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DIVERSITY AND FOCUS OF CMV SPECIFIC T-CELL RESPONSES IN PATIENTS POST-HSCT AND WITH SOLID TUMOR

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Diversity and focus of CMV specific T-cell responses in patients post-HSCT and with solid tumor

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SUMMARY

Human Cytomegalovirus (CMV) is a beta-herpesvirus that commonly infects humans. Most symptoms in healthy individuals are very mild. The virus is never eliminated and remains in the individual life-long. Thus, CMV and the immune response stay in coexistence until the protective response remains. CMV disease occurs when the immune system is either immature or immunocompromised, such as in hematopoietic stem cell transplant recipients. Over the last decades, CMV has been reported in diverse cancer including glioblastoma (GBM) that could suggest a potential link between CMV infection and cancer initiation or development.

The papers in this thesis investigated the CMV-specific immune response of individuals in different clinical settings: patients after hematopoietic stem cell transplantation (HSCT) and patients with brain tumor or pancreatic cancer. The thesis consists of the following studies:

In **Paper I**, we investigated the CMV-specific interferon-gamma (IFNγ) production in a cohort of 277 patients over time post-HSCT. Impairment of the CMV-specific immune response post-HSCT is associated with CMV infection. We monitored the IFNγ production in response to CMV-pp65 over a period of 2 years post-HSCT and observed a higher IFNγ production at the first month post-HSCT. Moreover, we identified that several clinical parameters such acute graft-versus-host disease (GVHD) and CMV infection affect the IFNγ production in response to CMV-pp65.

In **Paper II**, we aimed to characterize the CMV-specific CD8+ cytotoxic T-cells (CTL) with high affinity T-cell receptor (TCR) in patients post-HSCT using three different CMV-pp65 tetramers. High affinity CMV-CTL presented an effector memory phenotype and a stronger PD-1 expression as compared to CMV-CTL with lower affinity. Additionally, the high affinity CMV-CTLs were found at higher proportion in patients with chronic GVHD over time post-HSCT. Therefore, **Paper I and II** together may better characterize the CMV-specific immune response post-HSCT and potentially the bidirectional relationship between virus and GVHD.

In **Paper III**, we investigated the plasma interleukin 7 (IL-7) and the soluble IL-7 receptor (sIL-7R) levels post-HSCT. IL-7, through its receptor, is essential for T-cell proliferation, thus, to the response to infection. Patients presenting CMV infection exhibited a lower plasma level of sIL-7R and higher level of IL-7 in the early months post-HSCT. Furthermore, the plasma level of IL-7R was reduced in patients with acute GVHD. Together these observations suggest that sIL-7R may be associated with increased risk of GVHD and potential CMV infection.

In **Paper IV**, the clinical setting is different. We focused on the CMV-specific IFNγ production in a large cohort of patients with brain tumor and pancreatic cancer. Patients with brain tumor and more specifically those with GBM presented an impaired immune response towards viral and mitogen antigens. While survival was correlated with the CMV-

specific humoral response, no correlation between survival and CMV-specific IFNγ production could be observed. Contrarily, patients with high Epstein-Barr virus (EBV)- and PHA-specific IFNγ production showed an improved survival post-operation suggesting this immune response as potential marker of general immunocompetence.

LIST OF SCIENTIFIC PAPERS

I. **Poiret T**, Valentini D, Von Landenberg A, Liu Z Remberger M, Ringden O, Ernberg I, Ljungman P. CMV and EBV immune response characterized by specific IFN-gamma production post-HSCT correlates with grades of GVHD and CMV reactivation. *manuscript* 2018

II. **Thomas Poiret**, Rebecca Axelsson-Robertson, Mats Remberger, Xiao-Hua Luo, Martin Rao, Anurupa Nagchowdhury, Anna Von Landenberg, Ingemar Ernberg, Olle Ringden, Markus Maeurer. CMV-specific CD8+ T-cells with different TCR affinities segregate T-cell phenotypes and correlate with chronic GVHD in patients post-HSCT. *Front Immunol*. 2018; 9:760.

III. **Thomas Poiret**, Lalit Rane, Mats Remberger, Birgitta Omazic, Åsa Gustafsson-Jernberg, Nalini Kumar Vudattu, Raija Ahmed, Ingemar Ernberg, Jacek Winiarski, Isabelle Magalhaes, Olle Ringden and Markus Maeurer. Reduced plasma levels of soluble interleukin-7 receptor during graft-versus-host disease (GVHD) in children and adults. *BMC Immunol*. 2014; 15: 25.

IV. Zhenjiang Liu*, **Thomas Poiret***, Qingda Meng, Martin Rao, Anna von Landenberg, Esther Schoutrop, Davide Valentini, Ernest Dodoo, Inti-Harvey Peredo, Markus Maeurer. Epstein-Barr virus- and Cytomegalovirus-specific immune response in patients with brain cancer. *J Transl Med.* 2018; 16(1):182.

*shared first authorship

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LIST OF ABBREVIATIONS

A	astrocytoma
aa	amino acid
a/cGVHD	acute/chronic graft-versus-host disease
APC	antigen presenting cell
ATG	anti-thymocyte globulin
BCR	B-cell receptor
BM	bone marrow
CCR	cellular markers chemokine receptor
CB	cord blood
CD	cluster of differentiation
CMV	Cytomegalovirus
CTL	CD8+ cytotoxic T-cell
DLI	donor lymphocyte infusion
E	early
EBNA-1	Epstein-Barr nuclear antigen 1
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
GBM	gliobastoma multiforme
HHV	Human herpesvirus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
IE	immediate early
IFN-γ	interferon gamma
Ig	immunoglobulin

IL-	interleukin-
L	late
M	metastasis
MAC	myeloablative conditioning
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MUD	matched unfamiliar donor
NK	natural killer cell
OA/O	oligoastrocytoma /oligodendroglioma
Pan Can	pancreatic cancer
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
Pp65	phosphoprotein 65
PTLD	post-transplant lymphoproliferative disease
RIC	reduced intensity conditioning
Sib	sibling
TCM	central memory T-cell (CD45RA- CCR7+)
TCR	T-cell receptor
TEM	effector memory T-cell (CD45RA- CCR7-)
TEMRA	terminally differentiated T-cell (CD45RA+ CCR7-)
TGF-β	Transforming growth factor beta
TNF-α	tumor necrosis factor alpha
Treg	regulatory T-cell

1 INTRODUCTION

1.1 HUMAN CYTOMEGALOVIRUS

1.1.1 Overview

While the thesis is focused on the specific immune response to CMV, it is important to briefly describe the virus itself and its biology to better understand the clinical implication of the pathogens and, therefore, the necessity to continue the research on such a common, "inoffensive" virus.

Inclusion bodies in the nucleus, characteristic of human Cytomegalovirus (CMV) infection, were first observed in the late nineteenth century and then described in 1939 in lethal congenital cases (CID, called generalized cytomegalic inclusion disease). It was successfully isolated for the first time a decade later by three independent laboratories: Rowe, Smith, and Weller [1]. CMV infection, unnoticed in healthy individuals, infects a large proportion of the world's population [2], with a seropositivity ranging from 30% to 100% in adults depending on age, country, population density, and socioeconomic status [3]. Primary and persistent CMV infections are controlled by acquired immunity in healthy individuals and can be revealed by the presence of circulating antibodies specific against CMV. Both humoral and cellular immunity are involved in protective immune responses to CMV reactivation and CMV resolution [4]. CMV disease occurs in situation where the immune system is either immature or immunocompromised such as fetuses, HIV-positive patients, and recipients of solid organ or hematopoietic stem cell transplants. Over the last decades, association of human CMV in diverse cancer such as prostate cancer, colon cancer, or brain cancer has been reported suggesting a potential link between CMV infection and cancer initiation or development [5-8].

1.1.2 Biology

Human CMV is a member of the human herpesvirus family that includes also Epstein-Barr virus (EBV), varicella-zoster virus, herpes simplex virus 1 and 2, HHV-6 A and B, HHV-7, and HHV-8. It belongs to the β-herpesviridae subfamily and is also known as human herpesvirus 5 (HHV-5). Its genome of 235 kilobase pair, double-stranded DNA, contains 165 genes[9]. More than 200 open reading frames (ORF) encode over 200 proteins in three overlapping phases (immediate early (IE), early and late) and 50 ORFs are involved in viral replication and immune evasion [10]. The structure of the virion is similar to the other herpesviruses consisting of an extern lipoprotein envelope containing an icosahedral capsid (162 capsomers) formed by major and minor proteins. The envelope is composed of glycoproteins such as gB, gN, gO, gH, gM, and gL. The tegument, a layer between the envelope and the capsid, contains immunogenic proteins of which pp65 was the antigen used

in the so called antigenemia assay for monitoring CMV infection due to its abundance. Tegument proteins are important for viral gene expression, maturation of the virus and initiation of infection [11]. The capsid carries the genome which is single, linear double-stranded DNA [12].

1.1.3 CMV Infection

The infection can be acquired by different routes: i) Congenital infection, which is a vertical infection from the mother to the fetus during pregnancy. Congenital CMV infection may cause neurological damage such as sensorineural hearing loss, neurodevelopment disability, fetal or neonatal death in 10-20% of congenitally-infected children. The risk for mortality and severe sequalae is highest in primary infection of the pregnant woman. Intrauterine infection has a prevalence of 0.2% to 2.5% differing between countries [13]; ii) Perinatal infection is acquired during delivery from infected maternal cervix; iii) Postnatal infection can be obtained by breast feeding (milk). Another route is through viral excretion in saliva and transmission can most probably occur at an early-age from child-to-child in child care centers and playgrounds [14]. Occasionally, cases of CMV mononucleosis “kissing disease” (similar to EBV mononucleosis) are observed in young adults, representing 8% of all cases of infectious mononucleosis [15]. iv) CMV infection via blood transfusion was first observed during the 1960s. Nowadays, it is an uncommon event considering the progress in donor screening (CMV seronegative) and the use of leukoreducted blood products [16,17]. v) Human CMV is a significant pathogen in solid organ transplantation and symptoms are more severe in the seronegative recipient (R-) of an organ from a seropositive donor (D+). Thus, the patient experiences a primary infection and 60%-80% of R-D+ patients develop a CMV infection [18,19]. CMV’s role in HSCT is outlined in detail below.

1.1.4 CMV Pathogenesis

Human CMV pathogenesis is complex due to a tight conflictual relation with the immune system and a broad range of target cells. The cell tropism for CMV varies among strains and explains the numerous different clinical presentations of CMV disease [20]. CMV can infect a wide range of cells such as endothelial cells, epithelial cells, fibroblasts, smooth muscle cells as well blood cells including monocytes, macrophages and neutrophils [20]. Monocytes are the major blood cell-type infected by CMV during acute infection and can serve as transport before the differentiation into macrophages in the tissue where the viral replication occurs [21,22]. Therefore, most virus transmission through blood products can be prevented by removal of leukocytes. There is also evidence that supports the role of endothelial cells in viral dissemination and complete replication cycle [23]. To facilitate viral dissemination, the virus encodes chemokines to attract monocytes and neutrophils but the molecular mechanism remains unknown [24]. CMV might be found in bone marrow CD34+ cells and in all the organs in case of severe disseminated disease [25].

As mentioned earlier, studies have shown at least indirect involvement of CMV in several cancers but it has also been suggested that there is a correlation with various inflammatory diseases like atherosclerosis, systemic lupus erythematosus and rheumatoid arthritis [26-30].

1.2 THE ADAPTIVE IMMUNE SYSTEM

This thesis focuses mainly on the T-cell immune response toward CMV, but the immune system is a large, well-tuned network that provides defense against disease. Therefore, it is necessary to briefly introduce the different type of immune functions. The first line of defense against CMV is the innate immunity. Natural killer (NK) cells appear to be major players in early CMV control [31]. Innate immunity provides immediate response against infection and helps to initiate other immune responses through production of cytokines and expression of co-stimulatory molecules. Historically, NK cells were described to not being able to generate immunological memory like adaptive immune response but this idea was challenged recently as NK cells can acquire long-lasting and antigen-specific memory [32,33]. The adaptive immune system, by engaging both humoral (antibody production) and cellular (T- and B-lymphocytes) immunity, greatly improves the defense and specificity against a distinct pathogen such as CMV.

The adaptive immune system consists mainly of the B- and T- lymphocytes. Naïve B-cells migrate from the bone marrow (central lymphoid organ) to the germinal center of peripheral lymphoid tissues, such as lymph nodes, to complete the maturation process. B-cells recognize, bind and internalize specific antigens via the membrane B-cell receptor (BCR) which is identical to soluble immunoglobulin (Ig). After internalization and processing of the internalized molecules, B-cells present the major histocompatibility complex (MHC) class II – antigen on their surface. This allows the interaction with CD4+ T-cells to finalize the B-cell development by proliferation and differentiation. B-cells end their development by leaving the germinal center to become plasma cells or memory B-cells. Plasma cells are considered as effector B cells; they secrete a large volume of different immunoglobulins with variable functions: IgG, IgM, IgA, IgD and IgE. These antibodies contribute to antigen-neutralization by coating the infecting pathogen. The coating allows two ways to eliminate the pathogens: Antibody opsonization resulting in phagocytosis of the pathogen and the complement-mediated cytolysis of the pathogen. Thus, the humoral response orchestrated by B-cells play a major role in the extracellular pathogens and toxins neutralization towards lysis [34].

1.2.1 T-cell mediated immune response

Similar to B-cells, T-cells first develop in the bone marrow but, instead of migrating directly to the peripheral lymphoid tissues, T-cells have an intermediate step in the thymus where

education of the T-cell receptor (TCR) and selection of the T-cell repertoire occurs. Initiation of the T-cell response takes place in the lymph nodes (peripheral lymphoid tissues) where antigen-presenting cells (APC) display the target peptide driving the expansion and the differentiation of the antigen-specific T-cells that then migrate to the site of infection. T-cells are essential in eradication of intracellular pathogens.

1.2.1.1 CD4+ T-cells

CD4+ T-cells work in close collaboration with B-cells necessary for activation, clonal expansion and specific immunoglobulin production. Additionally, they are important to activate and expand cytotoxic T-cells (CTL) and contribute to macrophage activation; for those reasons, CD4+ T-cells are also called helper T-cells. CD4+ T-cells are activated in the peripheral lymphoid tissue by the interaction between their TCR and the major histocompatibility complex (MHC) II on APC surface, presenting the antigen peptide of a 10-30 amino acids length. Differentiation of CD4+ T-cells is dependent on the co-stimulatory signals between the APC and the T-cells and can lead into four major distinct CD4+ T-cells phenotypes: Th1, Th2, Th17 and Treg (regulatory T cell) characterized by their cytokine production and transcriptions factors as describe in Figure 1. Th1 cells produce interferon-gamma (IFNγ), interleukin- (IL-) 2 and lymphotoxin α making them particularly effective against intracellular pathogens; Th2 cells produce IL-4, IL-5 and IL-13 for a strong response to extracellular pathogens; Th17 cells, by producing IL-17 and IL-21, show ability to respond specifically to extracellular fungi and bacterial infections while Tregs, as the name indicates, regulate the immune response and provide self-tolerance by the production of IL-10 and TGF-β [35].

More than these four subsets exist and have been described in the past and recently: Thf, Th9, Th22 and Th1* but the phenotype of those CD4+ T-cells is not “fixed”, i.e not all terminally differentiated and instead present plastic ability [36].

