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Ferret TNF- α and IFN- γ Immunoassays

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1. Introduction

Despite the prominent use of ferrets in medical research, the immune system of ferrets remains poorly characterized (Svitek & von, V, 2007). Here we describe ferret TNF- α and IFN- γ immunoassays.

Specifically, this covers the following topics:

- Background: The use of ferrets in medical research
- TNF- α and IFN- γ cloning and sequencing
- Expression and purification of recombinant ferret TNF- α and IFN- γ proteins
- Cytokine real-time PCR based assays
- Development of IFN- γ and TNF- α hybridoma clones
- ELISA and ELISPOT assays for the ferret cytokine IFN- γ

2. Background

This Background describes the current use of ferrets in medical research but also includes a description of past uses. The importance of ferrets is highlighted in human disease modeling and in prophylactic and vaccine development. The various uses of ferrets in medical research demonstrate the need for immune profiling reagents and assays.

2.1 Biology of the ferret

The ferret, *Mustela putorius furo*, is a relatively small and inexpensive animal in terms of its potential for research use, although mice remain the traditional influenza model for virus pathogenesis.

Essentially, mice are low cost animals that have a broad availability of corresponding reagents for immunological investigation. Mice are easily mutated and there exists a plethora of currently available transgenic mice with immune targeted gene deletions or gene knock-ins (Belser, Szretter, Katz, & Tumpey, 2009). Two factors against the use of mice in influenza immune studies are 1) most human influenza strains must be mouse adapted prior to initiation of infection studies due to the inability of human influenza viruses to replicate in the mouse and 2) mice do not exhibit human-like clinical signs of influenza such as sneezing and temperature fluxes.

In contrast to mice, ferrets do develop respiratory illnesses that are similar to human disease. Although the ferret is not considered a large laboratory animal, it is able to provide many biological samples for pathological testing during an infection study. For instance, frequent and sizable blood sampling is feasible in the ferret that is not practical in smaller rodents such as in mice and rats.

Furthermore, clinical features of disease are easily observed. Such as clinical fever manifested as an elevation in body temperatures can be detected as early as 1 day following infection with many viruses. Our own as well as previously published studies have shown that high fevers can persist for many days following infection of viruses such as H1N1pdm influenza (Rowe et al., 2010b; Sweet et al., 1979; Zitzow et al., 2002). As well as fever, nasal discharge, sneezing and activity level can also be observed in ferrets infected with influenza viruses (Rowe et al., 2010b). Taken together, these clinical features along with the feasibility for blood and pathological sampling suggest the ferret to be an optimal animal for the study of human infectious diseases, including influenza viruses. For example, our group has recently used the ferret model successfully to characterize and compare immunopathology caused by several strains of currently circulating influenza A and B viruses (Huang et al., 2011).

2.2 Respiratory viral infections

Viral respiratory infectious diseases are a major worldwide concern which causes significant morbidity and mortality (Kolling et al., 2001). Respiratory viral diseases such as the severe acute respiratory syndrome coronavirus (SARS-CoV), avian influenza H5N1 and pandemic influenza H1N1 virus are potential epidemic and/or pandemic threats (Dushoff, Plotkin, Viboud, Earn, & Simonsen, 2006; Weiss & McMichael, 2004; Dawood, Dalton, Durrheim, & Hope, 2009; Dawood et al., 2009). Specifically, influenza is a significant contributor to morbidity and mortality worldwide and is the focus of our laboratory and the focus disease of this chapter.

The World Health Organization estimates the burden of season influenza to be approximately one billion cases annually, including 3-5 million severe cases and 300,000-500,000 deaths (Girard, Cherian, Pervikov, & Kieny, 2005). Influenza illness in humans is caused by an influenza RNA virus of the *Orthomyxoviridae* family. The influenza virus can be categorized as one of three types: A, B, or C (Steinhauer & Skehel, 2002). Importantly, the influenza viral genome is susceptible to two primary types of genetic mutations that cause variation in the immunogenic proteins and subsequently in disease presentation and clinical features (Kasowski, Garten, & Bridges, 2011; Steinhauer & Skehel, 2002). Firstly, antigenic drift is defined by minor changes to the viral genome introduced during virus RNA

replication (Steinhauer & Skehel, 2002). Second, antigenic shift is when a host is infected by various influenza strains at the same time allowing entire genome segments to be reassorted during co-infection. This results in novel influenza strains markedly distinct from their progenitors (Steinhauer & Skehel, 2002; Kasowski et al., 2011). From these reassortments, novel influenza strains may arise with new clinical symptoms and disease features that have the potential to be highly pathogenic, easily transmissible with pandemic potential.

The most significant recent reassortant to emerge was the 2009 pandemic H1N1 influenza A (H1N1pdm) strain (Perez-Padilla et al., 2009). H1N1pdm is closely related to the reassortant swine influenza A viruses previously isolated in North America, Europe, and Asia (Trifonov, Khiabani, & Rabadan, 2009). Infection with H1N1pdm resulted in diverse clinical outcomes. The majority of reported cases were mild and self-limiting (Gilsdorf, Poggensee, & Working Group, 2009; Nicoll & Coulombier, 2009; Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic, 2010) and typical symptoms include fever, sore throat, malaise, and headache (Health Protection Agency, Health, National Public Health Service for Wales, & HPA Northern Ireland Swine influenza investigation team, 2009). A small proportion of pandemic influenza cases required hospitalization and patient ventilator support (Centers for Disease Control and Prevention (CDC), 2009; Kumar et al., 2009; Perez-Padilla et al., 2009; Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic, 2010). Common complications in these severe cases were severe hypoxemia, shock, pneumonia, and acute respiratory distress syndrome (ARDS) (Perez-Padilla et al., 2009; Kumar et al., 2009; Centers for Disease Control and Prevention (CDC), 2009). Nonpulmonary acute organ dysfunction has also been reported (Uyeki, Sharma, & Branda, 2009; Kumar et al., 2009). Since ferrets show signs of illness and are easily infected with human strains of H1N1pdm, we are currently investigating and have published on the immunopathogenic mechanisms and possible therapeutics for H1N1pdm illness in ferrets (Rowe et al., 2010a; Huang S.S.H. et al., 2011; Cameron et al., 2008).

2.3 Host immune responses

Host immunity can be broken down into an innate and adaptive immune response. The innate immune response is a nonspecific attack on the invading agent while the adaptive immune response is an attack tailored to the individual pathogen (Ryan & Majno, 1977). What determines the type of triggered immune response is the invading agent itself. The agent is recognized first by the innate immune arm and together the innate and adaptive immune responses lead to a unique immune signature that for each pathogen. Furthermore, the clinical outcome is the biological consequence of the immune response that has developed toward the pathogen (Belz, Bedoui, Kupresanin, Carbone, & Heath, 2007; Zheng et al., 2007).

In order to evaluate the immune response during the course of viral infection, it is important to be able to determine the activity of the immune cells. Cell identity and their activation status are distinguished by the molecules expressed at the cell surface. Furthermore, cells of the immune system can be described as innate or adaptive immune cells. Innate immune cells include neutrophils, eosinophils, basophils, macrophages and NK cells. Adaptive immune cells include T lymphocytes, B lymphocytes and Dendritic cells (Hauge, Madhun, Cox, Brokstad, & Haaheim, 2007). Many of these cells have yet to be characterized in the ferret.

As well as understanding the cell activation and cellular populations during an immune response, it is also important to elucidate the intracellular activation and intercellular events which occur following infection. Cells are often activated by cytokines, soluble extracellular proteins that mediate signals from one cell to another. Once the cell has been in contact with a cytokine, intracellular signalling cascades are activated. The activation of these signalling cascades leads to cell effector function.

