Endothelial cells as targets for antigen-specific cytotoxic T lymphocytes

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I. Abstract

BACKGROUND: In this study we have examined human vascular endothelial cells as targets for antigen-specific cytotoxic T lymphocytes (CTL). Vascular endothelial cells (EC) act as the major interface between blood and tissues and are crucial for the maintenance of vascular integrity. It has been shown previously that EC are poor targets for immunodominant antigen-specific CTL. This feature was due to the impaired capacity of EC to present the cognate antigenic peptides to CTL. These findings fueled the hypothesis that EC present a substantially different repertoire of MHC class I ligands compared with syngeneic leukocyte derived cells and that they might be protected from CTL-mediated lysis by this mechanism. It was the object of this thesis to confirm or rule out this hypothesis.

METHODS: Our study consisted of three parts. First, the peptide repertoire from endothelial cells was compared with syngeneic leukocyte-derived cells. Cell type-specific HLA-A*02 restricted peptides were identified and characterized for several biochemical features like expression of source proteins, binding properties and decay that would explain preferential presentation by either one of the two cells. Next, a method for the induction of self-reactive CTL was established by using peptide-pulsed dendritic cells as antigen-presenting cells and the generation of CTL specific for the HLA-A*02 restricted endothelial self-peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ was attempted. In the third part, we modified the endothelial peptide repertoire and interfered with the peptide presentation pathway to test the hypothesis that abundant endothelial peptides indeed compete with immunodominant antigens for presentation on the surface of EC. The surface antigenic profile was altered by two approaches: a) by transfection of influenza virus A matrix protein 1 gene to make EC recognizable for $Flu_{(58-66)}$-specific CTL and b) by siRNA knockdown of the abundant EC-specific peptides.

RESULTS: We show for the first time that EC present a quantitatively different peptide repertoire with abundance of certain peptides, compared with leukocytes. The abundance of endothelial peptides is mainly caused by the preferential expression of the source proteins. This feature is immunologically interesting since it contributes twofold protection of EC from CTL-mediated lysis: a) by an extraordinary strong tolerance that exists against EC peptides and b) by competing with immunodominant peptides for the MHC class I binding site. Under conditions that were sufficient to generate CTL specific for certain immunodominant low abundant self-peptides induction of CTL against $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ failed. Improvement of culture conditions by stabilization of the pMHC I complex or interference with inhibitory pathways affected again only the induction of CTL against low abundant self-peptides but not against EC-specific peptides indicating that these peptides are particularly

tolerogenic. After modification of the endothelial peptide repertoire and knockdown of abundant EC peptides, EC became better targets for immunodominant CTL by increased presentation of endogenously processed peptides indicating that the selective presentation of $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ can protect EC by hiding them from CTL-mediated lysis.

CONCLUSION: Our in vitro findings are in accordance with the view that EC are protected from CTL-mediated lysis by presentation of a quantitatively different peptide repertoire. This protection results from peptides that compete with immunodominant peptides for the MHC class I binding site and, in addition, evoke extraordinary strong tolerance.

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IV. List of abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
APC	Allophycocyanin
BLC	B lymphoblastoid cells
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CC	Colon carcinoma cells
cDNA	Complementary desoxyribonucleic acid
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocytes
CTLA-4	CTL-associated antigen 4
DC	Dendritic cells
DDX5	DEAD box polypeptide 5
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent Assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisocyanate
FluM1	Influenza A virus matrix protein 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase

HUVEC	Human umbilical vein endothelial cells
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IL-2	Interleukin-2
LCMV	Lymphocytic choriomeningitis virus
LFA-3	Lymphocyte function-associated antigen-3
LPS	Lipopolysaccharide
MFI	Mean fluorescence index
mHA	Minor histocompatibility antigen
MHC	Major histocompatibility complex
MR	Maximal release
MS	Multiple sclerosis
NK	Natural killer cells
NKR	Natural killer receptors
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1/2	Programmed death ligand-1/2
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
pMHC	Peptide-MHC class I complex
PP1A	Protein phosphatase 1 alpha
PTRF	Polymerase I and transcript-release factor
RNA	Ribonucleic acid
RT	Room temperature
siRNA	Small interfering ribonucleic acid
SLE	Systemic lupus erythematosus
SR	Spontaneous release
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule-1
vWF	Von Willebrand factor

1. Introduction

1.1 Autoimmune diseases - with particular emphasis on $CD8^+$ T cells

Self-reactive lymphocytes always exist in the natural immune repertoire but are not often activated. In autoimmune disease, however, the loss of self-tolerance and expansion of those cells after activation by specific autoantigens lead to tissue injury. From a clinical perspective it is useful to distinguish between the following two major patterns of autoimmune disease: the diseases in which the expression of autoimmunity is restricted to specific organs of the body (organ-specific autoimmune disease); and those in which many tissues of the body are affected (systemic autoimmune disease) (Table 1). Systemic autoimmune diseases affect multiple organs and have a tendency to become chronic, because the autoantigens can normally never be eliminated from the body. Organ-specific autoimmune diseases include type 1 diabetes mellitus, multiple sclerosis (MS), myasthenia gravis, Grave's disease and Hashimoto's thyroiditis. In each case, autoantigens from one or a few organs only are targeted, and disease is therefore limited to those particular organs: insulin-producing β cells of the pancreas (diabetes), the myelin sheathing axons in the central nervous system (MS), and the thyroid-stimulating hormone receptor (Grave's disease). In contrast, systemic diseases like systemic lupus erythematosus (SLE) or scleroderma cause inflammation in multiple tissues because their autoantigens, which include chromatin and ribonucleoproteins, are found in every cell of the body. An overview of organ-specific and systemic autoimmune disease is shown in Table 1.

