Autoantibodies against Progranulin and IL-1 receptor antagonist in critically ill COVID-19

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STRUCTURED ABSTRACT

INTRODUCTION

Hyperinflammation is frequently observed in patients with severe COVID-19. Inadequate and defective IFN type I responses against SARS-CoV-2, caused by autoantibodies in a proportion of patients, lead to severe courses. In addition, hyperactive responses of the humoral immune system have been described so far.

RATIONALE

In the current study we investigated a possible role of neutralizing autoantibodies against anti-inflammatory mediators. Plasma from patients with severe and critical COVID-19 was screened by ELISA for antibodies against PGRN, IL-10, IL-18BP, IL-22BP and IL-1-RA. Autoantibodies were characterized and the antigens were analyzed for immunogenic alterations.

RESULTS

PGRN-autoantibodies were detected with high titers in 11 of 30 (36.7%), and IL-1-RA-autoantibodies in 14 of 30 (46.7%) patients of a discovery cohort with severe to critical COVID-19. In a validation cohort of 41 patients with critical COVID-19 high-titered PGRN-Abs were detected in 12 (29.3%) and IL-1-RA-Abs in 19 of 41 patients (46.2%). PGRN-Abs and IL-1-RA-Abs belonged to IgM and several IgG subclasses. In separate cohorts with non-critical COVID-19, PGRN-Abs and IL-1-RA-Abs were detected significantly less frequently and at low titers. Neither PGRN- nor IL-1-RA-Abs were found in 40 healthy controls vaccinated against SARS-CoV-2. PGRN-Abs were not cross-reactive against SARS-CoV-2 structural proteins or against IL-1-RA. Plasma levels of both free PGRN and IL-1-RA were significantly decreased in autoantibody-positive patients compared to Ab-negative and non-COVID controls. Functionally, PGRN-Abs from patients reduced PGRN-dependent inhibition of TNF-α signaling in vitro. The pSer81 hyperphosphorylated PGRN isoform was exclusively detected in patients with high-titer PGRN-Abs; likewise, a yet unidentified hyperphosphorylated IL-1-RA isoform was only found in patients with high-titer IL-1-RA-Abs. No autoantibodies against IL-10, IL-18BP or IL-22BP were found.

CONCLUSION

To conclude, neutralizing autoantibodies to IL-1-RA and PGRN occur in a significant proportion of patients with critical COVID-19, with a concomitant decrease in circulating PGRN and IL-1-RA, which is indicative of a misdirected, proinflammatory autoimmune response. The break of self-tolerance is likely caused by atypical isoforms of both antigens due to hyperphosphorylation. It remains to be determined whether these secondary modifications are induced by the SARS-CoV-2-infection itself, or are preexisting and predispose for a critical course.

INTRODUCTION

The disease COVID-19 caused by SARS-CoV-2 shows a very wide spectrum of manifestations and severity ranging from completely asymptomatic infection, to mild cold symptoms or attenuation of the sense of taste and smell, to acute respiratory distress syndrome (ARDS) often associated with thromboembolic complications (*1*)·(*2*)·(*3*)·(*4*). Patients requiring intensive care treatment often present with a hyperinflammatory state, which has been compared with hemophagocytic lymphohistiocytosis (HLH)(*5*). Correspondingly, anti-inflammatory or immunosuppressive drugs like dexamethasone or inhibitors of the IL-1ß-, IL-6-, JAK/STAT-, or the BCR-pathways have been studied in clinical trials (*6*)·(*7*)·(*8*)·(*9*)·(*10*)·(*11*)·(*12*). Among these drugs, though, only dexamethasone and IL-6 pathway blockade were so far found to provide a significant clinical benefit, in terms of a reduced 28-day mortality in patients with severe COVID-19 (*6*).

Defective IFN I (*13*)·(*14*)·(*15*) and overactive B-cell responses and autoantibodies against interferons were detected specifically in patients with severe courses of COVID-19 (*16*), but the role of pathogenetically or "self-attacking" antibodies has so far not yet been clearly established (*17*). Previously, we had identified neutralizing autoantibodies against progranulin (PGRN) in sera from patients with primary small vessel vasculitis (*18*). Subsequently we found progranulin-Abs in various rheumatic and other autoimmune diseases, but only very rarely in healthy controls, elderly or obese subjects, ICU patients and patients with melanoma (*19*)·(*20*)·(*21*).

PGRN, also called proepithelin, is a secreted precursor protein. Beside several other biological functions(*22*), a major property of PGRN is its anti-inflammatory effect (*23*)·(*24*), which is mediated by direct binding to TNFR1, TNFR2 and DR3 and thus antagonism of TNF-α and TL1-a (*25*)·(*26*). This has been demonstrated in vivo in several mouse models including collagen and collagen-antibody induced arthritis (*25*), OXA induced dermatitis (*27*) and more relevant for COVID-19 also in LPS-induced lung injury/ARDS mouse models (*28*)·(*29*)·(*30*). Both PGRN and TNF-α bind to cysteine-rich domain 2 and 3 (CRD2 and 3) of TNFR (*31*). The proinflammatory effect of neutralizing PGRN-antibodies was characterized in vitro by the analysis of TNF-α-induced cytotoxic effects by MTT-assays and by downmodulation of FoxP3 in $CD4^{+}CD25^{hi}$ Tregs in inflammatory bowel diseases and rheumatic diseases (*20*)·(*32*). Due to hyperinflammatory states and similarities of severe COVID-19 with vasculitis and autoimmune diseases(*4*), aim of the current study was to investigate the possible occurrence of antibodies directed against previously described anti-inflammatory antigens as progranulin or IL-10(*33*), but also against other secreted anti-

inflammatory mediators like IL-1-RA.

RESULTS

Occurrence, titers and IgG subclasses of anti-progranulin and anti-IL-1-RA antibodies

The anti-PGRN ELISA demonstrated the presence of PGRN-autoantibodies in plasma of 11 of 30 patients (11/30; 36.7%) with COVID-19 of the discovery cohort (21 critical, 9 severe disease) (Fig. 1 A). In controls of non-COVID-19 ICU patients only 1 of 28 patients (3.6%) had weakly detectable PGRN-antibodies (Fig. 1 B). In autoantibody-positive patients with severe or critical COVID-19, the titers ranged from 1:1600 up to 1:3200 (Fig. 1 C). PGRN-antibodies belonging to the IgM class were found in 10 of 11 patients with COVID-19, and in all of the 11 PGRN-Ab-positive patients IgG class autoantibodies were found, with IgG1 detectable in 8, IgG2 in 9, IgG3 in 6 and IgG4 in 8 of these (Fig. 1 D).

