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MERS-CoV Infection and Its Impact on the Expression of TSLP Cytokine and IgG Antibodies: An In Vivo and In Vitro Study

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Purpose: Thymic stromal lymphopoietin (TSLP) is a proinflammatory cytokine produced by epithelial cells that is involved in the activation of allergic disorders. To date, no study has examined TSLP induction during Middle East respiratory syndrome coronavirus (MERS-CoV) infection. Herein, we aimed to study the effects of the recombinant spike protein of MERS-CoV on TSLP production. Additionally, the effects of recombinant human TSLP (rhTSLP) on B cell survival and antibody production were investigated.

Patients and Methods: B cells were separated using the Human B Cell Enrichment Kit, and B cell survival was measured using the WST-1 Assay Kit. Enzyme-linked immunosorbent assay (ELISA) was used to measure TSLP levels in the sera of both MERS-CoV-infected (n=4; median age, 53 years) and healthy individuals (n=5; median age, 35 years).

Results: We showed that the group of infected patients had significantly higher levels of TSLP than healthy controls (37.6 pg/mL vs 19.8 pg/mL, $*p<0.05$). The levels of TSLP in A549 cells were remarkably increased after 48 h of stimulation with recombinant full-length spike protein (rSP) (32.2 pg/mL, $p=0.01$). B cell survival was greatly enhanced by rhTSLP alone or in combination with rSP (0.02 vs 0.046, and 0.045; $**p<0.01$, respectively). Our data also showed a significant synergistic effect of rhTSLP and rSP on the augmented response of IgG antibodies against the spike protein of MERS-CoV compared with unstimulated cells (0.156 vs 0.22; $*p<0.05$).

Conclusion: TSLP production is induced in vivo after MERS-CoV infection and in vitro after treatment with the rSP of MERS-CoV, which has a significant effect on the survival of B cells. Our data suggest that TSLP can be used as a strong mucosal adjuvant for vaccine development against MERS-CoV infection. However, further investigation is required to study the functional role of TSLP in MERS-CoV infection.

Keywords: MERS-CoV, TSLP, recombinants spike protein, B cells

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) causes a viral respiratory illness that was first reported in Saudi Arabian patients in 2012.[1] MERS-CoV causes a lower respiratory tract illness, which progresses from nonspecific influenza-like symptoms to severe pneumonia, multiple organ failure, and death. Exposure to dromedary camels has been associated with the primary transmission of MERS-CoV infections.[2,3] Human-to-human transmission may cause secondary infections, with nosocomial and household outbreaks accounting for most cases.[4,5] According to European Centre for Disease Prevention and Control (ECDC), to date, approximately 2622 cases, including 953 fatalities, have been documented in 27 countries, with a case fatality rate of approximately 36%.[6] Numerous studies have revealed that elevated systemic inflammatory cytokine/chemokine levels, along with associated immunopathology, are the main characteristics of MERS-CoV infection.[7–10] There

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was a link between high inflammatory cytokine and chemokine levels and poor clinical outcomes, immunopathology, as well as large infiltration of inflammatory immune cells into the lungs.[11–13]

The airway epithelium is the primary site of MERS-CoV infection and replication. Epithelial cells are known to generate various inflammatory mediators, such as a wide spectrum of cytokines and chemokines, which induce T helper 1 (Th_1) proinflammatory cells or T helper 2 (Th_2) anti-inflammatory cells. However, the importance of these mediators in fighting MERS-CoV infection remains unclear. Thymic stromal lymphopoietin (TSLP), an interleukin-7 (IL-7)-like cytokine, is primarily produced by epithelial cells.[14] It was first isolated from a mouse thymic stromal cell line and was found to be a growth factor for B lymphocytes.[15] TSLP exerts its biological activity by binding to its receptor TSLP-R. TSLP-R is expressed by several cells, including B cells, T cells, dendritic cells (DCs), natural killer (NK) cells, and epithelial cells.[16,17] TSLP has mostly been studied as a cytokine that affects the maturation, survival, and recruitment of many different cell types, including DCs, T cells, neutrophils, mast cells, eosinophils, and innate lymphoid cells (ILCs), and induces the proliferation of the human fetal liver, pro-B cells, and pre-B cells.[18–22]

