NF-kappaB pathway as a potential target for treatment of critical stage COVID-19 patients

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Abstract

Patients infected with SARS-CoV-2 show a wide spectrum of clinical manifestations ranging from mild febrile illness and cough up to acute respiratory distress syndrome, multiple organ failure and death. Data from patients with severe clinical manifestations compared to patients with mild symptoms indicate that highly dysregulated exuberant inflammatory responses correlate with severity of disease and lethality. Epithelial-immune cell interactions and elevated cytokine and chemokine levels, i.e. cytokine storm, seem to play a central role in severity and lethality in COVID-19. The present perspective places a central cellular pro-inflammatory signal pathway, NF-kappaB, in the context of recently published data for COVID-19 and provides a hypothesis for a therapeutic approach aiming at the simultaneous inhibition of whole cascades of pro-inflammatory cytokines and chemokines. The simultaneous inhibition of multiple cytokines/chemokines is expected to have much higher therapeutic potential as compared to single target approaches to prevent cascade (i.e. triggering, synergistic, and redundant) effects of multiple induced cytokines and chemokines in critical stage COVID-19 patients.

Introduction

Coronaviruses - enveloped positive-sense, single-stranded RNA viruses - are broadly distributed in humans and animals. While most human coronavirus (hCoV) infections show mild symptoms, there are highly pathogenic hCoV, including the severe acute respiratory syndrome virus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV), with 10% and 37% mortality, respectively. The novel coronavirus SARS-CoV-2 with more than 30 mio infected persons and more than 940.000 deaths worldwide (https://coronavirus.jhu.edu/) September 18, 2020 has become a global pandemic with enormous medical and socio-economic burden. Patients infected with SARS-CoV-2 show a wide spectrum of clinical manifestations ranging from mild febrile illness and cough up to acute respiratory distress syndrome (ARDS), multiple organ failure, and death, i.e. a clinical picture in severe cases that is very similar to that seen in SARS-CoV and MERS-CoV infected patients. While younger individuals show predominantly mild-to-moderate clinical symptoms, elderly individuals frequently exhibit severe clinical manifestations [1-4]. Post-mortem analysis showed Diffuse Alveolar Disease with capillary congestion, cell necrosis, interstitial oedema, platelet-fibrin thrombi, and infiltrates of macrophages and lymphocytes [5]. Recently, the induction of endotheliitis in various organs (including lungs but also in heart and kidney and intestine) by SARS-CoV-2 infection as a direct consequence of viral involvement and of the host inflammatory response was shown [6-7].

SARS-CoV-2 binds with its spike (S) protein to the angiotensin-converting enzyme-related carboxypeptidase-2 (ACE-2) receptor on the host cell using the cellular serine protease TMPRSS2 for S protein priming [8]. The ACE-2 receptor is widely expressed in pulmonary and cardiovascular tissues, hematopoietic cells, including monocytes and macrophages which may explain the broad range of

pulmonary and extra-pulmonary effects of SARS-CoV-2 infection including cardiac, gastrointestinal organs, and kidney affection [6].

Cytokine & Chemokine Storm As A Hallmark Of Covid-19

The morbidity and mortality of highly pathogenic hCoV is still incompletely understood. Virus-induced cytopathic effects and viral evasion of the host immune response play a role in disease severity. However, clinical data from patients, in particular those with severe clinical manifestations indicate that highly dysregulated exuberant inflammatory and immune responses correlate with severity of disease and lethality [1,5-7,9-11]. Significantly elevated cytokine and chemokine levels, *i.e.* cytokine storm, seem to play a central role in severity and lethality in SARS-CoV-2 infections, with elevated plasma levels of IL-1b, IL-7, IL-8, IL-9, IL-10, G-CSF, GM-CSF, IFNg, IP-10, MCP-1, MIP-1a, MIP-1b, PDGF, TNFa, and VEGF in both, ICU (Intensive care unit) patients and non-ICU patient. Significantly higher plasma levels of IL-2, IL-7, IL-10, G-SCF, IP-10, MCP-1, MIP-1a, and TNFa were found in patients with severe pneumonia developing ARDS and requiring ICU admission and oxygen therapy compared to non-ICU patients showing pneumonia without ADRS [1].

