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SARS coronavirus papain-like protease up-regulates the collagen expression through non-Smad TGF-β1 signaling

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A B S T R A C T

SARS coronavirus (CoV) papain-like protease (PLpro) reportedly induced the production of TGF-β1 through p38 MAPK/STAT3-mediated Egr-1-dependent activation (Sci. Rep. 6, 25754). This study investigated the correlation of PLpro-induced TGF-β1 with the expression of Type I collagen in human lung epithelial cells and mouse pulmonary tissues. Specific inhibitors for TGF-βRI, p38 MAPK, MEK, and STAT3 proved that SARS-CoV PLpro induced TGF-β1-dependent up-regulation of Type I collagen in vitro and in vivo. Subcellular localization analysis of SMAD3 and SMAD7 indicated that non-SMAD pathways in TGF-β1 signaling involved in the production of Type I collagen in transfected cells with pSARS-PLpro. Comprehensive analysis of ubiquitin-conjugated proteins using immunoprecipitation and nanoLC-MS/MS indicated that SARS-CoV PLpro caused the change in the ubiquitination profile of Rho GTPase family proteins, in which linked with the increase of Rho-like GTPase family proteins. Moreover, selective inhibitors TGF-βRI and STAT6 (AS1517499) ascertained that STAT6 activation was required for PLpro-induced TGF-β1-dependent up-regulation of Type I collagen in human lung epithelial cells. The results showed that SARS-CoV PLpro stimulated TGF-β1-dependent expression of Type I collagen via activating STAT6 pathway.

1. Introduction

Severe acute respiratory syndrome (SARS)-associated coronavirus (CoV), a member of betacoronaviruses in the Coronaviridae family, is identified as the causative agent for the outbreak of SARS in Asia and other countries in 2002–2003. Like other human coronaviruses (HCoVs) HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, and MERS-CoV (Li and Lin, 2013; Huang et al., 2015), SARS-CoV genome is an approximately 30 kb positive-strand RNA consisting of a 5′ cap, a 3′ poly (A) tract, and 14 open reading frames (ORFs). The largest ORFs ORF1a and ORF1b encode for the polyprotein replicases 1a and 1ab mainly involving in the SARS-CoV replication, as cleaved in cis and in trans by ORF1a-encoded papain-like protease (PLpro) and 3C-like protease (3CLpro). PLpro, a de-ubiquitinating/de-ISGylating enzyme (Barretto et al., 2005; Ratia et al., 2006), has the antagonistic activities of Type I interferon (IFN) by blocking IRF-3 and ERK1 phosphorylation, preventing the IκBα degradation, and de-ubiquitinating the STING-TRAF3-TBK1 complex (Li et al., 2011; Frieman et al., 2009; Sun et al., 2012). Recently, PLpro shows the inhibitory effect on Toll-like receptor 7 (TLR7) mediated cytokine production through removing Lys63-linked ubiquitin chains of TRAF3 and TRAF6 (Li et al., 2016a). SARS-CoV up-regulates pro-inflammatory cytokines like IFN-γ, IL-18, TGF-β1, TNF-α, IL-6, IP-10, MCP-1, MIG, and IL-8 (Huang et al., 2005; He et al., 2006), in which recruits immune responder cells into the lungs, triggers acute respiratory distress syndrome (ARDS), and even causes lung fibrosis in the late phase (Huang et al., 2005; He et al., 2006). Among SARS-CoV proteins, the nucleocapsid induces a Smad3-dependent induction of TGF-β1 expression (Zhao et al., 2008); spike protein stimulates the IL-8 up-regulation in lung cells (Chang et al., 2004); NSP1 provokes the expression of CCL5, CXCL10, and CCL3 (Law et al., 2007); and NSP11 inhibits the expression of ISG15 (Law et al., 2007).
et al., 2007); PLpro elevates the production of TGF-β1 and pro-fibrotic markers via ubiquitin proteasome, p38 MAPK, and ERK1/2-mediated signaling (Li et al., 2012). Recently, SARS-CoV PLpro notably initiates ROS/p38 MAPK/STAT3 pathway to activate Egr-1 dependent expression of TSP-1, TGF-β1 and vimentin in vitro and in vivo (Li et al., 2016b). Therefore, PLpro becomes a virulent factor in SARS pathogenesis.

