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Expression of Inflamatory Response Genes in Ferrets Challenged with H5N1 Avian Influenza Virus

Brock M. Miniard
Wright State University

EXPRESSION OF INFLAMATORY RESPONSE GENES IN FERRETS CHALLENGED WITH H5N1 AVIAN INFLUENZA VIRUS

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Master of Science

By

BROCK MICHAEL MINIARD
B.A., Ohio Wesleyan University, 2002

Wright State University
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WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Brock Michael Miniard ENTITLED Expression of Inflammatory Response Genes in Ferrets Challenged with H5N1 Avian Influenza Virus BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

Mill Miller, Ph.D.
Thesis Director

David Goldstein, Ph.D., Chair
Department of Biological Sciences

Committee on
Final Examination

Mill Miller, Ph.D.

Paula Bubulya, Ph.D.

Carol Sabourin, Ph.D.

Andrew T. Hsu, Ph.D.
Dean, School of Graduate Studies

ABSTRACT

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Expression of Inflammatory Response Genes in Ferrets Challenged with H5N1 Avian Influenza Virus

Influenza A H5N1 has emerged as a potential to be the next pandemic influenza. Ferrets are a promising model of H5N1 infection because the disease progression is similar to that known in humans, however reagents to characterize infections in ferrets are few. We developed real-time PCR assays for the ferret cytokines IL-1β, IL-2, IL-4, IL-8, IL-12p40, TNF-α, IFN-α, IFN-β, IFN-γ, TGFB-β1, and TGFB-β2. Forty-eight ferrets were vaccinated with either one of three vaccine formulations (Vaccine 1, Vaccine 2, or Vaccine 3), or saline (0.9% NaCl), then challenged intranasally with a lethal dose of H5N1 strain A/Vietnam/1203/04. Cytokine assays were then performed on blood drawn pre-vaccination, 0, 3, 4, and 14 days post infection, and brain, lung, and liver samples from ferrets euthanized either 4 or 14 days post infection or when found moribund. Ferrets vaccinated with Vaccine 1 showed an increase in IL-2, IL-6, IL-12p40, and IFN-β, and a decrease in TNF-α in the blood, an increase in IL-1β and TNF-α in the brain, an increase of IFN-β and IFN-γ in the lung, and an increase of IFN-γ in the liver during the course of infection. Ferrets vaccinated with Vaccine 2 showed an increase in IL-2, IL-12p40, IFN-α, IFN-β, IFN-γ, and TGFB-β2, and a decrease in TNF-α in the blood, an increase of IL-1β in the brain, an increase of IFN-β and IFN-γ in the lung, and little

variance of cytokine levels in the liver during the course of infection. Ferrets vaccinated with Vaccine 3 showed a decrease in IL-2, IL-6, IL-8, IL-12p40, and TNF-α in the blood, little variance of cytokine levels in the brain and liver, and an increase in IL-4 in the lung during the course of infection. Ferrets with the saline control showed an increase in IL-2, and a decrease in IL-12p40, TNF-α and IFN-β in the blood, an increase of IL-1β, IL-8 and TNF-α in the brain, an increase of IFN-β and IFN-γ in the lung, and an increase of TNF-α and IFN-γ in the liver during the course of infection. Vaccine 3 conferred total protection, as indicated by 100% survival of the ferrets in that group. Vaccines 1 and 2 conferred some protection, with 22% and 33% of ferrets, respectively surviving. This study exhibits a promising vaccine in Vaccine 3 as well a new reagents for characterizing ferret immune responses, which may be useful in evaluating potential vaccine or therapeutic efficacy.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

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Introduction

Influenza

Influenza is an upper respiratory tract infection caused by the influenza virus (WHO 2009). The disease is characterized by high fever, myalgia, headache and severe malaise, non-productive cough, sore throat, and rhinitis. The virus is endemic, with seasonal outbreaks which affect 5-15 percent of the world population, with three to five million cases per year and 250,000 to 500,000 deaths, especially in the elderly and chronically ill.

The virus is a member of the Orthomyxoviridae family of viruse, with this family divided into three genera: influenza A, influenza B, and influenza C (Maines 2008). Influenza A and B typically cause seasonal epidemics (WHO 2009, CDC 2009) but influenza C can also cause disease in humans. Influenza A, B, and C are classified based on the antigenic properties of matrix proteins or nucleoproteins (Zaraket 2009). Influenza A viruses are typed based on two surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA) (Maines 2008), and named based on which HA and NA the virus carries. For example, the current influenza A strains circulating among humans are H1N1 and H3N2 (CDC 2009). Influenza A has 15 sero-types of HA and 9 known sero-types of NA, while influenza B has only one sero-type of HA and NA (Maines 2008). Influenza C expresses a hemagglutinin-esterase (HE) instead of HA, but HE is homologous to HA in function (Zaraket 2009). Influenza virus infects host-cells by binding to sialic acid galactose on the cell surface via HA on the virion. NA cleaves sialic acid to release the virion from the cell (Maines 2008). Antigenic drift causes

frequent changes to the HA and NA proteins, which is due to the low proof reading capability of the RNA polymerase and to selective pressures exerted on the proteins by the host immune system.

Influenza has a segmented genome which allows antigenic shift that gives rise to reassortment of virion genes in the host when two antigenically different viruses co-infect the host (Maines 2008, Zaraket 2009). Influenza is an enveloped virus with ten gene products grouped into eight negative-sense RNA segments. Two of them are the HA and NA described above. Three polymerase proteins (PB1, PB2, and PA) form a RNA-dependent RNA polymerase. Nucleoprotein (NP) is involved in formation of the viral capsid, and along with the polymerase proteins, compose the ribonuclearprotein complex. The M segment contains two genes which encode for the matrix protein 1 (M1) and matrix protein 2 (M2). M1 forms a protein layer between the nucleocapsid and the envelope. M2 is found in the viron envelope which is obtained from budding through the cell membrane, and acts as an ion channel to acidify the virion during viral uncoating, which releases the nucleocapsid into the cell. The nonstructural proteins (NS1 and NS2) are involved in neutralization of host antiviral responses and nuclear export of viral ribonuclear proteins.

Influenza A, B, and C appear to have a common ancestor, with influenza C branching off first, followed by branching of influenza A and B (Maines 2008). A and B show more homology between genes than either does with influenza C, with the polymerase genes showing the highest homology in A and B of 60%. Influenza A shows the most antigenic diversity, with its many HA and NA types, while influenza B and C exhibit a more conserved and more slowly evolving genetic phylogeny.

The natural hosts of influenza A viruses are considered to be birds, with waterfowl being the most common carriers (Zaraket 2009). All HA and NA types of influenza A are found in avian hosts, while only a handful of types have been observed in humans (HA types H1, H2, H3, H5, H7, and H9, and NA types N1, N2, and N3). A viral-host equilibrium is expected to form between a host and infecting virus, resulting in little to no damage in the host but with a high proportion of the host population infected and efficient replication occurring in the host. Influenza A is ubiquitous in avian hosts and is usually asymptomatic in birds. This suggests a possible evolutionary equilibrium has been established between avian host defenses and viral replication, due to a long history of evolutionary back and forth. The disease the virus causes in humans reveals a possible relatively recent introduction of the virus to human hosts, which are not adapted to sustained viral replication. Also, influenza A types infecting humans tend to shift seasonally and a particular strain does not persist in human hosts.

Influenzas B and C do not infect avian hosts. Influenza B is restricted to humans, while influenza C is restricted to humans, dogs, and pigs. Both influenza B and C cause disease in their hosts and do not exhibit the equilibrium described above. Also, the absence of influenza B and C from avian hosts may be due to a longer history within the human host.

Emergence of Highly Pathogenic Avian Influenza

Avian influenza has recently emerged as a potential influenza pandemic, with the sero-type of concern being H5N1. H5N1 in humans was first observed in Guangdong, China in 1996 and Hong Kong in 1997 (Vijaykrishna 2008). This highly pathogenic strain causes a more severe infection, due to a mutation in the HA which allows it to be

cleaved by a wider variety of proteases, and thus it is able to infect a wider variety of tissues.

Pathology of H5N1

H5N1 infection typically has a 60% fatality rate (Guarner 2009). Histological examination of lung tissues shows inflammation, and the influx of T-lymphocytes and macrophages. Lung damage and inflammation are evident, with the attraction of T-lymphocytes and macrophages. Pathology can also be observed in bone marrow, spleen, kidneys, and liver, with some cases of necrosis in the brain reported. Alveolar damage is characteristic of other more pathogenic influenzas, while spread to other organs is common in H5N1 infections, but not infection with other strains of influenzas.

Cytokine Response to H5N1

Human Cytokine Response

In humans, a deregulation of cytokine expression or "cytokine storm" has been observed during the H5N1 infectious disease process. This was first described as a possible mechanism of human disease by Cheung and colleagues (2002). They found mRNA levels of IFN- β and TNF-α to be greatly elevated in H5N1-infected human macrophages, with some up regulation of RANTES, IL-1β, IL-4, IL-10, IL-12, MIP-1α, MIP-1β, and MCP-1. Chan et al. observed an increase in RANTES, IL-6, IP-10, and IFN-β mRNA in alveolar and bronchial epithelial cells infected with H5N1 virus (2005) in comparison to cells infected with a H1N1 strain. This up regulation was confirmed at the protein level as well (Chan 2005). Zeng and colleagues (2007) observed similar results when comparing H5N1 virus infection to H3N2 infection. IL-6 and TNF-α up

regulation has recently been observed in human astrocytes and other neuronal cells following infection with H5N1 virus (Ng 2010).

Some investigators have explored the idea that the up regulation of certain cytokines may contribute to the severity of H5N1 infection. The effects of TNF-α on cellular activity have especially been studied, since it is a signal protein involved in cellular apoptosis. The NS protein of H5N1 induces macrophages to produce TNF-α (Cheung 2002). H5N1 infection also activates p38 MAPK (Lee 2005) and TBK-1 (Hui 2009). TBK-1 then activates IFN3. IFN3 and p38 MAPK induce TNF-α expression (Hui 2009). H5N1 infection also up regulates TRAIL, which along with TNF-α activates the apoptosis pathway (Zhou 2006).

Rodent Cytokine Response

Mouse models have been used to study of H5N1 infection/pathogenesis. In sera from H5N1 infected BALB/c mice, an increase in IFN-γ, TNF-α, IL-1β, and IL-10 were observed, with a fluctuation in IL-6 (Evseenko 2007). Lipatove and colleagues (2005) showed that the NS gene was responsible for the increased cytokine expression in infected mice. When they inserted the NS gene of H5N1 into a H1N1 virus, the recombinant strain exhibited a higher pathogenicity in mice than the parent H1N1 virus, with an increase of IL-1α, IL-1β, IL-6, GM-CSF, and CXCL1. Up regulation of IL-1β, IL-6, and TNF-α has also been seen in the brains of H1N1 infected mice (Wang 2008).

The cotton rat has also been proposed as an influenza model, and it exhibits an increase in IL-1β, IL-6, TNF-α, GRO-α, MIP-1β, IL-4, and IL-12p40, with a delayed up regulation of IFN-α, RANTES, IFN-γ, and IL-10 when challenged with seasonal strains of influenza virus (Ottolini 2005).

Cytokines and Immunity

The increase in cytokine expression with H5N1 infection has led to the hypotheses that increased cytokines may contribute to the severity of the disease and attempts to control this expression may yield a treatment option. However, results are mixed. Salomon et al. tested this hypothesis by challenging BALB/c mice that were deficient in TNF-α, IL-6, or CCL2 with H5N1 virus. They did not demonstrate any decrease in morbidity, which suggests that increased expression of TNF-α, IL-6, or CCL2 did not contribute to viral pathogenicity (Salomon 2007). In addition, the use of glucocorticoids to suppress cytokines in infected mice did not decrease the pathogenicity of the virus. Szretter and colleagues (2007) using knockout mice for various cytokines, confirmed that IL-6 does not affect disease progression (as well as MIP-1α), but found that the absence of TNF-α delayed the onset of weight loss and other symptoms. However, the absence of TNF-α did not seem to affect viral titres or ultimate mortality. They also found that the absence of IL-1 yielded higher titres of virus. In a more recent study, Szretter and colleagues (2009) found that mice deficient in receptors for IFN-α or IFN-β succumbed to disease more rapidly than normal mice. Furthermore, treating murine lung epithelial LA-4 cells with IFN-α or IFN-β decreased viral replication however, there were some strains of H5N1 virus that are able to overcome the interferon response.

Ferret Model Development

The potential for avian influenza to become a pandemic has created a demand for research to characterize the disease and to develop possible vaccines and therapeutics. However, the pool of infected humans is low and therefore efficacy research is typically

limited to animal models and cell lines. In such cases, the FDA Animal Rule [21CFR 314.600-650 (drugs) and 21CFR 601.90-95 (biologicals)] requires that animal models be developed to characterize and test possible vaccines or therapeutics to determine efficacy against an infection. Ferrets are a promising model because the disease progression is similar to that known in humans (Barnard 2009). The ferret has been used to characterize disease progression at the molecular level with seasonal influenza (Svitek 2008) and morbillivirus (Svitek 2007). Ferrets infected with H5N1 have been shown to have a pathogenesis similar to humans, with similar mortalities (Zitzow 2002, Govorkova 2005, Maines 2005). Zitzow, et al. reported upper and lower respiratory tract infection, severe lethargy, fever, weight loss, lymphopenia and diarrhea (2002), and Maines, et al. also reported inflammation and virus present in the brain. Successful treatments of ferrets infected with H5N1 include Vaxfectin-formulated pDNA vaccines (Lalor 2005), oseltamivir (Govorkova 2007), and single- and multiple-clade H5N1 vaccines (Forrest 2009). However, reagents are not readily available to characterize avian influenza infection at the molecular level in the ferret (Barnard 2009). Some immunological characterization has been performed for H5N1 infection in ferrets using canine microarrays showing an upregulation of interferons and CXCL10 (Cameron 2008). The objective of this work was to develop molecular reagents for the characterization of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p40, TNF-α, IFN-α, IFN-β, IFN-γ, TGFB-β1, and TGFB-β2) in the ferret model of influenza infection and determine cytokine mRNA levels in blood and tissues from vaccinated and unvaccinated ferrets. If the ferret response to H5N1 infection is similar to the human response, then an increase in IL-1β, IL-4, IL-12p40, IFN-β, and TNF-α (Cheung 2002), an increase in IL-6, and IFN-β in

lungs (Chan 2005), and an increase in IL-6 and TNF-α in brains (Ng 2010) would be expected, with such expression being diminished or not observed with ferrets inoculated with an efficacious vaccine.

Materials and Methods

Reagent Design and Testing

Ferret DNA Sequences: All ferret gene sequences for the genes of interest in the NCBI database were examined for possible assays as below. The database had one or more annotated sequences for IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p40, TNF-α, IFN-α, IFN-β, IFN-γ, TGFB-β1, TGFB-β2, and GAPDH. Those genes that were represented by two or more entries in the database were aligned using Vector NTI® Software Version 10.3 (Invitrogen, Carlsbad, CA). Primers and TaqMan® minor groove binding (MGB) probes (Applied Biosystems, Foster City, CA) were designed with Primer Express® Software Version 2.0 (Applied Biosystems, Foster City, CA). The default parameters were used for MGB assays (primer: Tm 58-60°C, 30-80% GC, 9-40 bases in length; amplicon: Tm 0-85°C, 50-150 bases in length; TaqMan® MGB probe: Tm 10°C ≥ primers and not beginning with G). If possible two quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were designed for each gene targeting the 5′ and 3′ ends. If multiple sequences were available for a gene, qRT-PCR assays were designed to target conserved regions in the 3′ and 5′ mRNA (Table 1).

Ferret Peripheral Blood Mononuclear Cells (PBMCs): Whole blood was obtained from 35 naive ferrets. PBMCs were isolated from whole blood using a Vacutainer cell preparation tube (CPT tube) and stored in CTL wash medium in liquid nitrogen.

ConA Stimulation: Cryopreserved ferret PBMC samples were thawed in a 37°C water bath and approximately $1x10^6$ cells per well were stimulated in 96-well plates without and with 1 µg Concanavalin A (ConA). ConA stimulated PBMC served as a positive control to demonstrate cytokine production in response to a known potent mitogen. PBMCs were suspended in Compete RPMI (RPMI-1640 + 0.1% Penicillin/Streptomycin + 10% heat-inactivated fetal bovine serum) for overnight culture. After 20 h at 37°C, 5% CO_2, the stimulated PBMCs, as well as $1x10^6$ unstimulated PBMC, were harvested from the wells, washed with Complete RPMI and resuspended in 500 µL of RNA*Later* (Ambion, Austin, TX).

Nucleic Acid Isolation: Total nucleic acid was isolated from the ConA stimulated and unstimulated ferret PBMC samples using a bioMérieux NucliSENS® easyMAG™ (bioMérieux, Durham, NC) using the manufacturer's standard protocol. Briefly, 100 µL of cells were lysed in easyMAG™ Lysis Buffer for 10 minutes, then magnetic silica was added to bind the nucleic acid and incubated for 10 minutes, then the silica was washed by the easyMAG™ using easyMAG™ Buffer 1, easyMAG™ Buffer 2, and easyMAG™ Buffer 3. Purified nucleic acid was suspended in a total volume of 100 µL of easyMAG™ Buffer 3, and all samples were stored at -20°C.

