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MERS-CoV infection is associated with downregulation of genes encoding Th1 and Th2 cytokines/chemokines and elevated inflammatory innate immune response in the lower respiratory tract

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ABSTRACT

MERS-CoV, a highly pathogenic virus in humans, is associated with high morbidity and case fatality. Inflammatory responses have a significant impact on MERS-CoV pathogenesis and disease outcome. However, $CD4^+$ T-cell induced immune responses during acute MERS-CoV infection are barely detectable, with potent inhibition of effector T cells and downregulation of antigen presentation. The local pulmonary immune response, particularly the Th1 and Th2-related immune response during acute severe MERS-CoV infection is not fully understood. In this study, we offer the first insights into the pulmonary gene expression profile of Th1 and Th2-related cytokines/chemokines (Th1 & Th2 responses) during acute MERS-CoV infection using RT^2 Profiler PCR Arrays. We also quantified the expression level of primary inflammatory cytokines/chemokines. Our results showed a downregulation of Th2, inadequate (partial) Th1 immune response and high expression levels of inflammatory cytokines IL-1α and IL-1β and the neutrophil chemoattractant chemokine IL-8 (CXCL8) in the lower respiratory tract of MERS-CoV infected patients. Moreover, we identified a high viral load in all included patients. We also observed a correlation between inflammatory cytokines, Th1, and Th2 downregulation and the case fatality rate. Th1 and Th2 response downregulation, high expression of inflammatory cytokines, and high viral load may contribute to lung inflammation, severe infection, the evolution of pneumonia and ARDS, and a higher case fatality rate. Further study of the molecular mechanisms underlying the Th1 and Th2 regulatory pathways will be vital for active vaccine development and the identification of novel therapeutic strategies.

1. Introduction

Middle East respiratory syndrome (MERS) is a novel viral respiratory illness caused by the MERS-CoV that was first described in June 2012 in a patient who was hospitalized with signs of severe respiratory tract infection. The patient later died from respiratory and renal failure [1–3]. According to the statistics provided by the World Health Organization (WHO) on June 10, 2019, a total of 2428 laboratory-confirmed cases of human MERS-CoV infection have been reported, with an estimated 838 deaths in 27 countries. The accumulated evidence suggested that MERS-CoV infections are characterized by high levels of systemic inflammatory cytokines/chemokines as well as immunopathology [4–11]. High inflammatory cytokines and chemokines levels have been strongly correlated with poor disease outcomes, immunopathology and massive infiltration of the immune inflammatory cells into the lungs [10,12]. Moreover, the stimulation of various cytokines, chemokines and innate immune cells has been associated with the appearance of cytokine storms, which occur during infections with pathogenic coronavirus (CoV) and influenza virus [6,13,14]. In addition, increased IL-8 (CXCL8) levels play a key role in acute SARS

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infection, viral bronchiolitis pathogenesis, severe immunopathology, and disease enhancement during respiratory syncytial virus (HRSV) infection [10,12,15–17]. Likewise, increased levels of inflammatory IL-1β and IL-1α cytokines have been associated with tissue damage and acute inflammatory responses leading to mortality and severe pathogenesis, as well as induction of the inflammatory loop [14,18,19]. MERS-CoV evades and antagonizes the antiviral immune response, as well as the nuclear factor-κB (NF-κB) signaling pathway and the IFN signaling cascade [20–22]. The disease pathogenesis of MERS-CoV infection is complex, with various factors involved in the onset of severe pulmonary damage and dissemination of the virus to other organs. Th1 cells cytokines IFN-γ, TNF-α, TNF-β and IL-2 play a key role in antiviral immunity by enhancing the toxic effects of CD8 T cells, stimulate NK cells and contribute to the regulation of cellular immunity [9,23–29]. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 which mediate humoral immune response and stimulate antibody production [9,23–29]. Imbalanced Th1/Th2 cytokines/chemokines can contribute to the pathological change, inefficient clearing infections, and host susceptibility to immune-mediated diseases. In vivo data highlights the important roles of T cells in controlling and reducing the pathogenesis and decreasing MERS-CoV titers [30].

There have been no reports describing the complete cellular immune response of the lower respiratory tract to MERS-CoV. Therefore, it is still unclear whether the $CD4^+$ T-helper (Th1/Th2)-induced cytokines/chemokines are beneficial or harmful for MERS-CoV-infected patients. Moreover, the host immune responses which are activated in the lower respiratory tract during a MERS-CoV infection also remain unclear. In this study, we offer the first insight into the pulmonary molecular gene expression profile of Th1 and Th2 cytokines/chemokines (Th1 & Th2 responses) during acute MERS-CoV infection using RT^2 Profiler PCR. We also describe the expression level of primary inflammatory cytokines and chemokines in the lower respiratory tract.

