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Short communication

Middle East respiratory coronavirus (MERS-CoV) internalized by llama alveolar macrophages does not result in virus replication or induction of pro-inflammatory cytokines

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ABSTRACT

Severe Middle East respiratory syndrome (MERS) is characterized by massive infiltration of immune cells in lungs. MERS-coronavirus (MERS-CoV) replicates *in vitro* in human macrophages, inducing high pro-inflammatory responses. In contrast, camelids, the main reservoir for MERS-CoV, are asymptomatic carriers. Although limited infiltration of leukocytes has been observed in the lower respiratory tract of camelids, their role during infection remains unknown. Here we studied whether llama alveolar macrophages (LAMs) are susceptible to MERS-CoV infection and can elicit pro-inflammatory responses. MERS-CoV did not replicate in LAMs; however, they effectively capture and degrade viral particles. Moreover, transcriptomic analyses showed that LAMs do not induce pro-inflammatory cytokines upon MERS-CoV sensing.

The Middle East respiratory syndrome coronavirus (MERS-CoV) is a zoonotic pathogen causing diseases ranging from an asymptomatic or mild respiratory illness to severe pneumonia that might be fatal in humans. The virus caused major outbreaks in the Arabian Peninsula, where it is endemic, and is of current worldwide public health concern.

Severe MERS is microscopically characterized by diffuse alveolar damage, which mainly occurs due to massive infiltration of immune cells into the lungs. These cells produce an excessive and aberrant host-cytokine storm that exacerbates disease during late MERS-CoV infection stages [1]. High and prolonged secretion of pro-inflammatory cytokines (such as IL-6, IL-8, IL-17 and IL-1β), as well as discrepancy in levels of antiviral cytokines (IFNs or TNF-α), have been observed in sera and bronchoalveolar fluid lavages (BAL) of acutely affected patients [2–4]. Indeed, cytokine production positively correlated with the number of leukocytes in blood and disease severity [3,4].

Abortive MERS-CoV infection and induction of apoptosis have been described in T cells [5], suggesting that these cells do not play a major role in proinflammatory cytokine storm production. Moreover, while inefficient replication was described in plasmacytoid dendritic cells (pDCs) [6], productive MERS-CoV replication was only reported

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in monocyte-derived macrophages (MDMs) [7,8] and monocyte-derived dendritic cells (MDDCs) [7–9]. Upon viral infection, pDCs could elicit higher levels of antiviral responses than MDDCs, such as type I and III IFNs, but none of these cells triggered the secretion of inflammatory cytokines [6–9]. Instead, MERS-CoV replication in MDMs resulted in impaired antiviral responses (type I and III IFNs) but dysregulated and persistent production of inflammatory cytokines, such as IL-6 and TNF-α [7,8]. Thus, macrophages are thought to be the main drivers of the inflammatory cytokine storm leading to exacerbated lung tissue damage in human MERS-CoV infections.

Dromedary camels are the natural reservoir of MERS-CoV and primary hosts involved in virus transmission to humans [10]. Other camelid species, such as llamas, are also susceptible to MERS-CoV under natural and experimental conditions [10]. Contrary to humans, camelids experience a subclinical infection characterized by abundant viral replication in the upper respiratory tract [10,11]. The action of robust and timely innate immune responses at the nasal mucosa of camelids is thought to play a key role in MERS-CoV infection clearance, preventing disease development [12,13]. Furthermore, a transient MERS-CoV replication has been observed in the lower respiratory tract of experimentally-infected camelids [11–13]. Importantly, infiltration of mononuclear leukocytes at the lower respiratory tract was also observed in both naturally and experimentally infected camelids, although to a limited extent [12–14]. Nonetheless, besides being the key determinants of cytokine storms in human infections, the role of macrophages during MERS-CoV infection in camelids remains unknown.

The present work aimed to elucidate if llama alveolar macrophages (LAMs) are susceptible to MERS-CoV infection *in vitro* and could elicit a pro-inflammatory response potentially contributing to disease severity.

1. Materials and methods

1.1. Animal welfare and ethics

All animal and laboratory experimentation involving MERS-CoV were performed at the biosafety level-3 (BSL-3) facilities of the Biocontainment Unit of IRTA-CReSA (Barcelona, Spain). Animal experimentation procedures were evaluated and 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 (file No. CEA-OH/10942/1).