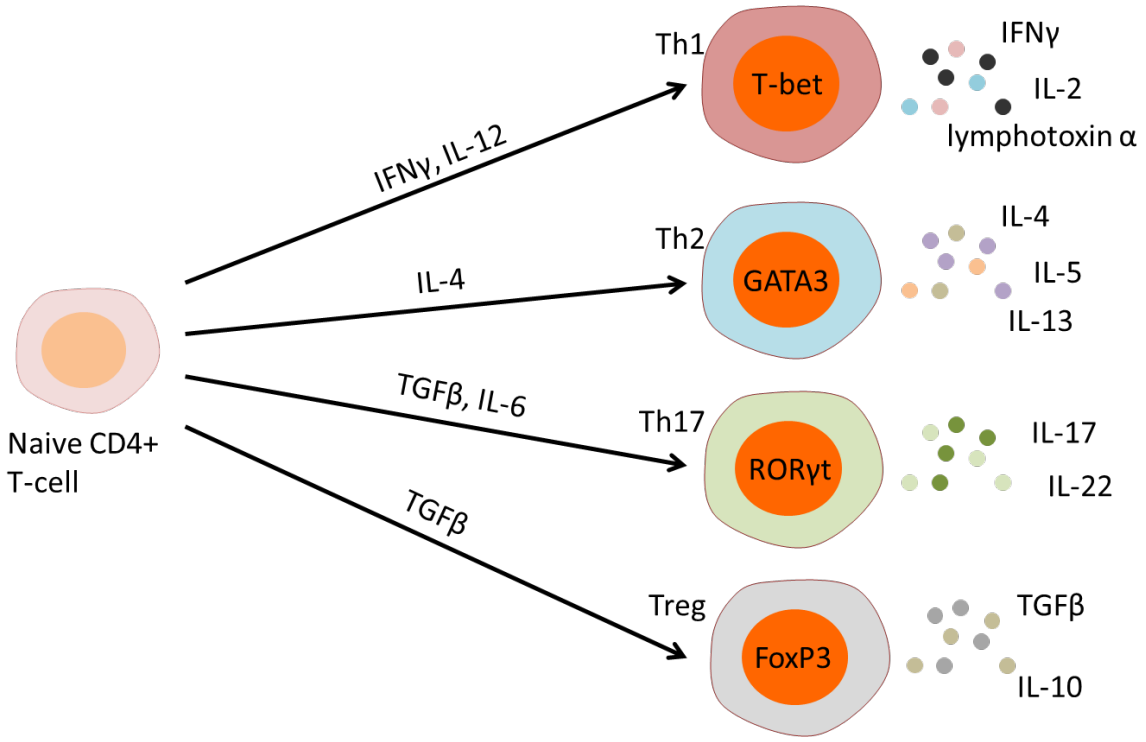

Figure 1: CD4+ T-cells subsets

1.2.1.2 CD8+ T-cells

Together with the IFN-γ producing Th1 cells, CD8+ T-cells are the main immune component of this thesis. TCR of the CD8+ T-cells recognize peptide antigens of 8 to 11 amino acid length presented by surface MHC II of APC that beforehand internalized the pathogen. Activated CD8+ T-cells are also called cytotoxic T-cells (CTL) due to their specific and effective response upon activation. Activated CD8+ T-cells present an effective cytotoxic activity toward target cells characterized by: i) production of perforin and granzymes (especially granzyme B) that disrupt the membrane and induce apoptosis via caspase activation of the targets cells; ii) IFN-γ and tumor necrosis factor alpha (TNF-α) production (pro-inflammatory cytokines) that contribute to resolution of infection [37].

1.2.1.3 Memory T-cells

One of the strong points of the adaptive immune response, in addition to its antigen specificity, is its ability to generate long-term memory response. The CD8+ memory T-cell can be achieved by the help of the CD4+ T-cells [38]. Different markers define a memory T-cell: CD45RA, CD45RO, CCR7 and CD62L allow the separation of 4 distinct subsets such as naïve, central memory (TCM), effector memory (TEM) and terminally differentiated effectors (TEMRA) T-cells as described below in Figure 2 [39]. Briefly, naïve T-cells expressing CD45RA, CCR7 and CD62L circulate in the peripheral blood until they encounter APC for antigen stimulation. Once activated, CD8+ T-cells loose the CD45RA expression to become TCM cells. In general, these cells no longer require co-stimulation for activation, produce IL-2 but keep the high proliferative capacity characteristic of stem cell-like

proprieties [40]. TEM cells express neither CD45RA nor CCR7; therefore they can migrate from lymphoid to peripheral tissues to target their specific antigens. Upon encountering antigen TEM cells produce IFNγ, TNFα, perforin and granzyme B. TEMRA cells are the final subset of the memory T-cell before apoptosis once the infection has been cleared. They retrieve the expression of CD45RA and produce a large quantity of perforin and granzyme B for optimal cytotoxic activity to pathogens. The proportion of memory subsets differs depending on the antigen stimulation. The pathogen seems to shape the memory immune response.

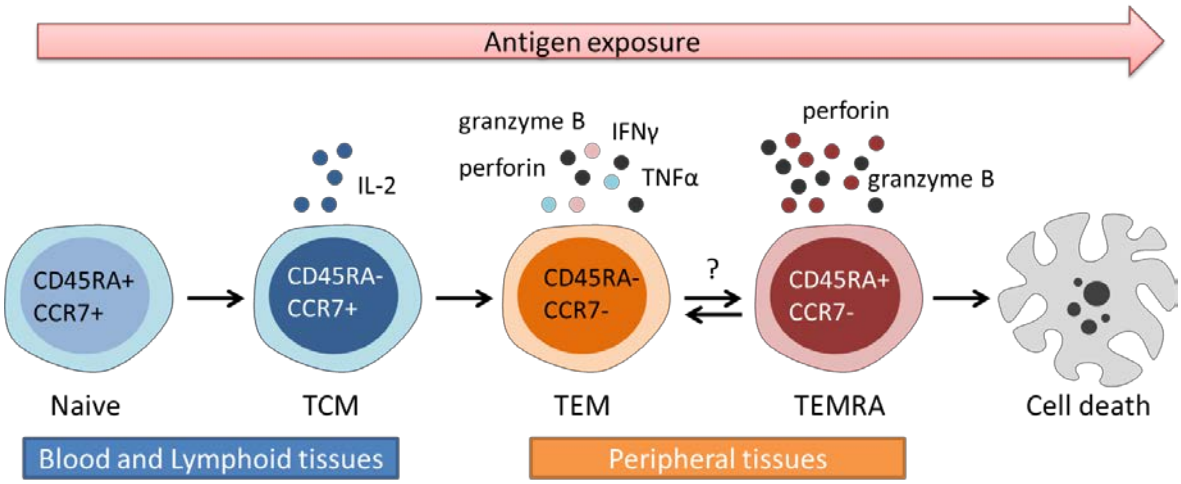

Figure 2: CD8+ memory T-cell subsets

1.2.1.4 MHC class I and II

Class I and class II major histocompatibility molecules (MHC), also called HLA (human leukocyte antigen) in humans, are molecules on the cell surface that present peptides to others (immune) cells. Displayed peptides can be self- or non-self-antigens. The two classes of MHC define two different functions: class I MHC (HLA-A, -B, -C) molecules are present on the surface of virtually all nucleated cells while class II MHC (HLA-DR, -DQ, -DP) molecules are expressed only on APCs such as dendritic cells, macrophages or B-cells. Class I MHC molecules display peptide residue from intracellular pathogens to CD8+ T-cells while class II MHC molecules display peptide residue from extracellular pathogens to CD4+ T-cells (Figure 3). Note that class I and class II MHC molecules are extremely diverse with over 15,000 MHC alleles that code for specific HLA receptors, making HLA the most polymorphic region of the human genome [41]. This high genetic diversity may allow humans to adapt to environmental challenges playing an important role in immune response.

1.2.1.5 TCR stimulation and signalization

As described above, APCs present the bound peptide via their MHC molecules and stimulate the T-cells through their TCR. The TCR consists of two transmembrane polypeptide, covalently-linked chains α and β. Those unique α and β chains recognize the antigen peptide presented by α1 and α2 domains of the class I MHC or α 1 and β1 domains of the class II

MHC. Co-receptors CD4 and CD8 augment the TCR signaling and stabilize the MHC-TCR interaction, enhancing TCR affinity [42,43]. Briefly, the MHC-antigen complex binds the TCR and initiates the signal via the CD3 complex. T-cell activation occurs by the intermediate of a co-receptor (CD4 or CD8) and co-stimulatory receptor such as CD3 and CD28 (Figure 3). Altogether, this leads to an intracellular phosphorylation cascade involving several proteins (zeta-chain-associated protein kinase 70 (ZAP-70), lymphocyte-specific protein tyrosine kinase (LCK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)) inducing the cellular activation characterized by cell proliferation, differentiation, survival and cytokine production [44].

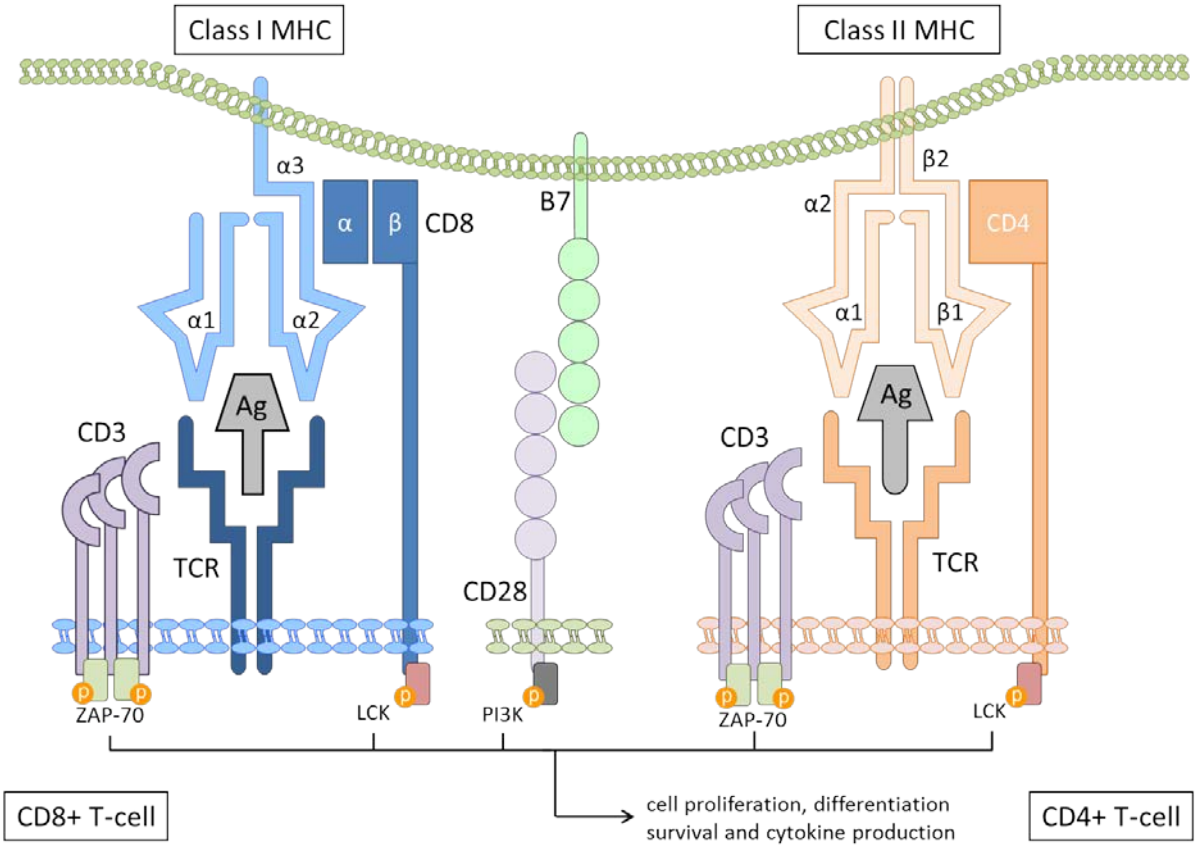

Figure 3: Representation of MHC – TCR interaction.

1.2.2 Cytokines

To orchestrate the most efficient immune defense, signaling between the different immune cells is essential. Cytokines are critical against viral infection but also have an in important role in cancer. A broken balance in the cytokine production or the network can lead to a life-threatening situation. Some were previously was mentioned and, in the scope of this thesis, it is important now to describe them in more detail.

1.2.2.1 Interferon gamma

IFNγ is a major cytokine that enhances the cellular immune response and is essential for host defense. IFNγ is produced by Th1 CD4+ T-cells, CD8+ T-cells and NK cells. Its receptor,

expressed on many cells [45], is composed of two chains: α chain (IFN-γRI) that plays the binding role and chain β (IFN-γRII) responsible for the biological response. Many cells express IFNγ receptor but macrophages are the main receptive population, significantly enhancing their activation and microbicidal function. Furthermore, IFNγ has an immunomodulatory effect on B-cells as it inhibits activation and induces isotype switching to IgG2a. This cytokine also drives the CD4+ T-cell differentiation to Th1 while inhibiting the proliferation of Th2 cells [46]. Importantly, the expression of MHC molecules is upregulated and antigen presentation to T-cells increased by IFNγ. Altogether it shows how important IFN-γ is against tumors and intracellular pathogens [47].

1.2.2.2 Interleukin 7 and Interleukin 7 Receptor

Interleukin-7 (IL-7) is an indispensable cytokine for the development of the immune system. IL-7 influences T-cell homeostasis, peripheral T-cell survival, memory induction and B-cell development. Therefore, IL-7 improves the immune response during infection but is also involved in the severity autoimmune disease due to its proinflammatory propriety, inducing production of TNFα [48,49]. IL-7 is produced by stromal cells in lymphoid tissues, intestinal epithelium, keratinocytes and few other cells. Its receptor IL-7R is expressed on many different cells but most importantly on T- and B-cells. Note that its expression varies depending on the maturation and lifetime of those immune cells [50]. IL-7R is a dimer composed of the common γc chain and CD127. γc chain is also a common sub-unit to other interleukin receptors: IL-2, IL-4, IL-9, IL-15 and IL-21. The binding of IL-7 to IL-7R results in a phosphorylation cascade leading to cell proliferation, survival, differentiation and activation. Due to alternative splicing at RNA level, different isoforms of soluble IL-7R (sIL-7R) have been described [51]. The exact function of the different soluble receptor forms is still elusive but different hypothesis and research present the soluble isoform as an inhibitor of the membrane receptor but it may also play as stabilizer of the cytokine in the extracellular environment [52,53]

1.3 CMV-SPECIFIC IMMUNE RESPONSES

Immunocompetent individuals can keep CMV suppressed into a latent state, however, the immune system cannot achieve a total clearance of the virus. Disturbing the balance between the virus and the immune response can lead to CMV replication with a potential CMV disease development. CMV-specific immunity includes cellular and humoral responses (Figure 4).

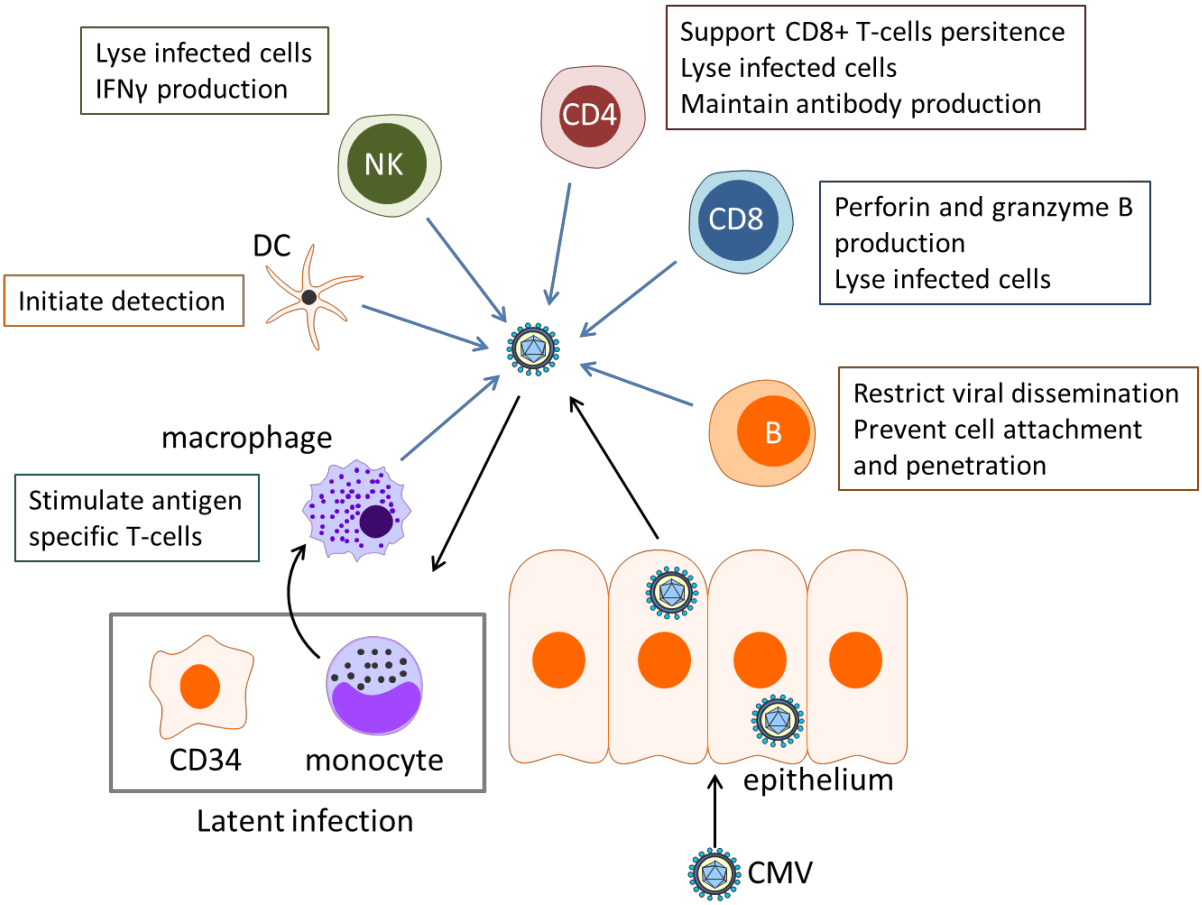

Figure 4: Innate and adaptive CMV specific immune response, adapted from Adland E et al. [54]

1.3.1 Humoral Response against CMV

Humoral immunity is crucial to restrict viral dissemination by producing neutralizing antibodies that block the virus infection [55].

