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Original article

Extraordinary GU-rich single-strand RNA identified from SARS coronavirus contributes an excessive innate immune response

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Abstract

A dangerous cytokine storm occurs in the SARS involving in immune disorder, but many aspects of the pathogenetic mechanism remain obscure since its outbreak. To deeply reveal the interaction of host and SARS-CoV, based on the basic structural feature of pathogen-associated molecular pattern, we created a new bioinformatics method for searching potential pathogenic molecules and identified a set of SARS-CoV specific GU-rich ssRNA fragments with a high-density distribution in the genome. *In vitro* experiments, the result showed the representative SARS-CoV ssRNAs had powerful immunostimulatory activities to induce considerable level of pro-inflammatory cytokine TNF-a, IL-6 and IL-12 release via the TLR7 and TLR8, almost 2-fold higher than the strong stimulatory ssRNA40 that was found previously from other virus. Moreover, SARS-CoV ssRNA was able to cause acute lung injury in mice with a high mortality rate *in vivo* experiment. It suggests that SARS-CoV specific GU-rich ssRNA plays a very important role in the cytokine storm associated with a dysregulation of the innate immunity. This study not only presents new evidence about the immunopathologic damage caused by overactive inflammation during the SARS-CoV infection, but also provides a useful clue for a new therapeutic strategy.

Keywords: SARS; Viral pathogenesis; ssRNA; TLR7; TLR8; Inflammation

1. Introduction

SARS coronavirus (CoV) is a novel etiological agent resulting in an atypical pneumonia followed with severe acute respiratory syndrome (SARS). With the whole genome sequencing of SARS CoV, the proteins (S, E, M, and N) have been identified [1,2], and their structure and function associated with evasion, virulence and immunity have been studied in great detail [3–6]. Although much has been learned since its outbreak in 2003, many aspects of the pathogenesis of the disease remain obscure [7].

Viral interactions with the host immune system always play a central role for the outcome of infection. Because cytokine storm has been observed in the rapid course of SARS [8], and the pro-inflammatory cytokines, such as IL-6, TNF-a, etc, are up-regulated in clinical serum or cultured supernatants [9–13], it is inferred that an overactive innate immune response should contribute to virus-induced immune pathology resulting in acute lung injury in SARS patients. Therefore, more detailed knowledge about how the viruses interact with the host innate immune system is very important for understanding the molecular mechanisms of pathogenesis and progress of the disease [14].

During the virus invasion, host toll-like receptors (TLRs) are able to recognize different pathogen-associated molecular patterns (PAMPs) and trigger innate immune response [15]. Recently, the spike (S) protein has been proposed to be recognized by TLR2 and provoke the pro-inflammatory cytokines release [16–18]. However, little is known about the role of single-strand RNA (ssRNA) in the SARS-CoV as a kind of potential PAMP. Since a GU (guanosine and uridine)

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rich ssRNA40 from the U5 region of HIV-1 was identified as a natural agonist of TLR7 and TLR8 [19], it is attractive if there are immune stimulatory ssRNAs existed in about 30 kb length of single-stranded SARS-CoV RNA genome, and the relevant immune effect is still unrevealed.

In this study, we attempt to make a comprehensive investigation on the SARS GU-rich ssRNA in the SARS-CoV RNA genome by bioinformatics scanning technique, evaluate their effect in the host innate immune response in order to reveal more detailed immunopathogenetic mechanism.

2. Materials and methods

2.1. TLR agonists

The phosphothioate-protected ssRNAs were synthesized by IDT, inc. (IA, USA), including ssRNA40 (5′-GCCCGUCUGUUGUGUGACUC-3′; at U5 region 108–127 nt of HIV-1 genome), ssRNA120 (5′-GUCUGAGUGUGUUCUUG-3′; at 24,524–24,540 nt in the SARS-CoV genome), ssRNA83 (5′-GUG CUUGUGUAUUGUGC-3′; at 16,174–16,190 nt in the SARS-CoV genome), and the U/A alternated fakeRNA120 (5′-GACAGAGAGAGAACAAG-3′). DOTAP (Liposomal Transfection Reagent *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate, Roche, Inc.), a liposomal transfection reagent, was used to complex with ssRNA before addition to cell cultures. The DOTAP/ssRNA mixture was prepared according to the following procedure: the ssRNA was diluted by HBS (HEPS-buffered saline) to a concentration of 0.025–0.6 μg/μl and the DOTAP was dilute by HBS with the ratio of 1:3, then the diluted ssRNA and DOTAP were mixed at the ratio of 1:2. In addition, Pam3CK4 were purchased from Invivogen (San Diego, USA) as a TLR2 agonist.

