Opposite influences of bacterial and fungal translocation on immune responses during COVID-19

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Abstract

Background

SARS-CoV-2 infection causes damage to the gut epithelium, allowing translocation of bacterial (lipopolysaccharide, LPS) and fungal (beta-glucan, BDG) products into circulation, contributing to inflammation. High circulating BDG levels are associated with immune activation and disease severity, underscoring the crucial role of gut permeability in COVID-19 disease progression. Currently, the influence of microbial translocation on the function of the immune system during COVID-19 infection remains unknown. We hypothesize that markers of mucosal damage, microbial translocation and inflammation will elevate during acute COVID-19 and associate with weaker anti-SARS-CoV-2 T and B-cells immune response.

Methods

Herein, we analyzed plasma and peripheral blood mononuclear cells (PBMCs) obtained from 19 acute and 15 recovered (> 6 months) COVID-19 participants. We measured plasma levels of BDG using the Fungitell assay, assessed plasma levels of I-FABP, LPS, REG3α via ELISA. PBMCs were stimulated with SARS-CoV-2 Spike peptides and T-cell production of IFN-γ, IL-2 and TNF-α were assessed using flow cytometry. Anti-SARS-CoV-2 IgG antibody levels were measured in plasma using MSD ELISA.

Results

In acute participants, spike-specific CD4 T-cell IL-2 response was positively correlated with plasma BDG levels. In recovered participants, polyfunctional spike-specific CD4 and CD8 T-cell responses were negatively correlated with BDG, while CD4 T-cell response was positively correlated with LPS levels. Antibody levels also negatively correlated with BDG and positively correlated with LPS in the recovered group. No correlation was found between gut permeability markers and spike-specific immune responses.

Conclusion

Our findings suggest contrasting effects of bacterial and fungal translocation on immune response in COVID-19. Further investigation is required to elucidate the link between these observations and the efficiency of the immune responses against SARS-CoV-2.

Résumé

Introduction

L'infection par le SRAS-CoV-2 provoque des dommages à l'épithélium intestinal, permettant le passage de produits bactériens (lipopolysaccharide, LPS) et fongiques (bêta-glucane, BDG) dans la circulation, contribuant ainsi à l'inflammation. Des niveaux élevés de BDG circulant sont associés à l'activation immunitaire et à la gravité de la maladie, soulignant le rôle crucial de la perméabilité intestinale dans la progression de la maladie COVID-19. Actuellement, l'influence de la translocation microbienne sur le fonctionnement du système immunitaire lors de la COVID-19 reste inconnue. Nous émettons l'hypothèse que les marqueurs des lésions muqueuses, de la translocation microbienne et de l'inflammation augmenteront pendant la forme aiguë du COVID-19 et s'associeront à une réponse immunitaire plus faible des lymphocytes T et B anti-SARS-CoV-2.

Méthodes

Ici, nous avons analysé le plasma et les cellules mononuclées du sang périphérique (PBMC) obtenus auprès de 19 participants atteints de COVID-19 en phase aiguë et de 15 participants guéris depuis plus de 6 mois. Nous avons mesuré les taux plasmatiques de BDG à l'aide du test Fungitell et évalué les taux plasmatiques d'I-FABP, de LPS et de REG3α via ELISA. Les PBMC ont été stimulées par les peptides de la protéine Spike du SRAS-CoV-2 et la production d'IFN-γ, d'IL-2 et de TNF-α par les lymphocytes T a été évaluée par cytométrie en flux. Les niveaux d'anticorps IgG spécifiques de la protéine Spike du SRAS-CoV-2 ont été mesurés dans le plasma à l'aide d'un ELISA MSD.

Résultats

Chez les participants en phase aiguë, la réponse T CD4 produisant de l'IL-2 en réponse aux peptides Spike était positivement corrélée aux niveaux de BDG. Chez les participants guéris, les réponses des lymphocytes T CD4 et CD8 polyfonctionnels spécifiques à Spike étaient négativement corrélées au BDG, tandis que la réponse des lymphocytes T CD4 était positivement corrélée aux niveaux plasmatiques de LPS. Les niveaux d'anticorps étaient également corrélés négativement avec le BDG

et positivement corrélés avec le LPS dans le groupe guéri. Aucune corrélation n'a été trouvée entre les marqueurs de perméabilité intestinale et les réponses immunitaires spécifiques aux peptides Spike.

Conclusions

Nos résultats suggèrent des effets contrastés de la translocation bactérienne et fongique sur la réponse immunitaire durant la COVID-19. Des recherches plus approfondies sont nécessaires pour élucider le lien entre ces observations et l'efficacité des réponses immune durant la COVID-19.

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Contribution of Authors

The project was initiated by Dr. Jean-Pierre Routy (supervisor) and Dr. Stéphane Isnard (mentor). Participant blood samples were processed into plasma and PBMCs by Tsoarello Mabanga. Dr. Stéphane Isnard proposed the experimental design and specific techniques to be used. All subsequent experiments, execution, data analysis, and reporting were performed by Simeng Bu under the guidance of Dr. Jean-Pierre Routy and Dr. Stéphane Isnard.

Statement of scientific integrity

I am familiar with the McGill University Academic Integrity Policy, and I understand the potential consequences should my thesis be found to contain plagiarized content or violate this policy in any other way. This study was approved by the research ethics board of the McGill University Health Centre.

Simeng Bu

List of Abbreviation

MERS-CoV	Middle East Respiratory Syndrome Coronavirus
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus.
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
COVID-19	Coronavirus disease 2019
I-FABP	Intestinal Fatty Acid-Binding Protein
REG3α	Regenerating islet-derived protein 3 alpha
BDG	β-D-glucan
LPS	Lipopolysaccharide
LBP	Lipopolysaccharide-Binding Protein
sCD14	soluble CD14
sST2	soluble suppression of tumorigenicity 2
IFN-γ	Interferon gamma
IL-2	Interleukin-2
TNF-α	Tumor necrosis factor alpha
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-21	Interleukin-21
IL-17A	Interleukin-17A
TGF	Transforming growth factor
CXCR5	Chemokine receptor 5
PD-1	Programmed cell death-1
ILCs	Innate lymphoid cells
CTLs	Cytotoxic T lymphocytes
NK	Natural Killer
Treg	Regulatory T
Th	T helper
Th17	T helper type 17
Tfh	T follicular helper cells
TLRs	Toll-like Receptors
RLRs	Retinoic acid-inducible gene I-like receptors
NLRs	Nucleotide-binding oligomerization domain like receptors
TCR	T-cell receptor
MHC	Major histocompatibility complex
APC	Antigen-presenting cell
CD	Cluster of differentiation
Foxp3+	Forkhead box p3+
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
IgD	Immunoglobulin D
ELISA	Enzyme-linked immunosorbent assay

PLWH	People living with HIV
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
ARDS	Acute respiratory distress syndrome
HCV	Hepatitis C virus
ART	Antiretroviral therapy
CMV	Cytomegalovirus
LCMV	Lymphocytic choriomeningitis virus
RPMI	Roswell Park Memorial Institute
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
DMSO	Dimethyl sulfoxide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GI	Gastrointestinal
IBD	Intestinal Bowel Diseases
CD	Crohn's disease
UC	Ulcerative colitis
RA	Rheumatoid arthritis
PRR	Pattern recognition receptor
PAMPs	Pathogen-associated molecular patterns
DAMPs	Damage-associated molecular patterns
GPI	Glycosylphosphatidylinositol
MFI	Mean fluorescence intensity
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
ChIP	Chromatin immunoprecipitation
CR3	Complement receptor 3

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Introduction

1.1 Human Coronaviruses

Coronaviruses are the largest known RNA viruses with enveloped, single-stranded, positive-sense genomes (approximately 26–32 kilobases in size) and belong to the family Coronaviridae (Weiss and Navas-Martin, 2005). The coronavirus family derives its name from the abundant spike proteins found on the virus' surface, which impart a distinctive crown-like appearance to the virions (Pellett et al., 2014). For numerous decades, coronaviruses have been extensively studied and recognized as significant respiratory and enteric pathogens affecting avian and mammalian species. Observations of coronavirus-like particles were also documented in reptiles (Crossley et al., 2012). Over the past few decades, several coronaviruses have crossed the species barrier into humans, causing outbreaks of severe, and often fatal, respiratory illness (Letko et al., 2020).

The coronavirus family is divided into four genera: α, β, γ, and δ coronaviruses. The α and β types primarily infect mammals, while the γ and δ types mostly affect birds or a few mammals (Tang et al., 2022). To date, only seven coronaviruses are known to infect human, causing from a mild cold to an epidemic of large-scale deaths and injuries, namely 229E, OC43, SARS, NL63, HKU1, MERS, SARS-CoV-2 (Tang *et al.*, 2022). NL63 and 229E are α coronaviruses, while OC43, HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2 are β coronaviruses (Tang *et al.*, 2022). 229E and OC43 were identified in the 1960s from individuals suffering common cold. Human coronaviruses were then not considered as deadly viruses until 2003, when the emergence of Severe Acute Respiratory Syndrome (SARS) in Guangdong, China, resulted in 774 deaths and thousands of infected participants, with a case fatality rate (CFR) of 9.6% (the CFR was around 50% among participants 65 or older) (Peiris et al., 2004; Peiris et al., 2003). In the same year, a 7-month-old kid was sent to the hospital in Amsterdam for fever, bronchiolitis and conjunctivitis. Complete sequencing of isolated virus was led to identification of the fourth human coronavirus, HCoV-NL63 (van der Hoek et al., 2004). Subsequently, HKU1 was officially classified documented by researchers at Hong Kong University in 2004 (Hu et al., 2017). Nine years later, the Middle East experienced an outbreak attributed to a

novel coronavirus known as Middle East Respiratory Syndrome Coronavirus (MERS-CoV). This time, approximately 2500 infected cases were reported including 861 deaths, resulting in a case-fatality rate of 34.4% (Cui et al., 2019). In 2019, an emerging coronavirus has presented unprecedented challenge to the global healthcare, economical and societal systems (Nicola et al., 2020). The causative agent identified from severe pneumonia cases in affected individuals identified as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which is lately known as the pathogen responsible for the Coronavirus disease 2019 (COVID-19) pandemic (Jackson et al., 2022). Symptoms associated with COVID-19 cases included acute pneumonia-related manifestations like fever, dry cough, chills, shortness of breath, and muscle pain (Lu et al., 2020). As of March 2023, more than 600 million COVID-19 cases have been declared in over 200 countries, causing more than 6 million deaths (COVID-19 Dashboard). Despite previous public health crisis caused by Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) outbreaks, the strategies for intervening against coronavirus infections are still at an early stage.

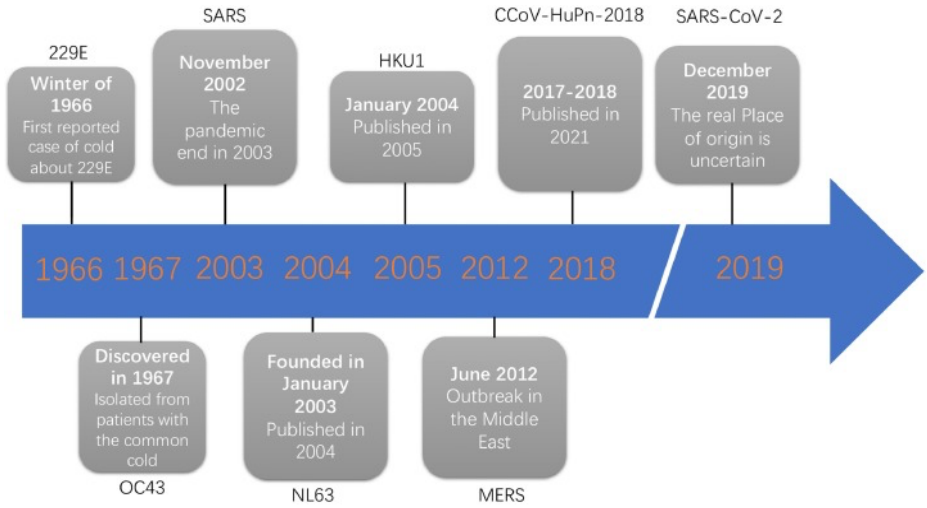

Figure 1: Timeline of all documented Human CoVs. The 229E coronavirus was initially identified in 1966 as the first known human-infecting coronavirus. Following this, OC43 was isolated from participants with common cold symptoms. Subsequently, the SARS outbreak emerged in November 2002 and concluded in 2003. The NL63 virus was identified using the virus discovery based on cDNA- amplified fragment length polymorphism (VIDISCA) and PCR methods in the same year. In 2004, HKU1 was reported and named by the University of Hong Kong. Seven years later, a novel coronavirus, MERS-CoV, caused an outbreak in the Middle East. In 2018, researchers examining nasal swabs from 301 pneumonia participants in an East Malaysian hospital discovered a new coronavirus. This virus, named CCOV-HuPn-2018 in their study, infected eight participants, excluding one child. In December 2019, the first case of SARS-CoV-2 was reported in Asia. However,

evidence from the New England Journal of Medicine revealed that cases of 2019-nCoV infection were diagnosed in Germany, suggesting transmission outside Asia. The original source of SARS-CoV-2 remains unidentified (Tang *et al.*, 2022).

1.2 SARS-CoV-2 and COVID-19

SARS-CoV-2 is an enveloped, positive-sense single-stranded RNA virus, classified upon the Betacoronavirus genus based on next-generation sequencing (Jackson *et al.*, 2022).

1.2.1 SARS-CoV-2 Lifecycle

Cross-species transmission is significantly facilitated by the pivotal process of cell entry. All coronaviruses possess a surface glycoprotein known as spike, which binds to the host-cell receptor and facilitates entry of the virus (Li, 2016). Specifically, the spike protein on viral surface binds to the host angiotensin-converting enzyme 2 (ACE2) receptor and the co-receptor TMPRSS2, promoting the fusion of the virus with the cell membrane and its endocytosis (V'Kovski et al., 2021). Subsequently, the RNA genome is released into the host-cells and translated into two major reading frames, ORF1a/b, generating polyproteins (pp1a/b) (V'Kovski *et al.*, 2021). These polyproteins are cleaved into non-structural proteins (nsps), which then assemble into the viral replication and transcription complex (V'Kovski *et al.*, 2021). Concurrently, newly translated structural and accessory proteins are integrated into the ER–Golgi intermediate compartment (ERGIC), where the assembly of virions takes place (V'Kovski *et al.*, 2021). Ultimately, these assembled virions exit the infected cells through exocytosis, completing the viral lifecycle and enabling further infection and dissemination within the host organism (V'Kovski *et al.*, 2021).

1.2.2 COVID-19 symptoms and clinical presentation

Under normal physiological condition, our body have balanced levels of pro-inflammatory and anti-inflammatory cytokines, which could be disrupted by activation of a variety of immune cells during viral infection, including SARS-CoV-2 (Tang et al., 2020a). These activated immune cells release large amounts of cytokines, and the pro-inflammatory cytokines promote activation of more immune cells in a positive feedback loop (Tang *et al.*, 2020a). In addition, large amounts of cytokines and chemokines could lead to hyperinflammation and onset of cytokine storm. The formation of cytokine

storm reduces further spread of virus in the body but causes tissues damages and causes acute respiratory distress syndrome (ARDS) and multi-organ failure (Tang *et al.*, 2020a).

Giron et al. previously demonstrated an association between the severity of COVID-19 and a state of hyperinflammation characterized by a cytokine storm (Giron et al., 2021). The systemic inflammation caused by a lung infection can indirectly disrupt other organs, including the gut barrier integrity, and increase gut microbial translocation, which further exacerbates systemic inflammation and lung damages in a positive feedback loop (Giron *et al.*, 2021). Previous studies have shown that immunopathological changes as well as diminished lymphocytes and elevated cytokines cause disease progression and death, especially in critically ill COVID-19 participants (Tang *et al.*, 2020a). Thus, early detection and control of cytokine storms are key to successfully prevent COVID-19 disease progression and minimize mortality rate.

1.2.3 COVID-19 Comorbidities

The clinical manifestations of COVID-19 are heterogeneous across populations. The term 'comorbidity' refers to any pre-existing health condition that coexists with COVID-19 (Russell et al., 2023). Numerous studies have underscored the connection between comorbidities and severity of COVID-19 illness by increasing the risk of complications and mortality. These include obesity, diabetes, chronic obstructive pulmonary disease (COPD) and asthma, cardiovascular diseases, cancer and HIV, as well as chronic kidney disease (Russell *et al.*, 2023). Obesity is proposed to be associated with chronic low-grade inflammation, characterized by increased secretion of pro-inflammatory cytokines from adipose tissue and the infiltration of leukocytes, which can impair immune function and increase susceptibility to severe COVID-19 outcomes (Muscogiuri et al., 2022). Patients with type 2 diabetes, particularly those with poorly controlled glycemia, often have co-existing cardiovascular diseases, which foster severe COVID-19 illness (Russell *et al.*, 2023). Moreover, individuals with COVID-19 and type 2 diabetes display peripheral blood monocyte activation and transition to a non-classical phenotype, alongside increased expression of interleukin-6 (IL-6) and the chemokine C-C ligand 2 (CCL2), which is indicative of a hyper-inflammatory state (Russell *et al.*,

2023). People with pre-existing respiratory conditions like COPD and asthma are more susceptible to pneumonia and ARDS upon SARS-CoV-2 infection, thus exacerbating COVID-19 outcomes (Chiner-Vives et al., 2022). Individuals with cardiovascular complications such as arrhythmias, coronary artery disease, heart failure, or a history of stroke are at higher risk of developing severe consequences from COVID-19 (Terzic and Medina-Inojosa, 2023). Immunocompromised individuals afflicted by COVID-19 due to conditions such as cancer, HIV, organ transplantation, or immunosuppressive therapy, exhibit an increased susceptibility to severe illness relative to their immunocompetent counterparts (Evans et al., 2023). Moreover, adverse outcomes have been observed in COVID-19 infected individuals undergoing recent chemotherapy and chemoimmunotherapy treatments for cancer treatment (Chavez-MacGregor et al., 2022). Recognizing these comorbidities is essential for assessing risks, planning treatments, and implementing preventive measures to mitigate the impact of COVID-19 on vulnerable groups. The transition to severe or fatal stages of the illness can happen rapidly and unpredictably. Therefore, identifying prognostic or diagnostic biomarkers to gauge COVID-19 severity is imperative. Early detection of these biomarkers is pivotal for prompt intervention and prevention of fatal outcomes.

1.2.4 COVID-19 and Gastrointestinal Presentation

Awareness of the progression of GI symptoms is essential, as these signs could potentially be among the first indications of a COVID-19 infection. Many COVID-19 participants display both GI symptoms and pneumonia-like illness, including fever, cough, and dyspnea (Pazgan-Simon et al., 2020). The spectrum of GI symptom is extensive, encompassing anorexia, diarrhea, nausea, vomiting and abdominal pain (Tian et al., 2020). Anorexia appeared to be the most commonly reported GI symptom in adults, occurring in 39.9% to 50.2% of confirmed cases, followed by diarrhea, reported for 2% to 49.5% of participants (Tian *et al.*, 2020). Previous report showed that diarrhea presents between 1 and 8 days post infection onset, with a mean onset at 3.3 days (Tian *et al.*, 2020). The prevalence of nausea and vomiting ranged between 1% and 29.4% in COVID-19–positive adults (Tian *et al.*, 2020). Abdominal pain has been less reported in the literature, with prevalence ranging

between 2.2% and 6% of participants with confirmed COVID-19 (Tian *et al.*, 2020). In a study of 116 participants in the U.S. revealed a significant proportion (31.9%) of participants experiencing concurrent GI symptoms related to SARS-CoV-2 (Cholankeril et al., 2020).

SARS-CoV-2 has been shown to directly infect epithelial cells, possibly directly leading to cell death and inflammation (Yuan et al., 2023).

1.3 Gut barrier function in healthy condition

The gut barrier is integral to preserving intestinal homeostasis and obstructing the transit of harmful substances from the intestinal lumen into systemic circulation (Isnard et al., 2021c; Takiishi et al., 2017). The mucus layer serves as the initial line of physical defense that external molecules encounter upon entering the gut lumen. It acts as a barrier, preventing direct contact between bacteria and the epithelial cells (Cornick et al., 2015).