TGF-β1 plays a crucial mediator of tissue fibrosis in lung, skin, liver, heart, and kidney through modulating the expression of pro-fibrotic proteins including type I collagen, fibronectin, α-SMA, and vimentin (Kubiczkova et al., 2012). In the canonical Smad signalling pathway, TGF-β1 interacts with the TGF-β type I receptor, activates receptor-regulated Smads (Smad2 and Smad3) complexed with Smad4, and then translocate to the nucleus to synthesize TGF-β1-induced transcriptional genes like Type I collagen (Leask and Abraham, 2004; Kubiczkova et al., 2012). In non-canonical TGF-β signalling pathways, TGF-β1 activates MAPKs (Erk, JNK and p38 MAPK), Rho-like GTPases (RhoA, Rac and Cdc42), PI3K/AKT, WNT/β-catenin, or Ca2+ signaling cascades in TGF-β1-induced transcriptional response (Zhang, 2009). The activation of p38 MAPK is responsible for TGF-β1-induced epithelial-to-mesenchymal transition of mouse mammary gland epithelial cells under a receptor independent of receptor-mediated Smad activation (Yu et al., 2002). The activation of RhoA in TGF-β1-induced EMT response is also independent of Smad2 and/or Smad3 (Bhowmick et al., 2001).

Our prior study demonstrated SARS-CoV PLpro triggering the TGF-β1 production in vitro and in vivo that linked with up-regulating the expression of pro-fibrotic proteins (vimentin and glial fibrillary acidic protein) (Li et al., 2016b). This study assesses possible effects and mechanisms of SARS-CoV PLpro-upregulated TGF-β1 upregulation on the expression of Type I collagen. The induction ability of SARS-CoV PLpro on in vitro and in vivo expression of Type I collagen was characterized. In addition, subcellular localization of Smad3, Smad7, and STAT6 was performed to elucidate the key factors involved in the induction of Type I collagen by PLpro. The relationship between TGF-β1 upregulation and the mechanism of Type I collagen induction by PLpro was validated by the specific inhibitors of TGF-β receptor kinase, p38 MAPK and STAT6.

2. Materials and methods

2.1. Cell culture and transient transfection

Human alveolar basal epithelial A549 cells grew in Dulbecco’s Modified Eagle’s Medium (HyClone Laboratories) and were transfected with control vector pcDNA3.1/His C (Invitrogen), or pSARS-PLpro containing SARS-CoV PLpro gene, as described in our prior reports (Li et al., 2011, 2012, 2016a, 2016b). In addition, pSARS-PLpro (H273A) that had the alanine substitution for histidine at position 273 by Ala within PLpro gene was constructed using PCR-based site-directed mutagenesis with a mutated primer pair (5′-GGTAACT ATCAGTTGGTGTACCTACTCATATACTCCTAAG-3′ and 5′-CTTACGTATATGGGTTGAAGCACCACACTGATAGTCACC-3′). A549 cells transiently expressing recombinant PLpro 2 days post transfection was analyzed using Western blotting, real-time RT-PCR, Sirius staining, and immunofluorescent staining assays.
2.2. Western blotting and immunoprecipitation assays

The lysate of transfected cells was performed by Western blotting with primary antibodies including rabbit anti-TGF-β1 (Cell Signaling), anti-E. coli synthesized PLpro mouse serum, anti-phospho STAT6 (Tyr641) (Cell Signaling), and anti-β-actin mAb (Abcam), and HRP-conjugated secondary antibodies like goat anti-mouse or anti-rabbit IgG. Immune complexes were detected using enhanced chemiluminescent β-actin mAb (Abcam), and HRP-substrate (Millipore).