2.2. Cell culture and stimulation with TLR agonists

The mouse macrophage-like cell line, RAW264.7 (American Type Culture Collection), was fed in DMEM medium (GIBCO, USA), the human acute monocytic leukemia cell line, THP1 (American Type Culture Collection), was fed in RPMI1640 (GIBCO, USA), the human PBMCs were isolated from normal human peripheral blood by a density gradient centrifugation with Lympholyte-H medium (Cedarlane Laboratories, Ltd., Canada), and resuspended in RPMI 1640. In addition, the culture media supplemented with 10% (v/v) low-endotoxin fetal calf serum (FCS) (Hyclone, USA), and 100 IU/ml penicillin and 100 g/ml streptomycin as well at 37 °C in a 5% CO_2 humidified incubator. Live cells diluted in the PBS (0.1 mM, pH 7.2) with 0.4% trypan blue were counted by a hemocytometer.

The prepared cells were resuspended at 1×10^6/ml in 0.2 ml culture media in 96-well plates. Before the addition of TLRs agonists, 4 h culture was needed for the adherence of RAW264.7 cells but not for THP1 cells. Each group consisted of four-well repeats for each sample. The group treated with ssRNA40 at a final concentration of 5 μg/ml was denoted as positive control of TLR7/8 stimulation, and that treated with 5 μg/ml fakeRNAs as the negative controls. Each of the experiment groups was treated respectively with 5 μg/ml different GU-rich ssRNA for an independent stimulation. A comparative analysis of immunostimulatory activity was performed between ssRNA40 and ssRNA120 respectively at the concentration gradient of 0, 1.25, 2.5, 5, 10 and 20 μg/ml.

2.3. Cytokine ELISA for TNF-a, IL-12, IL-6 and IFN-a

The above groups were cultured at 37 °C in a 5% CO_2 humidified incubator, the supernatants at each well were collected after 10 h culture for the measurement of TNF-a level, and collected once again after 24 h culture for the measurement of IL-6, IL-12 and IFN-a levels.

The cytokines were detected by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols of the relevant ELISA kits, The ELISA kits used in this study included the anti-mouse or anti-human TNF-a and IL-6 ELISA kit (eBioscience, Inc., USA), IL-12p40 ELISA kit (Boster, Inc., China) and IFN-a ELISA kits (Mabtech inc., USA). The cytokines were measured at 450 nm wavelength by Varioskan Flash spectral scanning multimode reader (Thermo Scientific, USA). Finally, one of representative experiment of at least two independent experiments would be shown in the results.

2.4. Gene knockdown

To knockdown the TLR7 and TLR8, the siRNAs templates were designed by BLOCK-iT™ RNAi Designer (Invitrogen inc.) based on the TLR7 and TLR8 mRNA sequences (GenBank accession no. NM_133211.3 and AF246971.1) and synthesized in Invitrogen inc. A preliminary test selected the best one for gene knockdown out of the three candidate templates from TLR7 (NM_133211.3_stealth_1560 5′-AAUAGUGUAAGGCCUCAAGGACCUG) and that from TLR8 (AF246971.1_stealth_1520 5′-AAGAGGAACUAUUUGCAUAACUCUG).

According to the standard siRNA transfection protocol provided by Invitrogen inc, the siRNA (5 pmol/ml) was transfected into cells in 96 wells plate (the cells are 60–80% confluent for each well) by Lipofectamine™ 2000 for 24 h culture, Then each well was replaced with fresh 1x normal growth medium, Meanwhile, the experiment groups were respectively treated with 5 μg/ml ssRNA120 and ssRNA83. The TNF-a levels were detected by ELISA after another 24 h culture.

