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ADAPTIVE IMMUNE RESPONSES TO TICK-BORNE ENCEPHALITIS VIRUS AND SARS-COV-2

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Adaptive Immune Responses to Tick-borne Encephalitis Virus and SARS-CoV-2

THESIS FOR DOCTORAL DEGREE (Ph.D)

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POPULAR SCIENCE SUMMARY OF THE THESIS

The ongoing COVID-19 pandemic has highlighted the threat emerging infectious diseases pose on public health and economic stability. This has resulted in increased public attention and funding in the field of virology and vaccinology, which will not only contribute towards combating the COVID-19 pandemic, but also towards the understanding and preventing other infectious diseases.

The primary focus of this thesis is two emerging viral infections – tick-borne encephalitis virus (TBEV) and SARS-CoV-2. TBEV is transmitted by ticks and leads to neurological symptoms, while SARS-CoV-2 is transmitted via respiratory droplets from infected people and primarily causes respiratory symptoms. Although highly different in the disease they cause, both viruses are recognized by the adaptive immune system once they enter the human body. The adaptive immune system is responsible for remembering and attacking any pathogen it encounters over the years and consists of two major types of cells – T cells and B cells. T cells can orchestrate the whole adaptive immune response or directly kill virus-infected cells, while B cells produce antibodies that can bind to the virus and prevent it from multiplying and spreading within the human body.

In this thesis, we studied TBE and COVID-19 patients, as well as TBE vaccinated individuals. In **Paper I**, we found a four times higher mortality in TBE patients that are over the age of 60 compared to the general population. This finding highlighted the importance of promoting TBE vaccination for people at risk, especially considering that TBE is a vaccine-preventable disease. TBE vaccination, however, does not lead to an as potent T cell response as TBEV infection, a finding highlighted by **Paper II**. This shows that currently licensed TBE vaccines could be improved to resemble the immunity induced by natural infection more closely. B cell responses towards TBEV were also investigated in this thesis. We showed that the cells that produce high levels of antibodies during early infection are not expanded in hospitalized TBE patients. The patients, however, already have high levels of antibodies and therefore are no longer in the early phase of infection. **Paper III** describes these events in detail.

Another aim of this thesis was to investigate if the adaptive immune system can remember SARS-CoV-2 once the infection is over. In **Papers IV-V** of this thesis, we showed that SARS-CoV-2 infection leads to a strong early T cell and B cell response in hospitalized COVID-19 patients. This results in immunological memory lasting for up to at least 9 months after the infection, likely protecting the patients from second infection with the virus.

The findings in this thesis contribute to the understanding of TBEV and SARS-CoV-2 infections in humans, and more specifically, how the adaptive immune system responds to these infections. These studies, along with others, will aid in the development of therapeutic interventions and effective vaccination strategies for TBE, COVID-19, and other infections.

ABSTRACT

Tick-borne encephalitis virus (TBEV) and SARS-CoV-2 are two unrelated viruses that currently cause substantially different public health burdens and distinct pathologies in humans. TBEV infection leads to neurological symptoms of varying severity, while SARS-CoV-2 primarily targets the respiratory tract. The aims of this thesis were to estimate the relative level of mortality due to TBE in Sweden (**Paper I**) and to describe the human adaptive immune responses to TBE vaccination (**Paper II**), TBEV infection (**Paper III**), and SARS-CoV-2 infection (**Papers IV-V**).

In **Paper I**, we measured the standardized mortality ratio (SMR) in TBE patients and found that, compared to a matched control population, TBE patients experience around a four-times higher mortality within 90 days after the diagnosis. Considering that TBE is a vaccine-preventable disease, this finding highlights the need for increased vaccination efforts for people at risk of exposure to TBEV.

In **Paper II**, we assessed memory T cell responses throughout the primary immunization schedule with TBE (three doses within one year). We observed a heterogenous magnitude of memory $CD4^+$ T cell response in the TBE vaccinated individuals, with the highest magnitude after the 2^{nd} dose. Compared to TBE patients, TBE vaccinees had fewer polyfunctional memory $CD4^+$ T cells and lower IFN-γ responses. This study suggests that the TBE vaccine elicits a lower quality of $CD4^+$ T cell memory compared to TBE infection and highlights the need for the development of improved TBE vaccines.

In **Paper III**, we assessed the antibody-secreting cell (ASC) responses and TBEV-specific antibody levels in TBE patients at varying timepoints after hospitalization. ASC expansion is typically a hallmark of early B cell responses during acute infections. Compared to dengue patients, who served as a control cohort in this study, low frequencies of ASCs were detected in TBE patients at all four sampling timepoints (i.e., <7, 7-13, 14-30 and >30 days after hospitalization). In addition, all TBE patients had detectable TBEV-specific IgM and IgG antibody levels throughout the course of the study. These findings indicate that the early B cell response may take place even earlier during TBE, likely before hospitalization.

In **Papers IV and V**, we investigated germinal center activity, ASC responses and antibody levels during the acute SARS-CoV-2 infection in hospitalized COVID-19 patients. We observed an increased germinal center activity and ASC expansion in COVID-19 patients. In **Paper V**, we subsequently detected polyfunctional memory T cell and memory B cell responses in previously hospitalized recovered COVID-19 patients at 5 and 9 months after symptom onset. This finding indicates that immunological memory to SARS-CoV-2 persists for at least up to 9 months regardless of COVID-19 severity at hospitalization.

In conclusion, this thesis contributes to the understanding of TBEV and SARS-CoV-2 infections, particularly in relation to the adaptive human immune responses to these viruses.

LIST OF SCIENTIFIC PAPERS

I. **Varnaitė R**, Gredmark-Russ S and Klingström J. Mortality in tick-borne encephalitis and other reportable zoonotic infectious diseases in Sweden. *Manuscript*.

II. **Varnaitė R**, Blom K, Lampen MH, Vene S, Thunberg S, Lindquist L, Ljunggren H-G, Rombo L, Askling HH and Gredmark-Russ S. Magnitude and functional profile of the human $CD4^+$ T cell response throughout primary immunization with tick-borne encephalitis virus vaccine. 2020. *The Journal of Immunology*. 204(4): 914-922

III. **Varnaitė R**, Mayola Danés N, Asgeirsson H, Ljunggren H-G, Blom K and Gredmark-Russ S. Characterization of human B cell responses and specific antibody levels in tick-borne encephalitis patients. *Manuscript*.

IV. **Varnaitė R**, García M, Glans H, Maleki KT, Sandberg JT, Tynell J, Christ W, Lagerqvist N, Asgeirsson H, Ljunggren H-G, Ahlén G, Frelin L, Sällberg M, Blom K, Klingström J and Gredmark-Russ S. Expansion of SARS-CoV-2–specific antibody-secreting cells and generation of neutralizing antibodies in hospitalized COVID-19 patients. 2020. *The Journal of Immunology*. 205(9):2437-2446

V. Sandberg JT*, **Varnaitė R*,** Christ W, Chen P, Muvva JR, Maleki KT, García M, Dzidic M, Folkesson E, Skagerberg M, Ahlén G, Frelin L, Sällberg M, Eriksson LI, Rooyackers O, Sönnerborg A, Buggert M, Björkström NK, Aleman S, Strålin K, Klingström J, Ljunggren H-G, Blom K, Gredmark-Russ S & The Karolinska COVID-19 Study Group. SARS-CoV-2-specific humoral and cellular immunity persists through 9 months irrespective of COVID-19 severity at hospitalisation. 2021. *Clinical & Translational Immunology.* 10(7):e1306.
**Contributed equally.*

CONTENTS

LIST OF ABBREVIATIONS

ASC	antibody-secreting cell
CD	cluster of differentiation
COVID-19	coronavirus disease 2019
cTfh	circulating T follicular helper cell
CXCL13	C-X-C motif chemokine 13
CXCR3	C-X-C chemokine receptor type 3
CXCR5	C-X-C chemokine receptor type 5
ELISA	enzyme-linked immunosorbent assay
ICS	intracellular cytokine staining
IFN-γ	interferon γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2	interleukin 2
mASC	memory B cell-derived antibody-secreting cell
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
N-protein	nucleocapsid protein
PBMC	peripheral blood mononuclear cells
S1	spike subunit 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SMR	standardized mortality ratio
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
Tfh	T follicular helper cell
Th1	T helper 1 cell
TNF	tumor necrosis factor
YFV	yellow fever virus

1 INTRODUCTION

1.1 TICK-BORNE ENCEPHALITIS VIRUS

Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE), a severe infection of the central nervous system that can result in long-term sequela affecting the quality of life and even in fatal outcome. TBEV is a positive sense RNA virus and encodes a single polyprotein. This polyprotein is processed into three structural proteins (i.e., capsid, membrane, and envelope), as well as seven non-structural proteins (i.e., NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). TBEV is a member of the *Flaviviridae* family and is related to several well-known viruses including yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV) and Japanese encephalitis virus (JEV). The distribution of these viruses around the world varies. In Sweden, for example, only TBEV is endemic and any other flaviviral infections are typically acquired abroad. Flaviviruses are arthropod-borne and are therefore transmitted to humans via mosquitoes or ticks. Despite a high morphology within the *Flavivirus* genus, the pathology caused by these viruses in humans results in a wide range of symptoms ranging from asymptomatic or mild febrile illness to severe neurological disease or hemorrhagic fever [1, 2]. Viruses such as TBEV, WNV, ZIKV and JEV are neurotropic and can therefore infect the nervous system leading to neurological manifestations. Of these four neurotropic viruses, only TBEV is transmitted to humans via ticks, or in rare occasions via the ingestion of contaminated milk from an infected animal [3].

1.1.1 TBEV transmission to humans

TBEV is an emerging virus and new TBE disease foci are appearing around Europe [4]. This is influenced by several socioeconomic, behavioral, and ecological factors [4, 5]. The risk of contracting TBEV increases with a lifestyle that involves recreational or occupational outdoor activities where ticks are abundant [4-6]. As TBEV is mostly transmitted to humans via bites of TBEV-infected ticks, yearly incidence of TBE is highly dependent on tick activity. In Europe, TBEV is transmitted to humans primarily by *Ixodes ricinus* tick species, particularly during the nymph and adult stage (Figure 1) [7]. TBEV persistence in tick populations is highly dependent on co-feeding of larvae and

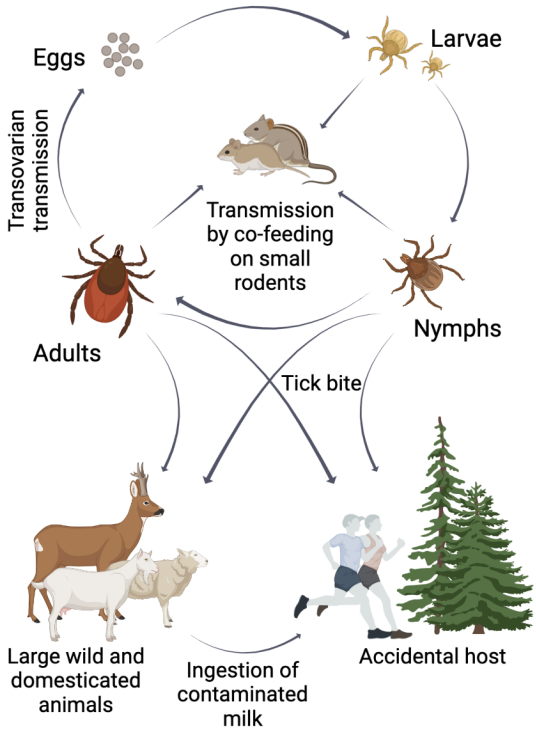

Figure 1. The life cycle of ticks and the TBEV transmission routes within the tick population, animals, and humans. Created with BioRender.com.

nymphs on small rodents, where the virus can be passed between the ticks [8]. Humans are entirely accidental and dead-end hosts and do not contribute to TBEV persistence within the tick populations (Figure 1).

1.1.2 TBE epidemiology

TBE is endemic in Europe and Asia and different virus strains are spread across the two continents. In Europe, the annual notification rate is around 0.41 to 0.65 cases per 100,000 population with the highest incidence in the Check Republic and Lithuania, accounting for 38.6% of all cases in Europe [9]. Three different TBEV strains exist: the European, the Siberian and the Far Eastern, that are classified based on the amino acid sequence of the envelope protein [10]. The genetic diversity of these viruses appear to affect their virulence and the ability to infect neurons, leading to different spectrums of disease severity [11-13]. The European TBEV strain causes the disease with the lowest mortality out of the three stains, and a more recent Europe-wide study estimated the case-fatality in TBE patients to be 0.5% [9]. Meanwhile, the Siberian and the Far Eastern TBEV strains are more fatal, leading to case-fatality rates of 6-8% and 20-60%, respectively [14]. In Sweden, only the European TBEV strain is endemic and the incidence of TBE is confined to the southern half of Sweden with a particularly high incidence in the Stockholm area [3, 15]. Despite relatively low case-fatality rates due to TBE in Europe, long-term sequelae after TBEV infection are common and substantially affect the quality of life of TBE patients [16, 17].

1.1.3 TBE disease progression

TBEV can be transmitted from the tick's saliva within minutes after a tick bite [18]. The exact mechanisms of TBEV dissemination after the bite is not fully understood, and the development of symptomatic disease likely depends on many genetic and immunological factors of the host. TBE is typically a biphasic disease with a mild febrile illness during the first phase, and moderate to severe neurological manifestations during the second phase.

Although TBEV primarily targets neurons in the central nervous system, other cell types can be infected [19, 20]. For example, cells residing in the skin, particularly mononuclear phagocytes, have been shown to be susceptible to TBEV, potentially facilitating the spread of TBEV to peripheral tissues via the lymphatics early after the tick bite (Figure 2) [21, 22]. Viral dissemination results in viremia and febrile symptoms observed during the first phase of disease. Most patients experience only mild symptoms at this stage and typically do not seek medical care. It is believed that TBEV enters the central nervous system during this phase and many potential mechanisms of TBEV entry have been proposed (reviewed in [23]).

