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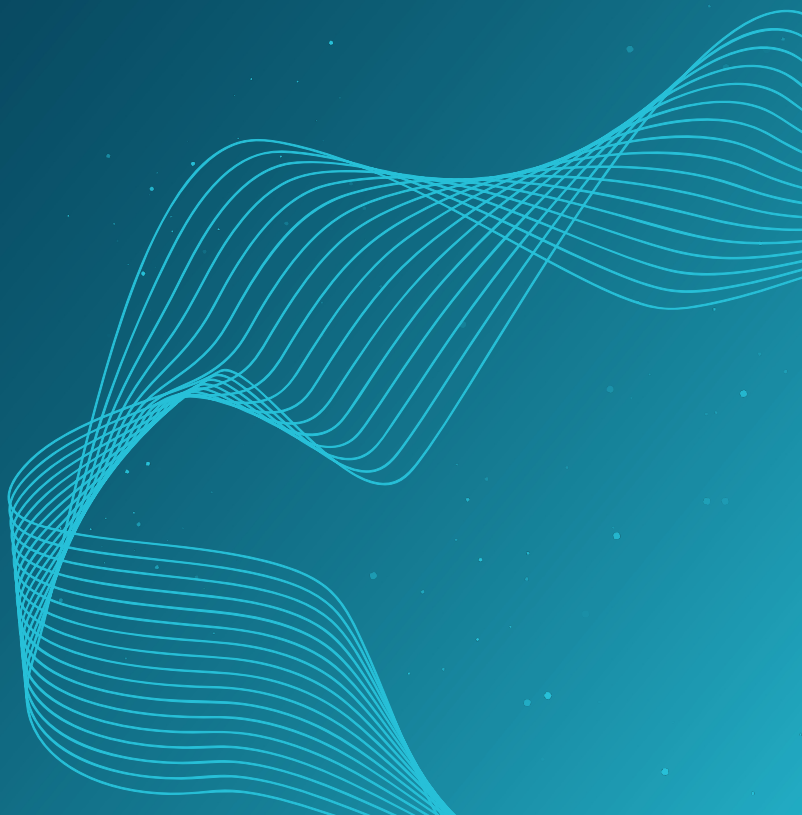
COVID-19: Biomarker Analysis

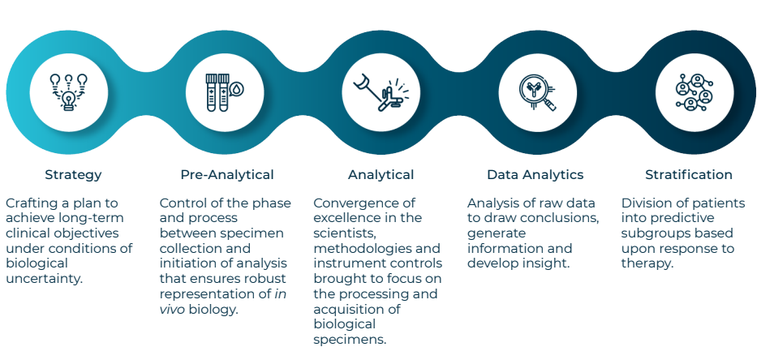


Contents

Introduction

At the dawn of 2020 it would have been near impossible to believe that our vocabulary would become so dominated by words such as “Pandemic”, “Quarantine”, “Isolation”, “Distancing” and “Unprecedented”. The global impact of COVID-19 and the attempts to curtail its spread occurred at an extraordinary pace. Despite the rapid scientific response to the disease the world still has a limited understanding of the biological extent of the disease and the long-term post-COVID health consequences.

Synexa Life Science’s expertise in immunology biomarker and translational research has allowed us to provide support to groups at the coal face of SARS-CoV-2 therapeutic and vaccine development programs. This COVID Biomarker eBook is an overview of our portfolio of COVID-19 related biomarkers and describes their utilisation in terms of the “Analytical Continuum”.



Strategy
Crafting a plan to achieve long-term clinical objectives under conditions of biological uncertainty.

Pre-Analytical
Control of the phase and process between specimen collection and initiation of analysis that ensures robust representation of *in vivo* biology.

Analytical
Convergence of excellence in the scientists, methodologies and instrument controls brought to focus on the processing and acquisition of biological specimens.

Data Analytics
Analysis of raw data to draw conclusions, generate information and develop insight.

Stratification
Division of patients into predictive subgroups based upon response to therapy.

We would welcome opportunities for collaboration in the use of biomarkers to help deepen the understanding of the pathobiology of COVID-19, predict disease severity and stratify therapeutic response.

We wish all readers of this eBook and their family’s health and well-being.

Dr. Justin Devine
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Chief Medical Officer

SARS-CoV-2 Serology Panel

INSIGHT

Antibody diagnostic assays play an important role in enhancing diagnostic capability of healthcare services and understanding the true scale of human-to-human transmission of the novel coronavirus.

These assays, which includes the SARS-CoV-2 serology assay, provide a wide range of applications which cannot be achieved by conventional molecular tests alone, such as measuring humoral responses, determining seroconversion and identification of convalescent individuals who may be at risk of re-infection.

At Synexa Life Sciences, we've developed an ELISA-based SARS-CoV-2 serology assay for the detection of several antibody isotypes, including IgG and IgM as well as IgA - the predominant antibody present in mucosal tissue. In addition to isotype detection, the assay is used to detect the presence of neutralising antibodies which prevent the receptor binding domain (RBD) of the SARS-CoV-2 spike (S) protein from binding to the target receptor (ACE2).

BIOLOGY

COVID-19 is the third known zoonotic coronavirus disease after SARS and Middle East Respiratory Syndrome (MERS). The limited availability of molecular testing for viral illness and the likelihood of a significant proportion of infected individuals having no or trivial symptoms may exacerbate the spread of the disease.

The coronavirus is a positive-sense, enveloped single-stranded RNA virus. Several structural glycoproteins are expressed on or in the lipid envelope of SARS-CoV-2, namely the membrane (M), envelope (E) and spike (S) proteins (Figure 1). The S protein is among the most immunogenic structural peptides of the virus and plays a pivotal role in viral entry. The trimeric S protein consists of two subunits, S1 and S2. The former is responsible for binding to the host cell receptor and contains a receptor binding domain (RBD) while the latter contains elements required for membrane fusion. The SARS-CoV and SARS-CoV-2 species of the virus specifically target the angiotensin-converting enzyme (ACE2) receptor, expressed on type II pneumocytes and endothelial, myocardial and gut mucosa cells.

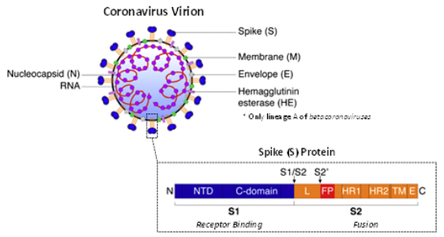

Figure 1. Schematic representation of the coronavirus virion (Reproduced from Amanat et al. 2020).

Conventional IgG and IgM serology assays are used in monitoring recovery in convalescent subjects as well as provide insight into the virus-targeted humoral response. IgM antibodies are generally the first and predominant antibody produced following infection and largely confined to the intravascular pool whereas IgG antibodies are the most abundant isotype in serum (Figure 2).

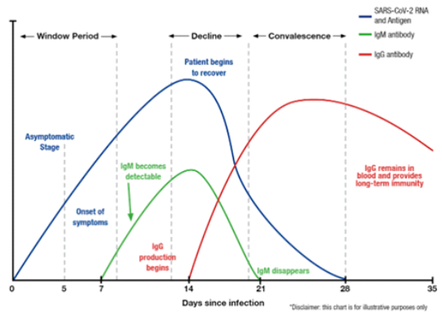

Figure 2. Schematic representation of immunoglobulin levels during the typical CoV infection. (Figure is for illustrative purposes only, http://www.diazyme.com/covid-19-antibody-tests)

Despite their abundance in serum, the presence of IgG is less prevalent in mucosal tissues. Neutralising anti-SARS-CoV-2 antibodies, specifically targeting S1, are a major target for current therapies and play an important role in interrupting entry and viral proliferation.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

Synexa Life Science's SARS-CoV-2 serology assay was designed as a versatile, high throughput diagnostic test which is compatible for multiple for use in detection of anti-SARS-CoV-2 antibodies in venous drawn samples (serum and plasma), saliva samples as well as dried blood spot (DBS) samples for enhanced diagnostics availability. Serum and plasma samples should be processed and stored at -70°C soon after collection to maintain sample stability. Samples stored in DBS are stable at 2-8°C for two weeks and thereafter should be stored at -20°C. Following elution, samples must be stored at -70°C.

ANALYTICAL

Antibody detection is performed using a sandwich ELISA format. The anti-viral antibodies are immobilised using a recombinantly expressed antigen, viral spike protein (S1 subunit), and isotype detection is achieved using the relevant HRP-conjugated secondary antibodies. A competitive ELISA format is used for the detection of anti-SARS-CoV-2 neutralising antibodies (nAb) where ACE2-conjugates function as the detection molecule (Figure 3).

