Vaccine responses after chemotherapy or stem cell transplantation in patients with hematological malignancies

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To my patients

Den mätta dagen den är aldrig störst.

Den bästa dagen är en dag av törst.

Visst finns det mål och mening i vår färd –

men det är vägen, som är mödan värd.

Karin Boye

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ABSTRACT

The prevention of infections in patients with hematological malignancies is important given the inherent immune deficiency associated with these diseases and the immunosuppressive effects of treatment. This thesis investigated the efficacy of vaccines against various pathogens among patients with hematological disease receiving chemotherapy or stem cell transplantation, including the longevity of vaccine responses. Paper I examines serum antibody levels against tetanus, diphtheria, and polio after standard chemotherapy in 104 patients treated for lymphoma or acute leukemia at median 18 months after completing treatment. Antibody levels were analyzed by enzyme-linked immunosorbent assay or neutralization tests and were compared with levels in age- and sex-matched healthy controls (n=47) and pretreatment levels (n=73). For tetanus, the number of seronegative patients increased during treatment (24 vs. 12 %) and antibody levels were reduced. A similar trend was observed for antibody levels against diphtheria. Immunity against poliovirus of serotypes 1 and 3 was preserved. Paper II describes a clinical vaccine trial that assessed the humoral response to four doses of vaccine against tick-borne encephalitis (TBE). A TBE vaccine (FSME-IMMUN®) was given starting nine months post-transplant to autologous (n=53) and allogeneic (n=51) stem cell transplant recipients. Serum samples were obtained prior to each vaccine dose (at nine, 10, 12, and 21 months) and three months after the last dose. Seventy-seven percent of patients after allogeneic stem cell transplantation (allo-HCT) and 80% after autologous stem cell transplantation (auto-HCT) achieved seropositivity following the last vaccine dose, compared with 100% among healthy controls. Graft-versus-host disease and ongoing immunosuppression were associated with poor vaccine responses. Paper III examines SARS-CoV-2 serum antibodies and T cell responses *ex vivo* before and after the third dose of an mRNA vaccine among 40 allo-HCT recipients. Many patients responded well, with antibodies above the upper detection limit. However, 16% were

seronegative following vaccination and 49% of patients showed no T-cell reactivity against SARS-CoV-2 peptides. Paper IV analyzes serum antibody levels against tetanus and diphtheria among 143 long-term survivors after allo-HCT who had been vaccinated according to standard protocol with three doses of diptheria and tetanus vaccine. Diphtheria immunity was poor and 40% of patients were seronegative. However, all patients had detectable antibodies against tetanus. To conclude, diphtheria immunity is poor in adult patients receiving chemotherapy as well as in long-term survivors after allo-HCT, and boosters may be considered. Vaccination against TBE is immunogenic when starting nine months after allo- or auto-HCT. The third dose of mRNA vaccine against COVID-19 elicits antibody responses in a majority of allo-transplanted patients. However, T cell responses remain poor in a significant proportion of these patients.

Keywords: vaccination, chemotherapy, stem cell transplantation, antibodies, tetanus, diphtheria, polio, TBE, SARS-CoV-2.

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SAMMANFATTNING PÅ SVENSKA

Hematologiska sjukdomar ger ofta upphov till långvarig nedsättning av immunförsvaret. Att förebygga och behandla infektioner är därför en viktig del av det hematologiska omhändertagandet. Vissa infektioner kan förebyggas eller lindras av vaccination. Syftet med avhandlingen har varit att bidra till ökad kunskap om vaccinationsbehov och vaccinsvar bland patienter med hematologisk sjukdom.

Barn med akut lymfatisk leukemi (ALL) som fått cellgiftsbehandling eller strålbehandling behöver revaccineras mot stelkramp och difteri efter avslutad behandling. Syftet med delarbete I var att undersöka om detta även gäller vuxna patienter. Vi mätte antikroppar mot stelkramp, difteri och polio bland 104 patienter som avslutat behandling för lymfom och leukemi och jämförde med antikroppsnivåer hos ålders- och könsmatchade friska kontroller och nivåer hos patienterna före behandlingsstart. En andel av patienterna saknade skydd mot stelkramp (24%) och difteri (21%). Antikroppsnivåerna var lägre än vid behandlingsstart. Skyddet mot polio var däremot tillräckligt även efter behandling.

Fästingburen encefalit (tick-borne encephalitis, TBE) är ett ökande problem i Sverige och kan orsaka livshotande sjukdom, inte minst hos immundefekta. Den viktigaste förebyggande åtgärden är vaccination. Vi undersökte i delarbete II antikroppssvar bland 104 patienter som vaccinerats med fyra doser TBE-vaccin (FSME-IMMUN®) med start nio månader efter autolog eller allogen stamcellstransplantation. Vi fann att nästan 80% av vaccinerade patienter svarade med antikroppar på vaccinet, jämfört med 100% av friska kontroller, som dock bara fick tre doser vaccin.

I delarbete III studerades specifika antikropps- och T-cellssvar före och efter den tredje dosen mRNA-vaccin mot SARS-CoV-2 till patienter som stamcellstransplanterats för mindre än tre år sedan och/eller hade pågående immundämpande behandling för graft-versus-host disease (GvHD). Vi fann att de flesta patienter (84%) svarade med bildning av antikroppar och att vissa uppnådde mycket höga nivåer. Hälften av patienterna hade dock inget mätbart T-cellssvar mot peptider från SARS-CoV-2.

Patienter som genomgår stamcellstransplantation behöver revaccineras mot stelkramp och difteri då tidigare förvärvad immunitet förloras. Patienterna får tre doser vaccin med start sex månader efter transplantation, men det har inte tidigare gjorts studier av vaccinationens effektivitet över tid. I delarbete IV

undersökte vi antikroppsnivåer mot stelkramp och difteri bland 143 patienter som genomgått allogen stamcellstransplantation i genomsnitt 14 år tidigare. Vi fann att skyddet mot difteri var svagt – 40% saknade antikroppsskydd trots vaccination. Däremot var skyddet mot stelkramp bättre - ingen patient saknade helt skydd mot denna infektion.

Sammanfattningsvis har studierna bidragit till ökad kunskap om immunitet, vaccinationsbehov och vaccinationssvar bland patienter med hematologisk sjukdom. De huvudsakliga slutsatserna är att (i) immuniteten mot difteri är svag både efter cellgiftsbehandling och bland långtidsöverlevare efter allogen stamcellstransplantation, och att vaccination kan övervägas, (ii) vid TBE-vaccination efter allo- eller autolog stamcellstransplantation är det viktigt att ge fyra doser vaccin som grundimmunisering, oberoende av ålder, (iii) den tredje dosen mRNA-vaccin mot COVID-19 resulterar i antikroppssvar hos de flesta patienter efter allogen stamcellstransplantation medan T-cellssvaret är betydligt sämre.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Einarsdottir S**, Ljungman P, Kaijser B, Nicklasson M, Horal P, Norder H, Bergström T, Brune M. "Humoral immunity to tetanus, diphtheria and polio in adults after treatment for hematological malignancies". *Vaccine.* 2020; 38(5): 1084-1088.

II. **Einarsdottir S**, Nicklasson M, Veje M, Bergström T, Studahl M, Lisak M, Olsson M, Johansson B, Andreasson B, Piauger B, Roth A, Friman V, Ljungman P, Brune M. "Vaccination against tick-borne encephalitis (TBE) after autologous and allogeneic stem cell transplantation". *Vaccine.* 2021; 39(7): 1035-1038.

III. **Einarsdottir S**, Martner A, Nicklasson M, Wiktorin H, Arabpour M, Törnell A, Vaht K, Waldenström J, Ringlander J, Bergström T, Brune M, Hellstrand K, Ljungman P, Lagging M. "Reduced immunogenicity of a third COVID-19 vaccination among recipients of allogeneic hematopoietic stem cell transplantation". *Haematologica.* 2022; 107(6):1479-1482.

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Other papers published, accepted, or submitted during the thesis work:

I. Ambati A, **Einarsdottir S**, Magalhaes I, Poiret T, Bodenstein R, LeBlanc K, Brune M, Maurer M, Ljungman P. "Immunogenicity of virosomal adjuvanted trivalent influenza vaccination in allogeneic stem cell transplant recipients*". Transpl Infect Dis.* 2015; 17(3):371-379.

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VI. **Einarsdottir S**, Martner A, Waldenstrom J, Nicklasson M, Ringlander J, Arabpour M, Törnell A, Wiktorin HG, Nilsson S, Bittar R, Nilsson M, Lisak M, Veje M, Friman V, Al-Dury S, Bergström T, Ljungman P, Brune M, Hellstrand K, Lagging M. "Deficiency of SARS-CoV-2 T-cell responses after vaccination in long-term allo-HSCT survivors translated into abated humoral immunity*". Blood Adv.* 2022;

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X. Al-Dury S, Waldenstrom J, **Einarsdottir S**, Saer H, Waern J, Martner A, Hellstrand K, Lagging M. “Hybrid immunity augments antibody response after third dose COVID-19 mRNA vaccine in at risk-populations”. Submitted 2022.

CONTENT

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BCG	Bacille Calmette-Guérin
BCR	B-cell receptor
BMT	Bone marrow transplantation
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
COVID-19	Coronavirus disease
DLI	Donor lymphocyte infusion
Hib	Haemophilus influenzae type B
HPV	Human papillomavirus
EBMT	European Society for Blood and Marrow Transplantation
ELISA	Enzyme-linked immunosorbent assay
FDC	Follicular dendritic cell
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HBV	Hepatitis B virus
HCT	Hematopoietic cell transplantation

HLA	Human leukocyte antigen
Ig	Immunoglobulin
IPV	Inactivated polio vaccine
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NGS	Next-generation-sequencing
NT	Neutralization test
NK	Natural killer
OD	Optical density
OPV	Oral polio vaccine
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SHM	Somatic hypermutation
TBE	Tick-borne encephalitis
TCR	T cell receptor
Tfh	T follicular helper cells
Tregs	Regulatory T cells
VAPP	Vaccine-associated paralytic polio
WHO	World Health Organization

1 INTRODUCTION

The development of vaccines for protection against infectious diseases is one of the most significant breakthroughs in the history of medicine. Edward Jenner noted that milkmaids did not contract smallpox if they had previously been infected by cowpox. Jenner spent his career performing scientific experiments and in 1796 showed that pre-inoculation with cowpox virus prevented smallpox upon challenge with the smallpox virus. This was the beginning of vaccinology. Jenner created the word "vaccination" from the Latin word for cowpox, *i.e.*, vaccinia. Modern era vaccinology has pursued this initial success by refining vaccines in cell culture, chemically, and by use of modern molecular methods.

Vaccination plays a key role in global preventive healthcare and is by far one of the best health investments that can be made. According to WHO, vaccination prevents two to three million deaths every year from diseases like influenza, tetanus, diphtheria, pertussis, and measles [1]. The mRNA vaccines licensed for COVID-19 in 2021 prevented approximately 500,000 deaths in their first year in use [2].

Preventing and treating infections in patients with hematological malignancies is essential given the humoral and cell-mediated immune deficiencies that depend on the underlying disease and the given therapies. Immune reconstitution following stem cell transplantation increases the risk of infection for years. Some of these infections are vaccine-preventable or the severity of the disease is substantially reduced by vaccines, thus avoiding hospitalization and death.

1.1 The immune system

The immune system is traditionally divided into innate and adaptive immunity, but these systems are closely interconnected [3].

Innate immunity, which is present in even the simplest animals, consists of barrier functions (physical and chemical) but mainly of cells such as neutrophils, monocytes, macrophages, dendritic cells, eosinophils, mast cells, and natural killer (NK) cells along with soluble factors such as the complement system, different cytokines, and acute phase proteins. The innate immune system acts as the first line of defense. The innate immune cells start inflammatory responses and act as a bridge to the adaptive responses.

The adaptive or acquired immune system consists of B and T lymphocytes. The adaptive response is more specific but slower and has a memory, allowing a vigorous response upon re-challenge with antigen.