One of the most prominent branches of cytokine-cell signalling events is of the inflammatory interferon (IFN) cytokines which connect the innate immune response with the activation of the adaptive immunity. The IFN family of cytokines can be categorized as either Type I IFN or Type II. IFN- α and IFN- β are of the Type I IFN cytokines and have a prominent role during viral infection. IFN- γ is of the Type II IFN family. IFN- γ also plays a role in viral infections but also functions during bacterial infections.

The release of IFN- γ leads to cellular activation through signalling pathways. Ligation of the interferon receptors 1 and 2 (IFNAR1 and IFNAR2 for IFN- α and IFN- β ; IFNGR1 and IFNGR2 for IFN- γ) with an IFN cytokine induces IFN signaling pathways and promotes IFN gene induction. Both the Type I and Type II cytokines signal through JAK-STAT pathways to activate IFN genes and promote immune responses (Marijanovic, Ragimbeau, van der Heyden, Uze, & Pellegrini, 2007). IFN induced JAK-STAT signalling often involves interferon regulatory factor 9 (IRF9) (Takaoka & Yanai, 2006). The interferon stimulatory factor 3 complex (ISGF3) binds to interferon-stimulated response element (ISRE) and activates transcription of IFN- α inducible genes, including 2'-5' oligoadenylate synthase 1 (OAS1), myxovirus resistance 1 (MX1), interferon stimulated gene 15 (ISG15) and many other IFN-response genes (IRGs) (Uddin & Platanias, 2004). The expression of IFN- γ -induced protein IP10, or CXCL10, following IFN stimulation, is often considered a hallmark of virus infection in host organisms. IFN- α stimulation ultimately promotes a cellular antiviral state which is hallmarked by the upregulation of IRGs (Chevaliez & Pawlotsky, 2009). Although IFN signalling gene upregulation during viral infection has been the subject of previous reports, there is little information regarding the host immune responses directly induced by viruses versus those that are upregulated due to secondary IFN stimulation (Chelbi-Alix & Wietzerbin, 2007; Haagmans et al., 2004; Loutfy et al., 2003; Cameron et al., 2007). Therefore there is a need for the study of IFN signalling and IFN stimulated events during viral infection which can be investigated using the ferret model.

TNF (Tumor Necrosis Factor) - α is a cytokine produced mainly by activated macrophages and T-lymphocytes, and exerts a multitude of biological activities including cytotoxic effects upon certain tumours and virus- infected cells, immunomodulation, and regulation of cellular proliferation (Vilcek & Lee, 1991). TNF- α is a potent inhibitor of influenza replication in vitro (Seo & Webster, 2002), and the induction of TNF- α expression has been associated with ARDS-like symptoms in H5N1 infected mice (Xu et al., 2006). Depletion of TNF- α in influenza or respiratory syncytial virus-infected animals significantly reduced pulmonary inflammation and cytokine production without compromising viral clearance, and almost completely abolished any associated weight loss and observable illness (Hussell, Pennycook, & Openshaw, 2001). There is evidence to suggest that hyper-production of TNF- α contributes to the high degree of virulence exhibited by H5N1 strains in humans. Using primary cultures of human monocyte-derived macrophages, Cheung et al. demonstrated a

significant increase in TNF- α gene transcription and protein expression in H5N1-infected cells compared to that of H1N1- or H3N2- infected cells (Cheung et al., 2002). Dysregulation of cytokines, including TNF- α , is thought to contribute to the immunopathogenesis of influenza and SARS CoV virus infections, however, the *in vivo* mechanism is unknown.

2.4 Examining the ferret host immune response in respiratory diseases

When infected with respiratory viruses ferrets display many of the symptoms and pathological features seen in infected humans (Darnell et al., 2007; Martina et al., 2003; Peltola, Boyd, McAuley, Rehg, & McCullers, 2006). The ferret model has been used in influenza research since the influenza virus was first isolated (Bouvier & Lowen, 2010; Lambkin et al., 2004; Small, Jr., Waldman, Bruno, & Gifford, 1976). Importantly, ferrets and humans have similar lung physiology allowing influenza to infect both species through a comparable mechanism, sialic receptors the host receptor for influenza (Maher & DeStefano, 2004; van et al., 2007). Furthermore, as ferrets are highly susceptible to influenza virus, they can also transmit the influenza virus from infected to healthy ferrets (Smith et al., 1933).

As well as influenza, ferrets have shown promise as a model for other respiratory viruses such as Severe Acute Respiratory Syndrome Corona virus (SARS Covirus), the BSL-4 Nipah virus and morbilliviruses and other pathogens such as gastro-intestinal bacteria and prions (Bouvier, Lowen, & Palese, 2008; van den Brand et al., 2008; Bossart et al., 2009; Svitek & von, V, 2007; ter et al., 2006; Martina et al., 2003).

2.5 The use of ferrets for the investigation of influenza therapeutics

Not only are ferrets useful for infectious disease modeling but they are also a good platform for testing and developing viral therapeutics. Currently ferrets are used for influenza drug testing; for example, neuraminidase inhibitors are effective during ferret influenza infection (Mendel et al., 1998; Govorkova et al., 2007; Yun et al., 2008). As well, ferrets display immunological memory and are thus useful for testing the safety and efficacy of vaccines (Bouvier & Lowen, 2010; Maher & DeStefano, 2004; Gupta, Earl, & Deem, 2006). Ferrets have been used to investigate SARS and influenza vaccines (Bouvier & Lowen, 2010; Maher & DeStefano, 2004; Gupta et al., 2006).

Previously, we sought to elucidate the ferret immune response during viral infection and identify potential therapeutic drug targets (Danesh et al., 2011). We investigated the genetic programs and cell signaling pathways that were regulated by SARSCoV infection compared to IFN- α 2b stimulation in the ferret model (Danesh et al., 2011). The phosphorylation status of signaling molecules in IFN- α 2b-stimulated peripheral blood mononuclear cells (PBMCs) was examined with the end of identifying kinase inhibitors that may be useful in SARS pathogenesis. We found IFN- α 2b caused STAT1 phosphorylation in *in vitro* experiments (Danesh et al., 2011). Importantly, gene expression profiles of PBMCs as well as lung necropsies of SARS-CoV-infected ferrets identified 7 upregulated IRGs that were similarly upregulated in response to IFN- α 2b injection (Danesh et al., 2011). In summary, IFN- α 2b injection and SARS-CoV infection led to both similar as well as unique gene expression signatures (Danesh et al., 2011). Taken together, increased knowledge of these gene expression signatures and signalling pathways will improve the understanding of the ferret immune system and lead to possible therapeutic drug targets.

2.6 Recent advances in ferret reagent development

Although researchers are able to use direct infection of human influenza strains and monitor biological clinical signs with the ferret model, there is a paucity of reagents for influenza investigative studies in the ferret. As well, there is a lack of information on the ferret immune system that has slowed the progress of this system. Specifically, the lack of ferret specific antibodies capable of detecting surface molecules of immune cells and reagents for ferret inflammatory mediators such as cytokines has hindered the immune profiling in ferret infectious disease models.

Recently, we reported the characterization of the ferret chemokines, CXCL9, CXCL10 and CXCL11, which are important in migration of mononuclear cells to sites of infection (Danesh et al., 2008). We have previously characterized ferret cytokine and chemokine genes as well as have developed immunological assays for evaluating the ferret immune system following SARS and influenza infection (Cameron et al., 2008; Danesh et al., 2008; Ochi et al., 2008). As both IFN- γ and TNF- α are significant hallmarks of adaptive immunity, these cytokines are useful markers when studying the viral immune response.

3. Ferret immunoassays

3.1 TNF- α and IFN- γ cloning and sequencing

The methods used for cloning ferret TNF- α and IFN- γ genes are covered in this section. ClustalW alignments of ferret genes with orthologues from other mammalian species such as canine are also shown.