Some autoimmune diseases seem to be dominated by the pathogenic effects of a particular immune effector pathway, either autoantibodies or activated autoreactive T cells. However, both of these pathways often contribute to the overall pathogenesis of autoimmune disease. The damage by autoantibodies is mediated through the complement and Fc receptor systems. Systemic lupus erythematosus (SLE) is an immune-mediated, multi-system disease characterized by pathogenic autoantibodies against nuclear antigens [1]. T cells contribute to autoimmune disease in two ways: by helping B cells to make autoantibodies, in an analogous manner to a normal T-dependent immune response, and by direct effector functions of T cells as they infiltrate and destroy target tissues such as skin, renal interstitium and vessels as would virus-infected cells.

The importance of $CD8^+$ T cells in the pathogenesis of organ-specific human autoimmune diseases has previously not been well recognized. There are several reasons for the neglect of the role of $CD8^+$ T cells in autoimmunity. First, it is much more difficult to demonstrate the

existence of autoreactive T cells than it is to demonstrate the presence of autoantibodies. In animal models, the disease can be transferred by injecting the active component of the immune response from one animal into another to obtain the most convincing proof that the immune response is causal in autoimmunity. Autoreactive human T cells cannot be used to transfer disease to experimental animals because T cell recognition is MHC-restricted. Second, it is difficult to detect the antigen recognized by a T cell. The identification of autoantigens is particularly difficult in autoimmune diseases in which $CD8^+$ T cells have a role, because autoantigens recognized by $CD8^+$ T cells are not effectively presented by MHC class I molecules. Third, there is a lack of measures to detect autoreactive T cells as the relevant disease-associated populations act in the tissue lesion, and circulate only at very low precursor frequencies in the peripheral blood.

Several experimental studies described the importance of autoreactive $CD8^+$ effector T cells in experimental autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, autoimmune thyroiditis, and multiple sclerosis. Autoreactive $CD8^+$ T cells can be responsible for the selective β cell destruction that is the hallmark of clinical type 1 diabetes. They are involved in the initiation of insulitis and in the destructive stage leading to insulin-dependent diabetes mellitus [2]. $CD8^+$ T cells are dominant among islet-infiltrating lymphocytes and are required for efficient diabetes induction upon adoptive lymphocyte transfer in the model of spontaneously diabetic NOD mice. CTL are involved both in initiation of β cell-directed autoimmunity and in ultimate destruction of β cells preceding manifest disease. Multiple sclerosis (MS) is a chronic human disease caused by inflammatory cell-induced demyelination in the central nervous system. Infiltrating $CD8^+$ T cells predominate over $CD4^+$ T cells, especially in regions of active demyelination, and these $CD8^+$ T cells appear to undergo local clonal expansion as assessed by analysis of TCR gene rearrangements at the single-cell level [3]. Autoreactive $CD8^+$ T cells responsive to myelin-derived peptides have been reported in MS patients that have the potential to kill HLA class I-matched oligodendrocytes *in vitro*, independent of exogenous peptide. Experimental autoimmune encephalomyelitis (EAE) is an experimental model for MS, induced in susceptible animals by immunization with myelin antigens. Although autoreactive $CD4^+$ T cells are implicated as major effectors of EAE, there is evidence pointing to a role for $CD8^+$ T cells in disease progression and severity. For example, a myelin oligodendrocyte glycoprotein-derived peptide (MOG) has recently been shown to elicit encephalitogenic $CD8^+$ T cells *in vivo* [4]. In addition, myelin basic protein (MBP) is processed and presented *in vivo* by the MHC class I pathway, and, in fact, responding $CD8^+$ T cell clones induce a CNS immunopathology in mice that resembles some forms of MS, implicating $CD8^+$ T cells as potential effectors of demyelination in MS [5]. All these findings, however, indicate that autoreactive $CD8^+$ T cells can contribute substantially to tissue damage in both murine and human autoimmune

disorders. As such, these T cells now become an attractive target for therapeutic intervention.

Table 1. Classification of autoimmune diseases.