TSLP is well recognized for its involvement in activating type 2 immunological responses such as allergic disorders. Recently, a monoclonal antibody targeting TSLP was approved for the treatment of severe asthma in 2021.[23] Viral nucleic acid analogs and proinflammatory cytokines associated with active viral infections are strong inducers of TSLP production[24] however it has not yet been shown whether TSLP can be induced after MERS-CoV infection. Here, we demonstrated that the human lung adenocarcinoma cell line (A549) produces TSLP in response to stimulation with the recombinant full-length spike protein (rSP) of MERS-CoV, Polyinosinic-polycytidylic acid (Poly I:C), and Respiratory Syncytial Virus (RSV). We also investigated the role of TSLP in B cell survival and antibody production.

Materials and Methods

Ethical Approval

This study was reviewed and approved by the Institutional Review Board of King Fahad Medical City (IRB number. 19–539). In accordance with the Declaration of Helsinki, all patients and healthy volunteers were informed of the purpose of the study, and signed consent forms were obtained before blood samples were collected.

Recombinant Proteins, Virus, and Cell Line

rSP from MERS-CoV (Sino Biological, China), recombinant human TSLP (rhTSLP) (R&D Systems, USA), and polyinosinic-polycytidylic acid.

(Poly I:C) (InvivoGen, USA) were used to stimulate the cells. Respiratory Syncytial Virus (RSV) was a gift from the Research Viral Group in the Microbiology Department at King Saud University and was used at 1:2 MOI. The human lung adenocarcinoma cell line (A549) was purchased from ATCC (CCL-185).

Clinical Samples and Criteria

In this study, MERS-CoV-infected patients (n=4; median age, 53 years) were admitted to the hospital with acute MERS-CoV infection in 2019. The MERS-CoV-infected group was confirmed as described in our previous study.[25] Healthy volunteers (n=5, median age 35 years) recruited to the blood bank were considered the MERS-CoV-noninfected healthy group. The exclusion and inclusion criteria were discussed in our previous work.[25] Blood samples (5–10 mL) were collected from both groups. Sera were separated from blood, aliquoted, and immediately stored at −80 °C for cytokine assessment.

Process and Separation of Peripheral Blood Mononuclear Cells (PBMCs)

Sera from the infected and healthy noninfected groups were collected directly from the hospital. Clinical blood samples from healthy subjects were collected in EDTA tubes for peripheral PBMCs separation. Briefly, peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque (Sigma-Aldrich), centrifuged at 400g for 25 min, and washed with cold PBS (containing 1% bovine serum albumin (BSA) for 10 min. Finally, PBMCs were counted and resuspended in complete RPMI-1640 medium for cell culture (Capricorn Scientific, Germany).

In vitro Stimulation of A549 Cells with Different Stimulants and TSLP Measurement by ELISA Assay

First, A549 Cells were cultured in high glucose (4.5g/L) Dulbecco's Modified Eagle Medium (DMEM) (Capricorn scientific, Germany), with L-glutamine supplemented with fetal bovine serum (FBS, 10%), antibiotic-antimycotic (1%) and then incubated in 5% CO_2 at 37 °C. Subsequently, the cells were sub-cultured after reaching 70–80% confluence. Subsequently, The A549 cell suspension (10^4 cells/mL) was seeded into a 96-well culture plate (Thermo fisher Scientific) and placed in a CO_2 incubator overnight at 37 °C. Then, the A549 cells were stimulated with rSP (5 µg/mL), poly I:C (500 ng/mL). RSV at a multiplicity of infection (MOI) 1:2 was used as a positive control, and unstimulated cells were used as a negative control. The plates were then incubated at 37 °C and 5% CO_2. Culture supernatants were collected at different time points post stimulation (4, 8, 12, 24, 48, 72, and 96 h) and stored at −80 °C. A human TSLP ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to measure TSLP in sera and cell culture supernatants, following the manufacturer's instructions.

