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IMMUNE EVASION OF HUMAN CYTOMEGALOVIRUS
STUDIES OF UL18 AND US2 FUNCTION

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ABSTRACT

The β-herpes virus cytomegalovirus (HCMV) infects human populations at a high frequency worldwide. Primary infection with HCMV, usually asymptomatic, is followed by lifelong latency. During the long co-evolution of virus and host, a fine balance has developed between viral immune evasion strategies and defence mechanisms of the immune system. Yet for individuals with a defective, immature, or compromised immune system, HCMV becomes a serious threat. The general aim of this thesis was to investigate functional and molecular aspects of the HCMV-derived immune-evasion proteins UL18 and US2.

One sophisticated immune evasion strategy of HCMV is interference with MHC class I presentation of viral peptides, using several unique short (US) proteins. We dissected the mechanisms underlying the allele specificity of US2 and demonstrated that a single arginine residue at position 181 (Arg_{181}) was critical for US2-mediated inhibition of HLA-A2 cell surface expression. Binding of US2 to HLA-A2 resulted in a unique, large conformational change of the side chain of Arg_{181}. Even though a prerequisite for the interaction of US2 with HLA-A2, the presence of this residue is not sufficient to guarantee binding to other MHC class I alleles.

Another suggested immune modulator with ambiguous function is the protein UL18, a viral MHC class I homologue that associates with β_2 microglobulin (β_2m). UL18 binds with high affinity to the leukocyte immunoglobulin-like receptor-1 (LIR-1). We tested the impact of several substitutions in UL18 proteins for binding affinity to LIR-1. Our results revealed that residues localized both in the α1 and α3 domain are important for LIR-1 binding, and demonstrated β_2m dependency of the UL18/LIR-1 interaction. Finally, two disulfide bridges, one of them unique for UL18, were essential for complex formation of UL18 with β_2m.

Since LIR-1 is widely expressed on immune cells, we investigated if UL18 could affect dendritic cells (DCs). We demonstrate that UL18 proteins specifically up-regulated CD83, while not influencing other maturation markers. UL18 also induced IL-10 production and to some extent other cytokines such as TNFα and IL-12. The presence of UL18 during DC maturation via CD40L inhibited DC migration and impaired subsequent T cell responses. We concluded that UL18 can alter phenotype and function of monocyte-derived DC.

The expression of LIR-1 particularly on HCMV specific T cells as well as on NK and T cells in lung-transplanted patients prior to development of CMV caused pneumonia supports the hypothesis that the UL18-LIR-1 interaction may be relevant during natural infection. Therefore, we focused on the role of LIR-1 in the immune response to HCMV and potential effects of UL18 on NK and T cells. Cells infected with a virus lacking the gene for UL18 induced less cytokine responses compared to parental virus, proposing an activating function for UL18. In contrast, isolated UL18 proteins inhibited LIR-1^+ T cells, which unlikely played a role in response to infected cells.

In summary, this thesis provides further insights into the mechanism underlying the binding of US2 to HLA-A2 and demonstrates that UL18 interacts with LIR-1 in a manner different from MHC class I ligands. Furthermore, our results regarding the effect of UL18 on several immune cells contributes to a better understanding of the complexity of viral evasion mechanisms.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their roman numerals:

I.
Thilo C, Berglund P, Applequist SE, Yewdell JW, Ljunggren HG, Achour A. Dissection of the Interaction of the Human Cytomegalovirus-derived US2 Protein with MHC Class I Molecules: Prominent Role of a Single Arginine Residue in HLA-A2.
Journal of Biological Chemistry, 2006 Mar 31;281(13):8950-57

II.
Wagner CS, Rölle A, Applequist SE, Cosman D, Ljunggren HG, Berndt K, Achour A. Structural Elements Underlying the High Binding Affinity of Human Cytomegalovirus UL18 to Leukocyte Immunoglobulin-like Receptor 1.
manuscript submitted

III.
Wagner CS, Walther-Jallow L, Buentke E, Ljunggren HG, Achour A, Chambers B. The Human Cytomegalovirus Protein UL18 Alters the Phenotype and Function of Monocyte-Derived Dendritic Cells.
manuscript submitted

IV.
Wagner CS, Riise GC, Bergström T, Kärre K, Carbone E, Berg L**.** Increased Expression of LIR-1 and Activating Role of UL18 in the Response to Cytomegalovirus Infection.
Journal of Immunology, 2007 Mar 15;178 (6)

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen-presenting cell
β_2m	β_2-microglobulin
CCL	CC chemokine ligand
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dUL18	CMV virus with the UL18 gene deleted
EBV	Epstein Barr virus
ER	endoplasmic reticulum
ERAD	ER-associated degradation
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp	glycoprotein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
HLA	Human leukocyte antigen
KIR	killer cell Ig-like receptor
LIR/LILR	leukocyte Ig-like receptor
LPS	lipopolysaccharide
MCMV	murine/mouse cytomegalovirus
MDC	myeloid DCs
MHC	major histocompatibility complex
MIC	MHC class I chain-related protein
NK	natural killer cell
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PIR	paired Ig-like receptor
PDC	plasmacytoid DC
PRR	pattern recognition receptor
rVV	recombinant vaccinica virus
siRNA	small-interfering RNA
SHP-1	Src homology domain 2-containing tyrosine phosphatase 1
TAP	transporter associated with antigen processing
TCR	T cell-receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
ULBP	UL16-binding proteins
VIPR	viral protein interfering with antigen presentation

1 AIMS OF THIS THESIS

The ubiquitously prevalent human cytomegalovirus employs a great variety of strategies to avoid immune recognition. The general aim of this thesis was to gain further insights into mechanisms used by HCMV to evade the immune system and thereby intervening with central processes of cell biology and function. The specific aims were:

To dissect at a molecular level how the viral protein US2, that mediates down-regulation of MHC class I molecules, interacts with specific MHC class I ligands (**paper I**).

To determine structural elements that control the high affinity binding of the viral MHC class I homologue protein UL18 to the inhibitory immune receptor LIR-1 (**paper II**).

To investigate whether UL18 may influence dendritic cell function (**paper III**).

To study the relationship of the inhibitory receptor LIR-1 to cytomegalovirus infection, with particular focus on the role of UL18 (**paper IV**).

2 INTRODUCTION

2.1 THE HUMAN IMMUNE SYSTEM

2.1.1 Innate and adaptive immunity – an overview

I start this thesis with a short introduction to immunology, the fascinating science about immunity. The term immunity is deduced from the Latin expression *immunitas*, originally used for the exemption from taxes, and later also for describing the capacity to withstand sickness. The phenomenon of immunity can be traced back until the notes of the Greek historian Thucydides, describing the plague of Athens 430BC, where people who recovered from disease did not get infected again. Immunology as a science started rather late, at the end of the 18^{th} century with Edward Jenner, disoverying that vaccination with cowpox could protect humans from smallpox. Almost 100 years later, Robert Koch discovered that microorganisms caused infectious diseases, and Louis Pasteur continued Jenner´s work in the field of vaccination, developing a remedy against rabies. Today, the field of immunology comprises many different disciplines, all working towards the final goal to understand how the immune system works. Ultimately, the gained knowledge shall be used to meet challenges posed by infectious diseases, allergic responses, cancer and autoimmune disorders.

One example for the amazing ability of our immune system to cope with demanding tasks is the following: Ten times more bacteria than human cells can be found in our body, and expressed in terms of organism, we are even outnumbered by a factor of 10^{13}-10^{14}– and still, those bacteria seldom cause disease. Thus, the immune system needs to distinguish between pathogens and commensal bacteria, and make sure the that latter do not become pathogenic by entering the wrong body compartment.

Traditionally, the immune system is divided into innate and adaptive immunity, which, like most artificial classifications, is an oversimplification. During last years, it has become more and more clear how deeply interwoven adaptive and innate immunity are, and that components classified as either of those categories do not merely influence, but can even be key players in the other category. For example, mast cells, part of the innate immunity and a well established role in allergic responses, were recently discovered to be responsible for induction of transplant tolerance mediated by regulatory T cells (1). In the following section, main features and constituents of adaptive and innate immunity shall be briefly outlined. For an in depth description I refer to the textbook of the late Charles Janeway and colleagues, “Immunobiology – the immune system in health and disease” (2).

Innate immunity is an ancient system, of which components like antimicrobial peptides can be found even in bacteria. This system provides an efficient first line of defence against harmful microorganisms, and is often sufficient to prevent infection. Even if innate immunity fails to cope with a pathogen, it promotes initiation of adaptive immune responses as well as confines propagation of the infectious agent for several days until adaptive immunity can step in (3). Besides the epithelial barriers, the innate

immune system relies on various cells that distinguish infectious foreign or abnormal self from normal self molecules, using germline encoded receptors, the so-called pattern recognition receptors (PRRs) (4, 5). Macrophages, granulocytes, mast cells, dendritic cells (DCs) and natural killer (NK) cells are all classified as innate immune cells. They operate by engulfing pathogens and secreting soluble factors such as cytokines, chemokines, cytolytic granules and other mediators. They therefore contribute to the direct elimination of the infectious agent, but also elicit subsequent adaptive immune responses. One important family of PRRs is the Toll-like receptor (TLR) family (6), named after the molecule "TOLL", meaning "great/amazing" in German, which was identified in the fruit fly *Drosophilia melanogaster*. Other examples of innate receptors found in humans and multiple other organisms are the family of C-type lectin receptors or the intracellular Nod-like receptors (7). The innate sensing mechanisms have the disadvantage that they cannot create memory. The response is therefore always of the same magnitude, even upon renewed infection with the same pathogen. An exception to this general view is a recent report about NK cells, which are able to function as memory cells in a mouse model for contact dermatitis, a phenomenon that will certainly be investigated further (8).

Even if less rapid, the adaptive immune system of vertebrates bears the advantage over innate immunity in that it is based on highly polymorphic receptors which, in the case of B cell receptors, can be adapted and fine-tuned according to the particular infectious agent the lymphocyte has to deal with. The main adaptive effector cells are B and T lymphocytes, which expand clonally after initial encounter of their receptors with matching antigen. They are able to create long-lived memory cells after termination of the acute response, where the infectious agent is usually removed. Thus, a second invasion by the same pathogen results in a faster and more efficient response. B cells control humoral immunity by producing antibodies, directed mainly against extracellular bacteria, whereas different subsets of T cells are responsible for elimination of infected cells and supporting B cell function.

Below, I will describe the cells I have been focusing on within the frame of my thesis studies more in detail, namely dendritic cells (**paper III**), NK and T cells (**paper IV**). But first, the major histocompatibility complex (MHC) class I molecules will be presented, which are crucial for the function of all three types of cells.

2.1.2 The Major Histocompatibility Complex class I

If there were a contest for the most important molecules in immunology, I would certainly nominate the MHC molecules. MHC class II molecules are important for the activation of $CD4^+$ T cell, whereas MHC class I complexes play a key role for $CD8^+$ T cell mediated immunity. MHC class I molecules deliver signals to $CD8^+$T cells to eliminate cells that have been infected or become malignant. Conversely the absence or reduction of MHC class I molecules can activate NK cells. In the following paragraphs I will mainly focus on MHC class I molecules since UL18, a major topic throughout this thesis, is a viral MHC class I homologue and the other CMV-derived molecule I have been working with, US2, binds to and results in the degradation of MHC class I molecules.

2.1.2.1 Nomenclature

The term 'major histocompatibility complex' (MHC) originates from the description of this gene locus in mice, determining transplant rejection or acceptance (9). Rolf Zinkernagel and Peter Doherty discovered the exceptional role of MHC class I proteins for presentation of viral antigens to T cells (10). In 1996, they were awarded the Nobel Prize for the discovery of this process, termed MHC restriction. MHC molecules are also named Human leukocyte antigen (HLA) in humans and Histocompatibility-2 (H-2) in mice. The classical MHC class Ia proteins in humans are HLA-A, -B and -C, in mice H-2D, H-2K and H-2L, and the MHC class II molecules are termed HLA-DP, -DQ and -DR in humans and H-2A and H-2E in mice. HLA-E, -G and -F are human non-classical MHC class Ib proteins. It has been suggested to reconsider the historical classification of MHC class I products, which is based on polymorphism and tissue distribution (11). It could be useful to group HLA-C with HLA-G and HLA-E, based on the importance for NK cell rather than T cell interaction and expression in the placenta, where HLA-A and B are absent (11).

CD1, which binds lipids instead of peptides, is also regarded as an MHC class Ib molecule, even though not encoded in the MHC gene complex. The first crystal structure of an MHC class I protein, namely HLA-A2, was determined by Pamela Björkman and colleagues in 1987 (12).

2.1.2.2 MHC class I structure

The MHC class I complexes are formed by a 45kDa heavy chain, a non-covalently linked β_2-microglobulin (β_2m) light chain of 12kDa, and a usually 8-10 amino acid long peptide **(Figure 1)**. The heavy chain is composed of three extracellular domains α1, α2, α3, each approximately 90 residues long, a transmembrane region and an intracellular portion. The overall structure is conserved between different alleles and species. The α1 and α2 domains (residues 1-182) form the peptide binding groove, with an α helix on each side and a ground formed by β-sheets (13). The allele specific peptide binding groove has six cavities (called pockets A-F), that determine the kind of peptide that can be accommodated (14). The α3 domain has an immunoglobulin (Ig)-like fold and is more conserved between different alleles than the α1 and α2 domain. Furthermore, the α3 domain can fold independently of the other domains or β_2m (15). The β_2m subunit, also with an Ig-like fold, is not only a structural component that contacts all three heavy chain domains **(Figure 1)**, but also serves as a chaperone during folding of the MHC class I heavy chain (15). In cells lacking β_2m, MHC class I heavy chain surface expression is severely impaired (16). β_2m harbours one disulfide bond (C25-C80) while the MHC class I heavy chain contains two disulfide bonds. One of them is situated in the α3 domain between C203 and C259. Similarly to β_2m, his disulfide bond in is part of the classical Ig-like fold, which is characterized by two connected parallel β-sheets, consisting of an overall set of 7-10 anti-parallel β strands (17, 18). The second heavy chain disulfide bridge, formed between residues C101 and C164 in the α2 domain, is unique for MHC class I molecules, and links the β-sheet floor with the α2 helix of the peptide binding groove (12, 19) **(Figure 1)**.

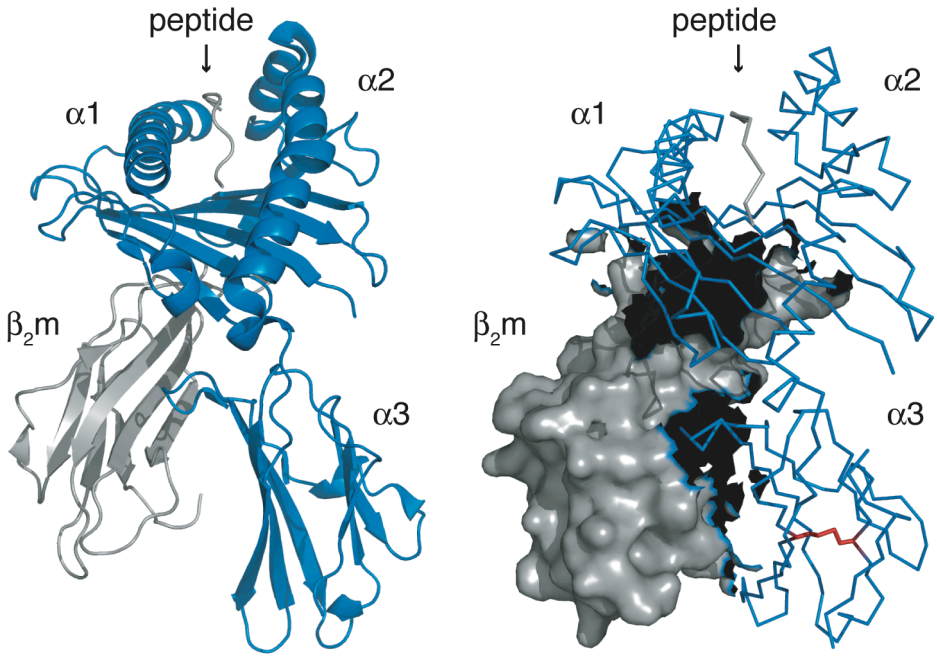

Figure 1. Overall three-dimensional structure of an MHC class I complex.
(Left and right) The three extracellular domains of the heavy chain (α1, α2, α3) are depicted in blue. The non-covalently linked light chain β_2-microglobulin is coloured grey. The peptide (in grey) is situated in the peptide binding groove. (Right) The surface of β_2m regions contacting the heavy chain (depicted as sticks) is coloured black. The two disulfide bonds in the α1 and α2 domain, respectively, are red.