During primary infection, Immunoglobulin M (IgM) antibodies are produced rapidly and persist for 3 to 4 months while IgG antibodies persist life-long. IgM antibodies are often not produced during viral reactivation but can occur in some individuals. The production of CMV-specific antibodies is maintained through the activation of the B-cells by the CD4+ T-cells. These antibodies are directed against specific viral glycoproteins such as gB and gH to neutralize viral particles and abort the infection but cannot target the CMV-infected cells [56]. CMV gB antibodies can be detected in all individuals infected by CMV, making gB one of the major targets for the antibodies and a promising vaccine candidate since it is a well-conserved protein and necessary for viral entry [57-59]. A study suggested that primary CMV infection induces an important functional exhaustion of B lymphocytes that may limit the production of antibodies and the control of viral dissemination [60]. However, the humoral immune response is believed to play a minor role after HSCT

1.3.2 T-cell Response against CMV

Cellular immune response is the predominant and critical immune mechanism that controls CMV. Suppression of CMV-specific T-cells can result in reactivation which is why CMV-disease occurs most commonly in patients with severe, impaired cell-mediated immunity such as those with HIV and organ transplants. The median frequency of circulating CMV-specific memory T-cells in healthy individuals is 10% of the circulating memory T-cells. This is considerably higher than the median observed for any other human viruses such as influenza, adenovirus or poxvirus, and similar to the HIV-specific T cells in active HIV infection [61]. CMV-specific memory CD8 T cells are composed by 50% of terminally differentiated cells (CD45RA+CCR7-) and 40% of effector cells (CD45RA–CCR7−) [62]. The specific T-cell frequency necessary to control the virus infection has not been determined. As mentioned earlier, pp65 matrix protein is one of the significant targets for the CMV specific T-cells (CMV-CTL). The percentage of healthy individuals with CD8 T-cells specific to pp65 is around 85% with a high degree of clonality for the protein target.

However, CMV-CTL responses are diverse containing multiple antigen-specific reactivities towards >70% of the CMV ORF. CD8+ T-cells specific to CMV seem to present a different profile regarding the virus infection i.e acute/primary (CCR7−CD27+CD45RA−) or chronic (CCR7−CD27-CD45RA+) that has been linked to terminal differentiation as well as dysfunction, defined by the re-expression of CD45RA [63,64].

Less is known about CD4+ T-cells responses after primary CMV infection. In young children infected *in utero*, CMV-specific CD4+ T-cell responses are very low but can rapidly expand after primary infection in adults [65]. ORF recognition pattern of CMV-specific CD4+ T-cells overlaps with the CMV-specific CD8+ T cells and as for the CD8+ T cells, responds to a large number of antigens [61]. Most healthy individuals have CMV-specific CD4+ T-cells directed against gB and pp65 antigens and seem to have a preferential recognition for immediate early (IE) genes. CD4+ T cell are mostly described as helper T cells, but a CMV-specific CD4+ T cell subset (CD28- and mainly CCR7-) associated with chronical viral infection shows some cytotoxic proprieties by producing high levels of anti-viral cytokines such as IFNγ and TNFα [66,67].

Primary CMV infection with an intense viral replication is associated with reduced CD4+ T-cell proliferation, increased PD-1 expression and defective IL-2 production whereas acquisition of CD4+ T cell proliferative response against CMV is associated with viremia control [68]. Interestingly, CMV-specific CD4+ T cell responses occurred earlier than CMV-CTL responses in asymptomatic individuals but were delayed in symptomatic individuals. The CMV-specific CD4+ T cell response was observable again after antiviral treatment suggesting that IFNγ–secreting CD4+ T cells are indispensable for CMV-protective immunity [69].

CMV-CTLs are dominant and persist at high frequency in peripheral blood after primary infection. This is in contrast to the CMV-specific CD4+ T-cells that decline rapidly following

an initial peak. During persistent/chronic infection, CMV-specific CD4+ T-cells appear to be more dominant to maintain an efficient CMV-specific immunity through cytokine production and by supporting antibody production [69-71].

1.3.3 T-cell Inflation

With aging, the T-cell population changes so that the naive population frequency decreases while there is an accumulation of differentiated T cells [72]. In addition to the immune senescence associated with aging, this may contribute to a survival decrease in the elderly due to infections. CMV is one of the major driving forces involved [73-75]. In an elderly population, the accumulation of CMV-CTL populations increase the number of dysfunctional CD8 T-cells characterized by their oligoclonal/monoclonal inflation [76,77]. This "memory T-cell inflation" towards CMV is associated with impaired immunity by narrowing the CD8 T-cell repertoire, constricting the immunological space for other antigens [78].

1.3.4 CMV Immune Evasion

CMV is a paradigm for viral immune evasion through its ability to utilize a multitude of immune modulatory strategies. Multiple genes are involved in viral immune evasion, interfering adaptive and innate immune response at every phase of the CMV life cycle allowing the virus to establish latency with lifelong shedding.

Briefly, CMV interferes by affecting the interferon-mediated immunity (induced early during infection) but also by encoding cmvIL-10. This cytokine homolog downregulates the production of IFNγ and TNFα and the expression of both class I and II MHC [79]. CMV is also able to prevent apoptosis to promote survival and to interfere with the T cell immune response to infection by restricting the presentation of viral antigen by both MHC class I and II molecules during every infection phase (reviewed in Table 1).

Infection Phase		Function
Intermediate Early	US3	Retains MHC I molecules in the ER, reduces MHC II presentation
Early	miR-US4-1	Suppresses translation of ERAP1 to limit loading of antigenic peptides
	US2	Induces degradation of MHC class I molecules
	US10	Retains MHC class I heavy chains, induces degradation of HLA-G
	US11	Induces degradation of MHC class I molecules
Early / Late	UL82 (pp71)	Delays progress of MHC class I complexes from the ER to the Golgi
	UL83 (pp65)	Prevents generation of viral antigenic peptides
Late	US6	Inhibits the translocation of peptides by the TAP complex

Table 1. CMV modulation of the T-cell immunity by interference of MHC class I and class II [80].

1.4 HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.4.1 Overview

The first HSCT was performed mid-twentieth century as a salvage against the lethal effects of radiation. Today HSCT provides an effective curative treatment against many diseases such as hematologic malignancies, severe combine immunodeficiencies, autoimmune disorders, and some inherited metabolic diseases. Different sources of stem cells are used: bone marrow, umbilical cord blood and peripheral blood stem cells [81]. Many parameters are essential to consider when performing a HSCT procedure such as the patient and donor age, the donor/recipient HLA match, and the general condition of the patient. As the procedure remains associated with morbidity and mortality, HSCT is used mainly for patients with life-threatening diseases. After HSCT, the major complications, besides relapse of the underlying disease, are infections and graft-versus-host disease (GVHD). Those complications can be influenced by the donor selection (sibling vs. unrelated, HLA-match vs. mismatch) and the conditioning of the patient before the transplant. Conditioning regimen varies significantly from myeloablative (MAC) to reduced-intensity (RIC) conditioning and are needed to eradicate the disease (malignant cells for example) and eliminate/suppress the recipient immune function to allow the engraftment of the donor cells.

The early period after HSCT is commonly characterized by an aplastic phase due to the conditioning regimen aimed to suppress/eradicate the immune system. The risks during this period are influenced by the intensity of the conditioning regimen. During this period there is a high risk of bacterial and fungal infections which remains until neutrophils and granulocytes recover at approximately 2 to 4 weeks after transplantation depending of the stem cell source.

Graft-versus-host disease is a common and severe complication after HSCT, consequence of an attack by the donor cells against the patient tissues. Acute GVHD (aGVHD) remains a major cause of morbidity and mortality post-HSCT and affected organs are skin, liver and gastrointestinal tract [82]. Acute GVHD occurs within the first 100 days post-HSCT and is classified into four grades (I to IV) depending on the severity and involved organs. Acute GVHD pathogenesis goes through a three-phase evolution and was first described by Billingham already in 1966 [83]. First, the afferent phase is a consequence of the conditioning regimen characterized by damage to the host tissue which creates an inflammatory response. The excessive release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α upregulates MHC expression on the APCs. Donor T-cells can then recognize the activated host APCs presenting host antigens leading to an alloimmune response and a cytokine storm. Pro-inflammatory cytokines induce the efferent phase of aGVHD which is the trafficking and expansion of more effector immune cells. Activated donor T-cells produce IL-2 (and IFN-γ

but its role is not well understood) that amplify the allogenic immune response. T-cells and NK cells induce the release of TNF-α by macrophages that damages the different organs (skin, gut, liver). Last, the effector phase that leads to the clinical manifestation of aGVHD: a cascade of cellular and inflammatory effects where activated donor T-cells, maintained by Th1 cells, mediate cytotoxic damage by production of perforin and granzyme and release of more TNF-α, further stimulating the "cytokine storm" effect. Altogether, this dysregulation causes end-organ damage and a continual loop of events involving neutrophils, NK cells and macrophages [84]. Increasing evidence supports that the microbiome composition is important for the development of aGVHD especially in the gut. Chronic GVHD (cGVHD) occurs later after the transplant and is the main factor affecting the long-term quality of life after HSCT. In most cases, cGVHD is preceded by aGVHD and the symptoms affect the skin, liver and oral mucosa. Chronic GVHD can be classified into mild, moderate and severe grades depending on organ manifestations and the number of organs affected.

To prevent GVHD, patients are usually given a combination of prophylactic immunosuppressive agents such as calcineurin inhibitors (cyclosporine or tacrolimus) with the addition of methotrexate, corticosteroids, or mycophenolate mofetil (MMF) [85]. Therefore, the challenge for the clinicians is to treat GVHD without diminishing the graft-versus-tumor (GVT) effect of the HSCT or increasing the risk of infections. Treatments of established GVHD vary depending on the severity of GVHD: from topical corticosteroids for grade I aGVHD to systemic corticosteroids with or without additional therapies for more severe grades mainly targeting T-cells. The treatment of chronic GVHD is more challenging as patients need immunosuppressive treatment for a long period of time but first line therapy remains a combination of calcineurin inhibitors and corticosteroids. Many second line therapies are being tested targeting either T-cells or B-cells.

1.4.2 T- and B- cell Reconstitution post-HSCT

Reconstitution of the innate immunity is faster (within weeks) than the adaptive immunity (within months to years): Natural Killer (NK) cells, monocytes, granulocytes and dendritic cells (DCs) are restored rapidly following HSCT, whereas B-cells and T-cells frequently show delayed and incomplete recovery [86,87]. Long-term immune reconstitution post-HSCT is influenced by several factors such as the conditioning regimen, T-cell depleting agents, the source of stem cell, but also by clinical events such as GVHD, infections and relapse [88].

The first months after HSCT are characterized by a cellular immune deficiency with a lack of cytotoxic T-cells/poor cytotoxic T-cell function. Memory T-cells are the first to expand, driven by cytokines and the presence of alloreactive antigens; they can be either from donor or recipient origin depending on the intensity of the conditioning regimen and if the graft was not T-cell depleted. Long-term, the immune system is reconstituted by cells of donor origin. CD8+ T-cells can regenerate within 2 to 3 months following HSCT as they are able to clonally expand but is, early in the process, associated with an impaired functional immunity

to specific antigens. CD4+ T-cell reconstitution relies on the thymic production of naïve T-cells. Therefore, CD4+ T-cells recover later than memory CD8+ T-cells leading to an inversion of the CD4/CD8 ratio. Thus, the CD8+ memory T-cells respond faster to previously encountered pathogens but present a skewed repertoire and limited response to infection. Naïve T-cells of donor origin issued from thymic maturation regenerate later (> 1 year) but present a broader repertoire and a better reactivity against infection (Figure 5). Broadening of the T-cell repertoire is delayed when the graft is T-cell depleted or issued from a CD34+ purification but also when clinical events and parameters such as aGVHD, age and total body radiation (especially in the elderly) impair the thymus function [89,90].

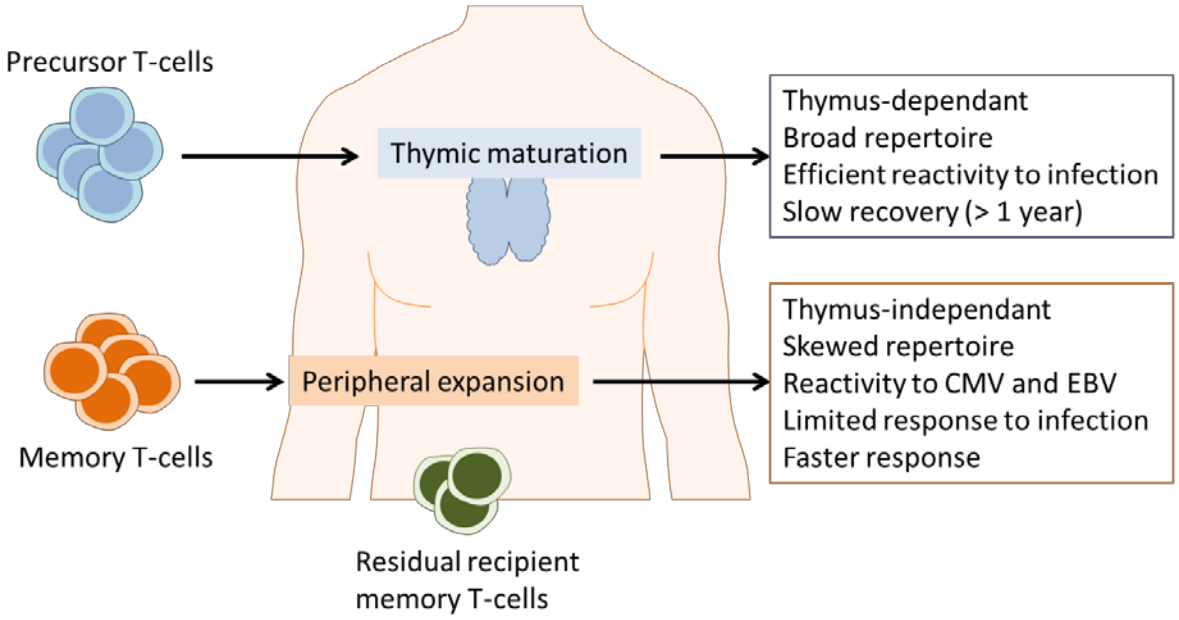

Figure 5: T-cell reconstitution (adapted from EBMT handbook)

Humoral immunity is vital to prevent exogenous infections. Functional humoral immunity, such as specific antiviral IgG, persists for several months after HSCT and some preparative regimen-resistant recipient plasma cells can persist for years following HSCT [91,92]. Early after HSCT, B-cells can also come from donor origin and present naïve and memory phenotype that have already undergone positive and negative selection in the donor [92]. These donor origin B-cells contribute to the early antiviral adaptive response and disappear after some months mainly if there is no antigen stimulation. B-cell reconstitution after HSCT is slow and may take up to 2 years after HSCT. This is particularly true in patients affected by chronic GVHD and/or its treatment and is related to the presence of functional helper CD4+ T-cell reconstitution since they are necessary for B-cell activation [93]. The slow B-cell reconstitution can be associated with development of late infections [94]. Furthermore, B-cells have been associated with cGVHD. Elevated plasma BAFF (B cell-activating factor, a TNF family member, role in peripheral B cell selection and survival) levels contribute to B-cell overactivation in chronic GVHD patients [95]. Note that B-cell reconstitution is faster in children. Functional humoral immunity to infections is essential to reduce the risk for late complications, therefore, vaccination initiated at 3–24 months is required post-HSCT to reach acceptable protective antibody levels [96].

