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TaqMan real time RT-PCR assays for detecting ferret innate and adaptive immune responses

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The ferret is an excellent model for many human infectious diseases including influenza, SARS-CoV, henipavirus and pneumococcal infections. The ferret is also used to study cystic fibrosis and various cancers, as well as reproductive biology and physiology. However, the range of reagents available to measure the ferret immune response is very limited. To address this deficiency, high-throughput real time RT-PCR TaqMan assays were developed to measure the expression of fifteen immune mediators associated with the innate and adaptive immune responses (IFNα, IFNβ, IFNγ, IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p40, IL17, Granzyme A, MCP1, TNFα), as well as four endogenous housekeeping genes (ATF4, HPRT, GAPDH, L32). These assays have been optimized to maximize reaction efficiency, reduce the amount of sample required (down to 1 ng RNA per real time RT-PCR reaction) and to select the most appropriate housekeeping genes. Using these assays, the expression of each of the tested genes could be detected in ferret lymph node cells stimulated with mitogens or infected with influenza virus *in vitro*. These new tools will allow a more comprehensive analysis of the ferret immune responses following infection or in other disease states.

1. Introduction

Ferrets are an outbred population widely used to study influenza virus infection (Belser et al., 2011; Laurie et al., 2010; Rockman et al., 2012; Hurt et al., 2010) as well as a range of other diseases, including SARS-coronavirus (CoV) (reviewed in (Roberts et al., 2008)) and henipaviruses, such as infection with Hendra virus and Nipah virus (Bossart et al., 2009; Pallister et al., 2009, 2011; Geisbert et al., 2012). The anatomical and physiological similarity between human and ferret lungs also enables ferrets to be used as a model to study lung carcinomas (reviewed in Baric et al., 2013). Recently, absence of the cystic fibrosis transmembrane conductance regulator (CFTR) was associated with spontaneous disease induction in the lung and pancreas in ferrets, showing similar pathology to that of cystic fibrosis in humans (Sun et al., 2010, reviewed in Keiser and Engelhardt, 2011). The broad utility of this model is highlighted by the use of ferrets to study pneumococcal transmission, reproductive biology and human fetal brain development (reviewed in Baric et al., 2013).

While the ferret is a good model for human respiratory virus infections, reagents to identify ferret leukocytes and immune mediators are limited. Studies have identified cross-reactive antibodies that recognize populations of ferret leukocytes, such as CD8, CD11β, CD44 and CD25, and cytokines IFNγ, TNFα, IL4 and IL8 (Rutigliano et al., 2008; Martel and Aasted, 2009; Pillet et al., 2011). Cloning and sequencing of ferret cytokine genes have enabled molecular approaches targeting the corresponding mRNAs (von Messling et al., 2006; Danesh et al., 2008; Nakata et al., 2008; Ochi et al., 2008; Qin et al., 2013). Expression of cytokine and chemokine genes has been assessed in *ex vivo* samples following infection of naïve or vaccinated ferrets with influenza virus or SARS-CoV by microarray analysis (Cameron et al., 2008; Fang et al., 2010; Rowe

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et al., 2010). Cytokine and chemokine gene profiles have also been assessed *ex vivo* and in *in vitro* epithelial cultures using SYBR green real time RT-PCR assays (Svitek and von Messling, 2007; Cameron et al., 2008; Danesh et al., 2008, 2011; Svitek et al., 2008; Kim et al., 2009; Fang et al., 2010; Hamelin et al., 2010; Kobinger et al., 2010; Rowe et al., 2010; Kang et al., 2011; Meunier and von Messling, 2011, 2012; Pillet et al., 2011; Huang et al., 2012; Maines et al., 2012; Meunier et al., 2012; Belser et al., 2013; Zeng et al., 2013). TaqMan chemistry incorporates target-specific fluorescent labeled probes enabling multiple genes can be assessed in a single real time PCR reaction (Giulietti et al., 2001). To date, TaqMan real time RT-PCR assays have only been developed for a smaller number of ferret-specific gene targets (Nakata et al., 2009; Suguitan et al., 2012).

To enable a broader characterization of the immune response in the ferret model, we developed a panel of TaqMan assays to detect mRNA of fifteen ferret cytokines, chemokines and immune mediators (IFNα, IFNβ, IFNγ, IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p40, IL17, granzyme A, MCP-1, TNFα) and four housekeeping genes (ATF4, GAPDH, L32 and HPRT). The cytokine and chemokine profile induced by stimulation of ferret leukocytes with mitogens or influenza virus was also assessed to investigate the relevance of the ferret immune response to human infection studies.

