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**Specific T- and B-cell Responses against
Human Cytomegalovirus after
Hematopoietic Stem Cell Transplantation**

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ABSTRACT

Human cytomegalovirus (CMV) remains an important complication in allogeneic hematopoietic stem cell transplantation (HSCT). CMV-reactivation may lead to CMV-disease associated with high morbidity and mortality in patients after HSCT. In this PhD thesis I have investigated how specific T- and B-lymphocyte responses against CMV reconstitute after HSCT. In my thesis I will therefore explain both how our studies were performed and what they have shown and furthermore I will give the reader some background in the fields of immunology, basic virology regarding CMV and the transplantation setting, which are all needed to understand the general aspect of the research.

In our 1st study we analyzed the effect of different pre-transplant related factors on the viral load (VL) and the effect of the VL and VL kinetics on the risk for CMV-disease in a series of consecutive allogeneic HSCT recipients. The VL influenced the risk for CMV-disease in univariate analysis but not when different factors were included in a multivariate analysis where only acute GVHD grades II-IV and the use of a CMV-negative donor to a CMV-positive recipient were significant risk factors. In patients, who required more than one course of preemptive therapy, acute GVHD and the rate of decrease in viral load during first preemptive therapy were significant risk factors for subsequent development of CMV-disease. The latter was a previously not described finding. Thus, the CMV VL kinetics is important after HSCT although we were unable to find a direct influence of the initial or peak VL on the risk for CMV disease.

In our 2nd study we evaluated the immune competence after HSCT by examining T-cell signaling and tested the phosphorylation of STAT5 in CD4⁺ T cells, CD8⁺ T cells and TCR $\gamma\delta$ T cells in response to stimulation with IL-7 or IL-2 after HSCT in association to CMV clinical outcome. Reduced responses to IL-7, reflected by STAT-5P may represent a clinically relevant functional biomarker for individuals at increased risk for CMV reactivation. This finding may also aid to design better strategies to improve anti-CMV immune responses without increasing the risk to develop GVHD.

For our 3rd and 4th study we wanted to investigate the reconstitution of humoral immunity against CMV after HSCT. We screened the entire CMV proteome to visualize the humoral epitope-focus profile in serum with a peptide microarray technology before and after HSCT in serum from patients divided into groups depending on CMV-serological status of donor and recipient (D+R+, D+R-, D-R+, D-R-). Data were analyzed using MaSigPro, PAM and the 'exclusive recognition analysis (ERA)' to identify unique CMV epitope responses for each patient group. Strongly (IgG) recognized CMV targets showed also robust cytokine production in intracellular cytokine staining (IL-2, TNF- α , IFN- γ and IL-17). To enable the global visualization of the entire intensity of Ig responses against CMV-epitopes a 3D model was used reflecting both the breadth and profile prior to and after reconstitution of the humoral immune response at different time points. Two different types of 3D graphs were constructed: CMV antigen regression surfaces (3DPOS) and peptide bulkiness /polarity regression surfaces (3DBP). We believe that high-content peptide microarrays allow epitope profiling of entire viral proteomes useful for diagnostics and therapy and that they may also be used to visualize the breadth of B-cell immune reconstitution after HSCT.

LIST OF PUBLICATIONS

- I. Ljungman P, **Pérez-Bercoff L**, Jonsson J, Avetisyan G, Sparrelid E, Aschan J, Barkholt L, Larsson K, Winiarski J, Yun Z, Ringdén O. ” Risk factors for the development of Cytomegalovirus disease CMV disease after allogeneic stem cell transplantation”. *Haematologica*, 2006 Jan; 91(1):78-83. PMID 16434374

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- III. **Pérez-Bercoff L**, Valentini D, Gaseitsiwe S, Mahdavifar S, Schutkowski M, Poiret T, Pérez-Bercoff A, Ljungman P and Maeurer MJ. “Whole CMV proteome pattern recognition analysis after HSCT identifies unique epitope targets associated with the CMV status”. Revised manuscript submitted to *PLOS ONE*, 2013 Oct 4.

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LIST OF ABBREVIATIONS

CMV	(Human) Cytomegalovirus
pp65	phosphoprotein 65
UL	Unique long
US	Unique short
IE	Immediate early
E	Early
L or LA	Late
HSCT	Hematopoietic stem cell transplantation
BM	Bone marrow
PBSC	Peripheral blood stem cells
CB	Cord blood
PBMC	Peripheral blood mononuclear cells
RIC	Reduced intensity conditioning
Sib	Sibling donor
MUD	Matched unfamiliar donor
ATG	Anti-thymocyte globuline
Cy	Cyclophosphamide
Bu	Busulphan
TBI	Total body irradiation
FTBI	Fractionated total body irradiation
MTX	Methotrexate
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
IEL	Intraepithelial lymphocytes
ILL	Innate-like lymphocyte
T _{reg}	T-regulatory cell
TSLP	Thymic stromal lymphopoietin
TCR	T-cell receptor
T _{CM}	Central memory (CD45RA ⁻ CCR7 ⁺) T-cell
T _{EM}	Effector memory (CD45RA ⁻ CCR7 ⁻) T-cell
T _{EMRA}	Effector or terminally differentiated (CD45RA ⁺ CCR7 ⁻) T cell
T _H cell	CD4 ⁺ T helper cell
TCL	CD8 ⁺ T cytotoxic cell
TLR	Toll like receptor

NK-cell	Natural killer cell
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
Ig	Immunoglobuline
cGVHD	Chronic Graft versus Host Disease
aGVHD	Acute Graft versus Host Disease
STAT5	Signal transducer and activator of transcription 5
TGF- β	Tumor growth factor beta
TNF- α	Tumor necrosis factor alpha
IFN- γ	Interferon gamma
BAFF	B-cell activating factor
EBV	Epstein-Barr virus
HIV	Human immunodeficiency virus
HHV	Human herpesvirus
IEL	Intestinal intraepithelial lymphocyte
PTLD	Post-transplantation lymphoproliferative disease
GCV	Ganciclovir
VGCV	Valganciclovir
CDV	Cidofovir
FOS	Foscarnet
MBV	Maribavir
PAM	Prediction analysis for microarrays
MaSigPro	Microarray Significant Profiles
3DPOS	3 dimensional antigen regression surfaces
3DBP	3 dimensional peptide/ bulkiness polarity regression surface
ERA	Exclusive recognition analysis
PCR	Polymerase chain recognition

1. THE HUMAN IMMUNE SYSTEM

1.1 OVERVIEW OF THE INNATE AND ADAPTIVE IMMUNITY

The immune system is traditionally divided into two complementary arms that work together protecting us from pathogens and tumors; the innate and the adaptive immune systems. The evolutionary older innate immune system provides a first line of defense that is immediate, but non-specific against invading pathogens, and it is often sufficient to prevent infection. Beside protection from epithelial barriers (the skin, the mucosal barriers and cilia of the respiratory tract), the innate immune system uses macrophages, granulocytes, mast cells, dendritic cells (DCs) and natural killer (NK) cells to perform phagocytosis, microbiocidal activities and to produce inflammatory mediators. Even though the innate system does not always succeed in removing a pathogen, it will promote initiation of the other arm of the immune system; the adaptive or acquired immune response that generates long-lived immunity (memory). The innate immune response cannot create memory and therefore the innate responses are not as fine-tuned to invading pathogens as compared to the adaptive immune responses [1-3]. The adaptive immune response can on the other hand expand memory immune cells that are able to respond to re-challenges by pathogens. The main effector cells of the adaptive immune response are the B- and T- lymphocytes, or B- and T-cells as they also are called. B-cells produce pathogen specific antibodies endowed with differential capacity to activate complement, while T-cells are responsible for both elimination of infected cells and support of B-cell function. After antigen encounter the antigen-specific receptor is activated and the B- and T-cells may clonally expand. The polymorphic immunoglobulin (Ig) receptors of B-cells can be adapted (maturation) according to the particular infectious agent, and they are able to trigger long-lived memory immune responses after termination of the initial response. Therefore, if the same pathogen invades, the immune response will be much faster and more effective. NK-cells have long been categorized as members of the innate immune response without immunologic memory. However, recent studies have suggested that NK-cells have the capacity to alter their behavior based on prior activation. [4]

1.2 T-LYMPHOCYTES

NK-cells, NKT-cells and T-lymphocytes (T-cells) contribute to the cell-mediated immune responses. As the precursor (naïve) T-cell encounters a specific antigen on the surface of an antigen-presenting cell (APC) in the context of an MHC class I/II antigen, which also expresses co-stimulatory molecules, the adaptive immune response is initiated. The effector phase is initiated when T-lymphocytes recognize their cognate target antigens, displayed as linear peptide stretches on the surfaces of infected cells bound to (self) MHC (in humans called HLA) molecules. Once activated, T-cells produce IL-2, driving them to proliferate and differentiate into several types of effector T-cells. [5]

T lymphocyte progenitor cells originate from lympho-hematopoietic stem cells in the bone marrow, but need to undergo maturation in the thymus [6, 7]. T-cell proliferation characterizing the early maturation is induced by cytokines, mainly by interleukin-7 (IL-7). The maturation involves the somatic rearrangement of antigen receptor gene segments. The combinatorial associations of multiple germline V, D and J genes generate the diversity of the T-cell-receptor (TCR) repertoires. During this maturation process thymocytes populate the cortex, acquire their antigen binding receptor, the TCR as well as the co-receptors CD8 and CD4. During the maturation in the thymus, T-cells pass two critical developmental steps: positive selection, showing that they can bind to HLA class I/II peptides and negative selection, showing that the affinity of the TCR to HLA-molecules is not too high [8]. As the surviving TCR⁺ thymocytes mature, they move into the medulla and become either CD4⁺CD8⁻ or CD8⁺CD4⁻ differentiating into either helper CD4⁺ T-cells (T_H cells) or CD8⁺ cytotoxic T lymphocytes (CTLs) or “killer T cells” and finally emigrate to peripheral lymphoid tissues [9].

CD8⁺ T cells/ CTLs recognize peptide:MHC class I complexes (see chapter 1.2.6 and 1.2.7) and once activated, they release perforin and often IFN- γ , TNF- α and carry membrane-bound effector molecule Fas ligand (CD178). Perforin helps to deliver granzymes (pro-proteases that are activated intracellularly to trigger) into the target cell. When Fas ligand (CD178) binds to Fas (CD95) it activates apoptosis in the Fas-bearing cell [10]. CD8⁺ effector cells fall into two subpopulations: type 1 CD8⁺ T cells (Tc1) and type 2 CD8⁺ T cells (Tc2). Both effector cell subpopulations display predominantly

perforin-dependent cytolysis in vitro. Production of IFN- γ , TNF- α is typical for a T_H1/Tc1 (cell-mediated) type response and IL-4 and IL-5 production is typical for a T_H2/Tc2 response, which favour more humoral immune responses [11].

CD4⁺ T helper cells (T_H cells) recognize peptide:MHC class II complexes (see chapter 1.2.7). T_H1 cells specialize in activating macrophages that have engulfed pathogens. The T_H1 cells will secrete IFN- γ to activate the infected cell. They can also express membrane-bound CD40 ligand (triggers activation of the target cell) and/or Fas ligand (triggers the death of Fas-bearing target cells) [12-14]. T_H2 cells specialize in promoting immune responses against parasites and also favour allergic responses by providing B-cell activation and secreting B-cell growth factors IL-4, IL-5, IL-9 and IL-13 [12]. T_H2 cells also express the membrane-bound effector molecule CD40 ligand, which induces B-cell proliferation and Ig isotype switching [15]. T_H17 cells produce cytokines of members of the IL-17 family and IL-6 and they promote acute inflammation by helping recruiting neutrophils to the site of infection [16]. T-regulatory (T_{reg}) cells produce inhibitory cytokines as IL-10, TGF- β and exert inhibitory actions that are dependent on cell contact [17]. (See chapter 1.2.3)

1.2.1 The T-cell receptor and CD4 and CD8 co-receptor

The TCR is a heterodimer composed of α and β chains (present on 90% of the T cells) or by γ and δ chains (present on 1-10% of T-cells; see chapter 1.2.2) and the disulfide-linked ξ chain (Xi chain), which is crucial for TCR signalling. T-cells that express functional γ and δ chains do not express α and β chains and vice versa. TCRs recognize peptides presented by the HLA class I molecule (if the T-cell has the CD8 co-receptor) or HLA class II molecules (if the T-cells has the CD4 co-receptor).

The CD4 and CD8 co-receptors both interact with the HLA-molecules and enhance the avidity of the TCR for antigen-HLA complexes by ensuring optimal orientation of TCR signalling molecules and signal transduction [18].

1.2.2 Gamma/delta ($\gamma\delta$) T-cells

$\gamma\delta$ T-cells express TCRs composed of γ and δ chains. Theoretically, the diversity of the $\gamma\delta$ T-cell repertoire is even greater than that of the $\alpha\beta$ T-cell repertoire, but the actual

diversity is limited because only a few of the available V, D and J segments are used in mature $\gamma\delta$ T cells. This may be due to the fact that $\gamma\delta$ T-cells serve as an early defence against limited number of commonly encountered microbes at epithelial barriers. For the $\gamma\delta$ T-cell development in the thymus IL-7 is required as it induces V(D)J recombination at the TCR γ locus by inducing histone acetylation regulating chromatin accessibility by recombinase (RAG) mediated cleavage [19]. Many of the intestinal intraepithelial lymphocytes (IEL) are $\gamma\delta$ T-cells, but $\gamma\delta$ T-cells also reside within other epithelia such as skin.

Due to this relative invariability of their receptors and because $\gamma\delta$ T-cells reside preferentially in specific locations in the body, they do not need to undergo clonal expansion before responding effectively to their antigens. They are therefore known as innate-like lymphocytes (ILLs) [20]. Moreover, $\gamma\delta$ T-cells may respond rapidly to molecules expressed by many different cell types because they recognize their target antigens directly instead of recognizing MHC-presented molecules as $\alpha\beta$ T-cells do. Some of the potential targets for $\gamma\delta$ T-cells are native protein targets, antigens presented by CD1 molecules and minor histocompatibility complex-presented antigens.

Activated $\gamma\delta$ T-cells display a broad cytotoxic activity and share certain effector functions with $\alpha\beta$ T-cells as well as with NK-cells and NKT-cells, but they are thought to have different aims [21-24]. Other proposed functions of $\gamma\delta$ T-cells are to limit the spread of infectious microorganisms by lysing infected macrophages [23, 25], to kill a broad variety of tumour cells [26] and to provide help to B-cells with altered clonotypes and inducing immunoglobulin hypermutation [27]. Gamma/delta T-cells may express a CD8^{dim} phenotype, but the majority are CD4⁻ and CD8⁻ [28]. It has been shown that a subtype of $\gamma\delta$ T-cells show a long lasting expansion and strong reactivity against CMV-infected cells in CMV-infected kidney transplant recipients [29, 30]. Recent data suggest a paradoxical association between CMV reactivation after HSCT and reduced leukemic relapse [31]. By expanding $\gamma\delta$ T-cells isolated from CMV-reactivating patients *ex vivo* and co-culture them with different malignant cell lines, it was shown that CMV reactivation after HSCT (from both CMV-positive and naive stem cell donors) associates with an increase in multipotent V δ 2^{neg} $\gamma\delta$ T-cell populations, which were able to recognize both CMV-infected cells and hematological tumor cells. $\gamma\delta$ T-cells isolated from patients with conventional stem cell grafts produced significantly

higher levels of IFN- γ upon contact with CMV-infected cells to compared with uninfected controls, and furthermore CD8 $\alpha\alpha$ expression appeared to be a signature of $\gamma\delta$ T-cells after CMV exposure [32].