1.4.3 CMV Infection/Reactivation post-HSCT

Despite the changes in patient conditioning (from MAC to RIC) and improved management of the immunosuppressive state, infections remains one of the main severe complications post-HSCT (Figure 6).

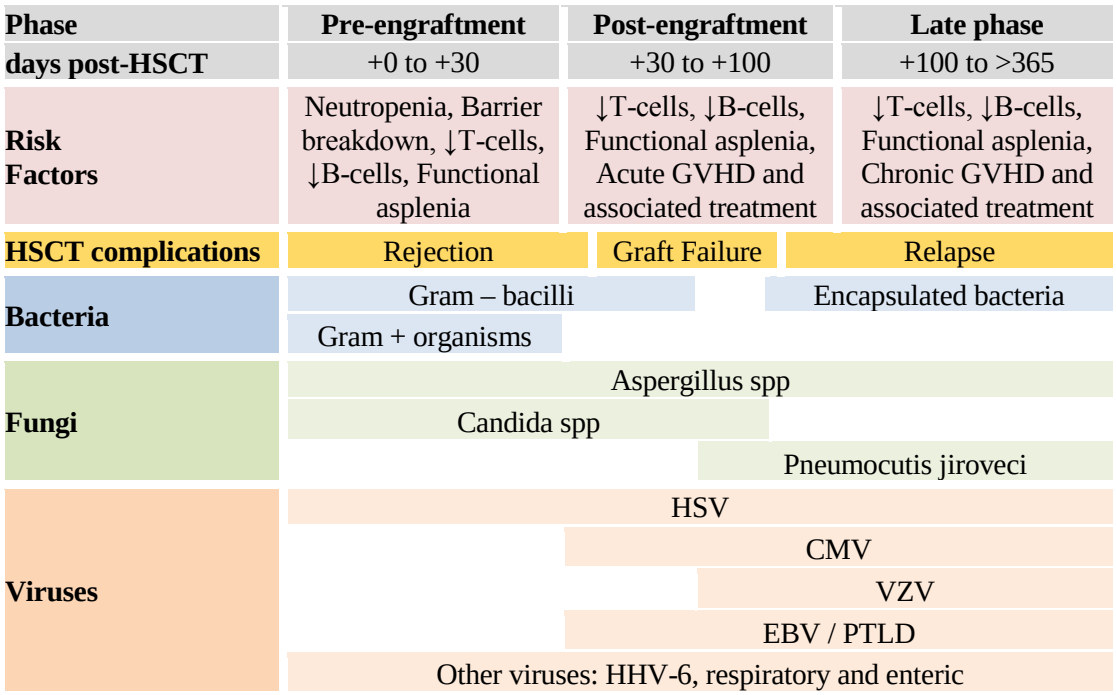

Figure 6. Chronology of predominant infections post-HSCT (adapted from EBMT handbook, and Tomblyn et al. [96]

In general after the first month following HSCT, patients are at risk for CMV. Primary infection is defined by the detection of CMV in the blood or plasma of patients with a pretransplant negative CMV serology or a seroconversion with the production of specific IgG or IgM. The latter is rarely seen early after HSCT. Reactivation of a latent CMV infection is defined by the detection of virus, viral antigen, or nucleic acid in patients with a pre-transplant positive serology. CMV disease is diagnosed when clinical manifestations occur and correlate to the presence of CMV in the organs detected by CMV DNA quantitation, viral isolation, rapid culture or immunohistochemistry [97]: CMV can cause end-organ disease such as pneumonia, retinitis, hepatitis, or gastroenteritis. Today, with improved control and management of CMV, the incidence of early CMV disease is rather low but the cumulative incidence of CMV disease at one year was found up to 10% due to the increased risk of developing late CMV disease [98,99]. Thus, despite improvement in management strategies, CMV remains a leading cause of morbidity and mortality also due to its indirect effects. It has, for example, been shown that patients with CMV infections have a higher risk for secondary bacterial and fungal infections either due to CMV itself or due to side-effects from antiviral therapy [100].

CMV infection and disease are directly correlated with the T-cell reconstitution through diverse mechanisms such as cytokine production and MHC expression. The main risk factor for CMV disease is the serological status of the donor (D) and recipient (R): The D-/R- combination has the lowest risk of CMV infection. Primary infection might occur through infusion of blood products from a CMV-seropositive donor or through other normal sources of CMV such as excretion of CMV in body fluids from children or through sexual transmission. Today, the risk for transfusion transmitted infection is very low due to the use of "CMV-safe" blood products. CMV-seropositive patients and especially those with a CMV-seronegative donor (D-/R+) have the highest risk for CMV disease. Without prophylactic treatment approximately 70% of patients would develop a CMV infection [101]. The D-/R+ combination in unrelated donor HSCT (with myeloablative conditioning only) is associated with decreased overall survival as compared to the D+/R+ combination, repeated CMV reactivations, and increased risk of CMV disease due to a delayed T-cell reconstitution[102,103]. Other factors for CMV reactivation after HSCT include the conditioning regimen (at least within the first year following the procedure), treatments that may affect the immune response such as treatment associated to treat GVHD (corticosteroids), and the use of unrelated or mismatched donors [104].

1.4.4 Acute and Chronic Graft-versus-Host Disease and CMV

After HSCT, CMV and GVHD often occur concomitantly. Thus, the correlation between acute GVHD with CMV has been studied to characterize the exact relationship between these two clinical events [105]. An epidemiological study showed a higher risk of CMV reactivation and pneumonitis in patients with aGVHD. The increased risk for CMV reactivation may also be explained by the immunosuppressive therapy used to treat aGVHD, such as high doses of corticosteroids [106-108]. An association between CMV infection and chronic GHVD has been proposed [109] as CMV reactivations seem to be a risk factor for the development of cGVHD possibly associated with the inflammatory environment [110]. Antibodies against CD13, a marker highly expressed on infected cells and present on all cells that may be infected by CMV, were found in patients developing cGVHD suggesting that CMV infection may trigger the humoral immune response toward autoantigens [111]. Therefore, removal of CD13+ cells was suggested to decrease the risk of CMV infection and thereby associated cGVHD [112]. Also other studies have shown that CMV infection is a risk for more severe cGVHD and early intervention based on PCR monitoring was suggested to decrease the risk [113]. In contrast to cGVHD, the question whether CMV increases the risk for aGVHD remains controversial [114-116]: A bidirectional relationship between CMV infection and aGVHD is feasible due to the pro-inflammatory function of the virus and the cross-recognition of the CMV-specific T-cells responsible for alloreactivity [117,118]

1.5 CMV-SPECIFIC T-CELLS IN PATIENTS WITH CANCER

1.5.1 Viral Infection and Cancer

A correlation between virus and cancer was proposed already the in early 1950s since virus can exchange genetic material with the host cell. Main oncoviruses known today in humans are papillomavirus (cervix cancer), hepatitis B and C virus (liver cancer) and EBV (lymphoma and post-transplant lymphoproliferative disorder).

Human CMV is not an oncovirus but its association with cancer has been investigated for decades as there is evidence of its role in several malignancies. It was described by Geder et al. during the 1970s to affect proliferation, survival, angiogenesis, invasiveness and immune modulation of tumor cells infected in vitro [119]. CMV could then be involved in "oncomodulation", a term and concept suggest by Michaelis et al. to explain the role of CMV in human cancer; that is, its ability to catalyze the oncogenic process [120]. CMV has been found at higher frequency in tumor tissues of several malignancies such as colon cancer, prostate cancer, breast cancer or glioma [5-7,121,122]. But is CMV a player in the cancer pathogenesis or CMV has only an increased tropism for tumor cells?

1.5.2 Molecular Effects of CMV leading to Oncomodulation

Many CMV immune evasion strategies are common to tumor escape mechanisms and oncogenesis: modulation of apoptosis, angiogenesis, cell adhesion and evasion, genomic injury and transcription factor activity. Therefore, CMV-infected tumor cells could see their oncogenesis proprieties improved. Many CMV proteins are involved in modulation of host cell controlled growth. IE1 and IE2 proteins have been described to inhibit the induction of apoptosis, which is a common feature of cancer cells [123]. CMV protein UL36 and UL37 promote an anti-apoptotic effect by inhibiting the Fas-mediated apoptosis and Bax/Bak activity [120]. CMV is also known to downregulate NCAM, a cell adhesion molecule that may lead to enhanced tumor cell migration [124]. Angiogenesis can be enhanced by CMV IE1 protein that stimulates the expression of IL-8, a promoter of tumor angiogenesis [125]. As mentioned earlier, CMV is able to decrease the host cell surface expression of MHC class I and II allowing the tumor cell to escape the T-cell immune response (Table 1) [126]. Finally, the viral protein UL76 has been shown to induce chromosome aberrations contributing significantly to genetic instability, which is a major cancer promoter (Figure 7) [127]. CMV has been detected and involved in various cancers, yet, the clear implication of the virus remains unclear. Does it lead to tumor transformation, improve the oncogenesis or only have an affinity for tumor cells that rapidly expand? Regardless, the idea to target CMV for cancer therapy has come back this last decade with several clinical trials using CMV-directed adoptive cell therapy [128].

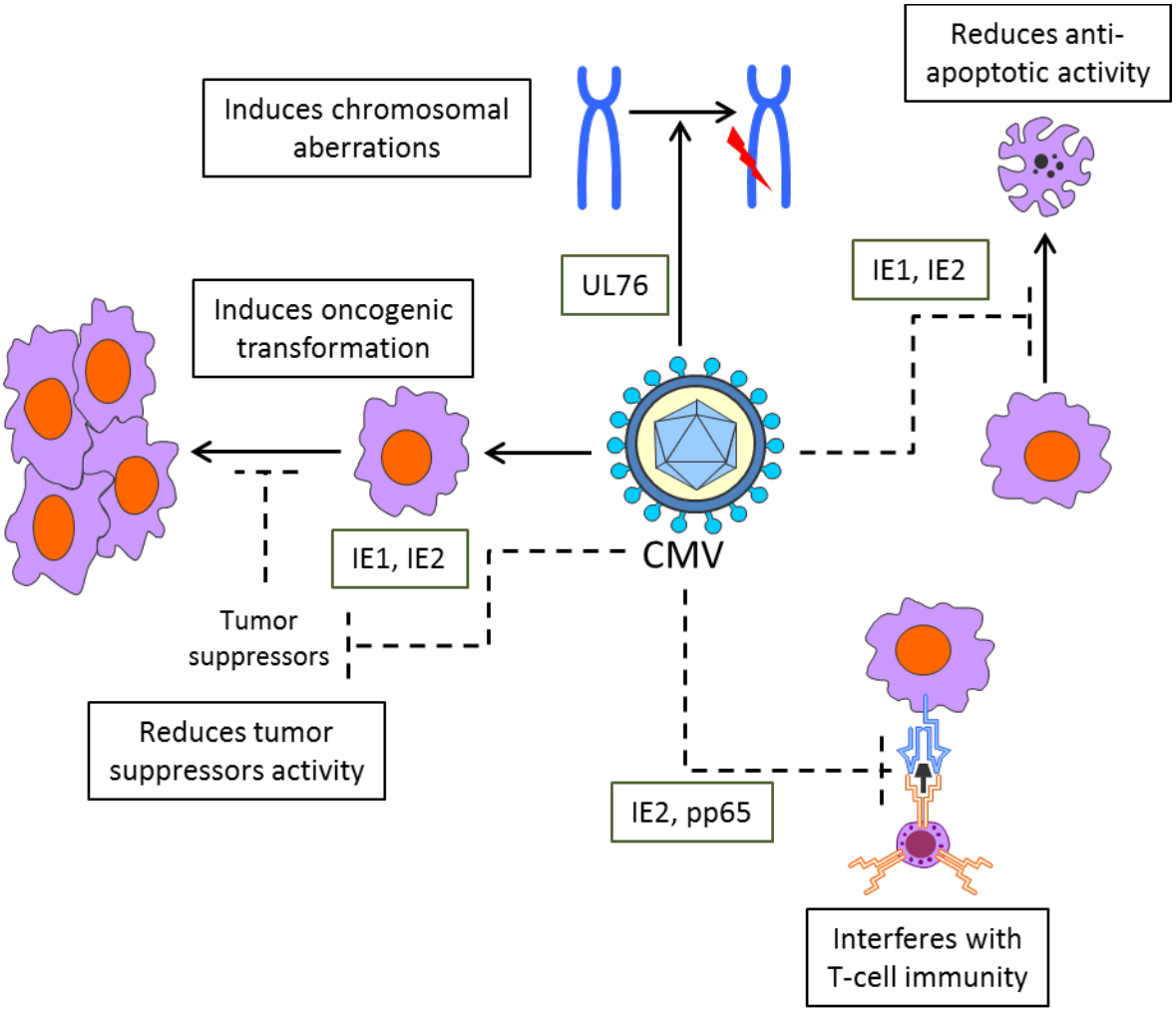

Figure 7 CMV proteome involved in modulation of host cell controlled growth, adapted from Herbein G et al. [121]

1.5.3 CMV in Cancer: Glioblastoma Multiforme and Pancreatic Cancer

Glioblastoma multiforme (GBM) are brain tumors usually highly malignant with a low median survival about 14,6 months and a two-year survival of 30%. Treatment options are few and inadequate: surgery, chemotherapy (temazolomide) and radiotherapy [129].

CMV infection and its role in GBM pathogenesis have been controversial since the first report and many studies have confirmed or refuted it [122,130]. The role of CMV in GBM is still unclear but few specific oncomodulatory proteins have been described to participate in the biology of GBM: angiogenesis (pp71, US28, gB), invasion (US28, gB), stem cell maintenance and immortalization (IE1) and immunomodulation (cmvIL-10) [131]. Furthermore, CMV specific T-cells demonstrated a specific killing of autologous GBM cells suggesting that a CMV-directed adoptive cell therapy for patients with glioma may be possible [132]. A phase II randomized trial using valganciclovir prophylaxis in a small cohort of patients with GBM showed a higher median survival of valganciclovir treated patients compared to the

untreated group [133,134]. A possible effect of the antiviral treatment is that CMV infection promotes the dissemination of tumor cells. Chemotherapy fails to induce tumor cell death while valganciclovir treatment may suppress CMV proliferation, which restores the sensitivity to chemotherapy. There are limitations to this study making interpretation of the results difficult. Therefore, additional studies of antiviral strategies and CMV-based immunotherapy for glioma are warranted and ongoing, suggesting that immunotherapy combined with chemotherapy after surgery may be a safe novel treatment option and may offer clinical benefit for patients with recurrent GBM [128].

Pancreatic adenocarcinoma (PDAC) is the most frequent form of pancreatic cancer and has the worst prognosis with a five-year survival of 5%. PDAC is usually diagnosed late and presents often a resistance to chemo and radiotherapy with only 10-20% of the patients with a resectable cancer [135]. No correlation between CMV and PDAC has been directly established.

1.6 MANAGEMENT OF CMV INFECTION

1.6.1 Monitoring

As described earlier, the risk for CMV infection can be estimated by the serological status of the recipient and donor [136].

