Neutralizing Anti-interferon-γ Autoantibodies; An Ameliorating Factor in COVID-19 Infection?

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Neutralizing anti-interferon-γ autoantibodies; an ameliorating factor in COVID-19 infection?

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Abstract

Background: Elevated levels of interferon-gamma (IFNγ) have been found in COVID-19 infection, however its role in this setting remains poorly understood. Cases of non-tuberculous mycobacterium (NTM) infections due to anti-IFNγ autoantibodies (Ab) were first reported in 2004. NTM and COVID-19 co-infection in a patient with acquired IFNγ deficiency has not previously been described. The impact of anti-IFNγ Ab on the severity of COVID-19 has not been previously explored.

Objective: We report a case of COVID-19 infection in a patient hospitalised with NTM infection due to acquired IFNγ deficiency caused by anti-IFNγ Ab. We also explore effects of IFNγ Ab on the severity of COVID-19 infection.

Methods: Detailed immunological investigations were performed. Bio-rad, Bio-Plex methodology was used to detect anti-IFNγ Ab, titration, IFNγ recovery assay and SARS-CoV-2 serology. Anti-IFNγ Ab were tested in patients with severe (COV-Pat) and health care workers with mild/asymptomatic COVID-19 infection (COV-Asx).

Results: Mycobacterium avium intracellulare was diagnosed following bone marrow examination and culture. High titre anti-IFNγ Ab were detected in patient's serum. The autoantibodies neutralized both endogenously produced and exogenously administered IFNγ. SARS-COV-2 infection was identified during routine inpatient testing. Despite prolonged SARS-COV-2 infection the patient showed only minimal additional symptoms, never developed any significant inflammatory complications and eventually mounted an adequate IgG antibody response to the SARS-COV-2 trimeric S-protein. Elevated titres of anti-IFNγ Ab were detected in COV-Pat and COV-Asx, compared to non-infected healthy controls. The titres were broadly similar between COV-Pat and COV-Asx groups, but much lower compared to patients with acquired IFNγ Ab deficiency.

Conclusion: IFN-γ is known to play a central role in hyperinflammatory disease states such as macrophage activation syndrome This study illustrates the potential value of inhibiting IFNγ to prevent pathological inflammatory response to COVID-19.

Abbreviations:

IFNγ– interferon gamma;
ARDS – acute respiratory distress syndrome;
JAK – Janus kinase;
MAS – macrophage activation syndrome;
NTM – non-tuberculous mycobacterium

Introduction

Interferon-gamma (IFNγ) is the central effector cytokine of cellular immunity. It is mainly produced by activated T-cells, natural killer cells and to a lesser extent by professional antigen presenting cells including macrophages, dendritic cells and B-cells. Its main roles include activation of macrophages, granuloma formation and augmentation of anti-tumour immunity[1]. In addition, it augments antiviral immunity by inhibiting viral entry, uncoating, translation and virion assembly[2].

SARS-CoV-2 virus attained pandemic proportions within months of its discovery in Wuhan, China, with over 50 million cases and 1.8 million deaths reported worldwide as of writing[3]. Although most patients experience mild symptoms, approximately 15% develop a moderate phenotype resembling viral pneumonia, with a further subset presenting with a severe illness characterised by hyperinflammation and acute respiratory distress syndrome[4]. These moderate and severe forms of COVID-19 typically arise after 7-10 days of illness and are characterised by hypercytokinaemia and cytokine storm, with high levels of TNF-α, IL-1β, IL-1Ra, and IL-6[5,6]. Although found to be elevated in COVID-19, the role of IFN-γ in this setting remains poorly understood with only a handful of studies describing its role as a pathogenic or prognostic factor[7]. Clinical trials have focused on specific (*via* IL-1β, IL-6 and JAK inhibition) and non-specific (*via* corticosteroids and chloroquine derivatives) suppression of the exuberant immune response, with little attention being paid to IFN-γ in this context[8–11].

Anti-cytokine autoantibodies have been described in health[12] and disease, targeting IFN-γ, IL-17A, IL-17F, IL-22 and GM-CSF among others [13,14]. Neutralizing anti-IFNγ autoantibodies were first described in 2004 in the context of selective susceptibility to non-tuberculous mycobacterial (NTM) infection[15,16]. Lymph nodes, bones/joints and lungs are most commonly affected, with soft tissue and skin involvement (in the form of neutrophilic dermatosis, erythema nodosum or exanthematous pustulosis) also commonly reported. [15,17,18]

Methods

Patients and controls

The patients reported here include: the main case with autoantibodies to IFNγ and concomitant Covid 19 disease, three disease control with high titre anti IFN gamma autoantibodies (but SARS-CoV-2 negative, N=3) and patients with PCR-confirmed COVID-19 (COV-Pat, N=39) who were being looked after at Addenbrookes Hospital NHS Foundation Trust and Royal Papworth Hospital NHS Foundation Trust (RPH) and RPH healthy staff who presented with either no history or a mild history of COVID-19 symptoms (COV-Asx, N=31). Data from healthy pre-pandemic healthy control sera are also shown (N=57). SARS-CoV2 positive sera for Luminex assays were collected following informed consent, then aliquoted and held at −70 °C before use. Samples were taken from Samples screened positive for SARS-CoV-2 N- and S-binding antibodies by Luminex assay as described PCR and /or were selected (ethics approval: IRAS project ID: 96194 REC: 12/WA/0148).