3.1.1 Methods

Animals

Six-month-old male ferrets (*Mustela putorius furo*) were obtained from Triple F Farms Inc. (Sayre, Pa. USA). Animals were housed at Toronto General Research Institute animal facility and the animal use protocol was approved by the animal care committee of the University Health Network, Toronto, Ontario. Animals were quarantined and monitored for one week before tissue, blood collection and project initiation. Animal diets are based on a low fat, high protein regimen, recommended by Triple F Farms for small carnivores.

Total RNA purification of ferret IFN- γ and TNF- α

Ferret whole blood was diluted at a ratio of 1:1 with RPMI (Invitrogen, Mississauga, Canada) and blood was stimulated with mitogens, LPS (1 μ g/ml, Sigma Chemicals, St. Louise, MO, USA), PMA (50 ng/ml, Sigma), ionomycin (0.1 mM, Sigma) or poly I:C (25 μ g/ml, Sigma) by incubating at 37°C in 5% CO₂ for 2, 4, 8, and 12 hrs. Following cell stimulation, RNA was isolated using the Paxgene RNA isolation method (Qiagen, Mississauga, Canada) according to manufacturer's protocols. cDNA was synthesized from purified total RNA by reverse transcriptase II (Invitrogen) according to supplier's instructions.

Cloning, sequencing and expression of ferret TNF- α and IFN- γ

Gene specific primers were used to amplify ferret TNF- α and IFN- γ by PCR. Primers were designed based on highly conserved regions of the nucleotide gene sequences. These

regions were identified through ClustalW-based multiple sequence alignments of the TNF- α and IFN- γ genes from several species (ClustalW 1.83, European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/>) and are shown in Table 1. Accession numbers used for ClustalW alignments of IFN- γ are as follows: Eurasian badger; Y11647, rabbit; P30123, cat; P46402, dog; P42161, mouse; P01580, and human; P01579. The Gene peptide accession numbers for IFN- γ are: badger, CAA72346; dog, AAD314233; panda, ABE02189; cat, BAA06309; rhinoceros, ABC18310; donkey, AAC42595; pig, ABG56234; dolphin, BAA82042; sheep, ABD64367; buffalo, BAE75855; cow, NP_776511; armadillo, AAZ57195; woodchuck, AAC31963; rabbit, BAA24439; human, P01579; monkey, AAM21477; mouse, P01580; rat, NP_620235; chicken, CAA69227; zebrafish, BAD06253.

Primers	5'-3' sequence
Consensus cloning primers*	
Forward	ATGAGCACTGAAAGCATGATCC
Reverse	GTCTACTTTGGGATCATTGCCCTGTGA
5' RACE Primers	
Adapter outer primer	GCTGATGGCGATGAATGAACACTG
Adapter inner primer	GCGGATCCGAACACTGCGTTTGCTGGCTT
	TGATG
Gene specific primers	
Inner primer	CGACGAGGAGGAAGGAGAAGA
Outer primer	CAGAGGTTTGATTAGTTGGAGGCC
3' RACE Primers	
Adapter outer primer	GCGAGCACAGAATTAATACGACT
Adapter inner primer	GCGGATCCGAATTAATACGACTCACTAT
	AGG
Gene specific primers	
Inner primer	ATCAACCTGCCTGCCTATCTCG
Outer primer	TCAACCTCCTGTCTGCCATCAAG
Expression-construct cloning primers	
Forward primer	AACGCCATGAGTACTGAAAGCATGATCC
Reverse prime	CAGGGCAATGATCCCAAAGTAGA

*In consensus primers, translation initiation and termination codons depicted in bold.

Table 1. Primers used to clone and express full length cDNA for ferret TNF- α

To ensure correct sequencing of 3' and 5' cDNA ends or the genes, we used RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) as per manufacturer's instructions (FirstChoice RLM-RACE Kit, Ambion, Ausin, Texas, USA). Briefly, 1-2 μ g of total RNA from mitogen-stimulated ferret blood cells (described above) was used as starting material. The RNA was treated with calf intestinal alkaline phosphatase (CIP) and subsequently with tobacco acid pyrophosphate (TAP). RNA adapter was ligated and the RNA was reverse transcribed to cDNA followed by PCR amplification with nested primers (outer and inner) to adapter and gene (for TNF- α see Table 1.). 3' RACE was also performed as per manufacturer's protocol with gene-specific nested primers (Table 1.).

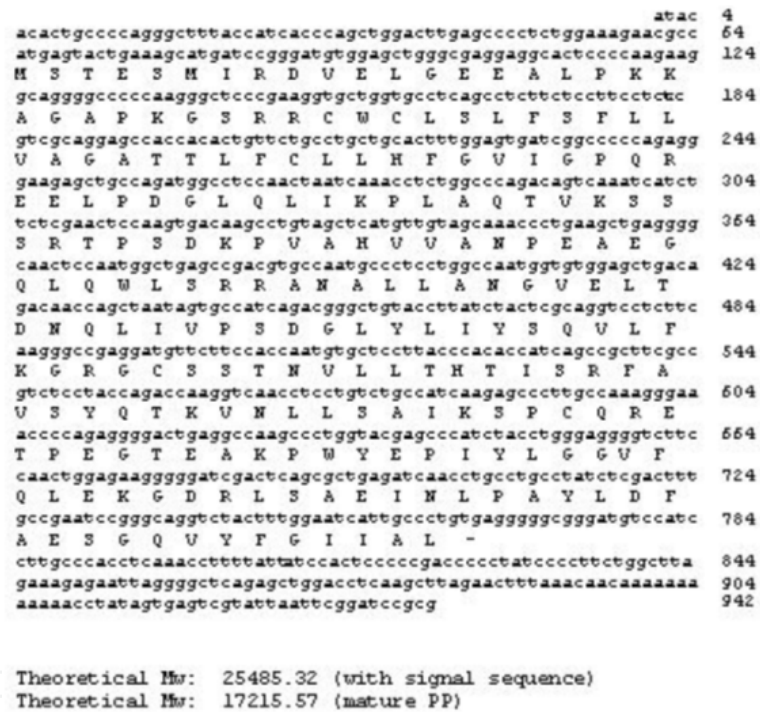
The primers were tested in silico using Primer Express (Applied Biosystems). Standard PCR was performed using these consensus primers and template cDNA. Bands at the appropriate size were excised, gel purified and sub-cloned into pCR2.1-TOPO vector (Invitrogen). DNA sequences of positive clones were confirmed by sequencing with ABI 3730XL DNA analyzers (Center for Applied Genomics, Toronto, ON, Canada). Gene identification was confirmed using Basic Local Alignment Search Tool (BLAST) analyses against the National Centre for Biotechnology Information databases.

3.1.2 Results

Cloning and sequencing of the ferret TNF-α and IFN-γ genes

To determine the consensus regions for both ferret TNF-α and IFN-γ ClustalW analysis was performed for human, cat and dog TNF-α and IFN-γ nucleotide sequences. The coding region for all three species was predicted to encode a 702 bp transcript and a 501 bp transcript, respectively for TNF-α and IFN-γ.

Using the consensus sequence for TNF-α and IFN-γ genes, forward and reverse primers (Table 1. for TNF-α) were designed and used to amplify full-length ferret TNF-α and IFN-γ from a ferret cDNA library derived from mitogen-stimulated ferret PBMCs. When necessary, RACE was used to identify the endogenous ferret sequences at the 5′ and 3′ ends of the transcript. The nucleotide sequence of ferret TNF-α and IFN-γ (previously published (Ochi et al., 2008)) are shown in Figure 1. and Figure 2., respectively. In addition to the 702 bp coding region, we have determined the sequence of the 65 bp 5′ and 175 bp 3′ untranslated regions, respectively.