1.2.3 T-regulatory cells

T-regulatory cells (T_{reg} cells or T-regs) were initially identified as a small percentage (10-15%) of murine CD4⁺ T-cells expressing CD25 (the α -chain of IL2R) [33, 34] and being critical for prevention of autoimmunity. It has been shown that deficiency in T-regs predispose to gastritis, thyroiditis, diabetes and graft-versus-host disease [35, 36]. Activation and expansion of self-reactive T-cells that managed to escape thymic clonal deletion are presumably suppressed by T-regs.

The T-reg phenotype includes expression of CD45RB, CD62L, CTLA-4, TNF-like receptor, CD103 and the intracellular transcription factor FOXP3 [37]. T-regs are "suppressive" or "regulatory", due to their ability to block proliferative responses of both T helper cells and CD25⁻ CD8⁺ T-cells [38, 39].

Human T-reg cells express low levels of IL-7R α . It was previously thought that T-reg cells were independent of IL-7 distinguishing them from most other T-lymphocytes that require IL-7 for development in the thymus as well as in the periphery. However, Mazzucchelli and Durum showed [37], that IL-7R α ^{-/-} mice did not develop T-regs in the thymus and T-regs were absent in peripheral lymphoid organs. IL-7^{-/-} mice developed normal numbers of T-regs suggesting that the IL-7 receptor is, most probably, activated by more than one ligand during T-reg cell development and implicating thymic stromal lymphopoietin protein (TSLP). Indeed, by promoting dendritic cell development, which in turn supported T-reg cell development, TSLP (but not IL-7) has been shown to indirectly induce T-reg cell development [40].

1.2.4 Memory T-cells

It is still under debate whether memory T-cells differentiate from effector cells or arise directly from precursor cells. Memory T-cells are more susceptible to antigen stimulation and are less dependent on co-stimulatory signals. In comparison to precursor T-cells, they display effector functions more rapidly and efficiently. The

different compartments of cells can be distinguished on the differential expression of cell surface markers.

Central memory T-cells (T_{CM}) express CCR7 but not CD45RA tyrosine phosphatase ($CD45RA^-CCR7^+$) and reside mainly in secondary lymphoid organs, i.e. spleen and lymph nodes. Effector memory T-cells (T_{EM}) are $CD45RA^+CCR7^-$ and are able to home to extralymphoid organs. Effector or terminally differentiated T cells (T_{EMRA}) express CD45RA but not CCR7 ($CD45RA^+CCR7^-$) and express large amounts of perforin [41]. See figure 1.

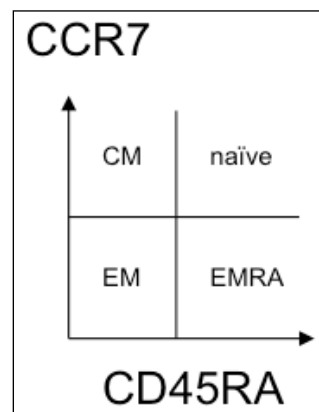


Fig. 1 T-cell compartments

1.2.5 $CD8\alpha\beta^+$ and $CD8\alpha\alpha^+$ T cells

The CD8 molecule is composed of two chains, one alpha and one beta chain. Most cytotoxic T-cells (CTLs) have CD8 molecules with one of each ($CD8\alpha\beta^+$ T-cells). However, a small subpopulation of CTL has two alpha chains, i.e. $CD8\alpha\alpha^+$ T-cells. The $CD8\alpha\beta$ heterodimers and $CD8\alpha\alpha$ homodimers share a similar structure [42]. However they interact differently with the HLA class I molecules. T-cells with low affinity TCRs require the co-stimulation of $CD8\alpha\beta$, whereas $CD8\alpha\alpha$ fails to support their activation. It has therefore been postulated that $CD8\alpha\alpha$ may serve as a modulating factor by affecting the ability of T-cells to efficiently transduce TCR signalling after activation and thereby modulating the avidity of T-cells by increasing the activation threshold [43].

$CD8\alpha\alpha$ homodimers can be co-expressed with $CD8\alpha\beta$ heterodimers and are found on many different cell types as T helper cells, CTLs, $\gamma\delta$ T-cells, “self-reactive” IELs, NK-cells, dendritic cells (DCs) and they can be found in the peripheral blood circulation [44].

1.2.6 HLA class I and class II molecules

In humans the major histocompatibility complex (MHC) is known as the human leukocyte antigen (HLA). The genes encoding for HLA molecules are highly polymorphic and are found on a 4-MB region, on the short arm of chromosome 6,

divided into two classes. HLA-A-G constitutes HLA class I and HLA-DR, -DQ, -DM and -DP constituting the HLA class II. The function of HLA class I and HLA class II molecules are to bind peptide antigens and to display them to antigen-specific T-lymphocytes. CD8⁺ CTLs recognize peptide antigens associated with HLA class I molecules, whereas CD4⁺ T helper cells recognize peptides associated with HLA class II molecules. HLA class I molecules are expressed on all nucleated cells. HLA class II molecules are expressed mainly on cells of the antigen-presenting cells of the immune system such as dendritic cells, macrophages, B-cells and thymic epithelial cells [45, 46].

HLA class I molecules are composed of a polymorphic α (or heavy) chain non-covalently attached to the non-polymorphic β 2-microglobulin (β 2m). Only the α chain is glycosylated. HLA class II molecules are composed of a polymorphic α chain non-covalently attached to the β chain. Both chains are glycosylated. Both classes are similar and both consist of an extracellular peptide binding cleft, a non-polymorphic IgG-like region, a transmembrane region, and a cytoplasmic region. The peptide binding cleft of class I molecules can accommodate peptides of 8 to 11 amino acids in a flexible, extended conformation. As the ends of the cleft are closed due to the presence of bulky tyrosine residues at both ends, larger proteins have to be “processed” to generate fragments small enough to bind to HLA class I molecules and be recognized by T-cells. The peptide-binding cleft of the HLA class II molecules is open and as a result longer peptides, of 12mers to 20mers, can be accommodated. The peptide binding off-rate is very slow, which ensures that there is enough time for T-cells to encounter and recognize the HLA: peptide-complex. HLA molecules are not specific to one single peptide but have a broad specificity for peptides with common features defined by certain anchor positions located in the peptide. [47-49]

1.2.7 Antigen processing and presentation pathways

B-cells recognize with their antigen receptors (immunoglobulins) a wide range of molecules in their non-processed form. T-cells however, can with their TCRs only recognize antigens in the form of a peptide bound to an HLA molecule on the cell surface, i.e. proteins from pathogens need to be degraded into peptides for the immune system to recognize it. This is called “antigen processing”.

Proteasomes, which are large protein complexes, are present in all human cells. They degrade damaged, “unwanted” or poorly folded proteins. Proteins from intracellular pathogens such as viruses also get degraded into peptide fragments by the proteasomes. Peptide fragments are then transported from the cytosol into the lumen of the endoplasmic reticulum (ER) by TAP, a transporter protein within the ER membrane. Inside the ER, HLA class I α (or heavy) chains are stabilized by calnexin, a membrane-bound protein, until the complex then binds to β 2-microglobulin (β 2m) and calnexin is released. The heterodimer of the α chain and the β 2m chain form a complex with tapasin, TAP and the chaperone protein calreticulin [50]. The HLA class I molecule remains in the ER until it binds to a peptide fragment delivered by TAP. The peptide: HLA class I molecule complex will then dissociate from tapasin and calreticulin, leave the ER and will be transported to the cell surface via the Golgi apparatus [51]. On the cell surface the peptide will be exposed to immune cells recognizing HLA class I molecules, i.e. $CD8^+$ T-cells [52]. See figure 2.

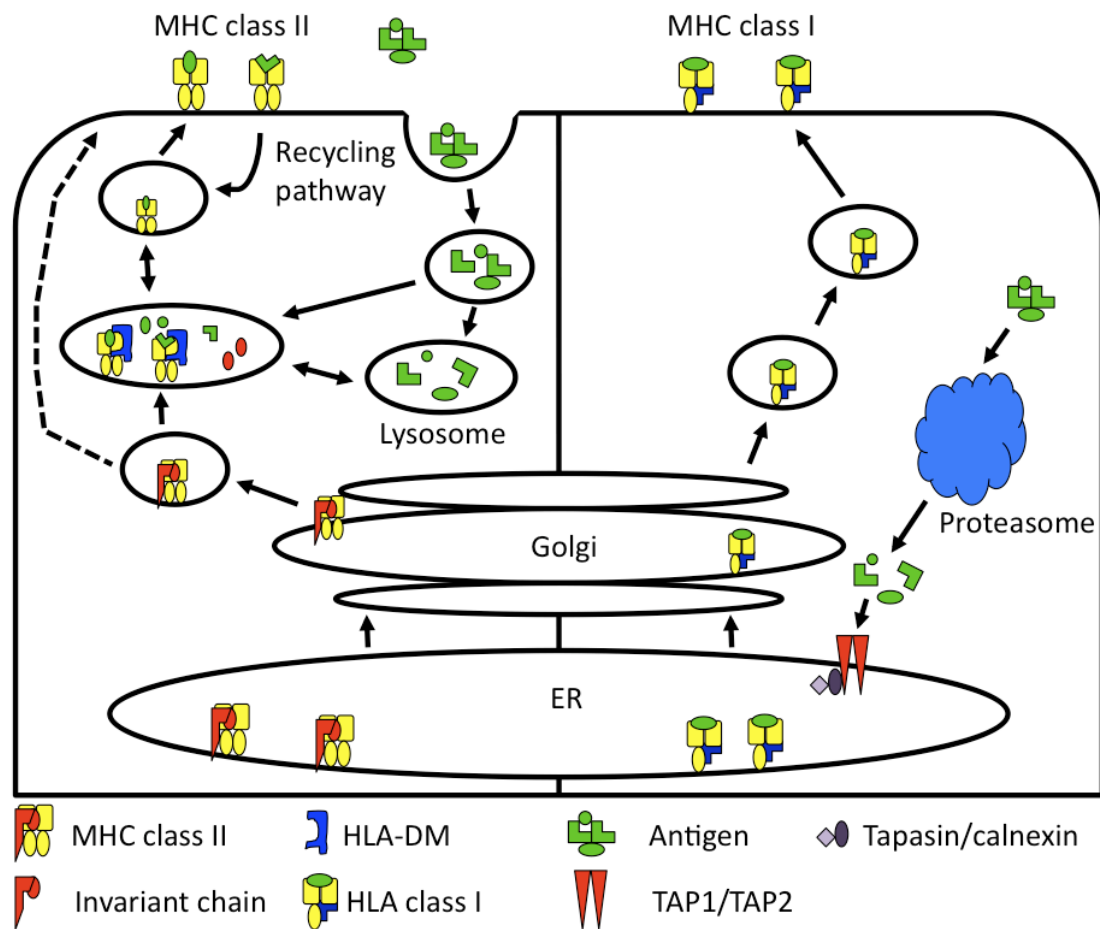


Fig. 2 Presentation pathways for HLA class II (left side) on APCs processing extra-cellular pathogens recognized by $CD4^+$ T-cells and HLA class I (right side) on all nucleated cells processing intracellular pathogens recognized by $CD8^+$ T-cells.

In general, extracellular antigens are processed differently and presented by the HLA class II molecules. Most cells continuously internalize extracellular fluid and material bound at their surface by the process of endocytosis. In addition, macrophages and other cells specialized in phagocytosis engulf larger objects, e.g. dead cells. The extracellular material is taken up to the vesicular system of the cell by endocytosis and phagocytosis. The proteins are then lysed into peptides by proteases in the lysosomes (vesicles for degradation of proteins) and the peptides can then bind to HLA class II molecules, which have been transported to the vesicles via the ER and the Golgi apparatus. The peptide: HLA class II complex is transported to the cell surface by outward-going vesicles. See figure 2. Thus, the HLA class I pathway samples the intracellular environment, complementing the HLA class II pathway, which samples the extracellular environment and presents antigenic peptides to T-cells endowed with the appropriate TCR. [52-55]

1.3 B-LYMPHOCYTES AND PLASMA CELLS

1.3.1 B-lymphocyte activation

Immature B-lymphocytes (B-cells) leave the bone marrow to populate peripheral lymphoid tissues, where they complete maturation. Antigens binding to the B-cell receptors (BCRs) are internalised and processed. After processing peptides are exposed on the cell surface in a complex with HLA class II molecules to interact with peptide specific CD4⁺ T-cells. The CD4⁺ T-cells are then activated and start to express co-stimulatory molecules on their surface that bind back to the B-cells, which in turn get activated and switch to produce a specific type of antibody (IgM, IgA, IgG, IgD, or IgE). B-cells can either transform to antibody secreting plasma cells or become resting memory B-cells, moving back to the bone marrow [56].

The principal function of B-cells is to produce antibodies (secreted immunoglobulins) and each antibody is highly specific for its corresponding antigen. The total antibody repertoire of each person is composed of several millions of different immunoglobulin molecules. B-cells also have cell-bound immunoglobulins (Ig) to captive antigens which are subsequently presented to CD4⁺ T-cells. Thus, B-cells are also antigen-presenting cells [57, 58].

Due to structural rearrangements, genes encoding for the immunoglobulin heavy- and light-chains (both κ and λ light chains) can be expressed in developing B-cells. This gene rearrangement is strictly controlled, ensuring that only one type of heavy and one type of light chain is expressed in each B-cell. The variable domains are encoded in two (C and J) or the three gene segments variable (V), diversity (D), and joining (J), which are brought together by recombination. Three different mechanisms contribute to the diversity in V-region sequences: (a) the random combination of the different segments, (b) the introduction of additional nucleotides at the junctions between the segments, and (c) the association of different combinations of heavy and light chains [59, 60].

The five different classes of immunoglobulins exhibit a different function. When the B-cell or plasma cell first encounters a pathogen, pentameric IgM antibodies are produced. These are made primary by plasma cells present in the bone marrow, spleen and lymph nodes. These antibodies are of low affinity and therefore multiple binding sites are needed. During the process of increasing affinity and antigen specificity, there will be a shift of the constant region of IgM that will be transformed into IgG, which is the most abundant antibody in blood and lymph. Monomeric IgA is made by plasma cells in lymph nodes, bone marrow and spleen and secreted into the blood. Dimeric IgA is found in extrinsic fluids like milk, saliva, sweat and tears. IgE antibodies are highly specialized towards activation of mast cells present in epithelial tissues and are thought to be involved in expulsion of parasites, and they are also involved in allergic reactions. IgD antibodies are produced in small amounts, and their effector functions are still unknown [61, 62].