2. Materials and methods

2.1. Ethical considerations

The institutional review board at King Fahad Medical City reviewed and approved the study protocol (IRB register number 17-182).

2.2. Biosafety considerations

The handling of respiratory samples, as well as aliquoting and viral and cellular RNA extraction were executed using appropriate personal protective equipment in a biosafety level 3 laboratory (Riyadh Regional Laboratory, Ministry of Health, Riyadh, Saudi Arabia)

2.3. Samples preparation and analysis

The lower respiratory samples were collected from 39 MERS-CoV positive patients and 30 healthy non-infected individuals. To exclude effects of antiviral therapy on the expression of cytokines/chemokines understudy, the samples were collected before the initiation of any antiviral treatment for MERS-CoV. For the sputum sample collections, the patients were asked to rinse their mouth and gargle with water twice immediately before obtaining the sample and instructed not to expectorate saliva or postnasal discharge into the container during collection to reduce the possibility of contamination with upper respiratory tract fluids. Subsequently, the patient was requested to breathe and expectorate deep cough sputum directly into a sterile sputum cup (screw cap). Tracheal aspirate (TA) was collected via the endotracheal tube as reported elsewhere [31,32]. Bronchoalveolar lavage (BAL) was collected by following a standardized protocol (technique, sampling, and procedure) as previously described [33–35]. The first BAL fluid aliquots recovered were discarded to reduce cross-contamination with upper respiratory tract fluids. In order to exclude the effect of viral co-infection with influenza A H1N1 on cytokines/chemokines expression levels, all MERS-CoV positive patients were screened for H1N1 by GeneXpert Flu (Cepheid, Sunnyvale, CA) according to the manufacturer's instructions. Samples were centrifuged at 1000 rpm at 4 °C for 5–10 min. The cell-free supernatants were frozen at −80 °C until they were used for subsequent analysis of the inflammatory cytokine and chemokine by ELISArray, as well as for the MERS-CoV viral load test. The pellets were used for total cellular RNA extraction using the RNeasy mini kit (Qiagen, Valencia, CA)

2.4. Lower respiratory tract MERS-CoV viral load estimation

To detect MERS-CoV RNA concentrations in the lower respiratory tract, viral RNA was isolated from 140 μL of lower respiratory specimens using the QIAamp Viral RNA extraction Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was dissolved in 60 μL of elution buffer and stored in separate aliquots at −80 °C. An Internal Control (IC) was included as a control for the procedure used for sample preparation (viral RNA purification). The extracted viral RNA was tested by real-time RT-PCR duplex assays targeting the upE and Orf1a regions of the MERS-CoV genome, using the RealStar® MERS-CoV RT-PCR Kit 1.0 (RT-PCR duplex assay). This system involves two independent assays, one targeting and quantifying a region upstream of the E gene (upE) and the other targeting the open reading frame regions 1a (orf1a) of the MERS-CoV genome. The analysis was performed using an ABI Prism® 7500 (Applied Biosystems).

2.5. Screening of the pro-inflammatory cytokine and chemokine profile by ELISArray

The main 12 human proinflammatory cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF) were measured in lower respiratory samples from both MERS-CoV infected patients and the healthy non-infected control group, using multi-analyte ELISArray (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The absorbance of the obtained products after 30 min was measured at 450 nm, and cytokine/chemokine levels were determined by comparing them to the negative (assay buffer) and positive control (cocktail containing all standard 12 cytokines or chemokines, respectively). The presence of a determined cytokine/chemokine was based on an absorbance value above the negative control. The mean absorbance of the samples and all standards was calculated as replicates. The concentrations were calculated using standard curves. Cytokine/chemokines levels were expressed as pg/ml.

2.6. Cellular RNA processing, quantification, and profiling of pulmonary Th1 and Th2 responses

Pulmonary expression genes involved in the Th1 and Th2 response was assessed. Cellular RNA was isolated from lower respiratory tract samples using the RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration (ng/μL) and purity (A260/A280) of the RNA was assessed using a NanoDrop2000 (Thermo-scientific). cDNA synthesis and genomic DNA elimination were performed using an RT^2 first-strand synthesis kit (Qiagen, Germantown, MD, USA), using 1.5 μL (0.5 μg) of RNA. mRNA expression levels of Th1/Th2 immune genes were measured using an RT^2-PCR Profiler Array kit (Qiagen, Germantown, MD, USA) on an ABI Prism 7500 system (Applied Biosystems) with the RT^2 SYBR Green ROX qPCR Master Mix (Qiagen, Germantown, MD, USA). Briefly, the cDNA template was combined with the RT^2 Real-Time SYBR Green Master Mix and RNase-free water. A final reaction volume of 25 μL was added to each well of the RT^2-PCR Profiler Array. Cycle-threshold (Ct) values were obtained using a constant baseline for all real-time RT-PCR runs as previously described [36]. Five endogenous control genes provided by the array (ACTB, B2M, GAPDH, GUSB, and HSP90AB1) were used to calculate the arithmetic mean, which was