In a validation cohort of patients with critical COVID-19 requiring mechanical ventilation, 12 of 41 (29.3%) patients had PGRN-Abs (Supplementary Fig. 1 A). Very similar to the discovery cohort, the PGRN-Abs often belonged both to the IgM class and simultaneously to different IgG subclasses and had similar titers (Supplement Fig. 1 B and 1C). Regarding 89 patients with predominantly moderate COVID-19 from the level 1 and 2 centers in the federal German state of Saarland, PGRN-Abs were only detected in 12 (13.5%) with titers of ≤ 1:400 (Supplementary Fig. 1 E and F). In patients with predominantly mild courses of COVID-19 PGRN-antibodies were detected with low titers in two of 56 (3.5%) convalescent plasma donors with an asymptomatic to mild course of the CAPSID-trial (supplementary Figure 1 I)) and in 2 of 49 patients (4.1%) with mild courses of COVID-19 obtained from the Institute of Virology from Saarland Medical Center (Fig. 1 E). If all critically ill patients with COVID-19 at time of blood test and ICU treatment are considered together, 21 of 62 patients (33.9%) had PGRN-Abs with titers ≥ 1:800, compared to 4 of 37 (1%) with severe and 0 of 69 moderate and 0 of 93 with asymptomatic or mild COVID-19 (Figure 1 F) (two tailed Fisher exact test: critical and severe compared to moderate and mild: $p < 0.0001$).

PGRN-Abs were not detected in sera or plasma of 40 healthcare-workers after 2 vaccinations against SARS-CoV2 (Supplementary Fig. 1 I).

Next, we analyzed autoantibodies against further anti-inflammatory mediators. IL-1-RA-antibodies were detected in 14 of 30 patients (46.7%) with severe to critical COVID-19 of the discovery cohort (including all 11 patients with PGRN-Abs), but not in ICU patients without COVID-19 (Fig. 1 G). In this discovery cohort with severe to critical COVID-19 titers of IL-1-RA-Abs ranged between 1:800 to 1:1600 (Fig. 1 H) and similar to PGRN-Abs, IL-1-RA-

Abs belonged to IgM and several IgG subclasses, except IgG4 (Fig. 1 I). The epitope region with the highest affinity was located within amino acid 63-123 (Supplementary Fig 2 A), but has to be narrowed down more precisely in the future.

In a validation cohort of patients with critical COVID-19 requiring mechanical ventilation of the second and third wave of COVID-19 in the federal German state of Saarland or included in the CAPSID trial, 19 of 41 (46.3%) patients had IL-1-RA-Abs. Similar to the discovery cohort, in the validation cohort IL-1-RA-Abs often belonged to both the IgM class and simultaneously different IgG subclasses and had similar titers (Supplementary Fig 2 C and D) Regarding plasma of 89 patients with predominantly moderate COVID-19 of level 1 and 2 hospitals of Saarland federal German state, no IL-1-RA-Abs were detected (supplementary Fig 2 F). IL-1-RA-Abs were not detected in 56 convalescent plasma donors with predominantly mild courses of the CAPSID-trial (supplementary Fig. 2 H) and only with a low titer of 1:400 in 2 of 49 patients with mild courses of COVID-19 from the Institute of Virology from Saarland Medical Center (Fig. 1 J and supplementary Fig 2 I). IL-1-RA-Abs were not detected in sera or plasma of 40 healthcare-workers after 2 vaccinations against SARS-CoV2 (Supplementary Fig. 2 J). As spontaneously occurring IL-1-RA-autoantibodies have not been described before, 188 healthy controls were screened, but none of these had IL-1-RA-antibodies (supplementary Fig 2 K) (two tailed Fisher exact test: critical and severe compared to healthy controls: $p < 0.0001$). To summarize, if all patients with critical COVID-19 and ICU treatment are considered together, 30 of 62 patients (48.4%) had IL-1-RA-Abs with titers $1 \geq 800$, compared to 4 of 37 patients with severe and 0 of 69 with moderate and 0 of 93 with asymptomatic or mild COVID-19 with titers $\geq$ 1:800 (Figure 1 K) (two tailed Fisher exact test: critical and severe compated to moderate and mild: $p < 0.0001$).

Autoantibodies against IL-10 were neither detected in patients with severe to critical COVID-19 nor in controls from patients from intensive care unit without COVID-19 (supplementary Fig 3).

Atypical antigen isoforms in PGRN- and IL-1-RA-antibody-positive patients

All 11 PGRN-Ab-positive patients of the discovery cohort with severe to critical COVID-19 had a double band of PGRN in isoelectric focusing (IEF), with an additional more negatively charged isoform of PGRN (11/30; 36.7%). None of the 19 PGRN-Ab-negative patients of this cohort and none of the 28 controls from ICU without COVID-19 had this distinct, more negatively charged PGRN isoform (Fig. 2 A). ELISA for the previously described pSer81 PGRN isoform(*32*) demonstrated that this more negatively charged isoform of PGRN was the

pSer81 isoform in all 11 PGRN-Ab-positive cases and only in these (Fig. 2 B). This pSer81 PGRN isoform was detected for all PGRN-Ab-seropositive patients with titers ≥ 1:800, but not in patients with titers ≤ 1:800 (Supplementary Fig. 1 G-H) Antibodies reactive against enriched pSer81 PGRN isoform belonged only to the IgG class, with IgG1 subclass being predominant (Supplementary Fig. 4 A).

In 89 patients form level 1 and 2 hospitals with mostly moderate COVID-19 (Supplementary Fig. 1 E), 11 patients had PGRN-antibodies. They had low PGRN-Ab-titers of 1≤400 and did not have the pSer81 isoform of PGRN (Supplementary Fig. 1 F-H).

In none of the IL-1-RA-Ab-positive patients with COVID-19 a visible difference in molecular weight of IL-1-RA was observed in conventional Western-blot compared to IL-1-RA-Ab-negative samples. However, in the IEF, an additional, more negatively charged third band of IL-1-RA appeared in samples from IL-1-RA-Ab-positive patients. This isoform of IL-1-RA was not seen in controls without IL-1-RA-Abs (Fig. 2 C). We then examined whether this additional isoform is related to a different phosphorylation state. Pretreatment with alkaline phosphatase before IEF led to the disappearance of both the normally occurring second and the atypical additional third IL-1-RA isoform (Fig. 2 D). This proved that the additional band represented a hyperphosphorylated IL-1-RA isoform, which was exclusively found in IL1-RA-Ab-positive patients. Patients with mostly moderate courses of COVID-19 from level 1 and 2 centers of the state of Saarland and seronegative for IL-1-RA-Abs did not have the third, more negatively charged, hyperphosphorylated isoform of IL-1-RA (Supplementary Fig. 2 G).