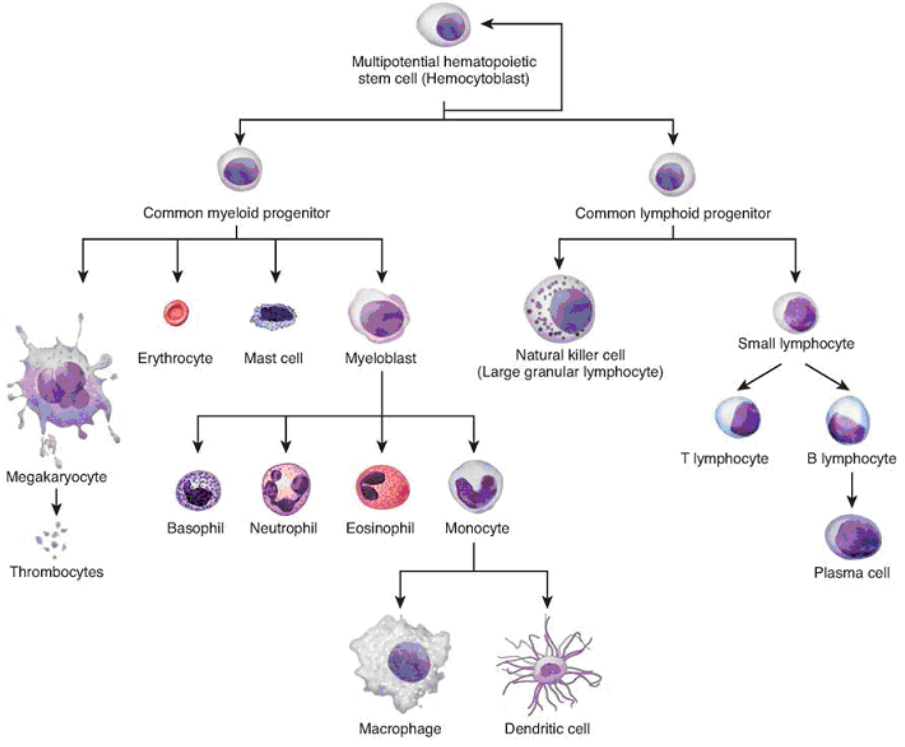


Figure 1. Hematopoiesis in adults. Reprinted with permission from Open Stax.

1.1.1 B cells and antibodies

B cells develop from progenitor cells in the bone marrow and remain in the bone marrow for their development and the rearrangement of the B cell receptor. The naïve B cells, *i.e.*, cells that have not yet encountered their specific antigen, are released into the circulation and populate the lymphoid organs, the lymph nodes, and spleen. Each naïve B cell released into the circulation has antigen-specific IgM expressed on its surface.

B cells produce antibodies. Antibodies typically do not kill pathogens on their own but act as enhancers of immunity. They neutralize toxins, prevent microorganisms adhering to mucosa, activate complement, opsonize bacteria for phagocytosis, and sensitize virus-infected cells for cytotoxic attack by killer cells [3].

Antibody diversity is fascinating and the number of different antibody specificities, B cell receptors, that the human body is capable of producing is almost infinite. There are four gene segments involved: variable (V), diversity (D), joining (J), and constant (C). There is a multiplicity of these genes within the genome of the developing B cell (V 25-100, D >20, J >50 genes) but only one of each is needed in a process named V(J)D recombination. During the recombination, for further diversity, splicing is “inaccurate”, leading to frameshift in base pairs, *i.e.*, “junctional diversity”. Additionally, there is an enzyme, deoxynucleotidyl transferase that inserts nucleotides to further alter the DNA sequences.

The basic structure of immunoglobulins comprises two identical heavy chains and two identical light chains. The chains have both variable and constant domains. There are five different variants of the heavy chain constant region, which is the basis for the five Ig-classes: IgG, IgA, IgM, IgD and IgE. The variable region, Fab, binds the antigen and the constant region, Fc, which is the trunk of the molecule or tail, binds to the Fc-receptor on the effector cell (Figure 2). [4].

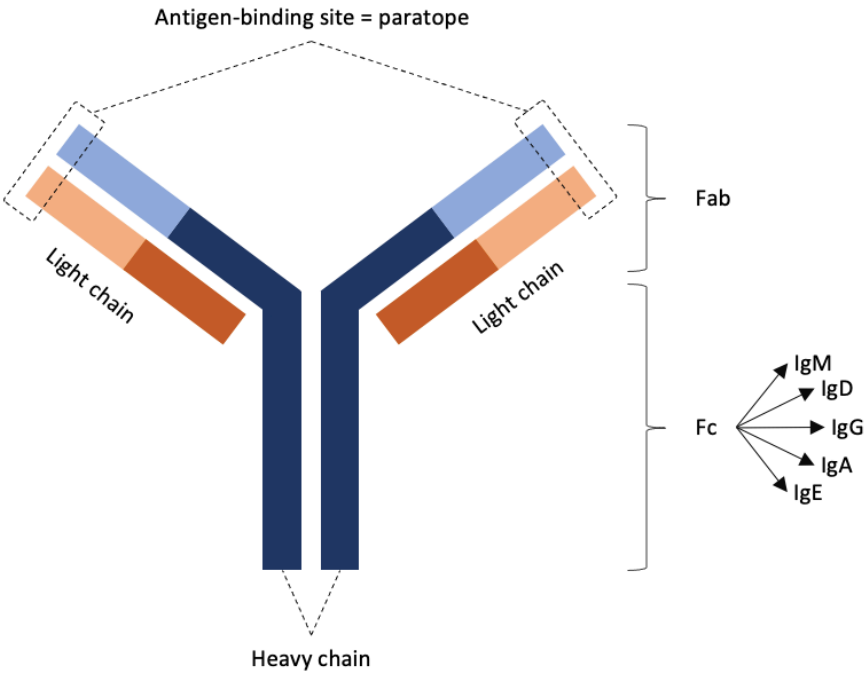


Figure 2. Immunoglobulins, basic structure

The isotypes of immunoglobulins have different functions in immunity. IgM is expressed as a monomer on the surface of the developing B cell. When IgM is secreted upon antigen stimulation, it is pentameric or hexameric (Figure 3). IgM is produced early in the B cell response and has broad antigen reactivity. The pentameric structure makes it effective in binding antigens with a

repetitive structure, *i.e.*, bacterial capsules or viral capsids. IgM is also the most efficient complement activator among immunoglobulins.

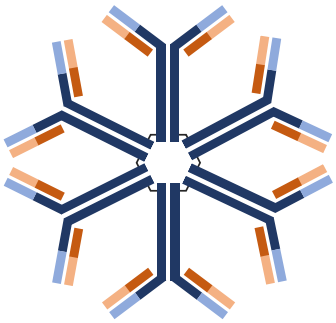


Figure 3. A secreted hexameric IgM.

IgD is also expressed on naïve B cells, and small amounts of IgD are detected in serum. IgD is known to activate basophils, but its role remains largely undefined. IgG, IgE, and IgA are produced after class-switching during the germinal center reaction and provide different functions in response to antigen.

IgA exists in a monomeric form in serum and as a secretory dimeric IgA on mucosal surfaces. IgA is considered the most important isotype in the respiratory tract, urogenital tract, and on the intestinal mucosa, and inhibits the binding of bacteria, toxins, and viruses to target cells. IgA is also found in saliva, tears, and breast milk.

IgE binds to Fc-receptors on mast cells and causes allergic and anaphylactic reactions. IgE participates in the defense against parasites and helminths.

IgG is the dominating isotype in serum. There are four subclasses, IgG 1-4. The subclasses share similarities, but each one has a distinct effector function, half-life, and capacity for complement activation. IgG is produced in large amounts during secondary immune responses. All four IgG subclasses can cross the placenta. IgG1 accounts for the majority of total IgG (60-70%) and a deficit often results in overall hypogammaglobulinemia. Adult IgG1 levels are reached at five years of age whereas IgG2, IgG3, and IgG4 remain lower until adolescence. Half-lives of IgG range from six to 21 days for IgG3 and are approximately 21-23 days for IgG1, 2 and 4 [5, 6]. Antibody responses to bacterial polysaccharide antigens mainly comprise IgG2. The antibody responses to protein and viral antigens are predominantly of the IgG1 and IgG3 subclasses.

1.1.2 The germinal center reaction

A naïve B cell expresses IgM or IgD on the cell surface as its B cell receptor. When the B cell encounters its specific antigenic epitope, the clone of B cells with the right antigen specific B cell receptor can be activated. T cell-independent antigens exist, mainly repetitive polysaccharide antigens, but most antigens are T cell-dependent, *i.e.*, the B cell requires a T helper cell that recognizes the cognate antigen presented on the B cell MHC II for activation. When B cells start to proliferate, the follicle can become a germinal center, which are small anatomical structures of the follicles that are formed during an immune response. They represent a collaboration between antigen-specific B cells, T follicular helper cells (Tfh), and specialized follicular dendritic cells (FDC). The germinal center can be viewed as a small immunological factory for mass-production of B cell clones that generate antibodies.

B cells proliferate during the germinal center reaction. In the dark zone of the germinal center, the cells undergo somatic hypermutation (SHM), a process where mutations occur in the variable genes creating diversified B cell receptors [3]. With newly formed BCR, the B cells exit to the light zone, where the B cells again are exposed to antigen expressed by FDCs, and the antibodies with the highest affinity for antigen are selected for proliferation. Further, the Tfh cells secrete cytokines that stimulate proliferation and the return of the B cells to the dark zones for further SHM. The B cell undergo several rounds of migration between these zones with the goal of producing high affinity antibodies [7] (Figure 4).

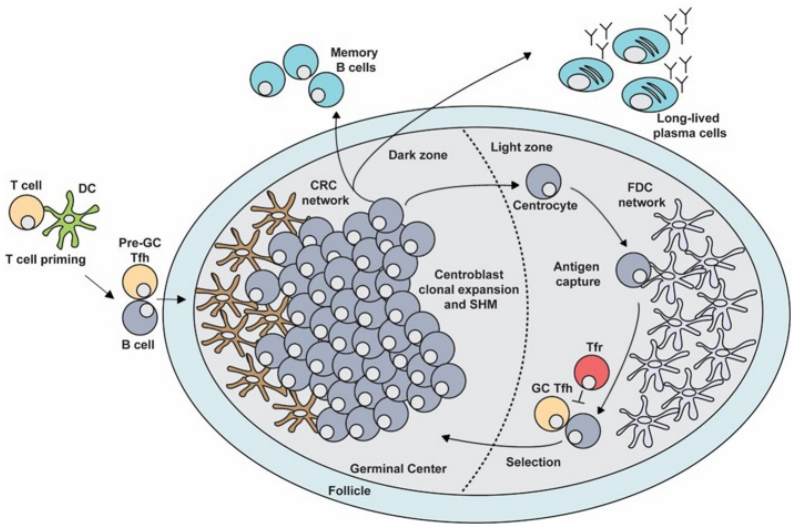


Figure 4. Regulation of the germinal center reaction. Stebegg et al*. Front of Immunology 2018 [8].*

A subset of the high-affinity B cell class switches and forms other antibody isotypes. In this process, B cells rearrange the genes coding for the constant part of the heavy chain, thereby evolving from IgM to IgA, IgE, or IgG. Some of the cells mature into effector B cells that secrete antibodies, in their most mature form called plasma cells, while some of the cells develop into memory B cells. Long-lived plasma cells reside in the bone marrow but are also found in the human intestine [9]. The memory B cells patrol the secondary lymphoid organs for their specific antigen. Most memory B cells are isotype-switched and produce mainly IgG. However, IgM memory B cells also exist.

The IgG response is longer lasting than the IgM response. IgG mostly reaches its peak within 4-12 weeks after infection but may persist for months or years and sometimes decades (see below).

The role played by memory B cells and long-lived plasma cells in immunological memory remains poorly defined [10]. Persistence of antigen in, for example, lymph nodes, has been suggested as an additional mechanism in creating immunological memory [11]. Some viruses, especially herpesviruses that persist in tissues, can induce life-long IgG production. Thus, it is likely that it is the antigen persistence that stimulates this long-term activity. Measles IgG and smallpox IgG have been shown to persist for life [10].

Generally, naturally acquired immunity confers stronger long-term protection against re-infection and symptomatic disease than vaccine-induced immunity [10]. However, immunity obtained through vaccination with a live attenuated vaccine against, for example, measles, mumps, and rubella, has been shown in some studies to be similar to that achieved after natural infection [10].

1.1.3 T cells

T cells are produced in the bone marrow and migrate to the thymus where the T cell receptor rearrangement occurs and the cells mature. The T cell receptor consists of two polypeptide chains in a majority of T cells, one alpha and one beta chain. The T cells able to bind to MHC receive survival signals in a process called positive selection. T cells that react to self-antigen are sorted out in a process known as negative selection.

The mature T lymphocytes that exit the thymus are either CD4+ or CD8+ and recognize foreign antigen presented on self-major histocompatibility complex (MHC). All nucleated cells in the body express MHC class I while MHC class II expression is largely restricted to antigen-presenting cells such as dendritic cells, monocytes, macrophages and B cells.

The TCR of T helper cells (CD4+) recognizes antigen presented on MHC class II while the TCR of cytotoxic (CD8+) T cells recognizes antigen presented on MHC class I. Upon TCR-specific antigen stimulation by an antigen-presenting cell, the naïve CD4+ T cells differentiate into subsets of T helper cells (Th1, Th2, Th17), regulatory T cells (Tregs) or Tfh cells (Figure 5), depending on co-stimulation and presence of cytokines in the environment. These effector subsets release different cytokines upon re-stimulation that have different effector functions and may activate a wide range of neighboring cells such as macrophages, B cells, and CD8+ cells [3, 12].