1.2. Cell culture and MERS-CoV

LAMs were cultured in Roswell Park Memorial Institute 1640 medium (RPMI; Lonza, Switzerland) supplemented with 10% fetal calf serum (FCS; EuroClone, Italy), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (all ThermoFisher Scientific, USA). Vero E6 cells (CRL-1586, ATCC, USA) were cultured in Dulbecco's modified Eagle medium (DMEM; Lonza, Switzerland) supplemented with 5% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine.

A passage-3 MERS-CoV Qatar15/2015 strain (GenBank Accession MK280984) stock was propagated on Vero E6 cells for 48 h at 37 °C and 5% CO_2. The infectious virus titer was determined in Vero E6 cells and calculated by determining the dilution that caused cytopathic effect (CPE) in 50% of the inoculated cell cultures (50% tissue culture infectious dose endpoint, $TCID_{50}$).

1.3. Isolation of llama alveolar macrophages

Two clinically healthy llamas were euthanized and bronchoalveolar fluid lavages (BAL) were performed with 1× PBS from the right lung lobe. Alveolar macrophages were concentrated by centrifugation and fluid was discarded. Red blood cells were removed using ACK lysing buffer (ThermoFisher Scientific, USA) according to the manufacturer's instructions, and LAM were resuspended in culture media. These cells were initially tested to ensure negativity to MERS-CoV.

1.4. MERS-CoV exposure assays

LAMs were isolated and cultured in triplicates. One million cells/well were seeded onto 24-well plates in 1 mL RPMI medium containing MERS-CoV (MOI of 0.1), or only cultured in media for 48 h at 37 °C and 5% CO_2. Culture supernatants and cells were collected at 0, 24 and 48 h post viral exposure (hpe), as schematically represented in Fig. 1A. Additional fresh control samples were also collected before seeding in culture plates.

1.5. Virus titration in cell culture

The presence of infectious MERS-CoV in supernatants of LAM cultures was evaluated on Vero E6 cells, as previously reported [15]. Ten-fold dilutions of the samples were transferred to Vero E6 monolayers and cells were monitored daily for the presence of virus-induced cytopathic effect under a light microscope. After 6 days, plates were evaluated, and infectious virus titers were calculated by determining the dilution that caused 50% CPE in cell cultures ($TCID_{50}$/mL).

1.6. Cellular RNA extraction

LAMs were detached from culture wells by mechanically pipetting and total RNA was extracted using the Direct-zol RNA Miniprep (Zymo research, USA), following the manufacturer's protocol. After RNA extraction, an additional HL-dsDNase treatment using the Heat&Run gDNA removal kit (ArcticZymes Technologies, Norway) was performed according to the manufacturer's protocol. Finally, 1 U/μL RNase inhibitors (Invitrogen, Life Technologies, Waltham, USA) were added to the RNA samples, which were stored at −75 °C until subsequent analyses. The purity and quantity of RNA were assessed using a BioDrop *μLITE* Spectrophotometer (BioDrop Ltd, UK). A260:A280 ratio ranged from 1.6 to 1.9, which are optimal values for RNA purity.

1.7. cDNA synthesis

cDNA was generated from 110 ng of total RNA using a combination of oligo-dT and random hexamers with the PrimeScript RT reagent Kit (Takara, Japan), according to the manufacturer's instructions. cDNA and the remaining RNA extracts were stored at −75 °C until required.

1.8. Transcriptomic analyses by microfluidic RT-qPCR

Expression of cytokines and immune-related genes, as well as normalizer genes, were quantified using a previously validated technique to monitor camelid immune responses [16]. A Fluidigm Biomark microfluidic RT-qPCR assay was used to quantify gene expression of LAMs. Additionally, specific primers for the detection of subgenomic viral RNA were added to the assay [17]. Each reaction was coupled with Tm analysis to ensure that specific amplifications occurred. Non-template controls with nuclease-free water were also included in the assays.

1.9. Relative immune response gene quantification and data analysis

Gene expression analyses were calculated as previously described [13,16]. Briefly, data were collected with the Fluidigm Real-Time PCR Analysis 4.1.3 (Fluidigm Corporation, USA) and analyzed with the DAG expression software 1.0.5.6 [18]. The relative standard curve method (see Applied Biosystems user bulletin #2) was applied to compare gene expression levels of LAMs cultured in different conditions against those of freshly collected ones (prior culture), using multiple reference gene normalization (GAPDH, HPRT1 and UbC). Relative expression of IFN-λ1 and IFN-λ3 was calculated according to the $2^{-\Delta\Delta CT}$ method [19] using the same normalizer genes, since expression levels of these genes in control samples were too low to generate standard curves. The relative expression of each studied gene was expressed in mean fold-change values (Fc) and is shown in Supplementary Table S1.