Randomly selected patients with either hyperphosphorylated PGRN or hyperphosphorylated IL-1-RA, did not have hyperphosphorylated SLP-2 (Supplementary Fig. 4 B), which is a previously described hyperphosphorylated antigen (at Ser17) of paraproteins in plasma cell dyscrasia and served as control(*34*)·(*35*).

ELISA for cross-reactivity of absorbed PGRN-antibodies against structural proteins of SARS-CoV-2 or Interleukin-1-Receptor Antagonist

Enriched PGRN-antibodies did neither show cross-reactivity in ELISA against recombinant HIS-tagged SARS-CoV-2 S1-, S2-, E- or M-proteins, nor against recombinant human FLAG-tagged IL-1-RA. In addition, antibodies against SARS-CoV-2 S1-, S2-, or M-proteins and against human IL-1-RA could not be adsorbed by immobilized PGRN, but were instead detectable in the eluate of samples from patients with severe or critical COVID-19, excluding cross-reactivity of PGRN-Abs (Supplementary Fig 5).

ELISA for plasma levels of PGRN and IL-1-RA and influence PGRN-antibody-serostatus on TNF-α-induced cytotoxic effect (MTT assay)

Using standard ELISA, we observed that progranulin-levels were significantly decreased by more than 90% in the plasma of PGRN-Ab-positive patients with COVID-19 (median: 15.12 ng/ml; SEM 3.6 ng/ml), compared to plasma of PGRN-Ab-negative patients with COVID-19 (median 161.23 ng/ml, SEM 48.14 ng/ml) (unpaired t-test: $p = 0.0015$) and to the plasma of PGRN-Ab-negative patients from the ICU without COVID-19 as control (median 206.05 ng/ml; SEM 10.59 ng/ml) (unpaired t-test: $p < 0.0001$) (Fig. 3 A). In a functional in vitro assay, the anti-TNF-α effects of plasma from patients with critical and severe COVID-19 with or without PGRN-Abs were examined. For this purpose, WEHI-S cells were incubated with TNF-α and plasma from a selected subset of patients at different dilutions. As shown in Fig. 3B, addition of plasma from patients without PGRN-Abs reduced the TNF-α-induced cytotoxic effect on WEHI-S cells at up to higher dilutions and to a significantly higher degree compared to PGRN-Ab containing plasma of patients with COVID-19. This anti-TNFα effect was significant up to a plasma dilution of 1:256 or 1:128 with PGRN-Ab-negative, and –positive samples, respectively (Fig. 3 B). Similarly, IL-1-RA plasma levels were significantly decreased by. 88% in IL-1-RA-Ab-positive patients with COVID-19 (median 320.7 pg/ml; SEM 66.2 pg/ml), compared to plasma of IL-1-RA-antibody-negative patients with COVID-19 (median 2728 pg/ml; SEM 346.7 pg/ml) (unpaired t-test: $p < 0.0001$) (Fig. 3C).

One patient with critical COVID-19 and high titers of PGRN-Abs and IL-1-RA-Abs required plasmapheresis due to exacerbating chronic inflammatory demyelinating polyneuropathy (CIDP). After 6 days of plasmapheresis titers of IL-1-RA and PGRN-Abs were strongly decreased from 1:1600 to 1:100, the additional atypic isoform of IL-1-RA was not anymore detectable by IEF in plasma, and IL-1-RA plasma levels rose from 34.5 pg/ml to 1022.5 pg/ml (Supplementary Fig. 6).

DISCUSSION

Here we report the occurrence of autoantibodies to PGRN and IL-1-RA in a considerable proportion (33.9% and 48.4%, resp.) of a cohort of patients with critical COVID-19. Importantly, such autoantibodies were not, or barely detectable in COVID-19 patients with only moderate, mild or asymptomatic disease, nor in non-COVID-19 ICU patients or healthy controls. Both IL-1-RA and PGRN are known to have anti-inflammatory activity, which is potentially interesting because at least critical forms of COVID-19 display a proinflammatory

phenotype. COVID-19 has been suggested to resemble hemophagocytic lymphohistiocytosis (HLH)(*5*), a condition associated with a hyperinflammatory dysregulation of the immune system. Misdirected, or defective IFN I responses were reported as a key factor for inefficient immune responses in patients with COVID-19 (*13*),(*14*),(*15*). Moreover, exuberant B-cell responses were regularly found in COVID-19 (*36*),(*37*), along with a high prevalence of antiphospholipid-(*38*), anti-Ro/La- (*39*)or anti-annexin-V-antibodies(*40*). It is noteworthy that PGRN-autoantibodies were first identified in plasma from patients with different forms of primary small vessel vasculitis. Thus, PGRN-Abs were detected in approximately 20-40% of patients suffering from primary small, middle and large vessel vasculitis (*18*). This is potentially relevant because critical forms of COVID-19 has have been shown to be frequently associated with vasculitis-like characteristics (*4*), which may contribute to some of the complications of this disease. In additional studies, PGRN-antibodies were detected in a wide spectrum of autoimmune conditions including rheumatoid arthritis (36% of patients), psoriatic arthritis (19% of patients) connective tissue disorders (15-40% of patients), and inflammatory bowel diseases (15-20% of patients), but only in 1 of 97 healthy controls, in 1 of 48 elderly residents of nursing homes, and not in 40 obese control patients, nor in 22 patients with sepsis requiring intensive care treatment, nor in 98 patients with melanoma. (*18*),(41)(41)⁴¹⁴¹⁴¹ 41, 38, 31,(*42*),(*19*),(*20*),(*43*),(*21*).

IL-1-RA is the physiologic inhibitor of IL-1α and IL-1β due to competition for IL-1R1. A proinflammatory imbalance between these proteins is found in gout crystallopathies, autoinflammatory or cryopyrin-associated periodic fever syndromes. Macrophage activation syndrome, which has many similarities to HLH, is a common complication in systemic juvenile idiopathic arthritis and adult-onset Still's disease. These conditions have in common the activation of the NLRP3 inflammasome(*44*). Therapeutically, colchicine, recombinant human IL-1-RA or monoclonal antibodies against IL-1β are used(*45*),(*10*). To our knowledge, except for IL-1-RA anti-drug-antibodies(*46*), spontaneously occurring IL-1-RA-antibodies have not yet been described in the literature. We have not detected them in healthy controls. They have to be characterized more precisely and their potential occurrence in rheumatologic or autoinflammatory disorders has to be investigated in future studies.