2.1.2.3 Antigen processing and presentation pathways

MHC class I molecules present 8-10 amino acid-long peptides derived mainly from endogenously synthesised antigens to $CD8^+$ T cells. These peptides are mainly generated through proteasomal degradation of cytosolic proteins and transferred into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where they are further optimized in length for binding to the MHC class I molecule. The loading complex bridges TAP with MHC class I and consists of several proteins: Tapasin connects the class I heavy chain in complex with β_2m to TAP and also supports peptide loading. The chaperone calreticulin is a lectin that binds a monoglycosylated N-linked glycan of the MHC class I heavy chain and associates non-covalently with Erp57, a thiol-oxidoreductase and member of the protein disulfide isomerase family. Erp57 may be involved in disulfide-isomerization in the α2 heavy chain and is covalently linked to tapasin through the formation of a disulfide-bond (19, 20). The properly loaded MHC class I complexes are transported to the cell surface. Some MHC class I alleles can assemble with peptides independent of tapasin or TAP (21, 22).

In contrast to MHC class I molecules, MHC class II proteins do not acquire peptides in the ER but are instead loaded in lysosomes (23). The peptide pool is derived from endocytosed exogenous proteins and from endogenous proteins that gain access to this

compartment (24). DCs can use a special pathway for MHC class I presentation of peptides, termed cross-presentation. Cross-presentation allows endocytosed antigens, which are normally loaded onto MHC class II molecules in lysosomes, to enter the MHC class I presentation pathway. Dendritic cells can thus activate MHC class I restricted $CD8^+$T cells without being infected themselves (25-28).

2.1.3 Natural Killer cells

2.1.3.1 Historical overview

NK cells are of lymphoid origin and constitute between 5-15% of peripheral blood lymphocytes in humans. They contribute to immune responses through the production of cytokines as well as direct cell lysis. Target cell killing is mediated either by secretion of perforin-granzyme containing granules or is receptor-mediated (reviewed in (29, 30)). Activated NK cells are also efficient producers of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF) α and interferon (IFN) γ, resulting in anti-microbial effects and modulation of differentiation/activation of other immune cells. In particular, IFNγ is implicated in viral defence mechanisms, e.g. by promoting a cell-mediated immunity and by increasing MHC class I surface expression, stimulating T cell recognition of infected cells (31, 32).

The history of NK cells and the Karolinska Institutet are strongly linked; therefore I will start here with a brief overview on NK cell discovery. NK cells were first described in 1975 by Kiessling *et al.* and independently by Herberman *et al.* (33-36). The name "natural killer" was designated because of their ability to eliminate tumor cells *in vitro* without prior sensitization (33, 34), in contrast to cytotoxic T cells. Today it is clear that NK cells are not in a constant steady killer state, but that activation results from integration of signals derived from both activating and inhibiting surface receptors **(Figure 2)**. Rolf Kiessling´s PhD student Klas Kärre postulated in his doctoral thesis 1981 the provocative and novel hypothesis of "missing self" (elaborated in (37)), which was based on the concept that NK cells kill target cells lacking self MHC class I molecules, rather than recognizing an activating surface ligand (38). Kärre *et al.* then demonstrated four years later that NK cells could reject tumour cells with reduced or absent levels of MHC class I *in vivo* in mice (39). This notion was of importance against the background that several tumours down-modulate MHC class I expression to escape T cell immunity (40), as do viruses (41, 42). The findings by Kärre and colleagues sparked off multiple studies on the relation of NK cell susceptibility and MHC class I expression levels both in mice (43-47) and in humans (48-51). The first mouse inhibitory NK receptor restricted by MHC class I, designated Ly49 (today Ly49A), was discovered by Yokoyama *et al.* (52, 53), followed by the discovery of the first inhibitory receptors on human NK cells, the p58 molecules (today KIR2DL) by Moretta and colleagues (54). For a more comprehensive summary of the history of the missing self-hypothesis I refer to articles by Kärre (41) and Borrego (55). Considering the knowledge gained during during more than 25 years of research, the original concept of "missing self" in ruling NK cell activity needs today to be broadened/extended, assigning more weight to the role of activating receptors and accepting that MHC class I molecules can also act as activating ligands (41).

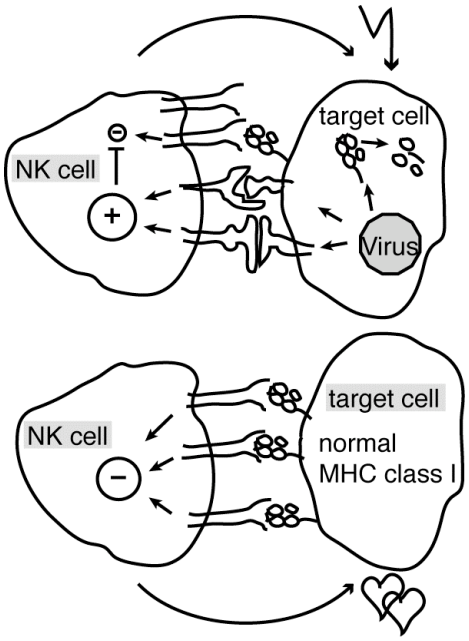

Figure 2. The principle of NK cell function.
NK cell activation is regulated by integration of negative and positive signals from different surface receptors. Virus infection reduces MHC class I levels on the cell surface on the target cell, diminishing interaction with inhibiting NK cell receptors. Concurrently, virus infection induces expression of ligands for activating NK cell receptors. As a result, NK cells receive predominantly activating signals and kill the infected target cell (top). Uninfected cells with normal MHC class I expression levels are protected from NK cell killing (bottom).

2.1.3.2 NK cell receptors

NK cells express two types of MHC class I receptors, belonging either to the C-type lectin family or to the immunoglobulin (Ig) superfamily (56). In humans, three different families of genes encode receptors for HLA class I, including the polymorphic killer cell Ig-like receptors (KIRs) on chromosome 19, the evolutionary related leukocyte Ig-like receptors (LIRs or LILRs), which will be discussed more in detail below, and finally the C-type lectin family, comprising the CD94-NKG2 heterodimers (56, 57). The CD94-NKG2A and CD94/NK2C receptors recognize the non-classical MHC class I molecule HLA-E in combination with leader peptides from classical MHC class I heavy chains (58-60). This system is also found in mice, where CD94-NKG2A, - C, or –E interact with the non-classical MHC class I molecule Qa-1^b (61, 62). Instead of KIRs, mouse NK cells express the highly polymorphic Ly49 C-type lectin family that – even though phylogenetically unrelated – exert similar functions as KIRs, i.e. recognizing a specific MHC class I allomorph (56).

Signalling of inhibitory NK receptors binding to MHC class I or MHC class I related molecules is regulated through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) V/IxYxxL/V in the cytoplasmic domains of the receptors. Phosphorylation of these motifs, e.g. via Src family kinases, allows docking of protein tyrosine phophatases such as SHP-1 and SHP-2, that mediate negative signalling (63). All

families of NK receptors mentioned above do not only comprise inhibitory, but also activating receptors, which usually depend on adaptor molecules for transmission of signals. The immunoreceptor tyrosine-based activation motifs (ITAMs) of the associated adaptor molecules recruit tyrosine kinases, mediating activation (63). An important activating receptor for recognition of CMV infected cells is NKG2D, that binds to the MHC class I chain-related proteins (MIC) -A and -B and the UL16-binding proteins (ULBPs) 1-3 in humans and related ligands in mice (64). CD16, the low affinity Fc-receptor for IgG, is another well characterized activating receptor mediating antibody-dependent cellular cytotoxicity (ADCC) (65). The ligands for many activating receptors that do not bind MHC class I molecules remain up to date unknown. Examples for additional activating receptors on human NK cells that do not belong to the MHC class I binding receptors are 2B4 and the natural cytotoxicity receptors NKp46, NKp30 and NKp44 (63, 66).

2.1.4 Dendritic cells

2.1.4.1 Classification

DCs are crucial for the initiation of immune responses. They are highly specialized for T cell stimulation and bridge innate and adaptive immunity. They are found in blood and most tissues and are heterogeneous in subtype, developmental origin, localization and function. Mouse and human DC subsets are rather similar in function, even though there are differences in surface markers and expression of certain cytokines within the various subgroups. However, since blood is the main available study material in humans, much less is known about tissue-resident human DCs compared to mouse DCs. The majority of DCs is derived from myeloid progenitors (67). The human Langerhans cell subset found in the epidermis was first described in 1868 by Paul Langerhans as a type of nerve cell based on morphological criteria. The other skin resident DCs are dermal DCs, and mucosal and submucosal DCs are found in mucosal tissue (68). Besides myeloid DCs (MDCs), one can also isolate plasmacytoid DCs (PDCs) from peripheral blood, which mainly are of lymphoid origin. PDCs are round-shaped cells, thus differing morphologically from other DC subsets. They produce high levels of type I interferons upon exposure to viral or microbial components (69). PDCs and different subsets of myeloid DCs can be distinguished by diverse cell surface markers. For example, PDCs are $CD123^+$ but do not express myeloid markers such as CD11c (67, 69). The individual subsets are also equipped with a distinct repertoire of TLRs and other PRRs, which makes them functionally diverse (69, 70).

Since DCs account for less than 1% of peripheral blood mononuclear cells (PBMC), it is common practice to isolate human monocytes instead, which can be differentiated through culture in the presence of GM-CSF and IL-4 into monocyte-derived DCs that resemble dermal DCs (71). The resulting DCs are of immature phenotype and can be further matured by addition of CD40L or lipopolysaccharide (LPS) (72). If cultured in IL-15 or IL-4 and TGFβ, monocyte-derived DCs resemble Langerhans cells, for which monocytes can be the direct precursors *in vivo*, at least in mice (73). This DC generation protocol is certainly not a reflection of the main DC differentiation pathway *in vivo*, even though it has been demonstrated that human monocytes are capable to

differentiate into both DCs and macrophages when cultured with a layer of endothelial cells without addition of cytokines (74).

2.1.4.2 Dendritic cell function

The main tasks of DCs are the uptake of antigen as well as the subsequent processing and presentation of antigenic peptides to T cells on MHC class I and class II molecules. Depending on localisation, cytokine milieu and maturation state of the DC, presentation of antigen elicits different forms of immune responses or induces tolerance. Cytokine secretion by DCs also influences innate immune cells such as NK cells. How different cytokines affect T cell polarization towards a certain type of immunity will be described in the T cell chapter below. Cytokines are also called 'signal 3' for T cells, in addition to 'signal 1', which is the interaction between the MHC-peptide complex and the TCR, and 'signal 2', which is the interaction between the co-stimulatory molecules and their ligands.

One can distinguish between migratory DCs and lymphoid-tissue resident DCs. The latter are of immature phenotype, do not migrate but are resident in lymphoid organs, continuously collecting and presenting antigens. The former sample antigens in the periphery and migrate only occasionally to lymph nodes under steady-state conditions. However, upon receiving danger signals, for instance in the form of infection or tissue injury, these DCs start a maturation programme and migrate to lymph nodes, where T cell stimulation takes place (75, 76). Besides different TLRs and other PRRs that sense microbial compounds, DCs express lectin-like receptors that recognize conserved carbohydrate motifs. For example, DC-SIGN, which is found on several DC subtypes, binds to the endogenous adhesion molecules ICAM-2 and -3 as well as to various pathogens, including CMV (77, 78). Engagement of Fc receptors, cytokine receptors and members of the TNF receptor family also induces DC maturation (77), as does ligation of CD40 on DCs by CD40L, which is mainly expressed by activated T cells (79).

Before DCs decrease all forms of endocytosis and migrate towards the lymph nodes, antigen uptake is transiently enhanced after TLR engagement, ensuring the right pathogen-derived cargo for subsequent presentation (80). The maturation process further includes a change in chemokine receptor repertoire, i.e. down-regulation of chemokine receptors for inflammatory chemokines such as RANTES or MIP-1α/-β, that guide DCs to the site of infection, and up-regulation of lymph-node homing-receptors like CCR7 (81). DCs also up-regulate other maturation markers such as CD83, MHC class I and class II molecules to ensure a high density of peptides complexes on the cell surface, as well as co-stimulatory molecules for T cells, such as CD80 and CD86 (B7.1 and B7.2), CD40 or OX40L (72) **(Figure 3)**.

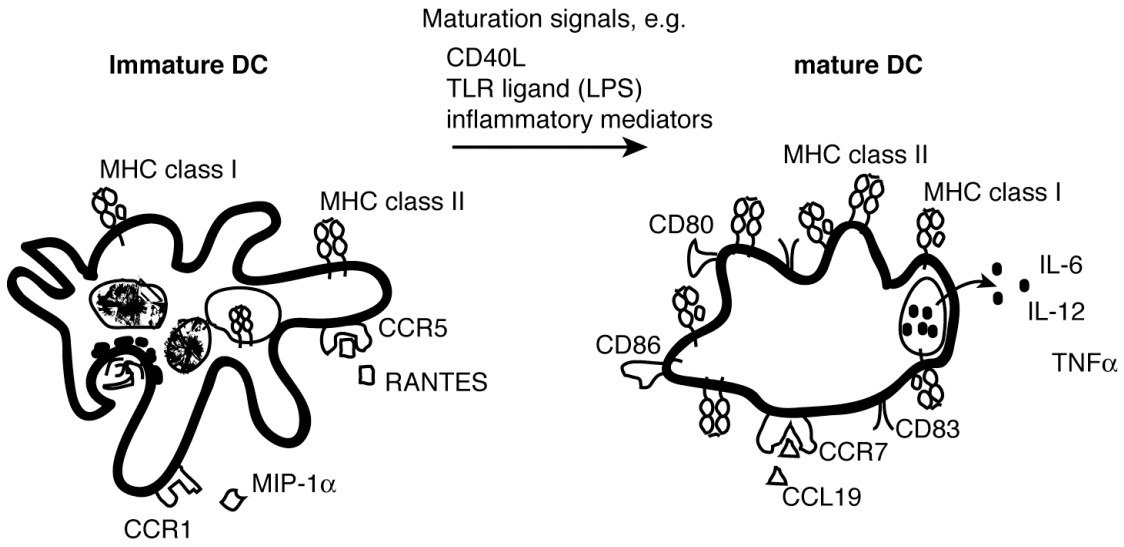

Figure 3. Comparison of immature (left) and mature dendritic cells (right).
Immature DCs (iDCs) express lower levels of surface MHC class I and II compared to mature DCs (mDCs). MHC class II molecules are stored intracellular in iDCs. Examples for different expression of chemokine receptors and other cell surface molecules are depicted. Functional differences are exemplified by the high endocytic capacity of iDCs versus secretion of immune regulating mediators by mDCs. Signals that induce maturation are indicated.

2.1.5 T cells

2.1.5.1 T cell maturation

T cell progenitor cells originate in the bone marrow and need to undergo a maturation process in the thymus (therefore the "T"), during which their antigen binding receptor, the T cell-receptor (TCR), is re-arranged and the co-receptors CD8 and CD4 are acquired. During this process, T cells need to pass two major control steps, termed positive and negative selection. The TCR must be able to bind to MHC class I molecules on thymocytes, otherwise the T cell dies by "neglect". Additionally, all T cells that express a TCR recognizing self-MHC class I in combination with a self-peptide with too high affinity are deleted, to prevent self-reactive T cells in the periphery. Most T cells express a TCR composed of one α and one β chain, while a minority is formed of δ and γ chains instead. T cells that express the co-receptor CD4 recognize MHC class II proteins, which are mainly expressed on antigen presenting cells, but can in humans also be expressed by various tissues following immune activation. In contrast, the co-receptor CD8 recognizes the ubiquitously (on all nucleated cells) expressed MHC class I molecules in combination with the TCR (2).
Lipid specific T cells that express NK markers are termed NKT cells and are restricted to CD1d. They exert regulatory functions and can both drive or reduce inflammatory responses (82).

2.1.5.2 Immune responses of different subtypes

$CD8^+$T cells differentiate into cytotoxic T lymphocytes (CTL), which mediate killing of e.g. virus-infected cells, using similar effector mechanisms to NK cells. $CD4^+$T cells

are also called T helper cells (Th), since they stimulate B cells, CTL, macrophages and other immune cells through the production of cytokines (2). T cells that become activated by DCs secreting cytokines such as interleukin (IL)-12, IL-18 and IL-23 get polarized towards a Th1 phenotype. Th1 cells subsequently produce IL-2, TNFα and IFNγ, inducing activation of macrophages, cytotoxic $CD8^+$ T cells and promoting an IgG2a dominated antibody response. Th2 cells, activated in the presence of IL-4 and for example CC chemokine ligand (CCL)-2, secrete IL-4, IL-5, IL-6 and IL-13, inducing a humoral antibody response, dominated by the production of IgA and IgE. DCs that produce cytokines such as IL-10 and TGFβ can induce regulatory T cells (83). A recently characterized subset involved in inflammation and autoimmunity is the Th-17 subset, which is induced by TGFβ in combination with IL-6, can be expanded by IL-23 and produces the cytokine IL-17 (84).

Again, the division into different T cell subtypes should be regarded as an over-simplification and the real situation is far too complex to be easily categorized. Therefore, more subsets and regulation factors are likely to be discovered. One example for breaking the paradigm is the existence of virus-specific $CD4^+$ T cells, e.g. recognizing Epstein Barr virus (EBV) or HCMV antigens, that can directly kill infected cells like $CD8^+$T cells, using the cytolytic effector proteins perforin and granzyme, or inducing apoptosis via Fas ligand (FasL) (85, 86).