then set as the Ct value for normalization. The online RT^2-PCR Profiler data PCR array analysis software was used to identify the most appropriate reference genes to use for the normalization of the RT-PCR array gene expression data. Real-time RT-PCR array results were analyzed using the online RT^2-PCR Profiler data PCR array analysis software (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php). Each sample was assessed for reverse transcription efficiency, PCR array reproducibility, and genomic DNA contamination using the quality control function of the software. Experimental gene expression was quantified as ΔΔCt and calculations were normalized to the housekeeping genes.

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 16 (SPSS, Inc., Chicago, IL). Variables were compared between MERS CoV infected patients and healthy non-infected controls. The *t*-test and One-way ANOVA analysis were used as appropriate. The data were presented as mean (± standard deviation) and counts (percentages). A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

The demographic data, outcomes, preexisting conditions, underlying chronic diseases, clinical characteristics of MERS CoV infected patients and disease outcomes are presented in Table 1.

3.2. Detection of high MERS-CoV viral load in the lower respiratory tract

The real-time RT-PCR results showed high viral loads in all MERS-CoV patients regardless of the type of specimen. The mean MERS-CoV RNA Ct values and standard deviations were 25.30 ± 5.1 and 26.3 ± 5.02 for upE and Orf1a, respectively (Table 3). The viral load of MERS-CoV and the expression levels of inflammatory cytokines/chemokines were found to be significantly correlated with the case fatality of MERS-CoV infected patients ($p < 0.002$). Furthermore, we found a significant correlation ($p < 0.0001$) between MERS-CoV viral load and expression levels of inflammatory cytokines IL-1α, IL-1β and neutrophils chemoattractant chemokines IL-8 (CXCL8). No Influenza A H1N1 co-infections were detected among MERS-CoV positive patients.

Table 2
Comparison of the cytokines/chemokines expression levels between MERS-CoV infected patients and healthy non-infected group.

Cytokines/ chemokines	MERS-CoV infected patients (n = 39)	Healthy non-infected group (n = 30)	*p*-value
	Mean concentrations pg/ml (± SD)	Mean concentrations pg/ml (± SD)	
IL-1α	1148.7 (688.2)	18.7 (3.8)	0.001
IL-1β	1230.8 (580.4)	23.2 (4.4)	0.001
IL-2	20.7 (20.5)	20.5 (18.4)	0.9666
IL-4	13 (8.5)	12.5 (9.1)	0.8150
IL-6	20.2 (28.8)	11 (8.6)	0.0957
IL-8	1956.4 (695.8)	18.9 (1.1)	0.001
IL-10	9.2 (1.1)	8.5 (2.7)	0.1461
IL-12	13.7 (6.3)	11.2 (6.8)	0.1191
IL-17A	19.3 (15.5)	15.5 (15.9)	0.3217
IFN-γ	32.4 (53.9)	12.3 (11.1)	0.0488
TNF-α	20.7 (32.8)	11.5 (11.8)	0.1481
GM-CSF	23.3 (25.5)	20.1 (25.1)	0.6046

3.3. MERS-CoV induced high expression levels of inflammatory cytokines and chemokines in the lower respiratory tract

The lower respiratory tract samples from MERS-CoV infected patients and healthy non-infected controls were used to quantify expression levels of the main 12 human pro-inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF). Among the panel of 12 inflammatory cytokines/chemokines, the mean expression levels of IL-1α (1148 pg/mL) ($p < 0.001$) and IL1-β (1230 pg/mL) ($p < 0.001$) were significantly higher in the lower respiratory tract of MERS-CoV infected patients when compared to healthy non-infected controls (Table 2). Furthermore, our results showed a significant correlation between IL and 1α ($p < 0.035$) and IL-1β ($p < 0.013$) levels and the case fatality of MERS-CoV infected patients. IFN-γ expression levels were detected in only four patients and TNF-α expression levels were increased only in five patients.

Table 1
Clinical characteristics and demographic data of MERS-CoV infected patients and healthy non-infected group.