2.5. Animal model and histological study

Adult male Kunming mice (18 ± 0.5 g) were obtained from the Animal Center of the Third Military Medical University (Chongqing, China) under the approval by the Animal Care and Use Committee. All the mice were injected with 800 mg/kg of D-GalN via tail vein [20]. One hour later, the mice were randomly divided into two groups ($n = 10$ for each), the mice in control group took a tail vein injection of 300 μl HBS and

DOTAP mixture (2:1), and the mice in treatment group took a tail vein injection of 300 μl HBS and DOTAP (2:1) mixed with SARS-CoV ssRNA83 at a dose of 1 mg/kg body weight. The activity and mortality of mice was recorded every 6 h for the first 3 days, and every 24 h for the last 4 days. During the experiment, the lung tissues from died mice in treatment group or the healthy mice in control group were cut off and fixed with 4% paraformaldehyde for at least 24 h and embedded in paraffin. After deparaffinization and dehydration, the lungs were cut into 4-μm sections and stained with hematoxylin and eosin. The Smith scoring was done in the lung tissue [21].

Meanwhile, in a paralleled experiment, the cytokines were detected by ELISA after the mice were treated for 4 h.

2.6. Bioinformatics analysis of GU-rich sequences

The GU-rich ssRNA fragments were detected by scanning the whole genome sequence of SARS CoV (accession number: NC_004718.3) and HIV-1 (accession number: NC_001802.1) by using our in-house Perl program, which was based on the Heil's description for GU-rich motif. The GU-rich sample sequences were screened out while met a threshold of the GU or UG pairs content of more than 40% and at least one 'GUGU' or 'UGUG' motif contained in every scanning window with a length of 18 bases. Moreover, the sequence similarity was checked by BLAST program of NCBI tools.

2.7. Statistical analysis

Data are expressed as mean ± standard error. Comparative studies of means were carried out using a *t*-test analysis with a statistical significance at p value < 0.05, and the graphical figures were drawn by Microsoft Excel.

3. Results

3.1. Identification of GU-rich regions of SARS-CoV

A comprehensive GU-rich fragment searching was performed by our in-house bioinformatics tool against the whole genome sequences. Because ssRNA40 was the first natural GU-rich ssRNA identified from HIV-1 genome [19], both of the whole genomes of SARS-CoV and HIV-1 were scanned in order to make a comparative analysis. Totally, 904 GU-rich ssRNA fragments were found to be located in 130 regions of the single strand SARS CoV genome, in contrast, only 130 GU-rich ssRNA fragments were found to be located in 16 regions of the HIV-1 genome, among which the previous ssRNA40 was detected as well. Interestingly, relative to the full length of each genome (SARS-CoV 29.75 kb and HIV-1 9.18 kb), SARS-CoV had about twenty-fold higher distribution density of GU-rich fragments than HIV-1. Therefore, it was obvious that GU-rich ssRNA fragments of SARS-CoV had much more chances to contact with the host immune system than that of HIV-1 did.

Based on these data, a set of representative GU-rich ssRNA fragments from SARS-CoV was selected out for the following functional examination. Firstly, the similarity analysis showed there was generally no similar sequence in nucleotide sequence database matching them. It suggested these ssRNAs were characterized by the SARS-CoV.

To investigate the immunological role of SARS-CoV ssRNAs, in the following section, we observed the immune characters from several aspects by *in vitro* and *vivo* experiments, including the TLR7, TLR8 recognition, the stimulatory activities and immunopathologic damage.

3.2. Immune stimulatory activity of SARS-CoV specific ssRNA through TLR7

The immune effect of SARS-CoV specific ssRNAs mediated by TLR7 was observed upon the RAW264.7 cells, which expressed TLR7 but no TLR8 [22]. The result showed that all the GU-rich ssRNAs, including ssRNA40, ssRNA120 and ssRNA83 induced significantly the TNF-a release, in contrast to the DOTAP control group (p value < 0.01) and the U/A alternated fakeRNA120 (p value < 0.01) (Fig. 1). Because the fakeRNA120 lost the stimulatory ability, it reflected GU-rich motif was the core structure for the TLR7 recognition. Interestingly, ssRNA120 led to two-fold of TNF-a release than the ssRNA40 did, and the ssRNA83 led to higher TNF-a level than ssRNA120 (p value < 0.05). However, the production of IL-6, IL-12 or IFN-a was not detected, so it seemed likely only the early NF-kB activation took place in the TLR7-mediated MyD88 signaling pathway. Meanwhile, After the TLR7 knockdown by siRNA with 50% transfection rate, The TNF-a level was significantly decreased (Fig. 3). Taken together, it suggested that both of the SARS-CoV GU-rich ssRNA83 and ssRNA120 had a higher stimulatory performance than HIV-1 specific ssRNA40 through early NF-kB activation after TLR7-mediated recognition.