B Cells Separation Using Magnetic Beads

PBMCs were collected from healthy volunteers and separated using Ficoll-Paque (Sigma-Aldrich). B cells were then separated by negative selection using the EasySep Human B Cell Enrichment Kit (Stem Cell Technologies, China) following the manufacturer's instructions. Briefly, the PBMCs were resuspended in PBS containing 2% FBS and 1 mm EDTA, and the enrichment cocktail was added and incubated at room temperature (RT) for 10 min. Subsequently, magnetic particles were added and incubated at RT for 5 min. The tube was placed on a magnet (EasySep^{TM} Magnet, Stem Cells Technology) and incubated at RT for 5 min. The magnet was inverted using a tube to pour the cell suspension into a new tube. The purity of the cells was > 99% (Figure 1B). It was measured through staining PBMC with an anti-human CD3 antibody (BioLegend) before cell depletion (as a whole-cell population) and after depletion (as a $CD3^{neg}$ population). Flow cytometry (Beckman Coulter) was performed for visualization and analysis.

IgG Detection Using Indirect ELISA

PBMCs from four healthy subjects were stimulated with or without rhTSLP (100 ng/mL) and rSP (5 µg/mL) in 96-well culture plates (Thermo Fisher Scientific) for 10 days. The cell culture supernatant was collected and stored at −80 °C for further analysis. Briefly, the ELISA plate (BioLegend) was coated with the optimal concentration of the full spike protein of MERS-CoV (2 µg/mL). The plate was washed to 3–5 times with washing buffer (PBS containing 0.05% Tween 20). The cells were then incubated with blocking buffer (PBS containing 1% bovine serum albumin, BSA) for 1 h. The culture supernatants were added to each well and incubated for 2 h. The plate was washed 3–5 times then anti-IgG-horseradish peroxidase (Invitrogen) was added and incubated for 1 h. The substrate was then added and incubated for 20 min, followed by the addition of a stop solution. The plate was read using an ELISA plate reader (ELX-808 microplate reader; BioTek Laboratories, USA) at a wavelength of 450 nm. All the readings were subtracted from those of the negative control (diluent).

Statistical Analysis

GraphPad Prism statistical software (version 9) was used for all data analysis. Mann–Whitney *U*-test was used to compare between healthy control and MERS-CoV infected patients. The data were presented as the standard error of mean (SEM) if normally distributed, or as median if non-normal distributed. To compare unstimulated and stimulated cells with different stimulants, Student's paired *t*-test was used. Differences between different time points were analyzed using one-way ANOVA test. Asterisks denote p-value (*p< 0.05, **p< 0.01, ***p< 0.001). A *p*-value of less than 0.05 was considered significant.

Results

MERS-CoV Infection Elevated the TSLP Response Among Patients' Group

All patients showed a remarkable increase in TLSP levels compared to those in the healthy, noninfected group (Figure 2). The TLSP levels in the infected group ranged from to 25.9–116.5 pg/mL and were nearly 2-fold

