The MHC II Ligandome

Mass Spectrometric Applications in Immunology

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Massenspektrometrische Anwendungen in der Immunologie

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Preface

Some of the chapters of this thesis are parts of publications. At the beginning of each chapter it is indicated which experiments where done by the author of the thesis, which persons contributed to the publication, and in which journals the work was published.

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1 Introduction

1.1 The immune system

Although our body is daily confronted with possible invasions of pathogens, severe infections occur only seldom, due to our highly efficient immune system. It is responsible for host defense against infectious agents. With the help of a complex system of humoral and cellular defense mechanisms it is able to discriminate between self and non-self protecting our body against pathogens. The immune system consists of all organs, tissues, cells and molecules involved in host defense. Immunocompetent organs can be broadly divided into central and peripheral. In the central organs immunocompetent white blood cells – leukocytes – are generated and major parts of their development take place: the bone marrow, where generation of leukocyte progenitors from pluripotent hematopoetic stem cells occurs and B lymphocytes mature, and the thymus, the organ of T lymphocyte maturation. The most important peripheral organs are the spleen, the lymph nodes and the mucosal lymphatic tissues. Here, lymphocytes are maintained and meet possible antigens.

Defense mechanisms of the immune system, the so called immune response, are based on two different but interacting principles, innate and adaptive immunity. Innate immunity provides a first line of defense against many common pathogens. It evolved before adaptive immunity and plays a crucial role in controlling infections in the first four to seven days, the time needed before an initial adaptive immune response can take effect. Part of an innate immune response are: the complement system, inflammatory cells – macrophages and neutrophils –, natural killer cells (NK cells), γ:δ T cells and B-1 B cells. The components of innate immunity are constitutively present in our body and do not generate an immunological memory.

Adaptive immunity is based on clonal selection of antigen-specific effector lymphocytes and on generation of memory cells that are able to prevent re-infection with the same pathogen. An adaptive immune response, also known as acquired immune response, is mainly made up by three different cell types:

professional antigen-presenting cells (APC), B lymphocytes and T lymphocytes. To raise such a response at least two of the above mentioned cell types have to participate. Adaptive immunity distinguishes humoral, or B cell-mediated, and cellular, or T cell-mediated, immunity. Humoral immunity is mainly based on antibodies (Ab) secreted by B cells which have to get activated by antigen-specific T helper cells (TH2), and is directed against extracellular pathogens and toxins. Cellular immunity, in general directed against intracellular pathogens or for example tumor cells, is based on cytotoxic T lymphocytes (CTL) and inflammatory TH1 cells. To become activated, CTL have to interact with APC and TH1 cells. Coordination and regulation of a specific immune response are maintained through subpopulations of T cells that either activate or inhibit other immune competent cells.

1.1.1 Major histocompatibility complex molecules and antigen processing

As already mentioned, the immune system is able to distinguish between self and non-self through humoral and cellular defense mechanisms, respectively. B cell receptors and Ab bind three dimensional, native structures, whereby nearly every chemical substance can be recognized by Ab. T cells, on the other hand, are more limited. Via their T cell receptor (TCR), they recognize parts of processed proteins, peptides, which have to be presented on special receptors, the major histocompatibility complex (MHC) molecules, on cell surfaces. The two different subsets of T cells, CD8+ and CD4+ T cells, recognize two different types of MHC molecules, MHC class I and MHC class II, respectively. The MHC is the most polymorphic gene cluster in humans and is located on chromosome six. MHC molecules, in humans called human leukocyte antigens (HLA), are a family of highly polymorphic glycoproteins. MHC I molecules are heterodimers formed of a heavy chain, in humans HLA-A, -B, -C, and of a noncovalently bound light chain, β2-microglobulin (β_2m). In contrast, MHC II molecules, in humans HLA-DR, -DQ, -DP, are made of two heavy chains, α and β.

In the classical view, MHC class I molecules present peptides from endogenously synthesized proteins on cell surfaces of the vast majority of cells, allowing

circulating CD8+ cytotoxic T cells to survey cells for possible infections or improper protein expression, such as might be seen during tumorigenesis. As MHC class I genes are highly polymorphic, proteins encoded by each allele will bind only a unique set of peptides. Anchor residues of the antigenic peptides determine the binding specificity to the peptide binding groove of a specific MHC molecule [1]. The MHC class I molecule defines the position of anchor residues which have to be occupied by specific amino acids. Most common, peptides of eight to ten amino acids in length are generated by normal cellular degradation of proteins and presented on the cell surface by class I [2]. The ubiquitin-proteasome system generates precursor peptides that have the correct C-terminus but may have N-terminal extensions of several amino acids [3]. The constitutive 20S proteasome consists of 14 non-identical subunits (α1-7 and β1-7) forming four stacked rings of seven subunits each (2 outer α rings and 2 inner β rings) and shows three different proteolytical activities: a chymotrypsin-like (β1, cleavage after hydrophobic residues), a trypsin-like (β2, cleavage after basic residues) and a caspase-like (β5, cleavage after acidic residues) [4]. In the presence of interferon γ (IFNγ) the subunits which harbor the active sites are exchanged by so called immunosubunits: MECL1, LMP2 and LMP7 [5]. The newly formed immuno-proteasome is able to enhance the generation of peptides which can be presented on class I [6]. The N-termini of the peptides are further trimmed by aminopeptidases residing either in the cytosol or in the endoplasmic reticulum (ER) [7]. Peptides generated by the proteasome are transported by the transporter associated with antigen processing (TAP) into the ER [8] where they are loaded on MHC I heavy chain-β_2m heterodimers through interactions in the peptide-loading complex, consisting additionally of the transmembrane glycoprotein tapasin, the chaperone calreticulin and the thiol oxidoreductase ERp57 [9]. Mature MHC I complexes consisting of MHC I heavy chain, β_2m and peptide are then transported on the cell surface where they can interact with CD8+ T cells.

MHC class II molecules – as polymorphic as class I – typically present peptides from exogenous proteins acquired by endocytosis or from internalized plasma membrane proteins to CD4+ T cells. Whereas for MHC class I the initial events of antigen processing and MHC peptide assembly take place in different cellular compartments, processing of exogenous antigens and MHC II peptide loading happen in the same [10]. Therefore it is not surprising that MHC II peptide

assembly differs substantially from that of MHC I. MHC II α- and β-chains are synthesized into the ER where they form nonameric complexes consisting of three MHC II dimers and three invariant chain molecules (Ii, CD74) [11], a type II membrane protein. Ii serves as both a targeting subunit into the MHC class II loading compartment (MIIC) [12;13], part of the endosomal-lysosomal system, and a guardian of the peptide binding groove [14]. On the way into the MIIC, Ii is removed by an ordered proteolytic reaction, leading to the generation of a fragment called class II invariant chain peptide (CLIP), lodged in the peptide binding site. Ii processing happens in a C-terminal-to-N-terminal direction and is dependent on lysosomal proteases. Cathepsin S, cathepsin L and asparagine endopeptidase (AEP) are three proteases involved in Ii processing [15], but further proteases may also play a role, as some processing steps are redundant. Dissociation of CLIP and loading of antigenic peptides is catalyzed by the chaperone DM, whose function is modulated by DO [16]. Antigen processing happens as Ii processing in the endosomal-lysosomal system. The principal cysteine proteases involved are cathepsin S, L, B, F, H and V and the unrelated AEP [17;18]. Compared to MHC I molecules, the binding grooves of MHC II molecules are open at their ends. Therefore, MHC II ligands show a much greater variety in their length, in general being 11 to 18 amino acids long [19;20]. Often, MHC II peptides encompass a core sequence, which is variably extended at both termini [21]. In contrast to MHC I molecules, binding of class II molecules and their ligands is mainly based on interactions between peptide backbone and MHC II binding groove [22]. MHC II molecules have also binding pockets specific for defined anchor amino acids, but compared to class I they are degenerated.

1.1.2 T lymphocytes and anti-tumor immunotherapy

CD4+ and CD8+ T pocess TCRs which cells recognize MHC-peptide-complexes presented by other cells. At the cell surface, the TCR is associated with the CD3 complex, responsible for signal transduction. To recognize MHC I- and MHC II-peptide-complexes, in addition the co-receptors CD8 and CD4, respectively, have to interact with conserved domains of the MHC molecules. Foreign peptides

presented on MHC molecules on surfaces of professional APC can be recognized by naive T cells, a process called priming. Primed T cells proliferate and differentiate into armed effector T cells, either CTL, in the case of CD8+ T cells, or TH1 and TH2, in the case of CD4+ T cells. To get primed, naive T cells have to recognize MHC-peptide-complexes on professional APC, as they need an additional co-stimulatory signal which is conferred for example by the receptors CD80 and CD86 on APC to CD28 on T cells. In general, without this additional co-stimulatory signal T cells recognizing MHC-peptide-complexes go over into anergy. Activated CTL destroy their target cells via the induction of apoptosis, either with the help of perforin and granzymes [23] or via Fas-ligand [24;25]. TH1 cells activate macrophages and CD8+ T cells – a cellular immune reaction – via the secretion of interferon γ (IFNγ) and interleukin 2 (IL-2) [26], whereas TH2 cells lead to an activation of Ab secreting B cells and the complement system via interleukin 4 (IL-4) [27] – a humoral immune reaction. Recently, another subpopulation of CD4+ T cells has been described: regulatory T cells (Treg) [28]. These cells express constitutively CD25 and inhibit T cell reactions in an, until now, not fully understood way.

In the last 10 years it has been shown in many different clinical trials that the immune system can be manipulated to specifically recognize and eliminate tumor cells [29;30]. Many different approaches have been used to activate the immune system towards the tumor, ranging from immunzations with whole tumor cell lysates to the administration of molecularly defined parts of tumor rejection antigens, in general peptides of proteins produced by the tumor. Tumor cells may differ from their surrounding by the expression of tumor antigens which are either tumor specific, meaning they occur exclusively in tumor tissue, as cancer-testis antigens, mutated antigens and tumor-virus antigens, or tumor associated which means they are highly overexpressed in tumors but can also be found in normal tissue, as for example differentiation antigens. Table 1 shows a classification of tumor rejection antigens.

Peptide based immunotherapy studies have shown that CTL are able to recognize tumor rejection antigens on tumor cells and are thus able to contribute to tumor regression [31;32]. Our knowledge about anti-tumor CD4+ effector T cells and their epitopes is far more limited which is in part due to the more difficult characterization. Degenerated class II binding motifs inhibit an as efficient class II

epitope prediction as for CTL epitopes, a process known as reverse immunology [33]. However, in order to elicit a long lasting anti-tumor immune response the effector CTL response should be accompanied by effector CD4+ T cells [34-36], giving the reason for the need of identification of class II epitopes of tumor rejection antigens [37]. In addition, it could be shown that CD4+ T cells are able to act tumor repressive without any CTL effector function. Normally, this happens in an indirect, cytokine dependent manner [38-40]. Thus, CD4+ T cells are able to inhibit tumor angiogenesis via IFNγ [41]. They can also counteract tumor development via the induction of an Ab response [42].

Table 1: Classes of tumor antigens*

class	examples	recognized by T cells	recognized by antibodies
point mutation of normal gene	CDK4 [43]	x	-
frame shift mutation of normal gene	TGFβRII [44]	x	-
anti sense transcript of normal gene	RU2AS [45]	x	-
expressed intron of normal gene	N-acetylglucosaminyltransferase V [46]	x	-
fusion protein caused by translocation	BCR-ABL [47]	x	-
fusion protein caused by posttrans. mod.	gp100 [48], FGF-5 [49]	x	-
altered posttranslational modifications	tyrosinase [50]	x	-
cancer/embryonic antigen	CEA [51;52]	x	x
overexpressed antigens - protein	Her2/neu, MUC1 [53;54]	x	x
overexpressed antigens – non-protein	ganglioside GD3 [55]	-	x
cancer testis antigen	NY-ESO-1 [56]		
	MAGE family [57]	x	x
oncogenes	ras [58-60]	x	x
tumor suppressor genes	p53 [61;62]	x	x
differentiation antigen	tyrosinase [63;64]	x	x
	gp100 [65;66]	x	x
viral proteins	HPV E7 [67;68]	x	x

* adapted from Rammensee et al. [69]

1.2 Mass spectrometry

Mass spectrometry (MS) is an analytical method which is able to determine molecular weights of ions in a high vacuum. Ions of inorganic or organic compounds are generated by different means, separated by their mass-to-charge ratios (m/z) and detected qualitatively and quantitatively by their respective m/z [70]. In principle, a mass spectrometer is made up by an ion source, for example an electrospray ionization (ESI) or a matrix assisted laser desorption/ionization (MALDI) source, by a mass analyzer, for example a quadrupole or a time-of-flight analyzer (TOF), and by a detector, for example a multichannel plate. With the invention of soft ionization methods such as MALDI [71] and ESI [72;73] MS became one of the most versatile tools in modern analysis of biomolecules. With these techniques it is possible to determine molecular weights of big biomolecules, for example of proteins, and to obtain sequence information, for example from peptides. In the remaining part of this chapter MS with respect to the analysis of proteins and peptides will be discussed. The different applications of MS in inorganic chemistry and in the analysis of small organic compounds will not be dealt with.

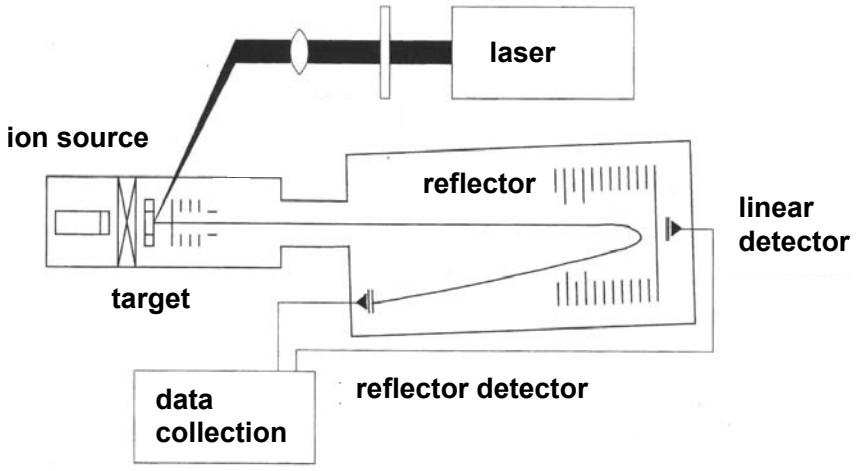

Figure 1: Schematic of a reflector-MALDI-TOF mass spectrometer (adapted from Bruker Daltonik, Bremen, Germany).

1.2.1 MALDI-TOF mass spectrometry

MALDI uses photons to deposit energy into a solid sample layer. The sample is co-crystallized with a matrix, usually small organic molecules that absorb at the wavelength of a given laser, on a metal target. A common laser in MALDI mass spectrometers is a nitrogen laser (337 nm). But not only ultraviolet (UV) lasers are in use, wavelengths ranging from UV to infrared (IR) are employed. For the analysis of peptides, matrices like α-cyano-4-hydroxycinnamic acid (CCA) [74] and 2-(4-hydroxyphenylazo)benzoic acid (HABA) [75] are used together with UV lasers. The energy uptake of matrix crystals upon laser irradiation causes evaporation and ionization of the sample. Once sample molecules have reached the gas phase, usually singly charged ions are formed by proton transfer from photo-ionized matrix molecules. Opposite of the target an electrode is mounted which creates an electric field pulling the ions towards the analyzer (Figure 1). A common analyzer used in MALDI mass spectrometers is a TOF analyzer. M/z are determined by the time ions take to trespass a high vacuum and to reach a detector. TOF analyzers have resolutions of up to 15,000 *full width at half maximum* (FWHM) in a mass range of up to 5000 Da. To reach such high resolution two devices are needed, a reflector [76] and an ion source with a delayed extraction [77]. A reflector is an ion mirror that is mounted opposite of an ion source and reverses flight directions of ions so that they reach the reflector detector. With its help ions of different kinetic energies are focused in time. Delayed extraction or time-lag focusing, a time delay between ion formation and extraction/acceleration, also counteracts the energy spread of emerging ions and the time distribution of ion formation. Usually, MALDI-TOF mass spectrometers are used to determine molecular weights of peptides and proteins.

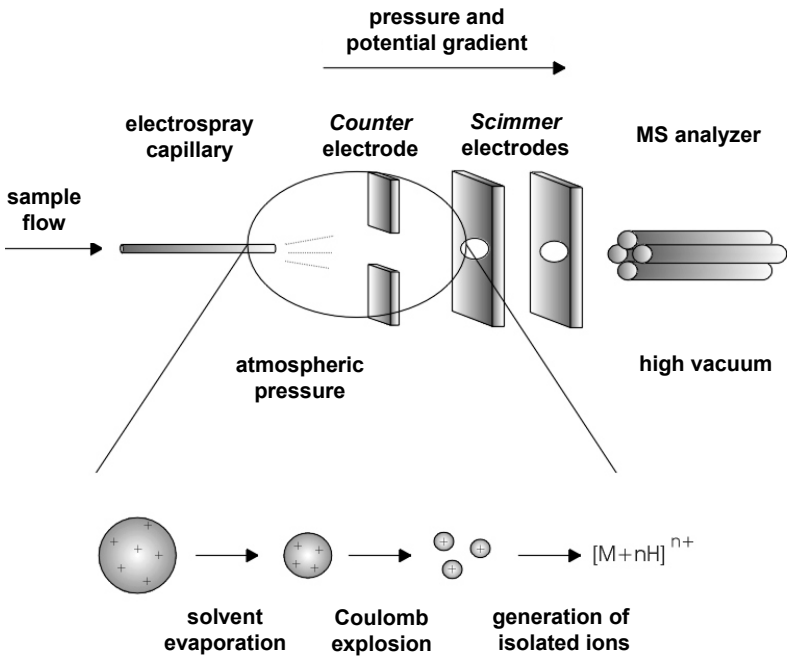

Figure 2: Disintegration of charged droplets and generation of single ion entities in an ESI-MS interface.

1.2.2 ESI-quadrupole-TOF mass spectrometry

In ESI MS gas phase ions are generated by the dispersion of a fluid in an electric field which is created between a capillary and the entrance of a mass spectrometer (Figure 2). In contrast to MALDI, ESI is not a real ionization process. Ions are already present in the fluidic phase and are only converted into the gas phase. An electric field creates a fine mist consisting of small highly charged droplets which follow a potential and pressure gradient into the analyzer of the mass spectrometer. Discrete, completely desolvated ions are generated through three interacting processes: desolvation of the solvent, Coulomb explosion, and escape of single ions out of droplets. A perpendicular gas flow, the so called curtain gas as for example heated nitrogen, supports the desolvation process. Commonly, multiple charged ions are generated in ESI MS. As the desolvation of the solvent is endotherme, it reduces the inherent energy of the ions and causes no fragmentation. A special application of ESI is nano-electrospray [78].

Miniaturization of the electrospray produces sample flow rates of 5 to 1000 nl/min which causes reduced sample consumption, and thus a higher sensitivity.

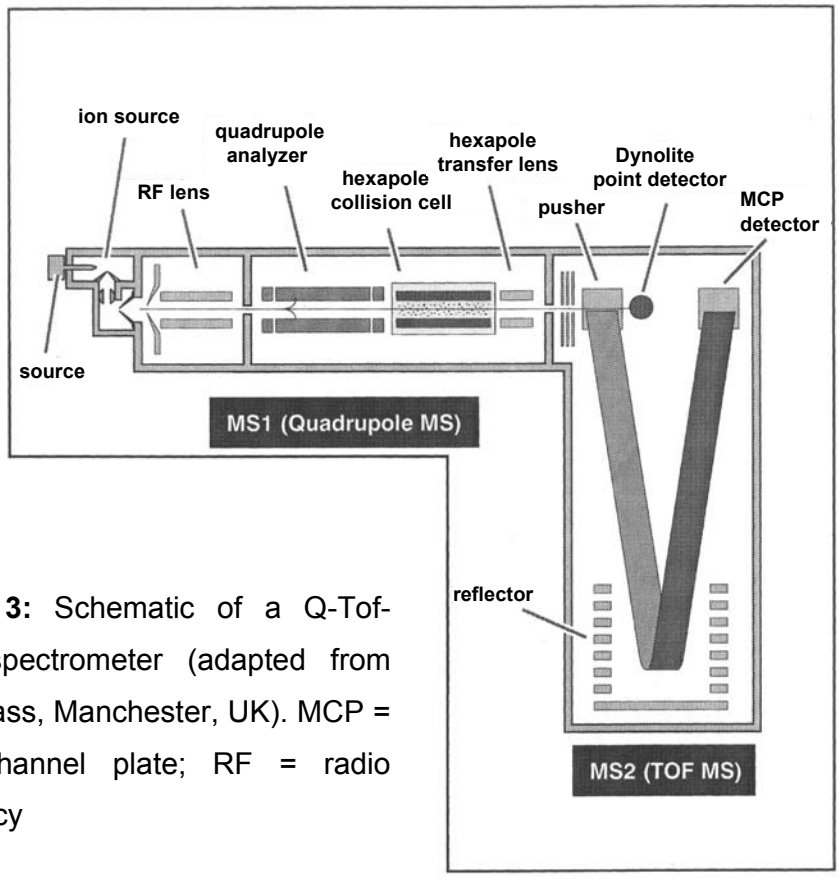

Figure 3: Schematic of a Q-Tof-mass spectrometer (adapted from Micromass, Manchester, UK). MCP = multi channel plate; RF = radio frequency

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With the help of tandem mass spectrometers structural information of ions can be obtained, in addition to mere mass analysis. In tandem mass spectrometry (MS/MS) two mass analyzers (MS1 and MS2) are coupled in a row. Mass selected ions (MS1) are subjected to a second mass spectrometric analysis (MS2) [78;79]. One possible setup of a tandem mass spectrometer is shown in Figure 3. Ions are selected by a quadrupole analyzer (MS1) and subjected to a hexapole collision cell, where they collide with argon gas and ion fragments are generated (collision induced dissociation, CID). These fragments are then further analyzed by a reflector-TOF analyzer (MS2).

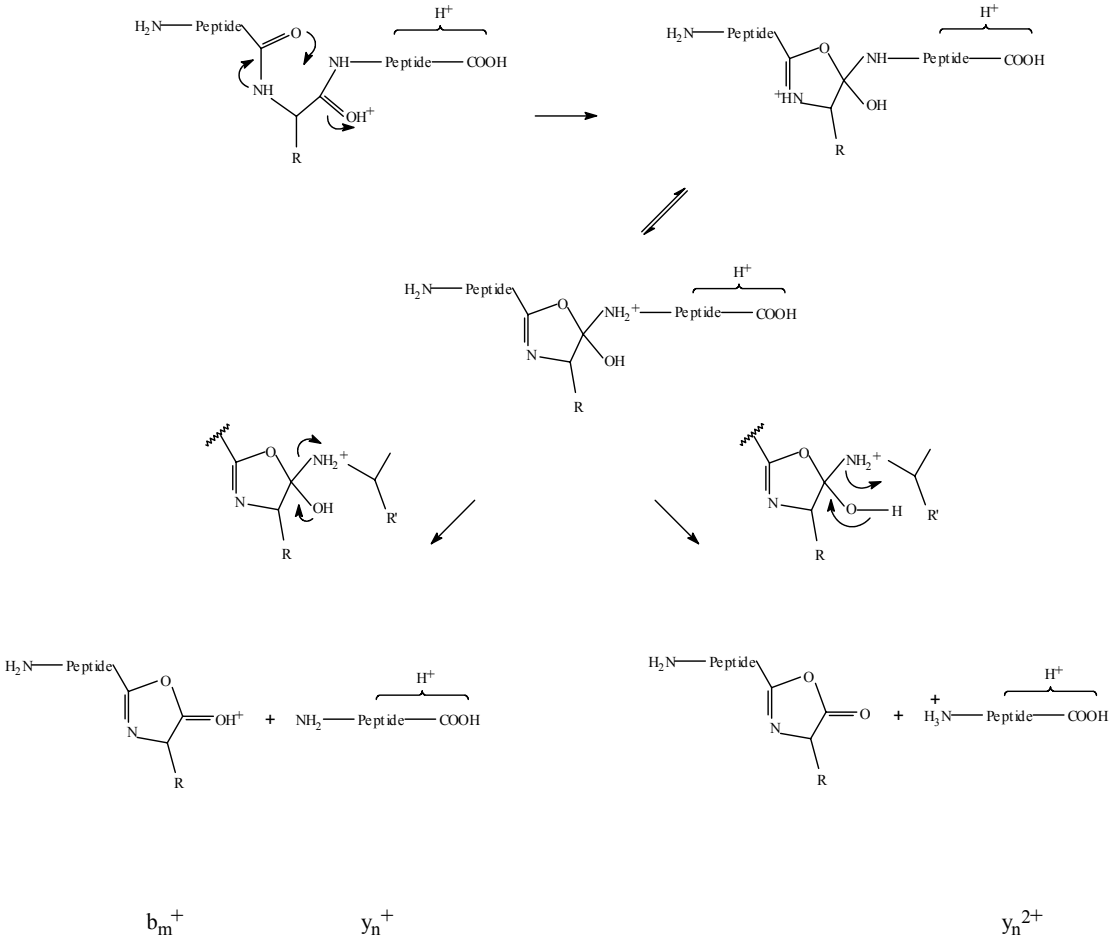

Figure 4: Generation of b- and y-ions during CID in the gas phase [80].

