

## Cross-reactivity of antibody against SARS-coronavirus nucleocapsid protein with IL-11

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### Abstract

Infection of SARS-associated coronavirus (SARS-CoV) induced a strong anti-nucleocapsid (anti-N) antibody response. However, the pathophysiological significance of the anti-N antibodies in SARS pathogenesis is largely unknown. To profile the anti-N antibodies, a phage-displayed scFv library was prepared from mice immunized with heat-inactivated SARS-CoV-infected Vero E6 cell lysate. Specific anti-N scFvs were isolated by panning against a recombinant nucleocapsid protein and reactivity was confirmed with phage-ELISA. Sequence analysis indicated that two of the isolated anti-N scFv clones were identical and displayed a high homology with an scFv specific for interleukin 11 (IL-11), an anti-inflammatory cytokine derived from bone marrow stroma cells. In a neutralization assay, IL-11-induced STAT 3 phosphorylation in rat intestinal epithelial IEC-18 cells was completely suppressed by the anti-N scFv clone L9N01.

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The outbreak of severe acute respiratory syndrome (SARS) in 2003 has spread to 29 countries, infected more than 8000 people, and resulted in 916 deaths worldwide [1]. A new type of coronavirus is identified as the causative agent for SARS [2–5]. Hematological studies indicated that SARS patients elicited a strong antibody response to viral nucleocapsid protein [6,7] and a moderate antibody response to viral spike protein and other viral envelop proteins in some patients [6,8]. In addition, phage-displayed peptide library has been used to profile the binding epitopes of anti-SARS-CoV antibodies from convalescent serum, identifying peptide sequences of various viral

proteins including nucleocapsid, spike, and proteins of predicted open reading frames (ORFs) [9,10].

Lines of evidence suggest that the anti-spike (anti-S) antibodies can neutralize SARS-CoV and block its infection of cells [11–14]. A protective effect on virus infection has been reported for non-neutralizing antibodies against hepatitis virus nucleocapsid protein [15]; however, the pathophysiological roles of anti-nucleocapsid (anti-N) antibodies in SARS patients are largely unknown.

In order to gain a better understanding of the characteristics of anti-N antibodies, an scFv library was constructed from mouse immunized with heat-inactivated SARS-CoV-infected Vero E6 cell lysate. Panel of anti-N scFvs was derived from the library. Intriguingly, one of the anti-N scFvs cross-reacted with interleukin 11 (IL-11) which is a

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bone marrow stroma-derived cytokine and a member of the interleukin 6 (IL-6) cytokine family [16,17].

## Materials and methods

**Cloning, expression, and purification of SCoV-N protein.** The full-length SARS-CoV nucleocapsid cDNA was amplified by PCR from the SARS-CoV genomic library (CUHK-W1) and cloned into the *NdeI* and *EcoRI* sites of pAC28m vector which places a His<sub>6</sub>-tag at the N terminus of the protein. Expression of the N protein was induced by adding 0.1 mM IPTG for 6 h at 25 °C in *Escherichia coli* strain B834. Bacterial cells were lysed by sonication and cell debris was removed by centrifugation. Nucleocapsid proteins in soluble fraction were purified using a combination of Ni-NTA agarose chromatography (Qiagen) and S-200 size exclusion chromatography (Amersham Biosciences).

**SARS-CoV-infected vero E6 cell lysate.** Vero cells (ATCC CRL-1586) were cultured in DMEM supplemented with 5% fetal calf serum (Gibco) at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. The Vero cells were infected with a SARS-CoV strain (CUHK-W1) for 16–48 h, and the cells were lysed in a Hepes buffer (10 mM; pH 7.0) supplemented with 40 mM KCl, 3 mM MgCl<sub>2</sub>, 5% glycerol, 0.2% NP40, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail (Sigma). After removing cell debris by centrifugation, the virus-infected cell lysate was heated for 30 min at 55 °C to inactivate any live virus. The heat-inactivated cell lysate was then kept at –70 °C until use.