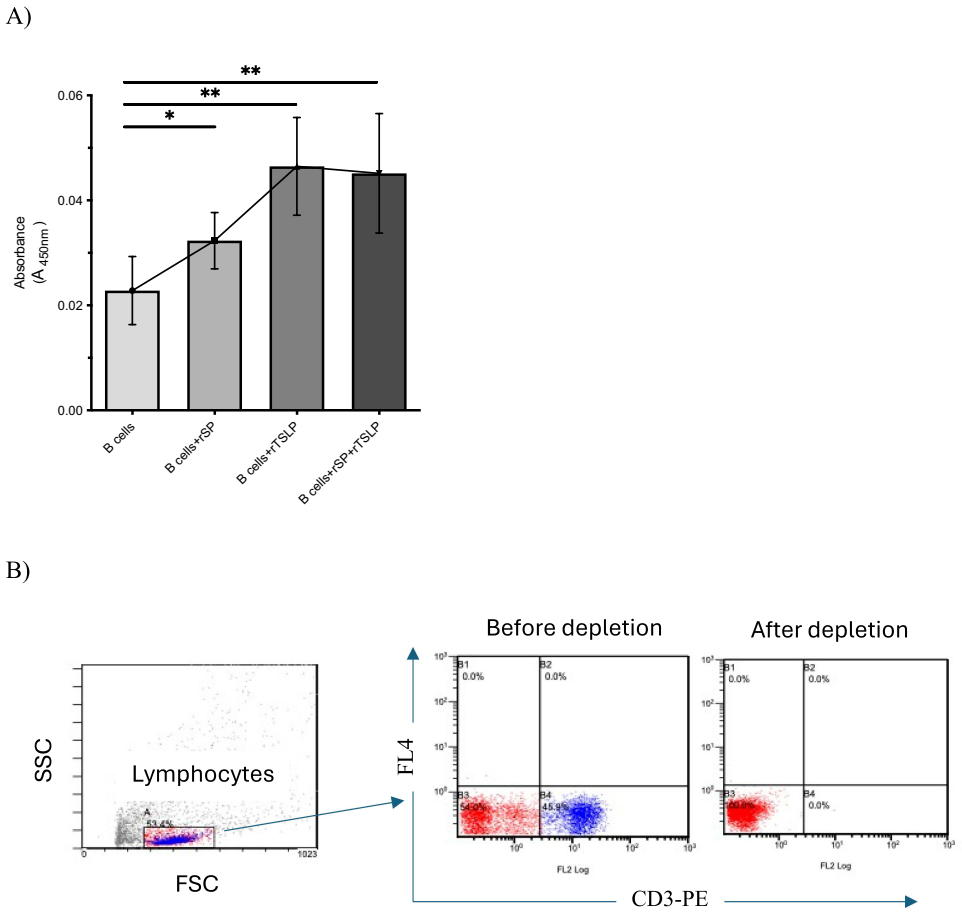

Figure 1 The rate of B cell survival is increases by rhTSLP with/without rSP. (**A**) B cells (10^4 cells/well) from healthy subjects were incubated for 4 days (n=10) with rhTSLP in the presence of rSP or not. Quick Cell Proliferation WST-1 Assay Kit was used to evaluate the B cell survival after 4 days. (**B**) PBMC were stained with anti-human CD3-PE antibody and gated from the lymphocyte population to determine the cell depletion was performed properly. The purity of cells was > 99.9% using flow cytometer. Student's paired *t*-test was performed to compare between both stimulants with B cells. Data are presented as the SEM.

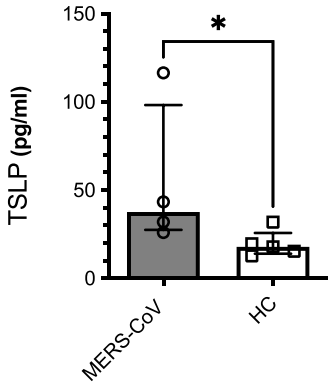

Figure 2 Level of TSLP in MERS-CoV infected patients. Sera were measured by ELISA for the TSLP concentration (pg/mL) in both groups; MERS-CoV-infected patients (n=4) and HC; healthy noninfected group (n=5). Mann–Whitney *U*-test was used, and the data are presented as the median (25th-75th percentile).

higher than TLSP levels in the healthy control (HC) group (37.6 pg/mL vs 19.8 pg/mL). The p-value for TSLP levels in patients with MERS-CoV relative to those in the control group was statistically significant (*p<0.05).

TSLP Levels Were in vitro Induced in A549 Cells by Different Stimulants

To measure the TSLP levels, A549 cells were stimulated with rSP, Poly I:C, and RSV and the supernatants were collected at different time points. TSLP levels were undetectable 24 h before rSP stimulation. However, low levels of TSLP were detected and measured after 24 h of stimulation by rSP, Poly I:C, and RSV at 1:2 MOI (7 pg/mL, 12.8 pg/mL and 16.5 pg/mL, respectively). Compared with the TSLP levels during the initial hours of stimulation, A549 cells showed a significant increase in TSLP levels after 48 h of stimulation with rSP (32.2 pg/mL, p =0.01). The concentration of TSLP increased slightly at 72 h (32.4 pg/mL) and began to decrease at 96 h (19.2 pg/mL) (Figure 3A). Notably, both Poly I:C (500 ng/mL) and RSV induced an increase in TSLP levels after 48 h; respectively (54.9 pg/mL; p =0.02, Figure 3B) and (76.7 pg/mL; p =0.01, Figure 3C). In addition, compared to unstimulated cells, all stimulants, rSP, Poly I:C, and RSV, remarkably induced TSLP levels after 48 h of stimulation (32.2 pg/mL, 54.9 pg/mL, 76.7 pg/mL). A comparison of the three stimulants and unstimulated cells (Un) is shown in Figure 3D.