Anti IFNγ serology – Titration of patient sera

Recombinant human IFNγ was covalently coupled to a carboxylated bead set (Bio-Plex; Bio-Rad Laboratories) as previously described[19]. The IFNγ- coupled beads were incubated with patient serum from for 1 h in 96-well filter plates (MultiScreenHTS; Millipore) at room temperature in the dark on a horizontal shaker. Fluids were aspirated with a vacuum manifold and beads were washed three times with 10 mM PBS/0.05% Tween 20. Successful coupling of the cytokines to their respective bead sets was verified by a specific mAb. Beads were incubated for 30 min with a PE-labeled anti–human IgG-Fc antibody (Leinco/Biotrend), washed as described, and resuspended in 100 µl PBS/Tween. They were then analyzed on an Luminex analyzer (Bio-rad, Bio-Plex) after titration in eight 1:5 dilution steps staring at 1:100.

IFN gamma recovery in the presence of autologous patient serum:

RPMI containing 20% Patient (Pt) or Control (Ct) serum was incubated for 1h in 96-well F plates (Corning) in the presence of IFNγ (2 x 104 IU/mL, Imukin, Boehringer Ingelheim). IFNγ levels were measured by particle based Flow cytometry on a Luminex analyser (Bio-Plex, Bio-Rad, UK) using an R&D Systems custom kit (R&D Systems, UK).

IFN gamma inhibition test

$1x10^5$ healthy control PBMC were incubated in 100 micl RPMI in the presence of 20% control (Ct) or patient (Pt) serum and activated by single stimulation with LPS (1 μg/ml, List Biochemicals) alone or in co-stimulating with IFNγ (2 x 104 IU/mL, Imukin, Boehringer Ingelheim) respectively. Supernatants were taken after 24 hours incubation at 37C/5%CO2. IL12 Levels (pg/ml) were measured by multiplexed particle-based Flow cytometry on a Luminex analyser (Bio-Plex, Bio-Rad, UK) using an R&D Systems custom kit (R&D Systems, UK).

Cytokine levels after whole blood activation

Cytokines were measured in whole blood after 24 hours stimulation using 10 μg/mL PHA or 1 μg/mL LPS alone or in combination with 20 μg/mL IL12 or 20,000 IU/mL IFNγ, respectively. To account for lymphopenia data were corrected for lymphocyte counts (in response to PHA) or monocyte counts (in response to LPS). Whole blood was diluted 1:5 in RPMI into 96-well F plates (Corning) and activated by single stimulation with phytohemagglutinin (PHA; 10 μg/ml; Sigma-Aldrich) or LPS (1 μg/ml, List Biochemicals) or in co-stimulating with IL12 (20 μg/ml, R&D Systems) and IFN-g (20000 U /mL, Imukin, Boehringer Ingelheim) respectively. Supernatants were taken after 24 hours. Levels (pg/ml) are shown for IFNγ, IL12. Cytokines were measured by multiplexed particle based Flow cytometry on a Luminex analyzer (Bio-Plex, Bio-Rad, UK) using an R&D Systems custom kit (R&D Systems, UK).

SARS-CoV-2 serology by particle-based flow cytometry (Luminex):

Antibodies to the trimeric S protein of SARS-CoV2 were determined by particle-based flow cytometry (Luminex) as previously described[20]. SARS-CoV2 trimeric S was covalently coupled to distinctive carboxylated bead sets (Luminex) as described above. All sera were tested at a dilution of 1/100.

T-(helper) cell (Th1/Th2/TH17) phenotyping

Whole EDTA blood was stained with a combination of CD3-V500, CD4-BV421, CCR6-Pe and CXCR3-Alexa-Fluoro 647 (all antibodies from Becton Dickinson, UK) at room temperature protected from light. Erythrocytes were subsequently lysed using BD red cell lysis solution and following a further 10-minute incubation the samples were washed by

centrifugation (1500rmp for 6 mins) using PBS/1% FBS. Following the final wash, cells were resuspended in 400ul of PBS +0.5% formaldehyde and cells analysed using a FACSCanto II flow cytometer (BD) using FACSDIVA software. The gating strategy is show in Figure 1D

Results

Case description

A previously fit 58-year-old Filipino man became unwell in December 2018. Over the following 12 months he developed folliculitis, episcleritis, intermittent rash with abscesses affecting his chest, arms and hands (Figure 1 A, B), weight loss, reduced appetite, tiredness, arthralgia (hands, shoulders and spine), cervical and supraclavicular lymphadenopathy, and intermittent fever with night sweats.