2.3. Quantification of mRNA expression of type I collagen, TGF-β1 and vimentin using real-time RT-PCR

To measure the expression of type I collagen, TGF-β1, and vimentin in transfected cells, total RNAs extracted from transfected cells 2 days post transfection and mouse lung tissues were analyzed using two-step real-time RT-PCR with SYBR Green I, as described in our prior reports (Li et al., 2016b). Primer pairs included (1) 5′-GTTCGTGACCGTGACCTCG-3′ and 5′-TCGGTCTCTGTGGTCTGC-3′ for human type I collagen, (2) 5′-GACGGGAGAGTACTGGATCG-3′ and 5′-CTCTCAACACTTAC-3′ for mouse type I collagen, (3) 5′-GGCTTCTCTGCTTCTAATG-3′ and 5′-CTCTGCTCTGCTGTCCT-3′ for human TGF-β1, (4) 5′-TCTCTGAGGCTGCCAACCG-3′ and 5′-CAGAGGTAGACGACCTT-3′ for human vimentin, (5) 5′-AGCCACATCGCTGACCC-3′ and 5′-GCCAATAGGACCAAATCC-3′ for human Rac1, (6) 5′-AGCCACATCGCTGACCC-3′ and 5′-GCCAATAGGACCAAATCC-3′ for human Rac1, (7) 5′-TGAGGCGGTCTGAGATGTCT-3′ and 5′-CCACAGGCTCTGGTGGACGT-3′ for mouse GAPDH. Specific PCR product was quantified using the ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). Relative mRNA levels of indicated genes were normalized relative to GAPDH mRNA.

2.4. Sirius stain assays

For the detection of collagen expression, the tissue sections were stained with Sirius red solution for 2 h, and then rinsed 10 times with 0.5% glacial acetic acid in PBS. After dehydrating with ethanol, stained sections were mounted on the glass slides, and then examined using light microscopy (Olympus, BX50).

2.5. Mouse model with a chest injection of recombinant plasmids

The mouse mode with a direct chest injection was performed as described in our prior report (Li et al., 2016b). Empty vector pcDNA3.1 or recombinant plasmid pSARS-PLpro (50 μg/100 μl) in 3% sucrose/PBS was injected into the right chest of 5 eight-weeks-old BALB/c male mice using a 1-ml syringe with a 28-gage needle every 2 days. After 15 injections, the mice were sacrificed; the lung tissues were fixed, dehydrated, embedded in paraffin, and cut at 4–5 μm thickness using a rotary microtome. For immunohistochemistry (IHC) staining, mouse lung tissues were performed with anti-E. coli synthesized PLpro serum, as descried in our previous report (Li et al., 2016b). For H&E staining, sections were stained with hematoxylin for 3 min, eosin for 3 min, dehydrated in ethanol, and then mounted as slides that were examined and photographed using light microscopy (Olympus, BX50). Sirius staining and SYBR Green real time RT-PCR assays were mentioned above.

2.6. Immunofluorescence staining assay

For determining the effects of SARS-CoV PLpro on the nuclear translocation of SMAD3, SMAD7, and STAT6, A549 cells grew on the glass coverslip in 6-well were transfected with pSARS-CoV PLpro or pcDNA3.1, and treated with or without 1 μM kartogenin (Sigma). For testing the role of Rac1 in STAT6 signal, the Rac1 mutant plasmid, pMX-IG-Rac1 T17N provided by Dr. Takehito Uruno (Kyushu University, Japan), was co-transfected into cells. After 2-day incubation, cells were fixed with 3.7% formaldehyde in PBS for 1 h, blocked with 1% bovine serum albumin in PBS for 1 h, and then incubated with specific primary antibodies against SMAD3, SMAD7, and STAT6 at 4 °C.
overnight. Subsequently, cells were reacted with FITC- or AF546-
conjugated secondary antibodies in a dark box for 2 h. Finally, cells
were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min.
After washing with PBS, stained cells were photographed using the
immunofluorescence microscopy (Olympus, BX50).

### 2.7. Identification of ubiquitin-conjugated proteins nanoLC–MS/MS

The lysates from PLpro-expressing and empty vector cells were
reacted with anti-ubiquitin antibodies for 4 h at 4 °C, and then
incubated with protein A-Sepharose beads. The ubiquitin-conjugated
proteins were collected after centrifugation, washed four times with
NET buffer, embedded in SDS-PAGE gel, and then digested in gel. The
peptides of ubiquitin-conjugated proteins were recovered for
NanoLC–MS/MS spectra. Proteins were identified according to mass
spectra obtained were compared to SwissPort database (release 51.0)
via MASCOT algorithm (version 2.2.07), as described in our prior
reports (Li et al., 2012). Peptides were identified if MASCOT individual
ion scores exceeded 30.

