High-throughput quantification of camelid cytokine mRNA expression in PBMCs by microfluidic qPCR technology

Authors: Jordi Rodon[1,2], Nigeer Te[2,#], Maria Ballester[3], Joaquim Segalés[1,4], Júlia Vergara-Alert[1,2,*], Albert Bensaid[1,2]

Author affiliations: [1]Unitat mixta d'investigació IRTA-UAB en Sanitat Animal, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Catalonia, Spain; [2]IRTA, Programa de Sanitat Animal, Centre de Recerca en Sanitat Animal (CReSA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Catalonia, Spain; [3]Animal Breeding and Genetics Program, Institute for Research and Technology in Food and Agriculture (IRTA), Caldes de Montbui, Spain; [4]Departament de Sanitat i Anatomia Animals, Facultat de Veterinaria, Universitat Autònoma de Barcelona (UAB), Campus de la UAB, 08193 Bellaterra, Catalonia, Spain.

Email addresses: J.R. (jordi.rodon@irta.cat); N.T. (tenigeer@hku.hk); M.B. (maria.ballester@irta.cat); J.S. (joaquim.segales@irta.cat); J.V.-A. (julia.vergara@irta.cat) and A.B. (albert.bensaid@irta.cat).

[#]Current affiliation: School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong.

*Correspondence should be addressed to J.V.-A. (julia.vergara@irta.cat).

ABSTRACT

Camelids are economically and socially important in several parts of the world and might carry pathogens with epizootic or zoonotic potential. However, biological research in these species is limited due to lack of reagents. Here, we developed RT-qPCR assays to quantify a panel of camelid innate and adaptive immune response genes, which can be monitored in a single run. Validation of the assays was performed with PHA, PMA-ionomycin, and Poly I:C-stimulated PBMCs from alpaca, dromedary camel and llama, including normalization by multiple reference genes. Further, comparative gene expression analyses for the different camelid species were performed by a unique microfluidic qPCR assay. Compared to unstimulated samples, PHA and PMA-ionomycin stimulation elicited robust Th1 and Th2 responses in PBMCs from camelid species. Additional activation of type I and type III IFN signalling pathways was described exclusively in PHA-stimulated dromedary lymphocytes, in contrast to those from alpaca and llama. We also found that PolyI:C stimulation induced robust antiviral response genes in alpaca PBMCs. The proposed methodology should be useful for the measurement of immune responses to infection or vaccination in camelid species.

Keywords: Camelid species, cytokines, immune responses, RT-qPCR, Fluidigm Biomark, PHA, PMA-ionomycin, PolyI:C, dromedary, camel, llama, alpaca

INTRODUCTION

Camelid species are livestock of great economic, sanitary, health and environmental importance in the north of Africa, central Asia, the Middle East, and South America. Number of these animals is expected to grow since the camelid industry is in transition from nomadism to intensive production (FAOSTAT, 2021). In Europe, camelids are used for fine wool production but also kept as pets, guardians of other livestock, or used for recreational or leisure purposes (Halsby et al., 2017). However, these species are susceptible to several viruses, bacteria and protozoan parasites affecting meat and milk production. As an example, Camelpox and Peste des Petits Ruminants viruses are causing recurrent epizootic outbreaks in Africa and Middle East (Khalafalla, 2017). Furthermore, there is limited information available about the role of camelids in the epidemiology of zoonotic diseases. In recent years, the most studied pathogens causing zoonotic diseases included the Middle East respiratory syndrome coronavirus (MERS-CoV), Rift Valley fever virus (RVFV), *Brucella* sp., and *Echinococcus granulosus* (Zhu et al., 2019). Many other less studied viruses known to be carried and transmitted by camelids, such as hepatitis E virus (HEV) or Crimean-Congo haemorrhagic fever virus (CCHFV), are of serious human health concern as well. Camelids are also hosts of many other fastidious bacterial diseases including tuberculosis, gastrointestinal illnesses caused by verotoxin-producing *E. coli*, campylobacteriosis, listeriosis and salmonellosis, among others, as well as protozoan parasites (*Cryptosporidium* spp., *Sarcoptes*, *Giardia duodenalis*, etc.) of veterinary and human health concern (Khalafalla, 2017).

Understanding disease pathogenesis and identifying protective immune responses are prerequisites for the rational development of new anti-microbial drugs and vaccines. In addition, comparison of immune responses between humans and domestic or wildlife

species would shed insights to delineate host factors involved in disease outcome. Upon pathogen infection, the host immune system is regulated by complex mechanisms in which cytokines play a pivotal role in determining the intensity and duration of the immune response (Dinarello, 2007; Svanborg et al., 1999). In some domestic species, such as pig, goat, cattle, and sheep, the quantification of cytokines, either at the protein or mRNA level, has become a widely used method to monitor immune responses upon pathogen infection (Smeed et al., 2007; van Reeth & Nauwynck, 2000). However, cytokine detection in camelids has been hampered by the lack of specific reagents.

To date, there are few reliable commercial ELISA kits available to study immune responses in camelids at the protein level. Nonetheless, camelid interferon (IFN)-α, and some Th1 and Th2 cytokines, and pro-inflammatory cytokine cDNAs have been cloned and sequenced (Nagarajan et al., 2012; Odbileg et al., 2006; Odbileg, Konnai, et al., 2005; Odbileg, Lee, et al., 2004, 2005; Premraj et al., 2020). Sets of primers have been derived from these sequences (Odbileg, Konnai, et al., 2004) to quantify cytokine mRNAs in peripheral blood mononuclear cells (PBMCs) of *Camelus bactrianus* upon vaccination with *Brucella abortus* strain 19 by reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays (Odbileg et al., 2008). Although these previous works provided tools to quantify a few camelid cytokine mRNAs, primer assays only allow for a cursory study of immune response pathways and were not optimized to function in high-throughput qPCR platforms. We previously took advantage of well-annotated camelid draft genomes (Wu et al., 2014) to design a comprehensive set of primers from genes encompassing several innate immune response pathways (Te et al., 2021, 2022), and demonstrated their usefulness in respiratory tract samples of alpacas. Here, we extended this panel of primers to characterize the expression of innate and adaptive immune response genes in

phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA)-ionomycin and/or PolyI:C-stimulated PBMCs from three different camelid species (dromedaries, llama, and alpacas). We optimized gene expression analyses in the highly sensitive and cost-effective Fluidigm Biomark microfluidic qPCR system. A full validation and standardization of these assays is provided together with an interspecies comparison, characterizing camelid cytokine expression with non-specific PBMC stimuli widely used in immunological research.

MATERIALS AND METHODS

The present work was performed using the nomenclature and following the validation protocols proposed by the Minimum Information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009).

Animal welfare, ethics, and experimental design

Experiments with animals were performed at animal facilities of IRTA Alcarràs farm (Lleida, Catalonia, Spain) or at the Biosafety Level-3 (BSL-3) facilities of IRTA-CReSA (Barcelona, Catalonia, Spain), and were approved by the Ethical and Animal Welfare Committee of IRTA (CEEA-IRTA) and by the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (approval No. FUE-2017-00561265 and FUE-2018-00884575).

Two llamas (L1, L2) and five alpacas (A1-5) were purchased from Belgium and The Netherlands, respectively, housed at IRTA-CReSA animal facilities and used for routine blood collection. L1 and L2 were used in a previously published study (Rodon et al., 2019), and blood was collected prior experimental infection with MERS-CoV. One healthy dromedary camel (D1) from a private zoo (Alicante, Valencian

Community, Spain) was also bled once for routine checking purposes, and extra blood samples were taken to perform this work.

Blood collection

Whole blood samples (40 to 50 mL) from each animal were collected from the jugular vein using EDTA BD Vacutainer® tubes (Beckton Dickinson, New Jersey, USA), following animal welfare protocols.

PBMC isolation

Prior PBMCs isolation, whole blood was diluted 1:1 with phosphate-buffered saline (PBS). PBMCs were harvested from blood by density-gradient centrifugation with Histopaque®-1083 (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. PBMCs were cultured in RPMI-1640 media supplemented with antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin) and glutamine (2 mmol/L) purchased from Life Technologies (Waltham, USA), β2-mercaptoethanol (5×10^{-5} M; Sigma-Aldrich, MO, USA), and 10% heat inactivated foetal calf serum (FCS; EuroClone, Pero, Italy). Cell viability was assessed by the Trypan blue staining exclusion method.

Cell stimulation assays

PBMCs from A1-2, D1, and L1-2 were seeded on 24-well plates at $5\cdot10^6$ cells/mL, and cultured in duplicates in medium alone (control condition), or stimulated with 10 µg/mL of PHA (Sigma-Aldrich, St. Louis, MO, USA), or with a combination of 10 ng/mL PMA (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/mL ionomycin calcium salt (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 37°C and 5% CO_2. Additionally, PBMCs from A3-5 were cultured with 250 ng/mL Poly(I:C)-LMW/LyoVec™ (PolyI:C; Invivogen, San Diego, USA) for 48 h at 37°C and 5% CO_2. Afterwards,

PBMCs were carefully collected by up and down pipetting and transferred to a DNase/RNase-free tube. After centrifugation, supernatants were removed and lysis buffer for RNA extraction was added to the cell pellet. All samples in lysis buffer were stored at -80□ until RNA extraction.

RNA extraction and quantification

Total RNA was extracted from PBMCs using the RNeasy® Mini Kit (Qiagen Ltd., Crawley, UK), according to the manufacturer's protocol. After RNA elution, an additional DNase I treatment was performed using the Heat&Run gDNA removal kit (ArcticZymes Technologies, Tromsø, Norway), following the manufacturer's protocol. Finally, RNase inhibitors (Invitrogen, Life Technologies, Waltham, USA) were added to the RNA samples in a final concentration of 1 U/µL prior storage at -80°C until reverse transcription (RT) reaction was performed.

