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DISCUSSION FORUM

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Perforin, COVID-19 and a possible pathogenic auto-inflammatory feedback loop

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Abstract

During COVID-19 infection, reduced function of natural killer (NK) cells can lead to both compromised viral clearance and dysregulation of the immune response. Such dysregulation leads to overproduction of cytokines, a raised neutrophil/lymphocyte ratio and monocytosis. This in turn increases IL-6 expression, which promotes scar and thrombus formation. Excess IL-6 also leads to a further reduction in NK function through downregulation of perforin expression, therefore forming a pathogenic auto-inflammatory feedback loop. The perforin/granzyme system of cytotoxicity is the main mechanism through which NK cells and cytotoxic T lymphocytes eliminate virally infected host cells, as well as being central to their role in regulating immune responses to microbial infection. Here, we present epidemiological evidence suggesting an association between perforin expression and resistance to COVID-19. In addition, we outline the manner in which a pathogenic auto-inflammatory feedback loop could operate and the relationship of this loop to genes associated with severe COVID-19. Such an auto-inflammatory loop may be amenable to synergistic multimodal therapy.

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by the virus named SARS-coronavirus-2 (SARS-CoV-2) originated in Wuhan in 2019 and rapidly became a global pandemic. It is a highly infectious viral disease with variable morbidity and mortality. Clinical presentations and complications range from asymptomatic infections to death. Host susceptibility appears to play a key role in determining clinical outcomes, but heterogeneity in susceptibility remains to be fully explained.

Type I and II interferons (IFNs), Fas ligand (Fas-L) and perforin/granzyme apoptotic pathways are three of the most important components of immune defence mechanisms against viral pathogens. Type I IFNs (α and β) are produced early in viral infection and are known to stimulate antiviral genes as well as being regulators of the innate response, including natural killer (NK) cells.[1] Type II IFN (IFN-γ) also forms a part of the innate antiviral response. It is produced by NK cells and activated T lymphocytes, other producers to include innate lymphoid cells type 1 and macrophages, and it promotes Th1 adaptive immune responses.[1] Fas receptor-Fas ligand signalling is involved in apoptosis of virus-infected target cells and contributes to the cytotoxic activity of both NK and cytotoxic T cells (CTL).[2] Perforin/granzyme-induced apoptosis, as discussed below, is the major pathway through which cytotoxic lymphocytes eliminate virally infected host cells.[3] Factors associated with alterations in NK and cytotoxic immune cell function, such as increased or decreased

perforin expression, are likely to impact on resistance or susceptibility to severe COVID-19 infection. Here, we examine the evidence for an association between the expression and function of perforin and susceptibility to severe COVID-19 infection. We also review how reduced cytotoxic lymphocyte function (with a particular focus on NK cell activity), associated with compromised perforin expression, might result not only in decreased virus clearance, but also dysregulation of the immune response, thereby creating a pathogenic auto-inflammatory feedback loop.

1.1 | Perforin expression and function

Perforin is expressed by both innate and adaptive NK cells, NKT cells, cytotoxic CD8+ T cells (CTL), Treg cells and γδ T cells.[4] NK cells and CTL, as cytotoxic lymphocytes, play key roles in the destruction of virally infected host cells. This is achieved via toxic proteases called granzymes (granzyme B having the most potent pro-apoptotic function) and the antimicrobial peptide granulysin.[4] Following creation of an immune synapse between cytotoxic lymphocytes and virally infected cells, perforin generates pores in the target cell membrane allowing entry of effector molecules and subsequent cell death.[4]

NK cells can engage with and kill target cells via the perforin pathway within hours of encountering foreign antigen (due to constitutively expressed perforin mRNA), whereas naive CTLs express the highest level of perforin mRNA and protein approximately one week after T cell receptor stimulation.[5] Perforin also plays a regulatory role in T cell activation as well as in destroying cancer cells.[4] It must be appreciated, however, that perforin is also expressed by other immune cells and that NK cell function generates antiviral effects through several mechanisms other than perforin/granzyme action.[6]