### 2.8. Statistical analysis

All data were collected from 3 independent experiments and
analyzed using Student’s t-test or \( \chi^2 \) test. Statistical significance was
considered at \( p < 0.05 \).

#### 3. Results

##### 3.1. Correlation of SARS-CoV PLpro-induced TGF-β1 production with Type I collagen up-regulation

To examine the association of SARS-CoV PLpro-induced TGF-β1
production with the collagen up-regulation, A549 lung epithelial cells
transiently transfected with pcDNA3.1 and pSARS-PLpro were analyzed
the production of TGF-β1 and type I collagen using Western blot, real-
time RT-PCR and Sirius red staining assays (Fig. 1). Transfected cells
with pSARS-PLpro, but not pcDNA3.1, secreted the active form of TGF-
β1, and significantly increased the mRNA and protein expression of
Type I collagen. Importantly, SB-431542 (a selective TGF-βRI inhibitor)
treatment at 100 nM caused the 4-fold reduction of Type I collagen
mRNA in transfected cells with pSARS-PLpro (Fig. 1B). In a mouse
model, the expression of Type I collagen in lung tissues of mice was determined using IHC staining with
anti-E. coli synthesized PLpro serum, and IHC positivity for PLpro
expression within lung tissues was observed in the group infected with
pSARS-PLpro (Fig. 2A). H & E and Sirius staining assays indicated that
pulmonary inflammation with the infiltration of immune cells and the
increase of Type I collagen was identified in the pSARS-PLpro group,
but not vector control and solvent groups (Fig. 2A). Real-time PCR
confirmed that PLpro triggered the mRNA expression of Type I collagen
in mouse lung tissues in comparison with vector control and solvent groups (Fig. 2B).

To examine the proteolytic enzymatic activity of PLpro on the production of TGF-β1 and type I collagen, the catalytic mutant of PLpro (H273A) was constructed, and then used to investigate whether PLpro (H273A) up-regulated TGF-β1 and type I collagen in vitro (Fig. 3A). Importantly, PLpro(H273A) with the catalytic mutation lose the ability to induce the expression of TGF-β1 and type I collagen in vitro. Since SARS-PLpro had been demonstrated to stimulate p38 MAPK/STAT3-mediated activation of TGF-β1 production (Li et al., 2016b), SB203580 (a specific p38-MAPKs inhibitor), U0126 (a MEK1/2 inhibitor), and Stattic (a small-molecule inhibitor of STAT3 activation) were used to further confirm the correlation between the TGF-β1 production and Type I collagen up-regulation in transfected cells with pSARS-PLpro and pcDNA3.1 (Fig. 3B-D). SB203580 significantly reduced the mRNA expression of TGF-β1, in which was linked with down-regulation of Type I collagen in transfected cells with pSARS-PLpro in presence of SB203580 or U0126 (Fig. 3B and C). Stattic also suppressed the mRNA expression of Type I collagen in transfected cells with pSARS-PLpro (Fig. 3D). Overall, results of the in vitro and in vivo data demonstrated that the proteolytic enzymatic activity was required for SARS-CoV PLpro-dependent TGF-β1-mediated up-regulation of pro-fibrotic gene Type I collagen.

### 3.2. Activation of STAT6 was responsible for SARS-CoV PLpro-induced Type I collagen up-regulation

To examine whether SMAD-dependent pathways involve in TGF-β1-mediated up-regulation of Type I collagen in response SARS-CoV PLpro, subcellular localization of receptor-regulated SMAD3 and inhibitory SMAD7 in transfected cells were detected using the immunofluorescent and DAPI staining (Fig. 4). Imaging analysis of transfected cells indicated that SMAD3 localized in the nucleus of pcDNA3.1-transfected cells, but not pSARS-PLpro-transfected cells (Fig. 4A). Moreover, SMAD7 was detected in the nucleus of both transfected cells (Fig. 4B). Interestingly, kartogenin, a stimulator for TGF-β1/Smad3 signal pathway (Wang et al., 2014), was used to verify the inhibitory effect of PLpro on TGF-β1/Smad3 signal in transfected cells (Fig. 4C).