Variable	Patients (n = 39) (%)	Deaths no (%)	Survives no (%)	Healthy non-infected group (n = 30) (%)
Age, Y, mean, ± SD (median)	67.31 ± 18.75 (73)			61 ± 20.4 (61.5)
19–59 year	10 (25%)	2 (20%)	8 (80%)	12 (40%)
60–100 year	29 (74%)	23 (793%)	6(20.7%)	18 (60%)
Male	29 (74%)	20 (69%)	9 (31%)	16 (53.3%)
Female	10 (25%)	5 (50%)	5 (50%)	14 (46.7%)
HCW	2 (5%)	0	2 (100%)	–
Contact with MERS-CoV infected patients	4 (10%)	4 (100%)	0	–
Chronic condition				
Heart disease	13 (33%)	4 (30%)	9 (70%)	–
Diabetes	16 (41%)	12 (75%)	4 (25%)	–
Hypertension	20 (51%)	13 (65%)	7 (35%)	–
Malignancy	4 (10%)	5 (100%)	0	–
Kidney disease	6 (15%)	4 (66.7%)	2 (33.3%)	–
Asthma	2 (5%)	2 (100%)	0	–
Pre-existing condition				
Kidney transplant	2 (5%)	2 (100%)	0	–
Central nervous system disease	4 (10%)	4 (100%)	0	–
End-stage renal disease	2 (5%)	1 (50%)	1 (50%)	–
Developed respiratory illness				
ARDS	20 (53.9%)	16 (80%)	4 (20%)	–
Pneumonia	26 (66.7%)	22 (84.6%)	4 (15.4%)	–
Case fatality rate	25 (64.1%)			–

Table 3
Comparison between MERS-CoV load, sample type, age, cytokines/chemokines expression levels and infection outcomes for each MERS-CoV infected patient.

Patients No	Age	Gender	Sample type	Ct value upE/Orf1a	IL-8 levels (pg/ml)	IL-1α levels (pg/ml)	IL-1β levels (pg/m)	Critical care	Infection outcome
1	27	M	BAL	29/29	1950	1000	2000	–	N/A
2	33	F	BAL	24/24	1250	200	800	–	N/A
3	88	M	BAL	25/25	2750	1000	1000	–	N/A
4	45	M	TA	23/27	2750	1200	1100	ICU	Survived
5	75	F	TA	23/28	2050	2000	1500	ICU	Died
6	77	M	Sputum	27/27	1050	1100	900	–	Survived
7	89	M	TA	20/21	2750	1500	1000	ICU	Died
8	72	M	BAL	21/22	2450	900	1200	ICU	Survived
9	67	M	TA	22/24	2750	800	1000	ICU	Died
10	76	M	TA	32/33	1050	900	2000	–	Died
11	100	F	TA	33/33	1750	1000	1100	ICU	Died
12	63	F	TA	34/33	1100	1000	1200	–	Survived
13	41	M	TA	31/32	1000	700	200	ICU	Survived
14	43	M	BAL	29/30	1050	2000	3000	ICU	Died
15	73	M	TA	21/20	2750	2500	2000	ICU	Died
16	73	M	BAL	21/22	2100	900	2000	ICU	Died
17	79	F	TA	19/20	2750	800	900	ICU	Died
18	88	M	BAL	30/31	1750	1000	700	ICU	Died
19	77	M	TA	26/26	1950	1100	1000	ICU	Died
20	31	F	BAL	30/31	1100	700	1000	ICU	Survived
21	80	M	BAL	22/29	1350	1100	1100	ICU	Died
22	70	M	TA	17/17	2250	1000	900	ICU	Died
23	62	M	BAL	22/21	2750	300	1000	ICU	Died
24	90	F	TA	27/30	1850	300	800	–	Died
25	80	M	BAL	22/22	2050	1000	2500	ICU	Died
26	79	M	BAL	18/20	2250	900	1000	ICU	Died
27	87	M	TA	33/33	1900	800	1200	–	Died
28	81	M	TA	22/25	2050	700	2000	ICU	Died
29	76	M	TA	30/32	1550	2000	900	–	Died
30	29	F	TA	33/33	1400	1100	1100	–	N/A
31	75	F	TA	22/22	2050	1000	1500	ICU	Died
32	47	M	Sputum	27/28	2300	600	900	–	Survived
33	69	M	TA	24/26	2050	1500	2000	ICU	Died
34	57	M	TA	17/19	2050	3000	1500	ICU	Died
35	77	M	TA	26/27	2750	2500	1200	ICU	Died
36	41	F	Sputum	30/30	1050	600	500	–	Survived
37	70	M	Sputum	32/32	1000	500	300	–	Survived
38	68	M	Sputum	28/27	1100	600	800	–	Survived
39	70	M	TA	15/15	3000	3000	1200	ICU	Died

3.4. MERS-CoV infection induces high expression levels of neutrophil chemoattractant chemokine IL-8 (CXCL8)

The expression of IL-8 (CXCL8) was found to be significantly increased (1956 pg/mL) ($p < 0.001$) in all MERS-CoV patients when compared to healthy controls (Table 3). Our results showed a significant correlation between the expression level of IL-8 and the case fatality of MERS-CoV infected patients ($p < 0.003$).