1.2 | Epidemiological factors associating perforin expression and resistance to severe COVID-19 symptoms and mortality

1.2.1 | Age and gender

Old age, particularly over 70 years, is a significant risk factor for mortality from SARS-CoV-2 infection.[7] Expression of perforin and NK cell cytotoxic function declines significantly after the age of 70 years.[8] Reduced release and binding of perforin with target cells at the immunological synapse appear to be a major cause of the age-related decline in NK cell cytotoxicity.[9] Perforin expression has been found to be significantly higher in children compared with both male and female adults.[8] Clinical manifestations of COVID-19 disease are much less common in children compared with adults, and hospitalizations for severe disease and deaths are rare.[10] Females have significantly higher levels of perforin expression compared with age-matched males.[8] Male gender is a prominent risk factor for COVID-19 mortality, whereas female sex is protective and gender mortality ratio M:F can be as high as 3:1.[7] FasL and IFN-γ expression follow similar patterns with some notable exceptions however, such as lower IFN-γ production in childhood.[11] There are, of course, many biological differences between males and females, and between the young and the elderly, that might also contribute to varying resistance to COVID-19, but it is intriguing that associations with disease severity mirror perforin levels in these groups.

1.2.2 | Obesity

Obesity, a prominent adverse risk factor for COVID-19, is associated with reduced perforin expression and NK cells from obese individuals have reduced cytolytic activity compared with those from lean individuals.[12] Aetiological factors for this include lipid accumulation in NK cells which reduces perforin, granzyme B and IFN-γ secretion and the negative inhibition of NK activity by adipokines (cytokines secreted by adipose tissue) including interleukin(IL)-6.[13,14] Conversely, and interestingly, perforin deficiency, related to its function in immune regulation, may predispose to obesity, glucose intolerance and insulin resistance as identified in studies with perforin-deficient mice.[15] Meta-analyses have revealed that obese individuals are at greater risk of: (i) testing positive for SARS-CoV-2, (ii) hospitalization, (iii) ICU admission and (iv) mortality from COVID-19 disease (by 48% increase in deaths).[16]

1.2.3 | Co-morbidities

The associations of perforin with heart disease are complex. However, reduced NK activity in coronary heart disease has been confirmed by several reports.[17-20] Cardiovascular disease has also been reported as having one of the highest co-morbid associations with COVID-19.[21] In both established Type 1 and Type 2 diabetes, lower levels of perforin expression are reported.[22,23] Diabetes, of either type, has also been reported as being among the commoner comorbidities with severe COVID-19 disease.[21]

1.2.4 | Drug medication

The relatively well-tolerated drug metformin has been shown to increase perforin and granzyme B expression at dose levels used in the treatment of diabetes.[24] Kow et al undertook a meta-analysis of the use of metformin and COVID-19 outcomes.[25] This revealed that there was a reduction in mortality among diabetic patients who were receiving metformin medication compared with diabetic patients who were not. Oestrogens have been shown to significantly increase perforin expression,[26] whilst anti-oestrogens reduce perforin expression.[27] Costeira et al, using a self-reporting 'mobile app' system, studied over 500 000 women for associations between COVID-19 infection and their oestrogen status.[28] Their findings support a protective effect of oestrogen on COVID-19 infection and severity.

In summary, several epidemiological factors appear to associate perforin expression and NK cell function with resistance to COVID-19.