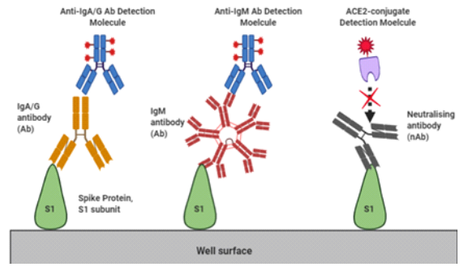

Figure 3. Schematic representation of serology assay design.

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DATA ANALYTICS

The assay provides qualitative detection of anti-SARS-CoV-2 antibodies (IgM, IgG, IgA and nAb) against a batch specific assay cut point derived from a panel of SARS-CoV-2 naïve subject samples. The performance of each assay will be assessed against three levels of quality (positive) control samples prepared from convalescent sera.

A cumulative assessment of the anti-SARS-CoV-2 antibody isotypes (IgA, IgG, IgM and nAb) will be conducted to improve the specificity and selectivity resolution of the humoral response against SARS-CoV-2.

STRATIFICATION

The temporal nature of the IgG, IgA and IgM isotype profile provides valuable information on the humoral response of the patient. Studies have identified anti-SARS-CoV-2 seroconversion at roughly day 5-8 of infection for IgM/A and day 14 for IgG (although variations in the order of isotype seroconversion have also been reported). IgM and IgA titer levels increased up to 15 days after exposure whereas IgG antibody levels continued to increase up until day 21.

There is a strong correlation between anti-S1 IgG response and nAb response. Furthermore, the intensity of isotype responses are heterogeneous and sparse evidence suggests that that mild or asymptomatic infection may result in substantially lower IgG responses. This assay will greatly improve screening of previously infected individuals to provide an understanding of a developed immunogenic response to infection.

> Synexa Life Sciences has developed an ELISA-based SARS-CoV-2 serology assay for the detection of several antibody isotypes, including IgG and IgM as well as IgA.

REFERENCES

1. Amanat et al. 2020. Nat Med. 26: 1033–1036
2. Food and Drug Administration, Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products. U.S. Department of Health and Human Services (2019). FDA, CDER, CBER.
3. Infantino et al. 2020. IMAJ. 22: 203-10.
4. Krishnamurthy et al. 2020. MedRxiv.
5. Long et al. 2020. Nat Med. 395(10236): 1527-9.
6. Sullivan et al. 2020. JMIR Public Health and Surveillance.
7. Woo et al. 2004. Clin Diagn Lab Immunol. 11:665-8.
8. Yuan et al. 2020. Science. 368(6491):630-633

Cellular Immune Responses to SARS-CoV-2

INSIGHT

Coronavirus disease (COVID-19) has been declared a world-wide pandemic and has affected over 8.5 million people globally with almost 1 000 000 deaths reported to date.

Knowledge regarding the pathogenesis of SARS-CoV-2 infection and subsequent immunological responses will be critical for the design and development of an efficacious vaccine. These immunological insights may also be important to inform future social distancing measures to control further outbreaks of the virus.

BIOLOGY

Due to the rapid emergence of the pandemic, information on the adaptive T cell responses to the virus is not fully understood.

Uncertainty exists about whether adaptive immune responses to SARS-CoV-2 are protective or contribute to disease pathogenesis. On one hand, as has been observed for SARS-CoV and other respiratory viruses, it is likely that substantial T cell and neutralising antibody responses develop to SARS-CoV-2 which contribute to viral clearance and the development of long-lived immune memory. On the other hand, protective immune responses can fail to develop due to a T cell or antibody response of insufficient magnitude and/or longevity, with the neutralising antibody response dependant on CD4+ T cells. CD4+ T follicular helper (Tfh) cells play a critical role in the generation of high affinity and long-lived antibody responses. Thus, there is need to understand the role of T cell-directed immunity in ameliorating the severity and/or duration of COVID-19, by examining the precise phenotype, magnitude and longevity of SARS-CoV-2-specific T cells.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

For flow cytometry and ELISpot analysis of cellular responses to SARS-CoV-2, the choice of assay matrix is critical.

Whilst ELISpot assays are typically run using cryopreserved PBMC, both whole blood and PBMC can be used for flow cytometry assays. The selection of sample matrix often relates to the analytical requirements as well as the logistical capabilities of the sites collecting and processing the samples. Whilst cryopreserved PBMC allow for shipping of samples and optimised batching of samples for analysis, the preparation of the PBMC is crucial to ensure high-quality samples for robust and accurate data analysis.

Adherence to strict SOPs for the isolation and cryopreservation of these cells is important and requires highly trained and competent staff, as well as specialised equipment (e.g. cell counters and liquid nitrogen storage facilities). The use of whole blood in flow cytometry assays can simplify the work flow for sites involved in clinical studies.

However, the shorter stability of the blood samples requires that they can be transported to a central lab within a specified time frame for analysis which can present logistical difficulties.

Irrespective of the sample matrix of choice, preliminary assessments regarding the stability of the chosen biomarkers for the assay read-out should be performed to establish suitability of the sample matrix for analysis.

ANALYTICAL

For the assessment of immune cell responses to SARS-CoV-2 antigens, both flow cytometry and ELISpot are acceptable techniques.

Flow cytometry offers the advantage of being able to multiplex markers of interest in a single experiment, either using a single or multiple flow cytometry panels depending on the instrument capabilities and selected markers. However, flow cytometry typically requires higher numbers of cells and more technical expertise than ELISpot which may influence the choice of analytical technique.

ELISpot assays remain the regulatory gold-standard for examining cellular immune responses to vaccine antigens, but are limited to one or two functional read-outs, usually IFN-γ and/or IL-2.

The design of flow cytometry panels is an important step in the development of assays used to assess cellular immune responses to disease vectors and/or vaccines or therapies to the disease in question.

> A DETAILED UNDERSTANDING OF THE IMMUNE RESPONSE TO SARS-CoV-2 IS IMPORTANT AT EVERY STAGE OF VACCINE DESIGN AND DEVELOPMENT.

The choice of markers that are informative for the study population in question are critical to ensure useful data from the assays performed. When assessing cellular immune responses to viruses and/or vaccines, a number of different approaches are available.

Traditionally, specific cytokines that are produced by different cell subsets are used to determine memory recall responses to antigen-stimulation and identification of antigen-specific cells (example shown in Figure 1). More recently, activation-induced markers (AIM) are being used in place of cytokines to delineate antigen-specific cells. AIM assays include selected surface markers of cellular activation to identify antigen-specific cell populations and have been shown to accurately capture the pool of antigen-specific cells.

For both flow cytometry and ELISpot assays, it is important to carefully select assay controls and specific antigens of interest. Typically, a mitogen (e.g. PMA/Ionomycin, PHA or SEB) is selected as the positive assay control. Study-specific antigens related to the disease or vaccine in question also need to be identified and qualified prior to being used to stimulate clinical samples.

CELLULAR IMMUNE RESPONSES TO SARS-COV-2

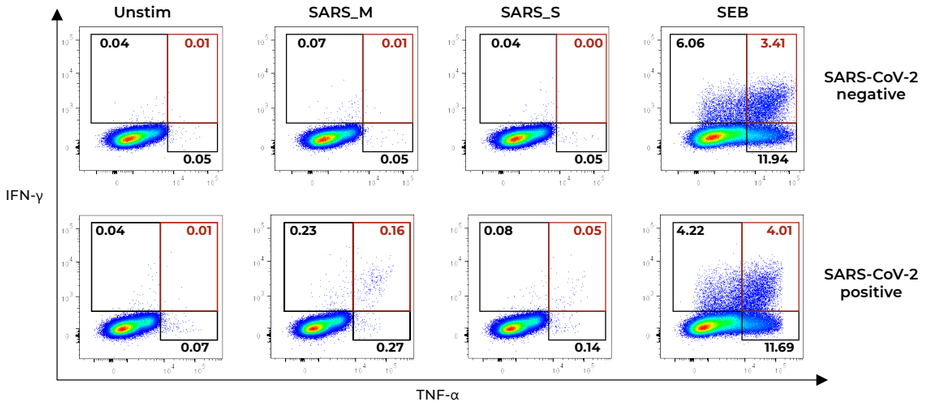

Figure 1: Cellular immune responses to SARS-CoV-2 peptides. Cryopreserved PBMC from COVID-19 convalescent donors were stimulated with peptide pools from either the membrane (SARS_M; second column) or spike proteins (SARS_S; third column) from SARS-CoV-2. SEB (last column) was used as the positive assay control. IFN-γ and TNF-α production by CD4+ T cells were used to characterise cellular responses in SARS-CoV-2 negative donors (top panel) and SARS-CoV-2 positive donors (bottom panel).

DATA ANALYTICS

Data analysis of flow cytometry data can be complex and should follow carefully designed processes to ensure robust reportable data.

Depending on the size and complexity of the flow cytometry panels used to process the samples and the gating strategies employed to analyse the acquired data, data sets can be large and intricate. The data analytics strategy should consist of tools and technologies to perform data exploration, standardisation, visualization, and potentially integration with other data sources (e.g. soluble cytokines). Strategies for quality control of the data, either manual or automated, should be implemented in order to make sure that only high quality “events” are analysed and reported.