Disease	Effectors	Antigens	Experimental model	References
Organ-specific autoimmune diseases				
Type 1 diabetes mellitus	Autoreactive T cells (Autoantibodies)	Insulin β-cell antigens	NOD (non-obese diabetic) mouse	[2, 6]
Goodpasture's Syndrome	Autoantibodies	Collagen Type IV	Fcγ receptor IIB– deficient mouse	[7]
Multiple sclerosis	Autoreactive T cells T helper cells Autoantibodies?	Myelin basic protein (MBP) Myelin oligodendrocyte glycoprotein (MOG)	Murine experimental autoimmune encephalomyelitis (EAE)	[3-5]
Grave's Disease	Autoantibodies	TSH receptor	Thyrotropin receptor-induced mouse	[8]
Hashimoto thyroiditis	Autoantibodies Autoreactive T cells	Thyroid peroxidase	Murine experimental autoimmune thyroiditis (EAT)	[9]
Myasthenia gravis	Autoantibodies	Acetylcholine receptor	Murine experimental autoimmune myasthenia gravis (EAMG)	[10]
Autoimmune hemolytic anemia	Autoantibodies	Rhesus antigens	Murine induced autoimmune hemolytic anemia (AIHA)	[11]
Systemic autoimmune diseases				
Scleroderma	Autoantibodies	Centrosome proteins, ribonucleoprotein antigens	Tight skin (TSK) mouse	[12]
Giant cell arteritis	Antigen-specific T cells Macrophages (Giant cells)	Viral antigens?	GCA (Giant cell arteritis)-SCID model	[13]

Table 1. Classification of autoimmune diseases (continued).

Disease	Effectors	Antigens	Experimental model	References
Polyarteritis nodosa	Autoantibodies Antigen-specific T cells	Antineutrophil cytoplasmic antibodies (ANCA) Antiendothelial cell antibodies (AECA)	SCG/Kj mouse	[14, 15]
Systemic lupus erythematosus	Autoantibodies Autoreactive T cells	DNA, chromatin proteins, ribonucleoprotein antigens	(NZBxNZW)F1,MRL*/lpr* mouse	[16-18]
Rheumatoid arthritis	Autoreactive T cells Autoantibodies	Antigens of joint synovium	Murine collagen-induced arthritis (CIA) in DBA/1	[19]
Sjögren's Syndrome	Autoantibodies Autoreactive T cells	Ribonucleoprotein antigens	MRL/MpJ-*lpr*/*lpr* mouse	[20]

1.2 Normal T cell activation, tolerance and autoimmunity

Pro-T lymphocytes arise from stem cells in the bone marrow and migrate to the thymus for maturation. The thymus is the primary lymphoid organ involved in T cell generation. There, mature T cells are selected from a pool of immature $CD4^+CD8^+$ double positive thymocytes dependent on how their T cell receptors (TCR) interact with self-peptide–MHC (self-pMHC) ligands [21-25]. The TCR is the highly variable antigen receptor of $CD8^+$ or $CD4^+$ T cells that recognizes antigenic peptide bound to major histocompatibility complex (MHC) class I or II molecules, respectively, on target cells [26]. The degree of inflammation, precursor frequency, antigenic signal strength and duration determines the efficiency of pre-T cell differentiation [27-31]. Thymocytes expressing TCRs that fail to recognize any self-pMHC ligand presented by thymic dendritic cells die by 'neglect', while weak recognition of self-pMHC complexes by the TCR results in the development of mature, single positive T cells (positive selection). Strong recognition of self-pMHC leads to thymocyte death or lineage deviation and removal of self-reactive cells from the T cell repertoire (negative selection) [32, 33]. Therefore, the peripheral T cell repertoire is both self-pMHC restricted and self-tolerant.

However, central tolerance is incomplete, and autoreactive T cells can escape negative selection [34]. In addition, some self-antigens do not access the thymus [35] or are not expressed in the thymus at levels required for efficient negative selection, while others may be expressed later in life, after the T cell repertoire has formed. Therefore, central tolerance needs to be buttressed by peripheral mechanisms [36, 37].

After leaving the thymus, each mature naïve T cell possesses a unique TCR specific for one antigen and a single coreceptor, either CD4 or CD8 for forming the interaction with MHC class II or I, respectively. T cells are then homing to the secondary lymphoid organs like lymph nodes, spleen or mucosa-associated lymphoid tissue (MALT) to encounter an antigen-presenting cell (APC) bearing the cognate antigen leading to activation and proliferation of the T cells into effector cells. For the activation of naïve $CD8^+$ T cells into cytotoxic effector T cells several concomitant signals are required. The first signal results from the interaction of the TCR with the peptide-MHC class I complex as described above. This interaction is further stabilized by the CD8 co-receptor. A second signal is delivered through costimulatory ligands that are highly expressed on activated APC as CD80 and CD86 that bind to the CD28 receptor. As a third signal for the induction of a CTL response, cytokines like IL-2 are critical to support T cell survival and differentiation [38, 39]. Generally, cytokines are not only produced by activated $CD8^+$ T cells but particularly effectively by T helper cells or other leukocytes.

Peripheral tolerance can be achieved by multiple means and can be classified into three main categories: ignorance, death by deletion and anergy (functional unresponsiveness). The most likely candidates for ignorance are low avidity self-antigens, which fail to initiate negative selection. For normal peripheral T cell activation a higher TCR/pMHC affinity is required than for what results in thymic deletion [40]. These antigens, therefore, might never induce peripheral immune activation, and the population of self-antigen specific naïve T cells could potentially remain untouched. Another possibility of achieving peripheral ignorance is when antigens are restricted to immune privileged sites, such as across the blood–brain barrier or across the fetal–maternal barrier [40]. However, this form of tolerance is not permanent, as there are situations where the antigen could be presented under the right costimulatory conditions lowering the activation threshold. This might result in fatal immune responses [41]. Both anergy and activation induced clonal deletion are effects of incomplete activation of T cells in the absence of costimulatory signals. Deletion of autoreactive T cells is the most permanent and irrevocable form of peripheral tolerance. The factors that influence the decision for one of the two cell fates are not completely understood. Antigenic persistence has been found to be an important variable in determination of either deletion or survival [42]. When relatively small numbers of T cells are confronted with an excess of antigen or the antigen is present in the system for a long time, the CTL disappear. This has