Investigations revealed a fluctuating leukocytosis (12-33x10^9/L), with neutrophilia, anaemia (nadir 87 g/L), persistent thrombocytosis, elevated CRP (peak of 100 mg/L) and low albumin (nadir 28 g/L). Screening tests for autoimmunity, blood-borne viruses and syphilis were negative. Anti-neutrophil cytoplasmic antibodies (ANCA) demonstrated an atypical c-ANCA pattern; cytomegalovirus and Epstein-Barr virus serology indicated past infection/mild reactivation only. He demonstrated borderline elevated rheumatoid factor and polyclonal IgG increase. Three IFNγ release assays for tuberculosis were indeterminate. Blood microscopy and culture were negative. PET-CT demonstrated widespread noncalcified lymphadenopathy in the neck, chest, and abdomen, with avid fluorodeoxyglucose uptake. Neutrophilic dermatosis seen in an initial skin sample raised the possibility of Sweet's syndrome, although dermal fibrosis suggested atypical mycobacterial infection. A cervical lymph node biopsy sample demonstrated none of the hallmarks of lymphoproliferative disease. A differential diagnosis of sarcoidosis or Sweet's syndrome was made and empirical treatment with prednisolone commenced in December 2019. This improved his rash, weight loss and lymphadenopathy, but not his fever.

On 10th February 2020 he developed right iliac fossa pain, vomiting, productive cough and rash. There was significant leukocytosis (26x10^9/L) and CRP was elevated at 243mg/l. CT imaging revealed new splenomegaly and splenic infarcts. Further dermatology review reported no features of neutrophilic dermatosis, instead resembling dermatitis (Figure 1C); repeat skin biopsy histology was suggestive of granulomatous interstitial dermatitis. In light of the patient's ethnicity, indeterminate IFNγ release assay for TB, and the evolving clinical picture, a provisional diagnosis of atypical mycobacterial infection and acquired IFNγ deficiency

(aIFNγ) was made. Bone marrow staining and culture demonstrated growth of Mycobacterium avium intracellulare (MAI) after 1 week of incubation. Subsequent mycobacterial blood culture was also positive for MAI.

Detection, functional analysis of anti-IFNγ autoantibodies and immunological investigations

Testing for anti-IFNγ Ab, using a Bio-Plex methodology as previously described[13], revealed high titre anti-IFNγ Ab (Figure 3 A, Figure 4) comparable to three other patients. We then verified the neutralising capacity of these antibodies and their ability to interfere with IFNγ signalling pathway in an *ex-vivo* setting by functional testing using whole blood or PBMC. Patient's response to and production of IFNγ was compared to 3 other aIFNγ/COVid-19 negative patients (N=3) and to healthy controls (N=15). After 24h stimulation with PHA, IL-12, or a combination of the two, we were unable to detect IFNγ in the supernatant from the patient sample, suggesting that anti-IFNγ Abs were neutralising the endogenously-produced IFNγ (Figure 3B, left panel). This was also observed in samples taken from patients with known aIFNγ. By contrast, the HCs demonstrated good production of IFNγ in response to both stimuli. In addition, under the same experimental settings, stimulation with IFNγ failed to induce IL-12 production in the patient sample (Figure 3B, right panel). To further demonstrate the neutralizing capacity of the anti-IFNγ Ab, we activated HC PBMC with LPS or LPS+ IFNγ in the presence of 20% control or patient serum, measuring IL-12 levels after 24h incubation. Stimulation with LPS or LPS + IFNγ triggered production of IL-12 from HC PBMC incubated with control serum, whilst HC PBMC incubated with the patient serum failed to produce IL-12 (Figure 3C). This demonstrated that patient serum neutralised both endogenously produced IFNγ and exogenous IFNγ. The capacity of patient serum to neutralise recombinant IFNγ was further confirmed by adding 20% of control or patient serum to RPMI-containing recombinant IFNγ and measuring IFNγ levels by Luminex. The patient serum completely neutralised recombinant IFNγ. Lastly, we analysed the patient's HLA type to determine if he carries the high-risk HLA-DR alleles associated with anti-IFNγ autoantibodies. The patient was found to carry DRB1*15:02, DRB1*16:02, and DQB1*05:02 (homozygous), all of which have all been previously been reported to increase the risk of developing anti-IFNγ autoantibodies[21].