**ScFv library construction and biopanning.** ScFv antibodies were prepared as described previously [18]. The *NotI*- and *SfiI*-restricted scFv antibodies were then cloned into pCANTAB 5E phagemid vector (Amersham). Library was constructed by chemical transformation using competent TG1 *E. coli*. Subsequently, log-phase TG1 transformants were superinfected with M13KO7 helper phage (Amersham) in a multiplicity of infection (moi) ratio of 3:1. Pool of scFv-phages produced in overnight culture was purified by polyethylene glycol precipitation (20% PEG8000 and 2.5 M NaCl). Purified phages were resuspended in 4 ml of a pre-blocking buffer (1× PBS, 0.2% Triton X-100, 0.01% NaN<sub>3</sub>, 0.1% BSA, and 10% non-fat milk) and incubated at room temperature for 30 min before panning process. Phages (0.5 ml/well) were panned against immobilized antigen in a 24-well plate of which each well was pre-coated with 7.5 µg of recombinant N protein in 0.5 ml of carbonate coating buffer overnight at 4 °C. After incubation at room temperature for 2 h with gentle shaking, bound scFv-phages were eluted with 100 µl of 0.1 M glycine-HCl, pH 2.2. After 10 min acid incubation at room temperature, the eluant was neutralized with 10 µl of 1 M Tris-HCl, pH 8.0. Specificity of eluted phage clones was confirmed by phage-ELISA.

**Phage-ELISA.** Phage-ELISA was carried out in a 96-well ELISA plate, and each well was coated with 50 µl of a carbonate coating buffer, pH 9.6, containing 2 µg of recombinant N protein overnight at 4 °C. After incubation with 100 µl scFv-phages at 37 °C for 1 h, bound phages were detected by incubation with 100 µl of a horseradish peroxidase-conjugated anti-M13 mouse antibody (Amersham) at 37 °C for 1 h. Activity of horseradish peroxidase was measured by a colorimetric method with *o*-phenylenediamine/H<sub>2</sub>O<sub>2</sub> as substrates. Color was allowed to develop for 1 h at room temperature, and absorbance at 450 nm was measured with a µQuant micro-plate reader (Bio-Tek).

**Nucleotide sequence analysis.** Nucleotide sequence determinations were performed by dye-terminator cycle sequencing using Beckman CEQ DTCS Kit as recommended by the manufacturer. Sequencing products were separated by capillary gel-electrophoresis and the nucleotide sequence was read by Beckman CEQ2000 sequencer (Beckman Coulter). Sequences obtained were compared with NCBI IgBLAST, and multiple sequence alignment was performed by ClustalW from EMBL-EBI server with the following default conditions: matrix, BLOSUM; gap opening penalty, 10.0; gap extension penalty, 0.05; gap separation penalty, 8; maxdiv, default; no end gap separation penalty. Alignment in the CDR3 was further adjusted manually in accordance with the physical property of amino acid residues.

**Western blot analysis of STAT 3 phosphorylation.** IEC-18 cells (ATCC CRL-1589) were cultured in DMEM supplemented with 5% fetal calf serum and 0.1 U/ml bovine insulin (Sigma) at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. For STAT 3 phosphorylation analysis, the IEC-18 cells were grown to confluence in 24-well plates. The cells were then serum-starved by reducing FCS supplement to 0.5% for 24 h followed by to 0.01% for 12 h. The cells were then exposed to 5 nM IL-11 (PeproTech EC) for 60 min at 37 °C in the absence or presence of various individual anti-N scFv clones ( $9 \times 10^{10}$  cfu) in a final volume of 100 µl. The cells were lysed in a lysis buffer (25 mM Tris-HCl; pH 6.8, 1% SDS, 1 mM sodium orthovanadate, and 1× protease inhibitor cocktail), boiled for 10 min, and then stored at –20 °C until use. Proteins of cell lysate (50 mg) were separated in a 12.5% SDS-PAGE gel and then electro-transfer to a nitrocellulose paper. The protein blot was probed with a 1000-fold diluted mouse monoclonal anti-phospho STAT 3 (Tyr 705) antibody (Cell Signalling) together with a 10,000-fold diluted mouse monoclonal anti-β actin antibody (Sigma) for 16–18 h at room temperature in an immunoblotting buffer (50 mM Tris-HCl; pH 7.4, 0.02% NaN<sub>3</sub>, 80 mM NaCl, 20 mM CaCl<sub>2</sub>, and 5% skimmed milk powder). After incubating with an alkaline phosphatase-conjugated anti-mouse Ig secondary antibody (2000-fold diluted, Zymed) for 2 h at room temperature, the immunoreactive bands were visualized by incubating with NBT/BCIP as suggested by the manufacturer (Boehringer-Mannheim).