2.2 HUMAN CYTOMEGALOVIRUS (HCMV)

HCMV belongs to the β-herpes viruses, which are highly species-adapted pathogens. HCMV is a ubiquitous virus, with seroprevalence varying from 40-100% between different countries. Primary infections are usually mild and occur early during childhood, by transmission through breast-milk or saliva. Occasionally, HCMV can cause mononucleosis when acquired later in life, with manifestations such as high fever and long-enduring fatigue. Primary infection is followed by life-long latency, implying silent persistence without production of viral progeny, which is interrupted by phases of reactivation that allow virus shedding and transmission to new hosts. The long co-evolution of virus and host has resulted in a rather peaceful co-existence, with very little pathogenesis but continuous high viral persistence in the population. This delicate balance depends on a functioning immune system that keeps viral replication under control. If the restraint of viral load fails in individuals with a defective or compromised immunes system, such as transplant recipients or AIDS patients, HCMV can cause life-threatening disease. The virus is also the most common infectious cause of congenital defects (87).

2.2.1 HCMV pathology

The risk for transmission of HCMV to the foetus through the placenta is high (around 40%) when the mother is primary infected during pregnancy. Newborns may suffer from growth retardation, jaundice, hepatitis, blindness, hearing loss or even permanent brain damage (87). HCMV disease in transplant patients occurs either through reactivation of latent virus in the host or, in case of a seronegative recipient, through transmission via the transplant. Clinical manifestations of HCMV pathology include

retinitis, hepatitis, encephalitis, gastrointestinal ulcerations, pancreatitis and pneumonitis. The grade of diseases correlates with the degree of immunosuppression, and is most severe in AIDS patients with low $CD4^+$ T cell counts and bone marrow transplant patients (87, 88). HCMV is also associated with autoimmune diseases, where the virus may either be one of the inducing agents or an opportunistic pathogen contributing to exacerbation of pathology. Molecular mimicry has been suggested for autoimmune type 1 diabetes or systemic sclerosis, where autoantibodies were found to cross-react with HCMV proteins (88).

2.2.2 HCMV biology

The 230 kilo base pair (kbp) double stranded DNA genome of HCMV is the largest of all characterised human viruses (89). It is enclosed by capsid-proteins and further surrounded by tegument-proteins. A lipid envelope derived from the trans-Golgi network forms the outermost layer, containing both host and viral glycoproteins. In total, the virion is composed of more than 140 proteins, half of which are of host cellular origin (90). The large and complex HCMV genome allows space for many genes that are dedicated to subvert the host immune system. The corresponding proteins are termed "immuno-evasins". Of the more than 200 open reading frames (ORFs), over 50 ORFs are dispensable for replication and therefore potential immune evasion genes (87, 91). The genome is organized in unique long (UL) and unique short (US) regions. Protein expression during replication can be divided into immediate early, early and late phases. During repeated fibroblast passage, the common HCMV laboratory strain AD169 has lost approximately 5% of its genome, which encodes several immune evasion genes that are superfluous for propagation *in vitro* (92).

HCMV can infect most cell-types *in vivo*, including monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, stromal cells, fibroblasts, smooth muscle cells and neuronal cells. Latency is thought to occur in myeloid lineage cells, but may also exist in endothelial cells of different locations, such as arterial vessels. Full replication cycle is not sustained in all cells i*n vitro*. Furthermore, replication in certain cell types depends on the use of cell-type adapted viral stains (93-96). Given the wide variety of cell types infected by HCMV, it is not unexpected that universal surface molecules such as heparan-sulfate proteoglycans, integrins and growth-factor receptors, e.g. epidermal growth factor receptor (EGFR), are involved in viral entry, besides cell-type specific receptor-ligand interactions. TLR2 stimulation during entry activates innate immunity (93). During acute infection, monocytes are the main cell-type in blood infected with CMV. However, monocytes are only abortively infected. Therefore, monocytes may serve as transport vehicles, while differentiation into tissue-macrophages permits the full replication cycle (97). Latently infected DC precursor cells also reactivate HCMV upon differentiation into mature DCs (98).

2.2.3 Immune responses to HCMV

As HCMV is host specific, it is not possible to use animal models for the analysis of HCMV. However, mouse cytomegalovirus (MCMV) is a good model to study instead, since immune responses to the virus are similar to those in humans. Furthermore, many MCMV genes, even though of different genetic origin, are still similar to HCMV genes

in mechanism and function (99). For example, both HCMV and MCMV elicit large CMV-specific T cell responses (100), and both viruses encode several immune-evasion proteins that interfere with the MHC class I antigen presentation pathway (99). Studies in mice have provided a vast and detailed knowledge about the importance of specific components of the immune responses to cytomegalovirus, and have helped us to better understand the course of HCMV infection.

As for most infections, the immune response to CMV includes both innate and adaptive components, such as NK cells and T cells that release inflammatory mediators and kill infected cells, as well as B cells that produce neutralizing antibodies. The main effector cells in immune control of CMV however are NK cells as well as $CD8^+$ and $CD4^+$ T cells (86, 99-102). The first report regarding the crucial role of NK cells for the initial control of herpes viruses was published in 1989, with the description of a patient that suffered from recurrent herpes virus infections, including CMV-induced pneumonitis, due to a complete lack of NK cells (103). Another case of selective NK cell deficiency resulting in fatal varicella infection was reported recently (104). Evidence for the key role of T cells for constraining CMV infection came in the 1990s from T cell depletion studies in mice as well as from adoptive transfer experiments of CMV-specific CTLs in bone marrow transplant patients (87).

The task to keep HCMV in check is rather costly for the immune system. A comprehensive study analysing the T cell response to HCMV by overlapping peptides covering the whole HCMV genome defined that around 5% of the total peripheral blood $CD4^+$ and $CD8^+$ T cells recognize HCMV determinants (105). These numbers rise up to 10% in the memory compartment (105). Surprisingly, the specificity of HCMV-reactive T cells was found to be much broader than anticipated, covering over 70% of the total ORFs. Certain individuals respond to only 5 ORFs while others respond to up to 55 ORFs, including determinants from proteins of all viral replication stages and functions (105). In the elderly, the frequency of CMV- specific $CD8^+$ T cells increases even more, and responses tend to become oligoclonal. Consequently, it has been hypothesized that the dominance in the T cell repertoire to HCMV antigens may lead to impaired responses against other pathogens (100, 106-108). However, a high frequency of CMV-specific T cells does not necessarily enhance susceptibility to heterologous infections (109).

2.2.4 Immune Evasion Strategies

2.2.4.1 Interference with the MHC class I presentation pathway

2.2.4.1.1 Human cytomegalovirus

$CD8^+$ T cells are crucial in combating viral infections (100) and rely on the presentation of viral peptides by MHC class I molecules. Therefore, many viruses, including the majority of herpes viruses, but also other DNA or RNA viruses such as adenovirus or HIV, respectively, have developed sophisticated mechanisms to disturb the MHC class I antigen presentation pathway. I will describe here below the broad array of cytomegalovirus proteins that exert this function. For a more general overview on this topic I refer to several reviews (110-112).

An early step in the antigen processing/presentation pathway is blocked by the HCMV tegument protein pp65. pp65 induces phosphorylation of the viral immediate early antigen-1 (IE-1), which limits access of IE-1 to the antigen-processing machinery and thus the generation of peptides for MHC class I presentation (113). IE-1 is an essential viral transcription factor, which is expressed very early upon infection, at a time when no other VIPERs are functioning yet. Furthermore, HCMV expresses several glycoproteins in the US region that disrupt MHC class I expression **(Figure 4)**. The US proteins are 20-28kDa type I glycoproteins of (predicted) Ig-like fold, with a body in the ER lumen, a single transmembrane domain and a short cytoplasmic tail (114). The Ig-like fold is not apparent from the sequences of US2 proteins, because they do not possess any significant similarity to sequences of other known proteins (114). However, members of the Ig-like fold family are often very different in sequence (<10% identity), despite sharing a similar structural core (18). The crystal structure of US2 revealed an Ig-like fold composed of seven β sheets (115). The characteristic disulfide bridge, commonly found buried in the hydrophobic core of Ig-like folded proteins, is positioned between two outer strands in US2 (115). The structure of US3 is suggested to be similar to US2, based on secondary structure analysis of the US3 luminal domain using nuclear magnetic resonance (NMR) followed by tertiary structure modelling (116).

US3 belongs to the immediate-early genes. It encodes for a protein of 23kDa that was originally described to cause retention of MHC class I complexes through direct interaction with MHC class I molecules (117, 118). Recently, US3 was also found to bind to tapasin, thus preventing MHC peptide-loading (119). Yet another function of US3 is impairment of MHC class II processing through association with HLA-DR, hindering binding of the invariant chain (120). Curiously, HCMV also encodes a short isoform of US3, that does not down-modulate MHC class I expression, but rather acts as a negative regulator of full-length US3 through competition for binding to tapasin. The expression of the short isoform may be an elegant way to temporally regulate general MHC class I down-regulation, thus avoiding not only T cells but also NK cells (121).

US6 inhibits peptide loading through binding to the luminal side of TAP (122-124), thereby preventing binding of ATP to TAP and consequently preventing peptide translocation (125). Compared to the other known US genes involved in impairment of antigen processing, the effect of US10, found to delay MHC class I trafficking, seems only modest and is not well characterized yet (126).

In contrast, the impact and mechanisms of US2 (127) and US11 (128) on down-regulation of MHC class I molecules have been extensively studied. Both molecules associate directly with specific MHC class I alleles, causing rapid dislocation into the cytosol and connection to the ubiquitin-proteasome degradation pathway. Despite the same outcome, i.e. destruction of MHC class I molecules, the mechanism used by the two proteins to achieve this goal is different. US2 and US11 use distinct adaptor proteins for coupling MHC class I to ER-associated degradation (ERAD) pathways (129, 130). They have different allele specificities (131-138) and requirements on assembly, folding and ubiquitination status of the targeted MHC class I molecules.

Even though both US2 and US11 are dependent on a functional ubiquitin system, poly-ubiquitination is required for US11 mediated dislocation (139, 140), whereas the US2 induced ubiquitin pattern is of lower molecular weight (141). Furthermore, US2 can initiate retro-translocation of modified MHC class I heavy chains that lack lysine residues in the cytotosolic tail. These mutated molecules are subsequently ubiquitinated at their luminal domain in the cytosol, although ubiquitination normally occurs at lysines localized in the cytosolic tail of MHC class I molecules during US2 mediated degradation (141). A recent study clarified that US2 requires the presence of lysines, at least in the ER-luminal domain, for dislocation to the cytosol, whereas US11 can even trigger the dislocation of lysine-free MHC class I heavy chains, as long as the ubiquitin system itself is not inhibited, suggesting the involvement of additional adaptor proteins (142). US11 can mediate degradation of MHC class I molecules prior to association with β_2m, and does not require proper folding of the heavy chain, whereas the formation of a tertiary structure is a prerequisite for US2 induced destruction (141). US2 can also associate with β_2m-free heavy chains and target them for degradation, as demonstrated in β_2m-negative cells (143). In contrast, an earlier study that used a different cell line and small-interfering RNA (siRNA) for β_2m depletion, found that US2 assembles only with MHC class I molecules in complex with β_2m (144). Both the cytosolic tail and the transmembrane domain of US2 are required for its function, whereas US11 can mediate MHC class I dislocation without its cytosolic tail (145).

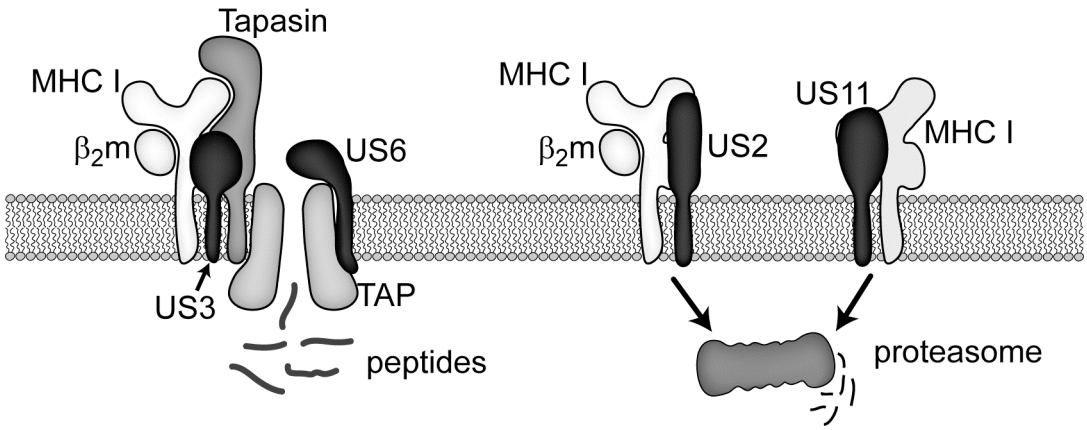

Figure 4. Interference of US proteins with antigen presentation.
US3 binds to MHC class I complexes and to tapasin, retaining MHC class I molecules in the ER and preventing interaction with the loading complex. Erp57 and calreticulin, components of the loading complex with TAP and tapasin, are omitted to preserve clarity. US6 binds to TAP, inhibiting peptide loading onto MHC class I molecules. US2 binds to folded MHC class I molecules, whereas US11 binds to MHC class I heavy chain independent of tertiary structure. Both US2 and US11 induce MHC class I transport into the cytoplasm, followed by proteasome degradation.

Similarly to US3, US2 can interfere with the MHC class II presentation pathway (146-148). Yet another target for US2 is HFE, a non-classical MHC class I molecule involved in the regulation of iron uptake (149, 150). Interestingly, CMV induced increase of cell size, i.e. the characteristic morphological change termed “cytomegaly”, is crucially dependent on iron (151). Apart from its remarkable multi-functionality,

US2 is also a unique type I membrane protein from a biochemical viewpoint, since its signal sequence is not cleaved upon proper insertion of US2 into the ER membrane (152).

Recently, a gene outside the US region has also been implicated in interference with MHC class I surface expression. The virion associated protein pp71, the product of UL82, is known to enhance viral replication by several ways, for example as a transactivator of genes and cell-cycle activation. Freshly synthesized pp71 is now suggested to also hinder transport of MHC class I molecules early between ER or cis-Golgi apparatus (153).

2.2.4.1.2 Mouse cytomegalovirus

MCMV has three genes involved in MHC class I down-modulation, that are evolutionary unrelated to the US genes of HCMV. i) *m152* encodes the glycoprotein (gp)40, which is an MHC class I homologue. This protein prevents MHC class I complex traffic beyond the cis-Golgi compartment, but also impairs NK cell recognition of the infected cell by down-regulation of RAE-1, a ligand for NKG2D. ii) m06 encodes gp48, which targets the fully assembled MHC class I complex in the ER for lysosomal degradation. iii) Finally gp34, the product of the gene *m04*, can bind to MHC class I molecules in the ER and remains associated all the way up to the cell surface ((91, 101) and references within). *m04*/gp34 has been thought to act as an inhibitor of CTL recognition, but a recent study about the concerted action of *m04*, *m06* and *m152* using different combinations of single and combined knock-out viruses, suggests that *m04* may instead act as a positive regulator of MHC class I expression (154).

2.2.4.1.3 The role of multiple immuno-evasins

In view of all these immuno-evasins impairing MHC class I presentation, or VIPRs (for viral proteins interfering with antigen presentation, pronounced "viper" (111)), one may ask the question why there is a need for several proteins exerting – at least in part – redundant or similar functions. Especially since epitopes derived from many VIPRs are also recognized by T cells, therefore enhancing immunity against the virus (99, 155). First, the existence of multiple VIPRS highlights the importance of T cells immunity in viral infection – and the need for the virus to avoid this pressure to survive. Second, one may envisage a scenario in which the host immune system overrides the effect of a certain immuno-evasin, which then in turn requires multiple cooperative approaches from the viral side to accomplish the task in question (112). Examples for host counter-measures are MHC polymorphism, allowing escape from the allele specific action of US2 or US11 proteins (138), and cell-type specific factors that also influence the efficacy of US2/US11 (137). Furthermore, escape from US6 and US3 mediated control can at least partly be achieved through tapasin-independent peptide-loading (21). The up-regulation of co-stimulatory molecules such as the MHC class I-like molecules MIC, binding not only to NK cells but also to NKG2D on T cells, is another way of compensating reduced MHC class I expression levels (91). Finally, the different

immuno-evasins collaborate: US2 and US11 can remove MHC class I complexes that are retained by US3 during the immediate early phase of infection (118).

An example for the *in vivo* relevance of VIPRs is evidence that *m152* can prevent presentation of viral epitopes which nevertheless elicit a strong $CD8^+$T cell response through cross-presentation in antigen-presenting cells (APCs). Thus, priming of T cells for epitopes that are not present on infected cells wastes host resources and is in favour of viral escape (156).