Numerous flow cytometry analysis software packages are available and should be used by experienced operators.

STRATIFICATION

Understanding cellular immune responses to natural SARS-CoV-2 infection as well as in the context of vaccination will help predict patient disease morbidity and potential for immunity. The ability to quantify virus-specific CD4+ and CD8+ T cells is important in providing insights into immunity and pathogenesis of SARS-CoV-2 infection, as well as vaccine design and evaluation of candidate vaccines.

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REFERENCES

1. Coronavirus disease (COVID-19) Situation Report – 152 Data as received by WHO from national authorities by 10:00 CEST, 20 June 2020.
2. Crotty, S. (2019) T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. Immunity 50, 1132-1148.
3. Grifoni, A., Weiskopf, D., Ramirez, et al. (2020) Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell, doi: https://doi.org/10.1016/j.cell.2020.05.015.
4. Guo, X., Guo, Z., Duan, C., et al. (2020). Long-Term Persistence of IgG Antibodies in SARS-CoV Infected Healthcare Workers. medRxiv, 2020.2002.2012.20021386.
5. Janetzki, S. (2015) Immune monitoring technology primer: the enzyme-linked immunospot (Elispot) and Fluorospot assay. J Immunother Cancer. 3:30, 1-2.
6. Kissler, S.M., Tedijanto, C., Goldstein, E., et al. (2020). Projecting the transmission dynamics of SARS-CoV-2 through the postpandemic period. Science, eabb5793.
7. Sallusto, F., Lanzavecchia, A., Araki, K., and Ahmed, R. (2010). From vaccines to memory and back. Immunity 33, 451-463.
8. Wong, C.K., Lam, C.W., Wu, A.K., et al. (2004). Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. Clin Exp Immunol 136, 95-103.
9. Zhang, X. Tan, Y., Ling, Y., et al. (2020) Viral and host factors related to the clinic outcome of COVID-19. Nature, https://doi.org/10.1038/ s41586-020-2355-0.

Investigating Immunological Response to COVID-19 using NanoString nCounter Technology

INSIGHT

COVID-19 symptoms are frequently compared to Severe Acute Respiratory Syndrome (SARS), also caused by a coronavirus, where cytokine storm and other dysregulated immune and inflammatory responses are associated with disease progression and severity.

In the absence of an effective antiviral treatment, alternative host-directed therapies are being explored, with focus on the control of runaway inflammation and the cytokine storm. However, more recent gene expression work suggests that the inflammatory and immune responses to COVID-19 are dynamic, and vary over the course of illness, finding that the peak in pro-inflammatory response lagged behind the nadir of respiratory function, implicating a different set of inflammatory cytokines to previous studies. While the sample size is limited, it highlights the need for controlled time-point sampling when searching for COVID-19 biomarkers.

BIOLOGY

COVID-19 Infection

The most common symptoms of COVID-19 are fever, shortness of breath, expelling mucous, fatigue, dry cough, and myalgia. Peripheral blood examination counts of COVID-19 patients have found that many patients have decreased counts of leukocytes, lymphocytes (T-cells in particular), eosinophils, platelets, and haemoglobin, but have higher neutrophil-lymphocyte ratio (NLR) and monocyte-lymphocyte ratio (MLR).

The virus is highly contagious, and the severity of infection ranges from asymptomatic or mild upper respiratory tract infection to severe infections. Cases of respiratory failure, severe viral pneumonia, or secondary haemophagocytic lymphohistiocytosis (sHLH) leading to organ failure may be fatal. This range in severity appears to be underpinned by differences in host-pathogen interaction, host immune response, and pathogen immune evasion strategy. The inflammatory response, in particular, is thought to underpin COVID-19 pathogenesis.

Host Immune Response

When the SARS-CoV-2 virus enters the cell, it does so via the angiotensin-converting enzyme-2 (ACE-2) entry receptor, triggering the adaptive immune response which attempts to control the viral infection. It is sensed by Toll-like receptor 7 (TLR7) which leads to activation of the TLR-mediated pro-inflammatory response, with production of alpha interferon, Tumour Necrosis Factor alpha (TNF-α), and the secretion of interleukin 12 (IL-12) and IL-6. This, in turn, results in the formation of CD8+-specific cytotoxic T cells, and the formation of antigen-specific B cells and antibody production through the CD4+ helper T cell.

When the body does not produce an adequate adaptive immune response to the infection, it results in persistent innate-induced inflammation.

This inflammation is hypothesised to lead to cytokine storm, acute respiratory distress syndrome (ARDS), and diffuse organ involvement, as has been observed in Severe Acute Respiratory Syndrome (SARS), caused by the SARS coronavirus. Reduced T-cell activation can potentiate TLR-mediated inflammation in a positive feedback loop.

In particular, IL-6 plays a pleiotropic role in immune response. While IL-6 is crucial for the formation of follicular helper T cells, TH17 subset deviation, and for the formation of long-lived plasma cells, it can also block CD8+ cytotoxic T cells by inhibiting the secretion of gamma interferon. IL-6 also induces the suppression of cytokine signalling (SOCS-3) and increases the expression of programmed cell death protein 1 (PD-1), which can incapacitate the cell-mediated antiviral response. Some studies have also found elevated plasma levels of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-7 (IL-7), interleukin-8 (IL-8), granulocyte-colony stimulating factor, interferon-γ inducible protein 10, monocyte chemo-attractant protein 1, macrophage inflammatory protein 1-α, and TNF-α in severe COVID-19 patients.

A limitation of much COVID-19 research is that the varied disease progression between patients complicates direct comparison. One genetic study called the widely-held link between COVID-19 mortality and the cytokine storm into question after finding that pro-inflammatory gene expression lagged behind the nadir of respiratory function in some cases.

This study found that in one patient only the expression of interleukin-1α (IL-1α) and interleukin-1β (IL-1 β) preceded the nadir of respiratory function. While no strong conclusions can be drawn from such a limited sample size, it highlights the need for controlled sampling over disease progression.

Risk Factors

Numerous factors play a role in increased risk of COVID-19 mortality, the greatest of which is age (thought to be due to immunosenescence, leading to a decreased number of naïve T-cells) followed by co-morbidites, including hypertension, diabetes mellitus, obesity, cardiovascular and cerebrovascular disease, and being immunosuppressed.

These at-risk populations may be unable to produce an adequate adaptive response (i.e., virus-specific CD8+ T cells), thus falling victim to innate immunity-driven inflammation. This can be particularly devastating in countries where the population demographic is skewed towards an older age distribution (e.g. Italy), or with high rates of pulmonary or immune disease such as tuberculosis or HIV/AIDS.

Under-served populations, especially hospitalised patients who require mechanical ventilation, vasopressors, or are on continuous renal replacement therapy (CRRT) or haemodyalisis (HD) have a much greater risk of inpatient mortality.

ACE2 gene polymorphisms have been suggested to play a role in susceptibility or resistance to SARS-CoV-2 infection, as well as suggesting that some polymorphisms may be more common in certain geographic areas or populations than others, but further population genetics and functional studies are required. It is also noteworthy that ACE2 modulates two of the most common COVID-19 co-morbidities – hypertension and diabetes mellitus. ACE2 disruption impacts vascular function, the renin-angiotensin system, and exacerbates cardiovascular complications associated with diabetes. ACE2 may also play a role in mediating inflammation post-infection.

Treatment

At present, there is no commercially available antiviral treatment for COVID-19, and various host-directed treatment and supportive options are being explored. These include:

- Immunosuppressive drugs such as IL-6 blockers (e.g. tocilizumab) or Janus kinase (JAK)-signal transducer and activator of transcription signalling inhibitors;
- Recombinant IFN to foster host antiviral response e.g. Type I IFNs (IFN-I);
- Corticosteroids in cases of severe hyperinflammation, where immunosuppression could improve mortality;
- Intravenous immunoglobulin;
- IL1 or IL1 receptor antagonists, e.g. canakinumab and anakinra;
- Chloroquine and hydroxychloroquine have been used to block viral replication and viral entry into the cell. Hydroxychloroquine is thought to also block TLR7 and TLR9 signalling, allowing the continuation of the CD8+ cytotoxic viral response;
- Immunomodulators (e.g. azithromycin) which blocks IL-6 and TNF-alpha;
- Angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) (usually used to treat hypertension) have been suggested as possible treatments for COVID-19. However, a retrospective clinical study found no strong association between ACEI/ARB treatment and COVID-19 health outcome in patients who were already receiving anti-hypertensive treatment.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

Blood samples should be drawn from subjects who have been COVID-19 positive (confirmed with RT-PCR testing) with varying disease/symptom severity, and have recovered (samples drawn 2 – 4 weeks after symptoms cleared). PBMCs are prepared from the blood samples, and frozen for batch processing once recruitment is complete. PBMCs from each subject should be divided into multiple stimulation groups, including a group stimulated with SARS-CoV-2 protein, a control viral protein, and an unstimulated control sample. Once samples are stimulated, the PBMCs can be lysed and RNA extracted.