What might have triggered the production of PGRN- and IL-1-RA-Abs? A noteworthy observation in this context is that in contrast to rheumatologic diseases, where PGRN-antibodies largely belong to the IgG1 class(*18*), in COVID-19 PGRN-Abs were distributed across the IgM and multiple IgG subclasses. Moreover, the PGRN-antibody titers were relatively high ranging from 1:1600 up to 1:3200 (Fig. 1 C-D). Similarly, IL-1-RA-Abs were

found at relatively high titers and were also distributed across IgM and IgG subclasses (Fig. 1 H-I). These observations suggest that a recent event might have triggered the formation of these antibodies – possibly the COVID-19 infection itself. No cross-reactivity of PGRN-Abs with SARS-CoV-2 structural proteins was seen, though, which is indicative of an indirect induction of at least the PGRN-Ab production. Atypical posttranslational modifications may represent a common mechanism provoking autoimmunity (*47*)·(*48*)·(*35*)·(*49*). In patients with severe to critical COVID-19 with relevant titers of PGRN-Abs (≥1:800), the pSer81 PGRN isoform was consistently found, whereas it was not detected in any PGRN-Ab-negative subjects. In previous studies, we had identified a posttranslationally modified pSer81 PGRN isoform exclusively in PGRN-Ab-positive patients, preceding antibody formation, as the reason for the immunogenicity of PGRN (*32*). Moreover, this second pSer81 isoform led to altered functions of PGRN, with a dramatic reduction in its affinity to TNFR1/2 and DR3 and consequently a loss of PGRN's ability to antagonize to TNF-α and TL1A effects (*32*). In autoimmune diseases PKCß1 was identified as the kinase and PP1 as the phosphatase relevant for phosphorylation and dephosphorylation of Ser81 PGRN and inactivation of PP1 seemed to be responsible for the observed phosphorylation of PGRN at the Ser81 residue (*32*).

Likewise, in the present work, we found that IL-1-RA showed an additional, i.e. a third hyperphosphorylated isoform. This was found exclusively and without exception in all patients with high-titers of antibodies against IL-1-RA. The exact site of differential hyperphosphorylation is currently still under investigation. Since for both PGRN and IL-1-RA an additional hyperphosphorylated isoform was found, one may assume that this similar posttranslational alteration reflects a more general mechanism of autoimmunity in the context of COVID-19. This view is further supported by the concomitant presence of PGRN-antibodies and IL-1-RA-antibodies. Further studies will be needed to clarify the processes underlying this autoimmune response to COVID-19, for instance by examining samples collected longitudinally, and also trying to identify kinase(s) and phosphatase(s), as well as the T- and B-cell immune responses involved in this context.

Whatever the primary cause of the loss of self-tolerance to PGRN and IL-1-RA, the present observations raise the question of what potential clinical implications the autoantibodies might have. Notably, PGRN and IL-1-RA plasma levels were substantially reduced (by more than 90%) in patients with PGRN-Abs as compared to PGRN-Ab-negative patients (Fig 3 A). This contrasts with the recent finding that patients with COVID-19 had elevated PGRN plasma levels, as detected by 96-plex proximity extension assay (*50*). The reason for this discrepancy is unclear, but it might be explained by different assay kits measuring free or

bound PGRN, and complete or converted fragments thereof. Functional confirmation of the neutralizing effect of PGRN-antibodies in our study was obtained in a TNF-α-induced cytotoxicity assay, which showed that plasma from PGRN-Ab-positive patients was less effective than plasma from PGRN-Ab-negative patients in inhibiting the effect of TNFα in vitro (Fig. 3 B). It thus appears plausible that the autoantibodies detected in the present study have caused the observed massive reduction in the circulating levels of PGRN and IL-1-RA. It is tempting to speculate that this reduction of two anti-inflammatory regulators might, in turn, contribute to a proinflammatory milieu in a relevant subgroup of critically affected patients with COVID-19. This resulting proinflammatory shift in the inflammatory balance due to PGRN-Abs and IL-1-RA-Abs represents a different mechanism compared to autoantibodies neutralizing type I IFN and thus weaken the antiviral immune response(*16*). Based on the current results, several issues are emerging for future investigations.

In particular, it will be interesting to clarify (i) if antibodies to PGRN and IL-1-RA and respective posttranslationally modified antigens act as predisposing factors for critical courses of COVID-19, and (ii) if the hyperphosphorylated immunogenic isoforms are induced during, or prior to, the SARS-CoV-2 infection (*51*), followed by a break of self-tolerance, and formation of these antibodies inhibiting anti-inflammatory mediators. The broad presence of IgM and various IgG subclasses tends to support a fresh B-cell response for both autoantibodies. Another question is whether the presence of PRGN-antibodies (and the ensuing reduction of PGRN) in patients with critical COVID-19 represents a causal factor in the development of an autoimmune-like vasculitis (*4*).

Another potential implication of autoantibody-induced downregulation of two key inflammatory pathways, is that targeted therapeutic approaches for this subgroup of patients might consist in reinforcing these impaired anti-inflammatory pathways, i.e. by substitution of recombinant blockers of the TNF-α, IL-6, IL-1-pathways in combination with immunosuppressive agents like dexamethasone. Another targeted approach might be to block the autoimmune response in the first place, for instance by BTK-inhibition, but this would require to first identify which patients are predisposed to develop PGRN- and IL-1-RA antibodies. However, for all these approaches, in addition to an increased risk of super- or opportunistic infections, it would be essential to what extent the immune response against SARS-CoV-2 itself would be affected. A possible next step towards evaluating the potential of these agents as targeted therapies for treating and/or preventing a critical course of COVID-19 would be to perform retrospective analyses of plasma samples from prospective trials of these agents and of correlations with clinical outcome (*6*)·(*9*)·(*8*)·(*7*)·(*52*)·(*11*)·(*53*).

As plasmapheresis for a patient of the discovery cohort with coincident CIDP led to a strong decrease of titers of both PGRN- and IL-1-RA-Abs (Supplementary Fig. 6), patients might benefit from this, plasma exchange by convalescent plasma or IVIGs, but probably not from cytokine absorption alone.

Future studies need to confirm the frequency of these autoantibodies in critical COVID-19, to analyze the time course of their occurrence and their clinical relevance including the prognostic and predictive value. Finally, the questions need to be addressed, whether these or other pathogenic autoantibodies play a role in long-COVID and in pediatric inflammatory multisystem syndrome.

MATERIAL AND METHODS

This study was approved by the local Ethical Review Board (Bu 62/20) and conducted according to the Declaration of Helsinki. Plasma samples and PBMCs from 30 patients with critical (n=21) or severe (n=9) COVID-19 of the first wave of the pandemic in the spring of 2020 and from patients without COVID-19 requiring intensive care treatment were obtained from the COVID-19 wards of the Department of Internal Medicine II and V and the ICU of the Department of Internal Medicine III of the Saarland University Hospital (Homburg/Saar, Germany) were analyzed in an extended in house diagnostics for proinflammatory autoimmunity.