1.3.2 Epitope binding to antibodies

The antigen-binding sites of antibodies may vary according to both size and shape of the epitope. Depending on the amino acid length of the antigen it will bind to the antibody in either a linear or a conformation-dependent (discontinuous) epitope. Binding of antibodies is based on non-covalent forces: hydrogen bonds, hydrophobic interactions, electrostatic forces and van der Waals forces. Even very small differences in shape and chemical properties of the binding site can give several antibodies specificity for the same epitope. However they will have different affinities to the epitope, i.e. they will bind to the epitope with different strength [63]. Corti and co-

workers showed that B-cell epitopes are closely related to CD4⁺ T-cell epitopes, since Ig, bound to specific epitopes, alters antigen processing [64].

1.4 NK-CELLS

NK-cells constitute less than 10% of the peripheral blood leucocytes and they are also produced in the bone marrow. NK-cells can be activated to kill virus infected or malignant cells by two different mechanisms: (I) disturbance of the balance between killing-activating and killing-inhibiting signals or (II) interaction between IgG-coated cells and NK-cell-receptors triggering antibody-dependent cellular toxicity [65]. When the HLA class I complex is down regulated or lost, due to e.g. herpesvirus infections or malignant transformation of cells, the inhibiting signal is lost and NK-cell mediated killing occur. (Also see chapter 2.4.1. “NK-cells responses against CMV”.)

1.5 INTERLEUKIN-7

Interleukin 7 (IL-7) was primarily identified as a pre-pro B-cell growth factor (PPBGF) and cloned on the basis of its ability to induce proliferation of B-cell progenitors in the absence of stromal cells [66]. IL-7 is secreted by stromal cells in the bone marrow and thymus and is irreplaceable in the development of both B- and T-cells [66, 67]. IL-7 is also essential for the survival of mature, naïve T-cells and memory cells, especially CD4⁺ memory cells, both of which express high levels of the IL-7 receptor [68-70]. Indeed, the non-redundant nature of IL-7 is underscored by the observation that ablation of IL-7 or parts of the IL-7 receptor in gene-knock-out mice, leads to a profound defect in lymphocyte development. In humans, mutations in a chain of the common gamma-chain of the IL-2R shared by receptors of several cytokines, including those for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, give rise to X-linked severe combined immunodeficiency [71].

Various anatomic sites i.e. the thymus, bone marrow, intestinal epithelium, keratinocytes, liver and dendritic cells and tissues, including many tumors, are sources of IL-7 protein production [72, 73].

IL-7 serves as a ligand for its receptor, the IL-7R (CD127), which is present in a dimeric form with the common gamma chain (CD132), which also serves as a part of the receptors for IL-2, IL-4 and IL-15. Furthermore IL-7R acts as a co-receptor for the TSLPR (thymic stromal lymphopoietin protein receptor). TSLPR is expressed preferentially in myeloid cells including dendritic cells, activated monocytes and it is weakly expressed on T-cells. Ligand receptor interactions have been implicated in the development of the hematopoietic system, dendritic cell maturation, and the maintenance and polarization of human CD4⁺ memory T-cells in allergic and autoimmune diseases [74, 75]. It has also been shown that CD4⁺ memory T-cells proliferate independently of IL-7, but they do not survive without IL-7 [76]. Furthermore, even several months after a viral infection, CD4⁺ memory T-cells require IL-7 for survival and homeostatic proliferation [77]. A recent report underscored the need for IL-7 in homeostatic proliferation of CD4⁺ T-cells, due to IL-7-regulation of MHC class II expression on dendritic cells [78].

After activation of IL-7R α , a series of intracellular phosphorylation events mediated by signalling molecules occurs. These signaling molecules include Janus kinases (JAK 1 and 3), Src kinases, STAT5a/b and PI 3 kinases [79-81].

IL-7 rescues T-cells from activation-induced-cell-death associated with the up-regulation of T-lymphocyte survival factors and induces phosphorylation of signal transducer and activator of transcription 5 (STAT5) in T-cells [82] activating STAT which then enters the nucleus and binds to specific DNA sequences in the promoter regions of genes, resulting in gene activation or suppression [83]. CD4⁺ or CD8⁺ T-cells from healthy individuals exhibit low constitutive STAT-5 phosphorylation (pSTAT5) and show a fast and strong response to IL-2 or IL-7 stimulation. Decreased response to IL-7 was defined by absence of pSTAT5, which has been associated with decreased immune functions such as decreased cytokine production [84]. Increased constitutive pSTAT5 has been associated with chronically activated T-cells [85]. Furthermore, the IL-7/IL-7R axis has been found to be crucial for maintenance and expansion of CMV-specific immune responses [86].

Increased IL-7 in infectious diseases may be advantageous. In contrast, increased IL-7 levels may lead to severe autoimmune responses. In the stem cell transplant setting it was shown that high levels of IL-7 and IL-15 precede Graft-versus-Host Disease

(GVHD), but low levels of IL-7 and IL-15 were observed before relapse of the primary malignancy [87].

IL-7R α expression is weaker in naïve precursor (CD45RA⁺CCR7⁺) and T_{EMRA} terminally differentiated (CD45RA⁺CCR7⁻) T-cells and stronger in T_{CM} central (CD45RA⁻CCR7⁺) and effector (CD45RA⁻CCR7⁻) memory T-cells [88]. This is apparent during an antiviral immune response, where a reduction is seen of IL-7R α expression on CD8⁺ effector T-cells. Differentiation of T-cells is dependent on antigen signalling strength: stronger stimulation leads to differentiation into effector cells and down-regulation of IL-7R α expression, whereas weaker stimulation leads to differentiation into memory cells [89]. CMV infections or other chronic infections such as Epstein-Barr virus (EBV) or human immunodeficiency virus (HIV) are associated with exhaustion of CD8⁺ T cells, which show reduced expression of IL-7R α , resulting in poor T-cell viability and function [90]. A correlation between EBV post-transplantation lymphoproliferative disease (PTLD) and decreased IL-7 signaling was previously described by our group [91].

2 HUMAN CYTOMEGALOVIRUS

2.1 CLINICAL PRESENTATION OF CMV INFECTION

The seroprevalence of human cytomegalovirus (CMV) varies between 40-90% in western countries. In Sweden approximately 70% of adults are infected. The virus will persist life-long and it is debated however CMV continuously slowly replicates or however it should be considered as a latent infection that can reactivate such as in immunosuppressed individuals [92, 93].

In immune competent individuals primary infections are usually mild and frequently occur early during childhood by transmission through breast-milk or contact with infectious body fluids such as saliva. However it is thought to account for approx. 8% of all cases of infectious mononucleosis [94]. Symptoms of CMV are persistent fever, myalgia, headache, cervical lymphadenopathy, splenomegaly, non-specific constitutional symptoms, and rash (30%). These symptoms may persist for weeks [95]. The major distinguishing feature of CMV infectious mononucleosis is the absence of the heterophilic antibodies that are found in infectious mononucleosis caused by Epstein-Barr virus.

In recent years, after the control of rubella, CMV is the major infectious cause of birth defects (causing neurological damage like e.g. hearing deficit, migrational disturbances/ cerebral cortical malformations and developmental disability in 10-20% of all children with congenital CMV) with an incidence of 0.2-2.2% per live birth [96-98]. CMV is also an important pathogen for immunocompromised individuals such as transplant recipients and HIV infected individuals. In the latter viral dissemination leads to multiple organ system involvement presented as pneumonia, hepatitis, gastroenteritis, retinitis, and encephalitis.

In patients undergoing HSCT, CMV-disease can appear both early and late after the transplant procedure [99-101].

2.2 CMV BIOLOGY

Human CMV has one of the largest genomes, 230 kilo base pair (kbp) double stranded DNA, of viruses known to infect man [102], belongs to the beta-herpesviridae (cytomegalovirus group), and it is designated as human herpesvirus 5 (HHV-5). It has many similarities to the human herpesviruses (HHV) -6 and -7 also belonging to the beta-herpesvirus group. CMV has more than 200 open reading frames (ORF) and of these, more than 50 are used for viral replication and may be involved in immune evasion while the rest is thought to be used for immune modulation of the host cell or other for the virus beneficial changes of the environment [95]. The proteins expressed during replication are divided into “immediate early”, “early” and “late”. The genome is organized in unique long (UL) and unique short (US) regions. There are several different laboratory and various clinical strains of CMV. It is speculated that the genomic variation between different strains may be implicated in CMV-induced immunopathogenesis [103].

2.2.1 Structure of herpesviruses

Herpesviruses replicate in the nucleus of the host cell (see section 2.2.4). Eight human herpesviruses are known. They are divided into three different groups: alpha-herpesviridae (consisting of Herpes Simplex Virus types 1 and 2 and Varicella Zoster Virus), beta-herpesviridae (consisting of CMV, HHV-6 and HHV-7, see above) and gamma-herpesviridae (consisting of Epstein-Barr virus and HHV-8).

The virions of herpesviruses consist of four structural elements, from inside to outside: core, capsid, tegument and the envelope (see figure 3). The icosahedral **capsid** consisting of 162 capsomers is enclosed in a lipoprotein **envelope**, forming the outer layer of the virion. The envelope is derived from the internal nuclear membrane of the host cell modifying it by a dozen unique viral glycoproteins. Most neutralizing antibodies will target these glycoprotein components of the envelope. The **tegument** lies between the envelope and the capsid and it contains transcription factors and virus-coded enzymes essential for initiation of the infectious cycle. The **core** consists of the **genome** consisting of a single stranded linear molecule of double-stranded DNA coiled around proteins [104].

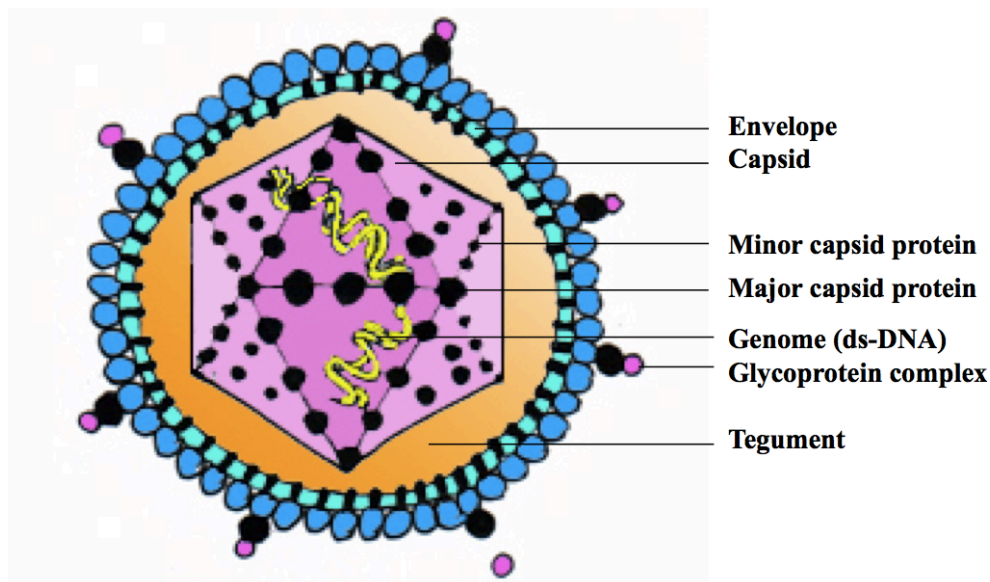


Fig 3. The CMV virion structure

2.2.2 The CMV genome

The genome of CMV lies in a single molecule of linear, double-stranded DNA. Nucleotide sequence comparisons of the three *in vitro* adapted human cytomegalovirus strains, Toledo, Towne, and AD169 show the complexity of the genome sequence that may explain the differences that these strains exhibit in virulence and tissue tropism.

The laboratory strain AD169 has lost approximately 5% of the wild type virus genome during the fibroblast passage. The Toledo strain of CMV contains 22 additional genes, which are absent in the highly passaged laboratory strain AD169 and additionally it contains a segment not found in the Towne genome. The Towne strain also contains a DNA segment absent on the AD169 genome, meanwhile, in the Toledo strain only about half of this segment was present but arranged in an inverted orientation. A large region of the AD169 genome was conserved in all three strains.

Ultimately these genome differences lead to proteome differences: the DNA segment unique to the Toledo genome contains 19 open reading frames (ORFs) not present in the AD169 genome and the additional DNA segment within the Towne genome contains 4 new ORF. One of these four ORF shares homology with the Toledo genome. The additional Toledo strain sequences are conserved in all clinical isolates [105].

2.2.3 The CMV entry, replication and proteome

The beta-herpesviridae have a relatively slow replication cycle resulting in the characteristic multinucleated giant host cells. The replication cycle of CMV is divided into 3 regulated classes: immediate early (IE), early (E) and late (L) genes. These three gene groups will also name the different types of CMV proteins. IE gene transcription occurs within the first four hours after viral infection / reactivation. IE proteins are key regulatory proteins allowing the take-over of the cellular machinery. IE1 and IE2 are the most abundantly expressed proteins in the initial phase activating both viral and cellular genes in the CMV infected cells [95, 106].

The E genes products include structural and replication proteins. Late gene products are produced twenty-four hours after infection / reactivation. L proteins are structural proteins involved in assembly and exit of the virion [106, 107].

Host neutralizing antibodies mostly target the glycoproteins of the envelope. Host cell-mediated immune responses often target the tegument proteins. One of the most important tegument proteins is UL83, also called phosphoprotein 65 (pp65) that is one of the main targets of the T-cell immune response.

2.3 CMV PATHOGENESIS

Human CMV is species specific and cannot easily be studied in animal models. The pathogenesis of CMV infection and disease is very complex with several interactions between CMV and the immune system presumably with the aim to suppress immune responses to the virus. The interaction is mediated through several mechanisms including the virus having effects on HLA-expression, cytokine production and expression of adherence molecules. Heparan-sulfate preoglycans, integrins and growth-factor receptors are involved in virus entry.

CMV can infect many cell-types *in vivo* including monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, stromal cells fibroblasts, smooth muscle cells, and neuronal cells explaining the different clinical presentations of CMV-disease. During the acute infection the main cell-type in blood infected with CMV is monocytes that may serve as transport vehicles, while differentiation into tissue macrophages

permits the full replication cycle [108]. Monocytes are the major sites of CMV latency in vivo [109, 110] and have been identified as the predominant infected cell type in peripheral blood [111]. Most likely CMV spread to different tissues either by utilising cells as a vehicle for virus shedding or by trafficking of free virions in the blood [112]. In bone marrow the CMV genome is carried by CD34⁺ progenitors [113]. Viral reactivation can occur after inflammatory stimuli when monocytes differentiate to macrophages or dendritic cells (DCs) [114, 115]. During latency the CMV genome does not produce infectious virus and there is a lack of IE transcription. Samples from tissues with CMV-disease show a high frequency of CMV-infected macrophages expressing late viral genes [116, 117]. Furthermore, the virus has developed strategies to delay viral replication, avoid lysosomal degradation and avoid destruction of the infected cells as well as other functions to remain persistent in macrophages. Therefore it is thought that CMV uses macrophages as “Trojan horses” [118].