1.3 | Natural killer phenotype and function in COVID-19

The literature regarding NK cell function and phenotype in patients with COVID-19 and COVID ARDS is not uniform. This perhaps is not surprising as the studies reported cover different definitions of 'severe', differing ethnicity and other patient demographics and the examination of different compartments of NK cells as well as time points in disease course. However, certain trends are observed consistent with perforin performing an important role in antiviral activity and reduced/dysregulated NK function in severe COVID-19. Li et al found reduced perforin and granzyme B expression by peripheral blood NK cells in hospitalised patients.[29] D'Allesandro et al reported reduced numbers of blood NK cells in patients with severe COVID compared with those with non-severe illness.[30] Maucourant et al found that absolute numbers of blood NK cells (both cytokine-producing $CD56^{bright}$ and cytotoxic $CD56^{dim}$ cells) were significantly reduced in COVID-19-infected patients, and this was more apparent in severe cases (ICU/high dependency unit, non-invasive/mechanical ventilation). The reduced number of remaining $CD56^{bright}$ and $CD56^{dim}$ NK cells did show 'arming' with significant perforin expression in COVID patients.[31] Patients with severe disease also had distinct adaptive NK immunotypes with NKG2C, perforin and ksp37 (killer-specific secretory protein that is usually co-expressed with perforin[32]) expression.[31] NKG2C interacts with its ligand HLA-E to mediate NK cytotoxic and cytokine response to virally infected cells.[33] Gene variants with deletion of NKG2C receptor are a highly significant risk factor for severe COVID-19.[33] Li et al demonstrated lower NK cells with 'exhaustion' of NK cells as demonstrated by programmed death, PD-1, expression in patients with advanced COVID-19 and severe symptoms.[29] Reduced NK cytotoxicity in severe COVID-19 was related to increased IL-6 levels and treatment with anti-IL6 biologic therapy restored cytotoxic potential of NK cells.[34]

1.4 | Perforin mutations and cytokine storms

The perforin amino acid substitution Ala91Val (A91V) impairs perforin activity in several ways: it reduces protein stability and slows intracellular trafficking, which can reduce cytotoxicity by 50%-90%.[4] Twenty-two previously healthy patients aged 24-52 who had been admitted to intensive care with COVID-19 infection were tested for the common perforin gene polymorphism A91V. Two of the patients harboured this variant and both died, whereas only one of the A91V-negative patients died.[35] Although it is tempting to interpret the association of this mutation with defective perforin function as contributing to the death from COVID-19, this early report comprising a very small number of cases awaits further corroboration

Perforin has a significant role in regulating T cell activation and activity, including regulating cytokine release.[5] Defective perforin function, as seen in patients with perforin gene mutations in familial haemophagocytic lymphohistiocytosis (fHLH), can be associated with 'cytokine storm'. Failed killing of the target cell leads to prolonged attachment, resulting in many successive rounds of Ca^{2+} flux into the cytotoxic cell, which triggers substantial secretion of pro-inflammatory cytokines and chemokines.[4] In turn, secreted IFN-γ causes hyperactivation of the myeloid cellular compartment and stimulates macrophages to secrete IL-6. This cytokine is considered the main instigator of fatal systemic inflammation in fHLH.[4] 'Cytokine storms' (also known as macrophage activation syndrome [MAS] or secondary HLH [sHLH]) are also one of the major factors leading to COVID-19-associated mortality.[36-38] Laboratory and clinical features of sHLH resemble fHLH (associated with homozygous mutations in the perforin cytolytic pathway). sHLH is a much more common entity and now recognized as being associated with heterozygous mutations (possibly functioning as hypomorphic or partial dominant-negative alleles) in the same perforin pathway genes that can predispose to similar cytokine storm-like manifestations including from specific viral triggers.[39] Indeed, examples of other causative genes include *SH2P1A* involved in viral control.[39] Of note,

mutations in perforin and related genes have previously been associated with sHLH/MAS-like presentations in patients who died from H1N1 influenza.[40] Recently, germline variants in *UNC13D* and *AP3B1*, both HLH-related genes, were found to be associated with COVID-19 plus high cytokine levels and mortality.[41]

Black and Asian ethnicity represents another substantial risk factor for severe COVID-19, and although racial inequalities are acknowledged as an important factor in this risk disparity (including inequitable access to health, overcrowded living conditions, engagement in essential work),[42] genetic susceptibility may also be relevant. Single nucleotide polymorphisms (SNPs) in the perforin gene, particularly those associated with decreased activity of the protein, have been linked with reduced population distance from the equator due to equator-associated selection pressure from infectious diseases.[43] It is proposed that in some diseases, such as falciparum-associated cerebral malaria, these SNPs can confer a host survival advantage (relating to a protective effect of perforin deficiency on pathogen-associated blood-brain barrier disruption).[43] We postulate that individuals of African or Asian ancestry possessing such SNPs (such as the higher prevalence of Arg232His in South Asian populations) have an elevated susceptibility to COVID-19 and/or cytokine storms via impairment of perforin function.