Treatment for MAI was started in early March 2020. Due to rifabutin-induced hyperbilirubinaemia, the patient was established on ethambutol, azithromycin and amikacin.

Prednisolone was continued throughout his hospitalisation. Injectable IFNγ-1b (Immukin) treatment was implemented alternatingly with plasma exchange in an effort to raise IFNγ levels and reduce titers of anti-IFNγ autoantibodies, respectively.

Further immunological investigations showed normal lymphocyte profile with reduced naïve T cells 23% (normal 49-90%). We were interested to determine if the presence of IFNγ Ab had effects on the distribution of CD4 T-helper-cells (Th)Th1, Th2 and Th17, since this could have played a part in patient's inability to clear the infection. Compared to HC the patient had similar proportions of Th1, and TH17 cells, and somewhat reduced Th2 (Figure 1 D, E).

Anti-IFNγ autoantibodies and COVID-19 infection-single case

On the 28th March 2020 the patient was tested for SARS-CoV2 prior to his transfer to another ward. SARS-CoV2 nucleic acid amplification test (NAAT) from a nose and throat swab was positive. A previous swab taken on admission was negative. At the time of screening the patient was pyrexial (38.1°C) but had no respiratory symptoms. Chest imaging demonstrated novel subpleural nodules, but no changes indicating COVID-19 pneumonia. Nose and throat swab COVID-19 NAAT tests were positive on 6 subsequent occasions between 9th April and 19th May 2020 (Figure 2), but were negative from 1st June 2020 onwards. His steroid treatment was uninterrupted throughout this period. The patient required no oxygen supplementation and had no significant respiratory symptoms. He continued to receive regular plasma exchange and IFNγ-1b supplementation. However, this combination therapy was unlikely to have made a substantial difference in correcting his acquired immunodeficiency. Serial measurements of anti-IFNγ Ab showed some reduction in titres, but levels remained high (Figure 3A). Experiments looking at effects of the patient serum on the levels of Immukin, showed that following incubation with the serum, no free IFNγ was detectable (Figure 3D).

He eventually mounted an adequate IgG antibody response to several viral antigens including the trimeric S protein (Figure 4). However, his MAI infection remained difficult to control. Plasma exchange and immukin were stopped, he continued azithromycin, ethambutol and rifabutin, and in October 2020 was treated with a course of rituximab to which a good initial response was seen.

Anti-IFNγ autoantibodies and COVID-19 infection-patient cohorts

To determine if autoantibodies to IFNγ are associated with severity of COVID-19 infection we tested sera from three groups. These included healthy volunteers, healthcare workers with asymptomatic or predominantly mild COVID-19 infection (COV-Asx) and patients who were hospitalized with COVID-19 (COV-Pat). Both, the COV-Asx and the COV-Pat group, showed similarly increased anti IFN gamma levels when compared to the healthy control group (HC). However, antibody levels remained low, when compared to the patient and the NTM disease controls. Interestingly, the two individuals from the COV-Asx cohort with the most severe disease presentation (acute respiratory failure and severe systemic symptoms, respectively) were found to have the lowest anti IFN gamma titers within their cohort (Figure 4).

Discussion

In this study we have explored the role IFNγ in COVID-19 infection by studying a single case of acquired IFNγ deficiency with NTM and COVID-19 co-infection and two cohorts of infected individuals, one with severe form of disease and the second group with a mild/asymptomatic disease course. In the case of a patient with NMT and COVID-19 co-infection, despite the combined immune suppression resulting from steroid therapy and concomitant IFNγ deficiency, the patient did not have a severe course of COVID-19 infection. This contrasts starkly with the experience of patients with primary and secondary immune deficiency disorders, who have increased COVID-19 associated mortality compared to the age, sex and ethnicity matched healthy population[22]. This mild course of the disease is particularly surprising given that compensatory hyper-activation of the IL-1/IL-6 pathways in the context of immunodeficient response to the virus is thought to be partly responsible for the cytokine storm seen in severe COVID-19[23].

The lethality of the closely related SARS-CoV and MERS-CoV correlates with their ability to impair type I IFN signalling *via* phosphorylation of STAT1. Such correlation remains to be confirmed for SARS-CoV-2 whose various proteins have been proven to interfere with the interferon response *via* their effect on type I IFN promotors[24,25].

