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STUDIES OF SPECIFIC IMMUNITY AGAINST VIRAL INFECTIONS AFTER STEM CELL TRANSPLANTATION

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Stockholm 2007

Published by Karolinska Institutet. Printed by [name of printer]

ISBN 978-91-7357-339-9

A conclusion is simply the place where one got tired of thinking

Albert Einstein

To my family &
Памяти любимой Люды посвящается

SUMMARY

Recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT) experience a prolonged period of immune deficiency, resulting in significant morbidity and mortality from the infections. Viruses such as cytomegalovirus (CMV) and influenza virus are frequent causes of infectious complications following allo-HSCT.

CMV is one of the most frequent pathogens causing life-threatening complications after HSCT. Factors like patient age, donor and recipient serostatus, type of conditioning, grade of graft versus host disease (GVHD), CMV viremia and number of CMV reactivations are all factors influencing virus reactivation and contributing to the development of the disease. Although CMV infection can be controlled by pre-emptive treatment based on sensitive diagnostic tests, there is a risk of unnecessary over-treatment, resulting in development of antiviral resistance and late onset of CMV disease. In our two CMV studies we aimed in paper I to use the immunological monitoring of CMV-specific responses to correlate transplantation factors to the recovery of cellular immunity in HSCT recipients, intending to understand how those factors influence the CMV-specific T cell reconstitution. The aim of paper II was to use the CMV-specific immunological monitoring to target antiviral therapy to patients at the highest risk of developing CMV disease. By correlating the intracellular interferon-gamma (IFN-γ) production by CMV-specific T lymphocytes with transplant factors (paper I) we observed that (a) CMV-specific T cell response is associated with a lower rate of CMV replication; (b) reduced intensity conditioning results in improved early T cell reconstitution; and (c) the recovery of CMV-specific immunity might be delayed in patients with CMV disease. Using the same method of monitoring CMV-specific immunity in the second study (paper II) we found that 25% of patients with late CMV DNAemia, who had CMV-specific immunity and lacked other risk factors of CMV disease, could be spared pre-emptive therapy without developing CMV disease or need of anti-viral therapy.

Annual vaccination is the main way to prevent influenza and its complications, but it is less effective in immunocompromised patients compared to healthy individuals. Since most previous studies of vaccination efficacy investigated the humoral immune response, we aimed to develop an Elispot technique for measuring the influenza-specific cellular response as a marker of vaccine responsiveness (paper III). We measured the IFN- γ production by T cells pulsed with influenza peptides in healthy volunteers and HSCT recipients. Influenza vaccination elicited strong cell-mediated immune response in the group of healthy volunteers and we concluded that Elispot is a sensitive and specific assay for measuring cell-mediated immunity to vaccination. To further explore the responses to vaccination, we applied two Elispot methods to characterize both the cell-mediated and humoral responses to influenza vaccination (study IV). The cell-mediated responses were strong both in the healthy volunteers and in the transplanted patients. However, we found a big difference in the number of influenza-specific antibody secreting cells between healthy controls and HSCT patients, both before and after vaccination, supporting the previously shown weak ability of transplanted patients to respond to vaccination with a protective antibody response.

In conclusion, routine immunologic monitoring will be helpful in guiding virological monitoring and therapeutic decisions in HSCT recipients. The inactivated split influenza vaccination elicits cell-mediated and humoral immune responses in HSCT recipients. In spite of that, the effectiveness of the vaccination in immunocompromised patients is low, which highlights the need of more immunogenic vaccine formulations for this population.

LIST OF PUBLICATIONS

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

I. **Avetisyan G**, Larsson K, Aschan J, Nilsson C, Hassan M, Ljungman P
Impact on the cytomegalovirus (CMV) viral load by CMV-specific T cell immunity in recipients of allogeneic stem cell transplantation. *Bone Marrow Transplantation*, 2006 Nov, 38(10):687-92

II. **Avetisyan G**, Aschan J, Hagglund H, Ringden O, Ljungman P
Evaluation of intervention strategy based on CMV-specific immune responses after allogeneic SCT. *Bone Marrow Transplantation*, 2007 Aug 27; [Epub ahead of print]

III. **Avetisyan G**, Ragnavolgyi E, Toth GT, Hassan M, Ljungman P
Cell-mediated immune responses to influenza vaccination in healthy volunteers and allogeneic stem cell transplant recipients. *Bone Marrow Transplantation*, 2005 Sep, 36(5):411-5

IV. **Avetisyan G**, Aschan J, Hassan M, Ljungman P
Studies of immune responses to seasonal influenza vaccination in healthy volunteers and in patients after stem cell transplantation. *Manuscript submitted*

CONTENTS

LIST OF ABBREVIATIONS

APC - antigen presenting cells
ASC - antibody-secreting cell
ATG - anti-thymocyte globulin
BM - bone marrow
CDV - cidofovir
CMV - cytomegalovirus
CsA - Cyclosporine
CTL - cytotoxic T-lymphocite
DC - dendritic cells
EBV - Epstein-Barr virus
ELISA - Enzyme-linked immunosorbent assay
Elispot - Enzyme-linked immunospot
FITC - fluorescein
FOS - foscarnet
G-CSF - granulocyte-colony stimulating factor
GCV - ganciclovir
GVHD - graft-versus-host disease
HA - hemagglutinin
HIA - hemagglutination ilhibition assay
HLA - human leukocyte antigen
HSC - Hematopoietic stem cells
HSCT - Hematopoietic stem cell transplantation
HSV - herpes simplex virus
IFN - interferon
Ig - immunoglobulin
IL - interleukin
mAb - monoclonal antibodies
MBV - maribavir
MC - myeloablative conditioning
MHC - major histocompatibility complex
NA - neuroaminidase
NK - natural killer cells
PBL - peripheral blood lymphocytes
PCR - polymerase chain reaction
PerCP - peridinin-chlorophyll-protein complex
PRR - pattern recognition receptor
RIC - Reduced intensity conditioning
RSV - respiratory syncytial virus
TBI - total body irradiation
TcR - T cell receptor
Th - T helper cells
TLR - Toll-like receptor
VZV - varicella zoster virus
EBMT - European Group for Blood and Marrow Transplantation

1 INTRODUCTION

1.1 STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) is a complex therapeutic procedure in the field of hematology and oncology. It is most often performed on the patients with hematological malignancies (*e.g.* leukemia, lymphoma, and multiple myeloma), solid tumors (*e.g.* sarcomas, neuroblastoma, breast or testicular cancer) and nonmalignant conditions (*e.g.* aplastic anemia, autoimmune disorders, myelodysplastic syndrome, immunodeficiency syndromes, and inborn errors).

The use of bone marrow for the treatment of various forms of anemia or leukemia was attempted as early as 1891. A first “true” transplant attempt, for severe aplastic anemia, was reported in 1939 (Osgood *et al.* 1939). Early clinical attempts were generally unsuccessful, mostly due to a lack of knowledge regarding the histocompatibility antigen system and appropriate supportive care. Observations of atomic bomb victims in Japan, August 1945, generated great interest in the effects of body exposure to high doses of irradiation, and in the late 1940s investigators began to experiment with total body irradiation (TBI) and later with cytotoxic drugs in animal models. It was in these animal models that most of the problems related to transplantation were first recognized. Aside from the toxicity related to the conditioning regimen, problems including hemorrhage, infections, electrolyte and fluid imbalance, and, most importantly, "secondary disease", subsequently called graft-versus-host disease (GVHD) were recognized (Deeg *et al.* 1999).

The modern era of hematopoietic SCT spans approximately three decades and was pioneered by E. Donnall Thomas mostly working at the Fred Hutchinson Cancer Research Center starting in the 1950s through the 1980s (Thomas *et al.* 1957; Thomas *et al.* 1975). During these years indications have been broadened, a variety of conditioning regiments have been developed, and not only marrow but also hematopoietic stem cells derived from peripheral blood, cord blood or fetal liver are used for the transplantation. Stem cell transplants are used as a rescue procedure to (a) re-supply the bone marrow when it has been damaged by disease, chemotherapy, or radiation; (b) as a vehicle for gene therapy; and (c) as a means of immunotherapy.

1.1.1 HSCT procedure

HSCT involves the harvesting of HSCs from a donor and infusion of these cells into a conditioned recipient. The HSCs are usually infused into the blood stream and then migrate into the bone marrow space where they can expand and start to produce blood cells. The HSCT can either be allogeneic or autologous. In allogeneic transplantation, the HSCs are harvested from a donor, which can be an HLA-matched sibling, HLA-matched unrelated donor, or a mismatched family member (Hansen *et al.* 1980; Powles *et al.* 1983; Beatty *et al.* 1985). Currently more than 10 million volunteer unrelated donors exist in several donor registries all over the world. Autologous transplants are often used as the primary choice of therapy for example, in patients with multiple myeloma or lymphoma, but are sometimes performed as an alternative to allogeneic transplants in patients without histocompatible donors (Stuart 1993; Bjorkstrand *et al.* 1995).

1.1.2 Stem cell sources

Since the first applications of hematopoietic transplantation, **bone marrow** (BM) has been the most frequently used stem cell source. BM harvesting is usually accomplished in one relatively fast collection from a stem cell donor's posterior iliac crest under general anesthesia. The clinical importance of mobilized **peripheral blood** as a stem cell source has increased during the past few years and this source of HSCs is now used in the majority of transplantations. The cells are mobilized from marrow to blood in greater numbers by injecting the donor with a cytokines such as granulocyte-colony stimulating factor (G-CSF) or granulocyte-macrophage-colony stimulating factor (GM-CSF) a few days before the cell harvest (Amos *et al.* 1995). The cells are collected by leukapheresis. The use of peripheral blood stem cells (PBSCs) for treatment of some hematological disorders was compared to BM transplantation and resulted in shorter times to neutrophil and platelet engraftment, but is associated with increased rates of chronic GVHD (Cutler *et al.* 2001). **Umbilical cord blood** is attractive as a stem cell source for the following reasons: (a) it is a resource that is usually being discarded; (b) it is rich in stem cells of fetal origin with considerable proliferative potential; (c) the stem cells have reduced immuno-competence, making them less likely to cause GVHD and therefore greater HLA-mismatches are permissible (Barker *et al.* 2002); (d) the cells are usually more readily available. The disadvantages of using cord blood include the relatively small size of collection resulting in a longer time to engraftment and thereby in an increased risk for infections after HSCT (Rocha *et al.* 2000; Barker *et al.* 2001).

1.1.3 Conditioning therapy

Shortly before the transplantation of hematopoietic stem cells, patients are given a course of chemotherapy, often with the addition of total body irradiation. This treatment is called conditioning and has at least two functions: immunosuppression of the recipient and reduction in the number of unwanted (usually malignant) cells. Chemotherapy drugs and irradiation damage DNA in the cell nucleus and rapidly kill dividing cells by triggering apoptosis, and since bone marrow cells divide frequently, they are particularly sensitive to this treatment. Two types of conditioning regiments are commonly employed: myeloablative and reduced intensity (or non-myeloablative) regiments. **Myeloablative** regiments include a combination of high-dose chemotherapy, usually cyclophosphamide, with TBI (Thomas *et al.* 1975) or busulphan (Santos *et al.* 1983). When the donor is unrelated or the underlying disease is nonmalignant, anti-thymocyte globulin (ATG) is commonly added to the conditioning therapy in order to avoid graft rejection (Storb *et al.* 1994; Remberger *et al.* 2001) and reduce the risk for GVHD (Remberger *et al.* 2004). **Reduced intensity** regiments are a newer treatment approach using lower doses of chemotherapy and irradiation, which mechanism of action mainly rely upon a graft-versus-malignancy effect mediated by donor-origin T cells (Antin 2003). Reduced intensity conditioning (RIC) offers the possibility of stem cell transplantation for patients not easily treatable with myeloablative conditioning (*e.g.* elderly patients, patients with medical co-morbidities precluding the use of standard conditioning) and is also used for some indications for which intensive chemo-radiotherapy confers more risks than benefits, for example solid tumors or non-malignant disorders. Reduced intensity regiments either use combinations of chemotherapy drugs such as fludarabine, together with ATG, busulfan, or cyclophosphamide (Georges *et al.* 2003; Sandmaier *et al.* 2007) or low dose TBI. RIC is associated with a decreased risk for early infectious complications after HSCT (Mohty *et al.* 2004; Ohnishi *et al.* 2005) due to a shorter duration of neutropenia, less mucosal membrane damage, and possible less damage of the thymus allowing better T cell reconstitution. However, this early reduction in the

rate of infectious complications observed during the first 3 months might be balanced by a higher risk later after RIC-HSCT, possibly due to a higher rate of chronic GVHD necessary to achieve an immune mediated tumor control (Busca *et al.* 2003; Maris *et al.* 2003; Jimenez *et al.* 2007). Several questions remain to be answered regarding the benefit of RIC in terms of immune reconstitution after transplantation.