For validation, blood samples from 42 patients with critical, life-threatening COVID-19 requiring mechanical ventilation and/or ECMO treatment of the Department of Internal Medicine V of the Saarland University Hospital (Homburg/Saar, Germany) and of patients treated within the CAPSID trial were analysed for proinflammatory autoimmunity. Plasma samples and whole blood lysates from a control group (n=89) with mostly moderate to severe COVID-19 at the time of blood sample collection (68% moderate, 31% severe and 1% mild) were obtained from level 1 and 2 hospitals in Saarland federal German state. None of these 89 patients was in critical condition at the time of blood collection. However, 7 of 89 (8%) patients died.

Regarding asymptomatic to mild courses of COVID-19, plasma and serum samples of 49 patients were obtained from the Institute of Virology of the Saarland Medical Center Homburg/Germany and plasma samples from 56 convalescent plasma donors of the CAPSID trial (2020-001310-38) with mostly mild courses were obtained from the Institute of Clinical Transfusion Medicine and Immunogenetics at the University Hospital Ulm (Germany).

Blood samples of 40 vaccinated healthcare workers were obtained from MVZ Mindelheim/Germany and from the institute of virology of Saarland Medical Center Homburg/Germany.

Except for two samples with moderate COVID-19, all samples were from adult patients. All plasma and PBMC samples were stored at -20°C or -80°C, respectively until use.

ELISA for autoantibodies against PGRN, IL-1-RA and IL-10

The ELISA for autoantibodies was performed as previously described.(*41*) In short, the antigens were obtained using the coding sequences of the *GRN* gene encoding PGRN, IL-10 and isoform 1 precursor and isoform 2 of *IL1RN,* and the fragments aa1-63, aa107-177 and aa47-123 of isoform 1 of IL-1-RA which were recombinantly expressed with a C-terminal FLAG-tag in HEK293 cells under the control of a cytomegalovirus promoter (pSFI). Total cell extracts were prepared and bound to Nunc MaxiSorp plates (eBioscience, Frankfurt, Germany) precoated with murine anti-FLAG mAb at a dilution of 1:2,500 (v/v; Sigma-Aldrich, Munich, Germany) at 4°C overnight. After blocking with 1.5% (w/v) gelatin in Tris-buffered saline (TBS) and washing steps with TBS with Triton X-100, the individual plasma samples were diluted 1:100. ELISA was performed according to standard protocols with the following Abs: biotinylated goat antihuman heavy and light chain immunoglobulin G (IgG) at a dilution of 1:2,500 (Dianova, Hamburg, Germany); subclass-specific sheep antihuman IgG1, IgG2, IgG3 and IgG4 (Binding Site Group, Birmingham, UK) at dilutions of 1:5,000; goat antihuman IgM (Dianova) at a dilution of 1:2,500; or goat antihuman IgA (Dianova) at a dilution of 1:2,500. Following this step, corresponding biotinylated secondary Abs were used for immunoassays carried out to detect IgG subclasses and IgM. Peroxidase-labelled streptavidin (Roche Applied Science, Indianapolis, IN, USA) was used at a dilution of 1:50,000. As a cut-off for positivity, the average of the optical density (OD) of the negative samples plus three standard deviations was applied.

Western blot, isoelectric focusing of PGRN and IL-1-RA, and ELISA for pSer81 or npSer81 PGRN

Western blotting and isoelectric focusing was performed as described (*32*). Whole blood lysates or lysates of PBMCs from PGRN- and/or IL-1-RA-antibody-positive patients and controls were analyzed by IEF for PGRN and SLP2 isoforms and plasma samples were analyzed for IL-1-RA isoforms. Whole blood cell lysates or lysates of PBMCs from IL-1-RA-antibody positive patients were treated with alkaline phosphatase as previously described

using FastAP thermo-sensitive alkaline phosphatase (Fermentas/VWR, Darmstadt, Germany).(*35*) For ELISA for the pSer81 isoform of PGRN Nunc MaxiSorb plates were coated overnight at 4°C with rabbit antihuman PGRN antibodies directed against the C-terminus at a dilution of 1:2500 (v/v; LsBio, Seattle,WA, USA), followed by blocking with 1.5% (w/v) gelatin in TBS and washing steps with TBS-Tx [TBS, 0.1% (v/v) Tx100]. Individual plasma samples were utilized at a dilution of 1:2, and individual whole blood cell lysates were utilized at a dilution of 1:100. ELISA was performed according to standard protocols. For the detection of the hyperphosphorylated pSer8 or the non-phosphorylated Ser81 PGRN isoform, phospho-Ser81- or non-phospho-Ser81 PGRN-specific Fabs, which had previously been selected by phage display screening (*32*), were used at a concentration of 10 mg/l. Following this, corresponding biotinylated antihuman Fab secondary antibodies and subsequently peroxidase-labeled streptavidin (Roche) were used.

ELISA for antibodies against pSer81 PGRN isoform and their Ig class

To detect antibodies against pSer81 PGRN, Nunc MaxiSorp plates (eBioscience, Frankfurt, Germany) were precoated with murine anti-HIS mAb at a dilution of 1:2,500 (v/v; Sigma-Aldrich, Munich, Germany) at 4°C overnight. After blocking with 1.5% (w/v) gelatin in Tris-buffered saline (TBS) and washing steps with TBS with Triton X-100, HIS-tagged pSer81-specific recombinant Fabs (which had previously been selected by phage display screening (*32*)), were added at 10 µg/ml followed by washing steps with TBS with Triton X-100 and addition of lysates of PBMCs of patients with the pSer81 isoform. This was followed again by washing steps with TBS with Triton X-100 and by addition of individual plasma samples were diluted 1:100. Biotinylated goat antihuman heavy and light chain IgG were used at a dilution of 1:2,500 (Dianova, Hamburg, Germany); subclass-specific sheep antihuman IgG1, IgG2, IgG3 and IgG4 (Binding Site Group, Birmingham, UK) at dilutions of 1:5,000; and goat antihuman IgM (Dianova) at a dilution of 1:2,500. Following this step, corresponding biotinylated secondary Abs were used for immunoassays carried out to detect IgG subclasses and IgM. Peroxidase-labelled streptavidin (Roche Applied Science, Indianapolis, IN, USA) was used at a dilution of 1:50,000.