Recombinant Human TSLP Has Positive Effect on B Cells Survival

As shown in Figure 1A, the survival rate of isolated human B cells from healthy individuals was measured after stimulation by rhTSLP (100 ng/mL) with or without rSP (5 µg/mL) for 4 d. Both rhTSLP and rSP from MERS-CoV enhanced the survival of B cells after 4 d, relative to unstimulated B cells (0.02 vs 0.045, $^{**}p<0.01$). In addition, B cells

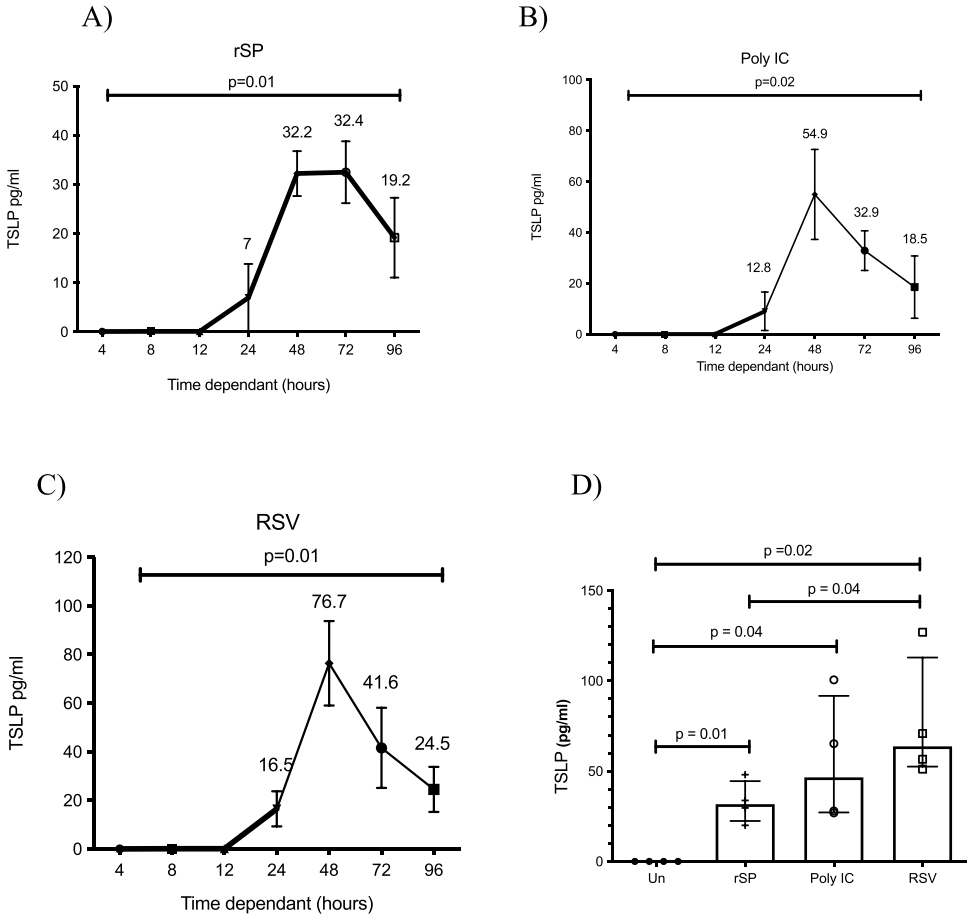

Figure 3 TSLP levels in A549 cell line in response to different stimulants. (**A**) rSP, (**B**) Poly I:C, and (**C**) RSV were used as stimulants for A549 cells and the collection of supernatants were performed at different timepoints (hours). (**D**) comparison between the three stimulants with unstimulated cells (Un) after 48 h of stimulation. Cell culture supernatants were collected and used for measurement of TSLP concentration by ELISA technique. The data analysis was performed in four independent experiments (each timepoints were performed in duplicate) for each stimulant. One-way ANOVA was used to compare between different time points. The data (**A**–**C**) are presented as the standard error of the mean (SEM). Mann–Whitney *U*-test was used to compare between different stimulants with unstimulated cells as indicated in (**D**), and the data are presented as the median (25th-75th percentile). A *p*-value of <0.05 was considered significant.

stimulated with rTSLP alone or rSP showed a significant effect compared to unstimulated cells (0.02 vs 0.046, $**p<0.01$, and 0.02 vs 0.032, $*p<0.05$). PBMC were stained with an anti-human CD3-PE antibody (surface marker) before the whole cell population (45.9%) and after cell depletion as the $CD3^{neg}$ population (0%) to ensure that all CD3 populations were depleted (Figure 1B).

rhTSLP Provoked in vitro Production of MERS-CoV-Specific IgG