Beneath the epithelium, the lamina propria houses the gut-associated lymphoid tissue, comprising Peyer's patches, lymphoid follicles, and intraepithelial lymphocytes, macrophages, DC and stromal cells which collectively coordinate immune surveillance and antigen sampling within the intestinal microenvironment (Mowat and Agace, 2014).

Maintaining the integrity and optimal functionality of the gut barrier is imperative for sustaining overall health and well-being.

1.3.1 Gastrointestinal Microbiota

The human body includes the gastrointestinal tract, integumentary system, and various mucosal environments, also harbors a diverse microbiota comprising bacteria, fungi, protozoa, and viruses (Zheng et al., 2020). In human, a significant proportion of the microbiota is acquired from the mother during birth (Dekaboruah et al., 2020). The microbial composition undergoes dynamic changes in the first three years of life, then stabilizes but continues to undergo minor changes in later life stages (Bhatt et al., 2017; Palmer et al., 2007). The complex gut microbiota exerts profound effects on human physiology (Thursby and Juge, 2017). The most extensively studied host-microbiota interface is the intestinal mucosa (Zheng *et al.*, 2020). Under healthy conditions, the host’s immune response to the

gut microbiota is strictly compartmentalized to the mucosa surface. Furthermore, the gut microbiota plays a substantial role in preserving gut barrier integrity through the modulation of immune responses, the generation of supportive metabolites, and the competitive inhibition of pathogen colonization (Takiishi *et al.*, 2017).

However, disruption of the gut microbiota due to environmental factors, compromised host-microbe interfaces, or immune system alterations may lead to the systemic spread of commensal microorganisms, increased susceptibility to pathogens, and abnormal immune responses (Zheng *et al.*, 2020).

1.3.2 Gut epithelium

The gut epithelium is a dynamic and multifunctional lining that serves as an interface between the external environment of the digestive tract and the internal milieu of the body. The gut epithelial barrier assumes a pivotal role in maintaining intestinal homeostasis by intricately controlling the entry of nutrients, while preventing translocation of microbes and their pro-inflammatory byproducts, across the mucosal barrier into the systemic circulation (Ouyang et al., 2023).

The epithelium comprises a single layer of distinct intestinal epithelial cells (IECs) with specialized functions, including enterocytes, goblet-cells, Paneth cells, and enteroendocrine cells (Takiishi *et al.*, 2017). Enterocytes and Paneth cells produce antimicrobial peptides crucial for pathogen control and regulation of the gut microbiota composition. Goblet-cells secrete mucins that lubricate and protect the intestinal epithelial surface, while also serving as antigen presenting cells (APCs) by delivering luminal antigens to DCs, which foster the development of Tregs (McDole et al., 2012). Tight junction complexes composed of claudins, occludin and zonulin, as well as junction adhesion molecules, establish a barrier between adjacent IECs, thereby governing permeability to water, ions, and nutrients, while also impeding pathogen entry (Takiishi *et al.*, 2017). Under normal conditions, these components are dynamically regulated, but sustained inflammation or infections can disrupt adhesion molecule expression, compromising the barrier and facilitating microbial entry.(Ouyang *et al.*, 2023).

Understanding the intricacies of the gut epithelium is crucial to unraveling key aspects of digestive health, immune function, and the delicate balance that underlies gastrointestinal homeostasis.

1.3.3 Gut Permeability and gut damage

The term gut permeability refers to the loss of barrier function of the gut epithelium. Transitory or permanent loss of tight junctions or cell integrity allows passage of microbial products into the mucosa and the systemic circulation, a process called microbial translocation (Ouyang *et al.*, 2023). Gut permeability encompasses gut damage, which refers to the death of epithelial cells through apoptosis, necrosis, or other types of cell death, which leads destruction of the epithelial barrier and gut permeability (Subramanian et al., 2020).

1.3.4 Markers of Gut permeability

Gut permeability markers are indicators used to assess the integrity and function of the gastrointestinal tract, particularly in the context of diseases or conditions affecting the gut (Ouyang *et al.*, 2023). These markers encompass various molecules or substances that reflect different aspects of gut health, including epithelial barrier integrity, mucosal inflammation, and tissue damages. Commonly studied gut damage biomarkers include I-FABP, REG3α, zonulin, soluble ST2, occludin and claudin, along with indicators of microbial translocation like BDG, LPS, LBP and sCD14, all offer valuable insights into gut permeability. Evaluating these gut damage markers offers valuable insights into gastrointestinal health and function, aiding in diagnosing, monitoring, and managing a range of gut-related disorders. Moreover, they are vital tools in research to uncover the mechanisms behind gut pathologies and the development of precise therapeutic strategies to restore gut health (Ouyang *et al.*, 2023).

1.3.4.1 I-FABP

I-FABP is a protein expressed by the enterocyte cells lining the small intestine (Gajda and Storch, 2015). I-FABP possesses a molecular weight of approximately 15 kilodaltons (kDa) and serves a pivotal role in the transport and metabolism of long-chain fatty acids, which are key in the maintenance and renewal of enterocytes (Ouyang *et al.*, 2023). When these cells are damaged, I-

FABP is released into the mucosa and in bloodstream, and its elevated levels in the blood can be indicative of intestinal injury and severity of multiple intestinal disease including ulcerative colitis, Crohn's disease (CD), mesenteric ischemia, coeliac disease and HIV (Adriaanse et al., 2013; Isnard et al., 2020b; Lieberman et al., 1997; Wang et al., 2015; Wiercinska-Drapalo et al., 2008). Consequently, it is recognized as a biomarker reflecting the disruption of the integrity of intestinal epithelial cells and serves as a prognostic marker in critically ill participants with COVID-19 (Tyszko et al., 2022).

1.3.4.2 REG3α

REG3α is a C-type lectin antimicrobial peptide that is excreted by Paneth cells into the luminal environment of the gut (Bevins and Salzman, 2011). Its function involves the containment of bacterial infections through the binding to peptidoglycans present in the cell wall of selected bacteria. Additionally, REG3α exhibits bactericidal activity against specific gram-positive bacteria, which indicates that REG3α participates in the regulation of the composition of the gut microbiota (Ouyang *et al.*, 2023). The expression of REG3α is induced in response to bacterial colonization and serves as part of the host defense mechanism against bacterial invasion (Isnard et al., 2020a). Upon gut permeability, REG3α can translocate from the gut lumen to the mucosa and systemic circulations. Hence, elevated circulating levels of REG3α are indicative of increased permeability or damage to the intestinal barrier in multiple inflammatory disease, such as CD, celiac disease, ulcerative colitis, non-alcoholic steatohepatitis, gastrointestinal graft-versus-host disease and HIV (Wang et al., 2016) (Zhao et al., 2018) (Ferrara et al., 2011; Isnard *et al.*, 2020b; Marafini et al., 2014). Plasma REG3α levels tended to be elevated in COVID-19 patients (Giron *et al.*, 2021).

1.3.4.3 Zonulin

Zonulin is specifically expressed and secreted by the intestinal epithelium; it is the only known physiological enzyme that regulates intestinal permeability by reversibly disassembling tight junctions between epithelial cells (Wang et al., 2000). It is elevated in inflammatory and autoimmune

disorders including type 1 diabetes, inflammatory bowel disease, and Crohn's disease (Fasano, 2020). Zonulin has been shown to be associated with disease progression in PLWH, and predict mortality in PLWH with past AIDS history (Hunt et al., 2014). In addition, it is also associated with systemic inflammation markers: CRP, IL-6, and D-dime in perinatally HIV-infected children who were breastfed (Dirajlal-Fargo et al., 2020). Circulating zonulin levels are elevated in intermediate and severe COVID-19 patients (Giron *et al.*, 2021).

1.3.4.4 Soluble ST2

Suppressor of tumorigenicity 2 (ST2) functions as the receptor for IL-33, which is an IL-1-like cytokine secreted by living cells in response to cell damage (Villacorta and Maisel, 2016). It exists in primarily two main isoforms: transmembrane or cellular (ST2L) and soluble or circulating (sST2) forms (Pascual-Figal and Januzzi, 2015). ST2 is further linked to inflammatory and immune mechanisms, particularly in the context of mast-cells and type 2 CD4 T-helper cell regulation, as well as the synthesis of Th2-associated cytokines (Villacorta and Maisel, 2016). Elevated levels of sST2 are observed in inflammatory conditions and serve as a predictor of cardiovascular outcomes in both chronic and acute heart failure (Villacorta and Maisel, 2016). Additionally, sST2 has been used as a marker of mucosal epithelial damage in early HIV infection and an indicator of disease severity and inflammation in COVID-19 (Mehraj et al., 2016; Zeng et al., 2020). As ST2 expression is not restricted to the intestine, soluble ST2 levels do not solely reflected gut damages.

1.3.4.5 Occludin and Claudin

Tight junctions consist of numerous protein complexes situated at the apical ends of the lateral membranes of intestinal epithelial cells. To date, four integral transmembrane proteins have been identified: occludin, claudins, junctional adhesion molecule (JAM), and tricellulin (Lee, 2015). The association between tight junction proteins and the actin cytoskeleton is essential for preserving tight junction structure and enables the cytoskeletal modulation of tight junction barrier integrity (Lee, 2015). Recent research has unveiled that diminished occludin expression results in increased

paracellular permeability to macromolecules, emphasizing occludin's role in the maintenance and organization of tight junctions, crucial for their assembly and integrity (Al-Sadi et al., 2011). Moreover, claudin is essential in barrier formation, as demonstrated by the fact that claudin knockout mice die within 24 hours of birth due to a dramatic loss of fluid and electrolytes through the impaired epidermal barrier (Fujibe et al., 2004).

Upon cell stress or death, occludin and claudin can be released into the mucosa and the circulation. Hence, circulating levels of these molecules can serve as markers of gut damage (Lee, 2015).

1.3.5 Microbial translocation markers

Microbial translocation is the phenomenon where microbial products transverse from the gut lumen into systemic circulation due to increased gut permeability. Microbial translocation is recognized as a key contributor to immune activation and inflammation in various pathological conditions (Brenchley and Douek, 2012). The identification of reliable markers for microbial translocation is crucial for understanding its pathophysiological implications and guiding therapeutic strategies. Commonly studied microbial translocation markers include β-D-glucan (BDG), lipopolysaccharide (LPS), soluble CD14 (sCD14), and bacterial DNA fragments such as 16S ribosomal DNA (16S rDNA). Elevated levels of microbial translocation markers in plasma or serum indicate heightened gut permeability and systemic immune activation, providing insights into disease progression, prognosis, and the efficacy of therapeutic interventions for addressing gut barrier dysfunction and associated complications. Giron et al. have shown that inflammation is associated with both bacterial translocation (using markers LBP and sCD14) and fungal translocation (BDG) and related to the severity of disease during acute COVID-19. Gut damage markers and BDG were also elevated in the blood of participants with long COVID-19 compared to those who recovered completely after acute disease (Giron *et al.*, 2021).

1.3.5.1 β-D-glucan

BDG is a polysaccharide commonly found in the cell walls of various fungi (Theel and Doern, 2013). Presence of BDG in the bloodstream may indicate fungal infection and/or increased permeability of

the intestinal barrier, allowing fungal components to pass through (Ouyang *et al.*, 2023). Serum BDG test aids in timely diagnosis and management of invasive fungal infections, which are life-threatening opportunistic infections that occur in immunocompromised or critically ill people, especially when conventional diagnostic methods are inconclusive (White et al., 2020). In absence of infection, translocation of BDG from the fungal microbiota triggers an immune response and can lead to systemic inflammation and complications. Therefore, monitoring BDG levels can provide valuable insight into the extent of fungal translocation and related health conditions (Isnard et al., 2021b). Increased blood levels of BDG have also been noted to correlate with immune activation and the propensity for developing non-AIDS-associated comorbidities such as cardiovascular and metabolic diseases, neurocognitive dysfunction, and cancer (Isnard *et al.*, 2021b). Essentially, BDG testing offers a non-invasive approach for identifying fungal translocation and evaluating gut barrier integrity across various clinical contexts.

Fungal PAMPs induce inflammation by binding to various cell receptors, including Dectin-1, toll-like receptor 2 (TLR2), and complement receptor 3 (CR3) (Isnard *et al.*, 2021b). Dectin-1 predominantly interacts with BDG on APCs such as macrophages, monocytes, dendritic cells, neutrophils, and B-cells. Similarly, TLR2 is expressed on macrophages, monocytes, and dendritic cells, activating the NF-κB pathway upon recognizing soluble or particulate BDG (Isnard *et al.*, 2021b). Stimulation of both Dectin-1 and TLR2 on macrophages and monocytes leads to the secretion of pro-inflammatory cytokines like IL-6, IL-8, TNF-α, as well as the anti-inflammatory mediator IL-10 (Bonfim et al., 2009). BDG also binds to CR3 on APCs, particularly after complement opsonization (McDonald et al., 2012). NK cells primarily employ NKp30 as their main fungal receptor, recognizing membrane-bound BDG and aiding in fungal cell elimination (Isnard *et al.*, 2021c).

1.3.5.2 Lipopolysaccharide

LPS constitutes the primary component of the outer membrane in most Gram-negative bacteria. Presence of LPS in the bloodstream can indicate bacterial infection and/or disruption in the intestinal

barrier and translocation of LPS from the microbiota into the circulation (Ouyang *et al.*, 2023). Furthermore, when circulating LPS binds with monocyte/macrophage receptor (i.e. CD14), it triggers activation of the immune system and systemic inflammatory response (Ikegame et al., 2022) (Ciesielska et al., 2021). The immune system detects LPS via PRRs like TLR4 presented on immune cells (Soares et al., 2010). Activation of these receptors by LPS initiates a cascade of signaling events, leading to the production of pro-inflammatory cytokines, chemokines, and other immune mediators (Soares *et al.*, 2010). This systemic immune activation is essential for mounting an effective defense against bacterial infections; however, excessive, or prolonged immune activation can lead to systemic inflammation and tissue damage (Diamond and Kanneganti, 2022). In conclusion, LPS serves as a critical marker for microbial translocation and systemic immune activation, providing valuable insights into the role of gut integrity in health and disease. Understanding the mechanisms underlying LPS-mediated immune responses is essential for developing targeted therapies to regulate immune activation and alleviate the adverse effects of microbial translocation on human health.

1.3.5.3 Soluble CD14

CD14 is a membrane glycoprotein anchored by GPI and is constitutively expressed on monocytes/macrophages and neutrophils. It binds to LPS and other ligands with LPS binding protein (LBP), activating TLR2 and proinflammatory pathways. Additionally, CD14 has a soluble form (sCD14), which can be derived from enzymatic cleavage of membrane bound CD14 or secreted from the liver (Reiner et al., 2013). sCD14 has been associated with heightened morbidity and mortality in HIV disease. Serving as a co-receptor for LPS, it is released from monocytes upon activation. LPS induces the secretion of sCD14 from immune cells, thus elevated plasma levels of sCD14 are indicative of LPS exposure (Mehraj et al., 2020a). Elevated levels of sCD14 are observed in conditions associated with heightened gut permeability, such as celiac disease, potentially resulting from bacterial translocation across the gut membrane (Al-Ayadhi et al., 2021). In PLWH, intestinal barrier disruption allows LPS to enter the bloodstream, which activates CD14 receptors on monocytes and macrophages and triggers systemic inflammation. (Ouyang *et al.*, 2023) Hence, elevated plasma

LPS and sCD14 levels are observed in PLWH, with sCD14 levels often positively correlating with circulating LPS levels (Ouyang *et al.*, 2023). Consequently, both circulating LPS and sCD14 are commonly employed as biomarkers of bacterial translocation, and their elevation is linked to systemic immune activation and the progression of HIV disease (Ciesielska *et al.*, 2021). In addition to LPS, IL-6 and CpGs could also induce sCD14 expression upon inflammation and infection (Marcos et al., 2010). Thus, monitoring sCD14 levels could provide valuable insights into the integrity of the gut barrier and the extent of microbial translocation.

1.3.5.4 16S rDNA

The presence of circulating microbial DNA fragments, particularly 16S rDNA, is widely acknowledged as indicative of bacterial infection or translocation and strongly correlates with ongoing immune activation in individuals with HIV (Ouyang *et al.*, 2023). 16S rDNA is present in bacterial chromosomal genes with homologous functions, and is the oldest of these genes, known as "bacterial fossils." 16S rDNA sequences correspond to bacterial ribosomal RNA and are highly conserved across bacterial chromosomal genes (Ouyang *et al.*, 2023). Due to its importance as a bacterial product, 16S rDNA from the gut is especially likely to translocate into the systemic circulation, especially when gut integrity is compromised. Thus, quantifying 16S rDNA levels in plasma offers a reliable method for evaluating microbial translocation (Ouyang *et al.*, 2023). Plasma 16S rDNA is strongly linked to persistent immune activation in HIV disease. Detecting 16S rRNA is a valuable method for assessing gut barrier integrity and microbial translocation, offering insights into the role of gut microbiota in health and disease.

1.4 Microbial Translocation in Health and Disease

The bidirectional interplay between the commensal microbiota and the mammalian immune system is integral to physiological balance and disease states. In a state of health, microbial translocation is occurring only to a limited extent due to the efficient epithelium barrier function (Hou et al., 2022). The host's immune response to the intestinal microbiota and its product is intricately regulated,

primarily targeting the mucosal surface (Zheng *et al.*, 2020). However, various factors such as infections, inflammation, and alterations in gut permeability can compromise this barrier function, leading to microbial translocation (Zheng *et al.*, 2020). Research has demonstrated that microbial translocation can induce immune activation and inflammation and studies have indicated that SARS-CoV-2 infection can lead to gut barrier dysfunction, allowing bacterial and fungal products to translocate into the bloodstream (Giron et al., 2022). Conditions such as HIV infection, IBD, systemic autoimmune diseases, and cancer are also associated with microbial translocation (Zheng *et al.*, 2020). Thus, understanding the mechanisms underlying microbial translocation in health and disease is essential for developing therapeutic strategies aiming at restoring gut barrier function and modulating immune responses. Targeting microbial translocation presents promising possibilities for the prevention and treatment of various immune-mediated and infectious diseases, which could ultimately improve patient outcomes and quality of life.

1.4.1 Microbial Translocation in COVID-19

SARS-CoV-2 can directly infect the gut and compromise its barrier function. Giron et al. have shown that inflammation is associated with both bacterial translocation (using markers LBP and sCD14) and fungal translocation (BDG). Higher BDG levels were found in severe COVID-19 cases and associated with markers of inflammation including TNF-α and IL-6 (Giron *et al.*, 2021). Serum levels of LPS and zonulin were found to be elevated in COVID-19 participants (Dorneles et al., 2023) (Oliva et al., 2021). Moreover, plasma levels of sCD14 were higher in severe COVID-19 patients (Giron *et al.*, 2021). Additionally, markers of gut damage and BDG were higher in the blood of individuals with long COVID-19 compared to those who fully recovered after acute illness (Giron *et al.*, 2021). Microbial translocation can trigger immune activation and inflammation and exacerbate the cytokine storm associated with severe COVID-19 and contributing to multi-organ dysfunction (Tang et al., 2020b). Understanding the role of microbial translocation in COVID-19 pathogenesis could provide valuable insights into disease severity and guide therapeutic strategies targeting the modulation of gut barrier integrity and immune activation.

1.4.2 Dysbiosis and Microbial Translocation in Other Diseases

Abnormal interactions between the microbiota and the host's immune system contribute to the onset of diverse immune-mediated disorders (Zheng *et al.*, 2020). While conditions like HIV, IBD, systemic autoimmune diseases, and cancer have been extensively studied, the causal relationship between microbiota and immune dysregulation in these diseases remains to be fully established (Isnard et al., 2021a) (Zheng *et al.*, 2020). Bacterial translocation and subsequent immune activation and inflammation are well documented. However, there is evidence that fungal translocation also induces immune activation and inflammation by binding to their receptors on the surface of immune cells, including macrophages, monocytes, and dendritic cells, to disrupt epithelial barrier permeability (Giron *et al.*, 2022).