first been shown in the context of infection with non-cytopathic viruses [43] but was also found to be induced by retroviral superantigens, by the injection of peptides in varying doses without or with adjuvant or of cells expressing a defined foreign antigen [44]. The details are poorly understood, but cell death by interleukin-deprivation and other mechanisms associated with apoptosis seem to be involved [45, 46]. A third mechanism of peripheral tolerance in naïve $CD8^+$ T cells is the induction of anergy. Anergy is a form of functional unresponsiveness, believed to occur when a T cell has been subjected to TCR/pMHC interaction in the absence of costimulation. On the basis of the *in vitro* anergy model systems it has been shown that the molecular mechanisms involved in anergy induction comprise interference in TCR signaling by downstream signaling molecules. Thus, excessive calcium/NFAT signaling results in transcriptional upregulation of negative regulatory proteins that inhibit correct TCR/CD28 signaling [47, 48]. An anergic T cell is rendered refractory to further stimulation, even in the presence of full costimulation [49]. The hallmarks of anergic cells are defects in proliferation and IL-2 production, but other effector functions show variable (or sometimes no) reduction [50]. Autoimmunity arises when these strategies fail and the lymphocytes are activated to mistakenly target 'self' (i.e. normal tissues).

Cytotoxic T lymphocytes (CTL) are one of the antigen-specific receptor arms of the adaptive immune system [51]. They were originally discovered as mediators of solid organ transplant rejection and graft-versus-host disease (GVHD) as prototypes of T cell-mediated immune diseases resulting from pMHC mismatch, but are physiologically important for immune defense against non-cytopathic intracellular microorganisms (e.g. EBV or CMV) and tumors [52]. Tissue injury by effector CTL critically depends on the binding avidity of the TCR for an antigen and the amount of antigen presented by target cells [51, 53]. Effector CTL kill antigen-bearing cells within minutes to hours by at least three different effector pathways [54]. Upon recognizing their cognate antigen, CTL release perforin and granzyme from preformed granules. Perforin forms pores in the plasma membrane of the target cell and allows granzymes to enter and cause apoptotic cell death [55]. The membrane-bound executor molecule CD95L is upregulated on CTL after TCR activation and induces apoptosis after binding to Fas (CD95) on target cells. Activated CTL synthesize and release cytokines, such as tumor necrosis factor-α (TNF-α). Their effect on target cells depends on the cell type and cellular activation state ranging in effect from apoptosis to survival. Fully differentiated effector CTL are capable to kill antigen-presenting target cells in the absence of any costimulatory signals or co-receptors.

The majority of reactive effector CTL that emerge after clonal expansion are short-lived and undergo apoptosis after their task is accomplished, i.e. the antigen-bearing target cells are eliminated. Only a small number of antigen-experienced cells survive for a long time as memory T cells. Upon re-encounter with their cognate antigen, memory T cells can very

efficiently respond by strong proliferation and undergo a rapid transition into effector CTL [56].

Studies of the triggers leading to activation of autoreactive T cells and further autoimmune disease revealed two important mechanisms. First, a previously described nonconventional mechanism of Ag presentation, “cross-priming” or “cross-presentation,” has been shown to be involved in the maintenance and/or amplification of autoreactive T cells [57]. Professional antigen-presenting cells are able to cross-present self-antigens to T cells and thereby activate them. Kurts et al. showed that adoptively transferred or thymically derived OT-I cells activated by cross-presentation are deleted from the peripheral pool of recirculating lymphocytes [57]. These findings were at odds with numerous reports showing that cross-presentation of exogenous antigen can prime class I-restricted CTL responses [40, 58]. However, the efficacy of cross-presentation leading to deletion may be affected by the antigen level in the target tissue, induction of apoptosis in self-antigen-expressing cells, presence of autoreactive $CD4^+$ T cells and immune complex-forming autoantibodies, and high T cell precursor frequency [59, 60]. A second dominant paradigm for explaining the apparent links between certain autoimmune diseases and infection is the molecular mimicry theory [61-63]. Triggering of autoaggressive T lymphocytes by non-self antigens during infection and subsequent cross-reactive T cell recognition of a similar self-antigen, together with selective up-regulation of certain host proteins, virus-induced changes in the processing of host proteins, enhanced APC function, and proinflammatory environmental conditioning can all facilitate induction of autoimmunity or immunopathology by viral infections [64, 65]. For example, antimyelin autoimmune reactions in MS may be induced by viruses that share cross-reactive T and B cell epitopes with myelin Ags [66].