CMV monitoring after HSCT can be done by different methods. Cell culture is a traditional method to detect CMV but is no longer used due to it being too insensitive and slow. The antigenemia assay is a method that can be used to rapidly detect the viral pp65 antigen with high sensitivity in the peripheral blood leukocytes [137]. Its use has decreased in recent years since it is semiquantitative and requires trained staff for interpretation. Quantitative PCR assays usually with real-time technology are the most sensitive method for detecting CMV DNA in whole blood or plasma and high levels of CMV DNA in blood can be a predictor of CMV disease [138]. However, especially with CMV gastrointestinal disease, CMV blood viral load can be negative [139]. Detection of CMV mRNA can also be used for monitoring but this method is less frequently used today. Immunochemistry for CMV detection can be performed on tissue samples and is useful for diagnosis of invasive CMV disease [140].

1.6.2 Antiviral Drugs

Anti-viral treatment to prevent and manage CMV infection has been improved over the past decades, mainly motivated by the post-HSCT risk for complications. Different management strategies can be used: Antiviral prophylaxis given especially during the 3 first months post-HSCT to prevent CMV replication, preemptive antiviral therapy to prevent the development of the disease when CMV reactivation has occurred, and treatment of established CMV disease. Different antiviral drugs are directed against different viral enzymes: ganciclovir,

valganciclovir, valacyclovir, high dose acyclovir and maribavir are directed against pUL97, a protein kinase. Foscarnet and cidofovir directly target pUL54, the viral DNA polymerase. Choice of treatment is based on the timing of CMV infection [141] (Table 3). More recently letermovir, an agent directed against CMV-terminase complex pUL51 and pUL56, was shown to be highly effective as prophylaxis reducing the risk for clinically significant CMV infection and also improving survival at 6 months after HSCT [142].

Viral replication			
	Diagnosis of CMV infection		**Viral Disease**
Timing of management	**Prophylaxis** (all patients)	**Pre-emptive therapy** (some patients)	**Treatment of established disease** (few patients)
Ganciclovir	Yes*	Yes	Yes
Acyclovir	Yes**	No	No
Valacyclovir	Yes**	No	No
Valganciclovir	Yes*	Yes	Yes
Foscarnet	No	Yes	Yes
Cidofovir	No	Yes	Yes
Maribavir	No	Yes	Yes
Letermovir	Yes	No	No

Table 3. Timing of CMV management and treatment. * Rarely used due to toxicity. ** Limited efficacy

1.6.3 Immunotherapy and Vaccines

As an alternative to antiviral drugs against CMV infection post-HSCT, adoptive T-cells therapy has been evaluated as a possible treatment options the last 25 years.

Already in the early 90s, adoptive transfer of CMV-specific CD8+ T cell clones proved the efficiency of the procedure by reconstituting the CMV-specific protective immunity [143,144]. This T-cell therapy has also been successful for patients with antiviral drug resistance [145]. HLA class I multimers and streptamers were used to select CMV-specific CD8+ T-cells before expansion and infusion into the patients, showing a reduction of their CMV viral load and some patients experienced clearance of CMV infection [146,147]. Less than 1 million/kg bodyweight of a single infusion of a pp65-specific cell dose was shown to be sufficient to clear the infection [148]. IFNγ capture T-cell technology, without MHC restriction, has proven its efficiency by adoptive transfer of CMV-pp65-specific (CD4+ and CD8+) T-cells in a pre-emptive therapy setting [149,150]. Advances in adoptive T-cell approaches allow new strategies for T-cell strategies for therapy. For instance, an Ad5f35pp65 vector was successfully used to create T-cells specific for CMV, EBV and several serotypes of adenovirus from a single cell culture [151]. Adoptive T-cell therapy is not yet able to be used as single therapy and acts most efficiently as adjunct treatment in anti-viral treatment but can be used as an alternative when primary antiviral therapy treatment fails [152].

Currently, no CMV vaccine has been licensed, although some vaccines are in late-phase clinical trials and its development is a high priority of the National Vaccine Program Office. A list of the CMV vaccines currently or recently in clinical trials has been published in 2016. This listed 26 trials in phases I and II using different vaccine technologies: DNA, vectored, attenuated, recombinant and peptides vaccines targeting mainly pp65, gB and IE1 antigens [153,154].

2 AIMS OF THE THESIS

The general aim of the thesis was to characterize and better understand the evolution of the immune response that targets one of the most common human viruses: CMV. For it, the investigation was done in different clinical settings: patients post-HSCT and patients with brain cancer.

We aimed, in **Paper I** and **II**, to gain insight into the CMV-specific immune response and the relationship between GVHD and CMV.

In **Paper III**, we aimed to define the function of IL-7 and soluble IL-7R in patients post-HSCT that are at risk of CMV reactivation and GVHD.

In **Paper IV**, we aimed to gain insight into the complex relationship between the CMV immune response and patients with cancer to identify the CMV specific immune response as a potential biomarker of immune fitness.

3 MATERIALS AND METHODS

3.1 ETHICAL IMPLICATIONS AND CLINICAL INFORMATION

For this thesis, all research was performed on healthy individuals' and patients' blood or peripheral blood mononuclear cells (PBMC). For each single study, the work was approved by the Regional Ethical Review Board in Stockholm (diary number: 2010/760-31/1 for **Paper I**, **II** and **III**, diary numbers 2013/576-31 and 2013/977-31 for **Paper IV** and 2009/1183-3 for the healthy individuals). All patients agreed to donate blood samples for research purposes and informed consent was obtained from them or from their parents or legal guardians in the case of children. Each patient was attributed an individual code to allow pseudonymization of data. Patients clinical information was collected by the staff from the clinical wards. For **Paper I** (N=277), **II** (N=23) and **III** (N=61), when possible, heparinized blood was taken before HSCT (time point 0) and at month 1, 2, 3, 6, 12 and 24 after HSCT. For **Paper II**, patients were selected based on HLA-A*02:01 positive and no ATG treatment. For **Paper IV** (N=374), blood was collected from patients with brain (n = 314) or pancreatic cancer (n = 60) on the day of surgery before anesthesia of the patient. Patients with brain tumor received corticosteroids at time of diagnosis. Samples from sex- and age-matched healthy individuals were used as controls for **Paper III** (n=26) and **IV** (n=244). All samples were processed in the laboratory within 24h.

3.2 WHOLE BLOOD ASSAY AND ELISA

Whole blood assay (WBA) used for **Paper I** and **IV** provides, in contrast to PBMC culture, a physiological environment closer to *in vivo* allowing the study of the immune response to specific antigen stimulation. Blood of the individual was first diluted with RPMI medium. Diluted blood was then added in duplicates to a pre-coated plate with CMV-pp65 and EBNA-1 proteins and incubated for a week. Phytohemagglutinin protein (PHA) and antigen-free medium were used as positive and negative controls. After the incubation period, supernatants of the cell culture were harvested. Note that for **Paper IV** only, the blood was conditioned with IL-2, IL-15 and IL-21 cytokines to gauge the ability of the immune cells of patients with cancer to respond to cytokine stimulation.

Enzyme-linked immunosorbent assays (ELISA) were used to quantify the antigen-specific IFNγ production following WBA (**Paper I** and **IV**), plasma level of soluble IL-7R (sIL-7R, **Paper III**), and level of antigen-specific plasma IgG (**Paper IV**). The exact cytokine and antibody concentration was evaluated by measuring the optical density correlated with a standard curve of known concentration.

3.3 CMV-REACTIVE TETRAMER AND FLOW CYTOMETRY

Flow cytometry was performed for **Paper II** and **III**. Tetramers provide the quantification, visualization and sorting of antigen-specific (mostly CD8+) T-cells without any *in vitro* manipulation. The three different anti-CMV pp65 HLA-A2*01:02$_{\text{NLVPMVATV}}$ tetramers were constructed in-house by Dr. Axelsson-Robertson. The mutations a245v and q226a were introduced into the sequence of the heavy chain. Mutant a245v, which due to the amino acid (aa) substitution from alanine to valine at position 245, reduces the MHC class I-CD8 co-receptor interaction. Mutant q226a totally abrogates the interaction between MHC class I and the CD8 co-receptor due to the aa substitution from glutamine to alanine at position 226 (figure 8). A tetramer was created from monomeric MHC class I-peptide complexes and labeled with the appropriate fluorochrome.

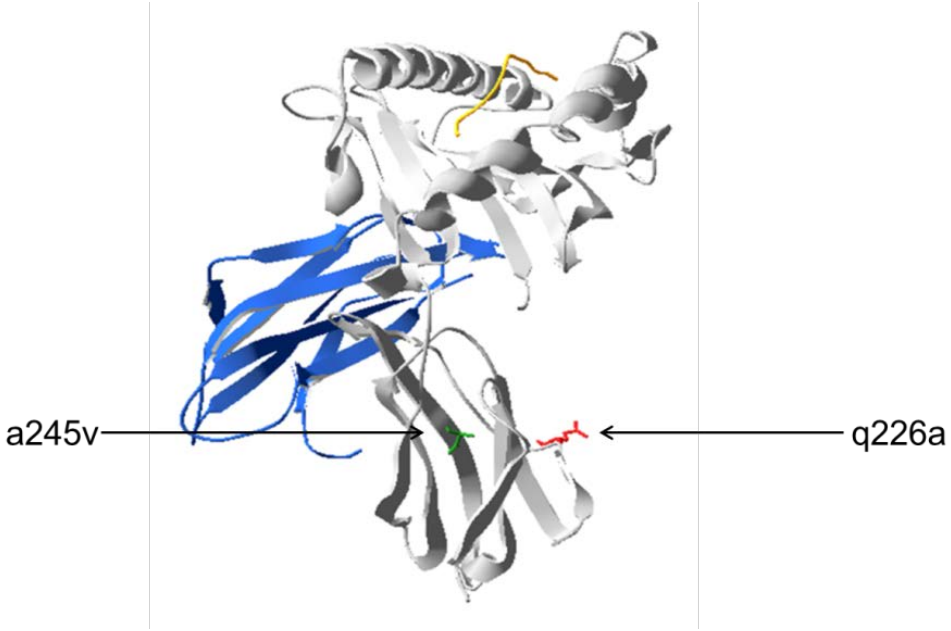

Figure 8. MHC class I molecule with the position a245 marked in green and the position q226 marked in red (from Dr. Axelsson-Robertson).

For **study II** and **III**, up to 12 different markers were used for a single panel (table 4, a non-exhaustive list of markers of interest used during this thesis). T-cell populations from patients who underwent HSCT were analyzed for IL-7R frequency, memory phenotype, exhaustion/activation and TCR affinity to CMV peptide antigen.

Marker	clone	Characteristic
CD3	UCHT1	identification of T-cell
CD4	RPA T4	identification of helper T-cell, binds class II MHC
CD8a	SK1	identification of cytotoxic T-cell, binds class I MHC
CCR7	G043H7	T-cell memory marker, homing marker to secondary lymphoid organs
CD45RA	HI100	T-cell memory marker
CD279 (PD-1)	EH12-2H7	activation/exhaustion marker
CD127 (IL-7R)	R34.34	IL-7 receptor
CMV wt tet		identification of all CMV-CTL
CMV a245v tet		identification of CMV-CTL with medium affinity TCR
CMV q226a tet		identification of CMV-CTL with high affinity TCR

Table 4: List of the markers analyzed by flow cytometry.

3.4 STATISTICS

For this thesis, most of the statistical analyses performed were univariate test. Differences within each group for comparison between time points post-HSCT were analyzed using Wilcoxon matched-pairs signed rank test. Differences between groups of unpaired samples were analyzed using Mann-Whitney U-test or Kruskal–Wallis test followed by Dunn's post-test. Differences between two groups over time post-HSCT were analyzed by two-way ANOVA followed by the Tukey's test for multiple comparisons to detect differences at specific time points. Linear regression was assed to determine the correlation. Survival was evaluated by the Kaplan-Meier survival analysis, log-rank test and cox univariate regression analysis.

Multivariate analysis was performed using Cox multivariate regression model (stepwise selection) for **Paper I** and multiple regression to analyze the clinical factors affecting the levels of sIL-7R at different time points in **Paper III**.

Statistical analysis was performed using GraphPad Prism software, R and Statistical software program (version 10).

4 RESULTS AND DISCUSSION

4.1 PAPER I

CMV and EBV immune response characterized by specific IFN-gamma production post-HSCT correlates with grades of GVHD and CMV reactivation.

Despite improvement in CMV management post-HSCT, CMV infection and reactivation remain major complications in the early months following the procedure. In this study, the aim was to analyze the CMV-specific immune response characterized by the IFNγ towards CMV-pp65 antigen and its clinical association with complications such as GVHD and CMV reactivation. Fresh blood from a large cohort of 277 patients (1244 samples with a median of 5 samples per patient) followed for 2 years was tested for CMV-pp65 stimulated specific IFNγ production.

Hundred twenty-eight of 277 (46.2%) patients with a pre-transplant positive CMV serology were treated at least once for CMV reactivation at a median time of 33 days post-HSCT. The median time to aGVHD development was 27 days and 43.7% of the patients presented symptoms of grade II-IV aGVHD. We observed a significant recovery of the anti-CMV immune response characterized by the specific IFNγ production at the first month post-HSCT. Normalization of the IFNγ production between patients and time points was established by evaluating the relative anti-CMV IFNγ response in relation to the response magnitude induced by the basal IFNγ response for each individual sample. Similarly, the relative CMV-specific IFNγ response presented as well the month 1 post-HSCT of interest with a significant higher relative IFNγ production the first month post-HSCT ($p<0.001$). Normalization of the IFNγ production may allow to evaluate the T-cells' ability to respond to a given antigen stimulation regardless the differences in cell number between patients and samples. Looking closer at the parameters that may affect the CMV-specific IFNγ response, we observed that patients with a positive CMV serology and those treated with ATG showed a higher IFNγ production especially at month 6 and 12 post-HSCT ($p<0.001$) compared to the CMV_{neg} patients that did not receive ATG. ATG treatment is used mainly in patients transplanted with unrelated donors but, as observed in our study, may create disturbances in the immune reconstitution [155].

Acute GVHD and the immunosuppressive treatment given for GVHD are known to increase the risk of CMV reactivation [106,141] while patients with CMV reactivation might have a higher risk of developing aGVHD [115]. Yet, the role of a CMV-specific immune response in GVHD development still requires understanding. We observed that the CMV-specific IFNγ production gradually decreased with increasing severity of the aGVHD ($p<0.001$) (Figure 9A-B). Since similar observations were made in response to EBV antigen and the positive PHA control, we assumed that the correlation was due to the prophylactic or therapeutic immunosuppressive treatment. Furthermore, patients who were unable to respond to antigen

stimulation (CMV and positive controls) showed a decreased survival. Akin to our previous observation, we assumed that this correlation revealed the impact of different grades of aGVHD on survival as the patients with the most severe grade of aGVHD (III-IV) presented survival rate much worse than any other grade of aGVHD (0-II).

Furthermore, CMV reactivation also appeared to stimulate the CMV-specific IFNγ production. Patients with CMV reactivation had greater CMV-specific IFNγ production regardless of aGVHD grade ($p<0.011$) as IFNγ response is one of the major arms of the immune defense toward CMV (Figure 9C) [156].