With a Q-Tof mass spectrometer it is possible to generate sequence information of peptides. Peptide ions are selected by the quadrupole and the corresponding fragment ions, generated in the hexapole, are analyzed by the TOF analyzer. Collision activated peptides preferably break between peptide bonds along the peptide backbone (Figure 4) which allows – with the knowledge of the masses of the amino acid residues (Table 2) – sequencing. Normally, during fragmentation the charge can either stay at the N-terminus or at the C-terminus of a peptide. Depending on the exact site of fragmentation, peptide ions are either called a, b, and c ions, or y, x, and z ions, respectively [78;81] (Figure 5).

Table 2: Masses of the common amino acid residues an their corresponding immonium ions.

amino acid residue	3-letter code	1-letter code	mass amino acid residue	mass immonium ion[a]
Alanine	Ala	A	71	44
Arginine	Arg	R	156	129
Asparagine	Asn	N	114	87
Aspartate	Asp	D	115	88
Cysteine	Cys	C	103	76
Glutamate	Glu	E	129	102
Glutamine	Gln	Q	128	101
Glycine	Gly	G	57	30
Histidine	His	H	137	110
Isoleucine	Ile	I	113	86
Leucine	Leu	L	113	86
Lysine	Lys	K	128	101
Methionine	Met	M	131	104
Phenylalanine	Phe	F	147	120
Proline	Pro	P	97	70
Serine	Ser	S	87	60
Threonine	Thr	T	101	74
Tryptophan	Trp	W	186	159
Tyrosine	Tyr	Y	163	136
Valine	Val	V	99	72

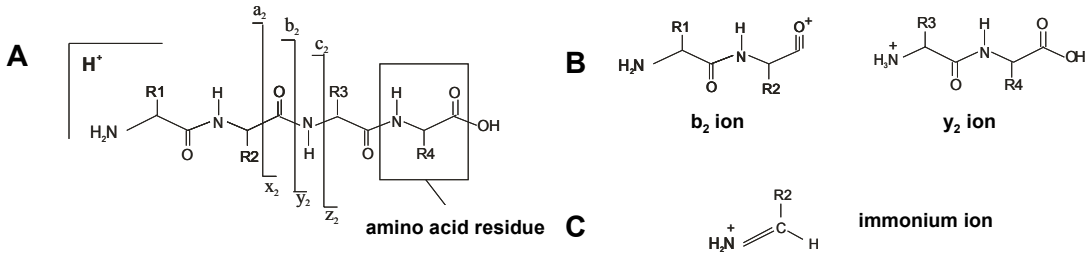

Figure 5: A. Nomenclature of peptide fragment ions. **B.** Simplified structures of b2 and y2 ions (see Figure 4). **C.** Structure of an immonium ion.

To analyze complex sample mixtures via MS, the combination of both separation techniques and MS is needed. One of the most important and common combinations is the coupling of a reversed-phase high performance liquid chromatography (HPLC) to an ESI mass spectrometer (LC-MS). Using small flow rates, the HPLC can be directly coupled online to the electrospray source, as is done with µ-capillary LC systems. Sample loss through extensive handling is reduced, contaminations like salts can be washed away, and the sample is concentrated in a sharply eluting peak. Usually, the sample is first loaded onto a C18 pre-column, where salts can be washed away and the sample is concentrated. In a second step, the sample is eluted in the opposite direction onto a fused silica µ-capillary separation column (for example 5 µm C18 material, 75 µm ID x 250 mm) which is directly coupled to the mass spectrometer via a nanoflow interface. Commonly, the selection of peptide ions for tandem MS is done automatically by the mass spectrometer.

1.3 Aims of the thesis

MHC class I ligands, especially in association with tumor development and immunity, have been extensively studied. Anti-tumor immunotherapy using class I peptides of tumor associated antigens in different vaccination combinations is also a research area which has been heavily explored. On the other hand, our knowledge about MHC class II peptides and their impact on anti-tumor immunity is far more limited. Thus, the aim of this thesis was to establish a better knowledge of the MHC class II peptide repertoire, the ligandome, in general, and to outline a procedure which helps in the identification of class II peptides from tumor associated antigens, in particular. To achieve these goals, state-of-the-art mass spectrometric devices, ranging from a MALDI-Reflector-TOF mass spectrometer to a LC-MS system, were used.

HLA-DR peptides from a tumor-like cell line should be identified by MS and analyzed using the rules of proteome analysis. The question to answer was: peptides from which source proteins are presented under normal conditions on MHC class II molecules on the cell surface? A further question was: has autophagy, one of the two major cellular degradation pathways and a process which plays a role in tumor development, an impact on the class II ligandome? To answer this question, a comparative MS analysis of MHC class II peptides isolated from cells undergoing autophagy and control cells had to be perforemd.

A special field of interest was posttranslational modified MHC peptides. It has already been described that T cell recognition of antigenic peptides can depend on posttranslational modifications of such peptides, but so far hardly any naturally presented posttranslational modified MHC II ligands have been identified. Therefore the class II ligandome should be searched for known modifications, such as glycosylation and deamidation.

Finally, as most tumors are class II negative and MHC II ligands cannot be directly isolated from solid tumor tissue, a strategy for the identification of naturally presented class II ligands from tumor associated antigens should be set up.

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2 Results and Discussion

2.1 Autophagy promotes MHC-II presentation of peptides from intracellular source proteins

This manuscript has been composed by Jörn Dengjel, Oliver Schoor, Rainer Fischer, Michael Reich, Marianne Kraus, Katharina Kreymborg, Florian Altenberend, Hubert Kalbacher, Roland Brock, Christoph Driessen, Hans-Georg Rammensee and Stefan Stevanović. The author of this thesis has performed the experiments leading to figure 1, 2, 3 and 4. All the mass spectrometric experiments and cell culture work were done by him.

2.1.1 Summary

MHC-peptide complexes mediate key functions in adaptive immunity. In a classical view, MHC I molecules present peptides from intracellular source proteins, whereas MHC II molecules present antigenic peptides from exogenous and membrane proteins. Nevertheless substantial crosstalk between these two pathways has been observed. We investigated the influence of autophagy on the MHC II ligandome and show that peptide presentation is strongly altered upon induction of autophagy. The presentation of peptides from intracellular and lysosomal source proteins was strongly increased on MHC II in contrast to peptides from membrane and secreted proteins. In addition, autophagy influenced the MHC II antigen processing machinery. Our study illustrates a profound influence of autophagy on the class II peptide repertoire and suggests implications in the regulation of CD4+ T-cell-mediated processes.

2.1.2 Introduction

Peptides of foreign and self proteins are presented on major histocompatibility complex class I (MHC I) and class II (MHC II) molecules at the cell surface and can be recognized by CD8+ and CD4+ T lymphocytes, respectively [1;2]. By this the MHC molecules transfer information about the current stock of proteins within a cell and its surroundings to the cell surface allowing the immune system to survey the cell's integrity and to react, if necessary. The definition of pools of peptides presented at the cell surface under specific conditions is vital for the understanding of the immune system. Especially if the interest lies in the manipulation of the immune system, for example in peptide based immunotherapy [3], it is absolutely necessary to understand which peptides are presented under which condition at the cell surface.

From a classical point of view, MHC I molecules present antigenic peptides derived from intracellular proteins whereas MHC II molecules do so for exogenous or membrane proteins [4]. This phenomenon is reflected in the two major cellular breakdown pathways for proteins: proteasomal degradation, relevant particular to the generation of MHC class I peptides [5], and degradation by the endosome/lysosome system, responsible for the processing of MHC class II peptides [6]. However, the separation of these distinct pools of source proteins is less stringent than originally thought. It is now well-established that MHC class I molecules are able to present peptides derived from exogenous antigens (Ag) by a process known as cross presentation [7]. On the other hand, intracellular proteins can be presented by MHC class II molecules [8] even though the underlying processes are less clear. It could be shown that a model protein, artificially introduced into cells, is presented on MHC class II molecules via autophagy [9]. Autophagy plays a role in the endosomal/lysosomal degradation pathway and is responsible for feeding intracellular components into this pathway. It is thought to be required for normal turnover of cellular components, particularly in response to starvation [10]. Against this background, we hypothesized that autophagy might mediate MHC class II presentation of intracellular Ag in general. Therefore, we performed a detailed characterization of the MHC class II ligand repertoire (ligandome) presented at the cell surface under normal conditions and after

increased autophagy, leading to a comprehensive overall picture of changes in peptide processing and presentation.

2.1.3 Materials and Methods

Cells and antibodies

The human B-lymphoblastoid cell lines Awells (IHW-No. 9090; HLA-DRB1*0401, HLA-DRB4*0101) and Awells-Ii-LGALS2 (Awells transfected with a fusion gene encoding the 80 N-terminal amino acids of Ii and LGALS2) were maintained at 37°C in DMEM (C.C.Pro, Neustadt, Germany) containing 10 % FCS (Pan, Aidenbach, Germany) and supplemented with 2 mM L-glutamine (BioWhittaker, Verviers, Belgium), 100 U/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker). During induction of autophagy, cells were kept in Hank's Balanced Salt Solution (HBSS). For autophagy inhibition, cells were kept in DMEM or HBSS supplemented with 10 mM 3-methyladenine (Sigma-Aldrich, Steinheim, Germany). The antibody L243 (anti–HLA-DR) [33] was purified from hybridoma culture supernatants using protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Antibodies used in flow cytometry analysis were from PharMingen (San Diego, CA, USA).

Analysis of monodansylcadaverine (MDC) labeled vacuoles

Autophagic vacuoles were labeled with MDC and analyzed using either fluorescence microspcopy [16;34] or fluorescence spectroscopy in cell lysates [16], essentially as described. Briefly, cells were incubated at 37°C for 10 min with 0.05 mM MDC and subsequently washed four times with PBS. Cells were either analyzed by live cell microscopy or lysed in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100 for fluorescence spectroscopy. After lysis, remaining cellular debris was spun down.

Fluorescence Microscopy

Live cells were immediately analyzed at room temperature by epifluorescence microscopy on an inverted microscope (Axiovert 63W; Carl Zeiss, Jena, Germany)

fitted with a 63 x 1.2 numerical aperture lens in eight-well chambered cover glasses (Nunc, Wiesbaden, Germany). Fluorescence emission and detection was performed with a filter system (excitation BP 365 nm, detection LP 397 nm, beam splitter FT 395 nm). Images were acquired with a Sensicam cooled 12-bit CCD camera (PCO Computer Optics, Kelheim, Germany) and processed using the program Axiovision 3.1 (Carl Zeiss).

Fluorescence Spectroscopy

MDC concentrations in whole cell lysates [16] were determined using an LS50B spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA), with excitation at 380 nm and detection of emission at 525 nm. The protein content of the lysates was determined using a commercially available Bradford protein assay kit (Bio-Rad Laboratories, München, Germany).

Gene expression analysis by high-density oligonucleotide microarrays

Total RNA was isolated from Awells using Trizol (Invitrogen, Karlsruhe, Germany) followed by an RNeasy cleanup (QIAGEN, Hilden, Germany) after autophagy induction for 6 h and 24 h and from cells cultured in normal medium for the same times as controls. High RNA quality was ensured by a 2100 Bioanalyzer (Agilent, Waldbronn, Germany) assay using the RNA 6000 Pico LabChip Kit (Agilent). Gene expression analysis of the four RNA samples was performed by Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) according to the Affymetrix manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, double-stranded cDNA was synthesized from 8 µg of total RNA using SuperScript RTII (Invitrogen) and the oligo-dT-T7 primer (MWG Biotech, Ebersberg, Germany) as described in the manual. In vitro transcription was performed with the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY), followed by cRNA fragmentation, hybridization, and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, The Netherlands). Images were scanned with the Affymetrix GeneChip Scanner 3000 and data were analyzed with the GCOS software (Affymetrix) using default settings for all parameters.

Normalization was performed by scaling all four arrays based on the overall fluorescence intensity of each array. Scaling factors differed by no more than a factor of 1.2 and all other quality control parameters provided by the array indicated a high data quality. For each of the two time points a pairwise array comparison was calculated with the autophagy array as the experiment and the respective normal medium control array as the baseline.

In order to identify functional categories or pathways for which a higher proportion of genes were up- or downregulated compared with the overall proportion of regulated genes, the following analysis was performed: First of all, genes were selected that were reproducibly up- or downregulated after 6 h and 24 h starvation according to the GCOS software. In order to be considered upregulated, a gene had to fulfill the following requirements: an "increase" in the change call algorithm, a "present" in the detection call algorithm for the autophagy array, and a log2 overexpression (signal log ratio, SLR) of at least 0.5 (approx. 1.4-fold overexpression). Downregulated genes had to show a "decrease", a "present" on the control array and a SLR equal to or smaller than -0.5. According to this, 1336 probesets were reproducibly upregulated, 1680 were downregulated. In a second step, these genes were analyzed using MAPPFinder [35] and EASE [36]. Both programs aim at the identification of overrepresented biological themes within lists of genes based on gene ontology (GO) categories. The 8-10% of GO terms with the best scores for overrepresentation were selected for each of the programs and for both, up- and downregulated genes. GO terms identified in common by both programs were further edited manually to avoid too much redundancy among overlapping terms and to exclude terms that were too general to draw any conclusions from them. The remaining GO categories are shown in supplementary tables 3 and 4.

Western blot

Cells/fractions lysed in NP-40/pH 7 lysis buffer (50 mM sodium acetate, 5 mM MgCl2, 0.5% NP-40) were resolved by 12.5 % SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA, USA), blocked, and probed with appropriate dilutions of the respective primary antibody, followed by a secondary anti-rabbit IgG antibody coupled with peroxidase (Southern Biotech, Birmingham, AL, USA). An ECL detection Kit (Amersham Pharmacia, Freiburg, Germany) was used to

visualize the Ab-reactive proteins. Anti cathepsin polyclonal antisera were provided by E. Weber (University of Halle, Germany).

Affinity-labelling of active cysteine proteases

Crude endocytic fractions were generated by ultracentrifugation of postnuclear supernatants as described [37]. 5 µg total endocytic protein were incubated with reaction buffer (50 mM citrate/phosphate pH 5.0, 1 mM EDTA, 50 mM DTT) in the presence of DCG-0N, a derivative of DCG-04 that shows the same labelling characteristics [38] for 1 h at room temperature. Reactions were terminated by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by 12.5 % SDS-PAGE, then blotted on a PVDF-membrane and visualized using streptavidine HRP and the ECL-detection kit [39].

Elution of MHC Class II bound Peptides

Frozen cell pellets (1×109 to 5.7×1010cells) were processed as previously described [40] and peptides were isolated according to standard protocols [11] using 5 mg to 25 mg HLA-DR specific mAb L243 [33].

Molecular analysis of HLA-DR-eluted peptides

Peptides were separated by reversed-phase high performance liquid chromatography (HPLC, SMART system, µRPC C2/C18 SC 2.1/10; Amersham Pharmacia Biotech, Freiburg, Germany), and fractions were analyzed by MALDI-TOF mass spectrometry (MS) using a Bruker Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Peptides were further analyzed by nano-ESI (electrospray ionisation) MS/MS either on a Q-TOF mass spectrometer (Micromass, Manchester, United Kingdom) or on a a QStar Pulsar i Qqoa Tof mass spectrometer (Applied Biosystems-MDS Sciex, Weiterstadt, Germany) as described [40].

For comparative peptide analysis between peptides eluted from 1-3×109 control cells and 1-2×109 cells undergoing autophagy, peptides were analyzed by a reversed phase Ultimate LC system (Dionex, Amsterdam, Netherlands), coupled to a Q-TOF. Samples were loaded onto a C18 pre-column for concentration and desalting. After loading, the pre-column was placed in line for separation by a fused-silica microcapillary column (75 µm i.d. x 250 mm) packed with 5 µm C18

reversed-phase material (Dionex). Solvent A was 4 mM ammonium acetate/water. Solvent B was 2 mM ammonium acetate in 80% acetonitrile/water. Both solvents were adjusted to pH 3.0 with formic acid. A binary gradient of 15% to 40% B within 120 min was performed, applying a flow rate of 200 µl/min reduced to approximately 300 nl/min by the Ultimate split-system. A gold coated glass capillary (PicoTip, New Objective, Cambridge, MA, USA) was used for introduction into the micro-ESI source. In MS/MS experiments, sequence information was obtained by interpretation of fragment spectra using computer-assisted database (NCBInr, non-redundant protein database) searching tools (MASCOT, Matrix Science, London) [41]. In order to differentially quantify the identified peptides, peptide signals in mass chromatograms from serial LC-MS runs (runs performed directly one after the other using the same settings) were summed and quantification was done from relative peak heights in the corresponding mass spectra.

2.1.4 Results

Proteomic analysis of the constitutive MHC II ligandome: Source proteins of HLA-DR presented peptides are allocated throughout the cell and are largely involved in responses to stimuli and normal cellular metabolism

We analyzed the constitutive human leukocyte antigen (HLA)-DR peptide repertoire of Awells human B-lymphoblastoid cell lines. Peptides were separated via high performance liquid chromatography (HPLC) as described previously [11] and subsequently analyzed by nanoflow electrospray tandem mass spectrometry (ESI-MS/MS). We were able to identify 404 peptides with 173 different core sequences (Table 1), some of them posttranslationally modified (Table 2). As expected many peptides with overlapping sequences were found, but there was also a substantial number of peptides (78) found only once. This is the largest number of MHC-presented peptides ever reported from a single experiment. In order to classify the source proteins according to their cellular localization (Figure 1) and function (Figure 2), we used the DAVID program [12] and the Gene Ontology (GO) classifications [13]. In contrast to the situation observed for MHC

class I peptides [14], the majority of MHC II source proteins, namely 41.1%, belonged to membrane proteins, which is in concordance with conventional MHC class II antigen processing via the endosomal/lysosomal pathway. However, with 34.9% a rather large proportion of source proteins localized intracellularly – meaning the contents of a cell contained within the plasma membrane, excluding large vacuoles and secretory or ingested material (GO classifications) –, the site where MHC class I peptide processing is expected to take place. Furthermore, we could identify peptides from proteins localized in virtually every cell compartment: 10.1% lysosome, 9.2% nucleus, 4.0% cytoskeleton, 3.0% Golgi apparatus, 2.0% ER, 1.2% ribosome, 0.7% peroxisome and 0.2% mitochondrion.

Regarding their biological function, source proteins were involved to a large extent in responses to stimuli (38.1%) and organismal physiological processes (37.6%) (Figure 2) indicated by the localization of many source proteins to the plasma membrane (26.5%). On the other hand, 33.4% of proteins took part in metabolic processes which mainly take place intracellularly. Thus, most peptides presented on HLA class II molecules derived from genes involved in normal cellular processes which should be commonly expressed.

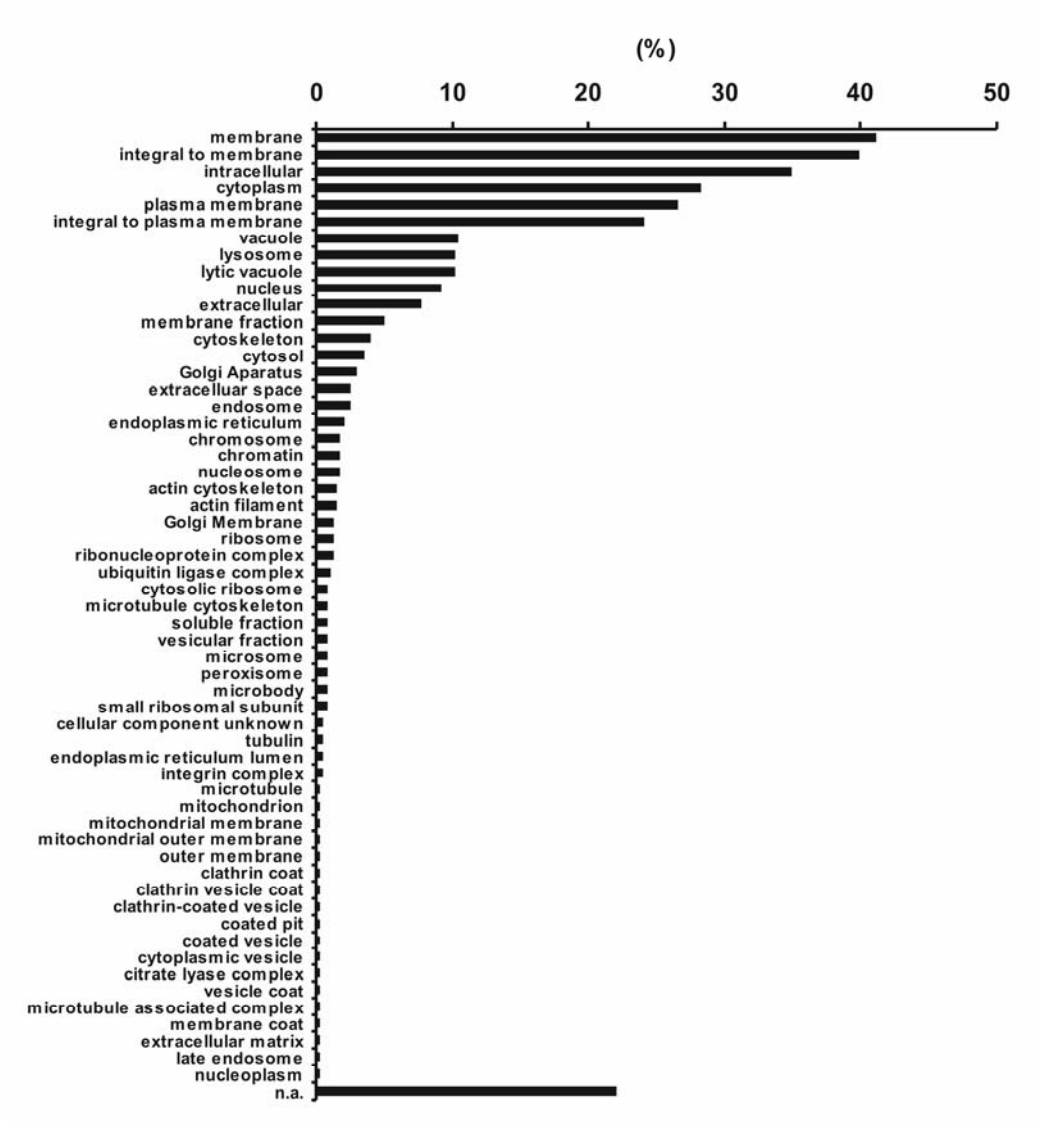

Figure 1: Cellular distribution of source proteins of HLA-DR peptides.