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**Table 1**

| Protein | UniProtKB ID | Gene ID | Mass | Matches | Percentage | Score |
|---------|--------------|---------|------|---------|------------|-------|
| Ras-related C3 botulinum toxin substrate 1 | RAC1_HUMAN | 51702787 | 21436 | 2 | 0.084912 | 53 |
| Ras GTPase-activating-like protein IQGAP1 | IQGA1_HUMAN | 4506787 | 189134 | 22 | 0.02419 | 85 |
| Ras-related protein Rab | RAB5C_HUMAN | 38258923 | 23468 | 6 | 0.2366412 | 116 |
| Putative Ras-related protein Rab | RAB1C_HUMAN | 193806493 | 22003 | 8 | 0.403333 | 140 |
| Ras GTPase-activating protein-binding protein | G3BP1_HUMAN | 14916572 | 52132 | 2 | 0.031842 | 51 |

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After 24 h treatment with kartogenin, Samd3 nuclear translocation was spotted within the nucleus in vector control cells, but not in PLpro-expressing cells. Most Samd3 was in the cytoplasm of pSARS-PLpro transfected cells treated with kartogenin. The result indicated that SARS-CoV PLpro inactivated SMAD-dependent pathways, implying that non-SMAD pathways in TGF-β signaling for the production of Type I collagen would be initiated by SARS-CoV PLpro.

Non-Smad pathways in TGF-β signaling include MAP kinase, Rho-like GTPase, and phosphatidylinositol-3-kinase/AKT pathways (Zhang, 2009). To examine the possible pathways involved in TGF-β1-dependent up-regulation of Type I collagen by SARS-CoV PLpro, the profiles of ubiquitin-conjugated proteins in transfected cells with vector control and pSARS-PLpro were determined using immune-precipitation and nanoLC–MS/MS. Interestingly, several proteins of the Ras GTPase family were identified (Table 1), in which indicated the ubiquitination of these Ras family GTPase proteins was influenced by SARS-CoV PLpro. Non-Smad pathways in TGF-β1 signaling include MAP kinase, Rho-like GTPase, and phosphatidylinositol-3-kinase/AKT pathways (Zhang, 2009). To examine the possible pathways involved in TGF-β1-dependent up-regulation of Type I collagen by SARS-CoV PLpro, the profiles of ubiquitin-conjugated proteins in transfected cells with vector control and pSARS-PLpro were determined using immune-precipitation and nanoLC–MS/MS. Interestingly, several proteins of the Ras GTPase family were identified (Table 1), in which indicated the ubiquitination of these Ras family GTPase proteins was influenced by SARS-CoV PLpro. Figure 5A represented the mass spectrum of Rac1 identified from one of up-regulated ubiquitinated proteins in PLpro-expressing cells with the calculated molecular weight (m/z values) along x-axis and relative intensity along y-axis (A). Relative mRNA levels of Rac1 were normalized by GAPDH mRNA (B). Rac1 protein level in transfected cells were detected by Western blotting (C). For analyzing STAT6 nuclear translocation (D), cells were co-transfected pcDNA3.1 or pSARS-PLpro with pMX-IG-Rac1 T17N for 24 h, washed, fixed, reacted with anti-STAT6 antibodies, followed by FITC-conjugated antibodies. Imaging was taken by immunofluorescent microscopy. ** p value < 0.01 compared to the vector control by student’s t-test.