In addition to the mechanisms described, there are also cell-based protection mechanisms against autoimmune disease. Naturally occurring $Foxp3^+CD4^+CD25^+$ T regulatory cells (Treg) are important for the establishment and maintenance of self-tolerance and the prevention of autoimmune and inflammatory manifestations [67, 68]. Treg exert a continuous control in the periphery of self-reactive T cells that have escaped central tolerance processes, as conditional ablation of Foxp3-expressing cells leads to overt autoimmune disorders in adult mice [68]. Treg can be used to promote antigen-specific tolerance for transplantation tolerance [69, 70], prevention of graft versus host disease [71, 72] and gene transfer applications [73-76]. While the ontogeny, phenotype and gene expression profile of Treg have been characterized in details, their mode of action and in particular the mechanisms by which they suppress CTL responses are still poorly understood.

The last group of modulators of autoimmunity are negative or inhibitory costimulatory molecules that regulate T cell activation and play a role in peripheral tolerance. The realization that efficient T cell activation requires two signals led to the search for the

costimulatory signal and identification of the CD28-B7 pathway in the early 1990s [77, 78]. Members of the B7-CD28 family play important roles in regulating T cell activation and peripheral tolerance [79]. Soon after the discovery of the CD28-B7 positive costimulatory pathway, it became apparent that CTL-associated antigen 4 (CTLA-4), a second inducible receptor that is homologous to CD28 and binds with higher affinity to CD80 and CD86, could function as a negative regulator of T cell activation [80, 81]. CTLA-4 is also constitutively expressed on Tregs [82] and is important for their function [83, 84] and generation [85]. In addition, CTLA-4–mediated negative signaling is required for establishing peripheral tolerance [86-88]. Fife et al. showed that selective expression of a single-chain, membrane-bound anti-CTLA-4 Ab on B cells leads to significant protection against disease in the NOD mouse by direct attenuation of autoreactive T cell activation [89]. Recently, novel CD28 family members, including ICOS, programmed death-1 (PD-1), and B and T lymphocyte attenuator (BTLA), and new B7 family members, including ICOS ligand (ICOSL) (also known as B7-H), PD-1 ligand (PD-L1) (also known as B7-H1), and PD-L2 (B7-DC), having varied expression patterns among both lymphoid and nonlymphoid (parenchymal) tissues, have been described, providing further insight into the complexity of the functions of T cell costimulatory pathways [90, 91]. PD-1 and its ligands regulate both the induction and maintenance of peripheral $CD8^+$ T cell tolerance [92] and indicate a previously unknown function for PD-L1 on non-hematopoietic cells in protecting tissues from autoimmune attack [93]. A further important role has been described for PD-1 in the restoring of exhausted T cells. Exhaustion of $CD8^+$ T cell function was originally described during chronic LCMV infection as the persistence of virus-specific $CD8^+$ T cells that lacked effector functions [94]. $CD8^+$ T cell exhaustion appears to be a prominent feature not only of experimental chronic infections in mice but also during chronic infections in primates and humans for example in HIV, Hepatitis B and C [95-98]. Blockade of the PD-1/PD-L1 pathway reinvigorates the exhausted T cells, allowing them to expand and produce effector cytokines.

In summary, an efficient CTL-mediated immune response is initiated if a naïve or memory CTL recognizes specific peptide-MHC class I complexes with enough avidity on an activated APC along with costimulatory signals. Under normal circumstances, central and peripheral tolerance mechanisms control the $CD8^+$ T cell repertoire which is prepared to respond vigorously against pathogen-derived or alloantigens but not against self-antigens.

1.3 Interactions between endothelial cells and CTL

Human vascular endothelial cells (EC) represent the major interface between blood and tissues. Forming the inner lining of blood vessels, they are crucial for the maintenance of vascular integrity, a prerequisite of organ homeostasis by preventing blood coagulation, regulating vasomotion and modulating permeability for molecules, particles and cells. EC are uniquely positioned between circulating lymphocytes in the blood and tissues. They are gatekeepers that regulate the trafficking of T lymphocytes from the bloodstream to sites of infection and inflammation and back to the lymphoid organs. EC express MHC class I molecules [99] and can therefore easily become targets of $CD8^+$ CTL.

Following transplantation of vascularized organs, EC are the first graft cells encountered by alloreactive host lymphocytes and therefore primary targets of alloreactive CTL [100, 101]. Different roles have been described for EC that interact with antigen-specific CTL [102]. It has been shown by several groups that EC are able to activate memory $CD8^+$ T cells to differentiate into allospecific MHC class I-restricted CTL *in vitro* [103-105] and *in vivo* [106, 107]. Moreover, EC exhibit important functions of professional APC, including expression of MHC class II and costimulatory molecules [108] and cross-presentation of minor histocompatibility antigens [109, 110]. The notion that EC may under particular circumstances act as APC has been supported by the finding that nonhematopoietic cells within the vascularized grafts – presumably EC – are able to initiate CTL responses that mediate allograft rejection [107].

