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Validation of assays to monitor immune responses in the Syrian golden hamster (Mesocricetus auratus)

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A B S T R A C T

The Syrian golden hamster (Mesocricetus auratus) is a valuable but under-utilized animal model for studies of human viral pathogens such as bunyaviruses, arenaviruses, flaviviruses, henipaviruses, and SARS-coronavirus. A lack of suitable reagents and specific assays for monitoring host responses has limited the use of this animal model to clinical observations, pathology and humoral immune responses. The objective of this study was to establish and validate assays to monitor host immune responses in the hamster including important pro-inflammatory, anti-inflammatory and innate immune responses, as well as markers of apoptosis, cell proliferation, cell junction integrity and coagulation. Commercially available mouse and rat ELISA and luminex panels were screened for potential cross-reactivity, but were found to be of limited value for studying host responses in hamsters. Subsequently, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays for the detection of 51 immune-related and four internal reference genes were developed. To validate the immune-related assays, hamsters were infected with vesicular stomatitis Indiana virus (VSV), Indiana species, or treated with lipopolysaccharide (LPS) and host immune responses were monitored in selected organs. Ribosomal protein L18 was identified as the most stable internal reference gene. In conclusion, these new assays will greatly improve the use of the hamster as an important small animal model in infectious disease research.

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1. Introduction

Syrian golden hamsters (Mesocricetus auratus; hereafter referred to as hamster) have been used in numerous studies as infection and disease models (Table 1) due to their ease of handling and disease development, which often mimics the natural course of human diseases. In particular, hamsters have been recognized as valuable animal models for studying emerging and high consequence acute human viral diseases caused by bunyaviruses (Niklasson et al., 1984; Hooper et al., 2001; Milazzo et al., 2002; Fisher et al., 2003) arenaviruses (Smee et al., 1993; Sbrana et al., 2006), henipaviruses (Wong et al., 2003), flaviviruses (Tesh et al., 2001; Xiao et al., 2001; Siirin et al., 2007) and SARS-coronavirus (Roberts et al., 2005). For some of these pathogens, such as Andes virus, a New World hantavirus and a causative agent of hantavirus pulmonary syndrome, hamsters are the only lethal disease model (Hooper et al., 2001). However, due to a lack of available reagents and specific assays to monitor host responses in hamsters, including disease-decisive acute and early innate immune responses, investigators are currently limited to studies on disease progression (clinical symptoms),
humoral immune responses (antibodies) and pathology. For other disease models using rodents, nonhuman primates and ferrets, expression microarray proteome and luminex technology-based quantification assays have been developed and utilized (Kayo et al., 2001; Datson et al., 2007; Bruder et al., 2010; lerna et al., 2010). However, for Syrian (Golden) hamsters similar specific assays are not available due to the lack of a complete genome sequence.

Here, we describe the testing of a large panel of ELISA and luminex based assays for cross-reactivity against hamster proteins and the development of an extended panel of real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays which were based on hamster mRNA sequences currently registered in Genbank. Due to limited cross-reactivity in ELISA and luminex based assays, the development of qRT-PCR assays seemed the most practical and useful strategy to quantify specific host responses in the hamster model. A few qRT-PCR assays for the quantification of a limited number of immunological responses have been previously reported (Melby et al., 1998; Muller et al., 2001; Vernel-Pauillac and Merien, 2006). We developed a unique extended panel of TaqMan® primer/probe assays for 51 different genes targeting specific markers in the pro-inflammatory, anti-inflammatory, innate immuni-

ty, apoptosis, cell junction and coagulation responses of the hamster. With ribosomal protein L18 we identified a more suitable and stable housekeeping gene. The assays were validated by monitoring host responses to treatment with lipopolysaccharide (LPS) and infection with vesicular stomatitis Indiana virus (VSV), both of which are known to strongly activate host responses in the hamster (Fultz et al., 1981b; Muller et al., 2001). The new assays will allow for a broader monitoring of host responses against infection and an advanced utilization of the hamster model.