In addition, whole blood samples (for PBMCs) and samples in PAXgene Blood RNA tubes are collected from subjects in the acute phase of disease, as well as post Day 14 of recovery, to study the efficacy of anti-inflammatory therapies aimed at suppressing the “cytokine storm”. PBMC samples should also be compared to PAXgene samples, as some research on asthma, comparing gene expression profiles between the two sample types, found that whole blood samples display a wider range of differentially expressed genes, and both sample types have uniquely expressed transcripts. Including both sample types ensures comparability with the samples from the recovered patients, as well as shed light on unique T-cell and monocyte-mediated processes in COVID-19.

INVESTIGATING IMMUNOLOGICAL RESPONSE TO COVID-19 USING NANOSTRING NCOUNTER TECHNOLOGY

ANALYTICAL

The panels used to quantify gene expression are the nCounter Host Response and PanCancer Immune Profiling Panels, and the COVID 19 Panel Plus codeset. The PanCancer Panel is a 770-plex expression panel covering a wide range of genes involved in immune response. While the panel was originally developed for use in immune oncology, it provides comprehensive profiling of immune cell types, common checkpoint inhibitors, CT antigens, and genes covering adaptive and innate immune response. The Host Response Panel targets genes involved in host susceptibility, interferon response, innate immune cell activation, adaptive immune response, and homeostasis.The nCounter COVID-19 Panel Plus includes a range of SARS-CoV-2 genes, in addition to probes for human and mouse ACE2.

STRATIFICATION

As there is no effective treatment currently available, and current management of infection is purely supportive in nature, understanding these responses and predicting which patients will experience more severe illness has become a focal point in COVID-19 research. The genetic landscape of a patient and the wider population seems to play a pivotal role in the immune response to COVID-19, as well as in the course of the disease. Possible stratification markers have emerged, including genetic factors, soluble biomarkers, as well as environmental factors.

Genetic polymorphisms in immune function-related genes such as human leukocyte antigen (HLA) are thought to be a possible predictor of COVID-19 severity. In a study on 28 COVID-19 patients with severe respiratory failure, the expression of HLA-DR was low and this was accompanied by profound reduction of CD4 lymphocytes, CD19 lymphocytes, and natural killer (NK) cells, 30 indicating that HLA might play an important immune-regulatory role in COVID-19. HLA genes may also affect the phenotypes associated with COVID19 infection.

C-reactive protein (CRP) is used in the diagnosis of early stages of pneumonia, and patients with severe pneumonia exhibit high levels of CRP. One study found a correlation between CRP levels and the diameter of the largest lung lesions in patients with COVID-19, suggesting that CRP may be a possible indicator for disease severity. Another study found a strong correlation between CRP levels and inpatient mortality. IL-6 and procalcitonin (PCT) have also been found to have some prognostic value, as patients with all three markers at higher levels were more likely to exhibit severe complications, including the need for ventilation.

Immunisation with the Bacillus Calmette-Guérin (BCG) vaccine, which is more commonly mandated in countries with high rates of tuberculosis, has also been found in some studies to play a role in resilience to respiratory infections, including COVID-19. Countries with high rates of BCG vaccination have experienced reduced morbidity and mortality from COVID-19, whereas countries with low rates of vaccination are more severely infected. In particular, the BCG vaccine seems to offer protection to the vaccinated elderly.

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REFERENCES

1. Allergy Asthma Clin Immunol. 2019 Nov 21;15:67
2. Cell Host Microbe. 2020 Jun 10;27(6):879-882.e2
3. Clin Infect Dis. 2020 Mar 12 : ciaa248
4. Emerg Microbes Infect. 2020 Mar 14;9(1):558-570
5. FASEB J. 2020 Jun 11 : 10.1096/fj.202001115R
6. Front Med. 2020 Jul 3;1-12
7. JAMA. 2020 Mar 23;323(18):1775-1776
8. J Allergy Clin Immunol. 2020 Jul; 146(1): 128–136.e4
9. J Clin Virol. 2020 Jun;127:104370
10. J Immunother Cancer. 2015; 3: 42
11. J Infect. 2020 Jul;81(1):e51-e60
12. J Med Virol. 2020 Jul 3 doi: 10.1002/jmv.26252
13. Lancet. 2020 Feb 15;395(10223):497-506
14. Lancet. 2020 March 28; 395(10229): 1033–1034
15. Med Mal Infect. 2020 Jun;50(4):332-334
16. MedRxiv. 2020 Jan 1
17. Nature medicine. 2007 Oct;13(10):1248-52
18. Transpl Int. 2020 Jul;33(7):824-825
19. Transpl Int. 2009 Nov;22(11):1041-50

Fighting COVID-19 & other Infectious Diseases with Immune Repertoire Sequencing

INSIGHT

The emergence and rapid worldwide spread of SARS-CoV-2 have underscored the need for the ability to quickly respond to novel biological threats in our current globalised society.

Early detection, social distancing, and basic hygiene measures have proven to be of paramount importance to mitigate the viral spread. However, a deeper understanding of the mechanistic underpinnings of new biological threats to human health is crucial. Recent studies of COVID-19 have demonstrated that repertoire sequencing of both the T- and B-cell compartments provides essential insights which are required to understand the pathogenesis.

These insights can, in turn, drive the development of novel therapies and vaccines. In this section, we explore how repertoire sequencing advances research and development in the field of infectious disease and explain how repertoire sequencing can contribute to therapy development.

BIOLOGY

Over 32 million COVID-19 cases have been reported worldwide with little under a million deaths, as of writing. Clinical symptoms vary from asymptomatic to very severe, many cases of which require mechanical ventilation. The disease severity varies considerably between age groups, with the elderly population and people with underlying medical conditions at a higher severity risk. Researchers across the world have been trying to unravel the factors that underlie this difference.

Recently, single-cell repertoire sequencing was used to chart the characteristics of respiratory immune cells in patients suffering from COVID-19. By analysing immune cells in bronchoalveolar lavage fluid from patients with varying severity of COVID-19, researchers could show enrichment of potentially SARS-CoV-2-specific, highly clonally-expanded CD8+ T cells in patients with moderate infections.

These clones had upregulated expression of genes related to tissue-residency and showed higher phenotypic variability in severe cases when compared to mild and moderate cases[1]. Using the same technology, others have shown that most immune cell types in patients with COVID-19 are characterised by a strong interferon-α response and an overall acute inflammatory response. Moreover, expansion of highly cytotoxic effector T-cell subsets, such as CD4+ and CD8+ effectors, and NKT, was associated with convalescence in moderate patients. In severe patients, the immune landscape showed an impaired interferon response, profound immune exhaustion with skewed T-cell receptor repertoire and broad T-cell expansion[2].

> RECENT STUDIES OF COVID-19 HAVE DEMONSTRATED THAT REPERTOIRE SEQUENCING OF BOTH THE T- AND B-CELL COMPARTMENTS PROVIDES ESSENTIAL INSIGHTS WHICH ARE REQUIRED TO UNDERSTAND THE PATHOGENESIS.

As the adaptive immune system plays a key role in protection from viral attack, the information required to develop new vaccines or therapeutic antibodies, is effectively contained within the immune cell repertoire. As such, the identification and characterisation of immune cells capable of recognising SARS-CoV-2 can aid in the development of novel therapeutics.

Preliminary studies of SARS-CoV-2 infections have found highly polyclonal responses with a notable degree of convergence and overlap to previous coronaviruses[2,3]. Of note, a significant proportion of individuals unexposed to SARS-CoV-2 have been found to have pre-existing T-cell clonotypes capable of recognising the pathogen[4]. Potentially, epitope overlap between SARS-CoV-2 and “common cold” coronaviruses are contributing to their higher prevalence.

Precise examination of the B- and T-cell repertoires of infected individuals could be used to guide the development of therapeutic vaccines and antibodies.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

DNA or RNA

When considering immune repertoire sequencing, there are a number of considerations regarding the starting material to be taken into account, such as: the genetic material to be analysed. Repertoire sequencing can be performed on both genomic DNA (gDNA) and RNA.

Using gDNA as input for library preparation and sequencing provides accurate quantification of relative abundances, as each cell provides a single template. In contrast, cells can contain multiple RNA copies of the same receptor sequence, leading to increased sensitivity of lowly abundant receptors, when compared to gDNA. This makes RNA well-suited as a starting material when studying rare cell populations. In addition, RNA transcripts have undergone splicing and post-transcriptional processing. This provides a number of distinct benefits, such as an increased likelihood of yielding functional receptor sequences and several advantages during library preparation.

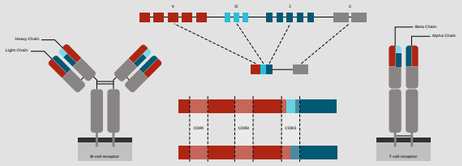

Figure 1. Schematic representation of genetic make-up of B- and T-cell receptors. V(ariable)-, D(iversity)- and J(oining)-gene segments depicted in red, yellow and blue, respectively.