1.1.4 GVHD

With a successful transplantation of HSCs and replacement of the recipient's lympho-hematopoietic system by donor-derived cells, these "new" immune cells can attack the recipient's tissues and cause an inflammatory disease – graft versus host disease. Different cell populations and cytokines, donor and host components are involved in the pathophysiology of GVHD: conditioning of the patient results in tissue damage and the release of pro-inflammatory cytokines (in acute GVHD T helper (Th)-1 cytokines, in chronic GVHD Th2-cytokines). These enhance activation of donor-derived natural killer (NK) cells and maturation of cytotoxic T cells (CTL), thereby causing further host tissue damage and clinical manifestations of GVHD (Ferrara *et al.* 2003). **Acute** GVHD (aGVHD) typically occurs in the first three months after transplantation and targets organs with epithelial surfaces – the skin, the gastro-intestinal tract, the biliary system, and the lungs (Glucksberg *et al.* 1974; Zeiser *et al.* 2004). Acute GVHD may be graded according to the classical Glucksberg-Seattle criteria (GSC) as following: overall grade (skin-liver-gut) with each organ staged individually from a low of I to a high of IV (Glucksberg *et al.* 1974). Patients with grade IV GVHD usually have a poor prognosis. **Chronic** GVHD (cGVHD) develops 3 month or later post-transplant and occurs in about 30 to 70% of all long-term survivors after allo-HSCT (Shulman *et al.* 1978; Sullivan *et al.* 1981; Bensinger *et al.* 2001; Mohty *et al.* 2002). Although previous aGVHD is the greatest risk factor for the development of cGVHD, 25-30% of cGVHD appears *de novo* (Lee 2005). The main target of cGVHD is the connective tissues (e.g. skin and exocrine glands), and the clinical symptoms in some respects resemble collagen vascular diseases such as scleroderma, systemic lupus erythematosus and rheumatoid arthritis (Atkinson *et al.* 1989). **Prophylaxis** of GVHD usually starts one day before HSCs infusion and continues for 3-12 month after HSCT. Commonly used regimens consist of combination of Cyclosporine (CsA) with Methotrexate or corticosteroids; however other drugs such as tacrolimus or sirolimus might also be used. **Treatment** of GVHD mainly consists of a combination of corticosteroids and increased and/or prolonged CsA. Other treatments include ATG, extracorporeal PUVA treatment (photosensitization with psoralen and UVA irradiation), tacrolimus, sirolimus, and more recently monoclonal antibodies (mAb) against CD3 (OKT3), CD52 (alemtuzumab), CD25 (antibody to IL-2 receptor) or cytotoxic T lymphocyte antigen (CTLA)-4 (Zeiser *et al.* 2004). If the GVHD is severe and requires intense immunosuppression involving steroids and additional agents to come under control, the patient may develop severe and potentially lethal infections as a result of the immunosuppression.

1.2 GENERAL IMMUNOLOGY

The contents of this section are mostly based on the following textbooks: *Immunology* (Kuby 1994), *Immunobiology: The Immune System in Health and Disease* (Janeway 1999), *Immunology* (Roitt *et al.* 1998), *Viral Pathogenesis and Immunity* (Nathanson 2002) and *Influenza Report* (Kamps *et al.* 2006).

1.2.1 The Immune System

Any organism is constantly being exposed to infectious agents and in most cases is able to resist these infections, owing to the immune system. The immune system is a remarkable collection of defense mechanisms within an organism that protects it from pathogenic invaders and tumor cells. It detects a wide range of pathogens and distinguishes them from the organism's own normal cells and tissues. The recognition and neutralizing of the microorganisms are activities that mount an immune response: once a foreign organism is recognized, the immune system enrolls a variety of cells and molecules to eliminate or neutralize the pathogen. Infection with an organism does not necessarily mean diseases, since the immune system in most cases will be able to eliminate the infection before disease occurs. Disease occurs only when the bolus of the infection is sufficient, when the virulence of the invading organism is large or when immunity is compromised. Although the immune system has unconditional beneficial effects, there can be detrimental effects as well. A hyperactive immune system attacking the organism's normal tissues causes autoimmune diseases, such as rheumatoid arthritis, lupus erythematosus, multiple sclerosis etc. On the contrary, immunodeficiency, which might be a result of genetic diseases, caused by pharmaceuticals or an infection, occurs when the immune system is less active than normal, and results in recurring and life-threatening infections.
The immune system consists of two major subdivisions, the *innate* or nonspecific immune system and the *adaptive* or specific immune system (Table 1). Although these two arms of the immunity cooperate in the production of a protective response, they differ in a number of ways. The adaptive immune system requires some time to react to an invading organism, whereas the innate immune system includes defenses that, for the most part, are constitutively present and ready to be mobilized upon infection. Second, the adaptive immune system is antigen specific and reacts only with the organism that induced the response. In contrast, the innate system is not antigen specific and reacts equally well to a variety of organisms. Finally, the adaptive immune system demonstrates immunological memory. It "remembers" that it has encountered an invading organism and reacts more rapidly on subsequent exposure to the same organism. In contrast, the innate immune system does not demonstrate immunological memory.

Table 1. Innate and adaptive immunities

Innate immunity	Adaptive immunity
Antigen-independent response	Antigen-dependent response
Direct maximal response	Interval between exposure and maximal response
Not antigen-specific	Antigen-specific
No immunologic memory	Immunologic memory

1.2.2 The innate immunity

The elements of the innate immune system include anatomical, humoral and cellular barriers. **Anatomical barriers** consist of (a) mechanical factors – the skin and mucous membranes, that prevent the entry of infectious agents; (b) chemical factors - fatty acids in sweat inhibit the growth of bacteria, lysozyme and phospholipase in tears, saliva and nasal secretions breakdown the bacterial membranes, the low pH of sweat and gastric secretions prevents growth of bacteria, surfactants in the lung act as opsonins, promoting phagocytosis; and (c) biological factors - the normal flora of the skin and gastrointestinal tract prevent the colonization of pathogenic bacteria by secreting toxic substances or by

competing with them for nutrients or attachment to cell surfaces. Once infectious agents have penetrated tissues, another innate defense mechanism activates, that is acute inflammation. **Humoral barriers** play an important role in inflammation and include soluble factors that are found in serum or are formed at the site of infection. These factors are: (a) complement system - a complex biochemical cascade containing over 20 different proteins that can be activated through different pathways, which leads to the activation of phagocytosis and lysis of bacteria; (b) lysozym – an enzyme that breaks down the cell wall of bacteria; and (c) cytokines - interferons (IFN) - the proteins that can limit virus replication in infected cells. **Cellular barriers** consist of cells that have germline encoded receptors termed pattern recognition receptors (PRR), which recognize molecular components of infectious agents, most of which are shared by large groups of pathogens and none of which are produced by the host. One important family of PRRs is the Toll-like receptor (TLR) family (Medzhitov *et al.* 1997). Ten human homologs of TLRs have been identified so far (Akira *et al.* 2001). TLRs recognize molecules derived from infectious agents once they have broken physical barriers and activate immune cell responses. TLRs are involved in the cytokine production and cellular activation in response to infection, but do not play a significant role in the adhesion and phagocytosis of microorganisms. The following cells of the cellular barrier are the main line of defense in the non-specific immune system that contribute to the direct elimination of the infectious agents: (a) neutrophils - are recruited to the site of infection where they phagocytose pathogens and kill them intracellularly; (b) macrophages - also function in phagocytosis and intracellular killing of microorganisms, are capable of extracellular killing of infected cells, and act as antigen-presenting cells, which are required for the induction of specific immune responses; (c) eosinophils - are effective in killing certain parasites; and (d) natural killer (NK) cells – cells of lymphoid origin that contribute to the innate immunity through the nonspecific lysis of virus infected and tumor cells and production of cytokines such as interferon-γ, tumor necrosis factor-α, interleukins and GM-CSF. The activity of NK cells is dependent on signaling through activating and inhibiting receptors, many of which interact with MHC class I molecules (Ciccone *et al.* 1992; Moretta *et al.* 1993; Smyth *et al.* 2002). Target cytolysis either is receptor-mediated, or occurs via the perforin-granzyme pathway (Lieberman 2003; Screpanti *et al.* 2005).

Many genes and proteins of the immune system are polymorphic. Although disease can result from mutations in single genes, most of the common diseases are due to complicated interactions between an unknown number of genes and environmental factors (Geraghty 2002). Functional polymorphisms might be expected in many genes that influence immunity. The genes of innate immunity, although highly conserved across a wide range of species, demonstrate substantial inter-individual variability predominantly in the form of single nucleotide polymorphisms (SNPs) (Lazarus *et al.* 2002). Recent insights into the complex mechanisms of human innate immunity suggest that genetic polymorphism in genes encoding its components may play a role in the development of some common diseases, which have an inflammatory component. Several groups have carried out genome-wide screens in a search for genetic linkage to a number of diseases. They found, that genetic polymorphism may play a role in determining susceptibility to a range of chronic diseases with inflammatory component, such as asthma, eczema, allergy, diabetes, chronic obstructive pulmonary disease, and inflammatory bowel disease (Lazarus *et al.* 2002; Cookson 2004). Whole-genome association strategy offers a new approach to gene discovery unbiased with regard to assumed functions or locations of causal variants. Using this approach were identified polymorphisms that validate genetic risk factors for a common disease like type II diabetes (Saxena *et al.* 2007), or explain why some people establish and maintain effective control of certain infections and others do not (Fellay *et al.* 2007). There are studies showing that the natural polymorphism of CMV contributes to development of anti-viral drug resistance (Fillet *et al.* 2004), and may counteract protective

responses by immune evasion (Garrigue 2007). Common human polymorphisms of surfactant protein D have been found in many human populations and are associated with an increased risk of certain infections, in particular with increased susceptibility to influenza A infection (Hartshorn *et al.* 2007). This novel field of immune genetic investigation might provide new insights in disease etiology, necessary for the development of new treatments for numerous diseases.

1.2.3 The adaptive immunity

The adaptive immune system, unlike innate immunity, is very specific and capable of recognizing and eliminating definite pathogens. The following major functions characterize adaptive immunity: the recognition of specific self/non-self antigens during the process of antigen presentation, the generation of diversity in its recognition of specific responses against billions of uniquely different antigens, and the ability to develop immunological memory.

1.2.3.1 Functions of humoral and cell-mediated immune responses

There are two fundamental branches of the adaptive immune system: humoral immunity and cell-mediated immunity, which are changeable and can adapt to better attack the invading antigen. The **cell-mediated** arm of the immune system is based on antigen recognition, cytokine production and cytotoxicity of infected cells; and involves the generation of various effector immune cells, both specific (Th cells and CTLs) and non-specific (macrophages, neutrophils, eosinophils, and NK cells). Cell-mediated immunity is directed against intracellular pathogens (most effective against viruses), tumor cells, and foreign grafts. The **humoral** arm of the immune system confers immune response by the secretion of antibodies specific for antigens on the surfaces of invanding pathogens (viruses and bacteria). The antibodies inactivate pathogens by binding to their specific antigens and causing: (a) complement fixation (cause holes to form cell lysis); (b) neutralization (prevent attachment); (c) agglutination (clumping); and (d) precipitation (forcing insolubility and settling out of solution) of antibody-antigen products. For a schematic view see figure 1.

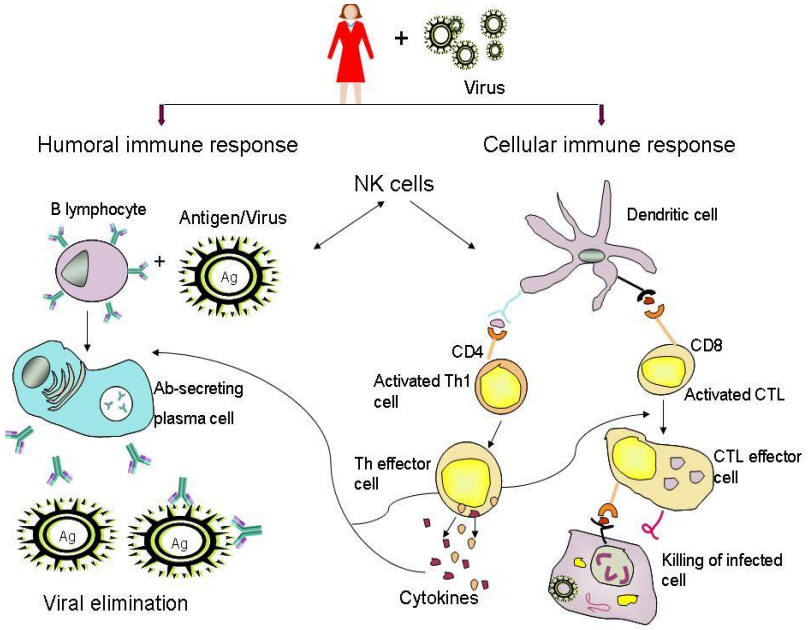

Figure 1. The humoral and cellular immune responses to virus infection. The humoral branch of the immune system comprises B-lymphocytes *(left)*, which after interaction with virus differentiate into antibody-secreting plasma cells. The cellular response *(right)* starts with antigen presentation via MHC I *(black)* and II *(blue)* molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8).

The main cell types of adaptive immunity are B and T lymphocytes and antigen presenting cells (APC).

B lymphocytes (B cells) represent approximately 12-15% of the normal peripheral blood lymphocytes, 50% of the spleen and 75% of bone marrow lymphocytes in normal individuals. These cells are derived in bone marrow from the pluripotential hemopoietic stem cells. B cells are involved in the humoral immune response by producing of antibodies to invading antigen. At the earliest developmental stages in the bone marrow, B cells develop a unique receptor - antigen-binding surface antibody (immunoglobulin), enter the circulation and travel around the blood and lymph through tissue and lymphoid organs. Once a naïve B cell meets an antigen recognized by the surface receptor it can further differentiate into (a) antibody secreting plasma cells - B cells that do not express the surface receptor, live for only few days, but secret vast amount of antibodies during this time; and (b) memory cells - B cells that remain resting in the bone marrow, express surface receptor and ensure that a more specific and faster response will be mounted following the re-infection with the same pathogen. Activated B cells produce 5 classes of immunoglobulins (Ig): IgA (about 15% of total antibody count), IgG (75%), IgM (about 10%), IgE (less than 1%) and IgD (less than 1%), based on differences in the amino acid sequences in the constant region of the heavy chains. The primary immune response consists of a transient IgM response, and follows by constantly elevated levels of IgG – the most abundant immunoglobulin. IgA antibodies are also secreted immediately after the exposure to an antigen, circulate for longer time than IgM, but significantly shorter than IgG. IgE is involved in allergic reactions and also plays a role in parasitic helminthes diseases. The role of IgD is still uncertain.