## Results

Recombinant nucleocapsid protein of SARS-CoV (SCoV-N) was cloned from the SARS-CoV genomic library (CUHK-W1) by PCR and tag with a His<sub>6</sub>-tag at its N-terminus for purification. Expression of recombinant N proteins in *E. coli* was construct-dependent and induced by addition of IPTG. Purified recombinant N protein was characterized with a molecular mass of ~52 kDa (Fig. 1A). The purified recombinant His<sub>6</sub>-N protein was recognized by convalescent serum of SARS patients but not by healthy subject (Fig. 1B), suggesting that the recombinant N protein shares the same antigenicity as the native viral N protein.

To profile the anti-N antibody response, we constructed a phage-displayed scFv library from a mouse that immunized with heat-inactivated SARS-CoV-infected Vero E6 cell lysate. As evidenced by Western blot analysis against the recombinant nucleocapsid protein, anti-N response in immunized mouse was detectable 8 days after the first immunization and displaying strong antibody response after two more boost injections (Fig. 2). No immunoreactive response was observed in mouse without immunization (Fig. 2), suggesting that the antibody response was induced by the heat-inactivated SARS-coronavirus, and consistent with the previous finding that inactivated SARS-CoV induces high level of neutralizing antibody in mice [19].

After the construction of phage-displayed scFv library, anti-N scFvs were retrieved by bio-panning the library ( $3 \times 10^6$  recombinants) against the recombinant SCoV-N protein, and 2100 anti-N scFvs were isolated. Among 14 selected strong SCoV-N protein binders, 2 scFv-phage clones (L9B3a and L9B4b, the clones were renamed as L9N01) gave the identical nucleotide sequence. To our surprise, BLAST search of the derived anti-N scFv sequences against NCBI database indicated that the L9N01 phage