RT-PCR reaction: RT-PCR reactions consisted of 12.5 µL 2X Reaction Mix [Deoxyribonucleotide triphosphates (dNTPs), ROX Reference Dye and optimized buffer components], 1 µL SuperScript III (SuperScript III Reverse Transcriptase and Platinum® *Taq* Polymerase) from the SuperScript III Platinum One Step RT-PCR Kit (Invitrogen, Carlsbad, CA), 1.25 µL 20X Gene Expression Assay mixture [900 nM forward primer, 900 nM reverse primer, and 250 nM probe (dual-labeled with FAM™ at the 5′ and a non-

fluorescent quencher at the 3′ end)], 8.25 µL nuclease-free water, and 2 µL isolated nucleic acid in a total volume of 25 µL. Real-time PCR was performed using an ABI PRISM® 7900HT Fast Sequence Detection System (Applied Biosystems Inc., Foster City, CA) with the following conditions: 15 min at 50°C, 2 min at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min. All reactions were performed in triplicate. Following acquisition, data was analyzed using Version 2.3 of the Sequence Detection System Software (Applied Biosystems Inc., Foster City, CA).

The ΔC_T for each mRNA cytokine result was calculated by subtracting the control C_T from the ConA stimulated C_T. An average of the GAPDH C_T was subtracted from the ΔC_T to normalize the results and calculate the $\Delta C_T \Delta C_T$ value. The mRNA fold change was calculated using the formula $2^{\Delta C_T \Delta C_T}$.

Vaccine Efficacy Testing

Animal Model: Forty-eight male ferrets (*Mustela putorius furo*) (Triple F Farms, Sayre PA), neutered and de-scented, were placed on study. The age of animals at Study day 0 (defined as day of first immunization) was 8-15 weeks (Table 2). Prior to study day 0, animals were randomized by weight to four cohorts of 11 animals, plus one extra animal per vaccine regimen and one extra animal for the control regimen (a total of four extra animals). Extra animals were treated the same as the other cohorts. In the event that an animal must be removed from study due to illness prior to challenge, the extra animal would undergo challenge, as previously described. During randomization, three animals per group were randomly identified to undergo a day 39 sacrifice (4 days post challenge). On study day 35, intranasal inoculation of challenge material was conducted on Telazol anesthetized ferrets (16-22 mg/kg, intramuscular). Challenge material (H5N1 strain

A/Vietnam/1203/04 target dose of 1 x 10^6 tissue culture infectious dose 50 ($TCID_{50}$) in a 600 µl volume) was slowly introduced into the ferret nasal cavity using a 200 µL pipettor with four 150 µL aliquots (alternating nostrils between instillations; each nostril receiving a total of two instillations of 150 µL each). Diluent was phosphate buffered saline (PBS) without divalent cations. H5N1 stock material was obtained from the Centers for Disease Control and Prevention (CDC) and had previously been tested in mice to characterize the virus.

Vaccinations and Blood Draws: The three vaccines (Vaccine 1, Vaccine 2, and Vaccine 3) that were tested were A/Vietnam/1203/04 strain-based, inactivated whole virus vaccines, with Vaccine 3 containing an alum adjuvant. Animals were vaccinated in the hip/thigh area, intramuscularly, once on day 0, and again on day 21 (35 days and 14 days pre-challenge), on the left and right sides, respectively. Vaccination was 0.5 mL of 15 µg/mL (7.5 µg per injection) of vaccine for Groups 1-3 and saline (0.9% NaOCl) for Group 4. Group 1 received Vaccine 1, Group 2 received Vaccine 2, and Group 3 received Vaccine 3. Animals were bled on days 0, 35, 38, and 49 (35 days pre-challenge, and days 0, 3, and 14 days post challenge). Animals that were found moribund had a terminal bleed. One mL of blood was drawn and stored in 2 mL of RNAlater for examination of cytokine mRNA levels.

Necropsy and Tissue Collection: On day 39 (4 days post challenge), three ferrets per group were euthanized and underwent full necropsy. Animals that survived to day 49 (14 days post challenge) were euthanized. All animals on the day of death (day 39, day 49 or moribund) had a gross necropsy performed. Whole blood, liver, brain, and lung (a 1 cm^3 portion of three right lung lobes) were taken. The excised pieces were approximately 1

cm^3. Tissues were placed into cryotubes containing 1 mL RNAlater and frozen at <-70ºC for examination of cytokine mRNA levels.

RNA Isolation and Real-time PCR on Samples Collected from Vaccine Efficacy Study

Isolation of RNA was performed using the Qiagen RNA Blood Prep Kit for the blood samples and the Qiagen RNA Tissue Kit for tissue samples. Tissues from ferrets found dead were not processed due to the determination that the mRNA in these samples may have already degraded significantly and thus yield unreliable results. Real-time PCR was performed as described above using one of each of the cytokine assays, with samples run in triplicate for each target. The ΔC_T for each cytokine was calculated by subtracting the C_T from study day 0 from the C_T for the respective time points. The GAPDH ΔC_T was subtracted from the cytokine ΔC_T to normalize the results and calculate the $\Delta C_T \Delta C_T$ value. The mRNA fold change was calculated using the formula $2^{\Delta C_T \Delta C_T}$.

Statistics

For each gene, the threshold cycles (C_Ts) recorded for each triplicate measurements were averaged by tissue sample type, study day, group, and animal for the statistical analysis. In order to determine if the mean changes from the housekeeping gene (GAPDH) were significantly different between the groups, the following analysis of variance (ANOVA) model was fitted separately for each gene:

$$Y_{tdij} - GAPDH_{tdij} = \mu + group_i + \varepsilon_{ij}$$

where Y_{tdij} is the observed CT for the *j*th animal in group *i* (*i*=1 to 5) associated with tissue sample type *t* (*t*=brain, liver, and lung) on study day *d* (*d*=39 and 49), $GAPDH_{tdij}$ is the corresponding observed control gene CT for the *j*th animal in group *i*, μ is an overall

constant, $group_i$ is the effect of group i, and ε_{ij} is the random error left unexplained by the model. Tukey's multiple comparisons procedure was also performed in order to determine which pairs of groups had mean changes from the housekeeping gene that were significantly different than each other. For blood samples, study day 0 was the group against which the fold change was calculated, while for brain, lung, and liver, group 5 was the control group.

Results

Reagent Design and Testing

To determine whether the designed real-time PCR assays would be effective, ferret PBMCs were thawed and incubated overnight both with and without ConA. The cells were harvested, RNA isolated, and PCR performed with the cytokine primer/probe sets. Table 3 shows the results of each target normalized to GAPDH. In general, the changes in mRNA expression induced by ConA were consistent among the three ferrets and the level of expression detected by assays designed to the 5' end of the transcript was similar to 3' end; however there were some notable exceptions.

The detection of the 3' end of IL-2 (IL2_41) was significantly higher in ConA stimulated PBMC from two ferrets than the 5' end (IL2_155). Eight of the 9 assays in the control PBMCs (ferrets 2 and 3) did not detect the IL2_155 target; therefore a fold change could not be calculated (Table 2). In contrast, the IL2_41 assay detected IL-2 in the control PBMCs for all three ferrets and showed a 3-4 fold increase in IL-2 induction in ConA stimulated PBMCs from all three ferrets. Possible reasons for the differences in the two IL-2 qRT-PCR assays are differences in the efficiency of the amplification due to degradation of the 3' end of the IL-2 gene, alternative splice variants, or a single

nucleotide polymorphism (SNP) in either the primer region or probe region. This study does not support any one possibility over the other. IL-8 expression was higher than most of the other targets and both IL-8 assays showed similar fold-changes. The detection of the 3' end of TGFB-β1 (TGFBβ1_324) was higher than for the 5' end (TGFBβ1_907), with a spike of TGFBβ1_324 detection for Ferret 3. The detection of the 3' end of TGFB-β2 (TGFBβ1_350) was higher than for the 5' end (TGFBβ2_1134)

All of the cytokine genes were induced in PBMCs treated with ConA except IL-1β. It is possible that the expression of IL-1β was induced by ConA treatment but had decreased before the time point of sample collection. The other targets showed a moderate increase in expression, and when two assays to the same cytokine target were developed there was a similar increase in expression.

Assays selected for use in the ferret model were GAPDH_21, IL1β_382, IL2_41, IL4_7, IL6_492, IL8_160, IFNα_25, IFNβ_39, IFNγ_97, TNFα_187, TGFBβ1_907, and TGFBβ2_1134.

Vaccine Testing

Ferret Groups and Day of Death:

The groups that the ferrets were placed into, as well as their days and manner of death are in Tables 4 and 5. All animals survived until at least the day 39 sacrifices (4 days post challenge), excluding ferrets scheduled for sacrifice on day 39. All the animals that succumbed to disease before day 49 were found dead or euthanized by day 42 (7 days post challenge). Group 1 (Vaccine 1) had two animals found dead and five animals euthanized before day 49 14 days post-challenge. Group 2 (Vaccine 2) also had three animals found dead, but only two animals euthanized before day 49. Group 4, the control

group, had five animals found dead; with the other three animals dead by day 41 (6 days post challenge).

Cytokine Responses in Blood Samples:

Tables 6-17 gives the fold change geometric means for each group and time point for each cytokine and the 95% confidence intervals. The data is plotted in Figures 1-12. Table 18 gives the ANOVA statistical modeling for each group and cytokine. A summary of the cytokine data is provided in Table 19.

On day 35 (day of challenge), cytokine expression was similar for each group. Most cytokines did not exhibit significant differences in expression between the groups. However, IL-1β did exhibit differences in expression, with higher levels in group 1 than group 3, and levels in groups 2 and 4 intermediate between groups 1 and 3. There was a trend of large confidence intervals on day 39 for all cytokines except IL-4 and TGFB-β1, indicating a high variance between animals in the group on that day. Day 49 results are discussed below, however, group 1 only had two animals by day 49, and group 2 only had four, so the any differences between the groups may be exaggerated.

There were no significant changes in expression in IL-2 (Figure 2) throughout the study. IL-4 expression was not detected in any blood sample (Figure 3). This may be due to low mRNA levels in the final sample, (GAPDH C_T values were around 30).

IL-1β expression was steady for days 35 and 38 (Figure 1). By day 39, group 2 had increased to the highest expression (2 samples), followed by group 1, group 4, and group 3, which dropped in expression, with the widest confidence intervals for groups 1, 3, and 4. By day 49, expression in groups 2 and 3 were similar to days 35 and 38, while group 1 had dropped. Terminal bloods had a higher level in group 1, with lower

expression in groups 2 and 4. Only day 49 differences were not significant in the ANOVA model (Table 18).

IL-6 expression was consistent for day 35, but by day 38, expression had increased in group 1 and decreased in group 3 (Figure 4). By day 39 and day 49, differences in expression were no longer detected. Differences in IL-6 levels between the groups on day 38 were significant in the ANOVA model (Table 18).

IL-8 expression was consistent for groups 1 and 2, throughout the time course. Expression in group 3 dropped by day 38 and day 39, but was increased back to day 35 levels by day 49. Expression in group 4 dropped slightly lower than group 3 by day 39. Terminal samples showed decreased expression in groups 1, 2, and 4. None of these differences were significant in the ANOVA model (Table 18).

Expression of IL-12p40 increased on day 39 for groups 1 and 2, but decreased for groups 3 and 4. By day 49, expression levels in groups 2 and 3 recovered to day 35 levels, while group 1 dropped below day 35 levels. Terminal blood expression was similar to day 39 for groups 1 and 3, but dropped for group 2. None of these differences were significant in the ANOVA model (Table 18).

TNF-α expression dropped slightly for all groups on day 38, and remained constant through day 49. For terminal samples, expression dropped in groups 2 and 4, but not in group 1. None of these differences were significant in the ANOVA model (Table 18).

IFN-α expression remained level on day 35 and day 39. By day 39, expression for group 2 increased, but no change was observed for the rest of the groups. By day 49,

expression had returned to day 35 levels. Terminal samples were similar to day 35. None of these differences were significant in the ANOVA model (Table 18).

IFN-β expression increased slightly from day 35 to day 38 for all groups. By day 39, expression had dropped slightly for group 4 and increased for groups 1 and 2. By day 49, expression had dropped in group 1, but expression was still elevated in group 2. Expression in terminal samples dropped in group 4, and less so in group 2, with expression in group 1 elevated similar to day 39. Only the differences in the terminal samples were significant in the ANOVA model (Table 18).

IFN-γ expression was similar in all groups on days 35 and 38. On day 39, expression increased in group 2, but was the same for the other groups. By day 49, expression had dropped in group 1, and group 2 had dropped to day 35 levels. Expression in terminal samples was decreased slightly for groups 2 and 4 when compared to day 35. None of these differences were significant in the ANOVA model (Table 18).

TGFB-β1 expression was similar for all groups throughout the time course. However, on day 39 expression was slightly elevated in groups 1 and 2. By day 49, expression in group 2 had dropped slightly, and in group 1 a little more. Expression in terminal samples was dropped in group 2, and slightly elevated in group 1 compared with day 35. The differences in days 39 and 49 were significant in the ANOVA model (Table 18).

TGFB-β2 expression was similar for all groups on days 35 and 38. By day 39, expression was increased in group 2, and decreased slightly in group 4. By day 49, expression in group 2 had lowered to slightly above day 35, but dropped for group 1. In

terminal samples, expression was low in group 4 similar to day 39, along with group 2. None of these differences were significant in the ANOVA model (Table 18).

Cytokine Responses in Tissue from Ferrets Challenged with H5N1:

Tables 20-29 give the geometric means of each group and time points for each cytokine and their 95% confidence intervals, which are shown graphically in Figures 13-22. TGFB-β1 and TGFB-β2 expression were not calculated, since cytokine data was not collected for group 5, which was the baseline for ΔC_T calculations. Table 30 gives the ANOVA statistical modeling for each group and cytokine (with summaries for brain, liver, and lung in Tables 31, 32, and 33 respectively). By day 49 expression levels were similar for each group for most tissues and cytokines, with exceptions noted below.

IL-1β expression was similar between days 39 and 49 for both lung and liver, while for the brain, expression levels were highest on day 39 in group 4, then group 1, and 2, with levels lowest in group 3 (Figure 13). However, differences in the mean were significant for day 49 in the ANOVA model (Table 30).

IL-2 expression was similar for all three tissues among all the groups and both days (Figure 14). However, all differences were significant in the ANOVA model (Table 30).

IL-4 expression was varied on day 39 in the lung between animals and groups, but was similar on day 49 and in the other tissues (Figure 15). IL-4 expression was highest in group 3, and then decreased in groups 1 and 2, with the lowest levels in group 4. However, all differences were significant in the ANOVA model (Table 30).

IL-6 expression was similar for all three tissues among all the groups and both days (Figure 16. However, all differences were significant in the ANOVA model (Table 30).

IL-8 expression in the lung and liver were similar for each group on each day, but levels by day 49 had decreased (Figure 17). However, expression in group 3 in the liver did not decrease. In the brain on day 39, expression was highest in group 4, and then decreased in groups 1 and 2, with levels lowest in group 3. Expression was similar for all three tissues among all the groups and both days. Differences in the brain and lungs on both days were significant in the ANOVA model (Table 30).

IL-12p40 expression was highest in the liver, but consistent between groups and days (Figure 18). In the lung, expression on day 39 was highest in groups 1 and 2 and lowest in group 3. In the brain, expression on day 39 was highest in groups 1 and 4 and lowest in group 3. In the brain and lung, expression dropped in groups 1 and 2 by day 49. Expression was similar for all three tissues among all the groups and both days. Differences in the brain and lung for both days, and the liver on day 39 were significant in the ANOVA model (Table 30).

TNF-α expression was similar for the lung among all groups on both days (Figure 19). In the liver and brain on day 39, expression was highest in group 4 and 1 and lowest in group 3. By day 49 in the liver, expression was highest in group 3 and lowest in group. Differences in all tissues for day 39 were significant in the ANOVA model (Table 30).

IFN-α expression was similar for all three tissues among all the groups and both days (Figure 20). However, all differences were significant in the ANOVA model (Table 30).

IFN-β expression was highest in the brain, but in the brain it was consistent between groups and both days (Figure 21). In the lung on day 39, expression was highest in groups 1 and 2, and lowest in group 3. By day 49, levels in groups 1 and 2 had decreased. In the liver, expression was highest in group 4, then decreases in groups 1 and 2, and was lowest in group 3. All differences were significant in the ANOVA model (Table 30).

IFN-γ expression in the lung on day 39 was highest in group 2, then decreased in groups 1 and 4, and was lowest in group 3 (Figure 22). By day 49, levels in groups 1 and 2 had IFN-γ levels decreased to the level of group 3. Expression in the liver on day 39 was highest in groups 1 and 4 and lowest in groups 2 and 3. By day 49, levels in groups 1 and 2 had dropped below group 3. In the brain, expression was similar between all groups on both days. Differences in the brain and lung on both days were significant in the ANOVA model (Table 30).

TGFB-β1 and TGFB-β2 results were not graphed, since expression levels were not obtained for the group 5 tissues, and thus $2^{\Delta C_T \Delta C_T}$ values could not be calculated. However, only expression of TGFB-β1 in the liver on day 39 and TGFB-β2 in the lung on day 39 were significantly different in the ANOVA model (Table 30).

Conclusions

Vaccine Efficacy

Ferrets inoculated with Vaccine 3 had 100% survival, with survival rates of 33% with Vaccine 2 and 22% with Vaccine 1. Vaccine 3 contained alum as an adjuvant, which is an effective adjuvant in increasing the immune response of the vaccine. As expected there were no survivors in Group 4, the saline control group, demonstrating that H5N1 at the challenge dose was lethal in ferrets.