ELISA for plasma level determination of PGRN and IL-1-RA

PGRN plasma levels were determined of 19 patients with COVID-19 without PGRN-Abs, 11 patients with COVID-19 with PGRN-Abs and 8 patients without COVID-19 and without PGRN-Abs treated on ICU with a commercially available ELISA kit (AdipoGen, Incheon,

South Korea) according to the manufacturer's instructions. IL-1-RA plasma levels were determined of 20 patients positive and 12 patients negative for IL-1-RA-Abs with a commercially available ELISA kit (Invitrogen) according to the manufacturer's instructions.

TNF-α-induced cytotoxic effect (MTT assay)

To assess the functional effects of PGRN-autoantibodies in vitro, a nonradioactive viability assay (EZ4U Cell Proliferation Assay; Biomedica, Vienna, Austria) was performed. For this TNF-α-induced cytotoxicity indicator assay, we used the highly TNF-α-sensitive mouse fibrosarcoma WEHI-S cell line as target cells. In short, $4x10^4$ WEHI-S cells were seeded into 200 µl of cell culture at 37°C and 5% CO_2. To detect possible differences of TNF-α inhibiting activity in plasma between patients with or without PGRN-Abs, plasma of patients with COVID-19 with or without PGRN-Abs was added in dilutions from 1:8 to 1:512 to cultured WEHI-S cells, followed by administration of TNF-α (100 pg/ml). WEHI-S cells without addition of TNF-α and plasma, or solely with addition of TNF-α (100 pg/ml), were used as positive and negative controls, respectively. After 48 hours of incubation at 37°C, chromophore substrate was added to each well. This chromophore substrate is converted only by vital cells. The adsorption of the product was measured at an OD of 450 nm.

ELISA for cross-reactivity of absorbed PGRN-antibodies against structural proteins of SARS-CoV-2

PGRN-Abs were enriched using plasma from patients listed below. For this purpose, 500 µl of lysate of HEK293 cells transfected with recombinant FLAG-tagged PGRN was incubated with 20 ml anti-FLAG matrix for 15 min at room temperature. PGRN-antibody positive patient's plasma (500 µl) was diluted 1:10 (v/v) in PBS and was incubated with the anti-FLAG matrix/FLAG-tagged PGRN complex and subsequently desorbed by elution with glycine buffer depleted. Elution was performed with 100 ml of 0.1 M glycine pH 3. The patient plasma, the flow-through, the eluted enriched PGRN-Abs and plasma of controls listed below were screened by ELISA for reactivity against recombinant HIS-tagged SARS-CoV-2 S1- and S2-proteins, N-protein and M-protein expressed in HEK293 cell (ABIN) and against reactivity against FLAG-tagged PGRN, precursor of IL-1-RA isoform 1 and IL-1-RA isoform 2. PGRN-antibodies were purified from plasma patient #10 and #20 of a cohort with moderate and severe COVID-19 infection, respectively. Plasma from two healthy control, from a patient with rheumatologic disease with PGRN-Abs and without COVID-19, from a patient with rheumatologic disease without PGRN-Abs and without COVID-19 served as controls.

COI: The University of Saarland with Lorenz Thurner, Klaus-Dieter Preuss and Michael Pfreundschuh as investigators had applied for a patent on the role of PGRN as a marker for autoimmunity in 2012. The patent expired in 2017.

Author contribution: LTh, NF, ER, SH, and BTh planned the study. NF and ER performed experiments. LTh, BTh, YF and SS wrote the manuscript. LTh, BTh, YF, NF, ER, KDP, SH, DKM, TR, CP, GG, JP, SB, MB, MR, KC, AB, VL, MA, MB, SS, FL, RB, SB, SM, MK and PL revised the data and manuscript. IK, MB, BTh, CS, FN, JP, TR, SM, AB, PA, CM. FS, JL, TA, SE, AL, CW, AT, MR, BF, GD, CP, TP, JR, MB, MH, RB, FL, SL, SM, CH, CL, SB, MK, PL, SK and HS provided samples of patients and controls and clinical data. FL, GG and BF contributed excellent medical assistance without which, the conduct of the study would not have been possible.

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Tables:

case #	PGRN-Ab	atyp. sec. modified PGRN (pSer81 PGRN)	IL-1-RA-Ab	atyp. sec. modified IL-1-RA	ICU	age	sex	blood group	immunologic relevant diagnosis	Oxygen / NIV	MV	vvECMO	Outcome
1	no	no	no	no	yes	69	m	B pos	no			yes	survived
2	yes	yes	yes	yes	yes	84	m	0 pos	Relapse of Guillain Barré Syndrom, Plasmapheresis for CIDP			no	survived
3	yes	yes	yes	yes	yes	61	m	0 pos	no			yes	died
4	no	no	no	no	yes	64	m	B neg	no	yes	yes	no	survived transfer to hospital close to home
5	no	no	no	no	yes	60	m	0 pos	no			yes	survived
6	yes	yes	yes	yes	yes	60	m	0 pos	no			yes	died
7	no	no	yes	yes	no	24	f	unknown	no	yes	no	no	
8	no	no	no	no	no	54	f	A neg	undifferentiated connective tissue disorder	yes	no	no	survived
9	yes	yes	yes	yes	yes	52	m	unknown	Erstdiagnose HIV, CMV-coinfection, PCP	yes	yes	no	survived
10	yes	yes	yes	yes	yes	60	m	B neg	no	yes	yes	yes	survived
11	no	no	no	no	no		m						
12	no	no	no	no	yes	57	m	unknown	hay fever, ANA 1:1280	yes	no	no	survived
13	no	no	no	no	no	58	f	A neg	Bilateral lung transplantation one year before, alpha1-Antitrypsin deficiency		no	no	
14	no	no	no	no	yes	64	m	A pos	Myasthenia gravis, thymectomy two years before, antibodies against acetylcholine receptor	yes	yes	no	survived
15	no	no	no	no	no	58	m	unknown		yes	no	no	survived
16	no	no	no	no	yes	61	m	0 neg	no	yes	yes	no	survived
17	no	no	no	no	yes	65	m	0 neg	no	yes	yes	no	survived
18	no	no	no	no	yes	53	m	A pos	no	yes	yes	no	
19	yes	yes	yes	yes	yes	68	m	A neg	no	yes	yes	yes	died
20	yes	yes	yes	yes	no	56	f	0 neg	5 months before bacterial pneumonia	yes	no	no	survived
21	yes	yes	yes	yes	yes	64	m	A pos	no	yes	yes	no	survived
22	no	no	yes	yes	yes	87	f	A pos	no	yes	yes	no	survived
23	no	no	no	no	yes	49	m	A neg	no	yes	yes	no	died
24	yes	yes	yes	yes	yes	71	m	O pos	kidney transplantation after Nephrolithiasis 6 years before, IS with Advagraf, Cellcept and Urbason	yes	yes	no	survived
25	no	no	no	no	yes	71	m	A pos	no	yes	yes	yes	died
26	yes	yes	yes	yes	yes	56	m	A pos	no	yes	yes	yes	died
27	no	no	no	no	no	87	m	A neg		yes	no	no	survived
28	yes	yes	yes	yes	no	72	m	unknown	MZL, 3 years before 6x Rituximab-Bendasmustin, until 1 year before Rituximab maintenance; hypogammaglobulinemia	yes	no	no	survived
29	no	no	no	no	yes	51	m	A pos	no, HIT	yes	yes	yes	survived
30	no	no	yes	yes	no	52	m	B pos	kidney transplantation after Nephrolithiasis 6 months before, FSGS	yes	no	no	survived