3.5. MERS-CoV infections downregulate genes encoding Th1 and Th2 cytokines and chemokines

In order to study the pulmonary Th1 and Th2 responses in MERS-CoV infected patients, molecular cytokine/chemokine profiles were assessed using a RT^2-PCR Array. Only genes with at least a two-fold change in expression when compared to the housekeeping gene were selected for further analysis. A side-by-side analysis of the data derived from the RT^2-PCR profiling of pulmonary Th1/Th2 responses showed that genes encoding Th1 and Th2-related cytokines and chemokines were largely downregulated in the lower respiratory tract of MERS-CoV infected patients. A total of 26 genes involved in regulating the Th1 and Th2 immune response were downregulated in MERS-CoV infected patients (Table 4). The mRNA expression levels of 10 Th1-related cytokines/chemokines were downregulated. In contrast, the expression of only 6 Th1 cytokine/chemokine mRNAs, namely IL-18, IL-18R1, SOCS5, CCR2, CD4, and CXCR3, were upregulated (Table 4). With regards to the Th2 immune response, we identified 15 cytokines/chemokines mRNAs expression levels were downregulated in MERS-CoV infected patients (Table 4). We did not observe any modulation or differences in Th1/Th2 responses and inflammatory cytokines/chemokines expression levels between MERS-CoV infected patients with underlying chronic medical conditions and MERS-CoV infected patients without underlying chronic medical conditions.

4. Discussion

The disease outcome, as well as the immunopathology and pathogenesis of MERS-CoV, SARS, and other respiratory viral infections in humans and other animals are strongly linked to the expression levels of inflammatory cytokines and chemokines [16,37,38]. Various cytokines, chemokines, and innate immune cells have been associated with the immunopathology during coronavirus (CoV) and influenza virus infection [5,6,13,14]. This study offers the first insight into the lung molecular gene expression profile of Th1 and Th2-related cytokines/chemokines (Th1 & Th2 responses) during acute MERS-CoV infection. Previous studies have shown that dysregulated and excessive immune responses may cause immunopathology and fatal disease [6,39–41]. As such, direct cytopathic effects, high virus titers, and viral evasion of host immune responses are believed to play a major role in the severity of the diseases resulting from SARS-CoV and MERS-CoV infections [6,39,40]. Our results showed high viral load (Ct) values in all MERS-CoV infected patients regardless of the specimen type (Table 3). Several pro-inflammatory cytokines, such as IL-8 and IL-1β, contribute to ARDS pathogenesis [6,15,42–44]. ARDS was shown to be the primary cause of

Table 4
Th1/Th2 cytokines/chemokines genes relative under/over-expressed in the lower respiratory tract of MERS-CoV infected patients.