The purity, quantity and integrity of the extracted RNA were assessed using a BioDrop *µLITE* Spectrophotometer (BioDrop Ltd, Cambridge, UK) and Lab-Chip analysis (Agilent Technologies, Santa Clara, USA). The A260:A280 ratio ranged from 1.6 to 2.1, and RNA Integrity Numbers (RIN) ranged from 7 to 9.6.

cDNA synthesis

Total RNA samples were used to generate cDNA as previously described (Te et al., 2021). Briefly, 110 ng of RNA were retrotranscribed in a final volume of 10 µL using the PrimeScript RT reagent Kit (Takara, Kusatsu, Japan) with a combination of oligo-d(T) and random hexamers, according to the manufacturer's protocol. No-reverse transcription controls (no-RT) with all buffers and reagents supplied by the kits, but omitting the reverse transcriptase, were prepared to assess non-specific amplifications and presence of genomic DNA (gDNA).

Additionally, control cDNA samples from stimulated PBMCs were obtained with the aim to generate standard curves and determine primer pair efficiencies. Samples were pooled by species at the same proportion per individual animal, except for the dromedary camel. For each species, pools contained cDNA samples from PMA-ionomycin and PHA-stimulated PBMCs at 1:1 proportion, while alpaca PBMCs stimulated with PolyI:C were pooled independently. Finally, samples were serially diluted by 1:4 steps (1/20, 1/80, 1/320, 1/1280, 1/5120) prior amplification reactions.

Primer design of immune associated and reference genes

Camelid genes and mRNA were found through bibliographic search or with described mRNAs in other species performing BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed through comparative genomics of sequences deposited at the National Centre for Biotechnology Information (NCBI) GenBank database of llama (*Lama glama*), alpaca (*Vicugna pacos*), dromedary camel (*Camelus dromedarius*), bactrian camel (*Camelus bactrianus*), and wild bactrian camel (*Camelus ferus*). Comparison of mRNA and genomic sequences of each studied gene were performed with the alignment tool ClustalW to determine exon boundaries. In some instances, exons were already annotated in camelid genomes.

Primer pairs were designed with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast), or Primer Express 2.0 (ThermoFisher Scientific, Life Technologies, Waltham, USA), according to the following desirable criteria: (i) to span two or more exons, and some of them were placed at the exon-exon boundaries, (ii) 17-23 nucleotides in length, close to the mRNA 3’ end when possible, (iii) GC-content percentage between 45 and 55%, (iv) leading to an approximate 80-200 bp PCR product, (v) melting temperature (Tm) of each primer between 57-63°C with less than 2°C difference within primer pairs, and (vi) avoiding

primer hairpin, self-primer dimer or cross-primer dimer formation.

The avoidance of primer secondary structure arrangement was assessed through the Beacon Designer™ program (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1), selecting for primers with ΔG greater than -3.5 kcal/mol when possible. Further, primer sequence specificity was assessed using the BLASTn alignment tool against all camelid genome sequences. Potential transcription of predicted pseudogenes was assessed by carrying out promoter region analyses through the VISTA (http://genome.lbl.gov/vista/customAlignment.shtml) and the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter) softwares. Finally, the primer position within exons was checked *in silico* with their respective camelid gene sequences from the NCBI GenBank using the MapViewer tool. All the primers designed in this study are summarized in Table1. Table S1 compiles GenBank accession numbers of camelid genes and mRNA used in this study. Table S2 compiles the principal characteristics of genes and derived primers used in this study. Oligonucleotides used in this study were supplied by Roche Diagnostics (Sant Cugat del Vallès, Barcelona, Spain).

Cytokine quantification by Fluidigm Biomark microfluidic RT-qPCR

cDNA obtained from PBMCs samples were used to validate the whole panel of primers designed for camelid species and to quantify gene expression levels by a microfluidic qPCR technique. Firstly, cDNA samples were pre-amplified using the TaqMan PreAmp Master Mix (Applied Biosystems, Life Technologies, Waltham, USA), following the manufacturer's recommendations, doing an initial activation step of the AmpliTaq Gold DNA Polymerase for 10 min at 95°C, followed by 16 cycles of 15 seconds denaturation at 95°C plus 4 min annealing and extension at 60°C. Pre-amplified products were treated with Exonuclease I (New England Biolabs, Ipswich, USA) for 30 min at 37°C

to eliminate the carryover of unincorporated primers. An inactivation step of the enzyme for 15 min at 80°C was included according to the manufacturer's protocol. The 96.96 Dynamic Array IFCs, the 96.96 DNA Binding Dye Sample/Loading Kit (Fluidigm Corporation, South San Francisco, USA) was prepared according to the manufacturer's instructions. Pre-amplified samples were diluted 1/20 in 1x TE Buffer, and aliquots of 2.25 µL of each sample and 0.6 µL of primer pairs at 100 µM were loaded in duplicates into their respective array inlets. Quantification of PCR reactions was performed on a Biomark HD system (Fluidigm Corporation, South San Francisco, USA). The PCR consisted in an initial activation step of 1 min at 95°C, followed by 30 cycles of 5 seconds at 96°C plus 1 min at 60°C. A dissociation step, increasing 1°C every 3 seconds from 60 to 95°C, was included for all reactions to confirm single specific PCR product amplification and define the Tm of each amplicon. Additionally, stimulated control samples were assayed in triplicates to create relative standard curves and calculate primer amplification efficiencies (see Table S3). No-RT controls and no-RNA template controls (NTC) were included in each assay to check for non-specific amplification or primer-dimer formation.

Relative quantification and data analysis

Expression data was collected with the Fluidigm Real-Time PCR analysis software 4.1.3 (Fluidigm Corporation, South San Francisco, USA). Cycle of quantification (Cq) threshold detection value was set at 0.020, quality threshold cut-off value was established at 0.65 and amplification specificity was assessed by Tm analyses for each reaction. Amplifications fulfilling the above criteria were analysed using the DAG expression software 1.0.5.6 (Ballester et al., 2013) to apply the relative standard curve method (Livak, 1997). Cq values obtained from pooled cDNA controls were used to create standard curves for each gene, species and PBMC stimulation condition, and to

extrapolate the relative quantity values. R-squared values were determined for each standard curve and the specific PCR efficiencies were calculated by applying the formula (10^(-1/slope value)-1)*100 (see Table S3). Multiple reference gene normalization was performed by using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase (*HPRT1*) and ubiquitin C (*UBC*) as endogenous controls. Their suitability for normalization procedures was assessed by control-gene stability analyses with the DAG expression software 1.0.5.6 (Ballester et al., 2013). The normalized quantity values of each sample and assay were used for direct comparison in relation to non-stimulated PBMCs samples cultured during 48 h. Therefore, the up- or down-regulated expression of each gene was expressed in fold changes (Fc).

Statistical analyses could only be applied in results from alpaca PBMCs cultured with PolyI:C stimulus, due to the sample size. Fc values were logarithmically transformed to achieve normal distributions. Means of the transformed fold changes obtained for stimulated and unstimulated conditions were compared using unpaired t-test analyses in R and GraphPad Prism softwares. Differences were considered significant at p-values < 0.05.

RESULTS

Selection of immune-related genes and primer design

The selected genes encompassed several functional categories representative of pathogen innate and adaptive immune responses, and comprised type I, II and III IFNs, pattern recognition receptors (PRRs), transcription factors (TFs), IFN-stimulated genes (ISGs), pro- and anti-inflammatory cytokines, enzymes, adaptors, cellular receptors, and other genes involved in Th1 and Th2 responses. In addition, three reference genes,

GAPDH, *HPRT1* and *UBC*, were selected to normalize gene expression. Table 1 summarizes genes and primers designed for subsequent expression analyses.

Primer sets were designed to anneal in conserved transcribed regions of genes from five camelid species (alpaca, Bactrian camel, dromedary camel, llama, and wild Bactrian camel). Some of the genes were not annotated in the genome of llama (see Table S1) but exon/intron boundaries could be found by performing a BLASTn with the mRNA of other camelid species. The main features of the designed primer pairs are listed in Table S2.

Primer amplification efficacy and specificity

Pools of cDNAs prepared from stimulated PBMCs were used to evaluate the whole panel of primers in samples from alpaca, dromedary, and llama. Number of dilutions used for the generation of each standard curve, slopes, coefficients of determination and amplification efficiencies are listed in Table S3. After 48 h of PBMC stimulation, all gene transcripts were sufficiently expressed to generate standard curves using 3 to 5 serial dilutions, except for *IFNAR1* in samples from dromedary and *IFN-λ1* in those from dromedary camel and llama (Table S3). *IFN-λ3* was not expressed in PBMCs from any species, regardless of the stimuli type (Table S3). All calibration curves produced linear standard curves, as evidenced by high coefficients of determination (>0.95, except for 1 sample that was 0.886). Primer pairs resulted in optimal amplification efficiencies in the different camelid species, ranging from 69 to 100%. Tm analyses confirmed a single specific amplicon for all amplified gene transcripts in all three camelid species. No amplifications occurred in no-RT and NTC samples included in the microfluidic RT-qPCR assays. Thus, most of the designed primer pairs targeting camelid cytokines and immune-related genes, as well as endogenous genes, displayed optimal specificity and efficacy suitable for immune response studies in

291 camelid PBMCs.