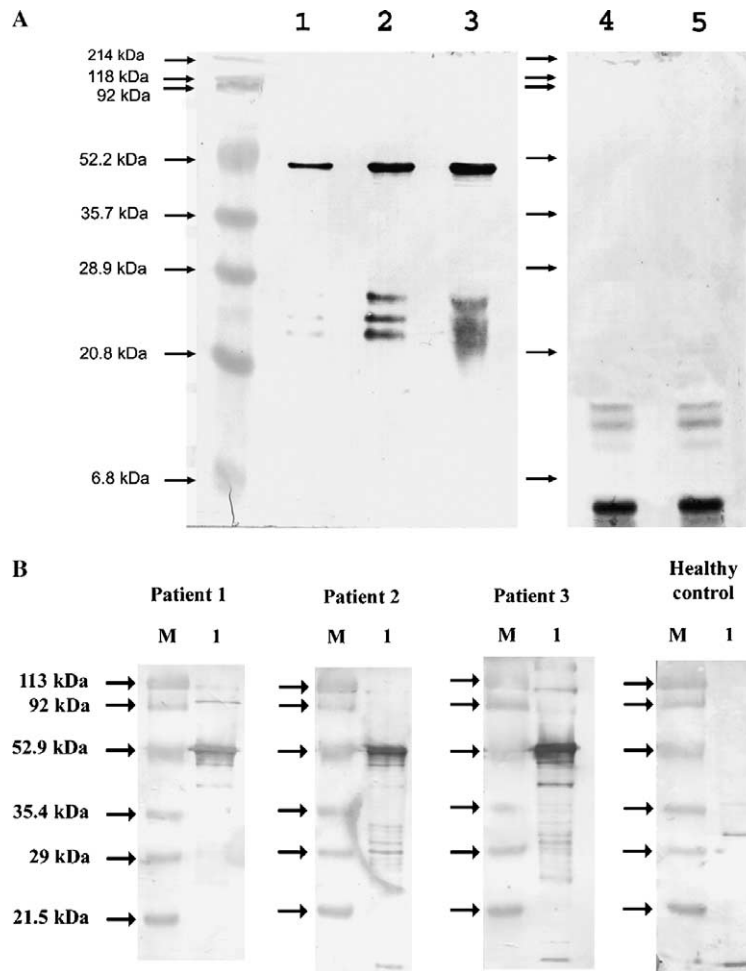


Fig. 1. Western blot analysis of recombinant SAR-CoV N protein. (A) Purified His<sub>6</sub>-tagged recombinant SCoV-N protein of 0.5 μg (lane 1), 1 μg (lane 2) and 1.5 μg (lane 3), and IPTG-induced vector-transfected bacterial cell lysate at 1 h (lane 4) and 3 h (lane 5) induction were separated on a 12.5% SDS-PAGE gel. After transfer to nitrocellulose paper, the recombinant N protein was probed with an anti-His tag antibody, revealing a major ~52 kDa immunoreactive protein with some possible degradative fragments with molecular masses ranging from 22 to 27 kDa. (B) Sera of a healthy subject and three convalescent SARS patients were 50× fold diluted and probed separately against purified recombinant N protein (0.8 μg/lane). Strong immunoreactive band at ~52 kDa was detected, suggesting that the recombinant N protein shares the same antigenicity as the viral N protein. Protein standards (lane M) and sera samples (lane 1).

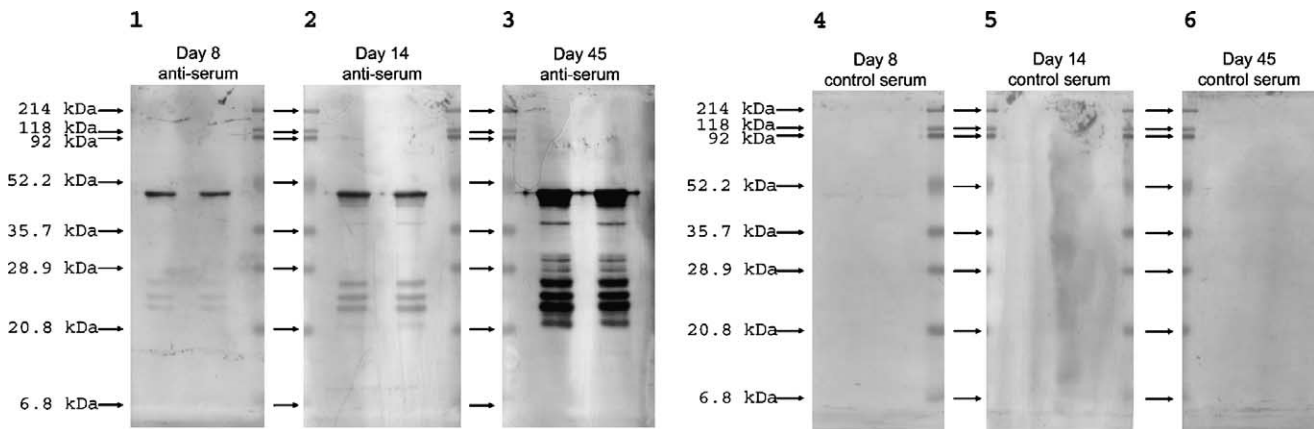


Fig. 2. Antibody response of individual mice that immunized with heat-inactivated SARS-CoV-infected Vero E6 cell lysate. Sera of immunized mice were collected at day 8 (lane 1), 17 (lane 2), 48 (lane 3), and the respective non-immunized controls at day 8 (lane 4), 17 (lane 5), and 48 (lane 6) were collected. Reactivity of individual mice sera (500× fold diluted) towards SCoV-N protein was tested against purified recombinant N protein (0.5 μg). Strong immunoreactivity was noted in immunized mice but not in controls, suggesting humoral response of mouse towards SARS-CoV-infected Vero E6 cell lysate mimicking antibody response to SARS-CoV infection.

clone shares a high sequence homology (84%) with an anti-IL-11 scFv (Accession No. AY171038) (Fig. 3).

In order to examine the cross-reactivity, L9N01 were tested against recombinant N protein and IL-11 by phage-ELISA. The L9N01 scFv-phage bound strongly both to the recombinant N protein as well as to a recombinant IL-11 (Fig. 4). However, L9N01 did not react with IL-6 which is structurally and functionally similar to IL-11 (data not shown).