Recently, immune profiling of COVID-19 patients revealed distinct immunotypes with therapeutic implications, *i.e.* immunotype 1 characterized by a robust CD4 T cell activation, proliferating effector CD8 T cells was connected to severe disease, immunotype 2 with more traditional effector CD8 T cell subsets, less CD4 T cell activation and memory B cells, showed intermediate clinical outcome, and immunotype 3 with only minimal lymphocyte activation response showed the least clinical symptomatic picture [12]. In the same line, asymptomatic SARS-CoV-2 infected individuals exhibited lower levels of a panel of 18 cytokines / chemokines [13].

Detailed insight into the underlying cellular interactions was recently published in Nature Biotechnology demonstrating by single-cell RNA sequencing analysis that COVID-19 severity correlates with the cellular airway epithelium-immune interaction. Critical COVID-19 cases - compared to moderate cases - exhibited stronger interaction between epithelial and immune cells, indicated by ligand-receptor expression profiles. Beside expression of pro-inflammatory cytokines, such as IL-1b and TNF-a the expression of chemokines CCL2, CCL3, CCL20, CXCL1, CXCL3, CXCL10, IL-8 was shown likely to contribute to clinical observation of excessive inflammatory tissue damage, lung injury and respiratory failure [14].

Interestingly, also for SARS-CoV and MERS-CoV infected patients, increased levels of pro-inflammatory cytokines in serum, including IL-1b, IL-6, IL-12, IFNg, TNFa, IL-15, IL-17 and chemokines including CCL2 (MCP-1), CXCL10 (IP-10), CXCL9 (MIG), CCL-5, IL-8 were associated with pulmonary inflammation and extensive lung damage [15-17]. Both, the nucleocapsid protein and the spike protein of SARS-CoV were shown to induce pro-inflammatory cytokines via activation of the NF-kB pathway [18,19]. Using comprehensive genomic analyses Smits et al showed that aged macaques have a stronger host response to virus infection compared to young macaques, with an increase in differential expression of genes associated with inflammation, with NF-kB as central player, whereas expression of type I interferon was

reduced indicating a possible negative-feedback cross-talk between the pro-inflammatory NF-kB pathway and IFN-induced antiviral pathways [20].

Furthermore, beside the three highly pathogenic hCoV, also H5N1 and certain H1N1 influenza virus infections with high lethality in humans, showed excessive alveolar immune inflammatory infiltrates and high levels of pro-inflammatory cytokines and chemokines including IP10/CXCL10, MIG, IL-6, IL-8 and RANTES in human cell lines, mice, and macaques [21-26] and in humans infected with H1N1[27]. Taken together, these multiple reports indicate a potential common pathophysiological mechanism of highly dysregulated exuberant inflammatory reactions in response to various acute respiratory RNA virus infections.

Recent transcriptome analysis from post-mortem lung tissue of COVID-19 patients and cell culture models infected with COVID-19, Respiratory Syncytial virus and influenza virus identified commonly regulated gene-expression modules of key inflammatory processes for all three viral infections. Key examples were TNF, NF-kB, IL-1 and ALOX5 signaling pathways [28]. Several recent reports demonstrating NF-kB pathway as *the* central signaling pathway for the SARS-CoV-2 infection-induced pro-inflammatory cytokine/chemokine response [29,30] and show that COVID-19 upregulate toll-like receptor (TLR)-mediated inflammatory signaling mimicking bacterial sepsis [31].