Ferret Cytokine Expression

Post challenge with H5N1 influenza, Group 1 showed an increase in IL-2, IL-6, IL-12p40, and IFN-β, and a decrease in TNF-α in the blood, an increase in IL-1β and TNF-α in the brain, an increase of IFN-β and IFN-γ in the lung, and an increase of IFN-γ in the liver. Group 2 showed an increase in IL-2, IL-12p40, IFN-α, IFN-β, IFN-γ, and TGFB-β2, and a decrease in TNF-α in the blood, an increase of IL-1β in the brain, an increase of IFN-β and IFN-γ in the lung, and no alteration of cytokine levels in the liver. Group 3 showed a decrease in IL-2, IL-6, IL-8, IL-12p40, and TNF-α in the blood, little variance of cytokine levels in the brain and liver, and an increase in IL-4 in the lung. Group 4, the saline control group, showed an increase in IL-2, and a decrease in IL-12p40, TNF-α and IFN-β in the blood, an increase of IL-1β, IL-8 and TNF-α in the brain, an increase of an increase of IFN-β and IFN-γ in the lung, and an increase of TNF-α and IFN-γ in the liver.

In the blood, the most variation in cytokine expression was observed at day 39 (four days after H5N1 challenge). Days 35 and 38 did not show significant alteration in cytokine expression suggesting that the disease may not yet affected the immune system

to a level that elicited a cytokine response. However, between challenge and day 39 showed some initial changes in cytokine expression were observed. On day 39, there was some variation in cytokine expression. Generally, Groups 1 and 2 showed higher expression of cytokines than Groups 3 and 4 (Figures 1-12). In general, Group 4 showed the lowest expression. This suggests that cytokine levels were modulated by the vaccines with partial efficacy, while the control group showed decreased or unaltered cytokine expression. Vaccines 1 and 2 may have induced sufficient immunity to delay the cytokine storm, resulting in delayed death in the animals However, since the ANOVA model did not show much significance in the differences between groups, these conclusions cannot be stated with full confidence.

An interesting note was cytokine expression on day 39 in all the tissue samples. The expression in group 4 animals varied greatly in almost all tissues for almost all cytokines, with brain showing the greatest variation in cytokine expression (Figures 13-22). This may be indicative of disregulation of cytokine expression which is thought to contribute to the cytokine storm. Some of this variance was also observed in for the other groups, especially in the lungs for IL-4, IL-12p40, and IFN-γ. The liver appears to have the least variance in cytokine expression, however, the expression of cytokines in Group 4 still varied on day 39. For all tissues and cytokines, expression levels returned to near baseline levels by day 49.

Previous studies have shown higher cytokine expression in mice by day 3 post challenge with increases persisting for nine days (Evseenko 2007, Lipatov 2005). In humans, H5N1 infection results in an increase in IL-1β, IL-4, IL-12p40, IFN-β, and TNF-α (Cheung 2002), an increase in IL-6, and IFN-β in lung (Chan 2005), and an increase in

IL-6 and TNF-α in brain (Ng 2010). Similar alterations in cytokine gene expression were not observed in the present study. We observed decreased expression of IL-12p40, TNF-α and IFN-β in the blood and an increase in IL-2, although these results were not statistically significant. There are several potential explanations for the differences observed. An issue with this study is that it was performed within the confines of another study, with different aims and objectives, thereby limiting sample availability. There were no naïve ferrets as a control for the blood samples. Also, the number of ferrets by day 49 was very low for Groups 1 and 2, and thus any differences between them and Group 3 were not statistically significant. This may have resulted in an incomplete picture of cytokine expression in ferrets infected with H5N1, and those treated with the vaccines.

This study would have benefited not only from additional timepoints but also more blood per timepoint. Optimally 1-2 mL of blood would be required to isolate sufficient RNA from the blood. This would also have allowed the use of the PAXgene kit (or similar kit) which is designed for RNA preservation in blood samples, but requires at minimum 1 mL of blood. The manufacturer of RNAlater cautions against the use of their product for whole blood, the use of which may have decreased the yield of mRNA. A more complete picture may have been obtained if timepoints occurred more often, even every six hours for at least 9 days. In cell lines, expression spikes between six and 24 hours (Cheung 2002, Chan 2005, Ng 2010), but in mouse models, spikes are seen between six and nine days (Evseenko 2007, Lipatov 2005). Data from blood drawn every six hours would help in determining to the kinetics of cytokine gene expression. Also, it would be beneficial in the elucidation of the model to have more blood and tissue sample

from earlier times post-challenge, perhaps at the same time points as the blood for comparison. Terminal data for tissues on non-scheduled sacrifice days were not collected because ferret necropsies on animals found dead or euthanized outside of the scheduled day 39 and day 49 sacrifices were not performed immediately. Immediate necropsies would have been beneficial for mRNA integrity, and may have yielded interesting results.

In summary, more frequent time points (every six hours) with increased amounts of blood would provide additional insight into the alterations in cytokine gene expression in both tissues and blood in the ferret model of highly pathogenic avian influenza. Future studies would include additional time points and scheduled sacrifices between day 35 and 38 to further define the time course of cytokine gene expression.

Further Experiments

The expression of additional cytokines is also up regulated during H5N1 infection in other species, primarily RANTES, MIP-1β, MCP-1, GM-CSF, and CXCL1 (Chan 2005 and Wang 2008). Other studies could be performed, to examine these cytokines, but sufficient sample would be required. Combining the results of a study such as this and the results of this thesis as well as future work based upon it could result in the elucidation of cytokine expression to characterize the immunological effects of H5N1 and other influenza infections such as the 2009 H1N1 influenza virus**.**

Primer ID	Length	Tm	%GC	Primer 5'-3'	Tm	%GC
IL-4_7	21	59	52	CTCACCTCCCAACTGATTCCA	81	52
IL4_29_MGB	18	68	50	CTCTGGTCTGCTTACTAG		
IL4_67	20	59	55	GGATGAAAGTGCCGGTGAGT		
IL4_250	21	58	48	TGCTCCAACAGATTGCTCAGA	80	49
IL4_273_MGB	16	70	50	ACTTCACAGGAACCTC		
IL4_312	22	58	45	ACAGGTCATGTTTGCCATGTTC		
GAPDH_738	20	58	50	CCTGGAGAAAGCTGCCAAAT	80	50
GAPDH_759_MGB	18	68	39	TGATGACATCAAGAAGGT		
GAPDH_797	20	59	60	CCCTCTGATGCCTGCTTCAC		
GAPDH_21	18	58	56	CGGATTTGGCCGTATTGG	81	53
GAPDH_42_MGB	15	70	73	CCTGGTCACCAGGGC		
GAPDH_88	28	59	36	CGACAATATCCACTTTACCAGAGT TAAA		
IFNγ_365	20	59	45	CAGCGCAAAGCGATAAATGA	76	40
IFNγ_386_MGB	19	69	37	CTCATCAAAGTGATGAATG		
IFNγ_432	27	59	41	TTCCTTAGGTTAGATCTTGGTGAGA GA		
IFNγ_97	25	58	40	TGCAAGTAATCCAGATGTAGCAGA T	77	43
IFNγ_124_MGB	15	69	47	TGGGCCTCTTTTCTT		
IFNγ_166	27	58	41	CTCCTCTCTCCAGTTCTTCAAAATA TC		
IFNα_25	20	60	60	TCACCTGCTCCCTGGAACAC	66	84
IFNα_51	17	69	53	CCTGGAGGAATTGTGCT		
IFNα_90	20	59	60	GCCCAGCTGCTCAGAAAGTC		
IFNβ_39	23	58	43	ATATTTCTCCACCACGGTTCTTG	77	43
IFNβ_63_MGB	25	69	32	CATGAACTATAACTTACTTCGATTC		
IFNβ_110	22	59	50	TCCACACTGCTGCTGCTTAGTT		
TNFα_187	20	60	60	CCTCTGGCCCAGACAGTCAA	80	49
TNFα_208_MGB	17	69	47	TCATCTTCTCGAACTCC		
TNFα_249	24	58	46	AACATGAGCTACAGGCTTGTCACT		
IL6_492	20	60	60	CCCCGACCCTACCACAGATT	84	60
IL6_516_MGB	13	69	69	CCTGCAGGCTCTC		
IL6_549	20	58	55	CCACTTGTCCTGCGACTTGA		

Table 1. (Continued)

Tm – Melting Temperature

%CG – Percentage of guanine and cytosine in amplicon

Primer ID	Length	Tm	%GC	Primer 5'-3'	Tm	%GC
IL8_8	22	59	50	CAGGAAGAAACCAGACCAAAGG	80	50
IL8_31_MGB	17	68	41	ACCTTGCATCAACATGA		
IL8_67	19	58	53	AGCAACAGCCAGCTTGGAA		
IL8_160	18	59	61	CCACTCCACGCCTTTCCA	79	48
IL8_179_MGB	19	68	32	CCCAAATATATCAAAGAAC		
IL8_220	21	59	52	TGGGCCACTGTCAATCACTCT		
IL1β_382	24	59	38	TTCCAGGACATAAACCAAAAATCC	79	48
IL1β_407_MGB	16	69	44	TGGTGCTGTATAACTC		
IL1β_441	18	59	61	GAGCGCCCGAAGCTCATA		
IL1β_24	26	58	42	CAGTGAAGTGATGTCTTATGGCTA CA	77	43
IL1β_51_MGB	17	68	41	TGACAATGAGAATGACC		
IL1β_90	22	59	45	AGGACCATCAGCCTCAAAGAAA		
TGFBβ2_50	18	60	67	CCGCGCTCAGCCTGTCTA	85	61
TGFBβ2_69_MGB	13	68	69	CTGCAGCACGCTC		
TGFBβ2_103	20	60	50	TGCGCATGAACTGGTCCATA		
TGFBβ2_1134	21	59	48	TTGCTGTGTGTCCCAGGATTT	80	50
TGFBβ2_1159_MGB	14	68	57	CCGCTCACCATTCT		
TGFBβ2_1195	20	58	55	TGGGTGTCTTGCCGATGTAG		
TGFBβ1_324	18	59	61	CACCCGCGTGCTAATGGT	79	48
TGFBβ1_348_MGB	17	70	41	CACCAACAGAATCTATG		
TGFBβ1_385	20	58	45	GCGGGCTTTTCTTGATTTTG		
TGFBβ1_907	20	59	55	CGTAAGGATCTGGGCTGGAA	83	57
TGFBβ1_928_MGB	16	69	63	TGGATCCACGAGCCCA		
TGFBβ1_967	20	60	50	GGCAGAAATTGGCGTGGTAA		
IL2_41	20	58	50	TCGCACTCTTCGCAAACAGT	80	49
IL2_64_MGB	17	68	47	CCTACTACTTCAAGCTC		
IL2_101	20	58	50	TGTTGCTGTGCTTCCTTCGT		
IL2_155	19	58	58	ATGAGAGCCCCAGGATGCT	79	47
IL2_176_MGB	20	70	40	CGTTTAAATTCTACATGCCC		
IL2_220	24	60	46	GATGAGTCAATTCTGTGGCCTTCT		

Table 1. Targeted Sequences in Selected Ferret Cytokine Genes.
Tm – Melting Temperature
%CG – Percentage of guanine and cytosine in amplicon

	$\Delta C_T \Delta C_T$			Fold Change ($2^{\Delta C_T \Delta C_T}$)		
PCR Assay	Ferret 1	Ferret 2	Ferret 3	Ferret 1	Ferret 2	Ferret 3
IL1β_24	2.6	2.1	1.2	0.2	0.2	0.4
IL1β_382	1.4	0.5	-0.2	0.4	0.7	1.2
IL2_41	-1.6	-2.0	-1.8	3.1	4.0	3.4
IL2_155	-5.5	NA	NA	44.0	NA	NA
IL4_7	-2.5	-3.1	-2.8	5.8	8.5	7.0
IL4_250	-2.2	-2.9	-2.5	4.7	7.3	5.6
IL6_492	-2.2	-2.6	-2.1	4.6	6.0	4.2
IL8_8	-4.0	-5.3	-5.1	16.3	38.3	35.0
IL8_160	-4.1	-5.1	-5.6	16.8	35.4	49.7
TNFα_187	-2.5	-5.2	-4.1	5.6	35.9	17.0
IFNα_25	-1.9	-2.5	-2.0	3.7	5.5	3.9
IFNβ_39	-2.7	-2.6	-2.5	6.7	5.9	5.8
IFNγ_97	-2.2	-2.5	-2.1	4.7	5.5	4.4
IFNγ_365	-1.6	-2.1	-2.1	3.1	4.4	4.2
TGFBβ1_324	-1.2	-4.5	-8.8	2.3	23.1	452.2
TGFBβ1_907	-1.3	-2.8	-3.6	2.5	6.7	12.4
TGFBβ2_50	-4.6	-6.3	-6.3	24.2	80.2	79.4
TGFBβ2_1134	-2.1	-2.5	-2.3	4.3	5.7	4.9

Table 2. Cytokine Induction in Ferret PBMCs following Stimulation with ConA.

	Vaccination					
Cohort	Vaccine	Vaccine Dose	ROA[1]	Days	Challenge Day 35	Blood Draw[2]
1	Vaccine 1	7.5 μg	IM	0, 21	A/VN/1203/04	0, 35, 38, 39, 49/t
2	Vaccine 2	7.5 μg	IM	0, 21	A/VN/1203/04	0, 35, 38, 39, 49/t
3	Vaccine 3 (contained alum adjuvant)	7.5 μg	IM	0, 21	A/VN/1203/04	0, 35, 38, 39, 49/t
4	Saline control	NA	IM	0, 21	A/VN/1203/04	0, 35, 38, 39, 49/t
5	None[3]	NA	NA	NA	NA	NA

Table 3. Ferret Vaccine Experimental Design.

[1]ROA—Route of Administration; IM--intramuscular

[2]49/t—Day 49 or terminal draw sample if animal is euthanized prior to Day 49.

[3]Three unchallenged ferrets from another study were used as baselines for tissue cytokine analysis. TGFB-β1 and TGFB-β2 data were not collected for that study.

Group	% Survival for Day Post Challenge					
	Day 4	Day 6	Day7	Day 8	Day 9	Day 14
1.0	88.9	55.6	22.2	22.2	22.2	22.2
2.0	100	66.7	55.6	44.4	33.3	33.3
3.0	100	100	100	100	100	100
4.0	75.0	50.0	0			

Table 4. Ferret Survival Post-Challenge with H5N1.
All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. For each group, three animals were scheduled for euthanasia on Day 39 (4 days post challenge).

Animal ID	Cohort	Sacrifice 39	Extras	Animal Death Date	Euthanized or Found Dead
1002	1	No	No	Day 42	EUTHANIZED
1000	1	No	No	Day 41	EUTHANIZED
1163	1	No	No	Day 42	EUTHANIZED
1031	1	No	No	Day 49	EUTHANIZED
1019	1	No	No	Day 39	FOUND DEAD
1033	1	No	No	Day 41	FOUND DEAD
1013	1	No	No	Day 41	EUTHANIZED
1010	1	No	No	Day 42	EUTHANIZED
991	1	Yes	No	Day 39	EUTHANIZED
995	1	Yes	No	Day 39	EUTHANIZED
1020	1	Yes	No	Day 39	EUTHANIZED
1014	1	No	Yes	Day 49	EUTHANIZED
996	2	No	No	Day 41	EUTHANIZED
1001	2	No	No	Day 42	FOUND DEAD
1024	2	No	No	Day 41	EUTHANIZED
1162	2	No	No	Day 49	EUTHANIZED
994	2	No	No	Day 49	EUTHANIZED
1023	2	No	No	Day 49	EUTHANIZED
1035	2	No	No	Day 41	FOUND DEAD
1016	2	No	No	Day 43	FOUND DEAD
998	2	Yes	No	Day 39	EUTHANIZED
1022	2	Yes	No	Day 39	EUTHANIZED
1027	2	Yes	No	Day 39	EUTHANIZED
1026	2	No	Yes	Day 49	EUTHANIZED
1003	3	No	No	Day 49	EUTHANIZED
993	3	No	No	Day 49	EUTHANIZED
997	3	No	No	Day 49	EUTHANIZED
992	3	No	No	Day 49	EUTHANIZED
1028	3	No	No	Day 49	EUTHANIZED
1009	3	No	No	Day 49	EUTHANIZED
1025	3	No	No	Day 49	EUTHANIZED
1030	3	No	No	Day 49	EUTHANIZED
1161	3	Yes	No	Day 39	EUTHANIZED
1029	3	Yes	No	Day 39	EUTHANIZED
1021	3	Yes	No	Day 39	EUTHANIZED
1004	3	No	Yes	Day 49	EUTHANIZED

Table 5. Days of Death in Ferrets Vaccinated with One of Three Candidate Vaccines or Buffer Control.

All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. For each group, three animals were scheduled for euthanasia on Day 39.