Symbol	Description	Fold regulation (± SD)			Category
		MERS-CoV infected patients	Deaths	Survives	*p*-value
EBI3	Epstein-Barr virus induced 3 (IL-27B)	1.8 (2.7)	−0.8 (2.5)	1.7 (3.5)	0.0233
IL12B	Interleukin 12B	−0.4 (1.8)	−0.4 (2.0)	−1.4 (2.3)	0.2091
IL1RL1	Interleukin 1 receptor-like 1	−0.3 (1.7)	−0.3 (2.0)	−0.7 (1.7)	0.6794 Th1 immune response
IL27	Interleukin 27	−0.4 (1.6)	−0.3 (2.0)	−0.9(1.5)	0.3991
TLR4	Toll-like receptor 4	−8.2 (4.1)	−8.2 (7.0)	−9.4 (4.3)	0.6184
TLR6	Toll-like receptor 6	−8.1 (4.1)	−8.2 (7.0)	−9.4 (4.3)	0.6184
IL2	Interleukin 2	−0.4 (1.6)	−0.3 (2.0)	−0.9 (1.5)	0.3991
TNF	Tumor necrosis factor	−16.5 (8.2)	−16.4 (14.0)	−18.8 (8.5)	0.6179
IRF1	Interferon regulatory factor 1	−2.2 (3.7)	−2.6 (4.6)	−1.9 (4.3)	0.6816
IFNG	Interferon, gamma	−10.2 (5.1)	−10.2 (8.7)	−11.7 (5.3)	0.6160 Th1 marker
IL13	Interleukin 13	−1.7 (2.5)	−2.1 (3.7)	−1.3 (2.1)	0.5266
IL25	Interleukin 25	−0.4 (1.6)	−0.5 (2.0)	−0.8 (1.5)	0.6720
IL4	Interleukin 4	−0.5 (1.6)	−0.4 (2.0)	−0.9 (1.5)	0.4815
IL5	Interleukin 5	−0.4 (1.6)	−0.3 (2.0)	−0.8 (1.5)	0.4815
IL10	Interleukin 10	−15.9 (8.0)	−15.8 (13.6)	−18.3 (8.3)	0.5931 Th2 immune response
IL6	Interleukin 6	−7.7 (3.3)	−7.4 (4.6)	−9.5 (4.3)	0.2231
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4	−52.9 (28.2)	−52.1 (48.7)	−62.5 (28.6)	0.5332
CCL5	Chemokine (C-C motif) ligand 5	−0.4 (1.6)	−0.3(2.0)	−0.9 (1.5)	0.3991
CCR3	Chemokine (C-C motif) receptor 3	−0.7 (1.5)	−0.6 (1.9)	−8.9 (1.5)	0.0001
GFI1	Growth factor independent 1 transcription repressor	−1.9 (1.9)	−2.2 (2.6)	−2.5 (2.4)	0.7549
PTGDR2	Prostaglandin D2 receptor 2	−0.3 (1.6)	−0.2 (2.1)	−0.9 (1.5)	0.3455
ICOS	Inducible T-cell co-stimulator	−0.3 (1.79)	−0.2 (1.9)	−0.9 (1.5)	0.3061
IL1R1	Interleukin 1 receptor, type I	−0.3 (1.6)	−0.3 (2.0)	−0.5 (1.6)	0.7801
IL9	Interleukin 9	−0.3 (1.6)	−0.2(2.0)	−0.9 (1.5)	0.3261
PCGF2	Polycomb group ring finger 2	−1.9 (1.2)	−1.6 (1.8)	−2.8 (1.2)	0.0617
CD80	CD80 molecule	−2676.1 (1355.3)	−2668.8 (2377.3)	−3088.5 (1405.7)	0.6064 $CD4^+$ T-cell marker
IL18	Interleukin 18 (interferon-gamma-inducing factor)	35.8 (24.0)	38.1 (36.3)	21.4 (22.2)	0.1861 Th1 immune response
IL18R1	Interleukin 18 receptor 1	143.6 (173.7)	170.8 (319.3)	44.3 (59.6)	0.2261
SOCS5	Suppressor of cytokine signaling 5	76.6 (58.5)	47.8 (40.2)	136.6 (187.3)	0.0286
CCR2	Chemokine (C-C motif) receptor 2	251.5 (293.8)	348.6 (657.5)	77.1 (121.7)	0.2074 $CD4^+$ T-cell marker
CD4	CD4 molecule	145.8 (124.7)	143.8 (210.7)	103.2 (93.7)	0.5669
CXCR3	Chemokine (C-X-C motif) receptor 3	3.9 (5.5)	0.7 (2.5)	4.4 (8.5)	0.0529

death among patients with SARS-CoV or MERS-CoV infections [6,45]. In this study, the majority of MERS-CoV infected patients progressed to ARDS, subsequently developing pneumonia and requiring admission to an intensive care unit (ICU).

In vitro studies have revealed that MERS-CoV induces significant but delayed modifications in the expression of both IFN and pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and other chemokines [7,10,46]. Our results showed high expression levels of IL-1α, IL-1β and IL-8 (CXCL8) in the lower respiratory tract of MERS-CoV infected patients. Evidence from studies investigating SARS and respiratory syncytial virus (HRSV) has revealed that IL-8 was associated with acute SARS infection, bronchiolitis, immunopathology and disease enhancement during HRSV infections [10,12,15–17]. IL-8 (CXCL8) is a critical chemokine involved in neutrophil recruitment, activation, and local neutrophil accumulation, and was shown to induce the formation of neutrophil extracellular traps (NETs) [44,47]. NETs are highly immunogenic and extremely toxic to the host tissue, being able to directly cause inflammation, pathological changes, and epithelial and endothelial cell death [44,48]. As such, lung NETs can increase inflammation by stimulating IL-8 expression, which leads to the recruitment of more neutrophils [44,47]. We hypothesize that high IL-8 expression levels may cause NETosis, which results in severe MERS-CoV infection and increases immunopathology.