Unpaired t-test analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, USA) to compare gene expression levels of LAMs exposed to MERS-CoV against those of cells cultured in media only. Differences were considered significant at p-values <0.05.

1.10. Transmission electron microscopy (TEM)

LAMs were chemically fixed at 24 h or 48 h post infection. Cells were mechanically detached from plates and transferred into 1.5 mL Eppendorf tubes. After centrifugation at 500 ×g for 10 min, supernatants were discarded, and pellets were fixed with 4% paraformaldehyde (PFA) for 2 h at 4 °C. In a second step, cells were fixed with 1% glutaraldehyde (GA) in PBS for 1 h at 4 °C. Post-fixation of cell pellets was done on ice with 1% osmium tetroxide +0.8% potassium ferrocyanide in water. Afterwards the pellets were dehydrated on ice with increasing concentrations of acetone and processed for embedding in the epoxy resin EML-812 (TAAB Laboratories, UK), as previously described [20,21]. After infiltration with epoxy resin at RT, samples were polymerized at 60 °C for 48 h. Ultrathin sections (50–70 nm) were obtained with a Leica UC6 microtome and collected on uncoated 300 mesh copper grids. Sections were contrasted with 4% uranyl acetate and Reynold's lead citrate. Images were taken with a Tecnai G2 TEM operated at 120 kV with a Ceta camera. At least 50 cells per condition were studied by TEM.

2. Results

LAMs were isolated and cultured in the presence of MERS-CoV for 0, 24 and 48 h, as summarized in Fig. 1A. The amount of infectious virus in supernatants constantly decreased throughout the study (Fig. 1B), evidencing that progeny viruses were not generated and released to the media. Consistently, cell-associated MERS-CoV RNA waned over time as determined by microfluidic RT-qPCR (Fig. 1C). Therefore, MERS-CoV was unable to productively replicate *in vitro* in LAMs.

Furthermore, we performed TEM analyses to confirm if MERS-CoV was able to interact with LAMs. As shown in Fig. 1D, E and F, non-exposed llama cells exhibited a size and round morphology with pseudopodia characteristic of macrophages, with good preservation of organelles, cytosol, and nuclei. Large vacuoles that contained electron-dense material and membranes were also observed in these cells. At 24 h post-MERS-CoV exposure (hpe), LAMs looked similar to non-exposed cell controls and no viral structures were seen (Fig. 1G, H and I). However, at 48 hpe (Fig. 1J, K and L), virus-like particles were detected inside vesicles, vacuoles, and dense globular compartments of LAMs (Fig. 1K and L). We found MERS-CoV virions attached to the plasma membrane or invaginations of it (Fig. 1K), as well as in larger membranous compartments eventually leading to viral degradation (Fig. 1L), as already described for other coronaviruses [21]. In addition, clusters of double-membrane vesicles (DMVs), which are virus-induced replication organelles of coronaviruses [22,23], were not formed in cells internalizing MERS-CoV throughout the study. Around 10% of the cells contained viral structures in cellular compartments in the plane of the section. Hence, MERS-CoV was successfully captured and internalized by camelid alveolar macrophages, being subsequently processed and degraded.

We also studied whether LAMs could induce cytokine mRNA expression upon MERS-CoV sensing, using a previously described microfluidic RT-qPCR array [16]. Expression levels of 43 immune-related genes from LAMs exposed to MERS-CoV and non-exposed controls were compared to those of freshly collected cells prior culture. The transcriptomic profiles included the analyses of type I, II and III IFNs, pattern recognition receptors, transcription factors, IFN-stimulated genes (ISGs), and cytokines involved in inflammatory responses, among other immune-related genes (Fig. 2A). Remarkably, most of the cytokines studied in LAMs inoculated with MERS-CoV were expressed at similar levels than in mock-treated cells. Moreover, when compared to freshly collected LAMs, expression of pro-inflammatory cytokines and chemokines associated with the cytokine storm occurring in humans, such as IL-6, IL-1β or TNF-α, decreased in MERS-CoV and mock-treated cells. Only the chemokine IL-8 was upregulated similarly in MERS-CoV and mock-treated cells upon culture (Fig. 2A). In agreement, transcription of genes involved in inflammasome complex formation (NLRP3, CASP1 and PYCARD) was not induced upon viral sensing (Fig. 2A). Moreover, the expression of anti-inflammatory IL-10 slightly increased over time compared to mock-treated cells (Fig. 2B). Thus, these data evidenced that LAMs internalizing MERS-CoV did not elicit antiviral nor pro-inflammatory responses.