To evaluate the biological effect of anti-N scFv, IL-11-stimulated tyrosine<sup>705</sup> phosphorylation of STAT 3 in rat intestinal epithelial IEC-18 cells was examined. IL-11 stimulated STAT 3 phosphorylation that lasted throughout the 60-min test period. In the presence of L9N01 phage

( $6 \times 10^{10}$  cfu), IL-11-induced phosphorylation was completely suppressed. By contrast, the presence of other specific anti-N scFvs exerted no inhibitory effect (Fig. 5).

**Discussion**

Induction of auto-antibodies has been reported in viral infection of human immunodeficiency virus [20,21], human-T-lymphotropic virus type-1 [22,23], hepatitis C virus [24,25], enterovirus [26,27], and Epstein–Barr virus [28,29]. Indeed, the generation of auto-antibodies against a phospholipid protein complex (Lupus anticoagulants) [30] and a glycan moiety of human serum glycoprotein asialo-orosomucoid [31] in SARS-CoV infection has been

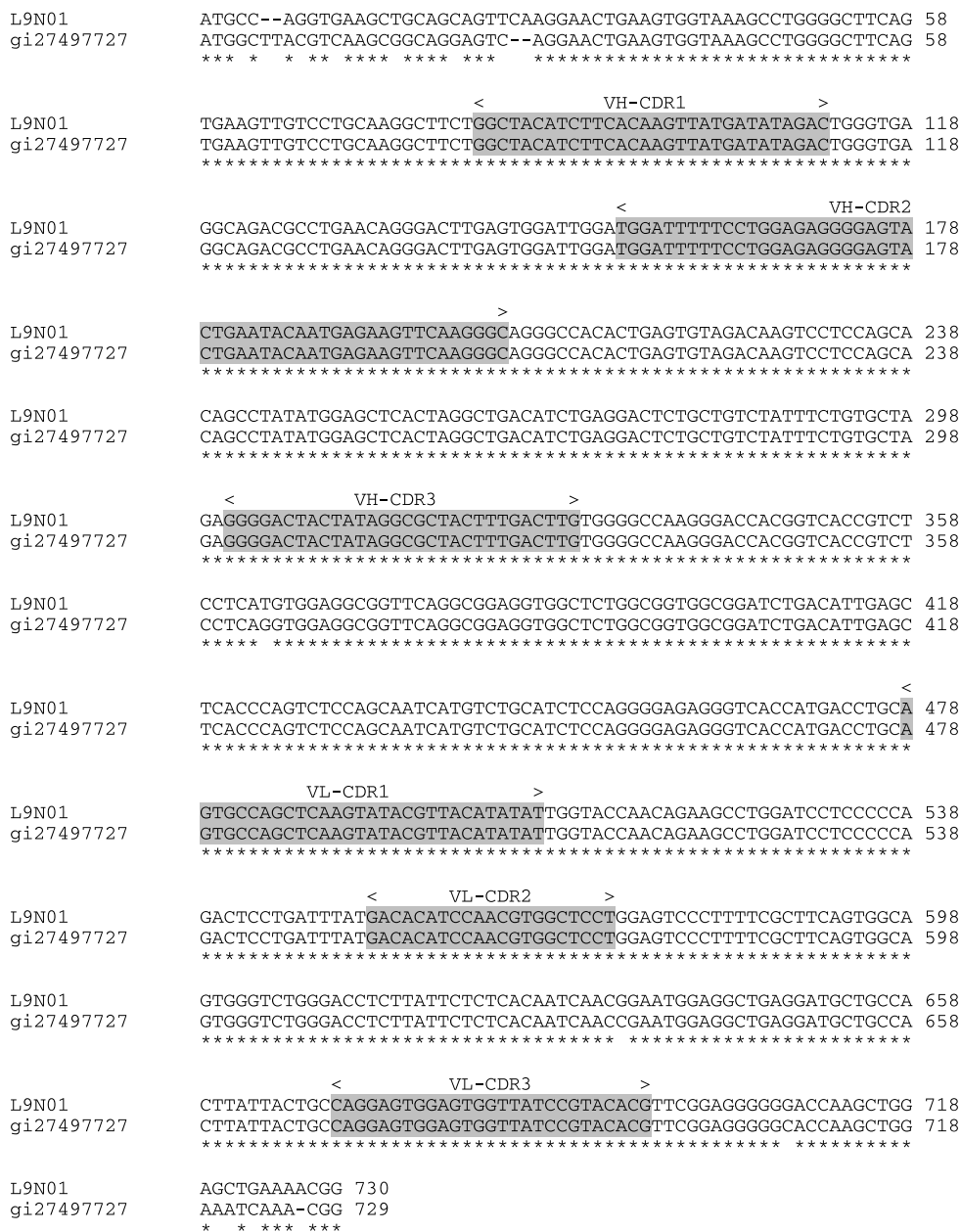


Fig. 3. Alignment of nucleotide sequences of anti-N scFv L9N01 with anti-IL-11 scFv (Accession No. AY171038, Version GI:27497727). The CDRs of heavy and light chains are highlighted with grey shade, and identical nucleotides are denoted with an asterisk.

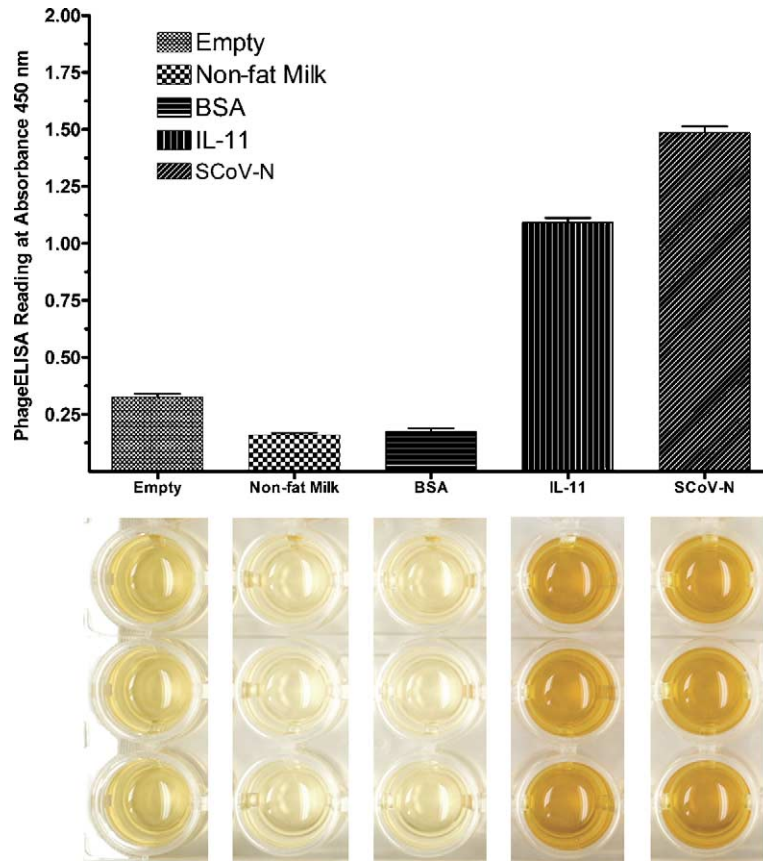


Fig. 4. Phage-ELISA of cross-reactivity of anti-N scFv L9N01 with IL-11. 96-well plates were coated overnight with 1 µg/well each of recombinant SCoV-N (▨), IL-11 (▩), BSA (▧), non-fat milk (▦) and buffer-only controls (▫). Freshly prepared scFv phage clone L9N01 was added and incubated for 1 h, and bound phage was detected with a peroxidase-conjugated anti-M13 as described in Materials and methods. Data shown are representative of three separate experiments with similar results.

