.167	2	1.583
Residual (within columns)	15.75	9	1.750
Total	18.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
JAM vs Column B	0.5000	0.7559	No	ns	-2.112 to 3.112
JAM vs Column C	1.250	1.890	No	ns	-1.362 to 3.862
Column B vs Column C	0.7500	1.134	No	ns	-1.862 to 3.362

Parameter

Table Analyzed MHC-IIa

One-way analysis of variance

P value	0.4629
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.8400
R square	0.1573

ANOVA Table	SS	df	MS
Treatment (between columns)	3.500	2	1.750
Residual (within columns)	18.75	9	2.083
Total	22.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MHC-IIa vs Column B	-0.2500	0.3464	No	ns	-3.100 to 2.600
MHC-IIa vs Column C	1.000	1.386	No	ns	-1.850 to 3.850
Column B vs Column C	1.250	1.732	No	ns	-1.600 to 4.100

Parameter

Table Analyzed MM2

One-way analysis of variance

P value	0.1020
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.077
R square	0.4348

ANOVA Table	SS	df	MS
Treatment (between columns)	12.89	2	6.443
Residual (within columns)	16.75	8	2.094
Total	29.64	10	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
MM2 vs Column B	-2.250	3.110	No	ns	-5.174 to 0.6736
MM2 vs Column C	0.0	0.0	No	ns	-3.158 to 3.158
Column B vs Column C	2.250	2.879	No	ns	-0.9079 to 5.408

Parameter

Table Analyzed Mx2

One-way analysis of variance

P value	0.0023
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	12.90
R square	0.7414

ANOVA Table	SS	df	MS
Treatment (between columns)	50.17	2	25.08
Residual (within columns)	17.50	9	1.944
Total	67.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Mx2 vs 8	4.750	6.813	Yes	**	1.997 to 7.503
Mx2 vs Column C	3.750	5.379	Yes	*	0.9967 to 6.503
8 vs Column C	-1.000	1.434	No	ns	-3.753 to 1.753

Parameter

Table Analyzed NOS2

One-way analysis of variance

P value	0.0977
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.045
R square	0.4036

ANOVA Table	SS	df	MS
Treatment (between columns)	11.17	2	5.583
Residual (within columns)	16.50	9	1.833
Total	27.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
NOS2 vs 8	0.5000	0.7385	No	ns	-2.173 to 3.173
NOS2 vs Column C	2.250	3.323	No	ns	-0.4235 to 4.923
8 vs Column C	1.750	2.585	No	ns	-0.9235 to 4.423

Parameter

Table Analyzed p27

One-way analysis of variance

P value < 0.0001

P value summary ****

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 88.20

R square 0.9515

ANOVA Table	SS	df	MS
Treatment (between columns)	98.00	2	49.00
Residual (within columns)	5.000	9	0.5556
Total	103.0	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
P27 vs 8	5.500	14.76	Yes	***	4.028 to 6.972
P27 vs Column C	6.500	17.44	Yes	***	5.028 to 7.972
8 vs Column C	1.000	2.683	No	ns	-0.4717 to 2.472

Parameter

Table Analyzed p75

One-way analysis of variance

P value 0.6338

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.4800

R square 0.09639

ANOVA Table	SS	df	MS
Treatment (between columns)	0.6667	2	0.3333
Residual (within columns)	6.250	9	0.6944
Total	6.917	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
p75 vs 8	0.0	0.0	No	ns	-1.645 to 1.645
p75 vs Column C	0.5000	1.200	No	ns	-1.145 to 2.145
8 vs Column C	0.5000	1.200	No	ns	-1.145 to 2.145

Parameter

Table Analyzed PECAM

One-way analysis of variance

P value	0.7479
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.3000
R square	0.0625

ANOVA Table	SS	df	MS
Treatment (between columns)	1.500	2	0.7500
Residual (within columns)	22.50	9	2.500
Total	24.00	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
PECAM vs Column B	-0.7500	0.9487	No	ns	-3.872 to 2.372
PECAM vs Column C	-0.7500	0.9487	No	ns	-3.872 to 2.372
Column B vs Column C	0.0	0.0	No	ns	-3.122 to 3.122

Parameter

Table Analyzed STAT1

One-way analysis of variance

P value 0.0261

P value summary *

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 5.618

R square 0.5553

ANOVA Table	SS	df	MS
Treatment (between columns)	17.17	2	8.583
Residual (within columns)	13.75	9	1.528
Total	30.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
STAT1 vs 8	2.250	3.641	No	ns	-0.1905 to 4.691
STAT1 vs Column C	2.750	4.450	Yes	*	0.3095 to 5.191
8 vs Column C	0.5000	0.8090	No	ns	-1.941 to 2.941

Parameter

Table Analyzed STAT1b

One-way analysis of variance

P value	0.2077
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.881
R square	0.2948

ANOVA Table	SS	df	MS
Treatment (between columns)	6.167	2	3.083
Residual (within columns)	14.75	9	1.639
Total	20.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
STAT1b vs 8	1.750	2.734	No	ns	-0.7777 to 4.278
STAT1b vs Column C	1.000	1.562	No	ns	-1.528 to 3.528
8 vs Column C	-0.7500	1.172	No	ns	-3.278 to 1.778