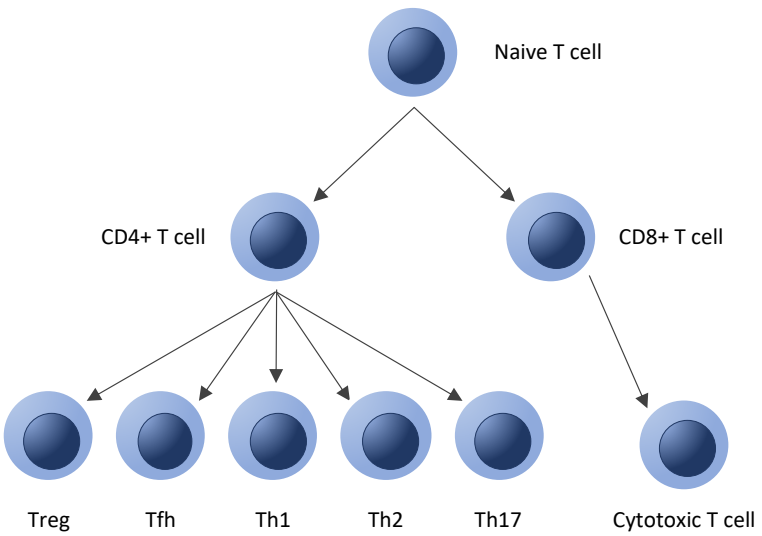


Figure 5. T-cell differentiation.

T follicular helper cells are essential in the germinal center reaction, by interaction with cognate B cells via CD40L and through expression of cytokines such as CXCL13 and IL-21. Also Th2 cells are important for activation of B cells, and Th2-derived cytokines such as IL-3 and IL-13 assist in isotype-switching of B cells, directing the response from IgM to other antibody subclasses [13]. As the switch from IgM to other subclasses occurs, some B cells develop into memory B cells [14].

In contrast to the CD4+ T cells, the CD8+ T cells carry T cell receptors that recognize specific antigens presented on MHC I. While all nucleated cells express MHC I, only antigen-presenting cells provide enough activating stimuli to trigger the activation of naïve CD8+ T cells. Upon antigen-specific activation by an antigen-presenting cell, the naïve CD8+ T cells differentiate into effector cytotoxic T cells that may lyse cells presenting their specific antigen on MHC I. Lysis comprises the induced release of perforin and granzyme B or other pathways inducing receptor-mediated apoptosis [3]. The activation of the naïve CD8+ cell is more efficient with simultaneous activation from an activated T helper cell. CD8+ T cell are essential in the defense against viral infections. Apart from directly killing virus-infected cells, CD8+ T cells also produce cytokines, including IFN-γ that modulate virus replication. A weak CD8+ T cell response favors virus persistence [15].

1.2 Immune reconstitution following stem cell transplantation

Reconstitution of a donor-derived immune system is essential in preventing infectious complications, in modulating graft-versus-host disease (GvHD), and in relapse control following allo-HCT. The intensity of the conditioning regimen varies depending on disease and patient factors but most protocols, in combination with the immunological effects of the donor cells post-transplant, destroy the recipient's immune system almost completely [16].

Many factors contribute to a successful immune reconstitution such as graft source, conditioning, HLA mismatch, *in vivo* or *in vitro* T cell depletion, acute and chronic GvHD and its treatment, and CMV seropositivity [17]. Disease status at transplantation, age, and comorbidities are additional factors of relevance to reconstitution.

Innate immunity, *i.e.*, granulocytes, monocytes, and NK cells, recovers within 1-2 months after allo-transplantation. However, functions of innate immunity, such as phagocytosis and chemotaxis, can be impaired for longer periods of time, especially if the patient develops GvHD. Antigen-presenting cells (APCs) are often chemo-resistant and may remain of recipient origin early after transplantation, but are gradually replaced by donor APCs.

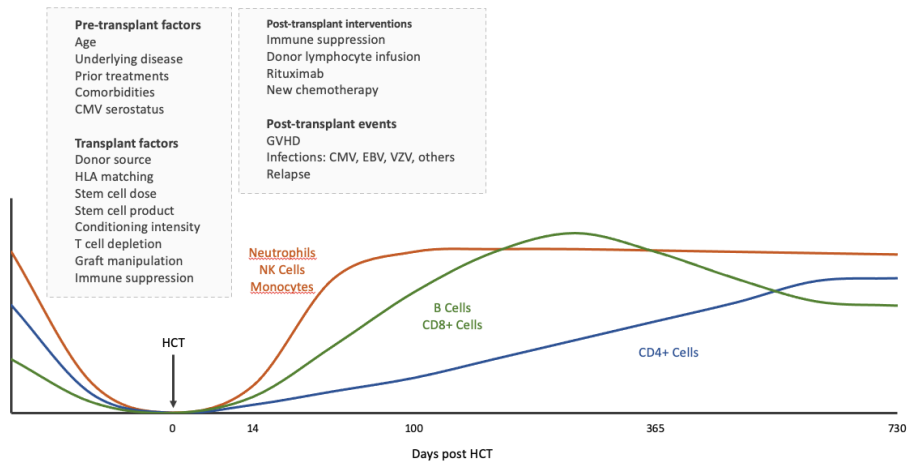


Figure 6. Overview of immune reconstitution following allogeneic stem cell transplantation. Adapted from Stern et al*, Front. Immunol. 2018 [18].*

In contrast, B and T cell immunity (adaptive immunity) recovers more slowly. The T cell compartment is repopulated in two distinct pathways; one is similar to the process in ontogeny when naïve donor T cells undergo thymic development. The other pathway comprises the expansion of mature donor T cells in the "empty" T cell compartment, which is thymus-independent [16]. This latter pathway is likely to be dominant in adult patients, in whom thymic function is low [17]. These expanded T cells, often CD8+, have insufficient function as their antigen specificities are limited and because these cells often have lost their homing receptors to interact with APCs in lymphoid organs. Thymus function is influenced by several factors, mainly age and the occurrence of GvHD [19]. The CD4+ cells reconstitute later and are more dependent on the thymic pathway, which may explain the inversed CD4/CD8-ratio seen following transplant [17]. A subset of CD4+ cells, Tregs, have been shown in experimental, pre-clinical and, recently, clinical models to ameliorate GvHD while preserving the GvL effect [17].

B cell numbers are low the first months after transplantation, reaching supranormal levels one to two years posttransplant, similar to the normal ontogeny in children [20]. Recovery of memory B cells is slower, leading to prolonged deficiency of humoral antibody responses, especially in patients with GvHD. The delayed recovery of CD4+ cells further hampers development of memory B cells. B cell recovery following allo-transplantation is diminished due to low numbers of circulating B cells, by the lack of CD4+ T cell help for isotype switching and memory B cell development, and by diminished somatic hypermutation [17, 20].

Normal levels of serum IgM are mostly detected three to five months following allo-HCT, followed by IgG and later IgA, similar to the development in the early years of life [21]. However, some patients develop secondary long-term antibody-class deficiencies [22]. Notably, IgG levels may derive from the recipient's plasma cells as plasma cells are relatively resistant to the current preparative regimens and may persist up to two years after transplantation [20]. Thus, most antibodies early posttransplant are of recipient origin [16]. However, occasional engraftment of memory cells of donor origin has been reported [23].

1.3 Vaccine immunology

Patients are vaccinated with the aim of preventing infection but mainly to prevent severe disease. Also, patients are, in some cases, vaccinated to not become a reservoir for the pathogen, as is the case with pertussis vaccination post-stem cell transplantation. In vaccinating the general population, the importance of inducing herd-immunity is vital, protecting the most vulnerable populations such as infants, the elderly, and immunosuppressed patients.

Many of the currently licensed vaccines are assumed to confer protection by antibody production [1]. However, the process of creating memory B cells requires T cell help. Some vaccines confer protection mainly by T cell immunity, such as BCG against tuberculosis or Shingrix®, licensed against shingles [24, 25]. For many vaccines, the protection arises from a combined induction of humoral and cell-mediated immunity [26]. For protection against severe disease, it is likely that T cells are of significant importance. In patients developing severe influenza, the T cell response to vaccination has been proposed to be more important than antibody levels [27]. In COVID-19, cell-mediated immunity is important in controlling disease severity [28]. To generalize, antibodies protect against infection whereas cellular responses control the infection once infection has been established [29]. Figure 7 is an overview of the immune response to a protein antigen.

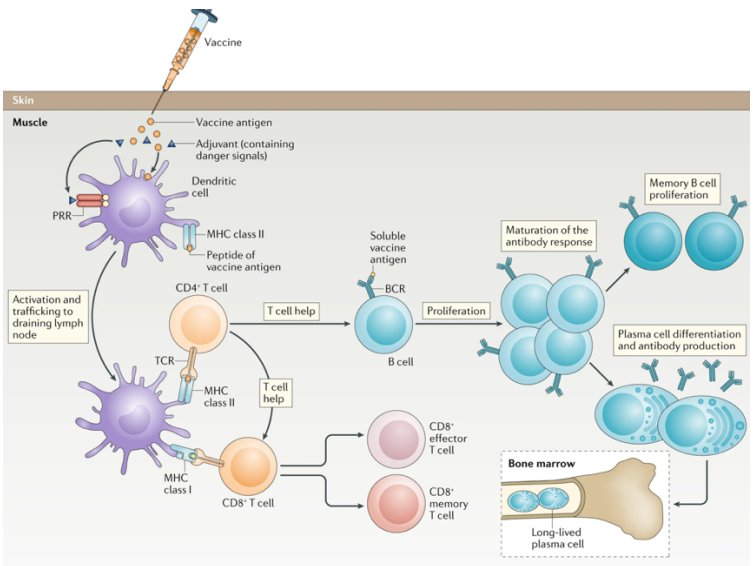


Figure 7. Overview of the vaccine response generated by vaccination. Pollard et al*, 2020. Nat. Rev. Immunol [1].*

A vaccine is typically injected into a muscle. Some vaccines are also given subcutaneously or intra-dermally; however, both these routes of administration may entail more pronounced local side effects [30]. Vaccines usually consist of an antigen and an adjuvant, where the latter is an ingredient that provides a stronger immune response to antigen. The antigen is either derived from the pathogen or synthetically produced to represent components of the antigen. Most vaccines contain one or more protein antigens [1].

Vaccines are divided into live attenuated vaccines or non-live, often referred to as inactivated. In live viral vaccines, attenuation is typically obtained by repeated passages of a viral strain in cell cultures, inducing amino acid mutations that confer non-virulence. The antigenic component in non-live vaccines may, for example, comprise killed whole virus (inactivated polio vaccine, IPV), recombinant proteins (hepatitis B vaccine, HBV), polysaccharides (some pneumococcal vaccines), or glycoproteins from the virus (shingles vaccine). Diphtheria and tetanus vaccines are toxoid vaccines, *i.e.*, formaldehyde-inactivated protein toxoids that have been purified from the pathogen.

In the past decades, new platforms have been developed, such as viral vectors and DNA and mRNA vaccines. Figure 8 shows an overview of currently available vaccines. When injecting a protein antigen intramuscularly, the antigen is taken up by an APC, typically a dendritic cell. The dendritic cells are activated by pattern recognition receptors (PRRs) aided by so-called danger signals in the adjuvant. The dendritic cell, carrying the antigen, travels to the draining lymph node where the protein antigen is presented to B and T cells on MHC molecules. The T cell recognizing the antigen through the specific T cell receptor (TCR) is activated alongside the B cell via its B cell receptor (BCR), are activated. The T cells drive the B cell development in the germinal center of the lymph node. This induces antibodies with high affinity to the particular antigen and the induction of different isotypes. Some of the cells develop into short-lived plasma cells that produce an increase in antibody levels for a few weeks. Long-lived plasma cells migrate to niches of the bone-marrow and secrete antibodies into the circulation for as long as decades [1]. A subpopulation of cells develops into memory B cells. The memory B cells can mature into antibody-producing plasma cells upon reencounter with an antigen; this process is much faster than that of a naïve B cell [31].

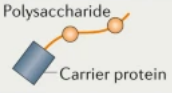
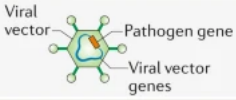
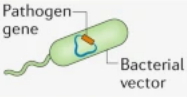
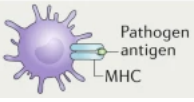
Type of vaccine		Licensed vaccines using this technology	First introduced
Live attenuated (weakened or inactivated)		Measles, mumps, rubella, yellow fever, influenza, oral polio, typhoid, Japanese encephalitis, rotavirus, BCG, varicella zoster	1798 (smallpox)
Killed whole organism		Whole-cell pertussis, polio, influenza, Japanese encephalitis, hepatitis A, rabies	1896 (typhoid)
Toxoid		Diphtheria, tetanus	1923 (diphtheria)
Subunit (purified protein, recombinant protein, polysaccharide, peptide)		Pertussis, influenza, hepatitis B, meningococcal, pneumococcal, typhoid, hepatitis A	1970 (anthrax)
Virus-like particle		Human papillomavirus	1986 (hepatitis B)
Outer membrane vesicle		Group B meningococcal	1987 (group B meningococcal)
Protein–polysaccharide conjugate		*Haemophilus influenzae* type B, pneumococcal, meningococcal, typhoid	1987 (*H. influenzae* type b)
Viral vectored		Ebola	2019 (Ebola)
Nucleic acid vaccine		SARS-CoV-2	2020 (SARS-CoV-2)
Bacterial vectored		Experimental	–
Antigen-presenting cell		Experimental	–

Figure 8. Overview of available vaccines. Pollard et al. *2020. Nature Rev Immunol [1].*

1.4 Donor vaccination

If lymphocytes transferred with the graft are antigen-specific, it is conceivable to vaccinate the donor pre-transplant, perhaps combined with vaccinating the recipient pre-transplant.