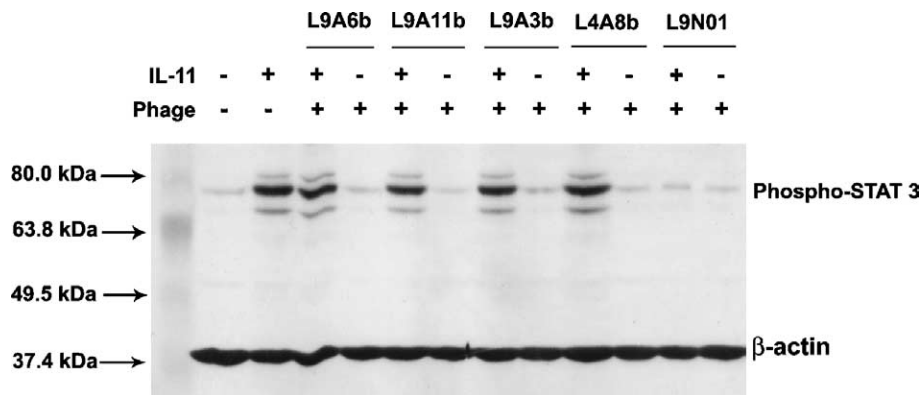


Fig. 5. Inhibition of IL11-stimulated STAT 3 phosphorylation. Serum-starved IEC-18 were grown in 24-well plates and stimulated with IL-11 (5 nM) in the presence and absence of various anti-N scFv phage clones ( $9 \times 10^{10}$  cfu) at 37 °C for 60 min in a final volume of 100 µl. Proteins of total cell lysate were separated in a 12.5% SDS-PAGE gel. After electro-transfer onto a nitrocellulose paper, the protein blot was then probed with a 1000× fold diluted mouse monoclonal anti-phospho STAT 3 (Tyr 705) antibody together with a 10,000× diluted mouse monoclonal anti-β-actin antibody. Data shown are representative of three to five separate experiments with similar results.

suggested. Patients with severe acute respiratory syndrome were characterized with a fast and strong anti-N antibody response [6,32]. The pathophysiological significance and clinical relevance of cross-reacting anti-N antibody with IL-11 are unknown. IL-11 is widely expressed in different

tissues including lung, thymus, bone, and central nervous system. Physiologically, IL-11 regulates hematopoiesis and bone metabolism, and inhibits the production of pro-inflammatory cytokines [16,17]. In this regard, it is of interest to note that thrombocytopenia and lymphopenia were



commonly observed in patients during the early phase of SRAS-CoV infection [33–35]. Furthermore, osteonecrosis has been reported in some convalescent SARS patients [36,37]. Significant levels of anti-N antibody were generally detected 1–2 weeks after the onset of symptoms [7,32], and therefore the inhibitory effect on IL-11 of those cross-reactive anti-N antibody on early SARS symptoms development might play a very limited role. On the other hand, the cross-reactivity of anti-N antibody with IL-11 suggests that the SCoV-N protein shares a similar structural motif with IL-11. The possibility cannot be excluded that the common structural motifs of N protein and IL-11 are important for mediating various biological activities of IL-11, and therefore the presence of N proteins in the early infection cycle may act as a functional antagonist of IL-11. However, SCoV-N protein itself did not stimulate STAT 3 phosphorylation, and the presence of SCoV-N protein also did not modulate IL-11-induced STAT 3 phosphorylation in IEC-18 cells (data not shown). Therefore, it is unlikely that SCoV-N protein and IL-11 share a common structural motif that binds and activates the STAT 3-coupled IL-11 receptor.

The administration of steroid in SARS patient has been suggested as the cause for osteonecrosis in convalescent SARS patients, however, the onset of osteonecrosis (91–143 days) is shorter than that of patients receiving chronic steroid therapy [37]. Furthermore, the cumulative prednisolone dosage of convalescent SARS children patients who showed radiologic evidence of osteonecrosis was not significantly different from those not showing any signs of osteonecrosis [36]. As shown in the present study that cross-reactive anti-N neutralized IL-11, the long-term significance of cross-reactive anti-N antibody on IL-11-mediated bone metabolism has to be evaluated.

A marked increase in proinflammatory cytokine IL-1 $\beta$  has been observed in children suffering from SARS [38]. Recently, Mizutani et al. [39] have demonstrated that phosphorylation of STAT 3 is suppressed in SARS-CoV-infected Vero E6 cells. In the present study, we document the anti-N antibody might cross-react with IL-11. These results suggest a possibility that SARS-CoV infection results in modulating cytokine responses. Hence, a detailed analysis of SARS patients' sera on anti-cytokine activities, in particular of those samples that derived from severely ill or deceased patients, might help us to have a better understanding of the pathogenesis of the SARS syndrome.

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