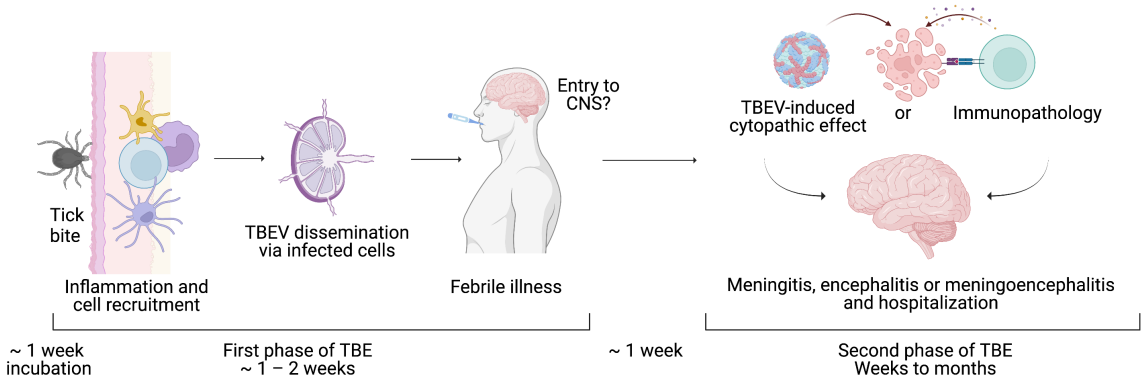

Figure 2. TBE disease progression from a tick-bite to the development of neurological symptoms, and potential mechanisms of pathogenesis in the central nervous system. Created with BioRender.com.

After the first (febrile) phase, TBEV-infected individuals either recover completely or develop neurological symptoms and progress into the second phase of disease. It has been estimated that only around one third of TBEV-infected individuals progress and develop clinical manifestations [24]. Patients with clinical manifestations suffer from neurological symptoms of different severity, i.e., meningitis, encephalitis, severe meningoencephalitis and in some cases myelitis [25]. It is not fully understood whether TBEV infection alone, or immune-mediated damage also, contribute to the development of neurological symptoms, but it is likely to be a combination of both (Figure 2). Cytotoxic $CD8^+$ T cells, for example, are suspected to have a potential role in immunopathology during TBE. One study demonstrated a prolonged survival of TBEV-infected mice lacking $CD8^+$ T cells compared to immunocompetent mice [26]. In addition, cytotoxic $CD8^+$ T cells were found in close proximity to TBEV-infected neurons in the brains of deceased TBE patients [19, 20].

Clinical course and outcome in TBE highly depends on the patient´s age, genetic background, TBEV subtype and other factors [27]. Post-encephalitic syndrome presenting as irritability, memory and concentration dysfunction, and disturbed sleep patterns is common, however, the severity and frequency of such symptoms decreases with time [28]. During the second phase of disease, most patients require hospitalization, however no TBEV-specific therapy exists and only symptomatic care can be provided [9]. Despite the lack of specific therapy, vaccines against TBE are available and highly recommended to people at risk of exposure to TBEV while travelling or working in endemic areas [29].

1.1.4 TBE vaccines

TBE is a vaccine-preventable disease and the evidence for the efficiency of TBE vaccines is highlighted by a successful national immunization program in Austria, which was initiated in 1981 and reduced the incidence of TBE to around one-fifth of that during pre-vaccination era [30-32]. In Europe, there are two available TBE vaccines: FSME-Immun (Pfizer) based on the Neudörfl TBEV strain and Encepur (GSK) based on the K23 TBEV strain [33]. These are

formaldehyde inactivated whole-virus vaccines with alum adjuvant and comparable dosing and administration schedules. The conventional immunization schedule for TBE vaccination consists of a primary immunization with three doses within a one-year period, followed by booster doses every 3 to 5 years (Figure 3).

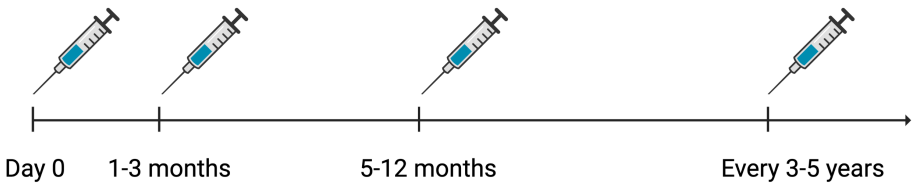

Figure 3. The conventional immunization schedule for TBE. Created with BioRender.com.

Unlike the yellow fever vaccine, which provides a lifelong immunity to the YFV [34], the TBE vaccine has to be frequently readministered to maintain protection, although in some cases antibody persistence has been shown to last for up to 10 years after booster vaccination [35]. Despite the fact that TBE vaccine is immunogenic in the majority of individuals and leads to seroconversion [36], vaccination breakthroughs have been reported, a phenomenon particularly common in older individuals [37-39]. The requirement for multiple doses of TBE vaccination and breakthrough events highlight the need for the development of more efficient vaccines against TBE.

1.2 SARS-COV-2 AND COVID-19

By the end of 2021, just two years after the emergence of SARS-CoV-2, there has been 270 million confirmed COVID-19 cases with 5 million deaths worldwide [40]. The COVID-19 pandemic reminded the world of the economic and public health dangers posed by viral infections, which has led to an increased interest and funding in the fields of virology, immunology, and vaccinology. The enormous scientific research output during the last two years and the swift development and licensing of vaccines against SARS-CoV-2 will likely benefit not only the current pandemic but will also guide the world on how to prepare for future communicable disease outbreaks.

1.2.1 The emergence of SARS-CoV-2

In December 2019, an outbreak of pneumonia of an unknown origin emerged in Wuhan, China [41]. The patients exhibited symptoms of lower respiratory tract infection, including fever, cough, shortness of breath, fatigue, and other symptoms. Some of the patients required a treatment in the intensive care unit and mechanical ventilation, developed severe acute respiratory distress syndrome (ARDS), or even died [42]. The outbreak was soon associated with Huanan seafood market where in addition to seafood, wild animals (potential reservoirs of zoonotic viruses) are traded [41]. Within weeks, a novel coronavirus SARS-CoV-2 (then termed 2019-nCoV) was identified as the causative agent of the pneumonia outbreak in Wuhan [43-45]. Coronaviruses had previously caused pneumonia outbreaks of global concern, namely SARS in 2002 and MERS in 2012 [46]. SARS-CoV-2 was found to have a high genetic similarity to a bat coronavirus suggesting a potential zoonotic origin [43-45]. Within weeks, the virus has spread around the globe causing a global health crisis, ultimately being declared as a pandemic by the World Health Organization [47].

1.2.2 COVID-19 disease progression and severity

SARS-CoV-2 causes a respiratory infection in humans named coronavirus disease 2019 (COVID-19), but clinical disease severity varies highly between the infected individuals. The virus enters the human body primarily via the respiratory tract and uses angiotensin-converting enzyme 2 (ACE2) as the receptor for the entry into the cells [48]. ACE2 is highly expressed on the epithelial cells of the respiratory tract and lungs, but also in other organs including heart and kidneys [49].

Not everyone infected with SARS-CoV-2 in their respiratory tract develops a symptomatic disease. Indeed, a systematic review estimated an overall proportion of asymptomatic individuals to be around 20%, but the true proportion is difficult to estimate [50]. Those who progress into symptomatic disease can exhibit a range of symptoms, with the most common initial symptoms including fever, cough, shortness of breath and fatigue. Symptomatic COVID-19 has a wide spectrum of disease severity and is typically categorized as mild,

moderate, severe and critical [51]. Mild COVID-19 (around 40% of the symptomatic cases) typically include fever, cough and fatigue without signs of pneumonia, while moderate COVID-19 (around 40% of the symptomatic cases) is characterized by the presence of pneumonia in the lungs, where fluid buildup causes shortness of breath, but does not greatly affect oxygen levels in the blood [52]. Severe disease (around 15% of the symptomatic cases) is characterized by a severe form of pneumonia where oxygen supplementation is required (Figure 4) [52]. Lastly, critical cases (around 5% of symptomatic cases) require life-sustaining interventions such as mechanical ventilation and these patients can also develop acute cardiac injury, ARDS, sepsis or septic shock [52-54].

Some of the hospitalized patients die due to the disease, and older individuals and patients with co-morbidities have an increased risk of fatal outcome [52, 55, 56]. The most common co-morbidities in hospitalized patients are cardiovascular diseases and diabetes [52, 55, 56]. Although the majority of COVID-19 patients recover, post-COVID-19 syndrome is often reported for weeks or months after the onset of clinical symptoms [57, 58]. The most common symptoms of post-COVID-19 syndrome are fatigue, shortness of breath and cognitive impairments, yet highly heterogenous manifestations at an individual level are observed [57, 58].

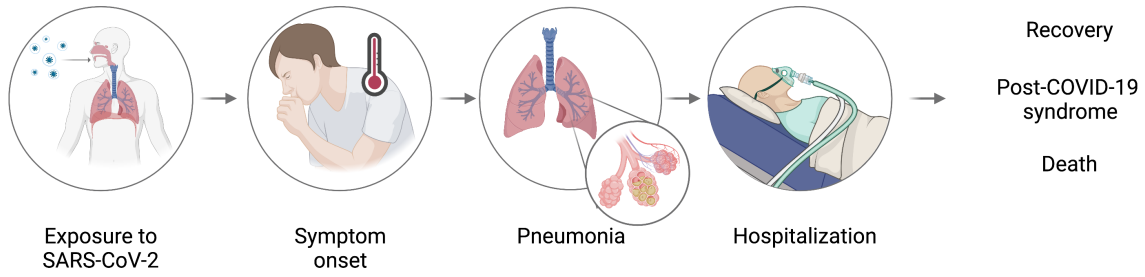

Figure 4. Disease progression in severe COVID-19. Upon the exposure to SARS-CoV-2, patients who develop severe symptoms (typically including pneumonia) often require hospitalization. During hospitalization, patients with severe pneumonia are typically administered supplemental oxygen. Some of the patients progress into a critical state where mechanical ventilation or other invasive treatments at the intensive care unit are required. The majority of hospitalized patients recover. However, some develop post-COVID-19 syndrome with lasting health impairments, and some die due to the disease. Created with BioRender.com.

1.2.3 COVID-19 vaccines

Although SARS-CoV-2 emerged only two years ago, vaccines against COVID-19 were developed and licensed at an unprecedented speed. As of December 2021, more than 8 billion COVID-19 vaccine doses have been administered worldwide with 137 vaccines in the clinical development and 194 in the pre-clinical development [40].

Classical vaccine technology relies on the inactivation or attenuation of the virus, as well as the use of adjuvants to enhance the immunogenicity of viral components. However, licensed COVID-19 vaccines and vaccine candidates in development rely not only on inactivated or live

attenuated virus, but also on a wide range of novel technologies [59]. These are some of the approaches used in licensed or candidate COVID-19 vaccines [60]:

- ***Inactivated virus*** – contains copies of "killed" SARS-CoV-2.
- ***Live-attenuated virus*** – contains copies of weakened SARS-CoV-2.
- ***Virus-like particles*** – contains only structural proteins of SARS-CoV-2 without the genetic material rendering the virus unable to replicate.
- ***Protein subunit*** – contains recombinantly expressed or isolated SARS-CoV-2 proteins.
- ***DNA or RNA*** – contains genetic material encoding SARS-CoV-2 proteins which are synthesized by our own cells after the vaccine is administered.
- ***Viral vector*** – contains the genetic material of SARS-CoV-2 packaged inside another virus.

RNA vaccines have perhaps drawn the most attention and have demonstrated very high efficacies against COVID-19 [61, 62]. The nationwide vaccination campaign in Israel, early after the roll out of Pfizer-BioNTech mRNA COVID-19 vaccine, highlighted the power of nationwide immunization in reducing COVID-19 incidence and controlling the pandemic [63, 64]. Waning antibody titers after vaccination, however, raised questions about the durability of protection by COVID-19 vaccines, especially against emerging SARS-COV-2 variants [65-67]. However, COVID-19 vaccination was also shown to induce a robust cellular immunity, which will likely contribute to at least partial protection during re-exposure to SARS-CoV-2 [68]. As new variants of SARS-CoV-2 continue to emerge and spread around the world, deciphering the longevity, quality and cross-reactive nature of vaccine-induced immunity is essential for controlling the ongoing COVID-19 pandemic.

1.3 ADAPTIVE IMMUNITY

In highly simplified terms, the adaptive immune system relies primarily on T cells and B cells, two large families of lymphocytes with many different subsets within each compartment. When the innate immune system fails to control an infectious organism, the adaptive immune system "kicks-in" and mounts a pathogen-specific response. This results in not only the clearance of the pathogen, but also a persistent immunological memory, which can protect from reinfection with the same pathogen and symptomatic disease. The formation of adaptive immunity to viral infections or following vaccinations, relevant to the studies presented in this thesis, are discussed below.

1.3.1 Helper $CD4^+$ T cells

$CD4^+$ T cells are highly heterogenous and can be divided into several distinct subsets; i.e., T helper 1, 2, 17 and 22 (Th1, Th2, Th17, Th22), regulatory T cells (Treg) and T follicular helper cells (Tfh) [69]. The Th1 subtype is characterized by IFN-γ secretion and is known to play a protective role in many viral infections [69]. IFN-γ can induce numerous interferon-stimulated genes that contribute to the establishment of an antiviral state [70, 71]. IFN-γ secreted by the Th1 cell subset has a direct effect on macrophages and activates them to kill intracellular pathogens [72, 73]. Polyfunctional $CD4^+$ T cells (cells secreting more than one cytokine at once in response to cognate antigens) are potent cytokine secretors and were previously shown to correlate with the control of viral infections and protection after vaccination [74-76]. HIV elite controllers, for example, have higher frequencies of IFN-γ secreting Th1 $CD4^+$ T cells as well as polyfunctional T cells compared to patients who succumb to HIV infection [77].