Vaccination against tetanus, given to the donor on day -20 in combination with vaccinating the recipient on day -1, and +50 and +365, was shown superior to only vaccinating the recipient using to the same schedule [32]. This strategy was not as immunogenic using polysaccharide antigen (against pneumococcus) or protein neo-antigen (hepatitis B). Two patients in the study developed severe local reactions after vaccines given on day -1; one of these patients subsequently developed necrotizing fasciitis that necessitated treatment with hyperbaric oxygen and surgery. Both patients recovered without long-term sequelae [32].

A benefit of donor vaccination was shown also for diphtheria [33]: 111 matched sibling donors were randomized to receive or not receive tetanus and diphteria-vaccine, Hib, and inactivated polio vaccine two to 10 weeks prior to harvest. The recipients were vaccinated according to standard schedule with vaccines given at 3, 6, and 12 months post-transplant. In the group of vaccinated donors, diphtheria antibody levels were consistently higher among the recipients. Additionally, a benefit of both donor and recipient vaccination was demonstrated for pneumococcal conjugate vaccine and Hib while data on HBV are conflicting [34]. Recently, Leclerc *et al.* reported a benefit of donor vaccination with mRNA-vaccines against COVID-19 on the recipient's humoral response early after transplantation [35].

1.5 Infectious diseases targeted by vaccines

1.5.1 Tetanus

Tetanus is caused by a highly potent neurotoxin produced by the anaerobic bacterium *Clostridium tetani.* Tetanus spores are present in nature worldwide, in soil and animal intestines, and often enter the human body through contaminated wounds or tissue injuries from puncture wounds. The toxin causes severe muscle contractions, particularly of the jaw and neck muscles. The case fatality rate approaches 100% in the absence of medical interventions [36]. The available vaccines are highly effective and inexpensive, and a four to five dose primary schedule is used in most countries. Although around 86% of the population worldwide is protected through vaccination, tetanus remains an important cause of death in developing countries. According to WHO, 34,000 neonatal deaths in 2015 were attributed to tetanus [36]. There is likely an underestimation of tetanus cases outside of neonatal care. Case reports have been published reporting severe tetanus in cancer patients, especially patients with ulcerating cancers and skin-cancers [37, 38].

Immunity against tetanus in patients with hematological diseases has not been extensively studied. In a report from 1998 of 206 patients treated for hematological malignancies, 36% of AML patients and 56% of ALL patients were seronegative against tetanus despite childhood vaccination. Increasing age, lymphoid malignancy, and advanced disease were risk factors for loss of immunity [39]. Most investigators have studied pediatric malignancies and report that 81% of patients treated for ALL had protective antibody levels (defined as 0.1 IU/ml) by ELISA at diagnosis, decreasing to 33% at six months after treatment. Following booster vaccination, the high-risk group showed an insufficient response with only 56% reaching the protective threshold [40]. Additional studies show that 18-20% of the patients were seronegative against tetanus following treatment for pediatric malignancy [41, 42]. A booster containing tetanus vaccine is recommended for all pediatric patients having completed ALL treatment according to international guidelines [43]. No such recommendations exist for adult patients.

Several studies have shown declining antibody levels against tetanus after allo-HCT [44, 45]. A satisfactory initial response (95-100%) to a three-dose schedule, starting at six to 12 months, has been reported [33, 44, 45] and revaccination with three doses of DT-vaccine is recommended, starting at six months, according to international guidelines [46]. However, the longevity of vaccine responses is largely unknown.

1.5.2 Diphtheria

Historically, diphtheria has been one of the most feared infections worldwide causing epidemics mainly affecting children. The case fatality rates in respiratory diphtheria during the pre-vaccination era in Europe and USA reached 50% in some areas [47]. A vaccination coverage of 85% in childhood immunization has been suggested to reach a threshold that confers herd immunity [48]. According to WHO, approximately 86% of children globally receive three doses of vaccine containing diphtheria toxoid [47].

Diphtheria is caused by the exotoxins produced by *Corynebacterium diphtheriae* or *Corynebacterium ulcerans*. Respiratory diphtheria carries high mortality and is the form reported to WHO. Progression of airway obstruction due to laryngeal infection causes 60-65% of deaths but toxic effects on the heart and central nervous system can also be fatal. Prompt administration of diphtheria antitoxin reduces mortality substantially. Vaccination does not prevent colonization but reduces transmission by 60% [49].

Diphtheria has rarely been reported in immunosuppressed patients [50, 51]. During a Swedish outbreak in 1986, many affected patients were alcoholics drinking from the same bottle [52].

A 47-year-old woman, with ongoing immunosuppressive treatment due to a kidney transplantation some years earlier, presented with fever and severe stridor requiring intubation and intensive care. Diphtheria (*C. ulcerans*) was diagnosed. She had not been abroad. An ambitious screening of healthcare workers and family was undertaken. The bacterium was later isolated from several ulcers on the woman's dog. The patient was fully vaccinated in childhood but had no antibodies against diphtheria toxoid (<0.1 IU/ml) on admission [50].

No data have previously been reported on diphtheria immunity in adults after completion of treatment for hematological diseases. The corresponding results are more robust in the pediatric population. Among pediatric patients with ALL, only 39% had protective antibody levels at diagnosis, decreasing to 17% after treatment [40]. This is in accordance with the study from von der Hardt [41] in which 38% of pediatric patients treated for various malignant diseases, mainly ALL, reached the protective threshold, defined as >0.1 IU/ml using ELISA, following treatment. A diphtheria-containing booster, in the form of high-dose diphtheria toxoid (D), is recommended for all pediatric ALL patients after completion of treatment [43] irrespective of antibody levels.

Diphtheria antibody levels wane following stem cell transplantation [53, 54] and the response to three doses of vaccine given post-transplant is 95% [33, 55]. The longevity of the vaccine response has not been studied.

1.5.3 Poliovirus

Poliovirus is an enterovirus and a member of the picornavirus family. There are three poliovirus serotypes. Wild-type poliovirus type 2 and 3 were eradicated in 2015 and 2019 respectively, but vaccine-derived poliovirus type 2 still circulates [56]. Wild-type poliovirus is endemic in Nigeria and Pakistan [56]. Infections occur via the fecal-oral route. Paralytic polio is caused by viral infection of motor neurons in the spinal cord and occurs in less than 1 % of infections. There are two available vaccines: oral polio vaccine (OPV), which is a live attenuated vaccine still used in many parts of the world, and an inactivated polio vaccine (IPV), which is used in most developed countries.

The live attenuated (OPV) polio vaccine has caused emergence of vaccine-associated paralytic poliomyelitis (VAPP) due to instability of the OPV-strains. Fatal vaccine-derived polio was reported in a 25-year-old ALL patient who had been in contact with a three-month-old nephew recently immunized with OPV [57]. The global polio situation has worsened in the past years [56], partly due to the COVID-19 pandemic, but also due to the global withdrawal of the trivalent OPV. The vaccine-derived strains, mainly serotype 2, have spread due to low mucosal immunity and removal of serotype 2 from the vaccines [58].

There are no data on immunity against poliovirus in adult patients having received treatment for hematological malignancies. In pediatric patients treated for cancer, non-protective antibody levels were seen in 18, 12, and 25% against serotype 1-3 respectively [59], and in another study, including mainly pediatric patients with hematological diseases, 7% were unprotected against polio [60].

Following stem cell transplantation, antibody levels against poliovirus have been shown to decline [54, 61]. The response to three doses of IPV is sufficient [61, 62] and the retainment of protective antibody levels in long-term survivors (median eight years following transplant) is reportedly >90 % [63].

1.5.4 Tick-borne encephalitis (TBE)

Tick-borne encephalitis (TBE) virus belongs to the flavivirus family. The virus is a small, enveloped virus with a single stranded RNA genome, and at least three subtypes have been described. TBE is a zoonotic disease and is mainly transmitted to humans through the bite of an infected tick of the species *Ixodes ricinus*.

The disease often manifests itself biphasically and severity ranges from asymptomatic infection to severe meningoencephalitis. Although many patients recover fully, long-term neurologic sequelae are common [64]. Severe disease and deaths have been described in immunosuppressed patients, mainly among patients receiving rituximab [65, 66].

TBE virus transmission from a deceased organ donor has been described. One liver and two kidney transplant recipients developed fever on day 17, 25, and 51, respectively, and subsequent fatal encephalitis. The diagnosis was established by next-generation-sequencing (NGS) from autopsy material from cerebrospinal fluid (CSF) and brain. An identical viral strain was found in both the donor and the three recipients [67]. Only one of the recipients had pleocytosis in CSF, which can suggest diagnostic difficulties in immunosuppressed patients.

Serological methods, detecting IgM and IgG in serum and CSF, are the main diagnostic tools. PCR can be positive early but a majority of patients with CNS symptoms are negative in samples from blood or from CSF [68]. However, the use of PCR for diagnosis in immunosuppressed patients, in whom antibody production is deficient, is important [65] due to higher viral load and most likely a deficient clearance of virus.

There is no antiviral treatment available for TBE. In Sweden there are two licensed vaccines, both based on inactivated whole virions. The primary schedule consists of three doses. Vaccine failures have been described in up to 5% of TBE cases [69] but since a fourth vaccine dose was recommended in 2010 in Sweden for individuals >60 years, no breakthrough infections have been reported in this group [70].

The vaccine confers protection mainly by producing antibodies against glycoprotein E and other proteins. Cell-mediated responses to vaccination are likely to be of additive importance [70].

1.5.5 SARS-CoV-2

The new coronavirus (SARS-CoV-2) that emerged from Wuhan in China shocked the world. In the early phase of the SARS-CoV-2 pandemic the mortality rate in allo-HCT recipients was high, exceeding 20% in the initial reports [71, 72]. The clinical presentation ranges from asymptomatic infection to severe pneumonitis, multiorgan failure and coagulopathy.

SARS-CoV-2 is an enveloped coronavirus, with glycoproteins giving the virus a crownlike or coronal appearance. The viral envelope is coated by several proteins, *i.e.*, the spike (S) glycoprotein, envelope (E), and membrane (M) proteins. The subunit of S1 contains the receptor-binding domain (RBD) that binds to the ACE receptor (ACE2) of the target cells [73].

Allo-HCT recipients, as well as other immunocompromised patients, were excluded from the initial registration studies of the mRNA-vaccines, and the recommendations for the most vulnerable patients were thus initially based on expert opinions only. Several studies now published have examined the response to the initial two dose schedule in allo-HCT recipients and humoral response rates, as defined by the authors, have ranged from 69-93% [74-79].

Among hospitalized patients with breakthrough infections, *i.e.*, infections despite full vaccination, 40% were immunocompromised [80]. Among hematological patients with documented infections despite two vaccine doses, the mortality was 8% [81] and the only factor predicting severe disease was seronegativity to SARS-CoV-2 prior to infection.

Despite vaccines, along with the emergence of monoclonal antibodies and antiviral treatment, there is still a considerable mortality in the stem cell transplant recipient population [81]. However, there is no data yet published on mortality rates in this population in the era of the omicron genotype.

The current recommendation from EBMT for stem cell transplant recipients is to receive three doses of vaccine in their primary schedule, starting at three or six months, depending on the transmission rate in the country. The timing of the third dose is not well established, and this dose can be given four weeks or up to five months following the second dose. One study assessed the response to the third dose in seronegative patients following the second dose, and only 48% of patients reached the putatively protective level of antibodies [82]. Patients are recommended to receive booster doses, but responses to a fourth or fifth dose in this cohort have not yet been published. The newer adjuvanted protein-based vaccine against SARS-CoV-2, Novavax®, can be used for patients not tolerating or not responding to the mRNA vaccines. However,

there are as yet no efficacy data from the immunocompromised patients receiving this vaccine.

2 AIMS

The aims of this thesis were to investigate the efficacy of vaccines in patients after chemotherapy or stem cell transplantation and to determine the longevity of vaccine responses.

Paper I: To clarify whether adult patients need to be revaccinated against tetanus, poliovirus, and diphtheria after conventional chemotherapy for acute leukemia and lymphoma.

Paper II: To examine the serologic response to TBE-vaccination in patients after auto- and allo-HCT to four doses of vaccine (FSME-IMMUN®) starting at nine months post-transplant.