1.5 | A pathogenic auto-inflammatory feedback loop?

Four important steps are involved in the potential production of a pathogenic auto-inflammatory feedback loop (Figure 1).

1.5.1 | Reduced NK/perforin function leads to dysregulation of the immune response to infection

Perforin-deficient mice have been shown to induce lymphocyte hyperactivation with increased production of cytokines in response to viral infection.[5] As outlined above, defective perforin function, as seen in patients with fHLH, is also associated with 'cytokine storm' resulting from dysregulation of the immune response to infection. In COVID-19 cytokine storm, NK cytolytic capacity and perforin protein levels may be consumed by effecting antiviral activity and individuals with lower baseline expression may be more prone to 'exhaustion', which could lead to defective regulation of the immune response.

1.5.2 | Overproduction of pro-inflammatory cytokines, increased neutrophil-lymphocyte ratio and monocytosis

Five studies assessing the cytokine release in COVID-19 reported increase of IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-17, IL-18, tumour necrosis factor-α (TNF-α), IFN-α 2, IFN-γ, granulocyte colony-stimulating factor (G-CSF).[44] Some are produced by neutrophils and IL-8, IL-17 and TNF-α promote or are chemotactic for neutrophils.[45-47] High-serum IL-6, IL-8 and TNF-α levels at the time of hospitalization were strong and independent predictors of patient mortality.[48] Severe COVID-19 is also associated with an increased neutrophil/lymphocyte ratio (NLR), suggestive of systemic inflammation and itself indicative of a poor prognosis.[49] Increased monocyte levels are also present, and these leucocytes are potent producers of IL-6 in severe COVID-19.[50] Neutrophils can promote the expression of IL-6[51] and also express cytokines such as TNF-α and IL-17 that can then, in turn, also promote IL-6 expression.[52]

1.5.3 | Increased IL-6 expression

IL-6 is a multifunctional cytokine synthesized by immune and stromal cells in response to danger signals[36] and an initiator of innate defence.[53] Raised IL-6 levels, as stated, are associated with COVID-19-related mortality,[36] and the daily change in the ratio of IL-6 to IL-10 (an anti-inflammatory cytokine) in patients hospitalized with COVID-19 forms the basis of a new prediction tool for disease severity (raised IL-6/IL-10 ratios being associated with a poorer prognosis).[54] Elevated levels of IL-6 have also been observed in conditions associated with a higher risk of severe COVID-19 including obesity, chronic heart disease and diabetes.[55,56] Raised IL-6 is associated with scarring,[57] thrombosis[58] and neutrophilia[59] —all features of severe COVID-19-related lung disease in particular.

1.5.4 | Raised IL-6 expression leads to reduced cytotoxic activity of NK cells and CTL

IL-6 is known to reduce both perforin and granzyme expression.[60] Raised IL-6 cytokine levels have the potential to impact negatively on both NK cells and CTL antiviral activity, which can lead to a reduction in both viral clearance and immunoregulation.[34] Therefore, a detrimental pathogenic feedback loop could evolve.

All four steps described above have been documented as having a poor prognosis.[34,61] It is possible also that this type of pathogenic feedback loop could