Recent research has shown that neutralising anti-IFN-I autoantibodies are present in a significant proportion of severe COVID-19 cases, highlighting the importance of a robust IFN-I response in COVID-19[26]. Our experience contrasts these observations, showing that complete absence of IFN-γ signalling does not result in a worse COVID-19 outcome and that IFN-g is not necessary to control this virus. Interestingly, in an experimental model using

human epithelial cells IFN-γ has been shown to promote SARS-CoV-2 infection[27]. Arguably, the mild disease phenotype in our patient could have also resulted from prolonged corticosteroid therapy rather than impairment of the IFNγ pathway or both, considering that corticosteroid therapy is a mainstay of COVID-19 treatment. [10]

Neutralizing antibodies to IFN-gamma have been approved for the treatment of paediatric primary hemophagocytic lymphohistiocytosis (HLH), a condition characterised by excessive cytokine induction similar to what can be found in some severe COVID-19 disease[28,29]. Combined TNF-α and IFNγ blockade results in abrogation of the inflammatory COVID-19 phenotype in murine models[30]; despite his SARS-CoV-2 infection, the patient displayed unremarkable serum cytokine levels when compared to COVID disease controls (data not shown), possibly indicating similar protective effects of such antibodies in humans.

When we explored the association of anti-IFNγ Ab with severity of COVID-19 infection in a larger cohort of patients we did not find an obvious link. Both groups of infected individuals, those with severe form of COVID-19 and hospital workers with mild or asymptomatic infection had similar levels of anti-IFNγ Ab. These autoantibodies seemed to develop during the course of infection, since the autoantibodies were not readily detectable in a non-infected cohort. Furthermore, the antibody titers were much lower compared to the patients with confirmed aIFNγ deficiency. This suggest that anti-IFNγ Ab seen in these two groups of patents, might be a transient phenomenon, with limited neutralizing capacity against IFNγ. It is interesting to note that two outliers with high titre anti-IFNγ Ab in COV-Asx group, on closer inspection, did in fact have more severe course of the disease.

Although we have not managed to replicate our findings from a single patient with acquired IFNγ deficiency in a larger cohort of COVID-19 infected individuals , our study offers some insights into the role of IFNγ in SARS-CoV-2 infection and provides an outline of what to expect when patients with this rare acquired immune deficiency encounter a viral illness characterized by immune hyperactivation. Given the higher prevalence of patients with IFNγ autoantibodies in South-East Asia, a future analysis of COVID-19 outcomes in those patients could provide additional insights into the role of IFNγ in (severe) COVID-19[31]. Furthermore, the utility of blocking IFNγ pathway in treatment of COVID-19 could be tested in an randomised controlled trial (RCT) using emapalumab, which is now licenced for treatment of primary and secondary HLH. One RCT (ClinicalTrials.gov Identifier: NCT04324021) which had emapalumab arm, unfortunately was terminated early, not due to safety of efficacy

concerns, but reportedly due to recruitment difficulties arising from ongoing changes to the standard of care for COVID-19. This is issue is not likely to be unique to emapalumab, but will affect other trials using novel immunomodulatory therapies for COVID-19.

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Ethical Approval

Ethics approval was granted by the Leeds (East) Research Ethics Committee. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. (ethics approval: IRAS project ID: 96194 REC: 12/WA/0148).

Consent to participate

Participants provided their consent to participate in this study

Consent to publish

Consent was sought and gained from the participant (the main case) to publish the findings of this study

Authors Contributions

MK, VP, HM, EGK collected clinical information; PCA, CC, PH, GBM performed experiments; RD, GBC, HEB and SS analysed data, MK, VP and SS wrote first draft of the manuscript. HEB and EGK provided sera for COVID-19 patients and healthy control groups. All authors read, edited and approved the manuscript. RD and SS design and funded the study.

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Competing Interests

The authors declare that they have no relevant conflicts of interest.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author

Figure legends

Figure 1. A and B) Pictures illustrating skin lesions at initial presentation C) Picture of skin lesions following admission to hospital in February 2020 D) T-helper (Th) 1, Th2 and Th17 distribution in peripheral blood. Whole EDTA blood was stained with a combination of CD3-V500, CD4-BV421, CCR6-Pe and CXCR3-Alexa-Fluoro 647 A) representative dot plot shows the gating strategy B) The results from 5 healthy controls (HC)-dark dots and the patient (aIFNγ)-red dots.