T lymphocytes (T cells) also arise in bone marrow from the pluripotent hemopoietic stem cells, but then migrate to the thymus gland for maturation. Approximately 70-80% of normal peripheral blood lymphocytes are T lymphocytes. During their maturation T cells start to express the T cell receptor (TcR) composed of two glycoprotein chains called α- and β- TcR chains. Unlike B cell receptor, which directly recognizes antigen, TcR requires the antigen to be presented in association with cell-membrane proteins on the surface of an antigen presenting cell known as major histocompatibility complex (MHC). There are two classes of MHC molecules, called MHC class I and MHC class II. The most immature T cells have a TcR associated with a protein called CD3. In the thymus, the cells then develop two other markers, CD4 and CD8. These cells are referred to as double positive. Eventually the cells lose either CD4 or CD8 to become one of the functional subsets. The cells with a CD4 marker are called *T helper cells* (Th) and bind to class II MHC-peptide complexes, while the CD8 positive cells are called *cytotoxic T cells* (CTL) and bind to the class I MHC-peptide complexes. Th cells and CTLs perform very different functions in the immune system. Upon activation Th cells are capable of differentiating either into Th1 or Th2 cell, and release distinctive patterns of cytokines, which play a very important role in the mounting of both cell-mediated and humoral immune responses. Th1 cells bind to antigen presented by APCs and start to release cytokines that attract other cells to the area. The result is inflammation: the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material. Th2 cells bind to antigen presented by B cells, which results is the development of clones of plasma cells secreting antibodies against the antigenic material. The main role of the CTLs, as the name suggests, is in recognizing and destroying cells that have been altered or infected. They enter the cell cycle and go through several rounds of mitosis (clonal expansion) followed by differentiation into effector (killer) cells. They do not secrete many cytokines, but their differentiation includes forming a large number of lysosomes pucked with proteins with cytolitic activity, such as perforin and granzymes. Most of these CTLs will undergo apoptosis when they have done their job, but

some (especially those that have received help from Th cells) will become memory cells - long-lived cells poised to respond to the cognate antigen in the case of re-infection.

Another important subset of T cells is *regulatory T cells* (T_{reg} cells), formerly known as suppressor T cells. T_{reg} cells are CD4+ T cells co-expressing a transmembrane protein CD25 and transcription factor Foxp3. This specialized subpopulation of T cells is crucial for the maintenance of immunological homeostasis. T_{reg} cells suppress activation of the immune responses after they have successfully fought invading pathogens and prevent pathological self-reactivity, i.e. autoimmune disease (Shevach 2002; Bluestone *et al.* 2003). T_{reg} cells, if activated, begin to secret large amounts of powerful immunosuppressive lymphokines (interleukin 9, interleukin 10 and transforming growth factor-beta (TGF-β)), thus inhibiting Th1 help for cell-mediated immunity, Th2 help for antibody production, and activity of CTLs. The higher frequencies of T_{reg} cells in CMV-stimulated compared with unstimulated PBMC from HIV-infected and healthy subjects suggest that these cells expand in response to CMV antigenic stimulation and may contribute to the lack of functional immunity (Jesser *et al.* 2006). *Gamma delta (γδ) T cells* represent the smallest subset of T cells found in the gut mucosa, within a population of intraepithelial lymphocytes. TcR of γδ T cells consist of one γ-chain and one δ-chain. γδ T cells are not MHC restricted and seem to be able to recognize whole proteins rather than requiring peptides to be presented by MHC molecules on antigen presenting cells. Recent studies suggest that γδ T cells participate in the initiation of the anti-viral immune response by producing pro-inflammatory cytokines, such as IFN-γ, since they can at least partly escape CMV immune evasion (Dechanet-Merville 2007; Roumanes 2007).

Antigen presenting cells are a functionally defined group of cells which are able to take up antigens and present them to T lymphocytes in a recognizable form. Although different cells can do this the cells which are most efficient, the so-called "professional antigen presenting cells", are *dendritic cells* (DC). DCs are heterogeneous in subtype, developmental origin, localization and function. DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Immature DCs constantly inspect the surrounding environment for pathogens such as viruses and bacteria. This is done through TLRs, which recognize specific chemical signatures found on subsets of pathogens. Once they have come into contact with such a pathogen, they become activated into mature DCs. Within different tissues, activated DCs become highly effective in the taking up and processing of antigens and their subsequent presentation on the cell surface linked to MHC molecules. Upon appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present antigen to T cells and induce an immune response. Since DCs play a crucial role in host-pathogen interactions they are of great scientific and clinical interest. The ability to culture these cells in vitro stimulated interest in dendritic cell immunotherapy – a potential method of treatment of chronic infections and a variety of malignancies. Its advantage is the induction of cellular-based immune response with potentially better cytotoxicity and constant effector cell effect. The potential efficacy of adoptive transfer of ex-vivo generated antigen-specific T cells was demonstrated by the regression of immunoblastic lymphoma in allo-HSCT recipients after the infusion of EBV-specific CTLs (Rooney *et al.* 1995) and by the successful treatment of CMV infection after allo-HSCT (Kleihauer *et al.* 2001; Grigoleit *et al.* 2007).

1.2.4 Cytokines

Cytokines are low-molecular weight proteins secreted by a wide variety of cell types, which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced *de novo* in response to an immune stimulus. They generally (although not always) act over short distances and short time period and at very low concentration. They function by binding to specific receptors on the membrane of target cells, which then signal the cell via secondary messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different target cell types (pleiotropy**)**. Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically (two or more cytokines acting together) or antagonistically (cytokines causing opposing activities). Schematic view of cytokine production is presented in figure 2.

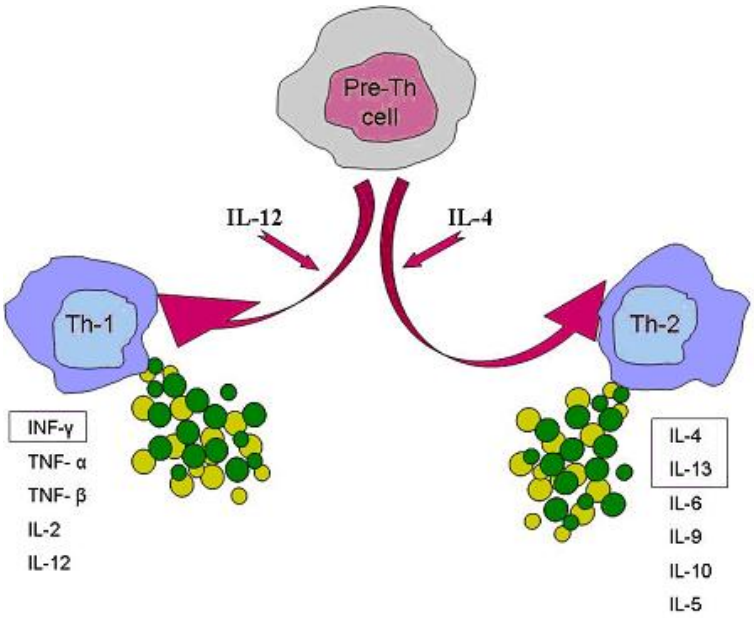

Figure 2. Th cells are able to differentiate from a precursor (preTh) cell into 2 apparently different types called Th1 and Th2. These subtypes differ in their cytokine secretion.

Cytokines, as a part of the innate immunity, favor the development of antiviral immune responses. Cytokines also influence the adaptive immune response and disease manifestation. In the following, I will give a short introduction on the cytokines that were studied in this project.

Interferons: Inferferons (IFN) are a group of cytokines produced by white blood cells, fibroblasts, or T cells as part of an immune response to a viral infection or other immune trigger. The IFN system is the most important nonspecific defense mechanism against viral infection. Of their function, the most important are inhibition of the viral (and some other parasites) replication, inhibition of certain tumor cell growth, and activation of the immune system. There are three types of interferons: alpha (α), beta (β), and gamma (γ). IFN-α and IFN-β, which are grouped together as type I interferon, are produced by white blood cells and fibroblasts, induce antiproliferative and antiviral activity, and increase expression

of class I MHC on cells. *IFN-* γ (or type II interferon) is produced by activated T cells (Th1 and CTL) and NK cells and, following production, activates CTLs and increases the cytotoxicity of NK cells. Its major functions are to activate macrophages and to upregulate the expression of class I and class II MHC molecules on APCs. IFN- γ secreted by Th1 cells has a cross regulatory role in controlling Th2 function, and induces immunoglobulin class switch.

Interleukins: Interleukins are a group of cytokines that are produced by a wide variety of bodily cells (*i.e*. Th1 and Th2 cells, macrophages, stromal cells of bone marrow and thymus, epithelial and endothelial cells) and actively involved in inflammation and immunity. *IL-4*: It has many biological roles and particularly down-regulates Th1 activity, thereby directing the immune response toward B lymphocytes. On resting B cells, and on macrophages, IL-4 increases MHC class II expression. On activated B cells, it stimulates their proliferation and differentiation, and induces an antibody class switch. Moreover, IL-4 induces differentiation of naïve T cells to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4. The cell that initially produces IL-4 and induces Th0 differentiation has not yet been identified. *IL-13* cytokine is secreted by many cell types, but particularly by Th2 cells. The functions of this cytokine are quite similar and overlap considerably with those of IL-4. The main target cells of IL-13 are macrophages. It also stimulates gamma-interferon production by NK cells, enhancing the effect of interleukin-2.

1.2.5 Anti-viral immunity

Resistance to viruses and recovery from viral infections depend on the interactions occurring between virus and host. The defenses mounted by the host may act directly on the virus or indirectly on virus replication by altering or killing the infected cell. The non-specific host defenses function quickly after an encounter with the virus and prevent or limit infection, while the specific host defenses function after infection in recovery from viral infection. Although the host defense mechanisms involved in a particular viral infection will vary depending on the virus, dose and portal of entry, below I have summarized some general principals of virus-host interactions, focusing on the immune responses to CMV and influenza virus.

Within their life cycle viruses have a relatively short extracellular period prior to infecting the cells, and a longer intracellular period during which they undergo replication. The immune system has both non-specific and specific effector mechanisms, which can attack the virus during both of these periods.

Non-Specific Mechanisms*: Interferons*: Infection of cells by CMV or influenza virus directly stimulates the production of type I interferons, leading to the induction of an "antiviral state" in the cells characterized by inhibition of both viral replication and cell proliferation. *Natural Killer Cells:* NK cells have the ability to recognize and lyse virally infected cells. Although not showing antigen specificity, they clearly have some selectivity in targeting "abnormal" cells for lysis, thanks to the receptors of yet unclear nature. The main advantage that NK cells have over antigen-specific lymphocytes in antiviral immunity is that there is no "lag" phase of clonal expansion for NK cells to be active as effectors. Thus NK cells may be effective early in the course of viral infection, and may limit the spread of infection during this early stage, while antigen-specific lymphocytes are being recruited and clonally expanded.

Both the humoral and cell-mediated arms of the immune response play a role as **specific effector mechanisms** in antiviral immunity. *Antibodies:* During the course of a viral infection, antibodies are most effective at an early stage, before

the virus has gained entry to its target cell. In this respect, antibodies are relatively ineffective in primary viral infections, due mainly to the lag phase in antibody production. The most effective type of antiviral antibody is "neutralizing" antibody able to bind to the virus, usually to the viral envelope or capsid proteins, and thereby blocking the virus from binding and gaining entry to the host cell. *T cells:* The principal effector cells involved in clearing established viral infections are the virus specific CD8+ CTLs. Since the recognition of antigen by CD8+ T cells is restricted by class I MHC molecules, which are expressed on almost all somatic cells, virtually any cell infected by virus can be a target cell for antigen specific CTLs. Th cells are involved in generation of CTLs and in assisting B cells to produce antibodies. In addition, cytokines secreted by T cells can recruit and activate macrophages and NK cells thereby mobilizing an intensive attack in the virus.

1.3 IMMUNE RECONSTITUTION AFTER HSCT

For a transplantation of HSC to be successful, a reasonably complete reconstitution of the immune system needs to occur. The patients experience an immune system dysfunction before and after engraftment that might continue for many months, resulting in significant morbidity and mortality from bacterial, viral, and fungal infections. Delay of immune reconstitution can also increase the risk for relapse of malignant disease. Following the myeloablative therapy and stem cell transplantation patients experience a period of severe neutropenia, which is the major risk for infection in the early post-transplant period. Disruption of mucosal surfaces, wide application of central and peripheral venous lines, and, most importantly, prolonged suppression of immunity contribute to infections. An expansion of donor granulocytes (engraftment) usually occurs within 14-21 days after SCT and reduces the risk for infection with bacteria and fungi. Almost all HSCT recipients lose most of the T and B lymphocyte function subsequent to the conditioning, consequently losing immune memory accumulated through a lifetime. Although some passively acquired immunity from the donor as well as some retained immunity from the recipient might exist, these cannot be relied upon to provide long-term protection against infectious diseases (Wahren *et al.* 1984). Even if recipients have normal total lymphocyte counts within two months after SCT, they have abnormal CD4/CD8 T cell ratios, reflecting their decreased CD4 and increased CD8 T cell counts (Guillaume *et al.* 1998). T cell reconstitution can involve both a thymus-dependent pathway (the generation of new naïve T cells from HSCs), and a thymus-independent pathway (peripheral expansion of pre-existing memory T cells) (de Gast *et al.* 1985; Bomberger *et al.* 1998). The number of naïve CD4 T cells in the peripheral blood of patients after HSCT is very low, most cells that routinely accompany stem-cell grafts are mature memory cells with restricted diversity of T cell receptors and therefore more limited immunocompetence (Douek *et al.* 2000). A decreased number of CD4 T cells and, consequently, a decreased number of secreting cytokines, lead to dysfunction of the remaining cell groups (Bowden *et al.* 1990; Storek *et al.* 1997). CD8 T cells, despite the high numbers, have weakened cytotoxic function. The release of mature B cells to the peripheral blood is restored approximately six months after HSCT, but their ability to produce antibodies is frequently decreased due to lack of T cell help. After one year, Ab responses to antigens frequently return to normal, although levels of IgG and IgA can remain low for years post-transplantation (Novitzky *et al.* 2001). The immunological recovery is even longer delayed in patients with chronic GVHD: levels of both IgG and IgA and subclass pattern did not normalize until 24 months after transplantation (Hammarstrom *et al.* 2000).