3. Discussion

Here we identified the ultrastructural and transcriptomic features of LAMs exposed to MERS-CoV *in vitro*. This virus causes a subclinical infection in the camelid reservoirs that is rapidly cleared, especially in the lower respiratory tract [10]. We confirmed that LAMs do not support MERS-CoV replication but can capture MERS-CoV particles, which are eventually degraded. Our findings suggest that the mild infiltration of macrophages into the lungs of naturally or experimentally infected camelids [12–14] can contribute to the efficient viral clearance observed in reservoir hosts. In this regard, depletion of alveolar macrophages in a human dipeptidyl-peptidase 4 *knock-in* mouse model resulted in increased morbidity and mortality to a mouse-adapted MERS-CoV [24].

We previously described that the mild infiltration of mononuclear cells in lungs of infected alpacas was concomitant to a moderate up-regulation of TNF-α, IL-1β and NLPR3. Moreover, chemotactic cytokines (CCL2, CCL3) were strongly correlated with the abundance of mononuclear cells in lungs [12,13]. Here, we used previously developed assays to quantify the expression of camelid innate immune genes [16] in LAMs sensing MERS-CoV. We determined that LAMs internalizing MERS-CoV did not induce effective antiviral or pro-inflammatory immune responses throughout the study, but slightly increased anti-inflammatory IL-10 transcription levels. The absence of ISG induction at 24 hpe might indicate that IFNs were not produced during initial stages after MERS-CoV internalization. Remarkably, our results suggest a minor contribution of macrophages in the transcriptional induction of pro-inflammatory cytokines in lungs of infected camelids. Indeed, contrary to human macrophages contributing to acute lung