Animal ID	Cohort	Sacrifice 39	Extras	Animal Death Date	Euthanized or Found Dead
1007	4	No	No	Day 41	EUTHANIZED
999	4	No	No	Day 41	EUTHANIZED
1006	4	No	No	Day 40	FOUND DEAD
1017	4	No	No	Day 39	FOUND DEAD
1005	4	No	No	Day 39	EUTHANIZED
1018	4	No	No	Day 40	FOUND DEAD
1012	4	No	No	Day 41	FOUND DEAD
1032	4	No	No	Day 41	EUTHANIZED
1008	4	Yes	No	Day 39	FOUND DEAD
990	4	Yes	No	Day 39	EUTHANIZED
1011	4	Yes	No	Day 39	EUTHANIZED

Table 5 (Continued).
All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. For each group, three animals were scheduled for euthanasia on Day 39.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	0.35 (0.24, 0.52)
		38	3	10	0.30 (0.16, 0.57)
		39	4	3	0.27 (0.08, 0.90)
		49	14	1	0.08 (--)
		Terminal	NA	4	0.51 (0.15, 1.73)
Vaccine 2	2	35	0	11	0.19 (0.08, 0.43)
		38	3	11	0.20 (0.12, 0.32)
		39	4	2	0.73 (0.00, 4378.74)
		49	14	4	0.28 (0.04, 2.06)
		Terminal	NA	2	0.05 (0.00, 47010.65)
Vaccine 3	3	35	0	11	0.07 (0.04, 0.11)
		38	3	11	0.07 (0.03, 0.12)
		39	4	3	0.04 (0.00, 0.66)
		49	14	8	0.17 (0.06, 0.47)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	0.21 (0.12, 0.38)
		38	3	11	0.38 (0.22, 0.66)
		39	4	2	0.14 (0.01, 3.15)
		49	14	NA	NA
		Terminal	NA	4	0.08 (0.01, 0.58)

Table 6. Fold-change in IL-1β mRNA Levels in Whole Blood from Ferrets Challenged with H5N1

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.45 (0.69, 3.08)
		38	3	10	4.90 (2.48, 9.68)
		39	4	3	11.18 (0.534, 233.89)
		49	14	1	2.19 (--)
		Terminal	NA	4	6.00 (2.04, 17.66)
Vaccine 2	2	35	0	11	2.17 (0.67, 7.05)
		38	3	11	4.55 (1.52, 13.60)
		39	4	2	5.64 (0.00, 2778872.19)
		49	14	4	4.81 (0.35, 65.69)
		Terminal	NA	2	5.59 (0.00, 23641708602)
Vaccine 3	3	35	0	11	3.58 (1.55, 8.26)
		38	3	11	2.85 (1.00, 8.07)
		39	4	3	1.96 (0.02, 176.80)
		49	14	8	5.33 (1.19, 23.93)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	1.74 (1.00, 3.01)
		38	3	11	2.55 (1.13, 5.80)
		39	4	2	1.30 (0.09, 18.28)
		49	14	NA	NA
		Terminal	NA	4	0.82 (0.01, 59.22)

Table 7. Fold Change IL-2 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	2.19 (0.91, 5.24)
		38	3	10	6.29 (2.76, 14.34)
		39	4	3	12.17 (4.35, 34.03)
		49	14	1	2.46 (--)
		Terminal	NA	4	7.98 (0.87, 73.56)
Vaccine 2	2	35	0	10	2.21 (0.89, 5.50)
		38	3	11	4.99 (2.40, 10.36)
		39	4	2	7.34 (0.10, 566.16)
		49	14	4	10.26 (3.67, 28.66)
		Terminal	NA	2	3.66 (1.59, 8.45)
Vaccine 3	3	35	0	9	2.22 (0.71, 6.97)
		38	3	11	4.83 (1.61, 14.45)
		39	4	3	5.76 (1.90, 17.42)
		49	14	8	6.48 (2.47, 16.95)
		Terminal	NA	NA	NA
Saline control	4	35	0	9	1.31 (0.67, 2.57)
		38	3	11	4.16 (1.79, 9.67)
		39	4	2	2.58 (0.19, 34.70)
		49	14	NA	NA
		Terminal	NA	4	0.49 (0.06, 3.86)

Table 8. Fold Change IL-4 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.44 (0.73, 2.82)
		38	3	10	3.93 (2.16, 7.15)
		39	4	3	2.91 (0.05, 164.77)
		49	14	1	1.13 (--)
		Terminal	NA	4	3.55 (0.86, 14.63)
Vaccine 2	2	35	0	11	1.79 (1.08, 2.96)
		38	3	11	2.01 (0.80, 5.03)
		39	4	2	3.30 (0.00, 421288.79)
		49	14	4	1.67 (0.59, 4.69)
		Terminal	NA	2	1.48 (0.00, 2145.55)
Vaccine 3	3	35	0	11	2.06 (0.75, 5.61)
		38	3	11	0.71 (0.21, 2.43)
		39	4	3	1.41 (0.04, 46.66)
		49	14	8	2.52 (0.49, 13.00)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	1.35 (0.42, 4.32)
		38	3	11	2.00 (0.98, 4.08)
		39	4	2	1.54 (0.15, 15.66)
		49	14	NA	NA
		Terminal	NA	4	0.43 (0.01, 20.74)

Table 9. Fold Change IL-6 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.75 (1.20, 2.55)
		38	3	10	2.76 (1.83, 4.15)
		39	4	3	3.17 (0.41, 24.37)
		49	14	1	2.06 (--)
		Terminal	NA	4	1.79 (0.50, 6.37)
Vaccine 2	2	35	0	11	2.14 (1.45, 3.15)
		38	3	11	1.98 (1.45, 2.70)
		39	4	2	2.91 (0.08, 106.16)
		49	14	4	2.88 (1.18, 7.05)
		Terminal	NA	2	0.98 (0.27, 3.58)
Vaccine 3	3	35	0	11	1.57 (0.71, 3.45)
		38	3	11	1.04 (0.50, 2.18)
		39	4	3	1.38 (0.15, 13.00)
		49	14	8	2.45 (1.04, 5.78)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	2.43 (1.11, 5.35)
		38	3	11	3.04 (1.34, 6.77)
		39	4	2	1.02 (0.01, 126.18)
		49	14	NA	NA
		Terminal	NA	4	1.65 (0.18, 14.73)

Table 10. Fold Change IL-8 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	3.13 (1.29, 7.59)
		38	3	10	10.87 (2.16, 54.71)
		39	4	3	19.65 (0.14, 2761.67)
		49	14	1	1.23 (--)
		Terminal	NA	4	31.37 (2.29, 429.54)
Vaccine 2	2	35	0	11	4.21 (1.59, 11.15)
		38	3	11	5.82 (1.75, 19.36)
		39	4	2	30.13 (0.00, 770391479.24)
		49	14	4	13.86 (2.00, 96.00)
		Terminal	NA	2	4.28 (0.00, 1187498200000)
Vaccine 3	3	35	0	11	5.19 (1.58, 17.01)
		38	3	11	2.28 (0.38, 13.81)
		39	4	3	3.83 (0.06, 229.10)
		49	14	8	11.92 (1.53, 92.99)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	4.13 (0.77, 22.28)
		38	3	11	5.22 (1.44, 18.93)
		39	4	2	3.65 (0.00, 274937.54)
		49	14	NA	NA
		Terminal	NA	4	4.38 (0.15, 127.34)

Table 11. Fold Change IL-12p40 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.65 (1.05, 2.58)
		38	3	10	0.61 (0.30, 1.25)
		39	4	3	0.52 (0.05, 5.78)
		49	14	1	1.04 (--)
		Terminal	NA	4	0.58 (0.21, 1.64)
Vaccine 2	2	35	0	11	1.23 (0.68, 2.21)
		38	3	11	0.50 (0.26, 0.93)
		39	4	2	0.82 (0.20, 3.42)
		49	14	4	0.87 (0.56, 1.37)
		Terminal	NA	2	0.18 (0.00, 160.63)
Vaccine 3	3	35	0	11	0.88 (0.40, 1.93)
		38	3	11	0.59 (0.35, 1.01)
		39	4	3	0.49 (0.17, 1.36)
		49	14	8	0.82 (0.39, 1.71)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	1.23 (0.72, 2.12)
		38	3	11	0.92 (0.52, 1.61)
		39	4	2	1.17 (0.06, 24.34)
		49	14	NA	NA
		Terminal	NA	4	0.23 (0.08, 0.68)

Table 12. Fold Change TNF-α mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	0.78 (0.24, 2.53)
		38	3	10	1.21 (0.23, 6.46)
		39	4	3	1.54 (0.00, 1009.03)
		49	14	1	0.17 (--)
		Terminal	NA	4	3.47 (0.09, 134.14)
Vaccine 2	2	35	0	11	1.07 (0.42, 2.72)
		38	3	11	1.53 (0.25, 9.42)
		39	4	2	18.94 (0.0000, 682698868806)
		49	14	4	1.85 (0.17, 20.51)
		Terminal	NA	2	1.12 (0.00, 1.2603276E14)
Vaccine 3	3	35	0	11	0.85 (0.21, 3.44)
		38	3	11	0.27 (0.04, 2.09)
		39	4	3	0.55 (0.00, 1950.14)
		49	14	8	2.22 (0.14, 35.59)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	0.89 (0.13, 6.17)
		38	3	11	1.04 (0.24, 4.52)
		39	4	2	0.66 (0.00, 1418.92)
		49	14	NA	NA
		Terminal	NA	4	0.49 (0.00, 171.11)

Table 13. Fold Change IFN-α mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.25 (0.49, 3.19)
		38	3	10	4.08 (1.64, 10.16)
		39	4	3	9.62 (2.42, 38.26)
		49	14	1	0.63 (--)
		Terminal	NA	4	5.11 (0.49, 53.72)
Vaccine 2	2	35	0	11	1.69 (0.80, 3.56)
		38	3	11	3.21 (1.35, 7.64)
		39	4	2	8.29 (0.00, 32189.94)
		49	14	4	7.66 (6.34, 9.25)
		Terminal	NA	2	1.66 (0.00, 87915.48)
Vaccine 3	3	35	0	11	1.60 (0.46, 5.50)
		38	3	11	2.41 (0.66, 8.78)
		39	4	3	3.18 (0.22, 44.96)
		49	14	8	4.27 (1.14, 16.00)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	1.41 (0.67, 3.00)
		38	3	11	3.00 (1.30, 6.91)
		39	4	2	1.57 (0.00, 12676.24)
		49	14	NA	NA
		Terminal	NA	4	0.42 (0.03, 5.53)

Table 14. Fold Change IFN-β mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	0.89 (0.51, 1.53)
		38	3	10	1.36 (0.36, 5.20)
		39	4	3	1.31 (0.03, 64.45)
		49	14	1	0.05 (--)
		Terminal	NA	4	2.19 (0.05, 87.46)
Vaccine 2	2	35	0	11	1.02 (0.62, 1.67)
		38	3	11	1.07 (0.2688, 4.2258)
		39	4	2	17.21 (0.00, 24195275699)
		49	14	4	2.73 (0.61, 12.16)
		Terminal	NA	2	0.26 (0.00, 3508836.98)
Vaccine 3	3	35	0	11	0.49 (0.18, 1.32)
		38	3	11	0.52 (0.17, 1.62)
		39	4	3	0.61 (0.01, 41.09)
		49	14	8	1.44 (0.31, 6.62)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	0.65 (0.21, 2.03)
		38	3	11	0.85 (0.38, 1.92)
		39	4	2	0.44 (0.00, 80.95)
		49	14	NA	NA
		Terminal	NA	4	0.47 (0.05, 4.23)

Table 15. Fold Change IFN-γ mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	1.13 (0.70, 1.81)
		38	3	10	0.71 (0.43, 1.16)
		39	4	3	1.43 (0.67, 3.04)
		49	14	1	0.47 (--)
		Terminal	NA	4	1.21 (0.46, 3.15)
Vaccine 2	2	35	0	11	1.09 (0.821, 1.44)
		38	3	11	0.89 (0.60, 1.33)
		39	4	2	2.01 (0.67, 6.03)
		49	14	4	1.32 (1.07, 1.62)
		Terminal	NA	2	0.09 (0.00, 1.7753446E13)
Vaccine 3	3	35	0	11	1.28 (0.77, 2.12)
		38	3	11	0.78 (0.51, 1.18)
		39	4	3	1.10 (0.37, 3.26)
		49	14	8	0.79 (0.49, 1.29)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	0.97 (0.56, 1.70)
		38	3	11	0.59 (0.36, 0.95)
		39	4	2	0.76 (0.38, 1.51)
		49	14	NA	NA
		Terminal	NA	4	0.40 (0.14, 1.15)

Table 16. Fold Change TGFB-β1 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Treatment	Group	Study Day	Day Post-Challenge	N	Geometric Mean (95% Confidence Interval)
Vaccine 1	1	35	0	10	0.85 (0.47, 1.54)
		38	3	10	1.43 (0.41, 4.96)
		39	4	3	2.15 (0.19, 24.85)
		49	14	1	0.06 (--)
		Terminal	NA	4	3.95 (0.22, 71.30)
Vaccine 2	2	35	0	11	1.14 (0.53, 2.45)
		38	3	11	1.24 (0.36, 4.26)
		39	4	2	19.34 (0.00, 2713578355.1)
		49	14	4	4.98 (0.58, 42.44)
		Terminal	NA	2	0.52 (0.00, 330585430.08)
Vaccine 3	3	35	0	11	0.45 (0.16, 1.22)
		38	3	11	0.55 (0.16, 1.86)
		39	4	3	0.65 (0.00, 156.99)
		49	14	8	1.74 (0.28, 10.66)
		Terminal	NA	NA	NA
Saline control	4	35	0	11	0.83 (0.20, 3.43)
		38	3	11	1.53 (0.46, 5.08)
		39	4	2	0.27 (0.00, 48.86)
		49	14	NA	NA
		Terminal	NA	4	0.36 (0.01, 21.04)

Table 17. Fold Change TGFB-β2 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1.

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Cytokine	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IL-1β	0	0.00*	0.41 (1<3) 0.01 2.37 (3>4) 0.01
	35	0.04*	2.41 (1>3) 0.03
	38	0.02*	0.41 (3<4) 0.02
	39	0.05*	
	49	0.52	
	Terminal	0.03*	6.11 (1>4) 0.03
IL-2	0	0.79	
	35	0.36	
	38	0.34	
	39	0.84	
	49	0.75	
	Terminal	0.07	
IL-4	0	0.26	
	35	0.78	
	38	0.65	
	39	0.85	
	49	0.56	
	Terminal	0.02*	11.04 (1>4) 0.02
IL-6	0	0.95	
	35	0.74	
	38	0.01*	4.05 (1>3) 0.01
	39	0.60	
	49	0.22	
	Terminal	0.06	
IL-8	0	0.42	
	35	1.00	
	38	0.13	
	39	0.83	
	49	0.43	
	Terminal	0.42	
IL-12p40	0	0.92	
	35	0.65	
	38	0.29	
	39	0.53	
	49	0.85	
	Terminal	0.28	

Table 18. Results of ANOVA Models Fitted to ΔCt for Cytokine Gene Expression in Whole Blood from Ferrets Challenged with H5N1.

Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
TNF-α	0	0.62	
	35	0.26	
	38	0.73	
	39	0.89	
	49	0.35	
	Terminal	0.19	
IFN-α	0	0.74	
	35	0.91	
	38	0.26	
	39	0.48	
	49	0.87	
	Terminal	0.19	
IFN-β	0	0.67	
	35	0.41	
	38	0.74	
	39	0.56	
	49	0.35	
	Terminal	0.03*	10.22 (1>4) 0.03
IFN-γ	0	0.69	
	35	1.00	
	38	0.77	
	39	0.10	
	49	0.51	
	Terminal	0.33	
TGFB-β1	0	0.64	
	35	0.94	
	38	0.57	
	39	0.00*	1.86 (1>4) 0.00 1.69 (2>3) 0.01 2.57 (2>4) 0.00 1.52 (3>4) 0.02
	49	0.01*	0.39(1<2) 0.04 1.78 (2>3) 0.02
	Terminal	0.12	

Table 18. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
TGFB-β2	0	0.53	
	35	0.99	
	38	0.65	
	39	0.10	
	49	0.46	
	Terminal	0.06	

Table 18. (Continued).

Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Days Post Challenge				
	0	**35**	**38**	**39**	**49**
IL-1β	+	+	+	+	-
IL-2	-	-	-	-	-
IL-4	-	-	-	-	-
IL-6	-	-	+	-	-
IL-8	-	-	-	-	-
IL-12p40	-	-	-	-	-
TNF-α	-	-	-	-	-
IFN-α	-	-	-	-	-
IFN-β	-	-	-	-	-
IFN-γ	-	-	-	-	-
TGFB-β1	-	-	-	+	+
TGFB-β2	-	-	-	-	-

Table 19. Summary of Significance of Differences in Cytokine Gene Expression Between Groups in Whole Blood from Ferrets Challenged with H5N1 virus

+ = Significant differences between groups (P-value <0.05)

- = No significant differences between groups (P-value ≥0.05)

Significance was determined by calculating P-values using ΔC_T. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. Data from terminal animals are not tabulated since significant differences cannot be determined.