1.4.2.1 HIV Infection

Microbial translocation contributes to immune activation during chronic infection with HIV. It results from factors like epithelial damage directly induced by HIV as well as inflammation induced damages to the gut environment: weakened junctions, immune abnormalities in the gut mucosa, including CD4 T-cell and Th17 lymphopenia, and neutrophil accumulation (Younas et al., 2019). Dr. Jean-Pierre Routy's team have previously shown that microbial translocation is detrimental in PLWH, including those receiving ART (Isnard *et al.*, 2021a). Elevated plasma levels of LPS and BDG are observed in both ART-naïve and ART-treated individuals with HIV and associated with levels of inflammation and increased risk of developing non-AIDS inflammatory diseases in this population. CMV is a common coinfection among PLWH and seropositivity against CMV is associated with increased risk of developing non-AIDS comorbidities despite long-term ART. Our team has shown that CMV-seropositive PLWH have higher levels of epithelial gut damage, microbial translocation, and inflammation compared to seronegative PLWH (Ramendra et al., 2020). Our team has also shown that extent of dysbiosis and a dysbalanced gut microbiota composition, is linked with microbial translocation in PLWH on antiretroviral therapy (Ellis et al., 2021).

1.4.2.2 Intestinal Bowel Diseases

IBD is a chronic, recurrent inflammatory disorder of the GI tract and mainly includes Crohn's disease and ulcerative colitis (Zheng *et al.*, 2020). Perturbation of the gut microbiota composition is associated with IBD pathogenesis including diminished diversity in intestinal bacterial and fungal and proliferation of certain bacterial and fungal taxa (Zheng *et al.*, 2020). These microbiota alterations are strongly associated with abnormal mucosal immune responses, including upregulated Th17, Th1 and Th2 type responses, downregulated T regulatory cells, and dysregulated humoral immunity (Sokol et al., 2017) (Zheng *et al.*, 2020). This may ultimately lead to persistent and clinically apparent intestinal inflammation and tissue injury, leading to microbial translocation of both fungal and bacterial products

1.4.2.3 Rheumatoid Arthritis

RA is a systemic autoimmune disorder characterized by joint inflammation and bone cartilage destruction (Zheng *et al.*, 2020). It has been shown that gut microbiota imbalance can lead to the migration of autoreactive cells to the joints, causing cartilage and bone damage. Bacterial antigens then trigger inflammation in the synovial membrane and activate T and B-cells in the lymphoid tissues, creating an imbalance between Th17 and Tregs that further expand the inflammatory response. Released inflammatory cytokines induce fibroblasts to generate MMPs and RANKL, leading to bone and cartilage destruction and the development of RA (Zhao et al., 2022).

1.5 COVID-19 Immune Responses

The host orchestrates a multifaceted immune response to SARS-CoV-2 during COVID-19. Upon viral infection, the innate immune system initiates a cascade of pro-inflammatory cytokines and chemokines and recruits immune cells to the infection site (Marshall et al., 2018). This initial response is pivotal for controlling viral replication and preventing further spread of the virus. However, dysregulated immune activation can precipitate a cytokine storm, exacerbating tissue damage and disease severity (Diamond and Kanneganti, 2022). Simultaneously, the adaptive immune system aids

in eliminating the virus by producing antibodies through B-cells and facilitating cytotoxicity by CD8 T-cells (Marshall *et al.*, 2018). The establishment of immunological memory following infection, , facilitated by CD4 T-cells, ensures lasting defense against subsequent exposures (Natoli and Ostuni, 2019). Individual variations in immune responses influenced by factors such as age, comorbidities, and genetic makeup contributes to variations in disease outcomes (Zimmermann and Curtis, 2019). In severe COVID-19 cases, SARS-CoV-2 impairs normal immune responses and triggers immune system dysfunction and excessive inflammation (Diamond and Kanneganti, 2022). Severe case individuals display lymphopenia, activated and dysfunctional lymphocytes, abnormal granulocytes and monocytes, elevated cytokine levels, and increased IgG and total antibodies (Yang et al., 2020). Understanding the intricates of the immune response to COVID-19 is essential for developing effective vaccines, treatments, and public health strategies to control the spread of the virus and mitigate its impact on global health.

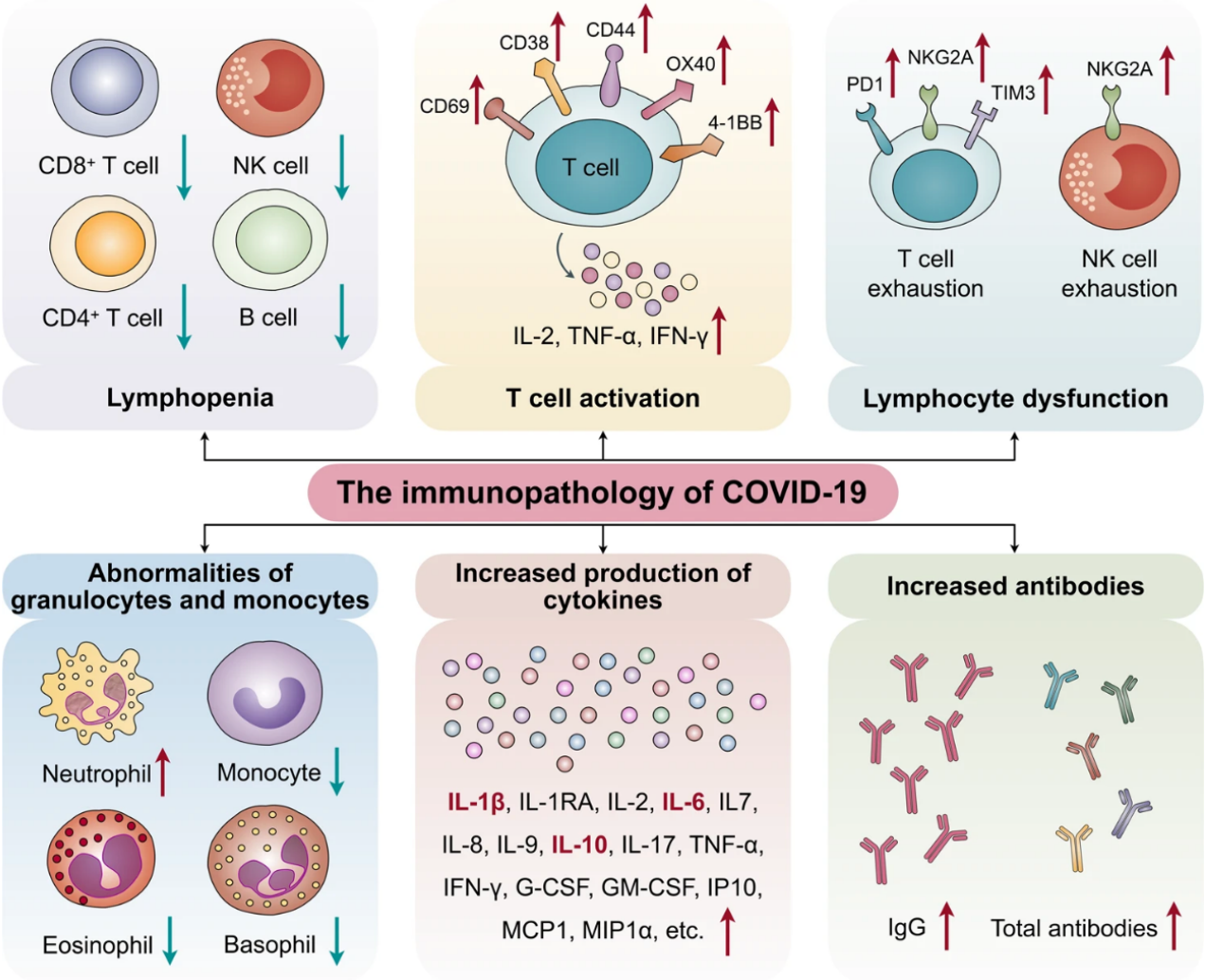

Figure 2: COVID-19 immunopathology. COVID-19 immune patterns include lymphopenia, lymphocyte dysfunction, granulocyte and monocyte abnormalities, increased cytokines, and elevated antibodies. CD69, CD38, and CD44 are highly expressed on CD4 and CD8 T-cells in participants, with virus-specific T-cells from severe cases displaying a central memory phenotype and elevated levels of IFN-γ, TNF-α, and IL-2. Despite this, lymphocytes exhibit signs of exhaustion. Severe participants show significantly higher neutrophil levels, reduced percentages of eosinophils, basophils, and monocytes. Elevated cytokine production, particularly IL-1β, IL-6, and IL-10, is a notable feature in severe COVID-19. Additionally, there is an increase in IgG levels and a higher titer of total antibodies (Yang *et al.*, 2020).

1.5.1 Innate Immune Response

The innate immune system is the first line of defence against pathogens and is often referred to as the "nonspecific" immune system. The ability of innate immune cells including monocytes, macrophages, dendritic cells, neutrophils, and NK cells use germ-line encoded PRRs to detect unique molecular patterns originating from pathogens, termed PAMPs, as well as endogenous molecules released from injured or dying cells, known as DAMPs (Kanneganti, 2020). The innate immune system works to restrict viral entry, translation, replication, and assembly, assisting in the identification and elimination of infected cells while also coordinating and expediting the development of adaptive immunity (Diamond and Kanneganti, 2022). The main PRR families include TLRs, RIG-1 like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin receptors, and absent in melanoma 2 (AIM2)-like receptors (Kanneganti, 2020). Activation of these receptors in innate immune cells triggers the production of inflammatory cytokines, chemokines, as well as the initiation of cell death mechanisms to eradicate infected cells (Diamond and Kanneganti, 2022). Additionally, primates have evolved specialized cells called plasmacytoid dendritic cells located in both blood and mucosal tissues, which are dedicated to producing type-I interferon, in addition to the production of type I interferon produced by all cell types (Tezuka et al., 2011). Beyond their direct antiviral effects, type-I interferon serves as the primary bridge connecting the innate and adaptive immune responses.

SARS-CoV-2 has evolved mechanisms to escape the host innate immune system for successful viral replication (Zhang et al., 2022). Thus, understanding the pathogenicity and developing innovative therapeutic approaches to limit the sequelae of viral infections.

1.5.1.1 Monocytes and Macrophages

One subgroup of leukocytes is the mononuclear phagocyte system, which comprises bone marrow-derived myeloid cells and includes monocytes and macrophages and are crucial for innate immunity (Chiu and Bharat, 2016). Their functions include phagocytosis, antigen presentation, chemokine secretion, and proliferation in response to infection and injury (Chiu and Bharat, 2016). Upon tissue recruitment, monocytes can differentiate into macrophages and dendritic cells. Macrophages are considered terminally differentiated cells that engage in pathogen phagocytosis, chemokines secretion to attract other immune cells, and migrate to local lymph nodes via lymphatics to present processed antigens (Chiu and Bharat, 2016). Both monocytes and macrophages express the β2 integrin, comprising CD11a, CD11b, CD11c, and CD11d paired with the β2 chain CD18 (CD11b and CD18 form the CR3 receptor of BDG) which play critical roles in regulating three fundamental aspects of immune cell function: recruitment to sites of inflammation, formation of cell–cell contacts, and downstream modulation of cellular signaling (Schittenhelm et al., 2017).

Macrophages can also be seeded in tissue since the fetal stage. Those embryonic originating macrophages have more often a regulatory anti-inflammatory function. However, plasticity is a key characteristic of macrophages, switching from pro- to anti-inflammatory function, and vice-versa, through mechanisms that remain unclear.

1.5.1.2 Dendritic cells

DCs constitute a unique subset of mononuclear phagocytes with specialized roles in presenting antigens to T-cells and orchestrating the initiation and regulation of immune responses (Geissmann et al., 2010). DCs are classified into classical DCs and plasmacytoid DCs. Classical DCs are adept at antigen processing and presentation, with high phagocytic activity in their immature state and robust cytokine production when mature (Geissmann *et al.*, 2010). They migrate efficiently to lymphoid organs and regulate T-cell responses during steady state and infection, though they are short-lived and replenished by blood-borne precursors (Geissmann *et al.*, 2010). In contrast, plasmacytoid DCs are long-lived and found throughout the body. They respond vigorously to viral infections by producing

type I interferons and also serve as antigen-presenting cells, which contribute to T-cell regulation (Colonna et al., 2004). DCs also express β2 integrins, with CD11c predominantly expressed along with CD11a (Schittenhelm *et al.*, 2017).

1.5.1.3 Neutrophils

Neutrophils are termed polymorphonuclear leukocytes and have long been characterized as short-lived, nonspecific cells contributing to pus formation and microbial eradication (Malech, 2007). Neutrophils were often marginalized in immunological discourse; however, they have been recently recognized to play pivotal role beyond microbial clearance, which encompass substantial involvement in modulating host responses to infection and preserving immune system equilibrium (Malech, 2007). Neutrophils also emerged as key mediators of the TH17-controlled pathway in pathogen resistance and immunopathology. TH17 cell-derived cytokines, including IL-17, IL-8, IFNγ, TNF, and GM-CSF, promote neutrophil recruitment, activation, and prolonged survival at inflammatory sites, thus amplifying their role in combating extracellular bacteria (Mantovani et al., 2011).

1.5.1.4 NK cells

NK cells constitute 5–20% of total PBMCs in humans and they possess the ability to directly detect infected cells independently of the MHC-I complex (Langers et al., 2012; Sun and Lanier, 2011). NK cells lack antigen-specific receptors and rely on killer cell immunoglobulin-like receptors to discern between normal and stressed cells by recognizing MHC class I molecules (which are typically abundant on healthy cells but often downregulated during stress), thereby eliciting a robust inhibitory signal that actively prevents NK cells from targeting healthy self-cells (Arnon et al., 2005). NK cells can also recognize “kill me” signals on stressed cells. Under normal circumstances, NK cells primarily reside in peripheral blood, spleen, and bone marrow, yet they possess the ability to migrate to inflamed tissues in response to various chemoattractants (Moretta et al., 2002). NK cells play a crucial role in regulating tumor growth, inhibiting metastatic tumor spread, and defending against

cytopathic viruses, particularly herpesviruses (Moretta *et al.*, 2002). Upon activation, NK cells release cytokines and chemokines that stimulate inflammatory responses, modulate hematopoiesis, regulate monocyte and granulocyte functions, and shape subsequent adaptive immune responses (Moretta *et al.*, 2002). While NK cell inactivation serves to safeguard against harming normal MHC-class I+ self-cells, NK cell activation relied on surface receptors responsible for triggering responses during natural cytotoxicity (Moretta *et al.*, 2002). Three members of the natural cytotoxicity receptor group specific to NK cells have been identified: NKp46, NKp30, and NKp44 (Moretta et al., 2001). Importantly, NKp46 and NKp30 facilitating precise identification of both resting and activated NK cells. Thus, NK cells should be regarded as specialized effector cells that operate in complementary to CTLs by selectively eliminating MHC-I deficient-cells that are invisible to CTL surveillance (Moretta *et al.*, 2002).

1.5.2 Adaptive Immune Response

The adaptive immune response has three main functions: distinguishing specific "non-self" antigens from "self" antigens, generating pathogen-specific immunologic effector pathways to eliminate pathogens or infected cells, and developing memory response to subsequent infections (Bonilla and Oettgen, 2010)

Virus infected cells present antigen onto their MHC classes I and II, which activate antigen-specific CD8 and CD4 T-cell responses, respectively. MHC-I are present on all nucleated cells. MHC-II molecules are located in the membranes of APCs like macrophages, monocytes, dendritic cells, and B-cells, and play a crucial role in activating both B-cells, supporting their proliferation and differentiation, along with CD4 T-cells (Giles et al., 2015). The adaptive immune response includes both cellular immunity mediated by T-cells, and humoral immunity mediated by B-cells (Marshall *et al.*, 2018). B-cells are responsible for antibody production, while CD4 T-cells exhibit various helper and effector functions, and CD8 T-cells possess cytotoxic killing mechanisms (Sette and Crotty, 2021). Understanding adaptive responses to SARS-CoV-2 is crucial as adaptive immune

responses play a vital role in controlling and eliminating nearly all viral infections that affect humans (Sette and Crotty, 2021).

1.5.2.1 Cellular Immune Response

T-cells are a type of white blood cell that originates from hematopoietic stem cells in the bone marrow and undergo maturation in the thymus (Marshall *et al.*, 2018). They express unique antigen-binding receptors, TCR, that bind to specific peptides on their membrane and play a central role in cell-mediated immunity (Marshall *et al.*, 2018). Activation of T-cells occurs upon interaction with an APC that has internalized an antigen and presents the corresponding antigen fragments bound to its MHC molecules (Marshall *et al.*, 2018). There are two main types of T-cells involved in the immune response against SARS-CoV-2: helper T-cells (CD4) and cytotoxic T-cells (CD8). Helper T-cells coordinate the immune response by secreting cytokines that enhance CD8 T-cell response and are vital for B-cell antibody production and plasma cell generation, while cytotoxic T-cells directly kill virus-infected cells (de Candia et al., 2021). Upon encountering virus-infected cells, cytotoxic T-cells recognize viral antigens presented on the surface of infected cells and release cytotoxic molecules, such as perforin and granzymes, to induce apoptosis in these cells (de Candia *et al.*, 2021). This helps limit viral replication and spread within the body. So far, five major subsets of CD4 T helper cells have been characterized: Th1, Th2, Th17, Treg and Tfh cells. Understanding the mechanisms underlying the cellular immune response against COVID-19 is essential for developing effective treatments to combat the disease.

1.5.2.1.1 CD4 T+ cell response

CD4 T-cells serve as important regulators, coordinators, and effectors in the context of antiviral immunity. Memory CD4 T-cells exhibit additional protective functions compared to naïve CD4 T-cells and they mount faster response upon re-exposure to the virus due to their enhanced effector

functions and rapid activation of innate immune defense mechanisms (Swain et al., 2012).

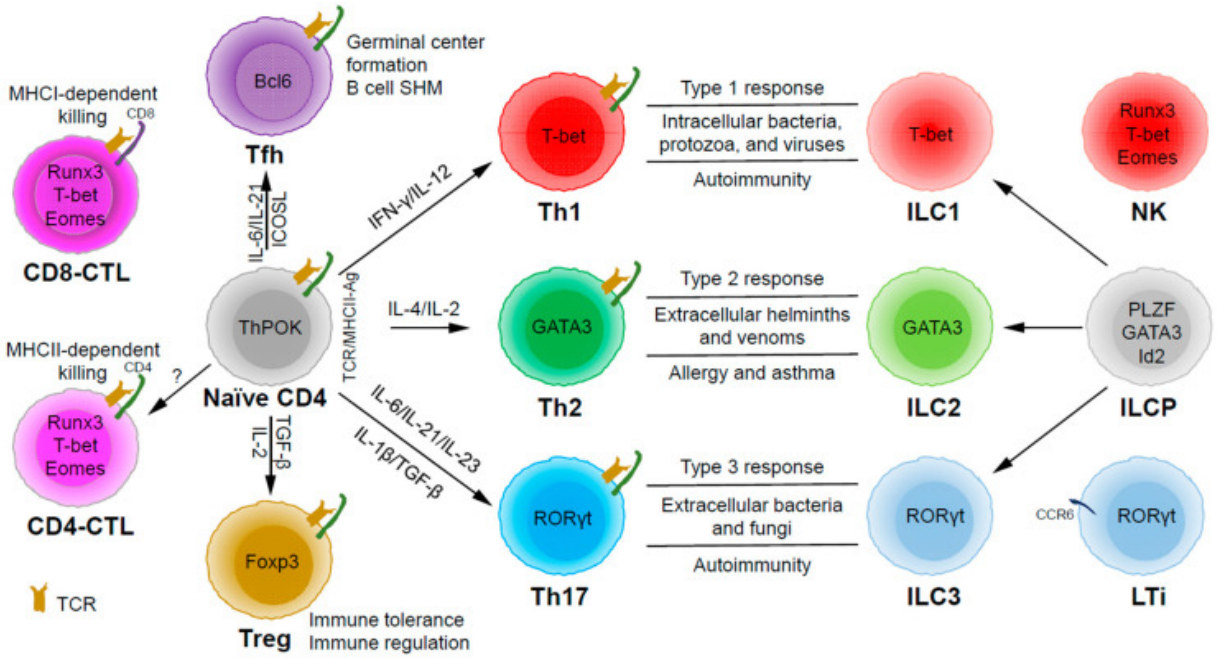

Figure 3: CD4 T helper cell and innate lymphoid cell subset development and functions. Upon TCR activation in various cytokine environments, naive CD4 T-cells can differentiate into Th1, Th2, Th17, Tfh, and Treg subsets. Additionally, they may become cytotoxic CD4 T-cells (CD4-CTLs) that target-cells in a MHCII-restricted manner. Innate lymphoid cell (ILC) subsets—ILC1, ILC2, and ILC3—mirror the functions of Th1, Th2, and Th17 cells, respectively, participating in type 1, type 2, and type 3 immune responses. These ILC subsets develop from distinct ILC progenitors (ILCPs), which are distinct from those that give rise to NK cells and lymphoid tissue inducers (LTis).