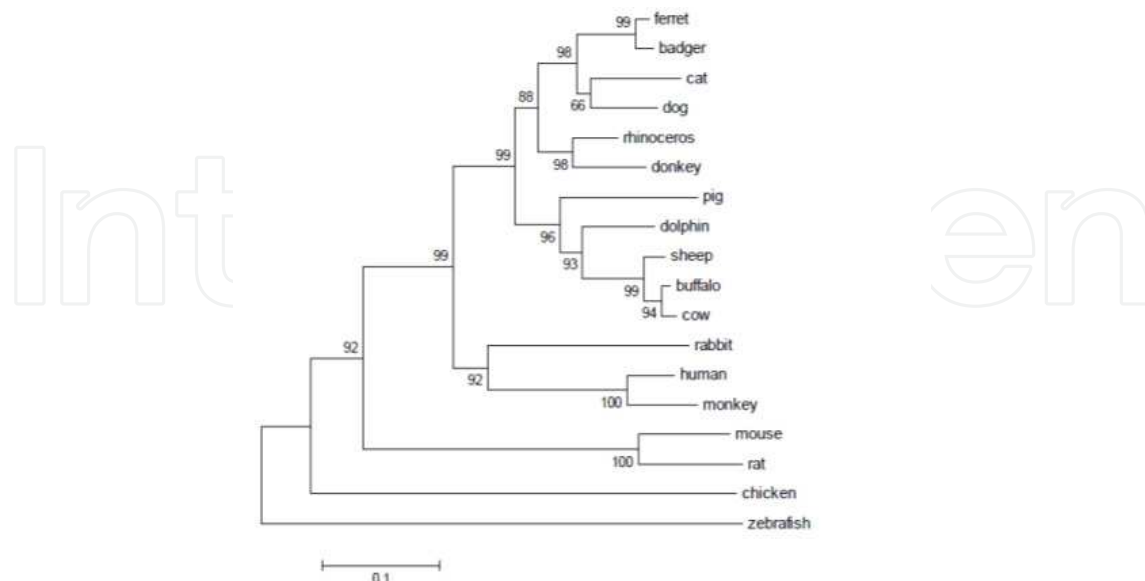


The ferret cDNA was 942 bp, and the predicted protein 314 aa. Numbers on the right indicate cDNA bp. The 5′-UTR was 65 base pairs, the complete coding region of 702 base pairs, and 175 bp of the 3′UTR. Calculated molecular weight (MW) of the predicted protein is indicated at the bottom.

Fig. 1. Full-length ferret TNF-α cDNA nucleotide sequence and predicted amino acid sequence.

Multiple amino acid sequence alignment revealed that the predicted ferret TNF-α protein shares a high level of homology with cat (91% similarity) and dog (95% similarity) sequences (Figure 3). The sequence similarity to ferret TNF-α was lower when compared to human (78%) and mouse (88%) TNF-α sequences. The TNF ligand family consists of 19 proteins characterized by a conserved C-terminal domain called the TNF-α homology domain (THD) (Bodmer et al., 2002). The ferret TNF-α THD domain was found to be significantly conserved with dog and cat TNF-α sequences (Figure 3).

the termination codon enabled the cloned gene to be expressed as a fusion protein with two C-terminal epitope tags, His6 and V5.



Phylogenetic tree analysis showing the relationship between ferret and other known vertebrate IFN- γ sequences. This tree was constructed using ClustalW and MEGA 3.1 packages and bootstrapped 10,000 times. †Bootstrapping confidence values are between 66 and 100.

Fig. 5. Ferret IFN- γ phlogenetic analysis (Ochi et al., 2008).

Transfection and purification of recombinant His-tagged TNF- α

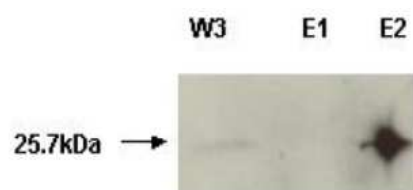
COS-7 cells (ATCC, Manassas, Virginia, USA) were maintained in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen) at 37°C, 5% CO₂. COS-7 cells (1x10⁷) were transfected with Effectene transfection reagent according to manufacturer's instructions (Qiagen, Mississauga, Ontario, Canada). Twenty-four to forty-eight hours following transfection, the cell culture media from the cells were run through Ni-NTA metal immobilized affinity columns to bind the His-tagged recombinant protein (Novogen, Oakville, ON, Canada). The columns were washed and the protein was eluted according to the manufacturer's protocols. Fractions containing the recombinant proteins were pooled and dialyzed against phosphate buffered saline (PBS) at 4°C and subsequently concentrated by spin column (Nanosep 10k OMEGA, Pall Life Science, East Hills, NY, USA). TNF- α and IFN- γ protein concentrations were determined by protein assay kit (Pierce, Rockford, IL, USA).

Expression and purification of GST-IFN- γ using a bacterial expression system

Ferret IFN- γ cDNA was subcloned into pGEX-6P1 vector (GE Healthcare) and purified as a GST fusion protein from *Escherichia coli* strain BL21 (DE3) by glutathione affinity chromatography.

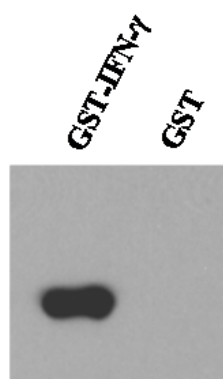
Western Blot analysis

SDS-Poylacrylamide gel electrophoresis (10-15% SDS-PAGE) was performed with pre-cast gels (Bio-Rad, Hercules, CA, USA) according to standard protocols. Protein blots were blocked with 5% milk protein in 0.01% Tween-20 in PBS (T-PBS) for 1 hour at room



The expression construct encoding full-length ferret TNF- α was transfected into COS-7 cells and purified using with ion (Ni²⁺) immobilized affinity chromatography. The eluted protein fractions were subjected to SDS-PAGE and western blotting. Arrow depicts the band at predicted molecular weight of ferret TNF- α . W3 denotes wash fraction 2, and E1 and E2 denote elution fractions 1 and 2.

Fig. 6. Purified recombinant ferret TNF- α protein molecular weight determined by western blot analysis.



Detection of recombinant ferret IFN- γ by polyclonal antibody-specific immunoblotting.

Fig. 7. Detection of ferret recombinant IFN- γ .

temperature followed by an incubation of 16 hours at 4°C with mouse-anti-His6 primary antibody (Invitrogen) at 508 ng/ml concentration or monoclonal anti-V5 Ab (1:1000) (Invitrogen). The blots were washed and incubated with goat-anti-mouse-HRP secondary antibody (Santa Cruz, Santa Cruz, CA, USA). Bands were visualized using enhanced chemiluminiscent (ECL) reagents according to manufacturer's protocol (GE Healthcare, Peterborough, Ontario, Canada).

3.2.2 Results

Expression and purification of the recombinant ferret TNF- α and IFN- γ

In order to obtain recombinant ferret TNF- α and IFN- γ protein to be used in immunoassays and monoclonal antibody generation, TNF- α and IFN- γ genes were first subcloned. Specifically, TNF- α and IFN- γ cDNA were individually subcloned into expression plasmid to generate expression tag fusion proteins (described previously). Ferret TNF- α was expressed and purified from a mammalian cell line and IFN- γ from a bacterial expression system.

The native human TNF- α polypeptide is cleaved at amino acid 76 to form the mature TNF- α signal peptide at 17215 Da (Wang et al., 1985). We predicted the ferret signal sequence cleavage site to be at the junction of 46 and 47 by SignalP 3.0 analysis. The observed molecular weight of ferret TNF- α according to our results was closer to that of human TNF- α at 25.7 kDa (including the His6 and V5 tags). In agreement, western blot analysis of the eluted fractions of ferret TNF- α protein purified from COS-7 transfected cell media resulted in a single band of approximately 25.7 kDa molecular weight (Figure 6).