IL-1β has been associated with tissue damage, neutrophil infiltration, acute inflammatory responses, higher case fatality and severe respiratory viral infection [10,14,19,49,50]. In this study, high expression levels of IL-1β were measured in the lower respiratory tracts of MERS-CoV infected patients. Previous studies have shown that the expression of IL-1β during influenza A H1N1 infection increased lung inflammation, and subsequent treatment with an IL-1β antagonist significantly reduced both inflammation and lung tissue damage, suggesting that IL-1β is a critical cytokine that contributes to lung inflammation [51,52]. Likewise, another study has shown that IL-1β mRNA levels were upregulated in Calu-3 cells infected with MERS-CoV and/or SARS-CoV [10]. IL-1α is an inflammatory cytokine that can be rapidly induced and quickly reaches high levels in response to a range of stimulants, such as IL-1β and pathogenic agents [18,53]. In mouse models, IL-1α was shown to be a key player in triggering neutrophilic inflammation [18,54]. Likewise, our results revealed high IL-1α expression levels in MERS-CoV infected patients. Therefore, we think that high levels of IL-8, IL-1α, and IL-1β may play a critical role in the immunopathology, severity, and case fatality of MERS-CoV infected patients.

IL-1α and IL-1β establish an inflammatory loop which activates downstream signaling and enables the IL-1α–IL-1R1 interaction, leading to further IL-1α and IL-1β production. Thus, a loop of continued and self-perpetuating inflammation occurs, resulting in extensive tissue damage and pathological changes [18]. As such, elevated IL-1β and IL-1α levels during acute MERS-CoV infection may cause an inflammatory loop that contributes to the extensive tissue damage and pathological changes associated with this disease, it is important to note that the IL-1α and IL-1β expression levels were not timely monitored at different intervals in this study. Thus, the proposed inflammatory loop may not be generalizable to cases with severe disease or patients treated by anti-inflammatory/antiviral drugs. It has been shown that effective control of host inflammatory response and antiviral treatment were associated with a reduction in inflammatory cytokine levels, steady improvement in disease condition, and pulmonary function [55–59]. Consequently, studying IL-1α and IL-1β expression levels at different intervals could assist in a better understanding of MERS-CoV immunopathology. We speculate that, monitoring of IL-1α and IL-1β expression levels at different time points during MERS-CoV infection may alter the proposed inflammatory loop.

TNF-α is an important innate and antiviral cytokine. High levels of

TNF-α were detected in an in vitro study of SARS-CoV and MERS-CoV at both 24 and 30 h [10]. In our study, the induction of TNF-α was largely absent. Similarly, in a recent study, TNF-α expression was not detected in most patients with MERS-CoV infections in the acute or convalescent stages [11]. This may indicate the limited early in vivo TNF-α response during MERS-CoV infections. One explanation for the differences between in vitro and in vivo studies could be the different kinetics of the MERS-CoV responses in the in vitro model, where there is a gradual increase in gene expression over time. However, a more likely explanation is that the complex interplay of target cell infection, MERS-CoV replication, viral load and time of sample collection. We measured cytokines/chemokines levels and viral load at a single time point in the early phase of infection; assessing cytokines/chemokines concentrations and viral load at different time points during MERS-CoV infection to create a kinetic profile of cytokines/chemokines might yield additional information. Overall, the distinct TNF-α responses in vitro and in vivo might impact on the in vivo pathogenesis and viral amplification.

$CD4^+$ T-cell immune responses during respiratory viral infections characterized by the production of signature cytokines, IFN-γ, TNF-α, and IL-2 for Th1 cells and IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 for Th2 cells [9,28,29]. The balance between the Th1 and Th2 responses is critical for the outcome of viral infections [60,61].

To our surprise, we found that the expression of genes encoding Th1 and Th2 cytokines/chemokines were largely downregulated in the lower respiratory tract of MERS-CoV infected patients (Table 4). The pulmonary Th1 and Th2 responses showed the downregulation of 26 genes encoding Th1 and Th2 cytokines/chemokines in MERS-CoV infected patients. IFNγ plays an important role in early immunity, inducing the apoptosis of infected cells and stimulating both $CD8^+$ and natural killer (NK) cells [62,63]. In this study, both IFNγ and main Th1-associated cytokine mRNA expression levels were downregulated in the lower respiratory tract of MERS-CoV infected patients. On the other hand, only 6 Th1-related cytokines and chemokines (IL-18, IL-18R1, SOCS5, CCR2, CD4, and CXCR3) were overexpressed in the lungs of MERS-CoV infected patients (Table 4). In regards to Th2-induced immune responses, 15 Th2 cytokines were downregulated. Th2 cytokines play an important role in the humoral immune response and promote antibody production. MERS-CoV infection completely downregulated the Th2 response in this study. We could not detect the mRNAs for the specific antiviral immune response in MERS-CoV infected patients. Reghunathan et al. also found a strong inflammatory response in acute SARS despite complete downregulation of the mRNA expression of genes involved in specific antiviral immune response [15].