Paper III: To investigate serologic and cellular responses before and after a third dose of COVID-19 mRNA vaccine in allo-transplanted patients within three years from transplantation and/or with ongoing immunosuppressive treatment for GvHD.

Paper IV: To determine serum antibody levels and provide data on the need for boosters against tetanus and diphtheria toxins among long-term survivors (>8 years post-transplantation) after allo-HCT.

3 PATIENTS AND METHODS

3.1 Patients and controls

Paper I: From local registries, we identified lymphoma (n=80) and acute leukemia (n=24) patients in complete remission in median 18 (four to 77) months after last chemotherapy at Sahlgrenska and Karolinska University hospitals. Patients were approached by a letter and, if needed, a phone call. The study was expanded twice, first with additional patients from Gothenburg and later with patients from Karolinska. Serum samples were obtained from 104 patients (Sahlgrenska n=60 and Karolinska n=44). No patient in the study had been treated with stem cell transplantation; thus, the included leukemia patients were mainly low risk leukemias. Stored serum samples from diagnosis were available for 73 patients. Sex- and age-matched healthy controls, n=47, were recruited for the first patients enrolled. Control sera (n=35) were obtained from the local blood bank. Additional controls were either staff at the hematology department or older relatives of the staff (n=12). All included patients filled in a questionnaire on previous vaccinations, relevant infections, and current medication. Corresponding data were not available from healthy controls.

Paper II: Patients after allo-HCT (n=51) and auto-HCT (n=53) were identified in local HCT-registries. Allo-HCT recipients were included at Sahlgrenska (n=35) and Karolinska (n=16). Patients after auto-HCT were included at Sahlgrenska (n=24) or in regional hospitals, *i.e.* Borås (n=10), Varberg (n=6) and Uddevalla, (n=13). Patients were vaccinated with four doses of FSME-IMMUN® starting at nine months post-transplant. Vaccine was given at zero, one, three and 12 months, and blood samples were obtained before each vaccination and three months following the last vaccination. Twenty-seven healthy controls (10 males/17 females, median age 39 years), all staff members at the Microbiology Department, received three doses of FSME-IMMUN® (zero, one, and 11 months). All controls were seronegative at baseline. Blood samples were obtained before vaccination, two to three months after the second dose, and one month following the last dose.

Paper III: All patients currently attending the outpatient allogeneic stem cell transplant clinic at Sahlgrenska, fulfilling the inclusion criteria, were invited to participate in the study. The majority of patients were contacted with a phone call and the rest when attending the clinic. Forty patients were included in the study. Ten patients were already included in another sub-study within the same study, assessing immune responses to the initial two doses [79]. Blood was collected in EDTA and heparin tubes and patients received the third dose of

mRNA vaccine immediately after blood sampling. The patients returned four weeks later for follow-up blood sampling. All patients filled in a questionnaire at baseline on current medication and previous COVID-19 infection. Three patients were excluded from the data analysis due to previously confirmed COVID-19. Data on side effects were collected at the follow-up visit. Information on GvHD was retrieved from the medical notes.

Paper IV: Long-term survivors, at least eight years after allogeneic stem cell transplantation, were identified in local HCT-registries at Sahlgrenska (n=98) and Karolinska (n=45). Most serum samples from the study (n=107) had been stored frozen. A smaller fraction of samples (n=36) was prospectively collected during 2021 at Sahlgrenska during routine visits. Information on the vaccine doses given after transplant, GvHD, and immunosuppressive treatment at the timepoint of sampling was extracted from the medical notes.

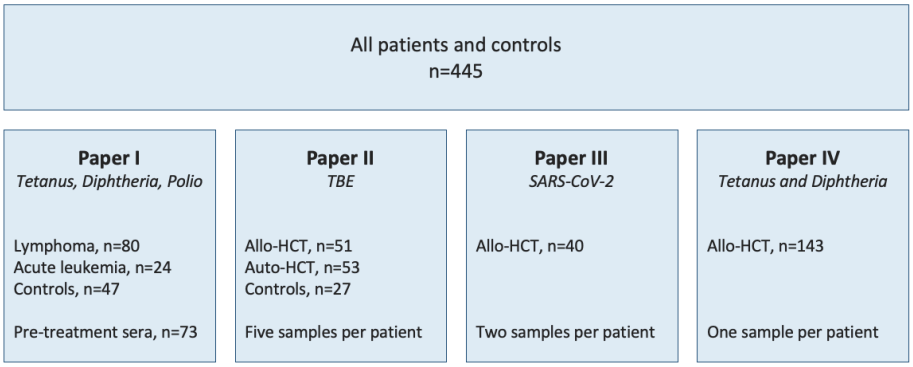


Figure 9. An overview of included patients and controls in papers I-IV.

3.2 Aspects on serology

Serological methods to assess the magnitude of antibody responses are frequently used in microbiology. Even if methods based on nucleic acid detection, mainly PCR, are largely replacing serology in acute diagnostics, serology remains the gold standard in certain situation, such as screening of blood donors, pregnant women, or patients and donors pre- transplant with the aim of assessing the risk of transmitting, or later developing, infections. For the diagnosis of certain infections, serological methods remain the method of choice and serology is the method used in clinical practice to assess the immunity after infection or vaccination. However, serology is often less useful in immunocompromised patients.

The main methods are enzyme-linked immunosorbent assay (ELISA) or virus neutralization tests (NT), and these assays are based on antigen-antibody reactions. Neutralization assays not only measure antibody titer but also the ability to neutralize pathogens, most often viruses. NTs are labor intensive, not suitable for large-scale automation, and require high safety precaution, as this method includes working with live virus or bacteria. Most serological assays detect IgG or IgM responses. ELISA methods have been improved in recent years and are now more sensitive, often automated, and easier to standardize and interpret.

When discussing the performance of serological diagnostic tests, the reliability is often described in terms of sensitivity and specificity. Sensitivity of a test is the ability to detect true positives. If the sensitivity of a test is low, patients with a specific disease will not be detected; so-called false negatives. Specificity is the ability of a test to detect true negatives. If the specificity of a test is low, patients without a specific disease will be categorized as having the disease; so-called false positives. If the specificity of an ELISA assay is low, this may depend on cross-reactivity, *i.e.*, antibodies reacting in the assay that are not specifically directed against the pathogen.

For serological tests, it is impossible to reach 100% specificity and sensitivity. Some true positive samples may thus contain antibody levels below the detection limit of the assay, and some of the true negative samples may show unspecific reactivity. The limits are established after analysis of many samples that have previously been defined as true negative or positive by several methods. Pathogens differ in their ability to induce antibodies, and the ability to mount antibody responses differs between individuals, depending on, for example, immune competence and age. In hematological patients it is also important to consider whether the individual has received intravenous or

subcutaneous immunoglobulins, as passive transmission of antibodies may cause confusion when interpreting serologic results. The dynamics of serological responses should also be considered: if an individual does not show specific antibodies, it mostly reflects that the individual is not infected but may also mean that the individual was sampled recently after infection, before antibodies are formed. The patient can be contagious and symptomatic during this window phase, which is why some western countries screen blood donors by PCR; for example, against HIV [83]. Serological methods can also be used to differentiate between whether a person has undergone infection or has been vaccinated, as exemplified by SARS-CoV-2 infection and hepatitis B [84].

There are many serological methods used: direct detection, indirect detection, sandwich assays, chemiluminescence assays, western blot, and more. Although different, these methods are founded on the same principle: there is a capture system (antibody or antigen), addition of the analyte (the substance the assay is designed to measure, which can be an antibody or an antigen), and some form of detection system. As an example, the ELISA method for serology will be described in more detail below.

3.2.1 Indirect ELISA

Indirect ELISA is useful in detecting human immunoglobulins. The principle is as follows: the wells of a plate are coated with antigen. After washing and blocking, the serum from the patient is added in a dilution series. If antigen-specific immunoglobulins are present in the patient sample, they will bind to the antigen. All unbound antibodies and other compounds are washed away. A conjugate is added. The conjugate is a secondary antibody, often from another animal species, that targets the Fc region of the primary antibody. These secondary antibodies can be targeted against IgM or IgG, depending on which isotype the assay is aimed to detect. The plate is washed again, to remove unbound secondary antibodies. For detection, a substrate is added. The substrate together with the secondary antibody give rise to fluorescence. The color intensity is measured by a spectrophotometer as optical density (OD) at defined wavelengths. The absorbance of color that is measured with the spectrophotometer is proportional to the concentration of antibodies in serum. A standard curve is constructed each time the ELISA is run. The standard curve is prepared by making the same serial dilutions of standard serum with known concentration. A background, negative control, containing only buffer, is always included.

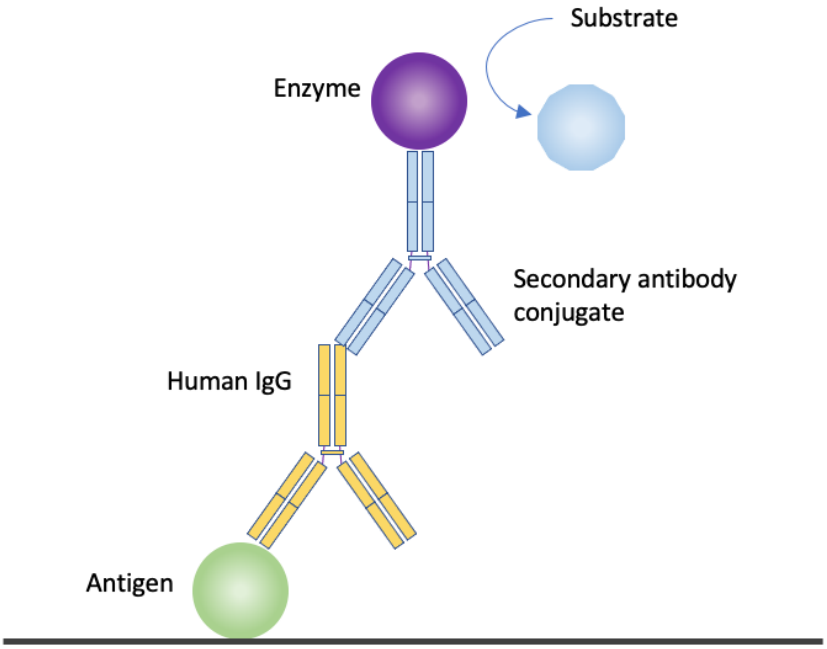


Figure 10. Indirect ELISA

3.2.2 Tetanus serology

In papers I and IV, the IgG against tetanus toxoid was measured using commercial ELISA kits. In paper IV, anti-tetanus toxoid ELISA IgG (EI 2060-9601G) from Euroimmun, Lubeck, Germany, was used. Optical density (OD) values were converted to International Units per ml (IU/ml) based on international WHO standard control sera.

In paper I, the minimal level for seroprotection ("cut-off") was set to ≥ 0.01 IU/ml. In paper IV, antibody levels < 0.01 IU/ml were considered "seronegative", values between 0.01-0.5 IU/ml were considered to represent "partial protection" and levels above 0.5 IU/ml were considered to confer "full protection". The cut-offs applied were per the manufacturer´s instructions and in accordance with WHO recommendations [36, 85].

3.2.3 Diphtheria serology

In paper I, diphtheria toxoid antibody levels were quantified using a neutralization test (n=60) or a commercial ELISA kit (n=44).

In paper IV, anti-diphtheria toxoid ELISA IgG (EI 2040-9601G) from Euroimmun, Lubeck, Germany, was used to determine IgG antibody levels. Optical density (OD) values were converted to international units per ml (IU/ml) based on international WHO standard control sera.

In paper I, the seroprotection was set to ≥ 0.01 IU/ml for both neutralization and the ELISA. In paper IV, levels below 0.1 IU/ml were considered "low" or "seronegative", levels between 0.1-1.0 were considered to confer "partial protection", and levels above 1.0 IU/ml were considered "high" and protective. According to WHO, using the toxin neutralization test, a diphtheria antibody concentration of 0.01 IU/ml is considered to be the minimum level required for some degree of protection [47]. The cut-offs for the ELISA kits are most often chosen by the manufacturers of the kits.

3.2.4 Poliovirus microneutralization

Antibody levels against poliovirus serotypes 1 and 3 were determined in a microneutralization test using 96-well plates. Serum samples, in two-fold dilutions, were incubated with poliovirus serotypes 1 and 3. A suspension of Green monkey kidney cell suspension was added and allowed to incubate for five days. The cells would be infected by the virus if, in the sample, there were not enough antibodies that bind to the virions to prevent entry into cells. The presence of antibodies was recorded microscopically by the absence of cytopathic cell effects.

The antibody titer was defined as the reciprocal of the highest dilution that neutralized the virus and all patient samples were diluted until the highest dilution was found. Seroprotection against poliovirus was defined as a microneutralization titer of ≥ 2.