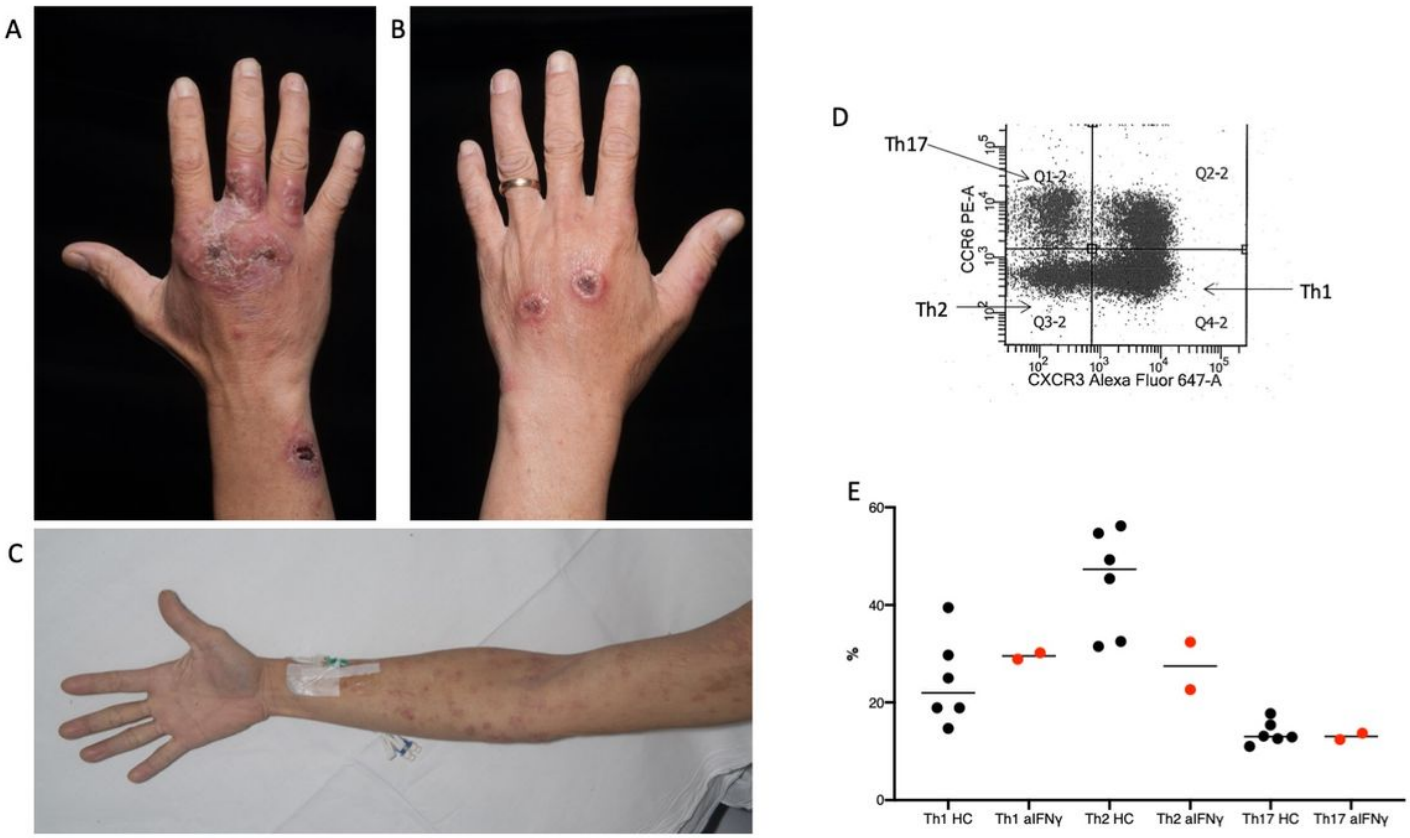
Figure 2. Graph depicting the patient's white cell count (WCC) and CRP over time, with the daily equivalent oral prednisolone dose, concomitant therapies and COVID-19 swab results superimposed

Figure 3 A) Detection and titration of anti-IFNγ antibody. Patient sera were diluted in seven 1/5 steps starting at 1/100. Anti IFNγ IgG was determined by particle based flow cytometry on a Luminex analyser and shown as mean fluorescence intensities (MFI). B) Cytokine levels after whole blood activation. Cytokines were measured in whole blood after 24 hours stimulation using 10 μg/mL PHA or 1 μg/mL LPS alone or in combination with 20 μg/mL IL12 or 20,000 IU/mL IFNγ respectively . To account for lymphopenia data were corrected for lymphocyte counts (in response to PHA) or monocyte counts (in response to LPS) . Controls are shown as grey circles (N=15), patients with acquired (a)IFNγ deficiency (N3) are shown as blue circles. For the aIFNγ/Cov19 patient data from four experiments are shown as red circles. Cytokine levels are shown as pg/ml. C) In vitro inhibition of IFNγ using patient serum. Healthy Control PBMC were activated with LPS or LPS+IFNγ in the presence of 20% control (Ct) or patient (Pt) serum and IL12 levels were measured in the supernatant after 24h incubation. D) IFNγ recovery in the presence of autologous patient serum: 20% control or patient serum were added to RPMI containing X U recombinant IFNγ and IFNγ levels were determined by Luminex. No IFNγ could be recovered from the well containing patient serum showing complete antigenic neutralization by the patients autoantibody.