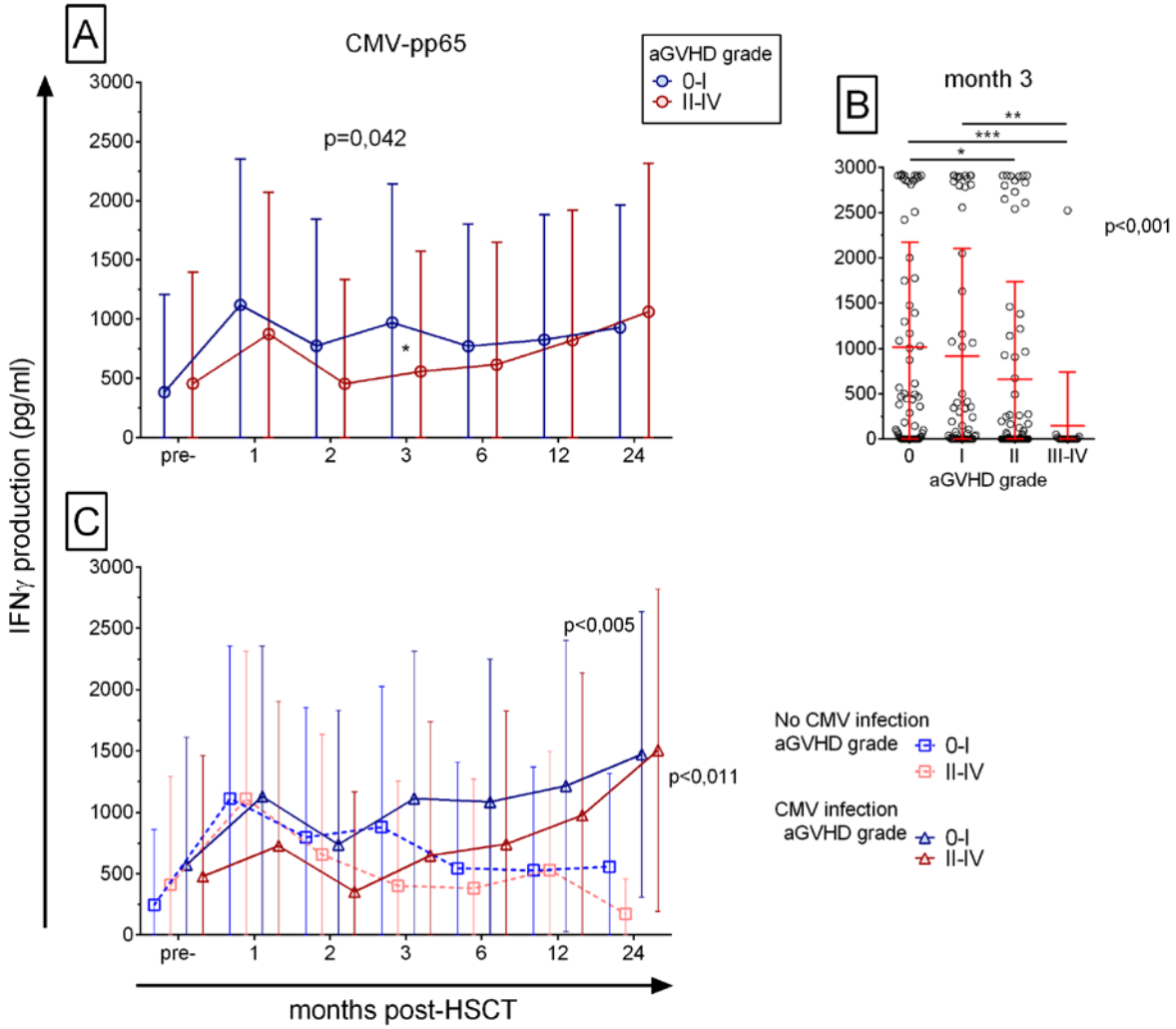

Figure 9. Impact of aGVHD and CMV reactivation in the CMV-specific IFNγ response in patients pre- and post-HSCT. **A**. Patients were grouped by aGVHD grades: 0-I and II-IV. **B**. CMV-pp65 specific IFNγ production at month 3 post-HSCT per aGVHD grades. **C**. Patients were separated into 4 groups by the CMV infection status and aGVHD grades.

Although we observed that the CMV-specific IFNγ response was greatly affected by many clinical parameters, this did not seem to be important for the patients' survival post-HSCT as the strongest clinical parameters in our cohort were aGVHD ($p<0.001$) or the use of donor lymphocyte infusion (DLI, $p<0.026$) that is given in relapse patients [157]. Mild grade of chronic GVHD had a beneficial effect on patients' survival, which could be explained by its protective effect on relapse [158]. Unexpectedly, female patients had a decreased survival post-

HSCT (p=0.01) (Table 5). This observation goes against previous data showing that male patients receiving a graft from a female presented a higher risk of both aGVHD and non-relapse mortality [159].

	beta	HR (95% CI for HR)	p.value
Gender	-0.63	0.53 (0.35-0.81)	0.003
DLI	0.58	1.8 (1.07-3)	0.026
aGVHD grade II	0.4	1.49 (0.92-2.4)	0.11
aGVHD grade III-IV	1.79	5.96 (3.2-11.12)	<0.001
cGVHD grade I	-1.13	0.32 (0.18-0.56)	<0.001
cGVHD grade II	-0.9	0.41 (0.16-1.03)	0.06
cGVHD grade III	-1.41	0.24 (0.07-0.83)	0.02

Table 5: Cox regression, multivariate analysis of the identified variables from the univariate analysis by stepwise selection.

This paper presents the anti-CMV immune response post-HSCT as characterized by the specific IFNγ production in association with crucial clinical parameters. In general, the anti-CMV response was similar to the response specific to EBV and the positive control which more reflects the general immune fitness of the different patients post-HSCT than an "antigen-specific" response. Further investigation is needed to better characterize what belongs to the general immune fitness and what belongs to the specific antigen response in this immunosuppressive clinical setting. The timing of intervention and clinical events also needs to be investigated to better understand the relationship and network between clinical parameters and the viral-specific immune response.

4.2 PAPER II

CMV-specific CD8+ T-cells with different TCR affinities segregate T-cell phenotypes and correlate with chronic GVHD in patients post-HSCT

Adoptive T-cell therapy (ACT) for CMV infection/reactivation has become a potential alternative to standard antiviral treatment to overcome the immunodeficient state of patients post-HSCT. Optimizing T-cell products for ACT might be achieved by a better characterization of the mechanism and biology providing an effective and long-lasting immune protection against transformed cells and pathogens like CMV. There is still room to improve the ACT, therefore, the aim of this second study was to characterize the CMV-CTL with TCRs of different affinities using three MHC class I-$CMV_{NLVPMVATV}$ peptide tetramers and to further correlate those different populations with clinical parameters such as CMV reactivation and GVHD.

The q226a mutant HLA-A2 tetramers ,which totally abrogates the interaction between MHC class I and the CD8 co-receptor, was functionally validated as we did not observe any inhibition of IFNγ production upon CMV-peptide stimulation while using CD8 blocking antibody. The wt tetramer binds to all CMV-CTL regardless of their TCR affinity, mutant a245v tetramer binds to CMV-CTL with medium and high-affinity TCR, and the mutant

q226a tetramer binds only to CMV-CTL with high-affinity TCR. Reconstitution of the CMV-CTL was then investigated in 23 patients during a 12 months period post-HSCT using the three different tetramers. CMV-CTL with high affinity TCR were found at higher frequencies over time post-HSCT (p=0.007) while CMV-CTL with medium-affinity TCR were present at a lower proportion as compared to CMV-CTL populations with low- and high-affinity TCRs (p<0.004) (Figure 10).

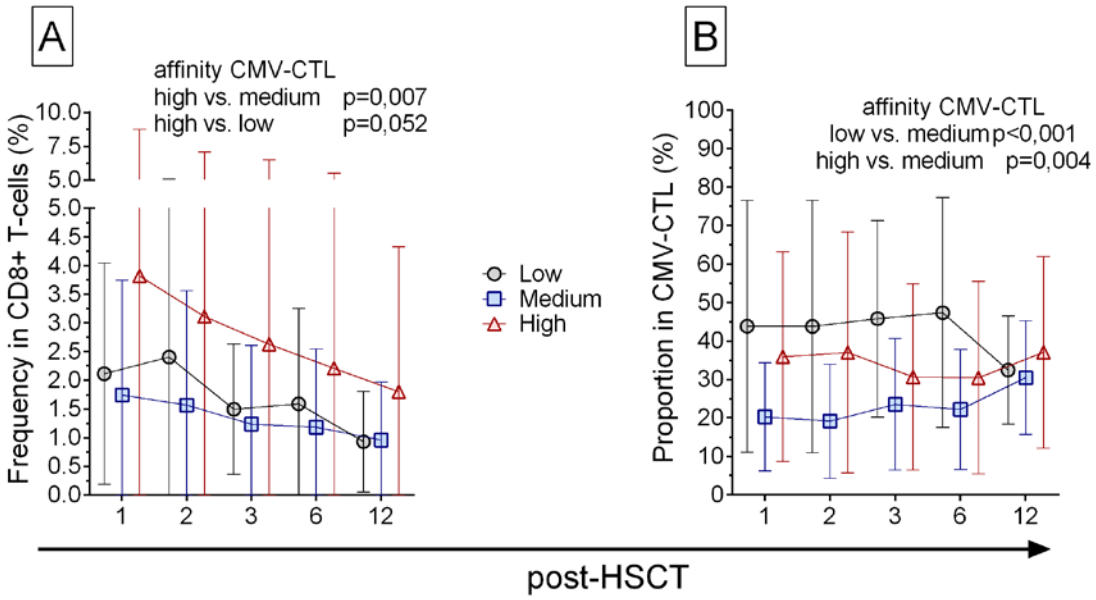

Figure 10. HLA-A2 CMV tetramer-reactive CD8+ T-cells and CMV-specific T-cells post-HSCT. **A**. Differences between the CMV-CTL subpopulations frequency in CD8+ T-cells. **B**. Proportion analysis of the different subpopulations (low, medium, high-affinity) in the total of CMV-CTL.

Next, we observed that CMV-CTL with high affinity TCR were mostly effector memory T-cells (CD45RA− CCR7−) as compared to CMV-CTL with low- and high-affinity TCRs which were mostly terminally differentiated (CD45RA+ CCR7−) phenotype. High-affinity T-cell clones were previously described to exhibit different memory phenotypes [160]. Furthermore, high-affinity T-cells enrich the memory pool during secondary response suggesting a long-lasting immune protection [161,162].

We observed a higher PD-1 expression among CMV-CTL with high-affinity TCR compared to the medium-affinity CMV-CTL (p=0.013). The proportion of high-affinity CMV-CTL in the CD8+PD1+ was higher than the low-affinity CMV-CTL at the first month post-HSCT (p=0.012). Within the CD8+PD-1+ T-cell population, high-affinity CMV-CTL were found at higher proportion compared to low-affinity CMV-CTL (p=0.012) at month 1 post-HSCT. Furthermore, high-affinity CMV-CTL were found at higher frequency, mostly early post-HSCT in the CD8+PD-1+ T-cell population compared to the total CD8+ T-cell population (p<0.001) (Figure 11). Expression of PD-1 can be a marker of cell exhaustion or T-cell dysfunction but also of antigen-experienced T-cells [163]: CMV-CTL expressing PD-1 were described to conserve their ability of produce cytokines without cytotoxic or proliferative function [164].

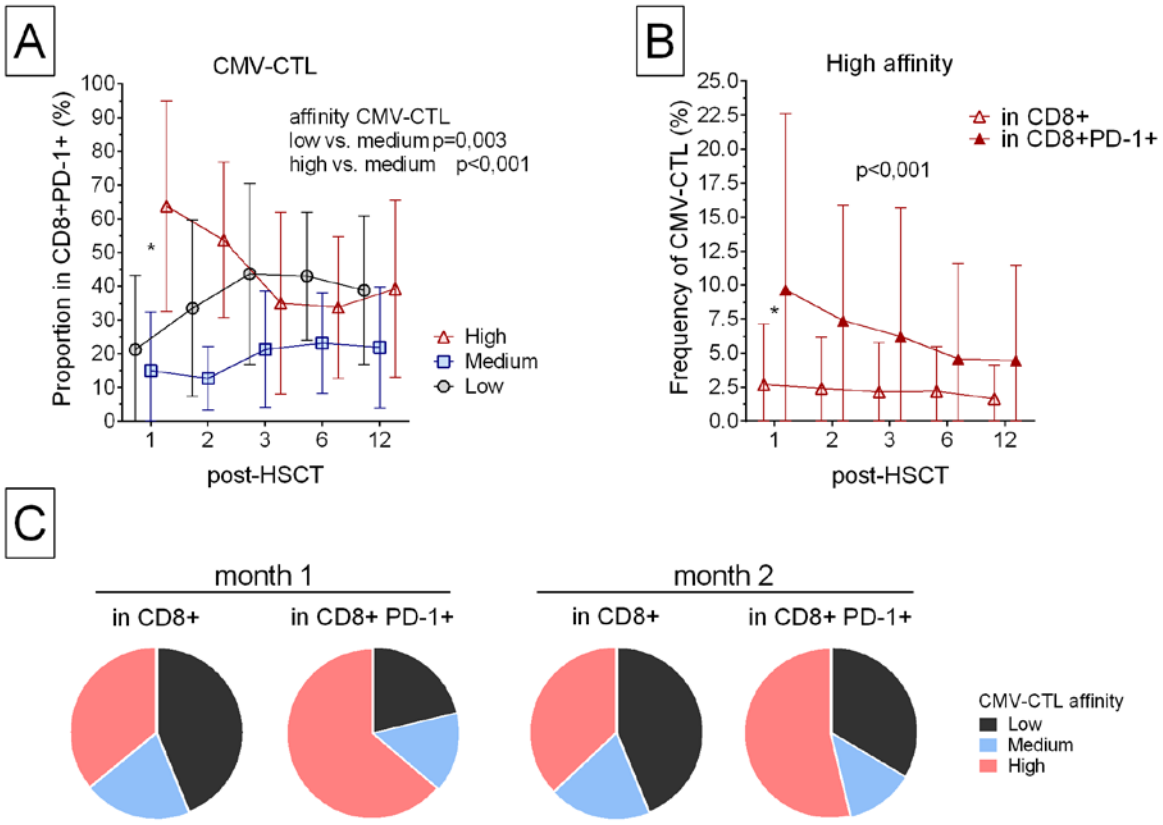

Figure 11. PD-1 expression of CMV-CTL with different TCR affinities over time post-HSCT. **A**. Proportion of the high/medium/low-affinity CMV-CTL in the CD8+ PD-1+ T-cell population. **B**. Comparison of the high-affinity CMV-CTL frequency between the total CD8+ T-cell population and the CD8+ PD-1+ T-cell population. **C**. Pie charts with the proportion of high/medium/low-affinity CMV-CTL the total CD8+ T-cell population and the CD8+ PD-1+ T-cell population at month 1 and 2 post-HSCT.

While analyzing the clinical factors that may affect the CMV-CTL, patients diagnosed for CMV reactivation (11/23) were found to have a non-significant (p=0.067) higher frequency of CMV-CTL compared to those who did not present CMV reactivation. No correlation between CMV reactivation and the different CMV-CTL affinities was found. We highlighted that patients with chronic GVHD showed a higher proportion of high-affinity CMV-CTL (p<0.001) compared to patients who did not show symptoms of cGVHD. Reversely, patients with cGVHD shower a lower proportion of low-affinity CMV-CTL (p<0.001) compared to those without cGVHD (Figure 12). Earlier studies have already suggested that T-cell populations with high affinity TCRs were the most susceptible to be cross-reactive [165,166].

This second manuscript presents the reconstitution of CMV-CTL with different TCR affinities in patients post-HSCT. CMV-CTL population with high-affinity TCR was at high proportion in the CD8+ PD-1+ population suggesting antigen-experienced T-cells. They had an effector memory phenotype while CMV-CTL with medium- and low- affinity TCR had a terminally differentiated memory phenotype. The correlation between cGVHD and high proportion of CMV-CTL with high-affinity TCR needs further investigation. Indeed, selection of cytotoxic T-cell population with high affinity TCR was suggested in the past to

be beneficial for ACT [167] but also associated with increased risk of alloreactivity like the present study suggests.