Epidemiological studies suggest that there is a correlation between CMV and development of cancers and various inflammatory diseases like atherosclerosis [119], inflammatory bowel diseases [120], rheumatoid arthritis [121], systemic lupus erythematosus [122] and Sjögren’s syndrome [123]. Even though the causality is more difficult to prove than the correlation in time and location, there are several studies where CMV proteins and nucleic acids are detected within the tissue of different cancer types, e.g. colon cancer [124], breast cancer [125], prostate cancer [126], salivary gland tumours [80], glioblastoma [127-129], neuroblastoma [130] and rhabdomyosarcoma [131].

2.4 IMMUNE RESPONSES SPECIFIC AGAINST CMV

The CMV-specific immunity includes both innate and adaptive components. The main effector cells in immune control of CMV are NK-cells, CD8⁺ and CD4⁺ T-cells. The primary infection, occurring in most cases during childhood, gives rise to a cell mediated defence. CMV infected cells will present CMV antigens on their surface to be recognized by T-cells specific against CMV.

2.4.1 NK-cell responses against CMV

Via binding of gB and gH to Toll like receptor 2 (TLR2) and subsequent TLR2 dependent activation of NF κ B, the initial stages of CMV infection trigger the innate immune responses with induction of interferons, inflammatory cytokines and recruitment and activation of NK-cells [132, 133]. Even though CMV has many mechanisms to avoid NK-cell mediated killing, NK-cells are thought to have an important role in the defence against CMV. Individuals lacking NK-cells suffer from recurrent herpesvirus infections and serious episodes of CMV-disease [134]. Studies of animal models point towards a great importance also of the innate immunity in the defence against CMV. After HSCT, NK cells play a crucial role in early immune responses because they are the first lymphoid population recovering after the allograft. In humans, Guma and colleagues showed that CMV seropositivity is associated with elevated frequencies of NKG2C⁺ NK cells [135] and furthermore, it has been suggested that cytomegalovirus promote NK-cell development after HSCT (with cord blood graft) as a more rapid NK-cell maturation together with expansion of NKG2C⁺ NK cells have been reported in patients experiencing CMV reactivation [136].

CMV-associated expansion of NKG2C⁺ NK cells have been reported in various human disease settings including immunodeficiency [137], HIV infection [138], acute hantavirus infection [139], and after HSCT [136, 140, 141]. NKG2C⁺ NK-cells transplanted from seropositive donors exhibit heightened function in response to a secondary CMV event compared with NKG2C⁺ NK-cells from seronegative donors. It would seem like NKG2C⁺ memory-like NK-cells are transplantable and dependant on CMV antigens in the recipient for clonal expansion of NK-cells previously exposed to CMV in the donor [140]. The expansion and differentiation of KIR-expressing NK-cells, visible as stable imprints in the repertoire [142] was induced by infection with human CMV, but not with other common herpesviruses.

2.4.2 T-cell responses against CMV

Even though merely all parts of the immune system contribute to control CMV, the predominant control mechanism is the cellular immune response. This is why CMV-disease occurs exclusively, with exception of congenital infection, in severely cellular immunosuppressed individuals. In patients with AIDS, CMV-specific INF- γ producing CTLs protect against CMV-retinitis [143] and in HSCT-patients the reconstitution of

CMV-specific CD8⁺ cells are shown to be correlated with protection against disease [144, 145]. CMV-specific T-cells have also been used in adaptive treatment with transfer of cellular immunity [146]. The humoral immunity develops through activation of B-cells by CD4⁺ T helper cells and CMV-specific antibodies will be produced. It is believed that these antibodies can neutralize viral particles but that they cannot help in the elimination of the CMV infected cells. [147, 148].

2.4.2.1 CMV-specific CD8⁺ T-cell responses

The CMV-specific CD8⁺ cytotoxic T-cells (CTL) will clonally expand and mature and kill infected cells. There is also evidence of the existence of virus-specific CD4⁺ T-cells that can kill infected cells directly just as CD8⁺ T-cells using the cytolytic effector proteins perforin and granzyme, or inducing apoptosis via Fas Ligand.

It is rather “costly” for the immune system to keep CMV under constant control. In adults (but not elderly) around 5-10% of the total peripheral blood CD4⁺ and CD8⁺ T-cells recognize CMV determinants and looking only at the memory cells, approximately 10% are CMV specific. The specificity of the CMV-reactive T-cells is rather broad, covering about 70% of the total ORFs of the CMV genome [149].

However, this picture tends to change when the individual gets older. In the Swedish longitudinal OCTO [150] immune study it was shown that a combination of high CD8⁺ and low CD4⁺ percentages and poor T-cell proliferation in peripheral blood lymphocytes (PBL) was associated with a higher 2-year mortality in very old (86-92 year old) Swedish individuals. These alterations were associated with evidence of CMV infection. The authors suggested that a combination of old age, lymphocyte activation due to chronic infection, such as CMV, and a related imbalance in unknown factors that regulate the homeostatic mechanisms in the immune system might have contributed to an increased risk for the decreased survival observed in the OCTO study. Khan et al. [151] showed that the clonally expanded memory CD8⁺ T-cells seen in CMV-seropositive healthy donors were characterized by the lack of CD28 expression and the increased expression of CD57 (CD28⁻CD57⁺CCR7) and he stated that such cells often are oligoclonal and directly correlated with poor immune response.

In a young adult, the CD8⁺ CMV-specific response is extremely diverse and is directed towards more than 70% of the CMV ORF [152]. The CTL recognize structural, early

and late antigens in addition to immunomodulators as pp28, pp50, pp150, pp65, gH, gB, US2, and others.

The impact of chronic CMV infection on memory T-cell homeostasis and the differentiation phenotype of antigen-experienced CTL has been examined in various studies. The main CD8⁺ effector T-cell population during acute CMV infection has the CD45RA⁻CCR7⁻CD45RO⁺CD27⁺CD28^{+/-} phenotype. In chronic CMV infections the markers CD27 and CD28 do not longer appear on the cell surface and the population is either CD45RO⁻ as in the effector memory cell population or shows the CD45RO⁺ as in terminally differentiated effector T-cells re-expressing CD45RA [153, 154].

2.4.2.2 *CMV-specific CD4⁺ T-cell responses*

CD4⁺ depleted mice get an increased incidence of recurrent CMV infections [155] and also in humans there is increasing evidence that not only HLA class I / CD8⁺ T-cell responses but also HLA class II / CD4⁺ T-cells are also important for the control of CMV [156, 157]. In lung transplant recipients low levels of CMV-specific CD4⁺ T-cells correlates with susceptibility to CMV-infections [157] and in HSCT-patients detectable CD4⁺ T helper responses protects against CMV-disease and the recovery of the CMV-specific T-cells is required for both the persistence of adoptively transferred CMV-specific CTL [158] and the endogenous reconstitution of the same [145].

In CMV-exposed individuals 9% of the circulating CD4⁺ memory T-cell population is directed against CMV epitopes. As for CD8⁺ T-cells, broad antigen recognition has been revealed also for CD4⁺ T-cells. In healthy individuals >30% of them are directed against glycoprotein B (gB) antigens. Most CD4⁺ T-cell responses against gB and gH are directed towards highly conserved regions [149].

2.4.3 Antibody mediated immune responses against CMV

Resistance to and recovery from CMV-induced disease may require both cellular and humoral CMV-specific immune responses. The humoral immunity develops through activation of B-cells by CD4⁺ T helper cells and CMV-specific antibodies may then be produced. It is believed that these antibodies can neutralize viral particles but can't help in the elimination of the CMV infected cells. Epitopes from the capsid and tegument proteins give a strong and durable antibody response *in vitro*. These are used as

diagnostic and serological assays. However, as these proteins are enclosed within the virion envelope and inaccessible to antiviral antibodies, they lack targeting signals *in vivo* recognizable by the cellular secretory system and are therefore not expressed on the cell surface of infected cells. Thereby they are not exposed to circulating antibodies. It is speculated that the most important antibodies *in vivo*, are those targeting surface-exposed virion glycoproteins in the envelope, including glycoprotein B and H (gB and gH) [159].

Glycoprotein B (gB) is a well-conserved protein among herpesviruses and is essential for viral entry and cell-to-cell spread [160]. CMV gB is believed to be a major target for neutralizing antibodies. Antibodies directed against gB can be detected in all naturally infected individual [161]. However, a vast majority of gB-specific antibodies secreted from B-cell clones do not have virus-neutralizing activity [162]. Five different antigenic domains (ADs) have been identified previously [162, 163]. AD-1 is located at amino acid (aa) position 560 – 640 of HCMV strain AD169 [164]. AD-2, located at the extreme amino terminus of the protein, consists of at least two distinct sites between aa 50 and 77 of gB [165]. Antibodies binding to AD-3 do not seem to be neutralizing antibodies. Most likely additional antigenic domains exist on gB. AD-4 and AD-5 have shown a neutralizing effect *in vitro*. AD-4, a discontinuous domain built from residues between aa 121–132 and 344–438 and AD-5 is located between amino acids (aa) 133–343 of gB [162].

3 HEMATOPOIETIC STEM CELL TRANSPLANTATION

3.1 THE TRANSPLANT PROCESS AND ITS HISTORY

In 1896 Quine performed the first attempt to do a transplantation of the lymphohematopoietic system but failed [166]. Today hematopoietic stem cell transplantation (HSCT) has developed to a curative treatment against a number of both malignant and non-malignant diseases such as different types of leukemias and lymphomas, myelodysplastic syndrome, severe immunodeficiencies, hemoglobinopathies (as sickle-cell anemia and Thalassemia major), metabolic diseases and severe aplastic anemia. It has also been used against some solid tumours with metastatic disease with varying results.

The transplant process starts with the conditioning regimen consisting of irradiation and/or cytotoxic chemotherapy combined with immunosuppressive agents. Today conditioning can be given with greatly varying intensity; myeloablative and reduced intensity [167]: The myeloablative conditioning has two main purposes: (i) to eradicate/reduce the malignant cells and (ii) to allow engraftment of donor cells by suppressing the recipient T-cell function [168]. With reduced intensity conditioning, the main aim is to suppress recipient immune function to allow engraftment and initiate a “graft-vs-malignancy” effect [169, 170]. In unrelated donor and mis-matched transplants, T-cell depletion either *in vivo* or *in vitro* is commonly used to facilitate engraftment and decrease the risk for acute GVHD: The stem cells are thereafter infused through a central venous line, and find their way to the marrow cavity. After engraftment, donor derived cells will slowly eradicate both the remaining hematopoietic cells and the immune competent cells of the recipient, along with the possible remaining tumour cells [171].

3.2 IMMUNE RECOVERY AFTER HSCT

3.2.1 Overview

After a conventional conditioning therapy most of the hematopoietic stem cells, and the B- and T-cell immunity, including most memory cells, are destroyed [172].

Reconstitution of the cell-mediated immune system following HSCT requires both successful engraftment and adequate thymic function and, moreover, it is dependent on the eventual development of GVHD and whether the graft is T-cell depleted or not [173].

3.2.2 Aplastic phase

The first period after HSCT, characterized by neutropenia and frequent mucosal damage especially after myeloablative conditioning, is called the aplastic phase and the typical infections are bacterial or fungal. Two to four weeks after HSCT the neutrophils gradually recover, even though they have subnormal functions until 3-4 months after HSCT [174, 175], and the mucosal barriers starts healing. The NK-cells proliferate rapidly after graft infusion and within the first months after HSCT, their numbers become normal [176, 177].

3.2.3 Recovery of cellular immune responses

After successful engraftment, there is a gradual reconstitution of the cellular immune responses. During this period the patient is in risk for pathogens whose control depend mainly on functioning T-cells such as CMV, HSV, HHV-6, adenovirus, EBV and fungi especially aspergillus. Several factors such as age, hormonal changes, damage caused by the conditioning regimen especially total body irradiation, T-cell depletion, earlier given therapy, and acute GVHD influence the thymic function that is needed for adequate reconstitution of the cellular immunity. CD4⁺ T helper cells require a competent thymus for maturation and are therefore dependent on the thymic function for expansion. Meanwhile CD8⁺ T-cells can rapidly regenerate in substantial number as they are able to clonally expand. A result of this might be prolonged imbalance between the two subsets of T cells. However, despite the high number of CD8⁺ T-cells during the first period after HSCT, their cytotoxic function is impaired [178, 179]. Other factors influencing reconstitution are the prophylaxis (T-cell depletion,

cyclosporine A, sirolimus, and tacrolimus) and the therapy of GVHD especially high dose corticosteroids that can further impair the T-cell function. The recovery is therefore often delayed and incomplete and it may take over a year after HSCT before cellular immune responses are completely restored and functional [180].

3.2.4 Recovery of humoral immune responses

It has been known for more than two decades, that both memory B-cells and antibodies can be transferred by HSCT. For several months after transplantation, antiviral IgG can be found in the pre-transplant seronegative recipient [181, 182]. This is thought to reflect the presence of functional B-cells transferred from the donor. However, the transferred antibody production ceases in most patients after three to twelve months. Long duration of transferred immune responses can, however, be found indicating the engraftment and expansion of memory B-cells especially after antigen exposure [181]. The opposite situation is also found, namely a persistence of antibody production for some time after HSCT in patients receiving grafts from for the antigen seronegative donors [181].

B-cell reconstitution after HSCT develops through certain stages. Four to eight months after HSCT the number of B-cells is normal. However, since most antigens are incapable of activating B-cells without the help from CD4⁺ T-helper cells the reconstitution of the humoral immune response is not possible as long as the CD4⁺ T-cells are defective. It can take up to 2 years for the humoral immunity to completely recover after HSCT [183, 184]. The B-cells are not properly functional showing a reduced ability to produce adequate responses to antigens independent of T-cell function. Transitional B-cells (CD19⁺CD24^{high}CD38^{high}) constitute only 4% of the circulating B-cells in adults, yet they account for 50% of B-cells in cord blood and for the majority of B-cells early after HSCT in the peripheral circulation. This B-cell population decreases as newly mature B-cells are formed, usually starting at 6 months after HSCT [185]. A large number of naïve B-cells contribute to the plasma immunoglobulin production, yet these immunoglobulins are believed to be of too low-affinity to be detected using microarray technology based on the assumption that a fluorescent-based Ig array detects approximately 10 attomoles of IgG [186]. There is a decrease in the diversity of the IgM repertoire [187], low levels of IgA, IgG₂, IgG₄ and there are also deficiencies the switch from IgM to IgG after antigen exposure [188]. In

patients with chronic Graft versus Host Disease (cGVHD) CD19⁺CD10⁻CD27⁻CD21^{high} naïve B-cells are highly elevated and many of these patients show hypogammaglobulinemia. Furthermore, in those cGVHD patients that have hypergammaglobulinemia more autoantibodies are seen [189]. Patients with cGVHD have delayed B-cell reconstitution and elevated levels of B-cell activating factor of the tumour-necrosis-factor family (BAFF) to B-cell ratios compared to patients without cGVHD. BAFF enhances B-cell survival, a function that is indispensable for B-cell maturation and has a role in enhancing immune responses [190]. B-cells from patients with active cGVHD have also been shown to have a heightened metabolic state and be resistant to apoptosis. Exogenous BAFF treatment amplified cell size and survival in B cells from these patients [191]. This is in accordance with another study showing that resistance to apoptosis was associated with high levels of B-cell activating factor (BAFF). It has also been suggested that BAFF regulates T-cell survival and that this dysregulation of T-cell survival and apoptosis is the common cause of autoimmune diseases [192].