3.2.5 TBE serology

To determine IgG antibodies against TBE virus in serum samples, Enzygnost®, anti-TBE virus ELISA (Siemens, lot 48842, Siemens Healthcare AB), was used, which is based on a whole virus antigen. All tests were performed according to the protocol that was provided by the manufacturer. Levels ≥ 12 U/ml were considered seropositive, according to the manufacturer's instructions. The cut-off was calculated by the mean of a non-reactive sample +0.2 optical density (OD) absorbance values. The results were given in units per ml (U/ml) and the analytical interval was 7-700 U/ml.

3.2.6 COVID-19 serology

Chemiluminescent microparticle immunosassay (CLIA) was used to determine IgG antibodies against the regional binding domain (RBD) of the spike protein of SARS-CoV-2.

CLIA is based on the same technique as ELISA with the difference that the enzyme coupled to the detection antibody catalyzes a light reaction (chemiluminescent reaction) that results in the emission of photons producing light, rather than a visible color change.

The assay used was SARS-CoV-2 IgG II Quant; Abbott, Abbott Park, IL in automated Alinity systems. Levels were reported in WHO international standard binding antibody units (BAU/ml) with a quantitative detection range of 14 to 5680 BAU/ml.

In paper III, we used the lower limit of the test, 14 BAU/ml, as the cut-off for seropositivity. Samples <14 BAU/ml were thus categorized as seronegative.

3.2.7 Interferon-γ release assay

The cell-mediated vaccine response was measured using an interferon (IFN)-γ release assay. The principle of this assay is to incubate whole blood with multiple peptides from the SARS-CoV-2 virus. The APCs in the sample take up these peptides and present them to the T cells. If there are reactive T cells in the sample they will respond with IFN-γ production, which is subsequently quantified by ELISA. The same process is performed with control samples from the same patient that are not incubated with peptides. The results from the control samples are later subtracted to obtain a measure of antigen-specific IFN-γ production.

A similar type of assay, IFN-γ release assay (IGRA), is used in clinical practice when assessing tuberculosis immunity using peptides from Mycobacterium tuberculosis. IGRA assays are also available for detecting CMV immunity [86].

Peripheral blood was collected in lithium-heparin tubes. Within 24h of collection, 1 ml of whole blood was transferred to 10 ml tubes and stimulated or not with 1 µg/ml of peptides spanning the S1 domain of the SARS-CoV-2 glycoprotein S1 (product number: 130-127-041; Miltenyi Biotec). The samples were incubated for 48 hours at 37 degrees with 5% CO2 to allow release of IFN-γ from peptide-responsive T cells. Thereafter, plasma was recovered after a five-min centrifugation of tubes at 1500 rpm. Recovered plasma was stored at -80 degrees C until analysis.

Recovered plasma was assessed for levels of IFN-γ by ELISA (DY285B; RD Systems) according to instructions provided by the manufacturer. To limit unspecific reactivity, plasma was diluted 1:2 in a phosphate buffered saline, containing 10% mouse serum and 1% bovine serum albumin. Optical density was measured at 570 and 450 nm with a FLUOstar omega plate reader (BMG, Germany). The lower detection limit of the assay was 10 pg/ml.

3.3 Statistics

Geometric mean titer (GMT) is often used for antibody titers because such data usually do not fit a linear scale. The arithmetic mean is therefore not an ideal representation of the results. The geometric mean is calculated by taking the average of the logarithms and then converting the mean back to a real value. As the logarithm of zero is undefined, a constant is typically added to the zero values, and the same constant is then added to all other values.

Paper I: For paired data, antibody levels in patients versus controls or patients pre- versus after treatment were compared using Wilcoxon signed rank test. The proportion of immune patients before versus after treatment was compared using McNemar test. Non-immune proportions and antibody levels were compared (between independent samples) by Wilcoxon rank sum test and Fisher's exact test. The correlation between antibody levels and age or time since last treatment was determined using Spearman rank correlation.

Paper II: GMT and the geometric mean fold rise (GMFR) were calculated. The GMFR is calculated as the mean of the fold rise between the pre-vaccination and postvaccination samples (after dose four). After log transformation, antibody titers were compared using t-test. A two-way ANOVA was used to adjust for the age difference between patients and controls.

Paper III: The patients were divided into two groups: responders and non-responders. Chi-square test was used to determine the correlation between the categorical variables in a contingency table. The Mann-Whitney U test was used to calculate differences in continuous variables between groups.

Paper IV: The nominal variables, "seronegative", "partial protection", and "full protection" were compared using a logistic regression model, with the results presented as odds-ratio (OR). For the continuous variables, GMTs were calculated, and a linear regression was used to determine predictors of immune response. Fisher's exact test was used to examine proportions of non-immune versus immune patients. Kaplan-Meier estimates were used to calculate the probability of remaining immune at certain timepoints, with loss of immunity used as events.

The statistical significance in all tests was set to < 0.05. All tests were two-tailed. Data was analyzed using SPSS statistical software package (version 24) or R (version 4.2.0). GraphPad Prism 8 for MacOS was used for the creation of box- and scatterplots.

3.4 Ethics

Ethical permits were obtained for all studies included in this thesis, from either the regional ethics review board (papers I and II) or the national ethics review board (papers III and IV). As papers II and III were clinical trials, permits were also obtained from the Swedish Medical Products Agency (Läkemedelsverket) and registered in EUDRA-CT. The permit numbers are listed below.

Paper I: Humoral immunity to tetanus, diphtheria, and polio in adults after treatment for hematological malignancies. Permit number: 008-11. Date of approval: 2011-02-07.

Paper II: Vaccination against tick-borne encephalitis (TBE) after autologous and allogeneic stem cell transplantation. Permit number: 415-14. Date of approval 2014-09-26.

Approval from the Swedish Medical Products Agency, permit number: 5.1-2014-78505. Date of approval: 2014-11-11. Eudra-CT number: 2014-003573-42.

Paper III: Reduced immunogenicity of a third COVID-19 vaccination among recipients of allogeneic stem cell transplantation. Permit number: 2021-00539. Date of approval: 2021-03-11.

Approval from the Swedish Medical Products Agency, permit number:5.1-2021-11118. Date of approval: 2021-03-21. Eudra-CT number: 2021-000349-42.

Paper IV: Long-term immunity to tetanus and diphtheria after vaccination of allogeneic stem cell transplant recipients. Permit number: 2020-02437. Approval date: 2020-08-19.

4 RESULTS

Paper I: Patients in remission, who had been treated for lymphoma (n=80) and leukemia (n=24) at median 18 months earlier, were included in the study. The patients had received a variety of chemotherapy regimens and some also with rituximab (n=48), a monoclonal antibody directed against CD20, which is a surface antigen expressed on a majority of B cells. The included patients answered a questionnaire regarding earlier vaccinations. Many patients were uncertain and the majority did not know their precise vaccine dates and numbers. No patient reported being vaccinated after treatment. For n=73, pretreatment serum was available. The healthy controls were from the local blood bank or staff at the hematology department or relatives of the staff.

For tetanus, we found an increased number of non-immune patients after treatment (24%) compared with before (12%; p=0.02) and antibody levels against tetanus were lower after treatment (p=0.02). For diphtheria there was a trend towards more non-immune patients after (21%) compared to before treatment (27%) (p=0.06). Antibody levels against diphtheria toxoid were lower after treatment (p=0.03).

For both diphtheria and tetanus toxoid, there was an inverse correlation between age and antibody levels, *i.e.*, older patients had lower antibody levels.

In the subgroup of patients where age- and sex-matched controls were available, diphtheria antibody levels were lower in patients (p=0.0005) and rate of non-immunity was higher in patients (p=0.01). However, we found no significant differences between rates of non-immunity or antibody levels for tetanus toxoid between patients and controls.

We found no impact of sex on antibody levels or rates of non-immunity against tetanus or diphtheria.

After treatment, 92% of patients were immune to poliovirus 1 (PV1) and 95% against serotype 3 (PV3). We found no significant differences in pre- versus post-treatment neutralization titers, or rates of non-immunity for PV1. For PV3, antibody levels were lower after treatment compared to before (p=0.006).

In the lymphoma group, rituximab treatment was not associated more with non-immune patients or lower levels of antibodies for any antigen (diphtheria, tetanus, PV1, and PV3).

So, do our results warrant the same recommendations in adults as in children after ALL treatment, are to give booster vaccine doses of tetanus and diphtheria to everyone who has been treated for lymphoma and acute leukemia?

When analyzing our data in a broader context, the problem with loss of immunity seems more pronounced in the pediatric cohorts published [40, 41] compared to in our adult cohort. It is possible that this is disease-specific, and the results would have been different in adult ALL patients, as ALL treatment is lengthy with high doses of steroids, compared to the shorter periods of myelosuppression that our patients have mainly received. This is supported by that children with high-risk ALL receiving more intensive treatment have a higher likelihood of losing immunity. We only had two patients with ALL in our study, thus precluding conclusions.

It would have been ideal to have access to serial samples from the control group to compare the decay of antibodies over time in patients versus controls. In our study, only one control sample was available, and the age of the controls was matched, within five years, with the age of the patient in the post-treatment sample. To run the samples at the same time, with the same methods, having controls and pretreatment sera for all patients would have improved the quality of the study. The heterogenicity of patients in the study is also problematic, but at the same time representative of the patients seen in the everyday hematology out-patient clinic.

How well-founded are the cut-offs chosen as indicative of protection? In commercial ELISA-kits there is often surprisingly little information available on the antigen used. Despite this, in most studies, concentrations above 0.01 IU/ml of tetanus toxoid antibodies measured by ELISA are considered to mediate some protection. However, cases of tetanus have been documented in individuals above these thresholds [36].

For diphtheria, there is more inconsistency in the literature regarding levels that confer protection [87] and there is some variability in the chosen cut-offs in different studies. Immunity is considered to depend mainly on anti-toxin antibodies, but cell-mediated immunity may also play a role [47].

In a comparative study of seven commercial ELISA kits for anti-diphtheria toxoid antibodies, the number of samples below 0.1 IU/ml varied between 0-25% depending on the kit used. However, when the curves were constructed using international standard serum, the tests were more consistent [88]. For diphtheria, there is generally a good correlation between levels of diphtheria toxoid antibodies in blood and clinical protection from the disease [47]. During

the Swedish outbreak of diphtheria in 1986, diphtheria antitoxin levels were studied in eight clinical cases and 36 carriers of Corynebacterium diphtheria. Of these carriers, 33 were seropositive, with a chosen cut-off of 0.01 IU/ml, while only one out of eight clinical cases had a titer above 0.01 IU/ml. This patient presented with a mild illness [89].

In conclusion, diphtheria and tetanus booster(s) can be considered, especially in older patients, following chemotherapy for acute leukemia and lymphoma. Polio immunity is apparently well preserved.

Paper II: Patients after allo-HCT and auto-HCT, and fulfilling inclusion criteria, were invited to participate in the study of humoral responses to TBE vaccination. One hundred and four patients were included in the study and answered a questionnaire at baseline regarding earlier TBE vaccinations. Four vaccine doses were given at nine, 10, 12, and 21 months after transplant and 84 completed the study and received all vaccine doses. Five consecutive samples were available in 84 patients, with a few samples missing, but the last sample was available for all patients that completed the study.

Notably, only 35/83 patients (42%) reached seropositivity after three doses of vaccine. After the fourth dose, 66/84 (79%) were seropositive; 33/43 (77%) after allo-HCT, and 33/41 (80%) after auto-HCT. In the younger healthy controls, 27/27 were seropositive following three doses, and GMT after three doses was higher in controls compared to patients after four doses (p=0.001). We adjusted for the age difference between patients and controls, but patients still had lower antibody levels (p=0.002).

Thirteen patients had detectable TBEV antibodies at baseline, but only seven above 12 U/ml, the threshold set for seropositivity in the study. Nine of these 13 reported earlier TBE vaccination pre-transplantation. Complete sampling was available in nine patients with detectable TBEV antibodies at baseline. The median difference between pre- and post-vaccination (after four doses) was lower in the patients with detectable antibodies at baseline. It is not ruled out that these very low levels of antibodies represent unspecific reactivity, *i.e.*, false positives.

Is the threshold for seropositivity in our study, 12 IU/ml, a relevant cut-off? The correlate of protection against TBE has not yet been determined. Neutralization tests (NTs) are considered the gold standard for assessing immunity, but no standardized NTs exist for TBE in clinical routine. The commercial ELISA-kits are mostly whole virion assays. There are problems with specificity and, in patients previously vaccinated or infected with other

flaviviruses, the results may be difficult to interpret. However, the results from commercial ELISA kits have been shown to correlate well with results from neutralization tests, especially when there is no recent flavivirus exposition [90, 91].

So, should we recommend TBE vaccines for everyone after a stem cell transplantation? For all immunosuppressed patients? For the general population?