4. Discussion

SARS-CoV PLpro has been demonstrated to trigger the TGF-β1-derived growth factor, respectively (Kippenberger et al., 2015; Lu et al., 2014). For analyzing the correlation between PLpro-induced Rac1 up-regulation and STAT6 activation, co-transfection of pcDNA3.1 or pSARS-PLpro plus the Rac1 mutant plasmid pMX-IG-Rac1 T17N was performed, and the STAT6 activation in co-transfected cells was measured using immunofluorescence staining with anti-STAT6 and FITC-conjugated secondary antibodies. PLpro expression caused the STAT6 expression and induced the translation of STAT6 in the nucleus. Remarkably, the Rac1 mutant slightly affected the STAT6 up-regulation by PLpro, but significantly reduced PLpro-induced STAT6 nuclear translocation (Fig. 5D). Later, the functional activity of STAT6 in TGF-β1-dependent collagen up-regulation was further characterized in vector control and PLpro-expressing cells (Figs. 6 and 7). Nuclear localization and phosphorylation of STAT6 was observed in transfected cells with pSARS-PLpro, but not vector control cells (Figs. 6 and 7 A). Importantly, SB-431542, a selective TGF-βR inhibitor, significantly reduced the entry of STAT6 in the nucleus of PLpro-expressing cells (Fig. 6). The result indicated that Rho GTPases/STAT6 was responsible for one of non-SMAD pathways in SARS PLpro-induced TGF-β1 signals. Furthermore, a STAT6 inhibitor AS1517499 markedly suppressed TGF-β1-dependent collagen expression in PLpro-expressing cells (Fig. 7). The results demonstrated that STAT6 activation was required for SARS PLpro-induced TGF-β1-dependent production of Type I collagen.
production in human promonocytes, human lung epithelial cells, and pulmonary tissues in mouse models (Li et al., 2012, 2016b). SARS-CoV PLpro induced the ROS-mediated p38 MAPK and STAT3 activation of Egr-1 expression, in which Egr-1 specifically bound to the TGF-β1 promoter region between −175 to −60, resulting in the increase of TGF-β1 production. This study indicated that SARS-CoV PLpro stimulated the production of Type I collagen in vitro and in vivo (Fig. 1–3). SARS-CoV PLpro-induced collagen deposition in pulmonary tissues was associated with lung inflammation and pulmonary fibrosis in mice injected with pSARS-PLpro (Fig. 3). The specific inhibitor for TGF-β receptor, SB-431542, blocked the up-regulation of Type I collagen in transfected cells with pSARS-PLpro. Moreover, the inhibitors for p38 MAPK and STAT3 that involved in Egr-1-dependent TGF-β1 production significantly reduced the expression of TGF-β1 and Type I collagen. TGF-β also activates non-canonical non-SMAD pathways, including MAPKs (ERK1/ERK2, JNK and p38), PI3K kinases, AKT/PKB, mTOR, and Rho-like GTPase family proteins (Ras, RhoA, Rac1, and Cdc42) (Zhang, 2009; Kubiczkova et al., 2012). Particularly, activation of Rac1 pathway was required for TGF-β-mediated process of epithelial-to-mesenchymal trans-differentiation in a dominant-negative Smad3 cells (Bhowmick et al., 2001). In addition, Rac1 activation promoted TGF-β-dependent collagen expression in mesangial cells (Hubchak et al., 2009). LC–MS/MS analysis demonstrated that SARS-CoV PLpro influenced the ubiquitination status of Ras-related C3 botulinum toxin substrate 1 (Rac1), Ras GTPase-activating-like protein IQGAP1, Ras GTPase-activating protein-binding protein (G3BP1) (Table 1). Real-time RT-PCR and Western blotting assays indicated the increased expression of Rac1 in transfected cells with pSARS-PLpro compared to vector control (Fig. 5). Since activation of Jak/STAT pathway in a Rac-dependent manner was identified in response to the agonist of protein-coupled receptors (Pelletier et al., 2003), STAT-dependent signals were investigated in this study. The TGF-βRI inhibitor SB-431542 significantly attenuated the phosphorylation and nuclear localization of STAT6 in transfected cells with pSARS-PLpro (Fig. 6). Meanwhile, the STAT6 inhibitor AS1517499 meaningfully reduced TGF-β1-dependent up-regulation of Type I collagen in PLpro-expressing cells (Fig. 7). The results indicated...
that STAT6 activation was required for PLpro-induced TGF-β1-dependent production of Type I collagen in human lung epithelial cells and mouse lung tissues. The result was accordant with the previous reports in that STAT6-dependent collagen production has been demonstrated in human skin fibroblasts and mouse airway fibroblasts (Kippenberger et al., 2015; Lu et al., 2014). SARS-CoV PLpro affected on the ubiquitination and expression profile of Rho-like GTPase family proteins that could link with the activation of STAT6 signaling in TGF-β1-dependent collagen expression, in which suggested that a new GTPase family proteins/STAT6 pathway might play a critical role in TGF-β1-dependent collagen production in PLpro-expressing cells.