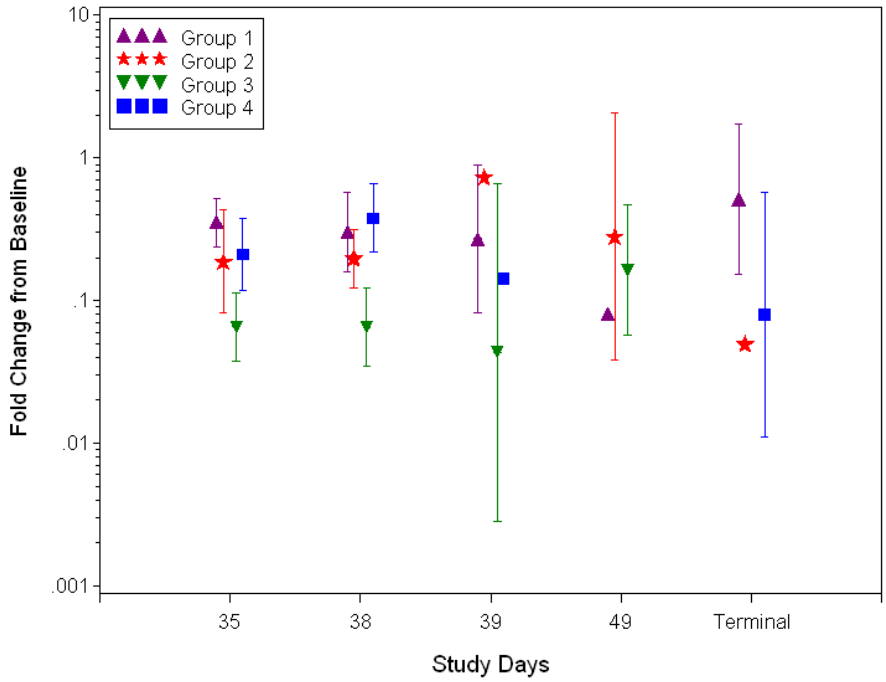

Figure 1. Fold Change in IL-1β mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

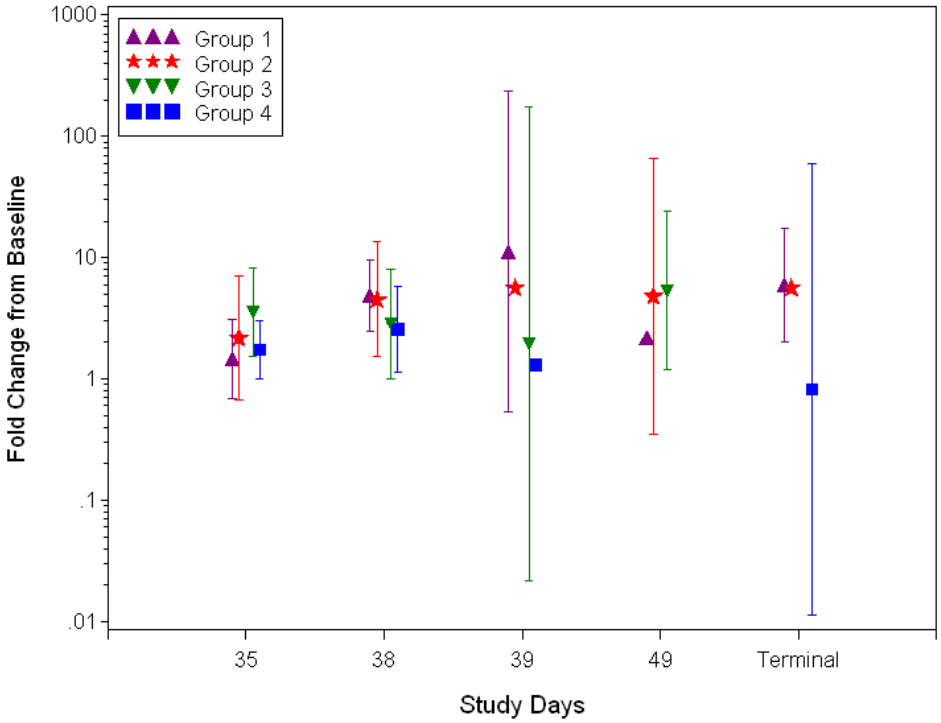

Figure 2. Fold Change in IL-2 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

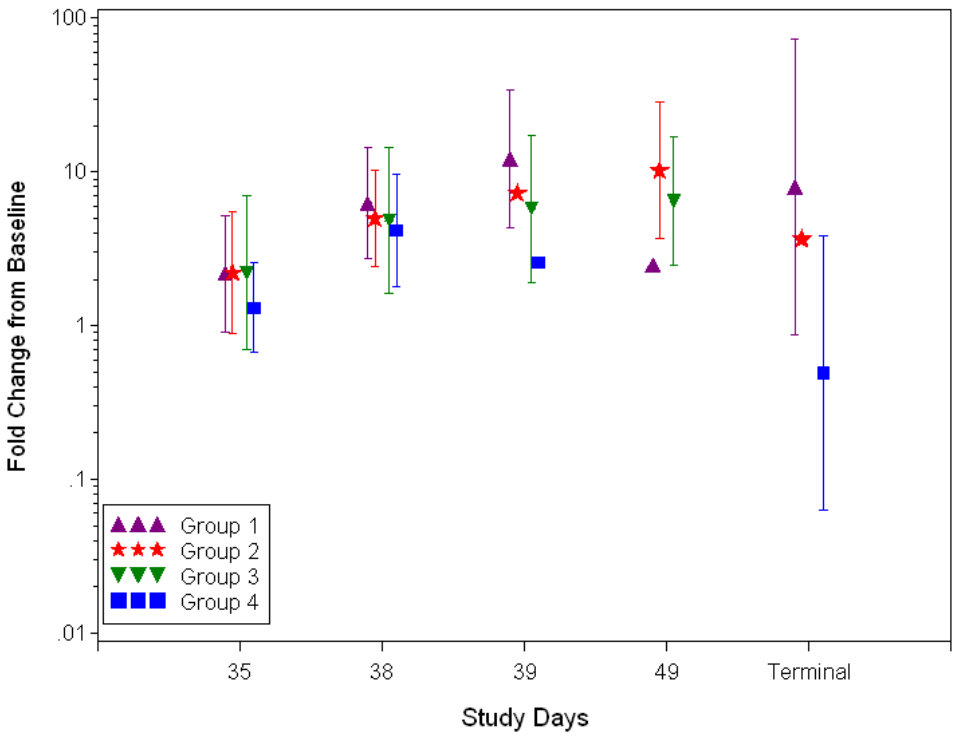

Figure 3. Fold Change in IL-4 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

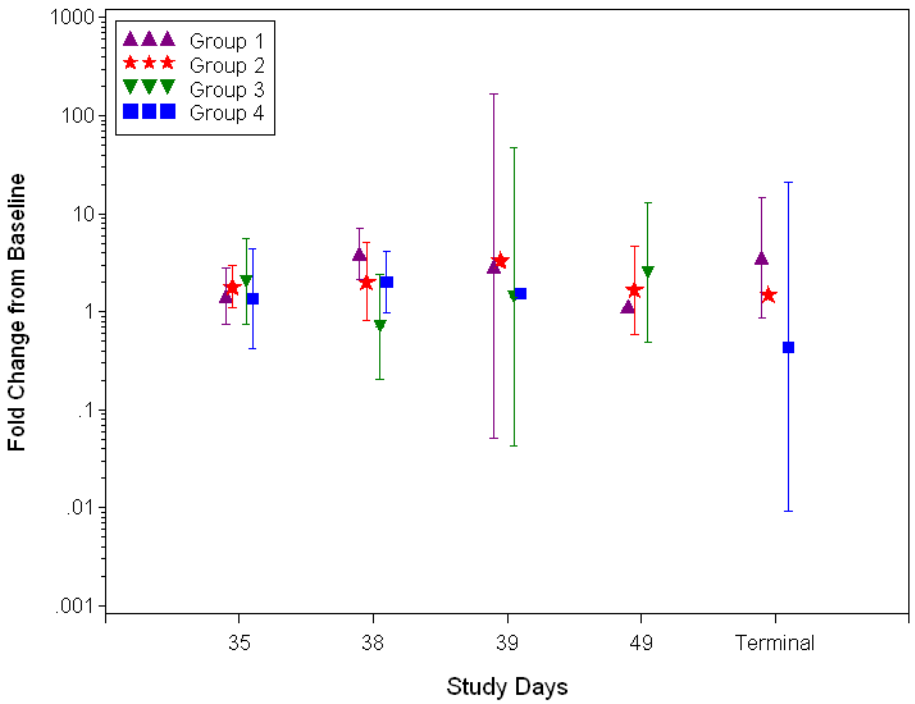

Figure 4. Fold Change in IL-6 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

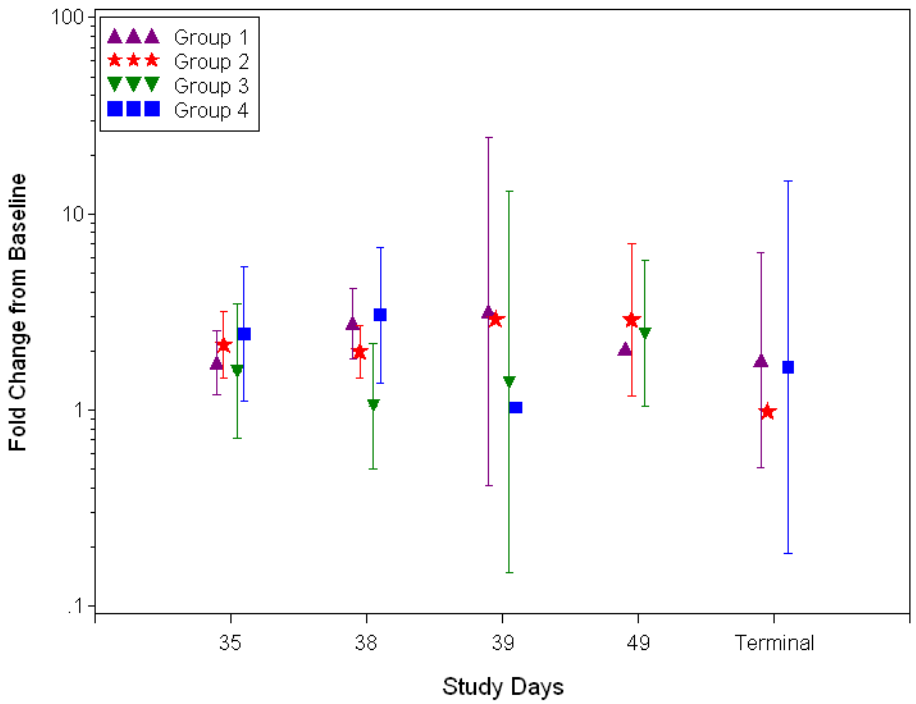

Figure 5. Fold Change in IL-8 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

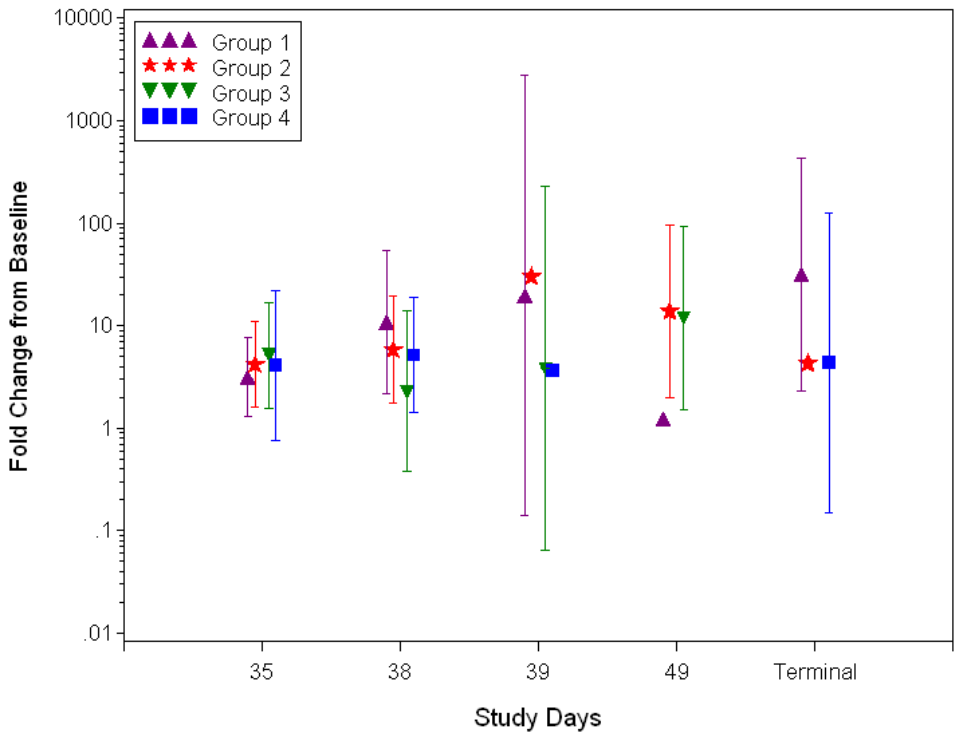

Figure 6. Fold Change in IL-12p40 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

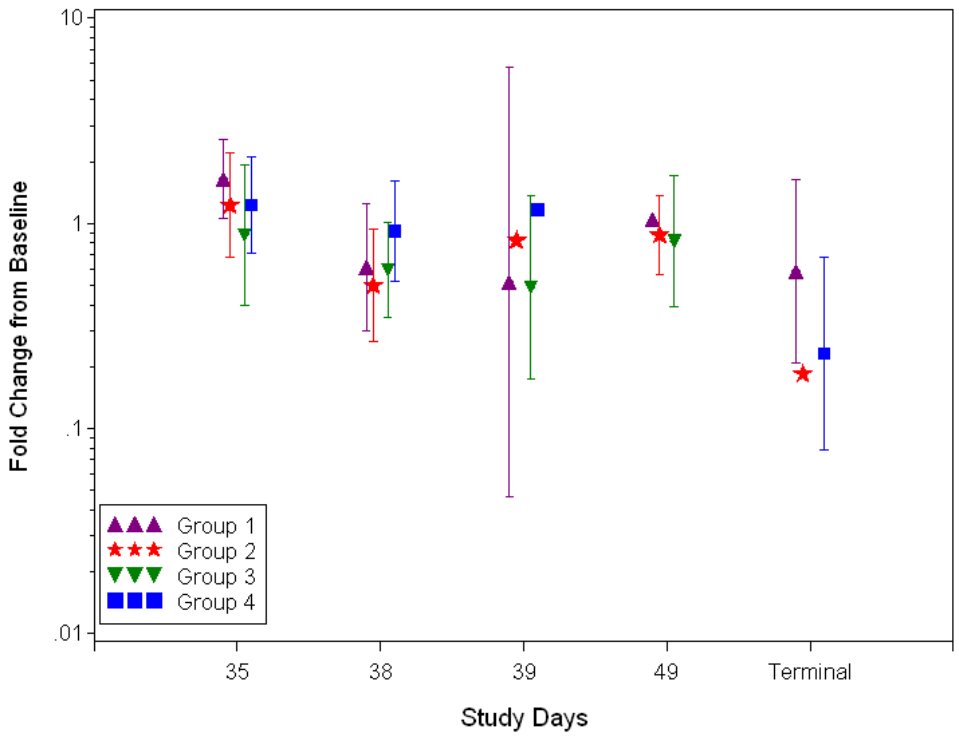

Figure 7. Fold Change in TNF-α mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

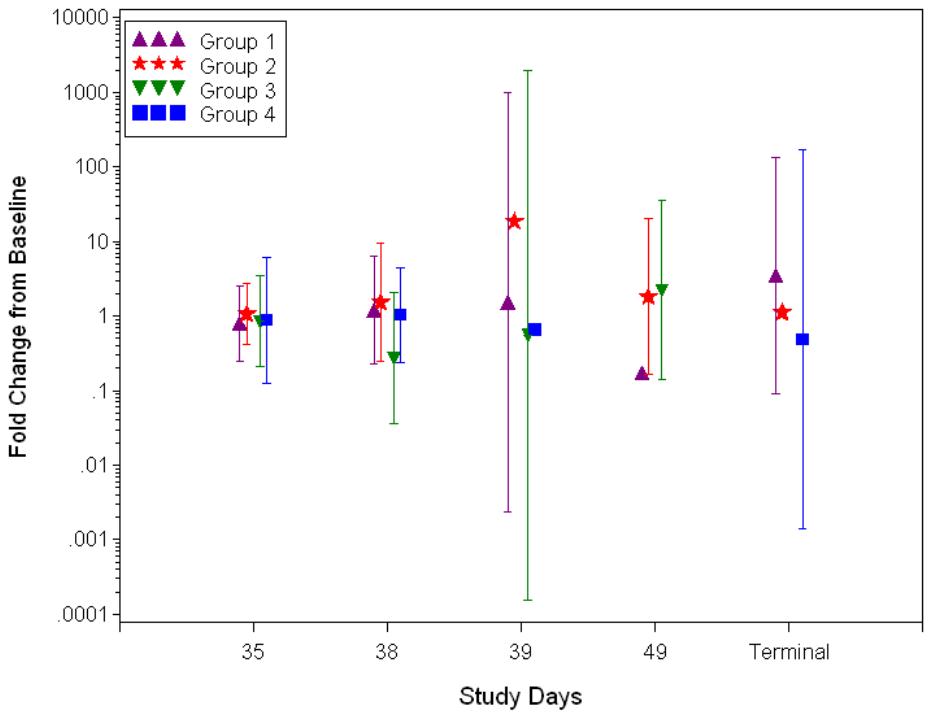

Figure 8. Fold Change in IFN-α mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

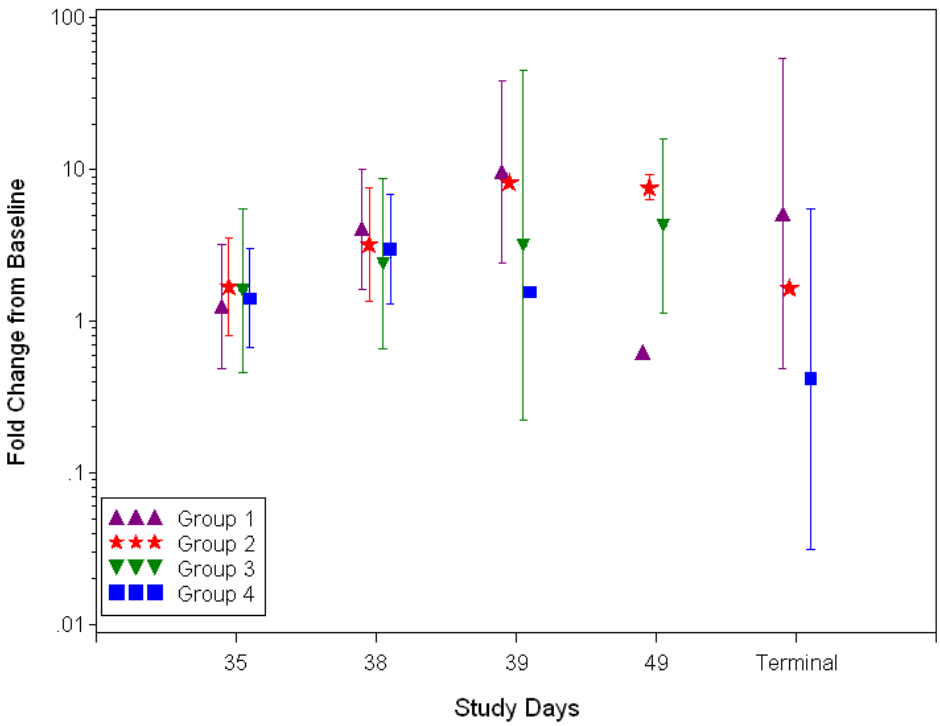

Figure 9. Fold Change in IFN-β mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