2. Materials and methods

2.1. Design of ferret cytokine and housekeeping gene primers and probes

Sequences for cytokine, chemokine and housekeeping genes of multiple species were obtained from Genbank (http://www.ncbi.nlm.nih.gov/genbank) and aligned. Regions of conservation were identified and primers were designed using PrimerSelect (DNASTAR Lasergene8, Madison, USA) or PrimerExpress (Applied Biosystems, California, USA) to amplify the region from ferret cDNA. Cloned genes were sequenced and TaqMan real time PCR primers and probes designed using PrimerExpress. All oligonucleotide primers and probes used in this study, including those previously published, are listed in Table 1. Primers for IFNα were designed to amplify multiple subtypes (1–12) (Easlick et al., 2010; Hillyer et al., 2012).

2.2. Oligonucleotide primer and probe generation

Lyophilized oligonucleotide primers were synthesized by Geneworks (Adelaide, Australia) and dissolved in nuclease-free water (Promega, Madison, USA) at 100 μM. All TaqMan® MGB™ probes were synthesized by Applied Biosystems with a 5′reporter dye (either FAM, NED or VIC) and a 3′non-fluorescent quencher (NFQ).

2.3. Ferrets

Adult male and female ferrets (weight 500–1500 g) were purchased from independent breeders and housed at CSL Limited (Victoria, Australia) using services provided under a Support Services Agreement. Serum samples were tested by hemagglutination inhibition assay to ensure seronegativity (titer <10) to currently circulating influenza strains before use. Experiments using ferrets were conducted with approval from the CSL Limited/Zoetis Australia Animal Ethics Committee, in accordance with the Australian Government National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

2.4. Influenza virus

A/Tasmania/2004/2009 (A(H1N1)pdm09) influenza virus was passaged in the allantoic cavity of embryonated hen's eggs and stored in aliquots at −80 °C. To heat inactivate, virus was incubated at 60 °C for 30 min.

2.5. In vitro culture of ferret lymph node cells with mitogens or influenza virus

Retropharyngeal lymph nodes were collected from naïve ferrets and placed in RPMI-1640 AQ media™ (Sigma–Aldrich, New South Wales, Australia) supplemented with 10% (v/v) fetal calf serum (Interpath Services, Victoria, Australia), 2 mM L-glutamine (SAFC Biosciences, USA), 50 U/ml penicillin/50 μg/ml streptomycin (Sigma–Aldrich) (complete-RPMI). Single cell suspensions were made by mashing the tissue and passing through a sterile 40 μM cell strainer (BD, San Jose, USA). Cell suspensions were washed twice then resuspended in complete-RPMI. Lymph node cells from each ferret (5×10^6 per well) were plated in a 24-well plate in 1 ml complete-RPMI with or without 10 μl of live or heat-inactivated virus (10^4 $TCID_{50}$) or 5 μg/ml Concanavalin A (ConA), Phytohaemagglutinin (PHA-P), Lipopolysaccharide (LPS), Ionomycin (Iono) or Phorbol 12-myristate 13-acetate (PMA) (all from Sigma–Aldrich) in duplicate or triplicate. Cell cultures were incubated at 37 °C in 5% CO_2 in a humidified incubator for the indicated periods.

2.6. RNA extraction

Total RNA was extracted from cultured cells using the RNeasy® Mini kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions. Briefly, cells from a single well were pelleted and resuspended in 600 μl RLT buffer. The sample was vortexed and then run through a QIAshredder column. RNA was extracted from the supernatant using the Animal Cells Spin protocol, without on-column DNase digestion and eluted with a 30 μl volume. RNA purity was assessed (A_{260}/A_{280}) using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Massachusetts, USA). RNA was stored at −80 °C.

2.7. Reverse transcription

For removal of genomic DNA, 800 ng RNA was incubated with 2 units DNase I (RNase-free) (New England Biolabs, Massachusetts, USA) in DNase reaction buffer (final volume 10 μ1) at 37 °C for 10 min. The reaction was terminated by the addition of EDTA (final concentration 5 mM, Sigma–Aldrich) and incubation at 75 °C for 10 min. cDNA was generated using the Superscript III First Strand synthesis System for RT-PCR (Invitrogen, California, USA) with random hexamer primers, according to the manufacturer's instructions. RNaseH treatment was performed. A simultaneous reaction without the reverse transcription enzyme was performed in parallel to generate a '-RT' control. The reaction volume resulted in 10 ng initial RNA/μl final cDNA preparation for culture samples, except at points indicated in the text, where 1 ng initial RNA/μl final cDNA preparation was used. cDNA standards were generated in parallel with cDNA test samples for each experiment. cDNA standards were prepared using RNA pooled from a range of test samples within each experiment, with at least 80 μl of 100 ng initial RNA/μl final cDNA standard prepared. Ten-fold and two-fold serial dilutions (five dilutions of each) were prepared and used for standard curve generation for efficiency calculations. cDNA was stored at −20 °C.

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