2. Materials and methods

2.1. Inoculations and sample preparation

Recombinant VSV was rescued from cloned cDNA and infectivity was titrated by conventional plaque forming assay, as described previously (Lawson et al., 1995; Garbutt et al., 2004). Three groups of six hamsters (female, 4–6 weeks old Harlan Laboratories Inc, Indianapolis, IN, USA) were injected intraperitoneally (ip) with either 50 μg LPS (Sigma, St. Louis, MO USA), 10⁶ plaque forming units (pfu) of VSV (both diluted in Dulbecco’s modified Eagle’s medium (DMEM)), or DMEM only (control) in a total volume of 400 μL. Three of the controls and VSV infected, and six of the LPS treated hamsters were euthanized by exsanguination while under deep anesthesia (inhalational isoflurane) one day post inoculation. The remaining control and VSV inoculated hamsters were euthanized at 3 days post inoculation. Whole blood was collected by cardiac puncture into EDTA vacutainers, after which plasma was separated by centrifugation and frozen at —80 °C. Lung, liver and spleen samples were harvested from euthanized hamsters, cut into approximately 50–100 mg pieces and submerged in 1 mL of RNAlater (Qiagen, Valencia, CA, USA) overnight at 4 °C. The following day RNAlater was removed and the tissues were frozen at —80 °C. Approximately 30 mg of the tissue samples were mechanically homogenized in RLT buffer and the RNA was extracted from the homogenate using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration of the extracted RNA was determined using a NanoDrop 8000 instrument (Thermo Scientific, Waltham, MA, USA) and adjusted to a final concentration of 10 ng/μL. All experiments were conducted under BSL2 conditions, and approval for animal experiments was obtained from the Institutional Animal Care and Use Committee. Animal work was performed by certified staff in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. ELISA and Milliplex panel preparation

Rat TNFα, IL-2, IL-6 and IL-10, mouse CXCL10/IP-10, IL-13 and IL-1β/IL-1F2, and mouse/rat/porcine/canine TGFβ1 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and tested according to manufacturer’s instructions with individual hamster plasma (Table 2). Individual control and VSV infected and pooled LPS hamster plasma were sent to Millipore (Billerica, MA, USA) for analysis by Milliplex® MAP Mouse (32 plex) and Rat (23 plex) Cytokine panels (Table 2).
Table 2
List of commercially available, rodent specific, ELISA and Luminex based assays examined for cross-reactivity with hamster proteins. Red indicates acceptable cross-reactivity (within the range of supplied standards), orange indicates marginal cross-reactivity (low or suspiciously high concentrations detected) and blue indicates no cross-reactivity.

| R&D Systems ELISA | Rat     | Mouse   |
|-------------------|---------|---------|
| TGFβ              | IL-10   | IL-13   |
| IL-1β             | IP-10   | IL-13   |

| Millipore Luminex Panels | Rat       | Mouse   |
|--------------------------|-----------|---------|
| RANTES                   | IL-1β     | IL-6    |
| VEGF                     | IL-2      | IL-5    |
| IFNγ                     | IL-4      | IL-5    |
| IP-10                    | IL-13     | IL-17   |
| GRO/KC                   | IL-18     | IL-18   |
| TRAIL                    | IL-8      | IL-10   |
| MIG                      | IL-10     | IL-13   |
| LIF                      | IL-8      | IL-10   |
| LIX                      | IL-12p70  | IL-13   |
| RANTES                   | IL-1β     | IL-6    |
| VEGF                     | IL-2      | IL-5    |
| IFNγ                     | IL-4      | IL-5    |
| IP-10                    | IL-13     | IL-17   |
| TRAIL                    | IL-8      | IL-10   |
| MIG                      | IL-10     | IL-13   |
| LIF                      | IL-8      | IL-10   |
| LIX                      | IL-12p70  | IL-13   |

2.3. Selection of target genes for development qRT-PCR assay

Over 800 hamster mRNA sequences have been registered in Genbank. Of those, 51 target genes related to host immunological responses elicited by pathogen infections were selected. These included pro-inflammatory, anti-inflammatory, innate immune (primarily Type I IFN-relating genes), and T-cell-mediated responses, as well as apoptosis, cell junctions and coagulation markers (Table 3) (Adler, 2008).