Austria is the only country in Europe where TBE vaccine is recommended for the general population, which has markedly (by at least 80%) reduced the incidence of TBE [92]. In Sweden, vaccination is recommended for everyone living in or traveling to endemic regions. TBE has increased dramatically in Sweden in the past decades, with 533 reported cases in 2021, which is the highest number recorded. It is increasingly difficult to determine which regions are endemic. A lack of clinical cases in a geographic region does not automatically translate into a low risk if vaccination coverage is high. In 2015, approximately 25% of the Swedish population had received at least one dose of TBE vaccine, with a higher coverage, 53 %, in the Stockholm area [93]. Two recent studies have shown the burden of TBE disease in Sweden, in terms of mortality, costs for healthcare, and sick-leave days, to be considerably higher than previously estimated [94, 95]. It seems likely that the severity of disease is higher in immunocompromised hosts [65-67]. A cost-effective model of a publicly-funded vaccination program in the Stockholm area has been published [96].

Are there any risks associated with TBE vaccines? Generally, the side effects are local pain and swelling at the injection site. Less frequently, malaise, fever arthralgia, nausea, and headache are reported. In a Cochrane review of vaccine trials on the four licensed TBE-vaccines, no side-effects were found to be severe or life-threatening [97]. There have been concerns regarding antibody-dependent enhancement, especially due to a report of some possibly severe vaccine breakthroughs [98]. This is a reality with some other flaviviruses, Dengue virus being the most typical example. Antibody-dependent enhancement is the major mechanism behind a more severe disease if a person is reinfected. A specific range of antibodies against Dengue have been shown to correlate with severe disease, whereas high levels could protect. Thus, it is not only lack of protection that is a concern [99]. However, antibody-enhancement in TBE has not been confirmed *in vivo* [100] and no reinfections have been reported.

There is one other study on humoral immune responses to TBE vaccine following allogeneic stem cell transplantation [101]. Thirteen patients received three doses of vaccine starting at median 12.5 (11-13.5) months after transplant and antibody responses were measured by both NT and ELISA. The primary endpoint was antibody response following two doses of vaccine, which was shown in 35% of patients compared to 93% of controls. However, 13/13 patients proceeding to the third dose obtained protective antibody titers by NT. In the control group, there was an excellent concordance between ELISA and NT (15/15), whereas in the patient group 15% of patient samples showed different results, mainly samples positive in ELISA that were subsequently negative in NT. Most patients, and sibling donors, were previously vaccinated in this high endemic area and 17/17 of patients had detectable antibodies pre-HCT, with a median GMT of 133 (71-248). At one year following HCT, prior to vaccination, 14/17 (82%), still had detectable antibodies but with a significantly lower GMT of 32 (15-67). The CD4-count was a predictor for immune response. Apart from being a smaller study than ours, it was performed in an endemic area and with the use of a three-dose schedule. Other studies of immunosuppressed patients have also used three-dose schedules for a majority of patients [102, 103]. A strength in our study is the use of a four-dose schedule that seems beneficial in this cohort.

In conclusion, TBE vaccination after autologous and allogeneic stem cell transplant can be started at nine months following transplant. It seems important to give four doses in the primary schedule, regardless of age. Whether to recommend TBE vaccination to everyone in Sweden is a difficult question but vaccination of the group with the highest morbidity and mortality *i.e.*, immunosuppressed patients, is likely warranted.

Paper III: The recommendation to give a third dose of COVID-19 vaccine to immunocompromised patients prior to the general population was rolled out in spring 2021. The selection criteria for the allo-HCT patients from the Swedish Public Health Authority was either (i) transplantation less than three years ago and/or (ii) ongoing immunosuppressive treatment for GvHD. The majority of patients, apart from the recently transplanted, had received the second dose several months earlier. Our aim was to investigate serologic and cellular responses before and after a third dose of COVID-19 mRNA vaccine in allo-grafted patients fulfilling criteria for receiving the third dose, prior to the general population.

Of the 40 included patients, three were excluded due to previous confirmed COVID-19. All patients had received two doses of mRNA-vaccine, at least eight weeks prior to the first available sample. The median time from transplant

was 23 months (min-max 6-191). Twenty-one (57%) had ongoing cGvHD and 25 (68%) were on immunosuppressive treatment, mainly for GvHD but also for disease-specific indication.

Of the 37 patients, 31 (84%) responded with detectable antibodies. A subgroup, 12/37 (32%), responded with antibodies above the upper detectable limit of the test (> 5680 BAU/ml). However, among 14 patients seronegative prior to the third dose, six (42%) remained seronegative following the third dose. Eighteen of the 37 (49%) showed no evidence of T cell reactivity following the third dose. T-cell responses were lower in patients with ongoing immunosuppression, (p=0.046). Of the six patients remaining seronegative following the third dose, five out of six (83 %) were also devoid of T cell reactivity. Seronegativity prior to the third dose predicted poor responses, both humoral and cell-mediated. See Figure 11.

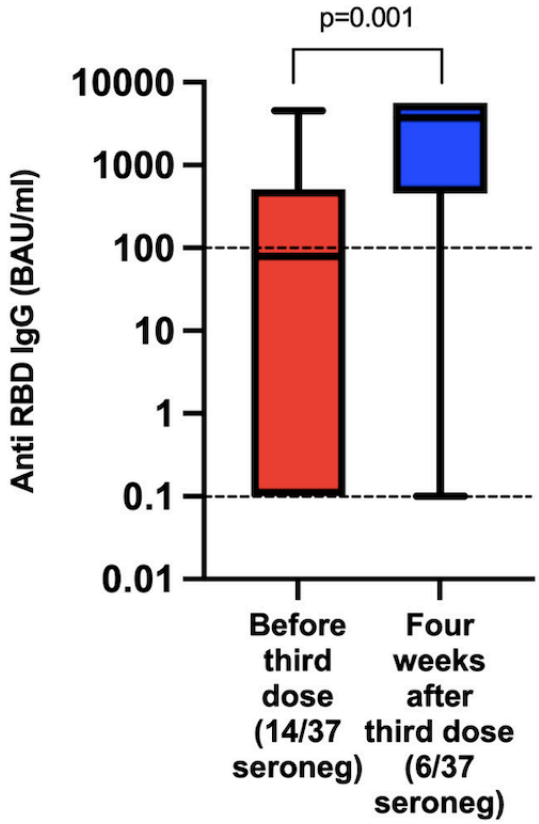


Figure 11. Antibody levels before and after the third dose of COVID-19 vaccination.

As a cut-off for seronegativity in this study, we used the lower detection limit of the assay, 14 BAU/ml. Another study of the response to the third dose in seronegative allo-HCT recipients used a 4.160 AU/ml, corresponding to approximately 590 WHO standard BAU/ml [82], considerably higher than the cut-off used in our study. In that study, 48 % of seronegative patients reached the putative protective level. Among our seronegative patients prior to dose three, 8/14 (57%) responded. It is thus possible that our choice of cut-off overestimated the number of responders. A cut-off of 100 BAU/ml has been used in some trials [79, 104], which derives from experimental data from non-human primates [105]. In a prospective trial of vaccine efficacy, 80% reduction in symptomatic infection (majority Alpha B1.1.7) was achieved in subjects with anti-RBD IgG above 506 BAU/ml. Interestingly, immune markers were not correlated with asymptomatic infection [106]. Healthcare professionals and younger patients were more likely to get infected.

Data on T cell responses to COVID-19 vaccines in allo-HCT recipients to the primary two-dose schedule are variably ranging from 19-100% [74, 79, 107].. There are no standardized methods to detect cellular immunity, thus making comparisons between studies and their conclusions difficult. Reports on T cell responses after the third or fourth doses in this cohort of patients are scarce. Ten allo-HCT recipients seronegative prior to the third dose all responded with cellular immune responses following the third dose. However, 60% remained seronegative after the third dose. The cellular immune responses were assessed with intracellular cytokine staining [108]. The importance of a cellular immune response in the absence of antibody response is yet to be determined.

Our study was a small, single center study. Further, there was no control group as a third dose was recommended by the Swedish Public Health Authority to be administered to the most vulnerable patients prior to the healthy population. There are as yet no data on T cell responses to the third dose in a healthy population using the same assay. However, this assay has been shown to elicit T cell responses in 13/13 in healthy persons, four weeks after the second dose [109].

Are transplanted patients more susceptible to side-effects from mRNA-vaccines? Several studies have reported new onset or worsening of GvHD ranging from 4-12 % of participants receiving two doses of vaccine [74, 77-79]. In our study we did not observe *de novo* or worsening GvHD. Cytopenias have also been reported following vaccination [77, 78]. Local or systemic reactivity following vaccination has, in some studies, been less frequent in allo-HCT recipients compared to control subjects [77, 78]. In allo-HCT recipients

with local or systemic adverse reactions to the vaccine, there was an association with stronger cellular immune responses [110].

In conclusion, most recipients of allogeneic stem cell transplantation respond well to the third dose of COVID-19 mRNA-vaccine. Vaccine-induced T cell immunity is more affected than antibody responses in patients vaccinated during immunosuppression or early after transplant.

Paper IV: It is well known that after allo-HCT patients need revaccination against diphtheria and tetanus following transplant, and the antibody responses following a three-dose immunization program are sufficient. The longevity of the immune responses has not earlier been studied. Vaccinations against tetanus and diphtheria are started at approximately six months after transplantation, when patients are not fully immune-reconstituted, and many patients are vaccinated during ongoing immunosuppression. The aim of this study was to determine antibody levels against tetanus and diphtheria among long-term survivors after allo-HCT and provide data that may inform the need for booster vaccination.

One-hundred and forty-three patients were included. The median time from transplantation to the obtained blood sample was 14 years (min-max, 8-40). Thirty-three out of 143 (23%) had ongoing chronic GvHD at sampling and 18/143 (13%) had ongoing immunosuppressive treatment (see Table 1).

Table 1. Diphtheria and tetanus immunity in long-term survivors. Results in all patients (n=143).

Diphtheria immunity	
High (>1.0 IU/ml)	13 (9 %)
Partial protection (0.1-1.0 IU/ml)	74 (52 %)
Low=seronegative (<0.1 IU/ml)	56 (39 %)
Tetanus immunity	
High (>0.5 IU/ml)	97 (68 %)
Partial protection (0.01-0.5 IU/ml)	46 (32 %)
Low=seronegative (<0.01 IU/ml)	0 (0 %)

The estimated probabilities to remain immune to diphtheria at 10, 15 and 20 years after transplantation were 77%, 57%, and 40% respectively (Figure 12).

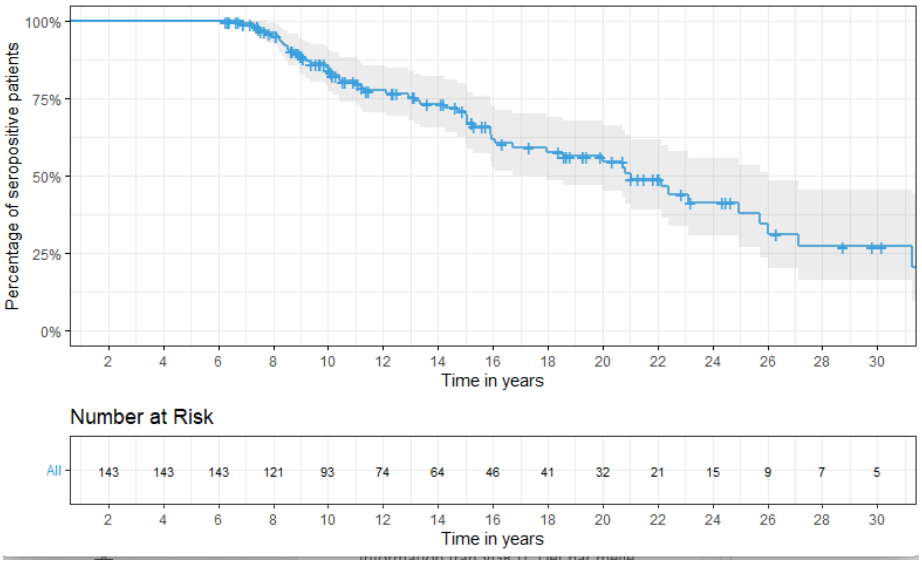


Figure 12. Probability of remaining seropositive to diphtheria following vaccination.

Titers of antibodies against tetanus were correlated with increased age at sampling, *i.e.*, older patients had higher antibody levels (p=0.02). This correlation was not found for diphtheria. Whether the older patients had received more doses of vaccine prior to transplant or if the higher tetanus antibody levels could depend on donor factors remains unknown. No correlation was found between sex, GvHD, or time from transplantation and diphtheria or tetanus antibody levels.

There was a trend towards a higher likelihood of seropositivity against diphtheria among patients known to have received one or more booster doses after HCT (p=0.053).

Due to the retrospective design of the study, only one sample was available for each patient. To properly report the decay of antibody levels, we would have needed more than one sample per patient. The number of booster doses given to the patients is likely to be underestimated. Better data on given boosters, through a digital vaccine record, would have added quality to the study. Furthermore, the transplant procedures in this study reflect practice several years ago, in regards to donor-choice, conditioning regimens and interventions post allo- HCT.