5’ RACE or multiplex TCR

Library preparation can be roughly divided into two approaches: multiplex PCR and 5’ RACE. Multiplex PCR can be performed on both DNA and RNA templates and makes use of degenerate primers to amplify the receptor locus and add the adapters required for next-generation sequencing. Many combinations of degenerate primers are necessary due to significant sequence diversity between receptor gene segments.

FIGHTING COVID-19 AND OTHER INFECTIOUS DISEASES WITH IMMUNE REPERTOIRE SEQUENCING

This can introduce PCR amplification bias when certain sequences are primed – and therefore amplified – more efficiently. In contrast, 5’ RACE methods start from an RNA template and exploit the polyA-tail as a universal priming site. During the first amplification round, a 5’ adapter is added to the sequence, which is subsequently used in the second round of amplification.

The use of universal primer binding sites greatly reduces the chance of PCR bias and virtually eliminates the confounding factor introduced by degenerate primer design in multiplex PCR approaches.

An additional advantage of RNA inputs over DNA, is related to the fact that the RNA template is intronless. This allows more sequence information to be captured during library preparation, enabling identification of e.g. the receptor constant domain, an important sequence characteristic for e.g. antibody development.

ANALYTICAL

Single cell or bulk

The immunological question at hand is the most important factor for choosing either single-cell or bulk sequencing. Conventionally, bulk sequencing is used to look at high-level changes to the immune system, following (therapeutic) intervention.

Examples are the detection of the “focusing” or “narrowing” of the repertoire by looking at changes in 1) repertoire diversity (“clonality”), 2) V/J gene usage or 3) clone frequency distribution.

Conversely, single-cell sequencing is highly suited for studies focusing on identifying the TCR or BCR involved in an antigen-specific response. While bulk sequencing gives a broad overview of the most abundant sequences in a repertoire, it does not provide pairing information. Reconstruction of a functional T-cell receptor requires identification of both alpha and beta (or gamma and delta) chains, while BCR’s need both the heavy and the kappa/lambda chains.

The availability of chain pairing information in single-cell sequencing comes with a trade-off in throughput: a maximum of several thousand cells per sample. Bulk sequencing, on the other hand, can identify up to millions of unique single-chain sequences per sample.

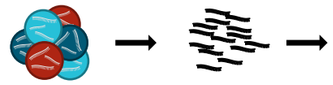

Figure 2. In bulk sequencing the genetic material is pooled, amplified and sequenced. Transcripts cannot be traced back to their original cellular context.

Figure 3. Single-cell sequencing uses molecular barcoding to ‘label’ every mRNA transcript of a cell, allowing grouping of transcripts originating from the same cell, after sequencing.

A unique feature of single-cell sequencing is the possibility to capture both the cellular transcriptome, as well as the receptor sequence of the same cell. The availability of both receptor sequence and cellular transcriptome allows for the inference of cellular phenotypes and clustering.

FIGHTING COVID-19 AND OTHER INFECTIOUS DISEASES WITH IMMUNE REPERTOIRE SEQUENCING

Finally, by performing single-cell and bulk sequencing on the same sample, both data types can be combined to couple the wider repertoire (as assayed by bulk analysis) with a detailed view on a more limited subset of cells, as provided by single-cell sequencing.

DATA ANALYTICS

Bulk repertoire sequencing experiments generate vast amounts of raw sequencing data, which not only require specialised algorithms to identify and quantify receptor sequences, but also powerful computational clusters which can handle multi-gigabyte input files.

A crucial part at the start of the analysis workflow is the normalisation of input data: when comparing samples, sequencing depth can introduce a major confounding factor, when not appropriately controlled for prior to data analysis. The interrogation of both bulk and single-cell data requires specialised knowledge of T-/B-cell immunology, bioinformatics and biostatistics.

Single-cell sequencing in particular can provide a multi-faceted view at the immune response, but the integration and interpretation of (paired) receptor and transcriptomic data presents significant analytical challenges. It should therefore be performed by experienced, multidisciplinary experts to ensure accurate and unbiased results.

STRATIFICATION

Single-cell repertoire sequencing allows researchers to combine expression data with (paired) receptor sequence and brings a deeper level of understanding of the pathogenesis and recovery in COVID-19.

Comparison of repertoires obtained from patients with different severity of disease aids the development of prognostic markers, enabling more informed treatment decisions and providing potential avenues for therapy development. Comparison of TCR and BCR repertoires of patients that have recovered from COVID-19, can help identify and quantify common, disease-specific sequences that are the result of convergent repertoire evolution.

These sequences can be used to feed vaccine development pipelines and ultimately help work toward ending the global pandemic.

> Repetoire analysis is performed in collaboration with Enpicom (www.enpicom.com)

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REFERENCES

1. A. Sette et al., "Pre-existing immunity to SARS-CoV-2: the knowns and unknowns," Nature Reviews Immunology, 2020.
2. J. D. Galson et al., "Deep sequencing of B cell receptor repertoires from COVID-19 patients reveals strong convergent immune signatures," bioRxiv, 2020.
3. S. C. A. Nielsen et al., "Human B cell clonal expansion and convergent antibody responses to SARS2 CoV-2," bioRxiv, 2020.
4. M. Liao et al., "Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19," Nature Medicine, 2020.

Cytokine Response to SARS-CoV-2 Infection

INSIGHT

The measurement of soluble cytokines and chemokines are currently the most important biomarkers to stratify SARS CoV-2 disease severity and the likely requirement for management in an intensive care unit.

These markers also are key targets and indicators of therapeutic response for drugs aimed at suppressing the “cytokine storm” response.

BIOLOGY

The immunological response to pathogens such as SARS CoV-2 involves the orchestrated mobilisation of both immune and non-immune cells.

Key to this process are the immunological signalling molecules called cytokines and chemokines. In the majority of individuals who contract SARS-CoV-2, the immunological response results in a relatively uncomplicated clinical course and disease resolution.

However, in a significant number of cases dysfunction occurs in the adaptive immune response resulting in an amplification of the innate immune response causes a hyperinflammatory state leading to a “cytokine storm” and the development of acute respiratory distress syndrome (ARDS).

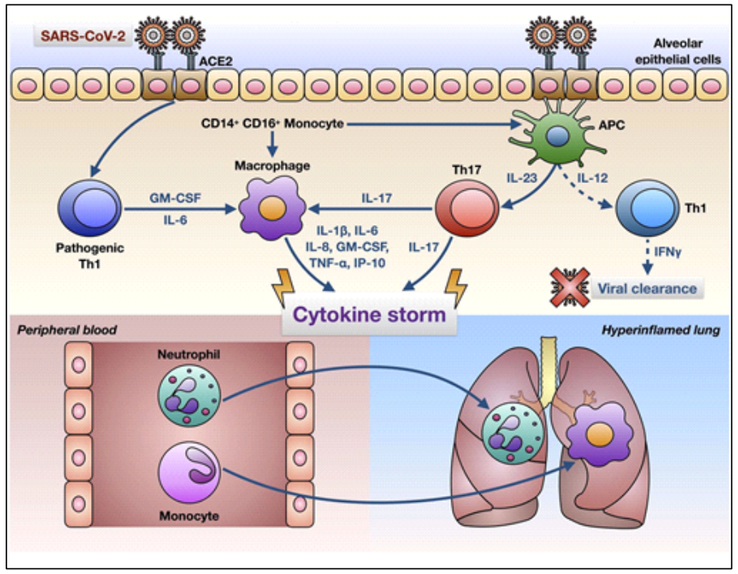

Figure 1: The figure describes the hypothesized mechanisms underlying the SARS CoV-2 cytokine storm. (Figure modified from Ther m; 2020, Vol. 14: 1–9)

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

Synexa Life Sciences offers a broad panel of validated cytokine and chemokine assays for use with serum, plasma, bronchoalveolar lavage (BAL) and culture supernatants. Serum and plasma cytokines and chemokines are generally stable, however it is recommended for samples to be processed by a transit lab as quickly as possible for freezing at -80°C.

The highly inflamed respiratory milieu of patients with ARDS may reduce the stability of cytokines and chemokines in BAL samples and it is recommended that additives are used to reduce protein degradation. Samples should be processed on ice and frozen as soon as possible.

The measurement of cytokines in PBMC culture supernatants in response to SARS CoV-2 and/or vaccine antigens provides valuable insight into the recall immune response. These samples are typically processed within specialised laboratories, reducing the time frame between supernatant harvesting and analysis or freezing.

Synexa has fully equipped cell culture facilities, capable of accommodating the analytical complexities of this sample type.

ANALYTICAL

The majority of the cytokine and chemokine assays are performed using MSD V-Plex technology. Synexa has fully validated these analytical methods which have been utilised to perform >200,000 cytokine and chemokine assays to date. Table 1 provides an overview of the key cytokines and chemokines and their role in SARS-CoV-2.

DATA ANALYTICS

The data analytics strategy consists of a portfolio of tools and technologies to perform data exploration, standardisation, visualization, and integration with other data sources such as flow cytometry.

STRATIFICATION

Cytokines and chemokines have been shown to be key biomarkers in the stratification of SARS CoV-2 disease severity, requirement for intervention (e.g. ventilation) and response to therapy. Please refer to Table 1 provided for more information.