1.4 INFECTIOUS COMPLICATIONS AFTER HSCT

Disruption of mucosal surfaces, wide application of central and peripheral venous lines, and, most importantly, prolonged suppression of immunity following stem cell transplantation contribute to development of infections. A broad variety of infectious organisms, including bacterial, fungal, viral and protozoal species, can cause major complications during the period of immune compromise after HSCT. During the *immediate post-transplant period* (pre-engraftment phase), defined as days 0 to +30, gram-negative (*e.g*. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and gram-positive (*e.g*. *Coagulase negative staphylococci*, *Staphylococcus aureus* and s*treptococci*) aerobic bacteria are the most commonly identified cause of infection (Bochud *et al.* 1994; Ninin *et al.* 2001). All through the aplastic period, fungal infections (mainly caused by A*spergillus* and C*andida* species) and reactivation of herpes simplex virus (HSV) in seropositive patients might also occur. In the *intermediate post-transplant phase* (post-engraftment phase, up to approximately three months after HSCT) the risk of developing bacterial and fungal infections decreases, although it may persist in the presence of GVHD, which is associated with additional suppression of immune function. During this period, characterized by poor T cell function, patients are at the greatest risk of infections caused by viruses such as cytomegalovirus (CMV) and adenovirus, and by aspergillus and other moulds (Williamson *et al.* 1999). This situation is worsened by GVHD (Aschan 2006). In the *late post-transplant phase* (after day +100), patients are at lower risk of serious infection, unless they have ongoing cGVHD (Kulkarni *et al.* 2000; Wingard *et al.* 2002). These late infections are usually due to varicella zoster virus (VZV), community-acquired infections, or, in patients with cGVHD, recurrent bacterial infections especially caused by pneumococci and other bacteria having a polysaccaride capsule (Ochs *et al.* 1995; Engelhard *et al.* 2002; Einsele *et al.* 2003).

1.4.1 Viral infections after HSCT

Viruses are small, intracellular parasites which cause infection by invading cells of the body and multiplying within them. Viruses range in size from less than 100 nanometers in diameter to several hundred nanometers in length. All viruses contain a nucleic acid genome (RNA or DNA) and a protective protein coat (capsid). The virus might also have a lipid bilayer membrane, an envelope, which is usually acquired by budding through a host cell membrane. Enveloped viruses do not necessarily have to kill cells in order to be released, since they can bud out of the cell - a process which is not always lethal to the cell. Hence, some budding viruses can set up persistent infections (Murray 2002). Enveloped viruses are readily infectious only if the envelope is intact, so agents which damage the envelope, such as alcohols and detergents, reduce infectivity. Since many viruses make few or no enzymes, they are dependent on host cell enzymes to produce more virus particles. Thus virus structure and replication are fundamentally different from those of cellular organisms. Viral dependence on the host cell for various aspects of the growth cycle has complicated the development of drugs since most drugs will inhibit not only viral multiplication but host cell growth as well (because the same cell enzymes are used).

Viral infections are frequent after HSCT. They may be life threatening, especially when affecting lungs, liver, or CNS. The following viruses have been recognized in causing infections after HSCT:

Herpesviruses - HSV, CMV, VZV, HHV-6, and Epstein-Barr virus (EBV).

Respiratory viruses - particularly influenza A and B, parainfluenza, RSV, metapneumovirus, adenovirus and picornaviruses
Papovaviruses - papilloma, polyomaviruses
Other viruses – e.g. measles, hepatitis B and C, enteroviruses, diarrhea inducing viruses, and parvovirus

The two viruses that were studied in this thesis are outlined below.

1.4.2 Cytomegalovirus

Human CMV is a ubiquitous agent that commonly infects individuals from diverse geographic and economic backgrounds. Although primary CMV infection generally does not produce symptoms in healthy adults, several high-risk groups, including immunocompromised HSC and organ transplant recipients and individuals infected with HIV, are at risk of developing serious illness and long-term consequences from CMV infection (Forbes 1989). In addition, CMV has became in recent years, after the control of rubella, the most important cause of congenital infection in the developed world, commonly leading to mental retardation and developmental disability.

1.4.2.1 History

In 1904, Ribbert first identified histopathological evidence of CMV in tissues from a congenitally infected infant and mistakenly assumed that the large inclusion-bearing cells he observed at autopsy were from protozoa (incorrectly named *Entamoeba mortinatalium*). In 1920, Goodpasture correctly postulated the viral etiology of these inclusions and used the term "cytomegalia" to refer to the enlarged, swollen nature of the infected cells. Human CMV (HCMV) was first isolated in tissue culture in 1956, and in 1960 the virus name was changed from cytomegalia to cytomegalovirus (Rowe *et al.* 1956; Smith 1956; Craig *et al.* 1957; Weller *et al.* 1960). First report of the role of CMV as a pathogen causing disease in adults was made in 1965 (Klemola *et al.* 1965) and later, during the 1970s and 1980s, knowledge about CMV as an important pathogen with diverse clinical manifestations increased steadily.

1.4.2.2 Pathophysiology and Epidemiology

CMV is one of eight human herpesviruses, designated as human herpesvirus 5 (HHV-5). Taxonomically, CMV is referred to as a Betaherpesvirinae, based on its tendency to infect mononuclear cells and lymphocytes and on its molecular relationship to other herpesviruses. CMV is the largest member of the herpesvirus family, with a double-stranded DNA genome of more than 240 kbp, capable of encoding more than 200 potential protein products. The function of most of these proteins remains unclear. The viral core is enclosed by capsid proteins and further surrounded by tegument proteins, bound by a lipid bilayed outer envelope. The pp65, also called internal matrix protein, is one of the major viral proteins that immediately after infection is localized in infected cells. Quantitative, phenotypical, or functional analysis of pp65-specific T cell can provide important information on the natural course of immune responses. In clinical specimens, one of the classic hallmarks of CMV infection is the cytomegalic inclusion cell. These massively enlarged cells contain intranuclear inclusions, which histopathologically have the appearance of owl eyes. The presence of these cells indicates a productive infection, although they may be absent even in actively infected tissues. In most cell lines, CMV is difficult to culture in the laboratory, but *in vivo* infection seems to mainly involve epithelial cells, and, with severe disseminated CMV disease, involvement can be observed in most organ systems. Although little is known about the molecular mechanisms

responsible for the pathogenesis of tissue damage caused by CMV, there are observation of CMV-induced alternations in the cell cycle and CMV-induced damage to chromosomes (Schleiss 2005). Dependent on the studied countries, between 50 and 100% of the population experience CMV infection during their lifetime. In developing countries, most children acquire CMV infection early in life, with seroprevalence approaching 100% by early adulthood. In contrast, in developed countries, the seroprevalence of CMV approximates 50% in young adults of middle-upper socioeconomic status (Stagno *et al.* 1982; Ho 1990). In a recent study, it was shown that the likelihood of being CMV seropositive in both HSCT recipients and healthy donors has decreased in Europe during the last decades but there are variations in the trends in different countries (Ljungman *et al.* 2007). CMV is transmitted to the neonate transplacentally or infection might be transmitted throughout life, mainly via contact with infected secretions (Forbes 1989).

1.4.2.3 Clinical symptoms and complications

In the majority of individuals infected with CMV the infection is usually asymptomatic. Congenital CMV infection is the most common and important of all infections of the newborn and might cause abnormalities such as microcephaly, brain calcification, hepatosplenomegaly, and may result in hearing loss (bilateral or unilateral) and retardation (Jones *et al.* 1995; Ahlfors *et al.* 1999).

Immunosuppressive treatment of transplanted patients frequently results in CMV infection with a variety of clinical symptoms. CMV disease causes high morbidity and mortality in HSC and organ recipients, and is one of the most important obstacles in successful transplantation (Meyers *et al.* 1990; Ljungman 1998; Nichols *et al.* 2000). Disease manifestations vary in severity depending on the degree of host immunosuppression. Infection may occur because of reactivation of a latent viral infection or may be newly acquired via organ or stem cell transplant from a seropositive donor, or blood transfusions. CMV infection develops in approximately 40% of seronegative patients transplanted from CMV seropositive donors and up to 70% of seropositive patients, who reactivate latent virus. Seropositive recipients of grafts from seronegative donors are at higher risk than those, receiving graft from seropositive donors (Meyers *et al.* 1986; Boeckh *et al.* 2004). CMV infection usually occurs 1 to 3 months following transplantation, but owing to the effective antiviral therapy developed in the early 1990s, the incidence of early CMV disease has been reduced from 20%-30% to less than 5% (Boeckh *et al.* 2003; Ljungman 2006). Meanwhile, the risk of late CMV disease, occurring after day 100 post-transplantation has increased, and is now in many centers more frequent than early CMV disease (Einsele *et al.* 2000; Boeckh *et al.* 2003). Viral dissemination leads to multiple organ system involvement, with the most important clinical manifestations being pneumonia, gastrointestinal disease, and retinitis. *CMV pneumonia* usually develops 1-3 months following transplantation and starts with symptoms of fever and dry, nonproductive cough. The illness can progress quickly with retractions, dyspnea, and hypoxia. The illness is an interstitial pneumonia, frequently showing a radiographic appearance of diffuse bilateral interstitial infiltrates. As the differential diagnosis of pneumonia is extensive in transplanted patients, bronchoalveolar lavage or open lung biopsy to confirm the diagnosis and direct appropriate therapy should be considered. *Gastrointestinal tract* disease caused by CMV can include esophagitis, gastritis, gastroenteritis, pyloric obstruction, hepatitis, pancreatitis, colitis, and cholecystitis. Characteristic signs and symptoms may include nausea, vomiting, dysphagia, epigastric pain, icterus, and watery diarrhea. Endoscopy and biopsy are necessary, and characteristic cytomegalic inclusion cells may be observed in GI endothelium or epithelium. *CMV retinitis*: CMV produces a necrotic rapidly progressing retinitis with characteristic white perivascular infiltrate with hemorrhage (brushfire retinitis).

Peripheral lesions may be asymptomatic, and even advanced disease does not cause pain, but can progress to total blindness and retinal detachment if left untreated. *Other manifestations* of CMV infection include encephalitis, nephritis, cystitis, myocarditis, and graft failure (Ljungman *et al.* 2002; Boeckh *et al.* 2003).

1.4.2.4 Laboratory diagnosis

Viral culture, serology, antigenemia, and PCR are commonly used methods for the diagnosis of CMV infection and disease. The classical method in the evaluation of suspected CMV disease is the *viral culture*. Blood, urine, saliva, cervicovaginal secretions, cerebrospinal fluid, bronchoalveolar lavage fluid, and tissues from biopsy specimens are all appropriate specimens for culture. The specimen is inoculated onto human cells (usually human foreskin fibroblasts), and the cell culture is monitored for development of the characteristic CMV-associated cytopathic effect. Although the culture is sensitive, clinical isolates of CMV may grow slowly, requiring up to six weeks of incubation in the virology laboratory. An adaptation of tissue culture that provides results more rapidly referred to as the *shell-vial assay*. In this technique, the clinical specimen is centrifuged onto a cell monolayer (in effect, concentrating the specimen). Then, following incubation in tissue culture, cells are stained with a monoclonal antibody to a CMV-specific antigen. A positive shell vial culture is presumptive evidence of active CMV infection, and the test is a useful adjunct to traditional viral culture. Newer diagnostic assays, including *qualitative* and *quantitative PCR* and CMV *antigenemia* test are today the most used for diagnosis of CMV infection in immunocompromised patients (Boeckh *et al.* 1996; Emery *et al.* 2000; Hebart *et al.* 2004; Ljungman *et al.* 2006). The detection of CMV-DNA by PCR is a rapid and sensitive method, based on selective amplification of CMV-specific nucleic acid sequences. It is used either qualitatively or quantitatively to measure the viral load, which is proportional to the level of viral DNA. By using quantitative PCR it was suggested that patients with higher CMV viral load have a higher risk of CMV disease (Emery *et al.* 2000; Razonable *et al.* 2002). The antigenemia assay is based on the detection of the tegument protein pp65 in leukocytes by immunofluorescence. Enzyme-linked immunosorbent assay (or ELISA) is the most commonly available serologic test for measuring antibody to CMV. Although theoretically helpful, the ELISA technique for CMV-specific IgG detection is unfortunately too nonspecific for a reliable diagnosis but is very important before HSCT to assess the risk for subsequent CMV infection. Different assays for immunological monitoring became available during the last few years, including detection of tetramers, intracellular cytokine staining, Enzyme-linked immunospot (or Elispot), and lymphocyte proliferation assays.

1.4.2.5 Definitions of CMV infection and disease in HSCT recipients (Ljungman et al. 2002)

CMV infection is defined as isolation of the virus or detection of viral particles in any body specimen. *Primary CMV infection* is detection of virus in an individual previously found to be CMV seronegative. *Recurrent infection* is detection of CMV infection in a patient with previously documented infection, with an interval of at least four weeks in-between. It may result from reactivation of latent virus (endogenous) or from reinfection (exogenous). Reinfection is detection of a CMV strain different from the one that caused the original infection in the patient. Reactivation is assumed if the previously detected CMV strain is found indistinguishable from the strain causing the new episode. **CMV disease** is defined by the presence of clinical signs and/or symptoms of an end-organ disease combined with the detection of CMV by virus isolation, hystopathologic testing, immunohistochemical analysis, *in situ* hybridization, or macroscopic findings.

1.4.2.6 Prevention and treatment

Knowledge of the epidemiology and transmission routs of CMV is key to the development of successful strategies for the prevention of CMV infection in the individuals at high risk for serious disease. One way to prevent CMV disease is to prevent transmission of the virus to seronegative patients by using exclusively CMV-seronegative stem cell donors. Another is to use "CMV safe" blood products that can be achieved either by using products from CMV seronegative donors or by transfusion of leukocyte-depleted blood products (Nichols *et al.* 2003; Visconti *et al.* 2004).