To evaluate the effect of rhTSLP on antibody production, PBMCs were stimulated with either rSP, or rhTSLP, and both rhTSLP + rSP. As shown in Figure 4. The combination of rSP and rhTSLP elevated the response of IgG antibodies against the full spike protein of MERS-CoV relative to unstimulated cells (0.156 vs 0.22, $*p<0.05$). In addition, rSP and rhTSLP showed no significant differences compared to the unstimulated control cells (0.156 vs 0.163, 0.156 vs 0.160; $p>0.05$). Thus, none of the stimulants induced IgG production or had the same effect on antibody production as in the unstimulated cells. All samples from healthy subjects were compared to control as reference sera (0.78) from MERS-CoV-infected patients.

Discussion

In this study, we sought to determine the expression of TSLP cytokines in the sera of MERS-CoV-infected patients relative to healthy controls in vivo. In addition, in vitro, we examined the effect of full-length spike proteins of MERS-CoV, RSV, and Poly IC on the induction of TSLP protein in the stimulated airway epithelial cell line (A549) in vitro. In addition, we examined the effects of rhTSLP on B cell survival and IgG production. To the best of our knowledge, no previous studies have examined TSLP expression after MERS-CoV infection. Our findings demonstrated that patients infected with MERS-CoV had significantly increased levels of TSLP protein in their sera compared to healthy controls (Figure 2).

Moreover, it is crucial to study cytokines and chemokines derived from airway epithelial cells (AECs), which can induce B cell responses and antibody production, especially following pulmonary infection, as we recently reported.[26] TSLP cytokines are produced mainly by AECs, as well as other immune cells and tissues, and have been shown to induce a local adaptive immune response against pulmonary infections.[27] Furthermore, in vitro, A549 cells were used to mimic the airway immune response and to examine whether TSLP proteins were produced post stimulation with various factors,