2.4. Internal control selection

Of the available hamster sequences on Genbank, the following four “housekeeping” genes were selected as internal reference genes: Hypoxanthine phosphoribosyltransferase (HPRT), β-actin, β-2-microglobulin (β2M) and ribosomal protein L18 (RPL18). β-actin and HPRT were chosen as these are seen as classical housekeeping genes and have been previously utilized by other researchers performing quantitative RT-PCR on hamster tissues (Melby et al., 1998). β2M and RPL18 were selected based on the work of Dheda et al. (2004). All 4 genes were tested in qRT-PCR experiments to determine which had the most stable expression in the tissues of infected/treated and control hamsters.

2.5. Primers and probes

qRT-PCR primers and probes were either designed with the aid of Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA, USA), or were directly designed and synthesized by TIB MOLBIOL (Adelphi, NJ, USA) based on the sequence information of target transcripts. Sequence information of primers and probes are shown in Table 3. Prior to use in qRT-PCR assays all the primer sets were tested to ensure the primers can amplify target transcripts by conventional reverse transcriptase PCR; amplicons were analyzed by agarose gel electrophoresis. Briefly, using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers, cDNA pools were generated according to manufacturer’s instructions from RNA derived from hamster spleen, lung or liver. The cDNA was used as template for conventional PCR. All PCR reactions were carried out using Taq polymerase (Qiagen Cat. No. 201203) at a denaturation temperature of 95 °C for 30 s, annealing temperature of 50 °C for 30 s, and an extension temperature of 72 °C with an extension time of 30 s for 40 cycles. If a band was evident at the correct size on an agarose gel, the primers were used for subsequent qRT-PCR runs. In total, 51 different primer/probe sets were validated on lung, liver and spleen tissues (Table 3). All experimental gene probes were labeled with 5’ 6FAM dye and quenched by 3’ BlackBerry Quencher (BBQ), and all internal control gene probes were labeled with Yakima Yellow (Yak) and quenched by BBQ (TIB MOLBIOL).

2.6. Primer and probe efficiency and limit of detection

The limit of detection and efficiency of cDNA amplification was determined by selecting a few representative cloned genes. The clones were altered to contain a T7 promoter directly upstream to the qRT-PCR forward primer binding site. Following amplification the PCR fragments were gel purified and in vitro transcribed by Riboprobe Combination System T3/T7 RNA Polymerase (Promega, Madison, WI, USA). Transcripts were DNase I digested and the RNA purified using TRIzol reagent (Invitrogen) according to manufacturer’s instructions. The purified RNA was diluted from 5×10¹ to 5×10⁻⁸ ng RNA and used as a template for qRT-PCR reactions. To determine the minimum amount of total hamster tissue RNA required for the experiments, representative genes were selected and run in a 2-fold dilution series (from 200 ng to 50 ng total RNA per reaction).

To test whether the efficiency of the primer/probe sets was suitable for direct comparison between reactions, approximately one third of the available genes were selected and cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer’s instructions. The inserts were verified by sequencing and entire plasmids were digested in series of log₁₀ from 5×10¹ to 5×10⁻⁸ ng of total DNA. Quantitative PCR and qRT-PCR were carried out on the plasmid and RNA dilution series, respectively, and the crossing threshold (Ct) values plotted and efficiency determined by measuring the slope of the best fit line through the Ct values. If the efficiencies were within 10% of each other the primer/probe sets were considered as being comparable (Ling et al., 2008).

2.7. qRT-PCR methods

All qRT-PCR experiments were performed on the Rotor-Gene 6000 thermal cycler (Corbett, San Francisco, CA, USA) using Rotor-Gene Probe RT-PCR kits (Qiagen) according to manufacturer’s instructions. Each reaction was multiplexed with RPL18 as the internal reference, using 50 ng of template RNA. The final concentration of each test primer and probe set was 0.4 μM and 0.1 μM, respectively. The final concentration
Table 3
Detailed sequence information of the primers and probes utilized in these studies. Primers and probes for the indicated genes were designed based on Syrian golden hamster specific sequences available on GenBank (accession number provided in brackets). The melting temperature of all these primers was ~55 °C and the melting point of the probes was ~65 °C.