For IFN-γ, purified recombinant ferret IFN-γ protein was expressed as a GST fusion protein and purified from chemically competent E. coli cells. Ferret recombinant IFN-γ protein migrated as a 40 kDa band when subjected to SDS-PAGE and western blotting using a polyclonal anti-IFN-γ antibody (Figure 7).

3.3 Cytokine real-time PCR based assay

Cytokines are important inflammatory and immune mediators. Here we present primers designed using ClustalW alignment analysis for studying ferret cytokines by real-time PCR.

3.3.1 Methods

ClustalW analysis for cytokine primer design

Cytokine Primers were designed as previously described (Section: Cloning, sequencing and expression of ferret TNF-α and IFN-γ in 4.2.1). Gene specific primers were designed based on highly conserved regions of orthologue nucleotide gene sequences. Conserved regions were identified through ClustalW-based multiple sequence alignments of the orthologues from several species using the ClustalW 1.83 from the European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/>). The Primers are presented in Tables 2, Table 3 and Table 4.

IL-1αF	ACCACTTCAIGAGACTCC
IL-1αR	TCTACIGATCIGGCTTCA
IL-1βF	CGACITCAAATTCAGACATAA
IL-1βR	TTCGTCACACTAGTCCGTGA
IL2B	CTTCGAAACAGIGCACCIA
IL2R	CCCTCTTCGGCAIGTAGAA
IL4F	TCACCGCACTTTCATCA
IL4R	TCTCTGCTGAGCAIGTICA
IL5F	CGCGACCTGICGATAAACT
IL5R	CAACTTCCGGIGTCCACIC
IL6F	AGTCCGAAACAGTAAACAATC
IL6R	ATGCCCCACGCTCAACT
IL8F	AAGACGAAAACITCCAAGAGA
IL8R	CCAGAAGAAACCTGACCAAAG
IL10F	CCIGTCCGAGATGATCCAGT
IL10R	CAAGCTCACATACGCTTIG
IL11F	CTGAGCTGIGCCACATA
IL11R	CGAATCCAGGTGIGGIC
IL12BF	CACCAGAGTCTTCAICA
IL12BR	AGGCTTGTCACCCAGAGT
IL13F	TGGTACCTGCGGATATCC
IL13R	GAIGCCGATCTCCTGGAT
IL16Fnew	CCGACCCGATCTAGAAAAC
IL16Rnew	CGACGAGGTCAGGICAG
IL17AF	CCACCAIGIGAAGGICAA
IL17AR	AACCAAGATCTTCTCTCGA
IL18F	GAGGATATCCCGATCTICA
IL18R	ATCATGCCCTCGAACACTTC

Table 2. Ferret Interleukine Primers

TNF-alpha F	CCAGATGGCCTCCAAC TAATCA
TNF-alpha R	GGCTTGTC ACTTGGAGTTCGA
IFN-gamma F	TCAAAGTGATGAATGATCTCTCACC
IFN-gamma R	GCCGGGAAACACACTGTGAC

Table 3. Ferret Inflammatory Cytokine Primers

Real-time PCR

Real-time PCR analysis was performed as previously described (Bosinger et al., 2004). Briefly, experiments were carried out using the SYBR Green qPCR kit (Applied Biosystems, Foster City, California, USA) on an an ABI 7900 System (Applied Biosystems). The PCR mixture contained 25 pmol primers and 250 nmol of cDNA. Amplification was performed with the following program: pre-heating for 15 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. Subsequent to the completion of PCR amplification, the temperature was raised from annealing temperature to 95°C for melting curve analysis.

CCL2 (MCP-1) F	GCICCCIATTCAC TIGCIGITTC
CCL2 (MCP-1) R	GATTGATAGCCCTCCAGCTT
CCL4 F	TGIGACCGTCC TTTCTCTCC
CCL4 R	GAATCTTCCGCAGGGTG TAA
CCL5 F	GCIGCTTTGCCTACAT TICC
CCL5 R	CCCATTTCITCTIG TGGGTTG
CCL7 F	TATCTCAACCACCTGCTGCT
CCL7 R	GCTTGGGTTTCTTGTCCAG
CCL8 F	CATCCCAATTACCIGCTGCT
CCL8 R	ACTGGCIGTTGGTGATCC TC
CCL11 F	AGGCTCCGCAGCACTTCT
CCL11 R	TATCCTTGCCAGTTTGGTC
CCL17 F	CGGGAGTGTGCTAGAGT A
CCL17 R	CTTACCCCTTGTCTTGG
CCL21 F	TCAGGCAGAGCTATG TGCAG
CCL21 R	TCAGTCCCTTGCAGCC TTT
CCL22 F	ACTGCACTCC TGGTTGICCT
CCL22 R	ATCTTACCCAGGGCACTCT
CCL23 F	ACGAATTCGATG TIGCAAACA
CCL23 R	AGCTGCCCCCTACTACCA TT
CXCL5 F	GAGCTCCGTTG TIGTGTGTT
CXCL5 R	ACTTCCACCTTGGAGCACTG
CXCL9 (MIG) F	GGTGGTGTTCCTCTTT GTTGAGT
CXCL9 (MIG) R	GGAACAGCGTCTATT CCTCATTG
CXCL10 (IP10) F	CTTTGAACCAAAGTGC TGTCTTATC
CXCL10 (IP10) R	AGCGTGTAGTTCTAGAGAGAGGTACTC
CXCL11 F	AGAGGACGCTGTCTT TGCAT
CXCL11 R	TGGGATTTAGGCATCG TGT
CXCL12 F	ACAGATGTCTTGCCGATTC
CXCL12 R	CCACTTCAATTTCCGGTCAA
CXCL13 F	TCCAAGGIGTTC TGGAGGTC
CXCL13 R	GGGAATCTTCTCTTAAACAC TGG
CXCL14 F	CCCTCCGGTCAGCATGAG
CXCL14 R	CCAGGCGTTGTACCACTTG

Table 4. Ferret CC and CXC Chemokine Primers

3.3.2 Results

Ferret cytokine and chemokine specific real-time PCR primers

Primers specific toward ferret cytokine and chemokine genes were designed by ClustalW analysis. Primers for ferret interleukins (Table 2), ferret inflammatory cytokines (Table 3) and ferret CC and CXC chemokines (Table 4) are described.

Induction of TNF-α target genes by treatment of ferret blood cells with recombinant ferret TNF-α

Once confirming that the recombinant TNF-α protein was of proper molecular weight, we then went on to assess the biological potential of the recombinant protein *in vitro*. Ferret whole peripheral blood was stimulated with recombinant ferret TNF-α and the RNA was extracted. Following extraction, the expression level of a panel of known TNF-α target cytokine/chemokine genes were measured by real-time PCR. CXCL8 (120-fold), IFN-γ (12-fold), IL-1R (6-fold), IL-6 (3-fold) and IL-1β (2-fold) were increased following stimulation (Figure 8). These results indicated that the recombinant ferret TNF-α protein had biological activity.