Inhibition Of Nf-kb Can Inhibit Virus-induced Cytokine Storm

We have previously shown that elevated cytokine release of IL-a/b, IL-6, MIP-1b, RANTES and TNF-a induced by highly pathogenic avian H5N1 influenza A virus was significantly reduced by application of the proteasome inhibitor VL-01 *in vivo* [32]. The underlying mechanism of this inhibitory effect of proteasome inhibitors is supposed to be mediated largely by the inhibition of one of the most prominent cellular transcription pathways, NF-kB. The inhibition of the nuclear translocation of the transcription factor NF-kB by proteasome inhibitors has been described [33-35]. It is mediated via the inhibition of the proteasomal degradation of the cytosolic inhibitor IkBa, this way keeping NF-kB sequestered by IkBa in the cytosol and thereby inhibiting the otherwise induced translocation of NF-kB to the nucleus where it would initiate the transcription of multiple pro-inflammatory proteins, such as cytokines, chemokines, adhesion molecules and growth factors (**see Figure 1**). Activation of the NF-kB pathway has been described for very different signal-receptor bindings, including binding of LPS to TLR4, binding of cytokines like IL-1 and TNFa to their respective receptors, or recognition of RNA viruses by Toll-like receptors, TLR7/8. Importantly, all these different signaling pathways join into a ***common*** downstream signaling sequence of phosphorylation of the cytosolic inhibitor IkBa which triggers its ubiquitination and proteasomal degradation resulting in release and translocation of NF-kB into the nucleus [35] (**see Figure 1**). These data suggest that interfering at these late stages (*i.e.* phosphorylation, ubiquitination, and/or proteasomal degradation of IkBa) of the pathway will inhibit NF-kB activation, irrespectively of the initial triggering signal. We could demonstrate the inhibitory effect of proteasome inhibitors on nuclear translocation NF-kB in various cell types such as human macrophages after stimulation with TNFa *in*

vitro. Without stimulation of the NF-kB pathway, p65/p50 (p65 FITC stained) is sequestered in the cytosol by its inhibitor IkB. Following stimulation by TNFa, NF-kB translocates to the nucleus (shown by coinciding p65 staining and nucleus staining by DAPI). NF-kB nuclear translocation after TNFa stimulation was inhibited by application of the proteasome inhibitor VL-01 showing p65 staining in the cytosol and only few cells with p65 positive nucleus (**Figure 2**).

The influence of VL-01 on the pro-inflammatory cytokine and chemokine response *in vivo* was demonstrated in a H5N1 influenza virus mouse model. A strong cytokine and chemokine response was induced in Balb/c mice intranasally infected with avian H5N1 virus A/mallard/Bavaria/1/2006 ($7x10^2$ pfu, i.e. 10-fold MLD_{50}). Mice were treated i.v. either with 25 mg/kg VL-01 or solvent (mock) two hours prior to virus infection. Serum samples for cytokine analysis were collected at different time points after infection. While some cytokines/chemokines such as TNFa, MIP-1b, and RANTES peaked very early after H5N1 infection (12 hrs), others, *i.e.* KC (neutrophil-activating protein-3) and IL-6, peaked later at 72 hrs after infection (**Fig. 3**). Treatment with proteasome inhibitor significantly inhibited the release of IL-1, IL-6, TNFa, MIP-1 and CXCL1 at the peak time-points in Balb/c mice after infection with the highly pathogenic avian H5N1 influenza A virus (**Fig. 3**). Importantly, proteasome inhibition significantly decreased the release for *all*, *early and late* cytokines and chemokines, and resulted in significantly increased survival of mice after infection with the highly pathogenic avian H5N1 influenza A virus [32].

In order to investigate whether the inhibition of cytokine and chemokine release by inhibition of the nuclear translocation of NF-kB is a general mechanism, an acute lung injury (ALI) mouse model with LPS challenge was used. This model provides a rapid and strong systemic induction of pro-inflammatory cytokines and chemokines. Balb/c mice were treated i.v. with 25 mg/kg VL-01, followed by i.p. application of 20 µg LPS. Serum samples for cytokine analysis were collected before (-4hrs) LPS treatment (control) and after LPS treatment (1.5 and 3 hrs). Again distinct release patterns were found for different cytokines/chemokines, with TNFa, IL-1b, MIP-1a and MIP-1b peaking already 1.5 hrs after LPS challenge, followed by others, such as IL-6, RANTES, IL-12p40 and KC peaking 3 hrs after LPS stimulus (**Fig. 4**). Importantly, treatment of mice with proteasome inhibitor significantly reduced release of the whole panel of pro-inflammatory cytokines and chemokines**.** Taken together, these data generated in different models demonstrate the principal potency of proteasome inhibitors to interfere with the pro-inflammatory effects, by inhibiting the translocation of NF-kB to the nucleus.