Figure 4

Levels of IgG anti-IFNγ autoantibodies and IgG antibodies to the SARS-CoV2 spike protein (S) where determined in index patient (bright green circles), non-tuberculous mycobacterium (NMT) disease controls (dark green circles), pre-pandemic healthy controls (HC, open circles) , Health care workers (COV-Asx) and a COVID hospitalised cohort (COV-Pat). Geometric means are shown +/- SD. P values were calculated assuming non gaussian distribution by Mann-Whitney Test

Figures



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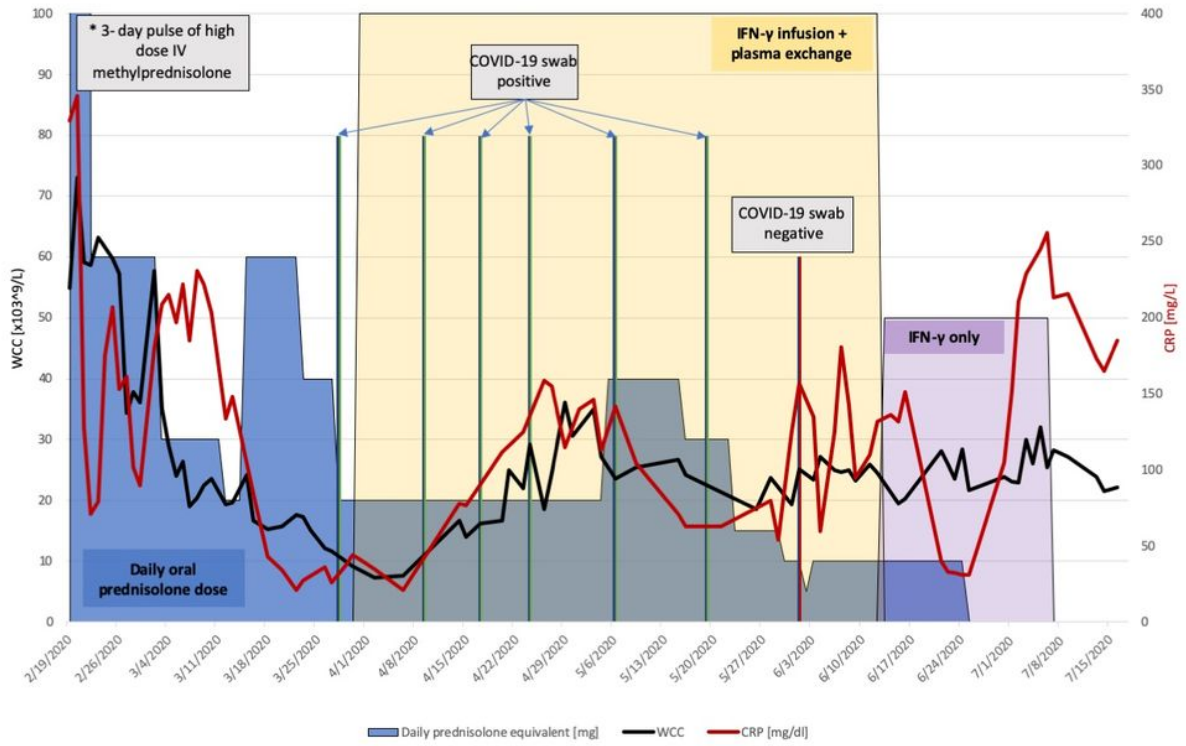

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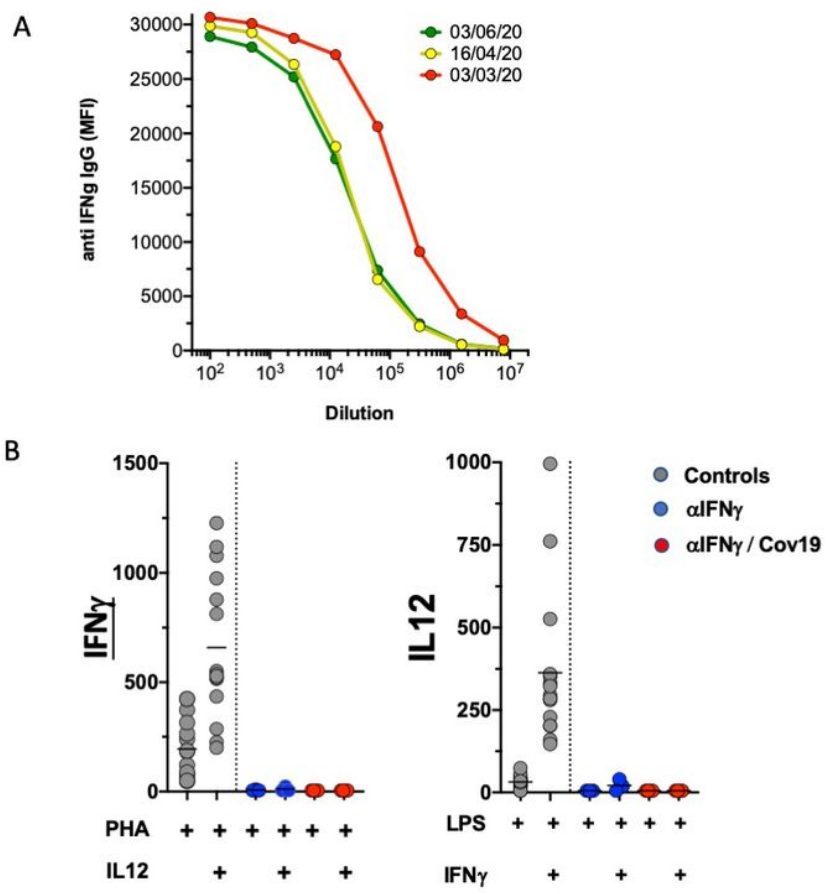