1.5.2.1.1.1 Th1 cells

Th1 cells are a subset of CD4 T-cells characterized by expression of the master transcription factor T-bet, and lineage cytokines such as IFN-γ and TNF-α upon activation. Primarily, Th1 cells induce type 1 responses by activating type 1 macrophages (M1), which enhance their ability to eliminate intracellular pathogens including virus and certain intracellular bacteria (Zhu and Zhu, 2020). IFN-γ is a key Th1 cytokine that stimulates other immune cells including CD8 T-cells, macrophages, ILC1s and B-cells during phagocytosis and boosts their ability to eliminate engulfed pathogens (Zhu and Zhu, 2020). Moreover, TNF-α from Th1 cells induces inflammatory mediator production and recruits additional immune cells to the infection site (Jang et al., 2021). Th1 cells also aid in CTL activation by producing cytokines like IL-2 to assist CD8 T-cells differentiation into CTLs (Schüler et al., 1999). Th1 cells are involved in autoimmune conditions like RA and IBD, as well as chronic inflammatory disorders such as atherosclerosis, and deficiencies in their generation and

function can result in chronic or recurrent infections due to their role in orchestrating type 1 immunity against intracellular pathogens (Zhu and Zhu, 2020). Altogether, the Th1 cell response plays a vital role in coordinating the immune defense against intracellular pathogens, ensuring their eradication, and establishing durable immunity.

1.5.2.1.1.2 Th2 cells

Th2 cells are characterized by expression of master transcription factor GATA3, and secretion of lineage cytokines such as IL-4, IL-5 and IL-13 upon activation (Zhu and Zhu, 2020). These cytokines orchestrate various immune processes: IL-4 stimulates B-cells class switching to IgE, which is crucial for defense against parasites and implicated in allergies; IL-5 enhances recruitment of eosinophils to sites of inflammation, which is vital for combating parasitic infections and allergic inflammation; and IL-13 regulates mucus production, smooth muscle contraction, and tissue remodeling (Gurram and Zhu, 2019). These cytokines were also produced by innate immune cells ILC2s. In a type 2 immune response, Th2 cells and ILC2s collaborate closely to promote immune responses against parasites and regulate allergic inflammation, making them essential components of the body's defense mechanisms (Zhu and Zhu, 2020).

1.5.2.1.1.3 Th17 cells

Th17 cells are key players of type 3 immune response that defend against extracellular bacteria and fungi and contribute to autoimmune diseases like multiple sclerosis due to their pathogenic effects (Zhu and Zhu, 2020). Th17 cells are characterized by expression of master transcription factor RORγt, and secretion of effector cytokines including IL-17A, IL-17F and IL-22. These cytokines play key roles in activating various immune and non-immune cells to enhance the clearance of extracellular pathogens through mechanisms like the production of antimicrobial peptides, cytokines, and other immune mediators (Zhu and Zhu, 2020). Th17 are also paramount in the maintenance of gut mucosa homeostasis through the induction of secretion of epithelium proliferation factor and anti-microbial peptides. Additionally, the Th17 response is implicated in the pathogenesis of various autoimmune and inflammatory diseases due to its dysregulation and

excessive activation (Zhu and Zhu, 2020), which highlights its complex role in immune homeostasis and disease.

1.5.2.1.1.4 Treg cells

Treg cells are mostly characterized by expression of master transcription factor Foxp3 and high levels of CD25 cell surface marker as well as lineage cytokines such as IL-2 and TGF- β (Zhu and Zhu, 2020). Tregs are crucial for maintaining immune homeostasis and self-tolerance, and defects in Treg cells could lead to autoimmune or chronic inflammatory diseases (Fiyouzi et al., 2023). Immune tolerance refers to the intricate regulatory mechanisms within the immune system aiming at discriminating between self and non-self-antigens, thereby preventing unwarranted immune responses against endogenous tissues and innocuous environmental elements (Fiyouzi *et al.*, 2023). Immune tolerance is particularly important in oral and gut mucosa, which serve as key portals for pathogen entry. As such, maintaining a finely tuned immune tolerance is essential to balance effective immune defense with self-tolerance at these sites (Fiyouzi *et al.*, 2023).

1.5.2.1.1.5 Tfh cells

Tfh cells are characterized by expression of master transcription factor Bcl6 and surface ligand CXCR5 and PD-1 as well as lineage cytokines such as IL-21 and IL-6 (Vinuesa et al., 2016). They are mainly found in the B-cell follicles in lymph nodes and spleen and play a key role in supporting B-cells differentiation into plasma cells to produce antibody and promoting affinity maturation, a process by which B-cells undergo somatic hypermutation in their immunoglobulin genes, leading to the generation of antibodies with increased affinity for the antigen (Vinuesa *et al.*, 2016). Tfh cells also induce class switching in B-cells, leading to the production of different antibody isotypes, each with distinct effector functions suited to combat specific types of pathogens (Zhu and Zhu, 2020). Tfh cell response is critical for the generation of effective and long-lived humoral immune responses and dysregulation of Tfh functions can lead to impaired antibody responses and contribute to the development of autoimmune diseases and immunodeficiencies (Zhu and Zhu, 2020).

1.5.2.1.2 CD8 T+ cell response

CD8 cytotoxic T-cells are activated via interaction between their TCRs and antigens presented on MHC class I molecules and play a pivotal role in targeting and eliminating pathogen-infected and antigen-expressing tumor cells (Marshall *et al.*, 2018). After activation, cytotoxic T-cells undergo clonal expansion to generate effector cells that target infected cells by releasing cytotoxic molecules, such as perforin and granzyme B which facilitate the formation of pores in the cell membrane and trigger apoptosis (Schmidt and Varga, 2018). CD8 T-cells also employ direct-cell-cell contact to eliminate target-cells via interactions involving surface molecules like Fas (CD95) and FasL (CD95L), which in conjunction with the perforin pathway, are recognized as the principal methods by which CD8 T-cells eliminate infected cells (Schmidt and Varga, 2018). Virus-specific CD8 T-cells can rapidly produce inflammatory cytokines including IFN-γ, TNF and IL-2, which can induce cell death in virus-infected cells either directly or indirectly (Schmidt and Varga, 2018). Following infection resolution, most effector cells undergo clearance by phagocytes, but a small portion persists as memory cells and enable rapid differentiation into effector cells upon re-exposure to the same antigen (Marshall *et al.*, 2018).

1.5.2.2 Humoral Immune Response

The humoral immune response is a critical component of the adaptive immune system that involves the production of antibodies by plasma cells that developed from B-cells (Bonilla and Oettgen, 2010). B-cells originate from hematopoietic stem cells and mature in bone marrow to acquire the antigen-binding receptors on their membrane, which enable them to directly recognize antigens without the involvement of APCs (Marshall *et al.*, 2018). Upon infection, B-cells recognize foreign antigens through their B-cell receptor and becomes activated and undergo clonal expansion and differentiation into antibody-secreting plasma cells or memory B-cells (Marshall *et al.*, 2018). Plasma cells are relatively “short-lived” and secrete large amounts of soluble antibodies that circulate throughout the body via the bloodstream and lymphatic system, where they recognize and bind to specific antigens on the surface of pathogens (Marshall *et al.*, 2018). This binding process

tags the pathogens for destruction by other immune cells or neutralizes their harmful effects directly. In contrast, the memory B-cells are “long-lived” cells maintained after previous infection that retain the expression of antigen-binding receptors and can promptly generate antibodies to eliminate antigens upon re-exposure, providing long-term immunity (Marshall *et al.*, 2018). Antibodies perform diverse functions that are critical in combatting pathogens. Antibodies can neutralize pathogens by blocking surface molecules on pathogens or prevent pathogen function and infectibility (Marshall *et al.*, 2018). Antibodies also perform opsonization by coating pathogens for ingestion and destruction by phagocytic immune cells like macrophages and neutrophils (Marshall *et al.*, 2018). Additionally, antibodies activate the complement system, which enhances pathogen destruction through membrane lysis and improved phagocytosis (Marshall *et al.*, 2018). Moreover, antibodies recruit other immune cells, such as NK cells, to eliminate antibody-bound target-cells via antibody-dependent-cellular cytotoxicity, thereby strengthening the immune response against pathogens (Pinto et al., 2022).

Following viral infection, the activated naïve B-cells also undergo class switching from expressing IgM and IgD on their surface to expressing IgG, IgE or IgA, which enhances the effector function of the antibody in combating the pathogen that initiated the immune response (Stavnezer and Schrader, 2014). Among the antibodies produced in response to SARS-CoV-2 infection, IgM is typically the first to appear, followed by a more sustained production of IgG antibodies. Additionally, IgA antibodies are primarily found in mucosal surfaces, such as respiratory and gastrointestinal tracts, were found to be primarily produced during the initial phase of SARS-CoV-2 infection (Abebe and Dejenie, 2023). These neutralizing antibodies target the Spike or Nucleocapsid glycoproteins of SARS-CoV-2, preventing viral entry into host-cells and counteracting their biological effects (Abebe and Dejenie, 2023). The humoral immune response is dynamic, with antibody levels peak in the weeks following infection and gradually declines over time (Abebe and Dejenie, 2023). Understanding the humoral immune response to COVID-19 is essential for the development of effective vaccines and therapeutic interventions.

1.5.3 T-cell cytokine production

In 1986, Mosmann and Coffman characterized two subsets of activated CD4 T-cells—Th1 and Th2 cells—distinguished by their cytokine production and functions (Mosmann et al., 1986). Cytokines produced by different T-cell populations have different functions.

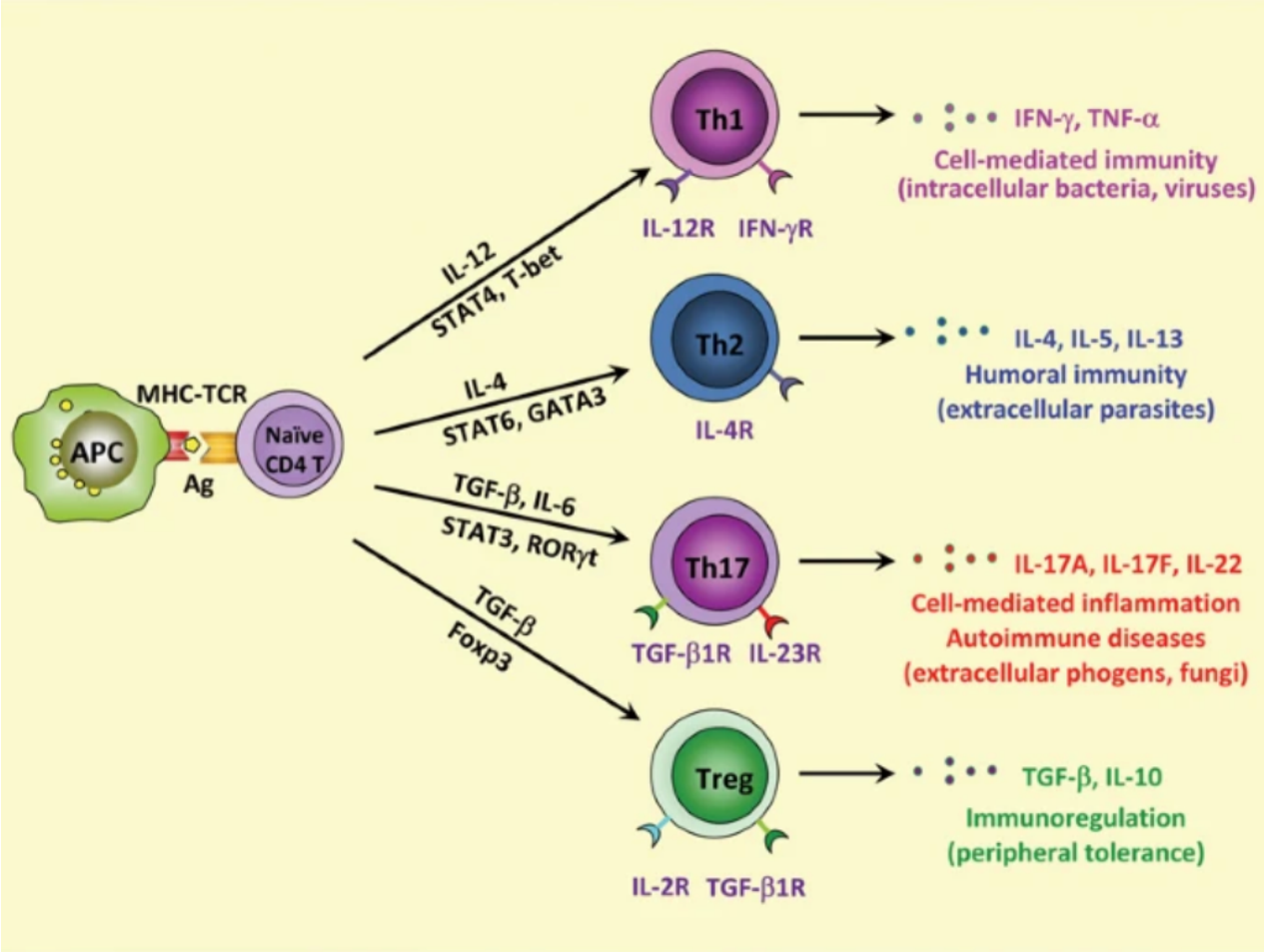

Figure 4: Model of Effector T-cell differentiation from naïve T-cells. IL-12 induces differentiation of naive CD4 T-cells into Th1 cells, which requires activation of the master regulator T-bet via STAT1. Th1 cells produce IFN-γ and TNF-α and contribute to cell-mediated immunity against intracellular pathogens. IL-4 activates STAT6 and GATA3 and promotes Th2 cell differentiation. Th2 cells play a crucial role in humoral immunity against parasites by secreting IL-4, IL-5, and IL-13. TGF-β, combined with proinflammatory cytokines like IL-6 and IL-23, directs naive CD4 T-cells to differentiate into Th17 cells, which is regulated by STAT3 and RORγt. Th17 cells are essential for host protection against extracellular pathogens and are implicated in inflammatory autoimmune diseases. Moreover, TGF-β induces naive CD4 T-cells to differentiate into Foxp3+ Treg cells. These Treg cells produce TGF-β and IL-10 and act as modulators of immune responses (Leung et al., 2010).

1.5.3.1 IFN-γ

Interferons are vital cytokines responsible for orchestrating the host's defense against viral infections (Akamatsu et al., 2021). Type II IFNs or IFN-γ are primarily produced by immune cells including

innate-like lymphocyte populations, such as NK cells and ILCs, and adaptive immune cells, such as Th1 cells and CD8 CTLs (Ivashkiv, 2018). IFN-γ pro-inflammatory cytokine promotes activation of macrophages and immune response against intracellular pathogens, including viruses (Ivashkiv, 2018). In the presence of IFN-γ and IL-12, naive CD4 T-cells undergo differentiation towards the Th1 subset, which is a process reliant on the activities of STAT1, the transcription factor T-bet, and STAT4. Th1 cells are characterized by their robust production of IFN-γ and serve a pivotal function in conferring protective immunity against intracellular pathogens by orchestrating the activation of macrophages (Leung *et al.*, 2010). Previously, IFN-γ has been shown to increase the susceptibility to influenza A infection and play a detrimental role in the pathogenesis of influenza through suppression of group II innate lymphoid cells (Califano et al., 2018). High levels of IFN-γ are detected in individuals with moderate to severe COVID-19, along with other proinflammatory cytokines (Akamatsu *et al.*, 2021). Moreover, the combination of IFN-γ and TNF-α prompts inflammatory cell death, characterized by pyroptosis, apoptosis, and necroptosis, in bone marrow-derived macrophages (Akamatsu *et al.*, 2021).

1.5.3.2 IL-2

IL-2 is a 15.5-kDa globular protein subject to variable glycosylation which was initially characterized as T-cells growth factor, exhibiting potent mitogenic and growth-regulatory effects on T-cells in vitro (Ross and Cantrell, 2018). The discovery of IL-2 has revolutionized immunology research, unveiling key signal transduction pathways like the calcium/calcineurin pathway, which is crucial for NFAT nuclear translocation (Macian, 2005). IL-2 modulates the fate determination of CD4 T-cells, promoting Th1 and Th2 lineage commitment while suppressing the differentiation of Th17 and Tfh subsets (Cote-Sierra et al., 2004; Laurence et al., 2007; Liao et al., 2008). IL-2 is predominantly produced by CD4 T-cells and plays a pivotal role in orchestrating the proliferation and specialization of CD4 T, CD8 T, NK cells, and other cellular populations via the IL-2R–JAK–STAT5 signaling cascade (Yang *et al.*, 2020). IL-2 also plays a critical role in CD8 effector cells. Upon infection, naive antigen-specific CD8 T-cells undergo proliferation and differentiate into effector CTLs that produce

proinflammatory cytokines such as IFN-γ and obtain the ability to eliminate infected cells (Ross and Cantrell, 2018). IL-2 further modulates CD8 T-cells by promoting the expression of IFN-γ, TNF-α, and lymphotoxin α, stimulating cytolytic effector molecules such as granzyme B and perforin, and augmenting target T-cells killing efficiency (Ross and Cantrell, 2018). Low-dose IL-2 therapy has been shown to be effective in accelerating virus clearance and promotes effector CD8 T-cell response in acute LCMV and influenza virus infection as well as reduces incidence of infection in systemic lupus erythematosus patients (Zhou et al., 2021). Studies have shown an elevated levels of IL-2 to be observed in severe COVID-19 patients as well as other types of coronavirus infection (Yang et al., 2021).

1.5.3.3 TNF-α

TNF-α is a pro-inflammatory cytokine involved in the regulation of immune cells and produced mainly by activated T-cells and macrophages in acute inflammation (Lee, 2015). TNF-α triggers apoptosis and inflammatory responses in intestinal epithelial cells, and recent studies have shown its capacity to disrupt the intestinal tight junction barrier through various mechanisms (Lee, 2015). Inappropriate or excessive activation of TNF-α signaling is linked to chronic inflammation and may ultimately result in the onset of pathological complications, including autoimmune diseases (Jang *et al.*, 2021). Previous study has demonstrated that TNF-α display robust antiviral effects against avian, swine, and human influenza viruses, surpassing the antiviral efficacy of both IFN- α/γ (Seo and Webster, 2002). Morris et al. and our group have established a link between elevated TNF-α levels and increased circulating BDG levels in PLWH (Mehraj et al., 2020b; Morris et al., 2012). Furthermore, individuals with severe COVID-19 exhibited higher systemic levels IL-6 and TNF-α (Dorneles et al., 2023)

1.5.4.4 IL-4

IL-4 is an anti-inflammatory cytokine that plays a critical role in allergic inflammation and parasite infection by facilitating the switch in immunoglobulin class from IgE to IgG4 (Iwaszko et al., 2021).

IL-4 can also influence the immune response to viral infections by promoting Th2 differentiation and inhibiting Th1 generation during T-cell activation in the lymph node. In areas of inflammation, IL-4 signals enhance the recruitment and activity of both innate and adaptive Type-2 immune cells, strengthening the local Th2 response (Lazarski et al., 2013). IL-4 has previously been shown to inhibit nitric oxide production, which is a potent antimicrobial mediator, and markedly suppresses the development of antiviral cell-mediated immune responses and impair viral clearance in vivo (Sharma et al., 1996). IL-4 also play a role in wound healing in which it stimulates extracellular matrix synthesis to restore damaged tissue barriers (Salmon-Ehr et al., 2000). Recently, serum IL-4 levels were significantly higher in non-severe COVID-19 infected individuals, participants than in severe cases (Chang et al., 2022).