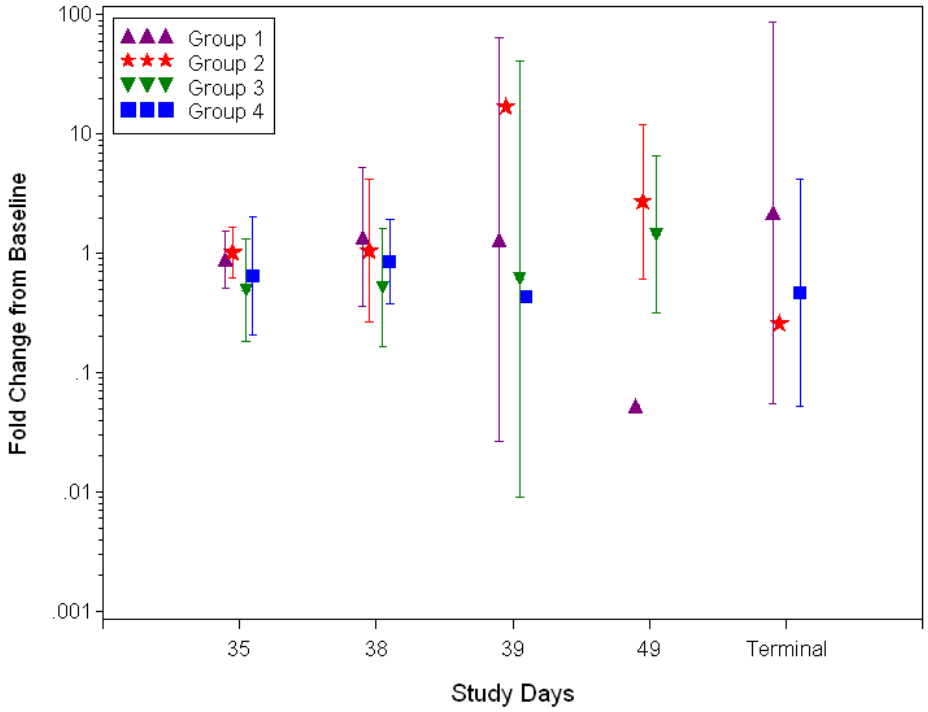

Figure 10. Fold Change in IFN-γ mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

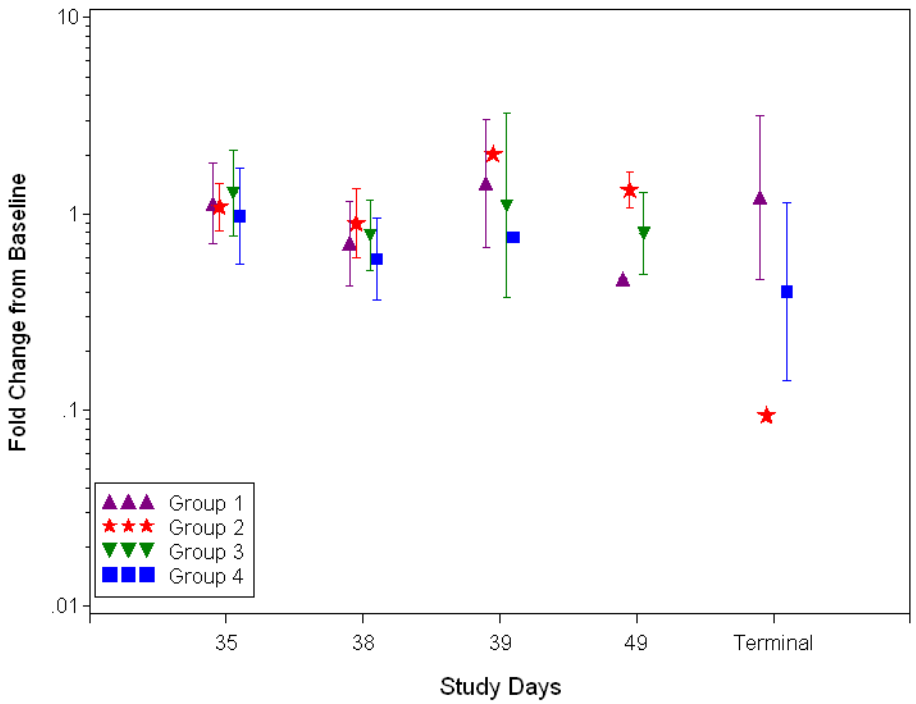

Figure 11. Fold Change in TGFB-β1 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

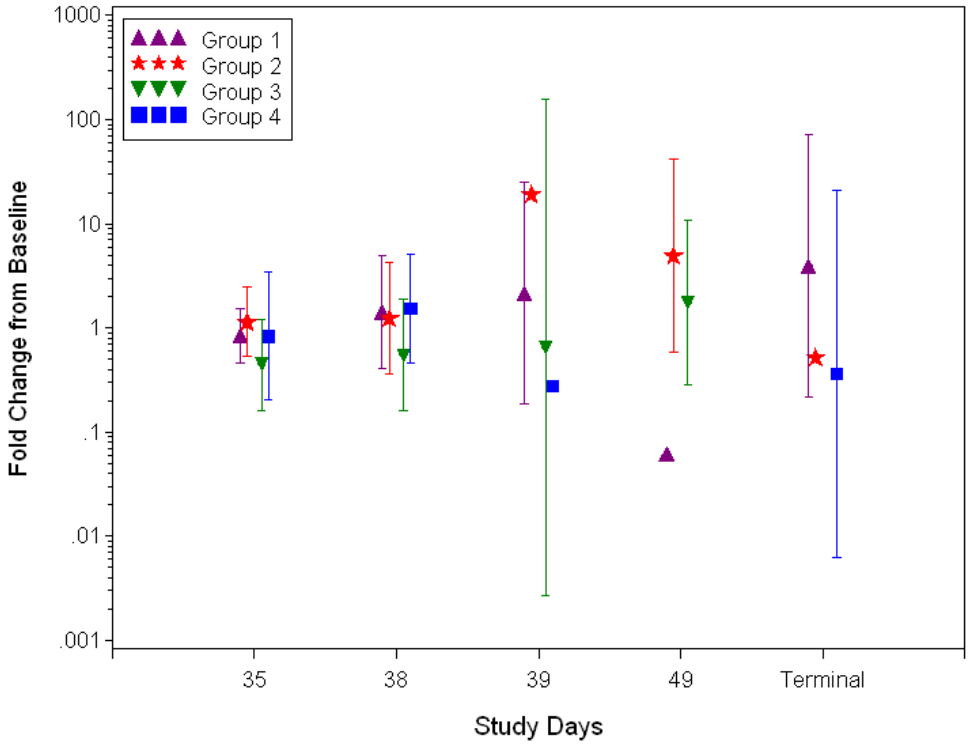

Figure 12. Fold Change in TGFB-β2 mRNA Levels in Whole Blood from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. Baseline is Day 0 (35 days before challenge). All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.94 (0.03, 31.76)
			14	49	1	0.10 (--)
	Vaccine 2	2	4	39	3	0.33 (0.06, 1.91)
			14	49	3	0.04 (0.01, 0.17)
	Vaccine 3	3	4	39	3	0.11 (0.00, 2.35)
			14	49	8	0.05 (0.02, 0.09)
	Saline Control	4	4	39	2	1.56 (0.03, 91.17)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.92 (0.155, 5.42)
			14	49	1	0.27 (--)
	Vaccine 2	2	4	39	3	0.60 (0.18, 2.07)
			14	49	3	0.22 (0.10, 0.52)
	Vaccine 3	3	4	39	3	0.64 (0.39, 1.04)
			14	49	8	0.50 (0.34, 0.74)
	Saline Control	4	4	39	2	0.50 (0.00, 100.11)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.63 (0.27, 1.48)
			14	49	1	0.14 (--)
	Vaccine 2	2	4	39	3	0.44 (0.04, 4.61)
			14	49	3	0.12 (0.03, 0.49)
	Vaccine 3	3	4	39	3	0.26 (0.02, 2.96)
			14	49	8	0.14 (0.06, 0.33)
	Saline Control	4	4	39	2	0.42 (0.00, 899.69)
			14	49	NA	NA

Table 20. Fold-change in IL-1β mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.01 (0.00, 0.09)
			14	49	1	0.01 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.02)
			14	49	3	0.00 (0.00, 0.02)
	Vaccine 3	3	4	39	3	0.01 (0.00, 3.44)
			14	49	8	0.00 (0.00, 0.01)
	Saline Control	4	4	39	2	0.00 (0.00, 27.62)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.02 (0.00, 0.10)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.01 (0.00, 0.05)
			14	49	3	0.01 (0.00, 0.02)
	Vaccine 3	3	4	39	3	0.01 (0.00, 0.02)
			14	49	8	0.01 (0.01, 0.03)
	Saline Control	4	4	39	2	0.02 (0.00, 7.73)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.01 (0.01, 0.09)
			14	49	1	0.01 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.03)
			14	49	3	0.01 (0.00, 0.04)
	Vaccine 3	3	4	39	3	0.01 (0.00, 0.04)
			14	49	8	0.01 (0.03, 0.03)
	Saline Control	4	4	39	2	0.00 (0.00, 0.04)
			14	49	NA	NA

Table 21. Fold-change in IL-2 mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.00 (0.00, 0.04)
			14	49	1	0.00 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.02)
			14	49	3	0.00 (0.00, 0.02)
	Vaccine 3	3	4	39	3	0.00 (0.00, 0.07)
			14	49	8	0.00 (0.00, 0.00)
	Saline Control	4	4	39	2	0.00 (0.00, 21.87)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.00 (0.00, 0.00)
			14	49	1	0.00 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.00)
			14	49	3	0.00 (0.00, 0.00)
	Vaccine 3	3	4	39	3	0.00 (0.00, 0.00)
			14	49	8	0.00 (0.00, 0.00)
	Saline Control	4	4	39	2	0.00 (0.00, 0.00)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.00 (0.00, 0.04)
			14	49	1	0.00 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.07)
			14	49	3	0.00 (0.00, 0.00)
	Vaccine 3	3	4	39	3	0.00 (0.00, 10.84)
			14	49	8	0.00 (0.00, 0.00)
	Saline Control	4	4	39	2	0.00 (0.00, 0.00)
			14	49	NA	NA

Table 22. Fold-change in IL-4 mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.21 (0.00, 9.54)
			14	49	1	0.05 (--)
	Vaccine 2	2	4	39	3	0.06 (0.01, 0.32)
			14	49	3	0.05 (0.01, 0.18)
	Vaccine 3	3	4	39	3	0.07 (0.01, 0.78)
			14	49	8	0.04 (0.02, 0.07)
	Saline Control	4	4	39	2	0.45 (0.00, 42107.03)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.07 (0.00, 5.93)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.02 (0.01, 0.07)
			14	49	3	0.02 (0.01, 0.04)
	Vaccine 3	3	4	39	3	0.03 (0.01, 0.14)
			14	49	8	0.03 (0.01, 0.07)
	Saline Control	4	4	39	2	0.16 (0.00, 150.92)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.63 (0.21, 1.84)
			14	49	1	0.04 (--)
	Vaccine 2	2	4	39	3	0.54 (0.06, 5.31)
			14	49	3	0.04 (0.00, 0.32)
	Vaccine 3	3	4	39	3	0.07 (0.01, 0.51)
			14	49	8	0.05 (0.03, 0.09)
	Saline Control	4	4	39	2	0.15 (0.00, 1346592.43)
			14	49	NA	NA

Table 23. Fold-change in IL-6 mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.25 (0.01, 6.21)
			14	49	1	0.05 (--)
	Vaccine 2	2	4	39	3	0.06 (0.01, 0.66)
			14	49	3	0.04 (0.01, 0.19)
	Vaccine 3	3	4	39	3	0.04 (0.00, 0.36)
			14	49	8	0.02 (0.01, 0.04)
	Saline Control	4	4	39	2	1.51 (0.00, 58384.75)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	1.18 (0.05, 27.99)
			14	49	1	0.10 (--)
	Vaccine 2	2	4	39	3	0.57 (0.32, 1.01)
			14	49	3	0.15 (0.02, 1.20)
	Vaccine 3	3	4	39	3	1.05 (0.11, 9.87)
			14	49	8	1.09 (0.25, 4.84)
	Saline Control	4	4	39	2	1.63 (0.05, 54.19)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.79 (0.14, 4.30)
			14	49	1	0.08 (--)
	Vaccine 2	2	4	39	3	1.16 (0.32, 3.91)
			14	49	3	0.08 (0.00, 1.50)
	Vaccine 3	3	4	39	3	0.13 (0.01, 2.21)
			14	49	8	0.04 (0.01, 0.13)
	Saline Control	4	4	39	2	0.38 (0.00, 12999.78)
			14	49	NA	NA

Table 24. Fold-change in IL-8 mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.30 (0.03, 2.92)
			14	49	1	0.04 (--)
	Vaccine 2	2	4	39	3	0.19 (0.01, 2.66)
			14	49	3	0.03 (0.01, 0.14)
	Vaccine 3	3	4	39	3	0.06 (0.00, 1.04)
			14	49	8	0.03 (0.01, 0.06)
	Saline Control	4	4	39	2	0.34 (0.00, 344.85)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	3.27 (0.91, 11.83)
			14	49	1	1.13 (--)
	Vaccine 2	2	4	39	3	3.77 (1.98, 7.18)
			14	49	3	0.51 (0.07, 3.53)
	Vaccine 3	3	4	39	3	0.70 (0.07, 6.65)
			14	49	8	1.01 (0.62, 1.63)
	Saline Control	4	4	39	2	1.47 (0.36, 5.96)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.13 (0.02, 0.92)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.12 (0.02, 0.78)
			14	49	3	0.02 (0.00, 0.19)
	Vaccine 3	3	4	39	3	0.02 (0.00, 0.10)
			14	49	8	0.02 (0.01, 0.05)
	Saline Control	4	4	39	2	0.06 (0.00, 2.99)
			14	49	NA	NA

Table 25. Fold-change in IL-12p40 mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	20.58 (2.010, 201.89)
			14	49	1	2.18 (--)
	Vaccine 2	2	4	39	3	8.56 (1.44, 50.98)
			14	49	3	1.47 (0.69, 3.12)
	Vaccine 3	3	4	39	3	2.56 (0.38, 17.46)
			14	49	8	1.51 (1.11, 2.06)
	Saline Control	4	4	39	2	37.21 (0.01, 138383.91)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	10.29 (4.15, 25.52)
			14	49	1	0.55 (--)
	Vaccine 2	2	4	39	3	6.33 (1.64, 24.46)
			14	49	3	1.08 (0.20, 5.76)
	Vaccine 3	3	4	39	3	1.99 (0.32, 12.38)
			14	49	8	2.78 (1.14, 6.79)
	Saline Control	4	4	39	2	10.65 (0.63, 179.65)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	4.02 (1.05, 15.35)
			14	49	1	0.99 (--)
	Vaccine 2	2	4	39	3	3.29 (1.04, 10.38)
			14	49	3	1.51 (0.28, 8.03)
	Vaccine 3	3	4	39	3	1.11 (0.48, 2.58)
			14	49	8	0.81 (0.55, 1.20)
	Saline Control	4	4	39	2	1.76 (0.10, 32.63)
			14	49	NA	NA

Table 26. Fold-change in TNF-α mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.02 (0.00, 0.10)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.02 (0.00, 0.06)
			14	49	3	0.02 (0.00, 0.07)
	Vaccine 3	3	4	39	3	0.03 (0.00, 0.92)
			14	49	8	0.01 (0.01, 0.03)
	Saline Control	4	4	39	2	0.03 (0.00, 4552.83)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.01 (0.00, 0.12)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.00 (0.00, 0.01)
			14	49	3	0.01 (0.00, 0.03)
	Vaccine 3	3	4	39	3	0.00 (0.00, 0.01)
			14	49	8	0.01 (0.00, 0.01)
	Saline Control	4	4	39	2	0.01 (0.00, 0.27)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.04 (0.01, 0.16)
			14	49	1	0.01 (--)
	Vaccine 2	2	4	39	3	0.03 (0.00, 0.17)
			14	49	3	0.00 (0.00, 0.01)
	Vaccine 3	3	4	39	3	0.01 (0.00, 0.03)
			14	49	8	0.01 (0.00, 0.03)
	Saline Control	4	4	39	2	0.04 (0.00, 0.21)
			14	49	NA	NA

Table 27. Fold-change in IFN-α mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.14 (0.06, 0.33)
			14	49	1	0.34 (--)
	Vaccine 2	2	4	39	3	0.22 (0.03, 1.64)
			14	49	3	0.37 (0.18, 0.75)
	Vaccine 3	3	4	39	3	0.25 (0.04, 1.65)
			14	49	8	0.29 (0.19, 0.44)
	Saline Control	4	4	39	2	0.28 (0.00, 577.14)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.03 (0.00, 0.27)
			14	49	1	0.02 (--)
	Vaccine 2	2	4	39	3	0.01 (0.01, 0.02)
			14	49	3	0.01 (0.00, 0.04)
	Vaccine 3	3	4	39	3	0.01 (0.00, 0.03)
			14	49	8	0.01 (0.01, 0.02)
	Saline Control	4	4	39	2	0.04 (0.00, 0.35)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.16 (0.05, 0.53)
			14	49	1	0.01 (--)
	Vaccine 2	2	4	39	3	0.10 (0.018, 0.51)
			14	49	3	0.01 (0.00, 0.02)
	Vaccine 3	3	4	39	3	0.01 (0.00, 0.04)
			14	49	8	0.02 (0.01, 0.04)
	Saline Control	4	4	39	2	0.06 (0.00, 22.84)
			14	49	NA	NA