Downregulation of the major Th1/Th2 cytokines/chemokines can contribute to the pathological change, inefficient clearing infections, and host susceptibility to immune-mediated diseases. The upregulated Th1 cytokines/chemokines (IL-18, SOCS5, CCR2, and CXCR3) have been shown to play an important role in virus-induced immunopathology and usually associated with acute lung inflammation. IL-18 was associated with acute lung inflammation [64]. In addition, IL-18 was shown to enhance the severity of HRSV disease [65]. SOCS proteins affect antiviral signaling pathways, allowing viruses to evade the host immune response, and facilitate viral replication. Moreover, SOCS5 has been suggested to shape the presentation of viral disease [66,67]. Previous data showed that, SOCS5 mRNA was significantly upregulated in response to H1N1, H3N2, H5N1, and H11N9. Suppressing SOCS signals improved influenza infection and inhibited HRSV replication [68,69]. CCR2 mRNA upregulation was correlated with HRSV-disease severity [70]. CCR2 antagonism reduced pathology, morbidity, and mortality during influenza A (H1N1) infection [71]. During HRSV infection increased CXCR3 was associated with the underlying pathology [72]. In addition, it has been shown that blocking CXCR3 reduced immunopathology during respiratory virus infections [73]. Therefore, we think IL-18, SOCS5, CCR2, and CXCR3 overexpression may play a critical role in immunopathology, severity, and case fatality of MERS-CoV infected patients. Factors contribute to CD4 molecules upregulation during MERS-CoV infection in this study need further study. Previous data showed that, CD4 and CD8 expression on T cells were not altered by MERS-CoV infection [74]. MERS-CoV could persistently induce the expression of pro-inflammatory cytokines/chemokines such as IL-8, which would up-regulate CD4 molecules to enhance helper T cell infection. Expression of high levels of IL-8 and the NET formation could have important effects on the CD4 expression.

We propose a mechanism, which can explain the role of lower respiratory tract cytokines/ chemokines during acute MERS-CoV infection. IL-1β and IL-8 (CXCL8) recruit and activate neutrophils. Subsequently, activated neutrophils undergo degranulation and NETosis, which contributes to pathological changes and leads to the recruitment of more inflammatory. Elevation of IL-1β and IL-1α during acute MERS-CoV infection may cause an inflammatory loop that contributes to extensive tissue damage and pathological changes. IL-8 and IL-1β are essential mediators in the development and pathogenesis of ARDS [6,15,42-44]. Moreover, overexpression of IL-1α, IL-1β, IL-8 (CXCL8), IL-18, CXCR3, SOCS5, and CCR2 may play a vital role in the severity, immunopathology, and case fatality of MERS-CoV infected patients.

It is important to note that the samples in this study were screened only for H1N1. We were not able to screen other respiratory viruses. Viral co-infections could result in specific virus-virus interactions [75]. However, the inflammatory response induced by one virus may not be significantly changed by the infection of a second or third virus [76]. We did not examine the influences of co-infections with other respiratory viruses on cytokines/chemokines expression levels. Any changes in immune profiles in MERS-CoV infected patients due to other respiratory pathogens are unknown. However, there is a possibility that other respiratory viral infections, which were not screened in our study, might skew immune profiles in MERS-CoV infected patients. We think that simultaneous study of other variables (ie, the interplay between MERS-CoV and other respiratory viruses), which could potentially skew the data is likely required to assess any alterations of the immune profiles in MERS-CoV infected patients.

Four limitations of this study should be noted. First, we measured cytokines/chemokines expression levels and viral load at a single time point in the early phase of infection; assessing cytokines/chemokines expression levels and viral replication at different time points during MERS-CoV infection to create a kinetic profile might yield additional information. Second, in our study design, we did not include controls from patients infected with other respiratory viral pathogens. Third, we have screened MERS-CoV positive samples only for H1N1, we did not screen our samples for other respiratory viruses. Fourth, we were not able to recruit health non-infected individuals with similar MERS-CoV infected patients underlying diseases/pre-existing conditions. Future study designs to close these gaps need to be considered.

5. Conclusion

Taken together, the high expression of inflammatory cytokines/chemokines IL-1 α and IL-1β, IL-8 (CXCL8), IL-18, CXCR3, SOCS5, and CCR2 in the lower respiratory tracts of MERS-CoV infected patients confirmed the lung immunopathology [46,77–80]. Therefore, the high expression of inflammatory cytokines and downregulation of the Th1 and Th2 immune responses in the lower respiratory tracts of MERS-CoV infected patients may contribute to a more severe infection, higher case fatality, lung inflammation, and immunopathology. Furthermore, high levels of inflammatory cytokines/chemokines could be a key point in the subsequent development of pneumonia and ARDS in MERS-CoV infected patients. As such, the characterization of pulmonary Th1/Th2 response in MERS-CoV infection could be valuable for effective vaccine development and novel therapeutic strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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