Peptides were isolated from 5.7×1010 cells, separated via HPLC, and subsequently analyzed by nanoflow ESI MS/MS. Displayed are percentages of peptides falling in each GO category of source proteins. The 404 identified peptides represent 100%. As some of the source proteins could be found in more than one compartment, the total is higher than 100%.

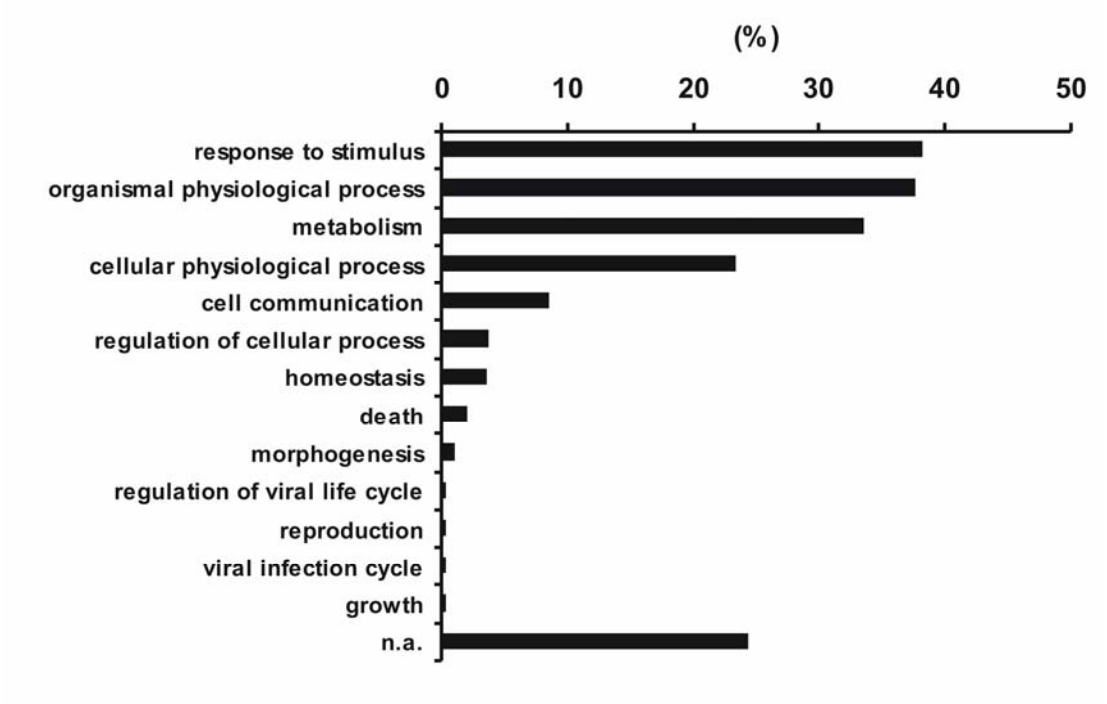

Figure 2: Biological processes in which HLA class II peptide source proteins take part. The major part of proteins was involved in responses to stimuli and in organismal physiological processes. Displayed are percentages of peptides falling in each GO category of source proteins (404 identified peptides represent 100%). As some of the source proteins could be found in more than one compartment, the total is higher than 100%.

Table 1. Sequences of peptides eluted from HLA-DR. Peptides are arranged according to their HLA-DR4 binding motive (http://www.syfpeithi.de), indicated by score and rank. Anchor amino acids are printed bold.

Gene Symbol	Peptide Sequence																					Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3									
HLA-A				**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R							3105	28	1/256
				F	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E					3105		
			Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E					3105		
		T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q								3105		
		T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R							3105		
		T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M						3105		
		T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P				3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q								3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R							3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M						3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E					3105		
	D	T	E	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E					3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P				3105		
	D	T	E	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P				3105		
	D	T	E	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	o	E	P			3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R			3105		
	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R	A	P	3105		
	D	D	T	**Q**	F	V	**R**	F	**D**	S	D	**A**	A	S	Q	R						3105		
D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E					3105		
D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P				3105		
D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R			3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R						3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M					3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P			3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R		3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R	A	P	3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R	A	P	W	3105		
V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R	M	E	P	R	A	P	W	I	E	3105		
V	D	D	T	**E**	**F**	V	R	**F**	D	**S**	D	A	**A**	S	Q	R						3105		
T	T	K	H	K	**W**	E	A	**A**	H	**V**	A	E	**Q**	L	R							3105	22	5/256
		K	H	K	**W**	E	A	**A**	H	**V**	A	E	**Q**	L	R							3105		
HLA-B				T	L	**F**	V	R	**F**	D	**S**	D	A	**T**	S	P						3106	28	1/362
		D	T	L	**F**	V	R	**F**	D	**S**	D	A	**T**	S	P	R	K	E	P	R	A	P	3106		
V	D	D	T	L	**F**	V	R	**F**	D	**S**	D	A	**T**	S	P	R	K	E	P	R	A	P	3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T							3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q						3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R					3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W			3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E		3106		
		L	S	S	**W**	T	A	**A**	D	**T**	A	A	**E**	I	T	E	R	K	W	E		3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T							3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q						3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R					3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W			3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E		3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E	A	A	3106		
	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E	A	A	R	V	A	3106		
E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R					3106		
E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T							3106		
E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W			3106		
E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E		3106		
E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E	A	A	R	V	A	3106		
N	E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W			3106		
L	N	E	D	L	S	S	**W**	T	A	**A**	D	**T**	A	A	**Q**	I	T	Q	R	K	W	E	3106		
	K	D	Y	I	A	**L**	N	E	**D**	L	**S**	S	W	**T**	A							3106	26	4/362
	G	P	E	Y	**W**	D	R	**E**	T	**Q**	I	S	**K**	T	N							3106	28	1/362

Table 1, continued.

Gene Symbol	Peptide Sequence																									Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3													
HLA-B				**L**	R	W	**E**	P	**S**	S	Q	**S**	T	V	P	I	V	G	I	V	A	G				3106	26	4/362
HLA-C				**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P								3107	28	1/366
			T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R											3107		
			T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R							3107		
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R											3107		
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G										3107		
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R							3107		
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P					3107		
	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R											3107		
	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R						3107		
	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P				3107		
	Y	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P				3107		
							D	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T								3107	18	35/366
							D	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A							3107		
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A							3107		
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T								3107		
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W									3107		
					G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T								3107		
					G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A							3107		
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T								3107		
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A							3107		
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A	A						3107		
	G	R	L	L	R	G	**Y**	N	Q	**F**	A	**Y**	D	G	**K**												3107	22	8/366
HLA-E				L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S										3133	28	1/358
				L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S	E	Q								3133		
			D	L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S	E	Q								3133		
IGHMBP2				E	P	R	R	**Y**	G	S	**A**	A	**A**	L	P	**S**											3508	22	27/993
CLN5							G	**H**	L	K	**I**	M	**H**	D	A	**I**	G	F	R								1203	18	45/407
						L	G	**H**	L	K	**I**	M	**H**	D	A	**I**	G	F	R								1203		
HLA-DRB1								**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y									3123	22	6/266
					Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R									3123		
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R										3123	22	6/266
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R	A								3123		
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R	A	V							3123		
			G	A	G	L	**F**	I	Y	**F**	R	**N**	Q	K	**G**	H	S										3123	22	6/266
HLA-DRA				A	Q	G	A	**L**	A	N	**I**	A	**V**	D	K	**A**	N	L	E	I							3122	20	13/254
	I	Q	A	E	F	Y	**L**	N	P	**D**	Q	**S**	G	E	**F**												3122	20	13/254
HLA-DQB1				D	V	E	**V**	Y	R	**A**	V	**T**	P	L	**G**	P	P	D									3119	20	9/229
HLA-DPB1			N	R	E	E	**F**	V	R	**F**	D	**S**	D	V	**G**	E	F	R										3115	22	1/58
				R	E	E	**F**	V	R	**F**	D	**S**	D	V	**G**	E	F	R										3115		
B2M							**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y									567	22	4/119
						Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y									567		
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**												567		
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D											567		
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E										567		
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y									567		
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A								567		
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**												567		
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D											567		
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E										567		
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y									567		
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A								567		
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D											567		
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y									567		
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A								567		
HLA-G		V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	S	**A**	C	P	R	M	E	P						3135	28	1/338

Table 1, continued.

Gene Symbol	Peptide Sequence (-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3)	Entrez Gene ID	Score	Rank
HLA-G	Y V D D T Q **F** V R **F** D **S** D S **A** C P R M E P R A P	3135		
HLA-DMA	F G P T **F** V S **A** V **D** G L **S** F Q	3108	22	5/257
ENO1	K E K **Y** G K **D** A **T** N V **G** D E G G	2023	22	9/433
	I K E K **Y** G K **D** A **T** N V **G** D E G G	2023		
	I K E K **Y** G K **D** A **T** N V **G** D E G	2023		
	V I K E K **Y** G K **D** A **T** N V **G** D E G G	2023		
	G V P L **Y** R H **I** A **D** L A **G** N	2023	16	66/433
	G V P L **Y** R H **I** A **D** L A **G** N S E V	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E V I	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E V	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E	2023		
	V P L **Y** R H **I** A **D** L A **G** N	2023		
GM2A	G N **Y** R I **E** S **V** L S **S** S G	2760	22	2/193
	G N **Y** R I **E** S **V** L S **S** S G K	2760		
	T G N **Y** R I **E** S **V** L S **S** S G	2760		
	T G N **Y** R I **E** S **V** L S **S** S G K	2760		
	T G N **Y** R I **E** S **V** L S **S** S G K R	2760		
	T T G N **Y** R I **E** S **V** L S **S** S G	2760		
	T T G N **Y** R I **E** S **V** L S **S** S G K	2760		
	L G C **I** K **I** A A **S** L K G I	2760	20	6/193
	R **L** G C **I** K **I** A A **S** L K G I	2760		
SLC2A14	V P M **Y** I G **E** I **S** P T **A** L R	144195	28	1/497
MIF	S P D R V Y I **N** Y Y **D** M **N** A A **N**	4282	20	5/114
	V P D G **F** L S **E** L **T** Q Q **L** A Q	4282	28	1/114
	V P D G **F** L S **E** L **T** Q Q **L** A Q A	4282		
TFRC	C P S D **W** K T **D** S **T** C R **M** V T	7037	28	1/760
	C P S D **W** K T **D** S **T** C R **M** V T S	7037		
	C P S D **W** K T **D** S **T** C R **M** V T S E	7037		
	F T Y **I** N **L** D K **A** V L G T S N	7037	22	19/760
	Y V A **Y** S K **A** A **T** V T **G** K L	7037	22	19/760
	N S Q **L** L S **F** V R D L N Q Y R A D I	7037	26	5/760
DHX34	I R **F** V V **D** S **G** K V **K** E M	9704	22	21/576
RAD23B	L **L** Q Q **I** S **Q** H Q **E** H F	5887	20	15/409
TUBB1	A K **F** W E **V** I **S** D E **H** G I D P T	7280	22	17/444
TUBB5	E P **Y** N A **T** L **S** V H **Q** L	10382	22	17/444
	E P **Y** N A **T** L **S** V H **Q** L V E	10382		
EEF1A1	**I** E K **F** E **K** E A **A** E M G K G	1917	20	18/463
	I E K **F** E **K** E A **A** E M G K G S	1917		
	I E K **F** E **K** E A **A** E M G K G S F	1917		
	T **I** E K **F** E **K** E A **A** E M G K G S F	1917		
	S K Y **Y** V T **I** I **D** A P **G** H R D	1917	16	60/462
HSPA5	V P T K K S Q **I** F S **T** A **S** D N **Q** P T V T	3309	20	29/654
	V M R **I** I N **E** P **T** A A **A** I A Y G	3309	26	5/654
HSPA6	G E R A **M** T K **D** N **N** L L **G** R F E	3310	20	23/643
HSPA1B	R **I** I N **E** P **T** A A **A** I A	3303	26	5/641
	R **I** I N **E** P **T** A A **A** I A Y G	3303		
	V L R **I** I N **E** P **T** A A **A** I A	3303		
	V L R **I** I N **E** P **T** A A **A** I A Y	3303		
	V L R **I** I N **E** P **T** A A **A** I A Y G	3303		
	N V L R **I** I N **E** P **T** A A **A** I A	3303		
	N V L R **I** I N **E** P **T** A A **A** I A Y	3303		
	N V L R **I** I N **E** P **T** A A **A** I A Y G	3303		
HSPA8	E G E R A **M** T K **D** N **N** L L **G** K F E	3312	20	15/646
	G E R A **M** T K **D** N **N** L L **G** K F E	3312		
	G E R A **M** T K **D** N **N** L L **G** K F E L	3312		
	E R A **M** T K **D** N **N** L L **G** K F E	3312		

Table 1, continued.

Gene Symbol	Peptide Sequence (-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3)	Entrez Gene ID	Score	Rank
HSPA8	E R A **M** T K **D** N **N** L L **G** K F E L	3312		
	R A **M** T K **D** N **N** L L **G** K F E	3312		
	G **I** L N **V** S **A** V D **K** S T G K E	3303	20	21/646
IL27RA	V P **Y** R I **T** V **T** A V **S** A S G	9466	22	9/636
	V G V P **Y** R I **T** V **T** A V **S** A S G	9466		
AHSG	I S R A Q **F** V P **L** P **V** S V **S** V E	280988	22	6/359
SYNGR2	N P K D V **L** V G **A** D **S** V R **A** A I T F	9144	26	3/224
MS4A1	S G P **K** P L **F** R **R** M S **S** L V G P T Q S F	931	18	41/297
	S G P **K** P L **F** R **R** M S **S** L V G P T Q S	931		
	G P **K** P L **F** R **R** M S **S** L V G P T Q S	931		
	G P **K** P L **F** R **R** M S **S** L V G P T	931		
	G P **K** P L **F** R **R** M S **S** L V G P	931		
	G P **K** P L **F** R **R** M S **S** L V G P	931		
		ox		
RAB6B	L I P S **Y** I R **D** S **T** V A **V** V V	51560	28	1/208
RAB7	F P E P **I** K L **D** K **N** D R **A** K A S A	7879	26	2/207
CTSC	D H N **F** V K **A** I **N** A I **Q** K S W	1075	28	1/463
	Y D H N **F** V K **A** I **N** A I **Q** K	1075		
	Y D H N **F** V K **A** I **N** A I **Q** K S	1075		
	Y D H N **F** V K **A** I **N** A I **Q** K S W	1075		
	Y D H N **F** V K **A** I **N** A I **Q** K S W T	1075		
	K Y D H N **F** V K **A** I **N** A I **Q** K S W T	1075		
	S G M D **Y** W I **V** K **N** S W **G** T G W G	1075	22	11/463
	K V **V** V Y **L** Q **K** L D **T** A Y D	1075	20	30/463
CTSC	K K V **V** V Y **L** Q **K** L D **T** A Y D D L G	1075		
TF	**F** V K **D** Q **T** V I **Q** N T D	7018	28	1/704
	D V A **F** V K **D** Q **T** V I **Q** N T D	7018		
	D V A **F** V K **D** Q **T** V I **Q**	7018		
	G D V A **F** V K **D** Q **T** V I **Q**	7018		
	G D V A **F** V K **D** Q **T** V I **Q** N T D	7018		
CNDP2	L A K **W** V A **I** Q **S** V S **A** W P E	55748	28	1/475
WBSCR1	D I D A **I** F K **D** L **S** I R **S** V R	7458	26	1/248
GEF2	A I F L F V **D** K T **V** P **Q** S S **L** T	11345	18	17/117
	A I F L F V **D** K T **V** P **Q** S S **L**	11345		
	A I F L F V **D** K T **V** P **Q** S S	11345		
	F V **D** K T **V** P Q S S **L**	11345		
	L P S E K A I F **L** F V **D** K T V P **Q** S S	11345	26	2/117
	L P S E K A I F **L** F V **D** K T V P **Q** S S	11345		
M17S2	S G T Q **F** V C **E** T **V** I R **S** L	4077		22/966
	S G T Q **F** V C **E** T **V** I R **S** L T	4077		
	S G T Q **F** V C **E** T **V** I R **S** L T L D	4077		
RAP1A	T E Q **F** T A **M** R **D** L Y **M** K N	5906	16	30/184
CTSZ	G T E **Y** W I **V** R **N** S W **G** E P W	1522	22	6/303
LGMN	V P K D **Y** T G **E** D **V** T P **Q** N	5641	22	11/433
	G V P K D **Y** T G **E** D **V** T P **Q** N	5641		
GAPD	L Q N **I** I P **A** S **T** G A **A** K A V G	2597	26	4/334
DKFZp43400 32.1	L **L** Q K **L** I **L** W R **V** L		20	24/415
HIST1H2BL	V N D I F E R **I** A S **E** A **S** R L **A** H Y N	8340	26	2/125
	V N D I F E R **I** A S **E** A **S** R L **A**	8340		
	D I F E R **I** A S **E** A **S** R L **A** H Y N	8340		
	D I F E R **I** A S **E** A **S** R L **A** H Y	8340		
	D I F E R **I** A S **E** A **S** R L **A** H	8340		
	D I F E R **I** A S **E** A **S** R L **A**	8340		
	D I F E R **I** A S **E** A **S** R L	8340		
APOB	S A S **Y** K A **D** T **V** A K **V** Q G			

Table 1, continued.

Gene Symbol	Peptide Sequence	Entrez Gene ID	Score	Rank
	-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3			
APOB	S A S **Y** K A **D** T **V** A K **V** Q G T			
	S A S **Y** K A **D** T **V** A K **V** Q G T E			
SCAMP2	S S R T **F** H R **A** A **S** S A **A** Q G A F	10066	28	1/329
	S S R T **F** H R **A** A **S** S A **A** Q G A	10066		
	S R T **F** H R **A** A **S** S A **A** Q G A	10066		
	R T **F** H R **A** A **S** S A **A** Q G A F	10066		
SCAMP3	Y G S **Y** S T **Q** A **S** A A **A** A T	10067	22	13/347
	Y G S **Y** S T **Q** A **S** A A **A** A T A	10067		
	Y G S **Y** S T **Q** A **S** A A **A** A T A E	10067		
DPP7	**L** P F **G** A **Q** S T **Q** R G H T E	29952	20	23/492
IFITM1	D R K **M** V G **D** V **T** G A **Q** A Y A	8519	26	2/125
	D R K **M** V G **D** V **T** G A **Q** A Y	8519		
	L G F **I** A F **A** Y **S** V K **S** R D	8519	26	2/125
	V P **D** H V **V** W **S** L F **N** T L	8519	18	25/125
SORL1	**K** P G **I** Y **R** S N **M** D G S A A Y	6653	18	231/2214
	R H P I N E Y **Y** I A **D** A **S** E D **Q** V F	6653	28	1/2214
UBE2L3	N P P Y D K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332		
	P P Y D K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332		
Unnamed protein	G P P **I** G S **F** T **L** I D **S** E V S Q L		20	37/626
DNPEP	**F** E L **F** P **S** L S **H** N L L V D	23549	22	12/475
PON2	S P D D K **Y** I Y **V** A **D** I L **A** H E I H	5445	22	8/354
GDI2	E P I E Q K **F** V S **I** S **D** L L **V** P K	2665	22	14/445
SLC1A5	V A A V F **I** A Q **L** S **Q** Q S **L** D F V K	6510	26	4/541
D4ST1	**L** P K **Y** I **L** D F **S** L	113189	14	73/376
	D V **L** P K **Y** I **L** D F **S** L	113189		
SIAT1	G **I** L I **V** W **D** P S **V** Y H S D I P	6480	20	18/406
ABCC4	A P **V** L F **F** D **R** N P **I** G R I L	10257	26	19/1325
MMS19L	L V A **F** R I **V** H **D** L I **S** R D Y S	64210	22	38/1030
LARGE	N P L H **F** H L **I** A **D** S I **A** E Q I L	9215	22	18/756
PPFIBP1	**M** E L **P** D **Y** V L **L** T A T	8496	14	193/1005
RNASET2	S L E L **Y** R E **L** D **L** N S **V** L L	8635	22	3/256
ITGA4	I D I S **F** L L **D** V **S** S L **S** R A E	3676	28	1/1038
	I D I S **F** L L **D** V **S** S L **S** R A E E	3676		
GNA13	L N I **F** E T **I** V **N** N R **V** F S	10672	28	1/377
TIP120A	L E A L D **I** M A **D** M **L** S R **Q** G G	55832	20	65/1230
	L E A L D **I** M ox A **D** M **L** S R **Q** G	55832		
ITGB7	L F F F L **V** E D **D** A **R** G T **V**	3695	26	10/798
PGK1	R V V **M** R V **D** F **N** V P **M** K N	5230	26	4/417
	G P V G V **F** E W **E** A **F** A R **G** T	5230	16	63/417
ATIC	L **V** E F **A** R **N** L T **A** L G L N L V	471	26	6/592
RPS13	L P P N **W** K Y **E** S **S** T A **S** A	6207	28	1/150
RPS10	D **R** D T **Y** R **R** S A **V** P P G A D	6204	12	31/165
	A D **R** D T **Y** R **R** S A **V** P P G A D	6204		
	R D T **Y** R **R** S A **V** P P G A D	6204		
CTSD	**L** S R **D** P **D** A Q **P** G G E	1509	14	88/412
	I F S **F** Y L **S** R **D** P D **A** Q P G	1509	16	75/412
NAPB	D Y **Y** K G **E** E **S** N S **S** A N K	63908	28	1/298
CCT2	S L M **V** T N **D** G **A** T I **L** K N	10576	20	24/535
CPD	V P G T Y K **I** T A **S** A **R** G Y **N**	1362	20	72/1380
	V P G T Y K **I** T A **S** A **R** G Y **N** P V	1362		
SLAMF6	D T G S **Y** R A **Q** I **S** T K **T** S A K	114836	22	6/331
KIAA1691	G S S **Y** G S **E** T **S** I P **A** A A H	80727	28	1/558
CPNE3	V A R **F** A A **A** A **T** Q Q **Q** T A	8895	28	1/537
LY6E	K P T **I** C S **D** Q **D** N Y **C** V T	4061	14	25/131

Table 1, continued.