1.3.2 Cytotoxic $CD8^+$ T cells

Viruses are obligate parasites and require the host cell for survival and replication. However, the nature of this dependency allows the cells to send a signal to the immune system about the ongoing infection. The signal is achieved by the presentation of processed viral peptides via the MHC class I molecule to the cytotoxic $CD8^+$ T cells. $CD8^+$ T cells can then recognize the infected cells via the TCR-MHC-I interaction and release cytotoxic molecules including perforin and granzymes that trigger apoptosis in the infected cells [78]. Cytotoxic $CD8^+$ T cells have been shown by numerous studies to play an important role in the control of viral infections [79].

1.3.3 Follicular helper $CD4^+$ T cells

In the early 2000s, follicular helper $CD4^+$ T cells were identified in humans based on CXCR5 expression and were shown to have an important role in germinal center reactions and B cell

responses [80-82]. However, Tfh cells were not widely accepted as a distinct subset of T cells until the identification of Bcl6 – a master transcription factor that determines Tfh cell fate [83-85]. Tfh cells are now widely accepted as an essential helper T cell subset for robust humoral responses to infections and vaccinations [86].

1.3.4 Antibody-secreting cells and memory B cells

Shortly following infection or vaccination, naïve B cells are activated and differentiate into antibody-secreting cells (ASCs) to produce pathogen-specific antibodies. ASCs are terminally differentiated B cells and include both plasmablasts and plasma cells [87]. Plasmablasts are short-lived, highly proliferative, and typically originate from an extrafollicular response. In contrast, plasma cells are long-lived, mostly quiescent, reside primarily in the bone marrow and have higher numbers of somatic hypermutations as they originate from germinal center reactions [88, 89].

ASCs expand in peripheral blood after both infection and vaccination in humans, secrete high levels of pathogen-specific antibodies, and are characterized by high antigen-specificity [90-92]. For example, during acute dengue virus and hantavirus infection, a massive ASC population is detected in peripheral blood, in some cases reaching 70-80% of all B cells in peripheral blood [91, 93]. Higher frequencies of ASCs in peripheral blood are observed in patients with a more severe course of dengue and COVID-19, raising speculations of a potential immunopathogenic role of ASCs [94-96]. In contrast to acute viral infections, lower levels of ASC expansion are observed after influenza and tetanus immunizations, typically below 20% of the B cells in peripheral blood, averaging around 2-6%, and peaking at around day 7 after vaccine administration [91, 97-99]. Very low frequencies of circulating ASCs are found in healthy state in humans, typically only around 1% of all B cells [91, 93, 100].

Most of the expanded ASCs shortly after infection and vaccination are plasmablasts, but these cells are short-lived, and the long-term production of pathogen-specific antibodies relies on plasma cells. Plasma cells reside in the bone marrow and produce high-affinity pathogen-specific antibodies [101]. Plasma cells are very long lived and can persist in human bone marrow for many years [102]. A study in rhesus macaques showed persistence of pathogen-specific plasma cells in the bone marrow for at least up to 10 years after vaccination [103]. A study that defined plasma cells in the human bone marrow found that the cells were specific for viral antigens including measles and tetanus, to which the subject was not exposed to for 40 years [104]. These plasma cells exhibited primarily $CD19^{-}CD38^{high}CD138^{+}$ phenotype [104]. Although plasma cells secrete pathogen-specific antibodies which, if at sufficient levels, can protect against reinfection, the secondary response to the same pathogen relies on memory B cell reactivation. Memory B cells are generated in the germinal centers, but this cell subset does not secrete antibodies. Instead, they circulate in peripheral organs or reside in non-lymphoid tissues and can differentiate into ASCs upon re-exposure to the same pathogen [105].

1.3.5 Germinal center reactions

Adaptive immune responses are initiated in the secondary lymphoid organs. After an infection or vaccination, free antigen and dendritic cells bearing the antigen from the inflamed tissue travel from the site of inflammation to the draining lymph nodes via the lymphatic vessels. Lymph nodes has specialized structures with different cell types located within specific areas. B cells are localized in the follicles (the B cell zone) within the outer cortex and T cells mostly in the paracortical areas (the T cell zone). Dendritic cells that encountered an antigen enter the paracortical area first where they can activate naïve T cells. Soluble free antigen that drains into the lymph node, reach B cells and activate them to migrate towards the T-B cell border where B cells can receive T cell "help" [78].

Dendritic cells present processed peptides via the MHC-II molecule to the cognate T cell receptor (TCR) on naïve $CD4^+$ T cells, and this interaction initiates $CD4^+$ T cell differentiation into the Tfh (Figure 5) [106]. The TCR-MHC-II interaction as well as the engagement of co-stimulatory receptors and cytokine production by DCs prime the differentiation of naïve $CD4^+$ T cells into Tfh cells via the induction of the Bcl6 transcription factor. This results in CXCR5 upregulation and downregulation of CCR7 allowing the cells to migrate to the T-B border [107, 108]. IL-2 expression by naïve $CD4^+$ T cells during the interaction with DCs and the strength of TCR-MHC-II determines the differentiation into cTfh cells while non-IL-2 producers differentiate into non-Tfh cells [109, 110]. At the T-B border, Tfh cells interact with activated B cells which act as antigen-presenting cells as they display processed antigen peptides via the MHC-II molecule to the cognate TCR of Tfh cells (Figure 5). This interaction with B cells is essential for the differentiation of Tfh cells into germinal center Tfh cells [111]. Tfh cells secrete IL-21 and express CD40L and ICOS which are required for B cell differentiation and progression to both the germinal center reaction and the extrafollicular reaction [112-114]. The extrafollicular reaction results in plasmablast expansion, which produce high levels of low affinity antibodies (Figure 5) [88]. In parallel, Tfh and activated B cells that interact in the T-B border subsequently migrate towards the germinal center. Germinal centers are polarized in two major sites, the light and the dark zone, with distinct cytokine and chemokine environments. During a germinal center reaction B cells move in and out of the dark zone, where they undergo clonal expansion and somatic hypermutations, while in the light zone B cells are being selected for survival by follicular dendritic cells (FDC) and germinal center Tfh cells based on the affinity of their BCRs towards the antigen (Figure 5) [115, 116]. This Darwinian selection results in the generation of memory B cells and long-lived plasma cells, the latter producing high affinity antibodies for many years after the infection or vaccination [117].

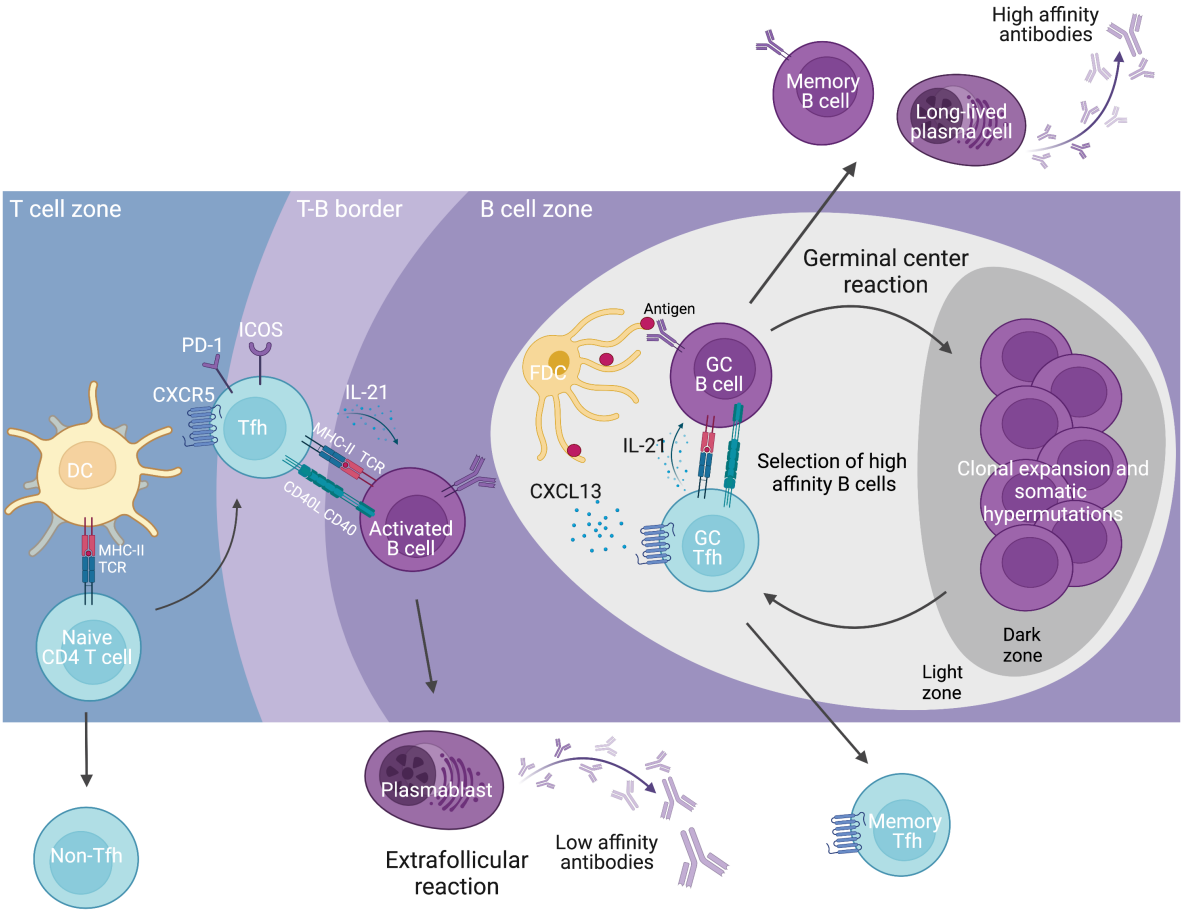

Figure 5. The adaptive immune responses initiated following infection or vaccination with a particular focus on germinal center reactions and the generation of immunological memory. Key cell types and cytokines are highlighted within the follicles (the B cell zone) and the paracortical areas (the T cell zone) of a lymph node draining the site of inflammation. Created with BioRender.com.

In peripheral blood in humans, circulating Tfh cells (cTfh) often identified by the expression of CXCR5, PD-1, and ICOS, were shown to be clonally and phenotypically related with germinal center Tfh cells, and their frequencies after vaccination correlate with virus-specific IgG levels [118, 119]. cTfh cells originate from the germinal centers in lymph nodes and may represent memory cells that can respond to secondary infection. In peripheral blood, cTfh cells are found in lower frequencies than in the secondary lymphoid organs [120]. However, their frequencies and activation increase in peripheral blood after infection or vaccination, and has been shown to correlate with the magnitude of antibody responses [121-124].

CXCL13 is a ligand for the CXCR5 receptor and is secreted by follicular dendritic cells and germinal center Tfh cells in the B cell follicles. Cells expressing CXCR5 can migrate towards the B cell follicles for germinal center reactions [125-127]. During germinal center reactions, the CXCL13 gradient is also necessary for B cells, that have undergone clonal expansion and somatic hypermutations in the dark zone, to move back into the light zone for selection. In humans, plasma CXCL13 levels have been shown to correlate with frequencies of germinal center Tfh cells [128]. Therefore, CXCL13 levels in peripheral blood can act as a surrogate marker of germinal center activity in humans.

2 RESEARCH AIMS

The overall aim of this thesis was to characterize human immune responses to emerging viruses, specifically tick-borne encephalitis virus and SARS-CoV-2. The specific aims of this thesis are as follows:

- Assess the mortality experience of TBE patients in Sweden (**Paper I**).

- Characterize the human $CD4^+$ T cell memory response to TBE vaccine throughout the course of primary TBE immunization (**Paper II**).

- Describe the B cell response to TBEV during the second phase of TBE following hospitalization (**Paper III**).

- Assess the magnitude and specificity of early B cell response to SARS-CoV-2 infection (**Paper IV and V**).

- Investigate the persistence and quality of immunological memory following SARS-CoV-2 infection (**Paper V**).

3 MATERIALS AND METHODS

This is a brief description of the methodology used in this thesis. Detailed descriptions of each method can be found within the individual manuscripts and papers.

3.1 ETHICAL CONSIDERATIONS

Research on human subjects requires a strict adherence to the ethical principles of medical research outlined by the World Medical Association in the Declaration of Helsinki. Therefore, all medical research projects involving human subjects must be approved by the local ethical review authority before the commencement of the study and the enrolment of study participants. **Paper I** is based on data from the Public Health Agency, Statistics Sweden and the Swedish National Board of Health and Welfare's Cause of Death Register. Meanwhile, **Papers II-V** relies on biological samples and clinical data from patients and healthy individuals.

The Regional Ethical Review Board in Stockholm, Sweden and the Swedish Ethical Review Authority has approved all the studies presented in this thesis. All of the participants provided written informed consent. Patient samples were coded, and personal information was handled and stored according to the General Data Protection Regulation.

3.2 STANDARDIZED MORTALITY RATIO

Many different epidemiological methods exist to measure a mortality experience of a study population. One of the most standard measures is case-fatality rate where a proportion of individuals with a fatal outcome within the whole study population (e.g., TBE-diagnosed individuals) is calculated. For example, if 10 people succumb to TBE out of a 100 people diagnosed, this would result in a case-fatality rate of 10%. However, the case-fatality rate does not account for baseline mortality of the study population, a particularly important consideration when the study population is at high risk of dying due to other causes, for example, old age.