> SYNEXA LIFE SCIENCES HAS A LARGE PANEL OF VALIDATED CYTOKINE AND CHEMOKINE ASSAYS PROVIDING INSIGHT INTO THE IMMUNOLOGICAL RESPONSE TO PATHOGENS SUCH AS SARS CoV-2.

CYTOKINE RESPONSE TO SARS-COV-2 INFECTION

Table 1: Overview of key cytokines and chemokines and their role in SARS-CoV-2

IL-1α Mainly produced by activated macrophages, neutrophils, epithelial cells, and endothelial cells. Together with IL-1β may predict the nadir of respiratory function.	**IL-1β** Inflammasome-derived cytokine, part of cytokine storm and ARDS. Elevated levels of IL-1β has been associated with SARS, hypercoagulation, and disseminated intravascular coagulation.	**IL-2** Significantly increased in patients requiring ICU admission. Levels correlate with disease severity.	**IL-4** Th2-derived cytokine. Acts through activation, proliferation, and differentiation of B lymphocytes. Proposed to have an anti-inflammatory function.
IL-5 Produced by Th2 and mast cells. Stimulates B-cell development and immunoglobulin secretion (mainly IgA). Increased in SARS-CoV-2 together with other Th2 cytokines.	**IL-6** Key proinflammatory cytokine in the development of ARDS, produced by lung epithelial cells. Levels correlate with disease severity.	**IL-7** IL-7 activates T cells, increases the production of proinflammatory cytokines, and negatively regulates TGF-β. Significantly increased in patients requiring ICU admission.	**IL-8** Key proinflammatory cytokine in the development of ARDS .
IL-10 Th2-derived cytokine which inhibits the production of proinflammatory cytokines. IL-10 may be hyper-expressed in anti-SARS-CoV-2 inflammatory response, being higher in patients of advanced age.	**IL-12/23p40** Produced by activated macrophages and induces Th1 development. Significantly increased in SARS-CoV-2 infection.	**IL-12p70** IL-12p70 has key functions in the development of Th1 and Th17 cells. IL-12 activates NK cells, which secrete IFN-γ and thereby inhibit viral replication.	**IL-13** Th2-derived cytokine. Little data on the role of this cytokine in SARS-CoV-2 however it has been observed that levels are proportional to viral load.
IL-15 IL-15 is a critical immunoregulatory cytokine with anti-viral properties. IL-15 is expressed by myeloid cells to aid in T cell responses, activate natural killer (NK) cells, and modulate inflammation.	**IL-16** Chemoattractant for cells expressing CD4. Role in SARS-CoV-2 infection is not yet understood.	**IL-17A** Contributes to severity of pneumonitis and is a key component of the cytokine storm response.	**IL-18** Inflammasome-derived cytokine, part of cytokine storm and ARDS.
IFN-γ Contributes to severity of pneumonitis. Elevated levels are associated with higher viral load and lung damage.	**TNF-α** Contributes to severity of pneumonitis with levels correlating with disease severity. Studies in SARS-CoV and MERS indicate that high levels of TNF-a are associated with a poorer prognosis.	**TNF-β** Important regulator of innate immune function. Role in SARS-CoV-2 is not yet understood.	**IP-10** Higher levels in patients requiring ICU admission and is associated with higher viral load and greater lung damage.
GM-CSF Secreted by endothelial and lung epithelial cells. Stimulates the proliferation and activation of macrophages, eosinophils, neutrophils, monocytes, and dendritic cells. Levels elevated in the acute phase of SARS-CoV-2 infection.	**MCP-1** Produced by a broad range of cells and plays an important role in the antiviral response.	**MCP-4** Induces chemotaxis in monocytes, eosinophils, T lymphocytes. Role in SARS-CoV-2 is not yet understood.	**MIP-1α** Powerful monocyte chemotactic factor expressed by a broad range of cells. Regulate migration of monocytes, memory T-cells and NK Cells. Significantly increased in the acute phase of SARS-CoV-2 in BAL and blood.
MIP-1β Powerful monocyte chemotactic factor expressed by a broad range of cells. Significantly increased at a transcriptomic level in BAL and PBMCs of SARS-CoV-2 infected individuals.	**MDC** Important mediator of lung inflammation and airway remodelling. Role in SARS-CoV-2 is not yet understood.		

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REFERENCES

1. Cytokine Growth Factor Rev. Aug 2020; 54: 62-75. https://dx.doi.org/10.1016%2Fj.cytogfr.2020.06.001
2. Emerg Microbes Infect. 2020; 9(1); 761-770. https://dx.doi.org/10.1080%2F22221751.2020.1747363
3. Front. Immunol., 16 June 2020; 11: 1441. https://doi/10.3389/fimmu.2020.01441
4. Front. Immunol., 27 May 2020; 11: 1206. https://doi/10.3389/fimmu.2020.01206

The Complement System and COVID-19

INSIGHT

Despite its origins in the respiratory epithelium, SARS-CoV-2 associated deterioration of lung function and multi-organ tissue damage in infected patients is attributed to a pathological immune hyper-response, rather than to viral particles. Hypercytokinemia and aggressive systemic inflammation in response to the virus are associated with increased morbidity and mortality, indicating the importance of targeting, and intercepting these pathological responses to a COVID-19 infection.

The complement system forms the foundation of the innate immune response to viral infection. Increasing evidence indicates that unrestrained complement activation plays a major role in epithelial/endothelial cell dysfunction, vascular injury, and clotting and thrombosis, contributing to multiple organ failure.

Inhibition of one or more components of the complement system may ameliorate or prevent host cytokine storms and pathological inflammation, representing a therapeutic intervention for COVID-19 associated tissue injury.

BIOLOGY

The complement system is comprised of over 50 proteins systematised into three activation pathways: the classical, the lectin, and the alternative pathway. These pathways converge on the cleavage of component C3 through enzyme complexes, generating complement activation peptides and fragments (C3a, C4a, C3b, and C4b).

These components perform pro-inflammatory immune functions through pathogen opsonisation, neutrophil/macrophage attraction and activation, and enhancement of T-cell response.

Studies performed in complement knockout mice demonstrate the importance of C3 activation in host protection against viral infection, and for adequate viral clearance. C3–/– mice infected with the H1N1 or H5N1 influenza A virus developed more severe bronchitis and vasculitis, and had greater numbers of macrophages in the lung parenchyma than their wild-type counterparts. Conversely, a C3–/– mouse model of infection with SARS-CoV (responsible for the 2003 coronavirus epidemic), exhibited less respiratory dysfunction and neutrophil/macrophage infiltration into the lung parenchyma, when compared to wild-type controls, despite the two groups having an equivalent viral load.

These contrasting results highlight the complexity of the complement system in severe viral infection. Complement is considered to be a “double-edged sword” of the immune system, having both a protective role, but an injurious impact when uncontrolled.

> THE COMPLEMENT SYSTEM FORMS THE FOUNDATION OF THE INNATE IMMUNE RESPONSE TO VIRAL INFECTION, AND PLAYS AN INTEGRAL ROLE IN THE SYMPTOMATIC RESPONSE TO SARS-COV-2.

In the setting of COVID-19, immunohistochemistry analysis of pulmonary and renal tissue from infected deceased patients revealed significant staining for the complement components C3, C4, and C5b-9, as well as increased serum C5a levels.

This confirms that complement over-activation may contribute to organ injury and dysfunction. Timely C3 inhibition may block downstream C3a and C5a generation, thereby preventing cytokine release in alveolar macrophages and other resident organ immune cells. In addition, systemic inflammation affecting organ microvasculature can be dampened.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

Whole blood is collected via routine venepuncture in citrate or EDTA-containing tubes. EDTA-plasma is suitable for quantification of complement factors and for assessment of activation products, while serum is better suited for analysis of complement function.

It is recommended that samples are frozen at ultra-low temperatures (ULT: -45°C to -90°C) within 2-4 hours post collection. For complement activation peptides and fragments, samples should be handled with additional caution: whole blood should be placed on ice, centrifuged at 2-8°C and stored at ULT immediately. Freezing samples at -20°C is not advised, as the slow freezing rate allows for further activation/inactivation of complement components.

Samples should be transported on dry ice and thawed/handled on ice prior to the analytical process, to prevent complement activation.

ANALYTICAL

C3 and C4

The complement proteins C3 and C4 are assessed in serum, using the Laser Nephelometry platform (Beckman). This platform is accurate and efficient, with large capacity and low variance. The platform offers a pre-validated assay by the manufacturer, but Synexa conducts these assays by including additional in-house QCs (at various analyte levels) over and above the controls provided by the supplier. These controls may vary according to the batch of reagents and are provided with each reagent order.

The validated quantification range for C3 is 5.830 – 12600.000 mg/dL and 1.670 – 4680 mg/dL for C4. Analyte stability is reliable up to 12 months in samples frozen at ULT.

Complement Activation Peptides/Fragments

For complement components with low plasma concentrations, ELISAs serve as a more appropriate platform. For assessment of complement activation peptides/fragments such as C3a, C4a or C5a, Synexa makes use of validated ELISA kits with sensitivities ranging from 15.625 pg/ml (LLOQ for C3a) to 20.000 ng/ml (ULOQ for C5a).