Gene Symbol	Peptide Sequence																					Entrez Gene ID	Score	Rank
					-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3					
LY6E					L	K	P	T	**I**	*C* *C*	S	**D**	Q	**D**	N	Y	**C**	V	T				4061		
PSAP						G	P	S	**Y**	W	C	**Q**	N	**T**	E	T	**A**	A	Q				5660	22	8/524
C19orf10						T	E	E	**F**	E	V	**T**	K	**T**	A	V	**A**	H	R	P	G		56005	22	5/173
NEDD4L					D	G	R	T	**F**	Y	I	**D**	H	**N**	S	K	**I**	T	Q				23327	28	1/854
UBQLN1					N	P	D	T	**L**	S	A	**M**	S	**N**	P	R	**A**	M	Q				29979	20	16/589
ALDOA			A	P	G	K	G	I	**L**	A	A	**D**	E	**S**	T	G	**S**	I	A				226	26	2/363
TNFAIP3							E	I	**I**	H	K	**A**	L	**I**	D	R	**N**	I	Q				7128	20	38/790
IMPA1				Y	P	S	H	S	**F**	I	G	**E**	E	**S**	V	A	**A**	G	E	K			3612	28	1/227
VDAC2						A	A	K	**Y**	Q	L	**D**	P	**T**	A	S	**I**	S	A				7417	28	1/347
PPGB						L	P	G	**L**	A	K	**Q**	P	**S**	F	R	**Q**	Y	S	G			5476	20	30/480
COCH							R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A			1690	16	84/550
					G	Q	R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A			1690		
				I	G	Q	R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A	L		1690		
TAX1BP1					H	K	G	E	**I**	R	G	**A**	S	**T**	P	F	**Q**	F	R				8887	26	2/789
SEMA7A						I	S	I	**Y**	S	S	**E**	R	**S**	V	L	**Q**						8482	28	1/666
STX6					N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R				10228	28	1/255
					N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R	K			10228		
					N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R	K	A		10228		
PTPRC			S	P	G	E	P	Q	**I**	I	F	**C**	R	**S**	E	A	**A**	H	Q	G			5788	20	65/1304
			S	P	G	E	P	Q	**I**	I	F	**C**	R	**S**	E	A	**A**	H	Q	G	V	I	5788		
IGLC1						K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T					3537	18	9/105
						K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V				3537		
				S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T					3537		
				S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V				3537		
				S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E	K	T	3537		
						K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E	K		3537		
						K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E			3537		
					H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V				3537		
					H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E			3537		
				S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E			3537		
			K	S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E			3537		
	T	P	E	Q	W	K	S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E	3537		
IGHM							G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K				3507	18	44/454
							G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	E			3507	18	44/454
						S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K				3507		
						S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	E	S	D	W	L	3507		
					E	S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	E	S	D	W	L	3507		
IGH@							Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**						3492	26	1/33
						T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D						3492		
						T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**					3492		
						T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A				3492		
					N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**					3492		
					N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A				3492		
				K	N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A				3492		
UBA52						S	D	Y	N	**I**	Q	K	**E**	S	**T**	L	H	**L**	V					7311	26	1/76
						D	Y	N	**I**	Q	K	**E**	S	**T**	L	H	**L**	V	L	R				7311		
ACLY					Y	P	E	E	A	**Y**	I	A	**D**	L	**D**	A	K	**S**	G	A	S			47	22	24/1001
HTGN29							R	G	**Y**	M	E	**I**	E	**Q**	S	V	**K**	S	F	K				56951	28	1/265
WDR1						A	P	S	G	**F**	Y	I	**A**	S	**G**	D	V	**S**	G	K	L	R			9948	22	12/606
						A	P	S	G	**F**	Y	I	**A**	S	**G**	D	V	**S**	G	K	L				9948		
ATP1A1							I	V	V	**Y**	T	G	**D**	R	**T**	V	M	**G**	R	I	A	T			476	22	31/1023
							I	V	V	**Y**	T	G	**D**	R	**T**	V	M	**G**	R	I	A				476		
CTSS							G	K	E	**Y**	W	L	**V**	K	**N**	S	W	**G**	H	N				1520	22	6/331
						T	T	A	F	**Q**	Y	I	**I**	D	**N**	K	G	**I**	D					1520	18	40/331
						T	T	A	F	**Q**	Y	I	**I**	D	**N**	K	G	**I**	D	S	D			1520		

Table 1, continued.

Gene Symbol	Peptide Sequence (-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3)	Entrez Gene ID	Score	Rank
CTSS	K N L K F V M L H **N** L E **H** S M	1520	22	6/331
CLTCL1	L E K **I** V L **D** N **S** V F **S** E H R N	8218	26	17/1640
RNF13	I P S V **F** I G **E** S **S** A N **S** L K D	11342	28	1/381
MTP18	I P I I **I** H P **I** D **R** S V **D**	51537	20	9/206
LAPTM5	L P S **Y** E E **A** L **S** L P **S** K T P	7805	28	1/262
	L P S **Y** E E **A** L **S** L P **S** K T P E	7805		
	L P S **Y** E E **A** L **S** L P **S** K T P E G	7805		
	G **Y** L R **I** A **D** L I **S** S F	7805	22	12/262
	V V L P S **Y** E E **A** L **S** L P **S** K T P E	7805	28	1/262
TPI1	**L** K P **E** F **V** D I **I** N A K Q	7167	20	10/248
YWHAE	R A S **W** R I **I** S **S** I E **Q** K E E	7531	28	1/255
GLG1	K V N **L** L K **I** K **T** E L **C** K K E V	2734	20	36/1179
	V N **L** L K **I** K **T** E L **C** K K E V	2734		
	D P E L D Y T **L** M R **V** C **K** Q M **I** K	2734	20	36/1179
	L G K **W** C S **E** K **T** E T **G** Q E	2734	22	20/1179
PPIA	V S F E **L** F A **D** K **V** P K **T** A E N	5478	20	6/164
CCR7	**I** A Y **D** V **T** Y S **L** A C V R	1236	26	6/378
	N **I** A Y **D** V **T** Y S **L** A C V R	1236		
PTGFRN	V P G **F** A D **D** P **T** E L **A** C R V	5738	28	1/879
SLC3A2	T G A **L** Y R **I** G **D** L Q **A** F Q G H G	6520	20	26/529
ALB	S P D **L** P K **L** K **P** D P **N** T L C D E F	280717	20	30/607
RAB4A	G A L **L** V Y **D** I **T** S R **E** T Y N	5867	20	19/213
CCT7	A T Q **Y** F A **D** R **D** M F **C** A G R V P	10574	16	106/543
	V A T Q **Y** F A **D** R **D** M F **C** A G R V P	10574		
GPC4	V T R A **F** V A **A** R **T** F A **Q** G L	2239	28	1/556
MAP1LC3B	T P **I** S E **V** Y **E** S E **K** D E D G F L	81631	20	11/124
	T P **I** S E **V** Y **E** S E **K** D E D G F L Y	81631		
TNFSF9	G P L S **W** Y S **D** P **G** L A **G** V S	8744	16	84/254
VCP	Q L I Y I P **L** P D **E** K **S** R V **A**	7415	26	5/806
MAN2B1	**V** D Y **F** L **N** V A **T** A Q G R Y Y	4125	26	7/1010
	H P P E **L** L F **S** A **S** L P **A** L G	4125	20	68/1010
	H P P E **L** L F **S** A **S** L P **A** L G F S	4125		
	H P P E **L** L F **S** A **S** L P **A** L G F S T	4125		
CLSTN3	N P P L **F** A L **D** K **D** A P **L** R Y	9746	22	21/956
Dlc2	M E K **Y** N I **E** K **D** I A **A** Y I K	140735	22	3/89
LNPEP	D V R K L **Y** W L **M** K **S** S L **N** G D N	4012	22	28/1025
ANXA2	V P K **W** I S **I** M **T** E R **S** V P H	302		28/338
	D V P K **W** I S **I** M **T** E R **S** V P H L	302		
	D V P K **W** I S **I** M **T** E R **S** V P H L Q	302		
C10orf128	T G K T P G A E I D **F** K Y **A** L **I** G T **A** V G V A	170371	22	3/155
C6orf211	**I** P W **F** V **S** D T **T** I H D F N	79624	26	4/441
IL6ST	I E V **W** V E **A** E **N** A L **G** K V T	3572	22	24/918
CD74	M H H W **L** L F **E** M **S** R H **S** L E	972	26	2/296
	A T P L **L** M Q **A** L **P** M G **A** L P Q G P	972	20	14/296
DDX1	G **Y** L P **N** Q **L** F R **T** F	1653		
CREG	**W** G A **L** A **T** I S **T** L E A V R	8804	28	1/220
VPS35	D P D P E D **F** A D **E** Q **S** L V **G** R F I	55737	22	31/796
HPCL2	A I P F V **I** E K **A** V **R** S S **I** Y	26061	26	2/578
	A I P F V **I** E K **A** V **R** S S **I** Y G			
ACAA1	L K P A **F** K K **D** G **S** T T **A** G N	30	28	1/424
KIAA0494	**F** S Q **F** L **G** D P **V** E K A A Q	9813	22	13/495
APOD	Q E **L** R A **D** G **T** V N **Q** I E G			
CD38	R D M **F** T L **E** D **T** L L **G** Y L A D	952	22	7/300
	R D M **F** T L **E** D **T** L L **G**	952		
	R D M **F** T L **E** D **T** L	952		
	V Q R D M **F** T L **E** D **T** L	952		

Table 1, continued.

Gene Symbol	Peptide Sequence (-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3)	Entrez Gene ID	Score	Rank
ACTG1	W I S K **Q** E Y **D** E **S** G P **S** I V H R K C F	71	18	40/375
	T D Y **L** M K **I** L **T** E R **G** Y S	71	20	20/375
	T D Y **L** M K **I** L **T** E R **G** Y S F	71		
	T D Y **L** M K **I** L **T** E R **G** Y S F T	71		
	R D L T D Y **L** M K **I** L **T** E R **G** Y S	71		
	G R D L T D Y **L** M K **I** L **T** E R **G** Y S	71		
RAB8	A **F** F T **L** A **R** D I **K** A K M D	4218	28	1/207
	N A **F** F T **L** A **R** D I **K** A K M D	4218		
AGRN	G R S **F** L A **F** P **T** L R **A** Y H T	375790	28	1/2026
	G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	P V P A F E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	A P V P A F E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
IGF2R	L I T **F** L C **D** R **D** A G **V** G F P E	3482	22	43/2491
	L I T **F** L C **D** R **D** A G **V** G F P	3482		
UBE2L3	K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332	28	1/154
	D K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332		

Table 2. Sequences of posttranslationally modified peptides eluted from HLA-DR. Peptides are arranged according to their HLA-DR4 binding motive (http://www.syfpeithi.de), indicated by score and rank. Anchor amino acids are printed in bold.
Modifications are printed in italics: E = deamidation; C = cysteinylation, GI = glycosylation

Gene Symbol	Peptide Sequence (-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3)	Entrez Gene ID	Score	Rank
HLA-A	D T *E* **F** V R **F** D **S** D A **A** S Q R M E	3105	28	1/256
	D T *E* **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D T *E* **F** V R **F** D **S** D A **A** S Q R M ox E P	3105		
LY6E	L K P T **I** *C* S **D** Q **D** N Y **C** V T *C*	4061	14	25/131
CD53	I H R **Y** H S **D** *N* **S** T K **A** A W D *GI*		28	1
	S I H R **Y** H S **D** *N* **S** T K **A** A W D *GI*			

Induction of autophagy by starvation

Next, we induced macroautophagy in Awells cells by deprivation of serum and amino acids [15;16] in order to perform a comparative quantitative ligandome analysis between cells undergoing autophagy and control cells by mass spectrometry. After 6 h and 24 h starvation, an increase in size and total number of autophagic vacuoles became evident compared to non-starved control cells (Figure 3a-c). After 6 h starvation, the formation of autophagic vacuoles, assessed by the overall incorporation of monodansylcadaverine (MDC), had already reached the maximum and could not be increased further by 24 h starvation (Figure 3d). We were able to inhibit MDC incorporation by 3-methyladenine, a specific inhibitor of autophagy (data not shown). These data indicate that the Awells cell line already displayed a constitutive level of autophagy, which could be considerably enhanced by starvation, as was already demonstrated for other cell lines [15;16].

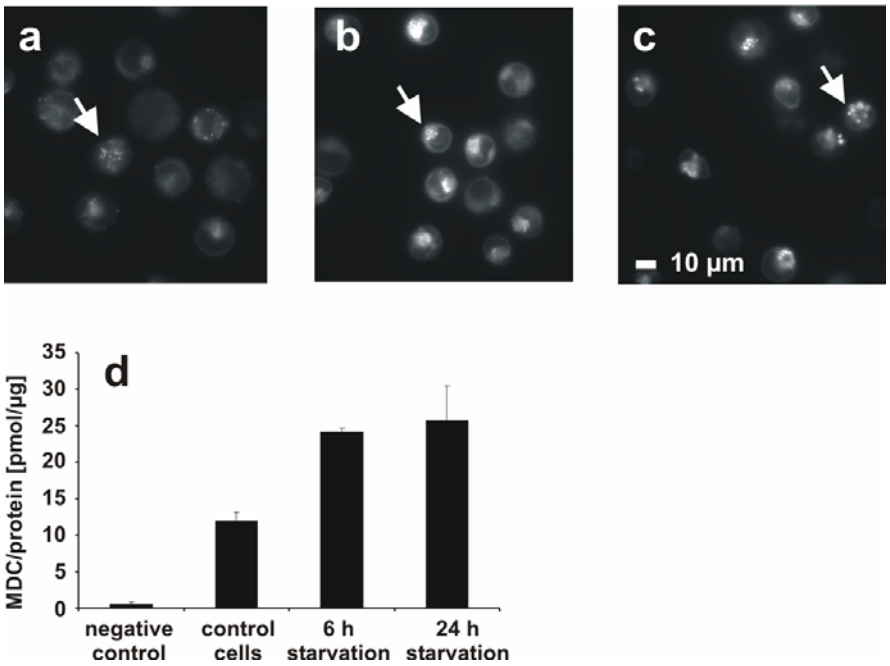

Figure 3: Starvation enhances the base level of autophagic vacuoles.

Autophagic vacuoles were stained with the specific dye monodansylcadaverine (MDC) [34] and analyzed by fluorescence microscopy or fluorescence spectroscopy. Awells were incubated for (a) 24 h in DMEM (control cells), (b) 6 h HBSS or (c) 24 h HBSS (starved cells), subsequently for 10 min with

monodansylcadaverine (MDC), and immediately analyzed by fluorescence microscopy. Autophagic vacuoles are marked with an arrow. (d) Intracellular MDC measurement by fluorescence spectroscopy, unstained cells were used as negative control.

A comparative gene expression analysis using oligonucleotide microarrays further supported these observations. Genes that were reproducibly up- or downregulated at 6 h and 24 h were assigned to functional categories based on the Gene Ontology (GO) classification system [13]. Categories showing a significantly enhanced proportion of regulated genes compared to the overall proportion of regulated genes were filtered out and are shown in tables 3 and 4. Several characteristics of autophagy as a process to ensure cell survival in a nutritionally deprived environment are reflected in these categories, exhibiting a distinct transcriptional signature of starved cells. The formation of autophagic vacuoles is illustrated by the GO terms "small GTPase mediated signal transduction" and "ARF guanyl-nucleotide exchange factor activity", which comprise upregulated genes involved in the control of vesicular transport and membrane trafficking, especially in the endosomal/lysosomal pathway, as for example Rab proteins [17]. In contrast, genes regulating rather exocytotic transport processes appear decreased under the term "Golgi stack". In response to amino acid starvation cells seem to upregulate in particular genes involved in amino acid transport. This has been described before in yeast [43]. Amino acid-dependent metabolic and proliferative activities can be expected to be reduced to a minimum under these conditions. This is reflected in the downregulation of various genes implicated in protein and nucleic acid synthesis. Gene categories like DNA replication and repair, ribosome biogenesis and assembly, or rRNA, tRNA, and mRNA transcription and processing among the downregulated biological functions demonstrate this phenomenon. Consequently, genes mediating cell cycle arrest are upregulated. Enduring starvation may ultimately cause cells to die. Autophagic phenotypes have been associated with programmed cell death different from apoptosis, suggesting a particular autophagic death pathway independent of caspase activation [44;45]. We observed an upregulation of genes related to apoptosis during starvation. However, most of them are classified in the context of apoptosis inhibition, supporting the model of autophagic death independent of

apoptosis. Interestingly, ceramide has been described as an important mediator of autophagy and autophagic cell death [46;47] and the GO terms "sphingoid metabolism" and "ceramide metabolism" appeared among the upregulated categories in our experiment.

Table 3. Gene ontology (GO) categories significantly upregulated under starvation. Genes fulfilling the upregulation requirements are shown (2 means 200% of mRNA was detected in starved compared with control cells). The first column indicates the GO category together with the GO ID and the GO system (BP: biological process, CC: cellular component, MF: molecular function).

Gene Category (GO ID, GO System)	Entrez Gene ID	Gene Symbol	Gene Title	x-fold over-expression	
				6h	24h
Vesicular Transport and Membrane Trafficking					
ARF guanyl-nucleotide exchange factor activity (5086, MF)	26269	FBXO8	F-box only protein 8	2.1	4.0
	9267	PSCD1	pleckstrin homology, Sec7 and coiled-coil domains 1	2.1	2.5
	9265	PSCD3	pleckstrin homology, Sec7 and coiled-coil domains 3	2.6	2.6
small GTPase mediated signal transduction (7264, BP)	399	ARHH	ras homolog gene family, member H	1.4	2.1
	23433	ARHQ	ras homolog gene family, member Q	2.6	3.7
	221079	ARL8	ADP-ribosylation factor-like 8	2.5	4.3
	55207	FLJ10702	hypothetical protein FLJ10702	2.1	3.0
	2669	GEM	GTP binding protein overexpressed in skeletal muscle	3.5	8.0
	2889	GRF2	guanine nucleotide-releasing factor 2	1.6	1.4
	3845	KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	1.5	2.6
	51762	LOC51762	RAB-8b protein	1.7	2.0
	51735	PDZGEF2	PDZ domain containing guanine nucleotide exchange factor (GEF) 2	4.0	4.0
	5862	RAB2	RAB2, member RAS oncogene family	1.6	2.5
	84932	RAB2B	RAB2B, member RAS oncogene family	1.4	2.1
	5868	RAB5A	RAB5A, member RAS oncogene family	1.5	2.0
	9367	RAB9A	RAB9A, member RAS oncogene family	2.3	2.3
	10890	RAB10	RAB10, member RAS oncogene family	1.4	2.3
	51552	RAB14	RAB14, member RAS oncogene family	1.4	1.7
	23011	RAB21	RAB21, member RAS oncogene family	1.4	2.5
	57403	RAB22A	RAB22A, member RAS oncogene family	1.6	2.1
	5898	RALA	v-ral simian leukemia viral oncogene homolog A	1.4	2.3
	57826	RAP2C	RAP2C, member of RAS oncogene family	1.6	2.1
	6009	RHEB	Ras homolog enriched in brain	1.6	1.5
	121268	RHEBL1	Ras homolog enriched in brain like 1	5.3	3.0
	6016	RIT1	Ras-like without CAAX 1	1.5	2.3
	64121	RRAGC	Ras-related GTP binding C	2.6	2.8
	22800	RRAS2	related RAS viral (r-ras) oncogene homolog 2	1.5	1.9
	8036	SHOC2	soc-2 suppressor of clear homolog (C. elegans)	1.7	2.1
	6478	SIAH2	seven in absentia homolog 2 (Drosophila)	2.1	3.0
	7248	TSC1	tuberous sclerosis 1	1.9	2.1
Amino Acid Transport and Metabolism					
amino acid transport (6865, BP)	6558	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2	1.4	3.0
neutral amino acid transporter activity (15175, MF)	6509	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2.1	4.0
	6510	SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5	1.4	3.0
amino acid permease activity (15359, MF)	81539	SLC38A1	solute carrier family 38, member 1	2.0	3.7
	54407	SLC38A2	solute carrier family 38, member 2	4.3	4.9
	6520	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.6	4.6
	6541	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	2.8	3.7
	23657	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	3.2	4.6
	8140	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+	3.0	4.9

			system), member 5		
serine family amino acid biosynthesis (9070, BP)	1491	CTH	cystathionase (cystathionine gamma-lyase)	5.7	18.4
	26227	PHGDH	phosphoglycerate dehydrogenase	2.6	6.1
	29968	PSAT1	phosphoserine aminotransferase 1	3.7	9.8
	5723	PSPH	phosphoserine phosphatase	2.3	6.1
Cell Cycle Arrest and Mitosis					
cell cycle arrest (7050, BP)	1026	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.9	2.6
	1027	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.5	3.2
	1647	GADD45A	growth arrest and DNA-damage-inducible, alpha	2.8	3.5
	8522	GAS7	growth arrest-specific 7	1.6	3.0
	283431	LOC283431	hypothetical protein LOC283431	1.6	4.3
	5325	PLAGL1	pleiomorphic adenoma gene-like 1	1.4	2.0
	23645	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.0	5.7
	83667	SESN2	sestrin 2	4.3	12.1
mitosis (7067, BP)	51434	ANAPC7	anaphase-promoting complex subunit 7	1.6	2.5
	701	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	1.4	1.6
	983	CDC2	cell division cycle 2, G1 to S and G2 to M	1.4	1.7
	1063	CENPF	centromere protein F, 350/400ka (mitosin)	1.5	2.0
	64151	HCAP-G	chromosome condensation protein G	1.6	2.6
	10403	HEC	highly expressed in cancer, rich in leucine heptad repeats	1.9	4.6
	4750	NEK1	NIMA (never in mitosis gene a)-related kinase 1	1.4	2.1
	5048	PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha	1.6	3.5
	5347	PLK	polo-like kinase (Drosophila)	1.6	2.0
	5885	RAD21	RAD21 homolog (S. pombe)	1.5	2.5
	10735	STAG2	stromal antigen 2	1.6	1.9
	6790	STK6	serine/threonine kinase 6	1.6	3.2
mitotic spindle elongation (22, BP)	51434	ANAPC7	anaphase-promoting complex subunit 7	1.6	2.5
	9055	PRC1	protein regulator of cytokinesis 1	1.7	3.0
spindle (5819, CC)	1063	CENPF	centromere protein F, 350/400ka (mitosin)	1.5	2.0
	6790	STK6	serine/threonine kinase 6	1.6	3.2
	7272	TTK	TTK protein kinase	2.3	2.3
Effects on Cell Cycle and Apoptosis					
protein phosphatase type 2C activity (15071, MF)	5494	PPM1A	protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	2.3	2.6
	8493	PPM1D	protein phosphatase 1D magnesium-dependent, delta isoform	2.1	4.6
Apoptosis					
apoptosis (6915, BP)	64651	AXUD1	AXIN1 up-regulated 1	2.6	4.6
	596	BCL2	B-cell CLL/lymphoma 2	1.6	4.9
	597	BCL2A1	BCL2-related protein A1	1.9	1.9
	598	BCL2L1	BCL2-like 1	1.4	1.9
	329	BIRC2	baculoviral IAP repeat-containing 2	1.6	2.8
	331	BIRC4	baculoviral IAP repeat-containing 4	1.5	1.5
	664	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.4	1.7
	665	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	2.1	2.6
	8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.7	1.5
	8087	FXR1	fragile X mental retardation, autosomal homolog 1	1.5	1.6
	1647	GADD45A	growth arrest and DNA-damage-inducible, alpha	2.8	3.5
	4616	GADD45B	growth arrest and DNA-damage-inducible, beta	2.6	4.6
	8870	IER3	immediate early response 3	1.4	1.5
	51747	LUC7A	cisplatin resistance-associated overexpressed protein	1.4	2.1
	4170	MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	2.1	1.7
	4790	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.6	2.1
	7262	PHLDA2	pleckstrin homology-like domain, family A, member 2	3.0	2.6
	23645	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.0	5.7
	5885	RAD21	RAD21 homolog (S. pombe)	1.5	2.5
	8767	RIPK2	receptor-interacting serine-threonine kinase 2	1.7	3.0
	64121	RRAGC	Ras-related GTP binding C	2.6	2.8
	6446	SGK	serum/glucocorticoid regulated kinase	9.2	1.7
	23411	SIRT1	sirtuin 1 (S. cerevisiae)	3.2	4.3
	9263	STK17A	serine/threonine kinase 17a (apoptosis-inducing)	1.9	2.5

	9262	STK17B	serine/threonine kinase 17b (apoptosis-inducing)	1.6	1.9
	7009	TEGT	testis enhanced gene transcript (BAX inhibitor 1)	1.5	2.5
	7124	TNF	tumor necrosis factor (TNF superfamily, member 2)	2.0	1.4
	7128	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	2.1	2.6
	8795	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	2.5	3.0
	8793	TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	9.2	13.9
	958	TNFRSF5	tumor necrosis factor receptor superfamily, member 5	1.9	1.4
	355	TNFRSF6	tumor necrosis factor receptor superfamily, member 6	1.9	2.1
	3604	TNFRSF9	tumor necrosis factor receptor superfamily, member 9	2.1	2.1
	970	TNFSF7	tumor necrosis factor (ligand) superfamily, member 7	1.6	2.0
	8626	TP73L	tumor protein p73-like	1.4	2.5
	7187	TRAF3	TNF receptor-associated factor 3	1.6	1.6
	8565	YARS	tyrosyl-tRNA synthetase	2.1	1.7
anti-apoptosis	596	BCL2	B-cell CLL/lymphoma 2	1.6	4.9
(6916, BP)	597	BCL2A1	BCL2-related protein A1	1.9	1.9
apoptosis inhibitor activity	598	BCL2L1	BCL2-like 1	1.4	1.9
(8189, MF)	329	BIRC2	baculoviral IAP repeat-containing 2	1.6	2.8
	331	BIRC4	baculoviral IAP repeat-containing 4	1.5	1.5
	664	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.4	1.7
	673	BRAF	v-raf murine sarcoma viral oncogene homolog B1	1.4	3.7
	8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.7	1.5
	25816	GG2-1	TNF-induced protein	1.9	2.6
	8870	IER3	immediate early response 3	1.4	1.5
	4790	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.6	2.1
	5663	PSEN1	presenilin 1 (Alzheimer disease 3)	1.7	2.8
	5055	SERPINB2	serine (or cysteine) proteinase inhibitor, clade B, member 2	1.9	3.7
	8887	TAX1BP1	Tax1 binding protein 1	1.4	2.0
	7124	TNF	tumor necrosis factor (TNF superfamily, member 2)	2.0	1.4
	7128	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	2.1	2.6
	355	TNFRSF6	tumor necrosis factor receptor superfamily, member 6	1.9	2.1
Sphingoid and Ceramide Metabolism					
sphingoid metabolism	427	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	1.4	2.6
(46519, BP)	8439	NSMAF	neutral sphingomyelinase (N-SMase) activation associated factor	2.5	1.7
ceramide metabolism					
(6672, BP)	5515	PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	1.4	1.5
	7357	UGCG	UDP-glucose ceramide glucosyltransferase	3.2	3.2
Regulation of Transcription					
transcription corepressor activity	467	ATF3	activating transcription factor 3	6.5	8.0
	22809	ATF5	activating transcription factor 5	1.4	1.5
(3714, MF)	1810	DR1	down-regulator of transcription 1, TBP-binding	1.4	2.3
	3726	JUNB	jun B proto-oncogene	1.7	2.0
	11278	KLF12	Kruppel-like factor 12	1.9	2.5
	4601	MXI1	MAX interacting protein 1	1.9	2.5
	23522	MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	1.9	1.7
	8554	PIAS1	protein inhibitor of activated STAT, 1	1.7	1.4
	5971	RELB	v-rel reticuloendotheliosis viral oncogene homolog B	1.5	1.4
	6478	SIAH2	seven in absentia homolog 2 (Drosophila)	2.1	3.0
	22797	TFEC	transcription factor EC	1.4	5.3
	7528	YY1	YY1 transcription factor	2.0	2.5
histone acetyltransferase activity	1387	CREBBP	CREB binding protein (Rubinstein-Taybi syndrome)	1.6	1.9
	2033	EP300	E1A binding protein p300	1.6	2.0
(4402, MF)	23522	MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	1.9	1.7
	8202	NCOA3	nuclear receptor coactivator 3	1.7	2.0
Nuclear Membrane					
nuclear inner membrane	4000	LMNA	lamin A/C	3.5	2.3
(5637, CC)	23592	MAN1	integral inner nuclear membrane protein	1.6	2.1
	5663	PSEN1	presenilin 1 (Alzheimer disease 3)	1.7	2.8

Table 4. Gene ontology (GO) categories significantly downregulated under starvation. Genes fulfilling the downregulation requirements are shown (-2 means 50% of mRNA was detected in starved compared with control cells). The first column indicates the GO category together with the GO ID and the GO system (BP: biological process, CC: cellular component, MF: molecular function).

Gene Category (GO ID, GO System)	Entrez Gene ID	Gene Symbol	Gene Title	x-fold over-expression 6h	24h
Vesicular Transport					
Golgi stack (5795, CC)	23062	GGA2	golgi associated, gamma adaptin ear containing, ARF binding protein 2	-1.5	-1.7
	23163	GGA3	golgi associated, gamma adaptin ear containing, ARF binding protein 3	-1.6	-3.2
	2778	GNAS	GNAS complex locus	-1.5	-1.9
	4952	OCRL	oculocerebrorenal syndrome of Lowe	-1.5	-2.3
	10040	TOM1L1	target of myb1-like 1 (chicken)	-1.9	-1.6
DNA replication and repair					
DNA replication origin binding (3688, MF)	4999	ORC2L	origin recognition complex, subunit 2-like (yeast)	-1.6	-1.6
	23595	ORC3L	origin recognition complex, subunit 3-like (yeast)	-1.6	-1.5
	5001	ORC5L	origin recognition complex, subunit 5-like (yeast)	-1.5	-2.0
damaged DNA binding (3684, MF)	672	BRCA1	breast cancer 1, early onset	-1.5	-2.3
	2237	FEN1	flap structure-specific endonuclease 1	-1.6	-2.6
	2967	GTF2H3	general transcription factor IIH, polypeptide 3, 34kDa	-1.4	-1.6
	7965	JTV1	JTV1 gene	-1.5	-4.0
	5383	PMS2L5	postmeiotic segregation increased 2-like 5	-1.7	-4.3
	5889	RAD51C	RAD51 homolog C (S. cerevisiae)	-1.7	-1.5
	5892	RAD51L3	RAD51-like 3 (S. cerevisiae)	-1.6	-1.7
	7508	XPC	xeroderma pigmentosum, complementation group C	-1.4	-1.4
	7517	XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	-1.4	-1.6
Ribosome formation					
ribosome biogenesis and assembly (42254, BP)	10969	EBNA1BP2	EBNA1 binding protein 2	-1.6	-2.5
	23212	RRS1	ribosome biogenesis regulator homolog (S. cerevisiae)	-2.5	-3.0
nucleolus (5730, CC)	1663	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)	-1.6	-2.6
	54606	DDX56	DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	-1.4	-4.0
	50628	GEMIN4	gem (nuclear organelle) associated protein 4	-3.2	-10.6
	23567	JAZ	double-stranded RNA-binding zinc finger protein JAZ	-1.4	-1.9
	84365	MKI67IP	MKI67 (FHA domain) interacting nucleolar phosphoprotein	-1.7	-1.9
	10514	MYBBP1A	MYB binding protein (P160) 1a	-1.9	-3.5
	4809	NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	-1.4	-1.6
	10528	NOL5A	nucleolar protein 5A (56kDa with KKE/D repeat)	-1.4	-1.7
	9221	NOLC1	nucleolar and coiled-body phosphoprotein 1	-1.4	-1.4
	54512	RRP41	exosome complex exonuclease RRP41	-2.5	-18.4
	6949	TCOF1	Treacher Collins-Franceschetti syndrome 1	-1.6	-1.7
	7343	UBTF	upstream binding transcription factor, RNA polymerase I	-2.5	-4.3
RNA synthesis and processing					
RNA cap binding (339, MF)	1973	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	-1.7	-1.7
	9470	EIF4EL3	eukaryotic translation initiation factor 4E-like 3	-4.3	-5.7
	4686	NCBP1	nuclear cap binding protein subunit 1, 80kDa	-1.5	-1.7
	22916	NCBP2	nuclear cap binding protein subunit 2, 20kDa	-1.4	-1.7
	10073	RNUT1	RNA, U transporter 1	-1.4	-2.3
RNA elongation (6354, BP)	5438	POLR2I	polymerase (RNA) II (DNA directed) polypeptide I	-1.5	-2.8
	51728	POLR3K	polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	30834	ZNRD1	zinc ribbon domain containing, 1	-1.6	-2.1
pre-mRNA splicing factor activity (8284, MF)	9416	DDX23	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	-1.9	-1.6
	25929	GEMIN5	gem (nuclear organelle) associated protein 5	-2.1	-6.1
	79833	GEMIN6	gem (nuclear organelle) associated protein 6	-3.0	-4.6
	84967	LSM10	U7 snRNP-specific Sm-like protein LSM10	-1.5	-2.5

	57819	LSM2	LSM2 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.5	-3.2
	23658	LSM5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.4	-2.0
	51691	LSM8	LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.7	-1.9
	8559	PRPF18	PRP18 pre-mRNA processing factor 18 homolog (yeast)	-3.2	-4.3
	9128	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)	-2.0	-5.3
	6426	SFRS1	splicing factor, arginine/serine-rich 1 (splicing factor 2)	-1.6	-3.2
	9169	SFRS2IP	splicing factor, arginine/serine-rich 2, interacting protein	-1.5	-2.1
	6432	SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	-1.4	-3.7
	8487	SIP1	survival of motor neuron protein interacting protein 1	-2.0	-4.3
RNA polymerase complex	5433	POLR2D	polymerase (RNA) II (DNA directed) polypeptide D	-2.1	-3.2
(30880, CC)	5438	POLR2I	polymerase (RNA) II (DNA directed) polypeptide I	-1.5	-2.8
	51728	POLR3K	polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	10622	RPC32	polymerase (RNA) III (DNA directed) (32kD)	-1.5	-2.6
	171568	RPC8	RNA polymerase III subunit RPC8	-1.9	-3.5
	9169	SFRS2IP	splicing factor, arginine/serine-rich 2, interacting protein	-1.5	-2.1
	23528	ZNF281	zinc finger protein 281	-1.5	-2.0
rRNA transcription	2971	GTF3A	general transcription factor IIIA	-1.7	-2.1
(9303, BP)	84365	MKI67IP	MKI67 (FHA domain) interacting nucleolar phosphoprotein	-1.7	-1.9
rRNA processing	10436	C2F	C2f protein	-1.5	-3.7
(6364, BP)	54606	DDX56	DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	-1.4	-4.0
	29960	FTSJ2	FtsJ homolog 2 (E. coli)	-1.7	-2.3
	50628	GEMIN4	gem (nuclear organelle) associated protein 4	-3.2	-10.6
	10528	NOL5A	nucleolar protein 5A (56kDa with KKE/D repeat)	-1.4	-1.7
	9221	NOLC1	nucleolar and coiled-body phosphoprotein 1	-1.4	-1.4
	22984	PDCD11	programmed cell death 11	-1.5	-2.6
	23404	RRP4	homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease	-1.9	-3.7
	51010	RRP40	exosome component Rrp40	-2.1	-1.9
	54512	RRP41	exosome complex exonuclease RRP41	-2.5	-18.4
transcription from Pol III promoter	672	BRCA1	breast cancer 1, early onset	-1.5	-2.3
	2971	GTF3A	general transcription factor IIIA	-1.7	-2.1
(6383, BP)	10625	IVNS1ABP	influenza virus NS1A binding protein	-1.7	-5.7
	51728	POLR3K	polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	10622	RPC32	polymerase (RNA) III (DNA directed) (32kD)	-1.5	-2.6
	171568	RPC8	RNA polymerase III subunit RPC8	-1.9	-3.5
	6619	SNAPC3	small nuclear RNA activating complex, polypeptide 3	-1.4	-2.0
	10302	SNAPC5	small nuclear RNA activating complex, polypeptide 5	-1.6	-1.7
tRNA processing	81627	C1orf25	chromosome 1 open reading frame 25	-2.1	-3.2
(8033, BP)	83480	FKSG32	hypothetical protein FKSG32	-1.4	-2.1
	79042	LENG5	leukocyte receptor cluster (LRC) member 5	-1.4	-2.1
	80746	MGC2776	hypothetical protein MGC2776	-2.0	-3.0
	10940	POP1	processing of precursors 1	-1.7	-3.2
	80324	PUS1	pseudouridylate synthase 1	-1.5	-2.0
	11102	RPP14	ribonuclease P (14kD)	-1.6	-1.4
	10248	RPP20	POP7 (processing of precursor, S. cerevisiae) homolog	-1.6	-3.2
	10557	RPP38	ribonuclease P (38kD)	-1.6	-1.7
	51095	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	-1.6	-2.5
	10785	WDR4	WD repeat domain 4	-1.6	-2.1
tRNA-specific ribonuclease activity	79042	LENG5	leukocyte receptor cluster (LRC) member 5	-1.4	-2.1
	80746	MGC2776	hypothetical protein MGC2776	-2.0	-3.0
(4549, MF)	10940	POP1	processing of precursors 1	-1.7	-3.2
	11102	RPP14	ribonuclease P (14kD)	-1.6	-1.4
	10248	RPP20	POP7 (processing of precursor, S. cerevisiae) homolog	-1.6	-3.2
	10557	RPP38	ribonuclease P (38kD)	-1.6	-1.7
Mitochondrial metabolism					
protein-mitochondrial targeting	26515	FXC1	fracture callus 1 homolog (rat)	-1.7	-2.6
(6626, BP)	80273	GRPEL1	GrpE-like 1, mitochondrial (E. coli)	-1.4	-2.0
	3329	HSPD1	heat shock 60kDa protein 1 (chaperonin)	-1.7	-4.0
	1678	TIMM8A	translocase of inner mitochondrial membrane 8 homolog A	-2.6	-3.7
	26521	TIMM8B	translocase of inner mitochondrial membrane 8 homolog B	-1.5	-2.0
	26520	TIMM9	translocase of inner mitochondrial membrane 9 homolog	-1.4	-1.6
	51095	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	-1.6	-2.5
protein translocase activity	51300	C3orf1	chromosome 3 open reading frame 1	-1.5	-2.0

(15450, MF)		26515	FXC1	fracture callus 1 homolog (rat)	-1.7	-2.6
		55176	SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	-1.4	-1.5
		1678	TIMM8A	translocase of inner mitochondrial membrane 8 homolog A	-2.6	-3.7
		26521	TIMM8B	translocase of inner mitochondrial membrane 8 homolog B	-1.5	-2.0
		26520	TIMM9	translocase of inner mitochondrial membrane 9 homolog	-1.4	-1.6
carnitine	*O-acyltransferase*	1375	CPT1B	carnitine palmitoyltransferase 1B (muscle)	-1.7	-1.9
activity		1376	CPT2	carnitine palmitoyltransferase II	-2.0	-7.0
(16406, MF)		54677	CROT	carnitine O-octanoyltransferase	-1.7	-1.7

Autophagy promotes the presentation of peptides from intracellular and lysosomal source proteins on MHC class II molecules

To determine whether autophagy contributes to the endogenous presentation of intracellular antigens on HLA class II in general or if this process represents a minor event followed by some model antigens [9], the presentation levels of peptides from different source proteins were quantified and compared between starved cells (6 h and 24 h) undergoing autophagy and non-starved control cells. For the quantitation experiments we had to use smaller cell numbers and consequently focused on a smaller subset of MHC class II ligands. In order to exclude influences caused by altered MHC surface expression on the subsequent ligandome analysis, we measured MHC class I and class II levels by flow cytometry and observed no significant alterations upon autophagy induction (data not shown). 54 HLA-DR-bound peptides from 31 different source proteins were sequenced and differentially quantified by liquid chromatography mass spectrometry and divided into two groups: peptides from membrane and secreted proteins, which should be preferentially presented on MHC class II molecules, and peptides from intracellular, especially nuclear, proteins, which should be preferentially presented on MHC class I molecules (Table 5). Additionally, we analyzed peptides from lysosomal proteins, as lysosomes take part in the autophagic turnover of the cell. After 6 h starvation, the presentation of peptides from intracellular and lysosomal proteins rose on average by 27%, after 24 h by 56% (Figure 4) compared to peptides from membrane and secreted proteins, which represents a remarkable change in the peptide repertoire. Upon application of unpaired two-tail student´s t-tests to the two groups of quantified ligands, the means turned out to be significantly different ($p<0.001$) with non-overlapping 99% confidence intervals. Enhancement of presentation seemed to be selective for the cellular localization of peptide source proteins. From the 4 source proteins that

showed the highest presentation levels of peptides after 24 h starvation, 3 are localized in the nucleus and 1 in lysosomes (Table 5, Figure 4). Regarding these 4 peptides, presentation levels were raised on average by 131% after 24 h autophagy.

Apart from an increased uptake into autophagic vacuoles, several other processes might contribute to an enhanced presentation of peptides derived from intracellular proteins under starvation. To control for the possibility that a higher mRNA expression for specific proteins upon autophagy induction led to an increased peptide presentation, gene expression for all 31 source proteins was assessed by oligonucleotide microarrays (Table 5). In general, mRNA levels of most genes were unchanged under starvation. Among the membrane proteins, only HLA-E and carboxypeptidase D displayed an increased expression. For intracellular and lysosomal proteins, the same could be observed for TNF alpha induced protein 3, heat shock 70 kDa protein 1, and cathepsin S. Peptides from the corresponding source proteins were also presented in higher amounts at the cell surface after induction of autophagy. Therefore, we cannot exclude that the overexpression of these particular proteins during autophagy was the reason for elevated presentation levels of the corresponding peptides at the cell surface. However, only intracellular source proteins from 7 of 24 analyzed peptides showed elevated mRNA expression levels during autophagy. It is therefore highly unlikely that altered source gene expression was a major contributor to the observed changes in presentation levels.

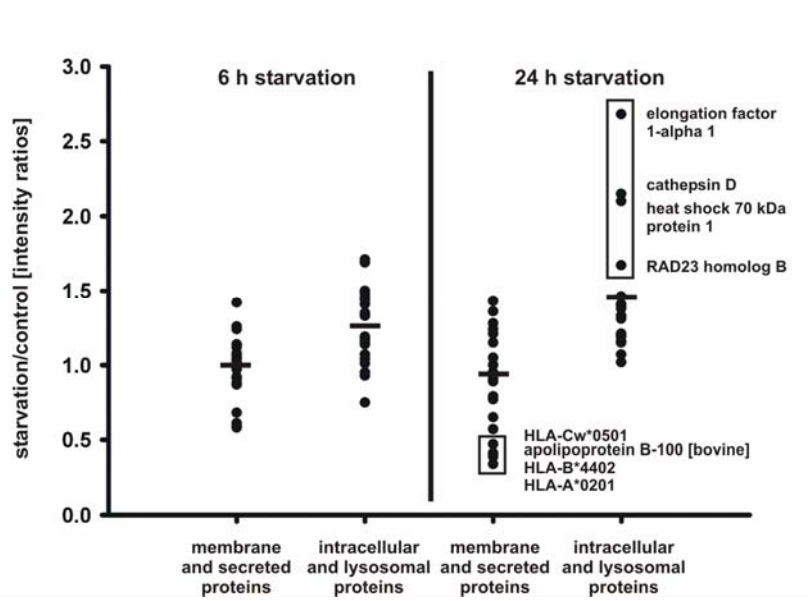

Figure 4: Altered peptide presentation on HLA-DR under starvation.
Displayed are the relative intensity ratios of peptides from starved cells (6 h and 24 h) and control cells as assessed by liquid chromatography (LC)-mass spectrometry (MS). Peptides were quantified by their relative peak heights in mass spectra and grouped according to the cellular localization of their source proteins: membrane plus secreted proteins and intracellular plus lysosomal proteins. Data of serial LC-MS runs were normalized to the abundant peptide LSSWTAADTAAQITQR, which showed only marginal differences in presentation levels (Table 5). Horizontal bars indicate the mean intensity ratios for each group. Marked in a box are the 4 peptides that showed the highest presentation levels after 24 h starvation. Their source proteins are localized in the nucleus and in lysosomes.

Table 5. Differential Presentation of peptides on HLA-DR molecules and corresponding mRNA data; the given peptide and mRNA ratios refer to the comparison of cells grown under starvation with control cells. For peptides, ratios were calculated from the signal intensities in LC-MS experiments. mRNA ratios were calculated from the signal log ratios given by the microarray analysis. "NC" (= no change) is displayed if no significant change in the expression level was observed according to the change algorithm.

Source Protein	Entrez Gene ID	Peptide Sequence	6 h Starvation		24 h Starvation	
			Peptide Ratio	mRNA Ratio	Peptide Ratio	mRNA Ratio
Membrane Proteins						
HLA-A*0201	3105	FVRFDSDAASQR	0.64	NC	1.53	NC
	3105	FVRFDSDAASQRME	0.68	NC	1.30	NC
	3105	DTQFVRFDSDAASQRME	1.58	NC	0.36	NC
	3105	VDDTQFVRFDSDAASQR	1.15	NC	0.82	NC
	3105	KHKWEAAHVAEQLR	1.09	NC	1.23	NC
	3105	DDTQFVRFDSDAASQRME	1.18	NC	0.95	NC
HLA-B*4402	3106	EDLSSWTAADTAAQITQRKWE	1.18	NC	0.42	NC
	3106	LSSWTAADTAAQITQR	1.11	NC	1.07	NC
HLA-Cw*0501	3107	VDDTQFVQFDSDAASPRGEPR	1.20	NC	0.50	NC
	3107	KDYIALNEDLRSWTA	1.11	NC	-	NC
	3107	DGKDYIALNEDLRSWTA	1.01	NC	0.61	NC
	3107	FVQFDSDAASPRGEPR	0.76	NC	1.12	NC
HLA-E	3133	DLRSWTAVDTAAQISEQ	0.97	1.87	0.70	2.46
HLA-DQB1*0301	3119	DVEVYRAVTPLGPPD	1.25	NC	-	NC
lymphocyte antigen Ly-6E	4061	KPTICSDQDNYCVT	1.18	NC	-	0.54
	4061	LKPTICCSDQDNYCVT	1.40	NC	0.97	0.54
immunoglobulin heavy chain	3492	YLQMNSLKTEDT	0.75	-	1.33	
	3492	TLYLQMNSLKTEDT	1.38	-	-	
immunoglobulin lambda chain	3537	SHKSYSCQVTHEGSTVE	1.02	-	1.45	
B-lymphocyte antigen CD 20	931	INIYNCEPANPSEK	1.16	NC	1.53	NC
class I cytokine receptor	9466	VGVPYRITVTAVSASG	1.20	NC	-	NC
transferrin receptor protein 1	7037	FTYINLDKAVLGTSN	1.18	NC	0.85	NC
carboxypeptidase D	1362	VPGTYKITASARGYNPV	1.27	1.23	1.37	1.52
	1362	VPGTYKITASARGYN	1.13	1.23	-	1.52
Extracellular Proteins						
serotransferrin [bovine]		FVKDQTVIQNTD	0.66	-	1.37	-
		DVAFVKDQTVIQNTD	1.13	-	-	-
		DVAFVKDQTVIQ	1.24	-	-	-
serum albumin [bovine]		SPDLPKLKPDPNTLCDEF	1.24	-	1.01	-
apolipoprot B-100 [bovine]		SASYKADTVAKVQGT	1.08	-	1.02	-
		SASYKADTVAKVQGTE	0.98	-	0.44	-
Intracellular Proteins						
heat shock 70 kDa protein 1	3303	NVLRIINEPTAAAIAYG	1.50	3.48	1.48	NC
	3303	VLRIINEPTAAAIAY	1.03	3.48	1.24	NC
	3303	RIINEPTAAAIA	1.49	3.48	2.25	NC
	3303	VLRIINEPTAAAIAYG	1.12	3.48	1.30	NC
heat shock cognate 71 kDa protein	3312	GILNVSAVDKSTGKE	1.67	NC	1.51	NC
	3312	ERAMTKDNNLLGKFE	1.19	NC	1.50	NC
	3312	GERAMTKDNNLLGKFE	1.48	NC	1.30	NC
elongation factor 1-alpha 1	1917	IEKFEKEAAEMGKGSF	1.49	NC	2.87	NC
TNF, alpha induced protein 3	7128	EIIHKALIDRNIQ	1.32	2.14	-	2.64
RAD23 homolog B	5887	LLQQISQHQEHF	1.88	NC	1.79	NC
actin, cytoplasmic 2	71	TDYLMKILTERGYS	1.30	NC	1.09	NC
NEDD4La	23327	DGRTFYIDHNSKITQ	1.26	NC	1.51	NC
T-complex protein 1, beta subunit	10576	SLMVTNDGATILKN	1.15	NC	-	NC
ubiquitin	7311	SDYNIQKESTLHLV	1.05	-	1.42	-
alpha enolase	2023	VPLYRHIADLAGNSEV	1.50	NC	1.14	NC
syntaxin 6	10228	NPRKFNLDATELSIRK	1.60	NC	-	NC
tubulin beta-5 chain	10382	EPYNATLSVHQL	1.50	NC	1.23	NC
Lysosomal Proteins						
cathepsin C	1075	YDHNFVKAINAIQKSWT	1.31	NC	1.28	NC
	1075	YDHNFVKAINAIQKSW	1.28	NC	1.27	NC
	1075	YDHNFVKAINAIQKS	1.56	NC	1.40	NC
cathepsin D	1509	LSRDPDAQPGGE	0.83	NC	2.30	NC
cathepsin S	1520	TTAFQYIIDNKGIDSD	1.61	2.30	-	4.92
	1520	TTAFQYIIDNKGID	1.90	2.30	1.56	4.92
lysosomal alpha-mannosidase	4125	VDYFLNVATAQGRYY	1.64	NC	-	NC

Autophagy leads to a time-dependent decrease of lysosomal proteases

Interestingly, presentation levels of peptides derived from the same source protein were differently affected by starvation. This applied to both, proteins processed by the classical MHC II pathway, as for example HLA-A*0201, as well as to intracellular proteins, as for example heat shock 70 kDa protein 1 (Table 5). This led us to hypothesize that activation of the autophagic pathway might concomitantly affect the MHC II processing machinery by altering the activity levels of lysosomal cysteine proteases. Therefore, we assessed the activity of the major cathepsins during autophagy by affinity labelling (Figure 5a). Active cathepsins Z, B, H, S and C could be detected in control cells using this method, largely in agreement with previous studies in other cells. Starvation of cells led to a time-dependent decrease of the activity signals for all cathepsins that occurred in a nearly linear fashion without a clear preference for any individual cathepsin. The same pattern of cathepsin downregulation was observed when control cells and cells undergoing autophagy were probed for cathepsin polypeptides by Western blot (Figure 5b). Of note, this effect was not due to non-selective breakdown of total cellular protein or lysosomal protein in general, because the amounts of β-actin as well as of the lysosome-resident protein LAMP-1 remained unaffected by autophagy.

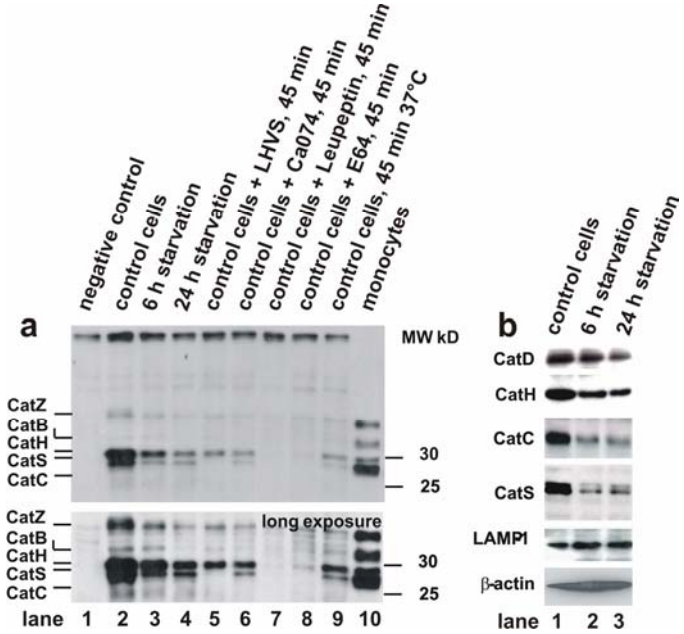

Figure 5: (a) Affinity labelling of active cathepsins. Endocytic extracts were generated from control cells, cells after 6 h and 24 h starvation, and from human

peripheral blood monoyctes, respectively, by differential centrifugation as reported [37;42]. 5 µg total endocytic protein (1.5 µg in monocytes) were either directly incubated with the active site-restricted biotinylated affinity label DCG-0N as described (lane 2: control cells; lane 3: 6 h starvation; lane 4: 24 h starvation; and lane 10: monocytes), or were subjected to 95°C as negative control (lane 1). In addition, control cells were incubated with the CatS-inhibitor LHVS (25 nM), the CatB-inhibitor Ca074 (1µM), the pan-cysteine protease inhibitors leupeptin (1 mM) and E64 (25µM) (lanes 5-8), for 45 min at 37°C prior to labelling, or for 45 min at 37°C without addition of protease inhibitors (lane 9) as further controls. Active cathepsins were visualized after resolution by SDS-PAGE by streptavidin-HRP blot: Cat Z, B, H, and S at 36, 33, 30, and 28 kD, respectively. (b) Cathepsin polypeptides probed by Western blot. Identical amounts of total cellular protein from control cells (lane 1) and cells undergoing autophagy (6 h and 24 h starvation, respectively; lane 2 and lane 3) were probed for CatS, CatC, CatD, CatH, β-actin and LAMP-1 by Western blot.

2.1.5 Discussion

In order to assess the impact of autophagy on the HLA class II ligandome, a proteomic analysis of the HLA-DR peptide repertoire of Awells cells was performed. So far no detailed proteomic analysis of the MHC class II self peptide repertoire was available. For the understanding of the immune system it is important to determine which peptides are presented under normal conditions on the cell surface and to know how the presentation pattern changes under different environmental conditions, e.g. under nutrient deprivation. 404 peptides with 173 different core sequences were identified showing that peptides from source proteins localized in almost all cell compartments and taking part in general cellular processes are presented on MHC class II molecules. Some examples of peptides from intracellular proteins presented on class II have been described before [18;19]. However in our case the number of such source proteins was surprisingly high. Thus, peptides from intracellular antigens are likely to have a larger impact on CD4+ helper T cell regulation than originally thought. It has

already been shown that CD4+ helper T cells are able to recognize peptides from intracellular melanoma [20;21] and viral antigens (EBNA1 [22]), and that under inflammatory conditions peptides from intracellular antigens are presented on HLA class II molecules on epithelial cells which are target cells in autoimmunity [23].

Autophagy is a constitutive process responsible for the turnover of intracellular proteins [24]. Basal levels have been observed in most tissues [25] and can be particularly enhanced by starvation. In addition, autophagy has implications on tumor development [25;26]. Starvation induced autophagy has been observed in lymphocytes isolated from patients with chronic lymphocytic leukaemia [27]. This might indicate an essential role of this process for tumor survival under nutrient-limiting conditions. In contrast, autophagy as a form of programmed cell death may accelerate tumor development if it is decreased [28]. Several genes are known to be required for autophagy [10;29], so far most studies have been done in yeast, however. To get a detailed impression of genes involved in autophagy in humans, whole-genome expression profiling using oligonucleotide arrays was performed. Inter alia, it could be shown that during autophagy genes are upregulated that are important for vesicular transport in the endosomal/lysosomal system and anti apoptotic genes, stressing the point that autophagy may be part of a programmed cell death different from apoptosis [30].

We could further show that autophagy constitutes a general pathway promoting the processing of intracellular proteins by lysosomes and presentation of the resulting peptides on MHC class II molecules. As the increase in presentation levels of cells undergoing autophagy was specific for intracellular, especially nuclear, peptides and not for peptides from membrane and secreted proteins, it is very unlikely that this presentation was due to an enhanced uptake of cellular debris by live cells, which would likely affect all proteins similarly, but rather to a feeding of intracellular proteins into the lysosomal system via autophagy. Autophagy has also been described as a constitutive process under nutrient rich conditions for several tissues in vivo, including thymic epithelial cells [31]. Therefore, it might play an important role in the presentation of intracellular self-antigens to CD4+ T cells during negative selection. Furthermore, some anticancer drugs potentially act via triggering autophagy [32] and could by this cause an enhanced presentation of CD4+ T cell epitopes in MHC class II expressing tumor cells.

To assess the impact of autophagy on the MHC II antigen processing machinery affinity-labelling and Western blot analysis of cysteine proteases was performed. It could be shown that autophagy decreases the amount of active cathepsins in the endocytic compartment in a time-dependent fashion and that the same pattern of cathepsin downregulation was observed on protein level. Decreased cathepsin levels might favor the generation of MHC II peptides due to a less efficient lysosomal protein digestion. This has been suggested as a mechanism to explain the superiority of dendritic cells over macrophages as antigen presenting cells (I. Mellman, paper presented at the 7th International Symposium on Dendritic Cells, Bamberg, Germany, Sept. 2002). Similarly, autophagy might subject the cell to an enhanced immune surveillance by CD4+ T cells under potentially dangerous stress conditions.

Recently, it has been described that a peptide from a cytosolic antigen [9] as well as from a cytosolic viral antigen (C. Munz, personal communication) can be presented via autophagy on MHC class II molecules. It was, however, so far unclear whether this represented a minor event or if autophagy contributes to the endogenous presentation of intracellular antigens on HLA class II in general. In this study, the analysis of a considerable proportion of the MHC II peptide repertoire in starved and control cells revealed a pattern of mechanisms that profoundly affect the MHC II ligandome. Autophagy induction leads to several changes in cellular metabolism, like altered mRNA expression or decreased activities of lysosomal proteases, all potentially contributing to modified MHC II presentation. Even though all those influences have to be considered, it is evident that autophagy is the key process that explains the overall changes observed in the MHC II ligandome. Thus, apart from its various known implications in stress responses and cell death, autophagy obviously plays an important role in the regulation of CD4+ T cell-mediated processes.

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2.2 Glycan side chains on naturally presented MHC class II ligands

This article is in press (state: December 2004) in the Journal of Mass Spectrometry by the authors Jörn Dengjel, Hans-Georg Rammensee and Stefan Stevanović. The author of this thesis performed all experiments.

2.2.1 Summary

The molecular characterization of unknown naturally presented MHC class II glycopeptides carrying complex glycans has so far not been achieved, reflecting the different fragmentation characteristics of sugars and peptides in mass spectrometric analysis. HLA-DR-bound peptides were isolated by affinity purification, separated via HPLC and analyzed by MALDI and ESI mass spectrometry (MS). We were able to identify two naturally processed MHC class II ligands, $CD53_{122\text{-}136}$ and $CD53_{121\text{-}136}$, carrying complex N-linked glycan side chains by a combination of in-source and collision-induced fragmentation on a Q-TOF mass spectrometer.

2.2.2 Introduction

Peptides of foreign and self proteins are presented on major histocompatibility complex (MHC) class I and class II molecules at the cell surface where they can be recognized by T lymphocytes [1;2]. By this mechanism MHC molecules transfer information about the current stock of proteins within a cell and its surrounding to the cell surface allowing the immune system to survey the cell's integrity and to react, if necessary. The definition of the pools of peptides presented at the cell surface is particularly vital for the understanding of the immune system, but also for the general understanding of cellular biology. It has been shown by direct or indirect evidence that MHC class I and class II molecules can present posttranslationally modified peptides, such as deamidated [3;4],

cysteinylated [5;6], glycosylated [7] and phosphorylated peptides [8], and that such posttranslational modifications can be essential for T cell recognition. However until now, the molecular characterization of naturally presented MHC ligands carrying glycans has not been achieved. Mass spectrometric approaches have been used in different ways to characterize glycosylated peptides [9;10] . Frequently, differential peptide mapping of tryptic digest mixtures before and after treatment of glycosylated peptides with endoglycosidases has been performed. Subsequently the peptide as well as the carbohydrate parts were analyzed [11;12]. Glycopeptides have also been dissected by sequential exoglycosidase treatment [13]. As peptide and sugar moieties have different fragmentation behaviors, the characterization of unknown oligosaccharide and peptide structures in single mass spectrometric experiments is very difficult. We used a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF) to identify glycosylated MHC class II presented peptides by a combination of in-source and collision-induced fragmentation.

2.2.3 Materials and Methods

Elution of MHC Class II bound Peptides

Frozen Awells cell pellets (IHW-No. 9090, ECACC 94082236, 3.5 to 5.7×10^{10}cells) were used to extract HLA-DR bound peptides. Awells is an HLA defined EBV transformed human B-lymphoblastoid cell line, homozygous for HLA-DR4 – the HLA of interest – presenting a high amount of HLA molecules at the cell surface. The cells were processed as previously described [14] and peptides were isolated according to standard protocols [15] using the HLA-DR specific mAb L243 [16], briefly: cells were lysed by incubation in PBS containing 0.6% 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate (CHAPS; Roche, Mannheim, Germany) (w/v) and Complete protease inhibitor tablets (Roche), followed by exposure to sonic waves. Cellular debris was ultra centrifuged at 151,000 *g* and the supernatant was passed over a 0.2 µm filter. The flow-through – containing the MHC-peptide-complexes – was run over an affinity column consisting of mAb L243 coupled to CNBr-activated sepharose 4B (Amersham

Bioscience, Uppsala, Sweden) and peptides were eluted by 0.1% TFA. Finally, peptides were passed over a 10 kDa cut-off filter and subjected to HPLC separation.

Molecular analysis of DR-eluted peptides

Peptides were separated by gradient elution from a reversed-phase column (HPLC, SMART system, µRP SC C2/C18-column, 100 x 2.1 mm, Amersham Pharmacia Biotech, Freiburg, Germany). A binary gradient of 10% to 60% B within 100 min was performed, applying a flow rate of 150 µl/min. Solvent A was 0.1% TFA/water (vol/vol). Solvent B contained 0.08% TFA in 80% acetonitrile/water (vol/vol). The peptides were fractionated in 150 µl aliquots. Before MS analysis each fraction, was completely dried and resuspended in 50% methanol/water/0.1 % formic acid (vol/vol).

MALDI-TOF analysis of the fractions from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on target using the fast evaporation method [17] was performed on a Bruker Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337 nm laser, gridless pulsed ion extraction and externally calibrated using synthetic peptides with known molecular weights. Spectra were recorded in positive ionization mode at acceleration voltages of 20 kV and 16.9 kV. PSD spectra were recorded using 10 reflector voltage steps from 23 kV to 0.9 kV. Peptides were further analyzed by nano-electrospray (ES) mass spectrometry (MS) on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) as described [14]. The ions were produced in a nanoflow electrospray ionization source. To the gold-coated glass capillary nanoflow needles (Proxeon, type Medium NanoES spray capillaries for the Micromass Q-TOF, Odense, Denmark) a potential of 1.2 kV was applied, resulting in sample flow rates of 20 to 50 nl/min. The cone voltage was 35 V for MS and tandem MS experiments and 80 to 100 V for in-source and collision-induced fragmentation experiments. A quadrupole analyzer was used to select precursor ions for fragmentation in a hexapole collision cell. The collision gas was argon used at collision energies of 24 to 50 eV. Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science [18].

Peptide N-glycosidase F digestion

Deglycosylation was performed using Peptide N-glycosidase F (PNGase F; New England Biolabs, Frankfurt, Germany) from *flavobacterium meningosepticum* essentially as described [19], briefly: Peptides were dissolved in 0.5 M sodium phosphate buffer (pH 7.5) and 0.1 µl PNGase F (50 U) was added. After incubation for 5 h at 37°C, samples were desalted on the target and analyzed by MALDI-MS as already described.

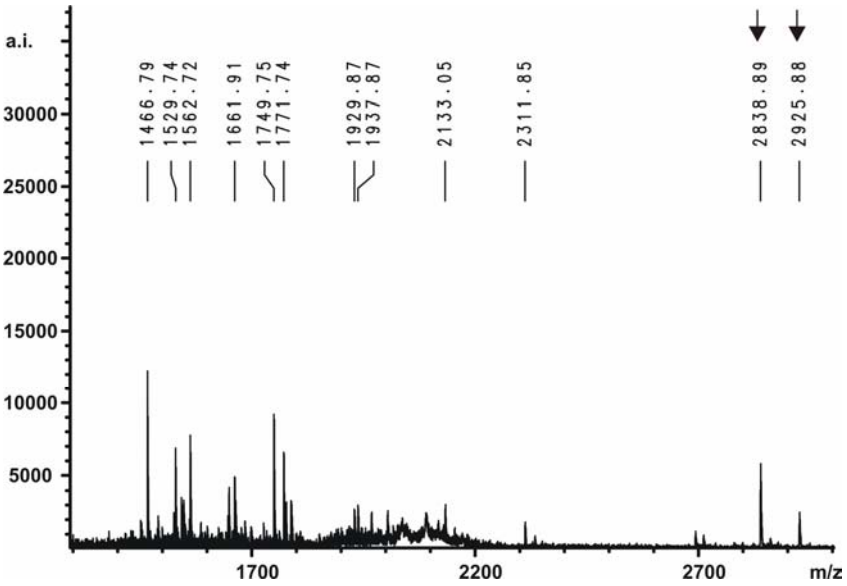

Figure 1: MALDI TOF mass spectrum of the HPLC fraction 10 containing several HLA-DR peptides as indicated by the annotated masses. The spectrum was recorded at a Bruker Reflex III mass spectrometer equipped with a N_2 337 nm laser. The ions at *m/z* 2838.57 and *m/z* 2925.55 (marked by arrows) were the only signals corresponding to glycopeptides.

2.2.4 Results and Discussion

HLA-DR-bound peptides isolated from Awells cells (IHW-No. 9090) were separated via HPLC. All HPLC fractions were analyzed by MALDI-TOF MS in order to identify peptide signals suitable for further investigation by ESMS (Figure

1). In positive ESI mass spectra doubly, triply and quadruply charged signals of the corresponding peptides could be observed and were chosen as precursors for MS/MS analysis to identify so far unknown HLA ligands (data not shown). The MS/MS spectra of the triply charged ions at *m/z* 947.05 and *m/z* 976.05, respectively, corresponding to the ions at *m/z* 2838.57 and *m/z* 2925.55 in the MALDI mass spectrum indicated the presence of N-acetylhexosamine residues (HexNAc) by the abundant ion at *m/z* 204.13 ($[HexNAc]^+$), further the loss of hexose residues (Hex, *m/z* 162) could be observed (Figure 2A). A detailed analysis revealed that the two peptides seemed to be modified by the same glycan, consisting of two HexNAc residues, three Hex residues and one deoxy hexose residue (DeoxyHex, *m/z* 146). The positive CID spectrum showed several overlying ions series yielding a complex picture. The ion at *m/z* 1177.69 represents loss of all three Hex residues from the parent ion. The ion at *m/z* 1075.72 corresponds to further loss of a HexNAc residue, leaving one HexNAc residue and the DeoxyHex residue still coupled to the peptide moiety. Therefore, the most abundant fragment ion at *m/z* 1002.64 is either generated through loss of a HexNAc residue from the ion at *m/z* 1104.72, or through loss of the DeoxyHex residue from the ion at *m/z* 1075.72. Loss of the last HexNAc residue can be observed from the ion at *m/z* 1002.64. In MS/MS experiments hardly any product ions corresponding to peptide parts of the glycopeptides could be observed, which has already been described for complex N-linked glycopeptides [10]. The major product ions corresponded to glycosidic cleavages from the nonreducing termini of the glycan. Mammalian glycopeptides commonly contain three types of glycans: asparagine bound N-linked and serine or threonine bound O-linked glycans. Both, N- and O-linked glycans are commonly linked via one N-acetylhexosamine residue to the peptide moiety. Nevertheless, as shown later, the peptides were unambiguously identified as carrying N-linked glycans. According to the three major subgroups of N-linked glycans and as it is a human sample, we propose that it is a complex-type oligosaccharide consisting of the pentasaccharide core of N-linked glycans, modified with one fucose residue [20-22] (Figure 2B).

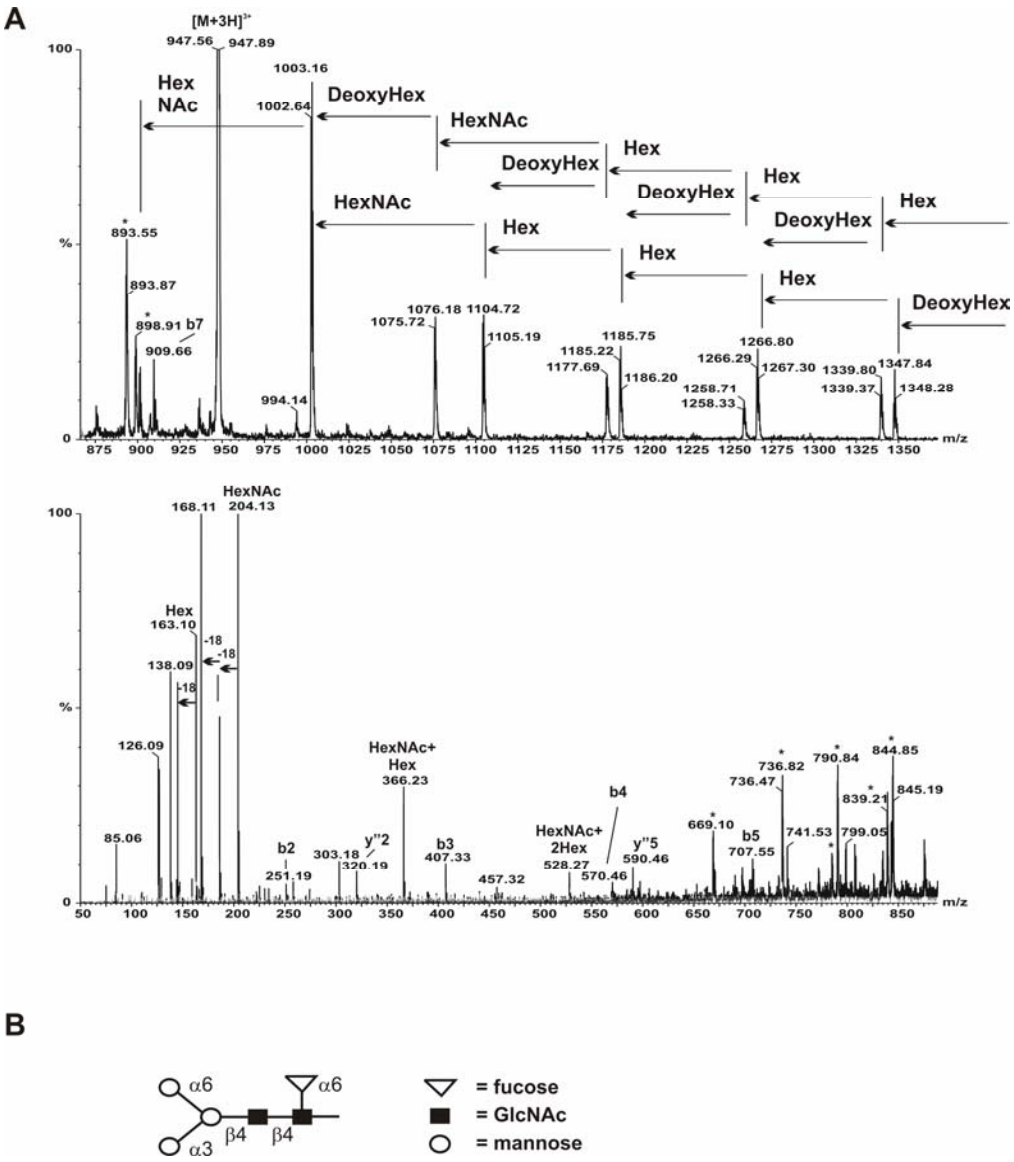

Figure 2: Molecular characterization of the glycopeptide $CD53_{122\text{-}136}$. **(A)** ESI MS/MS spectrum of the glycopeptide at *m/z* 947.05 corresponding to $[M+3H]^{3+}$ recorded at a Q-TOF mass spectrometer. The major product ions are doubly charged, corresponding to glycosidic cleavages. The corresponding 3+ series is marked by asterisks (*). Overlying ion series are annotated. The according MS/MS spectrum of the glycopeptide at *m/z* 976.05 is not shown. **(B)** Proposed glycan structure showing the core of human N-linked oligosaccharides with an additional fucose residue.

To elucidate the structure of the peptides we performed a combination of in-source and collision-induced fragmentation. The most abundant product ions at *m/z* 1002.6 and *m/z* 1045.9, respectively, corresponding to $[M+2H]^{2+}$ were selected for fragmentation. As expected, ions contained one N-acetylhexosamine residue (Figure 3) [23]. Further, b ion series with and without the glycosylation were identified, glycosylated b_m fragment ions appearing primarily as doubly charged species. Finally, the peptide sequences could be assigned to $CD53_{122\text{-}136}$ and $CD53_{121\text{-}136}$ (SwProt: P19397). The panleukocyte marker CD53 is known to be glycosylated, the two peptides belonging to the extracellular domain and containing one of the two potential glycosylation sites at Asn_{129} [24]. Unmodified peptides from the same antigen were not observed. The two peptides fit perfectly to the HLA-DR4 motif (www.syfpeithi.de), with Asn_{129} in position 5, which is usually exposed and available for T cell receptor interaction [25].

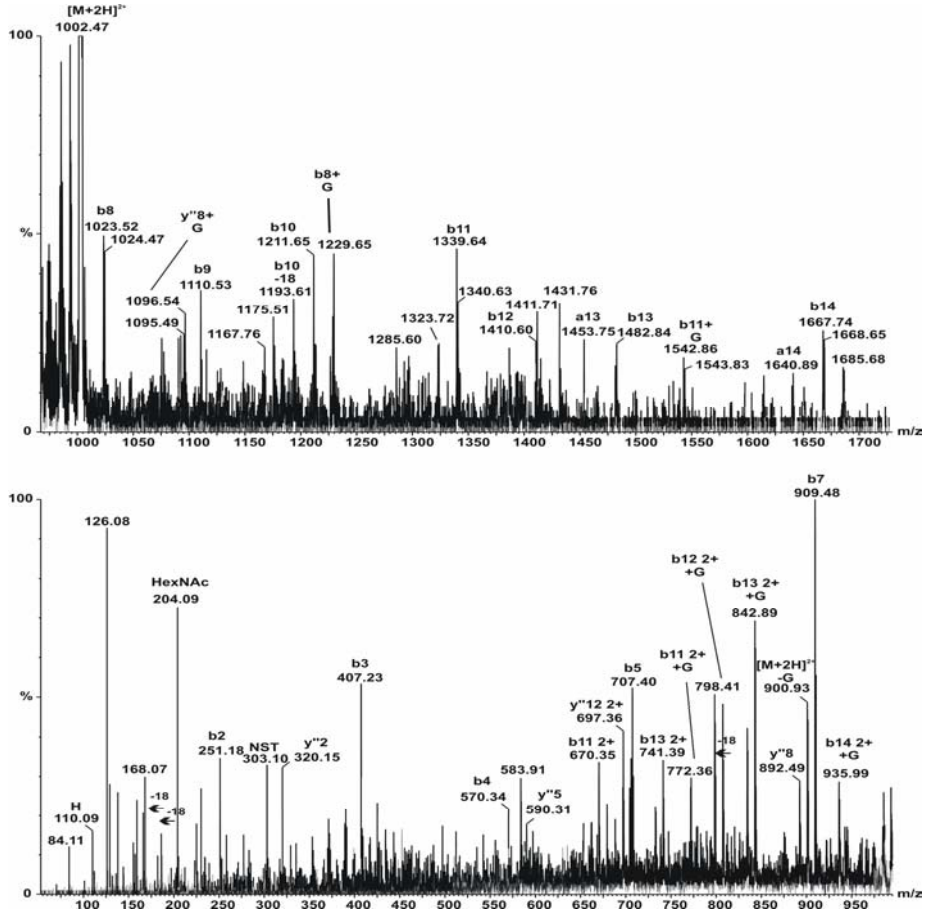

Figure 3: ESI fragment spectrum of the product ion IHRYHSDN(HexNAc)STKAAWD at *m/z* 1002.6 corresponding to $[M+2H]^{2+}$

recorded at a Q-TOF mass spectrometer using a combination of in-source and collision-induced fragmentation (G = HexNAc). In order to obtain enough information in the "MS^3" scans, rather large precursor selection windows (Δ *m/z* = 2) had to be chosen. Therefore, unassigned signals could be due to contaminating substances. The according MS spectrum of the peptide SIHRYHSDN(HexNAc)STKAAWD^{2+} at *m/z* 1045.9 is not shown.

To verify our results we performed a PNGase F digestion of the HLA peptides. As anticipated, the carbohydrate trees were cleaved giving rise to two peptides with a mass increase of 1 Da each (Figure 4). This proves that the peptides were indeed N-glycosylated, and further fixes the DeoxyHex residue as α1,6 linked. In addition, a PSD-MALDI spectrum of the peptide at *m/z* 1801.80 was recorded confirming the identity and the glycosylation site (data not shown).

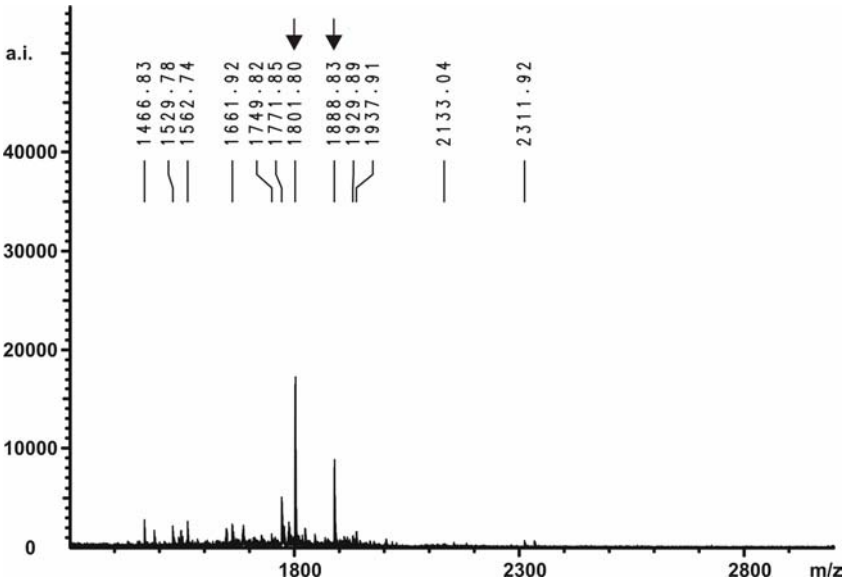

Figure 4: MALDI TOF mass spectrum of the HPLC fraction 10 after PNGase F treatment. The ions at *m/z* 2838.57 and *m/z* 2925.55 (Figure 1), corresponding to the two described glycopeptides, were deglycosylated yielding the two ions at *m/z* 1801.80 and *m/z* 1888.83 (marked by arrows). The glycosylated asparagine residues have been converted into aspartic acid residues, resulting in a mass increase of 1 Da.

Taken together all the information we propose that the MHC class II peptides $CD53_{122-136}$ and $CD53_{121-136}$ are glycosylated at Asn_{129} with the hexasaccharide shown in Figure 2B. It has been shown that MHC class II peptides can carry one monosaccharide [26], yielding the point of glycan attachment at the peptide moiety. But to our knowledge this is the first report of a molecular characterization of naturally presented MHC class II peptides carrying a glycan, the core structure of N-linked oligosaccharides. It has been shown that $CD4^+$ and $CD8^+$ T cells can recognize glycopeptides and that recognition can be carbohydrate specific. Knowing that MHC class II molecules present peptides modified by complex glycans amplifies the potential number of T cell epitopes drastically.

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2.2.5 References

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2.3 Identification of a naturally processed cyclin D1 T-helper epitope by a novel combination of HLA class II targeting and differential mass spectrometry

This article is published in the European Journal of Immunology, 2004 December, 34(12):3644-51, by the authors Jörn Dengjel, Patrice Decker, Oliver Schoor, Florian Altenberend, Toni Weinschenk, Hans-Georg Rammensee and Stefan Stevanović.

The author of this thesis generated the fusion protein expressing cell clones and examined the endosomal/lysosomal targeting of the corresponding fusion proteins. In addition, he performed the differential mass spectrometric screenings and characterized the two HLA peptides from cyclin D1 and keratin 18.

2.3.1 Summary

T-helper cells play an important role in orchestrating the effector function of CTLs in anti-tumor immunity. However, only a limited number of T-helper cell epitopes has been characterized. Here we describe a novel approach allowing to identify naturally processed and presented peptides derived from chosen antigens. This method combines a transfection step of antigen presenting cells with a vector encoding a fusion protein between the Ii chain and the antigen of interest, elution of the HLA-bound peptides and identification of the antigen-derived peptides by mass spectrometric comparison to the non-transfected cells. *In vitro* stimulated T-helper cells against the identified peptide of interest specifically recognize transfectants overexpressing the cognate antigen. Using this approach, we could identify the HLA-DR4-restricted T-helper cell epitope NPPSMVAAGSVVAAV derived from cyclin D1, which is frequently overexpressed in tumors. This method will help in identifying peptide candidates for vaccination studies for tumor immunotherapy.

2.3.2 Introduction

The identification of $CD4^+$ T-cell epitopes of tumor associated antigens has experienced much attention lately [1-8], as $CD4^+$T helper cells play an important role in orchestrating the effector function of anti-tumor T cells [1;3;9], even in the absence of CTL effector cells, by acting in an indirect, cytokine dependent manner [10-12]. They can inhibit tumor angiogenesis via IFNγ [13] and counteract tumor progression via the induction of an Ab response [14]. In addition, tumor-specific $CD4^+$ T cells, and particularly T-helper 1 (Th1) T cells, have also been shown to display cytotoxic activity [15;16].

In contrast to HLA class I ligands, only few class II ligands of tumor associated antigens are described. One reason for this might be their more difficult identification. Potential MHC class I ligands are often first predicted using peptide motifs [17;18] before primary T cell cultures are stimulated with the synthetic peptides, in order to define an actual T cell epitope. The prediction of HLA class II restricted peptides does not work as efficiently: firstly class II ligands show a greater variance in their length [19] and secondly the peptide motifs of most class II molecules are more degenerated as compared to MHC class I motifs [20]. As most tumors are HLA class II negative it is also not possible to isolate class II ligands directly from primary tumors in the search for ligands of tumor associated antigens, as can be done for class I ligands [21].

Until now numerous strategies to target antigens into the class II processing pathway have been described. It is possible to incubate antigen presenting cells (APCs) with the antigen of interest in order to be taken up and processed [2]. Other strategies use fusion proteins which contain lysosomal target sequences. Expressed in APCs, such fusion proteins direct the antigens into the class II processing compartment [22;23].

Here we describe a new and generally applicable combined approach for the identification of unknown naturally processed HLA class II ligands of defined – e.g. tumor associated – antigens in order to define new candidates for peptide-based immunotherapy. It has been shown that the 80 N-terminal amino acids of Ii are sufficient to direct proteins into the class II processing pathway [24;25]. We thus generated fusion proteins consisting of the 80 N-terminal amino acids of Ii and our antigen of interest, cyclin D1 (CCND1). Cyclin D1 is a cell cycle regulator involved

in the G1-S transition through interactions with cyclin-dependent kinases. Moreover, cyclin D1 is a proto-oncogene and has been shown to be overexpressed in several tumor types [26-28] whereas it is expressed at low level in a large panel of healthy organs and tissues without any particular distribution with the exception of liver and high aortic smooth muscle cells [21](WEINSCHENK unpublished data). In a differential mass spectrometric approach we compared mass spectra of purified HLA peptides from transfected and non transfected cells and used the resulting peptides of interest in *in vitro* priming experiments.

2.3.3 Materials and Methods

Cells and antibodies

The human B-lymphoblastoid cell line Awells (IHW-No. 9090; HLA-DRB1*0401, HLA-DRB4*0101) was maintained in RPMI 1640 (C.C.Pro, Neustadt, Germany) medium containing 10 % FCS (Pan, Aidenbach, Germany) and supplemented with 2 mM L-glutamine (BioWhittaker, Verviers, Belgium), 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker). In the case of the transfected cell clones 0.8 mg/ml G418 (PAA Laboratories, Linz, Austria) was added. Stable transfectants were generated by electroporation of Awells (280 V, 975 µF; Gene Pulser II, Biorad, München, Germany) cells, followed by cloning using the limiting dilution method. The antibodies L243 (anti–HLA-DR) [29] and W6/32 (anti HLA class I) [30] were purified from hybridoma culture supernatants using protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). The Th-cell line was induced and cultured in IMDM (BioWhittaker) containing 10 % human AB serum (Pel-Freez Clinical Systems, LLC, Milwaukee, WI, USA) and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 50 µM β-mercaptoethanol. Antibodies used in flow cytometry analysis were from PharMingen (SanDiego, CA, USA).

Plasmid DNA constructs

The cDNA encoding the 80 N-terminal amino acids of Ii (NCBI, GenBank X00497) was amplified in a PCR reaction out of the vector pBluescript II KS(+) 41-1

(Stratagen, Heidelberg, Germany) obtained from A. Melms [31] and subcloned into the Hind III and BamH I sites of pcDNA3 (pcDNA3-Ii; Invitrogen, Karlsruhe, Germany) using the 5` primer ATCGAAGCTTCCAAGATGCACAGGAG GAGAAGC and the 3` primer ATCGGGATCCTTTG TCCAGCCGGCCCTGCTG. The genes of interest were amplified in a PCR reaction from cDNA from malignant renal tissue using the 5` primer ATCGGAATTCTGAGCTTCACCACTCGCTCC and the 3` primer ATCGGCGGCCGCTTAATGCCTCAGAACTTTGGT for Keratin 18 (NCBI, GenBank X12881) and the 5` primer ATCGGAATTCTGGAACACCA GCTCCTGTGC and the 3` primer ATCGGCGGCCGCTCAGATGTCCACG TCCCGCAC for Cyclin D1 (NCBI, GenBank X59798), respectively. The obtained cDNA was subcloned using TOPO TA cloning (Invitrogen, Karlsruhe, Germany) and finally inserted into the EcoR I and Not I sites of pcDNA3-Ii, in frame with the Ii sequence.

Real-time quantitative PCR

RNA from cells was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. cDNA was synthesized from 1 µg of total RNA. Real-time quantitative PCR (qPCR) was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). SYBR Green PCR Master Mix (Applied Biosystems) was used for PCR amplification and real-time detection of PCR products. Primer sequences are as follows: 18S rRNA, 5` primer CGGCTACCACATCCAAGGAA and 3` primer GCTGGAATTACCGCGGCT; Keratin 18, 5` primer GAGCCTGGAGACCGAGAAC and 3` primer TTGCGAAGATCTGAGCCC; Cyclin D1, 5` primer CACGATTTCATTGAACA CTTCC and 3` primer TGAACTTCACATCTGTGGCAC. PCR reactions were carried out in 20 µl with 300 nM of each primer (18S reverse primer: only 50 nM). All samples were amplified in duplicate. Expression differences between transfected and wildtype cells for different genes were calculated from PCR amplification curves by relative quantification using the comparative threshold cycle (C_T) method (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). 18S ribosomal RNA was chosen as reference gene for normalizations.

Detection of fusion proteins

Fusion proteins were detected by Western blot analysis using the mAb PIN.1 (Stressgen, Biomol, Hamburg, Germany) which binds to amino acid residues 12-28 of Ii. Briefly, cells were lysed as described [32], lysates were boiled in Laemmli loading buffer, separated on a 12 % SDS-PAGE and transferred onto nitrocellulose membranes. After a saturation step with BSA, membranes were incubated for 1 h at room temperature with the mAb PIN.1 (1 µg/ml). Proteins were visualized using a peroxidase-coupled sheep anti-mouse IgG (Amersham Pharmacia, Freiburg, Germany). In some cases, transfected cells were cultured in the presence of 10-100 µM chloroquin (Sigma, Steinheim, Germany) in order to investigate the endosomal/lysosomal targeting of the fusion proteins. Cells were then lysed and proteins were detected by Western blot as described above.

Elution of MHC Class II bound Peptides

Frozen cell pellets (3.5 to 5×10^{10}cells) were processed as previously described [33] and peptides were isolated according to standard protocols [34] using the HLA-DR specific mAb L243 [29].

Molecular analysis of DR-eluted peptides

Peptides were separated by reversed-phase high performance liquid chromatoraphy (HPLC, SMART system, µRPC C2/C18 SC 2.1/10; Amersham Pharmacia Biotech, Freiburg, Germany), and fractions were analyzed by MALDI-TOF mass spectrometry (MS) using a Bruker Reflex III mass spectrometer (Bruker Daltonik). Differentially presented peptides were further analyzed by nano-ESI (electrospray ionisation) tandem MS on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) as described [33].

Peptide synthesis and analysis

Peptides were synthesized in an automated peptide synthesizer EPS221 (Abimed, Langenfeld, Germany) following the Fmoc/tBu strategy. After removal from the resin by treatment with TFA/phenol/ethanedithiol/thioanisole/water (90/3.75/1.25/2.5/2.5 by vol.) for 1 h or 3 h (arginine-containing peptides) peptides were precipitated from methyl-tert. butyl ether, washed once with methyl-tert. butyl

ether and twice with diethyl ether and resuspended in water prior to lyophilization. Synthesis products were analyzed by HPLC (Varian star, Zinsser analytics, München, Germany) and MALDI-TOF mass spectrometry (future, GSG, Bruchsal, Germany). Peptides of less than 80 % purity were purified by preparative HPLC.

Monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMC) were prepared according to classical procedures from an HLA-DRB1*0408-, HLA-DRB1*1101-, HLA-DRB3*0202-, HLA-DRB4*01-positive donor. Dendritic cells (DC) were obtained from plastic-adherent PBMC cultured in the presence of GM-CSF and IL-4 for 6 days as described previously [35], except that the medium used was X-VIVO 15 (BioWhittaker) without serum. At day 6, immature DC were analyzed by flow cytometry for CD1a, CD11c, CD14, CD40, CD83, CD86 as well as HLA-DR cell surface expression on a FACScalibur apparatus with CELLQuest software (Becton Dickinson, Mountain View, CA). DC were then matured in the presence of 50 µg/ml polyinosinic-polycytidylic acid (Poly I/C, Amersham Pharmacia, Uppsala, Sweden) and 10 ng/ml TNF-α (PharMingen) for two additional days and analyzed again by flow cytometry for CD14, CD80, CD83 and CD86 cell surface expression. Mature DC showed a clear up-regulation of CD80, CD83 and CD86 molecules.

Generation of peptide-specific T-helper cells

$3x10^5$ matured DC were loaded for two hours with 10 µM of peptide NPPSMVAAGSVVAAV in a 24-well plate and extensively washed. Then $4x10^6$ fresh autologous PBMC were added onto DC in the presence of 10 ng/ml IL-12p70 in order to favour Th-1 development. PBMC were weekly restimulated with peptide-loaded irradiated autologous PBMC in the presence of 10 U/ml IL-2 and 5 ng/ml IL-7. After 3 and 5 restimulations, T cells were pooled and tested against autologous PBMC in the presence of peptide. The T-helper cell line was then amplified every 1-2 weeks with irradiated allogenic PBMC in the presence of 1 µg/ml PHA, 25-50 U/ml IL-2 and 5 ng/ml IL-7 and then tested for the recognition of the transfected cell lines. Every three to four weeks, the T-helper cell line was restimulated with irradiated autologous PBMC in the presence of 10 µM peptide, 10 U/ml IL-2 and 5 ng/ml IL-7.

Functional assays and characterization of the T-helper cell line

T-helper cell activation was tested by cell proliferation as estimated by thymidine incorporation as well as cytokine secretion. Briefly, $2x10^5$ cells were incubated in triplicates in a 96-well plate with $2x10^5$ irradiated autologous PBMC in the presence or absence of 10 µM peptide or 3 µg/ml PHA. After 24 hours, two portions of 50 µl supernatant were harvested and frozen and 50 µl fresh medium was added to the cells. After 54 hours, 50 µl of tritiated thymidine-containing medium (0.074 MBq/well, Hartmann Analytic, Braunschweig, Germany) was added and thymidine incorporation measured at 72 hours using a scintillation counter (Microbeta, Wallac, Freiburg, Germany). Cell proliferation is expressed as a stimulation index (S.I.), which corresponds to the ratio: (mean cpm of stimulated T cells)/(mean cpm of unstimulated T cells). IL-2 secretion was measured using the IL-2 dependent CTLL-2 cell line. Briefly, 10^4 cells were incubated in the presence of supernatants for 20-24 hours. Then, thymidine-containing-medium (0.055 MBq/well) was added for 7-8 additional hours and thymidine incorporation was measured as described above. Results are also expressed as a S.I.

IFN-γ, IL-4 and IL-6 secretion was measured by sandwich ELISA using antibody pairs and peroxidase-conjugated streptavidin from PharMingen and according to Manufacturer's recommendations. We used the Supersensitive TMB (Sigma, Deisenhofen, Germany) as a substrate and the reaction was stopped using a 2 M H_2SO_4 solution. OD_{450} was then measured and results expressed in pg/ml according to the standards.

Recognition of cyclin D1-transfected cells by the peptide-specific T-helper cell line

Absence of detectable alloreaction of T cells against the transfectants was demonstrated by co-culturing fresh PBMC from the donor used to generate the T-helper line in the presence of different cell numbers of irradiated transfectants. Cell proliferation as well as IL-2, IL-4, IL-6 and IFN-γ secretion was measured as described above and the Effector/Target ratio to be used in further experiments was thus determined.

Recognition of the naturally processed peptide derived from cyclin D1 was tested by co-culturing the peptide-specific T-helper cell line ($2x10^5$ cells) in the presence of irradiated Awells ($4x10^4$ cells) transfected with a plasmid coding for either cyclin

D1 or keratin 18 as a negative control and according to the cell ratio determined above. Irradiated autologous PBMC in the presence of 10 µM peptide or 3 µg/ml PHA served as positive controls. Cell proliferation as well as IL-2 and IFN-γ secretion was measured as described above. In some experiments, cells were cultured in the presence of 20 µg/ml of purified L243 antibody.

2.3.4 Results

Expression and endosomal/lysosomal targeting of fusion proteins by cell clones

We cloned the cDNA encoding the 80 N-terminal amino acids of Ii in the vector pcDNA3 in such a way that the 3` end of the insert was followed by a general cloning site (GCS). This gave us a versatile vector to express fusion proteins of Ii and the genes of interest. In frame with Ii we cloned the cDNA of cyclin D1, as well as keratin 18 as a control.

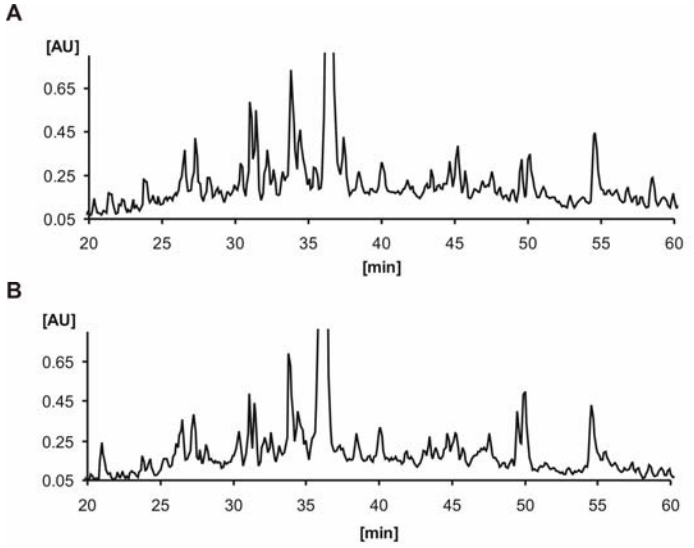

Figure 1: HPLC chromatograms of HLA-DR peptides purified using the mAb L243 and recorded at 214 nm. (A) shows the HLA-DR peptides purified from approximately 5×10^{10} Awells cells and (B) the HLA-DR peptides purified from approximately 3.5×10^{10} Awells-Ii-cyclin D1 cells.

The Awells cell line was stably transfected with vectors encoding the two fusion proteins using electroporation. Subsequently, single-cell-clones were generated and tested on their antigen expression on mRNA and protein level. Compared to the wildtype, the best Ii-keratin 18 clone expressed 5,700 times more keratin 18 and the best Ii-cyclin D1 clone expressed 1,200 times more cyclin D1 (data not shown), as determined by real-time quantitative PCR analysis. The data were normalized on 18S ribosomal RNA.

The Ii-fusion proteins were also detected by Western blot analysis, to test whether the constructs were targeted into the MHC class II pathway [24;25;31]. The bands, with the expected molecular weights, representing the Ii-keratin 18 and Ii-cyclin D1 fusion proteins, respectively, got more and more intense with increasing amounts of chloroquin, a cytotoxic drug which inhibits lysosomal degradation of proteins by raising the lysosomal pH [36-38] (data not shown). This indicated that fusion protein amounts increase with chloroquin concentrations and thus proved that the fusion proteins follow the MHC class II pathway of protein degradation.

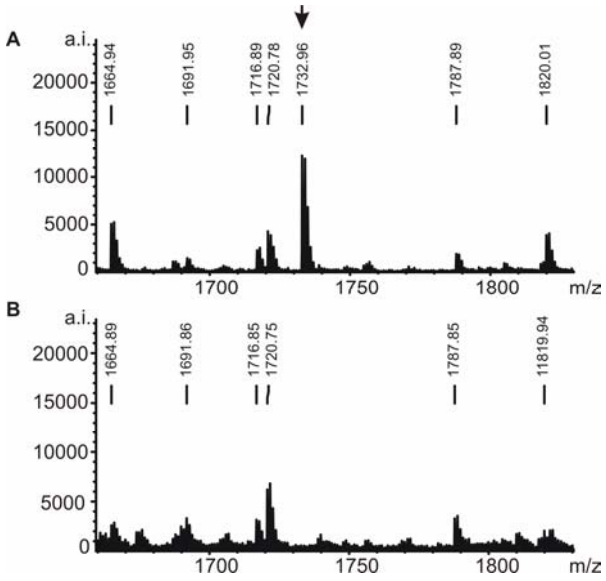

Figure 2: MALDI MS spectra of the HPLC fractions 44 (54 min) of purified HLA-DR peptides from (A) Awells-Ii-keratin 18 and (B) Awells. The peak at 1732.96 m/z in (A) represents the only significant differentially expressed HLA-DR ligand of Ii-KRT18.

The clones were also tested for their HLA class I and class II cell surface expression levels by flow cytometry, in order to determine whether the transfection

and cloning procedure interfered with it. Both clones showed normal expression levels of HLA class I and class II molecules, as compared to the untransfected cell line (data not shown).

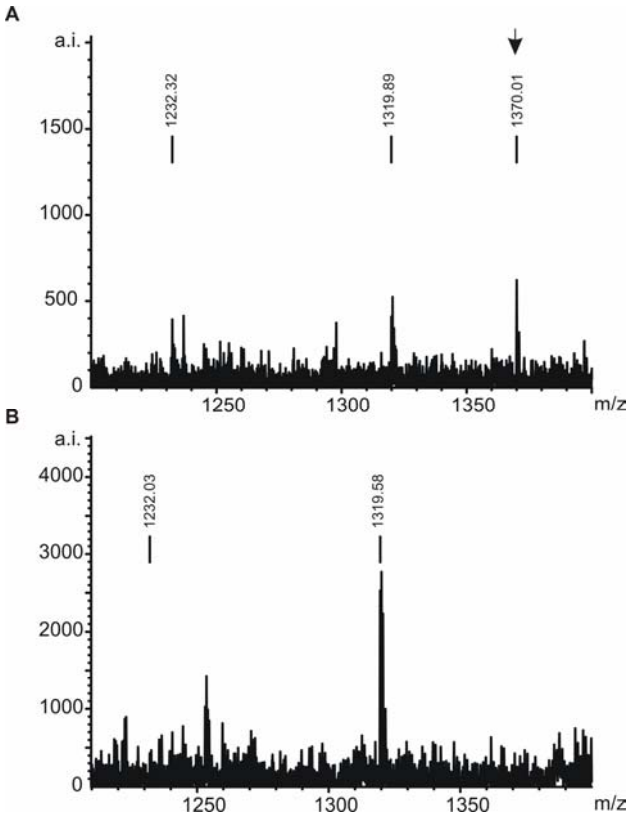

Figure 3: MALDI MS spectra of the HPLC fractions 26 (36 min) of purified HLA-DR peptides from (A) Awells-Ii-cyclin D1 and (B) Awells. The peak at 1370.01 m/z in (A) is differentially expressed.

Differential mass spectrometric analysis of HLA-DR-bound peptides

3.5 to 5×10^{10} cells from each clone and from the untransfected cell line were grown and the HLA-DR-bound peptides isolated and separated via HPLC as described previously [34]. The chromatograms of the untransfected cell line and the Awells-Ii-cyclin D1 clone were compared. Minor, mostly quantitative differences in the HLA-DR-presented peptide repertoire summed up to slightly different UV traces as shown in Figure 1. As expected from our experience, no distinct UV signals could be assigned to peptides exclusively presented by the transfectants. The only subtle differences in HLA-DR-restricted peptide presentation between Awells and the transfected lines became also visible by MALDI-TOF analysis where most of the HPLC fractions contained identical

patterns. Figure 2 shows the only fraction with a striking individual signal only occurring in the peptide mixture eluted from the keratin 18-transfected clone. In Figure 3, the m/z signal at 1370.1 indicates an exclusively presented peptide from the cyclin D1-transfectant. Both peptides were analysed in greater detail by nanoflow ESI MS/MS. We were able to identify the peptides NPPSMVAAGSVVAAV (cyclin $D1_{198-212}$) (Figure 4) and SHYFKIIEDLRAQI (keratin $18_{126-139}$, data not shown) derived from the two transfected fusion proteins, respectively. The sequences were verified by mass spectra of the corresponding synthetic peptides (data not shown).

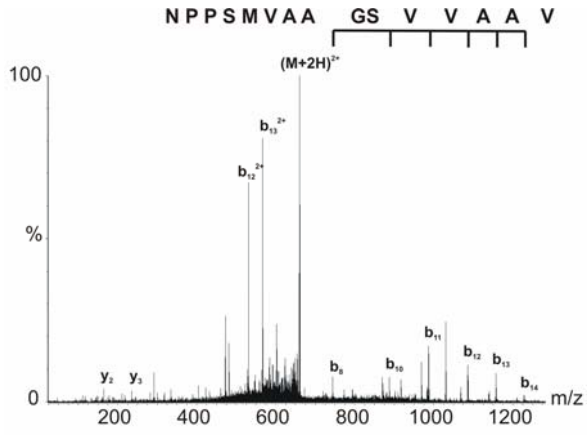

Figure 4: MS/MS spectrum of the HLA-DR ligand NPPSMVAAGSVVAAV from Ii-CCND1. This peptide is equivalent to the peptide 198-212 of Cyclin D1.

Generation and characterization of a peptide-specific T-helper cell line

T cells specific for the identified cyclin D1 peptide were induced by in vitro stimulation with the corresponding synthetic peptide loaded onto $DR4^+$ dendritic cells. After the third and the fifth stimulation, respectively, the specificity of the T-helper cell line was tested. T cells were specifically stimulated by the cyclin D1 peptide, as shown in Figure 5A. T cells proliferated in response to autologous PBMC loaded with the cyclin D1 peptide (S.I. = 5.4). As a positive control, PHA induced a strong T-cell proliferation (S.I. = 330). We next analyzed which type of T-helper cells (Th1 versus Th2) was stimulated in response to the peptide by examining the cytokine profile. As shown in Figure 5B, the T cells produced IL-2 in response to the peptide, although to a low extent, whereas they were still sensitive to PHA stimulation. On the contrary, peptide-induced T-cell stimulation resulted in

a strong IFN-γ secretion (3250 pg/ml, Figure 5C) but no IL-4 or IL-6 secretion, although T-cells were still highly and moderately sensitive to PHA-induced cytokine secretion, respectively. Stimulation with an unrelated peptide as a negative control for unspecific activation is presented in Figure 6. In conclusion, the T-helper cell line is specific for the cyclin D1 peptide and is of the Th1 type. This type is particularly important in helping specific CTLs for tumor cell elimination.

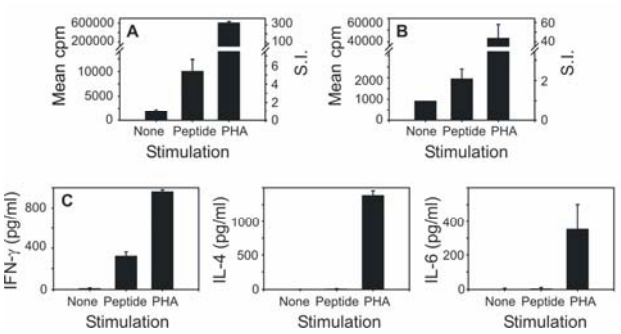

Figure 5: Characterization of the T-helper cell line. The specificity of the T-cell line was tested after the fifth restimulation in the presence of irradiated autologous PBMC and 10 µM cyclin D1 peptide. Unstimulated T cells were used as a negative control whereas 3 µg/ml PHA was used as a positive control. **A.** T-cell proliferation determined by incorporation of tritiated thymidine. Results are expressed as mean cpm of triplicates (left axis) or stimulation index (S.I., right axis). S.I. corresponds to the (stimulated T-cell cpm)/(unstimulated T-cell cpm) ratios. Standard deviations are included. **B.** IL-2 secretion was measured using the CTLL-2 cell line, the proliferation of which was determined as in A. **C.** IFN-γ, as well as IL-4 and IL-6 secretion were measured by ELISA. Results are expressed in pg/ml and standard deviations are included.

The peptide-specific T-helper cell line recognizes cyclin D1-transfected cells

Because Awells cells and the T-cell line are not perfectly HLA-matched, we first tested whether any alloreaction could arise by co-culturing both. Briefly, different cell numbers of irradiated Awells-Ii-keratin 18 or Awells-Ii-cyclin D1 transfectants were co-cultured in the presence of a fixed number of PBMC from the T cell donor, and cell proliferation as well as IL-2, IL-4, IL-6 and IFN-γ secretion were measured.

A moderate T-cell proliferation was induced by both transfectants at high cell numbers but no cytokine secretion was observed (data not shown). We thus decided to use an (effector T cells)/(target cells) ratio of 5/1, at which only a slight T-cell proliferation was observed in the absence of cytokine secretion. As a consequence, T-cell activation resulting in cytokine secretion could only be induced specifically by the cognate antigen presented by the transfectant.

The T-helper cell line specific for the cyclin D1 peptide was able to recognize the transfected cells over-expressing the cyclin D1 protein and naturally processing and presenting the cyclin D1 peptide in association with HLA-DR molecules. As shown in Figure 6, irradiated Awells-Ii-cyclin D1 tranfectants were able to specifically activate the T-helper cell line as observed by IL-2 secretion (S.I. = 4.0). On the contrary, Awells-Ii-keratin 18 transfectants (used as negative control for T-cell stimulation and known to present the unrelated keratin 18 peptide$_{126\text{-}139}$ in association with HLA-DR) did not induce T-cell activation, indicating that the peptide-specific T-helper cell line specifically recognizes the cognate antigen. Moreover, these results prove that the cyclin D1 peptide used in this study is a naturally processed peptide containing a T-cell epitope. This activation could be inhibited (by 71.2%) by the presence of the HLA-DR-specific blocking L243 Ab (data not shown).

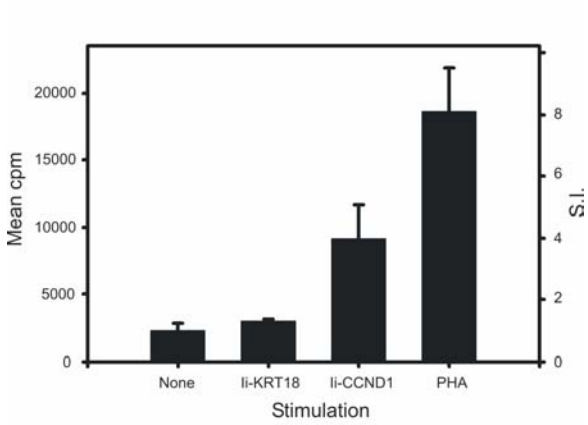

Figure 6: The T-cell line specifically recognizes the Ii-cyclin D1 transfected cells. T cells were cultured alone or in the presence of irradiated Ii-keratin 18-transfectants (KRT18), Ii-cyclin D1 transfectants (CCND1) or in the presence of PHA. IL-2 secretion was measured using the CTLL-2 cell line, the proliferation of which was determined as in Figure 7. Mean cpm as well as stimulation index (S.I.) are shown. Standard deviations are indicated.

2.3.5 Discussion

The identification of T-helper cell epitopes of tumor associated antigens remains an important task in anti-tumor immunotherapy. Here we report a new and generally applicable method based on differential peptide analysis by MS to identify naturally processed and presented MHC class II ligands of tumor associated antigens. This approach combines for the first time a transfection step of APC with a vector encoding for a fusion protein between the Ii chain and the Ag of interest, elution of the HLA-bound peptides and MS identification of the Ag-derived peptides presented by the transfectant by comparison to the non-transfected cells. Moreover, we could validate the method by showing that T cells induced against the identified peptide specifically recognize transfectants overexpressing the cognate Ag. Although the identified peptides still have to be tested for their immunogenicity *in vivo*, our approach leads to the exact characterization of naturally processed MHC class II ligands. Thus we avoid testing either synthetic overlapping peptides of tumor associated antigens, or a broad range of peptides selected by epitope prediction, which is less accurate as compared to class I epitope prediction. In contrast to laborious T-cell assays, which might lead to the identification of cryptic T-cell epitopes unable to induce T-cell activation *in vivo* [39], the work can be focused on the few peptides which are found to be presented. Moreover, using this method it is not necessary to produce the recombinant Ag or to possess Ag-expressing tumor cell lines in order to prove that the peptides are naturally processed.

We used the N-terminus of Ii to direct tumor associated antigens into the class II processing compartment of EBV-transformed B cells. In order to achieve this we constructed a versatile vector with which we can express any antigen as a fusion protein with Ii and which helps us to determine the expression level of the protein in transfected cells by Western blot analysis. It has already been shown that the N-terminus of Ii is sufficient to target proteins into the class II processing compartment. But until now this has only been described in a model using ovalbumin [24], in order to identify unknown Ag using fusion protein-encoding cDNA libraries [25] or to confirm the specificity of known T-cell clones [2]. To our knowledge this method has never been used before to identify naturally presented MHC class II bound peptides of known tumor associated antigens. The differential

analysis of class II ligands of transfected and non transfected cells by MALDI-MS and the further characterization of the differentially expressed peptides by ESI-MS results in a straightforward method for identifying class II ligands of antigens of interest. Transfection of cells with keratin 18 fusion proteins proved that our method is generally applicable for antigens of interest: We were also able to describe an HLA-DR-presented peptide of keratin 18.

We used our approach to identify an HLA-DR4-presented cyclin D1 peptide. We cannot exclude, however, that NPPSMVAAGSVVAAV is DRB4*0101-restricted, since this “second DR” is in linkage disequilibrium with DRB1*0401and shares a similar peptide motif. We were particularly interested in cyclin D1, because it has been described as a proto-oncogen [26] and because we found it overexpressed in renal cell carcinomas [21]. Thus, peptides from this antigen are possible candidates in vaccination studies using peptide based immunotherapies [21].

We think our new method will be helpful to identify new peptide candidates from tumor antigens to be used in clinical vaccination protocols.

Acknowledgments

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3 Summary

The aim of this thesis was to establish a better knowledge of the human MHC class II peptide repertoire, the HLA ligandome, in general, and to outline a procedure which helps in the identification of class II-presented peptides from tumor associated antigens, in particular. To achieve these goals, biochemical and biomolecular methods as well as state-of-the-art mass spectrometric devices were used.

To characterize the class II ligandome, HLA-DR peptides from the tumor-like cell line Awells, a human EBV transformed B lymphoblastoid cell line homozygous for HLA-DR4 – the HLA of interest –, were isolated and analyzed by MS using the rules of proteome analysis. 404 peptides with 173 different core sequences could be identified – the highest number of HLA ligands identified in a single experiment so far. Peptides from source proteins localized in virtually every cell compartment and participating in general cellular processes were presented under normal conditions on HLA class II molecules on the cell surface. In further experiments it could be shown that autophagy, a process involved in endosomal/lysosomal degradation and playing a role in tumor development, had a substantial impact on the class II ligandome. Cells undergoing autophagy over-presented class II peptides from intracellular source proteins by up to 131% in average as quantified by LC-MS. Thus, intracellular source proteins reach via autophagy the endosomal/lysosomal system and are there processed, corresponding peptides are loaded on class II molecules and presented on the cell surface.

Posttranslationally modified naturally presented class II ligands could also be identified. Deamidated, cysteinylated and glycosylated HLA-DR peptides were characterized showing for the first time that naturally presented class II peptides can carry complex N-linked glycans.

Finally, a strategy for the identification of naturally presented class II ligands from tumor associated antigens was set up. Fusion proteins targeting antigens of interest into the class II processing compartment were expressed in cells and the corresponding HLA-DR peptides isolated. By a differential mass spectrometric approach an HLA-DR4 ligand from cyclin D1 containing a $CD4^+$ T cell epitope could be identified.

Zusammenfassung

Ziel dieser Arbeit war es ein besseres Verständnis des HLA Klasse II Peptid-repertoires, dem so genannten Klasse II Ligandom, herzustellen. Insbesondere sollte eine Methode entwickelt werden, die die Charakterisierung von HLA Klasse II-Liganden Tumor-assoziierter Antigene ermöglicht. Um diese Vorgaben zu erfüllen, wurden neben molekularbiologischen und biochemischen Methoden moderne massenspektrometrische Technologien eingesetzt.

Zur Charakterisierung des Klasse II-Ligandoms wurden HLA-DR-Liganden von der Tumor-Zelllinie Awells, einer EBV-transformierten humanen B-lymphoblastoiden Zelllinie, die homozygot für HLA-DR4 ist, isoliert. Es konnten 404 unterschiedliche Peptide mit 173 Kernsequenzen, die bisher höchste Anzahl an identifizierten HLA-Liganden in einem einzigen Experiment, beschrieben werden. Eine Proteom-Analyse ergab, dass Peptide von Quellproteinen aus nahezu allen subzellulären Kompartimenten auf HLA-DR präsentiert werden. Des Weiteren nehmen die Quellproteine an generellen zellulären Mechanismen teil. In weiteren Experimenten konnte gezeigt werden, dass Autophagie, eine spezielle Form des endosomalen/lysosomalen Abbauweges, unter anderem involviert in der Tumorentwicklung, einen großen Einfluss auf das Klasse II-Peptidrepertoire hat. Autophagische Zellen überpräsentierten Peptide aus intrazellulären Quellproteinen durchschnittlich um 131%. Dies konnte mittels LC-MS gezeigt werden. Über Autophagie werden intrazelluläre Quellproteine in das endosomale/lysosomale System geschleust und dort abgebaut. Entsprechende Peptide werden dann auf Klasse II-Molekülen auf der Zelloberfläche präsentiert.

Zusätzlich konnten auch posttranslational modifizierte Peptide identifiziert werden. So wurden deamidierte, cysteinylierte und glycosylierte Peptide charakterisiert. Unter anderem gelang es zum ersten Mal die Struktur eines natürlich präsentierten Klasse II-Peptids, modifiziert mit einem N-gebundenen Hexasaccharid, aufzuklären.

Zur Identifizierung Klasse II-präsentierter Peptide aus Tumor-assoziierten Antigenen wurden in Zellen Fusionsproteine exprimiert, die Tumor-assoziierte Antigene in den Klasse II-Prozessierungsweg leiten, um anschließend HLA-DR Liganden der entsprechenden Antigene zu isolieren. Mit Hilfe einer differenziellen massenspektrometrischen Analyse konnte so ein HLA-DR4-Ligand aus Cyclin D1, der ein T-Helferepitop enthält, identifiziert werden.

4 Abbreviations

APC	Antigen presenting cell
Aa	Amino acid
β_2m	Beta-2-microglobulin
BCR	B cell receptor
BPI	Base peak ion
CD	Cluster of differentiation
CID	Collision induced decay
CLIP	class II invariant chain peptide
CTL	Cytotoxic T lymphocyte
EBV	Epstein-Barr-Virus
ER	Endoplasmic reticulum
ESI	Electrospray ionization
EST	Expressed sequence tag
FACS	Fluorescence activated cell sorter
HLA	Human leukocyte antigen
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain (CD74)
IL	Interleukin
mAb	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MHC	Major histocompatibility complex
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NK-cell	Natural killer cell
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	Potentia hydrogenii
RT	Room temperature
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
SDS	Sodium Dodecyl Sulfate
TAP	Transporter Associated with Antigen Processing
TCR	T cell receptor
TFA	Trifluoroacetic Acid
TIC	Total Ion Current
TIL	Tumor Infiltrating Lymphocyte
TOF	Time of Flight

One letter code for amino acids:

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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6 Academic Teachers

Prof. Baumann, Prof. Bisswanger, Prof. Bock, Prof. Bohley, Dr. Friedrich, Prof. Fröhlich, Prof. Fürst, Prof. Hamprecht, Prof. Hanack, Dr. Hudl, Prof. Jung, Prof. Kempter, Prof. Kleinpeter, Dr. Lehnhardt, Prof. Martin, Prof. Mecke, Prof. Nitschmann, Prof. Poralla, Prof. Probst, Prof. Rammensee, Prof. Reutter, Prof. Scheel, Prof. Scheller, Prof. Schild, Dr. Schmidt, Prof. Steup, PD Dr. Stevanović, PD Dr. Stoeva, Prof. Uhlemann, Prof. Voelter, Prof. Wallschläger, Prof. Walz, Prof. Weser, Prof. Willmitzer, Prof. Wohlleben

7 Publications

Dengjel,J., Rammensee,H.G., and Stevanovic,S., Glycan side chains on naturally presented MHC class II ligands. J.Mass Spectrom. *in press*

Dengjel,J., Decker,P., Schoor,O., Altenberend,F., Weinschenk,T., Rammensee,H.G., and Stevanovic,S., Identification of a naturally processed cyclin D1 T-helper epitope by a novel combination of HLA class II targeting and differential mass spectrometry. *Eur.J.Immunol.* 2004, 34: 3644-51

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Krüger,T., Schoor,O., Lemmel,C., Krämer,B., Reichle,C., **Dengjel,J.**, Weinschenk,T., Müller,M., Hennenlotter,J., Stenzl,A., Rammensee,H.G., Stevanovic,S. Lessons to be learned from primary renal cell carcinomas: Novel tumor antigens and HLA ligands for immunotherapy. Cancer Immunol. Immunother. *in press*

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