2.2.4.1.4 Viral immuno-evasins as useful tools

Many viral inhibitors are of interest beyond their role during viral infection, as they are useful tools to dissect general biological pathways (157). For instance, ICP47, the cytosolic herpes simplex virus inhibitor of TAP, and the ER- resident TAP inhibitor US6 of HCMV have been applied in investigations of cross-presentation pathways in DCs (158, 159). Furthermore, US2 and US11 are extensively studied for their exploitation of the ERAD system. Misfolded proteins are removed from the ER into the cytosol for subsequent degradation by the proteasome. The mechanism and factors involved in the dislocation procedure are just emerging. Through the study of binding partners of US11 (130), an ER-membrane protein named Derlin-1 was identified (130, 160). Surprisingly, US2-mediated dislocation is not dependent on Derlin-1, but requires the presence of signal peptide peptidase (129), supporting the idea that distinct pathways are coupled to ERAD, and exemplifying the strategic diversification of viral immune intervention. Using US2, it was demonstrated that a glycoprotein can be dislocated into the cytosol with an intact N-linked glycan, which is normally removed within the ER prior to dislocation (144).

2.2.4.2 Evasion of NK cells

2.2.4.2.1 MHC class I homologues of MCMV and HCMV

The ability to down-regulate MHC class I molecules to prevent T cell recognition requires additional strategies to also evade recognition by NK cells. Both MCMV and HCMV encode MHC class I homologues suggested to have evolved to prevent NK cell lysis of infected cells. In mice, the functions of these proteins are well defined, due to the possibility of studying their action both in cell-culture systems and *in vivo*. MCMV genes encoding proteins homologous to MHC class I molecules are *m144*, *m145*, *m152*, *m155* and *m157* (161).

The m144 protein binds β_2m but no peptides, and has been crystallized recently (161). Like MHC class I molecules, m144 is composed of three extracellular domains α1, α2 and an Ig-like folded α3 domain. The disulfide bonds in the α2 and α3 domain of m144 are also conserved compared to classical MHC class I molecules. However, the α1 domain of m144 is stabilized through an additional disulfide bond between the α1 helix and the β sheet floor (161). Interestingly, such an α1 intradomain disulfide bond occurs also in MICA (162). Peptide binding is probably impaired by the formation of a too narrow binding groove and the lack of crucial residues for the accommodation of

peptide anchor residues (161). m144 inhibits NK cell activity (163), yet the mechanism is still unknown.

m157, which is TAP and β_2m independent (164), can engage the inhibitory NK cell receptor Ly49I in a mouse strain that is susceptible for CMV. Surprisingly, m157 also binds to an activating member of the Ly49 receptors, namely Ly49H, which is expressed in the CMV-resistant mouse strain C57BL/6. In these mice, m157 triggers NK cells which is crucial for virus clearance. This was demonstrated by the introduction of a transgene for the Ly49H receptor into susceptible mice, inducing protection from the virus (91, 101, 165, 166). m157 is an excellent example for how the virus-host interaction may selectively shape the immune receptor repertoire as well as for how selective pressure of the immune system can steer expression of viral proteins. m157 helps viral immune evasion in hosts that express only inhibitory NK cell ligands recognizing m157. On the other hand, the CMV-resistant mouse strains have evolved a remedy to this viral evasion strategy through the use of an activating ligand for m157. In favour of this theory, it could be demonstrated that serial passage of MCMV through Ly49H expressing mice favoured viral immune escape mutants, resulting in loss of m157 binding to Ly49H (91, 101, 165). Most outbred mice lack the Cmv1 region, which encodes for Ly49H, and are CMV susceptible. Therefore, the expression of m157 may indeed be an advantage for the virus, working as an immune evasion strategy (167).

The aforementioned product of *m152*, gp40, interferes indirectly with NK cell activation by sequestering the NKG2D activating ligand RAE-1, in addition to retaining MHC class I complexes. Other activating ligands for NKG2D, such as MULT-1 and H60, are down-regulated by MCMV class I homologues m145 and m155 ((168) and references within).

For HCMV, two MHC class I homologues have been identified, UL18 (169) and UL142 (170). UL18 will be described in depth below. UL142 was discovered recently in clinical CMV isolates, encoded in a genomic region that is absent from common laboratory strains such as AD169 (170). UL142 is predicted to contain the extracellular α1 and α2 domains as well as a transmembrane and a cytoplasmic domain. However, the α3 domain is truncated compared to classical MHC class I molecules. Similarly to UL18, UL142 is heavily glycosylated. It confers protection from autologous NK cell lysis when expressed in fibroblasts, and causes enhanced NK susceptibility when knocked-down by siRNA in HCMV infected cells (170). One potential mechanism for the observed effect of UL142 on NK cells may be the down-regulation of the NKG2D ligand MICA (171).

2.2.4.2.2 Additional NK cell evasion strategies

Since the NK cell repertoire is polyclonal and varies between individuals, there is a need for multiple viral mechanisms to avoid NK cell recognition. Similarly to MCMV, HCMV uses several proteins to prevent NKG2D activation on NK cells and $CD8^+$ T cells. Upon stress, which can also be CMV infection, cells up-regulate various ligands for the activating receptor NKG2D, including MICA/B and ULBP1-4. The viral protein UL16 binds specifically to MICB, ULBP-1 and -2 (172) and sequesters them

intracellularly, preventing NK-cell-mediated lysis (reviewed in (101)). Certain MICA alleles can be down-regulated by UL142 as mentioned above, but also by other undefined mechanisms in AD169, which lacks the region encoding UL142 (171, 173). Interestingly, a common truncated form of MICA escapes HCMV- mediated down-modulation, which could reflect an evolutionary host response to HCMV immune intervention (171, 173). Another function attributed to UL16 is increasing resistance to cytolytic proteins (174).

HLA-E binds peptides derived from signal sequences of classical MHC class I molecules and is recognized by the inhibitory receptor CD94/NKG2A. The viral protein UL40 contains a peptide with the same motif, which can be loaded onto HLA-E in a TAP-independent way, therefore enhancing HLA-E expression despite viral mediated MHC class I down-regulation (175, 176). If this mechanism really plays a decisive role in NK cell modulation during infection is still a controversial issue (101, 177). Like many viral proteins, the viral tegument protein pp65 has distinct functions in immune modulation. Apart from its aforementioned role in preventing the generation of antigenic peptides, it can also reduce NK-mediated cell lysis through inhibition of the activating receptor NKp30 (178). The HCMV UL141 gene is found only in low-passage strains and is situated on the 15kb region that is deleted in AD169 (89). It prevents NK cell lysis by down-regulation of the constitutively expressed protein CD155, which binds to the activating receptors CD226/DNAM-1 and CD96/TACTILE (89).

It is noteworthy that two efficient NK cell evasion proteins, UL41 and UL142, have not been discovered until recently, because they are absent from widely used highly passaged HCMV laboratory strains. This exemplifies how selective pressure by the immune system influences genomic composition of the virus. Since research interest has just shifted towards clinical CMV isolates during the last few years, more data regarding specific genes or modification of genes important for immune escape will certainly emerge.

2.2.4.3 Impairment of dendritic cells

In mice, DCs are critically important for successful combat of CMV infection, and are therefore also targets for viral modulation (167). It is thus not particularly surprising that HCMV also impairs various DC functions, independently of the aforementioned inhibition of antigen presentation and NK cell recognition. This has been studied mostly in DC cultures *in vitro*, i.e. in monocyte-derived DCs or bone marrow progenitor derived Langerhans-type DCs. HCMV infection of DCs can impair expression of co-stimulatory molecules, reduce antigen presentation capacity, prevent up-regulation of the lymph-node homing receptor CCR7 and cause an alteration in cytokine production upon LPS or CD40L stimulation (179-184). Soluble CD83 released by HCMV-infected mature DCs is one of the factors responsible for the related reduction in T cell proliferation (185). Controversially, the activation of freshly isolated blood DCs is not compromised by the virus (186), which may explain why HCMV infection does not result in major immuno-suppression in healthy hosts. It may also suggest that diverse DC subtypes are not equally susceptible to functional impairment by the virus, and that evolutionary pressure may have created DC subtypes that avoid

viral modulation through specific mechanisms. On the other hand, the virus may have evolved ways to use certain DCs in favor of viral dissemination. It would be otherwise difficult to understand why CMV latency should occur in DC precursor cells (98). One way to circumvent direct suppression of antigen presentation in infected DCs is the ability of uninfected DCs to cross-present viral antigens from infected cells. Thus, effective initiation of T cell responses can still take place, allowing T cell priming for CMV proteins of all replication stages. A draw-back is that cross-primed effector T cells will not always be effective, since the corresponding antigen is not expressed in infected tissue dues to the action of VIPRs (156).

2.2.4.4 Additional immune modulators

I have already mentioned that US2 and US3 also interfere with the MHC class II presentation pathway. This is complemented by yet another strategy, namely the inhibition of the main MHC class I transcription factor in the early phase of viral infection, resulting in reduced levels of MHC class II (187). Similarly to EBV, HCMV encodes an IL-10 homolog. Despite only 27% identity to human IL-10, HCMV-derived IL-10 is nevertheless a potent functional agonist and may therefore be important in preventing immune responses, e.g. through the inhibition of antigen presentation and type I interferon production (188).

Furthermore, the US28 gene encodes a chemokine receptor homolog that binds with high affinity to several chemokines (189), and has been suggested either to function as chemokine sink or to sustain virus dissemination through direction of infected cells towards sites of inflammation (reviewed in (91)). Another mimicry of host receptors is the expression of two viral IgG Fc receptors, which may be involved in sequestration of CMV specific antibodies (91). Several HCMV strains, but not AD169, encode a potent analogue to the human chemokine CXCL1, inducing chemotaxis and effector functions of neutrophils (190). It can only be speculated that such pro-inflammatory consequences may help the virus to spread to distant body locations, by modulating trafficking of latently infected progenitor cells expressing the receptor for CXCL1 (91).

Regulation of cell-cycle progression and blocking of apoptosis are also important features of CMV infection. Four viral proteins are involved in preventing apoptosis induction, with vMIA being the most important one (91, 191). This protein prevents the release of cytochrome c in mitochondria, and is thus similar to the anti-apoptotic Bcl-2 familiy proteins in terms of functional outcome, yet acting through a different mechanism (192).

2.2.4.5 *Efficacy of immune evasion*

Considering the wide array of viral immune evasion strategies used by CMV, it is hard to understand at a first glance why the virus is so well controlled by the immuno-competent host. One explanation for this conundrum is that the effectiveness of immuno-evasins is not 100%, and not equally successful in different cell-types (99). Furthermore, effective T cell surveillance is possible through recognition of antigens that are processed prior to the expression of immune evasion genes. This applies to the tegument protein pp65, encoding for a major antigenic peptide (99), which is cross-

presented to T cells by dendritic cells (193, 194). Additionally, the evolutionary pressure on the immune system has led to the development of host counter-mechanisms, such as up-regulation of MHC class I molecules by IFNγ, which override or at least partly compensate the actions of viral evasion proteins (91, 195). Other examples of host adaptation have already been mentioned (see the chapter entitled 'The need for multiple VIPRs'), such as the expression of MHC class I alleles that are less dependent on the peptide-loading complex (21, 112). It is therefore likely that the viral strategies to escape immune recognition are important to ensure that reactivation from the latent stage can occur at all in healthy individuals. In other words, immune evasion proteins provide the virus with a certain window in which enough virus progeny is produced, allowing transmission to a new host before replication is terminated by immune responses (99).

2.3 THE VIRAL MHC CLASS I HOMOLOGUE UL18 AND ITS RECEPTOR LIR-1

2.3.1 Characterisation of UL18

In contrast to the recently described UL142, UL18 is an extensively studied MHC class I homologue, discovered already in 1988 by Beck and Barrell during genomic analysis of the AD169 sequence (169). So far, all analysed clinical CMV isolates have retained the gene for UL18 (170), which reflects its importance for the virus. UL18 is proposed to be involved in immune escape, since it is not needed for viral replication *in vitro* (196). However, the exact function of UL18 in HCMV infection still remains an enigma. Several attempts to shed light on this issue, including our own studies, will be described below.

UL18 is a 348-residue type-I membrane glycoprotein (119) which associates with β_2m (197) and, unlike m144 or UL142, can bind endogenous peptides (198). This suggests a remarkable structural resemblance to MHC class I complexes, despite the fairly low sequence identity: the proposed extracellular domains of UL18, corresponding to the α1, α2 and α3 regions, share only approximately 21% sequence identity with MHC class I molecules (169). Another difference to MHC class I proteins is the high degree of glycosylation. The UL18 sequence encodes thirteen potential N-linked glycosylation sites (169) versus one single glycan attached to asparagine residue N86 in MHC class I molecules (12). It is noteworthy that HCMV infection induces several glycosyltransferases (199), suggesting that the virus maybe able to alter the glycosylation system of the host. The large shell of carbohydrates is not unique for UL18, since the recently identified MHC class I homologue UL142 is also heavily glycosylated (with 17 potential N-linked glycosylation sites (170)). The MCMV MHC class I homologue m144 contains four N-linked carbohydrate addition sites in the α1 and α2 region, compared to mostly two in classical murine MHC class I molecules (161)**.** To my knowledge, reasons for the relatively higher glycosylation of the HCMV homologues have not been explored yet. One may speculate that it could be a way to protect the viral proteins from degradation, or act as steric hindrance for interaction with MHC class I receptors or co-receptors. Furthermore, the sugars could be implicated in binding to lectin-like receptors. In addition, the carbohydrates in U18

have been proposed to prevent recognition by viral US proteins that down-regulated MHC class I molecules but not UL18 (200).

2.3.2 Peptide binding to UL18

The peptides eluted from UL18 are similar to those bound by MHC class I molecules, and are derived from proteins degraded in the cytoplasm (198). In the absence of peptides, the stability of UL18 is significantly reduced (201, 202). The four conserved tyrosine residues that make hydrogen bond interactions with the N-terminus of the peptide in the so-called A pocket of the peptide-binding groove are also present in UL18 (201). The other end of the groove, interacting with the C-terminus of the peptide, is suggested to be different in UL18 compared to MHC class I molecules, explaining why UL18 binds also peptides of more than 9 residues in lenght (198, 201).

UL18 has been proposed to bind peptides both in a TAP-dependent (200) and -independent manner (203). Griffin *et al.* expressed UL18 via a recombinant adenovirus vector in a TAP-deficient fibroblasts line. UL18 was detected on the cell surface, yet in lower amounts than in fibroblasts with intact TAP. Park *et al.* used a recombinant vaccinia virus (rVV) for UL18 expression, which was significantly reduced upon TAP blockade by ICP47. The data obtained in these two studies are not contradictory, although the interpretation varies. Both studies detected lower levels of surface UL18 when TAP-dependent peptides were not available. This indicates that UL18 is able to utilize different peptide loading mechanisms, even though the TAP dependent pathway results in higher expression levels. Interestingly, UL18 expression can only be reduced by the herpes simplex virus inhibitor of TAP, ICP47, and not by the "brother" molecule US6 (200, 203). Obviously, cytomegalovirus has evolved strategies to ensure proper peptide loading on UL18, maybe through competition for TAP binding between UL18 and US6 (200).

2.3.3 UL18 expression using *in vitro* systems

The complete protein composition of the HCMV virion, the "HCMV proteome", has been determined (90), but UL18 was not among the 71 identified HCMV-encoded virion proteins. Therefore, UL18 should exert its functions in the infected cell.

Two differently glycosylated forms of UL18 can be detected in HCMV infected cells or when expressed by different vectors (203). A 67kDa form is short-lived and susceptible to endoglycosidase H (200, 203). It is thus glycosylated in the ER upon synthesis, but the carbohydrates are not further modified in the Golgi apparatus. However, the 67kDa form can be found at the cell surface, which was demonstrated by immunoprecipitation of biotinylated surface proteins of cells infected with rVV–UL18 (200) or recombinant adenovirus-UL18 (203). The other form of UL18, probably a processed and more stable form derived from the 67kDa protein, is even more glycosylated. It has a molecular weight of approximately 160kDa in HCMV infected cells, and appears later during infection, from 72h onwards (203). The 160kDa form is also found on the cell surface, but is endoglycosidase-H resistant (203). Removal of all glycosylation reduces the protein to a molecular weight of 35kDa (203), illustrating the contribution of carbohydrates attached to this protein.

UL18 mRNA is low abundant and appears late during infection (200, 204, 205). Due to difficulties in detecting the low levels of UL18 protein on the cell surface of infected cells (203, 206, 207), UL18 has been mostly studied in transfected cells (207, 208) or expressed by recombinant (retro-) viruses (200, 203). Detection of surface-expressed UL18 in HCMV-infected cells is further complicated by confounding antibody interactions with virus-encoded Fc receptors (203, 206). Another contributing factor to difficulties in detection may be low immunogenicity due to the shading effect of glycans. The use of different UL18 expression systems as an alternative to HCMV infection are not flawless either: it appears to be impossible to create long-term stable cell lines for UL18 (207-209) and recombinant viruses have confounding effects on cell metabolism and glycosylation machinery. In addition, any indirect effects of UL18 mediated through interaction with other CMV proteins can not be detected in these systems.