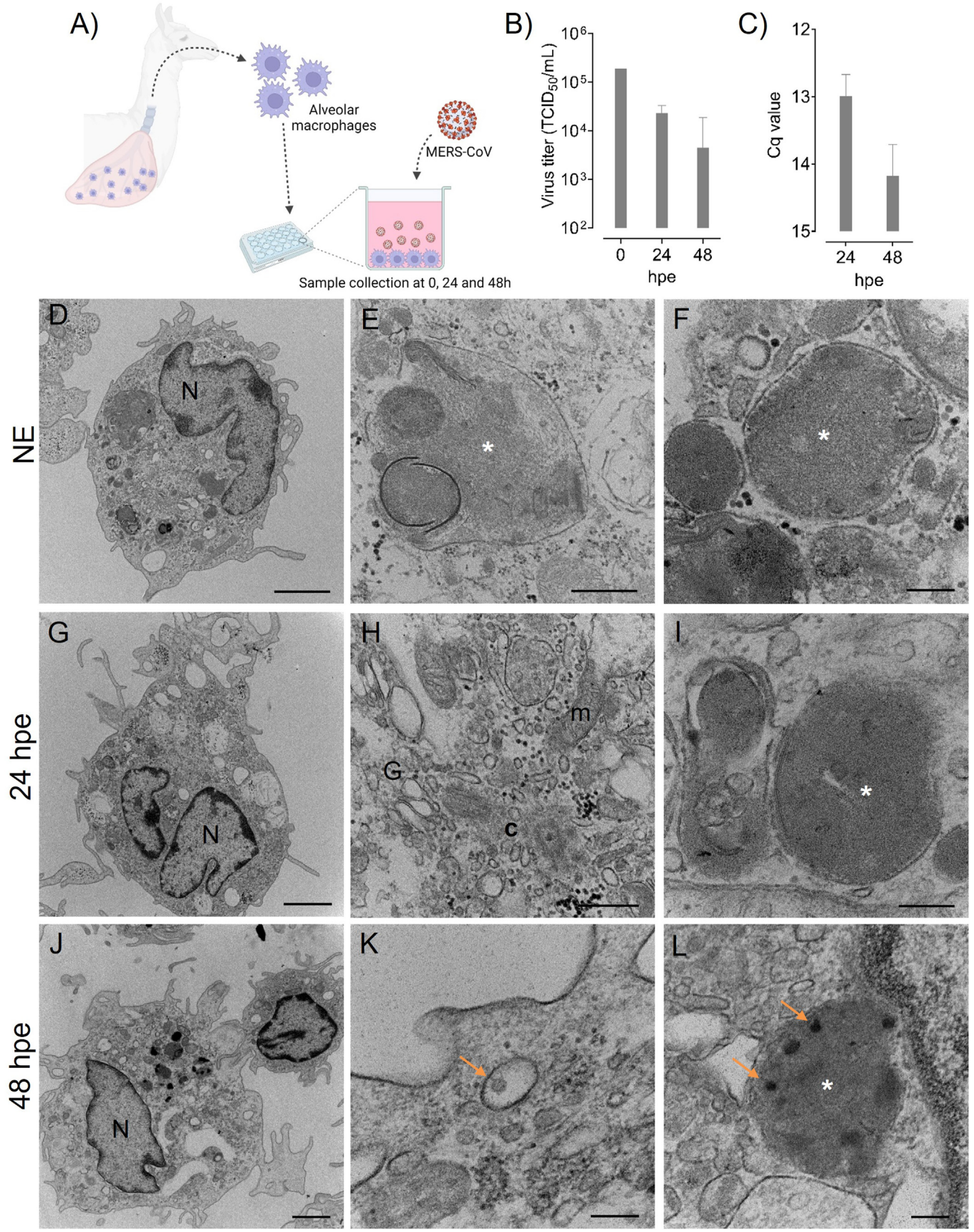

Fig. 1. Susceptibility of llama alveolar macrophages (LAMs) to MERS-CoV. LAMs were isolated and exposed to MERS-CoV as represented in panel (A). Mean values (±SEM) of infectious virus in culture supernatants (B) and cell-associated viral RNA (C) were monitored throughout the study. Transmission electron microscopy analyses of mock- and MERS-CoV-exposed macrophages were performed over time. Panels (D) to (F) show ultrathin sections of non-exposed (NE) cells. Low (D) and high (E, F) magnification images of NE