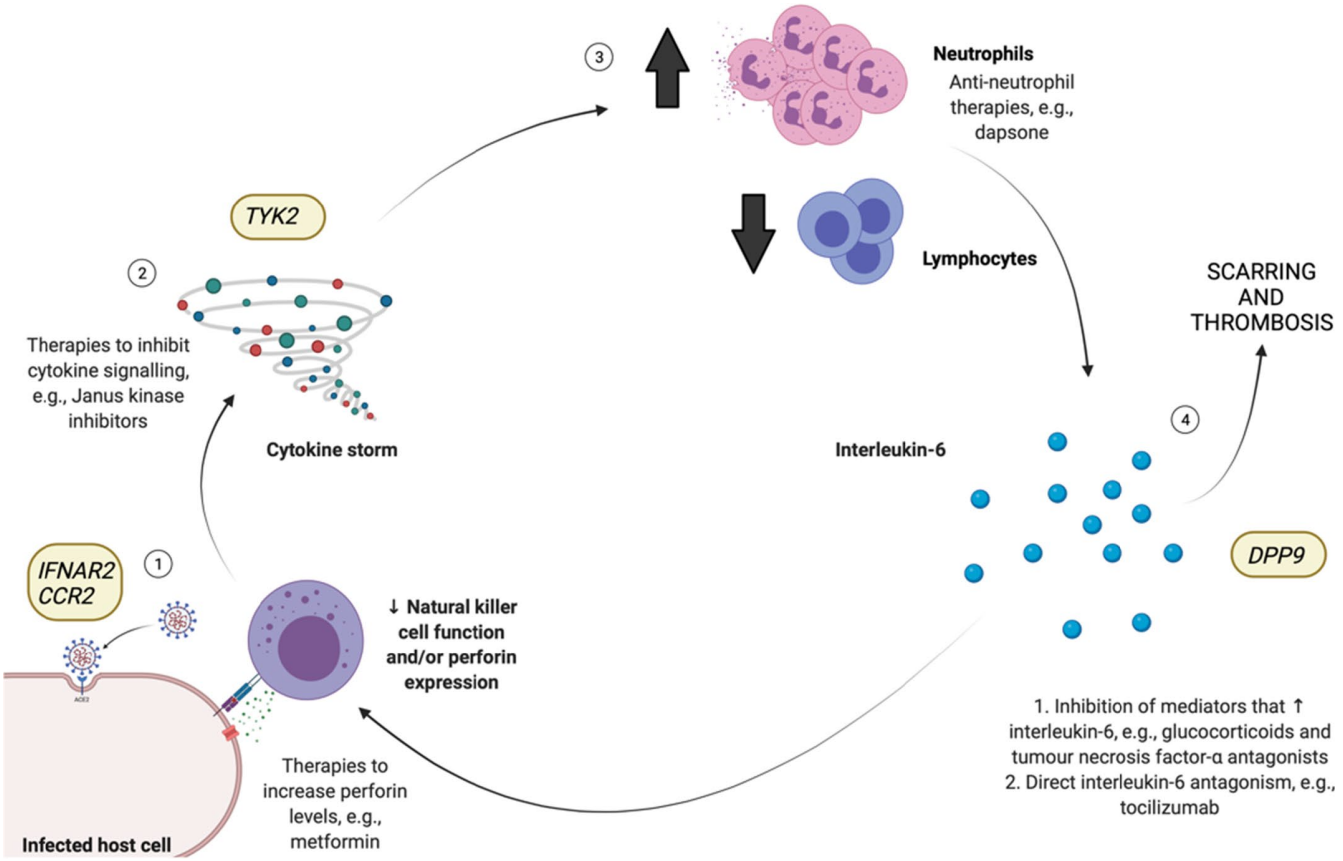

FIGURE 1 Model of a potential pathogenic auto-inflammatory cycle in COVID-19 infection. (1) Reduced perforin expression and/or function and defective natural killer cell function lead to decreased regulation of microbial-driven immune activation. (2) This results in the development of a cytokine storm characterized by increased levels of pro-inflammatory cytokines, eg tumour necrosis factor-α. (3) Pro-inflammatory cytokines cause neutrophil accumulation and activation, with an increased neutrophil/lymphocyte ratio. (4) Secretion of interleukin-6 is promoted, which contributes to scarring and thrombosis associated with severe COVID-19-related lung disease. Raised interleukin-6 secretion will also promote the suppression of perforin expression and decreased natural cell killer function, completing a pathogenic feedback loop. At each stage in the cycle, therapeutic strategies are described and relevant genes in which variants associated with severe SARS-CoV-2 infection have been identified are highlighted in yellow

also be operative in other diverse, neutrophilic auto-inflammatory diseases such as rheumatoid arthritis or hidradenitis suppurativa.