In most cases no treatment is necessary for CMV infection in the healthy individual since the majority of the infections resolve on their own. Antiviral agents may be administered therapeutically for established CMV disease or preventively, when the risk of development of CMV disease is high (*e.g.* in transplant recipients). **Prophylaxis** is prevention of the infection (either primary or reactivation), while **pre-emptive** therapy is early treatment of CMV infection before CMV disease has occurred (Goodrich *et al.* 1991; Goodrich *et al.* 1993; Ljungman *et al.* 1998). Both strategies effectively reduce the risk for CMV disease, although CMV disease might instead develop later after treatment has been discontinued. There is also a risk for development of antiviral resistance (Li *et al.* 1994; Boeckh *et al.* 1996; Nguyen *et al.* 1999; Boeckh *et al.* 2003). Most currently used antiviral agents active against CMV are nucleoside analogues. These agents share a common molecular target, namely, the viral DNA polymerase and, selectively activated by virus-specific enzymes, perform activity against virus-specific metabolic processes. The following antiviral agents - ganciclovir, foscarnet, and cidofovir have received marketing approval for the systemic treatment of CMV infection. *Ganciclovir* (GCV) is a synthetic acyclic nucleotide structurally similar to guanine and requires phosphorylation for antiviral activity. GCV is commonly used as pre-emptive therapy in transplant recipients at high risk of developing disease. It has virostatic activity and suppresses active CMV infection. The main side effect of GCV is myelosupression (neutropenia, anemia, and thrombocytopenia). *Foscarnet* (FOS) is an organic analog of inorganic pyrophosphate that inhibits viral replication at pyrophosphate-binding site on virus-specific DNA polymerases. FOS is the preferred drug for patients who are failing GCV therapy due to viral resistance, or those who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia (Reusser *et al.* 2002; Razonable *et al.* 2004). Nephrotoxicity and electrolyte disturbances are the main side effects of FOS. *Cidofovir* (CDV) is a broad-spectrum antiviral agent with potency against both herpesviruses and other DNA viruses. It is considered a second-line therapy, after failure of either GCV or FOS (Ljungman *et al.* 2001). The major limitation of CDV as an antiviral agent is nephrotoxicity. *Maribavir* (MBV) is a novel and promising anti-CMV drug. Unlike currently available anti-CMV agents that inhibit CMV DNA polymerase, MBV inhibits viral DNA assembly and inhibits egress of viral capsids from the nucleus of infected cells. According to a preliminary results from a phase II study, MBV shows advantages over existing anti-CMV drugs in its in vitro potency, bioavailability, safety profile, and the lack of cross-resistance inherent in its novel mechanism of action (ViroPharma Inc. 2006). The first phase III study of MBV was initiated in April 2007 in patients undergoing HSCT and is ongoing. An increased understanding of the mechanisms by which T cells recognize virus-specific antigens has stimulated much interest in the use of specific T cells in *adoptive immunotherapy* for viral and malignant diseases (Einsele 2002). Thus, generation of CMV-specific T cells is a potential alternative to antiviral treatment strategy (Riddell *et al.* 1992; Walter *et al.* 1995; Riddell *et al.* 1997; Peggs *et al.* 2003).

1.4.2.7 Immunity to cytomegalovirus

Although both arms of the immune response are involved in protection against CMV, cellular immunity, especially T cell-mediated, is the most important controlling factor (Quinnan *et al.* 1982; Quinnan *et al.* 1984). The key role of cellular immunity in mounting a protective response to CMV is shown also in immunocompromised patients, such as HSC and organ transplant recipients and AIDS patients (Reusser *et al.* 1991; Li *et al.* 1994; Riddell *et al.* 2000; Hebart *et al.* 2004; Lacey *et al.* 2004).

The cellular immune response to CMV includes both innate and adaptive components, with the CTLs, Th cells and NK cells as the main effector cell populations. CTLs recognize CMV tegument proteins, such as IE and pp65, expressed by infected cells and destroy the viral infected cells (Borysiewicz *et al.* 1988; McLaughlin-Taylor *et al.* 1994). Their activity arises within 3-4 days after infection, peaks by days 7-10, and then declines. Studies of the kinetics and characteristics of CMV-specific CD4 and CD8 T cells in immunocompromised patients suggest that both CD4 and CD8 lymphocytes are necessary for an efficient immune response to CMV and are required for the complete restoration of CMV immunity (Kalams *et al.* 1998; Gamadia *et al.* 2003). Although the primary effector activity is attributed to CTLs, there is an increasing appreciation of the role of Th cells in developing an effective and lasting immune response. Following specific antigen stimulation Th cells not only have direct effector activity through the production of cytokines such as IFN-γ, TNF-α and IL-2, but also are essential in initiating and maintaining CTL function and numbers (Riddell *et al.* 2000). In addition, the adoptive transfer of CMV-specific CD8 T cell clones to restore CMV immunity after transplantation showed that persistence of CTL cytolytic activity was correlated with the recovery of CD4 T cells (Greenberg *et al.* 1999). NK cells are important players in the innate immune response, characterized by strong cytolytic activity against susceptible target cells. They mediate direct lysis of CMV-infected cells by releasing cytotoxic granules containing perforin and granzymes, or by binding to apoptosis-inducing receptors on the target cell. Moreover, they secrete cytokines such IFN-γ and TNF-α, providing a first line of defense before the generation of a more specific T cell responses (Fletcher *et al.* 1998; Cerwenka *et al.* 2001; Lodoen *et al.* 2005). However, CMV as well as some other persistent viruses (HSV and VZV) have developed strategies to reduce the impact of host immune defenses, which allow them to escape immune recognition and replicate. These strategies include both molecular and cellular mechanisms to help the virus to avoid detection by CTLs and NK cells and consist of: downregulation of ligands, necessary for activating NK cell receptors; mimicry of host immune mediators such as cytokines; and the engagement of inhibitory receptors by viral proteins (Lodoen *et al.* 2005). Moreover, CMV also developed strategies to evade Th response: the virus interferes with CD4 T cells, directly suppressing T cell response, and indirectly through CMV-infected macrophages, which have decreased ability to stimulate Th response (Odeberg *et al.* 2001).

The cytokines, produced by Th cells and NK cells also support B-cell differentiation and IgM-IgG class switching. The neutralizing antibodies are directed against a variety of envelope proteins, but mainly against the most immunogenic phosphoprotein pp150. Though the antibodies against CMV do not clear the infection, they reduce the viral spread in the host and limit a symptomatic infection (Landini *et al.* 1991).

1.4.3 Influenza

1.4.3.1 Virology & Epidemiology

Human influenza viruses are members of the orthomyxovirus family. Out of the different genera of influenza virus, only the A and B viruses are of epidemiological interest in humans. Influenza viruses are enveloped viruses with a genome made of eight single-stranded RNA segments. Each segment codes a functionally important protein (Gürtler 2006) and two of these proteins, matrix protein (M) and nucleocapsid protein (NP), are found in infected cells (Cox *et al.* 1999). Hemagglutinin (HA) and neuroaminidase (NA) are the major antigenic determinants of influenza viruses; 15 hemagglutinin subtypes (H1-H15) and nine neuroaminidase subtypes (N1-N9) have been identified for type A virus and only one subtype of hemagglutinin and one subtype of neuroaminidase are recognized for type B virus. The HA protein is involved in attachment and membrane fusion in the endosome of the infected cell. The receptor binding site on the virus is in a pocket that is not exposed to the immune system, whereas the antigenic domains are on the surface. These later domains can be altered and the virus can thus avoid a humoral response without affecting its ability to bind to the receptor (Cross *et al.* 2001). The NA protein acts as an enzyme that digests sialic acid, a monosaccharide that most cells have on their surface and that also is a part of the virus receptor. Removing the sialic acid from the infected cell surface by the NA makes it easier for the progeny virions to diffuse away once they exit the cell. Neuraminidase is also involved in penetration of the mucus layer in the respiratory tract.

The epidemiological success of influenza viruses is mainly due to two types of antigenic variations that occur in HA and NA antigens and which results in sporadic outbreaks, limited epidemics, and boundless pandemics: *antigenic drift* is due to a mutation that both proteins undergo; *antigenic shift* is due to re-assortment, when "new" HA and/or NA are found in the circulating viral strains. Influenza viruses are usually transmitted via air droplets, which subsequently penetrate the mucin layer of the mucosa of the respiratory tract, and contaminate it by entering respiratory epithelial cells. Replication is very quick; after only six hours the first influenza viruses are shed from infected cells. Some viral proteins act as toxins and promote further production of influenza virus. Rapid bacterial growth, most commonly *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*, may begin in the very early phase of viral replication (Peltola *et al.* 2004; McNamee *et al.* 2006).

1.4.3.2 Clinical symptoms and complications

The risk of serious illness and death is highest among persons aged 65 years or older, children less than two years old, and immunocompromised persons such as HSC and organ recipients who have medical conditions that place them at increased risk of developing complications from influenza (Harper *et al.* 2005). Uncomplicated influenza typically presents with fever (38-40ºC) and chills, accompanied by headache and sore throat, mialgias, dry cough, nasal discharge, and photophobia. Pulmonary complications often occur in young children (croup or acute laryngotracheobronchitis) and in patients with underlying chronic obstructive pulmonary or heart disease (primary influenza virus pneumonia). Lack of mucociliary clearance in the respiratory tract provides a good environment for bacterial growth and development of secondary bacterial infection. Non-pulmonary complications of influenza include cardiac complications, encephalopathy, Reye's syndrome (acute encephalopathy with cerebral oedema and fatty degeneration of the liver), and Guillain-Barré syndrome (autoimmune attack on the peripheral nervous system) (Cox *et al.*

1999). The major causes of influenza-associated death are cardiac failure and bacterial pneumonia but can also be consequences of primary viral pneumonia in the absence of bacterial invasion (Louria *et al.* 1959). Although the impact of influenza on mortality was traditionally reported in terms of deaths from pneumonia and influenza, it is now recognized that influenza contributes to total (all-cause) mortality and deaths from circulatory diseases as well. Numbers of deaths attributable to influenza are difficult to estimate directly because influenza infections typically are not confirmed virologically or specified on patient discharge forms or death certificates. In addition, many influenza-associated deaths occur from secondary complications when influenza viruses are no longer detectable. However, using diagnostic virology and virologic surveillance, the morbidity and mortality attributed to influenza were calculated in the United States and it was shown that influenza-associated deaths have increased substantially during the last two decades especially in the elderly persons (Thompson *et al.* 2003). The published mortality rates in HSCT patients have varied between 17% and 57% (Nicholson *et al.* 1997; Ljungman *et al.* 2001; Raboni *et al.* 2003; Machado *et al.* 2005). In a prospective study of respiratory virus infections-related mortality after HSCT by EBMT (European Group for Blood and Marrow Transplantation) group was shown that influenza-attributable mortality in this group of patients reaches 23% (Ljungman *et al.* 2001).

1.4.3.3 Laboratory diagnosis

A definitive diagnosis of influenza requires laboratory tests like virus isolation, detection of viral nucleic acid, detection of viral proteins, and serology. The virus can be isolated from a nose or throat swab. This is used to infect cells in culture (or eggs) and subsequent detection of infected cells by immunofluorescence. By polymerase chain reaction (PCR) test it is possible to detect viral nucleic acid (RNA). Recently, rapid tests of protein detection based on enzyme immunoassay became available. These tests are less sensitive than virus isolation or PCR, but results are available in less than 1 hour, and can be used to guide the antiviral therapy. Serological diagnosis is based on the increase a of specific antibodies titer in serum samples, measured by hemagglutination inhibition, but the need of paired serum samples limits the usefulness of this test. Provisional diagnosis is often made clinically, based on knowledge of a current outbreak of influenza combined with appropriate clinical symptoms (Cox *et al.* 1999).

1.4.3.4 Prevention

Vaccination is one of the main, currently available, methods of preventing influenza and its complications. It exposes the immune systems to an inactivated strain of the virus that cannot cause disease, so when an organism is exposed to a real infection the immune system is prepared to fight it and clear it before the development of the disease. Even though vaccination is a cost-effective method to decreases the harm caused by influenza, about 20% of children and 5% of adults worldwide develop symptomatic Influenza A or B each year (Rowe *et al.* 1956). Two general types of influenza vaccines, live attenuated and inactivated vaccines, are currently licensed for use. Since both types of the vaccine are grown in embryonated hen's eggs, they are contraindicated for those with allergies to eggs. Live attenuated vaccine is approved for use in healthy individuals from 5 to 49 years of age. Current inactivated vaccines are trivalent formulations that contain 15µg of antigen from each of the three strains selected for that year's vaccine - two influenza A strains (H1N1 and H3N2) and one influenza B strain. The influenza vaccine has a short lived protective effect and is therefore usually given in the autumn so that protection is high in December/January - the usual peak months for flu in the northern hemisphere. It needs to be administered every year because of the short lived nature of the protection, resulting from changes in the circulating influenza strains due to the antigenic drift or shift. The inactivated vaccine induces immunity in 60-90% of

healthy children and adults (Nicholson *et al.* 1997; Bridges *et al.* 2000). However, the immunological response to the vaccine, or efficacy is lower in elderly people and in immunocompromised HSCT patients, varying between 10 – 50% (Pauksen *et al.* 2000; Robertson *et al.* 2000; Rapezzi *et al.* 2003; Mazza *et al.* 2005). National authorities of many countries recommend an annual influenza vaccination for the following population groups: (a) persons aged 65 years and older; (b) children aged 6 months – 5 years; (c) persons of any age with chronic medical conditions (e.g., asthma, diabetes, heart and lung disease, kidney failure); (d) immunosuppressed patients (those with HIV or who are receiving immunosuppressive drugs, such as chemotherapy and long-term steroids) and their household contacts; (e) women in the second or third trimester of pregnancy during the influenza season; (f) residents of nursing homes and other chronic care facilities; (g) children and teenagers on chronic aspirin therapy; (h) household contacts of persons at high risk, including children aged less than 2 years of age; and (i) health-care workers (Ljungman *et al.* 1995; Nicholson *et al.* 1995). The EBMT recommends an annual influenza vaccination for all allo-HSCT recipients no earlier than 6 month after HSCT and continued yearly as long as the patient has GVHD or ongoing immunosupression (Ljungman *et al.* 1995; Ljungman *et al.* 2005). Most previous attempts to improve the efficacy of influenza vaccination in HSCT patients, by adding a second dose of vaccine (Engelhard *et al.* 1993), or by using granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immunomodulating factor (Pauksen *et al.* 2000) had only a marginal effect. Therefore, vaccination strategies that can overcome the limitations of the impaired immune system are needed to improve vaccine efficacy for this population. In HSCT recipients, studies of vaccination efficacy, showing poor response to vaccination were mostly focused on detection of serological response. In a study conducted by Machado *et al*., a clinical benefit of influenza vaccination was assessed and an 80% rate of clinical efficacy, similar to the rates in healthy people, was observed (Machado *et al.* 2005). These data should encourage all HSCT centers to ensure good compliance with seasonal vaccination.