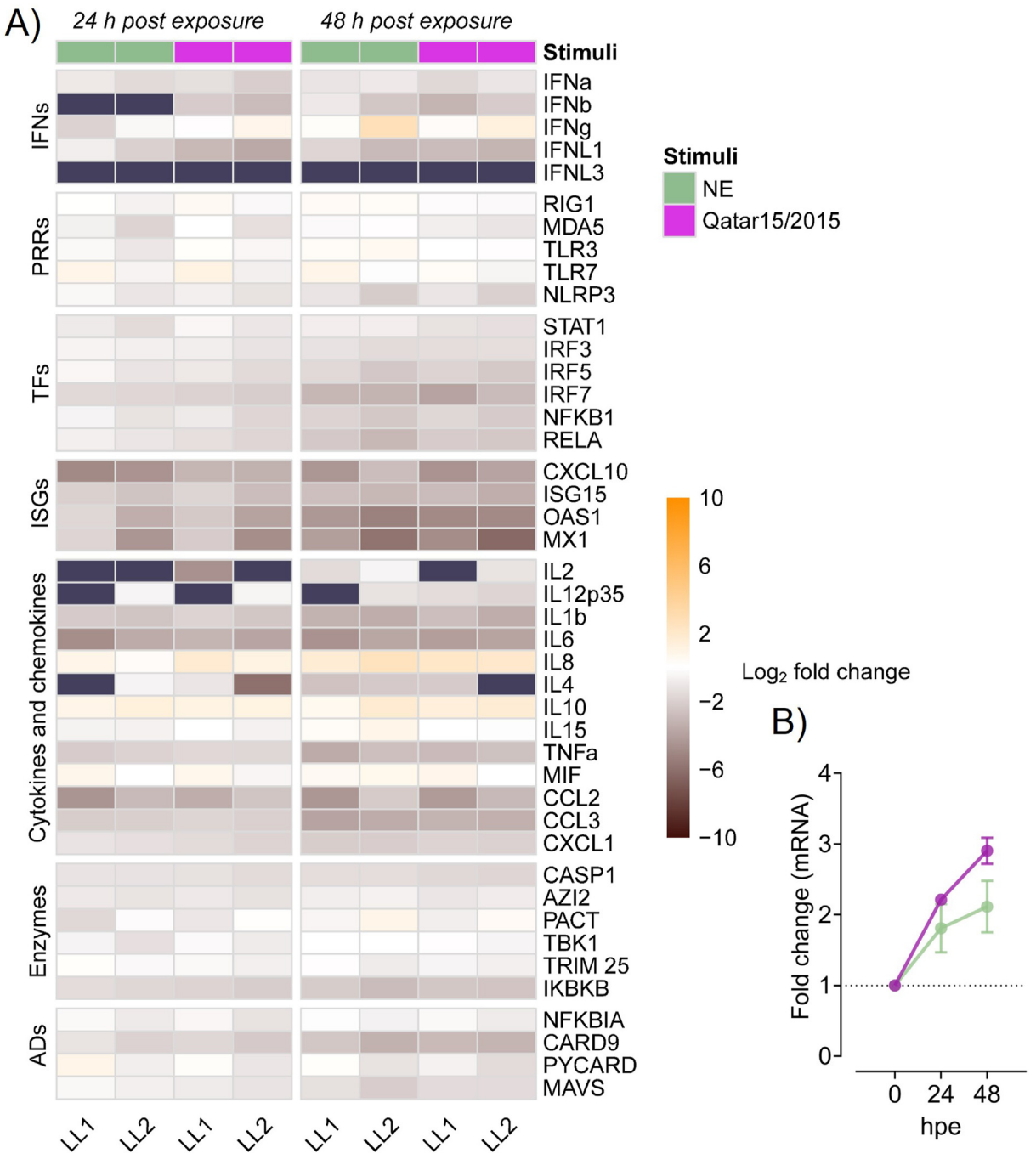

Fig. 2. Kinetics of immune response genes expressed by llama alveolar macrophages (LAMs) sensing MERS-CoV. The Fluidigm Biomark microfluidic RT-qPCR assay was used to quantify transcripts of immune-related genes at different h post-MERS-CoV exposure (hpe). After relative normalization, fold-change values of LAMs exposed for 24 and 48 h to MERS-CoV (Qatar15/2015, pink rectangles), or cultured in media only (NE, green rectangles) were calculated respective to freshly collected control LAMs. Panel (A) shows a heat-map plot with color variations corresponding to log2 fold-change values of expression for each studied gene; orange for upregulated and black for downregulated gene expression, respectively. Dark blue rectangles indicate absence of expression of the corresponding gene. IFNs, interferons; PRRs, pattern-recognition receptors; TFs, transcription factors; ISGs, IFN stimulated genes; ADs, adaptors; LL, llama. Panel (B) display the anti-inflammatory IL-10 expression over time in LAMs exposed to MERS-CoV (pink line) or cultured in media only (green line).

inflammation and cytokine storm [1,8], our findings support that camelid macrophages degrade MERS-CoV without activating a disproportionate pro-inflammatory response. Accordingly, IRF5, an important TF involved in M1 macrophage polarization [25], was downregulated in cultured LAMs. Consequently, together with robust antiviral innate immune responses occurring at the mucosal

macrophages with characteristic nucleus (N) and vacuoles with dense material and membranes (asterisks) are shown. Panels (G) to (I) display cells exposed for 24 h to MERS-CoV. Low (G) and high (H, I) magnification images of normal nucleus (N), mitochondria (m), Golgi complex (G), a centrosome (c) and vacuoles with dense material (asterisk) are shown. Panels (J) to (L) show cells exposed for 48 h to MERS-CoV. Low (J) and high (K, L) magnification images of cells with normal nuclei are displayed. Orange arrow in (K) points to a viral particle attached to an invagination of the plasma membrane. Orange arrows in (L) point to virus-like particles inside a dense vacuole (asterisk). Scale bars, 2 μm in D, G and J; 500 nm in E and H; 200 nm in F, I, K and L.

level [12,13], camelid reservoir species own unique effective mechanisms to impede disease development and experience asymptomatic MERS-CoV infection.

Overall, we show that LAMs are resistant to MERS-CoV infection, although these cells effectively capture, internalize, and degrade viral particles. Also, contrary to human MDMs, these cells do not induce pro-inflammatory cytokine responses upon viral sensing.

Author contributions

J.R., J.S., J.V.-A. and A.B. conceived and designed the study. J.R., M.S., N.T., J.S., A.B., C.R. and J.V.-A. 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.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105252.

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