3.3 GRAFT VERSUS HOST DISEASE

3.3.1 Acute GVHD

Acute GVHD is defined as occurring within the first 100 days after HSCT and is classified into four grades (I-IV) depending on the severity and the organ involvement [193]. Prophylaxis against GVHD is given to all recipients of allogeneic grafts and it is usually initiated during the conditioning and continued 3-6 months after HSCT for malignant diseases and for 1-2 years in patients transplanted for benign diseases [194, 195]. The needed immunosuppression lowers the GVHD-activity but as a side effect the patient becomes more vulnerable for opportunistic pathogens.

The exact actions of different cytokines, effector cells and regulatory cells on the development of acute GVHD are still incompletely understood. However, it is known that the initial damage of the tissues is caused by the conditioning regimen of the transplant procedure presumably inducing a cytokine cascade. The recipient dendritic cells (DCs) interacting with the donor derived T-cells induce the subsequent acute GVHD. This results in activation and clonal expansion of CD4⁺ and CD8⁺ T-cells [196], which then leads to inflammatory signals, cytolytic effects and apoptosis,

ultimately leading to host cell destruction. The inflammatory process can also involve the gut, causing transfer of LPS and endotoxins from the gut into the blood circulation where macrophages are activated [197]. This probably is influenced by the microbiota in the gut as the proper development and function of the intestinal immune system has been shown to be dependant on its specific microbiota [198]. An altered function and imbalances may lead to inflammatory bowel disease (IBD) [199], which have many similarities to intestinal GVHD. It has also been shown that intestinal inflammation due to GVHD after HSCT is associated with major shifts in the composition of the intestinal microbiota [200]. A recent hypothesis is that the inflammation in the gut like NEC is caused by injury to Paneth cells (PCs) in the intestinal crypts. PCs are specialized epithelia that protect intestinal stem cells from pathogens through their secretion of antimicrobial peptides, α -defensins, stimulate stem cell differentiation, shape the intestinal microbiota, and assist in repairing the gut [201]. In a mouse HSCT model, Paneth cells are targeted by GVHD, resulting in marked reduction in the expression of α -defensins, which selectively kill non-commensals, while preserving commensals. There was a significant correlation between alteration in the intestinal microbiota and GVHD severity. Oral administration of polymyxin B inhibited outgrowth of E coli and ameliorated GVHD. [202]. In accordance with this, one could hypothesize that CMV could cause inflammation in the gut, causing gut-GVHD, causing more inflammation and destruction of Paneth cells leading to more inflammation and more active CMV in the gut.

The inflammatory process in the gut leads to even more production of inflammatory cytokines as TNF- α and IL-1 leading to further activation of DCs (they express more co-stimulatory molecules such as CD80 and CD86), release of T_H1-activating cytokines and death of target cells [203]. In chronic GVHD, the mechanism is slightly different and has predominately auto-reactive features. T-cells with abnormal cytokine profiles secreting IL-4 and IFN- γ may be present [204]. As auto-reactive cells should be removed by the thymus, thymic damage (leading to release of auto-reactive cells to the periphery) and acute GVHD are believed to predispose to development of chronic GVHD. The Seattle BMT team could show already more than 25 years ago that chronic GVHD is usually preceded by acute GVHD [205].

Patients with GVHD are immunosuppressed during a longer period than patients without GVHD. The main reasons for that are that the immune system of the recipient

is one of the major targets of GVHD and that GVHD is treated with immunosuppressive medications in order to suppress the donor derived immune cells driving the GVHD. The major effect of cyclosporine A (CsA) and tacrolimus is the inhibition of production of interleukin-2 (IL-2) necessary for proliferation and activation of T-cells. Interestingly, the α -chain of the IL-2 receptor (CD25) is also expressed on T-regulatory cells, which are believed to counteract the development of GVHD. Again, the complex pathogenesis of GVHD is emphasized. High doses of corticosteroids are used as therapy of GVHD and results in multiple effects on the immune system. One mechanism is to induce apoptosis of CD4⁺ T-cells and enhance cytokine production promoting T_H2 cell immune responses through effects in macrophages/ monocytes [206, 207].

3.3.2 Chronic GVHD

After allogeneic HSCT, the primary causes of late morbidity are relapse and chronic GVHD predisposing to late infections [208]. Chronic GVHD is the main factor influencing quality of life in long-term survivors. Symptoms usually present within the first 3 years after allogeneic HSCT and in a majority of the cases they are preceded by acute GVHD. The main target organs are the skin, the liver, the mucous membranes, and the bronchi, the latter resulting in so called obstructive bronchiolitis. Risk factors for developing chronic GVHD includes acute GVHD, female donor for male recipient, older patients, donor lymphocyte infusions (DLIs), unrelated or mismatched donor and the use of peripheral blood stem cells for transplant. A recent study by Anasetti et al. indicated that peripheral-blood stem cells (PBSC) might reduce the risk of graft failure, whereas bone marrow may reduce the risk of chronic GVHD. In this study there were no significant differences between PBSC or bone marrow stem cells regarding the incidence of acute GVHD or relapse [209].

Many changes are seen to the immune responses in patients with chronic GVHD. They show macrophage deficiency, a poor ability to antibody switch (from IgM to IgG) on B-cells, low antibody levels [210] and impaired chemotaxis and opsonisation by neutrophils [211]. All in all, this leads to greater susceptibility to infections, especially from encapsulated bacteria such as pneumococci [212].

3.3.3 CMV and acute GVHD

A correlation between pre-transplant recipient CMV-seropositivity and increased frequency of acute GVHD has been suggested by epidemiological studies [213] although this remains controversial. In the other direction, acute GVHD doubles the risk for CMV-reactivation and increases the risk for CMV-pneumonitis by 2.6 fold [214, 215]. Also the risk of CMV-disease in other locations increases in patients with aGVHD [216, 217]. This could be explained by the allogeneic reaction assisting in reactivation of the virus [114] or by the immunosuppression given as therapy for acute GVHD.

3.3.4 CMV and chronic GVHD

Chronic CMV infections are, not surprisingly, related to chronic inflammation. Although there are clear clinical associations between early immunological complications to solid organ transplantations such as acute rejection, the mechanisms that account for the role of this virus in graft loss, are not well understood.

CMV has since many years been suggested as a risk factor for chronic GVHD [218, 219]. It is well known that CMV has a capacity of both persisting as well as increasing its replication in the midst of intense inflammation. It is suggested that this contribution to the inflammatory environment could serve as a trigger for the induction of both Host versus Graft and Graft versus Host responses causing chronic allograft rejection or chronic GVHD [159]. One could argue that the immunosuppression associated with acute or chronic GVHD paves the way for CMV infection / reactivation. Early studies have shown that CMV infection increases the risk for chronic GVHD [218] and patients with good control of CMV have a lower risk for developing extensive chronic GVHD than patients with more severe CMV associated problems [220]. Furthermore, patients receiving PCR based preemptive therapy have lower risk for severe chronic GVHD than patients monitored with less sensitive techniques [221]. Another fact supporting this theory is that it has been shown that CMV infection triggers autoantibody formation against CD13 in transplanted patients [222], which, in turn, has been shown to be associated with the development of chronic GVHD.

3.3.5 CMV and relapse

Reactivation of latent CMV in the transient state of immunodeficiency after HSCT is the most frequent and severe viral complication endangering leukemia therapy success. If CMV infects the bone marrow it can directly interfere with BM repopulation by the transplanted donor-derived hematopoietic cells and thus delay immune reconstitution of the recipient [223]. However, CMV infections/ reactivations after HSCT might not be entirely negative for the HSCT recipients. More than 25 years ago, it was suggested that CMV replication could reduce the risk for relapse [224]. Elmaagacli et al showed a reduction in the risk for AML relapse in patients with documented CMV replication [31]. In a recent study by Green et al the association between CMV reactivation and relapse was confirmed in a large cohort of patients with different haematological malignancies who underwent allogeneic HSCT showing a decreased risk for relapse in patients with acute myeloid leukemia (AML) by day 100. This association was not seen in patients with acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), lymphoma, and myelodysplastic syndrome (MDS). The effect was seen early since it disappears if a landmark analysis is performed from day +50 and appeared to be independent of CMV viral load, acute graft-versus-host disease, or ganciclovir-associated neutropenia. However it is not clear if the effect was independent to NK-cell development. One year after HSCT early CMV reactivation was associated with reduced risk of relapse in all patients (however not significant for any disease subgroup). [225]

3.4 INFECTIONS AFTER HSCT

The risk for infections is directly correlated to the different phases of immune recovery. The use of prophylaxis with antibacterial, antiviral and antifungal drugs have greatly influenced the presentation of infections after HSCT.

The first period reaches from day 0 until engraftment (day +15 to +30 after HSCT). Under this period the patient is neutropenic and the mucosal barriers are destroyed and all pathogens, even the normally not so virulent, can cause serious infections. Microorganisms present in the oral and GI-tract and skin, mainly gram-positive and gram-negative bacteria and candida, cause most of these infections. Furthermore, during the first 4 weeks after HSCT, reactivation of Herpes Simplex virus (HSV) is

very common. The early risk for infections has been reduced by the introduction of reduced intensity conditioning regimens [169, 170].

The period after engraftment up to day +100 is the second phase. Under this period the patient lacks mainly T-cell immunity and CMV is a key pathogen [226]. EBV, adenovirus, Respiratory Syncytial virus (RSV), *Pneumocystis jiroveci*, and *aspergillus* also cause significant problems during this second period especially in patients with acute GVHD. Furthermore the thymus is sensitive to both chemo- and radiation therapy and GVHD, and insufficient thymic recovery has been directly correlated with increased risk of opportunistic infections and poor clinical outcomes [227, 228].

The third phase starts after day +100. During this period, the patient still have impairment of both cellular and humoral immune systems. Therefore infections with encapsulated bacteria such as pneumococci can cause serious infections, since antibody-dependent killing is needed to clear the body from these bacteria. To reduce this risk, vaccination with conjugated vaccines against pneumococci and *H.influenzae* is recommended. Reactivation with Varicella Zoster virus (VZV) is also common and long-term antiviral prophylaxis is usually given [229]. Patients with moderate to severe chronic GVHD remain at risk for several types of infections including CMV [99, 230], pneumococci and *Pneumocystis jiroveci*.

3.4.1 Long-term survivors after HSCT and late infections

Approximately 6% of patients dying more than two years after transplantation, excluding patients dying from chronic GVHD, die from infection [231]. The main causes of late death in HSCT recipients are leukemic relapse and chronic GVHD [231]. CMV is a predisposing factor for late death. The exact mechanisms behind this are not known, but CMV has been shown to suppress cellular immunity, predisposing for bacterial and fungal infection [232]. Björklund et al. show that although the risk for late death due to CMV was low (0.7%), CMV infection was a significant risk factor for the development of late fatal infections [208]. Thus, the impact of CMV infection seems to last for a very long time after HSCT, but as CMV viral loads are not routinely measured for longer than 6 months after transplantation, it is unclear whether this is due to a lasting immune deficiency after a CMV infection or on-going low level CMV

replication. However, it must be stipulated that CMV reactivations lead to an impact on the late immune reconstitution.

3.4.2 CMV infection and disease after allogeneic HSCT

Historically, CMV-disease has been the main cause of death in HSCT-patients. Today better diagnostic techniques and preemptive therapy has lowered the incidence of the disease to 6%, but if CMV-disease develops the prognosis is still poor with a mortality of 50%. Despite these advances in management of CMV, it is still a negative factor for survival to be CMV seropositive when coming to HSCT [233-241].

The diagnostic criteria for CMV-disease require both clinical symptoms and signs compatible with CMV-disease, and detection of virus from the affected organ / body tissue. The only exception is in the case of CMV retinitis, where the characteristic appearance identified by an experienced ophthalmologist suffices to make the diagnosis [242].

CMV has direct effects on the affected organs, but is also associated with indirect effects. For example it appears to predispose immunosuppressed patients to secondary infections [208, 243]. In addition to these direct effects on endorgans, CMV has also been associated with various indirect effects as for example atherosclerosis, myocardial infarction and other autoimmune diseases in numerous studies [244, 245].

3.5 MANAGEMENT OF CMV INFECTIONS AFTER HSCT

The risk for transmission of CMV by a stem cell graft from a seropositive donor to a seronegative recipient is approximately 20-30% [246]. If a CMV seronegative patient receives a graft from another seronegative individual (D-R-) the risk for contracting an infection is quite low as long as leukocyte-reduced blood products or blood products from a seronegative donor is used [247, 248].

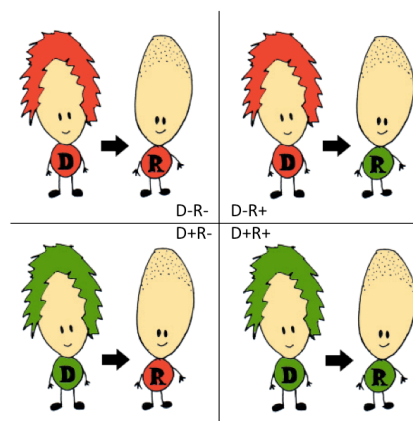


Fig 4. Showing the four CMV-serological groups D-R-; D-R+; D+R- and D+R+

The risk for both repeated CMV reactivations and CMV-disease is also increased in CMV seropositive recipients with CMV seronegative donors (D-R+) [249-251] (see figure 4).

There are three possible strategies for CMV management: prophylaxis, preemptive therapy, and therapy of CMV endorgan disease (see figure 5).