The most frequent side effects of an influenza vaccination are pain, redness, and swelling at the injection site, lasting 1-2 days, and occurring in about 10-64% of immunized persons, and systemic side effects such as headache, fever, and myalgia in about 5% (Musana *et al.* 2004). These side effects are mainly due to the local immune response, leading to systemic effects.

1.4.3.5 Treatment

Most patients with uncomplicated influenza, especially adolescents and young adults, can be treated symptomatically and do not need specific therapy. In high-risk individuals, especially patients with underlying medical conditions like HSCT recipients, treatment with antiviral drugs is a good option. All drugs are most effective if started within a few hours of the onset of symptoms and are generally licensed for use within 48 hours of the first symptoms. They can modify the severity of the illness, reducing the intensity of influenza symptoms and decreasing the duration of the illness by few days. There are four antiviral drugs currently available for the treatment of influenza A infection - two neuraminidase inhibitors and two M2 ion channel inhibitors. Only neuraminidase inhibitors are currently licensed for use in Sweden. The neuraminidase inhibitors, oseltamivir and zanamivir, introduced in 1999 and 2000, interfere with the normal function of the influenza neuraminidase by mimicking sialic acid, the natural substrate of the neuraminidase (Varghese *et al.* 1995). When started within the first 12 hours following the onset of fever, neuraminidase inhibitors shortened the illness by more than three days, in comparison to treatment that was started after 48 hours (Nicholson *et al.* 2000). The duration of the fever, severity of symptoms, and time to return to normal activity also correlated with the time of initiation of antiviral intervention (Aoki *et al.* 2003). Neuraminidase

inhibitors are generally well-tolerated, but in some patients oseltamivir can cause transient gastrointestinal disturbances (nausea, vomiting), and intranasal administration of zanamivir may result in local irritation (Hayden *et al.* 1997).
The M2 ion channel inhibitors rimantadine and amantadine block virus entry and interfere with virus release by blocking the ion channel formed by the M2 protein of the viral membrane (Hay *et al.* 1985). These drugs can help prevent more serious complications and reduce the duration of influenza A, if given within 48 hours of the onset of symptoms. Gastrointestinal symptoms and development of resistance are the major side effects associated with amantadine and rimantadine. Although the effectiveness of these two preparations is similar, rimantadine is safer, since amantadine has a wide range of toxicity and can cause neurological side effects. Apart from having fewer side effects, the advantage of neuraminidase inhibitors is activity against influenza B. The best symptomatic treatment is rest, liquids, and anti-febrile agents (not aspirin, since Reye's syndrome is a potential problem).

1.4.3.6 Immunity to influenza virus

Hosts may prevent the attachment of influenza virus by several mechanisms: (a) specific immune response and secretion of specific cytokines; (b) unspecific mechanisms, such as mucociliary clearance or production of mucoproteins that able to bind to viral hemagglutinin; and (c) genetic polymorphisms of the host receptor (sialic acid), which is highly conserved in the same species, but differs between avian and human receptors (Matrosovich *et al.* 2000). As a result, the avian virus needs to undergo mutations at the receptor binding site of HA to cross the interspecies barrier between birds and humans. Influenza causes an acute infection in the host and initiates numeral immune reactions involving different parts of the defense system. The initial innate immune response, which includes release of type I interferons and influx of neutrophils and NK cells, firstly limits viral replication, and secondly, is an essential precondition for the activation of the adaptive immune response. The adaptive response requires a number of days to be effective, but then helps to control the viral spread, to eliminate the virus and to establish a long-lasting memory to re-infection with homologous virus. Cross-protection within a subtype of influenza has only rarely been observed and infections essentially induce no protection across subtypes or between types A and B (Treanor 2005).

Influenza infection induces both humoral and cellular immune responses important in recovery from acute infection and resistance to reinfection. In response to influenza infection, the B cells produce antibodies to both influenza glycoproteins HA and NA, as well as to M and NP proteins. These antibodies help to lyse infected cells via complement or antibody-dependent cellular toxicity. HA-specific immunoglobulins, such as IgM, IgA and IgG, appear within 2 weeks of virus inoculation - primary infection is accompanied by a transient IgM response, followed by persistent elevated IgA and IgG-level. While IgA neutralizes and clears viral infection in upper airway (local, mucosal Ab), IgG is responsible for the protection of the lower respiratory tract (systemic, serum Ab). The mucosal immune response against influenza, as measured in nasal secretions, is characterized by the presence of IgA and IgG against HA. The mucosal anti-HA IgG levels correlate well with the respective serum levels, indicating passive diffusion from the systemic compartment, whereas IgA is produced locally. Either mucosal or systemic Ab alone can be protective in sufficient concentrations, but optimal protection occurs when both Abs are present. Serum inhibiting titers of 1:40 or greater should protect against infection. Higher levels of Ab required for protection in older individuals (Treanor 2005) and those with impaired immunity (Ring *et al.* 2002). In contrast to anti HA-Ab, anti NA-Ab does not neutralize virus, but instead reduces the release of virus from infected cells.

The cellular response starts with antigen presentation by DCs via MHC class I and class II molecules, which leads to activation, proliferation and differentiation of influenza-specific CD8 and CD4 T cells. The basic model is that lung-resident DCs acquire antigen from the invading pathogen, become activated, and subsequently travel to the local draining lymph nodes (Legge *et al.* 2003). In the lymph nodes, the now mature DCs trigger an immune response by any T cell with a receptor that is specific for the foreign-peptide-MHC complex on the DC surface (Shortman *et al.* 2002). Endogenous antigens from the viral infection of DCs are processed and presented to CD8 T cells on MHC I molecules. Exogenous antigens are presented via MHC II molecules to CD4 T lymphocytes. The activated T cells acquire effector cell functions and migrate to the site of infection in the lung where they mediate their antiviral activities. Both sub-groups of T lymphocytes are vigorous in mediating control of an influenza infection. Clearance of the primary infection depends on CD8 T lymphocytes. They recognize epitopes from HA or internal proteins M, NP, or PB2 presented on MHC class I molecules (Treanor 2005). Depending on their antigen specificity, CTLs may be subtype-specific or, in case they recognize internal antigens, broadly cross-reactive with influenza A. CD4 Th cells help B lymphocytes to generate anti-HA and anti-NA antibodies and promote the further generation of virus-specific CTLs. NK cells also contribute to the immunity against influenza: an activating NK cell receptor, NKp46, has been stated to recognize and kill influenza virus-infected cells (Mandelboim *et al.* 2001). Following recovery from influenza, antigen-specific T cells maintain long-lasting immunological memory that responds quickly to antigen re-stimulation. Memory is maintained by antigen-specific T cells that persist at increased frequencies, has reduced requirements for co-stimulatory signals in comparison to naïve T cells, and responds quickly to antigenic re-stimulation (Woodland *et al.* 2005). Hosts that survive an acute virus infection and clear the virus are in general immune to infections by the same virus. Nevertheless, acute infections caused by influenza virus occur repeatedly, despite active immune clearance. This is because influenza displays a structural plasticity as it can tolerate many amino acid substitutions in its structural proteins without losing its infectivity. These substitutions, which arise from copying errors, result in slight variations of HA that permit the virus to evade human immune responses (antigenic drift). These changes are the reason for the annual epidemic spread of influenza and require new vaccines to be formulated before each annual epidemic. Antigenic drift is possible every time a genome replicates. In contrast, antigenic shift can only occur under certain circumstances, is relatively rare and the likely reason for pandemics.

2 AIMS

The aims of this thesis were to study the immunological mechanism underlying viral complications after HSCT focusing mainly on the cellular aspects of anti-viral immunity.

- To monitor CMV-specific T cell reconstitution after allogeneic HSCT (Paper I)
- To evaluate an intervention strategy based on specific CMV immune responses after allogeneic HSCT (Paper II)
- To develop techniques for measuring specific T cell response to influenza vaccination (Paper III)
- To study the immune response to influenza in healthy volunteers and allogeneic HSCT patients, aiming to improve existent vaccination schemes in immunosuppressed HSCT recipients (Paper IV)

3 MATERIALS AND METHODS

In total 126 patients who received allogeneic HSCT for various hematological malignant and non-malignant disorders at Karolinska University Hospital/Huddinge were included in our studies. Informed consent was obtained from all participating patients. The studies were approved by the Ethics Committee of the Karolinska Institutet or the Regional Ethical Board in Stockholm. Thirty-six healthy volunteers, included in influenza vaccination studies, were recruited from the staff of the Departments of Medicine and Clinical Science at the Karolinska University Hospital. The detailed patient/volunteers information and description of the methods are given in the corresponding papers.

All assays were performed on the peripheral blood lymphocytes (PBL) isolated from heparinized blood by gradient centrifugation using Lymphoprep.

3.1 CMV MANAGEMENT POLICY

All HSCT patients at Karolinska University Hospital/Huddinge are monitored weekly for CMV by real-time PCR (on PBLs before October 2004 and after that time on whole blood). These techniques were compared and it was shown that a viral load of 100 genome copies/200 000 cells was comparable to 1000 copies/ml in the whole blood PCR. These levels were used as the basis for initiating pre-emptive antiviral therapy. The assay was performed in the virology laboratory at Karolinska University Hospital/Huddinge and results were obtained from the patients chart database. Pre-emptive antiviral therapy with either intravenous ganciclovir or foscarnet was given within three months after HSCT to all patients who have a viral load of 100 genome copies/200000 cells in PBL PCR or 1000 copies/ml in the whole blood PCR. Repeated courses were given if necessary, based on a new episode of CMV DNAemia.

In order to reduce the risk for CMV disease the blood transfusion policy (transfusion of leukocyte depleted blood products, no CMV seronegative blood products) is implemented at Karolinska University Hospital.

3.2 ANTIGENS AND PEPTIDES

For stimulation of T cells in studies I and II we used the laboratory strain of human cytomegalovirus AD-169. This antigen is a preparation of viral particles, purified from the culture medium of infected normal human dermal fibroblasts when the cells displayed maximal cytopathic effect (BioSite, Stockholm, Sweden). At the moment the study was initiated the only annotated, complete genetic sequence for HCMV was for the laboratory strain AD169.

For immunostimulation of CD8+ and CD4+ T cells (paper II) we have used a complete protein-spanning mixture of overlapping peptides PepMix (JPT Peptide Technologies GmbH, Berlin, Germany). The PepMix contains 138 peptides spanning the pp65 protein of CMV. The peptides are 15meres with an overlap of 11 amino-acids. Since pp65 is an immunodominant target of CD4 as well as CD8 T cells, it can be used for *in vitro* stimulation of CMV-specific T cells.

The synthetic peptides comprising the 306-341 region of human influenza A virus hemagglutinin [H1 (HA306-318), H2 (HA317-341), and H3 (HA316-341)]

were used to estimate specific helper T cell responses (papers III, IV). This region of human influenza virus is relatively conserved within subtype sequences and is not affected by antigenic drift (Rajnavolgyi *et al.* 1994). These peptides induce the full activation of antigen-specific Th cells and recognition of influenza virus HA by proliferating T cells. The peptides were synthesized at the Szentgyorgyi University, Szeged, Hungary (Nagy *et al.* 1994; Horvath *et al.* 1998).

Three lyophilized HLA-restricted influenza peptides, matched to the pentamers (A*0101 CTELKLSDY, A*0201 GILGFVFTL, and A*0301 ILRGSVAHK, ProImmune Ltd, Oxford, UK) were used for *in vitro* stimulation of patients' T cells in our IV study. These peptides are able to induce influenza-specific MHC class I-restricted CTL response *in vitro*, which, together with MHC class I pentamers specific for influenza that we used in the same study, gives a possibility for monitoring antigen-specific CD8 T cell response. Pentamer – is a complex of 5 MHC-peptides able to bind to TcR with very high avidity for detecting T cells. By labeling T cells with a specific pentamer, which mimics the MHC class I peptide complex that the T cell recognizes, it is possible to identify T cells of defined specificity (Kern *et al.* 2005). The addition of a fluorescent label allows the cells to be detected by flow cytometry.

3.3 FLOW CYTOMETRY (PAPERS I-IV)

Flow cytometry is one of the key research technologies that uses the difference in light scattering properties of cells and particles and is based on fluorescence signals to generate specific multi-parameter data. This information allows identifying and characterizing of various subpopulations of cells. One unique feature of flow cytometry is that it measures fluorescence on the single cell level.

3.3.1 Detection of intracellular IFN-γ production

We used previously described method of flow cytometric detection of lymphocyte subset cytokine responses to specific antigen (Jung *et al.* 1993; Maino *et al.* 1998). PBLs from HSCT patients were incubated with CMV antigen AD-169 for 6 hours (paper I) or with PepMix pp65 for 18 hours (paper II) at 37°C in 5% CO_2. The cells were permeabilized and stained with appropriate concentrations of fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8 and anti-INF-γ mAB. Intracellular INF-γ production by different populations of T cells was analyzed by flow cytometry. During each analysis 200 000 cells were acquired. A level of 0.05% (paper I) or 0.1% (paper II) of INF-γ-producing T cells was interpreted as a positive result for this test.