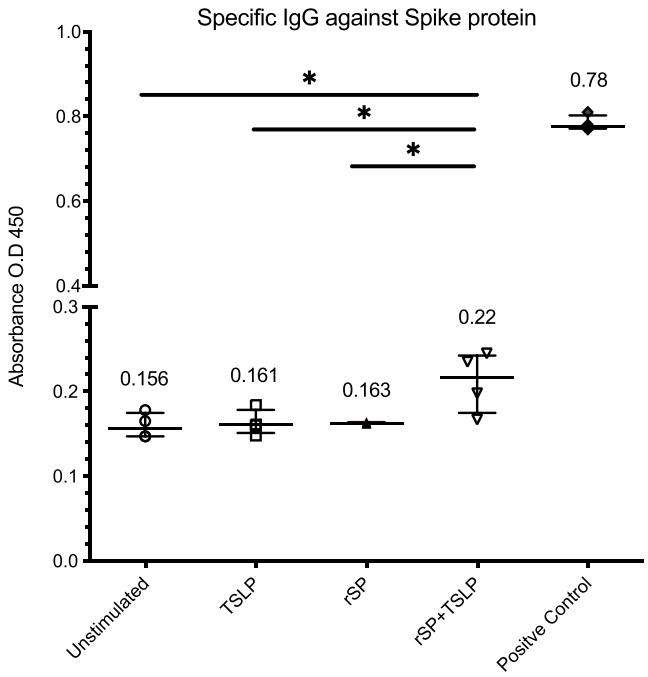

Figure 4 rhTSLP enhanced in vitro production of specific-IgG against MERS-CoV. PBMCs from healthy subjects were stimulated with rSP in the presence or absence of rhTSLP for 10 days (n=4). Culture supernatants were collected and measured by ELISA for full spike protein-specific IgG antibodies. All samples were performed in duplicate wells for each stimulant. For control, four different sera of MERS-CoV infected patients were measured for IgG concentration and then were used as reference sere. Mann Whitney *U*-test was used to compare between different stimulants with unstimulated cells. Data are presented as the median (25th-75th percentile).

including rSP, MESR-CoV, Poly IC, and RSV. Our results showed that low levels of TSLP were observed after 24 h of culturing A549 cells post stimulation with rSP. In addition, TSLP protein levels peaked at 48 h (Figure 3A).

Moreover, to determine whether TSLP protein was produced only as a result of MERS-CoV infection and rSP stimulation, different stimulants such as RSV and Poly IC were used. We found that TSLP protein levels increased significantly after RSV and Poly IC challenge in A549 cells compared to unstimulated cells (controls), suggesting that TSLP can be produced not only in response to MERS-CoV infection but also in response to other pulmonary viral infections (Figure 3B–D). Furthermore, RSV infection increases TSLP expression and is associated with asthma pathogenesis.[28] Thus, high TSLP production is considered an indicator of disease severity.[29]

In addition, to examine the role of TSLP in B cell proliferation during MERS-CoV infection, B cells were isolated from healthy individuals and stimulated with the rSP of MESR-CoV and rhTSLP alone or in combination with other rSP. We showed in vitro that rhTSLP alone or in combination with rSP can induce B cell survival (Figure 1). The current study aligns with other studies stating that TSLP is capable of enhancing the proliferation and differentiation of B cells in vitro.[18,30]

Furthermore, a previous study reported that the use of TSLP as an adjuvant vaccine against HIV resulted in a strong immune response and effective cellular and neutralizing antibodies that lasted for a long time in the serum and lungs of vaccinated mice compared to control animals.[31] Furthermore, a recent study showed that using TSLP as an adjuvant vaccine against influenza virus resulted in increased production of IgA in the airways, indicating the importance of TSLP expression in maintaining and protecting mucosal surfaces following respiratory viral infection.[32] This suggests that TSLP is involved, either directly or indirectly, in providing an effective local and systemic immune response to pulmonary viral infections.

Moreover, as shown in Figure 4, no significant IgG production was observed when PBMCs were stimulated with rhTSLP or rSP alone. However, when rhTSLP was combined with rSP, IgG levels increased relative to those in the unstimulated cells (Figure 4). In addition, the sera of MERS-CoV-infected patients, which were used as controls (reference sera), had higher amount of specific IgG by approximately 4-fold compared to rhTSLP + rSP. rSP itself was insufficient to produce a high production of antibodies, and it is considered a weak immunogenic protein that requires a potent adjuvant, such as rhTSLP, to induce a high antibody concentration. Moreover, rhTSLP alone maintained B cell survival but did not induce antibody production. Taken together, we believe that the significant and reasonable amount of specific IgG antibodies was due to the synergistic effect of rhTSLP and rSP on the survival rate of B cells, which may be considered memory B cells. Thus, we assumed that healthy individuals may have cross-reactivity with detectable IgG antibodies against MERS-CoV. This could be attributed to cross-reactivity with other coronavirus, as recently reported.[33] Collectively, our results suggest that MERS-CoV infection in the lungs can produce the TSLP protein, which can induce B cell response, survival, and antibody production, such as IgA and IgG. It has been shown that TSLP can directly activate B cells via receptor binding, promoting proliferation and differentiation into IgG-producing plasma cells.[34] Moreover, TSLP may indirectly activate B cells by improving the antigen presentation process in dendritic cells (DCs).[23] Furthermore, considering the ability of rhTSLP to induce B cell and IgG production and a more robust antibody response to the vaccine, potentially improving vaccine efficacy, we recently suggested that TSLP may be utilized as a vaccine adjuvant.[27] Additionally, TSLP may influence the development of long-lived memory B cells, leading to longer-lasting immunity. However, TSLP can activate Th_2 type immune responses and cause allergic reactions in vaccinated individuals.[27] However, further studies are required to determine the precise role of TSLP as a vaccine adjuvant.