3.3. Immune stimulatory activity of SARS-CoV specific ssRNA through TLR8

On the other hand, the immune effect of SARS-CoV specific ssRNA through TLR8 was observed upon the THP1 cells, which expressed TLR8 but no TLR7 [23].

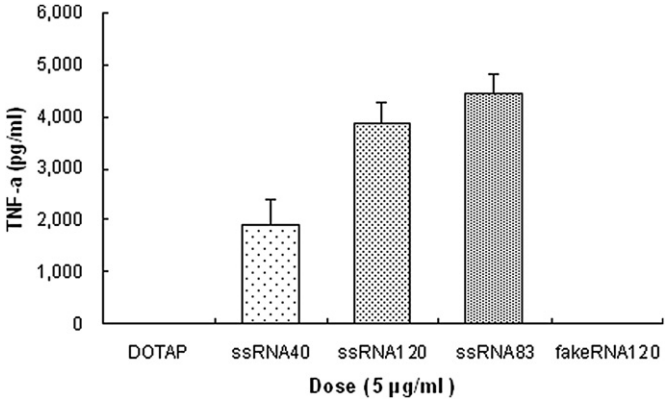

Fig. 1. TNF-a release induced by the ssRNA mediated by TLR7. The RAW264.7 cells in the experiment groups were treated by 5 μg/ml ssRNA for 24 h.