As a second line of evidence for the potential role of the NF-kB pathway in acute respiratory viral infection DeDiego et al. have demonstrated, that the inhibition of NF-kB-mediated inflammation in SARS-CoV infected mice significantly decreased the expression of pro-inflammatory cytokines including TNFa, IL-6 and chemokines including CCL-2, CCL-5, CXCL-1, CXCL-2, CXCL-10, correlating with increased survival. In their study four different NF-kB inhibitors, with different mechanism of inhibition, i.e. CAPE, resveratrol, Bay11-7082, and parthenolide, were used. All four inhibitors were shown to inhibit NF-kB activity, and to decrease the expression levels of pro-inflammatory cytokines and chemokines, without affecting viral titers or cell viability [36].

Moreover, Acetylsalicylic acid (ASA) and other salicylates – in contrast to pure (COX) cyclooxygenase inhibitors, such as indomethacin – are well-known inhibitors of NF-κB activation by acting as specific inhibitors of IKK2 – a kinase essential for phosphorylating IkB [37]. Furthermore, D,L-lysine-acetylsalicylate•glycine (LASAG) a water-soluble salt of ASA (licensed as Aspirin i.v.®) was shown to decrease activation of promoter constructs of NF-κB-dependent genes for IL-6 and IL-8 and to improve the time to alleviation of influenza symptoms in hospitalized patients in a phase II clinical trial [38]. The well-known analgesic, antipyretic, anti-thrombotic, anti-inflammatory, and antiviral effects of ASA have led to initiation at least 8 clinical studies investigating the effects of ASA in COVID-19 according to clinicaltrials.gov [39].

The concept of a central role of NF-kB pathway in critical stage SARS-CoC-2 infected patients is supported by two recently published studies showing pronounced clinical effect in critical COVID-19 patients by Bruton tyrosine kinase (BTK) inhibitors, correlating with significantly decrease in inflammatory parameters (C-reactive protein and IL-6), normalized lymphopenia, and improved oxygenation [40,41]. Bruton tyrosine kinase is known to be involved in TLR7/8-induced TNFa transcription via NF-κB recruitment at the stage of phosphorylation of p65 [42].

Finally, support for the role of NF-kB pathway in critical stage COVID-19 patients is provided by recent results from the RECOVERY trial. Dexamethasone was found to significantly reduce death in patients with severe respiratory complications of COVID-19 requiring ventilation by up to one third [43]. Dexamethasone – a broadly used glucocorticoid anti-inflammatory drug – is assumed to mediate its anti-inflammatory activity at least partially via downregulation of the NF-kB activity [44], probably by suppression of NF-kB expression [45] and/or increased expression of IkB in the cytoplasm [46].

All these data collectively strongly indicate that inhibition of the NF-kB signal pathway may be a promising target to control SARS-CoV-2 induced excessive immune activation associated with systemic cytokine and chemokine release, capillary leakage and multi-organ tissue damage (**Figure 1**).

Discussion

Reaching beyond the possibilities of currently evaluated drugs for single targets of the cytokine cascade, e.g. monoclonal antibodies against the IL-6 receptor [47-50] or IL-1 receptor antagonist [51] the inhibition of NF-kB pathway - preferably in parallel at several sensitive points (**Figure 1**) - could provide the unique potential to inhibit the release of multiple cytokines simultaneously, in particular strongly pro-inflammatory cytokines including IL-1, IL-6, TNFa and chemokines including MIP-1 and CXCL1.

Multiple approved medications with implicated NF-kB activity involving NSAIDs (*e.g.* acetylsalicylic acid, Aspirin), BTK inhibitors (*e.g.* Ibrutinib, Acalabrutinib), steroids (*e.g.* Dexamethasone), and proteasome inhinbitors are in wide-spread clinical use. Several registered proteasome inhibitors (Bortezomib, Carfilzomib or Ixazomib) are available for treatment of oncological indications [52]. In contrast to oncological indications where eight (or more) treatment cycles are routinely applied, it seems plausible

that just few applications of proteasome inhibitors will be sufficient to downregulate the acute cytokine storm in COVID-19 patients.