An alternative method to case-fatality rates is a standardized mortality ratio (SMR). SMR is a ratio between the number of deaths in the study population and the expected number of deaths estimated from the matched control population. SMR above 1 where 95% confidence intervals do not cross 1 indicates a significantly higher mortality in the study population compared to the control population (Figure 6A). For example, if 40 out of 100 TBE-diagnosed individuals had fatal outcome, while only 10 individuals died in the matched control cohort within the same time period, it would result in SMR of 4 (i.e., 4 times higher mortality in TBE-diagnosed individuals compared to controls) (Figure 6B). In **Paper I**, we measured SMR for TBE in

Sweden using all diagnosed TBE cases during 2004-2017, together with sex, age, and location of residence-matched controls. SMR is an important measure for understanding the true mortality experience of a study population and can be a valuable tool in guiding public health policies.

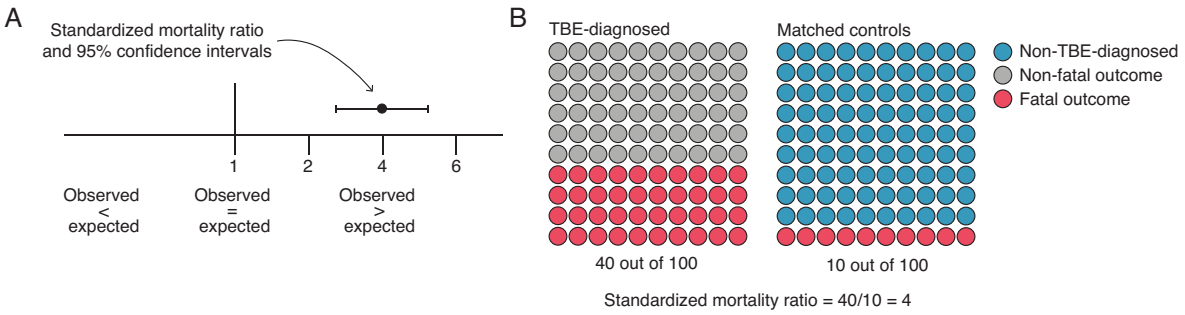

Figure 6. Standardized mortality ratio. (A) Mortality in the study population is higher than in the control population if the SMR is higher than 1 and the 95% confidence intervals do not cross the value of 1. (B) An illustration of when the mortality is four times higher in the study population (e.g., TBE-diagnosed individuals) compared to the controls.

3.3 BIOLOGICAL SAMPLE PROCESSING

Papers II-V of this thesis relied on peripheral blood samples from patients and healthy individuals. Peripheral blood from study subjects was collected by venipuncture into anti-coagulant-containing blood collection tubes and peripheral blood mononuclear cells (PBMCs), as well as plasma or serum were isolated. PBMCs were separated from erythrocytes and granulocytes by density gradient centrifugation and were either used in fresh experiments or cryopreserved for later use.

3.4 SEROLOGY ASSAYS

After an infection or vaccination, seroconversion is measured by assessing the levels of pathogen-specific antibodies in serum or plasma. The two major methods used in this assessment are enzyme-linked immunosorbent assay (ELISA) and virus neutralization assay. These methods are highly complementary to each other but measure different aspects of the serological response. In **Papers II-V** of this thesis, ELISA and virus neutralization assay were used in parallel to describe serological responses to TBEV and SARS-CoV-2.

ELISA can be either qualitative (provides a positive/negative result), or quantitative (provides antibody concentration). In viral infections, viral proteins or whole inactivated virus particles are typically used as targets to detect virus-specific antibodies in patients, including IgM, IgG or IgA isotypes. However, the sensitivity and specificity of such ELISAs vary depending on the target used and cross-reactivity between related viruses. ELISAs can detect all pathogen-specific antibodies, but it does not give any information about the functional quality of these

antibodies, i.e., the ability of the antibodies to neutralize the virus and prevent it from entering the target cells. Neutralization is an important measure as it describes the protective nature of antibodies following infection or vaccination. Neutralizing antibodies typically target viral proteins that are used by the virus for entering human cells. For example, the interaction between ACE2 and the spike protein of SARS-COV-2 can be blocked by antibodies binding to the receptor binding domain (RBD) of spike protein, making RBD the primary target of neutralizing antibodies (Figure 7) [130]. Neutralization assays can strongly complement ELISA in this functional assessment.

Neutralization assays typically measure the dilution of patient serum at which the virus is prevented from infecting or killing a specified fraction of target cells. Two different neutralization assays were used in this thesis: (i) cytopathic effect-based micro-neutralization assay for SARS-CoV-2 and (ii) rapid fluorescent focus inhibition test (RFFIT) for TBEV. The two assays are similar in principle but use different approaches for the readout. For SARS-CoV-2, a neutralizing titer is a dilution of serum at which less than 50% of the cell layer shows signs of cytopathic effect [131]. For TBEV, a neutralizing titer is a dilution at which less than ten areas out of twenty within a cell monolayer contain TBEV-infected cells (measured by immunofluorescence) [132].

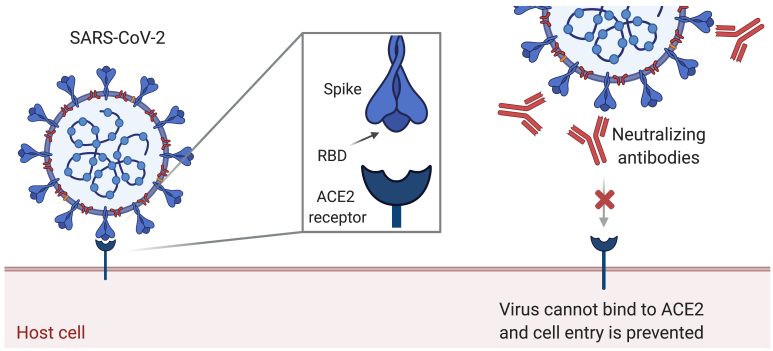

Figure 7. SARS-CoV-2 spike protein interaction with ACE2 receptor. If this interaction is blocked by neutralizing antibodies, SARS-CoV-2 virus entry into the host cell is inhibited. Created with BioRender.com.

3.5 FLOW CYTOMETRY

Flow cytometry allows phenotyping of a large pool of cells at a single-cell level, which contributed greatly to the advancement in the field of immunology (reviewed in [133]). Flow cytometry has many applications and has been used in this thesis as the key methodology to describe immunological events in TBE vaccinated individuals, TBE patients, COVID-19 patients and healthy individuals at a cellular level. Specifically, multicolor flow cytometry was used for the:

- Measurement of the absolute numbers of different immune cells in peripheral blood (**Papers IV and V**).
- Phenotyping of B cells, T cells and other immune cells (**Papers II-V**).
- Functional assessment of T cell responses to antigen stimulation (**Paper II**).

3.6 ANALYSIS OF IMMUNE CELLS BY FLOW CYTOMETRY

3.6.1 Antibody-secreting cells

This cell subset, also often referred to as plasmablasts or plasma cells, expands in many viral infections and following vaccinations to produce large quantities of virus-specific antibodies [91, 93, 134, 135]. In **Papers III-V**, ASCs were defined as $CD19^{+}$ $CD20^{low/-}$ IgD^{-} B cells that co-express high levels of CD38 and CD27 molecules on the cell surface. Typically, the magnitude of ASC expansion is measured as the proportion of ASCs within the total B cell pool. Immunoglobulin expression by ASCs can also be measured by flow cytometry to indicate which antibodies are secreted in response to the ongoing infection or after vaccination, but the true functional Ig secretion assessment is typically performed by the ELISpot assay [136].

Distinction between plasmablasts and plasma cells in peripheral blood is not straight forward and both subsets are characterized by high CD27 and CD38 expression, yet varying expression of CD138 [104, 137-139]. In the bone marrow, long-lived plasma cells primarily display $CD19^{-}CD38^{high}CD138^{+}$ [104]. $CD19^{-}$ ASCs were also identified in peripheral blood after vaccination, as potential precursors of long-lived plasma cells [140]. Due to the lack of an in-depth phenotype analysis, plasmablasts and plasma cells are referred to as ASCs throughout this thesis (Figure 8).

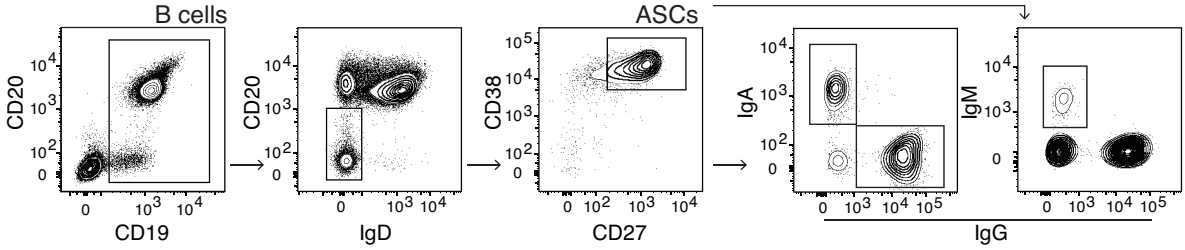

Figure 8. Flow cytometry gating strategy of antibody-secreting cells (ASCs) that was used in **Papers III-V** of this thesis (the figure is published in [100]).

3.6.2 Activated and proliferating T cells

T cell activation during infections and vaccinations can be assessed by the expression of many different molecules using flow cytometry. T cell activation during infections in this thesis (**Papers III and IV**) were assessed by co-expression of activation molecule CD38 and an intranuclear proliferation marker Ki-67.

3.6.3 Polyfunctional T cells

Although phenotypic characterization can indicate a T cell response to a pathogen, surface phenotyping does not directly assess functional capacity of T cells to produce cytokines. In **Paper II**, we stimulated PBMCs from TBE vaccinated individuals with peptide pools from TBEV structural proteins and assessed the memory $CD4^{+}$ T cell response via the production of intracellular IFN-γ, IL-2, and TNF, and the upregulation of CD154 (CD40L) and CD107a.

Using Boolean gating, we could describe the cytokine co-expression patterns by $CD4^+$ T cells and identify polyfunctional T cells (cells co-expressing at least two cytokines at once) [141].

3.6.4 Circulating follicular helper $CD4^+$ T cells

In **Paper V**, we investigated circulating follicular helper T cell (cTfh) frequencies [142-144]. cTfh cells were defined as $CD4^+$ $CXCR5^+$ cells and activated cTfh cells were considered as ICOS and PD-1 co-expressing cells. Based on CXCR3 expression, Th1-polarised cTfh ($CXCR3^+ICOS^+PD\text{-}1^+$) and Th2/Th17-polarised ($CXCR3^-ICOS^+PD\text{-}1^+$) subsets were identified.

3.7 IDENTIFICATION OF ANTIGEN-SPECIFIC T CELLS AND B CELLS

Following infection or vaccination, immunological memory is formed after the development of pathogen-specific memory B cells and T cells. The abundance and functional capacity of these cells in patients or vaccinees can be assessed using several techniques, but in this thesis two main methods were chosen: intracellular cytokine staining (ICS) assay (**Paper II**) and FluoroSpot (**Papers IV-V)**.

3.7.1 Intracellular cytokine staining (ICS) assay

Memory T cells can respond to cognate antigen stimulation by the production of cytokines (e.g., IFN-γ, IL-2, and/or TNF) and the upregulation of CD154 (CD40L) co-stimulatory molecule [145-148]. To assess the TBEV-specific memory T cell responses in TBE vaccinees (**Paper II**), PBMCs from the vaccinees were stimulated with an overlapping peptide pool based on structural TBEV protein sequences for 6 hours in the presence of the cytokine secretion inhibitors brefeldin A and monensin. The presence of cytokine secretion inhibitors during the stimulation reaction traps all the cytokines produced by individual memory T cells which can be analyzed by multicolor flow cytometry.

In **Paper II**, we characterized cytokine co-expression patters in TBEV-specific memory T cells using flow cytometry analysis software FlowJo and Simplified Presentation of Incredibly Complex Evaluations (SPICE) software [149].

3.7.2 Memory T cell FluoroSpot

T cell FluoroSpot assay can be used as an alternative method to ICS to assess memory T cell responses in patients or vaccinated individuals. The basis of FluoroSpot assay is the detection of individual T cells secreting cytokines after the cognate antigen stimulation. Similarly, to ICS assay, polyfunctional T cells can be identified by FluoroSpot, however, it is not possible to

phenotypically characterize single cells and therefore $CD4^+$ and $CD8^+$ T cell responses cannot be distinguished from one another.

In **Paper V**, we used an IFN-γ/IL-2/TNF FluoroSpot assay to measure memory T cell responses to SARS-CoV-2 in COVID-19 patients. Briefly, FluoroSpot plates were coated with anti-IFN-γ, anti-IL-2 and anti-TNF antibodies. PBMCs from COVID-19 patients were incubated on the FluoroSpot plates with different peptide pools based on SARS-CoV-2 protein sequences. A cell responding to a single peptide would secrete cytokines which would be immediately captured by the antibodies coating the plate. After the incubation, the cells were washed off and the secreted cytokine "spots" were detected with fluorescently labelled antibodies. The "spots" formed as the result of cytokine secretion by individual T cells were counted using a specialized fluorescence reader. Several measurements could be extracted from the data: (i) the number of responding T cells; (ii) relative amounts of cytokines secreted (based on fluorescence intensity of spots); and (iii) cytokine co-expression patterns of responding T cells (Figure 9).

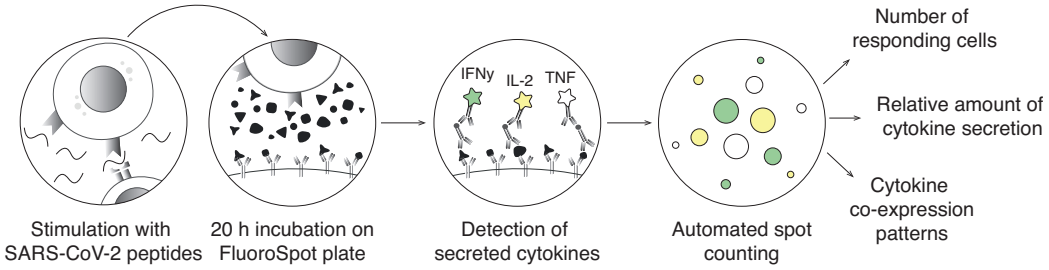

Figure 9. Flowchart of the FluoroSpot assay for the detection of memory T cells secreting IFN-γ, IL-2 and/or TNF in response to the stimulation with SARS-CoV-2 peptides in **Paper V** [150].