1.6 | Genes associated with severe COVID-19 infection

The results of the GenOMICC (Genetics of Mortality In Critical Care) genome-wide association study (GWAS) in 2244 critically ill COVID-19 patients from 208 UK intensive care units were recently reported.[62] The study identified and replicated novel genome-wide significant associations at four gene cluster sites associated with genes encoding antiviral restriction enzyme activators (*OAS1*, *OAS2, OAS3*), tyrosine kinase 2 (*TYK2*), dipeptidyl peptidase 9 (*DPP9*) and (the IFN-α receptor gene) *IFNAR2*. Using Mendelian randomization, the authors found evidence of a causal link for low expression of *IFNAR2* and high expression of *TYK2* with life-threatening disease. Furthermore, transcriptome-wide association in lung tissue revealed that high lung expression of the receptor *CCR2* was associated with severe COVID-19.[62] A more recent integrative genetic analysis re-affirmed the central role of inflammatory response and antiviral host response signalling, including *CXCR6, CCR9, CCR5, XCR1, IFNAR2, IL10RB, OAS1, OAS3 and JAK-STAT* signalling pathways.[63]

Whilst the implicated encoded proteins regulate a multitude of immunological pathways potentially underpinning host response to viral pathogens, four have the potential to impact on the feedback loop outlined in Figure 1. As regards *IFNAR2* (which encodes for a ubiquitously expressed receptor which binds Type 1 IFNs), IFN-α has been reported to increase both perforin and perforin mRNA expression.[64,65] TYK2, which is a part of the Janus kinase family of enzymes, is involved in promoting perforin-deficient associated

cytokine storm.[66] DPP9 can promote both IL-6 and TNF-α secretion.[67] CCR2 is expressed by different cells and can be involved in migration of cells such as monocytes/macrophages and neutrophils. It has been shown in the case of influenza that a significant proportion of NK cells migrate in a CCR2-dependent fashion.[68] Other genetic/epigenetic studies have highlighted the roles of IFN-mediated immune signalling, chemokine, chemotaxis and other impaired immune responses.[69] Meanwhile, interrogation of the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database confirms the central role of perforin in the protein network of the identified associations (Figure 2).

In essence, these identified genetic mutations associated with severe COVID-19 disease impact expression and/or function of key players in our proposed autoinflammatory pathogenic feedback loop (Figure 1).

1.7 | Therapeutic potential

The presence of a pathogenic feedback loop would suggest two pharmacological approaches. Firstly, combining drugs to 'attack' different parts of the loop, and secondly that earlier treatment may be more efficacious. Tocilizumab, an anti-IL6 receptor biologic, has already been given a UK license for COVID therapy. Dapsone, a drug with known anti-neutrophil and TNF-α properties,[70] has been advocated as a potential therapy[71] and is currently undergoing clinical trials to evaluate this further (DAP-CORONA). Janus kinase (JAK) inhibitors can ameliorate inflammatory models of cytokine storm amongst perforin deficient (−/−) mice.[66] IL-1 receptor antagonist anakinra has been effective in treating some cytokine storm syndromes.[72] Corticosteroid therapy has multiple potential points of action, including inhibiting neutrophilic function,[73] decreasing TNF-α and IL-6 levels[74] as