However, the direct interaction of $CD8^+$ T lymphocytes with allogenic endothelial cells results in unconventional effector CTL that are specific for EC but fail to kill leukocyte-derived target cells from the same donor [103]. Further, EC can be possible targets of CTL-mediated immune responses. When EC present cognate antigen that activates transmigrating effector CTL they should be targets and die rapidly from this interaction. However, the outcome of an interaction between effector CTL and EC can be manifold. Severe, immune mediated endothelial injury precipitates rapid organ dysfunction, such as seen in hyperacute solid organ transplant rejection [111, 112] caused by antibodies that non-specifically activate circulating T lymphocytes [113] or by certain cytokines, e.g. TNF-α [114]. However, CTL that are important mediators of acute graft versus host disease (GVHD) after allogeneic stem cell transplantation [115, 116] do not precipitate this type of rapid endothelial injury and EC are rather poor targets for peptide-specific CTL. Overall, endothelial injury is a rather uncommon event [106] as it occurs often only after long-during chronic stimulation, e.g. CTL-mediated injury of EC is found only late in the course of GVHD [117, 118]. Furthermore, T cells play an important role in the pathogenesis of common chronic inflammatory vasculopathies like

atherosclerosis [119] or different forms of large and medium vessel vasculitides [120] but it is unknown if EC are targets of autoreactive antigen-specific CTL in these diseases.
To identify the underlying molecular mechanism of cell-selective tissue injury, Kummer et al. [121] chose the HLA-A*02-bound male-specific peptide $SMCY_{(311-319)}$ from the broad spectrum of possible MHC class I restricted alloantigens in order to simplify the complex interaction of CTL with EC. Again, EC were poor targets for peptide-specific CTL compared to other target cells. This resistance to CTL-mediated lysis was explained by the inability of EC to present equal amounts of immunodominant antigenic peptides compared to the other target cells. Neither a general resistance of EC to CTL-mediated target cell lysis nor lack of costimulation or low MHC class I expression did explain the fact that EC escaped CTL-mediated lysis. The findings supported the accumulating evidence that EC present a substantially different repertoire of MHC class I ligands compared with syngeneic leukocyte derived cells and that they are protected from CTL-mediated lysis by this mechanism.

1.4 MHC class I-restricted antigen presentation

MHC class I molecules are essentially expressed on the surface of any nucleated mammalian cell and the copy number is estimated to be 50-100.000 per cell [122, 123]. The α-chain of the MHC class I molecule is highly polymorphic at all three MHC class I loci. The β-chain (β2-microglobulin) is non-polymorphic and not involved in peptide binding nor in direct contact with the TCR. EC express mostly HLA-A and B loci, but at a low baseline level. Upon activation with cytokines (TNF-α, IFN-γ), MHC class I molecules (and also various adhesion molecules) in EC can be rapidly upregulated [124]. IFN-γ and TNF-α act synergistically on vascular endothelial cells inducing a more than additional response of two signals [125]. Enhanced presentation of endogenous peptides and upregulation of adhesion and costimulatory molecules favor engagement of CTL [121].
MHC class I-restricted antigens are peptides of 8-9 amino acids length either derived from endogenous [126] or intracellular proteins that are degraded by the proteasome in the cytoplasma or within the ER [127] (Figure 1). Cytosolic peptides allocated to the MHC class I presentation pathway are transported via the transporter associated with antigen processing (TAP) [128] from the cytosol into the lumen of the endoplasmic reticulum (ER). TAP strongly affects the MHC class I ligand profile on cells [129]. Peptides are assembled with MHC class I molecules in a complex in the presence of chaperones [130]. Finally, the peptide-MHC class I (pMHC) complexes are transferred to the cell surface and there presented to other cells [131, 132]. The HLA-A*0201 molecule is carried by 40% of the caucasian individuals.

HLA-A*0201-restricted, immunodominant viral peptides are therefore extensively characterized [133, 134]. The efficiency of peptide presentation is influenced by numerous factors that act on three different levels: the processing of the antigen, the presentation of the peptide-MHC class I complex on the cell surface and the decay of the complex. The intracellular fate of source proteins for MHC class I ligands has been studied over the past years [131, 135]. The rate of source protein synthesis is one critical component that defines the amount of protein delivered to the proteasome for degradation [136, 137]. Due to the short half-life of peptides in the cytosol [138], the site of protein synthesis as well as TAP transport rates [139, 140] are also important components. The cellular protein degrading machinery consisting of the proteasome, cytosolic and ER proteases generates the antigenic peptides that can enter the ER [135, 138] and can thereby critically affect the MHC class I ligand repertoire [135]. The constitutive proteasome and the IFN-γ inducible immunoproteasome are shaping immunogenic peptides differently [141-143]. The immunoproteasome has been found to be important in particular for the generation of $CD8^+$ T cell epitopes during viral infections and inflammation [144]. The presentation of the pMHC complex on the surface depends mainly from the stability of the complex. Poor affinity for the MHC molecule can abolish a peptide's role as a surface antigen [145]. A further component influencing the quality of the presentation of the pMHC complex to CTL is its decay which is determined by the half-life of the complex [146, 147] but also by the internalization of MHC I molecules [148, 149].