For identifying the phenotype of antigen-specific T cells in study III, we stimulated PBLs of healthy volunteers with influenza peptides and stained them with different dye-conjugated monoclonal Abs.

3.3.2 Detection of CTLs by pentamer staining

The antigen-specific CD8+ T cell responses were monitored by the application of MHC class I restricted pentamers. Three MHC class I pentamers (A*0101, A*0201, and A*0301) were used in study IV for the detection of Influenza A-specific T cells. PBLs from the patients and healthy volunteers were stained

with allophycocyanin (APC) pre-labeled pentamer in combination with fluorescently labeled anti-CD8 (FITC) and anti-CD3 (PerCP) antibodies. After 20 minutes incubation on ice cells were washed, fixed and then analyzed by flow cytometry. During data collection 100 000 gated events from each sample tube were acquired.

3.4 ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY (PAPERS II-IV)

Elispot assay, being a combination of both bioassay and immunoassay techniques, is an accurate tool to monitor immune responses and vaccination efforts by quantification of cytokine- and antibody-secreting cells. With the Elispot assay, immune cell frequencies can be measured at the single cell level (Sedgwick *et al.* 1983; Czerkinsky *et al.* 1988).

3.4.1 T cell Elispot

Cytokine (INF-γ in paper III and IFN-γ, IL-4, and IL-13 in paper IV) Elispot assays were applied to detect influenza-specific immune responses. The PBLs stimulated with influenza peptides were plated onto a pre-coated by capturing antibody polyvinylidene difluoride (PVDF) microwell plates and incubated for 24 hours (INF-γ assay) or 48 hours (IL-4 and IL-13 assays) at 37^{o}C in 5% CO_2. Cytokine secreted by activated cells was captured locally by the coated antibody on the surface of PVDF membrane. A secondary antibody (biotinylated) reactive with a distinct epitope of the target cytokine was employed to detect the captured cytokine. The detected cytokine was then visualized using streptavidine and a precipitating colorimetric substrate. The colored end product (spot) represented an individual cytokine-producing cell. The spots were counted using an automated reader.

3.4.2 B cell Elispot

For the detection of antigen-specific antibodies the influenza vaccine (antigen of interest in this case) was bound to the membrane of PVDF plates during overnight incubation at 4°C. PBLs added to the wells were incubated for 24 hours at 37°C in a humid atmosphere containing 5% CO_2 and then washed thoroughly. Following addition of the secondary Ab and phosphatase-conjugated avidin, precipitates formed that were developed into spots by colorimetric substrate.

3.5 SERUM ANTIBODY TITERS

The hemagglutination inhibition assay (HIA) was performed on serum samples using a single stock source for each of the hemagglutinin antigens representing the strains of virus contained in the vaccine (Kendal *et al.* 1982). Immunity to influenza was defined as a HI titer ≥ 40.

4 RESULTS AND DISCUSSION

4.1 PAPERS I & II

Evaluation of CMV-specific immunity in HSCT recipients: a tool to lead antiviral treatment

In spite of the important improvements in the management of CMV infection, including introduction of new antiviral treatment strategies based on sensitive diagnostic tests, it remains one of the most frequent infections after HSCT (Ljungman *et al.* 1992; Boeckh 1999; Hebart *et al.* 2004). Numerous studies aiming to define the incidence and potential risk factors predicting the development of CMV complications after allo-HSCT, and to assess their impact on clinical outcome were carried out. Factors like patient age, CMV status of the donors and the recipients, type of conditioning, development and grade of GVHD, CMV viremia and number of incidences of CMV reactivation are all factors influencing virus reactivation and contributing to the development of the disease (Meyers *et al.* 1986; Emery *et al.* 2000; Patel *et al.* 2005; Ljungman *et al.* 2006). The aim of our first study was to correlate different transplantation factors with the recovery of cellular immunity in transplantation patients at different time points after HSCT. We investigated CMV-specific immunity in 48 HSC recipients by measuring the population of INF-γ-producing CD3+ and CD4+ T cells, stimulated *in vitro* with CMV antigen AD-169, and then correlated it with transplant factors such as viral load, type of conditioning, stem cell source, donor type and serostatus, and grade of aGVHD. Both the initial viral load and viral load kinetics are described as risk factors for the development of CMV disease (Emery *et al.* 2000). On the contrary, Ljungman et al. in a prospective study including 162 HSCT recipients, could not see that either the initial viral load or the rate of increase influence the development of CMV disease (Ljungman *et al.* 2006). A study to asses the onset and durability of CMV immune reconstitution in allo-HSCT, measured by T cell immune recognition of CMV pp65 and CMV IE-1 was performed by Gallez-Hawkins *et al*. (Gallez-Hawkins *et al.* 2005) They found, contrary to conventional knowledge, that the onset of detectable CMV immunity was not dependent on documented CMV viremia or DNA-emia, and there was no statistically significant difference between CMV immunity in patients with and without detectable CMV reactivation. This possibly indicates an occult CMV reactivation, which is sufficient to induce reconstitution of immunity in HSCT recipients.

We also studied the factors influencing the CMV peak viral load. Patients with INF-γ production by CD3+ cells at 4 weeks after HSCT had lower mean peak viral loads than patients who did not have such production (*p=0.03*). A similar tendency was observed regarding CD4+ cells (*p=0.08*). Analogous non-significant tendencies towards lower peak viral loads in patients who had detectable CMV-specific T cell immunity were observed at 8 and 12 weeks (table 2). In a multiple regression analysis, transplants from CMV seronegative donors were correlated to an increased $\log_{10}$ peak viral load (*r=0.68; p=0.03*), while a detectable CD3+ specific T cell response at 4 weeks after HSCT was correlated to a lower viral load (*r= -0.67; p=0.03*). Our finding supports the notion of a direct controlling effect on CMV replication kinetics by CMV-specific T cells. The only factor influencing the rate of decrease in the viral load during antiviral therapy was grade II-IV aGVHD (p=0.03), which is in line with study by Ljungman et al. (Ljungman *et al.* 2006), while the donor/recipient serological status, intensity of conditioning, type of donor, or CMV-specific immune response had no effect on the rate of viral load decrease.

The association between CMV disease and transplant factors like grade of aGVHD, use of corticosteroids, or transplantation from a CMV-seropositive donor to a CMV-seronegative recipient was previously described (Miller *et al.* 1986; Hakki *et al.* 2003; Ljungman *et al.* 2003; Remberger *et al.* 2004). Studies of correlation between donor and recipient serology and immune reconstitution after HSCT have suggested that CMV-specific CD8+ and CD4+ T cells regenerate faster in CMV seropositive patients who receive grafts from CMV seropositive donors (Li *et al.* 1994; Foster *et al.* 2002). A study by Lilleri et al. supports this suggestion by showing earlier (within 6 months) reconstitution of CMV-specific immunity in seropositive HSCT recipients compared to seronegative recipients (within 12 months) (Lilleri *et al.* 2006). Similar observations were made by Ganepola *et al*., who also found a protective effect and better outcome in CMV seropositive patients with CMV seropositive donors than in those with seronegative ones (Ganepola *et al.* 2007). Furthermore, they discovered that, regardless of their own serostatus, recipients with CMV seropositive donors have a low incidence of CMV infection and disease, *i.e.* the recipient serostatus had no impact on the risk of CMV infection and disease when the donor was CMV seropositive. There are also reports showing that recipients of PBSCs reconstitute immunity faster than BM recipients (Korbling *et al.* 2001; Abrahamsen *et al.* 2005).

We did not observe any effect on either CD3+ or CD4+ function by the stem cell source, donor type, or acute GVHD. However, the number of patients in our study was, unfortunately, too low to allow analysis of possible effects by these factors on the presence of CMV-specific immunity after HSCT.

Several groups have previously reported the earlier recovery of CMV-specific immunity in the RIC HSCT recipients when compared to recipients of myeloablative conditioning (Mohty *et al.* 2004; Ohnishi *et al.* 2005), but the overall risk to develop CMV disease and survival are similar (Junghanss *et al.* 2002). In our study patients who have received RIC HSCT more frequently had CD3+ (48%) and CD4+ specific immunity (56%) at 4 weeks after HSCT compared with patients who received MC (CD3+ 25%; CD4+ 35%). These results support the previous observation that recipients of RIC have better immunity early after HSCT that recipients of MC (Maris *et al.* 2003).
Thus, reconstitution of the immune system plays a critical role in the success of the transplantation and is influenced by numerous factors, and therefore detailed analysis of virus-specific immune responses in the context of allo-HSCT are essential for the development of adoptive immunotherapy strategies.

Table 2: Correlation of INF-γ production by CD3+ and CD4+ T cells with peak viral load

TRANSPLANT PARAMETER	WEEK 4			WEEK 8			WEEK 12		
	CD3+			CD3+			CD3+		
	INF-γ +	*INF-γ -*	*P value*	*INF-γ +*	*INF-γ -*	*P value*	*INF-γ +*	*INF-γ -*	*P value*
Peak Viral Load *log*10	2.1	2.8	0.03	2.1	2.6	0.26	2.4	2.6	0.45
	CD4+			CD4+			CD4+		
Peak Viral Load *log*10	2.3	2.9	0.08	2.1	2.7	0.15	2.3	2.7	0.21

The initiation of universal prophylaxis and pre-emptive therapy based on the sensitive methods of virus detection has generally improved the survival (Ljungman *et al.* 2006), but often results in unnecessary over-treatment with risk of development of antiviral resistance and late onset of CMV disease (Li *et al.* 1994; Boeckh *et al.* 1996; Boeckh *et al.* 2003). The timing of CMV disease has changed and, at present, patients develop it later after HSCT. Risk factors for late CMV disease such as antigenemia developing before day 100 after HSCT, presence of chronic GVHD, lymphocytopenia, low number of CD4+ cells, and absence of a CMV-specific T cell response were identified by Boeckh *et al*. (Boeckh *et al.* 2003). Techniques allowing immunological monitoring of virus-specific immunity such as the MHC-I tetramer assay, intracellular cytokine detection by flow cytometry, and Elispot have become available throughout the last few years (Cwynarski *et al.* 2001; Ozdemir *et al.* 2002). Immunological monitoring allows correlating transplantation factors with the recovery of immunity at different time points after HSCT and let development of an algorithm for monitoring the virus-specific immune status. This, in turn, might allow targeting prophylactic and pre-emptive treatment and target antiviral therapy for patients at the highest risk of developing late CMV disease.

Based on the observations from the first study we conducted a second in which we used the methods of immunological monitoring of HSCT patients' virus-specific immunity as a decision tool for patients' anti-viral management. The strategy consisted of the following: patients who had documented CMV-specific immunity but no symptoms of CMV disease or severe GVHD would not receive anti-viral treatment. Out of 58 patients included in the study, 15 patients reactivated CMV both before and after three months post-HSCT, and 2 patients had CMV detected only later than three months after HSCT. Thus, 17 patients of whom 2 had reactivated CMV twice (*i.e*. 19 episodes in total) were available for assessment of the study strategy of antiviral therapy based on immune status. The strategy was correctly applied in 16/19 episodes. Therapy was deferred in 5/19 and was given according to the strategy in 11/19 episodes. Two patients received antiviral therapy despite having T cell-specific immunity. We found a suggestive association between late CMV reactivation and weak CD8 T cell immunity at 3 months (p=0.06). Like in the first study, here we also investigated factors influencing the development of CMV-specific immunity. The donors' serostatus influenced the strength of both CD4 and CD8 immunity at three months after SCT (p<0.01) (figure 3), which is in accordance with the results of some other recent studies (Ljungman *et al.* 2003; Remberger *et al.* 2004; Ljungman *et al.* 2006).

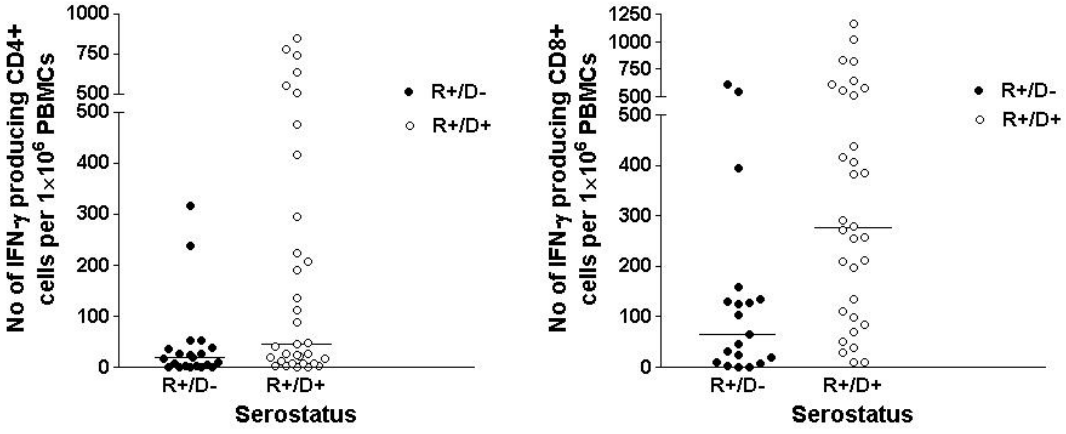

Figure 3: INF- γ productions by CD4+ (A) and CD8+ (B) T cells in relation to recipients'/donors' (R/D) serological status

We observed no effect as regards the type of conditioning, donor type, stem cell source, or acute GVHD, but this might be a result of the comparatively small number of patients included in the study. By applying the strategy, 25% of the patients who had late CMV DNAemia could be spared additional antiviral therapy without development of CMV disease and without the need of anti-viral therapy later on after HSCT. We found that immunological monitoring of virus-specific immunity may complete virological assays, and, thus, avoid unnecessary anti-viral treatment.