2.3.4 Characterization of LIR-1

While searching for a cell surface expressed receptor for UL18, Cosman *et al.* discovered a novel molecule, which was termed leukocyte immunoglobulin-like receptor -1 (LIR-1) (210). The discovery was probably facilitated by the extraordinary high affinity of UL18 to LIR-1, which is more than 1000-fold compared to host MHC ligands (202). Subsequently, LIR-1 was characterized as an inhibitory receptor recognizing a broad range of classical (HLA-A, -B, -C) (202, 210, 211) as well as non-classical (HLA-E, -F, -G) (210, 212-214) MHC class I molecules. The reason for this remarkable ligand diversity lies in the nature of the LIR-1 binding template, which was determined by the crystal structure of HLA-A2 in complex with LIR-1 domains D1 and D2 (215). LIR-1 recognizes the α3 domain of the MHC class I heavy chain, which is the least polymorphic domain between different alleles, and most importantly, the invariant β_2m contributes 70% to the total interaction surface (215). The tip of the LIR-1 domain D1 contacts six residues in the α3 domain of HLA-2, while the D1 and D2 regions make contact with fourteen residues of β_2m (215) (see **Figure 9** in the chapter 'Similarities in LIR-1 recognition between UL18 an MHC class I' further below).

LIR-1 is expressed predominantly on monocytes, macrophages, DCs and B cells, but is also found on the surfaces of a subset of NK and T cells (211). It is now known that this receptor belongs to a family of leukocyte Ig-like receptors (LIR or LILR), which contain between two and four extracellular domains (216). Some LIR receptors express ITIMs in their cytoplasmatic tail, while other members associated with ITAM containing adaptor molecules (216, 217). LIR-1 is the most broadly expressed member of this family. LIR-1 and LIR-2, both inhibitory MHC class I receptors, are best characterized (218). The systematic denomination assigned to LIR-1 is LILRB1 (leukocyte immunoglobulin-like receptor, subfamily B, 1) (218), other names used in the literature are ILT2 (immunoglobulin-like transcript 2) and CD85j.

2.3.5 LIR-1 signalling and function

LIR-1 is a cell surface expressed type-1 transmembrane protein with four extracellular domains and four ITIMs in its cytoplasmic tail (219). The co-ligation of LIR-1 with

activating receptors causes suppression of early events in the activating signalling cascade and the inhibition of effector functions (211, 220). However, LIR-1 signalling does not depend on co-ligation with activating receptors, because cross-linking of LIR-1 alone is sufficient to induce signalling (221). Upon ligand binding, ITIMs in the cytoplasmic tail of LIR-1 become phosphorylated by Src kinases and recruit tyrosine phosphatases such as Src homology domain 2-containing tyrosine phosphatase (SHP)-1, involved in negative signalling (211, 219, 221, 222). The molecular pathway of LIR-1 mediated inhibition was first studied in detail in monocytes, where cross-linking of FcγR1 and LIR-1 recruits phosphatases, which in turn impair tyrosine phosphorylation of the γ chain and of Syk kinase, preventing Ca^{2+} mobilisation (222) (**Figure 5)**.

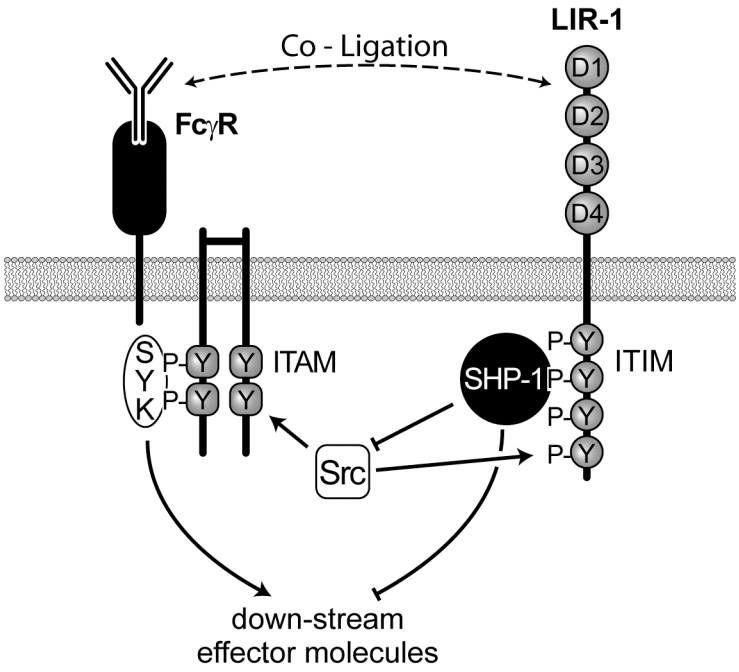

Figure 5. Negative regulation through LIR-1 signalling.
LIR-1 can interfere with activating signalling pathways downstream of ITAM- coupled receptors (exemplified by the Fc-receptor common gamma chain (FcγR)). Src- family kinases phosphorylate tyrosine residues within the ITAM or ITIM motifs upon receptor activation. Dual-phosphorylated ITAMs recruit members of the Syk protein tyrosine kinase family, initiating a signalling cascade that leads to cellular activation. Phosphorylated ITIMs of LIR-1 recruit the phosphatase SHP-1, which inhibits Src and decreases tyrosine phosphorylation of activating effector molecules. Modified from (234, 235).

Cross-linking of LIR-1 via antibodies on the surface of DCs suppresses Ca^{2+} mobilization, cytokine production and induction of antigen specific T cell proliferation (223). In T cells, LIR-1 signalling inhibits antigen specific proliferation, cytokine production and cytotoxicity (224-226). LIR-1 can compete with CD8 for MHC class I binding, and may serve as an inhibiting co-receptor during TCR-MHC interactions (214, 215). Co-ligation of LIR-1 with the B cell receptor prevents Ca^{2+} mobilization, cytokine production and isotype switching (211, 227). For primary NK cells, the LIR-

1/MHC class I interaction is too weak to inhibit lysis in the absence of other inhibitory receptors ((228) and references within, (229)), with the exception of HLA-G. The LIR-1/HLA-G interaction is stronger compared to other MHC class I molecules, although conflicting data from biochemical binding assays have been reported (202, 214). The ability of HLA-G to form disulfide-linked oligomers at the cell surface enhances avidity to LIR-1 which could explain the potency of LIR-1 mediated inhibition (230-232). The potential of HLA-G to inhibit NK cell plays a role during pregnancy, where HLA-G is expressed on the fetal trophoblast, and LIR-1 is highly expressed on surrounding maternal NK cells ((218) and references within). Elsewhere in the body, LIR-1 on NK cells may be important for fine tuning of responses through co-operation with KIR-signalling (228). A recent report described that LIR-2 co-localizes with MHC class I molecules on a human basophilic cell line, interacting in *cis* with MHC class I ligands, i.e. on the same cell (233). Similarly, the mouse orthologue of LIR-2, the paired Ig-like receptor (PIR)-B, was found to bind in *cis* to MHC class I ligands (233). This interaction regulates allergic responses on mouse mast cells. The importance of *cis* interactions for LIR-1 has not been investigated yet.

2.3.6 UL18 binding to LIR-1

Glycosylation of UL18 is probably not involved in binding to LIR-1, since an insect cell produced UL18 with modified shortened carbohydrates binds equally well to LIR-1 as UL18 produced in mammalian cells (202). Furthermore, when the glycosylation sites of UL18 are mapped onto the solved structure of HLA-A2 in complex with LIR-1 (domains D1D2), no glycans are situated in the LIR-1 contact site in the α3 domain (215). Peptide binding to UL18 does not either influence binding to LIR-1 according to Chapman and colleagues (202).

UL18 proteins from several clinical isolates differ in their binding affinity to LIR-1 when compared to AD169-UL18 (236, 237). The underlying variability of UL18 genes, with up to 20 amino acids deviation, is higher than that of other immune evasions ((237) and references within). Sequence variation is not only conferred to the α3 domain of UL18, the postulated main LIR-1 binding site (202, 215, 238), but mutations are also found in the α1 domain and scattered over the entire α2 domain (237). We could correlate certain variations within and outside the proposed LIR-1 binding site with alterations in binding strength to LIR-1 (**paper II**), as discussed below. It should be noted that UL18 proteins of AD169 and clinical CMV isolates bind solely to LIR-1, and not to other members the LIR-family (237). Using surface plasmon resonance binding assays, UL18 was found to bind to LIR-2 as well, yet with weak affinity (217, 238). However, UL18 may bind to a yet undefined receptor, and genetic mutations could provide an advantage for the virus by modification of the binding strength to this unknown molecule. If such a molecule was expressed as an activating receptor on NK or T cells, it could explain several findings where UL18 stimulated immune responses (206, 207, 239) (**paper IV**). Alternatively, mutations in the α2 domain may alter binding of peptides, modifying stability of the complex or its interaction with undefined receptors.

2.3.7 The role of UL18 in immune responses

Following the discovery of UL18 and prior to the identification of the US proteins accountable for this task, UL18 was originally proposed that this viral protein was responsible for MHC class I down-regulation through sequestration of β_2m (197). However, cells infected with a deletion virus lacking the UL18 gene (dUL18) still had reduced MHC class I expression levels (196). In contrast, the deletion of US2-US11 restored normal MHC class I expression in AD169, which argues against a contributing role of UL18 in interference with MHC class I expression (240). For several years, UL18 was then assumed to act as a decoy molecule for NK cells. Yet also these results were subject to controversy. Reyburn *et al.* found that protection from NK cell lysis could be conferred by transfection of UL18 and β_2m into the class I negative cell line 721.221 (208), whereas Leong *et al.* came to the opposite conclusion, that UL18 actually enhanced NK cell killing (207). When endothelial cells and macrophages were infected with AD169 or dUL18, no inhibitory effect of UL18 on NK cells could be detected (241), yet another study using UL18 transfected target cells in a xeno-situation supported an inhibitory role (209). These investigations did not take into account the expression of LIR-1 on NK cells. As already stated above, LIR-1 is expressed only on a subset of NK cells (242) but is also found on many other kinds of immune cells (211). During the preparation of this thesis, the effect of UL18 on NK cell lysis and degranulation in the context of LIR-1 expression was finally assessed. Using dUL18/AD169 infection and expression of UL18 by a replication deficient adenovirus vector, UL18 was demonstrated to inhibit LIR-1$^+$ NK cells but suggested to activate LIR-1$^-$ NK cells (206). The outcome of the UL18/NK interaction depends thus on the prevalence of LIR-1. In a polyclonal setting, the ratio of LIR-1 to other unknown activating receptors determines the net result. UL18 encounter may either lead to inhibition of NK cells or activation. It may also have no effect at all, if inhibitory signals are equalled by activating stimuli.

Although research regarding the function of UL18 has mainly focused on NK cells, the wide distribution of LIR-1 suggests that other cells may be a target for UL18 interference with immune functions (91, 101, 163). Therefore, we initiated the study of potential effects of UL18 on DCs (**paper III**). Saverino and colleagues postulated that UL18 could activate CD8$^+$T cells in a LIR-1 dependent fashion, yet no mechanism was provided (239). Our own results also favour an activating role for UL18, however independent of a potential LIR-1 interaction (**paper IV**).

3 RESULTS AND DISCUSSION

In the following section, I will discuss the results presented in papers I-IV in the context of the current literature. For details and original figures I refer to the articles included at the end of this thesis. For paper I, the experimental procedure will be mentioned here as well, since the read-out is not commonly used. The other methodological approach that I explicitly want to describe is the production of UL18-Fc fusion proteins, which were used in paper II-IV.

3.1 US2 AND MHC CLASS I DOWN-MODULATION

3.1.1 Study background

US2 is expressed from early to late phases during infection (243). It is of Ig-like fold and is composed of 199 amino acids (114, 115). It has a short non-cleaved signal sequence of 20 amino acids, one transmembrane domain (residues 163-185) as well as a 14 amino acid cytoplasmic tail (**Figure 6**) (138, 148). As described in the chapter 'Interference with antigen presentation', this protein plays an important role in MHC class I degradation. Interestingly, it is highly allele specific, and at the beginning of our study, literature regarding this topic was rather limited. Trophoblast-expressed MHC class I molecules HLA-C and HLA-G were thought to be US2 resistant (244). The HLA-A and HLA-B locus was proposed to be generally susceptible to US2 mediated down-modulation (138, 245), although the recombinant luminal domain of US2 could only bind to HLA-A2 or HLA-Aw68, but not to HLA-B7 or -B27 (138). The crystal structure of HLA-A2/US2 covered only around 50% of the full-length US2, with truncations both at the N- and C-termini (115) (**Figure 6**). Based on this novel structural information, we initiated the investigation of the molecular mechanism underlying US2 binding to HLA-A2 (**paper I**).

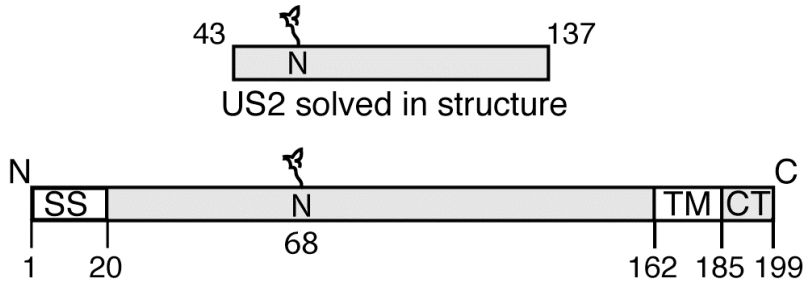

Figure 6. Schematic outline of US2.
The full-length 199 amino acid-long protein is depicted, composed of a non-cleaved signal sequence (SS), an ER luminal domain, a transmembrane domain (TM) and a short cytoplasmic tail (CT). A glycan is attached to arginine residue N68. The regions of US2 that have been structurally determined are sketched on top. Modified from (148).

3.1.2 Binding of US2 to HLA-A2

A preliminary analysis of the HLA-A2/US2 crystal structure suggested two contact sites (115). Site 1 is localized at the junction of the peptide binding cleft and the α3 domain **(Figure 7)** and covers a larger area (1320 Å), whereas site 2 comprises 697Å in the α2 domain. Gewurz and colleagues proposed site 2 to be an artefact of crystal packing, based on three mutations introduced in HLA-A2. Replacement of arginine residue R181 in site 1 by glutamic acid (E) significantly reduced the appearance of a cytoplasmic deglycosylated intermediate of HLA-A2, whereas two mutations localized in site 2 could not prevent US2 induced destabilization of HLA-A2 heavy chains in a pulse-chase experiment (115).

US2 binding site 1 is localized opposite the proposed binding region for the peptide-loading complex, potentially allowing US2 access to MHC class I complexes before and during association of MHC class I with the loading complex (115). Theoretically, the N-linked glycan at US2 residue 68 would not prevent association with the MHC class I heavy chain at either site (115). We decided to include US2 binding site 2 in our investigations, even though the ratio of US2 to HLA-A2 in solution was reported to be 1:1 (138). The ER is a crowded environment, where other chaperons besides proteins of the loading complex may temporarily mask site 1 and make it unavailable for US2. Site 2 could thus have been an alternative docking site, even though probably less efficient. Another possibility was that site 1 and site 2 were available for interactions at different times during synthesis. However, our thorough investigation using nine mutations in and surrounding the potential binding site 2 excluded this region as US2 interaction site, confirming the results by Gewurz *et al.* (**paper I**, (115)).

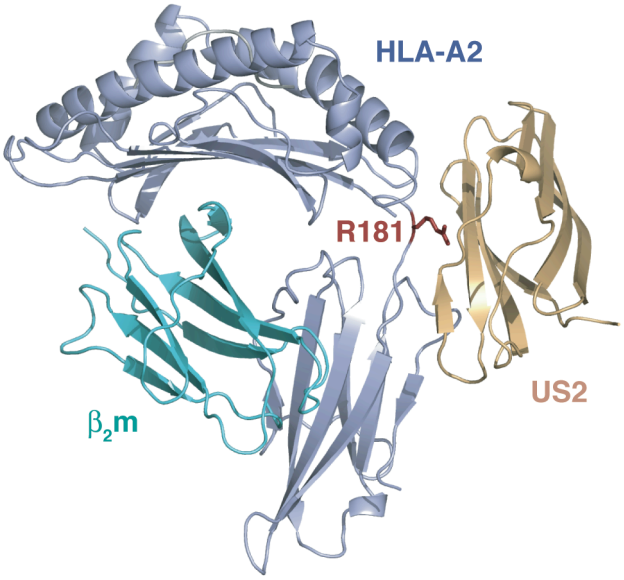

Figure 7. Overall structure of the HLA-A2/US2 complex.
The heavy chain of HLA-A2 is coloured blue, β2m aquamarine and US2 orange. Arginine residue R181, an anchor residue for US2 in HLA-A2, is highlighted red. The side chain of R181 is sketched as sticks.