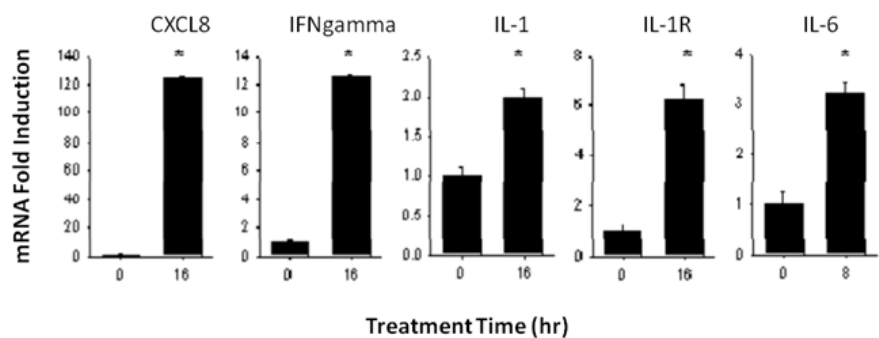


Fig. 8. Real-Time PCR analysis of ferret cytokines transcript stimulated by TNF-α

3.4 Development of IFN-γ and TNF-α hybridoma clones

The generation of monoclonal antibodies specific to ferret IFN-γ and TNF-α are vital to the development of ELISAs and ELISPOTs. In this section we outline the development of these monoclonal antibodies using recombinant IFN-γ and TNF-α conjugated to a carrier protein, KLH (keyhole limpet hemocyanin) using glutaraldehyde. Furthermore, the KLH-IFN-γ/TNF-α complexes injected and cell fusion to establish IFN-γ/TNF-α reactive B cell hybridomas is described along with downstream hybridoma clones selection.

3.4.1 Methods

Monoclonal anti-ferret TNF-α antibody production

Monoclonal antibodies to recombinant ferret TNF-α were manufactured by Open Biosystems (Birmingham, AL, USA).

Mouse B cell hybridoma preparation for anti-ferret-IFN-γ

Recombinant ferret IFN-γ (50 µg) along with 2 mg of keyhole limpet hemocyanine (KLH) (Calbiochem, San Diego, CA, USA) were diluted in 0.5 ml PBS. Five µl of glutaraldehyde

were added and the mixture was incubated for 1 hr at room temperature. The whole mixture was washed on a spin column (Nanosep 10k OMEGA, Pall Life Science) and then concentrated to 0.1 ml volume. PBS (0.5 ml) was then added and the mixture was centrifuged. After two PBS washes, the mixture was filled to 0.5 ml with PBS. This mixture was used as the priming antigen. Mice were immunized with 25 μ l antigen suspension in emulsified Complete Freund's Adjuvant and further injected at bi-weekly intervals with 5 μ g of recombinant ferret IFN- γ . Three days following the third injection, spleen cells were removed and isolated for fusion with Sp2/0-Ag14 using polyethylene glycol (Roche, Mannheim, Germany). HAT (hypoxanthine aminopterin thymidine) resistant hybridomas were selected. Hybridoma cells were screened for the reactivity against IFN- γ by ELISA using Nunc MaxiSorp 96 well plates coated with ferret IFN- γ (100 μ l, 0.1 μ g/ml).

3.4.2 Results

Monoclonal ferret TNF- α antibody recognizes endogenous TNF- α isolated secreted from mitogen-stimulated ferret blood cells

A monoclonal anti-ferret TNF- α antibody was commercially manufactured by immunizing mice with recombinant ferret TNF- α . Isolated ferret peripheral blood cells were then stimulated with the mitogens: SEB (Staphylococcal enterotoxin B), IFN- γ , ionomycin and PMA plus ionomycin. Following stimulation, cell supernatants were run on SDS-PAGE and analyzed by western blotting using the manufactured monoclonal ferret TNF- α antibody. Endogenous ferret TNF- α protein was recognized in samples that had been stimulated with SEB, IFN- γ and PMA plus ionomycin. In contrast, TNF- α was not present in the supernatant from cells treated with ionomycin alone or unstimulated cells (Figure 9). These results suggested that the manufactured ferret monoclonal TNF- α antibody was able to recognize endogenous secreted ferret TNF- α .



PBMCs were isolated and stimulated with SEB (100 μ g/ml), IFN- γ (2000IU/ml), ionomycin alone (1 μ g/ml) or PMA (50 ng/ml) with ionomycin (1 μ g/ml), for 24 hrs at 37°C. Cell supernatant was run on SDS-PAGE and subjected to western blotting with a monoclonal antibody (2 μ g/ml) against recombinant ferret TNF- α .

Fig. 9. Ferret monoclonal TNF- α antibody recognizes secreted endogenous TNF- α .

Generation of monoclonal Abs specific for ferret IFN- γ

Prior to the generation of ferret IFN- γ monoclonal antibodies, we attempted to monitor IFN- γ levels from ferret cell cultures using human and mouse commercially available cytometric bead arrays. Neither human nor mouse-specific arrays resulted in the positive detection of ferret IFN- γ . The failure in detection was most likely due to the inability of the human and mouse IFN- γ antibody to recognize the ferret IFN- γ protein. These results prompted us to generate our own monoclonal antibodies specific to ferret IFN- γ . To produce ferret IFN- γ specific antibodies, recombinant IFN- γ was conjugated to a carrier protein, KLH (keyhole limpet hemocyanin) using glutaraldehyde (as described in the methods). The resulting KLH-IFN- γ complex was injected intraperitoneal into Balb/c mice. Following fusion of splenocytes with hybridoma parent cells, IFN- γ -reactive B cell hybridomas were established and clones were selected by ELISA reactivity. Clone 1H1H12 and 4A4B7 recognized recombinant ferret IFN- γ evident by a 40 kDa band (Figure 10).

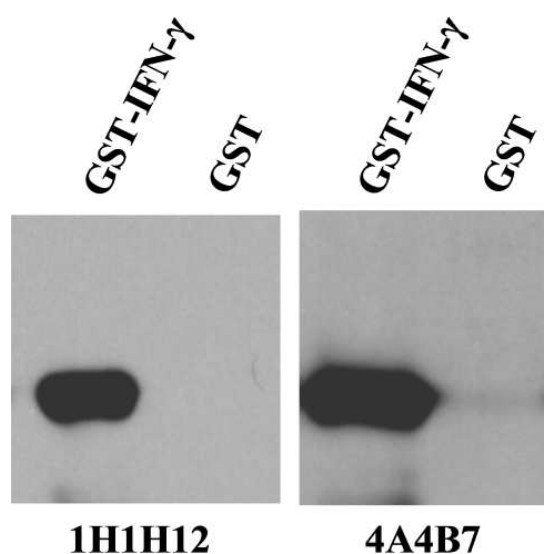


Fig. 10. Ferret IFN- γ monoclonal antibody detects recombinant and endogenous ferret IFN- γ protein.

Lysates from COS-7 cells transfected with recombinant ferret IFN- γ samples were analyzed by western blot using a monoclonal antibody established from mouse immunized by recombinant ferret IFN- γ (left panel). Supernatants derived from ferret PBMC cultures stimulated with PMA plus ionomycin, were also analyzed by western blot using anti-ferret IFN- γ monoclonal antibody (right panel). Arrows at the right of each panel indicate the dimers for lower molecular weight protein bands and putative tetramer as higher molecular weight species.