4.2 PAPERS III & IV

Studies of immune responses to influenza vaccination in HSCT recipients and healthy volunteers

The impact of influenza in the human population, whether measured by morbidity, mortality, or economic costs, is clear and significant. Annual influenza epidemics affect about 20% of population, having substantial impact in terms of hospitalization rates and mortality (Simonsen 1999; Simonsen *et al.* 2000). Individuals with weakened immunity, such as HSCT recipients are at higher risk to develop life-threatening complications of influenza (Ljungman *et al.* 2001; Raboni *et al.* 2003). The efficacy of the trivalent influenza vaccine is 70-90% in healthy adults and lower in the immunocompromise patients and elderly (Cox *et al.* 1999; Mazza *et al.* 2005). Recovery from influenza infection involves both antibodies and cell-mediated immune mechanisms. Since most previous studies of vaccination efficacy investigated humoral immune response, we conducted a study aiming to develop Elispot technique for measuring the influenza specific T cell response as a marker of vaccine responsiveness (Paper III). We stimulated PBLs obtained from 18 healthy volunteers and 6 HSCT recipients before and four weeks after influenza vaccination with 4 influenza peptides and measured intracellular IFN-γ production by the means of the ELispot assay, to determine the cell-mediated responses. We found that about 92% of healthy immunocompetent individuals responded to influenza vaccination with increased numbers of IFN-γ producing T lymphocytes, while only one HSCT recipient (17%) had increased post-vaccination number of IFN-γ producing cells (figure 4).

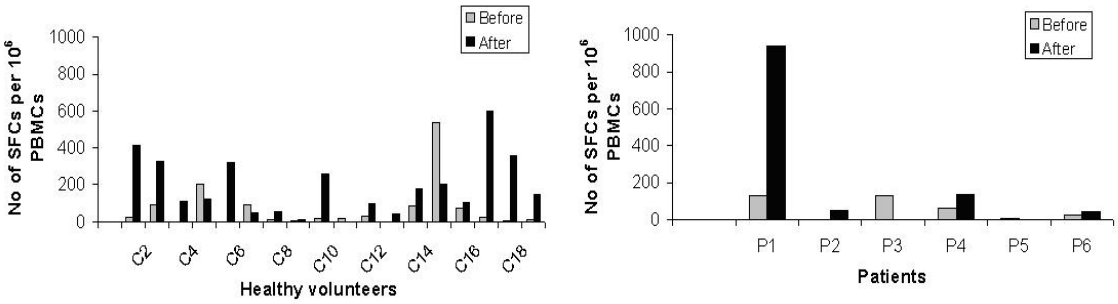

Figure 4: IFN-γ production after stimulation of PBLs from healthy volunteers (A) and HSCT patients (B) with H3 influenza peptide

These results suggest however, that Elispot is a sensitive and specific assay that can be used for measuring immunity to influenza vaccine.

In order to broaden the first influenza study and to investigate different this time components of the immune response to vaccination, we performed the next study (paper IV), where we intended to characterize both cell-mediated and humoral immunities. In 18 healthy volunteers and 14 HSCT recipients, vaccinated within 3 to 14 month after transplantation we evaluated cellular immune response using IFN-γ and IL-13 (only in patients) Elispot assays and pentamer technology. The humoral immune response was assessed by measuring ASCs using Elispot and by measuring serum hemagglutination inhibition (HIA) antibody response. Influenza vaccination elicited strong cell-mediated immune responses in the healthy volunteers ($p \leq 0.003$ for all peptides) and HSCT patients ($p \leq 0.008$) (table 3).

Table 3. Production of INF-γ by PBMCs stimulated with influenza peptides before and after influenza vaccination

Peptide	Median no. of SFCs before vaccination	Median no. of SFCs after vaccination	*p*-value
Healthy controls			
M1	5 (0–100)	160 (75–335)	0.0005
H1	0 (0–40)	67.5 (50–120)	0.003
H2	0 (0–25)	100 (35–190)	0.0004
H3	17.5 (5–95)	165 (90–355)	0.0002
Patients			
M1	0 (0-0)	15 (5-65)	0.003
H1	0 (0-0)	15 (10-25)	0.004
H2	0 (0-0)	12.5 (0-30)	0.008
H3	0 (0-0)	12.5 (5-15)	0.005

Our results are in line with reports by Guthrie *et al.* and Skowronski *et al*., who demonstrated significant boost of INF-γ production at 2 weeks and 6 months after vaccination (Skowronski *et al.* 2003; Guthrie *et al.* 2004). The percentage of CD8+ specific cells also increased significantly after vaccination in the group of volunteers ($p=0.005$) and patients ($p \leq 0.003$), confirming that vaccination not only boost the B-cell but also the CTL-responses (Boon *et al.* 2002; Peggs *et al.* 2003) (figure 5).

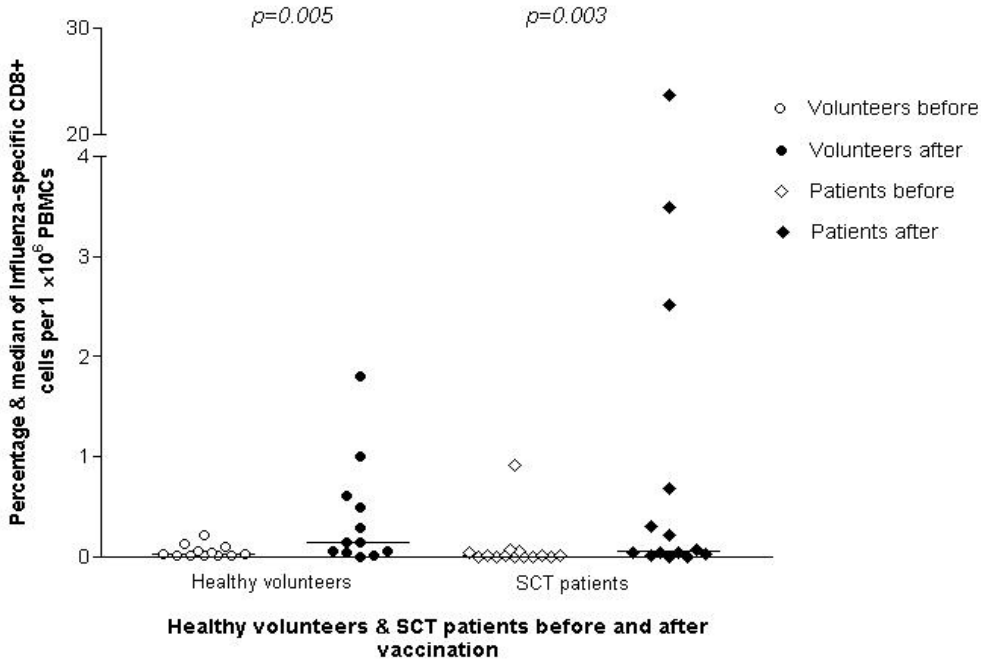

Figure 5. Influenza-specific CD8+ T cells by pentamer staining

To broaden the analysis of the humoral immune response to vaccination, we used Elispot assays to measure the number of antibody-producing cells as well as the IL-13 production in the patients group. The number of IL-13-producing cells increased significantly after vaccination in the entire group of patients (*p=0.03*). The number of influenza-specific ASCs enhanced after vaccination (*p=0.009* for volunteers and *p=0.01* for patients). No increase in the group of volunteers, but a significant one in patients (*p=0.004)* was found when looking at the number of non-specific IgG-producing ASCs after vaccination, suggesting a possible unspecific polyclonal B-cell activation. Only 29% of HSCT recipients had a protective antibody level to influenza A H1/N1 serotype, which is in accordance with previous studies, showing poor ability of imunocompromised patients to respond to vaccination with protective antibody response (Engelhard *et al.* 1993; Pauksen *et al.* 2000). Thus, we suggest that influenza vaccination boosts the cell-mediated response in HSCT patients and healthy volunteers, while B-cell responses in patients are suboptimal.

5 CONCLUSIONS

- The use of pre-emptive strategy against CMV significantly improves the outcome of HSCT, but often results in patients' over-treatment. The immunological monitoring of T cell reconstitution after HSCT and its correlation with certain transplantation factors is a useful complement to virological monitoring, and might be used for guiding the anti-viral treatment.

- The response to vaccination is frequently poor in immunosuppressed individuals. We developed a sensitive and specific Elispot assay for measuring of the virus-specific immune responses to influenza vaccination, to be able to investigate responsiveness to vaccine. We found that seasonal influenza vaccination was able to boost the cell-mediated response in HSCT patients and in healthy volunteers, while the humoral responses in patients were poor. Because of the tendency to suboptimal responses to vaccination in immunocompromise recipients of HSCT, there may be a need of chemoprophylaxis and/or development of more immunogenic vaccine formulations for this population.

6 FUTURE PROSPECTIVES

- Human cytomegalovirus has a major impact on recipients of hematopoietic stem cell transplantation, causing challenging infectious complications and is also immunosuppressive in its own right. The availability of rapid diagnostic tests has made treatment possible in the presympromatic phase of CMV infection, significantly reducing morbidity and mortality in HSCT patients. In recent years, technology in the field of antigen-specific T cells has taken several steps forward allowing studies of specific immune functions on the single T cell level. Increasing knowledge of CMV immune evasion mechanisms and genetic polymorphism might lead to the development of new anti-viral strategies and improvements in managing the virus infection. CMV-specific (*i.e*. dendritic cell based) immunotherapy is a promising, nontoxic alternative to existing treatments, but there are still several issues to be resolved before this method becomes a routine therapy. Meanwhile a strategy of routine immunological monitoring together with virological monitoring of transplanted patients can be beneficial in the management of antiviral treatment.

- Influenza is an important pathogen in HSCT recipients. Several challenges now exist in efforts toward the improving the control and prevention of influenza through vaccination Vaccination strategies that can overcome the limitations of the impaired immune system are needed. As the protective response to vaccination is lower in immunocompromised HSCT recipients, there is a need for more immunogenic and effective vaccines for this high risk population. Next generation of influenza vaccines will likely include novel adjuvants and delivery systems that targeting the immune system and trigger selective immune response. An interesting hypothesis, deserving further investigation is if transplanted patients would benefit from donor vaccination. To investigate if donors vaccinated before HSC donation may passively transfer anti-viral immunity to the recipients, we have initiated a study including allo-HSCT recipients with sibling donors that will be continued during the next influenza season.

7 ACKNOWLEDGEMENTS

I was looking forward to writing this part, not only because it means I am approaching the finish, but also because it gives me a chance to express my sincere gratitude to everyone who, in one way or another, helped me during the past five years. So, a huge thank you to all following:

Professor Per Ljungman, my supervisor, for accepting me in your research group, supporting and guiding me through my research and this thesis. Your prompt and sometimes mid-night e-mails were always a good stimulus for me to work harder! Thank you for your patience, generosity and optimism.

Professor Moustapha Hassan, my co-supervisor, for inviting me to Sweden, for always being friendly, easily approachable, and careful. Thank you for your jokes, your encouraging support and for creating a nice working atmosphere in the Hematology lab.

All the patients and healthy volunteers (often not free-will!), who repeatedly donated their blood for my experiments. Also the research nurses Marie, Gunilla, Kirsti and Sonja for helping with the collection of samples.

Taking in to account this section is the most often read and popular part of the thesis, and trying to avoid the risk to underestimate someone's input in my work/life during these years, I will skip specifying the person's merits. I simply want to thank, from all my heart, the following people for the help they gave me in every sense - technical support and practical help, discussions and criticism, advises and directions, parties, dinners, travels, conference trips and many other things, that made these years in Sweden fly by! You all have made an incredible difference to my time in Sweden and deserve many thanks.

Former and present prefects of the department of Medicine, professors Jan Palmblad and Jan Bolinder

My co-authors and members of our group: Lena, Andreas, Johan, Kajsa and professor Olle Ringdén

Professor Herman Einsele and Dr. Ulrich Grigoleit, my hosts in Tübingen

All the past and present members of the Hematology Lab: Sören, Lalla, Birgitta, Mari, Ann W, Sofia, Kerstin, Ann S, Kristina, Ulrika, Emma

My office mates: Christina – my first teacher in the lab, Monika, and Hamdy

My friends and companions-in-arms, PhD students Rasheed, Christian, Alexandra, Maryam, Martin and those who already completed their mission: Jan, Kyriakos, Evren, Ramin, Hayrettin

The members of the ECM group: Zuzana, Hairong, Behnam, Rana and Hatem

Hernan - a good friend of mine and emergency aid with FACS

Elispot experts Makiko, Sten and Therese

People and friends from different groups and departments in Novum and Huddinge Hospital: Natalia, Karin, Leonid, Natasha, Karolina, Pu, Jose, Anna, Signe, Inger W, Juhana, Jennifer, Eimantas, Victoria, Maria, Gaby, Gladys, Martin, Leonardo, Emelie, Xiaojin, Lev

My friend and external mentor Angel

My first and so far the only American and Swedish friends, Inger and Mia

My former office mates & lifetime friends Parvaneh and Marina

All my friends back in Armenia and all over the world: Zara, Anna, Armen & Gabriella, Narine, Naira, Lilit, Tatevik & Zara, Lena, Jacques, Anita, Albertas, Paulius, Kestas

My dear Armenian and Armenian by association friends in Sweden: Anahit & Paul, Marina & Souren, Vigen & Lia, Zara & Tobias, Narine & Alexander, Armen

My friend Lusine, the one who arranged for me to came to Sweden and the best friend one could wish to have

Ulf, whose only flaw is that he's perfect… ☺

My dearly-beloved Family: Mama, Papa, Gena, Arsen, Aram & Co

This work was supported by research funding from the Swedish Cancer Fund, Karolinska Institutet, European Leukemia Net, Stockholm Cancer Society, and a guest researcher scholarship from the Swedish Institute

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9 APPENDICES