292 **Gene expression analyses of llama PBMCs by microfluidic RT-qPCR**

293 Transcriptomic gene expression profile of llama PBMCs stimulated for 48 h with PHA
294 and PMA-ionomycin were compared to those of unstimulated cells. Relative expression
295 of the different genes grouped by functional categories is shown in Figure 1a-j. PHA
296 and PMA-ionomycin stimulation provoked similar gene expression profiles in llama
297 PBMCs. Both stimuli induced expression of *IFN-γ* at high levels (114 and 123 Fc,
298 respectively), but none of the other type I or III IFNs were upregulated (Figure 1a).
299 Within the category of ISGs, *CXCL10* expression was induced by PHA and PMA-
300 ionomycin and *MX1* only by PHA stimulated samples (Figure 1c). PHA and PMA-
301 ionomycin also provoked the upregulation of *CCL3*, *TNF-α*, *IRF7* and *NFKB1* (Figure
302 1d, e and f). High levels of *IL-6* (Fc of 79.82 and 62.47), *IL-2* (Fc of 71.32 and 80.30)
303 and *IL-4* (Fc of 195.78 and 145.58) expression were found of PHA and PMA-
304 ionomycin stimulation, respectively (Figure 1e and j). Transcription of other pro- or
305 anti-inflammatory cytokines, PRRs, adaptors, enzymes and IFN receptors were not
306 induced in llama stimulated PBMCs (Figure 1b, e, g, h and i). Globally, the results
307 obtained in llama cells were according to the expectancy that PHA and PMA-
308 ionomycin provoke a marked polyclonal stimulation of PBMCs.

309 **Gene expression analyses of alpaca PBMCs by microfluidic RT-qPCR**

310 We utilized the same methodology to study immune gene expression in alpaca. Gene
311 expression profile of stimulated PBMCs (PHA, PMA-ionomycin and PolyI:C) were
312 compared to that of unstimulated cells (Figure 2a-j). PHA and PMA-ionomycin
313 stimulation triggered an upregulation of *RIG-1*, *MDA5*, *MX1*, *ISG15*, *CXCL1*, and *IL-8*
314 compared to non-stimulated samples cultured for 48 h (Figure 2b, c, d, e). Moreover,

315 expression of *IFN-β*, *IFN-γ*, *IL-10*, *IL-6*, *STAT-1*, *IRF7*, *NFKB1*, *CASP1*, *IL-2* and *IL-4*
316 was more markedly upregulated in PHA-stimulated PBMCs (Figure 2a, e, f, h and j).

317 On the other hand, PMBCs from three additional alpacas were cultured with PolyI:C to
318 ensure functionality of primers targeting mRNA of impassive genes to PHA or PMA-
319 ionomycin stimulation. PolyI:C exposure for 48 h resulted in the upregulation of
320 different immune genes when compared with the previous polyclonal stimulations
321 (*IFN-α*, *IFN-β*, *RIG-1*, *MDA5*, *TLR3*, *TLR7*, *CXCL10*, *MX1*, *OAS1*, *ISG15*, *IL-10*, *IL-6*,
322 *STAT1* and *IRF7*), some of them being expressed at high levels such as *IFN-β* (83.72
323 Fc), *MX1* (30.68 Fc) and *ISG15* (62.34 Fc) (Figure 2a-f). Moreover, statistical analyses
324 determined a significant increase in *IFN-β*, *RIG-1*, *MDA5*, *TLR7*, *NLRP3*, all ISGs,
325 *STAT1* and *IRF7* expression levels compared to non-stimulated alpaca PBMCs.
326 Therefore, these results confirmed Poly I:C as a good *in vitro* immunostimulant of
327 antiviral responses in alpaca PBMCs.

328 **Gene expression analyses of dromedary PBMCs by microfluidic RT-qPCR**

329 Expression of dromedary immune genes in stimulated PBMCs were compared to that of
330 unstimulated cells (Figure 3a-j). Dromedary cells stimulated with PHA and PMA-
331 ionomycin displayed similar transcriptomic profiles with characteristics of classical
332 polyclonal stimulations. Relative to non-stimulated control samples, both PHA and
333 PMA-ionomycin stimuli provoked the induction of *IFN-β*, *IFN-γ*, *CCL3*, *CXCL1*, *IL-6*,
334 *IL-8*, *TNF-α*, *MIF*, *NFKB1*, *NFKBIA* and *IL-4* in higher relative expression levels than
335 unstimulated PBMCs (Figure 3a, d, e, f, g and j). In addition, specific PHA stimulation
336 led to increased mRNA levels of *IFN-α*, *IFN-λ1*, *RIG-1*, *MDA5*, *CXCL10*, *MX1*, *OAS1*,
337 *ISG15*, *IL-10*, *IL-1β*, *IRF7* and *TRIM25*, while transcription of *IL-2* was markedly
338 induced in PMA-ionomycin stimulated PBMCs of dromedary camels (Figure 3a, b, c, e,
339 f, h and j). Overall, dromedary PBMCs were properly stimulated by PHA and PMA-

ionomycin, enhancing immune-related gene expression in accordance with regular polyclonal stimulations.

DISCUSSION

Due to the lack of reagent availability (i.e., antibodies) in several animal species, analysis of gene expression has become a common method to determine the immune transcriptomic profile after infection and/or vaccination. In this work, we implemented an RT-qPCR method to monitor camelid innate and adaptive immune responses, which allow the quantification of forty-seven cytokines and immune-related genes in a single run. Importantly, the designed primer sets can be used for all camelid species using the same conditions (primer hybridization temperature) and methodology.

Several infectious diseases affect camelid species and threaten livestock productivity (Camel pox virus, trypanosomiasis, and gastro-intestinal helminthiases, among others). Also, camelids are a source for several zoonotic viral and bacterial pathogens (MERS-CoV, Crimean-Congo haemorrhagic fever virus, *Rickettsia* spp, and others) which threaten public health (Khalafalla, 2017; Wernery et al., 2014). However, immune responses elicited upon infections of camelids remain largely unknown. Although a previous work provided tools to quantify inflammatory cytokines mRNA from llama (Odbileg, Konnai, et al., 2004), the primers were designed before available annotation of camelid genomes (Wu et al., 2014). Therefore, we designed new primers sets to perform more accurate assays.

First, we focused on the requirements to ensure a specific amplification of the products (Taylor et al., 2010) and established criteria for the subsequent design of primers. Although not all primer designs fulfilled all parameters of the criteria (i.e., genes with a

single exon as IFNs α, β, and λ1), specific amplification was determined together with the absence of amplification in no-RT and NTC controls, which ensured that gDNA amplification did not occur. In addition, three reference genes, (*GAPDH*, *HPRT1* and *UBC*) were selected to normalize gene expression (Cicinnati et al., 2008; Mehta et al., 2010; Souza et al., 2013). These genes have been reported to be stable for data normalization in T lymphocytes (Albershardt et al., 2012; Bas et al., 2004; Dheda et al., 2004) and respiratory samples (Resa et al., 2014; Te et al., 2021). Choosing appropriate reference genes is crucial to achieve optimal data normalization, which is mandatory to discard sample-to-sample variations. Here, the selected normalizer genes were stable in PBMC samples from all the studied species, regardless the type of stimulation.

The designed primers would allow studying camelid immune responses in most laboratories worldwide, including those in developing countries, which have been infrastructurally and technically upgraded to perform PCR-based assays since the recent influenza virus outbreaks and the COVID-19 pandemics (Breiman et al., 2007). Nonetheless, to study expression analyses of a broad panel of genes in multiple samples by the gold standard RT-qPCR can result tedious and relatively expensive. Thus, we integrated the whole set of designed primers in a unique Fluidigm Biomark microfluidic qPCR assay. Gene expression analyses showed that PHA and PMA-ionomycin stimulation produced strong induction of some cytokines and immune gene transcripts in camelid PBMCs, which displayed profiles commonly found in polyclonally activated lymphocytes of bovine (Norian et al., 2015) and human (Rostaing et al., 1999). PBMCs from dromedary camel and llama underwent a robust increase in *IFN-γ*, *IL-4* and *IL-6* expression after PHA and PMA-ionomycin exposure, and the same phenomenon occurred in alpaca PBMCs stimulated with PHA but not with PMA-ionomycin. Transcription of the pro-inflammatory *IL-8* and the anti-

389 inflammatory *IL-10* was also upregulated in polyclonally-stimulated alpaca and
390 dromedary cells, but not in those from llama. Upregulation of *IL-8* in camelid cells was
391 expected since it is tightly regulated upon activation of the transcription factor NF-κB
392 (Liu et al., 2017). Similar to previous results in humans (Baran et al., 2001), *IL-10*
393 expression was only upregulated by PHA stimulation but PMA-ionomycin was
394 ineffective. Furthermore, it is known that type I and III IFNs activate a different
395 signalling pathway than *IFN-γ* does, which can lead to the expression of distinct ISGs
396 (Sen et al., 2018; W. Wang et al., 2017). *CXCL10* and *CCL3* are typically classified as
397 ISGs induced by *IFN-γ* (Rabin, 2003; Sen et al., 2018). Upregulation of these
398 chemokines was observed in all dromedary and llama samples expressing high levels of
399 *IFN-γ*, apart from *CXCL10* in dromedary cells stimulated with PMA-ionomycin. As
400 reported here, the stimulation of camelid cells using PHA and PMA-ionomycin did not
401 activate the transcription of PRRs, adaptors, enzymes and IFN receptors compared to
402 unstimulated controls. As observed in other mammalian species (Ciliberti et al., 2017;
403 Gao et al., 2010; Lin et al., 2021; Norian et al., 2015; Wagner & Freer, 2009), PHA and
404 PMA-ionomycin are also potent antigen surrogate activators of Th1 and Th2 cytokine
405 expression in alpaca, dromedary, and llama PBMCs. Our results support that PHA and
406 PMA-ionomycin can be used to monitor camelid T-cell activation, proliferation, and
407 effective cytokine production.

408 Curiously, a broader gene expression profile was observed in dromedary PBMCs 48 h
409 after PHA stimulation. Besides the activation of type II IFN signalling pathway, PHA
410 also upregulated the expression of type I and III IFNs, as well as downstream genes
411 regulated upon the IFN signalling cascade activation. Consequently, strong induction of
412 PRRs and ISGs occurred in dromedary samples but not in those from alpaca or llama.
413 Expression of *IRF7* and *IL-10* were also exclusively upregulated in PHA-stimulated

dromedary PBMCs, in agreement with previous reports showing that both type I and III IFNs increase the transcription of *IRF7* factor *in vitro* (Zhou et al., 2007), and that IL-10 production is enhanced as a type III IFN-stimulated gene (Stanifer et al., 2019). Further analyses involving samples from more animals would help to rule out individual animal variations to confirm whether dromedary camels have a broader immune response after PHA stimulation than other camelid species.