1.5.3.5 IL-10

IL-10 was initially characterized as a cytokine primarily secreted by Th2 cells, and later found to be produced by various myeloid and lymphoid cells, including CD4 Th1, Th2, and Th17 cells, Treg cells, dendritic cells (DCs), monocytes, and macrophages (Carlini et al., 2023). In vitro studies have revealed that IL-10, which is known for its anti-inflammatory properties and immunosuppressive effects, acts to counterbalance the cellular functions triggered by TNF-α and IFN-γ, emphasizing its role in regulating immune responses (Lee, 2015). IL-10 plays a protective role for preserving intestinal barrier integrity, as observed in IL-10 knockout mice, where heightened intestinal permeability and increased pro-inflammatory cytokine expression precede signs of inflammation. Moreover, studies reveal that total parenteral nutrition disrupts the intestinal barrier, yet IL-10 treatment effectively reverses these effects, which underscores its role in restoring barrier function (Lee, 2015). Upregulation of IL-10 expression plays both protective and harmful role on the organism. It regulates immune responses during acute infections to prevent excessive inflammation and aids in inflammation resolution post-pathogen clearance. However, excessive IL-10 can also prevent effective inflammation, promote immune tolerance, and facilitate microbial persistence, which could potentially lead to chronic infections (Carlini *et al.*, 2023). Previous studies have demonstrated

elevated circulating IL-10 levels in severe COVID-19 patients, which were positively correlated with increased exhaustion markers PD-1 and TIM-3 expression on T-cells, as well as a decreased total count of CD4 and CD8 T-cells (Carlini *et al.*, 2023).

1.5.3.6 IL-21

IL-21 is a pleiotropic cytokine that modulates various cellular functions proliferation, survival, differentiation, and function across lymphoid, myeloid, and epithelial cells. IL-21 is vital for B-cell differentiation into plasma cells and Tfh cell development, which is crucial for germinal center function and immunoglobulin production (Spolski and Leonard, 2014). It also enhances CD8 T-cells survival, antiviral and antitumor activity, while promoting the generation of pathogenic TH17 cells linked to various inflammatory diseases. (Spolski and Leonard, 2014). IL-21 plays a significant role in the immune response to acute HCV infection, which is characterized by increased levels of plasma IL-21 and increased IL-21- and IL-17-producing TH17 CD4 cells (Kared et al., 2013). IL-21 also enhances cytotoxic function in HIV-1-specific CD8 T-cells without inducing cellular activation, suggesting its therapeutic potential for modulating cytotoxic function in HIV patients (White et al., 2007). Previous research has demonstrated the predictive value of IL-21 in COVID-19 disease progression (Zhang et al., 2021).

1.5.3.7 IL-17A

IL-17A was first identified as cytotoxic T-lymphocyte antigen-8, which exhibits homology with the HSV13 gene from herpesvirus Saimiri (Pappu et al., 2011). IL-17 A is primarily produced by Th17 cells and is implicated in inflammatory and autoimmune disease including RA, multiple sclerosis and IBD (Lee, 2015). Th17 cells play a critical role in initiating the adaptive immune response against bacterial and fungal infections, as well as in the development of numerous inflammatory diseases. Their differentiation from naive CD4 T-cells depends on signals from IL-6 and TGF-β, while IL-23 and IL-21 are essential for their long-term maintenance (Pappu *et al.*, 2011). IL-17A stimulates innate immunity within tissues by initiating pro-inflammatory responses. Additionally, its synergistic

interaction with cytokines such as TNF-α, IL-1β, and IFN-γ amplifies pro-inflammatory responses across various target T-cells (Pappu *et al.*, 2011).

1.5.4 Polyfunctionality of Effector T-cells

Polyfunctionality refers to the capacity of T-cells to perform various functions concurrently, including the simultaneous secretion of multiple cytokines, chemokines, or cytotoxic granules at the individual cell level. The presence of polyfunctional T-cells has been proposed as a sensitive indicator of immune control in immunological diseases (Imai et al., 2020). In CD4 T-cells, this characteristic serves as a correlate for protection against various pathogens. For instance, comparing T-cell cytokine production profiles in different HIV-infected groups, from those controlling the infection to those with chronic progression revealed distinct molecular signatures associated with immune control (Betts et al., 2006). This suggests that the quality, rather than the quantity, of T-cell response may be correlated with immune protection. Polyfunctional T-cell development during CMV infection is associated with better prognosis and provides an immunological advantage against other pathogens (Pera et al., 2014). Additionally, polyfunctional CD4 T-cells are indicative of spontaneous viral replication control in CMV-seropositive liver transplant recipients (Carvalho-Gomes et al., 2022). Overall, this underscores the significance of assessing representative T-cell functions to identify and define immune protection correlates. A prior investigation revealed that individuals who recovered from severe COVID-19 exhibited elevated proportions of antigen-specific CD4 T-cells secreting Th1 and Th17 cytokines characterized by polyfunctional SARS-CoV-2-specific CD4 T-cells (Paolini et al., 2022). A similar cytokine profile was observed in patients with moderate COVID-19 pneumonia, while no significant differences were observed in polyfunctionality among CD8 T-cell compartments (Paolini *et al.*, 2022). Identifying these functional cell subsets can enhance our understanding of the intricate immune response to SARS-CoV-2 and its potential impact on protection.

1.6 Rational, experimental aims and hypothesis

Although linked with inflammation, the influence of gut permeability on immune function remains elusive during COVID-19 and other viral infections. By elucidating the relationship between gut

permeability and COVID-related inflammation and SARS-CoV-2 specific immunity, we aim to aid in the development of strategies to prevent or mitigate inflammation severity and symptoms during COVID-19, and promote establishment of long-lasting immune memory after infection or vaccine. Specifically, we seek to analyze how intestinal permeability influences inflammation and the immune response against SARS-CoV-2 during acute and recovered COVID-19 cases. We hypothesize that markers of mucosal damage, microbial translocation, and inflammation will increase during acute COVID-19 and correlate with decreased anti-SARS-CoV-2 cellular and humoral immune responses.

2. Methods

2.1 Study Design and Participant

In this cross-sectional analysis, a total of 55 adults were enrolled from the Biobanque québécoise de la COVID-19 (BQC19) and the Chronic Viral Illness Service at the McGill University Health Center. COVID-19 participants were categorized into hospitalized controls, acute, early recovered and late recovered groups. Hospitalized participants in the primary phase of the infection with recovery duration of either 0 or 2 days (n=19) were considered as acute group. 10 of the acute participants were also collected at day 30 post hospitalization and day-30 was classified as early recovery timepoint. People who recovered from COVID-19 were also recruited (n=15, median recovery duration: 229 days) from the CVIS and pool of HIV-negative donors. Healthy controls (n=3) who were PCR-negative for COVID-19 were also included. All test results from COVID-19 participants were compared with 11 controls (8 hospitalized and 3 non-hospitalized) who were also enrolled from the BQC19 and Chronic Viral Illness Service. Demographic data were extracted from medical charts and participant interview.

2.2 Clinical Laboratory Measurements

COVID-19 diagnostic was confirmed by RT-PCR at the time of infection.

2.3 Blood Samples Preparation

Whole blood was collected into ACD vacutainer tubes by venipuncture, and plasma was separated by centrifugation at 750g 10 min at room temperature less than 1 hour after blood collection. Isolated

plasma was aliquoted and frozen at −80°C until analysis. Concentrated blood was diluted in PBS and layered on Ficoll and subjected to centrifugation at 750g, without break, 20 min at room temperature to isolate the PBMC ring. Cells were then washed twice in PBS. A final centrifugation at 150g for 15 min was performed to eliminate most platelets. PBMCs were then stored in FBS containing 10% DMSO, in liquid nitrogen, until used.

2.4 Measurement of Plasma I-FABP, REG3α and LPS Levels

Plasma levels of I-FABP, REG3α and LPS were measured in duplicates for each sample by the ELISA assay (Hycult, R&D systems and Cusabio respectively) specific for each individual human markers as per manufacturer's instructions.

2.5 Measurement of plasma BDG levels using Fungitell Assay

BDG was quantified in plasma using the Fungitell TM assay (Associates of Cape Cod Inc.).

2.6 Quantification of spike-specific humoral response by MSD V-PLEX COVID-19 ELISA

Precoated multiplex assay plates were provided in MSD V-PLEX and plate 25 and 29 were performed as previously described (Patil et al., 2023). Briefly, each well on the 96-well plate was coated 10 different SARS-CoV-2 variants at the well base. First, assay plates were blocked with 150 µl/well of MSD Blocker A solution at room temperature with shaking for 30 min. Serum samples were diluted 1:5000 in MSD-Diluent 100, and calibrators were diluted according to the manufacturer's instructions. After blocking, plates were washed three times with 150 µl of MSD wash buffer. Samples (25 µl) and calibrators (25 µl) were added in duplicates to the plate and incubated for 2 hours at room temperature with shaking. Subsequently, 1× SULFO-TAG anti-human Ig antibody solution (25 µl) was added to the same wells containing serum samples, followed by incubation for 1 hour with shaking. This antibody competes with neutralizing antibodies present in serum samples for pre-coated antigen variants. After another three washes with MSD wash buffer, MSD GOLD Read Buffer B (150 µl) was added immediately, and plates were read in the MSD instrument. After wash and substrate addition, raw data was processed using MSD's Discovery Workbench version 4.0 and quantification was reported in Arbitrary Units/mL (AU/mL). As per manufacturer guidelines, cut-off values were

established as following: SARS-CoV-2 Spike, 1960 AU/mL; SARS-CoV-2 NC, 5000 AU/mL; and SARS-CoV-2 S1 RBD, 538 AU/mL. Value detected in Spot one, against the wild type virus that was infected our participants at the time of sampling, were taken into account.

2.7 Quantification of SARC-CoV-2 spike-specific T-cell responses

PBMCs were thawed in complete medium (RPMI containing 10%FBS), washed in PBS and incubated overnight in R10 medium. Cells were counted and then centrifuged at 400g 5 min at room temperature. Supernatants were discarded and cells were resuspended in R10 at 10 million cells/ml. One million PBMCs were incubated with peptides from (CMV, SARS-CoV-2 Spike, SARS-CoV-2 Nucleocapsid (0.6nmol/mL of each peptide) or PMA ionomycin (both at 1ng/mL) for 1h at 37°C. Brefeldin A (final concentration 5µg/mL) was added and plate was incubated for 5h at 37°C before proceeding to flow cytometry staining.

2.8 Spike-specific T-cell Cytokine Production Flow cytometry analyses

Extracellular labeling was performed in 50ul of FACS buffer (PBS 2mM EDTA, 0.5% FBS) with LiveDead Blue (Invitrogen), CD3, CD4, CD8 and CD45RA. The viability dye LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells (**Table 1**). Cells were kept on ice for 20 min in the dark. FACS buffer was added to each tube, and samples were wash by centrifugation at 2000 RPM for 3 mins at 4 °C in plate or 400g, 5 min at 4°C in tubes to wash. Supernatant was discarded. Cells were fixed by adding 100µl of PFA4% to each tube and incubating them on ice for 15 mins in the dark for intracellular staining. Then, 100ul of PERM buffer (PBS, 0.1%BSA, 0.1% Saponin) was added to each tube before subjected to two washes in the permeabilization buffer. Intracellular staining was performed in 50ul of PERM buffer with IFN-γ, IL-2, TNF-α, IL-4, IL-10, IL-21, IL-17A (**Table 2**) and incubated on ice for 45 mins in the dark. Cells were subjected to two rounds of centrifugation in the permeabilization buffer and resuspended in 150ul of FACS buffer to be analysed on the BD-LSRFortessa X-20 cytometer (BD Biosciences). FlowJo software (©Tree Star Ashland, OR) was used for analysis. Positivity gates were placed using the isotype strategy and compared to the unstimulated cells.

Table 1. Surface staining panel

Surface			
Fluorophore	Epitope	Manufacturer	Clone
Indo-1	LiveDead Blue	Invitrogen	
BUV737	CD8	BioLegend	SK1
APC-Cy7	CD45RA	BioLegend	HI100
BUV395	CD4	BD Biiosciences	RPA-T4
BV711	CD3	BioLegend	UCHT1

Table 2. Intracellular staining panel

Intracellular staining			
Fluorophore	Epitope	Manufacturer	Clone
BV510	IL4	BioLegend	MP4-25D2
BV421	IL-10	BioLegend	JES3-9D7
Percp	IL21	BioLegend	3A3-N2
FITC	IL-2	BioLegend	MQ1-17H12
PE-Cy7	IFN-g	BioLegend	B27
PE	IL17A	BioLegend	BL168
A700	TNFa	BD Pharmingen	MAb11

2.9 BDG Receptors Flow Cytometry Analyses

Frozen PBMCs were rapidly thawed and stained for 20 min at 4°C using 2 fluorochrome-conjugated antibody panels (**Table 3&4**). Cells were then washed and fixed in 2% paraformaldehyde for acquisition. Fluorescence minus one color controls were used to discriminate autofluorescence from positive signals. Cells were acquired using a BD Fortessa X20 (BD Biosciences) flow cytometer. Data were analyzed using FlowJo 10.0.7 (FlowJo, LLC).

Table 3. Monocyte/DC panel

Epitope	Fluorophore	Availability	Clone	Catalogue #
CD11b	PerCP	BioLegend	M1/70	101229
Dectin-1	PE	BioLegend	15E2	355403
CD38	BV605	BDBioSciences	HB7	562666
CD8	BUV737	BDBioSciences	SK1	564629
CD18	PE/Cy7	BioLegend	TS1/18	302117
CD14	BV785	BioLegend	M5E2	301839
HLA-DR	APC Cy7	BDBioSciences	L243	335796
CD123	APC	BioLegend	6H6	306011
CD16	BV510	BioLegend	3G8	302047
CD4	BUV395	BDBioSciences	RPA-T4	562724
CD3	PE/Dazzle 594	BioLegend	UCHT1	300449
CD11c	BV711	BDBioSciences	B-ly6	563130
PD-1	BV421	BioLegend	EH12.2H7	329919
CD56	FITC	BDBioSciences	B159	562794
LD	Blue	ThermoFisher Scientific	NA	L34961

Table 4. Lymphocyte/NK panel

Epitope	Fluorophore	Availability	Clone	Catalogue #
CD19	AlexaFluor700	BioLegend	HIB19	302226
CD14	BV785	BioLegend	M5E2	301839
NKp30 (CD337)	PE	BioLegend	P30-15	325208
NKp46 (CD335)	APC	BioLegend	9.00E+02	331918
CD16	BV510	BioLegend	3G8	302047
CD3	PE/Dazzle 594	BioLegend	UCHT1	300449
CD4	BUV395	BDBioSciences	RPA-T4	562724
CD56	FITC	BDBioSciences	B159	562794
LD	Blue	ThermoFisher Scientific	NA	L34961

2.10 Monocyte Isolation and Purification

PBMCs from healthy participants were washed and thawed in PBS and resuspended in PBS 2% FBS 1mM EDTA buffer to achieve a final concentration of 50×10^6 cells/ml. Monocytes were isolated and purified using the CD16+CD14+ negative selection kit as per manufacturer's instructions (Stemcell).

2.11 Monocyte Differentiation into Macrophages

Plated monocytes were stimulated in R10 medium with 20 ng/ml M-CSF and 20 ng/mL GM-CSF and incubated in 37 °C cell culture incubator with 5% CO_2 / 95% air for 7 days.

2.12 Measurement of Supernatant TNF-α and IL-6 Levels

ELISA kits from R&D Systems were used to quantify secreted TNF-α and IL-6 in cell culture supernatants as per manufacturer's instruction.

2.13 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad, CA, USA). Spearman's rank correlation test identified associations between 2 quantitative variables. Mann-Whitney U test and student t-test were used to compare two independent groups. Kruskal-Wallis one way ANOVA test was used to compare more than 2 independent study groups. Paired analysis was performed by Wilcoxon signed-rank test. P-values < 0.001 were considered significant for samples with $n > 250$, and $p < 0.05$ for samples less < 250. Logistic regression models were used to generate Receiver operating characteristic (ROC) curve. Multivariable analysis was performed using SPSS.

2.14 Ethics

All BQC19 study participants received ethics approval of from the institutional review board (IRB) of the Jewish General Hospital and the Centre Hospitalier de l'Université de Montréal (CHUM) in Montréal, QC, Canada. This project was approved by the research ethics board of the McGill University Health Centre (MUHC).

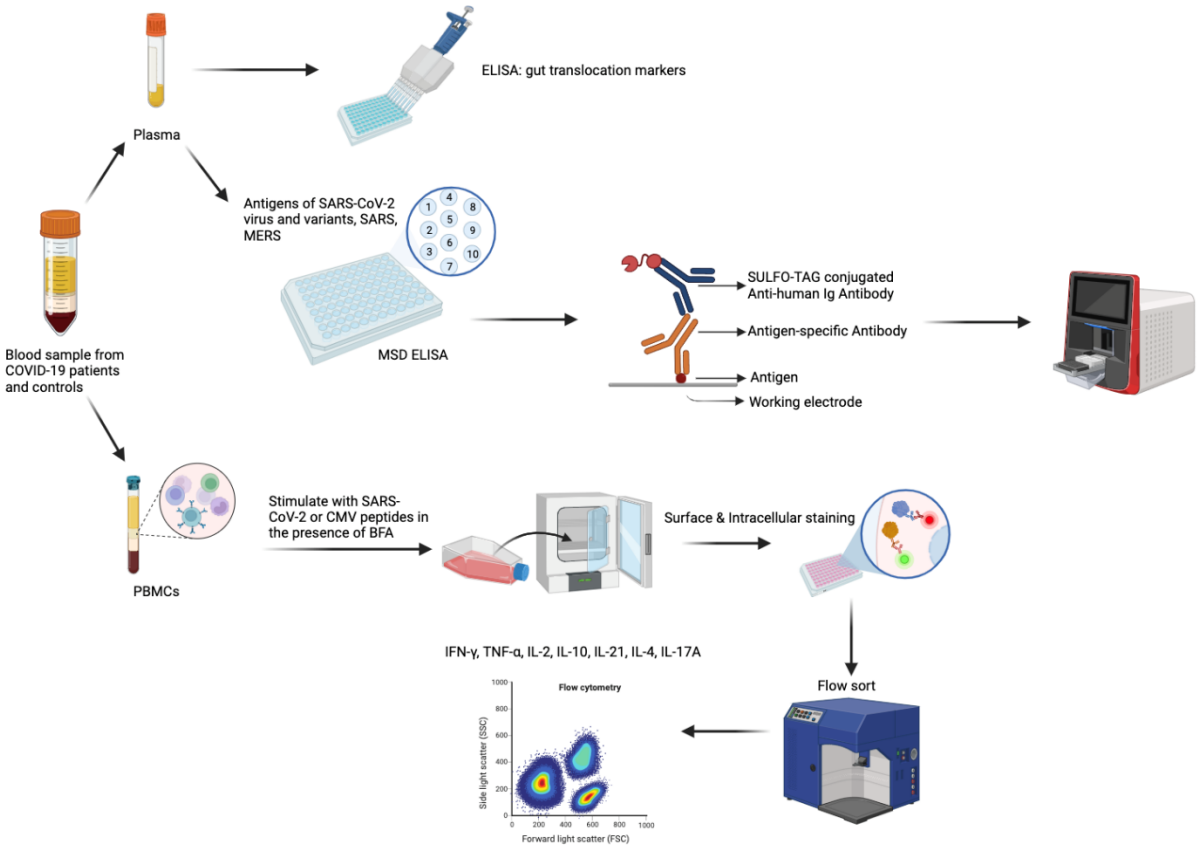

Figure 5: Experimental approach. Blood sample from COVID-19 infected participants and controls were processed into plasma and PBMCs. ELISA were used to quantify gut translocation markers (I-FABP, REG3α and LPS) levels in plasma. MSD ELISA were used to assess the spike-specific humoral response. PBMCs were stimulated with SARS-CoV-2 peptide and stained by cytokines including IFN-γ, IL-2, TNF-α, IL-4, IL-10, IL-21, IL-17A. Spike-specific T-cell responses were assessed by flow cytometry to decipher the percentage of pathogen responsive cells. Correlation analyses were performed to assess the influence of microbial translocation with each parameter. Macrophage stimulations were not shown because responses detected were very low.

3. Results

3.1 Study Participant Characteristics

A total of 55 adults participated in this study as shown in **Table 5**. Among them, 44 tested positive for COVID-19 and were categorized into three cohorts based on their recovery duration. The

remaining 11 participants were COVID-19 negative controls, comprising 3 healthy controls and 8 hospitalized controls. The youngest median age was observed in the healthy control cohort at 31 years (range: 30-51), followed by the late-recovered cohort at 35 years (range: 20-69). In contrast, the hospitalized controls, acute, and early recovered groups exhibited relatively older median ages of 55 years (range: 46-88), 55 years (range: 32-94), and 56 years (range: 30-78), respectively. Majority of the participants were female and all participants in the acute and early recovered cohorts were hospitalized.