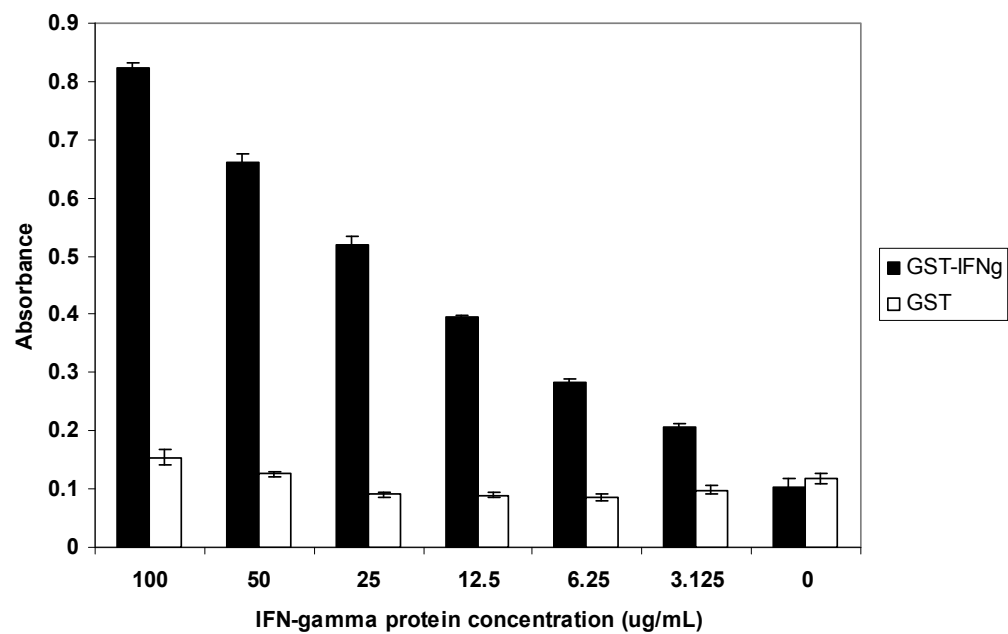
3.5 ELISA and ELISPOT assays for the ferret cytokine IFN- γ

An important application for monoclonal anti-ferret IFN- γ antibodies is the detection and quantitation of IFN- γ protein in biological samples. In this section the protocols for both IFN- γ ELISA and ELISPOT are described. This includes the selection and testing of the monoclonal antibodies and ELISAs to measure IFN- γ levels in sera obtained from influenza A-infected ferrets. Furthermore, a ferret-specific IFN- γ ELISPOT assay is outlined using the same set of monoclonal ferret IFN- γ antibodies used in the ELISA.

3.5.1 Methods

Ferret IFN- γ -specific ELISA

ELISA plates (96-well) (MaxiSorb, Nunc) were coated with 100 μ l/well with monoclonal anti-IFN- γ (2 μ g/ml) overnight at 4°C. Wells were blocked with 150 μ l 1% BSA in PBS for 1 hour at 37°C. Supernatants from mitogen-stimulated PBMC cultures or serum from influenza A virus infected ferrets were loaded into the wells at appropriate dilutions and incubated for 1 hour at 37°C. Wells were washed with PBS/0.5% Tween-20 and then incubated for 1 hour at room temperature with biotin conjugated anti-IFN- γ antibody (1 μ g/ml in 0.5% Tween-20/1%BSA). Following secondary antibody incubation, the wells were washed three times with PBS/0.5% Tween-20 before incubation with HRP-Avidin for 30 minutes. The substrate, (o-phenylenediamine, Sigma) was applied for 15 minutes at room temperature. Colorimetric changes were quantitated using an automated ELISA reader (μ Quant, BIO-TEK Instruments, Winooski, VT, USA).



ELISA plate wells were coated with a monoclonal anti-ferret antibody. Recombinant ferret IFN- γ was sequentially diluted and added to the wells. Ferret IFN- γ was detected by a second monoclonal anti-ferret IFN- γ biotin conjugated antibody. Logarithmic dilution was used to derive a standard curve for downstream applications of the ELISA.

Fig. 11. Recombinant IFN- γ protein quantification by ELISA.

Ferret IFN- γ -specific ELISPOT assay

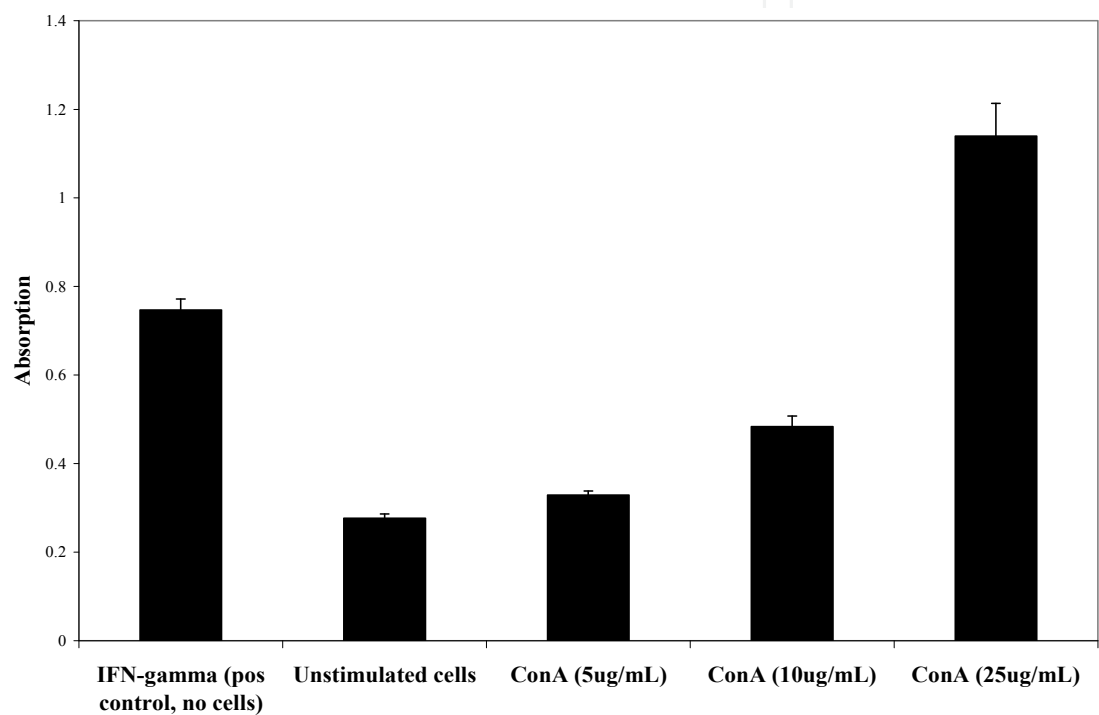
PVDF plates (Millipore, MAIPS4510) or MaxiSorp plates (Nunc) were coated with a monoclonal anti-ferret IFN- γ antibody, and subsequently blocked with 1% BSA in PBS. Isolated ferret PBMCs from peripheral blood were stimulated for 18 hours. The wells were then washed with water to remove cells and captured IFN- γ was detected by a biotin-conjugated detection antibody coupled to HRP-avidin (Sigma). The ELISPOT was developed using DAB (Vector Laboratories, Burlingame, CA, USA).

3.5.2 Results

Detection of ferret IFN- γ by ELISA and ELISPOT immunoassays

An important application for monoclonal antibodies is the detection and quantitation of protein in biological samples. Here we tested our monoclonal antibodies for use in a ferret IFN- γ -specific ELISA by first determining the antibody pair for IFN- γ recognition. Clone 4A4B7 was conjugated to biotin and used as the detection antibody against ferret IFN- γ . Clone 1H1H12 was used to coat the assay wells and be used as a capture antibody.

This clone pair showed an increased optical density that correlated directly with the concentration of purified recombinant IFN- γ (Figure 11).



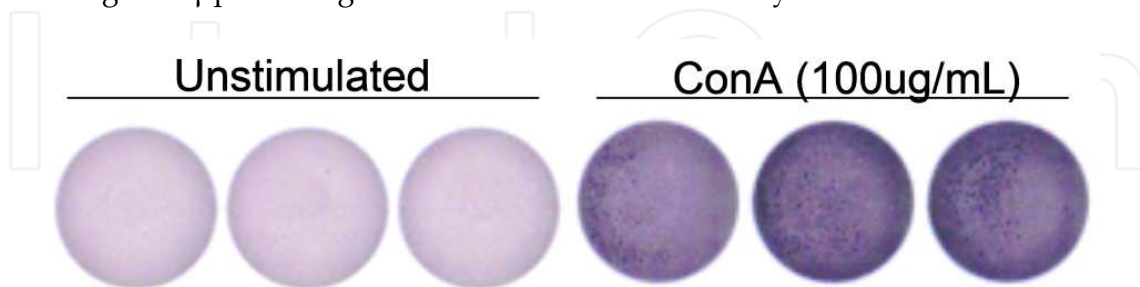
IFN- γ ELISA was performed on supernatant from isolated ferret PMBCs treated with ConA. Results represent the mean values of triplicate samples.