In vitro stimulation of PBMCs with PolyI:C is known to elicit the expression of cytokines, mimicking certain aspects of viral infection (Huang et al., 2006). After 48 h stimulation, alpaca PBMCs sensed PolyI:C and increased the expression of type I IFNs, all studied PRRs and ISGs, along with the transcription factors *STAT1* and *IRF7*. Contrary to human PBMCs (Murata et al., 2014), alpaca lymphocytes exposed to PolyI:C induced type I but not type III IFNs. Previous investigations indicated that *IFN-α* was not produced by ovine and bovine PBMCs treated with PolyI:C (Booth et al., 2010), hence future studies could shed light on differential antiviral immune responses among livestock species. Globally, PolyI:C stimulation of alpaca PBMCs yielded similar results than porcine and human PBMCs did (Docampo et al., 2017; Huang et al., 2006; J. Wang et al., 2016). Thus, Poly I:C is a good immunostimulant of antiviral responses in alpaca PBMCs. Further research is needed to determine if other camelid species are equally responsive to Poly I:C.

In the current work, regardless of the stimuli used to boost cytokine expression, none of the PBMC stimulation assays in any camelid species showed detectable levels of *IFN-λ3* expression. However, we previously demonstrated an optimal amplification of *IFN-λ3* mRNA in the nasal epithelium of MERS-CoV infected alpacas (Te et al., 2021, 2022) using the pair of primers reported here. Primers targeting *IFN-λ3* were also designed to anneal mRNA of all camelid species, therefore, a correct amplification is

439 also expected in samples from other camelids, although further studies are needed to
440 confirm this hypothesis.

441 In summary, we implemented a RT-qPCR method for the simultaneous quantification
442 of cytokines and immune-related genes involved in major immune response signalling
443 pathways of different camelid species. The novel assay was set up after the design of
444 primers targeting immune genes and performing data normalization with three
445 reference genes. The assays were validated using PBMCs from alpaca, dromedary
446 camel and llama PBMCs after stimulation with PHA, PMA-ionomycin or PolyI:C.
447 Microfluidic RT-qPCR results indicated that PBMCs from all camelid species
448 stimulated with PHA and PMA-ionomycin induce genes characteristic of Th1 and Th2
449 responses, besides PHA activation of type I and III IFN signalling pathways in
450 dromedary lymphocytes. PolyI:C stimulation produced a marked antiviral response in
451 alpaca PBMCs.

452

453 **ACKNOWLEDGEMENTS**

454 The authors thank Joana Ribes from the Centre for Research in Agricultural Genomics
455 (CRAG, Cerdanyola del Vallès, Barcelona) for her technical guidance in the use of
456 Fluidigm BioMark technology. This study was performed as part of the Zoonotic
457 Anticipation and Preparedness Initiative (ZAPI project) [Innovative Medicines initiative
458 (IMI) grant 115760] and the Veterinary Biocontained research facility Network
459 (VetBioNet) project (EU Grant Agreement INFRA-2016-1 Nº731014), with assistance
460 and financial support from IMI and the European Commission and contributions from
461 EFPIA partners. J.R. was partially supported by the VetBioNet project. IRTA is
462 supported by CERCA Programme / Generalitat de Catalunya.

AUTHOR CONTRIBUTIONS

J.R., J.S., J.V.-A. and A.B. conceived and designed the study. J.R., N.T., M.B., A.B., J.S., J.V.-A and A.B. performed the experiments and analyzed the data. All the authors discussed the results. The manuscript was written by J.R. and all the authors revised the manuscript.

REFERENCES

Albershardt, T. C., Iritani, B. M., & Ruddell, A. (2012). Evaluation of reference genes for quantitative PCR analysis of mouse lymphocytes. *Journal of Immunological Methods*, *384*(1–2), 196–199. https://doi.org/10.1016/j.jim.2012.07.020

Ballester, M., Cordón, R., & Folch, J. M. (2013). DAG expression: High-throughput gene expression analysis of real-time PCR data using standard curves for relative quantification. *PLoS ONE*, *8*(11), 8–12. https://doi.org/10.1371/journal.pone.0080385

Baran, J., Kowalczyk, D., Ozog, M., & Zembala, M. (2001). Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: Comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. *Clinical and Diagnostic Laboratory Immunology*, *8*(2), 303–313. https://doi.org/10.1128/CDLI.8.2.303-313.2001

Bas, A., Forsberg, G., Hammarström, S., & Hammarström, M.-L. (2004). Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scandinavian Journal of Immunology*, *59*(6), 566–573.

https://doi.org/10.1111/j.0300-9475.2004.01440.x

Booth, J. S., Buza, J. J., Potter, A., Babiuk, L. A., & Mutwiri, G. K. (2010). Co-stimulation with TLR7/8 and TLR9 agonists induce down-regulation of innate immune responses in sheep blood mononuclear and B cells. *Developmental and Comparative Immunology*, *34*(5), 572–578. https://doi.org/10.1016/j.dci.2009.12.018

Breiman, R. F., Nasidi, A., Katz, M. A., Njenga, M. K., & Vertefeuille, J. (2007). Preparedness for Highly Pathogenic Avian Influenza Pandemic in Africa. *Emerging Infectious Disease Journal*, *13*(10), 1453. https://doi.org/10.3201/eid1310.070400

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Cicinnati, V. R., Shen, Q., Sotiropoulos, G. C., Radtke, A., Gerken, G., & Beckebaum, S. (2008). Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. *BMC Cancer*, *8*, 350. https://doi.org/10.1186/1471-2407-8-350

Ciliberti, M. G., Albenzio, M., Inghese, C., Santillo, A., Marino, R., Sevi, A., & Caroprese, M. (2017). Peripheral blood mononuclear cell proliferation and cytokine production in sheep as affected by cortisol level and duration of stress. *Journal of Dairy Science*, *100*(1), 750–756. https://doi.org/10.3168/jds.2016-11688

Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G., & Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time

513 PCR. *BioTechniques*, *37*(1), 112–119.
514 https://doi.org/http://dx.doi.org/10.1016/j.ymeth.2010.01.003
515 Dinarello, C. A. (2007). Historical insights into cytokines. *European Journal of*
516 *Immunology*, *37*(S1), S34–S45.
517 https://doi.org/https://doi.org/10.1002/eji.200737772
518 Docampo, M. J., Cabrera, J., & Bassols, A. (2017). Hyaluronan mediates the adhesion
519 of porcine peripheral blood mononuclear cells to poly (I:C)-treated intestinal cells
520 and modulates their cytokine production. *Veterinary Immunology and*
521 *Immunopathology*, *184*, 8–17. https://doi.org/10.1016/j.vetimm.2016.12.008
522 Food and Agriculture Organization of the United Nations Statistics Division. (2021).
523 *FAOSTAT Statistical Database*. www.fao.org/faostat
524 Gao, Y., Flori, L., Lecardonnel, J., Esquerré, D., Hu, Z. L., Teillaud, A., Lemonnier, G.,
525 Lefèvre, F., Oswald, I. P., & Rogel-Gaillard, C. (2010). Transcriptome analysis of
526 porcine PBMCs after in vitro stimulation by LPS or PMA/ionomycin using an
527 expression array targeting the pig immune response. *BMC Genomics*, *11*(1), 1–25.
528 https://doi.org/10.1186/1471-2164-11-292
529 Halsby, K., Twomey, D. F., Featherstone, C., Foster, A., Walsh, A., Hewitt, K., &
530 Morgan, D. (2017). Zoonotic diseases in South American camelids in England and
531 Wales. *Epidemiology and Infection*, *145*(5), 1037–1043.
532 https://doi.org/10.1017/S0950268816003101
533 Huang, C. C., Duffy, K. E., San Mateo, L. R., Amegadzie, B. Y., Sarisky, R. T., &
534 Mbow, M. L. (2006). A pathway analysis of poly(I:C)-induced global gene
535 expression change in human peripheral blood mononuclear cells. *Physiological*
536 *Genomics*, *26*(2), 125–133. https://doi.org/10.1152/physiolgenomics.00002.2006
537 Khalafalla, A. I. (2017). *Emerging Infectious Diseases in Camelids BT - Emerging and*

538 *Re-emerging Infectious Diseases of Livestock* (J. Bayry (ed.); pp. 425–441).
539 Springer International Publishing. https://doi.org/10.1007/978-3-319-47426-7_20
540 Lin, Z., Huang, Y., Jiang, H., Zhang, D., Yang, Y., Geng, X., & Li, B. (2021).
541 Functional differences and similarities in activated peripheral blood mononuclear
542 cells by lipopolysaccharide or phytohemagglutinin stimulation between human and
543 cynomolgus monkeys. *Annals of Translational Medicine*, *9*(3), 257–257.
544 https://doi.org/10.21037/atm-20-4548
545 Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-κB signaling in inflammation.
546 *Signal Transduction and Targeted Therapy*, *2*(March).
547 https://doi.org/10.1038/sigtrans.2017.23
548 Livak, K. (1997). ABI Prism 7700 Sequence Detection System. *User Bulletin No. 2, PE*
549 *Applied Biosystems*, *4303859B*(bulletin reference 1997), 777802–778002.
550 Mehta, R., Birerdinc, A., Hossain, N., Afendy, A., Chandhoke, V., Younossi, Z., &
551 Baranova, A. (2010). Validation of endogenous reference genes for qRT-PCR
552 analysis of human visceral adipose samples. *BMC Molecular Biology*, *11*, 39.
553 https://doi.org/10.1186/1471-2199-11-39
554 Murata, K., Sugiyama, M., Kimura, T., Yoshio, S., Kanto, T., Kirikae, I., Saito, H.,
555 Aoki, Y., Hiramine, S., Matsui, T., Ito, K., Korenaga, M., Imamura, M., Masaki,
556 N., & Mizokami, M. (2014). Ex vivo induction of IFN-λ3 by a TLR7 agonist
557 determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients.
558 *Journal of Gastroenterology*, *49*(1), 126–137. https://doi.org/10.1007/s00535-013-
559 0814-1
560 Nagarajan, G., Swami, S. K., Ghorui, S. K., Pathak, K. M. L., Singh, R. K., & Patil, N.
561 V. (2012). Cloning and sequence analysis of IL-2, IL-4 and IFN-γ from Indian
562 Dromedary camels (Camelus dromedarius). *Research in Veterinary Science*, *92*(3),

420–426. https://doi.org/10.1016/j.rvsc.2011.03.028

Norian, R., Delirezh, N., & Azadmehr, A. (2015). Evaluation of proliferation and cytokines production by mitogen-stimulated bovine peripheral blood mononuclear cells. *Veterinary Research Forum*, *6*(4), 265–271.