Ultimately, our results in vivo and in vitro showed that TSLP levels were elevated in the sera of MERS-CoV patients compared to healthy individuals, and stimulation of the airway epithelial cell line (A549) with MESR-CoV rSP induces its expression. Furthermore, addition of rhTSLP enhanced B cell survival. However, the mechanisms underlying the regulation of IgG production has not been elucidated in this study. We hypothesized that TSLP indirectly enhances B cell survival and induces the production of IgG via the activation of Th_2 cytokines that induce antibody production. Furthermore, it has been shown that TSLP may regulate IgG production via multiple mechanisms. TSLP can activate DCs that enhance T follicular helper (Tfh) cell differentiation by increasing the expression of OX40-ligand, which results in the expression of Tfh markers, including CXCR5, IL-21, and BCL6, which are crucial for B cell activation and IgG production.[35] TSLP acts directly on B cells by exerting an effect similar to that of IL-7. It can provide essential signals for B lymphopoiesis and activating STAT5 pathways, which are important for supporting B cell survival and proliferation.[36,37] In addition, TSLP increases the production of Th_2 cytokines, in particular IL-13, resulting in the enhancement of IgG class switching.[18,34]

Conclusion

Our study concluded that MERS-CoV infection and the recombinant full-spike protein of MERS-CoV induce the production of TSLP*in vivo and* in vitro, respectively. This cytokine has a significant effect on B cell survival, which may enhance the local immune response via increased production of IgG antibodies in the airways following pulmonary viral infection. Although TSLP expression in the lungs enhances the immune response to pulmonary viral infection, uncontrolled expression of this cytokine has been implicated in allergic reactions. Thus, further investigations are needed to determine the precise role of TSLP during MESR-CoV infection and how this cytokine regulates the local immune response. Additional studies are required to evaluate the correlation between increased TSLP protein expression and IgG response.

Collectively, our findings suggest that TSLP can be used as a strong mucosal adjuvant for vaccine development against MERS-CoV infection. However, further investigation is required to identify the functional role of TSLP in MERS-CoV infection.

Limitations and Future Perspectives

Our small sample size was due to sample collection during the COVID-19 pandemic, which was the main concern. We performed a study in vitro and supported our results using a small number of clinical samples. Therefore, increasing the sample size will enhance the reliability of our study. Furthermore, measuring TSLP levels in the sera of MERS-CoV patients may not reflect the host immune response. Therefore, additional studies are required to determine TSLP expression in lower respiratory tract samples to gain a better understanding of the local immune response to MERS-CoV infection.

Abbreviations

BSA, bovine serum albumin; DCs, dendritic cells; DMEM, Dulbecco's Modified Eagle Medium; ECDC, European Centre for Disease Prevention and Control; ELISA, Enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HC, healthy control; IL-7, interleukin-7; ILCs, innate lymphoid cells; MERS-CoV, Middle East respiratory syndrome coronavirus; NK, natural killer; Poly I:C, Polyinosinic-polycytidylic acid; rhTSLP, recombinant human TSLP; rSP, recombinant full-length spike; RSV, Respiratory Syncytial Virus; SEM, standard error of mean; TSLP, Thymic stromal lymphopoietin protein; $Th_{1,}$ T helper 1; $Th_{2,}$ T helper 2.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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