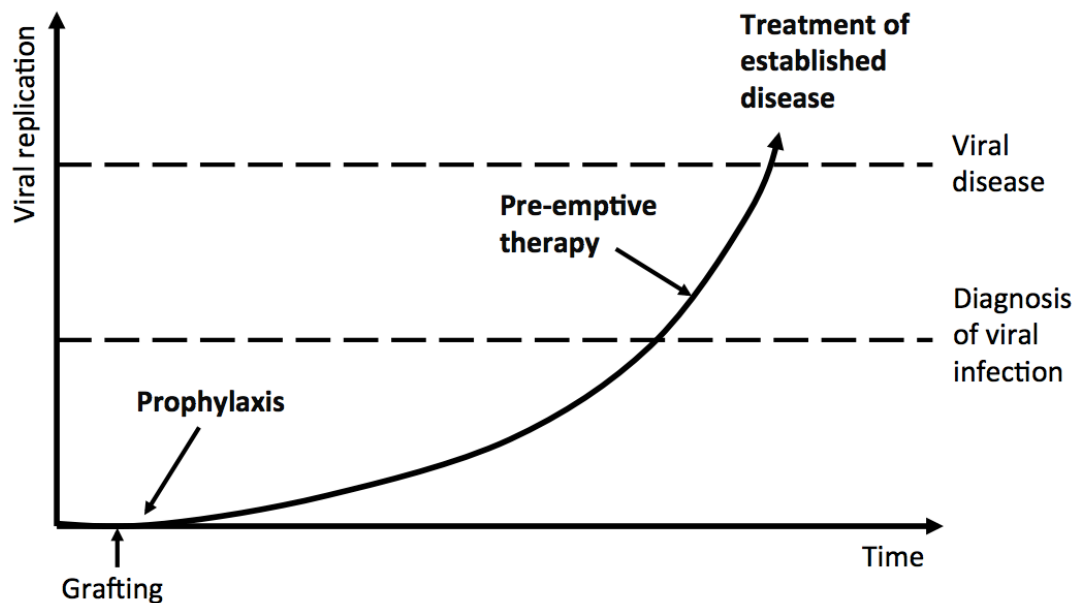


Fig. 5 The different strategies for timing of management options [adapted from *Alain, ILTS, 2008*].

Having to treat CMV endorgan disease in HSCT recipients should be regarded as a failure of strategy since the mortality in CMV-disease is still high. Most centres performing HSCT have adopted either the strategy of a) preemptive antiviral treatment, giving treatment with for example ganciclovir when CMV infection is first identified, or b) use prophylaxis strategies, with ganciclovir given to all patients at risk of CMV-disease from engraftment up to 3-4 months post-HSCT.

The strategy of preemptive therapy is defined as the introduction of antiviral therapy when CMV is detected by a sensitive diagnostic technique but before signs or symptoms of CMV-disease has occurred. These techniques include detection of CMV DNA by quantitative PCR, RNA by NASBA, or CMV proteins for example by the pp65 antigenemia assay. Today use of preemptive therapy is much more common in SCT due to the toxicity of ganciclovir/valganciclovir. However, in solid organ

transplants, antiviral prophylaxis is more commonly used. Both strategies are effective, but higher incidences of drug resistance particularly in high-risk patients (D+R-), have been observed on preemptive management than in those given prophylaxis in the solid organ transplant setting [252-256]. A recent Cochrane study by Owers et al showed that preemptive treatment against CMV in the solid organ transplantation setting was effective to prevent symptomatic CMV-disease compared with placebo, but showed no significant differences in preventing CMV-disease compared to prophylaxis. Leucopenia was significantly less common with preemptive therapy compared with prophylaxis. Other adverse effects did not differ significantly or were not reported. There were no significant differences in the risks of all-cause mortality, graft loss, acute rejection, and infections other than CMV [257]. There are also other strategies of which some, like the use of immunoglobulin to prevent CMV infection, are rarely used today in clinical practice due to poor efficacy [258].

3.5.1 Monitoring techniques and viral load

Prior to transplantation CMV IgG and IgM serology from the donor and the recipient are measured. The decision if a patient should be treated against CMV is usually based on measurement of CMV viral load combined with the clinical judgment of the physician [258]. One still commonly used technique is the so-called “CMV pp65 antigenemia”, which is a semi-quantitative test that has been shown to be helpful in initiating preemptive therapy, the diagnosis of clinical disease, and monitoring response to therapy [259]. CMV pp65 antigenemia does not require expensive equipment and it is easy to perform, although there are problems with a lack of assay standardization, including subjective result interpretation and therefore needs experienced staff to be performed correctly.

The today most commonly used technique for monitoring is quantitative PCR (QNAT test). QNAT, however, requires expensive equipment and specialized expertise. The viral load can vary significantly between whole blood and plasma specimens. CMV DNA is detected in greater quantitative amounts and earlier in whole blood. Therefore one and the same specimen type should be used when serially monitoring patients [260]. QNAT results can vary widely across different testing centers, due to variation in nucleic acid extraction, assay design and until recently, the lack of an international

reference standard. This is why an international standard for CMV was developed and approved in November 2010 by the World Health Organization [261].

An international standard composed of a standardized quantity of CMV, will allow laboratories and manufacturers to assess the accuracy of viral load values and to calibrate different individual assays such that results can be reported as international units (IU) (rather than copies) per millilitre and compared across various testing platforms [262]. In the future it will probably be difficult to publish results from research investigations if the WHO standards are not fulfilled. [263]

CMV culture is slow, expensive and can be less sensitive but rapid culture techniques remain the gold standard for diagnosis of CMV pneumonia from bronchoalveolar lavage (BAL). If CMV-disease is suspected an immunohistochemistry for CMV should be routinely performed on all biopsy specimens to maximize diagnostic sensitivity. Identification of inclusion bodies or viral antigens in biopsy material or in BAL [264, 265] specimen cells is specific for CMV disease. [263]

3.5.2 Antiviral drugs with effects against CMV

Currently available drugs for the treatment of human CMV-disease in the immunocompromised host include ganciclovir (GCV), its oral prodrug valganciclovir (VGCV), cidofovir (CDV), and foscavir (FOS). All these drugs target the viral DNA polymerase (pUL 54). Their common problem is dose-related toxicity and resistance. Relevant factors for the emergence of drug-resistant virus variants involve, for example, CMV serostatus of donor/recipient, lack of T-cells, severe immunosuppression, high levels of viral replication, inadequate dosing of antiviral compounds and prolonged antiviral therapy [266]. Persistent viremia at day 21, but not at day 14, was shown to be a risk factor for the emergence of resistance [253].

3.5.3 Acyclovir/ valacyclovir

These drugs have limited CMV efficacy and can only be used as prophylaxis. A reduced risk for CMV infection and possibly CMV-disease is seen when adults are given high dose treatment with 500 mg acyclovir/m² intravenously (i.v.) given 3 times daily, followed by 800 mg 4 times daily [267, 268]. One large randomized study has shown that high dose valacyclovir (2g four times daily) show reduction of CMV

infection from 40% to 28% in comparison to patients receiving acyclovir. Furthermore, the use of preemptive therapy was reduced by almost half but no difference was seen in either CMV-disease or survival [269]. Nowadays this strategy is less common.

3.5.4 Ganciclovir (GCV)

Ganciclovir can be used as prophylaxis, for preemptive therapy, or for treatment of CMV-disease. Prophylaxis with intravenous ganciclovir gives a reduction of the risk of CMV-disease compared to placebo [270, 271] but did not improve survival.

Ganciclovir given as preemptive therapy on the other hand was shown to improve overall survival [272, 273]. One important side-effect is the marrow toxicity of ganciclovir resulting in prolongation of the neutropenic period exposing the patient to the the risk of more invasive bacterial and fungal infections [274].

3.5.5 Valganciclovir

Valganciclovir is the valine ester pro-drug of ganciclovir. Despite the lack of a randomized study, valganciclovir is frequently used as preemptive therapy instead of intravenous ganciclovir due to easier logistics of therapy. No data exist for prophylaxis [258].

3.5.6 Foscarnet (FOS)

Foscarnet is administrated intravenously and is associated with dose dependent renal toxicity and electrolyte abnormalities [258]. A randomized study showed similar efficacy as ganciclovir when foscarnet was used for preemptive therapy [275]. It has also been used for CMV-disease [276]. No controlled studies have been published regarding the use of foscarnet for prophylaxis of CMV-disease.

3.5.7 Maribavir (MBV)

Maribavir, a competitive inhibitor of the UL97 protein, is a yet unlicensed treatment, that showed promising results in phase II drug trials with significantly lower risk for CMV infection and might also give a small reduction of CMV-disease compared to placebo [277]. However, results of a large randomized phase III trial failed to confirm these positive observations [278], which might be due to inadequate dosing regimen or

a wrongly chosen primary end-point. It is today being tested in larger doses for preemptive therapy [279].

3.5.8 Cidofovir (CDV)

Cidofovir (CDV) is administrated intravenously and requires cellular kinases for its activation. The major side effect is renal toxicity. Cidofovir has been used for preemptive therapy and for treatment of CMV-disease [280, 281].

3.5.9 CMX001

CMX-001 is administrated orally and delivers high intracellular levels of cidofovir (CDV)-diphosphate and exhibits enhanced *in vitro* antiviral activity against a wide range of double-stranded DNA viruses, including cytomegalovirus (CMV) [282, 283]. In HSCT-patients the most common side effect is diarrhea. Neither myelosuppression nor nephrotoxicity have been observed [284]. Mutations in the DNA polymerase of CMV that impart resistance to CDV also render the virus resistant to CMX001, and although it is rare, resistance have been reported [285].

3.5.10 AIC246/ Letermovir

AIC246/ letermovir is effective *in vitro* against human CMV laboratory strains, clinical isolates, and virus variants resistant to currently approved drugs. [286]. Letermovir inhibits the viral terminase complex (UL56/UL89), an enzyme that plays an important role in cleavage of viral deoxyribonucleic acid (DNA) into unit-length genome and packaging it into procapsids. Letermovir has been used as prophylaxis against CMV in a phase II study with promising results and is now entering phase III studies.

3.5.11 Fomivirsen

Fomivirsen was used to treat CMV-retinitis but is now discontinued /withdrawn from the market. Fomivirsen does not target the viral DNA polymerase UL54. Instead it is an antisense oligonucleotide inhibitor of IE mRNA and thereby blocks all classes of CMV gene expression [287]. As it was used to treat CMV-retinitis, it was administrated intravitreally. Its major toxicity is local (ocular toxicity).

3.5.12 Immunomodulatory CMV treatment /Adoptive CD8⁺ therapy

Today there are also several treatment options other than antiviral medication against CMV infection after allogeneic HSCT: i) adoptive immunotherapy with CMV-specific T-cells, which improves the elimination of CMV; ii) vaccination based on CMV-peptide pulsed dendritic cells, which improves control of CMV and iii) vaccination which are still on clinical trials [288].

CMV-specific CD8⁺ T cells are essential for the control of CMV and due to the toxic side-effects of the existing anti-viral treatment. Immunotherapeutic alternatives are an attractive tool for improvement of immune reconstitution in HSCT-patients. Adoptive immunotherapy, consisting of transfer of CMV-specific CD8⁺ T cells is an alternative to antiviral treatment to patients at high risk of CMV-disease [146, 158, 289]. Even though patients receiving CMV-specific CD8⁺ T cells do not develop CMV-specific CD4⁺ T-cell responses and the transferred cells gradually decrease, adoptive treatment does protect against CMV-disease. It is neither yet defined how many cells that needs to be transferred nor how their composition in respect of CD4⁺: CD8⁺ ratio, to require an effective transfer and prevention / treatment of viremia. Yet, 5×10^9 CMV-specific CD8⁺ T cells / m² were shown to be sufficient to reconstitute CMV-specific responses [288].

3.5.13 CMV vaccines

So far, there is no licensed prophylactic vaccine for CMV-associated diseases although a couple of vaccines are in late-phase clinical trials.

The ideal vaccine should induce both the innate (DCs, NK-cells and TLR) and the adaptive (CD4⁺, CD8⁺ and $\gamma\delta$ T cells) protective immune responses, because it is hypothesised that even though it may not be possible to prevent infection it should be possible to prevent or at least control CMV-disease.

The various strategies that have been developed have been extensively tested in animal models showing promising results and some of them have already been used for clinical trials with humans.

The currently most promising new vaccine candidate to enter clinical practice might be the so called TransVax vaccine, which promotes cellular immunity as well as the formation of neutralizing antibodies. TransVax includes two DNA plasmids and has in a phase 2 trial in HSCT recipients demonstrated significantly reduced occurrence and recurrence of CMV viremia and improved the time-to-event for viraemia episodes compared with placebo. The vaccine was well tolerated and the incidence of common adverse events after HSCT (e.g. GVHD or secondary infections) did not differ between groups [290].

Also a CMV glycoprotein-B vaccine with MF59 adjuvant has shown promising results in phase 2 trials in comparison to placebo. In this trial patients waiting for renal transplantation were given the vaccine at baseline, 1 month and 6 months later. If a patient was transplanted, no further vaccinations were given and serial blood samples were tested for cytomegalovirus DNA by real-time quantitative PCR (rtqPCR). Safety and immunogenicity were co-primary endpoints and were assessed by intention to treat in patients who received at least one dose of vaccine or placebo. The study showed that gB antibody titres were significantly increased in patients receiving the vaccine and the duration of viremia after transplantation, correlated inversely with glycoprotein-B antibody titres [291].

4 AIMS OF THE THESIS

There are many aims of this thesis. The main aim is to improve the understanding of how the CMV-specific immunity is reconstituted after HSCT. This could allow better prediction of patients who are at risk for CMV-disease or complicated (prolonged or recurrent) CMV-reactivations. Furthermore, we wanted to identify immunological endpoints allowing evaluation of immunomodulatory treatments, antiviral therapy, and possibly vaccine efficacy.

For the 1st study the aim was to analyze the effect of different pre-transplant related factors on the CMV viral load and moreover the effects of the viral load and viral load kinetics on the risk for CMV-disease in a series of consecutive allogeneic HSCT recipients.

In our 2nd study we aimed to investigate if the grade of phosphorylation of STAT5 after IL-7 stimulation could be used as a clinically relevant biomarker to identify patients at increased risk to develop CMV-associated complications.

Finally with our 3rd and 4th studies, we aimed to gain knowledge of how the humoral immunity against CMV is formed in the hematopoietic transplant setting to find novel peptides important for antibody recognition that ultimately could be used for prediction of HLA class II and CD4⁺ T-cell responses.

5 MATERIALS AND METHODS

5.1 PATIENTS

For the 1st paper we included 162 consecutive patients, who were CMV seropositive or had a CMV seropositive donor transplanted at Karolinska University Hospital Huddinge from the beginning of 2000 to the end of 2003. We used both patient computer charts to record the patient characteristics and to monitor viral load and clinical outcome. The patients were monitored at least once weekly by QNAT from engraftment until day 100 after the transplantation. Patients, who had experienced CMV reactivation or had severe GVHD, continued to be monitored weekly while other patients were monitored at each visit to the transplant center, usually every 2nd week, until 6 months after SCT.

For the 2nd paper 40 patients hematologic malignancies were studied. The patients and / or the stem cell donors were CMV-seropositive. Blood samples were drawn monthly during the first three months after HSCT (time point 1 -3). Additional samples (time point 4 – 6) were drawn from patients reactivating CMV or having a primary CMV infection. PBMCs from heparinized blood were isolated by Ficoll separation and immediately frozen and stored in liquid nitrogen. Samples from all time points from each patient were thawed and stained for flow cytometric analysis at the same laboratory session and analyzed with the Gallios flow cytometer (Beckman Coulter). Flow data analysis was performed using Kaluza software (Beckman Coulter).