Odbileg, R., Konnai, S., Ohashi, K., & Onuma, M. (2005). Molecular cloning and phylogenetic analysis of inflammatory cytokines of Camelidae (llama and camel). *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, *67*(9), 921–925. https://doi.org/JST.JSTAGE/jvms/67.921 [pii]

Odbileg, R., Konnai, S., Usui, T., Ohashi, K., & Onuma, M. (2004). Quantification of llama inflammatory cytokine mRNAs by real-time RT-PCR. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, *67*(2), 195–198. https://doi.org/JST.JSTAGE/jvms/67.195 [pii]

Odbileg, R., Lee, S. Il, Ohashi, K., & Onuma, M. (2005). Cloning and sequence analysis of llama (lama glama): Th2 (IL-4, IL-10 and IL-13) cytokines. *Veterinary Immunology and Immunopathology*, *104*(3–4), 145–153. https://doi.org/10.1016/j.vetimm.2004.11.002

Odbileg, R., Lee, S.-I., Yoshida, R., Chang, K.-S., Ohashi, K., Sugimoto, C., & Onuma, M. (2004). Cloning and sequence analysis of llama cytokines related to cell-mediated immunity. *Veterinary Immunology and Immunopathology*, *102*(1–2), 93–102. https://doi.org/10.1016/j.vetimm.2004.06.001

Odbileg, R., Purevtseren, B., Batsukh, Z., Konnai, S., Ohashi, K., & Onuma, M. (2006). Complete cDNA sequences and phylogenetic analyses of the Th1 and Th2 cytokines of the bactrian camel (Camelus bactrianus). *Journal of Veterinary Medical Science*, *68*(9), 941–946. https://doi.org/10.1292/jvms.68.941

Odbileg, R., Purevtseren, B., Gantsetseg, D., Boldbaatar, B., Buyannemekh, T.,

588 Galmandakh, Z., Erdenebaatar, J., Konnai, S., Onuma, M., & Ohashi, K. (2008).
589 Cytokine responses in camels (Camelus bactrianus) vaccinated with Brucella
590 abortus strain 19 vaccine. *The Journal of Veterinary Medical Science / the*
591 *Japanese Society of Veterinary Science*, *70*(2), 197–201.
592 https://doi.org/10.1292/jvms.70.197
593 Premraj, A., Aleyas, A. G., Nautiyal, B., & Rasool, T. J. (2020). Camelid type I
594 interferons: Identification and functional characterization of interferon alpha from
595 the dromedary camel (Camelus dromedarius). *Molecular Immunology*,
596 *119*(January), 132–143. https://doi.org/10.1016/j.molimm.2020.01.020
597 Rabin, R. L. (2003). CC, C, and CX3C Chemokines. In H. L. Henry & A. W. B. T.-E.
598 of H. Norman (Eds.), *Encyclopedia of Hormones* (pp. 255–263). Academic Press.
599 https://doi.org/https://doi.org/10.1016/B0-12-341103-3/00044-9
600 Resa, C., Magro, S., Marechal, P., Barranger, Cô., Joannes, M., Miszczak, F., & Vabret,
601 A. (2014). Development of an efficient qRT-PCR assay for quality control and
602 cellular quantification of respiratory samples. *Journal of Clinical Virology*, *60*(3),
603 270–275. https://doi.org/10.1016/j.jcv.2014.03.019
604 Rodon, J., Okba, N. M. A. A., Te, N., van Dieren, B., Bosch, B.-J. J., Bensaid, A.,
605 Segalés, J., Haagmans, B. L., & Vergara-Alert, J. (2019). Blocking transmission of
606 Middle East respiratory syndrome coronavirus (MERS-CoV) in llamas by
607 vaccination with a recombinant spike protein. *Emerging Microbes and Infections*,
608 *8*(1), 1593–1603. https://doi.org/10.1080/22221751.2019.1685912
609 Rostaing, L., Tkaczuk, J., Durand, M., Peres, C., Durand, D., De Préval, C., Ohayon, E.,
610 & Abbal, M. (1999). Kinetics of intracytoplasmic Th1 and Th2 cytokine
611 production assessed by flow cytometry following in vitro activation of peripheral
612 blood mononuclear cells. *Cytometry*, *35*(4), 318–328.

https://doi.org/10.1002/(SICI)1097-0320(19990401)35:4<318::AID-CYTO4>3.0.CO;2-4

Sen, N., Sung, P., Panda, A., & Arvin, A. M. (2018). Distinctive Roles for Type I and Type II Interferons and Interferon Regulatory Factors in the Host Cell Defense against Varicella-Zoster Virus. *Journal of Virology*, *92*(21). https://doi.org/10.1128/jvi.01151-18

Smeed, J. A., Watkins, C. A., Rhind, S. M., & Hopkins, J. (2007). Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. *BMC Veterinary Research*, *3*(1), 18. https://doi.org/10.1186/1746-6148-3-18

Souza, A. F. D., Brum, I. S., Neto, B. S., Berger, M., & Branchini, G. (2013). Reference gene for primary culture of prostate cancer cells. *Molecular Biology Reports*, *40*(4), 2955–2962. https://doi.org/10.1007/s11033-012-2366-5

Stanifer, M. L., Pervolaraki, K., & Boulant, S. (2019). Differential regulation of type I and type III interferon signaling. *International Journal of Molecular Sciences*, *20*(6), 1–22. https://doi.org/10.3390/ijms20061445

Svanborg, C., Godaly, G., & Hedlund, M. (1999). Cytokine responses during mucosal infections: role in disease pathogenesis and host defence. *Current Opinion in Microbiology*, *2*(1), 99–103. https://doi.org/https://doi.org/10.1016/S1369-5274(99)80017-4

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010). A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods*, *50*(4), S1. https://doi.org/10.1016/j.ymeth.2010.01.005

Te, N., Rodon, J., Ballester, M., Pérez, M., Pailler-García, L., Segalés, J., Vergara-Alert, J., & Bensaid, A. (2021). Type I and III IFNs produced by the nasal epithelia and dimmed inflammation are features of alpacas resolving MERS-CoV infection.

PLoS Pathogens, *17*(5), 1–28. https://doi.org/10.1371/journal.ppat.1009229

Te, N., Rodon, J., Pérez, M., Segalés, J., Vergara-Alert, J., & Bensaid, A. (2022). Enhanced replication fitness of MERS-CoV clade B over clade A strains in camelids explains the dominance of clade B strains in the Arabian Peninsula. *Emerging Microbes and Infections*, *11*(1), 260–274. https://doi.org/10.1080/22221751.2021.2019559

van Reeth, K., & Nauwynck, H. (2000). Proinflammatory cytokines and viral respiratory disease in pigs. *Veterinary Research*, *31*(2), 187–213. https://doi.org/10.1051/vetres:2000113

Wagner, B., & Freer, H. (2009). Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. *Veterinary Immunology and Immunopathology*, *127*(3–4), 242–248. https://doi.org/10.1016/j.vetimm.2008.10.313

Wang, J., Wang, Y., Wang, H., Wang, H., Liu, J. F., Wu, Y., & Guo, J. (2016). Transcriptomic analysis identifies candidate genes and gene sets controlling the response of porcine peripheral blood mononuclear cells to poly I:C stimulation. *G3: Genes, Genomes, Genetics*, *6*(5), 1267–1275. https://doi.org/10.1534/g3.116.028290

Wang, W., Xu, L., Su, J., Peppelenbosch, M. P., & Pan, Q. (2017). Transcriptional Regulation of Antiviral Interferon-Stimulated Genes. *Trends in Microbiology*, *25*(7), 573–584. https://doi.org/10.1016/j.tim.2017.01.001

Wernery, U., Kinne, J., & Schuster, R. K. (2014). *Camelid infectious disorders*. OIE (World Organisation for Animal Health).

Wu, H., Guang, X., Al-Fageeh, M. B., Cao, J., Pan, S., Zhou, H., Zhang, L., Abutarboush, M. H., Xing, Y., Xie, Z., Alshanqeeti, A. S., Zhang, Y., Yao, Q., Al-

Shomrani, B. M., Zhang, D., Li, J., Manee, M. M., Yang, Z., Yang, L., … Wang, J. (2014). Camelid genomes reveal evolution and adaptation to desert environments. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms6188

Zhou, Z., Hamming, O. J., Ank, N., Paludan, S. R., Nielsen, A. L., & Hartmann, R. (2007). Type III Interferon (IFN) Induces a Type I IFN-Like Response in a Restricted Subset of Cells through Signaling Pathways Involving both the Jak-STAT Pathway and the Mitogen-Activated Protein Kinases. *Journal of Virology*, *81*(14), 7749–7758. https://doi.org/10.1128/jvi.02438-06

Zhu, S., Zimmerman, D., & Deem, S. L. (2019). A Review of Zoonotic Pathogens of Dromedary Camels. *EcoHealth*, *16*(2), 356–377. https://doi.org/10.1007/s10393-019-01413-7

Table 1. Features of the selected cytokines and immune genes used for gene expression analyses, and their validated primer pair sequences for all camelid species.