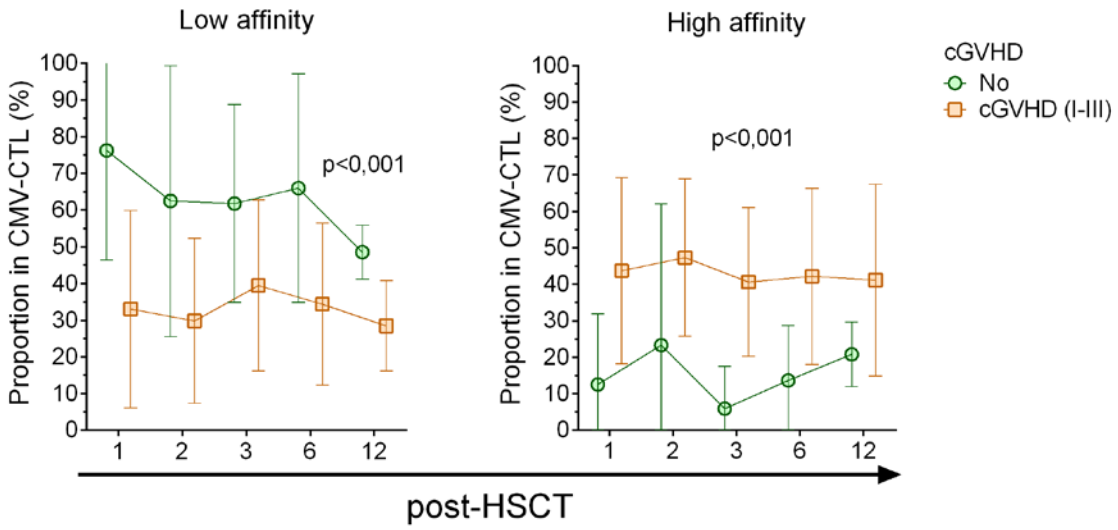

Figure 12. CMV-CTL frequency, low and high-affinity proportion correlated with cGVHD. Green circle: No cGVHD; orange square: cGVHD (I-III).

4.3 PAPER III

Reduced plasma levels of soluble interleukin-7 receptor during graft-versus-host disease (GVHD) in children and adults.

IL-7 is an essential cytokine which, via the IL-7R, induces T-cell homeostatic proliferation and survival. IL-7 is necessary for maintenance and expansion of immune responses against infection or reactivation post-HSCT. Reversely, increased IL-7 levels are associated with autoimmune immune responses and an increased risk of GVHD [168]. Soluble IL-7R is also associated with development of autoimmune responses when present at increased levels [169] but is also described to bind free IL-7 and consequently inhibit the IL-7/IL-7R signaling pathway. Therefore, we hypothesized that altered levels of sIL7R may be involved in GVHD and immune response towards viral reactivation post-HSCT.

Twenty-nine children and 32 adults were studied post-HSCT to determine the levels of IL-7 and soluble IL-7R in association with clinical events. At a membrane-bound level, CD4+ and CD8+ T-cells from children presented a higher expression of IL-7R over time post-HSCT compared to the adult cohort ($p<0.05$). No differences were found in the sIL-7R level between adults and children but both groups had an increased level of sIL-7R over time post-HSCT.

Correlation with clinical events showed that, in adults, a decreased level of plasma sIL-7R in patients was associated with any grade of acute GVHD ($p<0.05$) and CMV reactivation/infection (month 2 post-HSCT). Acute GVHD and associated

immunosuppressive treatment may be responsible for the delay in immune recovery and, consequently, is associated with the increased risk of CMV reactivation [115,141,155]. By multivariate analysis, ATG treatment was associated with lower sIL-7R levels in plasma (p=0.06) which is in line with our previous observation on GVHD and associated treatment (Table 6). Donor type was also an important factor associated with sIL-7R levels as those who received a graft from siblings had higher sIL-7R levels between two and six months post-HSCT (p<0.05) compared to patients receiving unrelated donor grafts. Stem cells from siblings may induce a better immune reconstitution than those from unrelated donors which could be represented by the higher level of sIL-7R level observed in our study [170]. This might also be due to the common practice to use ATG when transplants are performed from unrelated donors.

Factor	HR	95% CI	p-value
		1 month after HSCT	
Sibling donor	1.23	0.95–1.61	0.13
aGVHD	0.76	0.58–0.99	< 0.05
		2 months after HSCT	
Sibling donor	1.31	1.01–1.70	< 0.05
CMV infection	0.83	0.64–1.08	0.17
aGVHD	0.70	0.55–0.90	< 0.01
		3 months after HSCT	
Sibling donor	2.23	1.34–3.71	0.004
aGVHD	0.91	0.70–1.20	0.50
ATG	1.66	0.99–2.78	0.06
		6 months after HSCT	
Sibling donor	1.86	1.21–2.86	< 0.01
aGVHD	0.77	0.58–1.04	0.09
ATG	1.30	0.83–2.04	0.26
		12 months after HSCT	
Sibling donor	1.30	0.97–1.74	0.09
HLA-Mismatch	2.12	1.59–2.82	< 0.001

Table 6. Multivariate analysis of factors affecting sIL-7R levels over time post-HSCT.

This third study suggests an association of low level of plasma sIL-7R with acute GVHD. Soluble IL-7R could have a neutralizing role of free IL-7. We hypothesize that IL-7/sIL-7R complex is a "double-edged" sword as free IL-7 induces immune reconstitution and consequently promote the immune response towards infection such as CMV while increasing the risk of GVHD post-HSCT. In this setting, sIL-7R could play a role of "buffer" until release to ensure T-cell survival and proliferation.

4.4 PAPER IV

Epstein-Barr virus- and Cytomegalovirus-specific immune response in patients with brain cancer.

Antiviral immunity to common pathogens such as CMV and EBV, with prevalence above 80% in adults, is an integral and dominant component of immune competence in humans.

Patients with malignant glioma (GBM) and pancreatic cancer present the poorest prognosis and impairment of cellular immune responses may predict disease progression and consequently patient' survival. The last study presents the CMV-specific immune response in a different clinical setting: chemotherapy-naïve patients with advanced cancer. The aim was to investigate if CMV/EBV-directed immune reactivity characterized by the antigen-specific IFNγ production could be used as a clinically relevant biomarker of immune competence/exhaustion in patients with brain tumor (GBM and pancreatic cancer).

First, CMV-pp65-specific plasma IgG level were lower in patients with GBM compared to age- and sex-matched healthy individuals and those with pancreatic cancer ($p<0.05$ and $p<0.001$, respectively). Furthermore, among seropositive patients only, those with lower IgG titers (< median) at time of surgery displayed a decreased overall survival compared to those with higher titers of antibody ($p=0.017$) (Figure 13). An earlier study by Amirian et al. showed that patients with the lowest level of anti-CMV IgG exhibited the highest risk for glioma but no association with survival was made [171].

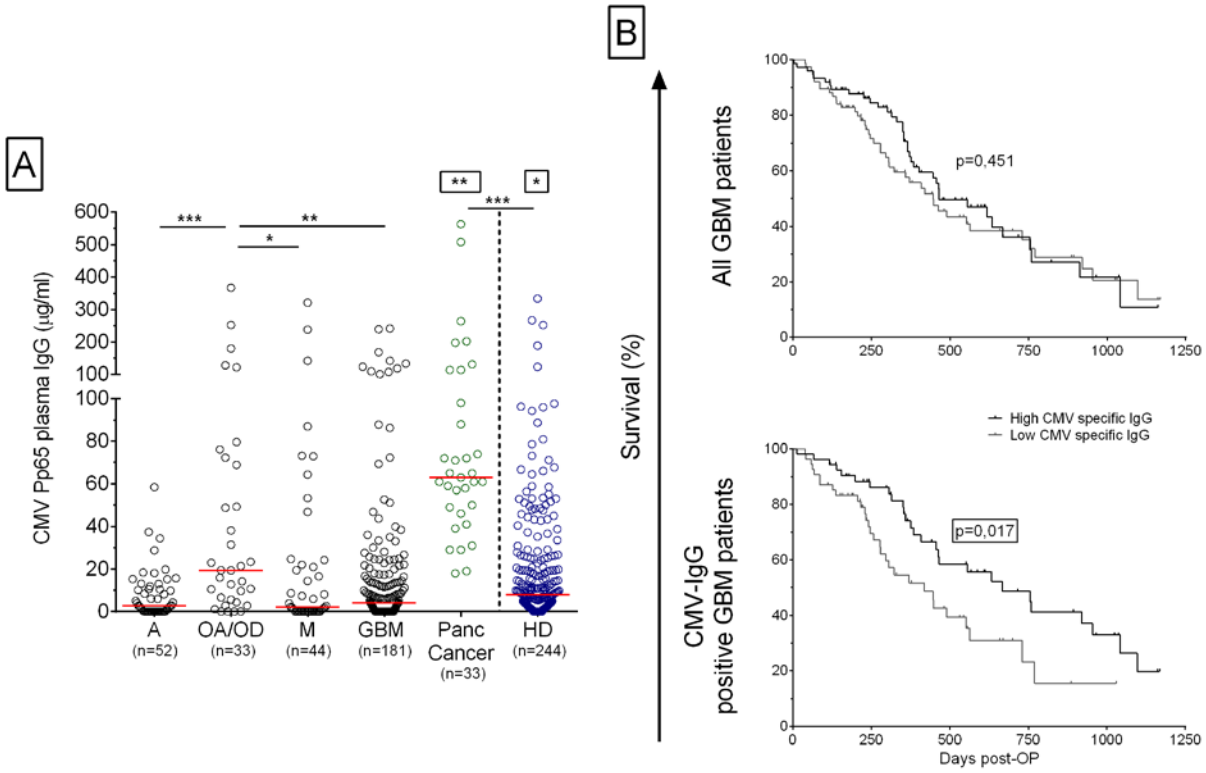

Figure 13. A. IgG recognition of CMV-pp65 in plasma of healthy donors (HD) and patients with brain tumor or pancreatic cancer. **B**. Survival of the patients with GBM based on the CMV-specific IgG levels. Top panel: all GBM patients. Bottom panel: GBM patients with detectable anti-CMV IgG. GBM glioblastoma multiforme, A astrocytoma, OA/OD oligoastrocytoma/oligodendroglioma, M metastatic disease, Panc Cancer pancreatic cancer

Secondly, the CMV-pp65 cellular immune response also appeared to be low in patients with GBM compared to healthy individuals and patients with pancreatic cancer. A similar observation was made toward the EBNA-1 and the positive control (PHA) cellular immune response suggesting that patients with glioma display a dysfunctional immunological control of latent infection. This dysfunction may be representative of the general immune impairment

due to the corticosteroids used to treat the brain inflammation in those patients but also from immunosuppressive agents secreted by the tumor itself [172,173].

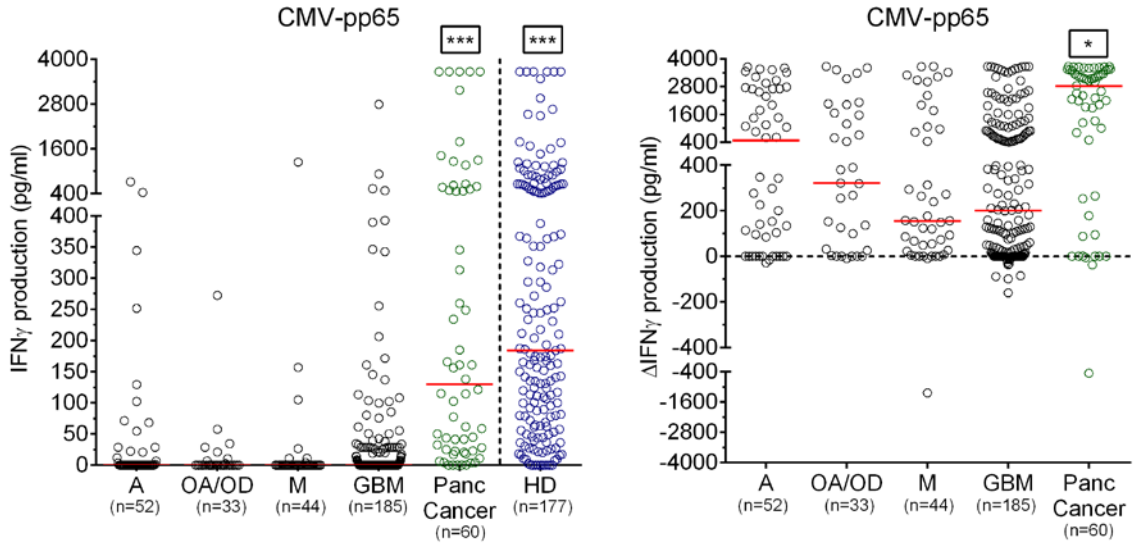

Figure 14. Absolute values and difference between unconditioned and IL-2/IL-15/IL-21 cytokine conditioning (ΔIFNγ production) of the CMV-pp65-specific IFNγ production for each group

Next, we observed that the responsiveness to cytokine conditioning characterized by the antigen-specific IFNγ production differences varied between patients groups, but also antigens themselves, suggesting different degrees of sensitivity to cytokine due to immune impairment that differs between cancer types. Yet, cytokine conditioning was able to improve the antigen-specific IFNγ response regardless of existing immunosuppression in patients with GBM (increased IFNγ production median above 200 pg/ml) (Figure 14). Interestingly, exclusively under cytokine conditioning, among the responding patients with GBM, those exhibiting a higher EBV-specific IFNγ production (> median) showed an increased overall survival compared to those with weak EBV-specific cellular immune IFNγ production (753 vs 370 days, $p < 0.001$). T-cell response to cytokine stimulation may differ due to the degree of exhaustion observed in patients with brain cancer [172].

In this report, the cellular immune response to viral antigens was different between groups of patients and did not seem to impact directly patients' survival. Altogether, this leads us to conclude that those observations reflected the general and complex immunological condition of patients with cancer in their ability to respond to common pathogens and to cytokine stimulation.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

It feels like this thesis is only a drop in the ocean of questions around the CMV specific immune response and its broad implications. And we come out from this with even more questions. Yet, our contribution brings some new information to our understanding of the CMV immune response in patients post-HSCT and with solid tumors. In this thesis, we looked at the relationship of CMV and the CMV specific T-cell response: the virus is triggering the immune system, shaping the phenotype, memory T-cells and frequency that may participate to clinical events while the general immune response together with CMV-specific one is crucial for the control of the virus reactivation:

In our studies post-HSCT, we observed that patients with an inexistent IFNγ production toward mitogen stimulation had a lower overall survival post-HSCT presumably due to deficient T-cell reconstitution. Deficient IFNγ production may be a marker for factors negatively affecting patient survival, mainly T-cell reconstitution, in patients post-HSCT [174]. The IFNγ have been earlier described to have a positive effect for CMV reactivation control: IFNγ is responsible for recruitment and activation of macrophages, T-cells, and NK cells [175]. It triggers the T-cell differentiation toward an antiviral effector function and increase the MHC expression and antigen presentation. Low level of CMV-specific IFNγ was correlated with high-level viremia and CMV disease post-HSCT using QuantiFERON-CMV-assay [176,177] and by intracellular staining: Patients with T-cells and more especially the CD4+ T-cell subset and that do not produce IFNγ upon CMV stimulation were showing a lower peak of CMV than patients who did not produced the cytokine. Furthermore, in a study by Avetisyan et al, no patient, who developed CMV disease, did have a detectable IFNγ production [178] To identify patients at risk for CMV disease, Özdemir et al reported that TNFα was a potential biomarker: While increased plasma level of TNFα during the first week post-HSCT may predict the development of CMV reactivation [179], inability of the CMV specific-CD8+ T-cells to produce TNFα upon CMV stimulation was associated with the reactivation of the virus [180]. In our study, however, low levels of IFNγ production were not correlated with CMV reactivation or disease, and did even show an opposite trend in after month 6 post-HSCT where the patients with CMV reactivation had a higher CMV-specific IFNγ production. This observation may be due that most CMV reactivations occur before the 6 month time point and patients controlling CMV develop IFNγ responses. However, mitogen-specific IFNγ production was found at higher level in patients that did not develop CMV reaction and aGVHD which reflects the general T-cell function of those patients from month 2 to 6 post-HSCT.