For the 3rd and the 4th paper, 54 serum samples were selected from 20 HSCT-patients (pat A to S), who had not received intravenous immunoglobulin infusions. CMV-negative recipients with CMV-negative donors (D-R-) were used as negative controls (pat A-E) and compared against the other groups (D+R+, D+R- and D-R+); each consisting of samples from 5 patients. Furthermore we recruited additional patients, designated at 1-7, from whom we had sufficient matching PBMCs available for testing T-cell reactivity.

For all our papers (I – IV), patients were monitored weekly in whole blood for HCMV DNA with a real-time polymerase chain reaction (PCR) (see chapter 5.6). Preemptive therapy was given according to existing cut-off level at the time of each study. The

intervention limit at the transplantation center changed during the collection of serum samples for paper no 3 and 4. It was 100 copies/200.000 leucocytes during the first period and later after change of PCR method the intervention limit changed to 1000 copies/ml. No patient including in paper no 3-4 developed CMV-disease and no preemptive antiviral therapy was given since the viral load was below the center's intervention limit.

5.2 FLOW CYTOMETRY

We used this technology for paper no 2. Flow cytometry is a laser-based technology to measure single cells and biomarkers and perform cell sorting of cells flowing through a detector system. Fluorescent-label antibodies specific to cell-surface markers of interest are used to characterize the cell population of interest. These cell surface markers can be different clusters of differentiation (e.g. CD3, CD4, CD8, CD19, etc.), the T-cell receptor, or glycoproteins showing that the cell recognizes a specific viral epitope (e.g. CMV pp65, CMV pp150, etc.). Each fluorophore has a characteristic peak excitation and emission wavelength, and the emission spectra often overlap.

Flow cytometry can be used on a variety of tissues as long as they are in liquid phase. In our studies we used heparinized whole blood, sorted out the PBMCs by Ficoll separation and performed fluorescent staining and thereafter flow cytometry.

The principle is that a beam of laser light(s) of one or several wavelengths is/are directed onto a stream of liquid with suspended cells. Each cell passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. A number of detectors will pick up this combination of scattered light (SSC; depends on the inner complexity of the particle like shape of the nucleus and granularity or the membrane roughness) and fluorescent light (FSC; correlated with the cell volume). It is thereby possible to derive various types of information about the physical and chemical structure of each individual particle by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak). A computer physically connected to the flow cytometer will be used for acquisition of the data and specialized software is used to adjust for parameters as voltage, compensation, etc. The generated data is then plotted in either histograms or in two-dimensional dot plots. The regions on these plots are usually sequentially separated, based on fluorescence intensity by creating a series

of subset gates. Further computational analysis will be used to see how many of each specific subpopulation of cells that were present in the sample.

5.3 PEPTIDE MICROARRAY

A peptide microarray is a collection of peptides displayed on a chip. The assay principle is similar to an ELISA protocol but a much larger scale. Thousands of peptides are attached to the surface of a glass chip, which is then incubated with patient serum or plasma. This allows the human antibodies present in the serum bind to the peptides that have an epitope they recognize. After several washing steps, all antibodies not binding to the peptides will be removed. After these steps a secondary antibody tagged by a fluorescence label will be added and it will bind to the Fc part of the bound patient antibodies. The fluorescence label, making each peptide spot visible if antibodies bind to it, will in turn be detected by a fluorescence scanner. After scanning the data have to be carefully normalized, evaluated and interpreted.

In our studies this method was used for papers no 3 and 4. We used CMV microarray slides manufactured by JPT, Berlin Germany with 15mer-peptides overlapping by 4 amino acids covering the whole proteome of HCMV. The slides consisted of two identical subarrays, each with 17496 spots arranged in 24 blocks of 729 spots arranged in columns and rows of 27. The peptide spots represented 17174 unique peptides, 305 control spots (4 repetitions each of IgG, IgA, IgM, and IgE), 268 negative controls and 31 other control spots. All slides belonged to the same batch.

After pre-processing and normalization of peptide responses [292], we used three different statistical analyses of the microarrays (all these analyses were made by a professional statistician in our group):

- (i) *PAM (Prediction Analysis for Microarrays)* [293]: a highly selective method which allows to examine in detail each time point of consecutive serum testing. This reveals only very ‘robust’ epitopes, i.e. the most predictive peptide targets associated with the differentiation of the patient groups;
- (ii) “*Exclusive recognition analysis*” (*ERA*): which epitopes predicted by PAM are recognized in serum from individuals exclusively in one group but never

in any individual in the D-R- patients (termed “in- and exclusive” epitopes)
and finally

- (iii) *MaSigPro (Microarray Significant Profiles)* [294]: to follow the dynamic changes over time between groups.

5.4 RECOM BLOT

Qualitative *in vitro* testing for detection and reliable identification of IgG antibodies against CMV were performed for paper no III using a commercially available Western Blot (Recom Blot CMV IgG test, Mikrogen Germany). It is used for the confirmation of uncertain and positive screening results.

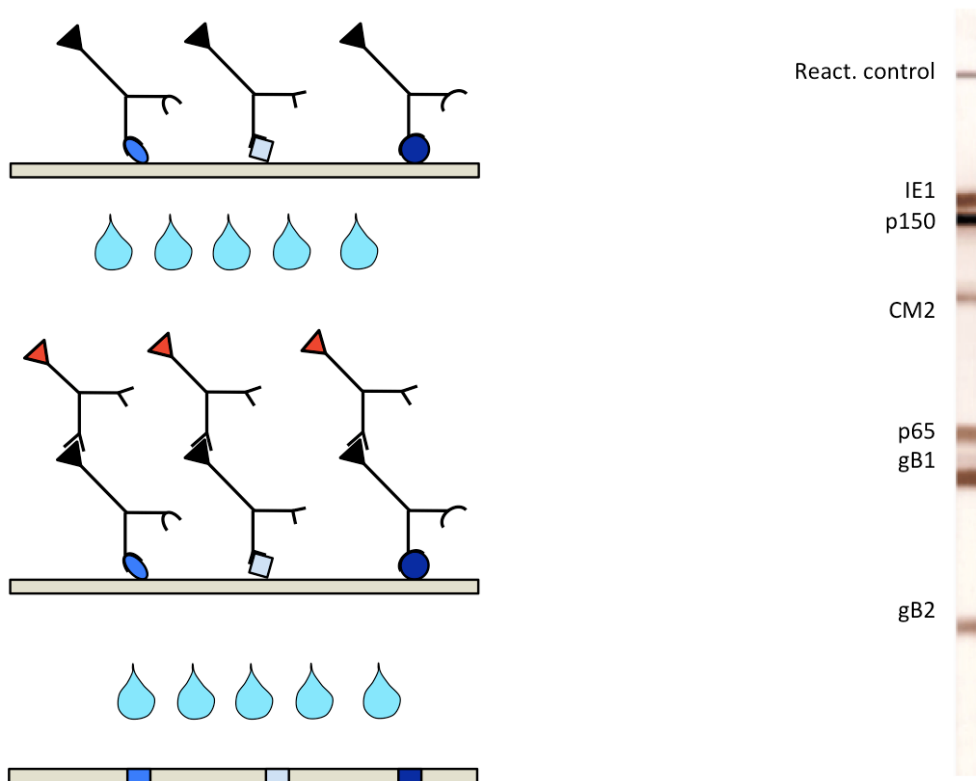


Fig. 6 On the left side the test procedure for Recomblot is shown. On the right side an example of a test strip showing the different antigen responses.

The test principle and procedure is simple. In the *first incubation* step a test strip loaded with CMV antigens is incubated with diluted serum or plasma in a dish for one hour and then thoroughly washed. During the following *second incubation* step peroxidase conjugated anti-human antibodies (IgG / IgM specific) are added. After more incubate and washing the color reaction step follows. Coloring solution is added and insoluble colored bands develop at the sites on the test strips occupied by antibodies. (See figure 6.)

5.5 INTRACELLULAR CYTOKINE STAINING

For paper no III cytokine production was analysed by intracellular cytokine staining (ICS) in frozen PBMCs, that were thawed, rested overnight and stimulated with peptide mixes from CMV covering the entire protein UL94 (P168000), CMV UL55/ glycoprotein B (P06473), CMV UL99/ pp28 (P13200) and CMVpp65. This was done in the presence of brefaldin A either with medium or medium and PMA/ionomycin as negative and positive controls, respectively as described by Magalhaes *et al* 2010 [295].

After washing, the cells were fixed and permeabilized and incubated with antibodies specific for intracellular cytokines (IL-2, IFN- γ , TNF- α and IL-17).

For quality reasons all samples were run in duplicate. The cells were analyzed using a Gallios Beckman Coulter flow cytometer and data analysis was performed using Kaluza software.

5.6 PCR

To monitor CMV reactivation the Karolinska University Hospital at the time of study no I routinely used a quantitative real-time polymerase chain reaction (PCR) [296, 297] for monitoring of CMV DNA from peripheral blood lymphocytes. When study no 2 was performed, instead whole blood was used as the basis for the PCR. For paper no 3 and 4 both these methods were used. HSCT-patients were monitored weekly during the first 3 months after HSCT but later after transplantation, sampling were done less frequently.

The general principle of quantitative PCR aims to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are the key components to enable selective and repeated amplification. As the PCR reaction progresses, the DNA generated is used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

The technique proceeds in three steps:

- (i) *Denaturation*: Causing melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.
- (ii) *Annealing*: Allowing annealing (=adduction) of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
- (iii) *Extension/elongation*: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

Real-time PCR is a modification of “normal” PCR and can be used for measuring viral loads. Short DNA fragments (probes) anneal to the middle region of the template DNA. These probes were a reporter dye (R) and a quencher (Q) that quench the fluorescence of dyes in proximity. Polymerases in the PCR solution break down the probes during the doubling of the DNA template, thereby freeing the reporter dye that migrates away from the fluorescence of the quencher. Hence the fluorescence is measured only if the polymerase has in fact copied the desired DNA strand and each free molecule of reporter dye represents a DNA strand that has been formed.

6 RESULTS AND DISCUSSION

6.1 PAPER I: RISK FACTORS FOR CMV DISEASE AFTER HSCT

CMV remains an important complication to allogeneic HSCT. Emery and co-workers showed that viral load kinetics were predictive for the development of CMV-disease with both the initial viral load and the initial rate of increase in viral load being independent risk factors [298]. A few years before this study was performed, new techniques had been introduced for HSCT such as the use of peripheral blood stem cells and reduced intensity conditioning. These changes have partly changed the timing of immune reconstitution after HSCT and thereby possibly the predictive value of viral load kinetics. The aims of this study were to analyze the effect of different pre-transplant related factors on the viral load and the effect of the viral load and viral load kinetics on the risk for CMV-disease in a series of consecutive allogeneic HSCT recipients.

162 consecutive patients, who either were CMV seropositive or had CMV seropositive donors, were included in the retrospective study. Quantification of CMV DNA was performed by real-time PCR. Although 105 of 162 patients had CMV DNA detected, the cumulative incidence for CMV-disease was only 1.6% during the first 100 days but it rose to 6.3% during the first year after HSCT. The viral load influenced the risk for CMV-disease in univariate analysis but not when different risk factors were included in the multivariate analysis. This analysis showed that only acute GVHD grades II-IV and the use of a CMV-negative donor to a CMV-positive patient were significant risk factors for CMV-disease. In patients, who required more than one course of preemptive therapy, acute GVHD and a slower rate of decrease (i.e. a slow response to antiviral therapy) in viral load during the first course of preemptive therapy were significant risk factors for subsequent development of CMV-disease. The likelihood of a repeated reactivation or the response rate did not seem to depend on the drug used.

Thus, the CMV viral load kinetics is important after HSCT although we were unable to find a direct influence of the initial or peak viral loads on the risk for CMV-disease. There are two possible explanations to that our findings differed from the study by Emery et al. [298]. The first is that their patients were mainly T-cell depleted and the second is that in their series preemptive therapy was not consistently used. Of course,

before comparing our results to studies performed at other transplant centers, it is important to realize that the overall risk for CMV-disease in this study was low and the mortality in CMV-disease was only 2 %. Furthermore, we were unable to find the high frequency of late CMV-disease seen in other studies [99]. In addition, we did not find a lower risk of CMV-disease in patients who underwent reduced intensity conditioning. The potentially most important finding in our study was that the rate of viral load decreases after the initiation of antiviral therapy was predictive of the risk of developing CMV-disease. This finding might be used for deciding management strategies after the first course of preemptive antiviral therapy.

6.2 PAPER II: REDUCED IL-7 RESPONSIVENESS DEFINED BY STAT5 PHOSPHORYLATION IN T-CELLS MAY BE A MARKER FOR INCREASED RISK TO DEVELOP CMV DISEASE IN PATIENTS AFTER HSCT

The aim of this study was to try to identify a new clinically relevant marker that might aid to identify patients at increased risk to develop CMV-associated complications. Our hypothesis was that the T-cell response to stimulation with IL-7 might be different in patients with and without CMV complications. This hypothesis was based on our results regarding IL-7 phosphorylation and EBV PTLD [91]. Therefore, we evaluated the phosphorylation of STAT5 in CD4⁺ T-cells, CD8⁺ T-cells and TCR $\gamma\delta$ T-cells after stimulation with IL-7 or IL-2 after HSCT by analyzing blood samples taken monthly 1 - 6 months after HSCT. Patients were monitored weekly with a quantitative PCR from the time of engraftment for CMV viral load in whole blood until at least day 100 after HSCT.

Thirty-seven CMV reactivations were documented occurring 17-711 days after HSCT and out of those reactivations 25 were treated preemptively with anti-CMV drugs. The patients were classified in 3 cohorts; those without CMV replication (CMV-), those with 1-2 CMV replication episodes (CMV+), and those with prolonged or repeated CMV replication episodes (CMV++). Risk factors for development of at least one CMV replication episode were the use of unrelated stem cell donors ($p<0.01$) and the CMV serological status combination of the donor and the recipient ($p=0.04$). Factors

not influencing CMV replication were age, stem cell source, conditioning intensity, acute GVHD, or chronic GVHD.

In general, the different patient groups, irrespective of the CMV reactivation pattern, exhibited strong phosphorylation of STAT5 in CD4⁺ T-cells, CD8⁺ T-cells, TCRγδ T-cells, CD8⁺CD4⁺ (activated) T-cells or (CD3⁺) CD4⁻CD8⁻ T-cells upon stimulation with IL-2 or IL-7 as compared to the constitutive pSTAT5.

We identified a correlation between CMV replication and the ability to respond to IL-7 and IL-2 defined by STAT-5 phosphorylation (pSTAT5). Patients with recurrent or prolonged CMV replications (CMV++) had significantly lower ΔpSTAT5 upon stimulation of T-cells with either IL-7 or IL-2 at time points 1-3 than the CMV- group ($p<0.05$). This was also found after stimulation of CD8⁺ T-cells at time point 2 ($p<0.05$).

An important controlling mechanism for CMV replication is specific T-cells and the absence of CMV-specific T-cells analyzed by tetramers is associated with more CMV complications and a higher transplant related mortality [299]. It has previously suggested that there is discrepancy between detection of T-cells by tetramers and their functional capacity possibly influencing control of CMV [300].