In summary, SARS-CoV PLpro induced p38 MAPK/STAT3-mediated TGF-β1-dependent up-regulation of Type I collagen, causing pulmonary pro-fibrotic responses. PLpro diminished the nuclear localization of SMAD3, changed the expression profiling of Rho-like GTPase family proteins, and activated STAT6-mediated TGF-β1-dependent production of Type I collagen. The results let us conclude that SARS-CoV PLpro induced non-SMAD signals including STAT6 activation in TGF-β1-dependent pulmonary pro-fibrotic responses.

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References

Barretto, N., Jukeliene, D., Ratia, K., Chen, Z., Mesecar, A.D., Baker, S.C., 2005. The papain-like protease of severe acute respiratory syndrome coronavirus has deubiquitinating activity. J. Virol. 79, 15189–15198.

Beijing Group of National Research Project for SARS, 2003. Dynamic changes in blood cytokine levels as clinical indicators in severe acute respiratory syndrome. Chin. Med. J. (Engl.) 116, 1283–1287.

Blomwick, N.A., Ghiasi, M., Bakin, A., Aakre, M., Lundquist, C.A., Engel, M.E., Arteaga, C.L., Moses, H.L., 2001. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell 12, 27–36.

Chang, Y.J., Liu, C.Y., Chiang, B.L., Chao, Y.C., Chen, C.C., 2004. Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: identification of two functional regions. J. Immunol. 173, 7602–7614.

Frieman, M., Ratia, K., Johnston, R.E., Mesecar, A.D., Baric, R.S., 2009. Severe acute respiratory syndrome coronavirus papain-like protease ubiquitin-like domain and catalytic domain regulate antagonism of IRF3 and NF-kappaB signaling. J. Virol. 83, 6689–6705.

He, L., Ding, Y., Zhang, Q., Che, X., He, Y., Shen, H., et al., 2006. Expression of elevated inflammatory cytokines in SARS-CoV-infected ACE2+ cells in SARS patients: relation to the acute lung injury and pathogenesis of SARS. J. Pathol. 210, 288–297.

Huang, K.J., Su, I.J., Theron, M., Wu, Y.C., Lai, S.K., Liu, C.C., Lei, H.Y., 2005. An interferon-γ-related cytokine storm in SARS patients. J. Med. Virol. 75, 185–194.

Huang, S.H., Su, M.-C., Tien, N., Huang, C.J., Lan, Y.C., Lin, C.S., Chen, C.H., Lin, C.W., 2015. Epidemiology of human coronavirus NL63 infection among hospitalized patients with pneumonia in Taiwan. J. Microbiol. Immunol. Infect. http://dx.doi.org/10.1016/j.jmii.2015.10.008.

Hubach, S.C., Sparks, E.E., Hayashida, T., Schnaper, H.W., 2009. Rac1 promotes TGF-beta-stimulated mesangial cell type I collagen expression through a PI3K/Akt-dependent mechanism. Am. J. Physiol. Renal. Physiol. 297, F1316–1323.

Kippenberger, S., Zoller, N., Kleemann, J., Müller, J., Kaufmann, R., Hofmann, M., Bernd, A., Meissner, M., Valesky, E., 2015. STAT6-dependent collagen synthesis in human fibroblasts is induced by bovine milk. PLoS One 10, e0131783.