Stability of these analytes is more limited than that of C3/C4, with confirmed stability up to 6 months in a frozen (ULT) state.

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Complement factor Bb has also been validated by Synexa in human plasma. Analysis is conducted on an ELISA platform with a quantifiable range of 0.039 – 0.600 µg/ml.

Complement functional assays such as CH50 are conducted using a Liposome Immunoassay. This assay is applicable to serum samples and is based on a colorimetric principle with OD signal readings. The assay is performed with known quality controls, as well as calibrators. The assay is robust with a dynamic measurable range of 10.00 – 60.000 U/ml. Stability of samples is validated up to 12 months at ULT conditions.

Synexa has also developed a flow cytometry-based assay for determination of C3d and C4d deposition on circulating red blood cells (RBCs). Briefly, red blood cells from healthy subjects were used as targets, and sera from clinical trial subjects (where complement activation was being investigated) were incubated, followed by detection using specific antibodies directed to complement fragments.

Results are expressed as an MFI readout (relative to isotype controls). The assay performs robustly with intra-day and inter-day variabilities of less than 20%. Sera are kept frozen at ULT until processing.

DATA ANALYTICS

It is important to investigate the effects of a complement system therapeutic intervention in the context of the full complement system, rather than an analyte in isolation, in order to gain insight into downstream effects. It may also be beneficial to include cytokine quantification as an additional assessment of immune modification.

STRATIFICATION

Due to its potential key effects in COVID-19 infection, and in a broad range of other diseases, inhibition or modification of the complement and downstream proinflammatory effects is now recognised as an important target in the prevention of patient demise following infection with SARS-CoV-2.

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REFERENCES

1. Am J Clin Pathol, Apr 2015; 143: 558-565. https://doi.org/10.1309/AJCPXPD7ZQXNTIAL
2. Circulation, Jun 2020; 141: 1739-1741. https://doi.org/10.1161/CIRCULATIONAHA.120.047419
3. Front. Immunol, Nov 2019; 10(2539): 1-13. https://doi.org/10.3389/fimmu.2019.02539
4. J Infection, Apr 2020; 80: 656-665. https://doi.org/10.1016/j.jinf.2020.03.041
5. J Kidney Int, May 2020; 1-9. https://doi.org/10.1016/j.kint.2020.05.013
6. J Med Virol, Jan 2020; 92:424-432. https://doi.org/10.1002/jmv.25685
7. medRXiv, Jun 2020; https://doi.org/10.1101/2020.03.29.20041962
8. Nature, Jun 2020; 20: 343-344. https://doi.org/10.1038/s41577-020-0320-7
9. PLoS ONE, Mar 2011; 6(3): e17377. https://doi.org/10.1371/journal.pone.0017377

Vascular Injury Biomarkers

INSIGHT

As research continues to uncover the novel pathophysiology of COVID-19, potential treatment targets continue to evolve. Recent literature suggests the importance of vascular injury in the development of the most severe symptoms of COVID-19 and is associated with poorer prognosis in patients.

Pulmonary endothelial cells have largely been overlooked as therapeutic targets; however, these cells contribute to the initiation and propagation of acute respiratory distress syndrome (ARDS). It is therefore critical in the continued effort to confront the pandemic, to identify markers of endothelial vascular injury in patient samples.

BIOLOGY

Pulmonary endothelial cells are enriched in transcriptomic signatures implicated in immunoregulation. Following a systemic inflammatory response which implicates a barrage of inflammatory cell migration and a cytokine storm, pulmonary complications, due to endothelial cell involvement, are associated with (see Figure 1):

- Alterations in vessel barrier integrity and subsequent barrier breach;
- Coagulation activation
- Endotheliitis
- Induction of further vascular inflammation
- Dysregulated inflammatory cell infiltration.

The binding of SARS-CoV-2 to human Angiotensin-converting enzyme 2 (ACE2) activates the kallikrein-bradykinin pathway, increasing membrane permeability (Figure 1). Together with cytokine cascades, disruption of the membrane barrier activates coagulation pathways, starting with platelet production. Platelets release vascular endothelial growth factor (VEGF), which in turn triggers endothelial up-regulation of tissue factor and the coagulation cascade (Figure 1). In response, the body triggers breakdown of fibrous clots, which have a high prevalence in the lungs of COVID-19 infected patients. This in turn increases the levels of fibrin breakdown products such as D-Dimer. Endothelial cells continue to promote inflammation, increasing the expression of vascular adhesion molecules. (Figure 1).

Analysis of lung tissue of patients who have died of COVID-19 indicates a clear difference in angiogenic gene expression as a feature of SARS-CoV-2 infection. This includes widespread vascular thrombosis, severe endothelial injury and significant new vessel growth, in response to a greater degree of endotheliitis and thrombosis. Angiopoietin 2 (Ang2), a marker of endothelial activation is a ligand for endothelial Tie2 tyrosine kinase, contributing to main pathway of angiogenesis.

Therefore, potential biomarkers to assess vascular injury in COVID-19 patients include:

- VEGF
- D-Dimer
- Ang2
- Soluble E-selectin
- Tie2 Tyrosine kinase

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

For the analysis of VEGF, soluble E-selectin, Ang2 and Tie2 tyrosine kinase, samples should be transported and stored frozen and analysed as soon as possible. Synexa has assayed VEGF in serum and plasma as part of a multiplex cytokine panel. D-dimer requires sample collection in platelet free matrix whilst haemolysed samples cannot be used for analysis. Care for sample collection is therefore important.

ANALYTICAL

Common technologies for the assessment of all markers include enzyme-linked immunosorbent assays (ELISA) in both colormetric and MSD electrochemiluminescent platforms. The upper and lower limits of quantification are dependent on specific calibrators used during validation.

DATA ANALYTICS

Plasma and serum concentrations of VEGF, D-dimer and soluble E-selectin have variable ranges in healthy populations. VEGF, Ang2 and Tie2 Tyrosine kinase levels correlate to the level of angiogenesis in study subjects. Elevated D-dimer and soluble E-selectin concentrations typically indicate raised levels of coagulation and inflammation, respectively.

It is important to interpret biomarker levels both in the context of the study population of interest and within the context of the broader cytokine and growth factor regulatory network.

STRATIFICATION

There has been increased attention on these biomarkers as predictors of disease progression and severity in COVID-19 patients. Ang2 and soluble E-selectin were shown as discriminate predictors of intensive care requirement in patients following Covid-19 diagnosis. Ang2 levels correlate positively with increased pulmonary oedema and mortality in patients with ARDS. As a result, a new trial has begun testing the effectiveness of an anti-Ang2 antibody therapy for COVID-19. Several other trials have re-purposed the anti-VEGF antibody, Bevacizumab, previously used in cancer treatments, as a novel treatment in COVID-19 patients owing to an increased disease severity in patients with raised VEGF levels. D-dimer concentrations have consistently distinguished COVID-19 patients from uninfected patients with concentrations at least double that in uninfected plasma. A recent systematic review suggested that increased D-dimer levels were associated with poorer prognosis in infected patients.

An additional consideration is that of the angiogenesis markers in biopsied patient lung samples. Synexa has validated multiple gene patterns using the NanoString platform and would therefore be suited to the measurement of a similar panel previously used to assess lung samples from COVID-19 infected patients in order to qualify genetic markers of angiogenesis.

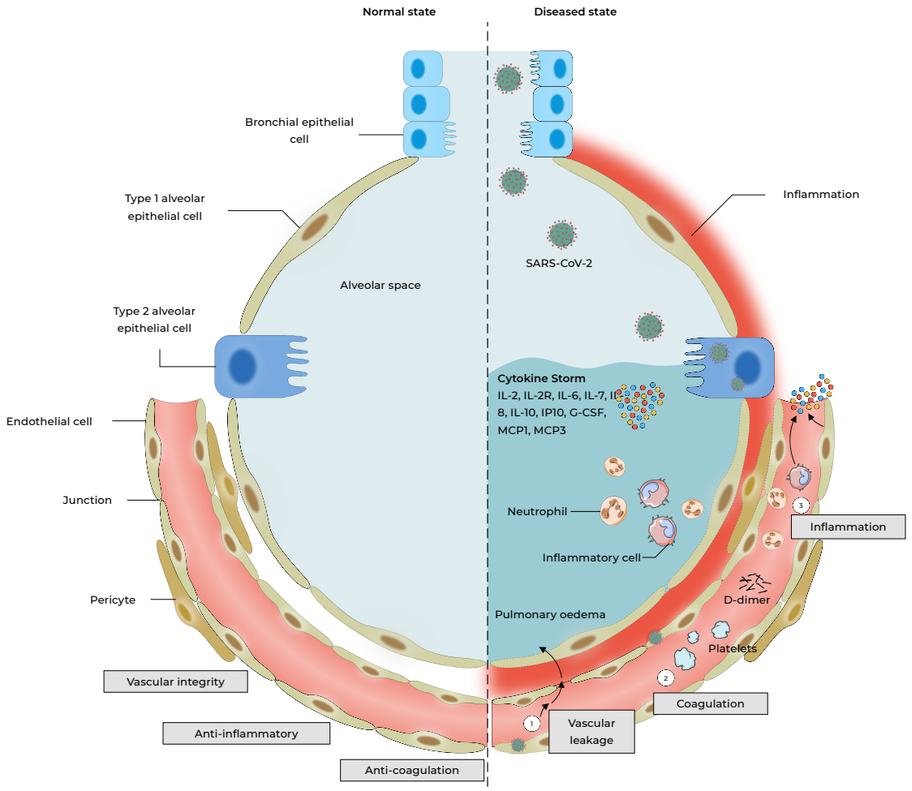

Figure 1: Proposed vessel-lung tissue interface in normal tissue and COVID-19 infection. Reproduced from Teuwen et al, 2020, Nat Rev Immunol