| Gene                                    | Forward Primer (5’-3’)                                  | Reverse Primer (5’-3’)                                  | Probe (5’-3’)                                | Forward Probe (5’-3’)                          | Reverse Probe (5’-3’)                          |
|-----------------------------------------|--------------------------------------------------------|--------------------------------------------------------|---------------------------------------------|------------------------------------------------|------------------------------------------------|
| B-cell lymphoma 2 protein (bcl-2) (AJ582074) | Bc2 F: GCCAATCTCAATGTGGG                                 | Bc2 R: CCACCGTGTGCTTGATG                              | Bc2 F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | Bc2 R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ        |
| Bcl-2 associated protein (AJ582075)     | Bax F: GCCAATCTCAATGTGGG                               | Bax R: CCCACCGTGTGCTTGATG                             | Bax F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | Bax R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ        |
| Complement C3 (complement C3d region)   | Cam C3d F: GCCACCACATCTAGCAGTA                        | Cam C3d R: TACACCGTGTTGCTTCTGAA                       | Cam C3d F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | Cam C3d R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ    |
| Complement component 5 (DQ369042)       | C5 F: GTATGCCTGCTGCTGGAATT                           | C5 R: GCTGCTGCTGCTGGAATT                             | C5 F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | C5 R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ         |
| Complement protein C1qBP (DQ367730)     | C1qBP F: CCATTAGTGATAGGCAATT                          | C1qBP R: TCGTGGCTGCTGCTGGAATT                        | C1qBP F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | C1qBP R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ     |
| E-cadherin (DQ237892)                   | Ecad F: TTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG | Ecad R: CATCCTTTCTTTGCTGGAACGAA                       | Ecad F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | Ecad R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ      |
| Epithelial mucin (Muc1) (L41545)         | Muc1 F: CGGAAAGCAATCACCTAGAAT                         | Muc1 R: GCCACTACTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG | Muc1 F: 6FAM-CCCTGGAGAAGAGCTATGAGCTGCCTGGAC-BBQ | Muc1 R: 6FAM-TCCTGGACTCTGGTACGGACGGA-BBQ      |
| Gene Name | Simple Name | Forward | Reverse | Primer Sequence |
|-----------|-------------|---------|---------|----------------|
| IL12p40   | Interleukin-12 p40 subunit (AB085792) | TGGTTACCTCCTAGCAGTCC | 6FAM-TCACGAAGATGATGACATCCTC | STAT1b F AGGTCCGTCAGCAGCTTAA |
|           |             | ATCTTGTAAGCCTCCTG | 6FAM-TCACGAAGATGATGACATCCTC | STAT1b R GCCCTTCACCAAAAT |
| STAT1b    | Signal transducer and activator of transcription-1 (AB177397) | AAACAGATGACATCCTCCTC | 6FAM-TCACGAAGATGATGACATCCTC | STAT1b TM 8FAM-TCTAAGTCAGTGCTGAGAGGACA-BBQ |
| STAT1b    |             | TGGTTACCTCCTAGCAGTCC | 6FAM-TCACGAAGATGATGACATCCTC | STAT1b TM 8FAM-TCTAAGTCAGTGCTGAGAGGACA-BBQ |
| Interleukin-2 receptor-α (DQ095372) | Interleukin-21 (FJ664142) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 F AATGCCTTCAGAGTGTACCG |
| STAT2     |             | TGGTTACCTCCTAGCAGTCC | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 R TGTTCACCGTACTATCCACTTCAT |
| STAT2     | Tissue inhibitor of matrix metalloproteinase-2 (AF260255) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Junction adhesion molecule (EU856104) | Junction adhesion molecule (EU856104) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Matrix metalloproteinase-2 (AF260254) | Matrix metalloproteinase-2 (AF260254) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| MHC class II antigen alpha chain (DQ092501) | MHC class II antigen alpha chain (DQ092501) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Myxovirus resistance protein-2 (EU161539) | Myxovirus resistance protein-2 (EU161539) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Nitric oxide synthase-2 (DQ355357) | Nitric oxide synthase-2 (DQ355357) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Occuldin (EU856106) | Occuldin (EU856106) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| p75 tumor necrosis factor membrane receptor (AF315291) | p75 tumor necrosis factor membrane receptor (AF315291) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Platelet endothelial adhesion molecule (AF508040) | Platelet endothelial adhesion molecule (AF508040) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Protein kinase R (DQ645944) | Protein kinase R (DQ645944) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Ribosomal Protein L 18 (DQ403027) | Ribosomal Protein L 18 (DQ403027) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
| Signal transducer and activator of transcription-1 (DQ092343) | Signal transducer and activator of transcription-1 (DQ092343) | TGAAGATACAGCATCTTCCCG | 6FAM-TCACTCCAGTAGCCTTTGCCATCCTC | STAT2 TM 8FAM-CTGACTTTCGCAAGGACCTGGGCT-BBQ |
of the internal control primer and probe set was 0.2 μM and 0.05 μM, respectively. Cycling conditions were as follows: 10 min at 50 °C (initial reverse transcription), 5 min at 95 °C (denaturation and HotStarTaq activation), and 40 cycles of 5 s at 95 °C (denaturation), followed by 10 s at 60 °C per cycle (annealing/amplification). Data acquisition was carried out at the end of the annealing/amplification step in the green (510 nm) and yellow (555 nm) channels.