The impairment seen in patients experiencing repeated or prolonged CMV- reactivations of T-cell responses defined by insufficient STAT5 phosphorylation seen in this study was most likely not associated with GVHD since we could not identify a correlation between the grade of GVHD and the functional ability of CD8⁺ T-cells to respond to IL-7 stimulation.

This could imply that gauging levels of phosphorylated STAT5 upon IL-7 stimulation *ex vivo* could aid to identify patients at higher risk to suffering CMV-complications. Already at 2 months after HSCT, patients at increased risk for contracting CMV complications might be identified by looking into the kinetics of the ΔpSTAT5 responses (i.e. the capacity of the T-cells to respond to IL-7 stimulation with STAT5 phosphorylation).

Our findings also confirm the pattern we found when investigating the correlation between EBV PTLID e.g. another viral complication to allogeneic HSCT and a decreased IL-7 signaling [91].

Furthermore, our study revealed that irrespective of CMV or GVHD, individual T-cell subsets, i.e. TCR $\gamma\delta$ T-cells and CD4⁺CD8⁺ T-cells, exhibited a higher frequency of constitutive STAT5 phosphorylation as compared to T-helper cells (CD4⁺) and CD8⁺ T-cells implying that certain immune cell subsets might be constitutively activated after HSCT. These subsets include CD4⁺CD8⁺ T-cells, representing activated CD4⁺ T-cells expressing the CD8 α chain [301], and TCR $\gamma\delta$ T-cells that may also play an important role in immune reconstitution [302] and in CMV-directed cellular immune responses [32].

6.3 PAPER III: WHOLE CMV PROTEOME PATTERN RECOGNITION ANALYSIS AFTER HSCT IDENTIFIES UNIQUE EPITOPE TARGETS ASSOCIATED WITH THE CMV STATUS

We screened the entire CMV proteome to visualize the humoral epitope-focus profile in serum after HSCT. IgG profiling from patients divided in the four possible pretransplant serological groups (donor and/or recipient +/- for CMV) was performed at 6, 12 and 24 months after HSCT using microarray slides containing 17174 of 15mer-peptides overlapping by 4 amino acids covering 214 proteins from CMV. Data were analyzed using MaSigPro, PAM and the “exclusive recognition analysis” (ERA) to identify unique CMV epitope responses for each group.

We found that the CMV donor positive groups (D+R+ and D+R-) had stronger capacity to develop antibodies to previously not recognized peptide targets and that the antibody profile against CMV seemed to be dependent on pre-transplant defined serological groups.

Comparisons over time show that the patterns of humoral responses changed after HSCT. ERA (the inclusive/exclusive analysis) segregated best 12 months after HSCT for the D+R+ group (versus D-R-). Epitopes were derived from UL123 (IE1), UL99 (pp28), UL32 (pp150), this changed at 24 months to 2 strongly recognized peptides provided from UL123 and UL100. Strongly (IgG) recognized CMV targets showed

also robust cytokine production in intracellular cytokine staining (IL-2, IFN- γ , TNF- α , and IL-17).

Our results would indicate that the early humoral response is not originating from the transplanted donor immune system, but rather represents a recipient derived response and is thereby a reflection of pre-existing antibodies or persistent recipient B-cells [303, 304]. To strengthen this hypothesis, we compared serum analyses from patients before and 6 months after HSCT founding no significant differences concerning CMV peptide recognition using PAM analysis.

One year after HSCT, the humoral CMV-specific response vanished in all groups with exception of the group where both donor and recipient were CMV seropositive. We believe that the observed phenomenon, already recognized almost 20 years ago by Wahren and coworkers when examining the transfer and persistence of specific B-cell responses in serologically disparate donor-recipient pairs [181], most likely reflects that the persisting antibody producing cells of recipient origin have a finite life-span. On the other hand, the new donor immune system has not matured enough to produce a wide spectrum of antibodies against CMV.

Two years after HSCT the CMV-specific humoral immune response was re-established in the groups that had a contracted humoral response 1 year earlier (D-R+ and D+R-) as the donor B-lymphocyte repertoire had been reconstituted. We did not monitor CMV DNA this far out after HSCT but this presumably represents the response of the new donor immune system to exposure to CMV proteins.

It is assumed that antibodies against epitopes from closely related amino acid sequences from different herpesviruses cross-react [305, 306] and this may in part account for the recognition pattern found in the D-R- group. We identified a higher percentage of potential cross-reacting CMV peptide targets from the PAM-analysis compared to the combination of PAM and ERA possibly indicating that antibodies directed against the epitope listed in the latter group are much more precisely directed against CMV. Most of these possible cross-reactive hits derived from HHV-8. However, the potential cross-reaction with HHV-8 is unlikely to be relevant since HHV-8 infections are quite rare in the Swedish population. It is therefore likely that these epitopes do in fact reflect an anti-CMV directed reactivity [307].

At 2 years after HSCT, the epitopes in the highly selective intersection of PAM and ERA in the patient group D-R+ mainly originate from CMV gene products already known to stimulate a strong immune response in CD8⁺ cells (e.g. CMV pp28, CMV pp150 and CMV IE2) reinforcing the hypothesis that CD8⁺ immune response and CD4⁺ immune responses, providing help to B-cells, are closely related.

As the presence of more than one CMV strain has been observed in immune competent individuals [308, 309], we chose to focus the statistical analysis on peptides recognized by all individuals in one group (for serum harvested at a single time point after HSCT but never in any individual from the D-R- group), in order to be able to find antibodies independent of the CMV strains circulating in the population [310-313]. However, since we used the D-R- group as a negative reference group, proteins that are rather conserved among human herpesviruses in general such as CMV glycoproteins gH, gB and gM [314] were not identified in our study even though they during natural CMV infection will stimulate to production of potentially neutralizing antibodies [315-317].

The MaSigPro analysis considering the dynamics of the peptide recognition over time enabled the identification of humoral CMV targets peptides distinguishing the seropositive donors/recipients against the D-R- control group.

To follow the development and the reconstitution of the CMV-specific humoral immune response over time after HSCT, we had to select a group of long-term survivors. However, as CMV has a major impact on the survival after allogeneic HSCT [208], a potential limitation to our findings is that this might represent a positive selection and that findings from patients with more CMV associated complications might have been different.

Our data also show that B-cell responses, identified by peptide microarrays, can be used to identify functional CD8⁺ and CD4⁺ T-cell responses directed against already described and newly identified cellular CMV targets in PBMCs from patients after HSCT.

To conclude, CMV epitope proteome mapping by peptide microarray technology with high resolution epitope-recognition matrix describes the breadth of humoral immune

reconstitution after HSCT allowing the description of both “common” and “private” CMV specific humoral recognition patterns [318]. This could be helpful in searching for anti-CMV specific B-cell responses associated to clinical outcomes possibly reflecting the breadth of B-cell immune reconstitution after HSCT. It also aids to appreciate in more general terms the nature of the B-cell immune reconstitution using the entire CMV proteome as a target matrix to describe the dynamic nature of the immune response in individuals over time [319].

6.4 PAPER IV: HUMORAL REACTOME PROFILES OF HUMAN CYTOMEGALOVIRUS

Serum antibody profiling was performed on samples from 18 patients at 6, 12 and 24 months after HSCT using CMV peptide microarray slides as described for paper III. To enable the global visualization of the entire intensity of Ig responses against CMV-epitopes a 3D model [320] was used. Two different types of 3D graphs were constructed: CMV antigen regression surfaces (3DPOS) and peptide bulkiness/ polarity regression surfaces (3DBP). By constructing 2D smoothed regression plots it is possible to recognize hotspots of IgG recognition within each protein.

Superficially, the regression surfaces (3DPOS) between the different serological groups (D+R+; D+R-; D-R+ and D-R-) seem to be very similar with hotspots on the same places. Some graphical characteristics differentiate the groups: In the D+R- group almost no response was seen at 6 months contrasting to a very intense response seen at 12 months possibly representing the expansion of CMV specific donor B-cells and subsequently of plasma cells. In the D-R+ group the intensity of the epitope response was rather low throughout all time points presumably representing the loss of pre-existing recipient CMV specific plasma cells. The average normalized intensity of the epitope response in the two groups D+R+ and D-R- were surprisingly quite similar.

The analysis of the peptide bulkiness/ polarity regression surfaces (3DBP) also revealed some interesting facts. Peptides that are more bulky tend to give rise to a more intense epitope response. The strongest intensity of epitope response was found in the peptides with most bulkiness but low relative polarity. The general base line for the intensity of the epitope response increased in all groups from 6 months to 12 months and stayed

more or less stable, with exception of the pattern in the D-R+ group where the general base line of intensity of the epitope response seemed to decrease at 6 months and then stabilize at this level.

Different approaches, including a detailed analysis of humoral reactivity against defined target antigens have been used to test the reconstitution of B-cells in patients after HSCT [321] and it has been shown that the patients often lose specific antibodies during follow-up [181]. In addition recurrent CMV reactivation or development of CMV disease after HSCT is linked to acute GVHD and the development of cGVHD [250].

On the other hand the large number of naive B-cells contributing to the plasma Ig production are believed to produce Ig of too low-affinity to be detected using microarray technology based on the assumption that a fluorescent-based Ig array detects approximately 10 attomoles of IgG [186]. There may be many reasons for the differences in Ig recognition of CMV targets: (i) Plasma half-life of IgG, (ii) B-cell reconstitution, (iii) transfer of mature B-cells with the graft, (iv) stimulation of developing B-cell repertoire by commensal microbes, (v) stimulation of B-cells by specific pathogens and autoantigens, (vi) the cytokine/inflammation milieu, (vii) the nature of immune suppression and (viii) the regimen used for conditioning of the host.

Spontaneous IgG production or transfer has been detected as early as 3 months after HSCT [181, 322, 323] reflecting most likely long-lived plasma cells, transferred with the graft homing to the recipient's bone marrow [324]. These 'natural antibodies' are generated in the absence of immunization and exposure to 'foreign antigens' [325]. However, in the case of CMV there are continuous reactivations of the virus most likely driving the reconstitution of the humoral immunity. The antibodies we found are directed against either native viral surface antigens (potentially neutralizing, i.e. preventing viral entry) or internal and denatured viral proteins (not neutralizing). The latter are found to a great extent *in vitro* and, as we can show, also *ex vivo*. This may be due to that cGVHD is seen in greater extent in patients with recurrent CMV reactivations, and therefore these patients can have more self-reactive antibodies and more necrosis of cells, thereby exposing internal viral proteins [220]. Several functions have been proposed for natural antibodies such as being an effective first line defense against infectious diseases [326]. Our data using the 3D analysis show that either

natural antibodies or cross-reactive Abs contribute to form a comprehensive matrix to recognize CMV epitopes in individuals with a D-R- background.

In general, analysis of B-cell responses rests on the ‘gain’ of reactivity, which also has determined our analysis presented in this report: We examined the IgG response directed against CMV and tested for Ig reactivities that would segregate the different patient groups. This view may be relevant, yet it may not reflect the biological situation. A *loss* of reactivity may also be critical and such a ‘negative’ profile may be much more difficult to demonstrate. For instance, cross-reactive and auto-antigen specific natural antibodies have been postulated to be protective against Alzheimer’s disease [327]. We have observed a similar loss of reactivity directed against certain CMV epitopes and this may warrant a more detailed re-examination of the role of natural antibodies and the selective loss of certain epitope specificities associated with CMV containment

7 CONCLUSIONS

Cytomegalovirus infections represent an important complication after hematopoietic stem cell transplantation. It is clinically relevant to try to identify which patients of those who reactivates CMV that have an increased risk of developing CMV-disease and thereby would be in need for more intensified or prolonged monitoring of CMV viral loads. Conversely, it is also important to determine the patients who might not need CMV-antiviral treatment.

From our 1st study, in which we investigated the risk factors for CMV-disease, the most important finding was that viral load kinetics after initiation of antiviral therapy predicted of the risk of developing CMV-disease: Patients who responded slower had a higher risk. Therefore we conclude that the assessment of antiviral response should be used for deciding management strategies after the first course of preemptive antiviral therapy.

From our 2nd study we conclude that reduced responses to IL-7, reflected by pSTAT5 may represent a clinically relevant functional biomarker for individuals at increased risk for CMV reactivation. Subjects with low Δ STAT5 phosphorylation could be followed with weekly monitoring of CMV viral loads for a prolonged period while shorter monitoring is required in patients with high Δ STAT5 phosphorylation. Our data may also aid to design better strategies to improve anti-CMV immune responses without increasing the risk to develop GVHD [328]. The findings in the present study, however, need to be assessed in a larger study.

From the 3rd and 4th study we conclude that high-content peptide microarray technology aids to describe the breadth of humoral immune reconstitution after HSCT by epitope profiling of entire viral proteomes and it might also be useful for mapping in an unbiased fashion, immune responses induced by CMV vaccines in a post-transplant setting.

The method we used in the 4th study displaying microarray results as 3D landscapes can be useful to determine and find new targets for humoral immune response, which may be biologically relevant in protection against CMV and thereby indirectly targets for

CD4⁺ T-cells. It will also aid to appreciate in more general terms the nature of the B-cell immune reconstitution using the entire CMV proteome as a target matrix. By constructing 2D smoothed regression plots it would be possible to recognize hotspots of IgG recognition within each protein.

8 FUTURE PERSPECTIVES

Human CMV has developed during the history of human evolution to a refined multi-talented virus with a large range of tools to escape the immune system. It is clear, however, that despite decades of research on CMV pathology and CMV-disease risk factors and CMV vaccine development, CMV is still a major clinical problem for certain patient groups. To understand how the human immune system balances the effects of human CMV is still a major research challenge.

One of the main aims and certainly one of the most important tasks for the future is to develop a clinically effective CMV vaccine. However, such a “solution” to the CMV problem would implicate that research regarding the developing and aging CMV-specific immune response should be better investigated.

One approach in order to enable this goal would be to examine a group of long time survivors after HSCT (here defined as patients 10 years or more after transplantation) combining the same methods and techniques described in all the papers of this thesis. The antigenic focus, the functional capacity, and the maturational differentiation state of CMV-specific CD8⁺ T-cells defined by flow cytometry and CMV-tetramer technology and CMV-specific antibodies (and indirectly CD4⁺ T-cell responses) defined by CMV peptide microarray profiling would be of particularly interest.

This situation may present an opportunity to define “markers of immune protection” and would allow investigations in detail of how humoral immune response develops over time after HSCT and if and eventually how the development of T-cell clonality and eventual B-cell and antibody clonality against CMV differs between hematopoietic transplanted patients from healthy individuals and between patients and healthy individuals having undergone vaccination against CMV. To associate these parameters to long-term clinical outcome like chronic GVHD, transplantation rejection, early and late relapse, and secondary malignancies would make this study unique and important. Finally it would be of greatest interest to see the effect of CMV-reactivation and CMV-vaccine on autoimmune inflammatory diseases and cancer.

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