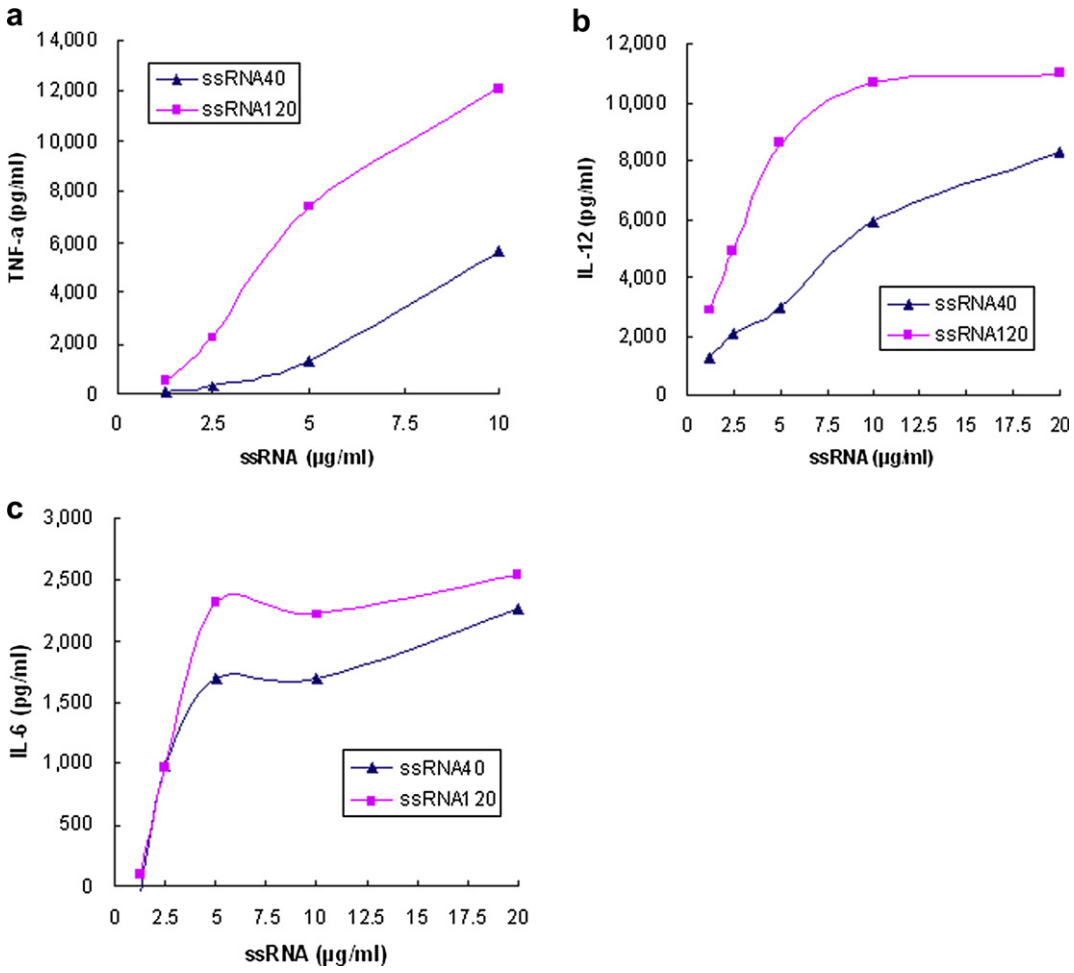

Fig. 2. Cytokines release induced by the ssRNAs at different dose mediated by TLR8. (a) TNF-a production after 10 h stimulation; (b) IL-12 production after 24 h stimulation; (c) IL-6 production after 24 h stimulation.

The data showed various cytokines were detected after ssRNA stimulation, including TNF-a, IL-6 and IL-12, but no IFN-a. Unlike the strong immune activity of ssRNA83 through TLR7, SARS-CoV ssRNA120 led to much higher levels of TNF-a through TLR8, in respect to the SARS-CoV ssRNA83 (p value < 0.01). It reflected that the TLR7 and TLR8 had different recognition capabilities to the ssRNA structure, although both of them mainly depended on the recognition of GU-rich motif.

In comparison with ssRNA40 previously identified from HIV-1, the immunostimulatory activity was evaluated under the treatment of SARS-CoV ssRNA120 at different dose. The data demonstrated that the GU-rich ssRNAs stimulated various cytokines release by TLR8 in a dose-dependent manner (Fig. 2), but the U/A alternated fakeRNA120 lost the stimulatory activity by TLR8 (Supplementary Fig. 1). Interestingly, the dose–effect curve of every typical inflammatory factor presented that the ssRNA120 was able to produce much stronger inflammatory effect, almost 2-fold higher than the ssRNA40. On the other hand, if the TLR8 knockdown, the product of cytokine was significantly decreased (Fig. 3). It testified that the ssRNA120 from SARS-CoV had a powerful stimulatory ability to induce high levels of TNF-a, IL-6 and IL-12 release through TLR8.

3.4. Pro-inflammatory response in hPBMC induced by SARS-CoV specific ssRNA

To investigate the integrate effect of SARS-CoV ssRNA120 on the human PBMC, the production of cytokines was measured by ELISA after 24 h stimulation by 5 μg/ml ssRNA. In contrast to the DOTAP control and ssRNA40, ssRNA120 induced multiple pro-inflammatory factors release with high levels (p value < 0.01 vs ssRNA40 and DOTAP) (Fig. 4). It testified that SARS-CoV ssRNA120 possessed a powerful immunostimulatory activity to the immune cells in the blood, and it implied that an excessive pro-inflammatory response could occur if the viral particles were swallowed up by host immune cells and exposed the GU-rich ssRNA fragments to TLR7 and 8.

3.5. Acute lung injury caused by SARS-CoV specific ssRNA

To assess overactive immune response and its consequence caused by SARS-CoV specific ssRNA *in vivo*, we observed the activity, mortality and lung histopathological changes between the treated mice and non-treated mice. The result showed that the general status of mice in the group treated with 1 mg/kg