3.7.3 B cell FluoroSpot for the detection of ASCs

Although flow cytometry is a valuable tool to detect ASC frequencies in peripheral blood after infection or vaccination, it does not directly assess antibody secretion capacity of ASCs. B cell FluoroSpot assay, a fluorescence-based variant of the enzyme-linked immunospot (ELISpot) assay, is a strong complementary approach to flow cytometry as it allows for the detection of the total and pathogen-specific ASC numbers secreting either IgA-, IgG- or IgM [151-153].

In **Paper IV** we combined flow cytometry and B cell FluoroSpot to assess ASC expansion in COVID-19 patients during the acute phase of disease. Briefly, to detect SARS-CoV-2-specific ASCs, the FluoroSpot plates were coated with the recombinant nucleocapsid protein (N-protein) of SARS-CoV-2 and freshly isolated PBMCs from COVID-19 patients were incubated on the FluoroSpot plate to allow for antibody secretion by the ASCs. The secretion of all three isotypes of antibodies was assessed including IgA, IgG, and IgM. In parallel, the total number of ASCs was measured by coating the FluoroSpot plate with anti-IgA, anti-IgG and anti-IgM antibodies. The fluorescent "spots", formed as the result of antibody secretion by individual

ASCs, were counted using a specialized fluorescence reader. We found that the numbers of ASCs detected by flow cytometry correlated strongly with the numbers detected by FluoroSpot (Spearman correlation coefficient [r_s] = 0.636; p = 0.003), highlighting the complementarity of these two methodologies [100].

3.7.4 Memory B cell FluoroSpot

Ex vivo detection of virus-specific memory B cells requires a highly sensitive method and is usually performed either by flow cytometry with fluorescently tagged viral proteins or by the ELISpot assay (reviewed in [154]).

In Paper V, we assessed whether the individuals who recovered from SARS-CoV-2 infection have circulating memory B cells specific for SARS-CoV-2 spike subunit 1 (S1) and nucleocapsid (N) proteins. Briefly, PBMCs from recovered individuals were stimulated with a polyclonal stimulus (i.e., IL-2 and TLR agonist R848 [155]) for 5 days leading to the differentiation of memory B cells into ASCs. Subsequently, memory B cell-derived ASCs (mASCs) were detected on SARS-CoV-2 protein-coated FluoroSpot plates. The secreted IgG and IgA "spots" formed by SARS-CoV-2-specific mASCs were counted using a specialized fluorescence reader (Figure 10).

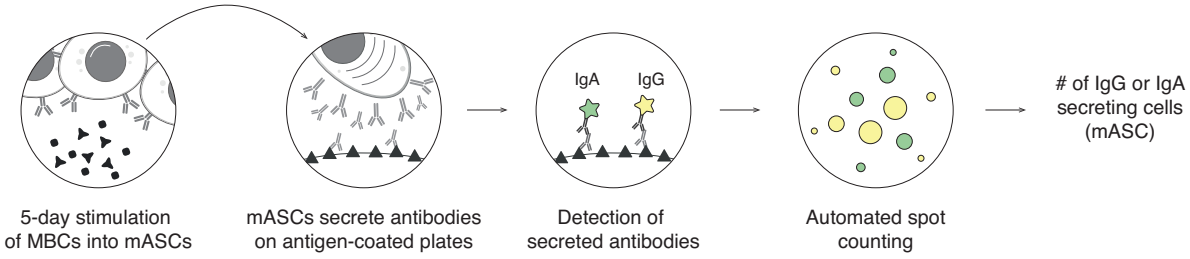

Figure 10. Flowchart of the FluoroSpot assay for the detection of memory B cells secreting IgG or IgA antibodies in response to a 5-day polyclonal stimulation in **Paper V** [150].

4 RESULTS AND DISCUSSION

4.1 MORTALITY DUE TO TBE IN SWEDEN

Case-fatality rates due to TBE vary depending on the strain, i.e., case-fatality rate for the European TBEV is 1-2%, while for the Siberian and the Far Eastern TBEV it is 6-8% and 20-60%, respectively [14]. A more recent estimation of case-fatality rates in TBE patients in Europe found the case-fatality rate to be 0.5% [9]. However, the case-fatality rates do not consider the baseline mortality experience of a study population. In **Paper I**, we aimed to estimate the mortality experience of TBE patients in Sweden compared to a matched general population to account for baseline mortality in non-TBE individuals. TBE cases were matched with controls based on sex, age and county of residence, and the standardized mortality ratio (SMR) was calculated. We found no fatal cases after TBEV infection within 90 days after the diagnosis in individuals under the age of 40, suggesting that TBE is not a highly fatal disease in this age group in Sweden. However, we found an increased SMR in people over the age of 60. An overall SMR of 3.96 (95% CI: 2.55-5.9; $p < 0.001$) for TBE was observed indicating an almost four times higher mortality in TBE-diagnosed patients compared to the control population. Our finding in **Paper I** highlights the need for an increased vaccination coverage of people at risk of TBEV infection, particularly older individuals.

Although we noticed a significantly increased mortality in TBE patients, our result may be an underestimation. In order to be exposed to TBEV-infected ticks, a person has to lead an active lifestyle that includes recreational or occupational outdoor activities. It is know that occupational and socioeconomic status can influence the risk of exposure to TBEV, via the activities such as forestry, hunting, farming or mushroom and berry picking [4-6]. In our study, TBE-diagnosed individuals were matched with controls only based on sex, age, and county of residence, while occupational information and socio-economic status were not considered. We speculate that TBE-diagnosed individuals may be healthier and lead a more active lifestyle than the matched control population and therefore SMR in TBE calculated in **Paper I** may be underestimated.

On the other hand, TBEV infections often go undiagnosed if the TBEV-infected individual is asymptomatic or only have mild symptoms, that do not require medical attention. A serological surveillance study in Sweden found that about two thirds of TBEV-infected individuals follow a subclinical course of infection [24]. Large seroprevalence studies for TBEV are lacking but considering that there may be a large proportion of asymptomatic cases which never get diagnosed, case-fatality rates, as well as SMR may be lower than estimated in **Paper I**.

4.2 ADAPTIVE IMMUNE RESPONSES TO TBE VACCINE AND TBEV INFECTION

Natural infection, if it results in recovery, usually leads to a long-lasting immunity from reinfection or symptomatic disease. Vaccines have the same goal. One of the best vaccine examples that requires only one dose for a life-long protection, is the yellow fever vaccine, which is made of live-attenuated YFV [34]. It was developed in 1936 and is still in use today. The vaccine has often served as a model for describing human immune responses to acute viral infections [156, 157].

4.2.1 TBE vaccine-induced T cell responses

In contrast to live-attenuated YF vaccine, TBE vaccines are made of inactivated TBEV together with an alum adjuvant, and multiple doses are required to maintain protective immunity. The primary immunization schedule consists of 3 doses within a one-year period, followed by booster doses every 3-5 years. The primary method of measuring vaccine-induced immunity is by serological assays, and the antibody responses to TBE vaccination have been well-characterized [158-164]. Less is known about T cells responses after vaccination and how those responses compare to natural infection. Robust T cell responses, specifically a high proportion of polyfunctional memory T cells, were previously described as a potential correlate of protection after vaccination and during viral infections, for example in HIV [74, 165-168].

In **Paper II**, we sought after describing the functional memory T cell responses throughout the primary TBE immunization schedule. Fifteen healthy volunteers with no history of TBEV infection or vaccination were recruited to undergo primary TBE immunization followed by four peripheral blood sampling timepoints (Figure 11). Eight recovered TBE patients were also included in the study as controls, sampled at 7-32 months after TBEV infection.

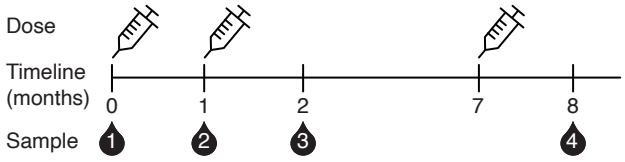

Figure 11. TBE immunization strategy and peripheral blood sampling of healthy volunteers in **Paper II** [170].

First, we wanted to assess if TBE vaccination induced a $CD4^+$ and/or $CD8^+$ T cell response and if the magnitude of this response increased after each vaccine dose. For this we used a flow-cytometric assay for specific cell-mediated immune response in activated whole blood (FASCIA) (Figure 12A) [169]. We stimulated freshly isolated blood of TBE-vaccinated individuals (samples 1-4) with the TBE vaccine FSME-IMMUN (the same vaccine as the one administered to the study participants) for 7 days and assessed the expansion of $CD4^+$ and $CD8^+$ T cell lymphoblasts by flow cytometry. The highest magnitude of T cell expansion was detected after the second dose of TBE vaccine, but the expansion was only detectable for $CD4^+$ T cell subset, and not $CD8^+$ T cells (Figure 12B-C). The magnitude of $CD4^+$ T cell lymphoblast response, however, varied highly between individuals, and was slightly lower after the 3rd dose compared to after the 2nd dose (Figure 12B).

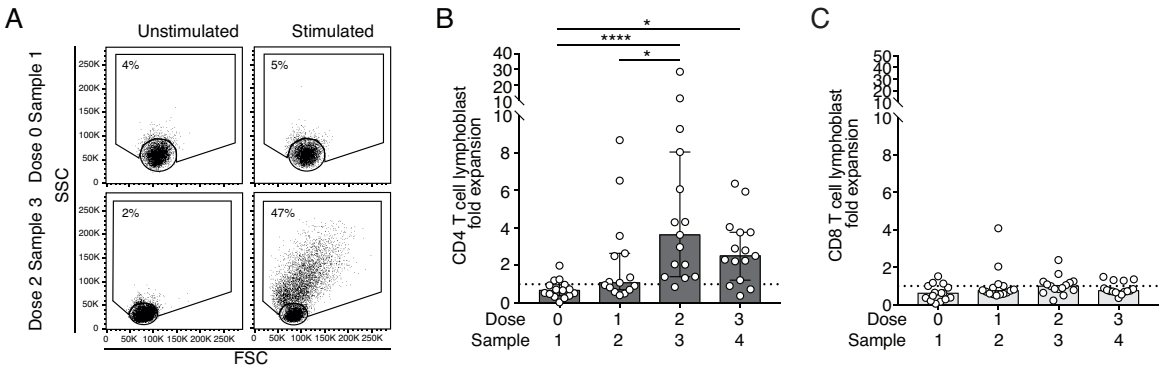

Figure 12. FASCIA results from TBE vaccinees throughout the primary TBE immunization schedule in **Paper II** [170]. (A) Flow cytometry FASCIA data from a representative TBE vaccinee. (B) Fold expansion of $CD4^+$ T cells in response to *ex vivo* stimulation with the TBE vaccine. (C). Fold expansion of $CD8^+$ T cells in response to *ex vivo* stimulation with the TBE vaccine. Horizontal dotted line is a positivity threshold of the assay. Median and IQR are plotted.

Considering a robust $CD4^+$ lymphoblast response following TBE immunization, we next wanted to assess the functional profile of TBEV-specific memory $CD4^+$ T cells. For this, we used intracellular cytokine staining assay followed by flow cytometry where we measured Th1 cytokine production (IFN-γ, IL-2, and TNF) by memory $CD4^+$ T cells in response to the stimulation with a peptide pool based on the TBEV structural protein sequences (Figure 13A-B). In agreement to FASCIA results, the highest magnitude of memory $CD4^+$ T cell response was observed after the 2nd dose of vaccine, consistent for all three cytokines measured (Figure 13C).