2.8. Data analysis

The data were analyzed using the ∆∆CT method (Livak and Schmittgen, 2001). Briefly, the CT of each test gene in a treated hamster was first normalized to the RPL18 CT (∆CT) and then compared to the same normalized gene in a mock-treated (calibrator) hamster to determine the ∆∆CT. The final value is displayed as the relative fold increase between the infected/treated and mock treated hamsters.

3. Results and discussion

3.1. Examination of cross-reactivities of ELISA and luminex based cytokine/chemokine detection assays

Many important genes are highly conserved between species; therefore cross-reactivity of antibodies isolated against other rodents may detect hamster proteins. First, we compared amino acid sequence homologies of representative cytokines and chemokines between hamsters, rats, and mice. We selected target proteins showing >90% homology at the amino acid level (data not shown) and tested cross-reactivities of ELISA kits for their detection. In total 63 antibody-based (55 luminex, 8 ELISA) assays were tested for cross-reactivity with hamster proteins and only 14 were determined to display cross-reactivity (Table 2). The mouse and rat ELISA kits tested in this study had limited cross-reactivity with hamster proteins. Of the eight ELISA kits tested, only three (mouse IL-1β, rat IL-10 and rat/mouse/porcine/canine TGFβ1) displayed cross-reactivity against the
In addition, mouse Eotaxin, MIP-1α, GRO/KC were detected with con-
only mouse IL-13 and MIG, and rat GM-CSF, IL-1β detected in comparison to mice. Thereby, we
concluded that the tested ELISA kits are unreliable and of limited use for monitoring proteins involved in immune
responses of hamsters. Similarly, the luminex rat and mouse panel data suggests that of the 55 different proteins tested
using mouse (n=17 per tissue, n=51 combined).

Fig. 2. Distribution of Ct values of the internal control genes in treated and
untreated hamster tissues. Ribosomal protein L18 (RPL18) has the lowest
variation between treated and untreated hamsters, followed by β-2-
microglobulin (β2M), Hypoxanthine–guanine phosphoribosyltransferase
(HPRT) and β-actin (βAct). The boxes indicate the 25th percentile to 75th
percentile values, the middle line is the median value and the whiskers the
total range of the values. 

3.2. Gene data mining and selection

Given the limited cross-reactivity (Table 2) and high costs of mouse and/or rat ELISA and luminex based assays, qRT-
PCR assays were considered and selected as the most practical detection system of choice to analyze hamster
immune responses until further genome sequences become available. For this we analyzed specific hamster sequences registered on Genbank. Of the ~800 sequences available, 51 hamster specific genes involved in pro-inflammatory, anti-
inflammatory, innate immunity, apoptosis, cell junction and coagulation responses, and four constitutively expressed reference genes were selected and TaqMan based qRT-PCR assays developed (Table 3).