Functional IFNγ producing CMV-CTL can provide protection against CMV reactivation and are essential to prevent multiple reactivation of the virus post-HSCT as Moins-Teisserenc et al. demonstrated 10 years ago [181,182]. Multiple reactivations are the hallmark of patients unable to control CMV and are also associated with antiviral resistance, development of

CMV disease, and increased not-relapse mortality [103]. Furthermore, patients, who experienced an early CMV antigenemia, presented a higher frequency of CMV-CTL as compared to those who did not [180]. Lack of IFNγ producing CD8+ and CD4+ CMV specific T-cells were also associated with a risk of developing active CMV infection [183]. Only a trend in this direction was observed in our second study but due to the small number of patients, we could not look at the timing of reactivation (early versus late). Due to the phenotype of the CMV-specific CD8+ T-cells with high-affinity TCR and the correlation with clinical events, we speculated that those cells may be more suitable for anti-CMV protection but also associated with alloreactivity (GVHD). Söderberg et al showed that patients with CMV disease later developed extensive cGVHD with the association of CD13 (marker of myeloid cells)-specific antibodies production towards affected organs[110]. We could speculate the involvement of the CD4+ T-cells, knowing their crucial involvement on the enhancement of antibody-mediated immunity, in the humoral immune response activation against the CD13 autoantigen [184]. Furthermore, early intervention against CMV infection based on PCR reduced the risk of extensive cGVHD [113]. Together, these information show that a possible correlation between immune response against CMV and cGVHD. T-cells with high-affinity TCR for peptide-MHC (pMHC) may have enhanced function as compared to low-affinity that require a higher level of pMHC and more co-receptor stabilization and stimulation for T-cell activation [185,186]. The severe down-side of those T-cells with high-affinity is the risk of TCR cross-reactivity to self-antigen as it happened in a clinical trial on patients with myeloma and melanoma using engineered T-cells expressing an affinity-enhanced TCR [187]. Along with this cross-reactivity risk of the T-cells with high-affinity TCR, in the context of HSCT, a recent study reported homology peptide sequences presented to TCR between CMV and human origin. The authors suggested that, given the distribution of those targeted peptides (skin, gastrointestinal tract) and the correlation with the patients symptoms of GVHD, an involvement of a cross-reactivity may contribute to the initiation of GVHD in some patients [118]. It should be noted, however, that adoptive transfer of CMV-specific T-cells using clones or lines (therefore possibly with higher TCR affinity) has been clinically applied over the last decades without showing any increased risk of cGVHD [143,144,188].

Patients at high risk for CMV reactivation (i.e those with aGVHD and treated with corticosteroids) were described to present an impaired function of the CMV-CTL while the number of these cells was not different to those who were not a risk of reactivation [180]. In our study, no difference in the frequency of CMV-CTL regardless of the incidence of aGVHD was observed. We found instead a correlation between a higher frequency of CMV-CTL and development of cGVHD but no time-dependent analysis was performed. Reversely, the number of CMV-specific IFNγ produced was correlated with the severity of GVHD as steroid treatment commonly is associated with delayed T-cell reconstitution and can also affect the function of the CMV-specific T-cells [180,189]. While the development of aGVHD was not associated with the PHA specific IFNγ production at month 3 post-HSCT, Yong MK et al. showed a strong association of the amount of PHA specific IFNγ production with the aGVHD severity and within patients with aGVHD; those with a low production had a

reduced overall survival at 12 months post-HSCT and it was predictive for increased non-relapse mortality [174].

The T-cell reconstitution is as well correlated with plasma level of IL-7 by expanding mature T-cells and enhancing production of naive T-cells but this cytokine was also involved in driving and worsening GVHD [168,190,191] thus IL-7/sIL-7R complex is involved in the delicate balance post-HSCT. sIL-7R is generated by polymorphism in the IL-7R gene (*rs6897932*, amino-acid substitution in exon 6) leading to increased splicing in the transmembrane domain of exon. IL-7 contributes to the immune response towards infection but the single nucleotide polymorphisms (SNP) of IL-7R responsible for the soluble form have been correlated with an increased risk of autoimmune disease [169] and alloreactivity manifested by GVHD [192]. Soluble IL-7R role on IL-7 is poorly understood and two models are discussed: 1) plasma sIL-7R limits the availability of IL-7 for T cells or 2) potentiates the bioactivity of IL-7 by protecting IL-7 with a short term inhibitory function for a later availability of IL-7 [193,194]. In the third study, we observed a lower level of sIL7-R in patients with aGVHD and with CMV reactivation but CMV reactivation was not significant in the multivariate analysis the low level of sIL-7R suggesting GVHD as a more important driving force on the level of sIL7-R. Recent studies from Kielsen K et al. suggested a similar finding with a low level of sIL-7R and high ration IL-7/sIL-7R may be predictive for the risk of GVHD and CMV reactivation on larger cohorts of patients [195,196]. They further showed that the specific donor *rs6897932* TT genotype of SNP in the exons of the IL-7Rα was associated with those increased risk as compared to *rs6897932* CC and *rs6897932* CT. This specific genotype of the donor were found to also be associated with an increased risk of disease relapse compared to CC and CT donors [192].

In our study in patient with solid tumors, we reported that the patients with GBM had generally a lower IFNγ production against viral and mitogen antigens and were less responsive to cytokine stimulation as compared to other types of brain tumors and pancreatic cancer. The IFNγ production to specific antigens was used as biomarker for immune fitness since impaired IFNγ production is also a characteristic of advanced immune exhaustion that follows the loss of IL-2 production and later on of TNFα production. Immune exhaustion can be the result from repeated antigen exposure such as chronic viral infection or cancer [197,198]. The sensitivity to cytokine stimulation may be due to the immune dysfunction such as an impaired expression of the cell surface cytokine receptor of the patients with cancer [172]. The CMV serostatus of the patients was reported by Baumgarten, P. et al. to not be associated with overall survival [199] and we confirmed this observation in our study as within the group of patients with detectable anti-CMV IgG only, GBM patients with a low humoral anti-CMV IgG response presented a decreased median survival as compared to those with anti-CMV IgG higher above the median as observed in previous study [171]. No such was observed regarding the cellular immune response toward CMV antigen unlike the one towards EBNA-1 and PHA. Like in study I, the IFNγ production against CMV-pp65 did not seem to be a factor or a biomarker for the survival of patients with GBM. Under cytokine conditioning only, the EBNA-1- and PHA-specific IFNγ production were associated with the patients'

survival. As mentioned for study I, impaired IFNγ production may be a marker for factors negatively affecting survival in patients with cancer and response to PHA was earlier described as a candidate for biomarker of immune exhaustion in patients with brain tumors [172,200,201]. Furthermore, a recent study by Mohme M et al. presented reduced production of IL-2 and IFNγ upon PHA stimulation of the peripheral blood lymphocytes indicating a dysfunctional response of the peripheral immune cells as signs of immune exhaustion [202]. Therefore, despite the recent interest of EBV in GBM pathogenesis [203], in the present study, the association between its specific IFNγ production and overall survival may be the consequence of the general immune fitness of the patients as the relative IFNγ production analysis lets suggest. It should be noted that we analyzed the patients only at the time of diagnosis and we have not followed the patients with repeated analysis to see what was the influence of therapy against GBM. Therefore, the antigen-specific IFNγ production may be affected by other clinical parameters that govern patient survival such as use of other chemotherapy or radiotherapy, Karnofsky performance status, complete vs incomplete tumor excision and the use of steroids but this association was not studied in the present manuscript [204,205].

CMV is a known pathogen for almost 80 years and its specific immune response has been extensively studied since the discovery of the severe symptoms generated by CMV reactivation and disease in individuals with immature or immunocompromised immune system. Yet, even after all those years, we do not have a total understanding of the CMV-specific immune response and all its clinical implications. In the most recent decades, we saw that the CMV-immune response was not only involved in the protective effect of viral reactivation but also in various inflammatory disorders and in cancer with the oncomodulation properties. Furthermore, CMV has been shown to shape the general immune system and enlist a non-negligible fraction of the memory T-cell population that becomes even more prominent in the elderly (memory T-cell inflation) which could enhance the immunosenescence.

It is amazing to see how, such a common “inoffensive” virus, could be involved in so much clinical pathology. From being a pathogen to a direct player in the shape of the general immune system, there is still so much to learn from the immune response to CMV that may extend our knowledge of the immune system to a larger extend.

Much more can be done following those four studies. First, this thesis obviously lack of functional immunology techniques that would help to better understand and characterize the complex network between the CMV-specific immune response and the observed clinical association. More specifically:

It is necessary to analyze the function of the different subset of CMV-CTL observed in paper II. What are their distinct cytotoxic activities? How do they proliferate? What are they producing? We observed differences in the expression of PD-1 between them; thus, it

could be interesting to look at the other marker of exhaustion and stimulation (TIM-3, CTLA-4, LAG-3 etc.) and analyze the impact of corresponding checkpoint inhibitor such as PD-1 blocking antibody on the function and proliferation.

The correlation between GVHD and high affinity CMV-CTL may as well be of interest: Low and high affinity TCR recognize the same epitope but show to have different alloreactivity. More investigations could be relevant to improve the product for adoptive T-cell therapy.

In the line of paper III, more functional assays are necessary to understand the IL-7/sIL-7R complex function and involvement in immune reconstitution. It could be interesting to analyze the STAT5 phosphorylation level and Bcl-2 expression of the T-cells over time upon IL-7 stimulation supplemented with different concentration of sIL-7R to better understand the soluble form of IL-7R mechanisms.

Many parameters post-HSCT are important to consider for a clear interpretation of the correlation between immune response and clinical implications. Thus, some of the studies (paper II and III) are limited by the few number of patients or samples analyzed. Those findings would be interesting to reproduce in a different center and in a larger cohort of patients.

Furthermore, post-HSCT the time-dependent analysis is essential to picture the cause-consequence cascade of clinical events that occurs post-HSCT. The study I, II and III will need further investigation to get the best picture of this complicated network. In study I, the timing of CMV reactivation and GVHD needs to be analyzed with the quantification of viral-specific IFNγ. In the study II, it is not possible to dissociate the cause and the consequence of low levels of sIL-7R or GVHD and other clinical events. In study III, the different classes of CMV-CTL need to be looked time-dependently of CMV reactivation and at closer time point around the clinical events.

Last, longitudinal study of the immune response in patients with advanced cancer such as GBM and pancreatic cancer would enable the characterization of the evolution and parameters involved in the immune impairment of those patients. Blood samples before and after treatment or surgery, and at early versus late stage of cancer for immunomonitoring in parallel of pathogen monitoring may contribute to the understanding of the immune fitness/exhaustion of the patients and the pathogens such as CMV and EBV implication.

6 FRENCH POPULAR SCIENTIFIC SUMMARY

A la question, "de quoi traite votre (*ou cette*) thèse ? la réponse est généralement adaptée en fonction de l'interlocuteur, mais pour simplifier, elle se réfère souvent à un programme télé éducatif et ludique de mon enfance : vous souvenez-vous de la série « *Il était une fois ... la Vie*" ? Cette thèse traite des personnages se déplaçant dans de petits vaisseaux qui défendent le corps humain contre les microbes (ceux qui avaient un gros nez): c'est de l'immunologie.

Commençons donc par-là : les personnages se déplaçant dans de petits vaisseaux de « *Il était une fois... la Vie* » sont appelés communément globules blancs, et une population de ces globules est appelée lymphocytes. Il en existe beaucoup de genres mais les plus connus et sur lesquels cette thèse porte sont les lymphocytes T et B. Ils sont indispensables à la défense du corps humain contre les attaques venant de l'extérieur comme les virus, les bactéries mais aussi pour le protéger, les cellules qui dégénèrent pouvant être initiatrices de cancer.

Les lymphocytes T et B participent à la défense immunitaire contre un virus, le Cytomégalovirus ou CMV. Ce virus est « inoffensif » pour les personnes saines au système immunitaire efficace. Dans le pire des cas, il provoque des symptômes comme ceux de la mononucléose, appelée aussi « maladie du baiser ». Il est tellement « inoffensif » que plus de 90% de la population française adulte est porteuse de ce virus. « Inoffensif » est certainement inapproprié car il ne l'est pas vraiment: il est inoffensif uniquement parce que les défenses immunitaires de la majorité de la population sont efficaces contre ce virus. Le CMV provoque des déficiences comme la surdité et le retard mental chez le nouveau-né en cas d'infection chez la mère enceinte. Chez les personnes au système immunitaire déficient, le CMV peut se multiplier et avoir des répercussions graves comme la perte de la vision, l'inflammation du système digestif, la pneumonie et l'encéphalite pouvant même être fatal dans certains cas. Les principales personnes concernées et à surveiller sont donc celles porteuses du HIV et celles qui ont subi une transplantation d'organe ou de moelle osseuse.

Dans les manuscrits I et IV, l'étude s'intéresse spécifiquement à la réponse des lymphocytes T contre le virus CMV chez les patients ayant subi une transplantation de moelle osseuse et ceux ayant eu un cancer du cerveau. La réponse immunitaire contre le virus reflète davantage l'efficacité générale des défenses immunitaires du patient plutôt que la réponse spécifique contre le virus. En pratique, si le système immunitaire du patient ne fonctionne pas efficacement, en général c'est que la réponse spécifique contre le CMV ne peut non plus être fonctionnelle. Les patients ayant une mauvaise réponse immunitaire ont un taux de survie plus faible que ceux ayant une réponse immunitaire fonctionnelle. Aussi, l'observation a mis

en évidence que la réponse immunitaire contre le CMV pouvait être diminuée par les traitements donnés aux patients pour traiter les inflammations ou le rejet de la greffe. De plus, à des stades avancés de cancer du cerveau (glioblastome), les patients se caractérisent par une faible réponse immunitaire contre le CMV. En conclusion, les traitements sembleraient donc être un facteur important dans l'augmentation des risques d'infection au CMV chez les patients transplantés. De plus la réponse immunitaire contre le CMV pourrait être un marqueur biologique permettant d'estimer l'évolution du cancer et la survie chez les patients atteints d'un cancer du cerveau.

Le manuscrit II décrit plus en détail la réponse des lymphocytes T spécifiques contre le CMV chez les patients ayant subi une transplantation de moelle osseuse. Nous avons observé qu'il existe différentes classes de lymphocytes T en lien avec son affinité envers le virus CMV. Trois classes sont décrites : lymphocytes T à faible, moyenne et haute affinité. Il estintéressant de noter que ces trois classes présentent diffèrents signes de fatigue et d'activation ; celle à haute affinité semblant être plus actives que les autres classes. Lors de l'analyse faisant la corrélation avec les paramètres cliniques, l'élément important issu de l'étude est que les lymphocytes T à haute affinité envers le CMV sont trouvés en plus importante proportion chez les patients présentant des symptômes chroniques de la maladie du greffon contre l'hôte alors que l'inverse est relevé pour les lymphocytes T à affinité moyenne et faible. La maladie du greffon contre l'hôte est une grave complication d'une greffe de moelle osseuse, elle se produit quand les cellules provenant du donneur s'attaquent aux organes du patient. La forme chronique survient au-delà des trois mois suivant la transplantation. Les symptômes sont variés et contraignants pour le patient : douleurs musculaires, troubles pulmonaires etc. En conclusion, il est important d'étudier plus amplement les implications cliniques des différentes classes de lymphocytes T venant du donneur : les lymphocytes T avec une haute affinité envers le CMV seraient plus efficaces contre les infections et leur contrôle mais pourraient aussi engendrer un risque accru de développement d'une maladie chronique du greffon contre l'hôte.

Le manuscrit III se focalise sur la production d'interleukine 7 (IL-7) et de son récepteur chez les patients ayant subi une transplantation de moelle osseuse. IL-7 est une cytokine, c'est à dire une molécule soluble qui agit à distance pour réguler les fonctions et activités de différentes cellules cibles. IL-7 intervient sur les lymphocytes : elle promeut la prolifération et la survie des lymphocytes T. Elle est donc nécessaire au système immunitaire et induit une meilleure défense contre les infections. Les cellules cibles sont sensibles à l'IL-7 car elles possèdent un récepteur spécifique à cette cytokine (IL-7R). Cependant, il existe aussi une forme soluble du récepteur, qui n'est donc pas attachée aux cellules. L'action de celle-ci (sIL-7R) n'est pas encore bien connue. Le but de cette étude était de décrire la concentration de sIL-7R et IL-7 dans le sang de patients et de la corréler avec les paramètres cliniques. Il a donc été observé qu'une faible concentration de sIL-7R est liée au risque accru d'infection

par le CMV mais aussi au développement de la forme accrue de la maladie du greffon contre l’hôte.

Cette thèse est donc assez large de par ses contextes cliniques. Le but était de mieux comprendre comment l’humain se défend contre un virus aussi commun que dangereux selon l'état de santé de ce dernier. Après ces années d’études, force est de constater qu'il en ressort finalement plus de questions que de réponses: osons espérer malgré tout que cette contribution répond à certaines interrogations et aideront le corps medical ainsi que les patients..

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