Kubíčková, L., Sedlariková, L., Hajek, R., Sevcikova, S., 2012. TGF-β: an excellent servant but a bad master. J. Transl. Med. 10, 185.
Law, A.H., Lee, D.C., Cheung, B.K., Yim, H.C., Lau, A.S., 2007. Role for nonstructural protein 1 of severe acute respiratory syndrome coronavirus in chemokine dysregulation. J. Virol. 81, 416–422.
Leask, A., Abraham, D.J., 2004. TGF-beta signaling and the fibrotic response. FASEB J. 18, 816–827.
Li, S.W., Lin, C.W., 2013. Human coronaviruses: clinical features and phylogenetic analysis. Biomedicine (Taipei) 3, 43–50.
Li, S.W., Lai, C.C., Ping, J.F., Tsai, F.J., Wan, L., Lin, Y.J., Kung, S.H., Lin, C.W., 2011. Severe acute respiratory syndrome coronavirus papain-like protease suppressed alpha interferon-induced responses through downregulation of extracellular signal-regulated kinase 1-mediated signalling pathways. J. Gen. Virol. 92, 1127–1140.
Li, S.W., Yang, T.C., Wan, L., Lin, Y.J., Tsai, F.J., Lai, C.C., Lin, C.W., 2012. Correlation between TGF-β1 expression and proteomic profiling induced by severe acute respiratory syndrome coronavirus papain-like protease. Proteomics 12, 3193–3205.
Li, S.W., Wang, C.Y., Jou, Y.J., Huang, S.H., Hsiao, L.H., Wan, L., Lin, Y.J., Kung, S.H., Lin, C.W., 2016a. SARS coronavirus papain-like protease inhibits the TLR8 signaling pathway through removing Lys 63-linked polyubiquitination of TRAF3 and TRAF6. Int. J. Mol. Sci. 17 pii: E678.
Li, S.W., Wang, C.Y., Jou, Y.J., Yang, T.C., Huang, S.H., Wan, L., Lin, Y.J., Lin, C.W., 2016b. SARS coronavirus papain-like protease induces Egr-1-dependent up-regulation of TGF-β1 via ROS/p38 MAPK/STAT3 pathway. Sci. Rep. 6, 25754.
Lu, J., Zhu, Y., Feng, W., Pan, Y., Li, S., Han, D., Liu, L., Xie, X., Wang, G., Li, M., 2014. Platelet-derived growth factor mediates interleukin-13-induced collagen I production in mouse airway fibroblasts. J. Biosci. 39, 693–700.
Pelletier, S., Duhamel, F., Coulombe, P., Popoff, M.R., Meloche, S., 2003. Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. Mol. Cell. Biol. 23, 1316–1333.
Ratia, K., Saikatendu, K.S., Santarsiero, B.D., Barretto, N., Baker, S.C., Stevens, R.C., Mesecar, A.D., 2006. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. Proc. Natl. Acad. Sci. U. S. A. 103, 5717–5722.
Sun, L., Xing, Y., Chen, X., Zheng, Y., Yang, Y., Nichols, D.B., et al., 2012. Coronavirus papain-like proteases negatively regulate antiviral innate immune response through disruption of STING-mediated signaling. PLoS One 7, e39802.
Wang, J., Zhou, J., Zhang, N., Zhang, X., Li, Q., 2014. A heterocyclic molecule kartogenin induces collagen synthesis of human dermal fibroblasts by activating the smad4/smad3 pathway. Biochem. Biophys. Res. Commun. 450, 568–574.
Yu, L., Hébert, M.C., Zhang, Y.F., 2002. TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses. EMBO J. 21, 3749–3759.
Zhang, Y., Li, J., Zhan, Y., Wu, L., Yu, X., Zhang, W., Ye, L., Xu, S., Sun, R., Wang, Y., Lou, J., 2004. Analysis of serum cytokines in patients with severe acute respiratory syndrome. Infect. Immun. 72, 4410–4415.
Zheng, Y.F., 2009. Non-Smad pathways in TGF-beta signaling. Cell Res. 19, 128–139.
Zhao, X., Nicholls, J.M., Chen, Y.G., 2008. Severe acute respiratory syndrome-associated coronavirus nucleocapsid protein interacts with Smad3 and modulates transforming growth factor-beta signaling. J. Biol. Chem. 283, 3272–3280.