Fig. 12. ELISA detection of IFN- γ from mitogen-stimulated PBMCs.

We then used the ELISA assay to detect endogenous IFN- γ from ConA-stimulated ferret PBMCs. Our assay showed that IFN- γ was only present in samples that were treated with ConA and no detection in the negative control unstimulated samples (Figure 12). These results indicated that the monoclonal antibodies selected are applicable for ferret IFN- γ -specific ELISA and that the pair was a reliable detection system.

ELISPOT assays are also key tools in the determining the activation of cellular immune responses. Specifically, ELISPOT assays directly quantitate the number of cytokine secreting cells as opposed to the ELISA which quantitates the amount of cytokine produced. To develop a ferret-specific IFN- γ ELISPOT assay we employed the same set of monoclonal ferret IFN- γ antibodies, clone 4A4B7 and 1H1H12. Isolated ferret PBMCs were subjected to an ELISPOT assay (as described in the methods) with stimulation of ConA. As expected,

increasing numbers of IFN- γ secreting cells were detected in direct proportion to the number of stimulated cells plated. Furthermore, the number of IFN- γ positive cells did not increase above background when increasing numbers of unstimulated cells were plated (Figure 13). These results suggested that 4A4B7 and 1H1H12 anti-ferret IFN- γ monoclonal antibodies will be useful for not only quantitating ferret IFN- γ levels but also for quantitating IFN- γ producing ferret cells in an ELISPOT assay.



ELISPOT assay was performed in the same manner outlined for Figure 12 for capture and detection. PBMCs were plated and stimulated with ConA 18 hours and IFN- γ secreting cells were captured and detected by biotinylated IFN- γ antibody.

Fig. 13. ELISPOT assay for the IFN- γ producing cells in mitogen-stimulated ferret PBMCs.

4. Conclusion

The development of ferret specific immune reagents will significantly improve knowledge gained by ferret infectious disease studies. The importance of the ferret as an animal model in the study of respiratory diseases is increasing. Currently, the use of the ferret model to investigate the immune response as well as immunotherapeutics in respiratory infections by viruses such as SARS CoV and influenza has been slowed by the lack of immunological reagents and immune response assessment tools. In this chapter we highlighted immune reagents and immunoassays that are successful at monitoring the ferret immune response, specifically focusing on cytokines.

Currently, there is much interest to determine the immunological role of TNF- α during influenza infection, as TNF- α is thought to play a pathogenic role in other respiratory infections (Headley, Tolley, & Meduri, 1997). Here we have described the cloning and sequence of the ferret TNF- α gene with the purpose of generating reagents and assays for the investigation of ferret immune responses. With the generated ferret TNF- α expression vector we were able to express and purify recombinant protein for downstream uses. Importantly, the recombinant ferret TNF- α protein was able to stimulate ferret peripheral blood cells and induce the expression of TNF- α target genes such as: CXCL-8, IFN- γ , IL-1 β , IL-6 and IL-1R (Vassalli, 1992; Baumann & Gauldie, 1994; Dinarello, 1996; Gabay & Kushner, 1999). Furthermore, we have successfully generated monoclonal antibodies that specifically recognized ferret TNF- α as well as primers that measured TNF- α transcripts in cells stimulated with LPS, a known stimulant of TNF- α (Curnis & Corti, 2004; Mestan et al., 1986; Wang et al., 1985). In summary, we have cloned, sequenced and expressed recombinant ferret TNF- α and demonstrated its biological activity. Our ferret TNF- α protein has enabled us to generate several useful reagents that will be important tools for immune modeling in respiratory diseases.

IFN- γ is an essential regulator in the viral host immune response. Here the cloning of a full length ferret IFN- γ and expression of the recombinant IFN- γ protein was described. Furthermore, we described the generation of two monoclonal antibodies specific for ferret IFN- γ and the subsequent development of immunoassays for the detection of native IFN- γ . We anticipate that the IFN- γ immunoassays established in this study will be useful in gaining insight into ferret antiviral responses and in other immune processes in general.

Previous studies on IFN- γ have shown that there is a strict species-specific activity of IFN- γ . IFN- γ genes isolated from diverse species such as guinea pig, turkey, rhino, and catfish have been previously described in the literature (Jeevan et al., 2006; Loa, Hsieh, Wu, & Lin, 2001; Milev-Milovanovic et al., 2006; Morar et al., 2007; Svitek & von, V, 2007). Our monoclonal antibodies detected recombinant IFN- γ in Western blotting. These two clones were paired successfully during the development of ferret IFN- γ -specific ELISA and ELISPOT bioassays. Previously, we infected ferrets with H3N2 influenza A strain (A/Panama/2007/99) and sampled serum from the animals 6 days post infection (Ochi et al., 2008). The ELISA showed a marked increase in the levels of circulating IFN- γ on day 6 post-infection compared to the uninfected control. Specifically, the level of IFN- γ in serum from the non-infected control ferret was below the detection limit. These results suggested that our developed ELISA assay for quantitation of ferret IFN- γ will be invaluable in monitoring systemic IFN- γ responses during a host response against virus infection.

There is an overwhelming need for experimental models that enable the evaluation of therapeutic treatments and vaccines for use in infectious diseases such as emerging influenza viruses. Ferrets have been used as an animal model of infection with influenza A viruses to test the severity of the disease and also to evaluate efficacy of potential vaccines (Suguitan, Jr. et al., 2006; Cameron et al., 2008; Danesh et al., 2011; Rowe et al., 2010b). Specifically, TNF- α and IFN- γ have been identified as major inducers of pathogenesis in respiratory illnesses which includes SARS (Cheung et al., 2005), (Roberts & Subbarao, 2006) and influenza virus (Maher & DeStefano, 2004; Cheung et al., 2002; de Jong et al., 2006). From these findings comes the hypothesis that these inflammatory cytokines may be the key to potential future therapies.

TNF- α antibodies have been shown to have therapeutic potential in disease either as therapeutic targets or as biomarkers. In a mouse model of influenza-induced pneumonia, a neutralizing antibody to TNF- α significantly reduced the lung pathology and prolonged survival of infected animals thereby suggesting TNF- α as a therapeutic target (Peper & Van, 1995). With the antibodies generated, it is now possible to investigate the therapeutic potential of targeting TNF- α to modulate the immunopathology during influenza infection. Alternatively, IFN- γ has been shown to be a useful biomarker. ELISPOT and ELISA assays are excellent techniques that can be employed in studies monitoring vaccine and therapeutic efficacy by measuring the levels of biomarkers such as IFN- γ . Taken together, these reagents will be helpful in the assessment of vaccine efficacy against influenza A and other emerging infectious viruses.

In conclusion, this work has expanded the potential of the ferret model for respiratory disease investigation as well as other diseases that involved the immune response.

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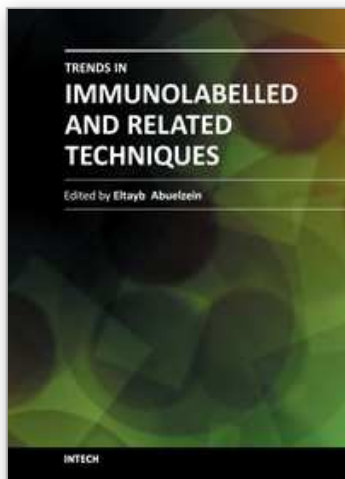
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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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