3.3. Evaluation of internal reference genes

The identification of a suitable reference gene was of paramount importance for the assay development. Studies
focusing on human tissues previously concluded that commonly used reference genes such as HPRT and β-actin may
not be suitable as internal references due to large variations in expression between tissues and in responses to stresses
(Dheda et al., 2004). Hamsters are outbred animals and therefore variability is inherent between individual animals.
This makes selection of an appropriate internal reference critical (Dheda et al., 2004). β-actin, β2M, HPRT, and RPL18 were selected as potential housekeeping genes and individually evaluated for their expression levels in lung, liver and spleen tissues of treated/infected and control hamsters (n = 17 per tissue, n = 51 total). Fig. 2 shows the distribution of the fluorescence curves within representative tissues of infected, treated and untreated hamster tissues. β2M and RPL18 vary less than HPRT and β-actin (β2M standard deviation (SD) ≤ 1 cycle within tissues, 1.29 cycles between all samples; RPL18 SD ≤ 0.5 cycles within tissues, 0.87 cycles between all samples; HPRT SD ≤ 4.6 cycles within tissues, 3.4 cycles between all samples; β-actin SD ≤ 7.2 cycles within tissues, 7.5 cycles between all samples). Though previous work in hamsters has used HPRT and β-actin as reference genes (Melby et al., 1998; Muller et al., 2001; Vernel-Paulliac and Merien, 2006), in this study these internal reference
genes showed some variation between infected/treated and untreated hamsters and in comparison of different tissues (Fig. 2). The variation of β2M and, especially, RPL18 was much less in all instances. Therefore we chose to use RPL18 as the most reliable internal reference gene and propose that it should be strongly considered for future work.

### 3.4. Primer/probe efficiency and limit of detection

The average efficiency or E value of the primers was determined to be 3.112 (range 2.904–3.321, n=15) (Fig. 3A), i.e. 3.112 cycles per log10 dilution, when used on DNA template and 3.178 (range 2.727–3.414, n=12) (Fig. 3B), i.e. 3.178 cycles per log10 dilution, when used on RNA template. The average r² values of the slopes were determined to be 0.9969 (range 0.9911–0.9996) from the DNA template (Fig. 3A) and 0.9616 (range 0.8148–0.9995) (Fig. 3B) from the RNA template. The signal generated per target template between the DNA template and 3.178 (range 2.727–3.414, n=12) (Fig. 3A), i.e. 3.178 cycles per log10 dilution, when used on RNA template.

The average r² values of the slopes were determined to be 0.9996 (range 0.9911–0.9996) from the DNA template (Fig. 3A) and 0.9616 (range 0.8148–0.9995) (Fig. 3B) from the RNA template. The signal generated per target template between the DNA template and 3.178 (range 2.727–3.414, n=12) (Fig. 3A), i.e. 3.178 cycles per log10 dilution, when used on RNA template.

The expression of pro-inflammatory genes was up-regulated with high transcript levels detected in the spleens and livers of the treated animals. Up-regulation of pro-inflammatory gene expression was less pronounced in lung tissue. Anti-inflammatory and immunoregulatory genes were up-regulated in all three tissues examined with liver being the strongest. The cell regulatory response was enhanced in

### 3.5. Target RNA levels following LPS treatment

Previous studies using hamsters as a sepsis model reported complete fatality within three days post inoculation of *E. coli* (Steinwald et al., 1999). Hamsters used in our study developed severe “sepsis-like” symptoms (lethargy, ruffled fur, hunched posture and labored breathing) within one day following LPS treatment (50 μg) and were therefore euthanized and their tissues harvested. Hamster immune modulation was studied in selected tissues on day 1 post treatment (Fig. 4). The expression of pro-inflammatory genes was up-regulated with high transcript levels detected in the spleens and livers of the treated animals. Up-regulation of pro-inflammatory gene expression was less pronounced in lung tissue. Anti-inflammatory and immunoregulatory genes were up-regulated in all three tissues examined with liver being the strongest. The cell regulatory response was enhanced in