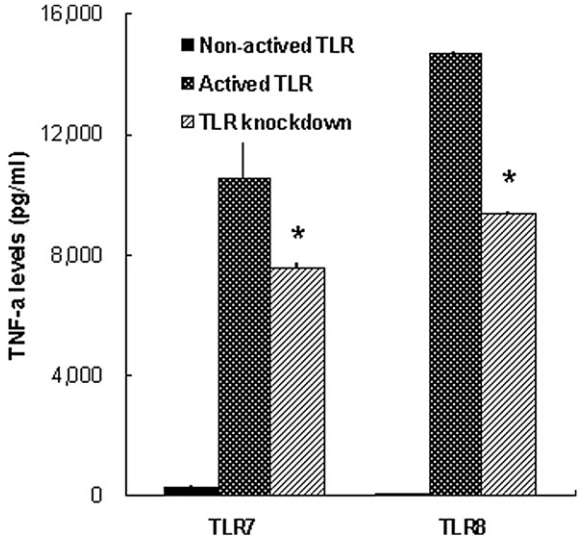

Fig. 3. TNF-a level changes after TLR7 or TLR8 knockdown at 50% siRNA transfection rates. Column 1 in the TLR7 group, RAW264.7 cells treated only with 100 μ/ml DOTAP/HBS. Column 2 in the TLR7 group, RAW264.7 cells treated with 5 μg/ml ssRNA83 for 24 h. Column 3 in the TLR7 group, the 5 pmol/ml TLR7 siRNA transfected into RAW264.7 cells before treated with 5 μg/ml ssRNA83 for 24 h; Column 1 in the TLR8 group, THP1 cells treated only with 100 μ/ml DOTAP/HBS. Column 2 in the TLR8 group, THP1 cells treated with 5 μg/ml ssRNA120 for 24 h. Column 3 in the TLR8 group, the 5 pmol/ml TLR8 siRNA transfected into THP1 cells before treated with 5 μg/ml ssRNA120 for 24 h.

ssRNA83 was decreased with symptoms of listlessness, sluggishness and narcolepsy, and more than half of the mice were died within 48 h, then the symptoms disappeared gradually in the survivors after 72 h. In contrast, there was normal in the control group (Fig. 5). The histopathological change of lung tissues in the dead mice demonstrated that pulmonary edema,

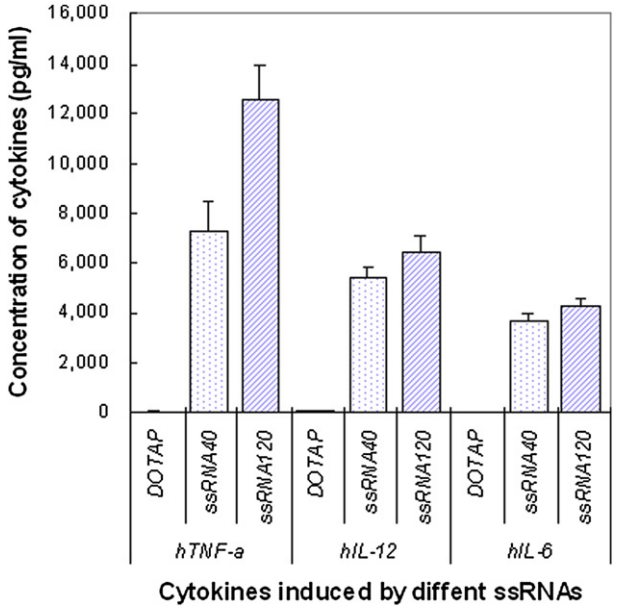

Fig. 4. Multiple cytokines release of hPBMC induced by ssRNA. hPBMC were treated by 5 μg/ml ssRNA for 24 h.

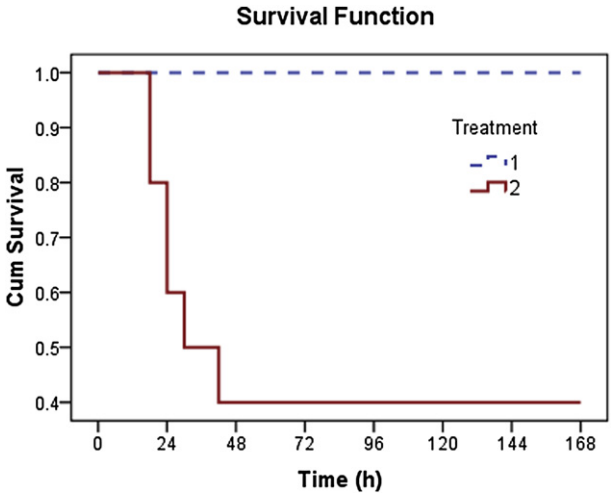

Fig. 5. Life table analysis of mice under attack of SARS-CoV specific ssRNA. 1. Control group ($n = 10$); 2. Treatment group with 1 mg/kg SARS ssRNA83 ($n = 10$).

infiltration of inflammatory cells and alveolar hemorrhage companied with alveolar damage (Fig. 6B). But there was no obvious change in lung structure in control group (Fig. 6A). And the smith pathologic scores of the treatment group (7.14 ± 1.35) were significantly higher than those of the control group (0.42 ± 0.23) ($P < 0.01$). Moreover, in comparison to the control group, the cytokines significantly increased in the treatment group at 4 h after ssRNA83 attack (Fig. 7). This experiment testified that a low-dose of SARS-CoV specific ssRNA was able to induce excessive inflammatory response and acute lung injury *in vivo* and lead to a high mortality rate.