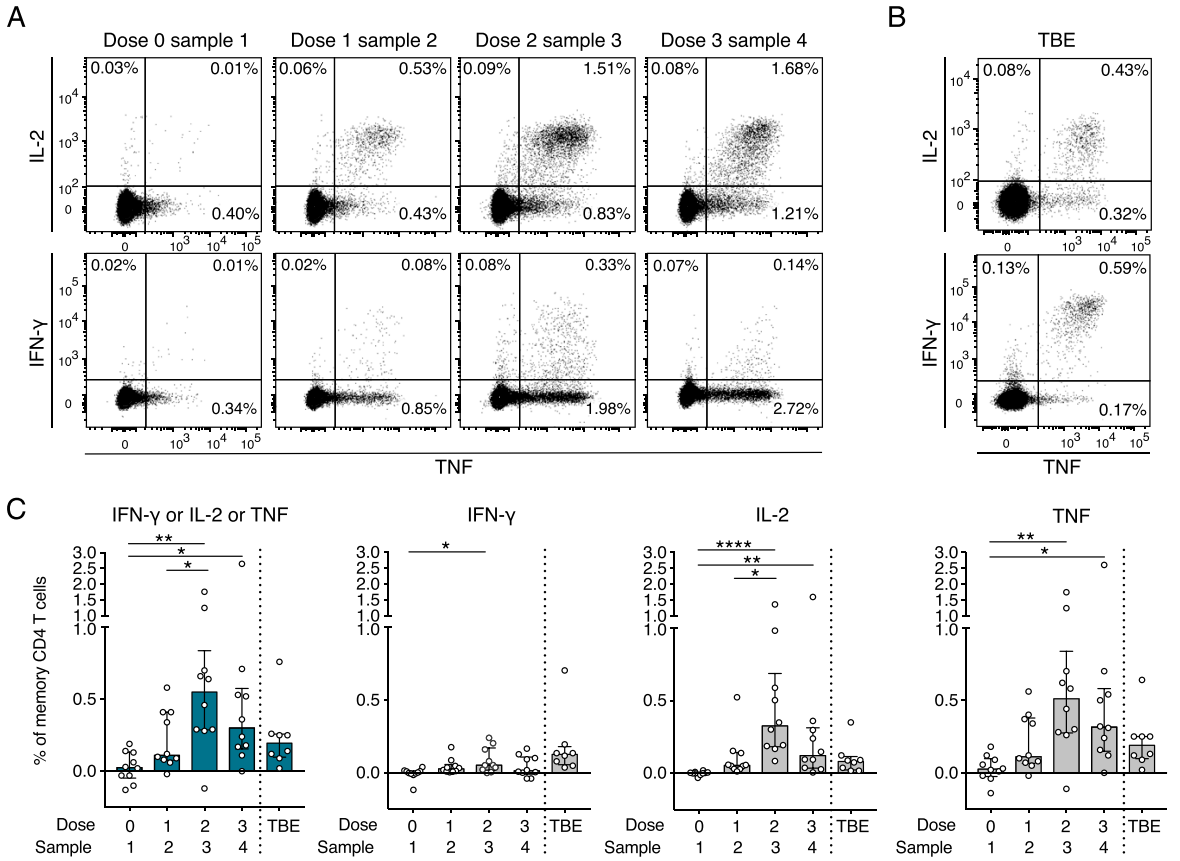

Figure 13. Memory CD4$^+$ T cell responses to TBEV peptide stimulation in TBE vaccinees and TBE patients in **Paper II** [170]. (A-B) IFN-γ, IL-2 and TNF secretion in response to the peptide stimulation in one representative TBE vaccinee and one TBE patient assessed by flow cytometry. (C) Frequencies of cytokine-secreting cells within all memory CD4$^+$ T cells in response to the peptide stimulation. Median and IQR are plotted in panel C.

When evaluating cytokine co-expression patters to identify polyfunctional CD4$^+$ T cells, we found a consistent dominance of IL-2$^+$TNF$^+$ or TNF$^+$ response after each dose of TBE vaccine (Figure 14A and C). Meanwhile, the recovered TBE patients had significantly higher IFN-γ response and, therefore, a larger proportion of IFN-γ$^+$IL-2$^+$TNF$^+$ co-expressing memory CD4$^+$ T cells (Figure 14B and D). Higher IFN-γ response in TBE patients compared to vaccinees is not an unexpected finding, as several other studies with inactivated vaccines show a similar T cell response pattern dominated by IL-2 and TNF [171, 172]. This pattern of cytokine response to structural TBEV proteins was also confirmed by an independent study which compared TBE booster vaccination with natural infection and found a predominance of IL-2 and TNF response, while the IFN-γ response was lower in vaccinees than in TBE patients [173]. Therefore, this shows that the functional profile does not change in TBE vaccinated individuals, regardless of how many TBE vaccine doses they receive. Although functional response patterns differ between vaccinated and infected individuals, immunodominance patterns to structural TBEV proteins were shown to be highly similar between patients and vaccinees [174].

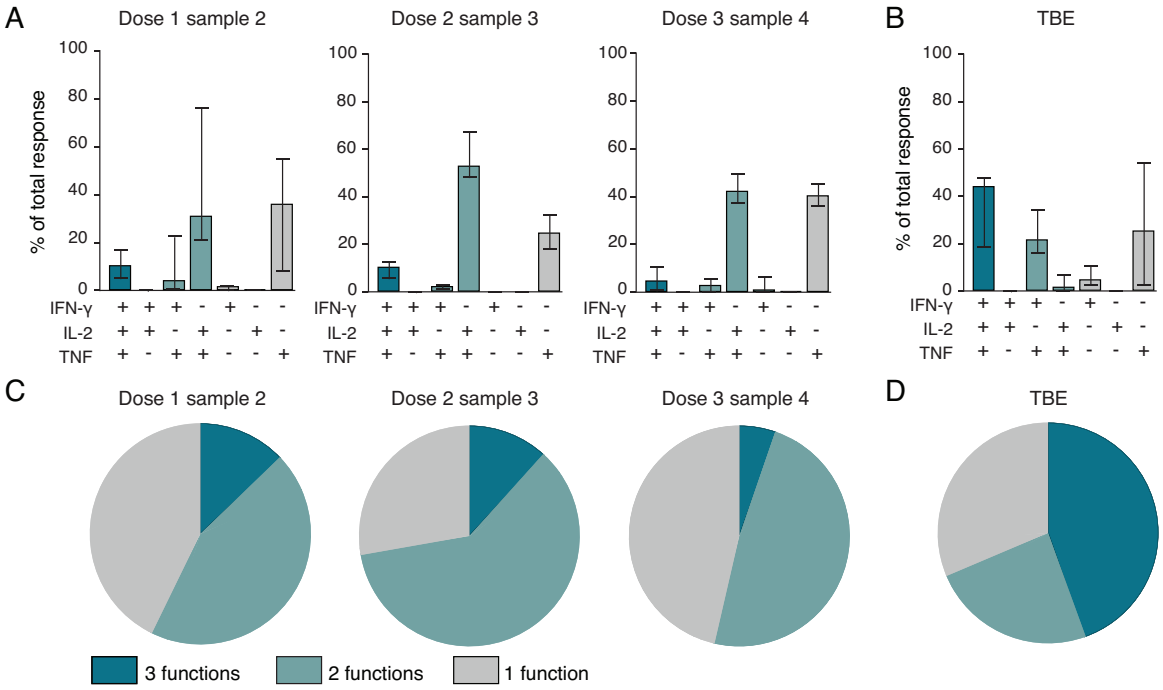

Figure 14. Functional profile of memory CD4$^+$ T cells responding to TBEV peptide pools in TBE vaccinees (A and C) and TBE patients (B and D) in **Paper II** [170]. Median and IQR are plotted in graphs A and B.

CD154 (CD40L) is a co-stimulatory molecule expressed by T cells and can activate dendritic cells and provide B cell help and, therefore, plays an important role in the initiation of adaptive immune responses [147, 148, 175, 176]. It has also previously been used as a valuable surface marker for the identification of antigen-specific CD4$^+$ T cells responding to stimulation [177]. Therefore, in **Paper II** we wanted to investigate if the expression of CD154 on cytokine-producing memory CD4$^+$ T cells after the stimulation is increased in vaccinated individuals (Figure 15A). CD154 is typically expressed at steady state, therefore a background level of expression on cytokine-producing cells was observed in unstimulated controls (Figure 15C) [178]. However, we found a significant increase of the proportion of CD154$^+$ cytokine-producing cells after each dose of vaccine (i.e., samples 2-4) compared to before vaccination (i.e., sample 1) in samples stimulated with the TBEV peptide pool (Figure 15C). Interestingly, a small proportion of CD154$^+$ cells did not secrete either of the cytokines measured, and this population was more abundant in TBE vaccinated individuals than in TBE patients (Figure 15D and E). We speculate that other cytokines than the ones measured in **Paper II** could be secreted in response to TBEV peptide stimulation in vaccinated individuals. cTfh cell responses were not assessed in this paper due to the lack of the key phenotypic markers (i.e., CXCR5, ICOS and PD-1) in the flow cytometry panel, however, we speculate that a large proportion of memory CD4$^+$ T cells responding to the stimulation are indeed memory cTfh cells.

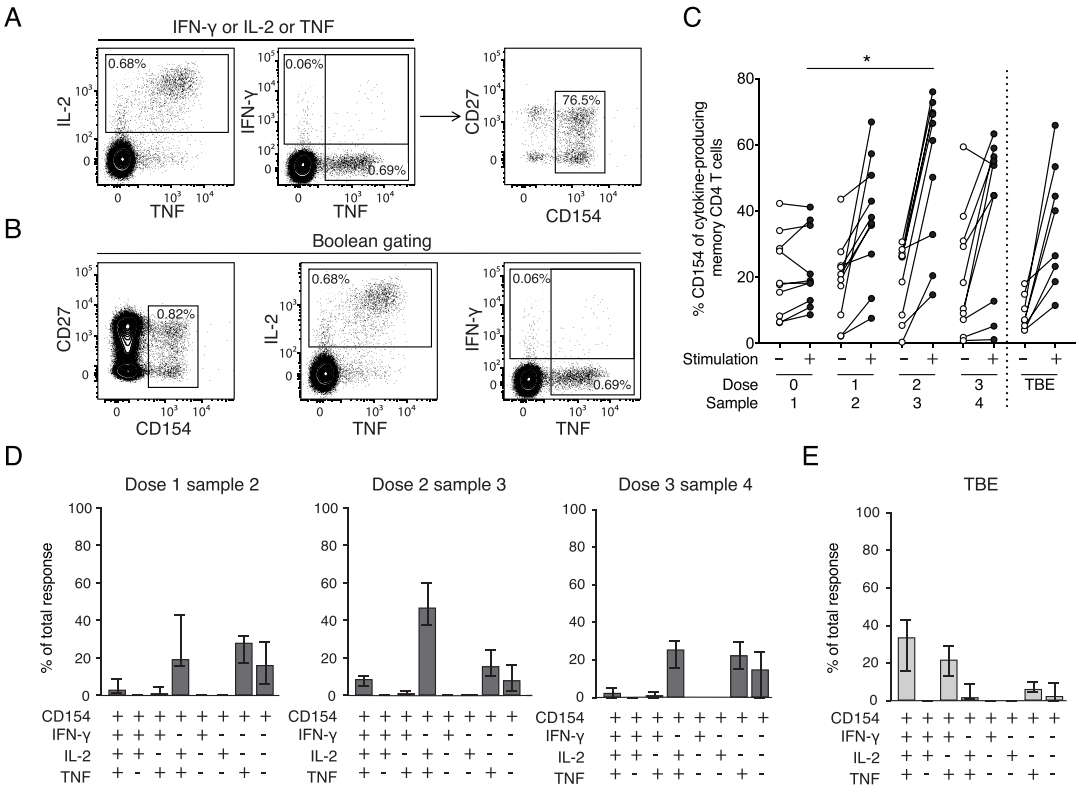

Figure 15. CD154 (CD40L) expression on memory CD4$^+$ T cells in response to TBEV peptide stimulation in TBE vaccinees and TBE patients in **Paper II** [170]. (A) Flow cytometry gating strategy for CD154 expression on cytokine-producing memory CD4$^+$ T cells. (B) Boolean gating to assess CD154 and cytokine co-expression patterns. (C) Upregulation of CD154 on cytokine producing memory CD4$^+$ T cells after the peptide stimulation. (D-E) CD154 and cytokine co-expression patterns after the peptide stimulation in TBE vaccinees (D) and TBE patients (E). Median and IQR are plotted in graphs D and E.

4.2.2 B cell responses to TBEV infection

Natural TBEV infection is known to elicit a robust serological response and TBEV-specific antibodies can be detected for many years after infection [179]. However, little is known about early B cell responses to TBEV and the kinetics of those responses in relation to the disease progression. Studies on early B cell responses are limited by the fact that TBEV-infected individuals do not seek medical attention until the emergence of neurological symptoms. Typically, neurological symptoms manifest a few weeks after the exposure to TBEV. Therefore, sampling of TBEV-infected individuals is only possible at later timepoints after the infection, primarily during hospitalization. By the admission to the hospital, most patients have already generated TBEV-specific antibodies and as the result, viral RNA is no longer detectable in peripheral blood [180-182].

ASC expansion is typically a hallmark of early B cell response following infection and vaccination and leads to a rapid increase in pathogen-specific antibodies [91, 93, 134, 135]. Although seroconversion is typical in TBE patients already by hospitalization, it has not been shown whether ASC response is detectable during neurological symptoms. In **Paper III**, we wanted to investigate if an ongoing ASC response could be detected. We hypothesized that the

ASC response may have taken place before hospitalization but considering a symptomatic disease during the second phase of TBE, and ongoing ASC response could not be dismissed.

We measured ASC frequencies in peripheral blood of TBE patients sampled at varying timepoints after hospitalization (<7 days, 7-13 days, 14-30 days, and >30 days), and compared the responses with dengue, an acute viral infection with a well-characterized ASC response (Figure 16). Throughout the follow up period, low frequencies of ASCs were detected in TBE patients. Median frequencies of 1.2% at <7; 2.16% at 7-13; 2.2% at 14-30; and 0.9% at >30 days since hospitalization were detected.

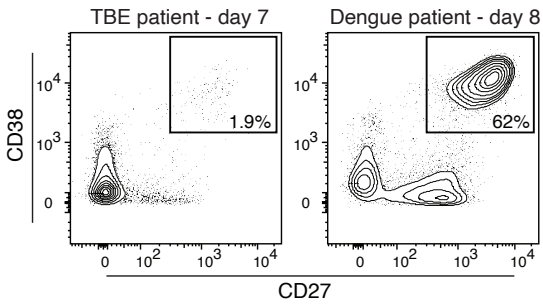

Figure 16. ASC frequencies within total $CD19^+$ B cell pool assessed by flow cytometry in a representative TBE and dengue patient (**Paper III**). Gated on $CD19^+$ $CD20^{low/-}$ IgD^- cells.

This level of ASC frequencies are relatively low and resemble frequencies observed in healthy donors where around 1% of B cells are ASCs [91, 100]. Meanwhile, a massive ASC expansion in dengue patients sampled at <14 days after symptom onset was detected with a median frequency of 28% of all B cells (Figure 16). A similar result was previously shown by another study on acute dengue virus infection [91].