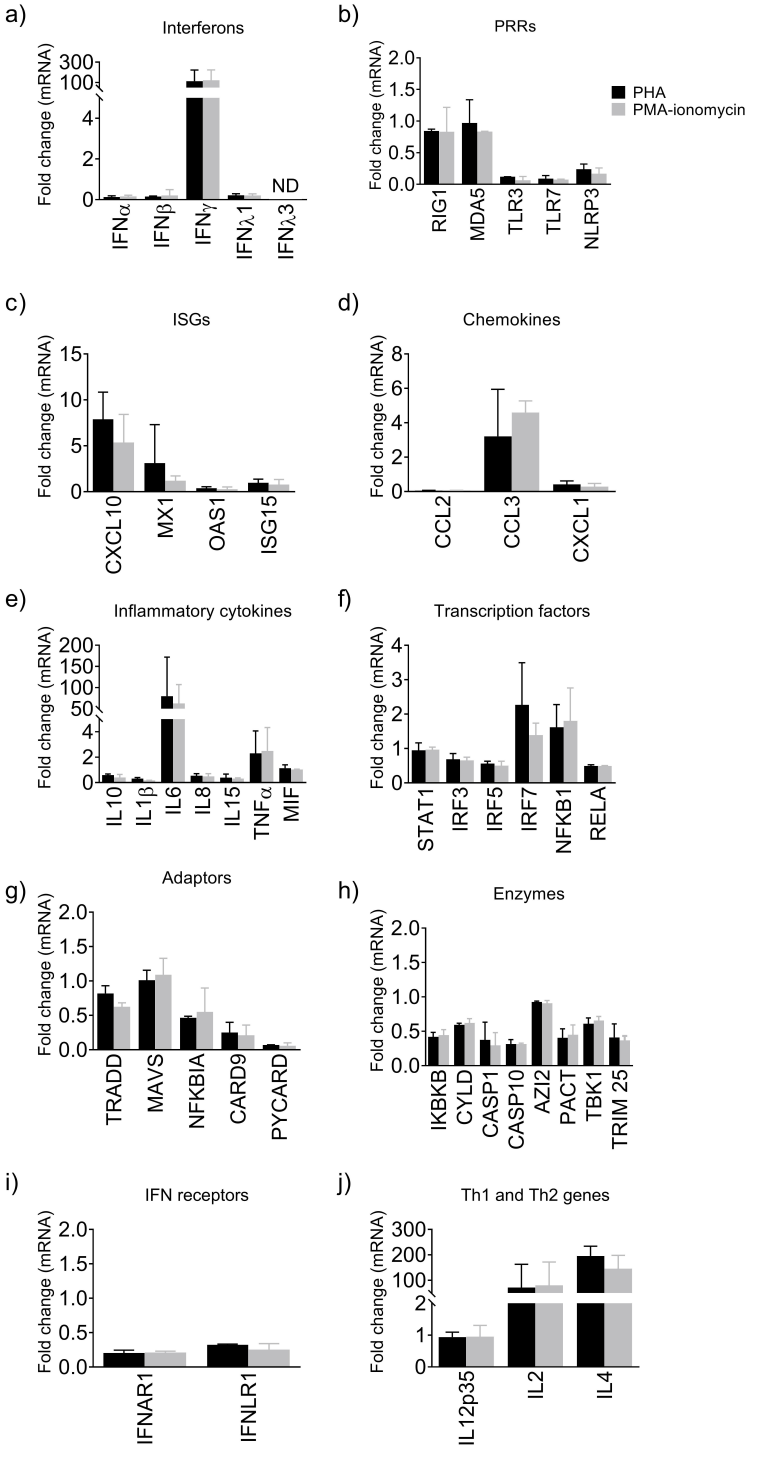
Table S1. NCBI accession numbers of camelid gene sequences used for comparative analyses and primer design.

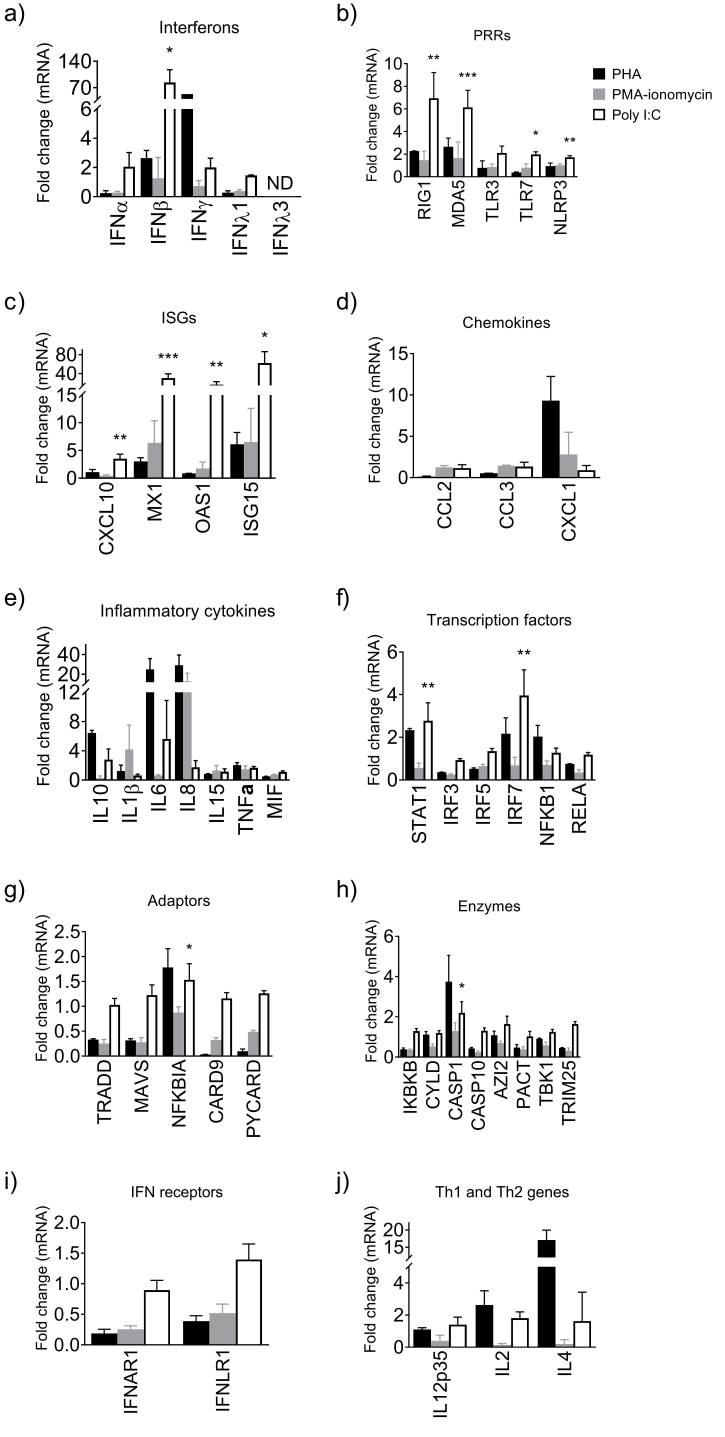
Table S2. Features of the primer pairs designed for the quantification of camelid immune and reference genes by RT-qPCR.

Table S3. Performance of the primer pairs designed for the quantification of mRNA expression of camelid immune and reference genes.

687 **Table S4**. Expression level of immune response genes from alpaca, dromedary camel
688 and llama PBMCs stimulated with PHA, PMA-ionomycin or PolyI:C.
689
690 **Figure 1**. Relative expression of llama immune genes by microfluidic RT-qPCR. Gene
691 expression profile of llama (L1-2) PBMCs stimulated for 48 h with PHA or PMA-
692 ionomycin were compared to that from unstimulated cells. The relative standard curve
693 method was applied for normalization purposes using multiple reference gene
694 normalization (*GAPDH*, *HPRT1* and *UbC*). Immune genes were grouped by functional
695 categories: (a) IFNs, (b) PRRs, (c) ISGs, inflammatory (d) chemokines and (e)
696 cytokines, (f) transcription factors, downstream signalling (g) adaptors and (h) enzymes,
697 (i) cellular receptors, and (j) cytokines involved in Th1 and Th2 response. Black and
698 grey bars display differential expression of PHA and PMA-ionomycin stimulated
699 PBMCs, respectively, relative to unstimulated cells. Relative expression data is
700 displayed as mean fold-change differences ± SD. IFN, interferon; ISG, interferon-
701 stimulated gene; PBMCs, peripheral blood mononuclear cells; PHA,
702 phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; PRR, pattern-recognition
703 receptor.
704
705 **Figure 2**. Relative expression of alpaca (A1-5) immune genes by microfluidic RT-
706 qPCR. Immune gene expression profile of alpaca PBMCs stimulated with PHA (A1-2,
707 black bars), PMA-ionomycin (A1-2, grey bars), or PolyI:C (A3-5, empty bars) for 48 h
708 were compared to that from non-stimulated cells. The relative standard curve method
709 was applied for normalization purposes using multiple reference gene normalization
710 (*GAPDH*, *HPRT1* and *UbC*). Immune genes were grouped by functional categories: (a)
711 IFNs, (b) PRRs, (c) ISGs, inflammatory (d) chemokines and (e) cytokines, (f)