3.1.3 Methodological Approach

In contrast to the biochemical approach by Gewurz and colleagues, we opted for a surface based read-out system in **paper I**, since surface expression of MHC class I complexes determines physiological outcome. Intracellular degradation events may not always reflect quantitatively the situation on the cell surface. Furthermore, if certain residues contribute differentially to the binding interaction between US2 and HLA-A2, small differences may be picked up easier through flow cytometry analysis of surface expressed HLA-A2. We studied the relative importance of individual HLA-A2 residues for US2 binding using 18 HLA-A2 variants expressed stably in J26 mouse fibroblasts (that produce human β_2m). Full-length US2 was expressed in the A2-transfected cell lines using rVV-US2. The advantages using rVV infection are the almost 100% infection rate and the high levels of protein production. However, a potential disadvantage is the relatively short time-window for analysis, determined by virus induced shut-off of host proteins (246). The residual MHC class I expression, consisting of molecules that reach the surface before US2 expression, could thus mask the effect of US2 on newly synthesized MHC class heavy chains and prevent a clear read-out. Therefore, to enhance resolution, pre-existing cell-surface MHC class I complexes were removed two hours post infection by acid treatment, upon reaching maximal levels of US2. This method provided us with a high sensitivity read-out system to assess the effect of the introduced mutations in HLA-A2 on US2 mediated down-modulation.

3.1.4 Interaction of US2 with HLA-A2

Each of the HLA-A2 residues predicted to form hydrogen bonds with US2 were mutated in our study (**paper I)**. Additionally, residues that differ between HLA-A2 and other HLA molecules that are not down-regulated by US2 were also substituted. HLA-A2 regions adjacent to the US2 contact sites could be important for binding to full-length US2 and were therefore included in the analysis. Our findings that the sole modification of HLA-A2 arginine residue R181 in US2 binding site 1 completely prevented US2 down-modulation, whereas none of the other introduced substitutions had any measurable effect at all was rather surprising. Apparently, this arginine residue **(Figure 7)** is used as an anchor position establishing five hydrogen bonds with US2 residues that form a narrow pocket, in which the side-chain of R181 fits well. The induced conformational change of the side chain of R181 upon binding to US2 (a rotation by almost 180°) compared to its conformation in most published HLA-A2 structures (pointing towards the core of the α3-domain) emphasizes the importance of this residue in the US2/HLA-A2 interaction. The pivotal role of R181 was confirmed in a subsequent study, in which introduction of R181 into the US2 resistant HLA-E rendered this molecule susceptible to down-regulation by US2 (134). HLA-E is one of the very few HLA molecules that expresses a histidine residue at position 181 instead of an arginine.

3.1.5 Allele specificity of US2

R181 is found in almost all MHC class I molecules, i.e. also in US2 resistant alleles. Therefore, the presence of this residue cannot explain the allele specificity of US2,

despite its crucial role for US2 binding to HLA-A2. Specific residues surrounding R181 presumably interfere with US2 binding to site 1 in MHC class I molecules that escape down-modulation. However, single or combined substitutions of candidate residues in site 1 did not have any effect on HLA-A2 down-regulation **(paper I)**. In contrast, the amino acid composition of the entire binding site 1 is a major factor for US2 mediated down-modulation since simultaneous exchange of multiple residues can convert a US2 resistant allele into one that is US2 susceptible, and vice versa (134). Other regions not present in the crystal structure may contribute to binding and influence overall affinity. Observations supporting this hypothesis are the results by Chevalier *et al.*, determining that domains of US2 that are not included in the crystal structure, such as US2 residues 140-160, are important for efficient binding to MHC class I molecules (148). These potential additional contact regions should be present within the ER-luminal domain of MHC class I molecules, which is solely responsible for US2 interaction (134).

In the course of our investigations, new data regarding the susceptibility or resistance of specific MHC alleles to US2 was published, with partially conflicting results. Llano and colleagues demonstrated that certain HLA-C allotypes (HLA-Cw7) were down-regulated by US2 (136), which stood in contrasted to results of an earlier report (244). Using a flow-cytometry based read-out system, Barel *et al*. demonstrated that HLA-G is also US2 susceptible (135), in contradiction to earlier results based on intracellular pulse chase experiments (244). Furthermore, HLA-B27 expression levels were reduced by US2 (131), in line with our findings (**paper I**). Apparently, full-length HLA-B27 can be targeted by US2 whereas soluble recombinant HLA-B27 cannot, despite presence of binding site 1 (138). In contrast, recombinant soluble HLA-A2 can be bound and down-regulated by US2 (138). The affinity of the interaction between US2 and HLA-A2 in binding site 1 may thus be stronger than between US2 and HLA-B molecules in the same region. In addition, US2 binding to HLA-A2 may depend less on other undefined US2 contact regions that are absent in soluble molecules. The magnitude of US2 mediated down-regulation of full length HLA-B27 is less striking compared to HLA-A2 down-regulation, supporting the view that overall binding affinity to US2 may vary between MHC class I molecules (131, 134). Based on a sequence comparison of MHC class I alleles, Barel *et al.* provided some general predictions regarding alleles that are likely to be down-regulated by US2 (131). For instance, HLA-B8 was predicted to be down-regulated by US2, as experimentally proven by our results (**paper I**). All HLA-A and HLA-G molecules as well as most HLA-B molecules were proposed to be down-regulated by US2 (131). However, HLA-C alleles were suggested to be generally US2-resistant (131), which stands in contrast to the finding that HLA-Cw7 can be down-regulated (136). The comprehensive analysis provided by the studies of Barel *et al* does not either identify the residues that define US2 susceptibility. Certain residues mentioned as typical for US2 down-regulated MHC molecules were demonstrated by our mutational study to not alter US2 mediated degradation of HLA-A2 (**paper I**).

So far, no study has been able to specify the exact molecular mechanism for how certain MHC class I molecules escape US2 while others do not. It is possible that no universal molecular signature exists that allows a general prediction on US2 susceptibility. Instead, elements that fine-tune US2 binding and ultimately define the

outcome of the interaction may be rather unique for different alleles, rendering predictions through sequence comparison difficult if not impossible. Other approaches, such as systematic region exchange experiments or random mutagenesis, defining unknown contact sites for full-length US2 in full-length HLA molecules, may provide a clearer answer to that question.

Concerning the mentioned contradictory results obtained for some MHC class I molecules in relation to US2 susceptibility, it is important to keep in mind that experimental set-ups, including MHC class I and US2 expression levels, cell type, read-out system and other factors can greatly influence the outcome of results. Therefore, a careful validation of the chosen assay system, the comparison of results obtained by different methods as well as repetition by independent laboratories are all important factors for reaching clear and unequivocal conclusions.

3.2 HIGH AFFINITY BINDING OF UL18 TO LIR-1

3.2.1 Production of UL18-Fc fusion proteins

To study the interaction of LIR-1 and UL18 under conditions close to physiological situations, we initiated the production of UL18 proteins in mammalian cells (293FT) ensuring proper post-translational modifications. For purification purposes, soluble fusion proteins, consisting of the extracellular domains of UL18 (α1-α3) connected to the Fc-portion of human IgG1 were used (210). In the following paragraph, the general procedure of UL18-Fc production will be outlined, with emphasis on major obstacles and corresponding solutions. For more experimental details I refer to the material and method section of **paper II**.

Since previous attempts to create long term stable cell lines expressing UL18 were unsuccessful (207-209), and since establishment of multiple stable cell lines is time-consuming, we used a transient transfection system (210, 247). Optimizations included testing of different cell lines, transfection reagents, plastic materials and fine-tuning of the transfection method. Soluble UL18-Fc proteins were collected from the cell supernatant, isolated over a protein A column and further purified through size exclusion chromatography. The latter purification step is important since it allows separation of dimers from multimers, aggregates and residual serum contaminants. Initially, up to one third of recovered protein could consist of contaminating serum Ig, depending on transfection efficiency. One remarkable enhancement of UL18-Fc yields was the usage of a commercially available serum-free medium formulation, allowing cell culture under drastically reduced serum conditions (1.8% instead of 10%). The other major breakthrough was the addition of recombinant β_2m after acid elution from the protein A column, ensuring proper complex formation and equal β_2m content in all UL18-Fc preparations. Additionally, eluates were supplemented with a UL18-binding actin-derived peptide (198, 202). In the CHO hamster cell line, only around 40% of UL18 complexes associate with endogenous peptides (201). Even though mammalian 293FT cells may provide a more suitable peptide repertoire, the addition of synthetic peptide in surplus ensured that all UL18 complexes were homogenously filled with adequate peptide. The entire process is illustrated in **Figure 8**.

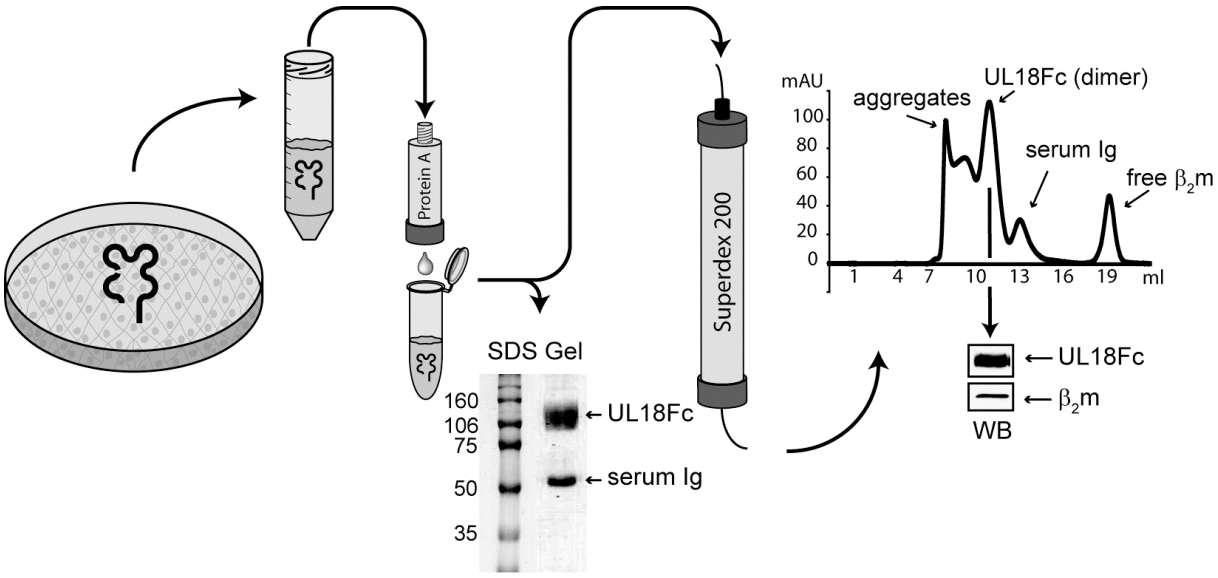

Figure 8. Production of UL18Fc fusion proteins.
UL18Fc proteins are harvested from transfected 293FT cells cultured in 10cm culture dishes and purified on a protein A column, followed by size exclusion on a superdex 200 column. Eluates from the protein A column, containing UL18Fc proteins as well as serum Ig (and β_2m, not shown), are visualized on an SDS gel. The broadness of the UL18Fc band is typical for highly glycosylated proteins. Size-exclusion fractions containing UL18Fc dimers in complex with β_2m are tested by Western Blot.

3.2.2 Molecular modelling of UL18

The UL18 structure has not been determined yet. Therefore, a three-dimensional model of UL18 was created (236) **(Figure 9)**, based on the sequence homology and the predicted secondary structure similarity to MHC class I molecules (201). The findings that UL18 can bind β_2m and peptides of similar composition to those binding classical MHC class I molecules further support the likelihood of a tertiary structure very similar to MHC class I proteins (197, 198). The crystal structure of HLA-Cw4 was used as a template for the UL18 heavy chain since this MHC displayed the highest sequence homology to UL18. Additional models of UL18 were created using the crystal structures of four other MHC class I molecules belonging to the HLA-A and HLA-B groups (data not shown). All created models were very similar to each other, which was not surprising due to the high structural resemblance of the entire MHC class I family. Comparison of the molecular model of UL18 with a classical MHC class I molecule revealed that their overall fold was indeed very similar **(paper II)** (236). However, specific gaps and insertions due differences between the sequences of UL18 and the MHC class I templates resulted in structural variations in well-defined regions **(Figure 9)**, which may reflect functional differences.

The molecular model of UL18 suggested that two loops in the α1 domain formed by residues 13-19 and 36-44 were longer than the corresponding loops in MHC class I

complexes. The model also indicated that a large additional loop (residues 153-164) was inserted between the two helixes of the α2 domain. One may speculate that modifications in the upper boundary of the peptide binding cleft could prevent/alter TCR or KIR recognition. Furthermore, the second helix of the α2 domain (residues 141 to 152) as well as the connecting section between the α2 and the α3 domain were suggested to be shorter in UL18. Importantly, the molecular model revealed that composition and conformation of the UL18 region corresponding to the US2 contact site in HLA-A2 was different. This provides a molecular explanation for how UL18 escapes its brother molecule. A loop (residues 228-235) connecting the two β-sandwiches of the α3 domain was proposed to be shorter in UL18 than in MHC class I molecules. An additional disulfide bond in the α3 domain of UL18 (C240-C255) not present in MHC class I molecules is localized in that very region and was demonstrated to be crucial for association with β_2m **(paper II** and discussion below**)**.

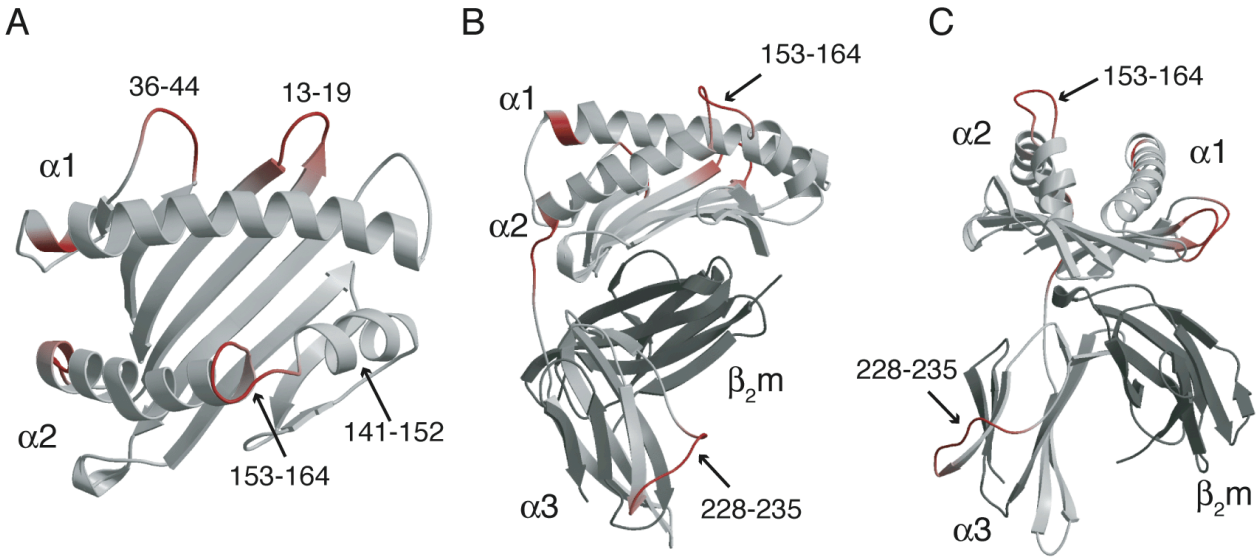

Figure 9. The model of UL18.
The UL18 heavy chain and β_2m are coloured light and dark grey, respectively. Regions that differ from MHC class I molecules as described in the text are shown in red. (A) View from above on the peptide binding cleft. (B) and (C) view on all three UL18 domains in complex with β_2m from different angles.

It should be noted that although the β_2m subunit was added in the model in a spatial positioning vis-à-vis the heavy chain of UL18 similar to the position found in classical MHC class I molecules, this may not reflect its exact positioning in the UL18 complex. Only future determination of the UL18/β_2m complex will provide an answer to this question.

3.2.3 Similarities between UL18 and MHC class I in LIR-1 recognition

Comparisons with structural analysis of MHC class I binding to LIR-1 combined with mutational approaches have been used to determine regions involved in the UL18/LIR-1 interaction. Using domain-swapping experiments with the non-classical MHC class I

molecule HFE which does not bind to LIR-1, the group of Pamela Björkman established that the LIR-1 domain D1 recognizes the α3 domain of both UL18 and MHC class I molecules (202). The low affinity of MHC class I molecules to LIR-1 compared to UL18 was reflected in experiments using LIR-1 proteins for immunohistochemical stainings. Monomeric LIR-1 proteins could label UL18 transfected cells, but failed to bind HLA-B transfected cells. HLA-B expression could only be detected using bivalent LIR-1Fc fusion proteins probably due to an increase in avidity (202). LIR-1 residues involved in binding to UL18 were identified through site-directed mutagenesis studies, based on the comparison of the sequences of LIR-1 and LIR-2 (238). The crystal structure of HLA-A2 in complex with LIR-1 domains D1D2 revealed two contact sites (215). Residues at the tip of the D1 domain contact six residues in the α3 domain of HLA-A2 **(Table I)**, while residues mostly localized in the D1D2 hinge region interact with β_2m (215) **(Figure 10)**. Although 70% of the contact surface area is formed with β_2m, the α3 domain is suggested to make essential energetic contributions to the total binding energy (215). Our mutagenesis study in UL18 that demonstrated the involvement of UL18 residue Q202 in binding to LIR-1 corroborates the importance of heavy chain residues for the interaction with this receptor (**paper II**). Surprisingly, LIR-1 can bind to H-2D^b in complex with mouse β_2m, whereas it does not bind to H-2D^k or H-2K^k complexes, which may support a critical role for the composition of the α3 domain (248).