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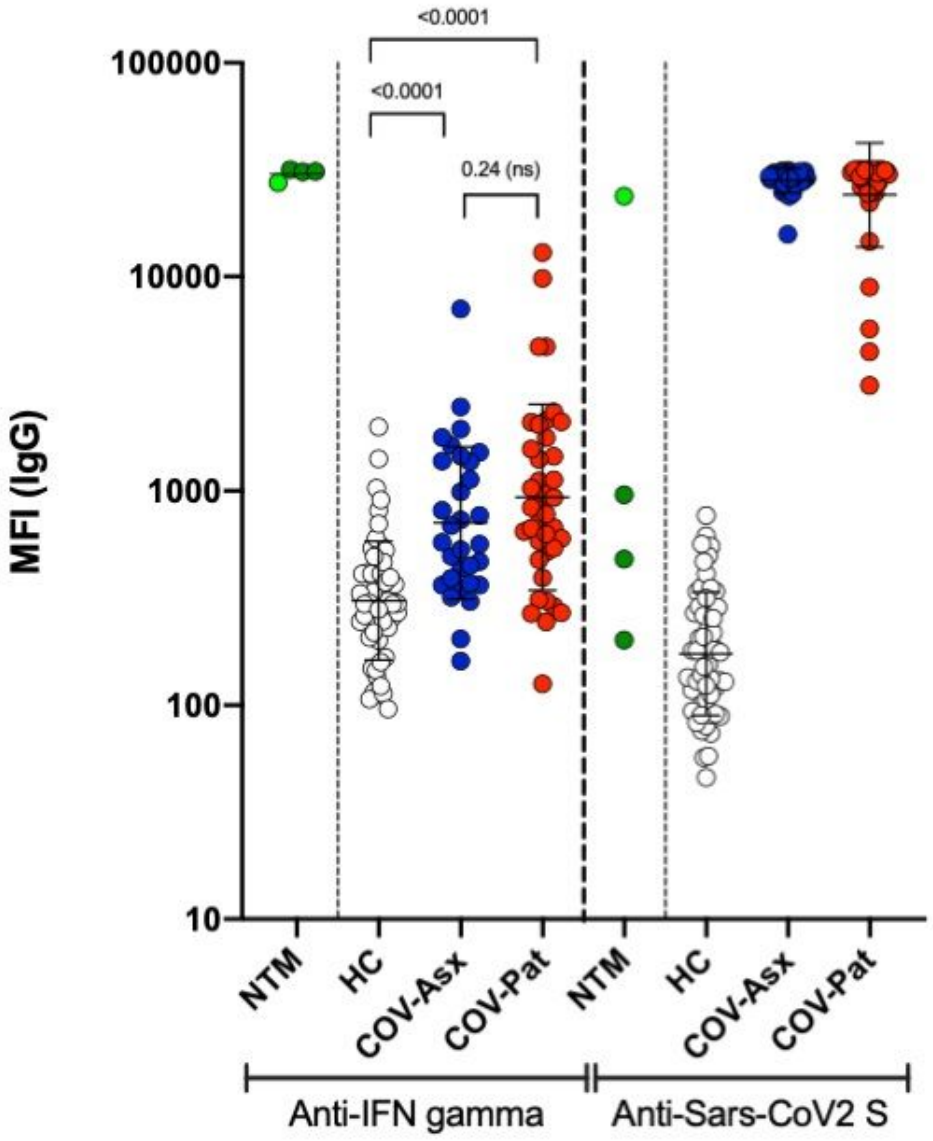

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