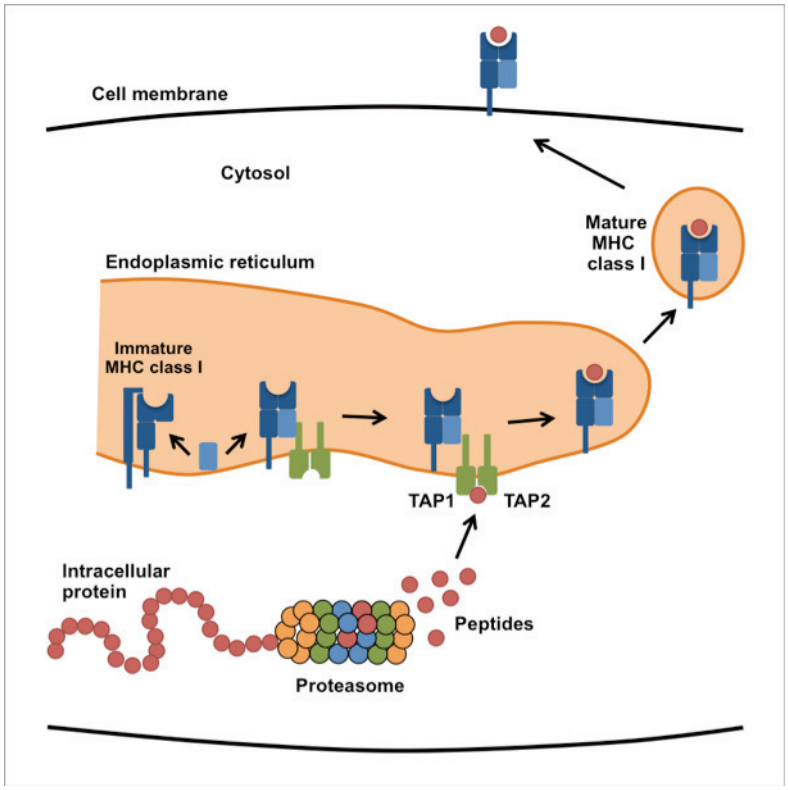


Figure 1. MHC class I presentation pathway.

1.5 The MHC class I-restricted peptides used in this study

In our study, we wanted to elucidate the special protection of EC in CTL-mediated lysis. Therefore, several MHC class I restricted peptides were used (Table 2). First, the endothelial cell-selective peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$. $PTRF_{(56-64)}$ is derived from the protein polymerase I and transcript-release factor (PTRF). The sequence for PTRF is located on chromosome 17q21. It was initially discovered as a murine nuclear factor involved in RNA polymerase I transcription termination [150] and by this mechanism enhances transcription [151]. It has been further shown to suppress type I collagen promoter activity [152]. Recent reports described PTRF to be enriched in associated with caveolae in adipocytes in humans and rats as well as endothelial cells [153, 154]. CD59 located on chromosome 11p13 is a GPI-anchored complement inhibitor mainly expressed on erythrocytes [155] and leukocytes [156], but some expression is found in most tissues. Its major function is to inhibit the formation of the membrane attack complex by binding to C8/C9 [157]. Recent studies showed that CD59 is also expressed in human EC and protective against complement-mediated lysis [158] and atherosclerosis [159]. One interesting feature is that the $CD59_{(106-114)}$ peptide is located in the proprotein (signal peptide) and not in the mature, GPI-anchored extracellular CD59. This suggests that the amount of $CD59_{(106-114)}$ presented on EC depends on the rate of synthesis and not on the rate of degradation.

Further, several control antigens derived from foreign and self-proteins were used. As a first control peptide, $Flu_{(58-66)}$ was chosen, a peptide that is derived from influenza A virus matrix protein [133, 160]. As most adults have been exposed to influenza virus, a high precursor frequency of naïve or memory CTL in the PBMC of healthy HLA-A*02 positive donors can be expected. The second antigen, $SMCY_{(311-319)}$, is a Y-chromosome encoded, male-specific minor histocompatibility antigen in sex mismatched, HLA-identical hematopoietic stem cell transplantation [161, 162]. It is a low abundant self-peptide that can be used as an alloantigen when stimulating female responder PBMC and as an autoantigen for male donors. As a control for highly abundant self-peptides we used $DDX5_{(168-176)}$ (DEAD box polypeptide 5, p68), a conserved and ubiquitously expressed member of the DEAD box family of proteins with RNA helicase activity [163] which was used as a self-antigen for male PBMC donors.

Table 2. peptides used for experiments

Flu$_{(58-66)}$	GILGFVFTL	influenza-virus derived antigen	*foreign*
SMCY$_{(311-319)}$	FIDSYICQV	alloantigen for females, autoantigen for males	*self, low abundance*
DDX5$_{(168-176)}$	YLLPAIVHI	autoantigen	*self, high abundance*
PTRF$_{(56-64)}$	SLLDKIIGA	autoantigen, **endothelial**	*self, high abundance*
CD59$_{(106-114)}$	SLSEKTVLL	autoantigen, **endothelial**	*self, high abundance*

1.6 Aims of this study

Several findings did support the hypothesis that EC present a substantially different repertoire of MHC class I ligands compared with syngeneic leukocyte derived cells and that they may be protected from CTL-mediated lysis by this mechanism. The major aim of this thesis project was to characterize the endothelial repertoire of MHC class I ligands, to investigate antigen-expressing EC as target cells for antigen-specific CTL *in vitro,* to assess the outcome of this interaction and to analyze immunogenicity of endothelial peptide-MHC class I complexes.

In this project, the following aims were pursued:

1. **To identify and characterize the endothelium-specific peptide repertoire**
   The peptide repertoire of endothelial cells and syngeneic leukocyte derived cells was compared. Therefore, MHC class I-restricted peptides were isolated from both cell lines and characterized by mass spectrometry.