![Fig. 4. Immune responses to LPS treatment in hamsters. Hamster immune gene expression profiles, with representative graphs (randomly selected), in spleen, lung and liver samples collected 1 day post-inoculation with 50 μg of LPS. Data is presented as fold increase over uninfected controls. Error bars indicate standard error of the mean (n = 6). For abbreviations see table in bottom part.](image-url)
spleen and liver tissues, but the anti-apoptotic gene expression was unaltered in all three tissues. Cell junction and integrity responses appeared to be minimally altered in the spleen, increased in the lung and nearly unaffected in the liver in response to LPS treatment and the expression level of vascular endothelial growth factor (VEGF) was strongly decreased in the three tissues examined, suggesting a lack of endothelial cell proliferation (Ferrara and Davis-Smyth, 1997). A previous study focusing on LPS induced sepsis in the hamster model noted that liver and spleen showed higher levels of gene expression than lung and other tissues (Muller et al., 2001). In our study here the responses to LPS inoculation were also more pronounced in liver and spleen than in lung tissue, suggesting a much greater impact on these organs.

3.6. Target RNA levels following VSV infection

Hamsters are especially susceptible to VSV infection. Previous studies report that, in hamsters, as few as 10 pfu of VSV inoculated i.p. will result in uniform lethality within 72 h (Fultz et al., 1981a, 1981b). Upon infection with VSV (10^6 pfu) pro-inflammatory, innate and anti-inflammatory responses were largely up-regulated in all three organs examined with some being down-regulated (Fig. 5). Down- and up-regulation were especially pronounced in liver and spleen tissue (Fig. 5A and C). Pro-apoptotic genes were minimally up-regulated in expression in all tissues, but anti-apoptotic gene expression was increased only in the livers of infected animals. Expression of genes encoding for cell junction proteins was generally unaffected by VSV infection in liver, increased in the lungs, and reduced in the spleen of infected hamsters. Expression of fibrinogen was increased initially followed by a substantial decrease in expression in spleen and liver in final stages of the disease. This suggests that after infection with VSV there is a deregulation of cytokine and chemokine expression, which may precede or induce coagulation abnormalities in spleen and liver tissues of hamsters. Interestingly, the opposite was found for lung tissue (Fig. 5B). VEGF expression was strongly reduced in all

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Fig. 5. Immune responses to VSV infection in hamsters. Hamster immune gene expression profiles, with representative graphs (randomly selected), in (A) spleen, (B) lung and (C) liver collected at 1 and 3 days post infection with 10^6 pfu VSV. Data is presented as fold increase over uninfected controls. Error bars indicate standard error of the mean (n=3 VSV day 1, n=2 VSV day 3). For abbreviations see table in bottom part.
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tissues monitored during both early and terminal stages of infection. Overall the data imply that hamsters mount more robust immune responses in the liver and spleen than in the lung during VSV infection. Previous work identified the spleen and liver as the organs displaying pathological changes during VSV infection without notable involvement of the lungs, kidney, brain or intestinal tract (Fultz et al., 1981b). The similarities between our results and previous studies demonstrate the usefulness of our assays for more in depth evaluation of host responses to infection in an important animal model for which tools are largely lacking.

4. Conclusions

Mouse and rat ELISA and luminex panels tested in these studies were found to be of limited value for detecting hamster host responses. Instead, qRT-PCR assays for selected host response genes were established and validated on LPS-treated and VSV-infected hamsters. The assays allow for broad range monitoring of hamster immune and other cellular activities in host response to stress factors and the identification of RPL18 as an internal reference gene allows for accurate standardization. Thus, the developed assays will be instrumental in future applications of the hamster as a disease model until the full genome sequence is available allowing for the development of large scale RNA microarrays or deep sequencing.

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| Protein | Day 1 | Day 3 | Day 1 | Day 3 |
|---------|-------|-------|-------|-------|
| Mx2     | 20- to 50-fold increase | 10- to 20-fold increase | 5- to 10-fold increase | 2- to 5-fold increase |
| IRF2    | 10- to 20-fold increase | 5- to 10-fold increase | 2- to 5-fold increase | <2-fold increase or decrease |
| Bcl-2   | 5- to 10-fold increase | 2- to 5-fold increase | <2-fold increase or decrease | 2- to 5-fold decrease |

Fig. 5 (continued).
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