Table 28. Fold-change in IFN-β mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Sample Type	Treatment	Group	Day Post Challenge	Study Day	N	Geometric Mean (95% Confidence Interval)
Brain	Vaccine 1	1	4	39	3	0.31 (0.02, 5.97)
			14	49	1	0.09 (--)
	Vaccine 2	2	4	39	3	0.09 (0.02, 0.49)
			14	49	3	0.09 (0.02, 0.32)
	Vaccine 3	3	4	39	3	0.12 (0.02, 0.82)
			14	49	8	0.07 (0.04, 0.14)
	Saline Control	4	4	39	2	0.26 (0.00, 227.26)
			14	49	NA	NA
Liver	Vaccine 1	1	4	39	3	0.79 (0.06, 10.28)
			14	49	1	0.05 (--)
	Vaccine 2	2	4	39	3	0.22 (0.05, 0.97)
			14	49	3	0.08 (0.02, 0.28)
	Vaccine 3	3	4	39	3	0.16 (0.01, 2.87)
			14	49	8	0.27 (0.06, 1.12)
	Saline Control	4	4	39	2	1.31 (0.15, 11.72)
			14	49	NA	NA
Lung	Vaccine 1	1	4	39	3	0.76 (0.01, 69.78)
			14	49	1	0.04 (--)
	Vaccine 2	2	4	39	3	1.73 (0.52, 5.67)
			14	49	3	0.03 (0.00, 0.32)
	Vaccine 3	3	4	39	3	0.03 (0.01, 0.09)
			14	49	8	0.03 (0.01, 0.05)
	Saline Control	4	4	39	2	0.24 (0.00, 11.81)
			14	49	NA	NA

Table 29. Fold-change in IFN-γ mRNA Levels in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus

-- Confidence interval was not calculated since there was only one observation available.
NA No data were available.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IL-1β	Brain	39	0.05	
		49	<0.00*	0.10 (1<5) 0.00 0.04 (2<5) <0.00 0.045 (3<5) <0.00
	Liver	39	0.44	
		49	0.00*	0.44 (2<3) 0.05 0.22 (2<5) 0.00
	Lung	39	0.30	
		49	0.01*	0.12 (2<5) 0.02 0.14 (3<5) 0.01
IL-2	Brain	39	0.00*	0.01 (1<5) 0.01 0.00 (2<5) 0.00 0.01 (3<5) 0.01 0.00 (4<5) 0.01
		49	<0.00*	0.01 (1<5) 0.00 0.00 (2<5) <0.00 0.00 (3<5) <0.00
	Liver	39	<0.00*	0.02 (1<5) <0.00 0.01 (2<5) <0.00 0.01 (3<5) <0.00 0.02 (4<5) <0.00
		49	<0.00*	0.02 (1<5) 0.00 0.01 (2<5) <0.00 0.01 (3<5) <0.00
	Lung	39	<0.00*	0.01 (1<5) <0.00 0.00 (2<5) <0.00 0.01 (3<5) 0.00 0.00 (4<5) <0.00
		49	<0.00*	0.01 (1<5) 0.00 0.01 (2<5) <0.00 0.01 (3<5) <0.00

Table 30. Results of ANOVA Models Fitted to ΔCt for Cytokine Gene Expression in Brain, Liver, and Lung from Ferrets Challenged with H5N1 virus.

Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IL-4	Brain	39	<0.00*	0.00 (1<5) 0.00 0.00 (2<5) 0.00 0.00 (3<5) 0.00 0.00 (4<5) 0.00
		49	<0.00*	0.00 (1<5) 0.00 0.00 (2<5) <0.00 0.00 (3<5) <0.00
	Liver	39	<0.00*	0.00 (1<5) <0.00 0.00 (2<5) <0.00 0.00 (3<5) <0.00 0.00 (4<5) <0.00
		49	<0.00*	6.08 (1>3) 0.01 0.00 (1<5) <0.00 2.86 (2>3) 0.01 0.00 (2<5) <0.00 0.00 (3<5) <0.00
	Lung	39	0.00*	0.00 (1<5) 0.01 0.00 (2<5) 0.00 0.00 (4<5) 0.00
		49	<0.00*	0.00 (1<5) <0.00 0.00 (2<5) <0.00 0.00 (3<5) <0.00
IL-6	Brain	39	0.03*	0.06 (2<5) 0.05
		49	<0.00*	0.05 (1<5) 0.00 0.05 (2<5) <0.00 0.04 (3<5) <0.00
	Liver	39	0.01*	0.07 (1<5) 0.05 0.02 (2<5) 0.01 0.03 (3<5) 0.01
		49	<0.00*	0.02 (1<5) 0.00 0.02 (2<5) <0.00 0.03 (3<5) <0.00
	Lung	39	0.02*	0.07 (3<5) 0.0233
		49	<0.00*	0.04 (1<5) 0.00 0.04 (2<5) <0.00 0.05 (3<5) <0.00

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IL-8	Brain	39	0.01*	0.04 (2<4) 0.03 0.06 (2<5) 0.03 0.03 (3<4) 0.01 0.04 (3<5) 0.01
		49	<0.00*	0.05 (1<5) 0.01 0.04 (2<5) 0.00 0.02 (3<5) <0.00
	Liver	39	0.64	
		49	0.18	
	Lung	39	0.04*	8.35 (2>3) 0.05
		49	0.01*	0.04 (3<5) 0.01
IL-12p40	Brain	39	0.04*	0.06 (3<5) 0.02
		49	<0.00*	0.04 (1<5) 0.00 0.03 (2<5) <0.00 0.03 (3<5) <0.00
	Liver	39	0.01*	4.70 (1>3) 0.03 5.41 (2>3) 0.02
		49	0.34	
	Lung	39	0.00*	6.27 (1>3) 0.03 0.13 (1<5) 0.02 5.78 (2>3) 0.04 0.12 (2<5) 0.01 0.02 (3<5) 0.00 0.06 (4<5) 0.00
		49	<0.00*	0.02 (1<5) 0.00 0.02 (2<5) <0.00 0.02 (3<5) <0.00

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
TNF-α	Brain	39	0.00*	8.04 (1>3) 0.04 20.58 (1>5) 0.00 8.56 (2>5) 0.03 0.07 (3<4) 0.02 37.21 (4>5) 0.00
		49	0.06	
	Liver	39	0.00*	5.18 (1>3) 0.01 10.29 (1>5) 0.00 6.33 (2>5) 0.01 0.17 (3<4) 0.02 10.65 (4>5) 0.00
		49	0.17	
	Lung	39	0.01*	3.62 (1>3) 0.02 4.02 (1>5) 0.01 2.96 (2>3) 0.04 3.29 (2>5) 0.03
		49	0.30	

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IFN-α	Brain	39	0.00*	0.02 (1<5) 0.00 0.02 (2<5) 0.00 0.03 (3<5) 0.01 0.03 (4<5) 0.01
		49	<0.00*	0.02 (1<5) <0.00 0.02 (2<5) <0.00 0.01 (3<5) <0.00
	Liver	39	<0.00*	0.01 (1<5) <0.00 0.00 (2<5) <0.00 0.00 (3<5) <0.00 0.01 (4<5) 0.00
		49	<0.00*	0.02 (1<5) 0.00 0.01 (2<5) <0.00 0.01 (3<5) <0.00
	Lung	39	<0.00*	5.38 (1>3) 0.03 0.04 (1<5) 0.00 0.03 (2<5) 0.00 0.01 (3<5) <0.00 0.01 (4<5) <0.00
		49	<0.00*	0.01 (1<5) 0.00 0.00 (2<5) <0.00 0.01 (3<5) <0.00

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Table 30. (Continued).

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
IFN-β	Brain	39	0.03*	0.14 (1<5) 0.02
		49	0.00*	0.34 (1<5) 0.01 0.37 (2<5) 0.00 0.29 (3<5) <0.00
	Liver	39	<0.00*	0.03 (1<5) 0.00 0.01 (2<5) <0.00 0.15 (3<4) 0.03 0.01 (3<5) <0.00 0.04 (4<5) 0.00
		49	<0.00*	0.02 (1<5) 0.00 0.01 (2<5) <0.00 0.01 (3<5) <0.00
	Lung	39	<0.00*	14.78 (1>3) 0.00 0.16 (1<5) 0.01 9.04 (2>3) 0.00 0.10 (2<5) 0.00 0.19 (3<4) 0.04 0.01 (3<5) <0.00 0.06 (4<5) 0.00
		49	<0.00*	0.01 (1<5) 0.00 0.01 (2<5) <0.00 0.02 (3<5) <0.00
IFN-γ	Brain	39	0.03*	0.0903 (2<5) 0.0265 0.1158 (3<5) 0.0470
		49	<0.00*	0.09 (1<5) 0.00 0.09 (2<5) <0.00 0.07 (3<5) <0.00
	Liver	39	0.04*	
		49	0.13	
	Lung	39	0.00*	29.97 (1>3) 0.01 68.01 (2>3) 0.00 0.03 (3<5) 0.01
		49	<0.00*	0.04 (1<5) 0.00 0.03 (2<5) <0.00 0.03 (3<5) <0.00

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Sample Type	Study Day	Group Effect P-value	Estimated Ratio of $2^{-\Delta Ct}$ (Relationship) Tukey's P-Value #
TGFB-β1	Brain	39	0.54	
		49	0.41	
	Liver	39	0.02*	2.72 (1>3) 0.03 0.31 (3<4) 0.03
		49	0.28	
	Lung	39	0.71	
		49	0.41	
TGFB-β2	Brain	39	0.09	
		49	0.15	
	Liver	39	0.35	
		49	0.17	
	Lung	39	0.05*	0.28 (1<3) 0.05
		49	0.60	

Table 30. (Continued).

\# Cells contain all significant pairwise group comparisons at the 0.05 level. The format within each cell is: (1) the ratio of geometric means, (2) the relationship between corresponding group geometric means, and (3) the Tukey-adjusted P-value.

* The overall group effect is significant at the 0.05 level.

Cytokine	Days Post Challenge	
	4	14
IL-1β	-	+
IL-2	+	+
IL-4	+	+
IL-6	+	+
IL-8	+	+
IL-12p40	+	+
TNF-α	+	-
IFN-α	+	+
IFN-β	+	+
IFN-γ	+	+
TGFB-β1	-	-
TGFB-β2	-	-

Table 31. Summary of Significance of Differences in Cytokine Expression Between Groups in Brain from Ferrets Challenged with H5N1 virus

+ = Significant differences between groups (P-value <0.05)

- = No significant differences between groups (P-value ≥0.05)

Significance was determined by calculating P-values using ΔC_T. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. Data from terminal animals are not tabulated since significant differences cannot be determined.

Cytokine	Days Post Challenge	
	4	14
IL-1β	-	+
IL-2	+	+
IL-4	+	+
IL-6	+	+
IL-8	-	-
IL-12p40	+	-
TNF-α	+	-
IFN-α	+	+
IFN-β	+	+
IFN-γ	+	-
TGFB-β1	+	-
TGFB-β2	-	-

Table 32. Summary of Significance of Differences in Cytokine Gene Expression Between Groups in Liver from Ferrets Challenged with H5N1 virus

+ = Significant differences between groups (P-value <0.05)

- = No significant differences between groups (P-value ≥0.05)

Significance was determined by calculating P-values using ΔC_T. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus was on Day 35. Data from terminal animals are not tabulated since significant differences cannot be determined.

Cytokine	Days Post Challenge	
	4	14
IL-1β	-	+
IL-2	+	+
IL-4	+	+
IL-6	+	+
IL-8	+	+
IL-12p40	+	+
TNF-α	+	-
IFN-α	+	+
IFN-β	+	+
IFN-γ	+	+
TGFB-β1	-	-
TGFB-β2	+	-

Table 33. Summary of Significance of Differences in Cytokine Gene Expression Between Groups in Lung from Ferrets Challenged with H5N1 virus

+ = Significant differences between groups (P-value <0.05)

- = No significant differences between groups (P-value ≥0.05)

Significance was determined by calculating P-values using ΔC_T. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Challenge with H5N1 virus was on Day 35. Data from terminal animals are not tabulated since significant differences cannot be determined.

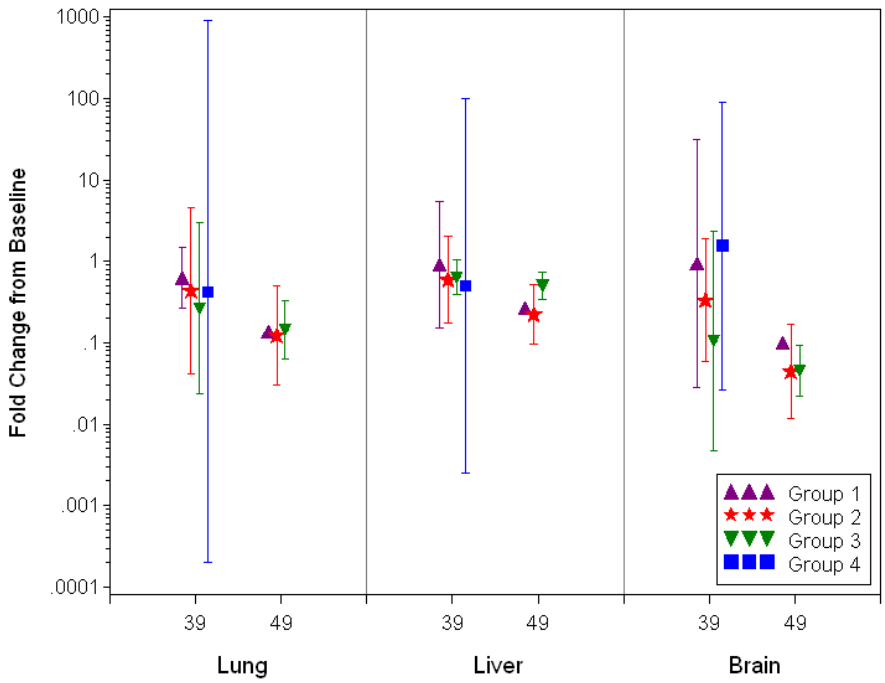

Figure 13. Fold Change in IL-1β mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

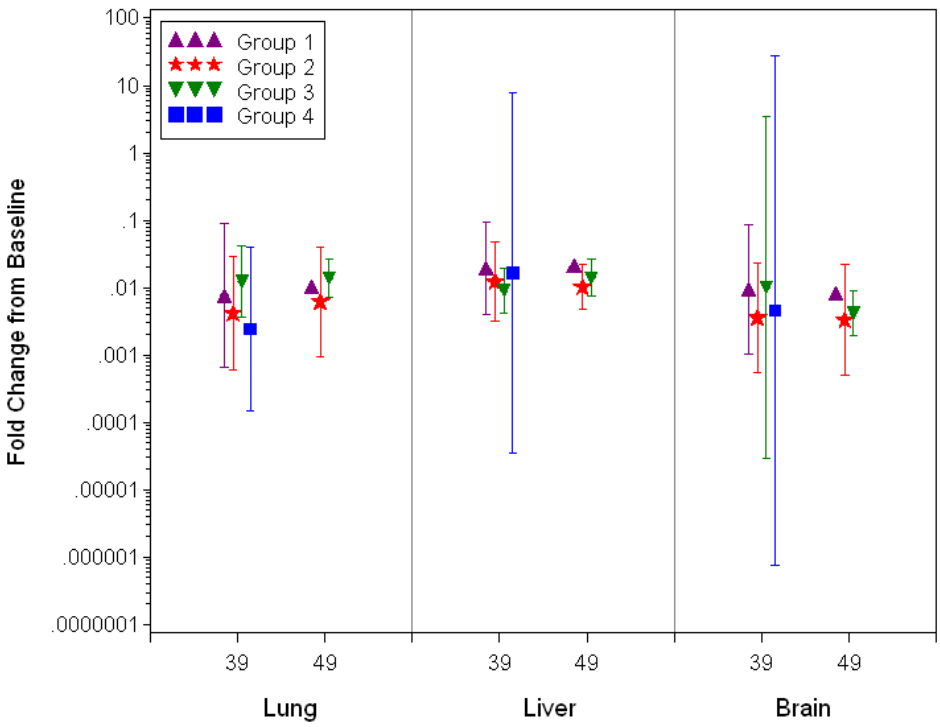

Figure 14. Fold Change in IL-2 mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