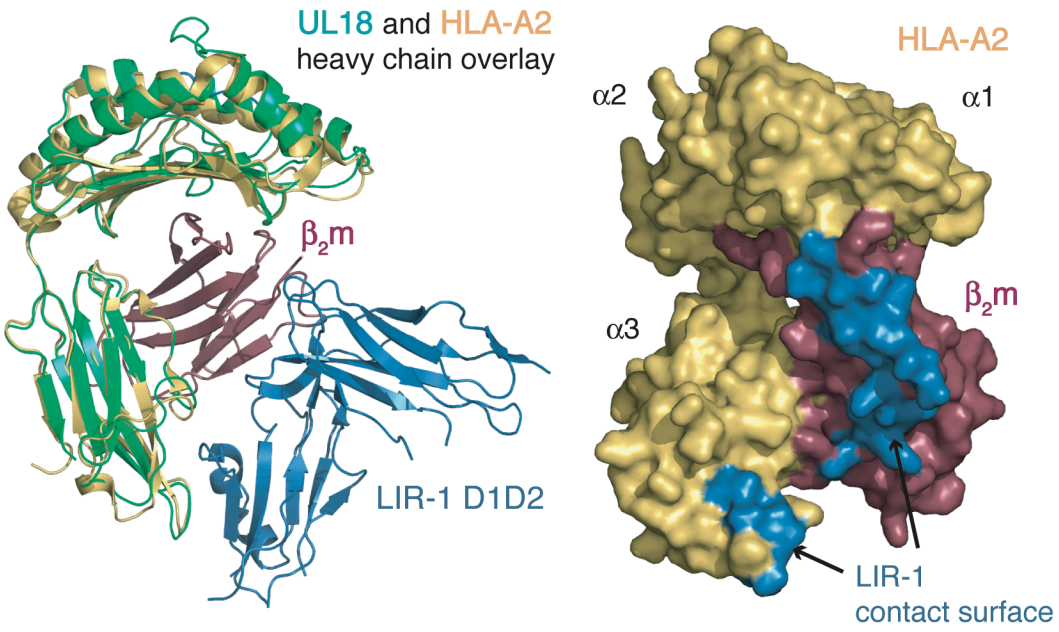

Figure 10. Overall structure of the LIR-1/HLA-A2 complex.
(Left) The LIR-1 domains D1 and D2, the HLA-A2 heavy chain and the β_2m subunit are in blue, yellow and raspberry, respectively. A model of the UL18 heavy chain, coloured green, is superposed on HLA-A2. (Right) The contact surfaces for LIR-1 in the α3 domain of HLA-A2 (yellow) and the β_2m subunit (raspberry) are visualized in blue.

The LIR-1 residues Y38 and Y76, localized in the D1 domain, are important for UL18 binding (238). The crystal structure of the HLA-A2/LIR-1 complex identifies these two amino acids as LIR-1 contact residues for the HLA-A2 heavy chain (215), suggesting

that LIR-1 uses the same region for binding to both MHC class I molecules and the viral homologue. The sequences of different MHC class I heavy chains corresponding to the contact region between LIR-1 and HLA-A2 are overall conserved except for some variation in HLA-G (215) **(Table I)**. Besides the change from a serine to a phenylalanine at position 195 **(Table I)**, HLA-G also encodes a tyrosine instead of a histidine at position 197, a residue localized between two LIR-1 contact residues at positions 196 and 198. The increased hydrophobicity of HLA-G in the expected LIR-1 contact region is likely to contribute to the three to four fold higher affinity to LIR-1 compared to other MHC class I molecules (214, 231). In contrast, the analogous region in UL18 differs in most residues **(Table I)**, which could be one factor for the higher binding affinity of UL18 to LIR-1 (215). The side-chain of LIR-1 residue Y76 contacts residue D196 in HLA-A2, which is conserved in the corresponding region in UL18 (215) **(Table I)**. However, amino acids V194 and S195 in HLA-A2 that contact LIR-1 residue Y38, correspond to Q202 and N203 in UL18 respectively (215) **(Table I)**. The substitution of residue Q202 in AD169-UL18 with alanine decreased binding affinity to LIR-1 (**paper II**), demonstrating the importance of this region also for the LIR-1/UL18 interaction. The molecular model of the UL18/LIR-1 complex suggested that the side chain of residue Q202 forms two hydrogen bounds with the side chains of LIR-1 residues K41 and T43, which may contribute to a stronger binding compared to the HLA-A2/LIR-1 complex (**paper II**). Interestingly, a UL18 protein derived from a clinical CMV isolate (13B-UL18), that encodes a histidine at position 202, binds with higher affinity to LIR-1 than AD169-UL18 (236).

	193	194	195	196	198	248
HLA-A2	A	V	S	D	E	V
HLA-B7	P	I	S	D	E	V
HLA-C7	P	L	S	D	E	V
HLA-G	P	V	F	D	E	A
UL18	N	Q	N	D	R	T

Table 1. Sequential comparison of MHC class I and UL18 residues identified as LIR-1 amino acid contacts in the HLA-A2/LIR-1 crystal structure. Note that numbers apply for MHC class I sequences. V194 in HLA-A2 corresponds to Q202 in UL18.

In summary, LIR-1 residues identified as contact residues for HLA-A2 are also involved in binding to UL18. Furthermore, we have demonstrated through site-directed mutagenesis that LIR-1 contacts the same region in the $\alpha 3$ domain of both the classical MHC class I molecule HLA-A2 and the viral homologue UL18.

3.2.4 The role of β_2m for LIR-1 binding

The pivotal role of β_2m for LIR-1 binding has been assessed for several MHC class I molecules. LIR-1 mediated inhibition of NK cell killing by HLA-G is dependent on β_2m (229). Likewise, HLA-B27 can only bind LIR-1 transfected cells when associated

with β_2m (249), and HLA-Cw7 in complex with a biotin-modified β_2m does not bind to LIR-1, while it still recognizes LIR-2 (214). The recently solved crystal structure of LIR-2/HLA-G indicates that LIR-2 binds to a much larger surface in the α3 domain of HLA-G compared to the contact of LIR-1 with the HLA-A2 heavy chain (250). This provides an explanation why LIR-2 binding is less dependent on β_2m.

We demonstrate that LIR-1 association with UL18 is critically dependent on β_2m **(paper II)**. The UL18-(ΔH36-S40) variant, in which residues 36-40 were removed, gradually lost binding to β_2m and simultaneously the ability to bind to LIR-1. Binding could be restored to original affinity through addition of exogenous β_2m **(paper II)**. Besides, in the early phases of our study we did not supply UL18-Fc proteins with exogenous β_2m during purification. As a consequence, different batches of the same UL18 variant displayed different binding affinities to LIR-1 in functional assays (data not shown). Subsequent comparison of these preparations by Western Blot analysis demonstrated that varying amounts of β_2m were associated with the different batches, correlating with binding affinity to LIR-1 (unpublished data), which indicates the crucial role of β_2m for LIR-1 binding.

Shortening of the α1 loop through deletion of residues H36-S40 also eliminates one potential N-glycosylation site at residue N38. Most glycosylation sites seem to be used during synthesis of UL18 since the protein migrates with higher molecular weight than predicted from the sequence (198, 202, 203) and the corresponding gel band is broad and diffuse (demonstrated for our fusion proteins in **Figure 8**). Assuming that N38 is indeed a functional glycosylation site, the removal of a carbohydrate may be one reason for the observed decrease in stability of this mutated protein.

3.2.5 Unique elements of UL18 important for LIR-1 interaction

3.2.5.1 The role of an additional disulfide bond in the α3 domain

The molecular model of UL18 suggested that the α3 domain of UL18 is stabilized by two disulfide bridges, in contrast to the highly conserved single disulfide bond found in all structures of classical MHC class I molecules (**paper IV**) (**Figure 1** and **Figure 11**). We demonstrated that the formation of both disulfide bonds in the α3 domain of UL18 was essential for association with β_2m (**paper IV**). In MHC class I molecules, establishment of the conserved α3 disulfide bond is required for intracellular trafficking from the ER to the cell surface (251) and substitution of involved cysteines alters tertiary structure (141). However, in contrast to UL18, MHC class I heavy chains can still bind to β_2m in the absence of an intact disulfide bond (251). Our molecular model of UL18 suggests that the additional disulfide bond may stabilize a stretch of residues in the α3 domain that is crucial for association with β_2m (**paper IV**) (252). The presence of two disulfide bridges in the α3 domain of UL18 may confer extra stability to this domain and indirectly strengthen binding to β_2m. The relative orientation of β_2m and the α3 domain is conserved in most MHC class I molecules (215). Even a slight modification in conformation of the α3 domain or in specific residues causing a different positioning of β_2m in UL18 may therefore be a critical element for binding affinity of UL18 to LIR-1 (**paper IV**). The altered orientation of β_2m in combination

with a stronger interaction of LIR-1 with residues in the α3 domain of UL18 may provide an explanation for the high binding affinity of UL18 to LIR-1.

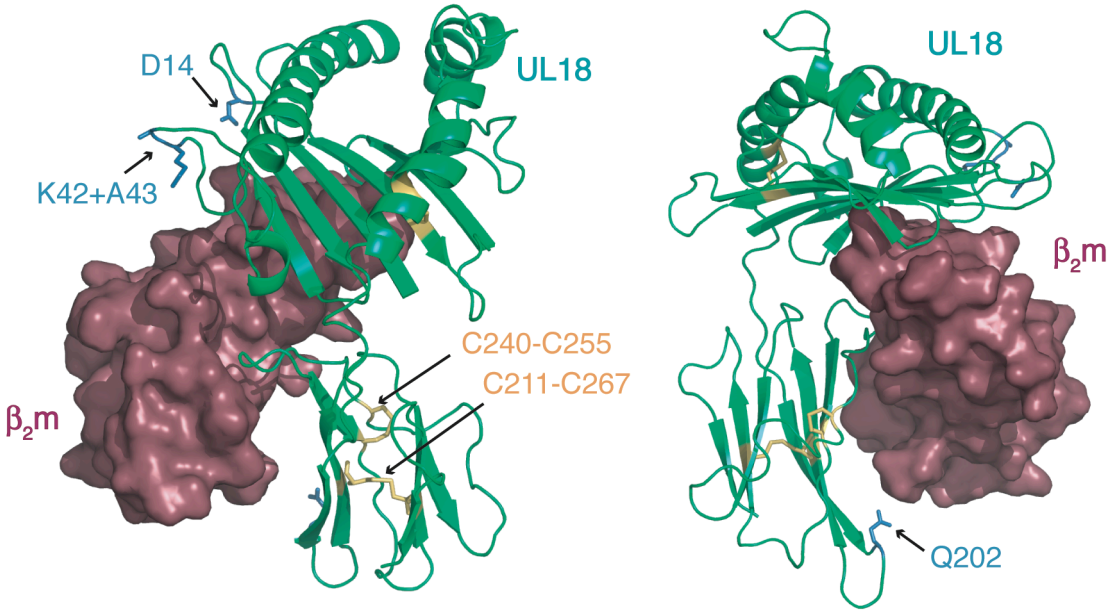

Figure 11. Localization of substituted residues in the model of UL18.
The UL18 heavy chain is coloured green, disulfide bonds are depicted yellow. The surface of β_2m is coloured raspberry. (Left) UL18 residues D14, K42 and A43 are coloured blue. Although situated in the α1 domain close to β_2m, neither of these two residues contacts β_2m directly. (Right) LIR-1 contact residue Q202 in the α3 domain of UL18 is depicted in blue.

3.2.5.2 Involvement of α1 residues in UL18-LIR-1 interactions

A third and unexpected region involved in regulating affinity to LIR-1 was discovered by site-directed mutagenesis of residues situated on one of the longer loops in the α1 domain of UL18 which were described above (**paper IV**) **(Figure 11)**. This domain has previously not been suggested to play a role in the LIR-1/MHC class I interaction. We demonstrated that the mutation of two α1 residues at position 42 and 43 in UL18 reduced LIR-1 binding affinity significantly (**paper IV**). Lysine residue K42 and arginine residue A43 were substituted in AD169-UL18 in paper IV with the corresponding amino acids (arginine and threonine) present in the clinical isolate 4636-UL18. The latter binds with a higher affinity to LIR-1 compared to AD169-UL18 (236). This particular combination of amino acids (R42+T43) is common among UL18 proteins from different clinical isolates, and occurs in UL18 molecules that bind better, equal or worse to LIR-1 compared to AD169-UL18 (237). However, UL18 proteins of clinical isolates deviate in several residues from AD169-UL18, often in regions not implicated in LIR-1 binding, rendering a direct prediction of the effect of a single residue substitution difficult (237). Thus, one strength of our study is that we were able to investigate the effect of single substitutions. Remarkably, shortening of the same α1 loop by five amino acids did not affect LIR-1 binding affinity (**paper IV**). It remains to be seen if residues at position 42 and 43 are directly involved in establishing contact with LIR-1, or indirectly modulate affinity, e.g. by affecting β_2m positioning. Granted

that these α1 residues interact directly with LIR-1, this may represent a third mechanism responsible for the enhanced binding affinity of UL18 compared to MHC class I molecules.

In conclusion, the combined effect of additional binding regions, improved α3 contact surface, different orientation of β_2m and better stabilization of α3 and β_2m could ultimately provide an explanation for the higher affinity of UL18 to LIR-1. Only a crystal structure of UL18/LIR-1 in combination with an extensive site-directed mutagenesis study will provide a definitive assessment of the molecular basis for the UL18/LIR-1 interaction.

3.3 MODULATION OF DENDRITIC CELLS BY UL18

3.3.1 UL18 and dendritic cells

Since UL18 binds to LIR-1 with such remarkable higher affinity than host ligands, it is tempting to speculate regarding a potential immune modulating role for the interaction of LIR-1 with the viral ligand. So far, research has mainly focused on the role of UL18 in relation to NK and T cells despite the fact that only a subset of these cells actually expresses LIR-1. Even though no experimental system has yet been devised, it has been recurrently speculated that UL18 could modulate myeloid cells or B cells through interactions with LIR-1 (91, 101, 163, 201). Our study (**paper III**) is to my knowledge the first of its kind, investigating the potential effects of UL18 on DCs. We used isolated UL18-Fc fusion proteins instead of whole virus, to avoid confounding effects by other CMV immune modulators. As mentioned above, LIR-1 is expressed at high levels on all DCs (211), (**paper III**). During immune responses, DCs could potentially meet cell-surface expressed UL18 at the site of infection. To be efficient, UL18 should therefore be able to affect both immature DCs that are freshly recruited to areas of pathology, and alter/inhibit the maturation of these cells (substantiated in **paper III)**. Alternatively, UL18-DC interaction could play a role when UL18 was expressed on differentiating DCs during reactivation of latent virus (98). In this scenario, UL18 could either interact with surrounding uninfected DCs or even with LIR-1 in intracellular compartments (217).

3.3.2 Inhibition of DC functions by UL18

At a first glance, it may seem obvious that UL18 proteins should inhibit DC functions through LIR-1 in analogy to LIR-1 antibodies (223). Yet this is not that self-evident after all. First, the contact of UL18 and LIR-1 could theoretically recruit yet undefined co-receptors that modulate intracellular signalling events. Second, engagement of LIR-1 by UL18 has been proposed to stimulate T cells (239), in contrast to reported inhibitory functions of LIR-1 in T cells (224-226). However, the molecular mechanism underlying the proposed activation by LIR-1 has not been determined. One such potential mechanism could be the engagement of a splice variant of LIR-1 lacking ITIMs (211, 239). Alternatively a yet undefined receptor for UL18 may be involved in mediating activation (**paper IV**, (206, 237)). Against this background, it was important to determine the outcome of UL18-LIR-1 interaction on dendritic cells. Our results,

demonstrating that UL18 can inhibit migration of immature DCs towards inflammatory cytokines as well as that presence of UL18 during DC maturation can reduce their capacity to stimulate T cells clearly points to the triggering of inhibitory signalling in DCs (**paper III**).