The difference observed between the TBE and dengue patients is most likely due to the differences in the timeline of disease progression. Dengue patients seek medical attention quite shortly after developing a febrile illness and they are often viremic at that stage [183]. Meanwhile, TBE patients admitted to hospital are no longer viremic as viral RNA can no longer be detected in most cases [180-182]. The difference between the two flaviviral infections may therefore be the key factor behind the differences in the magnitude of ASC response. Although we did not assess viral RNA levels in the serum of TBE or dengue patients, we expect the TBE patients to be negative for viral RNA, as all TBE patients in this study were seropositive already at hospitalization and both IgM and IgG antibodies specific for TBEV were detectable.

4.3 ADAPTIVE IMMUNE RESPONSES TO SARS-COV-2 INFECTION

4.3.1 Early B cell responses to SARS-CoV-2 infection

After the emergence of SARS-CoV-2 in 2019, the world was racing to understand the immune response to the virus, to aid treatments and vaccination strategies. In the early months of the pandemic, immune responses during COVID-19 were not well understood and we aimed to characterize the kinetics and specificity of the early B cell response to SARS-CoV-2 in hospitalized COVID-19 patients.

Twenty hospitalized patients were sampled between 7 to 19 days after the symptom onset, and we assessed the ASC response, as well as antibody levels in peripheral blood of these patients (Figure 17). Using flow cytometry and B cell FluoroSpot assays, we measured ASC frequencies and specificity for SARS-CoV-2 nucleocapsid (N) protein in COVID-19 patients. A significant ASCs response was detected in all COVID-19 patients by both methods (Figure 18 and 19). On average 3.5% of all ASCs were N-protein-specific. ASC response during ongoing infections or following vaccinations were previously shown to be highly pathogen-specific [90], and we speculate that the rest of the expanded ASC pool is also specific for SARS-CoV-2, but at the time we were not able to assess the specificity towards other SARS-CoV-2 proteins.

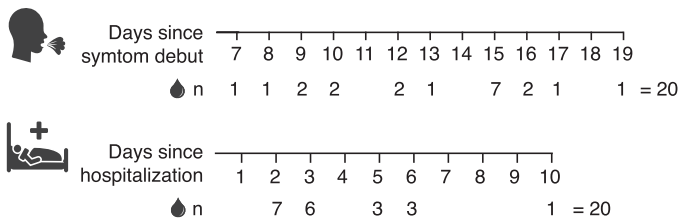

Figure 17. Peripheral blood sampling timepoints of COVID-19 patients in **Paper IV** [100] in relation to symptom debut and hospitalization.

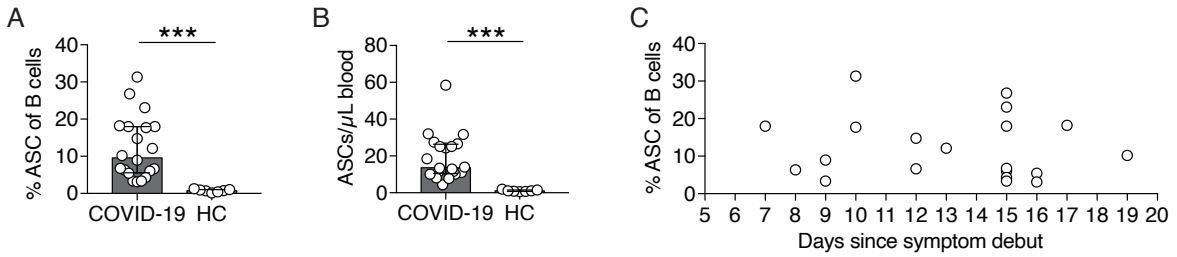

Figure 18. ASC expansion in COVID-19 patients in **Paper IV** [100]. (A) ASC frequencies within all B cells and (B) ASC numbers in peripheral blood of COVID-19 patients and healthy controls (HCs). (C) ASC frequencies in relation to symptom debut in COVID-19 patients. Median and IQR are plotted in graphs A and B.

ASC response was previously defined to be transient [90, 91, 97, 98]. However, in the COVID-19 patient cohort in **Paper IV**, ASC expansion could be detected as late as 19 days after the symptom onset (Figure 18C). A separate study where a single COVID-19 patient was sampled longitudinally during the first phase of disease also found a prolonged ASC response [184]. A sustained ASC response, detectable as late as 22-45 days after symptom onset, was observed in respiratory syncytial virus-infected patients who were shedding the virus in the airways, suggesting that the prolonged response may be stimulated by pathogen persistence [90].

Considering that COVID-19 patients in **Paper IV** were sampled at highly variable timepoints during the acute phase, antibody levels between patients also highly differed. Four of the twenty patients did not have detectable levels of SARS-CoV-2 IgG and neutralizing antibodies. A negative result in a serological assay, however, is based on a positivity threshold which varies between different assays. We showed that although the four patients in **Paper IV** lacked detectable antibody levels, N-protein-specific ASCs could be identified in all four patients in the FluoroSpot assay. This highlights that early during the infection, ELISA or neutralization assay may not be sensitive enough to detect a low-level B cell response to SARS-CoV-2, and that alternative methods such as B cell FluoroSpot may be more sensitive.

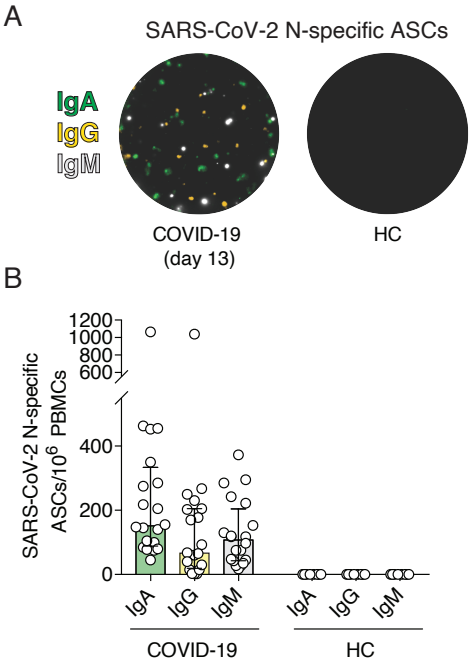

Figure 19. SARS-CoV-2 N-specific ASC numbers assessed by B cell FluoroSpot in COVID-19 patients and HCs in **Paper IV** [100]. (A) FluoroSpot wells from one representative COVID-9 patient (day 13) and one HC. (B) Numbers of N-specific ASCs in COVID-19 patients and HCs. Medians and IQR are plotted in graph B.

Accurate and sensitive serology assays are highly important for rapid diagnostics and national surveillance programs, yet neutralization assay – the most sensitive and functionally relevant serological assay – is time-consuming and often requires a high biosafety level laboratory. In **Paper IV**, we showed that antibody levels measured by ELISA strongly correlate with neutralizing antibody titers in COVID-19 (Spearman correlation coefficient [r_s] = 0.809; $p < 0.001$). Therefore, ELISAs utilized in **Paper IV** could also be used to predict the SARS-CoV-2-neutralization capacity of patient serum.

The focus of **Paper IV** was to describe the early B cell responses during the acute phase of COVID-19, and we did not address the relationship between the disease severity and the immune responses in this patient cohort. There were several reports emerging about the link between certain immune responses, for example antibody titers, and COVID-19 severity [185-187]. Therefore, we aimed to determine whether COVID-19 disease severity at hospitalization affects B cell responses. For this, two new cohorts of COVID-19 patients were collected in **Paper V**: 10 moderately sick patients (hospitalized and treated at the wards) and 16 severely sick patients (treated at the ICU) (Figure 20A-C). Peripheral blood from the two patient groups was sampled at comparable timepoints: at a median 14 days after symptom onset (Figure 20E). However, the ICU patients were treated with supplemental oxygen for longer compared to the moderate patients (median 21 days and median 2 days, respectively) (Figure 20F). Convalescence samples at 5 and 9 months were also collected for some of the patients. **Paper V** was part of the larger collaborative project *Karolinska KI/K COVID-19 Immune Atlas* where scientists with expertise in different immunology fields worked together to describe the major immunological events during acute COVID-19 [188-193].

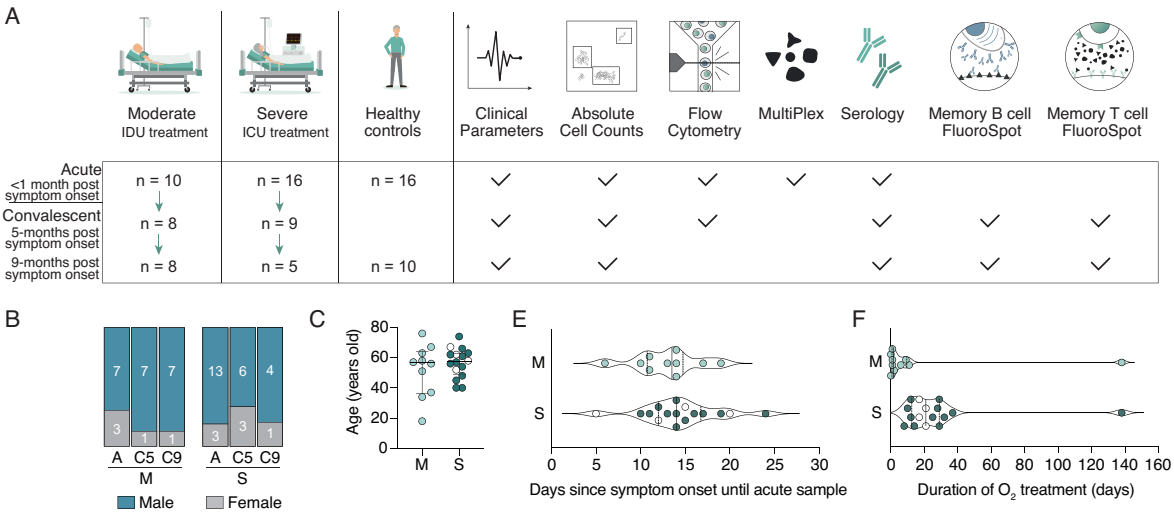

Figure 20. Study design and characteristics of COVID-19 patients in **Paper V** [150]. (A) Sampling of study participants and the experimental approach. (B) Sex and (C) age distribution in COVID-19 patients. (E) Acute sampling timepoints of COVID-19 patients in relation to symptom onset. (F) The duration of oxygen treatment in COVID-19 patients. A – acute; C5 – 5 months; C9 – 9 months; M – moderate COVID-19; S – severe COVID-19. Median and IQR are plotted in graphs C-F.

First, we aimed to investigate if a germinal center reaction is taking place during the acute phase using surrogate markers including CXCL13 concentration in plasma, as well as the frequencies of circulating follicular helper $CD4^+$ T cells (cTfh). We found increased frequencies of activated cTfh cells, as well as higher CXCL13 levels in COVID-19 patients (both moderate and severe) compared to healthy controls indicating an ongoing germinal center reaction (Figure 21). Many other studies demonstrated increased frequencies of cTfh cells in COVID-19 patients during the acute phase and $CD4^+$ T cell responses in general appear to be skewed towards cTfh profile persisting for many months after infection [188, 194-197].

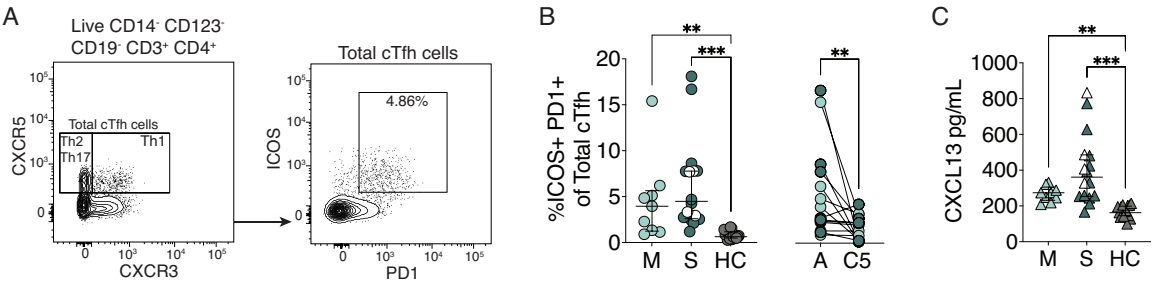

Figure 21. Germinal center activity in COVID-19 patients in **Paper V** [150]. (A) Gating strategy of cTfh cells. (B) Frequencies of activated ($ICOS^+$ $PD\text{-}1^+$) cTfh within the cTfh cell pool. (C) Plasma concentrations of CXCL13. A – acute; C5 – 5 months; M – moderate COVID-19; S – severe COVID-19; HC – healthy controls. Median and IQR are plotted in graphs B and C.

Next, we assessed the ASC expansion in moderate and severe COVID-19 patients and confirmed our findings in **Paper IV**. A substantial ASC expansion, dominated by the IgG^+ ASCs at the acute phase, was detected by flow cytometry in both patient groups compared to healthy controls (moderate COVID-19 - median 6.5% ASC of B cells; severe COVID-19 - median 9.6% of B cells; and healthy controls - median 0.76% of B cells) in **Paper V** (Figure 22). This expansion was no longer present in convalescence at 5 months after the symptom onset. Importantly, no significant difference between moderately and severely sick COVID-19 patients was observed regarding the germinal center activity or the ASC response.

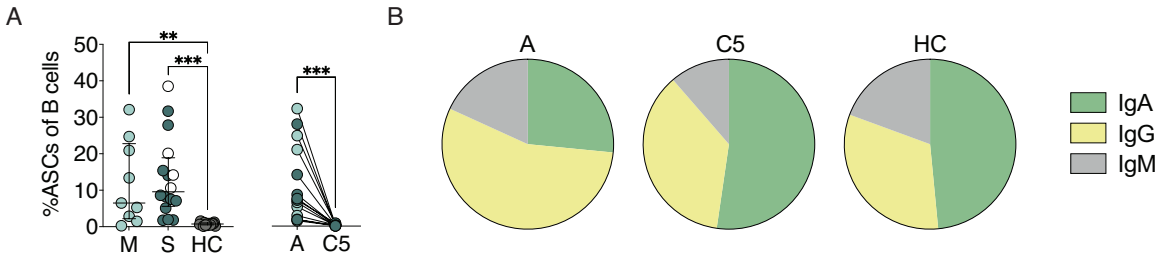

Figure 22. ASC expansion in COVID-19 patients in **Paper V** [150]. (A) ASC frequencies within all B cells in COVID-19 patients and HCs. (B) Immunoglobulin expression by ASCs. A – acute; C5 – 5 months; M – moderate COVID-19; S – severe COVID-19; HC – healthy controls. Median and IQR are plotted in graph A.