Parameter

Table Analyzed STAT2

One-way analysis of variance

P value	0.3695
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.114
R square	0.1985

ANOVA Table	SS	df	MS
Treatment (between columns)	2.167	2	1.083
Residual (within columns)	8.750	9	0.9722
Total	10.92	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
STAT2 vs 8	0.7500	1.521	No	ns	-1.197 to 2.697
STAT2 vs Column C	1.000	2.028	No	ns	-0.9469 to 2.947
8 vs Column C	0.2500	0.5071	No	ns	-1.697 to 2.197

Parameter

Table Analyzed TGFb1

One-way analysis of variance

P value	0.0638
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.794
R square	0.4574

ANOVA Table	SS	df	MS
Treatment (between columns)	7.167	2	3.583
Residual (within columns)	8.500	9	0.9444
Total	15.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFß vs Column B	0.2500	0.5145	No	ns	-1.669 to 2.169
TGFß vs Column C	1.750	3.601	No	ns	-0.1689 to 3.669
Column B vs Column C	1.500	3.087	No	ns	-0.4189 to 3.419

Parameter

Table Analyzed TGFb2

One-way analysis of variance

P value	0.2065
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.890
R square	0.2957

ANOVA Table	SS	df	MS
Treatment (between columns)	16.17	2	8.083
Residual (within columns)	38.50	9	4.278
Total	54.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFß2 vs Column B	-0.7500	0.7252	No	ns	-4.834 to 3.334
TGFß2 vs Column C	-2.750	2.659	No	ns	-6.834 to 1.334
Column B vs Column C	-2.000	1.934	No	ns	-6.084 to 2.084

Parameter

Table Analyzed TGFb3

One-way analysis of variance

P value	0.8936
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.1139
R square	0.02469

ANOVA Table	SS	df	MS
Treatment (between columns)	0.5000	2	0.2500
Residual (within columns)	19.75	9	2.194
Total	20.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFß3 vs Column B	0.2500	0.3375	No	ns	-2.675 to 3.175
TGFß3 vs Column C	0.5000	0.6751	No	ns	-2.425 to 3.425
Column B vs Column C	0.2500	0.3375	No	ns	-2.675 to 3.175

Parameter

Table Analyzed TGFR1

One-way analysis of variance

P value	0.1663
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	2.204
R square	0.3288

ANOVA Table	SS	df	MS
Treatment (between columns)	6.000	2	3.000
Residual (within columns)	12.25	9	1.361
Total	18.25	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TGFR1 vs Column B	0.0	0.0	No	ns	-2.304 to 2.304
TGFR1 vs Column C	1.500	2.571	No	ns	-0.8036 to 3.804
Column B vs Column C	1.500	2.571	No	ns	-0.8036 to 3.804

Parameter

Table Analyzed TIMM2

One-way analysis of variance

P value	0.9421
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0.06000
R square	0.01316

ANOVA Table	SS	df	MS
Treatment (between columns)	0.1667	2	0.08333
Residual (within columns)	12.50	9	1.389
Total	12.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TIMM2 vs Column B	-0.2500	0.4243	No	ns	-2.577 to 2.077
TIMM2 vs Column C	-0.2500	0.4243	No	ns	-2.577 to 2.077
Column B vs Column C	0.0	0.0	No	ns	-2.327 to 2.327

Parameter

Table Analyzed TJP2

One-way analysis of variance

P value	0.0572
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	4.000
R square	0.4706

ANOVA Table	SS	df	MS
Treatment (between columns)	10.67	2	5.333
Residual (within columns)	12.00	9	1.333
Total	22.67	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TJP2 vs Column B	0.0	0.0	No	ns	-2.280 to 2.280
TJP2 vs Column C	2.000	3.464	No	ns	-0.2800 to 4.280
Column B vs Column C	2.000	3.464	No	ns	-0.2800 to 4.280

Parameter

Table Analyzed TNF

One-way analysis of variance

P value	0.0679
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	3.682
R square	0.4500

ANOVA Table	SS	df	MS
Treatment (between columns)	4.500	2	2.250
Residual (within columns)	5.500	9	0.6111
Total	10.00	11	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
TNF vs 8	-0.7500	1.919	No	ns	-2.294 to 0.7935
TNF vs Column C	0.7500	1.919	No	ns	-0.7935 to 2.294
8 vs Column C	1.500	3.838	No	ns	-0.04354 to 3.044

Parameter

Table Analyzed VEGF

One-way analysis of variance

P value	0.3890
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	1.065
R square	0.2103

ANOVA Table	SS	df	MS
Treatment (between columns)	4.970	2	2.485
Residual (within columns)	18.67	8	2.333
Total	23.64	10	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
VEGF vs Column B	-1.000	1.309	No	ns	-4.086 to 2.086
VEGF vs Column C	0.6667	0.8081	No	ns	-2.667 to 4.000
Column B vs Column C	1.667	2.020	No	ns	-1.667 to 5.000