4. Discussion

A great deal of clinical and experimental evidence associated with the hypercytokinemia and systemic immunopathology has proved that a progressive immune-associated injury results in the severe acute respiratory syndrome. Especially, a hyper-innate immunity characterized with the cytokine storm is involved in the dysregulation of a series of pro-inflammatory cytokines during the viral infection [8]. Therefore, to discover the potential pathogenic factor of SARS is very important for understanding the interaction of host and virus.

In this study, based on the basic structural feature of PAMP, we are the first to create a new bioinformatics method for searching ssRNA fragments with pathogenic molecular pattern. After a comprehensive scanning for the SARS-CoV genome, a set of SARS-CoV specific GU-rich ssRNAs have been successfully figured out. The distribution density analysis displays SARS-CoV has a higher probability to release GU-rich ssRNAs to contact with the host immune system, in contrast to HIV-1. Moreover, the experimental data have confirmed that the GU-rich ssRNAs from the SARS-CoV are able to induce innate immune response through TLR7 and

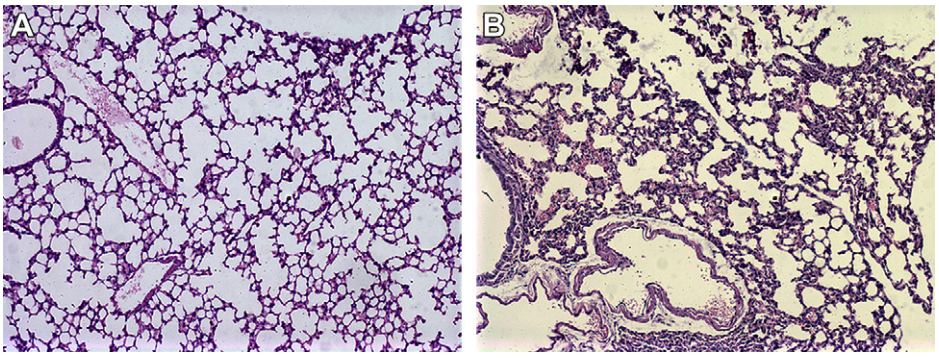

Fig. 6. Lung histopathological changes of mice (H&E × 100). (A) Control group; (B) treatment group with 1 mg/kg SARS ssRNA83.

TLR8 signal pathway. Especially, the SARS-CoV ssRNA120 and ssRNA83 with a powerful stimulatory ability could induce considerable productions of TNF-a, IL-6 and IL-12 *in vitro*, approximately 2-fold higher than the stimulatory ssRNA40 found in HIV-1 [19]. Furthermore, we have testified that acute lung injury occurred in the murine model with a high mortality, after attacked by a low-dose of SARS-CoV ssRNA, which is similar to the clinical pathological change of SARS.

During the period of SARS outbreak, many clinical investigations have showed a series of pro-inflammatory cytokine abnormally upregulated in the blood samples from SARS patients. For instance, TNF-a, IL-1 and IL-8 genes are updated in the acute severe cases [24], the productions of IL-6 and TNF-α are higher than normal in many early SARS patients [25], and similar report shows the Th1 cytokine, inflammatory cytokines such as IL-1, IL-6, and IL-12 are increased [26], but type I IFN is reported to be downregulated [27]. Although a lot of works focus on SARS-CoV protein to interpret these phenomena, the pathogenic factor is partly clarified till recent years, while Wang et al confirmed that spike protein as one of PAMPs is able to provoke the pro-inflammatory response via TLR2 signal pathway [16]. Now, we have newly identified that the SARS-CoV specific ssRNAs are able to induce various pro-inflammatory cytokines release with high performance, which is quite consistent with the immunopathological features of SARS previously reported. It is no doubt that this study provides more substantial evidence closely associated with the cytokine storm.