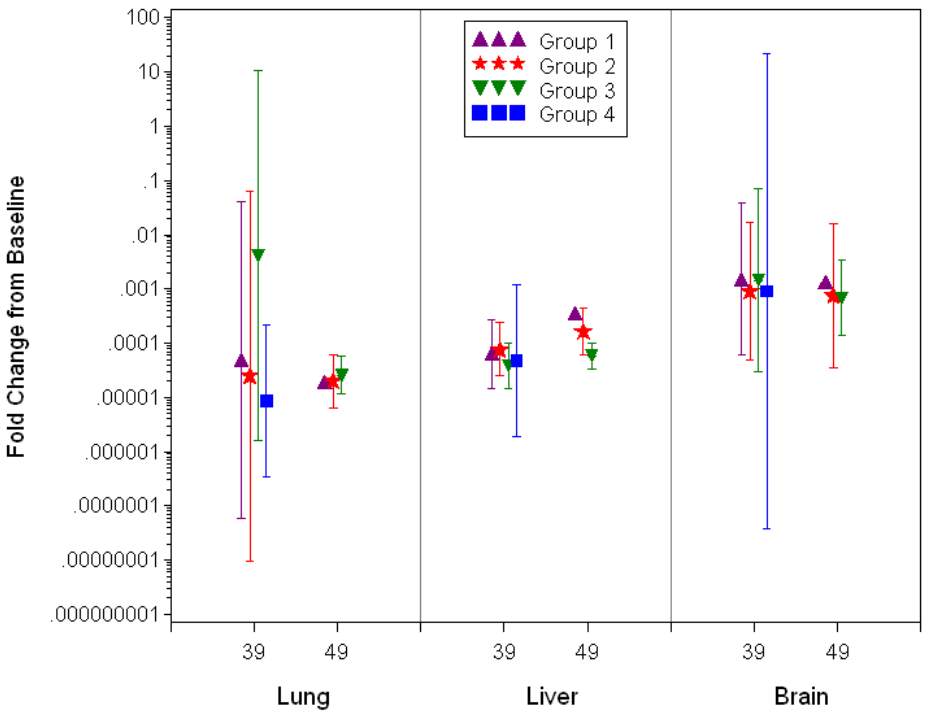

Figure 15. Fold Change in IL-4 mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

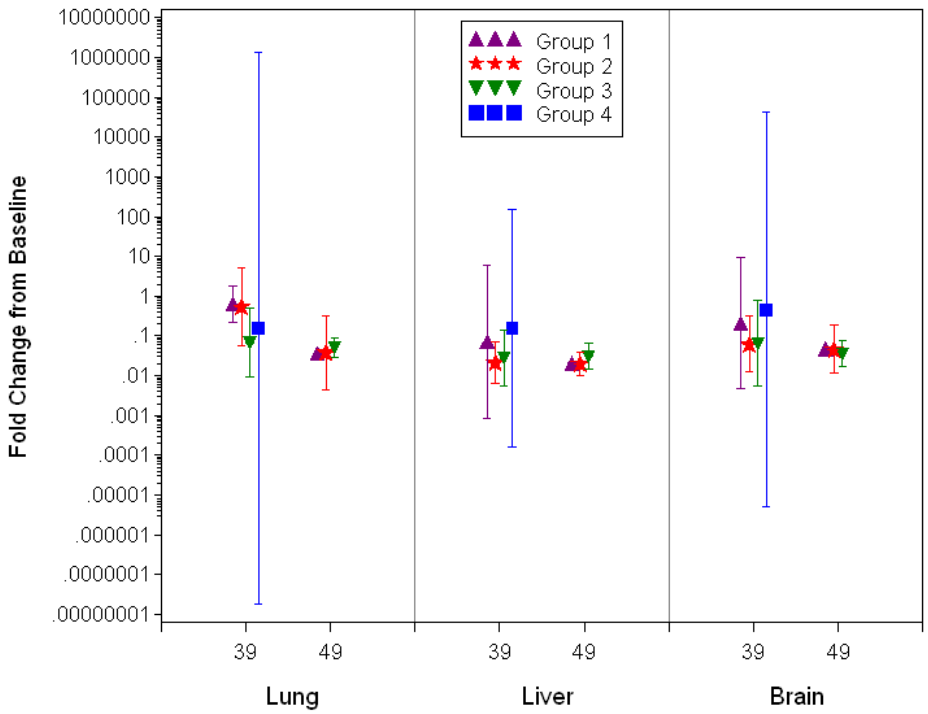

Figure 16. Fold Change in IL-6 mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

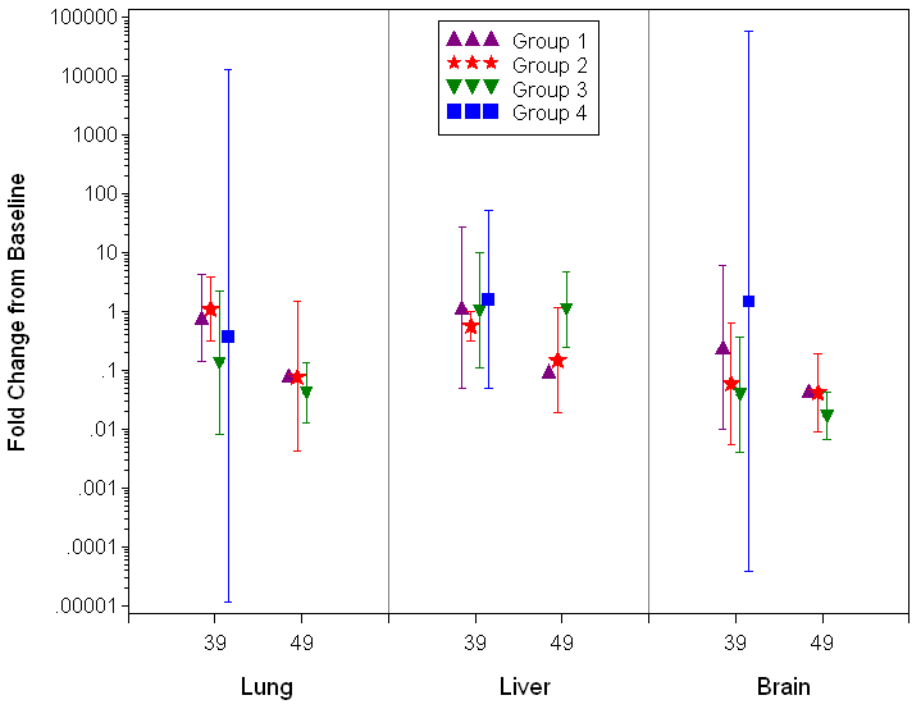

Figure 17. Fold Change in IL-8 mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.

Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

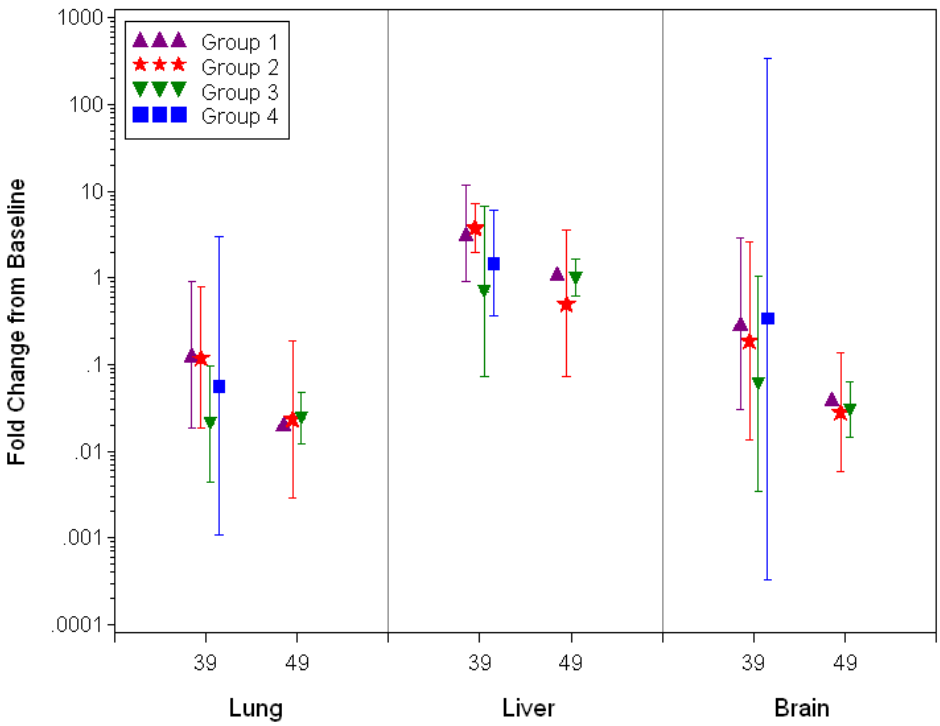

Figure 18. Fold Change in IL-12p40 mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

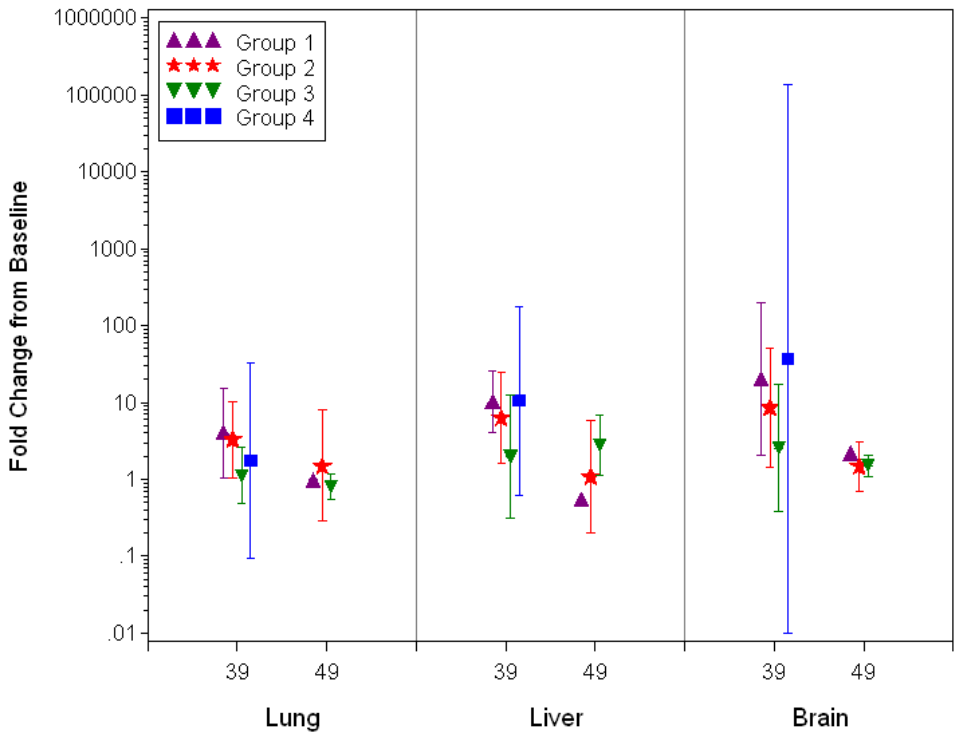

Figure 19. Fold Change in TNF-α mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

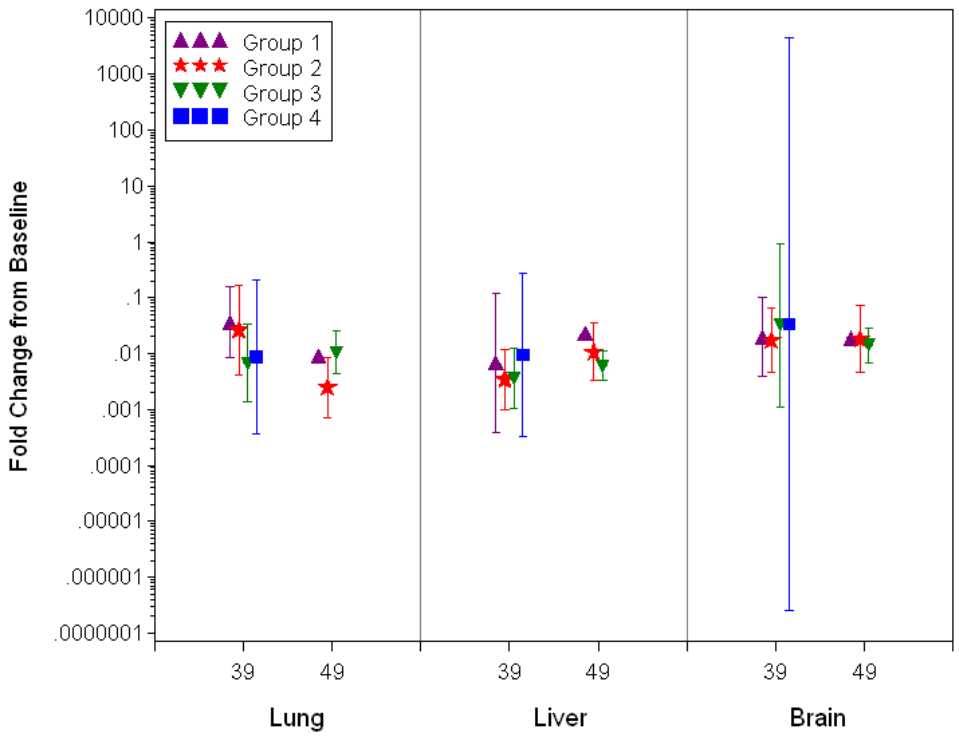

Figure 20. Fold Change in IFN-α mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

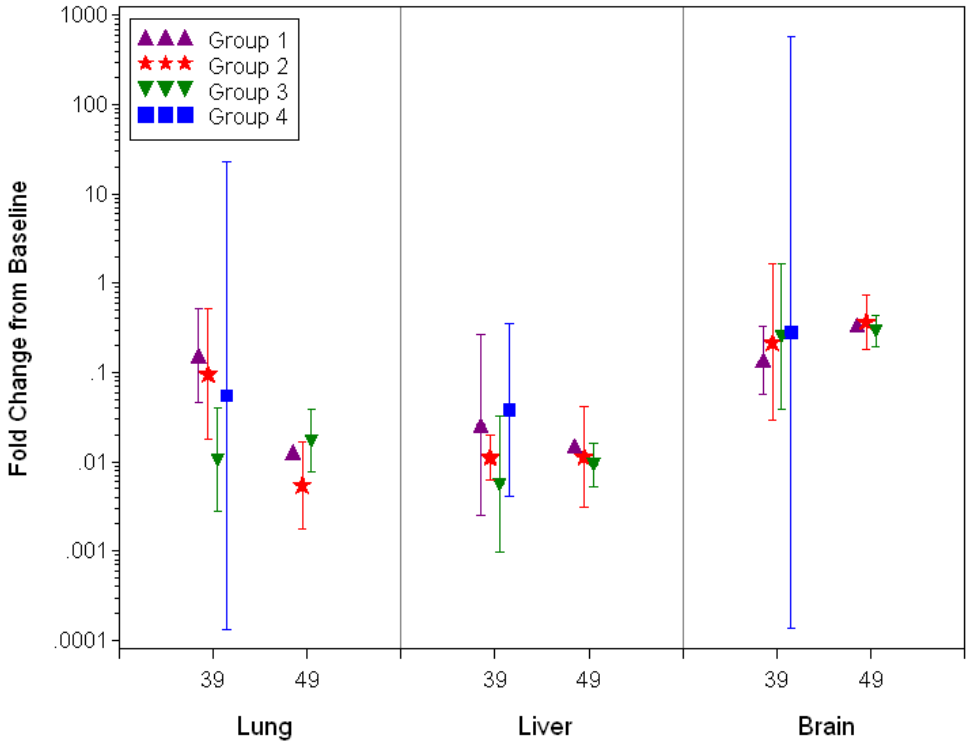

Figure 21. Fold Change in IFN-β mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

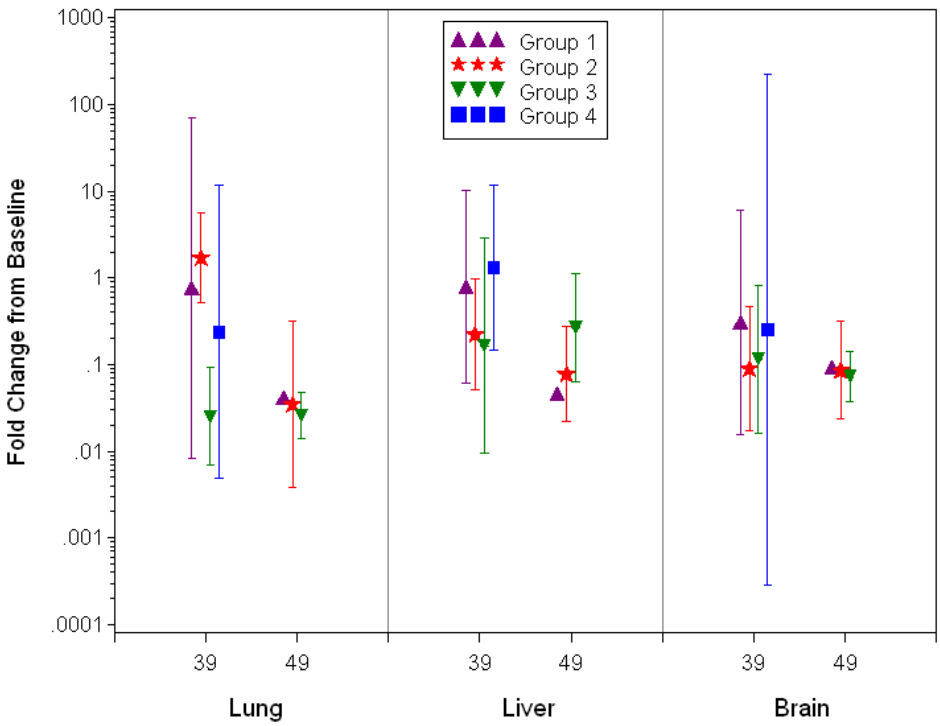

Figure 22. Fold Change in IFN-γ mRNA Levels in Lung, Liver, and Brain from Ferrets Challenged with H5N1 Virus.
Fold changes are expressed as $2^{-\Delta\Delta CT}$, using GAPDH expression as the calibrator. The baseline is expression from 3 naïve ferrets. All treatments were on Day 0 and Day 21 (35 and 14 days before challenge). Group 1 was treated with Vaccine 1, Group 2 was treated with Vaccine 2, Group 3 was treated with Vaccine 3, and Group 4 was treated with a saline control. Ferrets were challenged with H5N1 virus on Day 35.

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