REFERENCES

1. Ackermann et al. 2020. NEJM. 383(2):120-128
2. Akwii et al. 2020. Cells. 8(5):471
3. Chintharlapalli et al. 2020. JCI. 130(4):1977-1990
4. Ghazizadeh et al. 2020. Biol Rep. 47(2):867-875
5. Kalucka et al. 2020. Cell. 180:794-779
6. Kong et al. 2020. Crit Care. 24:373
7. Labussière et al. 2016. 34(1):39-44
8. Li et al. 2020. Euro J Clin Microbiol Infect Dis. Jul 1:1-9
9. Moreno et al. 2020. Med Intensiva
10. Pober & Sessa, 2007. Nat Rev Immunol. 7:803-815
11. Roviezzo et al. 2005. J Pharmacol Exp Ther. 314(2):738-744
12. Smadja et al. 2020. Angiogenesis. May 27:1-10
13. Spiezia et al. 2020. Thromb Haemost. 120(6):998-1000
14. Teuwen et al. 2020. Nat Rev Immunol. 20:389-391
15. Varga et al. 2020. The Lancet. 395:1417-1418
16. Zhang et al. 2020. J Throm Thrombolysis.

Microbiome Analysis and SARS-CoV-2

INSIGHT

Intestinal ACE2 functions as a chaperone for the amino acid transporter B^0AT1. It believed that B^0AT1/ACE2 complex in the intestinal epithelium may play an important role in the regulation of gut microbiota (GM) composition and function, which in turn will impact local and systemic immune responses against pathogens such as SARS-CoV-2.

BIOLOGY

The intestinal microbiota is not only involved in digestion but has a dynamic relationship with the host's immune and nervous systems as well.

The complex interplay between the microbiota and the immune system is currently the focus of much research. This interplay starts early in life, where the steadily evolving microbiota trains the intestinal immune system to recognise and tolerate the commensal organisms, while at the same time keeping them from escaping the gut. The immune system is also taught to differentiate between commensal and potentially pathogenic bacteria, directing secretory IgA preferentially against the pathogens. In the absence of microbial stimulation, for instance in gnotobiotic mice, the intestinal immune system is largely underdeveloped, e.g. by having fewer intra-epithelial lymphocytes than normal controls. This leads to a life-long attenuation of the intestinal immune response that can only partly be overcome by restoring the gut microbiota.

Vaccination success is dependent on a well-developed immune system, including the intestinal part.

People react differently to the same vaccine, with the level of antigen-specific antibodies varying over a wide range. Among the many factors potentially responsible for generating this differential response, the makeup of the intestinal microbiota is a prime suspect. Studies on infants in several countries have confirmed that responders to rotavirus vaccine (RVV) had more abundant Gammaproteobacteria while non-responders had higher levels of Bacteroides and Prevotella. The exact mechanism has not been elucidated yet, but it is tempting to speculate that some components of the gram-negative cell (e.g. flagella, LPS etc) may act as natural adjuvants to stimulate an immune response. However, if the Proteobacteria are too abundant, dysbiosis and accompanying inflammation and increased intestinal permeability occur, once again highlighting the importance of balance in the intestinal microbiota.

New information emerging from China suggests that an appreciable fraction of recovered COVID-19 patients may lose their anti-SARS-CoV-2 IgG antibodies within a few months after recovery. This does not bode well for the development of a successful vaccine for large-scale use. It could be very informative to analyse the intestinal microbiomes of vaccine test subjects to see if there is any relationship between dysbiosis and non-optimal immune response to the vaccine, and if so, whether it can be addressed by adding probiotics to the vaccination schedule.

ANALYTICAL CONTINUUM

PRE-ANALYTICAL

It is important for reproducibility that the faecal samples are collected in a standardised way. Currently, there are no standard methods of sampling and current methods range from taking stool samples using a scoop and freezing directly, to adding the scooped stool to a transport buffer followed by freezing or taking rectal swabs followed by freezing. Synexa has obtained favourable results using a single rectal swab inserted into a tube containing ZYMO stool preservative. Under these conditions the sample is stable for 7 days at room temperature and indefinitely stable at -80°C.

> **PEOPLE REACT DIFFERENTLY TO THE SAME VACCINE, AN IMPORTANT CONSIDERATION FOR VACCINE DEVELOPMENT. THE MICROBIOME HAS BEEN TARGETED AS A PRIME SUSPECT IN THIS DIFFERENTIATION.**

ANALYTICAL

Since it is practically impossible to cultivate the bacteria making up the intestinal microbiota, only DNA-based techniques can be used to study the populations. There are two main lines of investigation into the makeup of a complex bacterial population. The first is using a targeted approach, using a conserved gene that should be present in all bacteria, e.g. the gene coding for 16S rRNA. This gene is ideal as it contains both conserved and variable regions.

The conserved regions are used to design PCR primers to amplify the gene from virtually all bacteria, while the variable regions are used to identify the different taxa by deep sequencing. This approach has been the mainstay of worldwide efforts at exploring the microbiomes of humans, other animals, plants, insects etc. Although extremely useful, it suffers from a lack of resolving power for some organisms, where it can only identify higher taxa since those lower down the taxonomic ladder have identical sequences for the 16S genes.

The second, metagenomics approach, consists of sequencing all DNA in a sample, both host and microbiota. Powerful software removes all non-bacterial DNA, followed by reconstruction of the genomes of all bacteria present. For this reconstruction it uses both overlapping sequences as well as reference genomes of sequenced species. Metagenomic reconstruction provides rich data, not only about the taxa present, but also about which genes (e.g. enzymes in certain metabolic pathways) are present.

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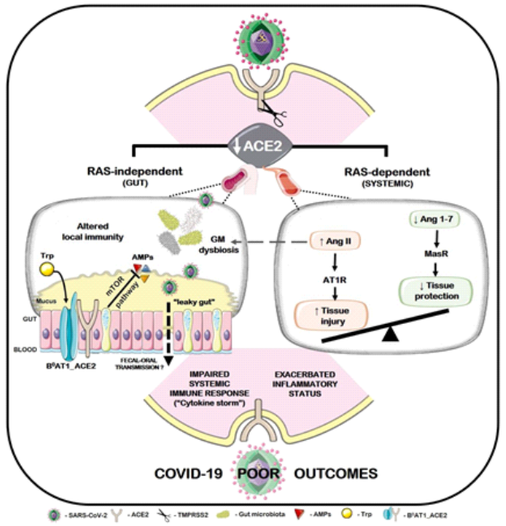

Figure 1: Putative association between ACE2 shedding after SARS-CoV-2 infection and poor outcomes in elderly COVID-19 patients with pre-existing age-related cardiovascular, cardiometabolic and cardiorenal diseases. An imbalanced Ang II/Ang 1-7 ratio, due to the loss of ACE2 protective function, favors Ang II-AT1R signaling and subsequent vasoconstrictive, pro-hypertensive, pro-oxidant, pro-inflammatory and pro-fibrotic events (RAS-dependent pathway, right). Furthermore, ACE2 shedding may also dictate a dysbiotic gut condition (RAS-independent pathway, left). Briefly, loss of ACE2 integrity, a chaperone of intestinal B0AT1 carrier, may negatively impact neutral amino acids transport with subsequent mTOR-dependent AMPs disrupted synthesis, altered local immunity and gut microbiota dysbiosis. Concurrently, increased Ang II levels may also impair GM composition and function. Altered gut barrier permeability may add an extra-level of complexity to this scenario, allowing the translocation of local microbiota components to the bloodstream, including resident SARS-CoV-2 viral particles.
Figure modified from publication: Ageing Res Rev. 2020 Sep; 62: 101123

DATA ANALYTICS

A massive amount of data is generated during sequencing runs, which requires extensive analysis before the data becomes useful. Synexa makes use of both commercial software packages providing turnkey solutions, as well as open-source programs and databases.

These are complemented by in-house software for report generation.

The aim is to obtain an overview of the population in terms of taxonomy and the relative contribution of the individual taxa for each sample.

STRATIFICATION

Gut microbiota are vital players in immunological homoeostasis and pathology. Analysis of the gut microbiome is a powerful stratification tool of differential immune response to pathogens and therapeutic interventions.

REFERENCES

1. Ageing Res Rev. 2020 Sep; 62: 101123. https://doi.org/10.1016/j.arr.2020.101123

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