712 transcription factors, downstream signalling (g) adaptors and (h) enzymes, (i) cellular
713 receptors, and (j) cytokines involved in Th1 and Th2 response. Relative expression data
714 is displayed as mean fold-change differences ± SD. Statistical significance was
715 determined by unpaired t-test. *indicates *p*-value < 0.05; **indicates *p*-value < 0.01;
716 ***indicates *p*-value < 0.001 compared with control samples obtained prior cell culture.
717 IFN, interferon; ISG, interferon-stimulated gene; PBMCs, peripheral blood
718 mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate;
719 PRR, pattern-recognition receptor.
720
721 **Figure 3**. Relative expression of dromedary camel (D1) immune genes by microfluidic
722 RT-qPCR. Immune gene expression profile of dromedary PBMCs stimulated with PHA
723 (black bars) or PMA-ionomycin (grey bars) for 48 h were compared to that from non-
724 stimulated cells. The relative standard curve method was applied for normalization
725 purposes using multiple reference gene normalization (*GAPDH*, *HPRT1* and *UbC*).
726 Immune genes were grouped by functional categories: (a) IFNs, (b) PRRs, (c) ISGs,
727 inflammatory (d) chemokines and (e) cytokines, (f) transcription factors, downstream
728 signalling (g) adaptors and (h) enzymes, (i) cellular receptors, and (j) cytokines involved
729 in Th1 and Th2 response. Relative expression data is displayed as mean fold-change
730 differences ± SD. Statistical significance was determined by unpaired t-test. *indicates
731 *p*-value < 0.05; **indicates *p*-value < 0.01; ***indicates *p*-value < 0.001 compared with
732 control samples obtained prior cell culture. IFN, interferon; ISG, interferon-stimulated
733 gene; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA,
734 phorbol 12-myristate 13-acetate; PRR, pattern-recognition receptor.


a)
Interferons
Fold change (mRNA)
300
100
4
2
0
ND
IFNα
IFNβ
IFNγ
IFNλ1
IFNλ3
b)
PRRs
Fold change (mRNA)
2.0
1.5
1.0
0.5
0.0
PHA
PMA-ionomycin
RIG1
MDA5
TLR3
TLR7
NLRP3
c)
ISGs
Fold change (mRNA)
15
10
5
0
CXCL10
MX1
OAS1
ISG15
d)
Chemokines
Fold change (mRNA)
8
6
4
2
0
CCL2
CCL3
CXCL1
e)
Inflammatory cytokines
Fold change (mRNA)
200
150
100
50
4
2
0
IL10
IL1β
IL6
IL8
IL15
TNFα
MIF
f)
Transcription factors
Fold change (mRNA)
4
3
2
1
0
STAT1
IRF3
IRF5
IRF7
NFKB1
RELA
g)
Adaptors
Fold change (mRNA)
2.0
1.5
1.0
0.5
0.0
TRADD
MAVS
NFKBIA
CARD9
PYCARD
h)
Enzymes
Fold change (mRNA)
2.0
1.5
1.0
0.5
0.0
IKBKB
CYLD
CASP1
CASP10
AZI2
PACT
TBK1
TRIM 25
i)
IFN receptors
Fold change (mRNA)
2.0
1.5
1.0
0.5
0.0
IFNAR1
IFNLR1
j)
Th1 and Th2 genes
Fold change (mRNA)
300
200
100
2
1
0
IL12p35
IL2
IL4


a)
Interferons
Fold change (mRNA)
IFNα
IFNβ
IFNγ
IFNλ1
IFNλ3
ND
b)
PRRs
PHA
PMA-ionomycin
Poly I:C
RIG1
MDA5
TLR3
TLR7
NLRP3
c)
ISGs
CXCL10
MX1
OAS1
ISG15
d)
Chemokines
CCL2
CCL3
CXCL1
e)
Inflammatory cytokines
IL10
IL1β
IL6
IL8
IL15
TNFa
MIF
f)
Transcription factors
STAT1
IRF3
IRF5
IRF7
NFKB1
RELA
g)
Adaptors
TRADD
MAVS
NFKBIA
CARD9
PYCARD
h)
Enzymes
IKBKB
CYLD
CASP1
CASP10
AZI2
PACT
TBK1
TRIM25
i)
IFN receptors
IFNAR1
IFNLR1
j)
Th1 and Th2 genes
IL12p35
IL2
IL4

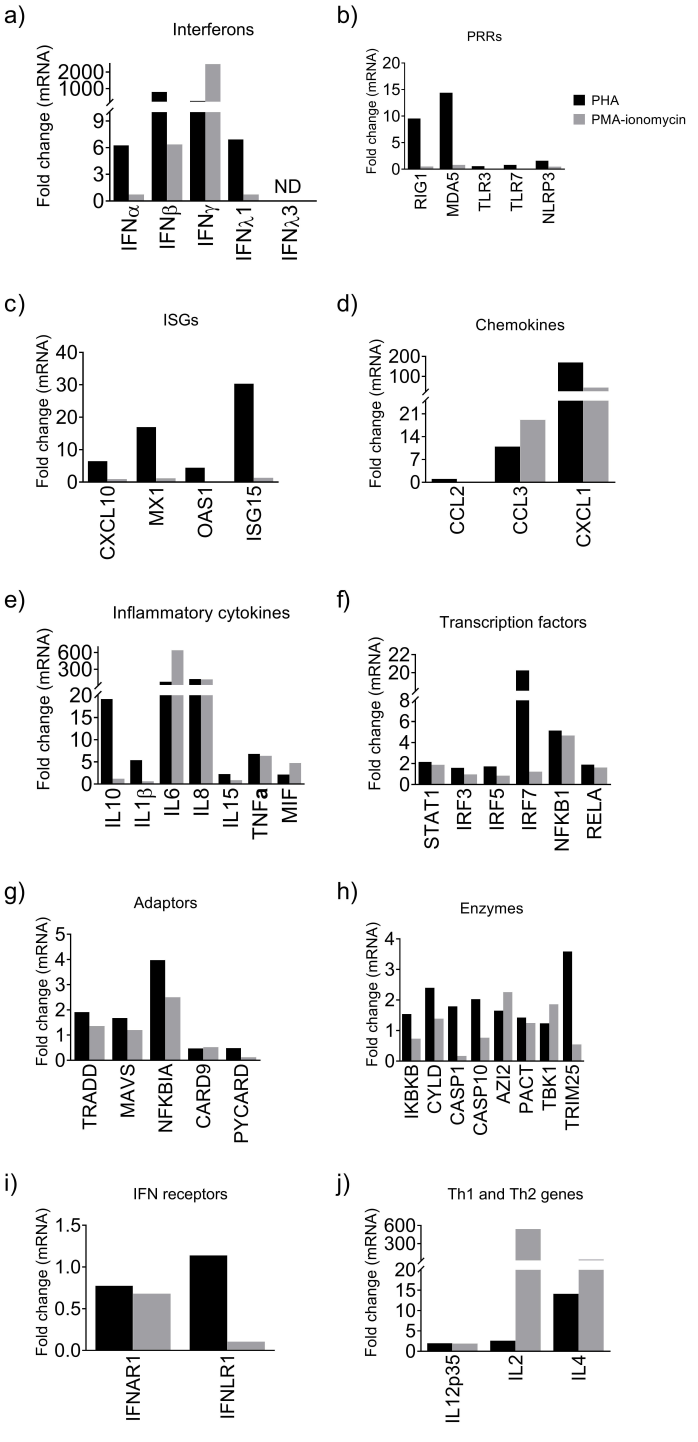

a)
Interferons
Fold change (mRNA)
2000
1000
9
6
3
0
ND
IFNα
IFNβ
IFNγ
IFNλ1
IFNλ3
b)
PRRs
Fold change (mRNA)
20
15
10
5
0
PHA
PMA-ionomycin
RIG1
MDA5
TLR3
TLR7
NLRP3
c)
ISGs
Fold change (mRNA)
40
30
20
10
0
CXCL10
MX1
OAS1
ISG15
d)
Chemokines
Fold change (mRNA)
200
100
21
14
7
0
CCL2
CCL3
CXCL1
e)
Inflammatory cytokines
Fold change (mRNA)
600
300
20
15
10
5
0
IL10
IL1β
IL6
IL8
IL15
TNFa
MIF
f)
Transcription factors
Fold change (mRNA)
22
20
8
6
4
2
0
STAT1
IRF3
IRF5
IRF7
NFKB1
RELA
g)
Adaptors
Fold change (mRNA)
5
4
3
2
1
0
TRADD
MAVS
NFKBIA
CARD9
PYCARD
h)
Enzymes
Fold change (mRNA)
4
3
2
1
0
IKBKB
CYLD
CASP1
CASP10
AZI2
PACT
TBK1
TRIM25
i)
IFN receptors
Fold change (mRNA)
1.5
1.0
0.5
0.0
IFNAR1
IFNLR1
j)
Th1 and Th2 genes
Fold change (mRNA)
600
300
20
15
10
5
0
IL12p35
IL2
IL4

Table 1. Features of the selected cytokines and immune genes used for gene expression analyses, and their validated primer pair sequences working for all camelid species.