Importantly, compared to single target approaches, a simultaneous inhibition of multiple cytokines/chemokines using (preferably a combination of) inhibitors of the NF-kB pathway, may be highly advantageous compared to single target approaches to compensate for redundant, synergistic, and triggering effects of multiple cytokines (i.e. cytokine cascade) released in critical cases of highly pathogenic hCoV infection (but also H5N1 or H1N1 infection). Whereas some clinical efficacy in COVID-19 patients has been recorded [47-51] also several notable caveats and limitations to the efficacy of single-cytokine targeting approaches have been seen and have led to the question which cytokine to target in a raging storm, calling for a systemic approach for simultaneous inhibition of multiple cytokines, including also early expressed cytokines and chemokines [53].

In contrast to another recently suggested systemic approach for simultaneous inhibition of cytokines by JAK inhibitors [54], NF-kB inhibition will inhibit predominantly highly pro-inflammatory cytokines and chemokines, such as TNFa, IL-1, IL-6, MCP-1, MIP-1, which are expected to be primarily involved in exuberant systemic inflammatory responses (as proven at the cellular level for COVID-19 patients by the study of Chua et al. [14]) rather than cytokines primarily involved in antiviral responsiveness, such as IFNg - which is primarily dependent on other pathways, i.e. JAK/STAT [20].

Although there are still many open questions regarding e.g. which compound class – or which combination of - would be most effective, as well as the optimal timing to start treatment [53], the potential to control the cytokine storm-induced severe lung failure and systemic organ failure by using already registered inhibitors of the centrally involved NF-kB pathway may be a real chance to get additional treatment options, hopefully decreasing the number of cases in need for artificial ventilation, multi organ failure, and death.

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Declarations

The experimental work was performed at ViroLogik GmbH, Innovation Centre for Medical Technology and Pharmaceuticals, Henkestr. 91, 91052 Erlangen, Germany and at the Institute of cell Biology and Immunology, Eberhard Karls University Tuebingen, Germany.