Table 1: Patients' characteristics and antibody-status of cohort of 30 patients with severe or moderate COVID-19

Figure legends:

Figure 1: A) Occurrence of anti-PGRN antibodies in plasma from patients with moderate to severe COVID-19 of the discovery cohort and B) in control patients from intensive care unit without COVID-19. C) Titers of PGRN-antibodies from patients with COVID-19 of the discovery cohort. D) Ig classes and IgG subclasses of autoantibodies. E) PGRN-antibodies were detected at low titers in two patients with asymptomatic or mild COVID-19. F) Collectively, PGRN-Abs with relevant titers ≥1:800 were detected in patients with severe COVID-19 of the discovery and validation cohorts with severe COVID-19 in 22 of 65 patients (33.8%), compared to an occurrence of 2 in 205 patients (1%) with asymptomatic, mild or moderate COVID-19. G) Occurrence of IL-1RA-antibodies in patients with moderate to severe COVID-19 of the discovery cohort and in controls from patients from intensive care unit without COVID-19. H) Titers of IL-1-RA-antibodies from patients with COVID-19 and I) Ig classes and IgG subclasses. J) Only weak, but no relevant IL-1-RA-antibodies were detected in patients with asymptomatic or mild COVID-19. K) Collectively, IL-1-RA-Abs with relevant titers ≥1:800 were detected in patients with severe COVID-19 of the discovery and validation cohorts with severe COVID-19 in 30 of 65 patients (46.2%), compared to an occurrence of 4 in 205 patients (2%) with asymptomatic, mild or moderate COVID-19.

Figure 2: A) IEF of PGRN in plasma from 30 (#1-#30) patients of the discovery cohort with moderate to severe COVID-19 and in 28 controls from patients from ICU without COVID-19. An additional and more negatively charged PGRN isoform appeared in the gel, which was not detected in a healthy control and PGRN-Ab-negative patients. B) ELISA for pSer81 PGRN isoform and for non-phosphorylated Ser81 PGRN isoform. C) WB and IEF of IL-1-RA in IL-1-RA-antibody-positive patients with moderate to severe COVID-19. An additional and differentially charged IL-1-RA isoform appeared, which was not detected in a healthy controls and IL-1-RA-Ab-negative patients. IEF of IL-1-RA isoforms from two IL-1-RA-Ab-positive patients (#19 and #20) and a healthy control without IL-1-RA-Abs before and after alkaline phosphatase treatment. Alkaline phosphatase treatment led to disappearance of the second hyperphosphorylated isoform, which occurs also in the healthy control, and of the third, atypical isoform.

Figure 3: A) PGRN plasma levels in patients with COVID-19 and ICU controls. PGRN plasma levels in controls from ICU without COVID-19 and without PGRN-antibodies (206.05 ng/ml), and in patients with COVID-19 without PGRN-antibodies (161.23 ng/ml)

were higher compared to high-titer PGRN-antibody-positive patients with moderate to severe COVID-19 (15.21 ng/ml). Data are represented with median and interquartile range. B) Effect of PGRN-antibody status on inhibition of TNF-α-induced cytotoxicity. WEHI-S cells were incubated with TNF-α (or PBS) and plasma of PGRN-antibody-positive patients (COVID-19 #9 and #26) or matched PGRN-antibody-negative patient (COVID #5), as indicated. The plasma of the patients #9 and #26 with PGRN-antibodies resulted in a weaker inhibition of TNF-α-mediated cytotoxicity. The adsorbance of colored Formazan, as a marker for cell viability was detected at 450 nm. C) IL-1-RA plasma levels in patients with COVID-19 and ICU controls. IL-1-RA plasma levels were determined in patients with COVID-19 without IL-RA-antibodies (median 2566 pg/ml) and in IL-1-RA-antibody-positive patients with COVID-19 (median 192 pg/ml). Data are represented with median and interquartile range.
≤0.01; * ≤ 0.001; ****≤0.0001

Figures:

Figure 1:

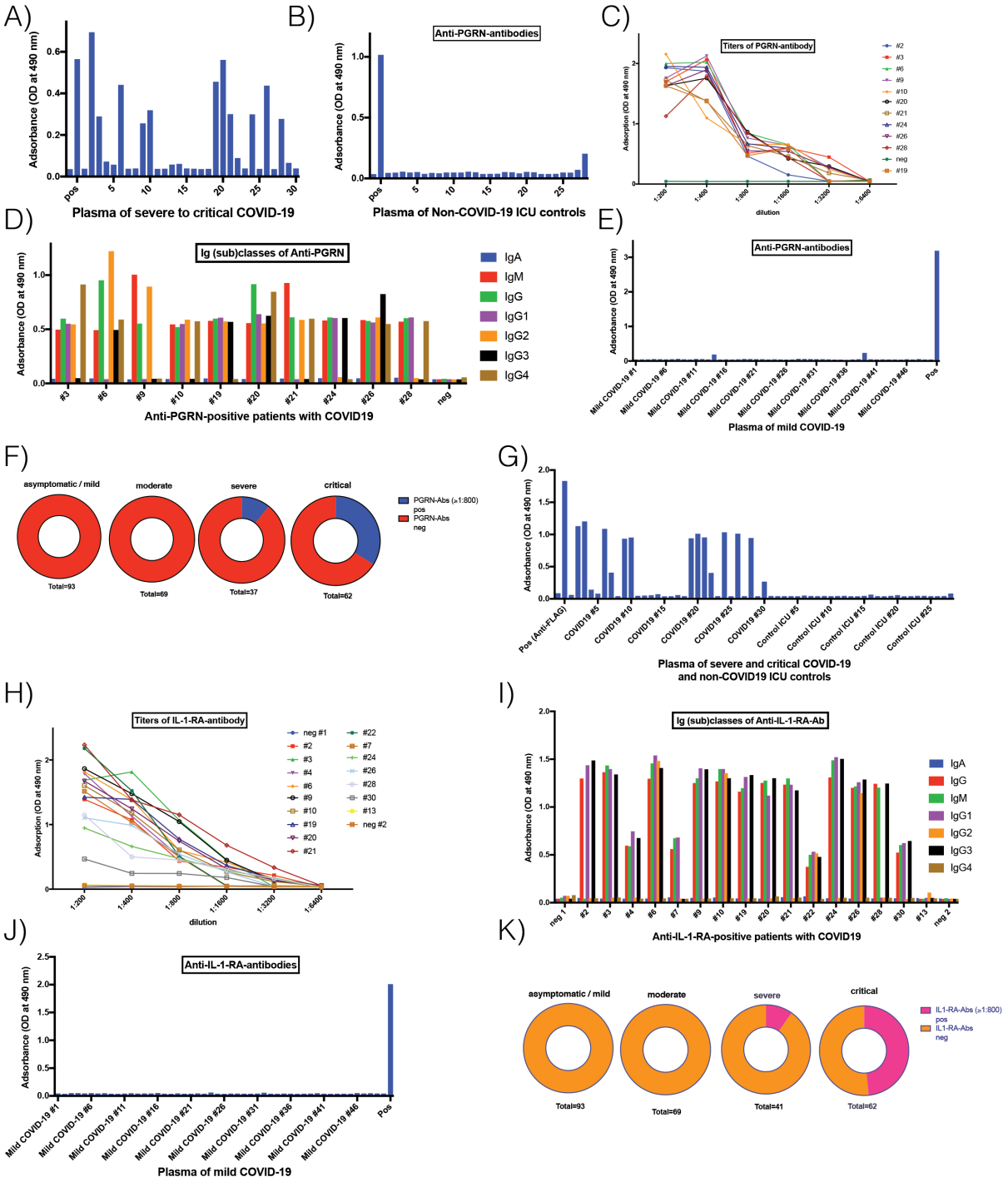
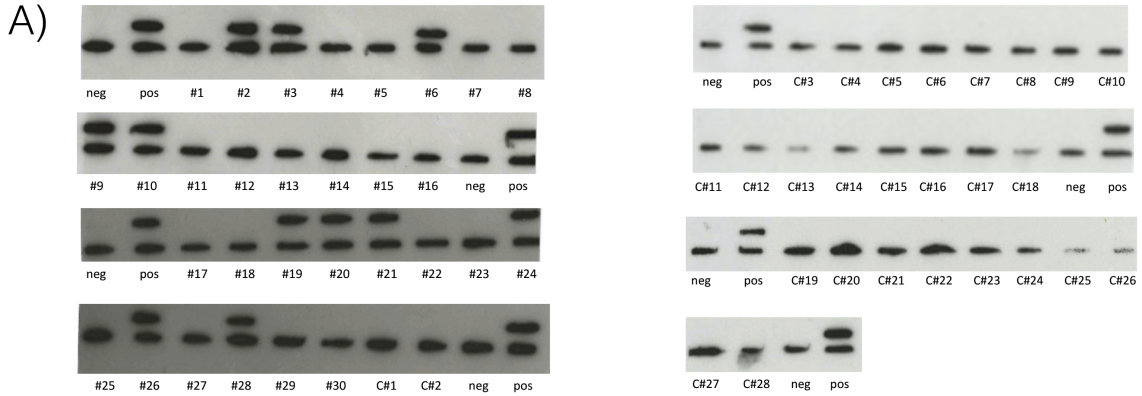
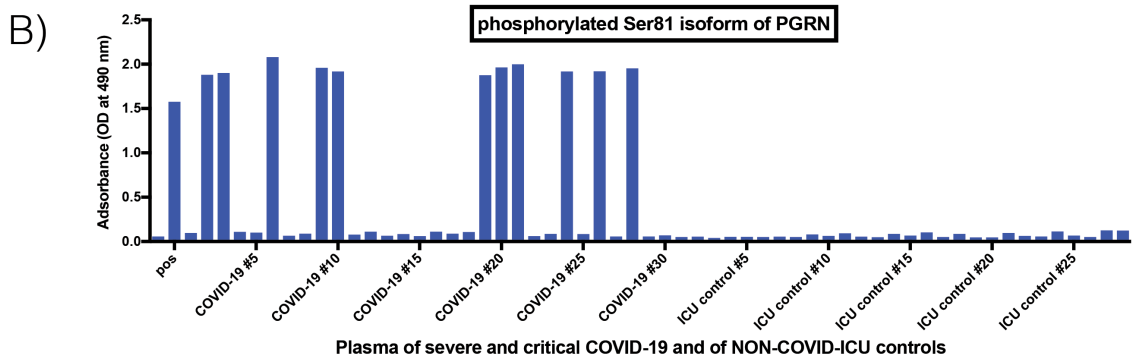


Figure 2:





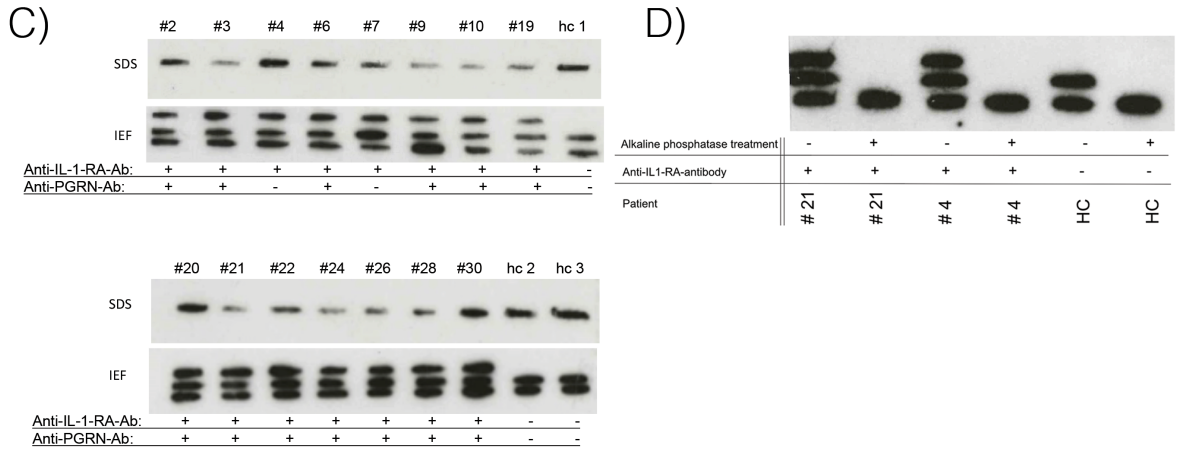


Figure 3: