

**Selective analysis of specific HLA ligand repertoires:  
poxviral CD8<sup>+</sup> T cell epitopes and  
phosphorylated HLA ligands of tumor cells**

Gezielte Analyse von spezifischen HLA-Liganden-Repertoires:  
CD8<sup>+</sup> T-Zellepitope von Pockenviren und  
phosphorylierte HLA-Liganden von Tumorzellen

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## Abbreviations

APC	antigen presenting cell	MV	mature virion
ARE	AU-rich element	MVA	modified vaccinia virus Ankara
B-LCL	B lymphoblastoid cell line	NCBI	National Center for Biotechnology Information
CEF	chicken embryo fibroblasts	NIC	nicotinic acid
CID	collision-induced decomposition	NK	natural killer
CLIP	class II-associated invariant chain peptide	ORF	open reading frame
CPXV	cowpoxvirus	p.b.	post boost
CTL	cytotoxic T cell	PAGE	polyacrylamide gel electrophoresis
CVA	chorioallantois vaccinia virus Ankara	PBMC	peripheral blood mononuclear cell
D <sub>4</sub> NIC	deuterated NIC	PBS	phosphate-buffered saline
DC	dendritic cell	pDC	plasmacytoid DC
DMSO	dimethylsulfoxide	PE	phycoerythrin
DNA	deoxyribonucleic acid	pep	peptide
ds	double-stranded	PFU	plaque forming unit
EBV	Epstein Barr virus	PMA	phorbol myristate acetate
ECTV	ectromelia virus	PRR	pattern recognition receptor
EDTA	ethylenediaminetetraacetic acid	pS	phosphoserine
EEV	extracellular enveloped virion	pT	phosphothreonine
ELISPOT	enzyme linked immunosorbent spot	Q-TOF	quadrupole-time of flight
ER	endoplasmic reticulum	RCC	renal cell carcinoma
ESI	electrospray ionization	RNA	ribonucleic acid
FACS	fluorescent-activated cell sorting	s.c.	subcutaneous
FCS	fetal calf serum	SCX	cation exchange chromatography
FITC	fluorescein-5-isothiocyanate	SDS	sodium dodecyl sulfate
Gua	guanylated	SEM	standard error of the mean
H <sub>4</sub> NIC	hydrogenated NIC	SEREX	serological identification of antigens by recombinant expression cloning
HCMV	human cytomegalovirus	SFC	spot forming cells
HIV	human immunodeficiency virus	SITE	stable isotope tagging of epitopes
HLA	human leukocyte antigen	ss	single-stranded
HPLC	high performance liquid chromatography	stim.	stimulation
i.m.	intramuscular	TAA	tumor-associated antigen
IFN	interferon	TAP	transporter associated with antigen processing
Ig	immunoglobulin	TCR	T cell receptor
IL	interleukin	T <sub>H</sub>	T helper
IMAC	immobilized metal affinity chromatography	TiO <sub>2</sub>	titanium dioxide
IMV	intracellular mature virion	TLR	Toll-like receptor
IU	international unit	VACV	vaccinia virus
LC-MS/MS	HPLC-coupled tandem MS analysis	VARV	variola virus
m/z	mass to charge ratio	WHO	World Health Organisation
mDC	myeloid DC	WR	Western Reserve
MHC	major histocompatibility complex	wt	wild type
MOAC	metal oxide affinity chromatography	y	year
MOCV	molluscum contagiosum virus		
MPXV	monkeypoxvirus		
MS	mass spectrometry		

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# 1 General introduction

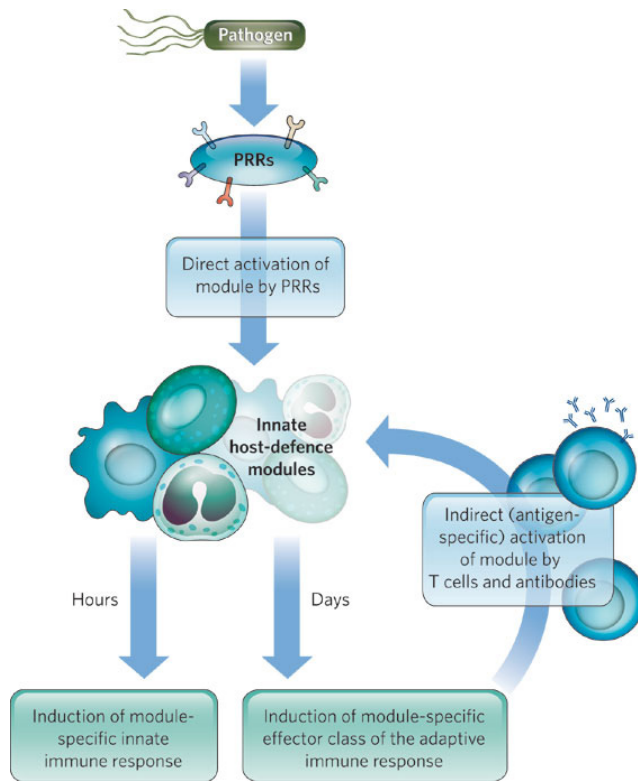
## 1.1 The sixth sense - immune receptors

A diversity of cellular sensors have evolved in order to discriminate between self, altered self (tumors) and non-self (viruses, bacteria and eukaryotic parasites) in or around mammalian cells. Basically, there are two types of defense mechanisms, which differ in the receptors used to recognize pathogens: innate and adaptive (also known as acquired).

Innate immune recognition is mediated by germline encoded pattern-recognition receptors (PRR), which are characterized by broad specificities for conserved and invariant features of microorganisms (1, 2). PRR engagement leads to host inflammatory, anti-pathogen and cell death effector mechanisms mediated by macrophages, neutrophils, natural killer cells (NK cells), and the complement system. This provides a first line of defense, which optimally controls an infection in the first four days before an initial adaptive immune response takes place.

Adaptive immune recognition is mediated by antigen-specific receptors. The genes encoding these receptors are assembled from gene segments in the germ line, and somatic recombination of these segments yields a diverse repertoire of receptors with random but narrow specificities (3). This diversity is further increased by additional mechanisms, such as non-templated nucleotide addition, gene conversion and (in the case of B cells) somatic hypermutation, generating a highly diverse repertoire of receptors with the potential to recognize almost any antigenic determinant in a specific manner. These specific antigen receptors are clonally distributed on T and B lymphocytes, which allows clonal selection of pathogen-specific receptors and is the basis for immunological memory. Since, in a non-immune individual, only an extremely small fraction of the adaptive immune cell repertoire (e.g., one in a million) will have an antigen receptor capable of recognizing a specific antigen, clonal amplification of these specific cells takes days to evolve. T-helper ( $T_H$ ) cells marked by the co-receptor CD4 on the cell surface and cytotoxic T cells (CTL) expressing CD8 recognize antigenic peptides bound to major histocompatibility complex (MHC) class II and class I molecules (also called human leukocyte antigens, HLA), respectively. They are the cells of the so-called cellular immunity. As the term HLA indicates, these

molecules are also involved in rejection of transplanted non-self tissue based on the fact, that every person has an individual set of HLA alleles, which bind different types of peptides and are co-recognized by T cells. B cells can recognize almost any type of antigen by binding to a specific three-dimensional molecular determinant. They constitute humoral immunity.



**Figure 1.1 Recognition of pathogens and activation of the immune response (1).** Pathogens induce a fast innate immune response through PRRs, which activates the appropriate set of cells of the antigen-specific adaptive immune response. An adaptive immune response results in boosting of the same innate immune response that instructed the adaptive immune response.

The innate and adaptive immune system complement each other (4) (Figure 1.1). Microbial antigens are taken up by professional antigen-presenting cells (APCs), such as dendritic cells (DCs), in the peripheral tissues and are delivered to the lymph nodes or spleen through the lymph or blood, respectively, where they are recognized by antigen-specific B and T cells. Since specificity of B and T cell antigen receptors is not directly linked to the origin of the antigen, the differentiation of lymphocytes into a particular effector-cell type and their localization to the site of infection are regulated by the instructions provided by the innate immune system, generally in the form of cytokines and chemokines, respectively: for specific T cell activation, and in the case of  $T_H$  ( $CD4^+$ ) cells for differentiation into one of several types of effector  $T_H$  cell ( $T_{H1}$ ,  $T_{H2}$  and  $T_{H17}$ , characterized by different cytokine production), it is essential that PRRs induce DCs to produce cytokines and to express cell-surface signals; for specific B cell



activation against so called T-dependent antigens (mostly proteins) effector T<sub>H</sub> cells, which have previously been activated by DCs, are necessary.

In addition to the conventional T and B cells, there are lymphocytes with innate-like characteristics. The innate-like B-1 cells reside in the peritoneal and pleural cavities and produce mainly antibodies of the IgM class against some common bacterial polysaccharides and some self antigens (5). Innate-like T cells recognize the non-classical MHC molecules class Ib molecules, which present bacteria-specific ligands, for example, bacterial lipids or formylated peptides in the case of the CD1 and H-2M3 families, respectively. In a way, these MHC-like molecules function as PRRs, presenting microbial ligands to specialized T cells (6). Some non-classical MHC molecules might themselves be ligands for T cell receptors, without presenting any other molecules. In this case, the expression of these molecules is thought to be inducible by the engagement of PRRs on specific cell types, such as mucosal epithelial cells (7).

### 1.1.1 Vaccination

Prophylactic vaccines aim to generate an effective, specific and long-lasting cellular and humoral immunological memory against infectious agents for protection against future infection and disease caused by the pathogen. There are three basic types of prophylactic vaccines: live attenuated vaccines, inactivated whole cell vaccines and subunit vaccines. Live attenuated vaccines are made from weakened, or attenuated, viruses or bacteria that are designed to mimic some of the early stages of infection without causing disease. Inactivated whole cell vaccines are made by growing the infectious organism in culture media or mammalian cells and then inactivating the organisms. Subunit vaccines are derived from individual antigens that can be purified. Although several serious pathogens have been successfully fought by vaccination yet, for example smallpox virus (see chapter 1.2.2) or poliomyelitis virus ([www.polioeradication.org](http://www.polioeradication.org)), there are numerous pathogens which remain a great challenge for the human organism.

Therapeutic vaccines aim to strengthen or modify the immune response in patients already suffering from the disease. Great effort is being devoted to developing therapeutic vaccines against tumors, AIDS, hepatitis B, tuberculosis, malaria, and autoimmune diseases (8). The vaccination-based induction of a specific anti-tumor

immune response bears the potential to reject malignant cells or at least to support current therapies in a highly specific manner.

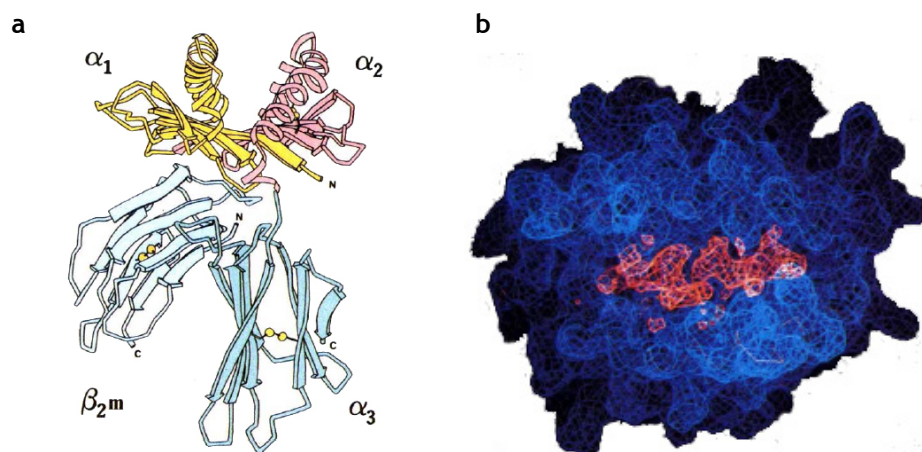
### 1.1.2 T cell induction by MHC class I and II antigen presentation

Adaptive cellular immunity by T cells is mediated by binding of the specific T cell receptor (TCR), which is composed out of an  $\alpha$ - and  $\beta$ -chain, to MHC-peptide complexes (9). CD8<sup>+</sup> T cells recognize the complex of MHC class I molecules with peptides of 8 to 12 amino acids derived mainly from intracellularly produced and degraded proteins (10). CD4<sup>+</sup> T cells recognize peptides of 9 to 25 amino acids, which are mainly derived from membrane-bound proteins or from extracellular proteins internalized by endocytosis, on MHC class II molecules, which, physiologically, are exclusively expressed by APCs, e.g., DCs (11).

Molecules of each MHC class are expressed by three genetic loci (called HLA-A, -B, and -C for human class I and HLA-DR, -DQ and -DP for human class II) that encode structurally similar but non-identical proteins. Each of these loci shows a tremendous genetic polymorphism, with hundreds of different allelic forms having been defined ([www.anthonynolan.com/HIG](http://www.anthonynolan.com/HIG)). This creates a very large genetic pool of structurally distinct HLA class I and II, from which a small subset (six alleles each) are represented in any individual human being. Most of the polymorphic differences among MHC molecules encoded by different genes and alleles alter the structure and specificity of the peptide-binding groove. Hence, the MHC polymorphism results in an increase in the overall information content displayed population-wide on cells for inspection by the immune system. Any of the HLA-A, -B, and -C alleles binds a repertoire of peptides, which share an amino acid sequence motif corresponding to two or more essential amino acids, the so-called peptide anchor residues (10, 12, 13). Pockets in the peptide-binding groove of MHC molecules contain particular amino acids corresponding to the anchor residues of the peptide ligand (14-16). Most HLA class I require anchor residues at position 2 and at the C-terminus ([www.syfpeithi.de](http://www.syfpeithi.de)). A B lymphoblastoid cell displays an estimate of 10,000 to 20,000 different peptides at greater than one copy by an individual class I and II MHC allelic form (12), representing a potential total display of 30,000 to 120,000 peptides.

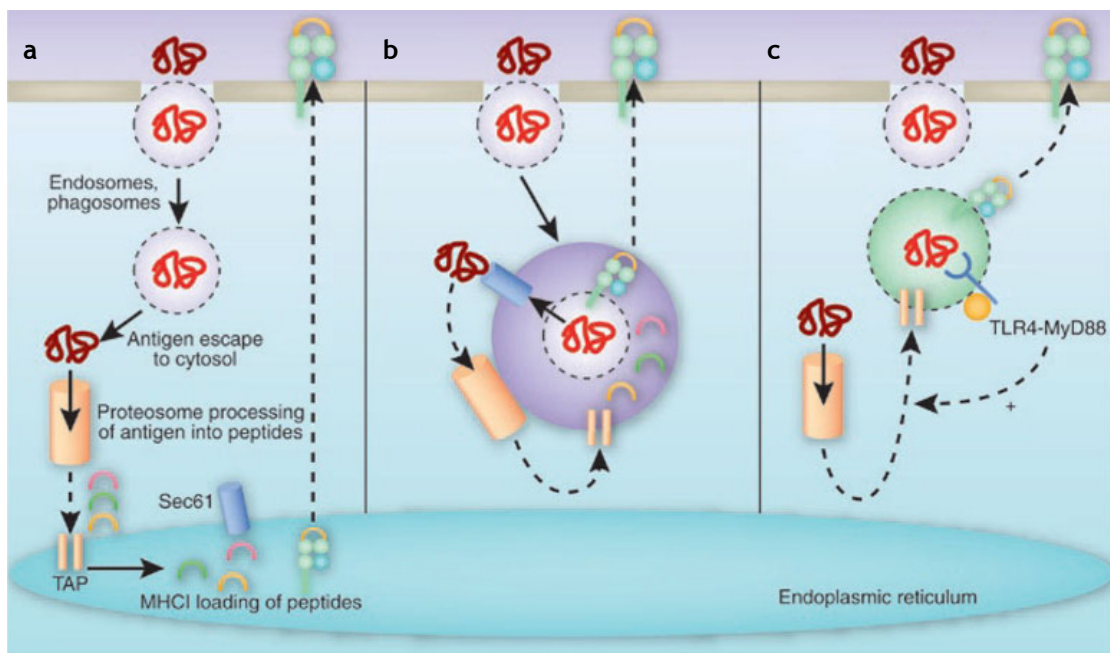
Peptide fragments binding on HLA class I are generated in the cytoplasm of any nucleated cell by proteasome-mediated proteolytic cleavage of cellular endogenous

proteins (self-HLA ligands), of viral proteins in case of infection (non-self-HLA ligands), and of products of self-genes that have undergone mutations (altered HLA ligands), e.g., during malignant transformation (17). After targeting of misfolded or nonfunctional proteins (18, 19) as well as of proteins designated for degradation (20) by ubiquitination (21, 22), they are recognized by proteasomes, which are multi-proteinase complexes (23). In case of interferon (IFN)- $\gamma$  stimulation, a cell switches from synthesis of the constitutive 20S core proteasome to synthesis of the immunoproteasome by exchange of the subunits harbouring the catalytic sites by immunosubunits called MECL1, LMP2 and LMP7 (24). The immunoproteasome is able to enhance generation of peptides presented on HLA class I (25). Proteasomes generate precursor HLA ligands with the final C-terminus, which are further trimmed by peptidases either in the cytosol (26) or in the endoplasmic reticulum (ER) (27). The peptides generated in the cytoplasm are transported into the ER by the transporter associated with antigen processing (TAP) (28). In the lumen of the ER, MHC class I molecules composed out of the polymorphic  $\alpha$ -chain and the non-polymorphic  $\beta_2$ -microglobulin ( $\alpha/\beta_2m$ -heterodimers) (Figure 1.2a) are loaded with peptides (Figure 1.2b) through interactions in the peptide-loading complex, which includes the transmembrane glycoprotein tapasin, the chaperone calreticulin and the thiol oxidoreductase ERp57 (29). Accumulated MHC class I  $\alpha/\beta_2m$ -peptide-complexes are then transported to the cell surface.



**Figure 1.2 HLA class I structure (14).** a) Side view of the complex composed of the membrane-spanning  $\alpha$ -chain (domains  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ) and the non-covalently bound  $\beta_2$ -microglobulin (schematic). Peptide binding occurs in the groove between domains  $\alpha_1$  and  $\alpha_2$ . b) Peptide (red) bound in the peptide binding groove (top view; electron density).

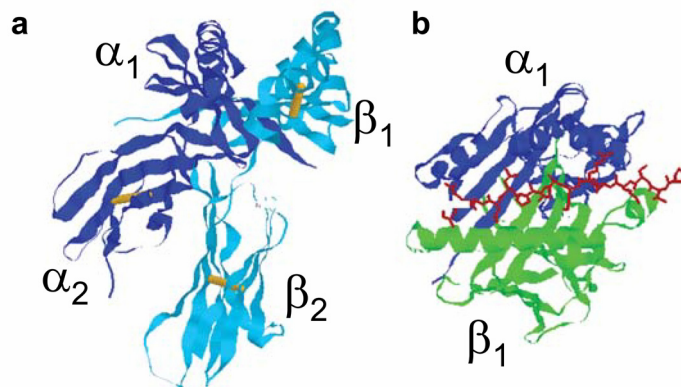
DCs can potentially initiate CTL responses through the process of direct antigen presentation (as just described) or through cross-presentation, which refers to the generation of HLA class I-peptide complexes from exogenous protein, e.g., from apoptotic cells (30-33). The priming of anti-pathogen or anti-tumor responses is often dependent on cross-priming, which is therefore an important issue in the context of vaccinations. The fact that, first, many viruses show strict tissue tropism and do not detectably infect DCs and, second, viruses often interfere with antigen presentation, may have contributed to the evolution of cross-priming (34). Both direct and cross-presentation lead to cell surface expression of HLA class I-peptide-complexes along with costimulatory molecules, such as CD80 and/or CD86, needed to induce naïve CD8<sup>+</sup> T cells. However, when a TLR ligand activates a DC, rapid maturation occurs and thus uptake of exogenous antigen is substantially decreased (35, 36). This finding is particularly critical for protein-based vaccines.



**Figure 1.3** Proposed pathways for cross-presentation by MHC class I molecules (37). For explanation see text below.

Presently, three mechanistic pathways are proposed for cross-presentation by HLA class I (Figure 1.3, (37)): The first model (Figure 1.3a) proposes that endocytosed or phagocytosed antigens are translocated into the cytosol, where they are degraded into antigenic peptides by the proteasome before being transported into the lumen of the ER by TAP. In the ER, the peptides are loaded onto nascent MHC class I molecules for

presentation at the cell surface. An important revision (Figure 1.3b) of the first model was made by studies showing that phagosome formation in macrophages and DCs might involve fusion with the ER, as the phagosomes contain ER-associated molecules such as calnexin, calreticulin, TAP and glucose-6-phosphatase, and Sec61. Sec61 is known to be involved in retrotranslocation of misfolded proteins from the ER to the cytosol for degradation, and thus it has been proposed that Sec61 might be involved in the translocation of proteins located in phagosomes to the cytosol. Recent findings provide evidence of an indispensable function for the early endocytic compartment in the cross-presentation of soluble antigens (Figure 1.3c) (32). Furthermore, this work establishes that recruitment of TAP to the early endosomal compartments is regulated by signaling through Toll-like receptor (TLR) 4 and MyD88 (32). Additional recent research demonstrates that human plasmacytoid DCs cross-present exogenous antigens to memory CD8<sup>+</sup> T cells through the early endocytic compartment (33).

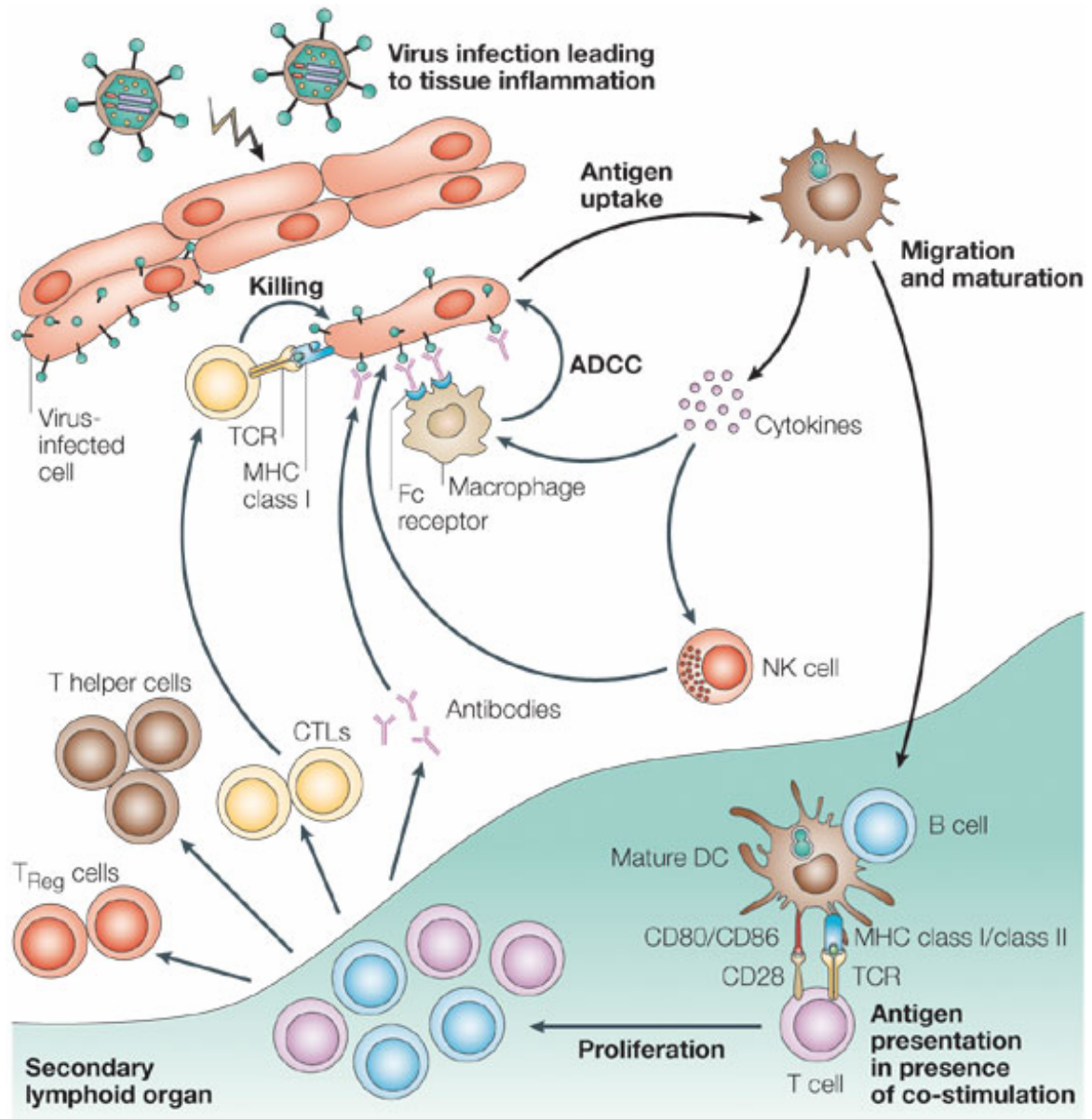


**Figure 1.4 HLA class II structure (38).** a) Side view of the complex composed out of the membrane-spanning  $\alpha$ -chain (domains  $\alpha_1$  and  $\alpha_2$ ) and  $\beta$ -chain (domains  $\beta_1$  and  $\beta_2$ ). Peptide binding occurs in the groove between domains  $\alpha_1$  and  $\beta_1$  (schematic). b) Peptide (red) bound in the peptide binding groove (top view; schematic).

Peptide fragments binding on MHC class II molecules (Figure 1.4b, (38)) are generated in the endocytic pathway (39). The two polymorphic membrane-spanning chains  $\alpha$  and  $\beta$  of HLA class II (Figure 1.4a, (38)) are synthesized into the lumen of the ER and associate with preformed trimers of the invariant chain (Ii) to form nonameric  $(\alpha\beta Ii)_3$  complexes (40-42). Ii occupies the peptide-binding site of the HLA class II, therewith preventing premature peptide binding. Ii is also required to direct the complex out of the ER and to chaperone the HLA class II from the biosynthetic pathway to endosomes, either directly via the trans-Golgi-network or via the plasma membrane

(dominant) (43-45). Upon arrival in acidic endosomal compartments, the Ii luminal domain is progressively degraded by lysosomal proteases, breaking up the nonameric complex but leaving a contiguous internal segment of Ii, the class II-associated invariant-chain peptide (CLIP), associated to the peptide-binding groove of the now liberated HLA class II- $\alpha\beta$  dimers (46). CLIP is then replaced from the peptide-binding groove by locally generated peptides of 9 to 25 amino acids, with the aid of the chaperone HLA-DM (47, 48). In contrast to HLA class I, the peptide binding groove of HLA class II is open at both ends and thus allows for binding of longer peptides with generally up to 25 amino acids ((38), [www.syfpeithi.de](http://www.syfpeithi.de)). Although antigen loading occurs predominantly in late endosomes, some HLA class II-peptide complexes can be generated in early endosomes or lysosomes, or even at the plasma membrane (49-51). Macropinocytosis and degradation of pathogenic antigens into peptides is stimulated in maturing DCs (52, 53).

CD8<sup>+</sup> and CD4<sup>+</sup> T cells are primed in the lymph nodes upon recognition of a specific non- or altered-self peptide presented by HLA class I or class II, respectively, together with costimulatory molecules on matured DCs, which have migrated into the lymph nodes after uptake of antigen (Figure 1.5, (54)) (55). Activated CD8<sup>+</sup> and CD4<sup>+</sup> T cells migrate into the periphery to exert their effector function as CTL or T<sub>H</sub> cells. Cytotoxicity is mediated by CTL through indirect killing of target cells by release of the cytokines TNF- $\alpha$  and IFN- $\gamma$ , through induction of apoptosis in target cells via death receptor triggering (binding of the CTL-expressed Fas ligand FasL to the Fas receptor CD95, which is upregulated in target cells by IFN receptor signaling), or through direct killing of target cells by release of perforin and granzyme B into the intercellular space between CTL and target cell (56). T<sub>H</sub>1 cells secrete IFN- $\gamma$  and interleukin (IL)-2 leading to activation of macrophages and CD8<sup>+</sup> T cells (57). T<sub>H</sub>2 cells support a humoral immune response by secretion of IL-4 leading to activation of antibody secreting B cells and the complement system (58). Regulatory T cells (T<sub>reg</sub>), which are able to inhibit autoreactive T cell responses, are also CD4<sup>+</sup> and in addition constitutively express CD25 (59).



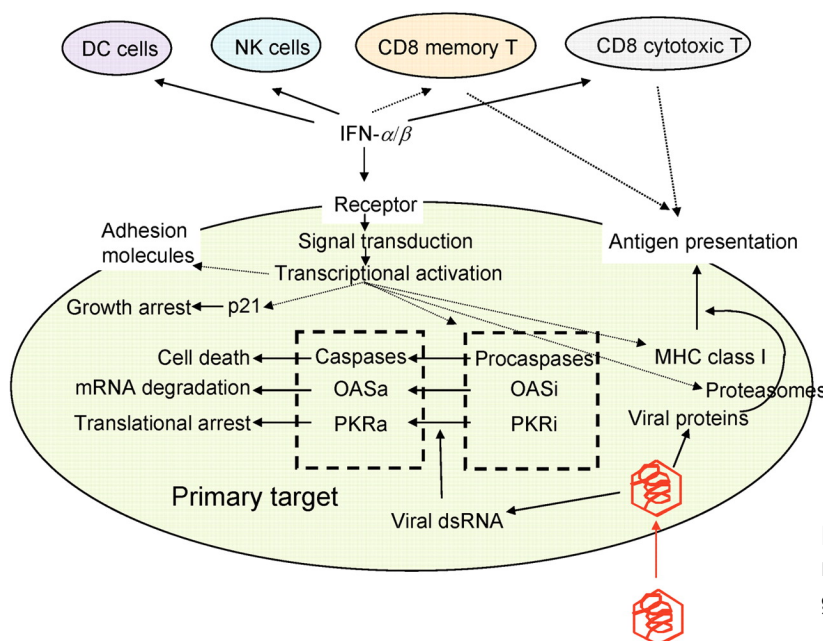
**Figure 1.5 T cell induction by MHC class I and II antigen presentation (54).** Antigen taken up by immature DCs in the periphery is processed and presented by matured DCs to naïve T cells in the lymphnodes in the form of MHC class I and II-peptide-complexes together with costimulatory proteins. Activated T cells proliferate and differentiate to effector T cells (CTL,  $T_H$ ,  $T_{reg}$ ), which exert their function in the periphery.

### 1.1.3 Anti-viral immune response

Since all viruses replicate within host cells, the main targets of innate immune recognition are viral nucleic acids, which are discriminated from self nucleic acids on the basis of specific chemical modifications and structural features that are unique to viral RNA and DNA, and on the basis of the cellular compartments where only viral nucleic acids are normally found (60, 61). This sensing of viral infection within cells results in the production of type I interferons IFN- $\alpha$  and IFN- $\beta$ , which induce the

expression of more than hundred IFN-inducible genes both in the infected cell and in neighbouring cells by autocrine and paracrine IFN-mediated signaling, respectively (62-64). In addition, a special subset of DCs, plasmacytoid DCs (pDCs), are triggered by the intracellular PRRs TLR3, TLR7/8 and TLR9 recognizing viral dsRNA (65), ssRNA (within endosomes) (66) and DNA (rich in CpG) (67, 68), respectively, which results in the production of systemic levels of type I IFN (69). Another type of DCs, myeloid DCs (mDCs), also express subsets of TLRs and, when stimulated, express high levels of IL-12, but little type I IFN (70). mDCs are crucial for the activation of T cells during viral infections (71).

The type I IFN-induced transcripts encode proteins that mediate the antiviral response (Figure 1.6, (72)). Some of these proteins (e.g., PKR and OAS) are enzymes whose activities are dependent upon viral co-factors (e.g., dsRNA). When such co-factors are provided, enzyme function evokes cellular changes, e.g., translational arrest. Other type I IFN-inducible factors trigger cell-cycle arrest (e.g., the G<sub>1</sub>/S phase-specific cyclin-dependent kinase inhibitor p21) and others promote the presentation of viral antigens to adaptive immune cells (e.g. by upregulating MHC class I and the antigen-processing machinery). IFN- $\alpha/\beta$  also has immunomodulatory functions: promotion of DC maturation, upregulation of activities of NK cells and CD8<sup>+</sup> T cells, and induction of synthesis of IL-15, a factor that promotes the division of memory CD8<sup>+</sup> T cells.



**Figure 1.6 Antiviral effects mediated by IFN-induced gene transcripts (72).**

NK cells play an important role in bridging the innate and adaptive immune system during viral infections by mediating elevated cytotoxicity during early viral infections



(73, 74). Upon priming by various soluble factors including type I IFN and by recognizing host stress proteins (MICA, MICB, ULBP1) by the NKG2D receptor, they can kill virus-infected target cells under particular conditions and boost the maturation and activation of DCs, macrophages, and T cells through a combination of cell surface receptors and cytokines including IFN- $\gamma$  and TNF- $\alpha$ . Conversely, NK cells can also kill immature DCs, activated CD4<sup>+</sup> T cells and hyperactivated macrophages. These NK cell regulatory functions are kept in balance by the recognition of constitutively expressed self molecules (e.g., MHC class Ia and MHC class Ib molecules) by means of inhibitory receptors (e.g., KIR or CD94-NKG2A).

The early proinflammatory cytokine responses promote a classical T<sub>H1</sub> response of mixed phenotypes with high IFN- $\gamma$ , moderate IL-2, and low IL-4 levels, which leads to activation of macrophages, stimulation of CTL, and upregulation of MHC-based antigen presentation on DC (75). CD8<sup>+</sup> T cell responses can be induced to extremely high levels with intense expansion. Activated CTL halt the spread of infection by killing virus-infected cells through direct cell-to-cell contact and by the release of soluble mediators (e.g., cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , as well as perforin and granzyme) (76-78). In addition, antibody production (e.g., for neutralization of virus) by B cells is aided by CD4<sup>+</sup> T cell cytokine responses (57, 58).

#### **1.1.3.1 Immune response to poxviruses**

The immune response against poxviral infections (see chapter 1.1.3.1) is reliable on IFN- $\gamma$  and other T<sub>H1</sub> cytokines provided by NK cells and macrophages to promote an effective CTL response including granzyme and perforin, and an effective antibody response (79). Recovery from secondary infection is dependent on the generation of neutralizing antibody.

Viruses employ multiple strategies to evade or modulate the host immune system including antigenic variation (antigenic drift by mutations, or antigenic shift, e.g. Influenza virus), interference with antigen processing and presentation, modulation of cytokine production, prevention of viral antigen presentation, and abrogation of the induction of cell death. Vaccinia virus (VACV) secretes soluble proteins to bind the host cytokines (B15R), complement factors C3b and C4b, chemokines, interferons (B18R/B19R, B8R), and their receptors (80). Furthermore, the virus has strategies to inhibit intracellular antiviral effects of class I interferons (E3L, K3L; (81, 82)) and to interfere in various intracellular steps of the IL-1R and TLR signaling pathways,

ultimately regulating the NF- $\kappa$ B pathway and interferon regulatory factors (IRFs) (A52R, A4R, K1L, N1L; (83-85)). In addition, VACV produces proteins directly interfering with apoptosis of the host cell and thereby impairing CTL mediated lysis (F1L).

### 1.1.3.2 The broad CTL response induced by poxviruses

Immunization with VACV induces lifelong protection from smallpox disease and can serve as a benchmark for the type of immunity that other vaccines should induce. After immunization, CD8<sup>+</sup> T cells are induced against VACV or attenuated strains such as MVA (see chapter 1.2.3.1) (86-88). They are polyfunctional (IFN- $\gamma$ , MIP-1 $\beta$ , TNF- $\alpha$ , CD107a, IL-2, perforin, and granzyme), make much more IFN- $\gamma$  than cells with fewer functions and express an unusual memory phenotype (CD45RO<sup>-</sup> CD27<sup>intermediate</sup>) (89). This polyfunctional CD8<sup>+</sup> T cell profile is induced in response to both the vector and the inserts (see chapter 1.2.3.2) and is consistent with virus-specific CD8<sup>+</sup> T cell responses observed in well-controlled persistent infections such as CMV, EBV, and nonprogressive HIV-1 infection (90-92). This suggests that maintenance of highly polyfunctional, virus-specific CD8<sup>+</sup> T cells is beneficial and contributes to effective antiviral immunity.

More than 200 human and mouse VACV- and/or MVA-specific CTL epitopes have been identified covering several different HLA alleles as well as the mouse alleles K<sup>b,d</sup> and D<sup>b,d</sup> (79, 93-108). These epitopes have been found in about hundred different VACV/MVA proteins meaning that half of the VACV/MVA proteome is targeted by the cellular immune response (93). Two thirds of the proteins contain a single epitope, while 10% of the antigenic proteins contain three or more epitopes. This broad epitope repertoire is contrary to smaller genome viruses which exhibit marked immunodominance (109).

Although a high number of CTL epitopes has been identified by many different research groups, there was only little overlap between the studies (93, 110). Several reasons could explain this observation: first, most groups attempted to narrow their focus to a selected group of antigens; second, different virus strains (permissive and non-permissive), different model systems (humans and HLA-transgenic mice (110)), and different methods (T cell screening by IFN- $\gamma$  ELISPOT, Tetramer-staining, intracellular cytokine staining and HLA ligand analysis) were used for the analyses; third, vaccinations modes (prime and boost), routes of infection, virus dose and time

points of analysis after vaccination were inconsistent (93). For example, analyses using permissive versus non-permissive strains, prime- versus boost-vaccinations, T cell screening versus HLA ligand analysis are likely to detect different percentages of CTL primed by cross-presentation and by direct presentation (111, 112).

### 1.1.3.3 Identification of viral T cell epitopes

Essentially, two basic methodologies are being employed for identification of T cell epitopes, which are either based on identification of epitope-specific T cells in peripheral blood mononuclear cells (PBMCs) of immune donors, or on sequencing of viral MHC ligands isolated from virus-infected cells:

The first method to identify T cell epitopes uses stimulation of PBMC of immune individuals followed by the measurement of the T cell response *ex vivo* or after expansion of low abundant memory T cells by peptide stimulation or of naïve T cells by *in vitro* priming. Analyzed are cytokine production (by intracellular cytokine staining (ICS; e.g. IFN- $\gamma$ ) or by IFN- $\gamma$ -enzyme-linked immunosorbent spot assay (ELISPOT)), cytotoxicity (by chromium ( $^{51}\text{Cr}$ ) release “kill” assay or granzyme B-ELISPOT) or presence of TCR-specific T cells (by tetramer staining) (113-116). Several techniques have been utilized to screen PBMC, including genome wide scanning (117), phage display/DNA libraries (118, 119), combinatorial peptide libraries (120-122) and expression cloning strategies (123). Less PBMC have to be screened by predicting peptides that may bind on a certain MHC allele using computer algorithm-based epitope prediction, for example SYFPEITHI ([www.syfpeithi.de](http://www.syfpeithi.de), (124)) and Bimas ([www-bimas.cit.nih.gov/molbio/hla\\_bind](http://www-bimas.cit.nih.gov/molbio/hla_bind)) (125-128). The bioinformatic algorithm of SYFPEITHI is based on identified amino acids commonly occurring at anchor positions in naturally MHC-presented peptides, whereas the algorithm of Bimas is based on binding studies of synthetic peptides. Using this still quite laborious approach, immunodominant epitopes are detected that are frequent in the population and induce a measurable T cell response *in vitro*. However, detection is often restricted to the most immunogenic viral epitopes. Epitopes resulting from posttranslational modifications or frame shifts are not detected. A more recent development in screening of PBMC is the identification of T cell responses by the loading of HLA tetramers with arrays of peptides using conditional HLA ligands (129). This technique allows for rapid high throughput detection of epitope-specific T cell

responses. Like all other approaches based on flow cytometry, this method enables only detection of frequent CD8<sup>+</sup> T cells (>0.01% of CD8<sup>+</sup> cells).

The second methodology is based on isolation and sequencing of viral HLA ligands from cells infected *in vitro* by mass spectrometry (MS) (130-134). An analytical approach to apply MS for the identification of viral epitopes in the plethora of self-epitopes was first described using an *in silico* subtractive method, which compares the MS spectra from infected cells and non-infected cells (132). The sensitivity of the technology was considerably improved through the use of stable isotopes, for example by the SITE technology (stable isotope tagging of epitopes) for the detection of viral HLA class I epitopes (133) and a similar strategy for identification of class II epitopes (135). SITE is based on metabolic labeling of endogenously synthesized proteins during infection and can therefore not be utilized to identify MHC ligands from infected organisms. MS-based approaches are generally less biased than T most cell-based methods, since no predictions are used and immunogenic epitopes of all viral proteins are detected irrespective of inducing a detectable T cell response in an individual. However, MS analyses imply a technical bias: individual peptides might not be detected by the mass spectrometer and for detection of low abundant viral MHC ligands sensitive instrumentation and high numbers of infected cells may be needed. Yet, epitopes resulting from posttranslational modifications or frame shifts may be detected. Most importantly, only MHC ligand analysis identifies the epitopes, which are actually presented by infected cells and which thus may provide protection from infection.

Mass spectrometry also enabled estimates of the abundance of individual peptides on the cell surface and of the complexity of the overall repertoire. By standardizing against the ion current of known amounts of model peptides spiked into extracts, many MHC-peptides have been estimated at one copy or less per cell (136, 137), at 10 to 400 copies per cell (138, 139), and a single viral peptide from cells infected with measles virus has been found at 100,000 copies per cell (132). The number of peptide-MHC complexes required for T cell recognition varies from several thousand per target cell to as few as one (137, 140-145). Some studies have shown a direct correlation between cell surface densities of individual peptide antigens and the magnitude of the immune response to them (146-150), but other studies have shown exactly the opposite (132, 137, 151). Higher doses of peptide antigen can actually reduce the magnitude of an immune response *in vivo* (132, 152). Finally, there are

examples of self-peptides that become antigens on cancer cells (153, 154), suggesting that the immune response may be initiated because their level of presentation is altered.

Criteria to rate how 'defined' a potential epitope is, which has been found by any of the described approaches, have been proposed by Yewdell (155):

- No star: Responses are measured by overlapping peptides; no effort is made to characterize determinants in a systematic manner.
- One star ★: The highest affinity peptide in a suspected peptide sequence is determined by testing various lengths of synthetic peptides compatible with class I-binding algorithm predictions, and peptides bind to class I molecule with a  $K_A$  of  $<10^{-7}$  M or activate  $CD8^+$  T cells at concentrations of  $<10^{-9}$  M.  $CD8^+$  T cells induced by the peptide are shown to recognize histocompatible cells expressing the source protein. While this evidence provides confidence that cells can generate the determinant from its source protein, it is not definitive, since  $CD8^+$  T cells might still cross-react with another peptide from the same protein.
- Two stars ★★: One-star criteria plus one of the following: peptides eluted from cells co-elute with the synthetic putative peptide via HPLC analysis that offers reasonable discrimination between distinct peptides. Half-star bonus for using multiple solvent systems to increase the resolution of peptide separation, or for demonstrating that  $CD8^+$  T cell recognition of cells synthesizing source protein is abrogated by introducing a missense mutation into the sequence encoding the putative determinant, with another half-star bonus for introducing the mutation into the source pathogen itself.
- Three stars ★★★: Two-star criteria plus mass spectroscopy identification of determinant from source-organism-infected cells.
- Four stars ★★★★: Three-star criteria, but attained using cells directly obtained from animals infected with the source organism.

#### 1.1.4 Anti-tumor immune response

Certain human tumors, particularly melanoma and renal cell carcinoma (RCC), can occasionally undergo spontaneous regression (156, 157). Several observations support the view that CTL are the major anti-tumor effectors in humans. First, immunosuppressed transplant recipients display higher incidences of non-viral tumors, such as melanomas, colon, lung, pancreas, bladder, kidney, and endocrine

system cancers, than immunocompetent control populations (158). Second, the presence of lymphocytes within the tumor is often a positive prognostic indicator of patient survival (159). Third, a minority of cancer patients (<5%) are able to develop spontaneous innate and acquired immune responses to the tumors they bear (160, 161). Therefore, stimulation of tumor-specific CD8<sup>+</sup> T lymphocytes has become the focus of many clinical trials (so called ‘cancer immunotherapy’), in which multiple antigen delivery strategies have been tested in hundreds of patients (summarized in (162)). These trials have demonstrated that vaccines are safe, immunogenic, and yield a low frequency of objective clinical responses. Several immune evasive mechanisms account for a limited effectiveness of endogenous or vaccine-induced immune responses to tumors (Figure 1.7, (163, 164)). Induction of a broad immunity to multiple tumor-associated antigens (TAAs), and activation of persistent T cells may be important. Additional modes of amplifying immune responses (lymphodepletion, cytokine support, inhibition of negative immune self-regulation) are now being tested and should improve clinical responses from 5-10% response seen currently (162). Importantly, CD4<sup>+</sup> T<sub>H</sub> cells have been shown to be required for generating and maintaining potent antitumor immunity (165).

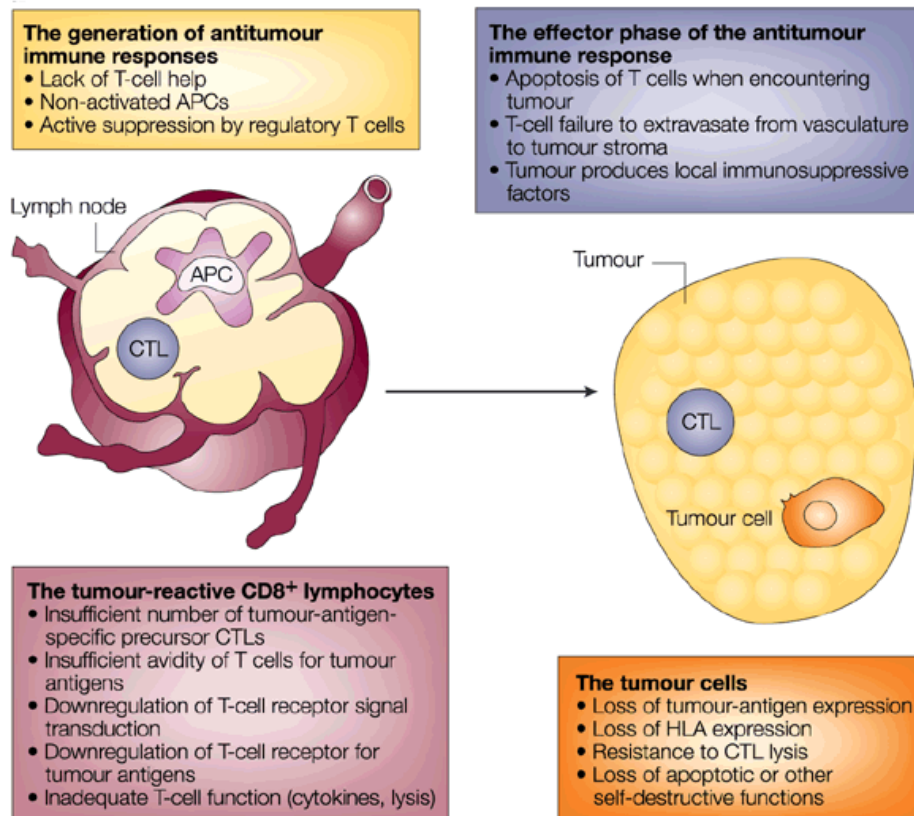


Figure 1.7 Mechanisms that limit immune responses against tumors (163).

#### 1.1.4.1 Tumor-associated antigens

Peptide antigens associated with MHC class I or class II molecules are the molecular targets for T cell recognition of cancer. Since, in 1991, the first molecularly defined human TAA recognized by CTL was described (166), several studies utilizing expression cloning of TAA cDNAs, reverse immunology, HLA ligand analysis, genetic approaches, and serological identification of antigens by recombinant expression cloning (SEREX) have been performed to identify a number of TAAs, which can be presented by HLA class I and II on tumor cells or APCs ((167-171), [www.cancerimmunity.org/peptidedatabase/Tcellepitopes](http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes)). Reverse immunology refers to prediction of epitopes from an already identified TAA on the basis of known HLA-peptide motifs. HLA ligand analysis involves eluting and fractionating of TAA peptides naturally presented by HLA on tumor cells by reverse-phase high performance liquid chromatography (HPLC) and mass spectrometry (MS) (LC-MS/MS). Genetic approaches are used to identify tumor genes coding for the epitopes recognized by isolated patient T cell clones reactive against autologous tumors. SEREX is based on the recognition of TAA by cancer patients' autologous sera. According to the pattern of expression in neoplastic and normal tissues, TAAs can be classified into five major categories: 1) proteins expressed only in testis/placenta (which lack HLA expression), 2) proteins specific for the (tumor) tissue, 3) proteins with altered amino acid sequence, 4) proteins derived from oncogenic viruses (for example, herpesviruses) 5) ubiquitous proteins over-expressed in tumor cells, 6) proteins with tumor-associated/specific alterations in posttranslational modification of amino acid side chains ((162, 172-176), Table 1.1).

For cancer immunotherapy it has to be taken into account that TAAs, which are highly overexpressed in tumor tissue but are also found in at least some normal tissues, bear the risk of inducing autoimmunity in contrast to TAA, which have emerged by mutation. TAAs are generally tumor- and patient-specific. In addition, presentation of epitopes is dependent on the patient's set of HLA alleles. Cancer immunotherapy is therefore a rather individual therapy against tumors.

Most TAAs used for epitope-based cancer immunotherapy are considered to be 'self-antigens'. Therefore, one of the main challenges is to effectively and safely break tolerance to TAA, for example by adjuvants, addition of heterologous helper peptides, inclusion of cytokines, autologous DCs pulsed with different TAAs, adoptive transfer

of *ex vivo* cultured effector T cells, or viral vectors producing a set of epitopes in parallel to viral antigens (162).

**Table 1.1 Tumor Antigen Categories**

TAA category	Antigen characteristics	Genes
Cancer - testis	Expressed in various tumors but not normal tissues except testis and placenta	MAGE, GAGE, BAGE, NY-ESO-1
Differentiation	Shared between tumors and normal tissues from which they arose	Tyrosinase, Melan-A/MART-1, gp100, TRP-1, TRP-2
Tumor-specific	Generated by point mutations or splicing aberrations in ubiquitous genes	p53, Ras, CDK4, $\beta$ -catenin, TRP-2/INT2
Tumor virus	Produced by oncogenic viruses (e.g., HPV)	HPV16 E7
Tumor-associated	Over-expressed in histologically different types of tumors	Survivin, MUC1/2, AFP, EphA2
Posttranslational modification	Containing altered posttranslational modification in malignant cells	glycosylated MUC-1, tyrosinase(D370N), $\beta$ -catenin(pS33), IRS2(pS1100)

One subclass of the sixth category of TAAs (Table 1.1) is special in the way that they are not defined by altered protein expression but by altered cellular signaling, and thereby they are directly linked to cellular growth control processes: proteins differentially modified by posttranslational phosphorylation in transformed cells compared to non-malignant cells (176, 177). Degradation of these proteins generates so called phosphopeptides that are uniquely or differentially presented on malignant cells by MHC class I molecules. CTL against tumor-specific phosphopeptides derived from some of these proteins have been generated and their ability to selectively recognize tumor cells has been demonstrated ((176), see chapter 1.1.4.2). Three observations support an intensified search for phosphopeptide epitopes as TAA candidates for cancer immunotherapy. First, malignant transformation leads to alterations in protein kinase pathways regulating cell growth, differentiation, and cell death (136, 178). Second, deregulated signaling cascades often lead to increased protein phosphorylation (179). Third, rapid protein degradation by the proteasome is an important mechanism for regulating the activity of many transcription factors, cell growth modulators, signal transducers, and cell cycle proteins (171, 180, 181). This



degradation often depends on E3 ubiquitin-ligases whose activity in turn depends on phosphorylation of the target protein (21, 22, 171).

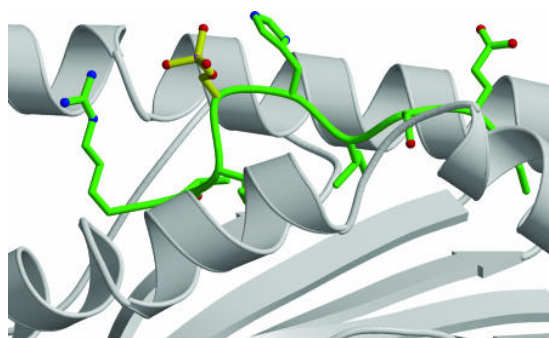
Signalling pathways and hence phosphorylation of proteins might be changed by several ways in the case of cellular transformation. First, up-regulation of phosphorylation at specific sites has been associated with transformation. Certain phosphorylation sites in some proteins become constitutively phosphorylated during oncogene activation, for example, in c-Jun and in the tumor suppressor gene product p53 (182). c-Jun becomes activated by phosphorylation of the two serines S<sup>63</sup> and S<sup>73</sup>, and has been reported to be phosphorylated in both leukemia and lung cancer cells. Phosphorylation of the p53 tumor suppressor protein has been described for a number of serine residues, among these S<sup>9</sup>, S<sup>15</sup>, S<sup>37</sup>, S<sup>315</sup>, S<sup>392</sup>, and S<sup>378</sup> (183, 184). Second, upon the critical event resulting in activation of the transforming phenotype, some protooncogenes characteristically acquire novel kinase activities leading to the appearance of novel phosphorylation sites (185, 186). For example, in leukemia, c-abl protooncogene becomes activated by its translocation to chromosome 22 giving rise to a BCR-ABL fusion protein (136, 187). A crucial difference between the fusion protein and the normal c-abl is that only the chimeric protein exhibits tyrosine kinase activity (185), resulting in autophosphorylation of a number of tyrosine residues of the BCR-ABL fusion protein (136, 188), as well as causing a constitutive increase in tyrosine phosphorylation of other cellular proteins. Third, transformation-associated posttranslational modification of a cellular protein might alter the pattern of peptide fragments that are generated from it.

#### **1.1.4.2 HLA ligands with differential posttranslational modifications as CTL inducers - phosphopeptides**

Only little is known about the effect of naturally occurring posttranslational modifications of peptides on MHC class I restricted antigen presentation *in vivo*. They may affect antigen processing, MHC binding, and interaction with the TCR. So far, it has been described that T cells can specifically recognize MHC class I and class II restricted peptides which have been posttranslationally modified (189-191). MHC class I presented peptide modifications include change of asparagine to aspartate (175), modification of cysteine (192), glycosylation of serine (174, 193, 194), and phosphorylation of serine, threonine or tyrosine (176, 195, 196).

It has been shown, that phosphorylated peptides can be transported by TAP, can bind to MHC class I molecules, and can be discriminated by CTL from the corresponding unphosphorylated ligands (195). Naturally processed HLA class I ligands have been identified from several cell lines by LC-MS/MS (196), and CTL against HLA-presented phosphopeptides specifically found on tumor cell lines have been detected (176).

Consistent with the ability of CTL to discriminate between phosphorylated and non-phosphorylated peptides, molecular modeling of one of the HLA-A\*0201 phosphopeptide complexes suggests that the phosphate moiety is accessible for the TCR (Figure 1.8, (177)). Moreover, the prominence of this functional group and its electronegativity may make it an effective immunogen eliciting a diverse CTL response. Whereas some phosphopeptides are involved in TCR contacts, others may enhance binding to the HLA. The solution of HLA-bound phosphopeptide structures in complex with specific T cell receptors has not been shown so far.



**Figure 1.8** Structural model of HLA-A\*0201 complexed to the phosphorylated peptide RTLpSHISEA (177). The phosphate moiety points away from the HLA binding groove.

Phosphorylated HLA ligands are of great interest, since phosphorylation of tyrosine, serine, and threonine residues (Figure 1.10a) by cellular kinases is a tightly regulated posttranslational cytosolic event, which can be deregulated by inflammation, intracellular infection, cellular activation, and malignant transformation. This may result in the generation of sufficient amounts of phosphopeptides for presentation on HLA to induce T cells (see 1.1.4.1). Moreover, a large number of phosphorylated viral proteins have been characterized, which also might generate phosphopeptide fragments for HLA presentation. Viruses with multiple known protein phosphorylation sites include EBV, adenovirus, HIV, and influenza virus (197-199).

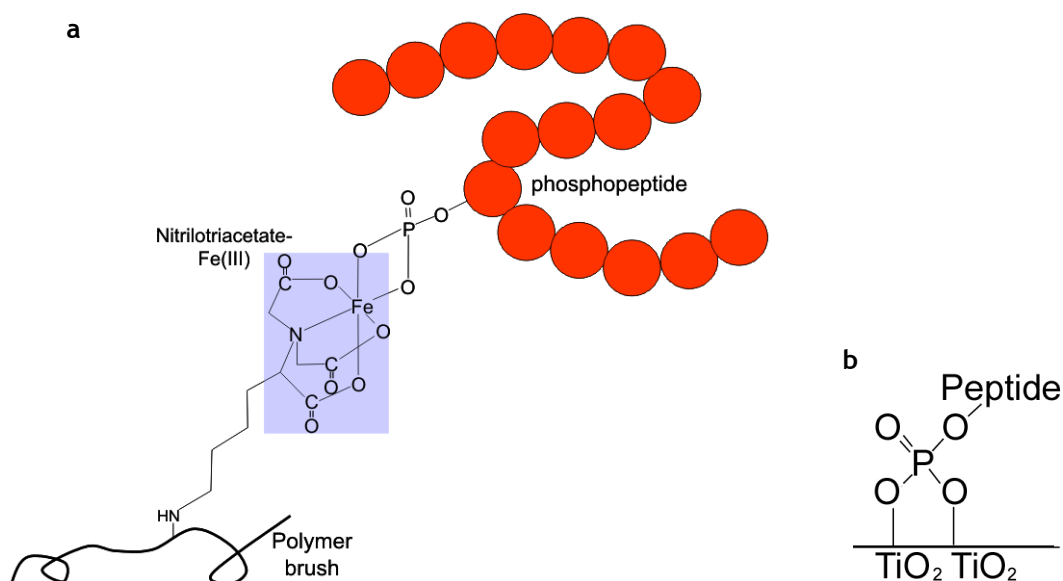
Several phosphorylation sites of proteins and their functional roles are known ([www.phosphosite.org](http://www.phosphosite.org)). The identified phosphorylated HLA ligands have added several novel ones to the list (176, 196).

### 1.1.4.3 Identification of phosphorylated HLA ligands

Stoichiometry of protein phosphorylation is often low. Therefore, in a standard LC-MS/MS experiment, the chance of detecting and sequencing phosphopeptide MHC ligands is quite low. Phosphopeptide enrichment is consequently a prerequisite to study phosphorylation on a global level.

Several phosphopeptide enrichment methods have been developed in the field of proteomics (200): 1) Antibodies raised to phosphorylated residues that are independent of the surrounding sequence can be used to purify phosphorylated proteins and peptides (201, 202). 2) Immobilised metal affinity chromatography (IMAC) is the most widely used method for enriching phosphorylated peptides or proteins (203). The positive charge of transition metals such as  $\text{Ga}^{3+}$  or  $\text{Fe}^{3+}$  bind negatively charged phosphate groups with high affinity (Figure 1.9a) and elution is readily achieved by competition with sodium phosphate or EDTA. 3) Metal oxide-affinity purification (MOAC) is particularly performed with titanium dioxide ( $\text{TiO}_2$ ) (204) (zirconium and aluminum metal oxides are also applied (205, 206)) used in the form of porous  $\text{TiO}_2$  beads or microspheres (Figure 1.9b). Phosphopeptides bind at low pH. Specificity has been further improved by adding 2,5-dihydroxybenzoic acid (DHB). Elution is achieved at high pH with ammonium bicarbonate or hydroxide (207). 4) Strong cation exchange chromatography (SCX) separates proteins or peptides based on their solution charge state. At low pH, phosphopeptides are charged more negatively because of the negative phosphate group and therefore elute earlier in a salt gradient than non-phosphopeptides (208). Phosphorylated MHC ligands have been enriched by  $\text{Fe}^{3+}$ -based IMAC, so far (176, 196).

In addition to these native phosphorylation strategies, several chemical derivatisation strategies have been developed exploiting various aspects of phosphate groups, such as their labile nature and the subsequent reactivity of the dephosphorylated group to nucleophiles, for tagging (200, 209). Other chemistries such as methyl-esterification were developed to improve the specificity of metal affinity chromatography (210). Additionally, methyl-esterification may be used to isotopically label peptides for relative quantification purposes (209, 211).

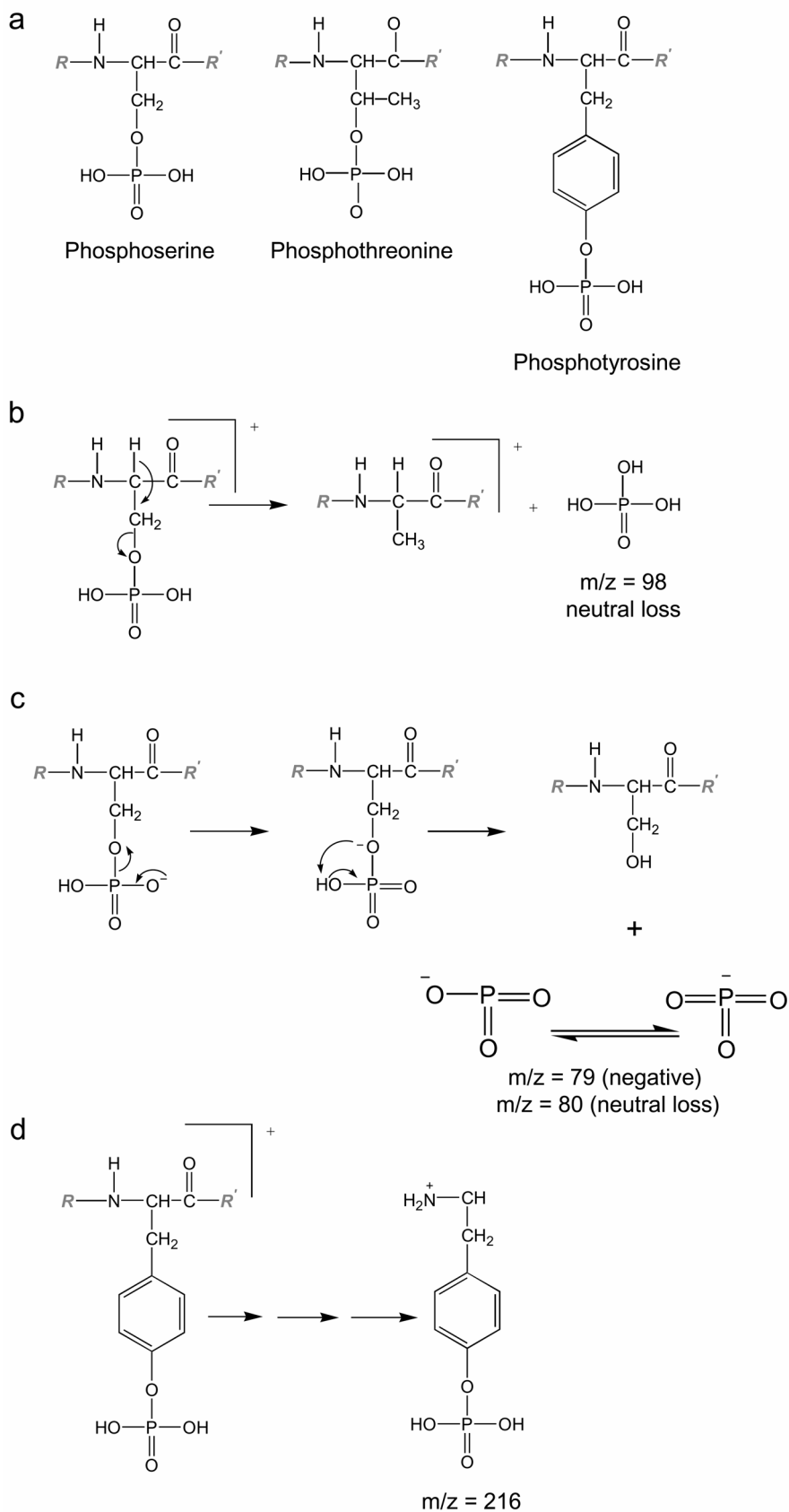


**Figure 1.9 Phosphopeptide enrichment.** a)  $\text{Fe}^{3+}$ -based IMAC (203); b)  $\text{TiO}_2$ -based MOAC (207)

Phosphorylation of amino acid side chains often shows greater susceptibility to cleavage by collision-induced fragmentation (CID) in MS than the peptide backbone (200). This characteristic may be used in different analytical strategies: 1) Detection of the low mass ‘marker’ ions generated from the modification itself, 2) Detection of the loss of the modification from the peptide precursor.

In positive ionisation CID MS/MS experiments, phosphopeptide ions are labile and thus produce a significant neutral loss of phosphoric acid ( $\text{H}_3\text{PO}_4$ ), corresponding to mass depletion of 98 Da, via gas-phase  $\beta$ -elimination from phosphoserine and phosphothreonine residues (200), and less often a neutral loss of  $\text{HPO}_3$  corresponding to mass depletion of 80 Da (Figure 1.10b, c). The number of phosphate groups on the phosphopeptide can be determined from the frequency neutral loss occurs from the precursor ion. Unique product ions of dehydroalanine (69 Da) from phosphoserine (pS), and dehydroaminobutyric acid (83 Da) from phosphothreonine (pT) are generated (200).

Techniques such as precursor ion monitoring, neutral loss monitoring and neutral loss triggered  $\text{MS}^3$  fragmentation are frequently applied in phosphopeptide MS-analysis (200). Phosphotyrosine (pY)-containing peptides yield a characteristic immonium ion at  $m/z$  216.04 in the positive ion mode (Figure 1.10d). When phosphopeptides are fragmented in the negative ion mode, a characteristic product ion ( $\text{PO}_3^-$ ) is generated giving rise to a peak at  $m/z$  79 in the product spectrum (Figure 1.10c). These two precursor ion-scanning modes are most commonly implemented on triple quadrupole mass spectrometers.



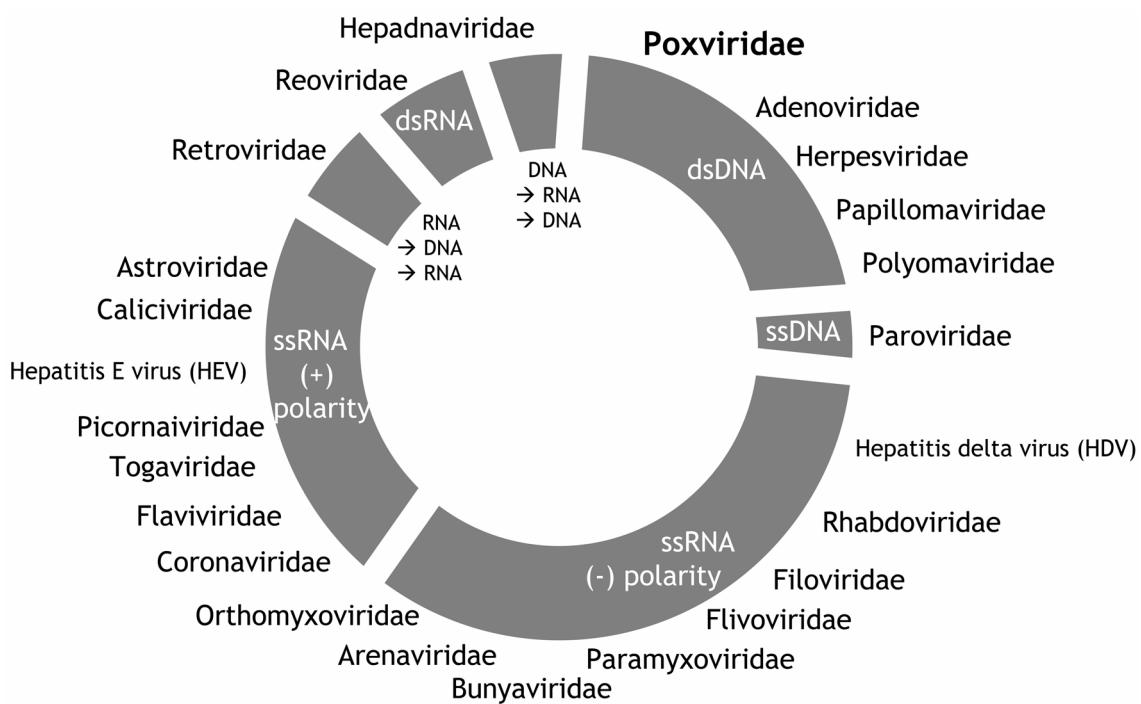
**Figure 1.10 Phosphopeptide characteristics.** a) Phosphorylated amino acids; b) Neutral loss of 98 Da from phosphoserine (equivalently for phosphothreonine, not shown); c) Generation of the negative precursor ion -79 Da / neutral loss of  $\text{HPO}_3^-$ , 80 Da; d) Phosphotyrosine immonium ion generated by a combination  $\alpha$ - and  $\gamma$ -type-cleavage.

## 1.2 Vaccinia Virus

### 1.2.1 Poxviridae - Orthopoxviruses

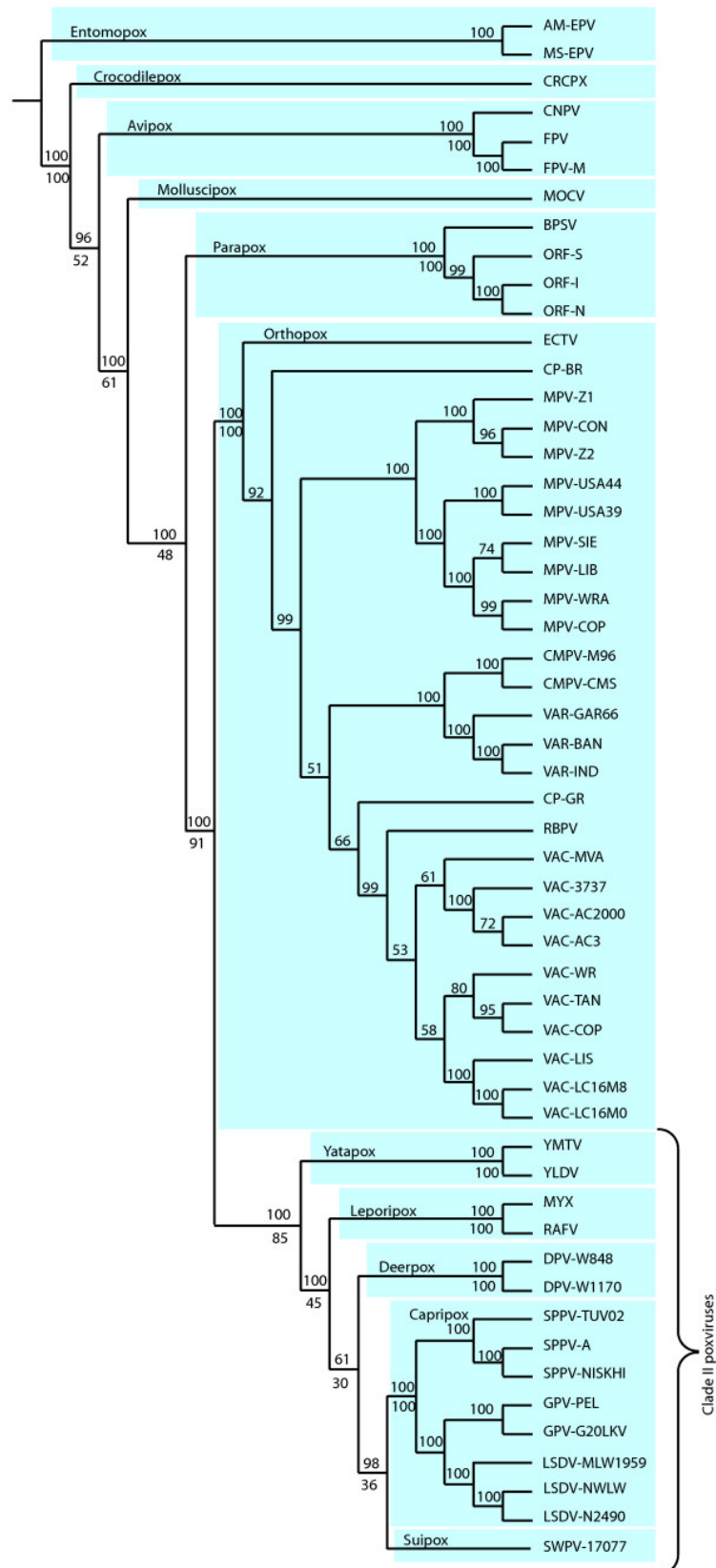
#### 1.2.1.1 Classification

The *Poxviridae* comprise a family of complex DNA viruses that replicate entirely in the cytoplasm of vertebrate or invertebrate cells (Figure 1.11).



**Figure 1.11 Taxonomy of main viruses infecting humans** (based on the International Committee on Taxonomy of Viruses Database (ICTVdB))

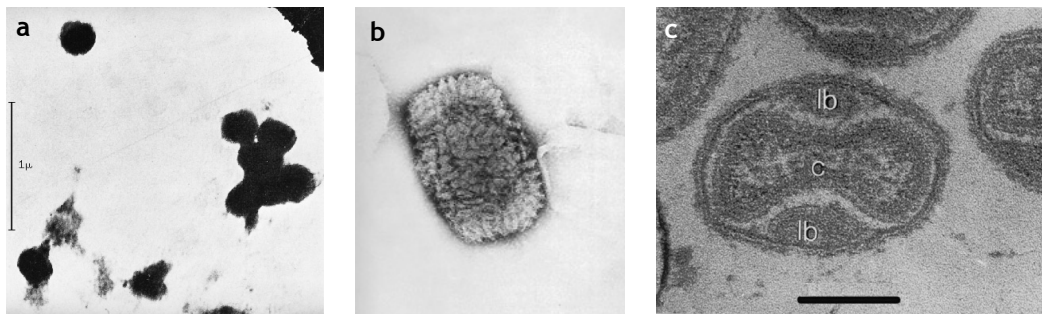
The distinguishing properties of the family *Poxviridae* include a cytoplasmic site of replication and a large complex virion, which contains enzymes that synthesize mRNA and a genome composed of a single linear dsDNA molecule of 130 to 300 kb containing nonoverlapping genes and a hairpin loop at each end. Poxviruses are divided into the subfamilies *Chordopoxvirinae* and *Entomopoxvirinae*, based on vertebrate and insect host-range (Figure 1.12). DNA sequencing and bioinformatic analysis confirm the genetic relationship between the poxvirus subfamilies.



**Figure 1.12 Poxvirus phylogenetic tree as described in (212):** Branch lengths are not to scale. Taxonomic groups are labelled and shaded. The term "clade II" poxviruses is refers to the group of yatapox, deerpox, capripox and suipox, as per convention (213). Numbers above branches are percent bootstrap values, numbers below the branches indicate the percentage of gene trees that supported the branch.

The subfamily of *Chordopoxvirinae* consists of eight genera: *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Caripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus*. Members of the same genus are closely related genetically and have a similar morphology and host-range. Two members of this family, smallpox causing variola virus (VARV) and the dermatotropic molluscum contagiosum virus (MOCV), are obligate human pathogens, but others can be transmitted from animals to humans as zoonoses.

Orthopoxviruses have been studied intensively. The prototype orthopoxvirus vaccinia virus (VACV) has been propagated as the smallpox vaccine for 200 years. VACV has a broad cellular tropism *in vitro* and potential host-range *in vivo*, but there is no clearly identified animal reservoir for the virus in nature. VACV was the first animal virus seen microscopically (Figure 1.12), grown in tissue culture, accurately tittered, physically purified, and chemically analyzed.



**Figure 1.13 Poxvirus morphology.** a) First microscopic documentation of VACV (214); b) Electron microscopic image of VACV (215); c) Thin section of CPXV (electron microscopy by Frank Fenner). lb, lateral bodies; c, core; bar represents 100 nm

DNA sequencing reveals that genes common to VARV, ectromelia virus (ECTV), camelpoxvirus (Figure 1.14, (215)), VACV, and cowpoxvirus (CPXV) are greater than 90% identical. CPXV contains all genes present in other orthopoxviruses, suggesting that it most closely resembles the ancestral member of this genus, whereas the other orthopoxviruses have lost genes.



**Figure 1.14 Examples of host-restricted poxviruses (215).** Some poxviruses, like variola virus (smallpox) (a), ectromelia virus (mousepox) (b) or camelpoxvirus (c) remain largely restricted to one host species and rarely, if ever, cause zoonotic infections outside that species. Other poxviruses can infect multiple zoonotic host species.



### 1.2.1.2 Virion structure

Virions of poxviruses are large compared to other animal viruses (360 x 270 x 270 nm) (Figure 1.13b). They are generally brick-shaped, or pleomorphic and consist of a lipoprotein structure, the outer membrane, a biconcave core, and lateral bodies (Figure 1.13c). During replication, two infectious forms of virus are produced: intracellular mature virion (IMV) and extracellular enveloped virion (EEV), which acquires an additional envelope composed of host cellular membrane and virus-specific proteins.

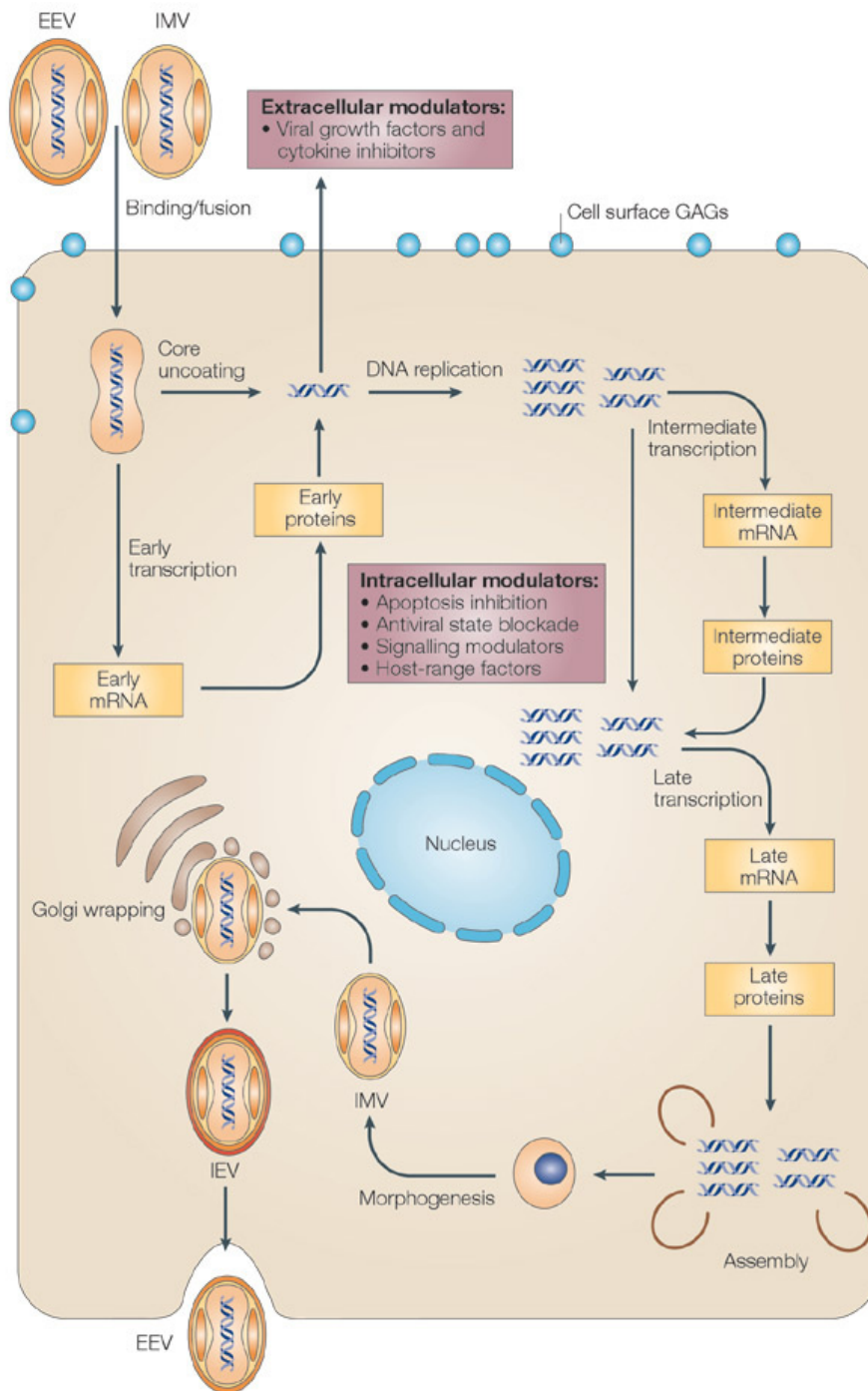
### 1.2.1.3 Virus entry into host cells

VACV mature virions (MVs) use macropinocytosis and apoptotic mimicry to enter host cells (216): Virus particles move along actin-containing filopodia to the cell body, where they are internalized after inducing the extrusion of large transient membrane blebs. The induction of blebs, the endocytic event, and infection are all critically dependent on the presence of exposed phosphatidylserine in the viral membrane. Surface-exposed cellular phosphatidylserine triggers the uptake of cellular apoptotic debris (217). Late-stage vaccinia-infected cells undergo apoptosis. MV spread is therefore likely to be connected with apoptosis and a preprogrammed macropinocytic response of neighboring cells to apoptotic bodies. This allows the virus to enter many different cell types, because phosphatidylserine-mediated clearance of apoptotic material is common to most cells (217, 218). By mimicking an apoptotic body, MVs may avoid immune detection as they spread to surrounding cells, because macropinocytosis of apoptotic debris suppresses the activation of innate immune responses (218).

### 1.2.1.4 Virus replication

The virus replicates and matures in the cytoplasm of the host cell within so called viral factories, distinct sites that are surrounded by membranes derived from the rough ER that support viral replication (Figure 1.15, (215)) (219, 220). Fully permissive viral replication is characterized by three waves of viral mRNA and protein synthesis (known as early, intermediate and late), which are followed by morphogenesis of infectious particles (221). This type of genetic programming is known as a cascade mechanism, because the products of each stage regulate the next. A complete early transcription system is present within the core of the virus particle, providing a mechanism for the synthesis of viral early mRNA soon after infection and accounting

for the fact that purified poxvirus DNA is not infectious. VACV encodes its own enzymes and proteins required for gene transcription, genome replication, virion production, and morphogenesis and, for the most part, does not depend on host cell proteins for these processes. In addition, VACV infection induces a rapid and massive shutdown of host gene expression that acts at several levels (222-225).



**Figure 1.15 Fully permissive poxvirus replication cycle (215).** EEV, extracellular enveloped virion; IMV, intracellular mature virion; IEV, intracellular enveloped virion

Following entry into the cytoplasm, virus cores are transported on microtubules to sites of transcription, and mRNA transcription is detected within 20 minutes (226). After the core is released into the cytoplasm, early mRNAs encode growth factors, immune defense molecules and enzymes and factors needed for replication of the viral DNA and for transcription of the intermediate class of genes (221). About half of the viral genome is transcribed prior to DNA replication (227). The core is uncoated and DNA replication begins 1-2 hours after infection and results in the generation of about 10,000 genome copies per cell (228). Following DNA replication, intermediate gene transcripts encode enzymes and factors for late gene expression. Products of the late genes form virion structural proteins, enzymes including RNA polymerase, and early transcription factors (221).

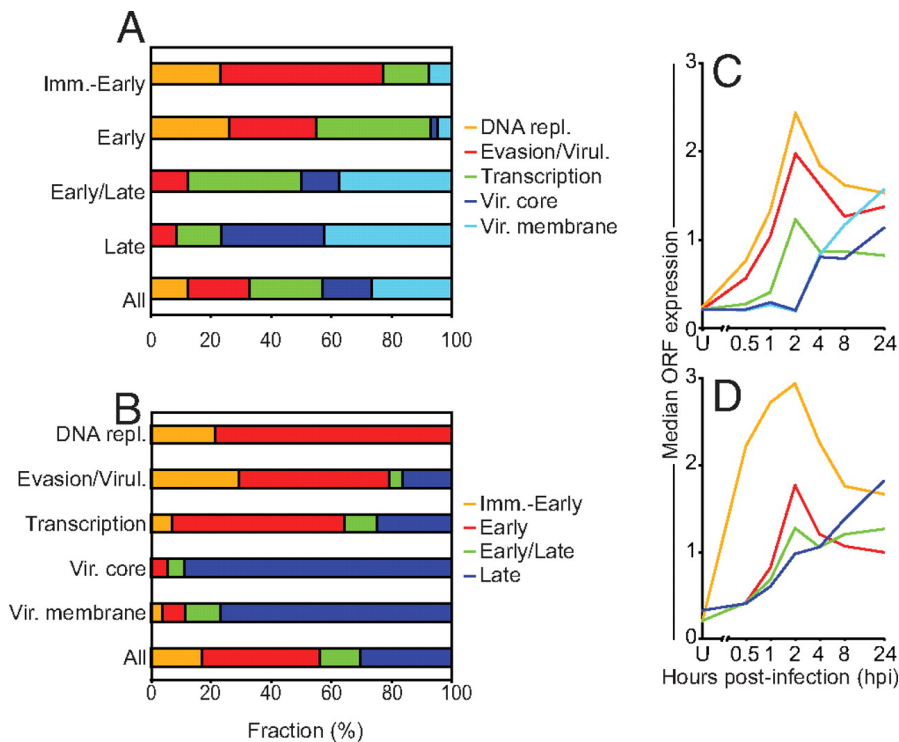
Assembly begins with the formation of discrete membrane structures (229). The concatemeric DNA intermediates are resolved into unit genomes and are packaged in immature virions. Maturation proceeds to the formation of infectious IMV. These are wrapped by modified trans-Golgi and endosomal cisternae and are then transported to the periphery of the cell along microtubules. Fusion of the wrapped virions with the plasma membrane results in release of EEV. After fusion with the plasma membrane, stimulation of actin tails beneath extracellular virion particles acts to enhance cell-to-cell virus spread (230).

#### **1.2.1.5 Gene expression**

Expression kinetics have been described for a variety of the 223 annotated VACV genes. These studies, and work done to define promoters and transcription complexes, have led to the definition of four temporal gene classes and three distinct promoter types. The promoters have been named early, intermediate, and late, with each promoter associated with one gene class (221). In addition, some genes have elements of early and late promoters in their upstream region, giving rise to a fourth class referred to as early/late.

A recent analysis of expression kinetics using a genome tiling array approach has revealed another class of genes: 35 genes of mostly unknown function exhibited immediate-early expression (Figure 1.16, (231)).

All poxvirus genes consist of a continuous open reading frame (ORF) and there is no evidence of RNA splicing (221).



**Figure 1.16 Function and expression levels of genes in the different classes (231).** A) The four gene classes with the fraction (%) of each class having a certain function indicated. B) Groups of genes associated with a specific function, with the fraction (%) of each group belonging to each kinetic class indicated. C, D) Median ORF expression at each time point for genes within each functional category (C) and kinetic class (D).

### 1.2.2 Smallpox Disease and its eradication by VACV-based vaccination

Smallpox disease is believed to have originated over 3,000 years ago in India or Egypt. For centuries, repeated epidemics swept across continents, decimating populations and profoundly influencing human history.

The disease is caused by VARV and was a febrile rash illness and had two main forms: variola major and variola minor (World Health Organization (WHO), [www.who.int/mediacentre/factsheets/smallpox/en/](http://www.who.int/mediacentre/factsheets/smallpox/en/)), both forms showing similar lesions. The disease followed a milder course in variola minor, which had a case-fatality rate of less than 1%. The fatality rate of variola major was around 30%. Between 65–80% of survivors were marked with deep pitted scars (pockmarks), most prominent on the face. Blindness was another complication. No effective treatment was ever developed against the disease.

In 1798, the English physician Edward Jenner reported the observation, that infectious agent, which caused lesions on the skin and mucosal surfaces on cows and their human caretakers could be used to prevent smallpox infection (232). This process,

termed variolation, involved the intentional introduction of dried CPXV induced pus/scabs into healthy individuals and led to a more benign course of disease with a death rate of approximately 1%. This was the beginning of both the eventual eradication of VARV and of vaccination in general. The vaccination procedure was later refined so that people were inoculated with pure preparations of live VACV, an orthopoxvirus of unknown origin, closely related to VARV and CPXV, and containing sufficient antigenic cross reactivity to provide protection from VARV. In 1977, through a world-wide vaccination campaign headed by the WHO, VARV was declared eradicated (233, 234). The seed virus (VACV strain Lister Elstree) used to produce the vaccine is being held for WHO by the WHO Collaborating Centre for Smallpox Vaccine in Bilthoven, the Netherlands.

Vaccination usually prevents smallpox infection for at least ten years (WHO). If symptoms appear, they are milder and mortality is less in vaccinated than in non-vaccinated persons. Even when immunity has waned, vaccinated persons shed less virus and are less likely to transmit the disease.

#### **1.2.2.1 Adverse effects of VACV vaccination**

VACV-based smallpox vaccination has some adverse effects (235-238). Historically, 14-52 people per million primary vaccinees had serious or life-threatening adverse reactions to the vaccine, and one to two people per million died because of the vaccine. Serious side-effects included inadvertent inoculation, eczema vaccinatum, progressive vaccinia, fetal vaccinia, generalized vaccinia, and erythema multiforme major. In particular, VACV occasionally infects the brain and causes postvaccinal encephalitis, which can be lethal or result in permanent brain damage.

Consequently, as smallpox became rarer, the dangers of vaccination began to outweigh its benefits. Routine smallpox vaccination stopped in the US in 1972, and in 1980 the World Health Organization recommended that all countries stop vaccination.

#### **1.2.2.2 Inflammatory autoimmune myocarditis**

Individual cases of cardiac complications, including myocarditis, pericarditis, and arrhythmias were reported in the four decades before routine vaccination was stopped, usually diagnosed after death, since cardiac enzymes and echocardiograms used to diagnose myocarditis today were unavailable in the 1960s and 1970s (239, 240). Most reports were from Europe and Australia, where the VACV strain Lister

Elstree was used, which was thought to be more “reactogenic” than the New York City Board of Health (NYCBOH) strain (Dryvax<sup>®</sup>; Wyeth Laboratories Inc, Marietta, Pa) used in the US (235).

However, during a Dryvax<sup>®</sup>-based smallpox vaccination campaign in the US started in December 2002 (see chapter 1.2.3.1), the frequency of adverse events that was anticipated on the basis of historical data was lower than expected, but there were higher-than-anticipated vaccination-related myopericarditis cases, which led to much publicity of and controversy about the program (134, 236, 241). Autoimmune eosinophilic-lymphocytic myocarditis could be diagnosed *in vivo* by biopsy shortly after vaccination providing histological evidence for eosinophil-mediated cardiac myocyte necrosis (242). The lymphocytic component consisted mainly of CD3<sup>+</sup> T cells, of which about 25% were CD8<sup>+</sup>. PCR showed that myocytes were virus-free.

### **1.2.3 The impact of Orthopoxviruses today**

#### **1.2.3.1 The new fear of smallpox disease - vaccine update**

By the end of 1983 all known remaining stocks of VARV were held in only two WHO Collaborating Centres - the U.S. Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, and the Research Institute for Viral Preparations in Moscow, Russia (in 1994, the Russian stocks were transferred to Kotsovo, Russia) (233). After 9/11 and the anthrax letters, however, there is concern that VARV may exist outside the WHO designated repository laboratories and may be used as a bioweapon (243). If this happened, exposed individuals and their contacts, possibly even whole populations, would have to be vaccinated as quickly as possible, since today very few people are probably fully protected against smallpox (244, 245).

Even if VARV never again infects humans, there are other poxviruses that can cause serious human disease. In 2003, an outbreak of human monkeypox occurred in the US due to the inadvertent importation of monkeypox virus (MPXV) in a shipment of rodents from west Africa (246, 247). An even more pathogenic variant resulting in mortality rates of 10–15% is found in central Africa (113, 248, 249). The animal reservoir for MPXV in Africa remains unknown, although several indigenous members of the squirrel species are likely candidates, but the features that predispose this virus to zoonotically infect man and other primates are unknown. If MPXV were

to establish a reservoir status in a susceptible north American rodent species, such as prairie dogs (250), the public health consequences would be considerable.

For these eventualities, many countries still have stockpiles of first-generation calf-lymph-derived smallpox vaccines, but these contain different VACV strains causing different frequencies of adverse effects (236, 238).

In October 2002, the US Department of Defense recommended smallpox vaccine for eligible volunteers whom public health authorities might designate to investigate initial smallpox cases. Then, in December 2002, a large-scale smallpox vaccination program, mainly involving army and health care personnel, but also interested civilians, has been started (134). The program is still ongoing with more than 1 million having been vaccinated so far. Unexpected issues, such as the development of vaccination-related myopericarditis were discovered during the implementation of this program (134, 241, 251).

The smallpox vaccines currently licensed in the USA and UK are live, attenuated VACV derived from calf lymph (Dryvax<sup>®</sup>, last produced in 1982 by Wyeth), a first-generation vaccine (238). Although this type of vaccine was used to eradicate smallpox worldwide, previous manufacturing methods using calf lymph are no longer acceptable because of the absence of controls in the process and the potential risk of contamination with the infectious agent associated with the prion disease bovine spongiform encephalitis.

Therefore, a second-generation cell-cultured smallpox vaccine (CCSV) produced by using the NYCBOH or Lister-Elstree VACV strains have been developed and seem to be a safe and immunogenic alternative to the first-generation calf-lymph derived vaccine for both VACV-naïve and non-naïve people (134, 252, 253).

However, the serious adverse effects of VACV vaccination prevent the widespread use of a VACV-based vaccine in a civilian population without an outbreak. Furthermore, the vaccine is contraindicated in up to 30% or more of the population, including infants, pregnant women or women who are breastfeeding infants, the immunocompromised, those with eczema or exfoliative skin disorders, people who live in the same house or are in intimate contact with people with the above conditions, and people with cardiovascular conditions (such as a history of myocardial infarction, angina, congestive heart failure, cardiomyopathy, stroke or transient ischaemic attack, chest pain or shortness of breath with activity, or any cardiac condition under the care of a physician) (US Centers for Disease Control and

Prevention, [www.bt.cdc.gov/agent/smallpox/vaccination/contraindications-clinic.asp](http://www.bt.cdc.gov/agent/smallpox/vaccination/contraindications-clinic.asp); (251, 254)).

A third-generation vaccine based on modified vaccinia virus Ankara (MVA), a highly attenuated replication-deficient strain of VACV, is currently being tested (89, 243, 255, 256). MVA was attenuated by Mayr *et al.* towards the end of the campaign for the eradication of smallpox in 1975 by more than 570 passages of the VACV strain chorioallantois vaccinia virus Ankara (CVA) in chicken embryo fibroblasts (CEF) (257). Thereby, MVA has lost about 15% of the CVA genome (ca. 30 kb) including several host-range genes and with it the ability to replicate efficiently in primate cells; limited replication has been demonstrated in certain mammalian cell lines, like BHK and BS-C-1 cells (258-262). The 193 ORFs mapped in the MVA genome (178 kb) probably correspond to 177 genes, 25 of which are split and/or have suffered mutations resulting in truncated proteins particularly affecting the host interactive proteins, but also involving some structural proteins. MVA no longer encodes many of the soluble inhibitors of cytokine and chemokine function as well as other factors that play a role in immune evasion. Its safety and its ability to protect against the development of poxvirus infections in several animal models has been demonstrated (263-265). MVA was used in almost 120,000 Caucasian individuals with no reported side effects, although many of the subjects were among the population with high risk of developing complications (263).

### **1.2.3.2 Modified vaccinia virus Ankara as viral vector**

Viruses are natural gene delivery systems inducing immune responses and have been developed as such (for review see (266)). Several features have made poxviruses highly attractive for use as viral antigen delivery systems: the capacity to stably carry up to 25 kb of recombinant DNA (267), precise virus-specific control of target gene expression, lack of persistence or genomic integration in the host, wide tropism, high immunogenicity as vaccine, no yet identified interference mechanisms with host cell antigen processing or presentation, and ease of vector and vaccine production (268-270). Even if there might exist low preexisting anti-vector immunity in formerly vaccinated individuals (245), this appears to be a less significant problem (271).

In 1982, it was first shown that genes coding for immunogenic proteins can be inserted into VACV DNA without impairing the ability of the virus to grow in tissue culture (272). Animals infected with these recombinant VACV containing genes



coding for a variety of immunizing proteins were protected against challenge infection with the corresponding infectious agent (Hepatitis B virus (273), rabies (274), malaria (275)).

Concerns about the safety of VACV as viral vector have been addressed by the development of non-replicating vectors (268, 276), such as MVA (see chapter 1.2.3.1, (269-271)), other highly attenuated strains of VACV (e.g. NYVAC, a derivative of VACV strain Copenhagen, from which 18 ORFs were specifically deleted (277)) and avipoxvirus vectors being replication-competent only in avian tissue (278).

Because of their well-established safety (see chapter 1.2.3.1, (263)), recombinant MVA are today among the most promising live viral vector systems for humans, capable of evoking potent cellular and humoral immune responses against their insert immunogens, especially when used in a DNA-prime / virus-boost immunization schedule (270, 279, 280). Despite its limited replication, MVA provides similar levels of recombinant gene expression as replication-competent VACV in human cells (269), and levels of antibody and T cell responses, and protection proved to be at least equal to that induced by recombinant VACV in the same systems. The enhanced immunogenicity of recombinant MVA may largely be attributed to the deletion of certain immune evasion genes (258, 259).

Numerous phase I/II clinical trials with recombinant MVA as vaccine against infectious diseases, such as HIV, malaria, or tuberculosis, and tumors have been performed or are under way (95, 279-288).

### **1.2.3.3 Orthopoxviruses as oncolytic viruses**

Oncolytic virotherapy is an emerging biotherapeutic platform based on genetic engineering of viruses capable of selectively infecting and replicating within cancer cells (289-291). Although VACV shows no specific preference to bind and infect transformed cells, several studies have shown increased viral replication levels in tumors (292), and a VACV strain with deletions of the genes that encode thymidine kinase and the vaccinia growth factor showed preferential replication in rapidly growing tumor cells while becoming attenuated for overall virulence (293, 294). VACV has many of the features thought necessary for an effective oncolytic virus: a short life cycle, rapid spread, strong lytic ability, well-defined molecular biology (295). The ability to insert multiple genes into poxviruses in order to increase their therapeutic potential or to assist in the virus' visualization is further a great advantage

of poxvirus-based vectors (292, 296). Importantly, a broad spectrum of clinical experience has been obtained with VACV. The virus produces no known disease in humans, and has already demonstrated anti-tumor efficacy in trials with vaccine strains (295).

Although attempts have been made to target VACV binding to specific cell types by engineering virion surface proteins that mediate host cell binding (297), such attempts have never circumvented the ability of the virus to bind to and enter mammalian cells promiscuously. It is likely that future use of oncolytic poxviruses will involve exploiting the signaling differences between normal and transformed cells so that the oncolytic virus will spread efficiently in tumor cells, as well as deliver therapeutic transgenes to assist in tumor killing and immunotherapy (215, 297, 298).

Other poxviruses have also been tested as oncolytic viruses, for example, myxoma virus, which is normally restricted to non-human cells, but replicates nevertheless robustly in human tumor cells (299, 300).

### 1.3 Aims of thesis

Viral and phosphorylated HLA ligands are difficult to identify in the multitude of self- and non-phosphorylated HLA ligands, respectively. Low abundance of these special peptides compared to other ligands may be a further complication. Both subsets of peptides require additional strategies for their identification. The aim of this thesis was to establish techniques which enable the specific analysis of the two HLA ligand repertoires, viral HLA class I ligands (Part I) and phosphorylated HLA class I and II ligands (Part II), and which avoid analysis of the complete HLA ligand repertoire of cells.

Questions to be answered by this thesis were:

Part I:

- Which HLA class I ligands are presented by MVA-infected cells?
- Are these ligands immunogenic? Do they provide protection against viral infection?
- Is there a correlation between presentation of viral HLA ligands and the steady state amount of viral intracellular protein?
- Does MVA-infection induce presentation of individual human self-HLA ligands?

Part II:

- Which phosphorylated HLA ligands are presented by tumor tissue (renal cell carcinoma)? Are the phosphorylations contained in these ligands described to be associated with malignant cellular processes?
- Does the tumor present phospholigands which are lacking on corresponding healthy renal tissue?
- Are phosphorylated peptides presented by HLA class II?

## 2 Materials and Methods

TFA, acetonitrile, formic acid, HPLC water, DMSO, and  $\beta$ -mercaptoethanol were from Merck (Darmstadt, Germany).

### 2.1 Materials and Methods of Part I

#### 2.1.1 Cell lines and antibodies

The human B lymphoblastoid cell line (B-LCL) JY expressing HLA-A\*0201 and B\*0702, (ECACC Cat.no. 94022533) and the human HLA-A\*0201 transfected CML cell line K562/A\*0201 (301) were used as described (113) and maintained in RPMI 1640 (C.C.Pro, Neustadt, Germany) containing 10% FCS (Pan Biotech, Aidenbach, Germany) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Bio Whittaker, Verviers, Belgium).

IgG2a antibodies B1.23.2 (anti-HLA-B, C) (302) and W6/32 (anti-HLA-A, B, C) (303) were purified from hybridoma supernatants using protein A Sepharose beads (GE Healthcare, Uppsala, Sweden).

#### 2.1.2 Virus

MVA was routinely propagated and titrated following standard methodology as described (304) by Wolfgang Kastenmüller (Institute of Virology, Technical University Munich and Helmholtz Center Munich).

For infection with MVA,  $1.4 \times 10^{10}$  JY cells were incubated at  $2.8 \times 10^{10}$  cells/l with MVA to obtain a multiplicity of infection of 7. After 2 h, infected cell suspensions were diluted to  $5.2 \times 10^9$  cells/l and maintained for 10.5 h at 37°C.

#### 2.1.3 Donors

Donors 1 and 2 (HLA-A\*0201 positive) were immunized twice with MVA in an interval of 30 d. Blood was taken as indicated in Figure 3.4. This was done by Ingo Drexler (Institute of Virology, Technical University Munich and Helmholtz Center Munich). Dryvax<sup>®</sup> vaccinees were HLA-A\*0201 positive and blood samples were taken

25 y, 29 y, and 44 y post vaccination for donors 3, 4, and 5, respectively. PBMC were isolated using Ficoll density centrifugation (Lymphocyte Separation Medium, PAA Laboratories, Pasching, Austria). This study was approved by the Ethics committees of the University of Tübingen and of the Technical University Munich.

#### **2.1.4 Isolation of HLA class I ligands**

HLA ligands were obtained by immunoprecipitation of HLA molecules from  $1.4 \times 10^{10}$  cells of MVA- and mock-infected JY using a slightly modified protocol (305) that involves the antibodies B1.23.2 and W6/32 coupled to CNBr-activated sepharose (Roche, Mannheim, Germany) followed by acidic elution and size exclusion ultrafiltration. To separate HLA-A and -B ligands affinity chromatography based on two distinct columns was performed: the first column was loaded with B1.23.2 (an antibody binding to HLA-B/C) and the second one with antibody W6/32 (binding to HLA-A/B/C). Therefore, HLA-B\*0702 bound to B1.23.2 first whereas HLA-A\*0201 bound to W6/32 and ligands from both HLA molecules were eluted separately.

#### **2.1.5 Peptide modification and analysis**

Modification of peptides was carried out as described (306). In brief, lysine side chains of peptides were blocked by O-methyl isourea hemisulfate (Acros Organics, Geel, Belgium). Peptides were desalted using Peptide Cleanup C18 Spin Tubes (Agilent, Palo Alto, CA, USA) and subsequently nicotinylated on the column for 15 min at room temperature using a 20 mM light ( $H_4$ ) or heavy (deuterated,  $D_4$ ) 1-([ $1H_4/2D_4$ ] nicotinoyloxy)succinimide solution in 50 mM phosphate buffer pH 8.5. After aminolysis of undesirable nicotinylated tyrosine esters, peptides were eluted by 50  $\mu$ l of 50% acetonitrile, 1% formic acid.

Peptide analysis was carried out as described (307) using an Ultimate HPLC system (Dionex, Amsterdam, Netherlands) with a gradient ranging from 15 to 55% solvent B within 170 min. The mix of the peptide samples from MVA- and mock-infected JY cells was recorded in an LC-MS experiment without fragmentation using a hybrid quadrupol orthogonal acceleration time of flight mass spectrometer (Q-TOF I, Micromass, Manchester, UK), equipped with a micro-electrospray ionization source, as described (305, 307). For peptide sequence analysis the sample derived from MVA-

infected cells was analyzed in a separate LC-MS/MS experiment. Fragment spectra were evaluated manually and database searches (NCBI, Expressed Sequence Tag) were carried out using the MASCOT search engine ([www.matrixscience.com](http://www.matrixscience.com)) (308). All viral HLA ligand sequences are available at [www.syfpeithi.de](http://www.syfpeithi.de).

### 2.1.6 Peptides

HLA-A\*0201 restricted peptides derived from MVA proteins (Table I), HCMV pp65<sub>495-503</sub> (NLVPMVATV), EBV BMLF1<sub>259-267</sub> (GLCTLVAML), Influenza virus M1<sub>58-66</sub> (GILGFVFTL) and HIV-1 RT<sub>476-484</sub> (ILKEPVHGV) were synthesized by standard Fmoc chemistry using an Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). Purity of peptides was analyzed by HPLC (Varian Star; Zinsser Analytics, Munich, Germany) and identity confirmed by MALDI-MS (GSG future; GSG, Bruchsal, Germany). This was done by Patricia Hrstić (Department of Immunology, Institute of Cell Biology, University of Tübingen). MVA synthetic peptides were further measured by LC-MS/MS (modified as described above) and fragmentation spectra were compared to the spectra obtained from endogenous MVA peptides. For T cell experiments all peptides were dissolved in 10% DMSO at 1 mg/ml.

### 2.1.7 Recombinant HLA molecules and fluorescent tetramers

Biotinylated recombinant HLA class I molecules and fluorescent HLA tetramers for CD8<sup>+</sup> T cell analysis were produced as described (309). Briefly, fluorescent tetramers were generated by co-incubating biotinylated HLA-A\*0201 monomers with PE- or Allophycocyanin-conjugated streptavidin (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

### 2.1.8 In vitro sensitization of human CD8<sup>+</sup> T cells using synthetic peptides

PBMC were cultured in IMDM (Bio Whittaker, Verviers, Belgium) containing 10% heat-inactivated human serum (PAA) and 50  $\mu$ M  $\beta$ -mercaptoethanol (Merck, Darmstadt, Germany). IL-4 and IL-7 (5 ng/ml; R&D Systems, Wiesbaden, Germany) were added after thawing PBMC in DNase (3  $\mu$ g/ml; Sigma-Aldrich) containing

medium and washing them once in medium without DNase. On d1 peptides (1 µg/ml) prepared as mixtures of five peptides each were added to PBMC: mix 1: F12L<sub>404-412</sub>, G5.5R<sub>27-35</sub>, B19R<sub>207-215</sub>, A23R<sub>273-281</sub>, B8R<sub>18-27</sub>; mix 2: C7L<sub>74-82</sub>, A48R<sub>187-195</sub>, B22R<sub>79-87</sub>, H3L<sub>184-192</sub>, A47L<sub>155-163</sub>; mix 3: O1L<sub>247-255</sub>, E5R<sub>93-101</sub>, D12L<sub>62-70</sub>, D12L<sub>251-259</sub>, B22R<sub>178-186</sub>; mix 4: O1L<sub>335-344</sub>, J6R<sub>303-311</sub>, B15R<sub>91-101</sub>) and IL-4 and IL-7 (5 ng/ml) were added. On d3, d5 and d7, IL-2 (2 ng/ml, R&D Systems) was added. Medium was exchanged after d9 when necessary. On d12, part of the cells were used for IFN-γ ELISPOT assay. The remaining cells were restimulated by addition of peptide (1 µg/ml), followed by IL-2 (2 ng/ml) 24 h later and analysis by combined tetramer / intracellular IFN-γ staining on d20.

### 2.1.9 IFN-γ ELISPOT assay

IFN-γ ELISPOT was performed essentially as described (113) except 5 x 10<sup>5</sup> PBMC/well were seeded in coated 96-well nitrocellulose plates (MSHAN4B50, Millipore, Bedford, MA) and 5 x 10<sup>4</sup> K562/A\*0201 cells/well and 1 µg/ml peptide were added. An HLA-A\*0201 restricted HIV-peptide was used as negative control; positive control wells contained PHA (Roche). After 26 h at 37°C cells were transferred into a 96 well plate and maintained for further investigation. Remaining cells were removed by washing once with PBS/0.05% Tween 20 (Serva, Heidelberg, Germany), once with sterile water and five times with PBS Tween. Captured IFN-γ was detected by incubation for 2 h at room temperature with biotinylated mAb anti-hIFN-γ (7-B6-1, 0.33 µg/ml; Mabtech, Nacka Strand, Sweden) in PBS 0.5% BSA (Sigma-Aldrich). After washing with PBS Tween the plates were incubated with ExtrAvidin Alkaline phosphatase (1:100; Sigma-Aldrich) for 1 h at room temperature. Unbound complex was removed by washing. Peroxidase staining was performed with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidine and nitroblue tetrazolium; B5655, dissolved in water; Sigma-Aldrich) for 7 min. Spot numbers were automatically determined (Immunospot Image Analyzer, series 1; ImmunoSpot Software Version 3.2e; both Cellular Technology, Cleveland, OH). To calculate the number of cells responding to a particular peptide, the mean spot numbers induced by the control peptide were subtracted from mean spot numbers induced by MVA peptides.

### **2.1.10 Tetramer staining**

1 day after ELISPOT analysis the transferred cells were stained by tetramers (in each case using a tetramer containing a peptide other than the peptide used for stimulation in ELISPOT analysis) using PE-tetramers for MVA peptides, Allophycocyanin-tetramers for control peptides, anti-CD8-PE Cy7 and anti-CD4-FITC (BD Biosciences, Heidelberg, Germany). Cells were incubated 30 min with fluorescent HLA-A\*0201 tetramers, followed by 20 min incubation with the antibodies in the dark at 4°C. After resuspending with PBS containing 1% paraformaldehyde, 2% FCS (inactivated at 56°C for 30 min; Pan, Aidenbach, Germany), 2 mM EDTA and 0.01% sodium azide cells were analyzed on a FACSCalibur cytometer (BD Biosciences).

### **2.1.11 Combined tetramer / intracellular IFN- $\gamma$ staining**

After two rounds of peptide/IL-2 in vitro sensitization PBMC were washed in IMDM, resuspended at  $2 \times 10^7$  cells/ml, and cultured for 7 h in IMDM containing either one of the MVA peptides or a control HLA-A\*0201 restricted HIV peptide (10  $\mu$ g/ml) and Golgi-Stop solution (BD Biosciences). Stimulation with PMA/Ionomycin was used as positive control. Cells were stained using the PE-tetramers mentioned above, anti-CD8-PE Cy7, the Cytofix/Cytoperm Plus kit for permeabilization and anti-IFN- $\gamma$ -FITC (BD Biosciences). Cells were analyzed on a FACSCalibur cytometer (BD Biosciences).

### **2.1.12 Proteomic analysis**

At 12.5 h post infection JY cells were used for analysis of intracellular proteins as described (310) with slight modifications. Approximately 200  $\mu$ g of intracellular proteins extracted from MVA- and mock-infected cells were separated by 2D-PAGE (first dimension: pH 3-10NL, 24 cm (Biorad, Hercules, USA), 70 kVh; second dimension: 12% SDS-PAGE). Gels were stained by Flamingo fluorescent staining (BioRad) and scanned using a laser scanner (FLA 5100, Fujifilm, Tokyo, Japan) and by silver staining as described (311) and scanned on a flatbed scanner (Powerlook 2100 XL, UMAX, Willich, Germany). Protein preparations from two infection experiments were subjected to 2D-PAGE in duplicates. All eight gels were comparatively evaluated using differential image analysis software (Progenesis SameSpots, Nonlinear Dynamics,



Newcastle upon Tyne, UK). All spots representing proteins which were unique or overexpressed upon MVA infection were excised manually from the gels, digested with trypsin, and peptide fragments were analyzed by LC-MS/MS as described (310). Significant overexpression was defined for spots detected >1.6-fold higher upon MVA infection and an ANOVA value of <0.05 comparing the gels of both conditions. Even though the majority of analyzed proteins was overexpressed in both experiments, differential spots detectable on gels of only one of the two experiments were also analyzed. Peptide sequences were identified using the MOWSE algorithm as implemented in the MASCOT software (Matrix Science, London, UK) (308) and using the NCBI database (as of 30/04/2007) containing human and MVA protein sequences. The proteomic analysis was done by Mirita Franz-Wachtel, Inga Buchen, Johannes Madlung, Claudia Fladerer, and Tobias Lamkemeyer (Institute of Cell Biology, Proteome Center Tübingen, University of Tübingen).

### **2.1.13 Vaccination of HLA-A\*0201-transgenic mice against a lethal challenge with VACV strain Western Reserve (VACV WR)**

For peptide vaccination, HLA-A\*0201 transgenic HHD<sup>+/+</sup>  $\beta$ 2m<sup>-/-</sup> D<sup>b</sup><sup>-/-</sup> HHD II mice (95) were immunized subcutaneously with pools of synthetic peptides (0.03 mg/peptide; Biosynthan, Berlin, Germany) and synthetic CpG-ODN 1668 (10 nMol; TIB-Molbiol, Berlin, Germany).

Quantification of antigen-specific CD8<sup>+</sup> T cell responses. PBMC isolated on d7 from vaccinated mice were stimulated with peptide pools (Early/Late pep) or a control peptide (Control pep) for 5 h. HLA-A\*0201 restricted control peptides were Tyr<sub>369-377</sub> (derived from human tyrosinase), FluM<sub>58-66</sub> (derived from the A/PR/8/34 Influenza virus matrix protein M1) and pp65<sub>495-503</sub> (derived from the human cytomegalovirus internal matrix protein pp65). Brefeldin A (1 mg/ml; Sigma-Aldrich) was added for the last 3 h. Cells were live/dead stained with ethidium monoazide bromide (Molecular Probes, Leiden, the Netherlands) and blocked with anti-CD16/CD32-Fc-Block (BD Biosciences). Surface markers were stained with Allophycocyanin-conjugated anti-CD8 and anti-CD62L-PE (Caltag, now Invitrogen, Carlsbad, USA). Intracellular IFN- $\gamma$  staining was performed with anti-IFN- $\gamma$ -FITC (clone XMG1.2) using the Cytotfix/Cytoperm kit for permeabilization (BD Biosciences Pharmingen). Data were

acquired by FACS analysis on a FACSCanto (BD Biosciences) and were analyzed with FlowJo (Tree Star, Ashland, USA) software.

Protection assays. 8 days after immunization with virus or after peptide immunization (see A), mice were infected intranasally with VACV WR (originally provided by Bernard Moss (NIH, Bethesda, USA)) diluted in 30 µl PBS, and monitored for more than 3 weeks with daily measurement of individual body weights as described previously (95). Mice suffering from severe systemic infection and having lost >30% of body weight were sacrificed. The mean change in body weight was calculated as percentage of the mean weight for each group on the day of challenge. This experiment was performed by Georg Gasteiger, Wolfgang Kastenmüller, Anya Krefft, and Ingo Drexler (Institute of Virology, Technical University Munich and Helmholtz Center Munich).

## **2.2 Materials and Methods of Part II**

### **2.2.1 Tissues and cell lines**

Renal cell carcinoma tissue (RCC414) (classified T<sub>1b</sub>N<sub>x</sub>M<sub>1</sub>) and surrounding healthy renal tissue were excised from a patient (HLA-A\*02, -A\*24, -B\*07, -B\*35) at the University clinic Tübingen by Jörg Hennenlotter and stored as described (305). This study was approved by the Ethics committees of the University of Tübingen.

The human B lymphoblastoid cell line (B-LCL) JY (ECACC Cat.no. 94022533; HLA-DRB1\*04, -DRB1\*13) and the human melanoma cell line MaMel-8a (European searchable Tumour Line Database ESTDAB-105; expressing HLA-DRB1\*01) were maintained in RPMI 1640 (C.C.Pro, Neustadt, Germany) containing 10% FCS (Pan Biotech, Aidenbach, Germany).

Monoclonal IgG2a antibodies W6/32 (anti-HLA-A, B, C) (303) and L243 (anti-HLA-DR) (312) were purified from hybridoma supernatants using protein A Sepharose beads (GE Healthcare, Uppsala, Sweden).

### 2.2.2 Peptides

Peptides were synthesized by standard Fmoc chemistry using an Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). Purity of peptides was analyzed by HPLC and identity confirmed by MS. This was done by Patricia Hrستیć.

### 2.2.3 Isolation of MHC ligands and stable isotope labeling

MHC ligands were obtained by immunoprecipitation of MHC molecules from approximately  $2 \times 10^{10}$  cells (JY, MaMel-8a) or from RCC and healthy renal tissue using a slightly modified protocol (305) that involves the antibodies B1.23.2 and W6/32 coupled to CNBr-activated sepharose (Roche, Mannheim, Germany) followed by acidic elution and size exclusion ultrafiltration. Phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO, USA) were added to the lysate of JY cells. Modification of peptides was carried out as described (306). Isolation of MHC ligands from RCC and healthy renal tissue was done by Anneke Neumann.

### 2.2.4 Phosphopeptide enrichment

Samples of modified peptides were taken to dryness and resolved in 40  $\mu$ l of loading buffer (50% acetonitrile/water, 6% trifluoroic acid, pH<1). TiO<sub>2</sub> (Titansphere 5 micron, loose media, GL Sciences, Tokyo, Japan; GELoader Tips, Eppendorf, Hamburg, Germany) microcolumns were packed as described (207). For centrifugation in a microcentrifuge, TiO<sub>2</sub>-microcolumns were inserted into a cut tip (epT.I.P.S. 2-200  $\mu$ L, 53 mm, Eppendorf), a bottom-cut 0.5 ml tube and a 1.5 ml tube (Safe-Lock micro test tubes, Eppendorf) (see Figure 4.1). Columns were sequentially loaded with solutions using GELoader tips (Eppendorf) and centrifuged in the tip-holding tubes (Figure 4.1) at 10,000 rpm (MHC class I peptides) or 11,000 rpm (MHC class II peptides) in a conventional tabletop microcentrifuge without letting the TiO<sub>2</sub> run dry. Centrifugation times depended on the amount of peptides contained in the sample (approximately 20 s for 10  $\mu$ l, 30 s for 20  $\mu$ l and 50 s for 40  $\mu$ l for MHC class I peptides at 10,000 rpm; for MHC class II peptides centrifugation times were about three times as long at 11,000 rpm). Solutions used for enrichment were essentially as described (207, 313). Columns were equilibrated three times with 20  $\mu$ l loading buffer and the

sample was loaded on the column three times. The column was washed twice with 20 µl of loading buffer, and twice with 10 µl of water. The flowthrough of the sample was pooled with the flowthrough of washing solutions for analysis of unbound peptides. Column-bound peptides were eluted by 5 times 10 µl and once 30 µl of elution buffer (1% ammonia, 20% acetonitrile, pH~11). The eluate was taken to dryness and resolved in solvent A for LC-MS/MS analysis.

### **2.2.5 Peptide analysis**

Peptide analysis was carried out as described (307) using a reversed phase HPLC system (CapLC, Waters, Manchester, UK; C18-column PepMap 75 µm x 25 cm (Dionex LC Packings, Sunnyvale, CA, USA); solvents: A: 4 mM ammonium acetate adjusted to pH 3.0 by formic acid, B: 80% acetonitrile/water and 2mM ammonium acetate adjusted to pH 3.0 by formic acid) with a gradient ranging from 10 to 50% solvent B within 90 min. LC-MS/MS- and LC-MS-analyses were performed using a Q-TOF Ultima (Waters) equipped with an ESI source as described (305, 307). Fragment spectra were evaluated manually and database searches (NCBI, Expressed Sequence Tag) were carried out using the MASCOT search engine ([www.matrixscience.com](http://www.matrixscience.com)) (308). Identity of several endogenous peptides was confirmed by synthetic peptides.

## 3 Results and Discussion Part I

### 3.1 Long-term immunity against actual poxviral HLA ligands as identified by differential stable isotope labeling

This chapter (except Figure 3.3) was submitted by the authors below and has been accepted by the *Journal of Immunology* on August 27, 2008 for publication.

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The author of this thesis designed and performed all experiments except the experiments described in chapters 3.1.3.2 and 3.1.3.5.

Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; LC-MS, *nano*HPLC-coupled MS analysis; LC-MS/MS, *nano*HPLC-coupled tandem MS analysis; MPXV, monkeypox virus; MS, mass spectrometry; MVA, modified vaccinia virus Ankara; m/z, mass to charge ratio of peptide ion; NIC, nicotinic acid; ORF, open reading frame; p.b., post boost; SFC, spot forming cells; VACV, vaccinia virus; VARV, variola virus; WR, Western Reserve

#### 3.1.1 Abstract

Viral peptides are presented by HLA class I on infected cells to activate CD8<sup>+</sup> T cells. Several immunogenic peptides have been identified indirectly by epitope prediction and screening of T cell responses to poxviral vectors including modified vaccinia virus

Ankara (MVA) currently being tested as recombinant or smallpox vaccines. However, for the development of optimal vaccination and immunomonitoring strategies it is essential to characterize the actual viral HLA ligand repertoire of infected cells. We used an innovative approach to identify naturally processed MVA HLA ligands by differential HPLC-coupled mass spectrometry. We describe twelve viral peptides presented by HLA-A\*0201 and three by HLA-B\*0702. All HLA-A\*0201 ligands participated in the memory response of MVA-immune donors and several were immunogenic in Dryvax<sup>®</sup> vaccinees. Eight epitopes were novel. Viral HLA ligand presentation and viral protein abundance did not correlate. All ligands were expressed early during the viral life cycle and a pool of three of these mediated stronger protection against a lethal challenge in mice as compared to late epitopes. This highlights the reliability of the comparative mass spectrometry-based technique to identify relevant viral CD8<sup>+</sup> T cell epitopes for optimizing the monitoring of protective immune responses and the development of effective peptide-based vaccines.

### **3.1.2 Introduction**

Viruses are one of the main factors which modify the repertoire of HLA ligands, the peptides associated with human MHC molecules. Viral HLA ligands are presented to T cells carrying the appropriate TCR in order to elicit a specific cellular immune response, thus making infected cells visible to the immune system. T cell responses were shown to play an essential role in clearance of poxvirus infections (238, 254, 314). Smallpox disease, caused by variola virus (VARV), was eradicated in the 1970s by vaccination with cross-protective vaccinia virus (VACV, Dryvax<sup>®</sup>) (237). Although, in general, vaccinations aim to induce a strong antibody response to achieve viral clearance, vaccinees with T cell defects failed to control the infection after immunization (238, 254).

Despite the eradication of smallpox, there are several reasons for studying the cellular immune response to VACV. First, there is a constant threat that VARV may be reintroduced by acts of bioterrorism or that forms of new pathogenic poxviruses may evolve from, for example, zoonotic human monkeypox virus (MPXV) (243, 249). Since smallpox vaccination was stopped in the late 1980s, a large part of the population is unprotected. Dryvax<sup>®</sup>, the only currently licensed vaccine against smallpox, carries the highest rate of side effects of any approved vaccine. Therefore, modified vaccinia

virus Ankara (MVA), an attenuated replication-deficient strain of VACV, is currently being tested as a safer third-generation vaccine (89, 243, 255, 256). A more detailed understanding of the CTL response to MVA allows both the development of epitope-based vaccines promising a safe, stable and handy alternative to traditional vaccination strategies, as well as the monitoring of clinical trials by following MVA-specific T cell responses (93, 315). Furthermore, MVA has been successfully introduced as a highly immunogenic recombinant viral vector vaccine for immunotherapy of infectious diseases and cancer (270), which requires the assessment of T cell epitope-specific responses elicited against vector and recombinant antigens.

Several CD8<sup>+</sup> T cell epitopes of MVA and other VACV strains have been identified by indirect approaches applying epitope prediction (e.g. using [www.syfpeithi.de](http://www.syfpeithi.de)) and subsequent T cell screening of immune donors, both human (95-104) and mouse (79, 105-108). Despite the increasing number of epitopes published, there is an ongoing need for further investigation. First, although thousands of peptides have been screened, covering approximately 35% of the large viral DNA genome which encodes more than 200 non-overlapping open reading frames (ORF), the published determinants represent only a fraction of the total anti-viral CTL response (93). Second, only a small number of the identified epitopes have been validated so far (93). Third, our recent work challenges the indirect strategy to identify epitopes which is based on the monitoring of T cell responses alone: upon a first vaccination with MVA, T cells were primed against viral peptides that were not necessarily presented by infected cells but cross-presented by non-infected cells (316). During a second infection, however, only T cells with the ability to recognize viral peptides that were efficiently presented on infected cells participated in the recall response and mediated survival (112). Therefore, the identification of the viral HLA ligands which are actually presented on infected cells appears crucial for the design and monitoring of protective prophylactic vaccines.

Thus, we set out to identify peptide ligands presented on HLA class I of virus-infected cells by mass spectrometry (MS). MS allows the identification of the exact chemical composition of T cell epitopes, including those generated by posttranslational modifications. Thus, directly sequenced HLA ligands have been considered to be more reliable than determinants identified solely by T cell analysis (155), which is mandatory to accurately determine the magnitude of specific CD8<sup>+</sup> T cell responses or the functionality of the CD8<sup>+</sup> T cells that respond to an epitope. So far, only one MVA-

derived HLA ligand has been identified by mass spectrometry, however, without demonstrating immunogenicity (134). Ideally, immunologically relevant viral T cell epitopes are confirmed both as HLA ligands and as T cell stimulators.

The challenge in MS-based discovery of viral HLA ligands is to pinpoint the signals derived from viral sequences among the hundreds of signals produced by human self-peptides. In the past, *nano*HPLC-coupled tandem MS analyses (LC-MS/MS) performed with chemically synthesized predicted epitopes and endogenously processed peptides isolated from virus infected cells were compared in order to reveal the presence of the predicted epitope (131). In order to systemically search for viral HLA ligands, two strategies relying on the comparison of HLA ligands from virus-infected and non-infected cells have been applied. First, *in silico* subtraction of the two respective *nano*HPLC-coupled MS analyses (LC-MS) (132), and second, metabolic stable isotope labeling of HLA ligands prior to purification (133, 135).

Here, we describe a novel approach to identify viral HLA ligands by differential stable isotope labeling of HLA ligands purified from MVA infected and mock-infected cells. This strategy is based on a technique recently established in our laboratory (307) to compare the repertoires of HLA ligands of tumor and healthy tissue (306, 317). We found 15 viral peptides of which twelve were presented by HLA-A\*0201 and three by HLA-B\*0702. Nine peptides have not been described as CTL epitopes so far. All HLA-A\*0201 ligands were actual memory CTL epitopes in MVA vaccinees. Eight of these epitopes were novel. All ligands were expressed early during the viral life cycle, although late protein synthesis was not impaired. Importantly, early viral HLA ligands mediated protection against a lethal respiratory challenge in mice while late viral peptides previously described as CTL epitopes were inefficient.

### 3.1.3 Results

#### 3.1.3.1 Identification of 15 MVA-derived HLA-A\*0201 and HLA-B\*0702 ligands

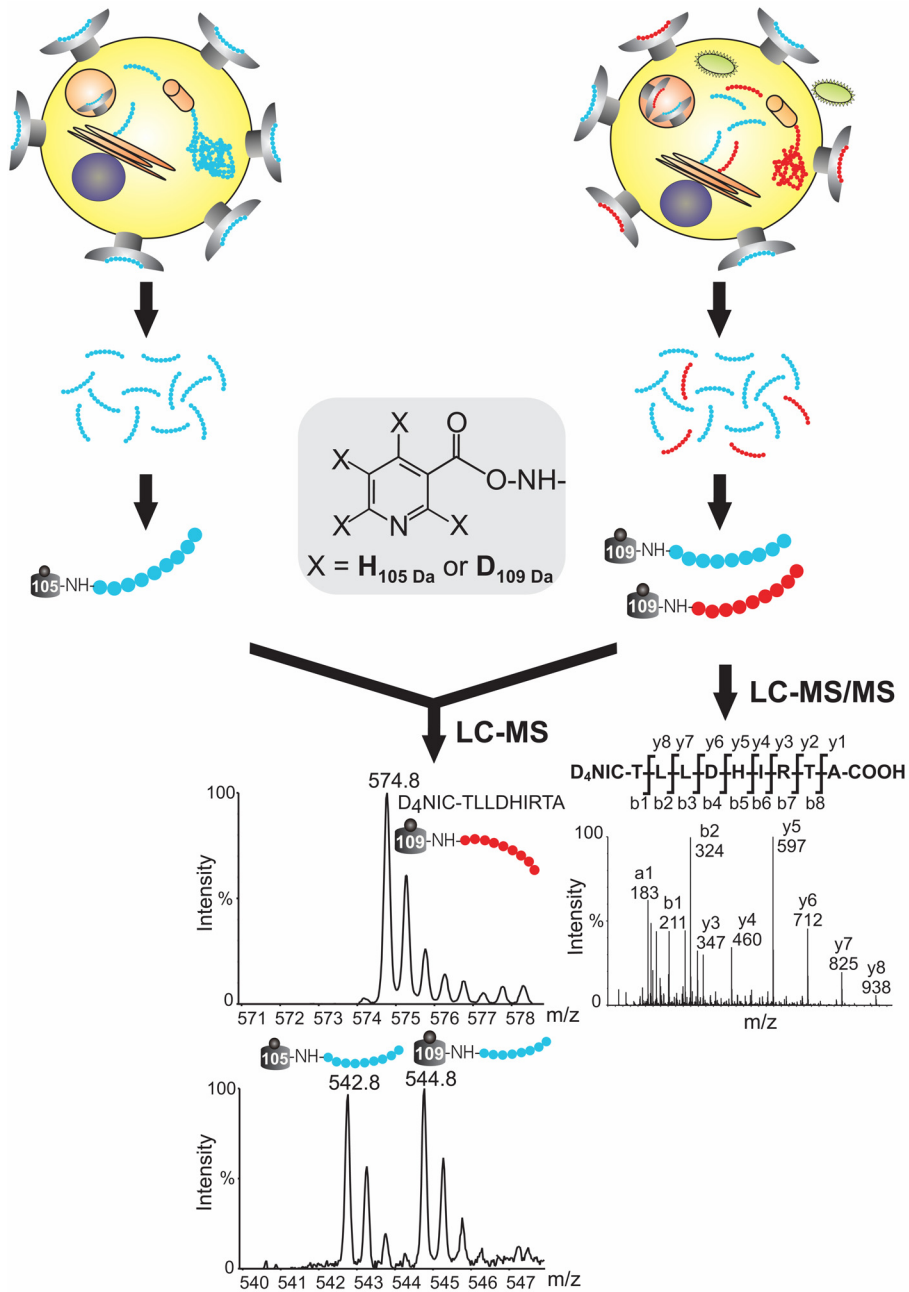
In order to identify MVA-derived HLA ligands presented by HLA-A\*0201 and B\*0702, we differentially analyzed the ligands isolated from MVA- and mock-infected cells of the human B-LCL JY after 12.5 h of infection. Figure 3.1 schematically illustrates the further experimental procedure. HLA-presented peptides were chemically modified by covalently-linked stable isotope tags: peptides isolated from MVA-infected cells



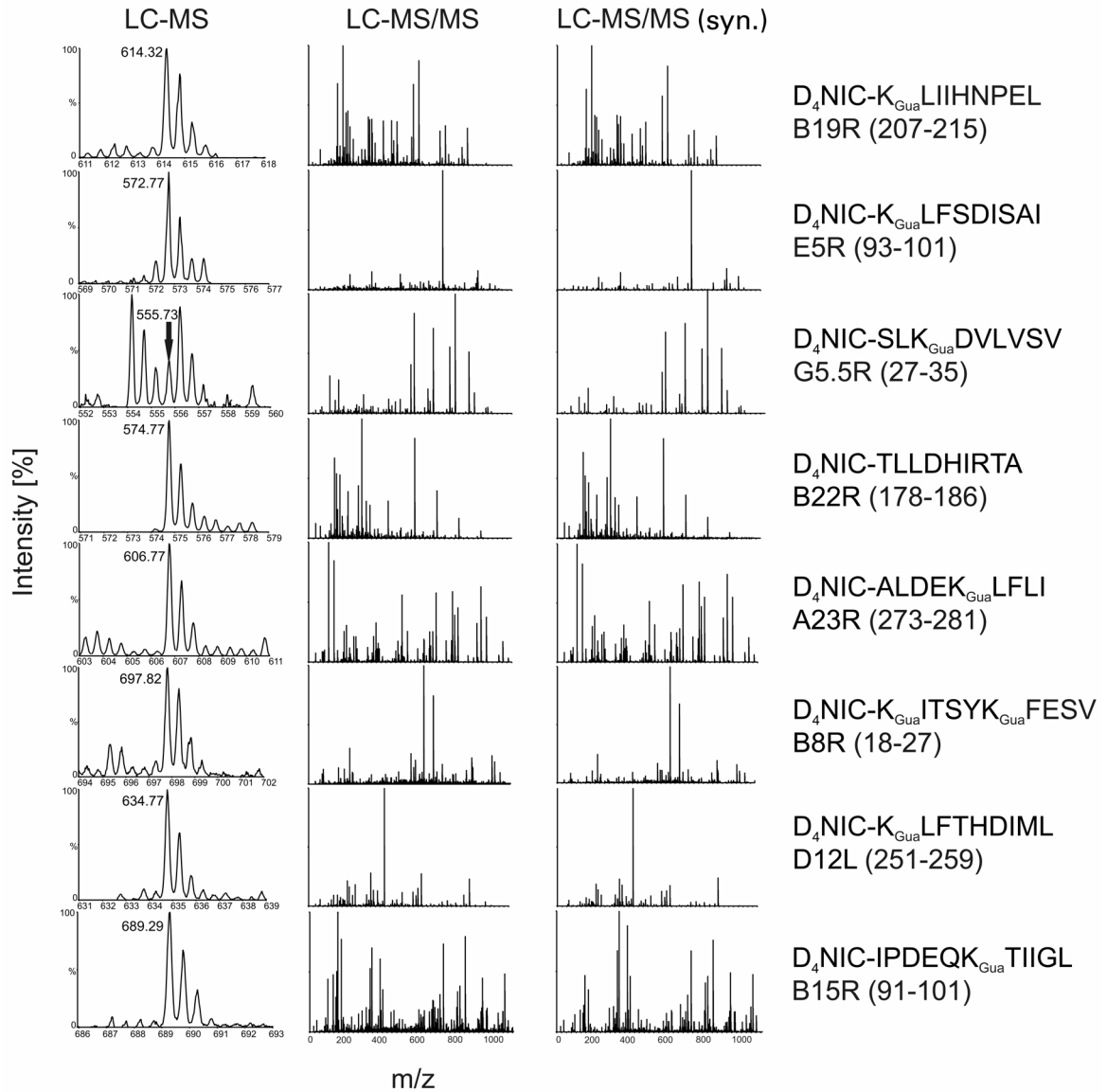
with heavy (deuterated, D<sub>4</sub>) nicotinic acid (NIC) and peptides isolated from mock-infected cells with light (hydrogenated, H<sub>4</sub>) NIC. The two pools of tagged peptides were mixed, and the peptides were separated by *nano*HPLC and analyzed online by MS. HLA ligands present on both MVA- and mock-infected cells were detected as doublets with a mass difference of 4 Da, due to the four deuterium atoms of D<sub>4</sub>NIC (Figure 3.1, lower central panel) replacing four hydrogen atoms present in H<sub>4</sub>NIC. In contrast, viral HLA ligands presented only by infected cells appeared as single peaks (Figure 3.1, upper central panel; Figure 3.2). The peptides corresponding to single peaks were then sequenced by fragmentation using LC-MS/MS analysis (Figure 3.1, right panel; Figure 3.2). All viral peptides identified were synthesized chemically and analyzed by the same procedure to verify their sequences (Figure 3.2; data not shown). We discovered twelve viral HLA ligands among the peptides isolated from HLA-A\*0201 (Table 3.1). Seven of these were novel (Figure 3.2), while four had previously been published as CTL determinants (see Table 3.6 for references), and one peptide (A48R<sub>187-195</sub>: IVIEAIHTV) had been described before as an MVA-derived HLA-A\*0201 ligand identified by LC-MS/MS (134), but had not been confirmed as CTL epitope. In addition, we detected three viral HLA ligands among the peptides isolated from HLA-B\*0702 (Table 3.1), one of which was also novel (Figure 3.2).

In total, we confirmed six established CTL epitopes as well as one MVA-derived HLA ligand, and additionally found eight novel ligands which represent potential CTL epitopes. All twelve viral HLA ligands were detected within an intensity range of one order of magnitude, suggesting an absolute quantity of about 150-1500 specific peptide/MHC-complexes per cell. This is in line with the absolute quantity of viral HLA ligands published for other viruses ((12), reviewed in (130)). Peptides presented at lower levels may be missed by this approach.

The lower number of viral peptides identified for HLA-B\*0702 in comparison to HLA-A\*0201 reflects the more stringent HLA-B\*0702 peptide motif, which requires the relatively infrequent proline residue in position 2 within its ligands ([www.syfpeithi.de](http://www.syfpeithi.de)). The HLA-A\*0201 motif, in contrast, is less restrictive, with frequent amino acids as anchors in position 2 (leucine, methionine, valine, isoleucine) and 9 (valine, leucine, isoleucine, alanine).



**Figure 3.1 Strategy of differential MS-based HLA ligand analysis.** HLA ligands were purified from extracts of MVA-infected (right) and mock-infected (left) cells. Peptide ligands were labeled differentially by chemical modification of the N-terminus with light isotopes (mock-infected, 105 Da, H<sub>4</sub>NIC) and heavy isotopes (MVA-infected, 109 Da, D<sub>4</sub>NIC) of nicotinic acid, giving respective peptides a difference in mass to charge ratio ( $\Delta m/z$ ) of 2 Da ( $z = 2$ ). LC-MS analysis of a mix of both pools revealed the quantity of a peptide in the MVA-infected sample relative to the quantity in the mock-infected sample (double peak with  $\Delta m/z = 2$ , lower central panel; isotopic peaks appear in  $m/z = 0.5$  Da intervals). HLA ligands potentially derived from MVA proteins are present in only one pool and are found as single peaks (upper central panel). The right panel representatively depicts the sequencing of a peptide of interest by separate LC-MS/MS-analysis, which generates fragmentation spectra. Identification of other newly identified MVA peptide sequences is shown in Figure 3.2.



**Figure 3.2** Identification of MVA HLA ligands by LC-MS and LC-MS/MS. syn. = synthetic peptide; z = 2 in LC-MS data, z = 1 in LC-MS/MS data; Gua = guanylated (as described in (307), reactivity of lysine side chains was blocked by chemical reaction with O-methyl isourea hemisulfate before nicotinylation (D<sub>4</sub>NIC) of peptide N-termini)

**Table 3.1 MVA-derived HLA ligands identified by differential MS analysis of MVA- and mock-infected B-LCL**

Sequence	ORF <sup>a</sup>	Epitope location <sup>b</sup>	Protein <sup>a</sup>	Locus tag <sup>c</sup>	Temporal expression <sup>d</sup>
HLA-A*0201:					
KLIHNPPEL <sup>d</sup>	B19R <sup>e</sup>	207-215 (234)	IFN- $\alpha$ / $\beta$ -receptor-like secreted glycoprotein	187	early
KLFSDISAI	E5R	93-101 (317)	abundant component of the virosome	052	early
SLKDVLSV	G5.5R	27-35 (63)	DNA-dependent RNA polymerase subunit rpo7	075	early
TLLDHIRTA	B22R, C16L <sup>f</sup>	178-186 (188)	hypothetical protein <sup>f</sup>	189, 004.5	early
ALDEKLFLI	A23R	273-281 (382)	intermediate gene transcription factor VITF-3 45kDa large subunit	134	early/late
KITSYKFESV	B8R	18-27 (226)	soluble interferon-gamma receptor-like	176	early/late
IVIEAIHTV	A48R	187-195 (204)	thymidylate kinase	161	early/late
KLFTHDIML	D12L	62-70 (287)	mRNA capping enzyme small subunit	109	early/late
RVEALYYV	D12L	251-259 (287)	mRNA capping enzyme small subunit	109	early/late
KVDDTFYYV	C7L	74-82 (150)	possible host defense modulator	018	early
FLTSVINRV	F12L	404-412 (635)	involved in plaque and EEV formation	042	early/late
GLNDYLHSV	O1L	247-255 (405)	hypothetical protein	059	early
HLA-B*0702:					
IPDEQKIIGL <sup>g</sup>	B15R	91-101 (143)	hypothetical protein	183	early
MPAYIRNTL	J6R	303-311 (1286)	DNA-dependent RNA polymerase subunit rpo147	090	early
RPMSLRSTII	O1L	335-344 (405)	hypothetical protein	059	early

<sup>a</sup> ORF, temporal expression and protein description according to VACV WR nomenclature (NCBI: NC\_006998) and as described in (231).

<sup>b</sup> Amino acid position in protein according to VACV strain Acambis 3000 Modified Virus Ankara (MVA) (NCBI: AY603355). In brackets, the number of amino acids of the protein is indicated.

<sup>c</sup> Locus tag according to Vaccinia virus strain Acambis 3000 Modified Virus Ankara (MVA) (NCBI: AY603355).

<sup>d</sup> Sequence in VACV WR: ELIHNPEL

<sup>e</sup> Previously B18R

<sup>f</sup> ORF and protein description according to VACV COP nomenclature (NCBI: M35027), since protein is deleted in VACV WR.

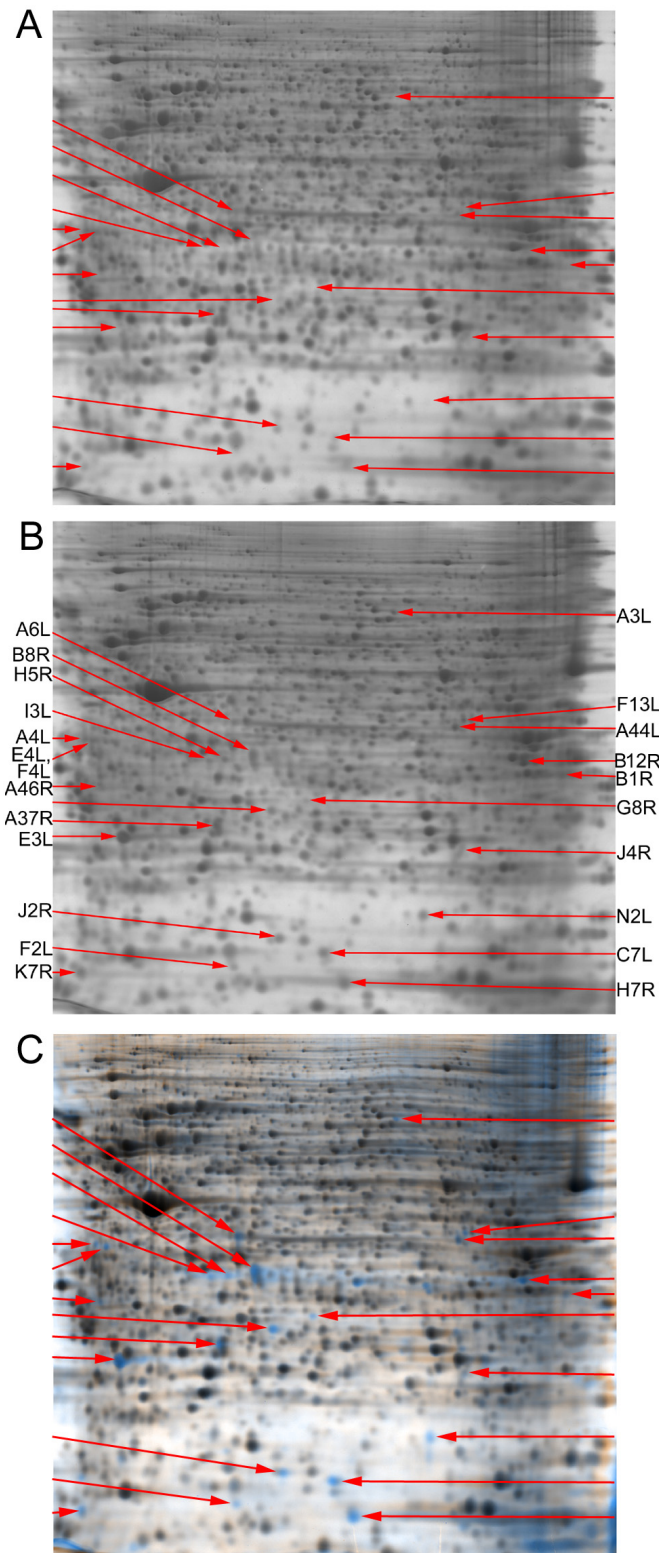
<sup>g</sup> Sequence in VACV WR: IPDEQKT[IREISA]IIGL

### 3.1.3.2 Comparison between the levels of viral protein expression and viral HLA ligand presentation

An intriguing characteristic of the proteins processed to the identified HLA ligands was their exclusive temporal expression early or early/late during the viral life cycle in MVA infected B-LCL (Table 3.1) despite the unimpaired expression of late genes in these cells (data not shown). This was surprising, since it had been shown for cells other than DC that proteins with late temporal expression can already be detected 4 h

after infection, 6.5 h earlier than the time point at which the infected B-LCL were harvested for HLA ligand purification (231, 304). Therefore, we analyzed the levels of viral protein expression and viral HLA ligand presentation in infected B-LCL simultaneously. Comparative differential image analysis of 2D gels containing proteins from MVA- and mock-infected cells revealed several abundant proteins solely or predominantly expressed in the infected cells (Figure 3.3). Furthermore, we specifically checked for expression of the proteins which gave rise to HLA ligands by predicting their theoretical spot coordinates on the gels. Differentially detected protein spots were digested by trypsin and fragments were sequenced by LC-MS/MS. Peptide digestion products derived from proteins of both viral (Table 3.2) and human origin (data not shown) were detected. Six of 24 identified viral proteins were late viral gene products (Table 3.2) indicating that late proteins were available for proteasomal processing at the time cells were harvested for HLA ligand analysis. Under the conditions used for this experiment, several viral proteins would not be detected due to their extreme size or pI: B22R and G5.5R would be missed due to their low molecular weight of 7.3 kD each, while detection of E5R is unlikely due to its high pI of 10. Proteins detectable on the 2D gels had a MW of more than 14 kD and a pI between 4 and 9. Altogether, 69 MVA proteins do not match the above characteristics and thus most likely should not be detected by this method.

Interestingly, there was little or no correlation between the relative abundance of intracellular viral proteins and directly processed viral peptides presented on HLA. Only two of the 24 most abundant MVA proteins were source proteins for identified HLA ligands, namely B8R (ligand KITSYKFESV<sub>18-27</sub>) and C7L (ligand KVDDTFYYV<sub>74-82</sub>), although CTL determinants for other proteins had been described previously (Table 3.2). Interestingly, B8R and C7L provide immunodominant epitopes in mice and humans, respectively (96, 97, 99, 100, 107). On the other hand, eleven out of 13 source proteins for which we found HLA ligands were not detected using this approach suggesting that most viral HLA ligands were derived from proteins of low abundance at 12.5 h post infection.



**Figure 3.3** Analysis of differentially expressed intracellular proteins 12.5 h post infection. Representative 2D-PAGE images of experiments performed with protein extracts of mock-infected (A) and MVA-infected (B) B-LCL JY. (B) MVA proteins identified by LC-MS/MS of tryptic digests are designated. Differentially expressed proteins identified as human are not indicated. (C) Alignment of gels A and B (gel A: red; gel B: blue; merged: black). The proteomic analysis was performed by Mirita Franz-Wachtel, Inga Buchen, Johannes Madlung, Claudia Fladerer and Tobias Lamkemeyer (Institute of Cell Biology, Proteome Center Tübingen, University of Tübingen).

**Table 3.2** Intracellular MVA proteins identified in B-LCL by proteomic analysis 12.5 h post infection<sup>c</sup>

ORF <sup>a</sup>	Temporal expression <sup>a</sup>	M <sub>w</sub> theor./exp.	pI theor./exp.	Number of tryptic peptides detected	Number of known HLA-A*0201 or B*0702 epitopes	Number of other known HLA-A and -B epitopes
A3L	late	72.6/61.0	6.37/6.30	13	-	2 (101)
A4L	late	30.9/39.0	4.91/4.90	7	-	-
A6L	late	43.1/40.0	5.71/5.60	19	2 (98, 99)	-
A37R	early	29.8/27.0	5.61/5.50	10	-	-
A44L	early	39.3/39.0	6.71/7.00	12	-	-
A46R	early/late	27.6/32.0	4.85/4.90	4	1 (98)	-
B1R	early	34.3/32.0	8.95/8.80	8	-	-
<b>B8R</b>	<b>early/late</b>	31.1/35.0	6.81/5.70	8	<b>1<sup>b</sup></b>	<b>4 (99)</b>
B12R	early	33.3/34.0	8.11/8.10	3	-	-
<b>C7L</b>	<b>early</b>	18.0/18.0	5.95/5.90	3	<b>1 (96-100),<sup>b</sup></b>	<b>1 (99)</b>
E3L	early/late	21.5/26.0	5.19/5.00	11	-	1 (101)
E4L	early/late	29.8/38.0	5.17/4.90	2	-	-
F2L	early	16.4/17.0	8.53/5.60	6	-	-
F4L	early	36.9/38.0	4.92/4.90	11	1 (99)	-
F13L	late	41.8/41.0	6.55/7.10	10	-	-
G8R	early/intermediate	29.9/29.0	6.60/5.80	7	-	1 (99)
H5R	early/intermediate	22.3/34.0	6.86/5.60	3	-	-
H7R	late	16.9/16.0	6.73/6.00	4	-	-
I3L	early	30.0/34.0	5.68/5.50	11	-	2 (98, 99, 101)
J2R	early	18.6/19.0	5.55/5.80	4	1 (98)	1 (110)
J4R	early	20.7/25.0	8.56/7.10	3	-	-
K7R	early	17.5/17.0	4.75/4.70	4	-	-
L4R	late	28.4/28.0	6.64/5.70	7	-	-
N2L	early	20.3/21.0	6.95/6.60	8	1 (99)	-

<sup>a</sup> ORF description and temporal expression according to VACV WR nomenclature (NCBI: NC\_006998) and as described in (231).

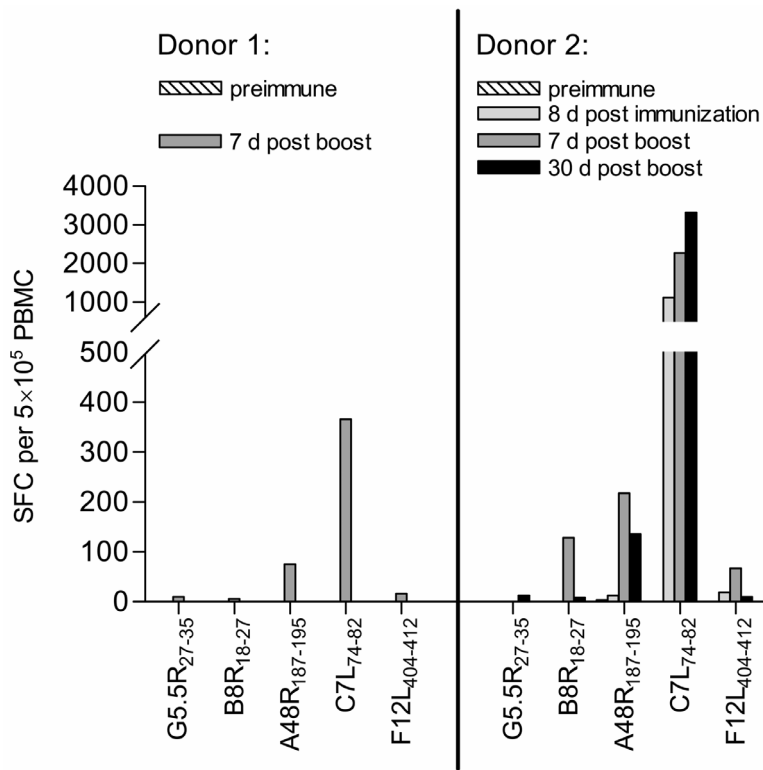
<sup>b</sup> Shown in this study, printed bold.

<sup>c</sup> The proteomic analysis was performed by Mirita Franz-Wachtel, Inga Buchen, Johannes Madlung, Claudia Fladerer and Tobias Lamkemeyer (Institute of Cell Biology, Proteome Center Tübingen, University of Tübingen).

### 3.1.3.3 Long-term recognition of HLA-A\*0201 ligands by specific IFN- $\gamma$ producing CD8<sup>+</sup> T cells in MVA vaccinees

We studied the immunogenicity of the identified HLA-A\*0201 ligands by IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay of PBMC derived from two HLA-A\*0201 positive donors immunized twice with MVA. An initial screen of PBMC taken before vaccination and up to 30 days post boost (p.b.)<sup>2</sup> indicated that immunization induced specific IFN- $\gamma$  production in response to four out of five HLA ligands tested (Figure 3.4). Since specific responses against two peptides, A48R<sub>187-195</sub> and C7L<sub>74-82</sub>, were seen in both donors and no responses were detected in PBMC isolated from

preimmune samples of either donor, we concluded that these responses were induced by immunization and were not generated by *in vitro* peptide/IL-2 stimulation prior to analysis.

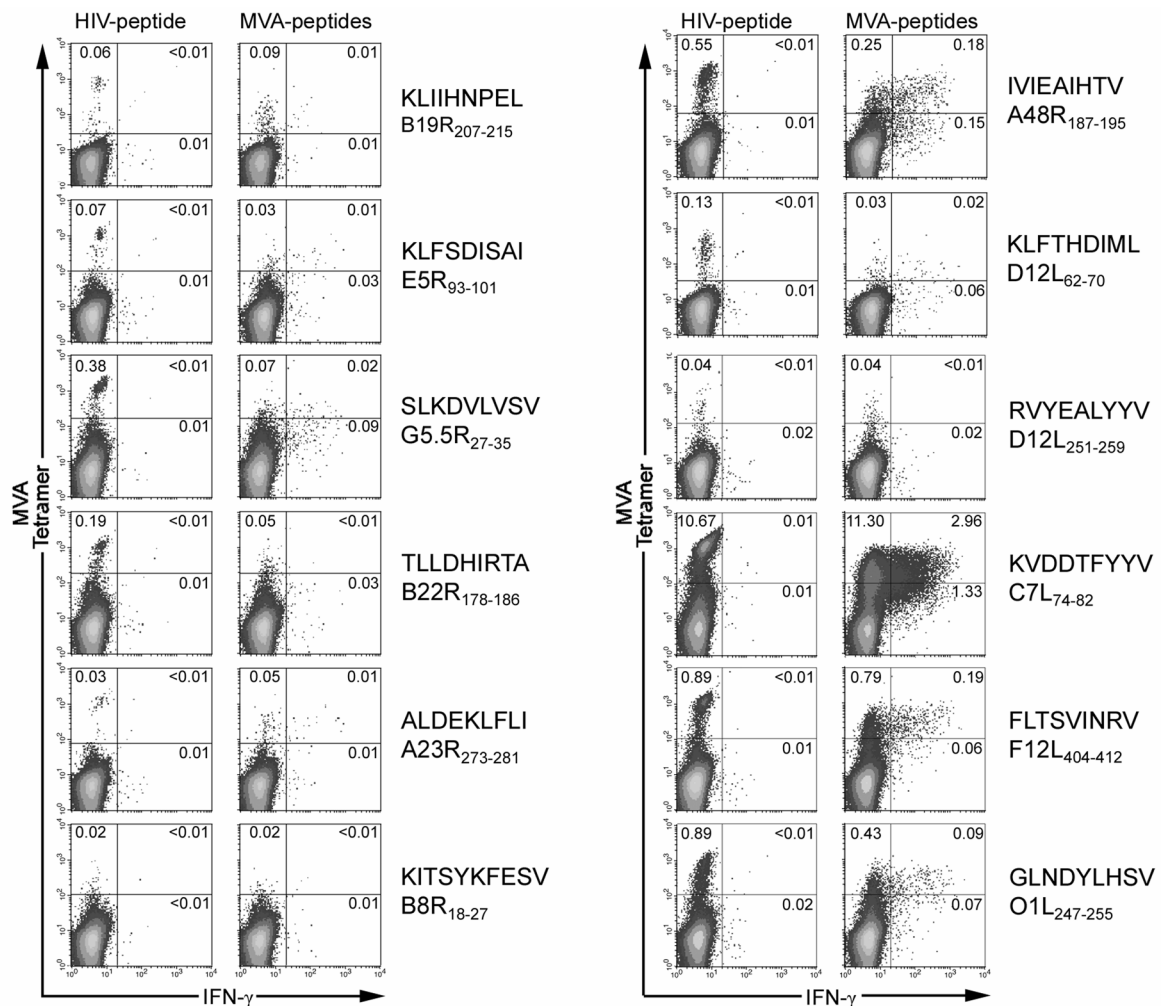


**Figure 3.4 IFN- $\gamma$  responses of MVA-immunized donors to MVA-derived HLA-A\*0201 ligand peptides varies with time post vaccination.** Blood was taken from donors at the indicated time points. PBMC sensitization and IFN- $\gamma$  ELISPOT assay were performed essentially as described in Materials and Methods except: PBMC were expanded by administration of peptide on d1 and a single dose of IL-2 on day 3; the assay was performed without adding K562/A\*0201 cells as APC; data was collected from single or duplicate measurements; spot forming cells (SFC) were calculated by subtracting the number of spots induced by an irrelevant HIV HLA-A\*0201 epitope.

We extended our study to the complete panel of HLA ligands and analyzed *in vitro* expanded PBMC taken 2.5 years p.b. by flow cytometry using combined MHC/peptide-tetramer and intracellular IFN- $\gamma$  staining (Figure 3.5, Table 3.3). For all peptides except B8R<sub>18-27</sub>, tetramer positive CD8<sup>+</sup> T cell populations were found in donor 1 (Figure 3.5, left panels), indicating that these HLA ligands are A\*0201 restricted CTL epitopes and are simultaneously recognized as part of the long-term memory response to MVA. CD8<sup>+</sup> T cell populations identified by tetramers specific for eight out of eleven epitopes were functional and responded to *in vitro* stimulation with the specific MVA peptide by downregulating the TCR and producing IFN- $\gamma$



(Figure 3.5, right panels). Since the populations recognized by tetramers specific for the remaining three peptides, B19R<sub>207-215</sub>, A23R<sub>273-281</sub> and D12L<sub>251-259</sub>, were small (less than 0.06%), our assay may not have been sensitive enough to detect production of IFN- $\gamma$  by a fraction of these cells reliably. However, TCR downregulation as an indication of activation was clearly observed in response to two of these HLA ligands: B19R<sub>207-215</sub> and A23R<sub>273-281</sub>. In donor 2, CD8<sup>+</sup> T cell populations specific for eight out of twelve HLA ligands were detected by tetramer staining (Table 3.3). Six of these populations specifically responded to *in vitro* stimulation with TCR downregulation and IFN- $\gamma$  production. Stimulation with E5R<sub>93-101</sub> induced IFN- $\gamma$  producing CD8<sup>+</sup> T cells, which, however, were not stained by the matching tetramer, possibly due to a low affinity TCR (Table 3.3).



**Figure 3.5** CD8<sup>+</sup> T cells specific for MVA-derived HLA ligands are HLA-A\*0201 restricted and produce IFN- $\gamma$ . Cells from donor 1 restimulated as described in Methods were treated for 7 h with either the MVA peptide indicated (right panels) or an irrelevant HIV HLA-A\*0201 restricted epitope (left panels) prior to combined tetramer / intracellular IFN- $\gamma$  staining. Gates were set on CD8<sup>+</sup> lymphocytes and numbers indicate the percentage of cells in each quadrant.

**Table 3.3 Comparison of CD8<sup>+</sup> T cell responses specific for MVA-derived HLA ligands in MVA vaccinees**

Peptide <sup>a,b</sup>	Donor 1			Donor 2			
	ELISPOT (1 stim.) IFN- $\gamma$ <sup>c</sup>	Tetramer / intracellular IFN- $\gamma$ (2 stim.)		ELISPOT (1 stim.) IFN- $\gamma$ <sup>c</sup>	Tetramer / intracellular IFN- $\gamma$ (2 stim.)		
		TCR <sup>d</sup>	TCR $\downarrow$ <sup>d</sup>	IFN- $\gamma$ <sup>d</sup>	TCR <sup>d</sup>	TCR $\downarrow$ <sup>d</sup>	IFN- $\gamma$ <sup>d</sup>
B19R <sub>207-215</sub>	-	+	+	-	-	-	-
E5R <sub>93-101</sub>	-	+	+	+/-	+	-	+
G5.5R <sub>27-35</sub>	++	+	+	+	-	+	+
B22R <sub>178-186</sub> <sup>e</sup>	+	+	+	+/-	-	+	+/-
A23R <sub>273-281</sub>	+	+	+	-	-	-	-
B8R <sub>18-27</sub>	-	-	-	-	-	-	-
A48R <sub>187-195</sub>	+++	+	+	+	+	+/-	+/-
D12L <sub>62-70</sub>	-	+	+	+	-	+	-
D12L <sub>251-259</sub>	-	+	-	-	-	+/-	-
C7L <sub>74-82</sub>	+++	+	+	+	+++	+	+
F12L <sub>404-412</sub>	++	+	+	+	+	+	+
O1L <sub>247-255</sub>	++	+	+	+	+	+	+

<sup>a</sup> ORF description according to VACV WR nomenclature (NCBI: NC\_006998).

<sup>b</sup> Amino acid position in protein according to VACV strain Acambis 3000 Modified Virus Ankara (MVA), (NCBI: AY603355).

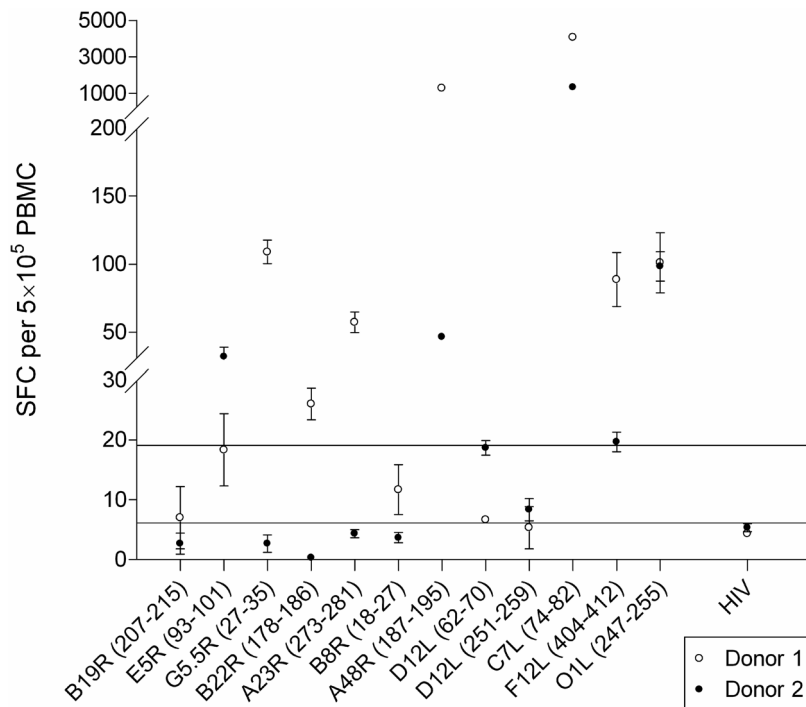
<sup>c</sup> Relative amounts of spot forming colonies determined by IFN- $\gamma$  ELISPOT after 1 round of *in vitro* peptide/IL-2 stimulation: + 18-60, ++ 61-200, +++ >200 per 5 x 10<sup>5</sup> PBMC

<sup>d</sup> Relative percentage of CD8<sup>+</sup> lymphocytes determined by combined tetramer / intracellular IFN- $\gamma$  staining after 2 rounds of *in vitro* peptide/IL-2 stimulation: TCR: + tetramer-positive cells, - tetramer-negative; TCR $\downarrow$ : + TCR downregulation detectable by tetramer staining for majority of tetramer-positive T cells, +/- TCR downregulation detectable for minority of tetramer-positive cells, - tetramer-negative; IFN- $\gamma$ : + IFN- $\gamma$  producing cells, +/- few IFN- $\gamma$  producing cells, - no IFN- $\gamma$  producing cells.

<sup>e</sup> ORF description according to VACV COP nomenclature (NCBI: M35027), since protein is deleted in VACV WR.

Analysis of the same samples by IFN- $\gamma$  ELISPOT assay after a single round of *in vitro* expansion gave similar results: eight epitopes were recognized in at least one donor and four were recognized in both (Figure 3.6). In addition, IFN- $\gamma$  production in response to A23R<sub>273-281</sub> by PBMC from donor 1 was observed, confirming this HLA ligand as a T cell epitope.

In summary, all twelve MVA-derived HLA-A\*0201 ligands which we identified were immunogenic, eleven of these provided long-term T cell memory. We demonstrate that the cellular immune response to MVA infection is based on simultaneous recognition of many different CTL epitopes with donor-specific variations in the epitope-specific CD8<sup>+</sup> T cell frequencies and in the epitope-hierarchy. This finding is consistent with earlier analyses of human T cell responses to MVA (100) and other viruses such as CMV (318), EBV (319) and Influenza virus (160). Four HLA ligands proved to be common epitopes in the long-term response to MVA vaccination: C7L<sub>74-82</sub>, the immunodominant epitope, as well as A48R<sub>187-195</sub>, F12L<sub>404-412</sub> and O1L<sub>247-255</sub>.



**Figure 3.6** MVA-immunized donors show IFN- $\gamma$  production in response to a broad repertoire of MVA-derived HLA-A\*0201 ligands. PBMC expansion and IFN- $\gamma$  ELISPOT assay were carried out as described in Materials and Methods. SFC were determined in triplicate; error bars indicate the SEM. Values three times higher than the HIV-peptide induced background were considered as positive.

### 3.1.3.4 Long-term recognition of MVA-derived HLA-A\*0201 ligands by CD8<sup>+</sup> T cells from Dryvax<sup>®</sup> vaccinees

Since we observed T cells specific for the MS-identified HLA ligands more than 2 years after immunization in MVA vaccinees, we investigated long-term memory T cell responses specific for these peptides in PBMC from donors vaccinated with the VACV Dryvax<sup>®</sup> vaccine more than 25 years ago. Since the sequences of the MVA-derived HLA-A\*0201 ligands are identical to those in VACV Dryvax<sup>®</sup> (Table 3.4), we anticipated that these ligands could also be immunogenic in the course of classical smallpox vaccination. Analysis of tetramer-specific CD8<sup>+</sup> T cells derived from *in vitro* expanded PBMC of three HLA-A\*0201 positive Dryvax<sup>®</sup> vaccinees at 25 to 45 years p.b. revealed specificity for seven of the identified HLA-A\*0201 ligands (Table 3.5). All three donors contained CD8<sup>+</sup> T cells specific for two of the common epitopes identified in the previous experiments with MVA vaccinees, C7L<sub>74-82</sub> and F12L<sub>404-412</sub>, while two donors contained T cells specific for G5.5R<sub>27-35</sub> and O1L<sub>247-255</sub>. These results suggest that one round of prime-boost vaccination with MVA or Dryvax<sup>®</sup> was

sufficient to induce a long-lived cellular immune response to several identical epitopes.

To determine if the MVA epitopes described in this study can potentially cross-protect against infection by other VACV strains or orthopoxviruses, we compared the sequences of the MVA-derived HLA ligands to those derived from VACV Dryvax<sup>®</sup>, VACV WR and VARV, as well as monkey pathogenic MPXV, which has been described recently to cause human disease (320). Seven sequences were conserved between all strains (Table 3.4), including three of the common epitopes shown to be cross-reactive between MVA and VACV Dryvax<sup>®</sup> (Table 3.5). While the immunodominant epitope C7L<sub>74-82</sub> is identical in the VACV strains, it differs by two amino acids in MPXV, making it unlikely that this epitope can provide protection against MPXV.

**Table 3.4 Comparison of peptide sequences of MVA-derived HLA ligands among orthopoxviruses**

Peptide	MVA <sup>a</sup>	VACV Dryvax <sup>b</sup>	VACV WR <sup>c</sup>	VARV <sup>d</sup>	MPXV <sup>e</sup>
<b>HLA-A*0201:</b>					
B19R <sub>207-215</sub>	KLIHNP <u>EL</u>	*	<u>EL</u> IHNP <u>EL</u>	<u>EL</u> IHNP <u>AL</u>	<u>EL</u> IHNP <u>EL</u>
E5R <sub>93-101</sub>	KLFS <u>DISAI</u>	*	*	*	KLFS <u>DISV</u> I
G5.5R <sub>27-35</sub>	SLKD <u>VL</u> SV	*	*	*	*
B22R <sub>178-186</sub>	TLLD <u>H</u> IRTA	*	deleted	*	TLLD <u>H</u> ILTA
A23R <sub>273-281</sub>	ALDE <u>K</u> FLI	*	*	*	*
B8R <sub>18-27</sub>	KITSY <u>K</u> FESV	*	*	<u>T</u> ITSY <u>K</u> FESV	*
A48R <sub>187-195</sub>	IVIE <u>A</u> IHTV	*	*	*	*
D12L <sub>62-70</sub>	KLFT <u>H</u> DI <u>M</u> L	*	*	*	*
D12L <sub>251-259</sub>	RVYE <u>A</u> LY <u>Y</u> V	*	*	*	*
C7L <sub>74-82</sub>	KVDD <u>T</u> F <u>Y</u> Y <u>Y</u> V	*	*	*	KVD <u>Y</u> <u>T</u> <u>L</u> Y <u>Y</u> V
F12L <sub>404-412</sub>	FLTS <u>V</u> IN <u>R</u> V	*	*	*	*
O1L <sub>247-255</sub>	GLND <u>Y</u> L <u>H</u> SV	*	*	*	*
<b>HLA-B*0702:</b>					
B15R <sub>91-101</sub>	IPDE <u>Q</u> K <u>T</u> I <u>I</u> GL	IPDE <u>Q</u> K <u>T</u> -I <u>I</u> GL	IPDE <u>Q</u> K <u>T</u> -I <u>I</u> GL	IPDE <u>Q</u> K <u>T</u> -I <u>I</u> GL	IPDE <u>Q</u> K <u>T</u> -I <u>I</u> GL
J6R <sub>303-311</sub>	MPAY <u>I</u> R <u>N</u> T <u>L</u>	*	*	*	MP <u>T</u> Y <u>I</u> R <u>N</u> T <u>L</u>
O1L <sub>335-344</sub>	RPMS <u>L</u> R <u>S</u> T <u>I</u> I	*	*	*	*

<sup>a</sup> VACV strain Acambis 3000 MVA (NCBI: AY603355)

<sup>b</sup> VACV strain Acambis 2000 (NCBI: AY313847), substrain isolated from the Dryvax<sup>®</sup> vaccine.

<sup>c</sup> VACV strain Western Reserve (NCBI: NC\_006998)

<sup>d</sup> VARV strain Bangladesh 1975 (NCBI: L22579)

<sup>e</sup> MPXV strain Zaire (NCBI: NC\_003310)

\* = identical sequence to MVA

- = IREISA; amino acid sequence deleted in MVA

**Table 3.5 Comparison of CD8<sup>+</sup> T cell responses to shared vaccinia epitopes in VACV Dryvax<sup>®</sup> vaccinees<sup>a</sup>**

Peptide <sup>b,c</sup>	Donor 3 (25 y)	Donor 4 (29 y)	Donor 5 (> 40 y)
B19R <sub>207-215</sub>	<b>0.08</b>	0.02	0.02
E5R <sub>93-101</sub>	0.03	0.04	0.02
G5.5R <sub>27-35</sub>	<b>0.10</b>	<b>0.06</b>	<0.01
B22R <sub>178-186</sub> <sup>d</sup>	<b>0.32</b>	0.03	0.01
A23R <sub>273-281</sub>	0.01	0.02	<0.01
B8R <sub>18-27</sub>	0.04	0.01	0.03
A48R <sub>187-195</sub>	0.04	0.02	<b>1.19</b>
D12L <sub>62-70</sub>	0.01	0.02	0.01
D12L <sub>251-259</sub>	0.03	0.02	0.01
C7L <sub>74-82</sub>	<b>0.14</b>	<b>0.28</b>	<b>1.33</b>
F12L <sub>404-412</sub>	<b>0.08</b>	<b>0.08</b>	<b>0.19</b>
O1L <sub>247-255</sub>	<b>0.16</b>	<b>0.09</b>	0.01

<sup>a</sup> Tetramer staining was carried out after one round of in vitro peptide/IL-2 stimulation. Tetramer<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> lymphocytes >0.05% are considered significant and indicated in bold.

<sup>b</sup> ORF description according to VACV WR nomenclature (NCBI: NC\_006998).

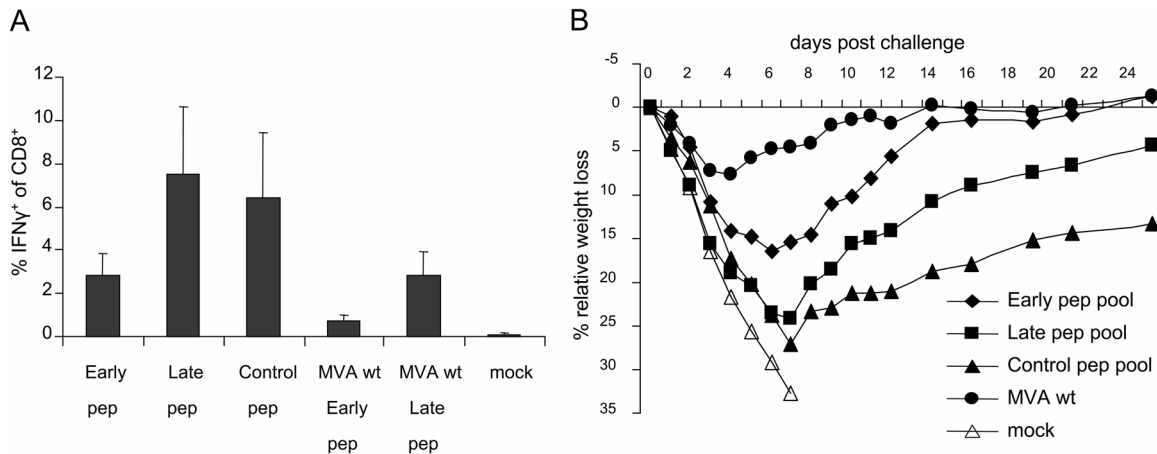
<sup>c</sup> Amino acid position in protein according to VACV strain Acambis 3000 Modified Virus Ankara (MVA), (NCBI: AY603355).

<sup>d</sup> ORF description according to VACV COP nomenclature (NCBI: M35027), since protein is deleted in VACV WR.

### 3.1.3.5 Vaccination with actual HLA ligands provides protection against a lethal VACV challenge in HLA-A\*0201-transgenic mice

All of the HLA ligands identified in this study were peptides derived from early viral proteins. Recently, we found that T cells recognizing such peptides were capable of dominating the response to a secondary VACV infection. Therefore we tested whether vaccination with the identified peptides would be able to clear an orthopoxviral infection. Importantly, HLA-A\*0201 transgenic HHD mice were fully protected against a lethal respiratory challenge with the virulent VACV strain WR after a single immunization with a pool of three peptides derived from early gene products identified in this study (B8R<sub>18-27</sub>, G5.5R<sub>27-35</sub> and C7L<sub>74-82</sub>) (Figure 3.7b). In contrast, peptides from late viral proteins (A6L<sub>6-14</sub>, H3L<sub>184-192</sub> and I1L<sub>211-219</sub>) which dominate the primary response in this mouse model after MVA immunization ((112), Figure 3.7a) or induced much higher CD8<sup>+</sup> T cell frequencies when applied in this study as pooled peptide vaccine (Figure 3.7a), were less protective. These animals showed a dramatical loss of weight (similar to the control group) and suffered prolonged disease progression (> 25 days), while all mice in the early peptide group were fully recovered by day 14.

Of note, the addition of CpG ODN as an adjuvant in all peptide vaccine preparations showed some unspecific protective capacity as demonstrated by survival of control peptide vaccinated mice which was most likely mediated by the innate immune response.



**Figure 3.7 Vaccination with HLA ligands provides protection against a lethal VACV challenge in HLA-A\*0201-transgenic mice.** Mice were immunized s.c. with pools of peptides derived from either early (Early Pep pool: B8R<sub>18-27</sub>, G5.5R<sub>27-35</sub>, C7L<sub>74-82</sub>) or late viral gene products (Late Pep pool: A6L<sub>6-14</sub>, H3L<sub>184-192</sub>, I1L<sub>211-219</sub>) or control peptides (Control Pep pool: Tyr<sub>369-377</sub>, FluM<sub>58-66</sub>, pp65<sub>495-503</sub>) or i.m. with MVA wt ( $10^8$  IU) or PBS. On day 7 mice were bled and PBMC were tested for reactivity against the immunized peptides using intracellular IFN- $\gamma$  staining. Reactivity against control peptides was below 0.1%. MVA-immunized mice were tested for reactivity against the early or late peptide pool (A). On day 8 mice were challenged with VACV WR ( $10^6$  PFU) intranasally. (B) Relative weight loss over time. In the mock-immunized group, all mice were dead by day 7, one mouse in the control and one mouse in the late group also died on day 7 (n=5). This experiment was performed by Wolfgang Kastenmüller, Georg Gasteiger, and Ingo Drexler (Institute of Virology, Technical University Munich and Helmholtz Center Munich).

### 3.1.4 Discussion

Identification of viral HLA ligands by LC-MS/MS analysis has resulted in a better understanding of the cellular anti-viral immune response. The major challenge has been to find a limited number of signals derived from the virus in the multitude of self-peptides. Planz et al. used a tedious “predict-calibrate-detect” strategy to identify an HLA ligand from borna disease virus (131), while de Jong and van Els have developed elegant approaches based on *in silico* subtraction and metabolic labeling to study measles and respiratory syncytial viruses (132, 133). Unfortunately, these strategies have not become routine and the number of viral HLA ligands known is still

very limited. So far, only one MVA-derived HLA ligand has been found by LC-MS/MS analysis of peptides isolated from infected cells (134).

The strategy described in this study, differential analysis of HLA ligands by chemical stable isotope labeling after purification of peptides, combines several advantages. First, comparative measurements of HLA ligands from infected and mock-infected cells eliminate the need for ligand prediction. Second, identification of single peptide peaks in a survey LC-MS scan is time-effective using manual evaluation, and algorithms providing automatic evaluation are expected to become available shortly. Third, the presence of a constant normalizing signal based on the self-peptides limits the requirement for reproducibility in chromatographic retention, peptide ionization and selection for fragmentation. Finally, this approach can be applied to tissue taken from any organism, and may allow comparative analysis of different sites of infection. Using differential stable isotope labeling of HLA ligands purified from infected and mock-infected cells, we discovered fifteen MVA-derived ligands, twelve restricted to HLA-A\*0201 and three to HLA-B\*0702 (Table 3.1). Eight ligands represent novel sequences. One peptide, A48R<sub>187-195</sub>, had been described by Johnson et al. (134), and six ligands matched known CTL epitopes (references see Table 3.6). Nine proteins from which HLA ligands were derived are among the 29 previously described immunogenic early proteins (93), and four proteins, B19R, E5R, G5.5R and B15R, were newly identified in this study to contain relevant human CTL epitopes (Table 3.6). The proteins bearing HLA ligands functionally belong to two groups: proteins with immunomodulatory or host range and virulence function (B8R, B15R, B19R, C7L and F12L (231, 321, 322)) and proteins functionally connected to DNA replication or transcription (E5R, A48R, G5.5R, A23R, D12L and J6R (231)). The function of O1L is unknown. The novel immunogenic proteins B15R and B19R are known cytokine receptors of VACV and play a pivotal role in the VACV mediated interference with the immune response (321, 323). Less is known about the function of the two other proteins newly identified as T cell epitope sources: E5R is located in cytoplasmic sites of viral DNA replication, where it associates with the proteins H5R and E3L, which were both detected as abundant proteins in this study (324); G5.5R has been described as a subunit of the DNA-dependent RNA polymerase (325). Two proteins, O1L and D12L, bear two HLA ligands each, suggesting high immunogenicity.

Notably, we exclusively detected HLA ligands from viral gene products expressed early or early/late during the viral life cycle (231) (Table 3.1). This is consistent with

our previous observation that presentation of peptides derived from late viral antigens to specific T cells by infected mouse target cells was very inefficient, but could be restored by the expression of the same viral antigens under the control of early promoters (112). This finding indicates a bias for early viral antigens to be processed and presented on MHC class I molecules of infected APC. However, since T cell responses against late viral proteins are found in humans and mice, the data support the concept that T cells are efficiently crossprimed upon MVA vaccination (316), particularly, when considering that late protein synthesis is blocked in MVA infected DC (304).

Several mechanisms can be invoked to explain the inability to detect HLA ligands from late proteins on infected cells. Early and late viral gene products are transcribed and translated in distinct cellular compartments (219, 220) possibly resulting in variable availability for antigen processing. Alternatively, the initiation of cell death during the course of infection may reduce the loading capacity of the cellular antigen-presenting machinery thus reducing the abundance of HLA ligands from late viral proteins (188).

Several studies have described a number of early as well as late epitopes of MVA and replication-competent VACV strains based largely on T cell analysis of immunized donors (95-100, 103). Our data concurs with the finding that VACV-specific CTL epitopes are predominantly derived from early proteins (99). We also confirm C7L<sub>74-82</sub> as the immunodominant epitope, and F12L<sub>404-412</sub> and O1L<sub>247-255</sub> as subdominant epitopes. However, using the MS-based technique we were able to detect only four of the 24 published HLA-A\*0201 restricted MVA epitopes as HLA ligands, but then only two out of the remaining 20 were derived from source proteins, from which we found HLA ligands (Table 3.6). One reason might be that different virus strains have been used for these studies. Particularly, replication-competent VACV strains might differ in pattern of antigen presentation or immunogenicity compared to MVA. In addition, several other factors may affect this limited overlap between the actual repertoire of MVA HLA ligands described here and previously identified HLA-A\*0201 restricted CTL determinants described for MVA or replication-competent VACV. First, technical restrictions within the LC-MS/MS analysis are likely to prevent the detection of all MVA-derived HLA ligands. Even if a peptide is presented by a sufficient number of HLA molecules to produce a signal with sufficient intensity, a peptide peak may be missed due to co-elution of peptides of similar molecular weight



or suppression of peptide ionization by co-eluting peptides (326). Furthermore, some peptide sequences are difficult to detect due to their chemical characteristics, as may be the case for the epitope B22R<sub>79-87</sub> (96, 100) containing a cysteine residue which can react by oxidation. Another possible reason for the limited overlap might be inherent to the *in vitro* infection model that we chose to generate the material for our HLA ligand analysis. We used one defined cell type and analyzed one time point post infection. In addition, cross-presentation of epitopes might add to the repertoire of CTL determinants *in vivo*. A third explanation may be the individual heterogeneity of subdominant epitopes, e.g. many VACV Dryvax<sup>®</sup> epitopes were characterized solely by IFN- $\gamma$  production in a single donor (99). In contrast, ten out of twelve HLA-A\*0201 ligands identified in this study were recognized by more than one of the five donors tested suggesting that they are immunologically highly relevant. Finally, the limited overlap might also be a result of differing T cell assay protocols. In contrast to some other groups, we restimulated the PBMC of vaccinees twice in order to clearly detect the T cells with specificity for the HLA ligands presented by MVA-infected cells.

In summary, the MS-based technique used here seems to be a reliable method to identify clinically relevant viral CTL epitopes and could be applied to other large-genome pathogens or recombinant antigens expressed by MVA. We identified twelve HLA-A\*0201 and three HLA-B\*0702 ligands derived from MVA. Nine of these 15 peptides were novel. All HLA-A\*0201 ligands were shown to be actual CTL epitopes in MVA-immune donors. These peptides, preferably common and more dominant epitopes such as C7L<sub>74-82</sub>, F12L<sub>404-412</sub>, G5.5R<sub>27-35</sub>, O1L<sub>247-255</sub> and A48R<sub>187-195</sub>, are essential to monitor CD8<sup>+</sup> T cell responses to MVA-based vaccines in clinical trials and may be used as correlates of protection. In addition, they seem suitable to be included e.g. as an epitope-based component in a smallpox vaccine which might be considered as a low-cost, safe and stable alternative to traditional vaccines against bioterrorist smallpox threats.

**Table 3.6 Newly identified and published epitopes of the viral HLA ligand source proteins**

ORF (temporal expression) <sup>a</sup>	Epitope location <sup>b</sup>	Sequence	MHC restriction	Ligand on B-LCL JY	CD8 <sup>+</sup> T-cells in MVA-vaccinees	References
B19R (early)	207-215	KLIHNPPEL <sup>d</sup>	HLA-A*0201	yes	yes	-
E5R (early)	93-101	KLFSDISAI	HLA-A*0201	yes	yes	-
G5.5R (early)	27-35	SLKDVLSV	HLA-A*0201	yes	yes	-
B15R (early)	91-101	IPDEQKTIIGL <sup>e</sup>	HLA-B*0702	yes	n/a	-
B22R (early) <sup>c</sup>	178-186	TLLDHIRTA	HLA-A*0201	yes	yes	-
	79-87	CLTEYLWV	HLA-A*0201	no	yes	(96, 100)
	72-80	TVADVRLHCL	HLA-B*07	n/a	n/a	(110)
A23R (early/late)	273-281	ALDEKFLFI	HLA-A*0201	yes	yes	-
	287-295	HDVYGVSNF	HLA-B*4403	n/a	n/a	(101)
	297-305	IGMFLNFTI	H2-D <sup>b</sup>	n/a	n/a	(107)
B8R (early/late)	18-27	KITSYKFESV	HLA-A*0201	yes	yes	-
	110-118	TEYDDHINL <sup>f</sup>	HLA-B*4001	n/a	n/a	(99)
	124-132	DMCDIYLLY	HLA-A*2601, HLA-A*2902, HLA-A*0101	n/a	n/a	(99)
	138-147	FGDSKEPVPY	HLA-A*2601, HLA-A*2902, HLA-A*0101	n/a	n/a	(99)
A48R (early/late)	262-271	FLSMLNLTKY <sup>g</sup>	HLA-A*2902, HLA-A*0101	n/a	n/a	(99)
	20-27	TSYKFESV	H2-K <sup>b</sup>	n/a	n/a	(97, 107)
	187-195	IVIEAIHTV	HLA-A*0201	yes	yes	(134)
D12L (early/late)	58-66	TYNDHIVNL	HLA-A*2301	n/a	n/a	(101)
	62-70	KLFTHDIML	HLA-A*0201	yes	yes	-
C7L (early)	251-259	RVYEALYYV	HLA-A*0201	yes	yes	(98)
	14-22	VLLPFYETL	H2-K <sup>b</sup>	n/a	n/a	(107)
	74-82	KVDDTFYYV	HLA-A*0201	yes	yes	(96, 97, 99, 100)
F12L (early/late)	31-40	KLKISNDYK	HLA-A*0301	n/a	n/a	(99)
	404-412	FLTSVINRV	HLA-A*0201	yes	yes	(99)
J6R (early)	286-295	NLFDIPLLTV	HLA-A*0201	no	n/a	(99)
	303-311	MPAYIRNTL	HLA-B*0702	yes	n/a	(99, 103)
	332-340	NQVKFYFNK	HLA-A*0301	n/a	n/a	(99)
O1L (early)	542-551	AGLLSDHKS	H2-K <sup>b</sup>	n/a	n/a	(105)
	247-255	GLNDYLHSV	HLA-A*0201	yes	yes	(99)
	335-344	RPMSLRSTII	HLA-B*0702	yes	n/a	(99)

<sup>a</sup> ORF description and temporal expression according to VACV WR nomenclature (NCBI: NC\_006998) and as described in (231).

<sup>b</sup> Amino acid position in protein according to VACV strain Acambis 3000 Modified Virus Ankara (MVA), complete genome (NCBI: AY603355) or as described by the reference, if sequence differs in MVA.

<sup>c</sup> ORF and protein description according to VACV COP nomenclature (NCBI: M35027), since protein not present in VACV WR.

<sup>d</sup> Sequence in VACV WR: ELIHNPEL

<sup>e</sup> Sequence in VACV WR: IPDEQKTI---

<sup>f</sup> VACV Dryvax<sup>®</sup> CTL epitope; homologous sequence in MVA: TEYDDH---

<sup>g</sup> No homologous sequence in MVA.

n/a, not applicable

### **3.1.5 Acknowledgements**

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## **3.2 MVA infection upregulates presentation of cytoskeleton-derived self-peptides on HLA-A\*0201**

### **3.2.1 Introduction**

Myocarditis is an inflammatory heart disease often associated with a previous viral infection (327-331). VACV-based smallpox vaccination has been reported to induce autoimmune eosinophilic-lymphocytic myocarditis as a serious side effect (134, 236, 240, 241). It has been diagnosed by biopsy in human individuals shortly after vaccination providing histological evidence for eosinophil-mediated cardiac myocyte necrosis (242). The lymphocytic component consisted mainly of CD3<sup>+</sup> T cells, of which about 25% were CD8<sup>+</sup>. PCR showed that myocytes were virus-free.

It has been suggested that myocarditis may develop due to autoimmune responses directed against cardiac tissue (332-336). The inflammatory immune response caused upon infection may break tolerance by mechanisms of molecular mimicry, bystander activation, and loss of immune regulation (337-342). The innate immune response to infection and release of cardiac myosin or other cardiac antigens may contribute to the overall enhanced inflammatory state in the myocardium (331). Once initiated, the immune responses leading to myocarditis can be perpetuated by exposed and presented cardiac antigens in the presence of inflammatory cytokines (330, 343, 344). A marked increase in expression of MHC class I and II molecules by the myocardium of patients with active myocarditis has been shown (327).

Recently, CD8<sup>+</sup>-based autoimmunity has been demonstrated to be induced by DCs during HIV infection by cross-presentation of caspase-cleaved apoptotic self antigens mainly derived from the cytoskeleton (345). The chronic T cell activation in HIV-patients has been shown to be largely attributable to T cells specific for multiple subdominant self peptides rather than HIV-specific T cells.

We hypothesized that presentation of human self-HLA ligands on virus-infected cells, which are lacking on non-infected cells, might induce self-reactive T cells during infection. The technique used to identify MVA-derived HLA class I ligands, differential analysis of peptides presented by MVA infected and mock-infected cells, is ideal to identify not only viral HLA ligands but also self-ligands which are specifically presented by infected cells or whose presentation is upregulated upon viral infection.

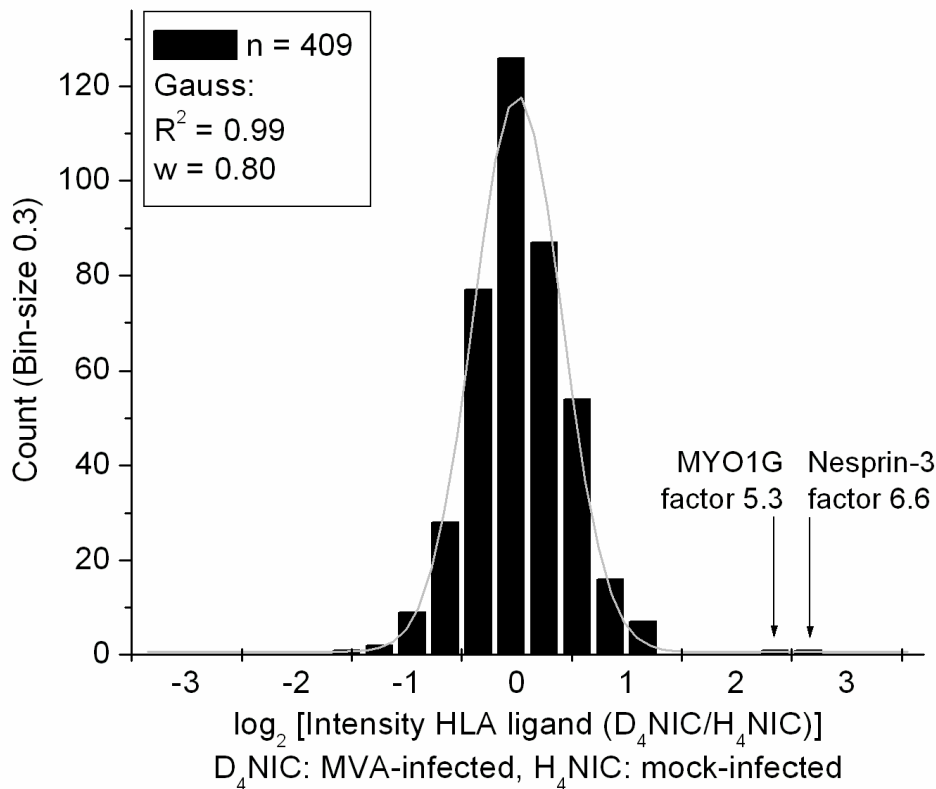
No myocarditis cases have been reported with MVA so far, suggesting that the attenuated virus is not capable of inducing autoimmune-reactive cells. Yet, cells infected by replication-competent VACV might upregulate the same human self-ligands as the attenuated VACV strain MVA. Therefore, we analyzed the relative abundance of human self-HLA class I ligands on MVA-infected versus mock-infected cells.

### 3.2.2 Results

#### 3.2.2.1 Identification of differentially overpresented human HLA-A\*0201 ligands upon MVA infection

In order to identify human self-ligands specifically or overpresented by HLA-A\*0201 on the human B-LCL JY upon infection with MVA, we differentially analyzed the ligands isolated from MVA- and mock-infected cells after 12.5 h of infection. For this purpose, HLA-presented peptides were chemically modified by covalently-linked stable isotope tags: peptides isolated from MVA-infected cells with heavy (deuterated, D<sub>4</sub>) nicotinic acid (NIC) and peptides isolated from mock-infected cells with light (hydrogenated, H<sub>4</sub>) NIC. The two pools of tagged peptides were mixed, and the peptides were separated by *nano*HPLC and analyzed online by MS. HLA ligands present on both MVA- and mock-infected cells representing human self-peptides were detected as doublets with a mass difference of 4 Da due to the four deuterium atoms of D<sub>4</sub>NIC (Figure 3.1, lower central panel) replacing four hydrogen atoms present in H<sub>4</sub>NIC. Single peaks may either represent peptides derived from viral proteins or from human self-proteins which are specifically synthesized and/or degraded upon infection. Peptides were sequenced by fragmentation using LC-MS/MS analysis.

Although no human self-HLA ligands exclusively presented on the infected cells were identified, two out of 409 self-ligands evaluated were found highly overpresented upon MVA-infection (Figure 3.8). The HLA-A\*0201-restricted peptides were derived from two proteins associated with the cytoskeleton, myosin 1G (ALVDHVAEL, upregulated by factor 5.3, and C14orf49/nesprin-3 (ALAQRLLEV, upregulation by factor 6.6). The abundance of 95% of all ligands differed maximally by the factor of 1.7 ( $\log_2$  [Intensity HLA ligands(D<sub>4</sub>NIC/H<sub>4</sub>NIC)] = -0.8 to 0.8; Figure 3.8). Taking into account that only about 50% of analyzed cells were infected, upregulation of the ligands was likely even more pronounced than by the evaluated factors.



**Figure 3.8** Two out of 409 human HLA ligands evaluated were overrepresented on B-LCL JY upon MVA-infection. Normalized intensity ratios of peptide doublet peaks in the LC-MS analysis performed with an 1:1 mix of the peptide pools isolated from MVA-infected (D<sub>4</sub>NIC-modified) and mock-infected (H<sub>4</sub>NIC-modified) cells were evaluated. Almost all peptides were presented in equal abundancies on infected and mock-infected cells. Peptides upregulated upon MVA-infection were identified by LC-MS/MS analysis as derived from myosin 1G (factor 5.3) and nesprin-3 (factor 6.6).

### 3.2.3 Discussion and Outlook

By analyzing the relative abundance of directly presented HLA class I ligands of MVA-infected versus mock-infected cells, two human self-HLA ligands were found highly upregulated in MVA-infected B-LCL, suggesting either specifically induced synthesis and/or degradation upon infection. Strikingly, both peptides were derived from proteins associated with the cytoskeleton. Nesprin-3 is an outer nuclear membrane protein binding to the cytoskeletal linker protein plectin which can directly cross-link the actin and the intermediate filament cytoskeletal system (346). Myosin 1G is a member of the class I myosin proteins, which are nonfilament-forming myosins thought to play a role in intracellular transport and locomotion (347). It is

expressed in cardiac tissue (see [www.genecards.org](http://www.genecards.org): GeneCard for protein-coding MYO1G, Microarray Integrated Expression by GeneNote and GNF GeneAtlas Data). Cytoskeletal alterations have been shown upon VACV-infection: formation of virally-induced microvilli, disruption of actin stress fibres and formation of actin tails in the cytoplasm of the host (348, 349). Moreover, cytoskeletal derived proteins have been shown to play a major role in virally induced autoimmune reactions including myocarditis (345, 350). It is therefore imaginable that the increased presentation of the self-HLA ligands derived from nesprin-3 and myosin 1G in association with infection might be capable of inducing an autoimmune response in case the infection is virulent. As MVA, replication-competent VACV might upregulate the two human self-HLA ligands in host cells, perhaps even in a more pronounced manner due to complete synthesis and egress of virions. It remains to be shown, whether VACV Dryvax<sup>®</sup>-vaccinees, who had suffered from autoimmune reactions following vaccination, carry memory CD8<sup>+</sup> T cells recognizing these two ligands.

## 4 Results and Discussion Part II

### 4.1 Identification of natural MHC class II presented phosphopeptides and tumor-derived MHC class I phospholigands

This chapter has been submitted by the authors below for publication.

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The author of this thesis designed, performed and evaluated the experiments leading to the results described in this chapter. Tissue was excised by Jörg Hennenlotter<sup>†</sup>. Peptide isolation from tissue and synthesis of peptides were done by Anneke Neumann\* and Patricia Hrštic\*, respectively.

Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; HLA, human leukocyte antigens; LC-MS, *nano*HPLC-coupled MS analysis; LC-MS/MS, *nano*HPLC-coupled tandem MS analysis; MHC, major histocompatibility complex; m/z, mass to charge ratio of peptide ion; pS, phosphoserine; pT, phosphothreonine; RCC, renal cell carcinoma

#### 4.1.1 Abstract

MHC molecules present protein-derived peptides to T lymphocytes. By developing TiO<sub>2</sub>-based microcentrifugation columns, we identified the first phosphorylated MHC class I ligands from tumor tissue (renal cell carcinoma) and, by comparison to healthy renal tissue, found one Brf1-derived ligand as potentially tumor-associated. We further discovered the first natural phosphorylated MHC class II ligands. They revealed several novel phosphorylation sites of significant transmembrane receptors, such as Frizzled 6, CXCR4 and CD20.



## 4.1.2 Introduction

Major histocompatibility complex (MHC) molecules, also referred to as human leukocyte antigens (HLA), present peptides to be recognized by the T cell receptor of T lymphocytes. There are two classes of MHC molecules differing in the origin and length of the peptides presented and the T cells recognizing the peptide/MHC-complexes (351). Peptides which are produced endogenously and are degraded to a length of 8 to 12 amino acids by the proteasome and other proteases in the cytoplasm and endoplasmic reticulum are presented by MHC class I molecules (352-354). In contrast, MHC class II molecules bind peptides which are mostly derived from exogenous or transmembrane proteins but also from cytosolic proteins and are degraded to a more variable length of 9 to 25 amino acids by various proteases originating from the lysosomal compartment (355-361). Peptide/MHC class I-complexes are recognized by CD8<sup>+</sup> T cells, also called cytotoxic T cells (76-78), those of MHC class II are recognized by CD4<sup>+</sup> T cells including T<sub>H</sub>1 cells, which exert helper activity for the induction and maintenance of CD8<sup>+</sup> T cells via activation of antigen presenting cells and secretion of cytokines (57, 58). Recognition of a peptide derived from a disease-associated protein, e.g., a viral or a tumor-specific protein, in presence of a costimulatory signal triggers a T cell-mediated immune response (for review see (54)). Anti-tumor immunity optimally requires the participation of both tumor cell-directed cytotoxic CD8<sup>+</sup> T cells and CD4<sup>+</sup> T helper cells (362-367).

Tumor cells differ from non-malignant cells in the activity of protein kinase pathways regulating cell growth, differentiation, and apoptosis (136, 178, 179). Endogenous MHC class I peptides containing a posttranslational phosphorylation are discriminated from non-modified peptides by CD8<sup>+</sup> T cells *in vivo* (176, 195, 196). Hence, peptides containing a tumor-associated phosphorylation represent potential tumor antigens (176). By comparing the repertoires of MHC class I bound phosphopeptides of cancer cell lines with the one of a B lymphoblastic cell line (B-LCL), phosphopeptides restricted to the tumor cell lines and common to all tumor cell lines studied could be identified (176).

Phosphopeptide enrichment is essential for specifically identifying MHC-presented phosphopeptides in the multitude of non-phosphorylated peptides. Several methods have been developed to enrich phosphorylated peptides (200). Yet, most approaches have been used in the field of proteomics investigating peptides generated typically by

tryptic digestion. These peptides share a certain physicochemical behaviour, since digestion products contain lysine at the C-terminus and are typically longer than MHC class I peptides. Zarling et al. (176, 196) have been successful in enriching MHC class I phosphopeptides from cell lines by using  $\text{Fe}^{3+}$ -immobilized metal-affinity chromatography linked to  $\text{C}_{18}$ -HPLC-coupled mass spectrometry (LC-MS). However, MHC class II presented phosphopeptides have not been found using this technique although several cell lines have been searched for (196). Yet, it has been demonstrated that  $\text{CD4}^+$  T cells can distinguish a phosphorylated MHC class II epitope from the non-phosphorylated peptide (368).

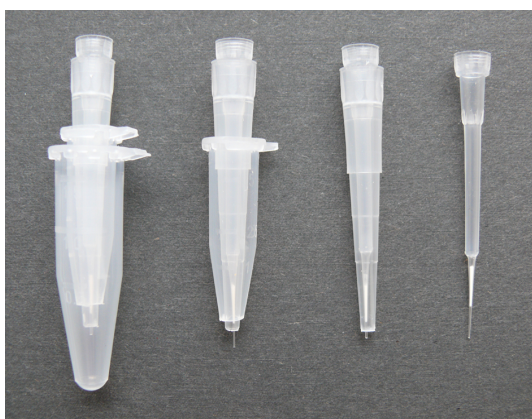
For application in immunotherapy, it is necessary to investigate whether a peptide containing a potentially tumor-associated phosphorylation is presented *in vivo* by a patient's tumor. Thus, it is crucial to not only investigate cell lines (176) but actual tumor tissue. In this study, we show that MHC class I presented phosphopeptides can be isolated from renal cell carcinoma (RCC) tissue and can be identified by HPLC-coupled mass spectrometry after *offline*-enrichment by a newly developed  $\text{TiO}_2$ -based centrifugation technique. In order to identify potentially tumor-restricted phosphopeptides we compared the repertoire of phosphopeptides presented by a patient's tumor tissue to the one presented by the same patient's healthy renal tissue. Moreover, by applying the  $\text{TiO}_2$ -microcentrifugation technique, we enriched and identified the first phosphopeptides naturally presented by MHC class II molecules of an EBV-transformed B-LCL and a tumor cell line. This finding clearly contradicts the former observation that phosphorylated peptides may only be presented by MHC class I molecules (196). We demonstrate that phosphorylated MHC class II ligands contain phosphosites of plasmamembrane receptors involved in cancerogenesis and thus represent a potential new source for tumor-specific  $\text{CD4}^+$  T cell antigens.

### 4.1.3 Results

#### 4.1.3.1 *Offline*-enrichment of MHC presented phosphopeptides by $\text{TiO}_2$ -microcentrifugation columns

To perform this study, we advanced an *offline*-technique using  $\text{TiO}_2$ -microcolumns, which was established by Larsen et al. (207), by the development of centrifugation tubes (Figure 4.1). In contrast to air pressure created manually with a syringe as described by Larsen et al. (207), centrifugation allows for a stable flow of solvents

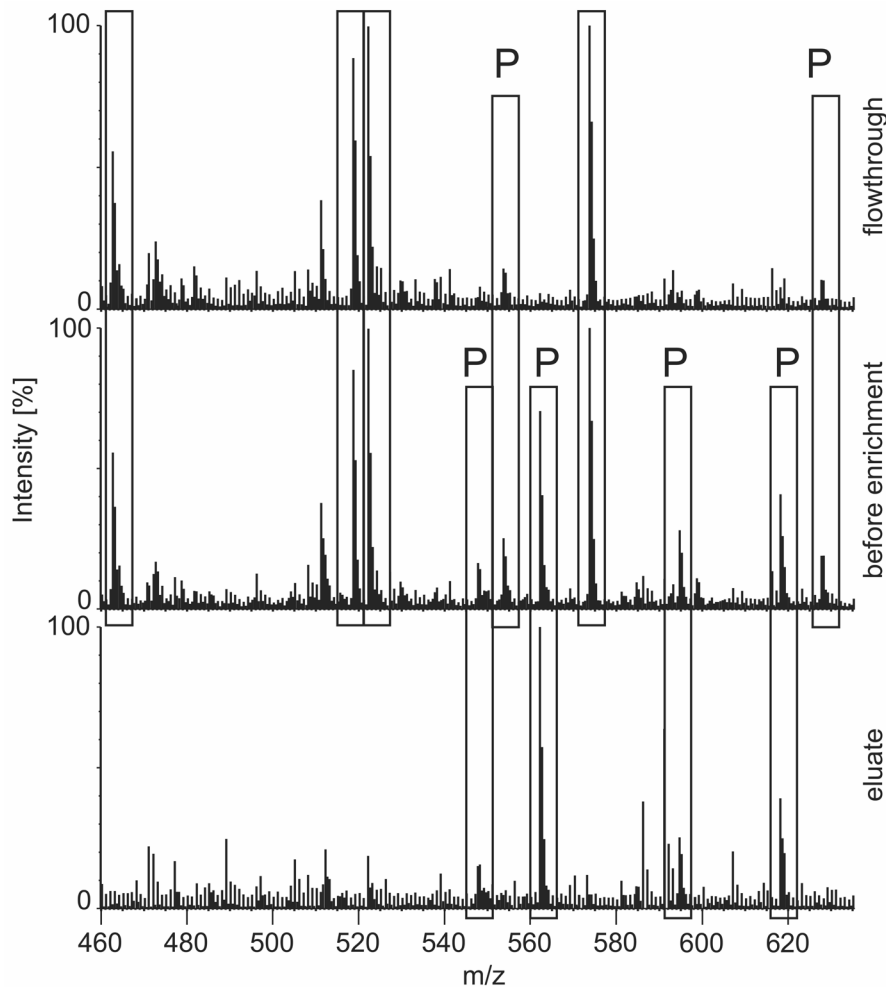
through the microcolumns. Enrichment of synthetic peptides (Table 4.1) showed quantitative separation of phosphopeptides from non-phosphorylated peptides (Figure 4.2). Yet, two phosphorylated peptides were not retained by the column: SYVKpTKMGL and KRFpSFKKSF. Both peptides contained basic amino acids next to or indirectly surrounding the phosphorylated residue (basic amino acids printed bold in Table 4.1). In contrast, a peptide equivalent to SYVKpTKMGL but containing acidic residues instead of the basic ones next to phosphothreonine (peptide SYVDpTEMGL, (Table 4.1) was retained by TiO<sub>2</sub>. This suggests that enrichment of peptides with two or more basic residues next to or surrounding the phosphogroup might be missed by this method. Importantly, peptides in the flowthrough which have not bound to TiO<sub>2</sub> may be analyzed as well (Figure 4.2). Thus, the method allows analysis of both phosphorylated and non-phosphorylated MHC ligands of a sample using the eluate and the flowthrough, respectively. We established a centrifugation protocol both for MHC class I and class II peptides, the latter requiring longer centrifugation times and higher *g* values due to their length (see Material and Methods).



**Figure 4.1** Preparation of TiO<sub>2</sub>-microcentrifugation columns (from right to left). GELoader tips are loaded with TiO<sub>2</sub> as described by Larsen et al. (207).

**Table 4.1** Synthetic peptides used for phosphopeptide enrichment (Figure 4.2)

Peptide	[M+H] <sup>2+</sup>	eluate	flowthrough
ALASHLIEA	462.8	-	+
ALNELLQHV	518.8	-	+
RVAPEEHPVL	573.8	-	+
VLRENTSPK	522.3	-	+
VLRENTpSPK	562.3	+	-
RLDpSYVRSL	594.8	+	-
MPHEKHYPpTL	618.3	+	-
SYVDpTEMGL	547.7	+	-
SYVKpTKMGL	553.8	-	+
KRFpSFKKSF	627.9	-	+



**Figure 4.2 MS analysis of the ten synthetic peptides listed in Table 4.1.** Before enrichment, flowthrough (combined flowthrough of the sample and washing solutions), eluate. P = Phosphopeptide. Synthesis of peptides was done by Patricia Hrstić (Department of Immunology, Institute of Immunology, University of Tübingen).

#### 4.1.3.2 Identification of phosphorylated MHC class I ligands from tumor tissue and corresponding healthy tissue

To identify potentially tumor-restricted phosphorylated HLA ligands, MHC class I-peptide complexes were extracted by immunoaffinity chromatography from a patient's RCC tissue (classified T<sub>1b</sub>N<sub>x</sub>M<sub>1</sub>) and from surrounding healthy tissue expressing HLA-A\*02, -A\*24, -B\*07, -B\*35. Peptide ligands were separated from MHC molecules by acidic elution and size exclusion filtration. Peptides derived from tumor and healthy tissue were differentially labeled by stable isotopes (deuterated and hydrogenated nicotinic acid) giving respective peptides a mass difference of 4 Da. Phosphopeptides from both modified pools were enriched using the TiO<sub>2</sub>-microcentrifugation columns and analyzed by HPLC-coupled ESI-Q-TOF-MS/MS (LC-MS/MS).

In total, we found 16 phosphorylated peptides as determined by detection of the neutral loss of 98 Da per phosphate group from the parent ion, which corresponds to phosphoric acid and occurs at phosphorylated serine or threonine residues but not tyrosine residues (data not shown). We were able to identify sequences of eleven phosphoserine (pS)- or phosphothreonine (pT)-peptides, out of which eight peptides were fragmented in the LC-MS/MS analysis of the tumor tissue and nine were fragmented in the analysis of the healthy tissue (Table 4.2). Two phosphopeptides were exclusively fragmented in the tumor tissue analysis, three exclusively in the healthy tissue analysis. Eight peptides matched the peptide motif of HLA-B\*0702, and one peptide each was HLA-A\*02, -A\*24 and -B\*35 restricted. Phosphorylation sites of nine peptides were known, two have been identified newly: pS<sub>1044</sub> of the protein V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ErbB3, alternative name Her3) and pS<sub>139</sub> of the protein Family with sequence similarity 128, member B (Fam128b). One peptide was a known HLA ligand: KPRpSPVVEL from the protein Adrenergic beta receptor kinase 1 (Adrbk1) (196).

Presence of a peptide in one of the samples was further investigated by an LC-MS analysis (without fragmentation of peptides) performed from a mix of the two differentially labeled peptide pools (Table 4.2). In this analysis, which allowed direct comparison of both samples, two peptides were detected as single peaks representing one peptide exclusively present in the tumor tissue (with an intensity ratio of 5.7 to background) and one peptide being present only in the healthy tissue (with an intensity ratio of 7.7 to background) (Table 4.2). All other peptides, which had been found fragmented only in the analysis of one of the samples, appeared as pairs (i.e., peaks with a 4 Da difference in the mass to charge ratio (m/z)) in the LC-MS run. Hence, out of eleven phosphopeptides one peptide was detected as tumor-restricted: RPRLQHSFpSF presented by HLA-B\*35 and derived from the protein butyrate response factor 1 (pS<sub>203</sub>-Brf1<sub>195-204</sub>). Interestingly, the HLA-B\*07 restricted phosphopeptide FPRRHpSVTL found in both the tumor and healthy tissue derives from the same protein (pS<sub>54</sub>-Brf1<sub>49-57</sub>). The two MHC presented peptides contained two out of three known phosphorylations of Brf1 (291, 369, 370). Yet, phosphorylation of S<sub>54</sub> had not been confirmed by MS so far. Brf1 is a zinc finger protein that – in the unphosphorylated state – regulates mRNA levels by targeting transcripts containing AREs (AU-rich elements) into the decay pathway (371).

**Table 4.2 Phosphorylated HLA class I ligands identified from RCC tumor tissue and the corresponding healthy tissue<sup>b</sup>**

Peptide <sup>a</sup>	LC-MS/MS			LC-MS		Gene	GeneID	Position	Phosphosite
	HLA	Tumor	Healthy	Tumor	Healthy				
RPRLOHSF <u>p</u> SF	B*35	+	-	+	-	ZFP36L1 (BRF1) zinc finger protein 36, C3H type-like	677	195-204 (338)	pS 203
FPRRH <u>p</u> SRTL	B*07	+	+	+	+	ZFP36L1 (BRF1) zinc finger protein 36, C3H type-like	677	49-57 (338)	pS 54
KAF <u>p</u> SPVRSV	A*02	+	+	+	+	ID2 inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	3398	2-10 (134)	pS 5
RFK <u>p</u> TQPVT	A*24	+	+	+	+	LOC387763 hypothetical LOC387763	387763	365-373 (435)	pT 368
RPRSL <u>p</u> SSPTVTL	B*07	+	+	+	+	NEDD4L neural precursor cell expressed, developmentally down-regulated 4-like	23327	423-434 (955)	pS 428
SPFKRQ <u>Lp</u> SL	B*07	+	-	+	+	NUMB numb homolog (Drosophila)	8650	288-296 (651)	pS 295
MPRQP <u>p</u> SATRL	B*07	+	+	+	+	FAM128B family with sequence similarity 128, member B	80097	134-143 (158)	pS 139
KPR <u>p</u> SPVVEL	B*07	+	+	+	+	ADRBK1 (GRK2) adrenergic, beta, receptor kinase 1	156	667-675 (689)	pS 670
RPF <u>p</u> SPREAL	B*07	-	+	+	+	LUZP1 leucine zipper protein 1	7798	742-750 (1076)	pS 745
RPQR <u>Ap</u> TSNVF	B*07	-	+	+	+	MYL9 (MRLC1) myosin, light chain 9, regulatory	10398	14-23 (172)	pT 19
RPRG <u>p</u> SQSL	B*07	-	+	-	+	ERBB3 (HER3) v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	2065	1040-1048 (1342)	pS 1044

<sup>a</sup> Underlined residues indicate known phosphosites (www.phosphosite.org).

<sup>b</sup> Isolation of peptides from tissue was done by Anneke Neumann (Department of Immunology, Institute of Cell Biology, University of Tübingen).

#### **4.1.3.3 Naturally presented MHC class II ligands contain phosphorylations**

Although we were able to isolate MHC class II presented peptides from the RCC tissue used for analysis of phosphopeptides presented by MHC class I, the amount of peptides isolated was seemingly too little to detect phosphopeptides after enrichment. Therefore, we analyzed MHC class II bound peptides extracted from two cell lines, a human EBV-transformed B-LCL (JY: HLA-DR4, -DR6) and a human melanoma cell line (MaMel-8a: HLA-DRB1\*01), for presence of phosphopeptides.

We detected 57 phosphopeptides in the LC-MS/MS analysis of JY, and 48 in the analysis of MaMel-8a (data not shown); identification of sequences was possible for 27 and 20 phosphopeptides, respectively (Table 4.3, Table 4.4). As typical for MHC class II ligands, most peptides were found in differing length variants, i.e., peptides can be grouped to seven ligands discovered from JY and ten from MaMal-8a (denoted as such in the following); one ligand was found in both analyses. We found peptide variants containing one, two or three phosphorylations. In the majority of peptides serine residues were phosphorylated; only one ligand carried the phosphorylation at a threonine residue. Nine out of 16 ligands were derived from transmembrane proteins. The source proteins of the other seven ligands were known to be located either in the cytoplasm and/or the nucleus. In contrast to the phosphorylation sites of the identified MHC class I ligands described above, the phosphorylation sites of the MHC class II ligands were mostly unknown: we newly defined twelve phosphorylation sites, four of which were the first phosphorylation sites described for the respective source proteins at all (pS<sub>35</sub>-CD20, pS<sub>624</sub>- and pS<sub>629</sub>-Frizzled 6, and pS<sub>414</sub>-Actin-related protein 10 homolog). Strikingly, the majority of the newly described phosphorylation sites were contained in transmembrane proteins (nine out of twelve), whereas most of the known phosphorylation sites were contained in soluble proteins (four out of seven).

**Table 4.3 Phosphorylated HLA class II ligands presented by the EBV-transformed B-LCL JY**

Peptide <sup>a</sup>	Gene	GeneID	Position	Phosphosites	Protein location/function (phosphosite location)
GpSpSLKILSKGKRGG	CXCR4 chemokine (C-X-C motif) receptor 4	7852	323-336 (352)	pS324, pS 325	plasmamembrane receptor (cytoplasmic)
GpSpSLKILSKGKRGGH			323-337 (352)	pS324, pS 325	
GpSpSLKILSKGKRGGHS			323-338 (352)	pS324, pS 325	
GpSpSLKILSKGKRGGHS <sub>pS</sub>			323-339 (352)	pS324, pS 325, pS 339	
RGpSpSLKILSKGKRGGHS			322-338 (352)	pS324, pS 325	
SRGpSpSLKILSKGKRGGHS			321-338 (352)	pS324, pS 325	
GSpSLKILSKGKRGGHS			323-338 (352)	pS 325	
GPKPLFRRMpSSLVG	MS4A1 (CD20) membrane-spanning 4-domains, subfamily A, member 1	931	26-39 (297)	pS 35	plasmamembrane receptor (cytoplasmic)
GPKPLFRRMpSSLVGP			26-40 (297)	pS 35	
GPKPLFRRMpSSLVGPT			26-41 (297)	pS 35	
GPKPLFRRMpSSLVGPTQ			26-42 (297)	pS 35	
SGPKPLFRRMpSSLVGPTQ			25-42 (297)	pS 35	
pSPTIEAQGTSPAHDN	LRMP (JAW1) lymphoid-restricted membrane protein	4033	75-89 (499)	pS 75	ER transmembrane (cytoplasmic)
pSPTIEAQGTSPAHDNI			75-90 (499)	pS 75	
pSPTIEAQGTSPAHDNIA			75-91 (499)	pS 75	
ApSPTIEAQGTSPAHD			74-88 (499)	pS 75	
ApSPTIEAQGTSPAHDN			74-89 (499)	pS 75	
ApSPTIEAQGTSPAHDNI			74-90 (499)	pS 75	
ApSPTIEAQGTSPAHDNIA			74-91 (499)	pS 75	
SApSPTIEAQGTSPAHDN			73-89 (499)	pS 75	
KSVKALSSLHGDDQD <sub>pS</sub>	IGF2R insulin-like growth factor 2 receptor	3482	2394-2409 (2491)	pS 2409	lysosomal transmembrane receptor (cytoplasmic)
TTKSVKALSSLHGDDQD <sub>pS</sub>			2392-2409 (2491)	pS 2409	
pSKQDIRGKTEQSTIG	KIAA1109	84162	1682-1697 (5500)	pS 1682	transmembrane
pSNVTASPTAPACPSDKPA	TNIP1 TNFAIP3 interacting protein 1	10318	94-111 (636)	pS 92	cytoplasm, nucleus
DpSNVTASPTAPACPSDKPA			93-111 (636)	pS 92	
GKDpSNVTASPTAPACPSDKPA			91-111 (636)	pS 92	
FDKHTLGD <sub>pS</sub> DNES	FTH1 ferritin, heavy polypeptide 1	2495	171-183 (183)	pS 179	cytoplasm

<sup>a</sup> Underlined residues indicate known phosphosites (www.phosphosite.org).



**Table 4.4 Phosphorylated HLA class II ligands presented by the melanoma cell line MaMel-8a**

Peptide <sup>a</sup>	Gene	GeneID	Position	Phosphosites	Protein location/function (phosphosite location)
ASISRLpSGEQVDG	FZD6 frizzled homolog 6 (Drosophila)	8323	623-635 (706)	pS 629	plasmamembrane receptor (cytoplasmic)
ASISRLpSGEQVDGK			623-636 (706)	pS 629	
ASISRLpSGEQVDGKG			623-637 (706)	pS 629	
ApSISRLpSGEQVDGKG			623-637 (706)	pS 629, pS 624	
SPAASISRLpSGEQVDGKG			620-637 (706)	pS 629	
SPAApSISRLSGEQVDGKG			620-637 (706)	pS 624	
KYpSPGKLRGN	MXRA7 matrix-remodelling associated 7	439921	142-151 (204)	pS 144	transmembrane
YDLMDNKDKGSpS	FAM62A extended-synaptotagmin-1	23344	1093-1104 (1104)	pS 1104	intracellular membranes (cytoplasmic)
VSKVMIGpSPKKV	TNS3 tensin 3 (tumor endothelial marker 6)	64759	1434-1445 (1445)	pS 1441	putative transmembrane
VMIGpSPKKV			1437-1445 (1445)	pS 1441	
FSDpSEEEGEGGRKNSSN	HDAC1 histone deacetylase 1	3065	420-436 (482)	pS 423	nucleus
FSDpSEEEGEGGRKN			420-433 (482)	pS 423	
YTLPRQATPGVPAQQpSPSM	AOF2 (LSD1) amine oxidase (flavin containing) domain 2	23028	834-852 (852)	pS 849	nucleus
FDVGKTQPPLMKRAFpSTEK	ACTR10 actin-related protein 10 homolog ( <i>S. cerevisiae</i> )	55860	399-417 (417)	pS 414	cytoplasm
TPSQHSHSIQHpSPERSGSGSVGN	BCLAF1 BCL2-associated transcription factor 1	9774	257-279 (920)	pS 268	cytoplasm, nucleus
IRSRpTPSASNDDQQE	SGTA small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	6449	299-313 (313)	pT 303	cytoplasm
RSQIRSRpTPSASNDDQQE			296-313 (313)	pT 303	
LRSQIRSRpTPSASNDDQQE			295-313 (313)	pT 303	
FDKHTLGDpSDNES	FTH1 ferritin, heavy polypeptide 1	2495	171-183 (183)	pS 179	cytoplasm
LFDKHTLGDpSDNES			170-183 (183)	pS 179	

<sup>a</sup> Underlined residues indicate known phosphosites ([www.phosphosite.org](http://www.phosphosite.org)).

#### 4.1.4 Discussion

Altered cellular signaling is often associated with malignant transformation (136, 178, 179). Since signaling pathways are regulated to a large part by phosphorylation, the phosphorylation status of several cellular proteins differs between normal and malignant cells (136, 179). In addition, increased turnover of individual proteins is also marked by an increase of phosphorylation, since phosphorylation may target a protein for ubiquitinylation and thus degradation by the proteasome (20). Phosphorylated MHC class I ligands, which are derived mainly from cytoplasmic proteins, have been shown to exist, and the phosphorylation has been demonstrated to be due to differential phosphorylation of the corresponding source protein (176). MHC ligands containing phosphorylations which are specifically relevant for the proliferation of tumor cells represent potential targets for T cell-based cancer immunotherapy. To identify such ligands, in former studies, the repertoires of phosphorylated MHC class I ligands presented by tumor cell lines were compared to the repertoire presented by an EBV-transformed B-LCL (176). However, tumor cell lines bear the risk of presenting MHC ligands which contain phosphorylations reflecting *in vitro* conditions. With regard to application in cancer immunotherapy, it is crucial to investigate which phosphopeptides are actually presented *in vivo*. Therefore, we advanced a phosphopeptide enrichment technique for identifying phosphorylated MHC class I ligands from a patient's RCC tissue. In order to assess whether a phosphopeptide may be cancer-related, we equivalently analyzed the phosphorylated peptides presented by the corresponding healthy renal tissue. Unexpectedly, nine out of eleven phosphopeptides identified were presented both by the tumor and by the healthy tissue. This suggests that these ligands might rather be typical for kidney tissue than associated to malignant transformation. Alternatively, the healthy tissue might have been influenced by the same growth factors as the tumor tissue, since both tissues were derived from the same kidney. Only two peptides were specific for either tissue. One phosphorylated MHC ligand was exclusively found presented by the tumor tissue: a peptide derived from the protein butyrate response factor 1 (Brl1): pS<sub>203</sub>-Brl1<sub>195-204</sub>. Interestingly, another phosphorylated MHC ligand derived from Brl1 was found in both tissues, tumor and healthy: pS<sub>54</sub>-Brl1<sub>49-57</sub>. We cannot exclude that the peptide pS<sub>203</sub>-Brl1<sub>195-204</sub> was present below detection limit in the healthy tissue. Yet, according

to literature phosphorylation of pS<sub>203</sub> and pS<sub>54</sub> may be regulated by two distinct pathways: it has been shown that phosphorylation is carried out *in vivo* by PKB/Akt (pS<sub>203</sub>) (369) and *in vitro* by MK2 (both pS<sub>203</sub> and pS<sub>54</sub>) (370). Unphosphorylated Brf1 regulates mRNA levels by targeting transcripts containing AU-rich elements (AREs) into the decay pathway (291). AREs containing mRNAs include highly regulated mRNAs, in particular transcripts from genes expressed upon stimuli by growth factors, such as cell-cycle genes and oncogenes (372-376). It has been demonstrated that PKB/Akt mediated phosphorylation of S<sub>203</sub> and S<sub>92</sub> inhibits Brf1 from promoting its regulatory mRNA decay activity (369). MK2-mediated phosphorylation of S<sub>203</sub>, S<sub>92</sub> and S<sub>54</sub> inhibits mRNA decay activity as well (370). The fact that we found the MHC ligand containing pS<sub>203</sub> exclusively in the tumor tissue points into the direction that PKB/Akt activity might be upregulated specifically in the tumor tissue, whereas MK2 activity seems to be equal in both tissues. PKB/Akt regulates many key effector molecules involved in cell survival (377) and its activity has been shown to be frequently increased in RCC (378). The kinase is regulated by the PI3K signaling pathway, a pathway suppressed by tumor suppressor protein PTEN, which is often mutated or deleted in tumors, including RCC (379-383). Increased mRNA stability has been implicated in malignancy (reviewed in (376, 384, 385)). Thus, we conclude that the Brf1-derived phosphopeptide pS<sub>203</sub>-Brf1<sub>195-204</sub> might represent an MHC ligand reflecting a cellular phenomenon connected to tumor progression. This finding demonstrates that the identification of phosphorylated MHC class I ligands from tumor tissue compared to healthy tissue appears to be a valuable strategy to identify tumor-associated MHC ligands for immunotherapy.

Surprisingly, we did not find any of the phosphorylated HLA-A\*02 ligands, which were identified by Zarling et al. on three tumor cell lines (melanoma and ovarian carcinoma) but not on EBV-transformed B-LCL (176). A reason for the lack of overlap might be that we used a different phosphopeptide enrichment technique and chemically modified the peptides in a different manner before analysis. However, we found several of these phosphopeptides presented by an HLA-A\*02 expressing RCC cell line (Table 4.5, peptides were modified chemically as well). This rather indicates that the peptides might have been below detection limit in the analysis of the RCC tissue. Yet, we detected the one HLA-A\*02 ligand, which we identified from the renal tissue (pS<sub>5</sub>-ID2<sub>2-10</sub>), also in the analysis of the RCC cell line with similar signal intensity. The most probable explanation for the lack of overlap is that we examined

primary tumor tissue instead of tumor cell lines. The repertoires of phosphorylated MHC ligands seem to differ less between tumor cell lines cultured *in vitro* than to tumor tissue *in vivo*. This observation emphasizes the need to analyze actual tumor tissue rather than tumor cell lines in order to define tumor-associated phosphopeptides for immunotherapy.

**Table 4.5 Phosphorylated HLA-A\*02 ligands presented by the RCC cell line RCC68**

Peptide <sup>a</sup>	Gene (GeneID)	Position	Phosphosite
VMIG <u>p</u> SPKKV	TNS3 tensin 3 (64759)	1437-1445 (1445)	pS 1441
TLA <u>p</u> SPSVFKST	C13orf34 chromosome 13 ORF 34 (79866)	38-48 (559)	pS 41
AVV <u>p</u> SPPALHNA	BRD4 bromodomain containing 4 (full length transcript variant) (23476)	855-865 (1362)	pS 858
RQI <u>p</u> SQDVKL	AMPD2 adenosine monophosphate deaminase 2 (isoform L) (271)	165-173 (879)	pS 168
RL <u>L</u> pSPLSSA	RAVER1 ribonucleoprotein, PTB-binding 1 (125950)	581-589 (756)	pS 584
RQD <u>p</u> STPGKVFL	NR2C1 nuclear receptor subfamily 2, group C, member 1 (7181)	61-71 (467)	pS 64
RTFSP <u>p</u> TYGL	DMN desmuslin (23336)	426-434 (1253)	pT 31
SMTR <u>p</u> SPPRV	SFRS2B splicing factor, arginine/serine-rich 2B (10929)	248-256 (282)	pS 52

<sup>a</sup> Underlined residues indicate known phosphosites ([www.phosphosite.org](http://www.phosphosite.org)).

The majority of phosphorylated MHC class I ligands identified were presented by HLA-B\*07, while only one HLA ligand each was found HLA-A\*02-, A\*24- and B\*35-restricted (Table 4.2). This bias might be due to the fact that basic amino acids at the N-terminus (applicable for eight out of eleven phosphopeptides identified) seem to be favorable for the fragmentation and detection of phosphopeptides by ESI-MS and that N-terminal arginine or lysine residues are found more frequently among HLA-B\*07 ligands than among ligands of other HLA alleles (see peptide motifs at [www.syfpeithi.de](http://www.syfpeithi.de)). This suggests that some combinations of HLA alleles might be more beneficial for the identification of tumor-associated phosphopeptides from a patient's tumor tissue than others.

Proteins which are normally not degraded by the MHC class I but the MHC class II antigen processing pathway are membrane proteins (386). This class of proteins includes plasmamembrane receptors which play a major role in cellular signaling and are frequently phosphorylated upon stimulation. Presented on the cell surface by MHC class II molecules, phosphorylated peptides derived from such receptors might be of similar relevance for cancer immunotherapy as MHC class I phosphopeptides, since anti-tumor immunity is dependent not only on CD8<sup>+</sup> T cells but also on CD4<sup>+</sup> T cells (362-367). Although MHC class II molecules are normally only expressed by cells of the immune system, tumors have been shown to express MHC class II molecules as well (171, 387). Former studies pointed into the direction that phosphorylated MHC

class II ligands do not exist (196). In this study, we could show the contrary. Using the TiO<sub>2</sub>-microcentrifugation columns, we enriched and identified MHC class II phosphopeptides from both an EBV-transformed B-LCL and a melanoma cell line. In summary, we identified 47 peptides, which were partly length variants of the same core ligand and contained up to three phosphorylations, mostly of serine residues. Twelve out of 19 phosphorylation sites identified were unknown; of three proteins we discovered the first phosphorylation sites at all. As expected, the majority of ligands were derived from transmembrane proteins and most of the novel phosphorylation sites are contained in these ligands. On the other hand, most known phosphorylation sites were contained in soluble proteins. Thus, the analysis of phosphorylated MHC class II ligands represents a method to identify novel phosphorylation sites in particular of transmembrane proteins, which seem to be undiscovered to a higher extent than those of soluble proteins.

Several of the MHC class II ligands identified were derived from receptors which play a significant role in cellular signaling: Chemokine receptor 4, CD20, and Insulin-like growth factor 2 receptor (B-LCL JY); Frizzled 6 (melanoma cell line MaMel-8a). Phosphorylations contained in these ligands were located at the cytoplasmic part of the receptors. Thus, a function of these phosphorylations in signaling is likely. The two novel phosphorylation sites found within the ligand derived from the receptor Frizzled 6 are particularly noteworthy; they are the first phosphorylation sites identified for this protein. Frizzled 6 is a member of the Frizzled family of 7-transmembrane-domain-receptors, which bind secreted Wnt proteins (388, 389). The Wnt signaling pathways are involved in the regulation of tissue and cell polarity, embryonic development, and the regulation of proliferation (390). Inappropriate activation of the Wnt signal transduction pathway plays a role in a variety of human cancers. The tumor suppressor protein APC (Adenomatous polyposis coli), a negative regulator for Wnt signaling, has been shown to be inactivated in most colon cancers (391-393). The majority of Frizzled receptors are coupled to the  $\beta$ -catenin canonical signaling pathway, which leads to the activation of Dishevelled proteins (Dvl), inhibition of GSK-3 kinase, nuclear accumulation of  $\beta$ -catenin and activation of Wnt target genes (390); yet, transduction of signals by Frizzled receptors is largely unknown (394). In contrast, Frizzled 6 has been shown to abrogate Wnt signaling in a ligand-dependent manner by inhibiting the ability of  $\beta$ -catenin to activate transcription of Wnt target genes (395). Accordingly, Frizzled 6 has been suggested as

a tumor suppressor protein. Cytoplasmic C-terminal phosphorylation, as found in this study within the Frizzled 6-derived MHC class II ligand of a melanoma cell line might possibly inactivate the receptor and promote its internalization and degradation with the consequence of increased expression of  $\beta$ -catenin target genes. This hypothesis would be consistent with previous observations that Frizzleds transduce signals through G-proteins (396-398) and that phosphorylation of the C-terminus by G-protein-coupled receptor-associated protein kinases (GRKs) is associated with attenuation of signaling, receptor internalization, and protein turnover (399). In any case, knowledge of the first two phosphorylation sites within the C-terminus of Frizzled 6 will likely advance investigation of the Wnt/Frizzled/ $\beta$ -catenin signaling pathways.

Similarly, within the MHC class II ligands identified from a B-LCL, we have found two additional phosphorylation sites and confirmed the only one known of the chemokine (C-X-C motif) receptor 4 (CXCR4), a G-protein coupled receptor, which is internalized upon ligand-dependent phosphorylation (400). All three phosphorylation sites identified in the MHC class II ligand (pS<sub>324</sub>, pS<sub>325</sub>, pS<sub>339</sub>) and phosphorylation of S<sub>338</sub> have been proposed to be critical for internalization of the receptor (400). We demonstrate that internalized CXCR4 is indeed phosphorylated at S<sub>324</sub>, S<sub>325</sub> and S<sub>339</sub> as well as degraded and presented on MHC class II. S<sub>338</sub> was not found phosphorylated within the MHC class II ligands and one ligand lacking phosphorylation of S<sub>324</sub> was detected, suggesting that phosphorylation of these two serines might not be essential for internalization and degradation. CXCR4 is expressed in many cancers where it may regulate tumor cell growth and migration and therefore might be a target in cancer treatment (401, 402). CXCR4 has further been shown to act as (co-)receptor for the entry of some HIV strains and to promote Env-mediated fusion of the virus and thus has been proposed as target for antiviral drug development (403-405).

Also worth mentioning, we have found the first phosphorylation site within the receptor CD20, a B-cell antigen located in proximity to MHC class II molecules, against which therapeutical antibodies are directed in treatment of B-cell non-Hodgkin's lymphoma and autoimmune disorders (406-409). In summary, our results show that analysis of phosphorylated MHC class II ligands represents a convenient way to increase knowledge of natural phosphorylation sites within significant plasmamembrane receptors.

One crucial question remains to be answered in the next step: can all phosphorylated ligands be distinguished from their unphosphorylated counterparts by CD4<sup>+</sup> T cells? Discrimination has been shown previously (368), however, it seems possible that recognition of the phosphorylation is dependent on its location within an MHC class II ligand: a phosphorylation in the central part of a peptide and thereby within the binding groove of the MHC class II molecule might be recognized rather than a phosphorylation located at the peptide's termini. Among the peptides identified were both cases. The answer to this question will determine the relevance of individual phosphorylated MHC class II ligands for application in cancer immunotherapy.

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## 5 Summary / Zusammenfassung

Human leukocyte antigens (HLA) present protein-derived peptides to T lymphocytes. The knowledge of HLA ligands presented by certain types of cells (e.g., virus infected or cancer cells) allows for stating against which molecular compounds, so called epitopes, a T cell-based immune response may be directed.

This thesis concentrated on the selective identification of viral and phosphorylated HLA ligands by HPLC-coupled mass spectrometry. Peptides of viral origin are presented on HLA class I by virus-infected cells to CD8<sup>+</sup> cytotoxic T cells (CTL), which kill the infected cell upon recognition. Phosphorylated HLA class I ligands have raised hope to play a role in the immune response to cancer, since cellular signaling, which is often altered in malignant cells, includes phosphorylation of proteins. Identification of viral or phosphorylated HLA ligands is challenging due to their low frequency and potential low abundance compared to that of self-ligands and non-phosphorylated peptides.

A novel approach using differential stable isotope labeling of HLA ligands purified from infected and mock-infected cells was applied to identify naturally processed viral HLA ligands. The virus analyzed was modified vaccinia virus Ankara (MVA), which is currently being tested as recombinant and smallpox vaccine. 15 viral peptides of which twelve were presented by HLA-A\*0201 and three by HLA-B\*0702 were found. Nine peptides have not been described as CTL epitopes so far. All HLA-A\*0201 ligands were actual memory CTL epitopes in MVA vaccinees. Eight of these epitopes were novel. All ligands were expressed early during the viral life cycle, although late protein synthesis was not impaired. Importantly, early viral HLA ligands mediated protection against a lethal respiratory challenge in mice while late viral peptides previously described as CTL epitopes were inefficient. Thus, knowledge of the peptides which are actually presented by infected cells is crucial for optimizing the monitoring of protective immune responses and for the development of effective peptide-based vaccines.

In addition, this differential analysis revealed two human self-HLA ligands which were highly upregulated in the MVA-infected cells, suggesting either specifically induced synthesis and/or degradation of the source proteins upon infection. Both ligands were derived from cytoskeletal proteins. Unusually high presentation of self-ligands in association with infection might potentially induce self-reactive T cells playing a role in autoimmune responses observed during vaccinia virus vaccination.

By developing TiO<sub>2</sub>-based microcentrifugation columns, the first phosphorylated HLA class I ligands presented by tumor tissue (renal cell carcinoma) were identified. By comparison to the ligands of healthy renal tissue one phosphorylated Brf1-derived HLA ligand was found as potentially tumor-associated. Moreover, the first natural phosphorylated HLA class II ligands were discovered. They revealed several novel phosphorylation sites of transmembrane receptors playing a significant role in malignant signaling, such as Frizzled 6, CXCR4 and CD20.



*Zusammenfassung:*

Humane Leukocyten-Antigene (HLA) präsentieren aus Proteinen stammende Peptide den T-Lymphocyten. Die Kenntnis von HLA-Liganden, die von bestimmten Zelltypen (z.B. Virus-infizierten oder Krebszellen) präsentiert werden, ermöglicht es Aussagen zu treffen, gegen welche molekularen Komponenten, so genannte Epitope, eine T-Zell-Antwort gerichtet sein kann.

Fokus dieser Arbeit war die gezielte Identifizierung von viralen und phosphorylierten HLA-Liganden mittels HPLC-gekoppelter Massenspektrometrie. Peptide viralen Ursprungs werden von HLA Klasse I auf virus-infizierten Zellen den CD8<sup>+</sup> cytotoxischen T-Zellen (CTL) präsentiert, die die infizierte Zelle nach Erkennung abtöten. Bezüglich phosphorylierten HLA Klasse I-Liganden besteht die Hoffnung, dass sie in der Immunantwort gegen Krebs eine Rolle spielen, da die zelluläre Signalweiterleitung, die in malignen Zellen oft gestört ist, Phosphorylierung von Proteinen umfasst. Die Identifizierung von viralen oder phosphorylierten HLA-Liganden ist dadurch erschwert, dass diese im Vergleich zu Liganden aus zelleigenen Proteinen und nicht-phosphorylierten Liganden in geringer Anzahl und möglicherweise auch Menge auftreten.

Zur Identifizierung von natürlich prozessierten viralen HLA-Liganden wurde ein neuer Ansatz verfolgt, der auf der differentiellen Markierung von HLA-Liganden aus infizierten und nicht-infizierten Zellen mit stabilen Isotopen beruht. Bei dem untersuchten Virus handelte es sich um das Modified Vaccinia Virus Ankara (MVA), das zurzeit als rekombinanter und Pocken-Impfstoff getestet wird. Es wurden 15 virale Peptide identifiziert, davon wurden zwölf auf HLA-A\*0201 und drei auf HLA-B\*0702 präsentiert. Neun dieser Peptide wurden zuvor noch nicht als T-Zell-Epitope beschrieben. Alle HLA-A\*0201-Liganden erwiesen sich tatsächlich als CTL-Epitope in MVA-Impfungen. Acht dieser Epitope waren neu. Alle Liganden stammten aus Proteinen, die früh im viralen Lebenszyklus gebildet werden, und dies obwohl die späte Proteinsynthese nicht gehemmt war. Eine wichtige Beobachtung war, dass Mäuse durch Impfung mit frühen viralen HLA-Liganden gegenüber einer letalen eingeatmeten Viruslast geschützt waren, während späte Peptide, die zuvor als CTL-Epitope beschrieben worden waren, diesen Schutz nicht boten. Um den Verlauf von schützenden Immunantworten optimal zu überwachen und um effektive Peptid-basierte Impfstoffe zu entwickeln, ist es also unverzichtbar, die Peptide, die tatsächlich von infizierten Zellen präsentiert werden, zu kennen.

Durch diese differenzielle Analyse wurden zusätzlich zwei humane HLA-Liganden gefunden, die auf den MVA-infizierten Zellen stark überpräsentiert waren, was darauf hinweist, dass die Proteine, aus denen diese HLA-Liganden stammten, durch die Infektion hervorgerufen verstärkt synthetisiert und/oder abgebaut wurden. Beide Liganden stammten aus Proteinen des Cytoskeletts. Ungewöhnlich starke Präsentation von Liganden körpereigener Proteine in Verbindung mit einer Infektion könnte selbst-reaktive T-Zellen aktivieren, die eine Rolle in den Autoimmunreaktionen spielen könnten, die bei Impfungen mit dem Vaccinia Virus aufgetreten sind.

Mittels selbst entwickelter TiO<sub>2</sub>-Mikrozentrifugationssäulchen gelang es, die ersten phosphorylierten HLA Klasse I-Liganden aus Tumorgewebe (des Nierenzellcarcinoms)

zu identifizieren. Durch den Vergleich mit Liganden, die aus gesundem Nierengewebe identifiziert wurden, wurde ein phosphorylierter HLA-Ligand aus dem Protein Brf1 gefunden, der möglicherweise in Verbindung mit dem Tumorwachstum stehen könnte. Außerdem wurden die ersten endogenen phosphorylierten HLA Klasse II-Liganden entdeckt. Sie enthielten einige bisher nicht bekannte Phosphorylierungsstellen von Transmembranrezeptoren, die eine bedeutende Rolle in der Signaltransduktion von Krebszellen spielen, wie zum Beispiel Firzzled 6, CXCR4 und CD20.

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## 7 Publications

**Meyer VS**, Hennenlotter J, Rammensee HG, Stevanovic S, Identification of natural MHC class II presented phosphopeptides and tumor-derived MHC class I phospholigands, *submitted*

**Meyer VS**, Kastenmüller W, Gasteiger G, Franz-Wachtel M, Lamkemeyer T, Rammensee HG, Stevanovic S, Sigurdardottir D, Drexler I, Long-term immunity against actual poxviral HLA ligands as identified by differential stable isotope labeling, *J. Immunol.*, 2008, 181: 6371-6383

Schlarb-Ridley BG, Mi H, Teale WD, **Meyer VS**, Howe CJ, Bendall DS, Implications of the effects of viscosity, macromolecular crowding, and temperature for the transient interaction between cytochrome f and plastocyanin from the cyanobacterium *Phormidium laminosum*, *Biochemistry*, 2005, 44: 6232-6238

### Oral and poster presentations

- 07/2008      20<sup>th</sup> International Congress of Genetics, Berlin, Germany (poster presentation)
- 06/2008      17<sup>th</sup> International Poxvirus and Iridovirus Conference, Grainau, Germany (oral presentation)
- 03/2008      18<sup>th</sup> Annual Meeting of the German Society of Virology, Heidelberg, Germany (oral presentation)
- 10/2007      5<sup>th</sup> Meeting of the working group “Immunology of viral diseases” of the German Society of Virology, Deidesheim, Germany (oral presentation)
- 09/2007      37<sup>th</sup> Annual Meeting of the German Society of Immunology, Heidelberg, Germany (poster presentation)
- 09/2006      16<sup>th</sup> European Congress of Immunology, Paris, France (poster presentation)
- 05/2006      Summer School in advanced immunology of the European Network of Immunology Institutes, Capo Caccia, Sardinia/Italy (poster presentation)

## 8 Appendix

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