Table 5. Study Participant Characteristics

Characteristics	Controls (n=3)	Hospitalized controls (n=8)	Acute COVID-19 (n=19)	Early Recovered COVID-19 (n=10)	Late Recovered COVID-19 (n=15)
Age	31 (30-51)	55 (46-88)	55 (32-94)	56 (30-78)	35 (20-69)
Sex: Women	2	4	13	7	9
Men	1	4	6	3	6
Recovery Duration Median (IQR)	N/A	0	2 (0-2)	30	229 (135-406)
Hospitalization	1.74 (1.45-2.34)	0.37 (0.02-0.99)	0.64 (0.01-1.19)	10	2

N = 55, 44COVID-19 participants and 11 controls

3.2 Lower plasma levels of I-FABP and higher plasma levels of REG3α in acute COVID-19

We first assessed plasma levels of different markers of the main gut permeability and microbial translocation in our groups of acutely infected or recovered COVID-19 patients. Cross-sectional analysis showed higher plasma I-FABP levels in late recovered COVID-19 cohorts compared to acute COVD-19 cohorts. ($p = 0.0084$) **(Figure 6A)**. Cross-sectional analysis showed higher plasma REG3α levels in hospitalized controls and acute COVID-19 cohorts compared with healthy controls ($p < 0.05$ for both) **(Figure 6B)**. No distinction of plasma levels of BDG or LPS were observed in all groups tested **(Figure 6C, D)**. Longitudinal assessment of 10 pairs of acute COVID-19 participants showed varied levels of gut damage and fungal translocation markers at a 30-day-follow-up period (**Figure 6E-G**). LPS levels were not tested in two groups due to insufficient samples.

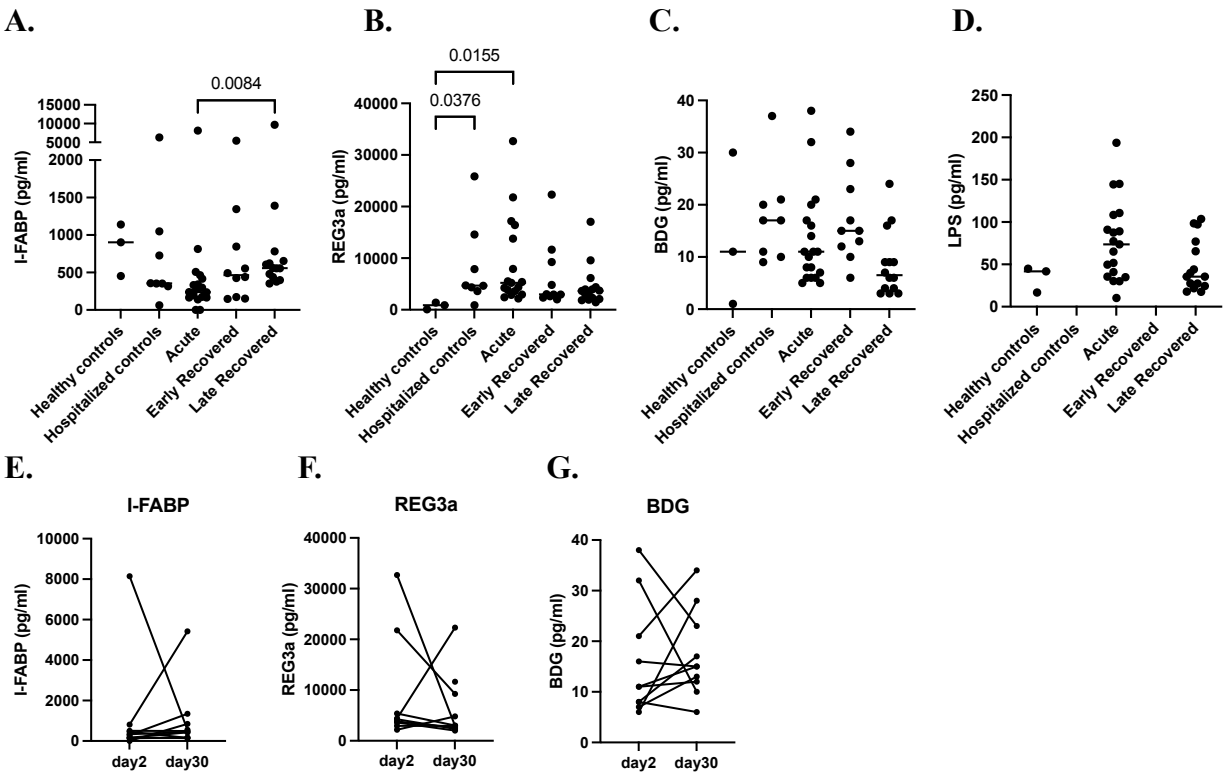

Figure 6: Cross-sectional and longitudinal plasma levels of I-FABP, REG3α, BDG and LPS among different groups of COVID-19 infection. Higher plasma levels of I-FABP (**A**) were observed in late recovered compared to acute COVID-19 cohorts (p = 0.0084 Kruskal-Wallis' test with Dunn's post-test). Cross-sectional analysis showed higher plasma levels of REG3α (**B**) in hospitalized controls and acute COVID-19 cohorts compared with healthy controls ($p < 0.05$ for both, Kruskal-Wallis' test with Dunn's post-test). No distinction of plasma levels of BDG (**C**) or LPS (**D**) were observed in all groups tested (samples were insufficient for LPS testing). Longitudinal assessment of 10 pairs of acute COVID-19 participants showed varied levels of I-FABP (**E**), REG3α (**F**) and BDG (**G**) at a 30-day-follow-up period.

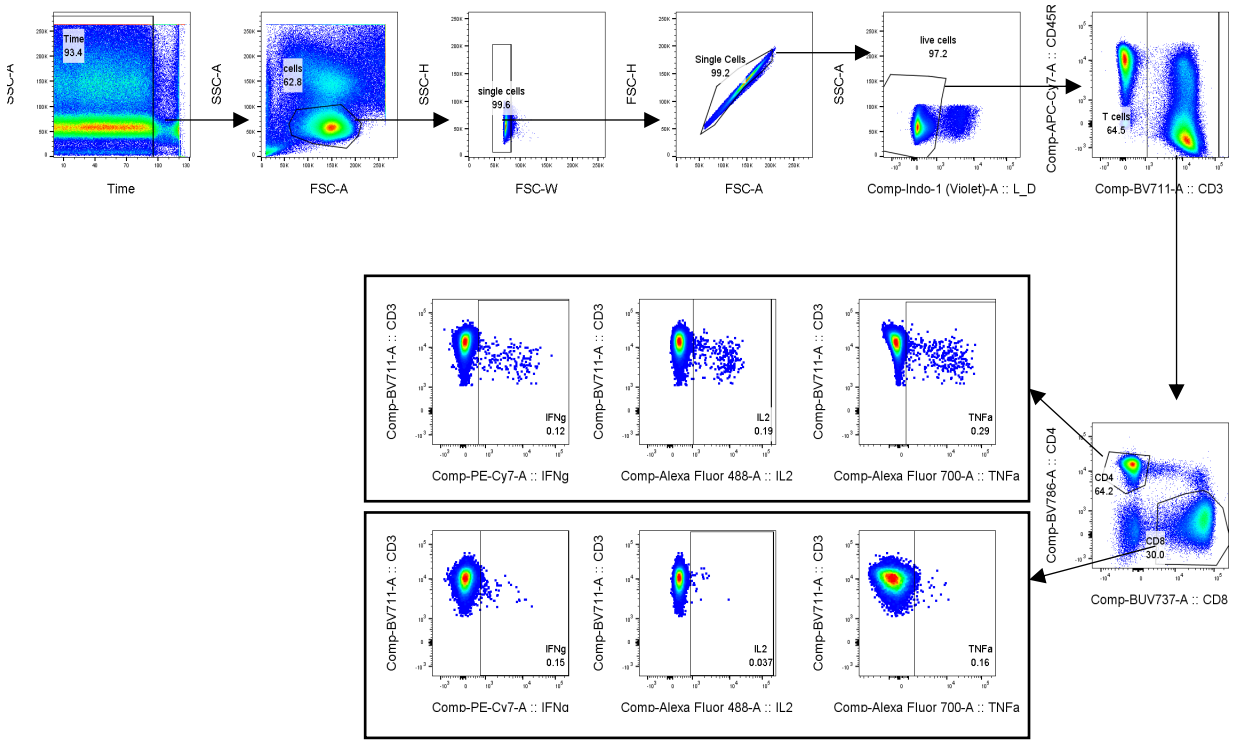

Figure 7: Gating Strategy upon SARS-CoV-2 stimulation. Time gate was applied to exclude shifting scatter created by nonlaminar flow and gate on good acquisition interval (Staats et al., 2019). Height versus width and height versus area singlet gates using SSC and FSC was applied to exclude doublets and aggregates. Live cells were selected by viability dyes. CD3 was used as a parental gate prior to analysis of T-cell subsets., then CD4 versus CD8 T-cell gating were employed to discriminate CD4 and CD8 populations. Intracellular cytokine productions were gated in CD4 and CD8 activated T-cells.

3.3 SARS-CoV-2 spike-specific T-cell responses in different groups

We then quantified the frequency of SARS-CoV-2 spike specific CD4 and CD8 T-cells between groups. Flow cytometry gating strategy of spike-specific T-cell responses was shown in **Figure 7**. T-cell responses were consistently low across all cohorts, with a notable increase observed in the COVID-19 groups compared to controls. The highest T-cell responses were identified in individuals from the early or late recovered COVID-19 cohorts **(Figure 8A-F)**. Longitudinal assessment of 10 pairs of acute COVID-19 participants showed varied levels of SARS-CoV-2 spike-specific CD4 or CD8 T-cell inflammatory cytokine at a 30-day-follow-up period **(Figure 9A-F)**. T-cell counts were not available, thus we were not able to compute absolute count of specific cells.

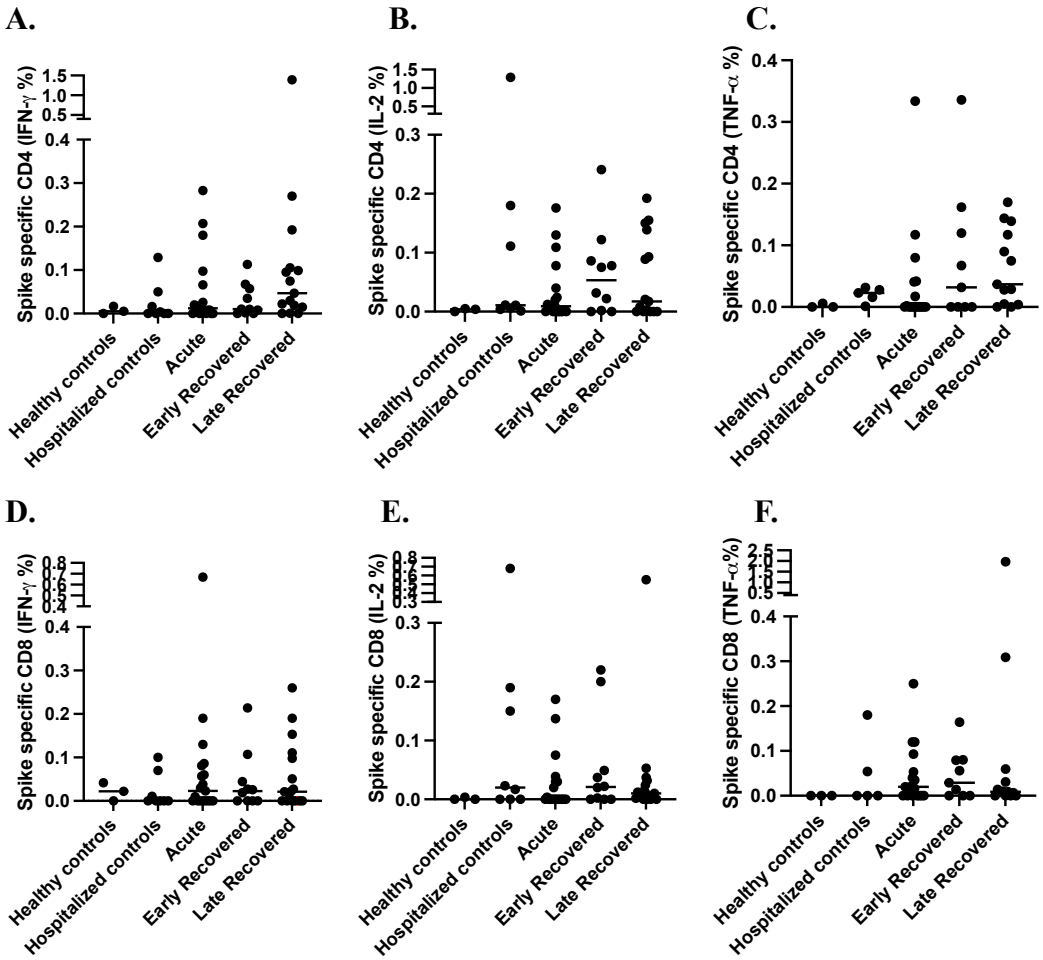

Figure 8: SARS-CoV-2 spike-specific cytokine production by CD4 and CD8 T-cells. Out of the 7 intracellular cytokines tested, only IFN-γ (**A,D**), IL-2 (**B,E**) and TNF-α (**C,F**) yielded detectable production. However, according to our flow cytometry results, the overall T-cell responses are still quite low. Highest responses were detected in early or late recovered COVID-19 participants.

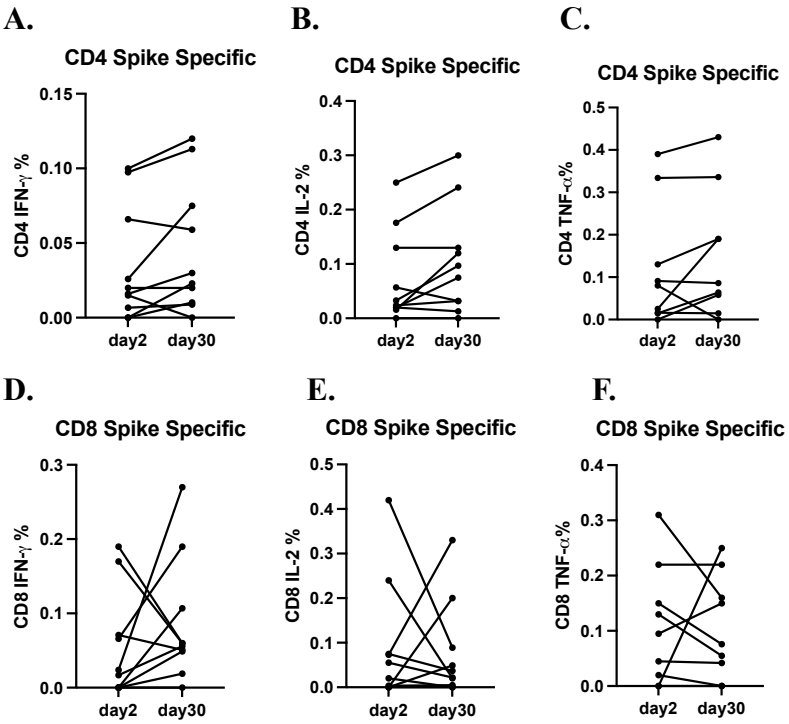

Figure 9: Day 2 versus Day 30 gut markers and cytokine production in T-cells in response to SARS-CoV-2 peptides. Longitudinal assessment of 10 pairs of acute COVID-19 participants showed variation in frequency of SARS-CoV-2 spike-specific CD4 or CD8 T-cell cytokine productions at day 30 follow up.

3.4 SARS-CoV-2 spike-specific B-cell responses

To assess the B-cell response against SARS-CoV-2, we quantified Spike-specific IgG levels in plasma. Spike-specific B-cell responses remained consistently low in both the acute and late recovered groups, while the early recovered COVID-19 cohort exhibited significantly higher responses compared to the healthy controls (p=0.0064) **(Figure 10)**. Anti-SARS-CoV-2 IgG levels were also found to be elevated in severe cohorts compared to hospitalized controls (p=0.0494) and were also higher in early recovered cohorts than in hospitalized controls (p=0.0368) **(Figure 11)**.

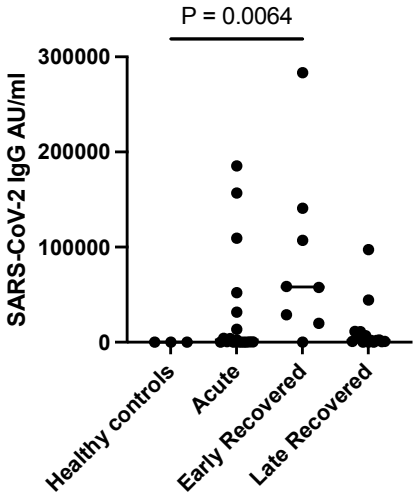

Figure 10: SARS-CoV-2 spike-specific IgG production by B-cells. According to our MSD ELISA results, B-cell responses were consistently low in the acute and late recovered groups with highest responses detected in early recovered COVID-19 groups. Significant differences were found between early recovered COVID-19 participants and healthy controls (p=0.0064).

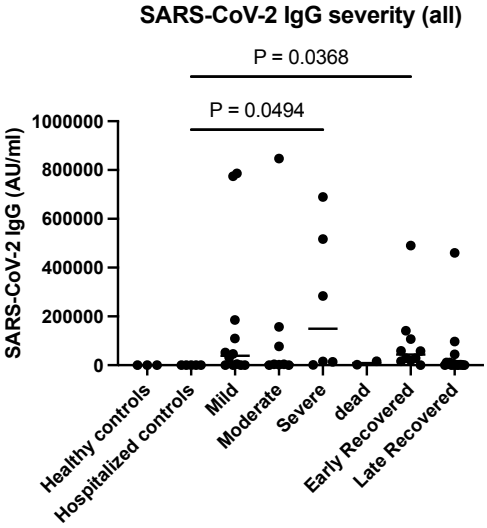

Figure 11: SARS-CoV-2 spike-specific IgG levels and disease severity. IgG levels were higher in severe cohorts compared to the hospitalized controls (p=0.0494 Kruskal-Wallis one way ANOVA test), and higher in early recovered cohorts than the hospitalized controls (p=0.0368 Kruskal-Wallis one way ANOVA test).

3.5 BDG and LPS correlation with spike-specific T-cell and B-cell responses

To assess links between gut permeability and SARS-CoV-2 specific cellular and humoral immune responses, we performed correlation between gut permeability markers and T-cell and antibody responses. All correlations between spike-specific T-cell responses, B-cell responses, gut damage, and microbial translocation markers are summarized in **Tables 6** and **7**, with significant correlations ($p < 0.05$) highlighted in red. Plasma BDG concentration positively correlated with percentage of

spike-specific CD4 T-cell response in acute COVID-19 **(Figure 12)** and negatively correlated with percentage of spike-specific CD4, CD8 T-cell and B-cell response in late recovered COVID-19 cohort **(Figure 13A-C)**. In the acute cohort, BDG levels positively correlated with percentage of spike-specific CD4 producing IL-2 T-cells **(Figure 12)**. Conversely, in late recovered COVID-19, BDG levels negatively correlated with percentage of IL-2 and TNF-α produced by SARS-CoV-2 spike-specific CD4 T-cells **(Figure 13A, B)**. Anti-SARS-CoV-2 IgG levels also negatively correlated with plasma BDG levels in late recovered COVID-19 cohort **(Figure 13C)**. Additionally, plasma LPS levels positively correlated with percentage of CD4 producing IL-2 T-cells in late recovered cohort (**Figure 14)**.