Unlike the spike protein directly contacting with TLR2 on the surface of host immune cells in the early viral evasion, GU-rich ssRNAs must be recognized by TLR7 and 8, which depend on the SARS-CoV replication manner, so the stronger pro-inflammatory response should occur after a latent period. Except for that, the host immune cell uptaking viral particle is an essential condition for the intracellular TLR7 and 8 to recognize the GU-rich ssRNA fragments. Although the pulmonary alveolar epithelium is the chief target of SARS-CoV [20], SARS-CoV is able to infect both immature and mature human monocyte-derived DCs and the incomplete viral replication has been observed followed with low expression of antiviral cytokines (IFN-a, IFN-β, etc) and up-regulation of pro-inflammatory cytokines (TNF-a and IL-6) [13]. More interestingly, TLR7 is predominantly expressed in lung, placenta, and spleen, while TLR8 is predominantly expressed in lung and peripheral blood leukocytes [28]. These factors may provide SARS-CoV a shortcut to trigger innate immune response through the TLR7/8 and ultimately contribute to development of immune pathology within the lungs.

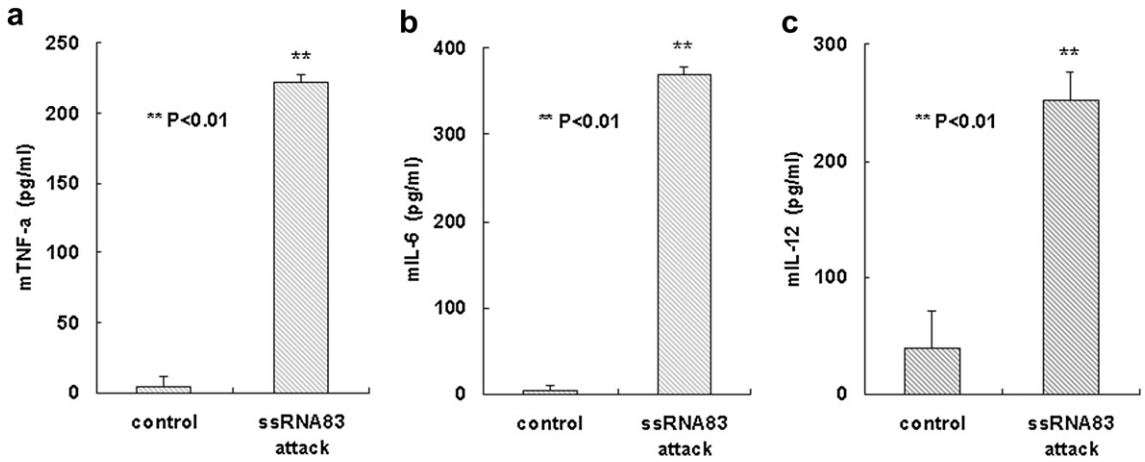

Fig. 7. Cytokines release under ssRNA83 treatment. Treatment group with 1 mg/kg SARS ssRNA83 for each mouse for 4 h.

On the other hand, although TLR7 and TLR8 are phylogenetically and structurally related, TLR7 and TLR8 specific agonists trigger a different cytokine induction profile. TLR7-specific agonists generally induce IFN-regulated cytokines, but TLR8-specific agonists primarily lead to the production of pro-inflammatory cytokines, such as TNF-a and IL-12 [29]. In this study, the cytokine profiles show similar pro-inflammatory responses between TLR7 and TLR8 pathway, but the IFN-a is not detected after either TLR7 or TLR8 recognition, so the activation process induced by SARS-CoV GU-rich ssRNAs is mainly depended on the MyD88-NF-kb signal pathway but not the MyD88-IRF pathway. It implies the over-inflammation occurs together with a dysfunction of anti-virus immune response related to type I IFN in the acute severe period. All together, with the accumulation of new evidence, we are closer to the clinical truth of the dramatic cytokine storm and inflammatory response in SARS patients.

In conclusion, here we have successfully identified the extraordinary GU-rich ssRNAs with powerful immunostimulatory activity from SARS-CoV, which are able to provoke a strong pro-inflammatory response via the TLR7/8 recognition and cause acute lung injury leading to death. These findings testify that GU-rich ssRNAs of SARS-CoV play a key role in the overactive innate immune response. This study not only presents new evidence to comprehensively interpret the occurrence of cytokine storm and the immune pathogenetic mechanism behind the SARS-CoV infection, but also provides an important clue for a new therapeutic strategy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2012.10.008.

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