Gene[(a)]	Cytokine/Protein type and function	Primer	Sequence (5' - 3')
GAPDH	Normalizer gene	GAPDH F	GGTCGGAGTGAACGGATTTGG
		GAPDH R	TTGAGGTCAATGAAGGGGTCG
UbC	Normalizer gene	Ubc F	AGGCGAAGATCCAAGACAAGG
		Ubc R	CCAAGTGCAGAGTGGATTCCT
HPRT1	Normalizer gene	HPRT1 F	CAAAGATGGTCAAGGTCGCAA
		HPRT1 R	TCAAATCCAACAAAGTCTGGTCT
IFN-α	Type I IFN, antiviral	IFN-α F	TCTTCAGCGAGACACTTGCAA
		IFN-α R	GTTGGTCAGTGAGAATCATTTCCA
IFN-β	Type I IFN, antiviral	IFN-β F2	GCATCCTCCAAATCGCTCTCC
		IFN-β R2	ATGCCAAGTTGCTGCTCCTTT
IFN-γ	Type II IFN, antiviral activity, and mediator of cellular immunity	IFN-γ F	ACTGGAAAGAGGAGAGTGACAAAA
		IFN-γ R	CAACCGGAATTTGAATCAGCT
IFN-λ1	Type III IFN, antiviral	IFN-λ1 F	CTGCCACATGGGCTGGTT
		IFN-λ1 R	CGATTCTTCCAAGGCATCCTT
IFN-λ3	Type III IFN, antiviral	IFN-λ3 F	CCACCTGGCCCAATTCAA
		IFN-λ3 R	AGTGACTCTTCAAAGGCGTCCTT
RIG-1	PRRs and ISG, recognises dsRNA and ssRNA; induce IFN production and ISG	RIG-1 F	ACAAGTCAGAACACAGGAATGA
		RIG-1 R	CTCTTCCTCTGCCTCTGGTTT
MDA5	PRRs and ISG, recognises dsRNA and ssRNA; induce IFN production and ISG	MDA5 F	ACACCAGAGTTCAAGAGACTGTAT
		MDA5 R	CACCATCATCGTTCCCCAAGA
MAVS	PRRs, interacts with RIG-1	MAVS F	CAGCCTCCACAACTGCTACAGA
		MAVS R	CTGTGGGACTTTCTTTGAACTCTCT
TLR3	PRRs and ISG, recognises dsRNA; induce IFN production and ISG	TLR3 F	AGAAATAGACAGACAGCCAGAG
		TLR3 R	TGCTCCTTTTGATGCTATTAACGA
TLR7	PRRs and ISG, recognises ssRNA induce IFN production and ISG	TLR7 F	AGAGAGGAGTCACCAGCGTAT
		TLR7 R	GACACAAATGCAAATGGAGAC
NLRP3	PRRs, increased expression of pro-inflammatory cytokines	NLRP3 F	ATGGCCACATGGATTTTTGC
		NLRP3 R	AAACATTGGCATTGTCCCATTC
STAT1	Transcription factor activated by IFNs; increased expression of ISG	STAT1 F	TCTCTGTGTCTGAAGTTCACCCT
		STAT1 R	GGGAATCACAGGTGGGAAGGA
IRF3	Transcription factor and ISG, activated by IFNs; increased expression of ISG	IRF3 F	TCACCACGCTACACCCTCTGGT
		IRF3 R	GAGGCACATGGGCACAACCTTGA
IRF5	Transcription factor and ISG, activated by IFNs; increased expression of ISG	IRF5 F	TCAGAAGGGCCAGACCAACACC
		IRF5 R	TGCTACGGGCACCACCTGTA
IRF7	Transcription factor and ISG, activated by IFNs; increased expression of ISG	IRF7 F	CGTGATGTTGCAAGACAACTCA
		IRF7 R	TGGTTAACGCCTGGGTCTCT
NFKB1	Transcription factor activated by IFNs; increased expression of pro-inflammatory cytokines	NFKB1 F	GGGACAGTGTCTTACACTTAGCAATC
		NFKB1 R	CATCAGAAATCAAGCCAGATGTG
RELA	Transcription factor, binds to NF-κB	RELA F	AGAGTCCTTTCAATGGCCCCACCG
		RELA R	GGATGGAAGTTGAGCTGCGGGA
IKBKB	Transcription factor activated by IFNs; increased	IKBKB F	TAATGAACGAAGACGAGAAGATGGT

	expression of pro-inflammatory cytokines	IKBKB R	ACCTTGCTACACGCAATCTTCAG
CXCL10	ISG, activation and migration of immune cells to the infected sites	CXCL10 F	CGTGTTGAGATTATTGCCACAATG
		CXCL10 R	GAGGTAGCTTCTCTCTGGTCCT
MX1	ISG, GTPase with antiviral activity	MX1 F	GAAGATGGTTTATTCTGACTCG
		MX1 R	TTCTCCTCGTACTGGCTGT
OAS1	ISG, antiviral enzyme; degrades viral RNA	OAS1 F	TGAAGAAGCAGCTCGGGAAAC
		OAS1 R	AGTAACTGTCTTTTCTGGGCAGC
ISG15	ISG, antiviral activity	ISG15 F	CACAGCCATGGGTGGAATC
		ISG15 R	CAGCTCCGATAACAGCATGGA
IL-10	Interleukin, inflammatory antagonist	IL-10 F	CTGCTGGAGGACTTTAAGGGT
		IL-10 R	AGGGGAGAAATCGATGACAGC
IL-1β	Interleukin, pro-inflammatory response	IL1-beta F	AGGATATGAGCCGAGAAGTGGT
		IL1-beta R	CCCTTTCATCACACAAGACAGGT
IL-6	Interleukin, pro-inflammatory response	IL-6 F	TCTGGGTTCAATCAGGAGACCT
		IL-6 R	AGGGGTGCTTACTTCTTCTGGT
IL-8	Interleukin, pro-inflammatory response	IL-8 F	TGTGTGAAGCTGCAGTTCTGT
		IL-8 R	GCAGACCTCTCTTCCATTGGC
IL-15	Interleukin, induces proliferation of antiviral natural killer cells	IL-15 F	CAGCCTACAGAAGGTCATGAAGTACTC
		IL-15 R	GGGTAACTCCTTAAGTATCGAAGAAGAG
IL-2	Interleukin, cell-mediated immunity	IL-2 F	AAACTCTCCAGGATGCTCAC
		IL-2 R	TTTCAGATCCCTTCAGTTCC
IL-4	Interleukin, humoral immunity mediator	IL-4 F	CCCTGGTCTGCTTACTGGTTT
		IL-4 R	TCTCAGTCGTGTTCTTTGGGG
IL-12p35	Interleukin, cell-mediated immunity	IL-12p35 F2	AATCACCTGGACCACCTCAGT
		IL-12p35 R2	TCTAGGGTTTGTCTGGCCTTC
TNF-α	Cytokine, pro-inflammatory response	TNF-α F	TGGCCCAGACCCTCAGATCA
		TNF-α R	TTCCAGCTTCACACCATTGGC
CCL2	Chemokine, recruite monocytes and dendritic cells at the sites of inflammation	CCL2 F	CCAGTAAGAAGATCCCCATGCA
		CCL2 R	GTGTGGTCTTGAAGATCACAGCTT
CCL3	Inflammatory chemokine, attract monocytes, macrophages and neutrophils	CCL3 F	GCTCAGCGTCATGCAGGTGCC
		CCL3 R	AGCAGGCGGTTGGGGTGTCAG
CXCL1	Chemokine, attracts neutrophils	CXCL1 F	CGTGCAGGGAATTCACTTCAA
		CXCL1 R	GAGAGTGGCTACGACTTCCGTTT
MIF	Anti-inflammatory cytokine, macrophage migration inhibitory factor	MIF F	GCGAGTTGGTCGGTTCCTGTGTT
		MIF R	ACCACGTGCACTGCGATGTACT
CASP1	Enzyme, initiates inflammatory responses	CASP1 F	ACTCCACCAAGACCTCAACCAGT
		CASP1 R	GGGTAAATCTCCGCTGACTTCTCG
CASP10	Enzyme, involved in apoptosis and inflammation	CASP10 F	CGGTAGCCACGGGAACTGAGTCAT
		CASP10 R	ATCTTGCCAGGACCCCTCCGAT
CYLD	Enzyme, involved in transcription factor NF-κB activation	CYLD F	TCGGGATGGTGGTCAGAATGGC
		CYLD R	AGTCTTCGTGCACAGCCCTGGAT
AZI2	Enzyme, NF-κB-activating kinase-associated Protein 1	AZI2 F	TGAGCGTCTCCAGCGCTAA
		AZI2 R	CTGCACTTGCGTCACCAGAT
PACT	Enzyme, protein kinase activated by double-stranded RNA	PACT F	TGCAGTTCCTGACCCCTTAATG
		PACT R	GATGAATAGCCAGTTCCTGTAGTGAA

TBK1	Enzyme, activates the transcription factor IRF3	TBK1 F	GTACAGAAAGCAGAAAATGGACCAA
		TBK1 R	AACTTGAAGGCCCCGAGAAA
TRIM25	Enzyme and ISG, ubiquitination of RIG-1	TRIM25 F	GCCCGAGCTCCTACAGTATGC
		TRIM25 R	GAAGCGACGGTGTAGGTCTTG
NFKBIA	NF-κB inhibitor	NFKBIA F	TCCCTCTTTTCCCCGCAGGTT
		NFKBIA R	TGGAGTGGAGTCTGCTGCAGGT
TRADD	Adaptor, mediates NF-κB activation and apoptosis	TRADD F	CGGCCAGGAAGCAAGATG
		TRADD R	TGAAGACTCCACAAACAGGTATGC
CARD9	Adaptor, activates pro-inflammatory and anti-inflammatory cytokines through NF-κB	CARD9 F	GGCAGTGCAAGGTCCTGAAC
		CARD9 R	CAGGAGCACACCCACTTTCC
PYCARD	Adaptor, activates caspases and inflammasome	PYCARD F	CAAGCCAGCACCGCACTT
		PYCARD R	TCTGTCAGGACCTTCCCATACA
IFNLR1	Cellular receptor of type III IFNs	IFNLR1 F	CAGGGTGTGTGATCTGGAAGAG
		IFNLR1 R	GTCTGTGTCCAGAGAAATCCAGG
IFNAR1	Cellular receptor of type I IFNs	IFNAR1 F	TGCGAGGAAACCAAACCAGGAAAT
		IFNAR1 R	ACGACGACGATACAAAACACCGC

(a) Genes have been grouped in functional categories: Normalizer genes, IFNs, pattern recognition receptors, transcription factors, IFN-stimulated genes, pro- and anti-inflammatory cytokines, enzymes, adaptors and cellular receptors. dsRNA, double-stranded RNA; IFN, interferon; ISG, interferon-stimulated gene; PRR, pattern recognition receptor; ssRNA, single-stranded RNA.