Table 6. Acute Correlation with Gut Markers

		CD4 IFN-g		CD4 IL-2		CD4 TNF-a		CD8 IFN-g		CD8 IL-2		CD8 TNF-a		REG3a		I-FABP		BDG		LPS	
		p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r
CD4	IFN-g		1.0000	0.4339	-0.1540	0.0020	0.5597	0.0828	0.3335	0.8874	0.0280	0.6185	0.0984	0.6016	-0.1353	0.8520	-0.0459	0.2981	0.2519	0.1226	-0.5570
	IL-2	0.4339	-0.1540		1.0000	0.6452	0.0910	0.1306	-0.2927	0.7941	0.0516	0.3375	0.1882	0.4863	-0.1803	0.9527	-0.0146	0.0053	0.6124	0.8889	0.0685
	TNF-a	0.0020	0.5597	0.6452	0.0910		1.0000	0.9768	-0.0058	0.9352	0.0161	0.5468	-0.1189	0.8726	0.0430	0.6008	0.1283	0.0654	0.4311	0.1226	-0.5570
CD8	IFN-g	0.0828	0.3335	0.1306	-0.2927	0.9768	-0.0058		1.0000	0.1754	0.2636	0.0047	0.5187	0.7361	0.0877	0.7696	0.0720	0.5204	-0.1572	0.8611	-0.0693
	IL-2	0.8874	0.0280	0.7941	0.0516	0.9352	0.0161	0.1754	0.2636		1.0000	0.2713	0.2152	0.6496	-0.1180	0.5361	-0.1514	0.7617	-0.0745	0.8889	0.1369
	TNF-a	0.6185	0.0984	0.3375	0.1882	0.5468	-0.1189	0.0047	0.5187	0.2713	0.2152		1.0000	0.9628	0.0127	0.6048	-0.1269	0.2394	0.2836	0.5556	0.2510
SARS-CoV-2 IgG		0.8678	0.0461	0.2051	-0.3344	0.2527	-0.3578	0.1023	-0.4243	0.2856	-0.2827	0.8135	-0.0763	0.5862	-0.1471	0.2487	0.3059	0.4934	-0.1834		

Table 7. Late Recovered Correlation with Gut Markers

		CD4 IFN-g		CD4 IL-2		CD4 TNF-a		CD8 IFN-g		CD8 IL-2		CD8 TNF-a		REG3a		I-FABP		BDG		LPS	
		p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r
CD4	IFN-g		1.0000	0.2366	0.3240	0.2401	0.3213	0.3389	0.2630	0.9770	-0.0090	0.8621	0.0491	0.2778	-0.2985	0.2127	0.3542	0.0820	-0.4828	0.6261	-0.1360
	IL-2	0.23664	0.3240		1.0000	4.84E-06	0.9257	0.0718	0.4808	0.0803	0.4689	0.1566	0.3852	0.7984	0.0727	0.2036	0.3619	0.0016	-0.7841	0.0319	0.5626
	TNF-a	0.24013	0.32131	4.84E-06	0.9257		1.0000	0.2201	0.3343	0.0389	0.5431	0.0923	0.4518	0.5689	0.1587	0.2420	0.3334	0.0075	-0.6955	0.0895	0.4549
CD8	IFN-g	0.3389	0.2630	0.0718	0.4808	0.2201	0.3343		1.0000	0.7098	0.1040	0.3571	0.2535	0.1227	-0.4168	0.7362	-0.0984	0.1417	-0.4129	0.1978	0.3507
	IL-2	0.9770	-0.0090	0.0803	0.4689	0.0389	0.5431	0.7098	0.1040		1.0000	0.0111	0.6484	0.6739	0.1187	0.4220	-0.2328	0.4562	-0.2152	0.1541	0.3870
	TNF-a	0.8621	0.0491	0.1566	0.3852	0.0923	0.4518	0.3571	0.2535	0.0111	0.6484		1.0000	0.4326	0.2182	0.9504	-0.0202	0.2576	-0.3227	0.4032	0.2312
SARS-CoV-2 IgG		0.1320	0.4075	0.0007	0.7965	0.0439	0.5722	0.0553	0.5092	0.6835	0.1151	0.4431	0.2312	0.3335	-0.2679	0.1553	0.4022	0.0233	-0.6089	0.1115	0.4290

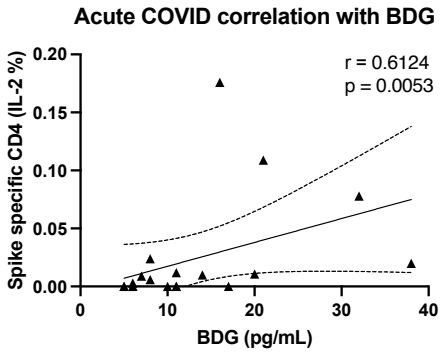

Figure 12: BDG correlation analysis in acute cohorts. In acute COVID-19 participants, plasma levels of BDG positively correlated with the percentage of IL-2 produced by spike-specific CD4 T-cells (p=0.0053). All correlations were independent of age and sex.

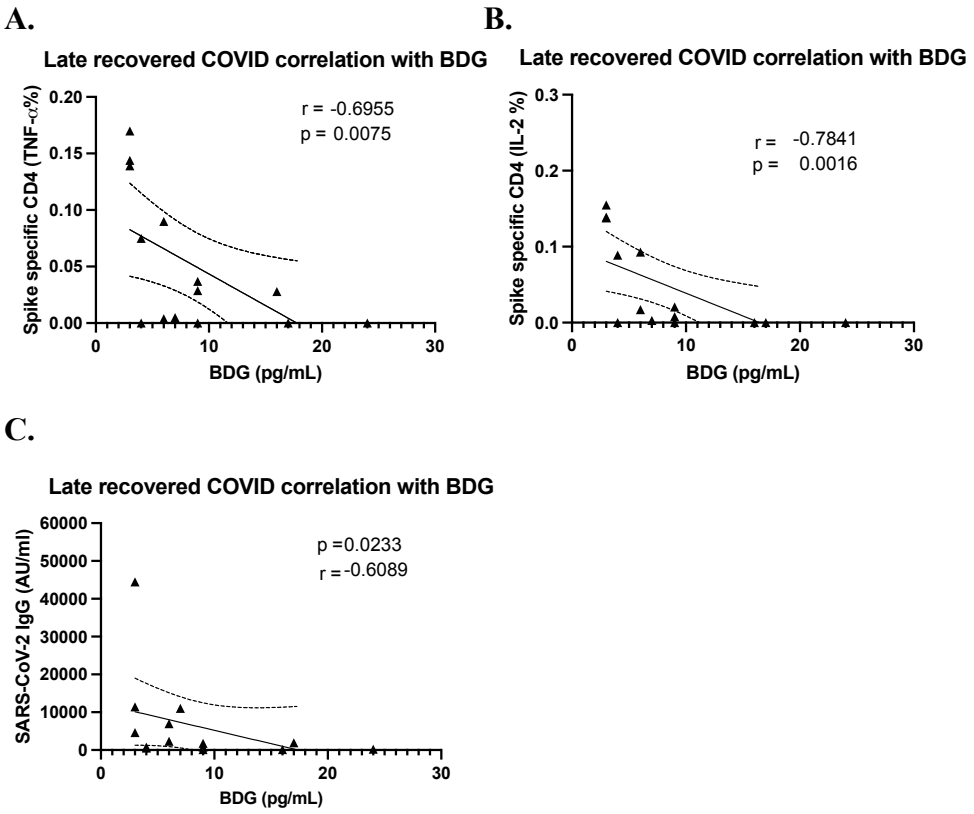

Figure 13: BDG correlation analysis in late recovered cohorts. In late recovered COVID-19 participants, plasma levels of BDG were negatively correlated with percentage of TNF-α (p=0.0075) (**A**) and IL-2 (p=0.0016) (**B**) produced by spike-specific CD4 T-cells. (**C**) Plasma levels of BDG were negatively correlated with SARS-CoV-2 IgG levels (p=0.0233). All correlations were independent of age and sex.

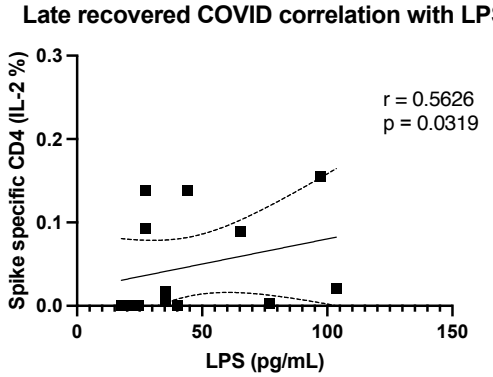

Figure 14: LPS correlation analysis in late recovered cohorts. In late recovered COVID-19 participants, plasma levels of LPS positively correlated with the percentage of IL-2 produced by spike-specific CD4 T-cells (p=0.0319). All correlations were independent of age and sex.

3.6 BDG receptor correlation

We have previously shown in people living with HIV that BDG binds to its receptors NKp30 on NK cells and Dectin-1 on monocytes. Binding of BDG to its receptor decreased expression of these receptors and a negative correlation was observed between BDG levels and cellular expression of NKp30 and Dectin-1. Thus, we compared BDG receptor levels on different cells on leftover PBMCs

from late recovered COVID-19 participants. BDG receptor, Dectin-1 and CD18 levels were also found to be elevated in late recovered COVID-19 participant with BDG levels <7 pg/mL compared to whom with BDG levels > 7 pg/mL in CD4, CD8 T-cells and B-cells and non-classical monocytes. BDG receptor CD11b levels appeared to be higher only in non-classical monocytes **(Figure 15)**. BDG receptor Dectin-1 dMFI tended to be consistently lower in late recovered COVID-19 participants with BDG levels greater than the median levels of 7 pg/mL in CD4, CD8, PD1+CD8 T-cells and B-cells compared to cells from participant with BDG levels lower than median levels of 7 pg/mL **(Figure 16A-E)**. Differences were only significant in PD+CD8 T-cells and B-cells (p=0.02 for both). Percentage of Dectin-1+ non-classical monocytes were also lower in late recovered COVID-19 participants with BDG levels > 7 pg/mL **(Figure 15 F).**

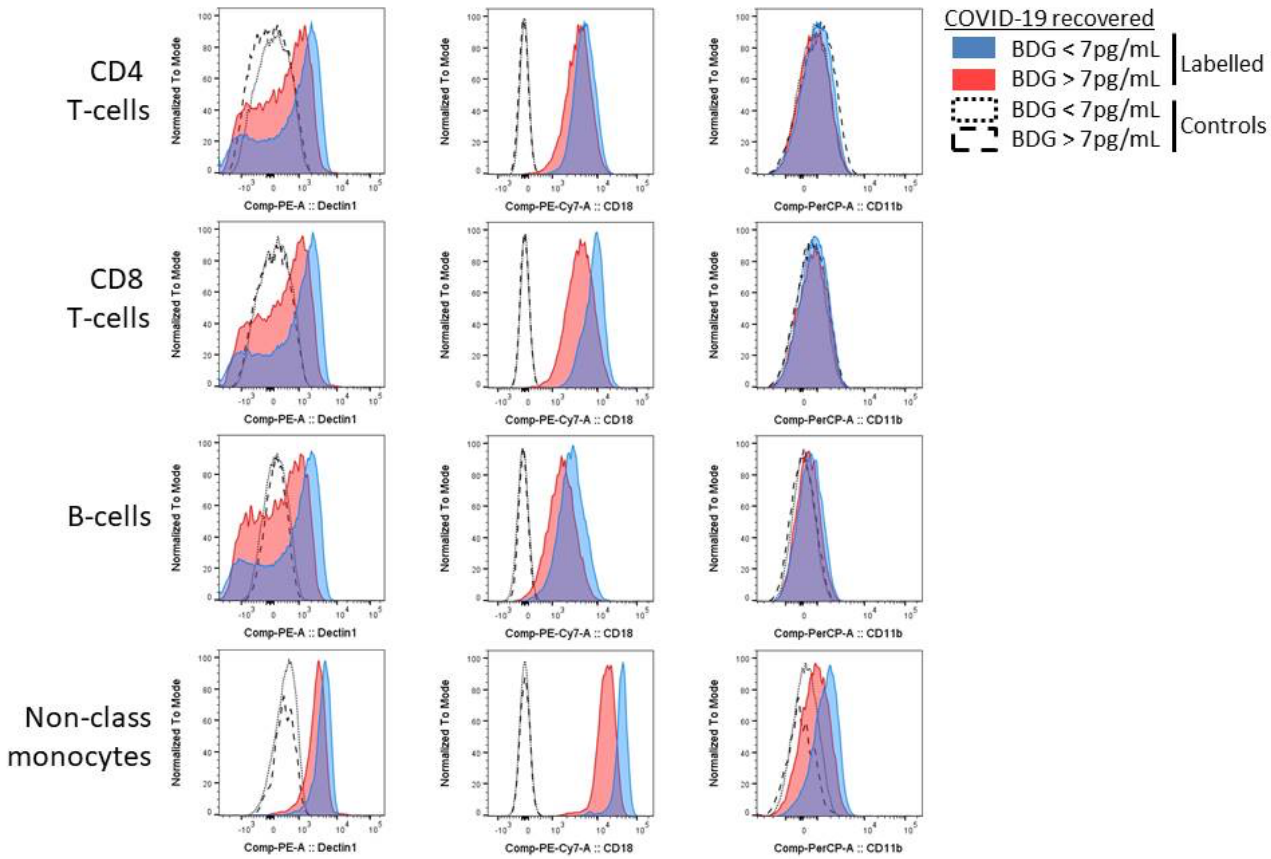

Figure 15: BDG receptor levels on immune cells from late recovered COVID-19 participants with different BDG levels. Representative histograms showed different expression of Dectin1, CD18, CD11b in LAP13 with low (<7 pg/mL, blue) or LAP16 with high (>7 pg/mL, blue) BDG expression in CD4, CD8 T-cells, B-cells and non-classical monocytes

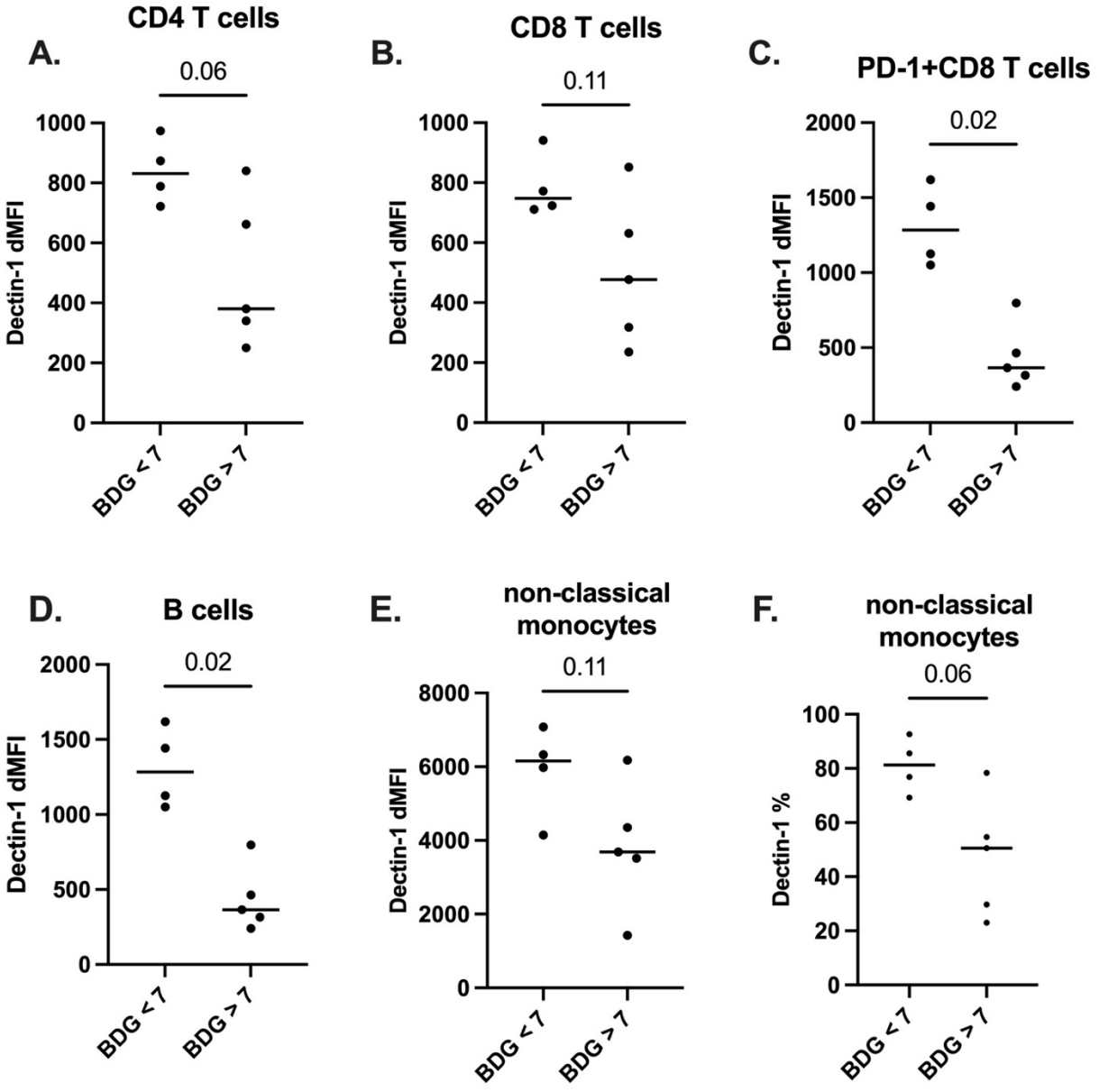

Figure 16: BDG receptor levels on immune cells from late recovered COVID-19 participants with different BDG levels. (**A-E**) Dectin-1 dMFI levels were higher in cells from late recovered COVID-19 participants with BDG levels below the median of 7pg/ml than in participants with BDG levels > 7ug/ml in CD4, CD8, PD-1+ CD8 T-cells, B-cells and non-classical monocytes (Mann-Whitney's test). (**F**) Percentage of Dectin-1+ non-classical monocytes were higher in late recovered COVID-19 participants with BDG levels < 7pg/mL than in participants with BDG levels > 7pg/mL (Mann-Whitney's test).

4. Discussion

SARS-CoV-2 primarily infects the respiratory tract, particularly the upper respiratory tract, while also directly invading the gastrointestinal tract and impairing its mucosal barrier function. Concurrently, acute COVID-19 inflammation has been associated with the translocation of both bacterial and fungal components across the gut barrier, as evidenced by elevated biomarkers such as LBP, sCD14, and BDG, which demonstrate a positive correlation with disease severity. Notably, persistent elevation of markers indicative of gut mucosal damage and fungal translocation persists in individuals with long COVID-19 (Giron *et al.*, 2022). It is hypothesized that microbial translocation serves as a trigger for increased immune activation and subsequent inflammatory cascades, which further exacerbates the cytokine storm, contributes to multi-organ dysfunction and ultimately leads to severe COVID-19.

Prior to COVID-19, our research focused on microbial translocation's effects on HIV. We discovered that microbial translocation is harmful in PLWH, with elevated plasma levels of LPS and BDG observed in both ART-naïve and ART-treated individuals, correlating with increased inflammation and increased risk of developing non-AIDS inflammatory diseases in this population (Isnard *et al.*, 2021b). While COVID-19 research has predominantly focused on its respiratory aspects, the impact of microbial translocation on the immune response during COVID-19 remains underexplored. Globally, the influence of microbial translocation on antigen specific immune function is poorly known, in the context of acute or chronic infection. We postulate that markers of mucosal damage, microbial translocation, and inflammation will increase during acute COVID-19 infection, potentially correlating with a weakened anti-SARS-CoV-2 T and B-cell immune response. This study aims to specifically assess gut damage markers I-FABP and REG3α, and microbial translocation markers BDG and LPS, due to our previous knowledge of their roles in HIV infection.

In this study, 55 adults were recruited, with 44 testing positive for COVID-19 and classified into three cohorts based on recovery duration. While the majority of participants were female and all

participants in the acute and early recovered cohorts were hospitalized, multivariate analysis revealed that all outcomes remained unaffected by age and sex variables.

We discovered that plasma levels of I-FABP were significantly lower in acute COVID-19 cohorts compared to late recovered participants. Levels of I-FABP were similar in late recovered and uninfected controls, indicating a decrease in plasma I-FABP during acute COVID-19, which was unexpected. An hypothesis for such observation is the high levels of inflammation during acute COVID-19 could lead to a decrease in the rate of epithelial cells multiplication, decrease migration and shedding at the villi apex.

Additionally, elevated plasma REG3a levels were observed in hospitalized controls and acute COVID-19 cohorts compared to healthy controls, which suggests an acute inflammatory response and gut permeability, particularly in those with severe illness requiring hospitalization. These findings contrast with our previous observations in HIV, where plasma REG3a levels were higher in chronic HIV infections compared to early-stage infections (Isnard *et al.*, 2020a). In chronic HIV infections, prolonged immune activation and ongoing viral replication contribute to sustained inflammation and elevated REG3a levels over time. This discrepancy in the role of REG3a between COVID-19 and HIV underscores the distinct physiological dynamics between acute and chronic viral infections and highlights the importance of understanding context-specific gut barrier function for precise disease characterization and management. Furthermore, the diverse levels of gut damage and fungal translocation markers observed in the longitudinal assessment of 10 paired acute and early recovered COVID-19 participants indicate individual variability in the recovery process.