Declarations of interest: none

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Figures

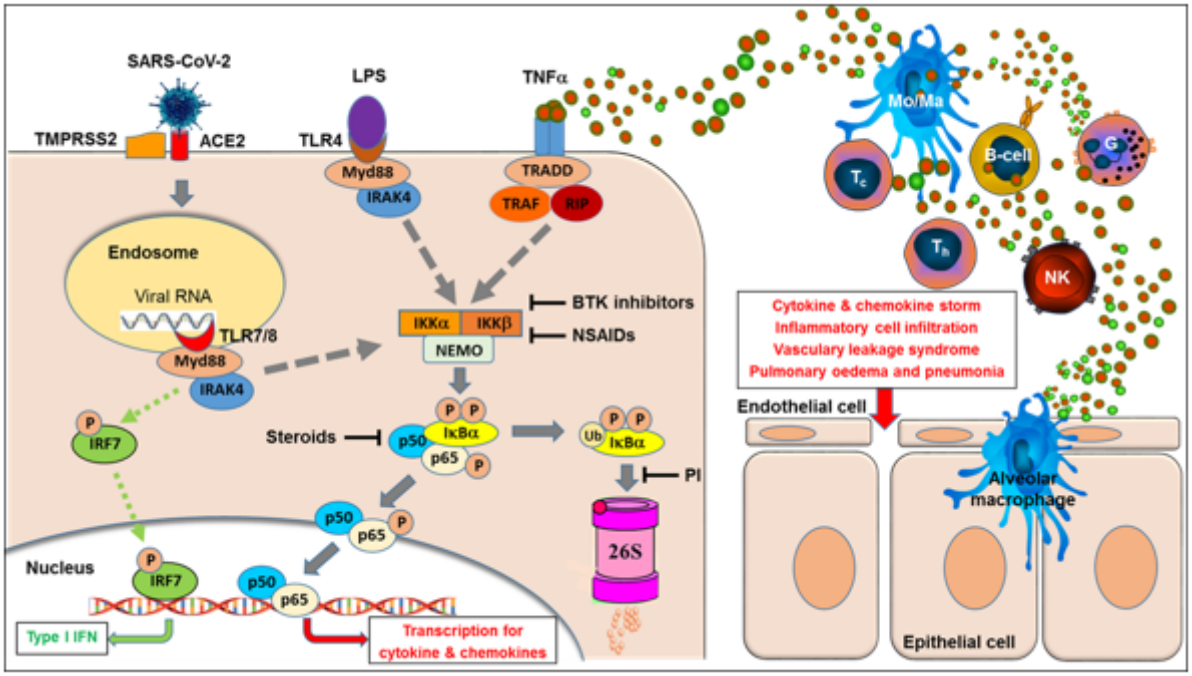

Figure 1

Activation pathway of NF-κB and linkage to acute respiratory RNA virus induced cytokine storm Binding of SARS-CoV-2 to its receptor, i.e. the angiotensin-converting enzyme 2 (ACE2) and the help of the cellular serine protease TMPRSS2 trigger endocytosis into the host cell. Within the endosomes, RNA from single-

stranded RNA virus is generally known to activate the Toll-like receptors TLR7 and TLR8. This can lead to activation of transcription of the interferon-regulator factor (IRF) family and antiviral responses (green dotted lines). However, as a second major effect the activation of the TLR7/8 can trigger - via various intermediates – the activation of IKK (I□B kinases) (grey dotted lines) resulting in phosphorylation of the cytoplasmic inhibitor factor I□B□□triggering ubiquitination followed by its degradation by the 26S proteasome, thereby NF-□B (a heterodimer complex consisting of protein subunits p50 and p65) is released from I□B□□and can now enter the nucleus and initiate transcription of various genes coding for pro-inflammatory proteins such as cytokines, chemokines, adhesion molecules, and growth factors. Importantly, this final sequence of NF-□B activation is shared with a multiple range of cytokine receptor- and Toll-like receptor mediated signal cascades, including binding of TNF□ or IL-1 to their receptors or binding of LPS (e.g. from secondary bacterial infections) to the TLR4. In contrast, interferon-response factor (IRF)-related responses are largely independent on NF-□B translocation. Excessive cytokine release triggered by NF-□B activation leads to inflammatory cell activation and infiltration, vascular leakage syndrome, finally leading to pulmonary oedema and pneumonia. This figure presents a hypothesis combining various information from literature 5-11,28-32,35-51, 55-57 and the present work. BTK inhibitors – Bruton Tyrosine Kinase inhibitors, PI – Proteasome inhibitors; NSAIDs – Nonsteroidal anti-inflammatory drugs