Pneumococcal immunity has been studied in long-term survivors after allo-HCT [111], as well as poliovirus immunity [63] and measles immunity [112]. Regarding pneumococcal immunity in adult patients at a median of nine years following transplant, only 50% of patients were protected against 7/7 serotypes and 70% against 5/7 serotypes. Lack of seroprotection was associated with transplant not performed in complete remission, from a cord blood unit (CB), relapse after transplant, or chronic GvHD [111]. No cases of clinical, invasive, pneumococcal disease (IPD) were observed after start of vaccination.

For polio immunity, studied in 134 patients, at a median of eight years post-HCT vaccination, 16% of patients were seronegative to at least one of the poliovirus serotypes. The only risk factor for loss of immunity was low age. There was a trend ($p=0.07$) for patients with chronic GvHD to lose immunity more rapidly [63].

In the study of long-term retainment of measles immunity, 62% of allo-HCT recipients were seropositive at a median of nine years after transplantation [112]. The probability of remaining seropositive was higher in patients with a history of measles infection prior to transplant, compared with patients vaccinated prior to transplant. The factors associated with seropositivity were myeloproliferative disorders, reduced intensity conditionings, and absence of acute GvHD all grades [112].

Unlike other studies, we found no association with chronic GvHD and loss of immunity against tetanus and diphtheria. The number of patients with ongoing GvHD was small, 33/143 (23%), and only 18/143 (13%) were on systemic immunosuppression, thus most patients had a mild chronic GvHD. It is not ruled out that GvHD may have influenced the time to becoming seronegative, but as we lack serial sampling this remains unknown.

In conclusion, many long-term survivors after allo-HCT lack protection against diphtheria, and booster(s) or serologic testing may be considered. Tetanus immunity is, on the other hand, well preserved.

5 DISCUSSION

This thesis has investigated mainly humoral immune responses to different vaccines in patients after receiving either chemotherapy for hematological malignancies or stem cell transplantation, and the longevity of these responses.

As discussed previously, the cut-offs for seropositivity are often arbitrary and vary between different trials. There are several distinct ways of reporting vaccine efficacy such as seropositivity, seroconversion, geometric mean titer (GMT), or geometric mean fold rise (GMFR). Efforts have been made to standardize vaccine responses; for COVID-19 this has resulted in a consensus statement, and most studies now report antibody levels in international standard units [113].

What is the protective antibody level? Is the patient immune? Even though both the patient and clinician demand straightforward answers, the reality of determining immunity must be viewed in the right context. An antibody level is just one piece of the puzzle and other aspects such as cellular immunity, virulence of different strains of pathogens, infective dose, age, comorbidities, methodology etc. have to be considered. In the transplant setting, the situation is even more complex. Correlates of protection are studied in healthy populations and their relevance in immunosuppressed patients are often unknown. Some studies have shown reduced functionality of reported vaccine responses in immunosuppressed patients. For example, opsonophagocytosis in multiple myeloma patients was poor despite acceptable levels of antibodies shown with ELISA [114]. ELISA assays, mostly used in our studies, are not functional tests, which is a limitation as regards to interpretation of immunity.

A previous study did not find correlations between memory B cell frequencies and antibody levels against tetanus and diphtheria [10]. It is not ruled out that despite seronegativity, persistence of memory B cells, perhaps in combination with cell-mediated immunity, could mediate protection. Seronegativity against diphtheria is reportedly common in healthy populations [87, 115]. In our healthy control group in paper I, consisting of blood donors, healthcare staff, and relatives of healthcare staff, the seronegativity rate was 17%. Despite this, outbreaks of diphtheria are rare.

Most vaccine studies use serological endpoints. How can we be sure that we are protecting our patients through vaccination if there are no studies with clinical endpoints? All clinicians working with immunosuppressed patients during the rollout of the COVID-19 mRNA-vaccines noted a striking drop in

hospitalizations and deaths when vaccinations started. To prove a clinical efficacy, you need a prevalent disease and often a large number of patients. Clinical efficacy has been proven for influenza vaccines post allo-HCT [116, 117]. Influenza was less frequent in the vaccinated groups, but more importantly, vaccination reduced the risk of severe disease, hospital admission, and ICU admission [116, 117].

The field of HCT is rapidly evolving. The use of maintenance therapy, especially in AML, post allo-HCT has increased, such as FLT-3 inhibitors, other multikinase inhibitors, hypomethylating agents and prophylactic DLI, as well as combination strategies in high-risk patients [118, 119]. The use of haploidentical donors has also rapidly increased over the last ten years. In both papers II and IV, the transplant procedures reflect practice used several years ago, which must be held in mind when interpreting the results.

Despite the initial hypotheses that immunosuppression could perhaps mitigate the cytokine storm in COVID-19 disease, mortality among immunosuppressed patients has been consistently high during all waves of the pandemic. It is becoming increasingly evident that the pandemic has had a huge impact on hematological patients, sometimes delaying treatment and diagnosis, and restricting social contact. The current practice in our department is to give three doses of mRNA-vaccine in the primary schedule starting at three months after transplantation. Booster doses are then administered at three months following the third dose and four months following the fourth dose, meaning five doses in the first year, regardless of vaccinations pre-transplantation. Apart from protecting our patients from severe disease, it is important to inhibit ongoing viral replication which poses a risk for other patients in the hospital. Persistent viral shedding, common in immunosuppressed individuals, has also been shown to be a reservoir for new variants of the virus [120].

In the stem cell transplant patient populations, international guidelines have recommended vaccination programs for several years; however, compliance with the program has, in some reports, been low [121]. In a large study of compliance, 38% of patients had received the first series of vaccines (influenza, pneumococcal conjugate, tetanus, diphtheria, polio, pertussis and Hib) at six months and 60% at one year. Reasons identified for withholding vaccines were: (i) relapsed disease, (ii) ongoing treatment for GvHD, (iii) treatment with IVIG, (iv) treatment with rituximab, or (v) inpatient care when vaccines were due. In 25% of the cases, no plausible explanation for withholding vaccines was found. Non-English-speaking individuals, African Americans, and Hispanic patients were less likely to be vaccinated [121]. This study highlights the need for clear vaccine recommendations in complex

clinical scenarios as well as the importance of being more attentive when patient barriers are present. Although we have not specifically studied compliance with vaccination in our studies, we have collected data on the number of vaccine doses and noted that compliance to the vaccination program seemingly is better than in the study reported by Ariza-Heredia *et al*. Perhaps this could be due partly to organizational or cultural factors, as we know there are differences in vaccine hesitancy between countries [122] as well as in how health care is organized or financed, or an improvement over time.

A more personalized strategy for vaccination post allo-HCT has, in some publications, been suggested, using, for example, individual serological testing [123-126]. The problem with this approach, especially in the first years following transplant, is that we do not know if we are measuring waning recipient antibody levels, waning donor levels, and, most importantly, we do not know which levels are protective in the individual patient. Serological testing should be reserved for cases where there is a clinical suspicion of a poor response. Pneumococcal serology, for instance, can be difficult to interpret as the serotypes tested might not be the serotypes in the vaccine. In clinical practice, awaiting serological results can sometimes also delay vaccinations. Another proposed option is to use markers of immune reconstitution. This option also has advantages and disadvantages. It might improve vaccine responses, but requires a carefully organized program. Furthermore, as discussed above, the strength of the response required is often unknown.

For the non-transplanted population, even though several immunization regimens are recommended in international guidelines [43], vaccines are often not administered. The reasons are likely manifold *i.e.*, unawareness and lack of infrastructure for vaccination. Many patients are not followed in the hematology department for more than a few years following treatment, and other patients are rarely followed-up. Whether the responsibility for booster vaccination falls on the patient, the hematologist, or the primary care physician is often unclear. The lack of a national digital vaccine record easily accessible by patients and healthcare providers is also a major shortcoming. In paper I, none of 104 patients reported vaccination against tetanus, diphtheria, or polio after treatment.

How should we approach the patients asking about TBE vaccination after transplantation? The seasonal variation of TBE is one aspect, as the ticks are active during spring and summer and most cases are thus reported in September. Global warming is likely to contribute to an increase in incidence of disease, as the areas where the ticks thrive are expanding [127, 128]. Patients that spend much time in nature are likely at increased risk. Our study, starting

at nine months after transplantation, shows poor responses following the first three doses, which patients should be aware of and take additional precautions prior to completing the full four-dose schedule.

Donor immunizations have been shown to improve the recipient's vaccine responses. Donor vaccination in the unrelated donor setting is generally not feasible. In the related donor-setting, the strategy is partly limited by ethical concerns, although when considering the safety of most vaccines, the risk-benefit ratio is most often beneficial. This strategy has not yet been prospectively studied in the haploidentical setting with T cell depleted grafts. Donor serostatus against CMV in T cell depletion *in vivo* using ATG has been shown to reduce the risk of recurrent CMV reactivation, CMV disease, and death. CMV-specific CD4+ cells were consistently higher in recipients transplanted from seropositive donors, highlighting the importance of transferred T cell immunity [129]. We have not specifically studied donor vaccination but not to mention vaccinations at all to the related donors is probably a missed opportunity for enhancing recipient immunity.

How about pretransplant recipient vaccination? For patients with malignant disease, the window for vaccination pre-transplant is often small, mainly depending on treatment of the underlying disease. The risk of side-effects when vaccinating recipients close to transplant, in worst case by postponing HCT, must also be considered.

The world is increasingly globalized and during 2012 one billion international tourist travels were reported, which is a tripling over the past two decades [130]. Immunocompromised patients do travel, and they do not always seek pre-travel medical advice. Travel to developing countries in Africa and Asia has particularly increased [130].

Is the immunity against diphtheria, tetanus, and polio merely of theoretical interest? Although few cases have been reported in immunosuppressed patients, worldwide diphtheria and tetanus are still a great concern [36, 47]. The polio situation globally has worsened in the past few years with the spread of pathogenic vaccine-derived strains [56]. Polio outbreaks, as well as of other vaccine preventable diseases, are common in conflict zones [131]. The war of Russia on Ukraine has further increased the risks in Europe as Ukraine has the lowest vaccination coverage in Europe [132]. Tick-borne encephalitis is an increasing problem on the Eurasian continent and SARS-CoV-2 is probably here to stay.

These thesis papers, although not practice changing, have added knowledge and created new hypotheses on vaccine responses in patients with hematological diseases, and have described the longevity of these responses. Increased knowledge and awareness of preventing infections will hopefully improve the supportive care for these patients.

6 CONCLUSIONS

- Diphtheria and tetanus booster(s) should be considered, especially in older patients, following chemotherapy for leukemia and lymphoma. Polio immunity is well preserved.

- TBE vaccination after autologous and allogeneic stem cell transplant is safe. Vaccination can be started at nine months following transplant. It seems important to give four doses in the primary schedule, regardless of age.

- Most recipients of allogeneic stem cell transplants respond well to the third dose of COVID-19 mRNA-vaccine. T cell immunity is more affected than antibody responses in patients vaccinated during immunosuppression or early after transplantation.

- Many long-term survivors after allo-HCT lack protection against diphtheria, and booster(s) or serologic testing may be considered. Tetanus immunity is well preserved.

7 FUTURE PERSPECTIVES

Patients with hematological diseases are facing novel therapies that likely entail reduced vaccine efficiency. For example, vaccine responses in CAR-T cell treated patients are not well studied. Guidelines vary with some centers using an ambitious vaccination program post-treatment, similar to the program after allo-HCT. Vaccination pre-CAR-T treatment in combination with vaccination after treatment could be compared to the strategy used in some centers, where vaccination is performed when serum antibody levels are low or at the attending physician's discretion.

HPV vaccination post allo-HCT is likely beneficial given the increased risk of cervical dysplasia in female survivors and the increased incidence of secondary malignancies. The safety profile of the vaccine available is excellent. The best timing for vaccination is unknown and there are no data from male allo-HCT recipients.

Rituximab had been used for many years before the data on low or absent immune responses to vaccination following treatment were published. Vaccine responses in patients treated with newer therapies, such as ruxolitinb, ibrutinib, new generation TKIs, and daratumumab, are not well established. Likewise, vaccine responses in patients on maintenance FTL3-inhibition post allo-HCT have not yet been reported.

Data on vaccine responses in patients receiving haploidentical grafts, with the use of intensive T-cell depletion are scarce and prospective trials are needed.

All cases of tetanus and diphtheria diagnosed in Sweden are reported to the Swedish Public Health Agency. Some basic statistics have been published on its website but a more thorough review of these cases is not available. A collaboration has now been initiated to review all cases reported in Sweden for the past 10 years, with the aim to describe clinical presentations, vaccine-status, number of immunosuppressed patients etc.

In national treatment protocols for hematological disease, there should be evidence-based guidelines for vaccination for each particular disease. In the national CLL (chronic lymphocytic leukemia) guidelines, a practical schedule is suggested, leading to an increased likelihood of vaccines being given. Many other guidelines in hematology will likely benefit from more precise vaccination advice.

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