Contrary to Giron et al, we found no differences in plasma levels of BDG and LPS between groups of acutely infected or COVID-19 recovered participants. This highlights the complex and dynamic nature of the immune response and gut health in COVID-19, which emphasizes the importance further study of the role of microbial translocation during COVID-19 and other acute viral infection, as well as personalized approaches to patient care and monitoring.

We assessed the frequency of spike-specific T-cells in PBMCs from all participants using a combination of 7 cytokines after SARS-CoV-2 spike protein incubation. Spike-specific cytokine production was determined by subtracting the percentage of cytokine-producing cells in the non-stimulated condition from that in the spike-stimulated condition. Results showed that four of these cytokines—IL-10, IL-4, IL-22, and IL-17A—elicited low to undetectable levels of response. As indicated by the results using PMA and ionomycine stimulation, the frequency of cells producing these cytokines is lower than those producing IL-2, TNF-α or IFN-γ. Moreover, anti-viral response is expected in the Th1 compartment of CD4 T-cells and CD8 T-cells, and to a lower extent in Th17, Th2 or Tfh cells, which fits our observation. (Ai et al., 2013). Nevertheless, we found a low frequency of spike-specific CD4 and even lower levels of specific CD8 T-cells. This finding suggests that the subsets of SARS-CoV-2 responsive T-cells are present in relatively low frequencies within the peripheral blood.

Regarding the three cytokines with observable production, we observed consistently low spike-specific T-cell responses across all cohorts, with a notable increase detected in the COVID-19 groups compared to controls. Remarkably, the highest T-cell responses were identified among individuals in the early or late recovered COVID-19 cohorts, which implying a potential persistence or augmentation of T-cell immunity post-recovery. Moreover, longitudinal assessment of 10 pairs of acute COVID-19 participants unveiled varied levels of SARS-CoV-2 spike-specific CD4 or CD8 T-cell inflammatory cytokines over a 30-day follow-up period, suggesting dynamic fluctuations in T-cell responses during the course of infection and recovery. These findings underscore the intricate nature of the immune response to SARS-CoV-2 infection and emphasize the necessity of longitudinal studies to capture the nuanced dynamics of T-cell immunity throughout the COVID-19 trajectory.

We found no discernible differences in plasma BDG levels across all COVID-19 and further investigation employing larger sample sizes and longitudinal assessments could yield more profound insights into the role of BDG in the context of COVID-19 pathogenesis and immune response. Additionally, the lack of distinction in plasma levels of LPS across all COVID-19 and control groups

could stem from the constraint posed by a restricted number of patient samples in this study, which restricts our ability to conduct thorough testing for LPS levels across all COVID-19 cohorts. This limitation significantly impedes our capacity to fully analyze the impact of LPS on the immune response within different COVID-19 groups. Increasing sample size could establish a stronger basis for investigating the complex correlation between LPS levels and the immune responses witnessed in different manifestations of the disease.

In this study, we conducted a comprehensive analysis encompassing all parameters across various cohorts, with a specific focus on delineating individuals in the acute phase of COVID-19 from those who had recovered from the disease. Our investigation revealed no significant correlations between markers indicative of gut damage, namely I-FABP and REG3a, and the immune responses elicited by spike-specific T-cells or B-cells. Interestingly, we noted divergent associations between microbial translocation markers BDG (of fungal origin) and LPS (of bacterial origin) and the spike-specific CD4 T-cell response among recovered COVID-19 patients. In the late recovered COVID-19 cohort, we identified a negative correlation between BDG levels and the spike-specific CD4 T-cell response, while a positive correlation was observed between LPS levels and spike-specific CD4 T-cell response. The positive correlation between LPS levels and spike-specific CD4 T-cell response could potentially be explained by the presence of outer membrane vesicles (OMVs). OMVs are produced by bacteria and known to primarily comprise bacterial outer membrane constituents, including LPS, proteins, lipids and nucleic acid (Tian et al., 2023). These key antigenic components could serve as natural "adjuvant", fostering anti-SARS-CoV-2 immune responses (Micoli and MacLennan, 2020). These OMVs may contribute to the activation of CD4 T-cells specific to the SARS-CoV-2 spike protein directly or through the stimulation of APC, thereby influencing the immune response during COVID-19 infection. However, further research is required to fully elucidate the mechanisms underlying this correlation and its implications for disease progression and treatment. Moreover, fungi also produce OMVs containing BDG, which role remains to be studied.

Our previous studies on HIV, a chronic infection, highlighted similar pro-inflammatory capacity of both LPS and BDG, where we observed a positive correlation between plasma levels of BDG and gut damage markers I-FABP, as well as immune activation markers IL-6 and IL-8 in PLWH (Isnard *et al.*, 2021a). Such findings diverged from our observations in COVID-19 cohorts. To unravel the mechanisms underlying the negative correlation between BDG and the spike-specific CD4 T-cell response, as well as the contrasting profiles for BDG and LPS, we then investigated the role of B-cell immune responses against SARS-CoV-2 across all cohorts. Interestingly, we found a negative correlation between IgG levels against SARS-CoV-2 and plasma BDG levels, which is consistent with our observations regarding spike-specific T-cell response in late recovered COVID-19 cohorts. This negative correlation may suggest a potential immunosuppressive effect of BDG on both spike-specific T-cell and B-cell functions. This phenomenon is significant in COVID-19, as the disease often leads to immune dysregulation with reduced CD4 and CD8 T-cells (Huang et al., 2023), as well as a decrease in anti-SARS-CoV-2 IgG antibodies over 6 months post-infection (Li et al., 2023). Chronic exposure to higher levels of BDG and viral antigens may exacerbate the immune dysregulation and ultimately result in suppressed T-cell and B-cell responses. One plausible mechanism for this negative correlation is that BDG may exert a direct inhibitory effect on spike-specific T-cell and B-cell activation or functionality. Alternatively, BDG-induced persistent immune activation in recovered COVID-19 participants which might induce immune exhaustion or tolerance, thereby attenuating spike-specific T-cell and B-cell responses progressively (Schietinger and Greenberg, 2014). Furthermore, it is noteworthy to consider that underlying health conditions of individuals may contribute to this correlation. For example, individuals with compromised immune function or pre-existing health conditions, and higher pre-COVID-19 BDG levels, may exhibit increased susceptibility to both elevated BDG levels and diminished IgG production.

In addition to inflammation, microbial translocation has been demonstrated to trigger changes in epigenetics, particularly in monocytes, altering their response to a subsequent infection by either enhancing (trained immunity) or reducing (tolerance) the reaction to the same pathogen. (Netea et al.,

2020). Literature indicates that BDG can activate immune cells, particularly monocytes, and prompt epigenetic alterations that enable these cells to mount a stronger response upon subsequent stimulation (Divangahi et al., 2021) (Isnard *et al.*, 2021b). Trained immunity describes the phenomenon observed in innate immune cells, such as monocytes and macrophages, where exposure to certain stimuli leads to enhanced responsiveness and memory-like characteristics without requiring antigen specificity (Netea et al., 2011). A key feature of trained immunity is the long-term epigenetic remodeling of the chromatin accessibility within the innate immune cells, which results in alterations in gene expression and functional traits, such as enhanced cytokine production upon re-exposure to the microbial pathogens or inflammatory stimuli (Ochando et al., 2023). Interestingly, although both LPS and BDG can initiate metabolic and epigenetic changes in immune cells, they elicit contrasting trained immunity programmes (Saeed et al., 2014). Previous study showed that exposing LPS-treated monocytes to BDG from *C. albicans* cell walls maintains their ability to produce pro-inflammatory cytokines upon LPS re-exposure compared to non-BDG-treated cells (Saeed *et al.*, 2014). Due to limited availability of patient samples, our study did not allow an exploration of whether the observed opposite influences of LPS and BDG on spike-specific immune responses are associated with trained immunity. We found that BDG receptors are expressed and possibly triggered on CD4 and CD8 T-cells as well as B cells and monocytes, which indicates that BDG could have a direct and indirect role on these cells. In future studies, it would be worthwhile to use ATAC-seq to investigate epigenetic changes in chromatin accessibility associated with the induction of trained immunity in innate immune cells, as well as T and B-cells from COVID-19 patients. By profiling chromatin accessibility using ATAC-seq, we could identify key regulatory elements and pathways involved in immune tolerance, which may ultimately lead to the development of novel therapeutic approaches for long-lasting immune protection against COVID-19. In addition, it is valuable to integrate the ATAC-seq data with other omics data, such as RNA-seq and ChIP to gain a comprehensive understanding of the molecular mechanisms underlying immune tolerance.

In this study, we face the challenge of limited comorbidity data for all participants, which is crucial given the potential impact of these underlying health conditions would have on the contrasting immune responses triggered by BDG and LPS. The presence of comorbidities, including obesity, diabetes, COPD and asthma, cardiovascular diseases, cancer and HIV, as well as chronic kidney disease, holds paramount importance in the analysis of COVID-19 outcomes, given its substantial impact on disease severity, treatment response, and disease prognosis (Russell *et al.*, 2023). Individuals infected with COVID-19 with pre-existing comorbidities face an elevated risk of developing severe complications and adverse outcomes of COVID-19 (Russell *et al.*, 2023). Having comprehensive information on participants' comorbidities would enable us to evaluate how underlying health issues interact with immune system activation, thus enhancing our ability to accurately interpret study outcomes and account for potential confounding factors, which ultimately enhances the validity and applicability of our findings. Unfortunately, comorbidity information was not available for the acute participants of our study.

In our analysis, we also noticed a contrasting spike-specific CD4 T-cell response associated with BDG in both acute and late recovered COVID-19 cohorts. A positive correlation was found between BDG levels and the spike-specific CD4 T-cell response, which indicates that microbial translocation may have adverse effects during acute COVID-19. This disparity might stem from the differing stages of infection represented in these cohorts. In the acute cohorts, participants were sampled during the early stages of infection, either on day 0 or day 2 post-infection, when the immune system is actively mounting a response to the invading pathogen. It is plausible that T-cell activity is robust during this time and serves as a crucial element in the initial defense against the virus. Whether T-cell-induced inflammation and activity participates in gut permeability and microbial translocation during the acute phase remains to be studied. Conversely, in the late recovered COVID-19 cohorts, individuals had progressed beyond the acute phase of infection and recovered for a significant amount of time, with an average recovery duration of 229 days. By this stage, the initial peak of T-cell activity has subsided, and the immune system has transitioned from an acute response to a long-term immune

memory state. Previous study has demonstrated that spike-specific memory T-cell responses have an estimated half-life of 200 days (Cohen et al., 2021). We observed a decline in spike-specific T-cell response alongside increased levels of BDG, which suggests the link between the weakened immunity and increased susceptibility of infection. Given the uncertainty surrounding the protective nature of spike-specific T-cell responses, future studies shall prioritize investigating the memory immune response in individuals who have recovered from COVID-19 and subsequently encounter a second infection, which will provide crucial insights into long-term immune protection and inform strategies for managing recurrent infections. The influence of persistent microbial translocation on the efficiency of the memory response should be studied in this context as well.

Giron et al. previously showed increased BDG levels in long-COVID patients, indicating that the extended presence of SARS-CoV-2 antigens might have lasting effects on immune responses (Giron *et al.*, 2022). Further investigation is needed to validate the presence of spike-specific T-cell responses in individuals with long-COVID-19.

No notable disparities were detected in the cytokines generated by spike-specific T-cells. The most robust CD4 spike-specific T-cell response was identified in the production of TNF-α, which, in a sub analysis, exhibited higher levels in severe COVID-19 participants as opposed to those with mild or moderate symptoms, as well as control participants as shown in **Figure 17**. This suggests that percentage of CD4 producing TNF-α T-cells may be particularly pronounced in severe cases of COVID-19 and highlights its potential role in the pathogenesis and progression of COVID-19 disease.

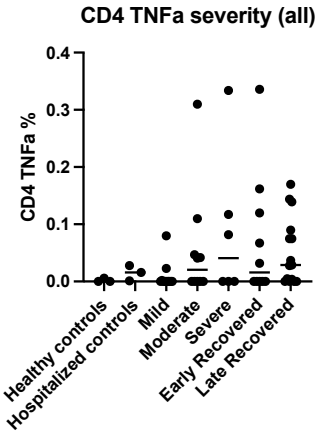

Figure 17: SARS-CoV-2 spike-specific CD4 producing TNF-α T-cells and disease severity. Percentage of CD4 producing TNF-α T-cells were higher in severe cohorts compared to mild or moderate cohorts as well as controls.

To date, the precise impact of microbial translocation on immune responses in diseases remains incompletely understood. Hence, investigating its role across a spectrum of acute and chronic disorders holds significant value. In acute diseases like pancreatitis and myocardial infarction, sudden physiological changes disrupt the gut barrier and leads to microbial translocation (Luo et al., 2021). Gut dysbiosis is directly associated with an elevation in intestinal permeability due to the breakdown of the epithelial barrier (Stolfi et al., 2022). Presence of microbial products in the bloodstream can trigger inflammation and exacerbate tissue damage. In chronic disorders such as chronic kidney disease, IBD, and cancer, microbial translocation may result from prolonged immune dysregulation or tissue damage. Likewise, in IBD, persistent inflammation compromises the composition and functionality of the gut microbiota (Sokol *et al.*, 2017). The gut microbiota's influence on cancer is increasingly recognized, with potential implications for its onset, progression, treatment, and prognosis across various cancer types (Sun et al., 2023). Understanding its functional role in cancer is crucial for advancing personalized medicine strategies. However, the influence of microbial translocation on cancer prognosis and response to treatment remains poorly studied. Exploring the impact of microbial translocation in these varied conditions offers valuable insights into the intricate relationship among the gut, the immune system, and disease progression. Such investigations may uncover potential targets for therapeutic intervention aimed at regulating immune responses and enhancing patient outcomes in both acute and chronic disorders. Thus, thorough examination of microbial translocation across diverse diseases is vital for advancing our knowledge of disease mechanisms and developing more effective treatments.

The increased anti-SARS-CoV-2 IgG levels observed in early recovered COVID-19 individuals compared to healthy controls suggest a robust and sustained immune memory formation, where the immune system "remembered" the encountered pathogen and mounts a more rapid and potent response upon re-exposure. Similarly, the elevated levels of anti-SARS-CoV-2 IgG antibodies in

severe COVID-19 cases compared to hospitalized controls and in early recovered individuals compared to hospitalized controls signify an intense humoral immune response elicited by the virus. In severe cases of COVID-19, the immune system may respond more vigorously due to a higher viral load and increased tissue damage, which leads to elevated antibody levels. Understanding these immune response dynamics is crucial for providing insights into the effectiveness of natural immunity following COVID-19 infection, and convalescent plasma therapy, which relies on transfer of pathogen-specific antibodies from a recovered patient for the purpose of preventing or treating disease (Casadevall and Scharff, 1995). Finally, it also aids in identifying biomarkers for disease severity and prognosis and allows for risk stratification and personalized patient care.

As BDG has opposite link with the cellular and humoral SARS-CoV-2 Spike immune responses in acute and recovered COVID-19, we assessed the levels of BDG receptors on effectors cells in late recovered participants. Expression of BDG receptors on NK cells were generally low (data not shown). However, in monocytes/DCs staining, an elevation in BDG receptor Dectin-1 and CD18 levels was noted in CD4 and CD8 T-cells, B-cells, and non-classical monocytes populations when BDG levels were below the median of 7 pg/mL. Notably, CD11b only showed higher expression specifically in non-classical monocytes when BDG levels were below 7 pg/mL. We detected tendencies towards lower levels of Dectin-1 on activated/exhausted PD1+CD8 T-cells and B-cells of participants with plasma BDG levels below the median. These results highlight the complex relationship between BDG levels and the expression of particular BDG receptors in diverse immune cell populations. Although without normal range of BDG levels, these differences indicate that higher BDG levels could trigger BDG receptors and then influence the function of these immune cells. They imply that BDG concentrations might regulate immune cell function and activation status even during the post-recovery stage of COVID-19. Further investigation is warranted to elucidate the precise molecular pathways and functional implications of BDG-mediated modulation of immune cell receptor expression in the context of COVID-19 recovery.

Blocking LPS or BDG receptors and monitoring subsequent inflammatory cytokine production holds promise for elucidating the distinct inflammatory capacities of BDG and LPS. Prospective studies should consider this approach to dissect the nuanced roles of these receptors within the intricate inflammatory cascade induced by BDG and LPS in the context of COVID-19. This approach offers the opportunity to discern how the blockade of specific receptors modulates macrophage responses, thereby elucidating the underlying mechanisms of inflammation associated with microbial translocation in the disease. Additionally, implementing a long-term follow-up strategy to evaluate memory responses in individuals who have recovered from COVID-19 holds significant importance. Tracking memory responses over an extended period and repeating serological tests and cellular immune assays at regular intervals (e.g., every 6 months) will allow us to monitor changes in antibody levels, memory T-cell responses, memory B-cell frequencies, and assessing the durability and evolution of immune responses over time. It's important to note that all samples in this study were collected from participants without any COVID-19 vaccinations. Given that a significant portion of the population is now vaccinated against SARS-CoV-2, it's crucial to acknowledge that vaccination status may influence immune responses.

In this study, we solely relied on blood samples, which may not fully depict the immune status or pathology present in the lungs or other infected regions. To gain a more comprehensive understanding of the impact of COVID-19 on human body, integrating blood sample data with imaging studies and lung tissue biopsies or bronchoalveolar lavage could offer a more holistic perspective, and assess the link between spike-specific immune responses and tissue damage. In this study, we are confronted with the limitation of having a restricted amount of samples, which prevent us to examine LPS levels in acute COVID-19 participants. The inclusion of such data could offer intriguing insights to enhance our correlation analysis. It is also valuable to use animal models for the validation of the findings presented in this study. Moreover, we obtained samples from a limited number of participants. Future studies should focus on evaluating the potential impact of COVID-19 vaccination on the spike-specific immune responses and gut translocation in vaccinated COVID-19 population. Additionally,

it's essential to note that correlation analysis performed here does not establish causation. It's crucial to conduct further studies like randomized controlled trials to explore potential causal relationships and mechanisms underlying the observed correlations.

In future studies, we would like to validate the impact of BDG on the persistence and efficiency of the anti-SARS-CoV-2 immune response in recovered and in vaccinated individuals. Due to the small amount of samples were collected, we were not able to perform quantification of pro- and anti-inflammatory cytokines. Additionally, our efforts to evaluate the inflammatory potential of BDG and LPS in the plasma of both acute and recovered participants using filtration, cell stimulation, and antibody blocking did not yield specific responses. These experiments could be replicated using samples from a separate cohort for comparative analysis. We are also looking whether the variation in the expression of LPS receptors in PBMCs is linked with circulating levels in acute COVID-19 and recovered individuals.

Conclusion

In summary, this study has shown that microbial translocation markers BDG (fungal) and LPS (bacterial) influence SARS-CoV-2 spike-specific T-cell response in late recovered COVID-19 in opposite ways. BDG is negatively linked to SARS-CoV-2 spike-specific CD4 T-cell responses while LPS positively linked with SARS-CoV-2 spike-specific CD4 T-cell response. Negative correlation was also found between plasma BDG levels and anti-SARS-CoV-2 IgG levels in late recovered COVID-19 participants. Additionally, a consistent pattern of higher expression of BDG receptors was noted in late-recovered COVID-19 participants with lower BDG levels. However, it's important to note that our findings do not allow us to definitively determine whether BDG or LPS has a protective or harmful role in SARS-CoV-2 infection. Future investigations should prioritize evaluating the role of memory immune responses and assessing the impact of immune exhaustion and tolerance on the divergent profiles of BDG and LPS with immune responses observed in COVID-19 participants. By elucidating the relationship between gut permeability and inflammation, we aim to contribute to the development of strategies aimed at preventing or mitigating inflammation and symptoms in both acute and recovered cases of COVID-19.

Note:

During my Masters, I analyzed data from the BQC-19 proteomics analyses to investigate the association between inflammation and disease progression. Our findings revealed elevated levels of GDF-15, a growth factor from the TGF-β family, in the plasma of severe COVID-19 cases, indicating a correlation with inflammation. A manuscript detailing these results is presently undergoing review in Frontiers in Immunology, where I serve as the first author.

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