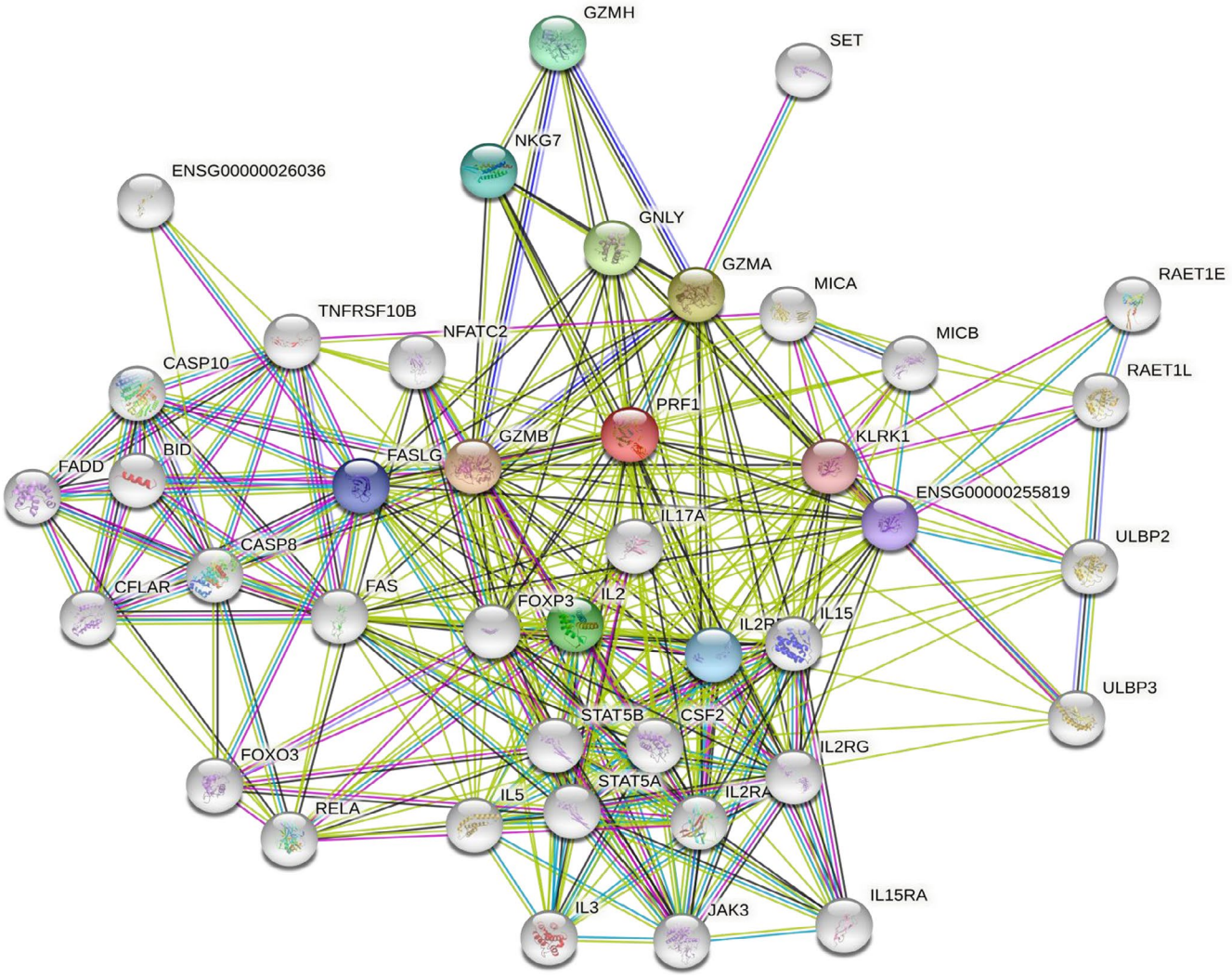

FIGURE 2 Protein network association analysis for perforin (PRF1) using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database; network nodes represent proteins; edges represent protein-protein associations

well as inhibiting scar formation.[75] Initial studies looking at combined and/or early drug therapy, such as the addition of anakinra or tocilizumab to systemic corticosteroids, have shown promise.[76,77]

2 | CONCLUSIONS

Since the onset of the COVID-19 pandemic, several factors have been identified which are associated either with disease resistance (including childhood, female gender, normal range body mass index, oestrogen medication) or with susceptibility (aged 70 years and over, male gender, obesity, cardiovascular disease, diabetes). All these factors also have in common an association with raised or decreased perforin expression/function, suggesting that perforin is an important mediator of resistance to COVID-19 infection. Integrating this information with pathways involving NK cell/perforin suggests the possibility of a pathogenic auto-inflammatory feedback loop (Figure 1).

CONFLICTS OF INTEREST

None

AUTHOR CONTRIBUTIONS

All authors contributed to the writing, editing and reviewing of this paper and approved the final version.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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