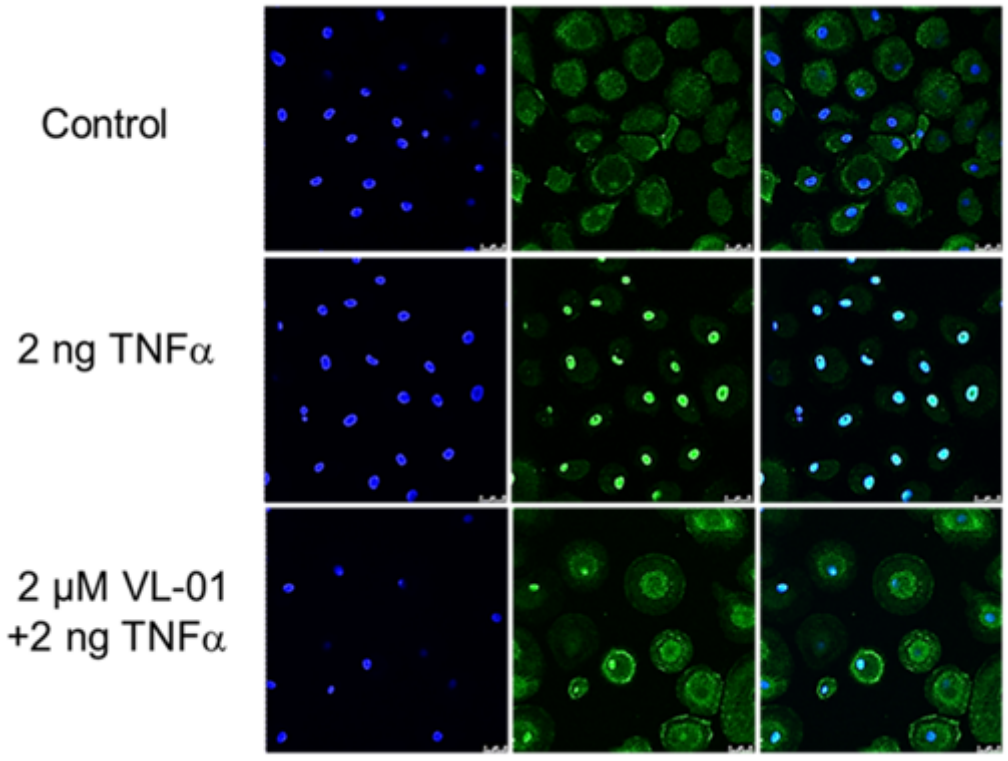

Figure 2

Inhibition of TNF□ induced nuclear translocation of NF-□B by the proteasome inhibitor VL-01 Human monocyte-derived macrophages were were seeded on cover-slides, incubated overnight, and incubated with increasing concentrations of proteasome inhibitor and stimulated with TNF□ (2 ng/ml) for 30 min.

Immunofluorescence staining of NF-□B was done using a p65 specific antibody, and cell nuclei were counterstained with DAPI and evaluated by confocal microscope (Leica, LSM3).

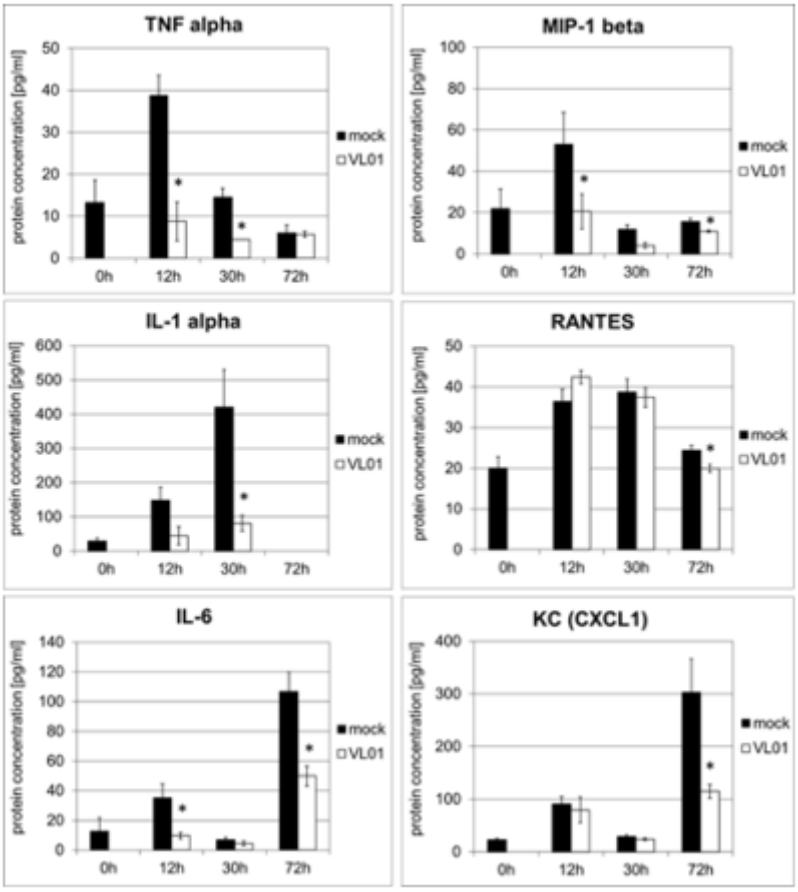

Figure 3

Inhibition of cytokine release in BALB/c mice infected with H5N1 by treatment with the proteasome inhibitor VL-01 Balb/c mice (n=4) were intranasal infected with avian H5N1 virus A/mallard/Bavaria/1/2006 (7x102 pfu, i.e. 10-fold MLD50). Mice were i.v. treated with 25 mg/kg VL-01 or solvent (mock) two hours prior to virus infection. Cytokine levels in blood were determined before (0h) and 12h, 30h or 72 h after infection using the Bio-Plex Pro Mouse Cytokine 6-Plex Panel (Biorad). * $p<0.05$.