Although no significant differences between the patient groups were found regarding cTfh cell activation, CXCL13 levels, or ASC frequencies, we noted significantly higher levels of SARS-CoV-2-specific and neutralizing antibodies in severely sick patients compared to moderately sick patients, an observation previously reported by other independent studies (Figure 23) [185-187]. It is possible that the incubation period varies between the two cohorts, but as we rely on self-reported date for symptom onset when assessing the kinetics of antibody response, the timeline may not be completely accurate. Importantly, the difference between the two patient cohorts in terms of antibody titers was only observed during the acute phase, while at 5 and 9 months this difference was no longer apparent. This suggests, that regardless of disease severity at hospitalization, comparable antibody levels persist through to convalescence.

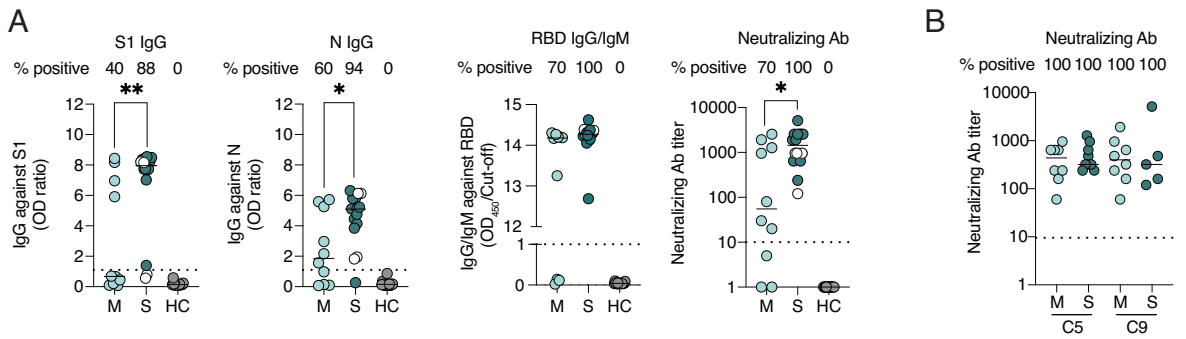

Figure 23. SARS-CoV-2-specific antibody levels in COVID-19 patients and HCs in **Paper V** [150]. (A) SARS-CoV-2 antibody levels during the acute phase of COVID-19 and in HCs. (B) SARS-CoV-2-neutralizing antibody titers in COVID-19 patients at convalescence. C5 – 5 months; C9 – 9 months; M – moderate COVID-19; S – severe COVID-19; HC – healthy controls. Median and IQR are plotted, horizontal dotted lines indicate the positivity threshold.

4.3.2 Immunological memory persistence after COVID-19

One of the most important questions during the COVID-19 pandemic was whether immunological memory is formed in recovered individuals. The presence of SARS-CoV-2 antibodies, as well as memory B cells and T cells in recovered patients were thought to provide protection upon re-exposure to SARS-CoV-2. It was therefore a highly relevant research question not only to understand the disease, but also to guide vaccination strategies for recovered individuals. Using the follow up samples from the two patient cohorts in **Paper V** we aimed to assess if memory B cells and T cells specific for SARS-CoV-2 could be detected at 5 and 9 months after symptom onset.

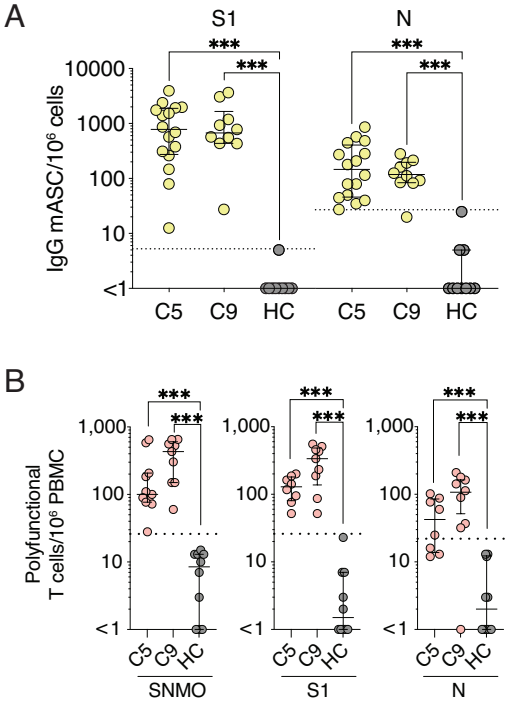

Figure 24. SARS-CoV-2 memory B cell and T cell responses in COVID-19 patients and HCs assessed by FluoroSpot assays in **Paper V** [150]. (A) Numbers of memory B cell-derived ASCs specific for S1 or N proteins of SARS-CoV-2. (B) Numbers of polyfunctional T cells (secreting at least two cytokines at once) responding to SNMO, S1 and N peptide pools. C5 – 5 months; C9 – 9 months; HC – healthy controls. Median and IQR are plotted, horizontal dotted lines indicate the positivity threshold.

To do this, we utilized memory B cell and memory T cell FluoroSpot assays. To detect memory B cells, PBMCs from 5 and 9 months were stimulated with a polyclonal stimulus for 5 days to stimulate memory B cell differentiation into ASCs. The PBMCs were plated on FluoroSpot plates coated with either spike subunit 1 (S1) or nucleocapsid (N) protein of SARS-CoV-2. Memory B cell-derived ASC (mASCs) secreting antibodies specific for S1 and N proteins were detected in all but one COVID-19 patient at both 5 and 9 months after symptom onset, but at highly heterogenous magnitude

(Figure 24A). Higher numbers of mASCs were specific for S1 compared to N, and almost no background responses were detected in pre-pandemic controls.

In addition to memory B cells, polyfunctional memory T cell responses to at least one SARS-CoV-2 peptide pool (SNMO, S1 or N) could also be detected in all COVID-19 patients (Figure 24B). Lower numbers of memory T cells were responding to N compared to S1, a finding similar to the memory B cell responses. We also found that triple cytokine producing cells (highly polyfunctional T cells) also secrete more of each cytokine compared to double or single cytokine-producing memory T cells, further contributing to the concept of polyfunctional T cells being superior in establishing an antiviral state (Figure 25) [74-76].

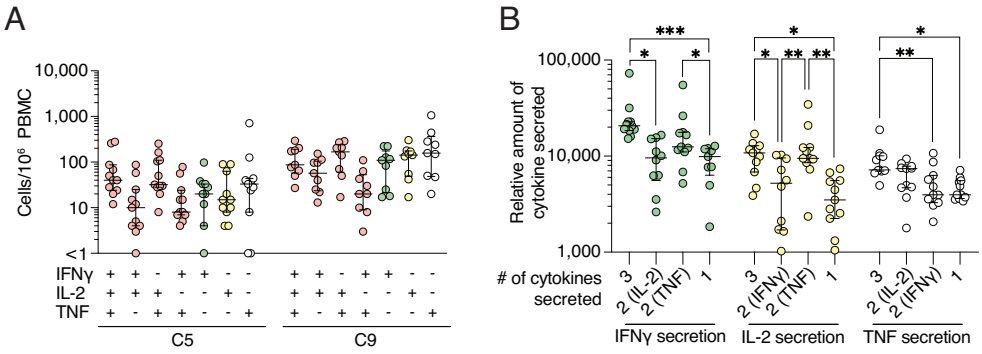

Figure 25. Functional profile and relative amounts of cytokine secretion by memory T cells responding to SNMO SARS-CoV-2 peptide pool stimulation assessed by FluoroSpot in **Paper V** [150]. (A) Cytokine co-expression patterns of responding memory T cells. (B) Relative amounts of cytokines secreted in relation to the functional profile of responding memory T cells. C5 – 5 months; C9 – 9 months. Median and IQR are plotted.

We found no differences in the memory B cell or memory T cell magnitude of response between moderate and severe COVID-19 patients (Figure 26). Our results suggest that regardless of disease severity at hospitalization, immunological memory can persist in COVID-19 patients for at least 9 months after symptom onset.

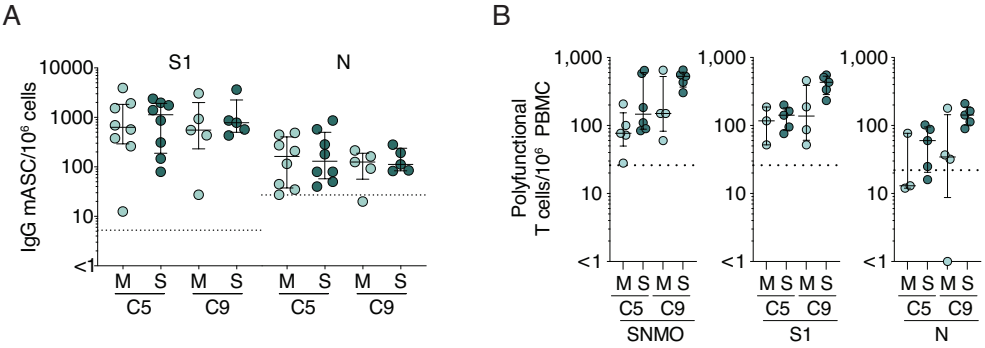

Figure 26. SARS-CoV-2 memory B cell and T cell responses in COVID-19 patients at convalescence assessed by the FluoroSpot assays in **Paper V** [150]. (A) Numbers of mASCs specific for S1 or N proteins of SARS-CoV-2. (B) Numbers of polyfunctional T cells (secreting at least two cytokines at once) responding to SNMO, S1 and N peptide pools. C5 – 5 months; C9 – 9 months; M – moderate COVID-19; S – severe COVID-19. Median and IQR are plotted, horizontal dotted lines indicate the positivity threshold.

Even if antibody titers decrease after the acute SARS-CoV-2 infection, a robust persistence of antibodies, as well as cellular immunity, have been shown by numerous studies [196-203]. Long-lived plasma cells have also been shown to be generated and to reside in the bone marrow of recovered COVID-19 patients, likely providing a long-term production of SARS-CoV-2-specific antibodies [204]. Infection-induced immunity can also be efficiently boosted by COVID-19 vaccination [205-207]. However, several genetic variants of SARS-CoV-2 have emerged during the pandemic with escape mutations in the spike protein, that raise concerns over the protective immunity against these variants in vaccinees or recovered COVID-19 patients. At the time of writing this thesis, the Omicron variant (B.1.1.529) is spreading around the world at an unprecedented speed, but its transmissibility, and the severity of disease it causes are still not fully understood [208]. The future of COVID-19 pandemic is uncertain and further research is needed to understand the protective immunity against SARS-CoV-2, particularly in relation to vaccines and their protectiveness against the SARS-CoV-2 variants of concern.

5 CONCLUSIONS

Understanding the immune responses to infectious agents can aid the development of therapeutics and vaccines and is a highly relevant topic in the midst of the ongoing COVID-19 pandemic. The aim of this thesis was to describe the adaptive immune responses to emerging human viral pathogens such as TBEV and SARS-CoV-2.

These are the key findings from the studies presented in this thesis:

- Mortality in TBE-diagnosed individuals is around four times higher than in the general population in Sweden, particularly in people over the age of 60, highlighting the need for increased immunization efforts against TBE (**Paper I**).

- Primary TBE immunization induces a heterogenous magnitude of $CD4^+$ T cell response dominated by IL-2, TNF and CD154 expression, and a lower IFN-γ response compared to TBEV infection (**Paper II**).

- TBE patients have detectable TBEV-specific IgM and IgG antibodies already at hospitalization and have low frequencies of circulating antibody-secreting cells (ASCs) during the second phase of disease (**Paper III**).

- Germinal centers are activated, and the ASC population is expanded in COVID-19 patients during the acute phase of disease (**Paper IV and V**).

- SARS-CoV-2-neutralizing antibodies, memory B cells and memory T cells persist in hospitalized patients up to at least 9 months after the symptom onset irrespective of disease severity at hospitalization (**Paper V**).

6 FUTURE PERSPECTIVES

Although the research summarized in this thesis answered many questions regarding the adaptive human immune responses to TBEV and SARS-CoV-2 infections, several further questions have been raised.

Paper I

- What is the true incidence of TBEV infection in Sweden and how many individuals are asymptomatic after the exposure to the virus?
- What are the behavioral and socioeconomic features of TBE patients in Sweden and are the individuals exposed to TBEV healthier than the general population?

Paper II

- How can the current TBE vaccine be improved to induce a robust $CD4^+$ and $CD8^+$ T cell memory in TBE-vaccinated individuals?
- Could a novel vaccine technology, for example mRNA-based vaccine, induce stronger and longer-lasting immunity towards TBE compared to the currently licensed inactivated TBE vaccines?

Paper III

- Is a robust B cell memory generated after TBEV infection and which TBEV proteins are targeted the most? Are the proteins targeted by B cells different after infection compared to after vaccination? Which TBEV proteins should be included in the future vaccine formulation?
- Are the B cell responses induced by TBEV infection superior to the responses induced by TBE vaccination?
- What are the characteristics of the B cell responses during the first phase of TBE?

Paper IV and V

- What is the nature of the anamnestic B cell response upon the re-exposure to SARS-CoV-2 in previously infected or vaccinated individuals?
- What specific antibody levels and memory B cells and T cell responses are required for protection from SARS-CoV-2 reinfection in previously infected or vaccinated individuals?

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