When trying to demonstrate that LIR-1 is the only ligand for UL18 on DCs we faced several difficulties. Blocking of UL18-Fc binding to DCs by the LIR-1 antibodies HP-F1 or M405 clearly reduced staining intensity in flow cytometry experiments but could not totally prevent binding (unpublished data). There could be several reasons for this: UL18 may bind unspecifically to the DC cell surface or to some lectin-like receptors through its multiple attached glycans. Alternatively, the LIR antibodies may not block the epitope for UL18 or UL18 may successfully compete for binding due to its high affinity to the receptor. For HP-F1, we demonstrated that this antibody could not block UL18 binding to LIR-1 on T cells at all (**paper IV**). The reduction of UL18-Fc binding to DCs by HP-F1 compared to no blocking effect for binding to T cells may depend on antigen density of LIR-1 as well as on the ligand concentration that was used. Immunoprecipitation of biotinylated DC surface proteins by UL18-Fc or HP-F1 did not reveal any band specific for UL18-Fc arguing against the existence of another receptor for UL18 on monocyte-derived DCs (unpublished data). However, a low-affinity binding may not be detectable by this method.

3.3.3 Influence on phenotype and cytokine profile

The UL18 mediated inhibition of DC migration and reduction of T cell stimulation is probably caused by LIR-1 induced phosphatases interfering with activating tyrosine kinase pathways downstream of chemokine receptors and CD40, respectively (**paper III**). However, the effects of UL18 on DC phenotype, namely the up-regulation of CD83 without up-regulation of other maturation markers, and the induction of cytokine production, occur after UL18 contact with immature DCs in the absence of other stimuli (**paper III**). It is known that LIR-1 signalling can be triggered without concomitant cell activation (221), so the effect of UL18 in the absence of activating signals does not necessarily need involvement of another receptor. The change of DC chemokine and cytokine profile induced by UL18 may be in favour of the virus and disturb the natural balance of these mediators, thus deregulating normal immune responses. The exclusive up-regulation of CD83 may either be a way of inducing an incomplete matured phenotype, unsuitable for T cell stimulation, or CD83 may be used to inhibit immune responses after shedding from the surface. Recombinant soluble CD83 can inhibit DC maturation and prevent induction of T cell responses (253). Senechal *et al.* reported that CD83 can play a role in HCMV-induced immune modulation, where HCMV infected monocyte-derived DCs lose surface expressed CD83 which inhibits T cell stimulation by surrounding non-infected DCs (185). However, it is not necessarily granted that all effects of UL18 are in favour of viral immune escape, but UL18 may also induce processes that actually help viral clearance. Consistently, it is known that membrane bound CD83 has immune stimulatory function, like activating T cells, in contrast to the inhibitory effects of soluble CD83 (254, 255). Herpes-simplex virus 1 therefore down-regulates surface-bound CD83 upon DC infection, to prevent activation of T cells and inhibit immune responses (256).

Having established that UL18 indeed can influence monocyte-derived DC phenotype and function, it will be important to extend these findings on other DC subsets as well as to establish a system in which the physiological role of UL18 can be studied during CMV infection. Even if I may not be able to do this myself, I hope that our study can provide an impulse and provide a starting point for further investigations on these particular topics.

3.4 UL18 AND LIR-1 DURING HCMV INFECTION

3.4.1 Relation of HCMV and LIR-1

Increased expression of LIR-1 on lymphocytes, in particular T cells and NK cells, and HCMV infection are obviously linked. A higher proportion of LIR-1 expressing NK and T lymphocytes has been detected in lung-transplanted patients that develop CMV caused pneumonitis compared to those that do not (257) (**paper IV**). Furthermore, individuals that are carriers of HCMV have higher numbers of LIR-1 expressing T cells than seronegative persons. LIR-1 is particularly expressed on HCMV specific $CD8^+$ T cells, but not on T cells specific for other viruses such as EBV or influenza (258, 259). The reason for the increased expression of LIR-1 on lymphocytes in relation to CMV is not clear yet, but it may be in favour of the virus, since expression of LIR-1 is associated with impaired T cell function during infection (224). In theory, an increased number of LIR-1^+ lymphocytes could be caused either by up-regulation of LIR-1 expression on previously LIR-1^- cells, or by proliferation of LIR-1^+ cells. For T cells, LIR-1 is a marker of an effector-memory phenotype, and is acquired during differentiation. LIR-1^+ memory cells do not proliferate well and are difficult to clone *in vitro* (259, 260). We show that LIR-1 can be induced by cytokines such as IL-15 or IL-2 on NK cells (**paper IV**), and that it is a matter of *de novo* up-regulation, because we did not observe an increased proliferation of LIR-1^+ NK cells upon cytokine stimulation (**paper IV**). In line with these results, it has been proven difficult to clone LIR-1^+ NK cells (206).

One suggested explanation for the increased numbers of LIR-1^+ cytotoxic T cells in CMV-carrying individuals is that recurrent viral reactivation, providing low doses of viral antigen, drives accumulation of these cells (259). Even though attractive, the validation of this proposal is presently lacking. An increase in the proportion of LIR-1 expressing T cells is seen after 10 days of *in vitro* PBMC co-culture with either live or UV-irradiated HCMV-infected fibroblasts, or even with virus alone (261), pointing towards specific T cell activation by CMV-derived antigens as the driving force for LIR-1 expression. This would involve a process independent of LIR-1 interaction with its ligands (MHC class I or UL18). In contrast, we did not observe an increase in number of LIR-1^+ cells or LIR-1 expression level on either NK or T cells co-cultured with AD169 infected fibroblasts (**paper IV**). The discrepancy may lie in details of the experimental set-up.

In NK cells, LIR-1 expression levels are lower than on myeloid cells and, as mentioned above, LIR-1 ligation with classical MHC class I molecules alone does not trigger strong inhibitory signals, but can enhance KIR signalling. One explanation for the

observed higher number of LIR-1$^+$ NK cells in CMV disease patients (257) (**paper IV**) could be that interaction with ligands, such as HLA-G (see below) or later during productive infection UL18, directs the up-regulation of the receptor. Alternatively, the cytokine milieu in response to CMV infection or general inflammation may influence LIR-1 expression on NK cells (**paper IV**).

Whatever the cause, the increased LIR-1 expression on lymphocytes may lead to a greater level of general inhibition through MHC class I molecules, and at sites of CMV replication, low levels of the high affinity ligand UL18 may directly inhibit T cells (**paper IV**) and LIR-1$^+$ NK cells (206).

Although the full replication cycle is not supported, infection of human myeloid cell lines with AD169 allows expression of immediate-early genes, which induces transcription of various genes. Among these, LIR-1 and LIR-2 genes are transcribed at an enhanced level compared to non-infected cells (262). Because myeloid progenitors are site for latency and reactivation, LIR-1 induction may be important under physiological settings. Yet this is unlikely to be an explanation for the observed link between high LIR-1 expression and CMV, because the increased proportion of LIR-1 positive cells observed during acute or persistent CMV infections consists of T and NK cells, which are not targets for viral infection (257-259) (**paper IV)**. On the other hand, induction of LIR-1 in myeloid cells may be an additional way to increase the threshold for activation of DCs and macrophages, through interaction with MHC class I ligands and potentially also through UL18. In this respect, it is interesting that HCMV can induce HLA-G surface expression in macrophages (263). The HLA-G/LIR-1 interaction is stronger and more potent than that with classical MHC class I (214, 232), and may therefore be critical for viral induced suppression. Interestingly, down-regulation of HLA-G by US2 or US11 upon HCMV infection of trophoblasts is prevented by exclusion of these viral proteins from their normal ER-localization (264). Possibly because of the importance of HLA-G in trophoblast protection from NK cell killing, the virus has evolved ways to preserve non-classical MHC class I expression in these cells, allowing viral persistence.

3.4.2 Activating effects of UL18

When comparing the cytokine production of PBMC co-cultured with either AD169 or dUL18 infected human lung fibroblasts, a marked decrease of both IFNγ and IFNα was observed in the absence of UL18, suggesting an activating effect of this viral protein (**paper IV**). The comparison of NK cell killing of AD169 and dUL18 infected cells also favoured the idea of UL18 induced activation, however without correlation to LIR-1 expression (207). In our study, LIR-1$^+$ T cells were responsible for IFNγ production, together with NK cells irrespective of LIR-1 phenotype (**paper IV**). This response is most likely driven by CMV derived antigens, since it was only observed in CMV positive individuals (**paper IV**), and fits to the reported LIR-1 expression on CMV specific T cells (258, 259). Additionally separated T cells, i.e. in the absence of antigen presenting cells, showed the same UL18 dependent activation. Whether UL18 stimulates responses through direct interaction with an unknown ligand, as proposed by Prod´homme *et al.* (206), or indirectly, e.g. through intracellular interaction with cellular or viral proteins, remains to be determined (**paper IV**). It is clear, however, that

the presence of UL18 alone is not sufficient to activate PBMCs, otherwise we should have observed some cytokine production in seronegative individuals as well. Thus, UL18 rather enhances an ongoing antiviral response, unless it is UL18 specific T cells that contribute to much of the cytokine production. UL18 specific T cells have been detected in humans, but are not present in all individuals, and UL18 is not a major antigen against which responses are directed (265, 266). It is therefore unlikely that the observed activating effect related to UL18 is mediated by responding T cells specific for this viral protein, since we observed a diminished cytokine response against dUL18 infected fibroblasts for all 17 blood donors (**paper IV**). Likewise, UL18 specific T cells cannot explain the activating effect of UL18 on NK cells (206, 207).

In our study, NK cell activation by AD169 infected fibroblasts compared to dUL18 was LIR-1 independent (**paper IV**), whereas Prod´homme *et al.* show that UL18 inhibits LIR-1 expressing NK cells (206). Differences in the experimental set-up can explain this discrepancy. Prod´homme and colleagues look either at killing of AD169 or dUL18 infected fibroblast by the LIR-1 expressing cell line NKL, or at polyclonal NK responses of three different donors in an autologous cytotoxicity assay, where no UL18 induced activation is observed (206). Furthermore, degranulation and cytotoxicity of NK cell clones and polyclonal NK populations were tested against cells infected with UL18 expressing recombinant adenovirus or control adenovirus. Adenoviral expression of UL18 greatly increases cell surface levels compared to CMV infection (203, 206). In contrast to that, UL18 surface expression was below the detection limit of flow cytometry in our study (**paper IV**). Furthermore, we focused on cytokine production of NK cells in PBMC. Purified NK cells did not secrete IFNγ upon co-culture with virus-infected fibroblasts, whereas a considerable part of the cytokine production in combined T and NK cell cultures came from NK cells, arguing for a T cell driven NK response (**paper IV** and unpublished data). In summary, the low levels of surface UL18 possibly interacting with LIR-1 do not play a role for total responses in our set-up. It is also possible that the activating effect is not directly mediated by surface expressed UL18 (discussed in the following paragraph).

3.4.3 LIR-1 mediated inhibition of T cells through UL18

We used isolated UL18-Fc proteins to assess the effect of UL18 on T cells, and to clarify if the observed activating response could be directly mediated by UL18 (**paper IV**), having in mind the reported activation of T cells via UL18-LIR-1 interaction (226). In our setting, UL18 proteins could clearly inhibit IFNγ production of LIR^+ T cells stimulated by antibodies against the TCR, in line with published inhibitory functions of LIR-1 (224-226). When comparing the effect of UL18-Fc and UL16-Fc on T cell stimulation, the proportion of $LIR\text{-}1^+$ T cells was consistently found reduced after contact with UL18, pointing towards ligand induced receptor internalization (**paper IV**). If this process is followed by receptor degradation or recycling remains to be determined. LIR-1 down-regulation upon contact with UL18 was recently confirmed in NK cells (206). Co-staining of T cells with anti-LIR-1 and UL18-Fc proteins demonstrates that the same antigen is recognized, and makes the existence of another receptor for UL18 on T cells unlikely (**paper IV**). Addition of anti-UL18 (10C7) to co-cultures of PBMC with virus infected cells could not prevent the activating effect of UL18 (**paper IV**), even though this particular antibody has been used previously to

block the interaction of UL18 and T cells (239). Low-level surface expressed UL18 on virus infected fibroblasts did therefore not play a dominant role in our co-culture setting, since IFNγ was produced by LIR-1^+ T cells, which would have been inhibited by UL18 rather than activated.

3.4.4 About the impact of UL18 on NK and T cells

The multiple studies about UL18 and NK cells can be summarized as follows: If UL18 is expressed to a sufficient level at the cell surface, which is always the case when this protein is expressed in isolation by means of transfection or a suitable vector, then UL18 can inhibit NK cells (206, 208, 209). LIR-1 presence is a prerequisite for the UL18 mediated inhibitory effect, which is granted when LIR-1^+ NK clones or LIR-1^+ NK cell lines are utilized as effector cells, but may be an issue if polyclonal NK cells with varying subsets of LIR-1 expressing cells are used (206). The situation is more complex in CMV infection, because UL18 is expressed at very low levels, often not even detectable on the cell surface (203)(**paper IV**). UL18 may activate certain NK cells, yet no details about involved receptors or UL18 localisation are known (207) (206).

If UL18 protein is accessible for interaction with LIR-1 on T cells, then an efficient inhibitory signalling cascade is initiated, abrogating TCR mediated activation, which in turn drastically reduces effector functions like cytokine production (**paper IV**). However, comparison of AD169 with dUL18 infected cell lines again points towards an activating function of UL18 with unknown mechanism (**paper IV**). An activating effect of UL18 on certain cytotoxic T cell clones has also been reported (239).

4 CONCLUDING REMARKS

In conclusion, UL18 may be able to exert both activating and inhibitory functions, depending on the receptor repertoire of the target cell population and possibly involving different localisations (surface expressed versus intracellular). Depending on which function ultimately dominates, the outcome may thus differ a lot with experimental set-up. The inhibitory function mediated via LIR-1 is rather clearly defined, whereas the mechanism of UL18 induced activation remains obscure. The situation certainly becomes more complex *in vivo*, where UL18 may possibly be found at higher levels on the cell surface of infected cells than during *in vitro* experiments. Support for UL18 expression during active CMV replication is provided by a study that found UL18 mRNA to be translated in patients with high viremia (204), and by the fact that UL18 specific T cells can be detected (265, 266). Together with the notion that all clinical CMV isolates have retained the gene for UL18, this argues for a role of UL18 during natural infection. However, if the protein mediates its function intracellular or extracellular is not proven. Theoretically, T cells could be primed for UL18 by cross-presentation of dendritic cells, without ever seeing the antigen on the surface of infected cells. On the other hand, several arguments favour an extracellular role for UL18: its extremely high affinity for LIR-1, an inhibitory receptor so widely expressed on the cell surface of different immune cells, makes UL18 an ideal candidate to exert immuno-suppressive functions. It would be quite a coincidence if the virus has evolved this option of inhibiting immune responses via UL18/LIR-1 interactions on the cell-surface without using it. Even though multiple variations were found in UL18 proteins from clinical isolates, mutations never prevented LIR-1 binding (208), supporting an *in vivo* role for the UL18-LIR-1 interaction. Furthermore, from a biochemical point of view, the fact that UL18 contains more disulfide bonds than classical MHC class I molecules could be a sign that UL18 is meant to function extracellularly. Proteins stabilized by disulfide bonds are often found extracellularly, where a higher degree of structural stability helps to resist extracellular proteases and other challenges in a rather disruptive milieu compared to intracellular locations (19). Because many viral immuno-evasins can exert multiple functions, this may also be true for UL18. Some of the effects induced by UL18, like inhibition of T cells, NK cells or dendritic cells, would be in favour of viral immune escape, whereas activation may actually support host defence. The net effect should be balanced towards the virus, otherwise UL18 ought to be lost from the viral genome in the far future…

Until the issue about UL18 is solved, our results obtained in **paper IV**, proposing an activating role for UL18 independently from a UL18-LIR-1 interaction, should not prevent further investigations regarding the effect of UL18 on other LIR-1 expressing cells, like DCs studied in **paper III**. Neither should investigations about the interaction of UL18 with LIR-1 (**paper II**) be neglected, not least because knowledge about the biology underlying the high affinity interaction between the viral MHC class I homologue and LIR-1 could help in understanding general principles of the function of this receptor. The establishment of suitable model systems to continue the study on UL18, and the characterization of the diverse functions of this protein in CMV infection may finally shed light on its true role in viral immune escape.

Although our knowledge about viral immune evasion is increasing rapidly, a lot of structural information is still missing. Structural analysis of viral proteins interacting with components of their respective hosts can provide a basis for selective functional studies. Molecular approaches such as those employed in our study on the molecular mechanism for US2 binding to HLA-A2 are a valuable complement to structural studies. With this thesis and the studies of US2 and UL18 I have hopefully contributed a piece of the puzzle in the constantly progressing knowledge of how viral immune evasion proteins work.

5 ACKNOWLEDGEMENTS

To start with, I would like to express my sincere gratitude to all friends, colleagues and collaborators, who contributed to making the past years in Stockholm a pleasant and unforgettable time in my life. For reasons of consistency and simplicity, I will follow the Swedish tradition and refrain from addressing people with their respective title. Yet, I have to admit that this feels almost disrespectful in certain cases, an impression that certainly originates from my German background.

In particular, I want to thank

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