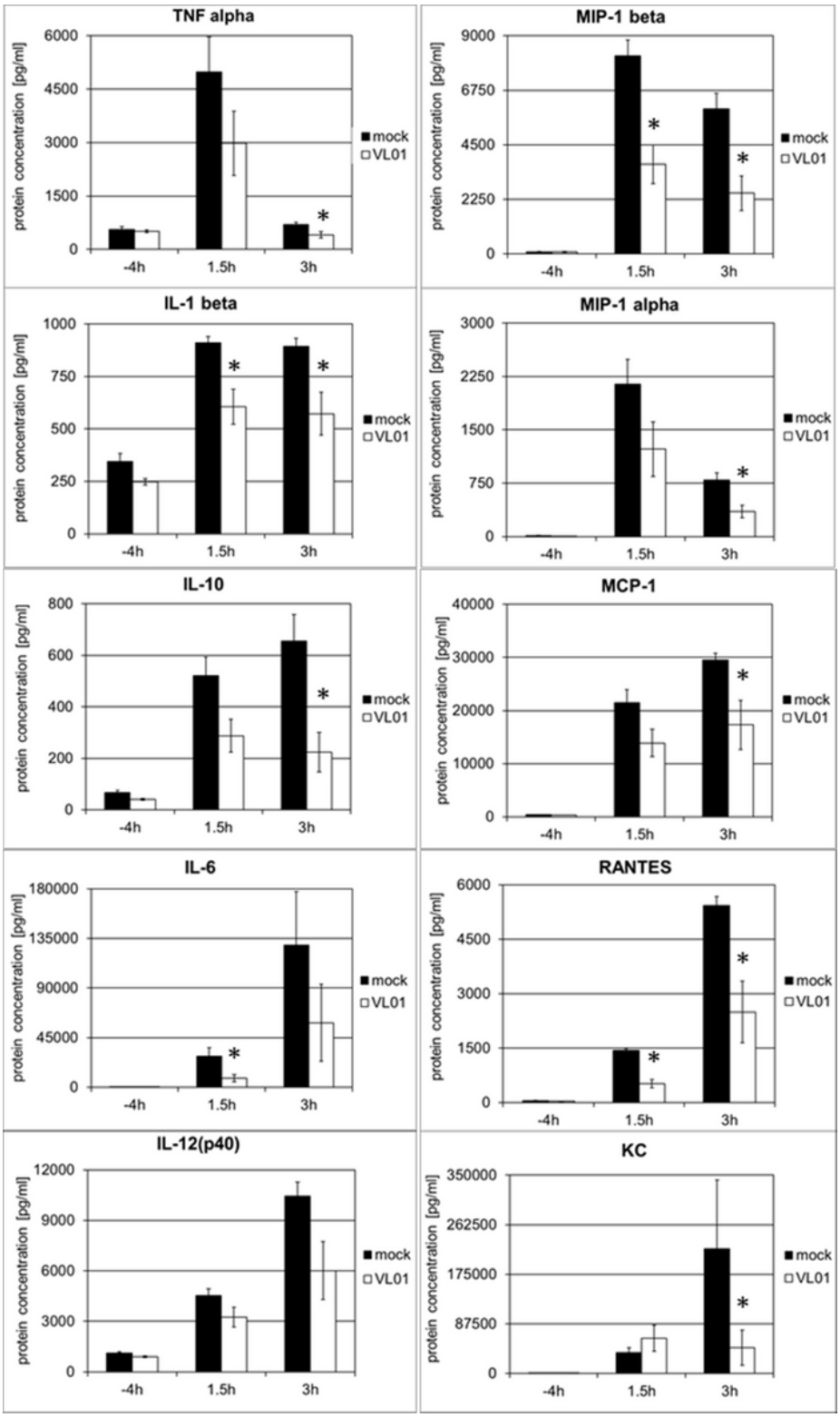

Figure 4

Inhibition of LPS-induced cytokine release in BALB/c mice by the proteasome inhibitor VL-01 Balb/c mice (n=7) were injected i.v. with VL-01 (25 mg/kg, in 200µl i.v.) two hours prior LPS stimulation (20 µg/mouse. 200 ml i.p.). Cytokine levels were determined for the time points before (-4h), and 1.5h or 3h after LPS injection, using the Bio-Plex Pro Mouse Cytokine 23-Plex Panel (Biorad). * $p<0.05$