2. **To elucidate which molecular mechanisms affect endothelial peptide presentation**
   Several biochemical features of the identified peptides were characterized. Levels of protein expression and turnover, antigen processing by the proteasome and presentation and decay of the pMHC complex that are all critical steps of the MHC class I presentation pathway were analyzed whether they are involved in the selective expression of endothelial peptides.

3. **To grow endothelial cell peptide-specific CTL from the blood of healthy donors**

   First, a method for induction of self-reactive CTL was established. Peptide-pulsed dendritic cells were used as antigen-presenting cells to induce peptide-specific CTL. The induction of CTL specific for the HLA-A*02 restricted endothelial self-peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ was attempted under various culture conditions including stabilization of the pMHC complex and interference with inhibitory costimulatory molecules like PD-1.

4. **To modify the endothelial peptide repertoire with an attempt to break immunoprotection of these cells**

   The surface antigenic profile was altered by two approaches: a) by transfection of influenza virus A matrix protein 1 gene to make EC recognizable for $Flu_{(58-66)}$-specific CTL and b) by RNA interference to knockdown the abundant endothelium-specific peptides.

2. Material and Methods

2.1 Culture media

For culturing BLC complete RPMI 1640 medium, on the basis of RPMI 1640 (52400025) containing 10% FCS (10270106), 2 mM L-glutamine (25030024), 100 U/ml penicillin and 100 µg/ml streptomycin (15140122), 5 mM nonessential amino acids (11140035), 5 mM sodium pyruvate (11360039) (all reagents from Invitrogen Life Technologies, Carlsbad, CA, USA) was generally used. RPMI 1640 washing medium is based on RPMI 1640 containing 2.5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. As cloning medium complete RPMI1640 medium supplemented with 200 U/ml IL-2 (Proleukin, gift from Roche, Basel, Switzerland) was used.

DC medium was AIM-V Medium (Gibco, Invitrogen) supplemented with 500 U/ml GM-CSF (PeproTech, Rocky Hill, NJ, USA) and 10^4 U/ml IFN-α (Intron A, Essex Chemie AG, Luzern, Switzerland). LPS (final concentration 0.5 µg/ml) (E.coli 026.B6, Sigma-Aldrich, Saint Louis, USA), TNF-α (final concentration 50 ng/ml) and IFN-γ (final concentration 100 ng/ml) (both from PeproTech) were added for the last 24 hrs of culture.

CTL Assay medium is based on medium 199 (31153026, Invitrogen) containing 2% FCS, 5 mM HEPES (15630056, Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin.

Complete medium 199 containing 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, supplemented with fibroblast growth factors (20 ng/ml human acidic fibroblast growth factor and 20 ng/ml human basic fibroblast growth factor; both from PeproTech) and heparin (0.2 mg/ml; Sigma-Aldrich) was generally used for culturing endothelial cells. EC washing medium is based on medium 199 containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Special media for particular experiments are indicated in the corresponding section.

2.2 Peptides, cytokines, reagents and Abs

The following HLA-A*02 restricted peptides were synthesized as described [164] (generous gifts from St. Stevanovic, Tübingen, Germany): GILGFVFTL ($Flu_{(58-66)}$), FIDSYICQV ($SMCY_{(311-319)}$), the ubiquitously expressed YLLPAIVHI ($DDX5_{(148-156)}$), and the two endothelial cell selective peptides SLLDKIIGA ($PTRF_{(54-64)}$), and SLSEKTVLL ($CD59_{(106-114)}$).

Peptides were dissolved in DMSO (Sigma-Aldrich) at indicated concentrations and stored at 4°C.

The following cytokines were used: GM-CSF (PeproTech, Prod.-Nr. 300-03), IL-2 (Proleukin, gift from Roche), IFN-α (Intron A, Essex Chemie AG), IFN-γ (PeproTech, Prod.-Nr. 300-02) and TNF-α (PeproTech, Prod.-Nr. 300-01). LPS (E.coli 026.B6) was from Sigma-Aldrich. All stock solutions were stored in aliquots at -70°C.

Calcein-AM (C1430, Molecular Probes, Invitrogen) was dissolved in DMSO at 5 mM and stored at -20°C.

Antibodies were used for flow cytometry, western blot and immunofluorescence. The source, specificity and conjugation are shown in Table 3.

PE-labelled Pro5^{TM} MHC Pentamers for the HLA-A*02-binding peptide SMCY$_{(311-319)}$ were purchased from ProImmune (Oxford, UK).

Table 3. Antibodies used for different experiments.

Antigen	Species	Conjugate	Supplier	Cat. No.	Usage	Titer
CD3	mouse IgG	FITC	BD Pharmingen	555332	FC	5µl/tube
CD4	mouse IgG	PE	BD Pharmingen	555347	FC	5µl/tube
CD4	mouse IgG	FITC	BD Pharmingen	560133	FC	5µl/tube
CD8	mouse IgG	PE	BD Pharmingen	555635	FC	5µl/tube
CD8	mouse IgG	APC	BD Pharmingen	555369	FC	5µl/tube
CD14	mouse IgG	PE	BD Pharmingen	345785	FC	5µl/tube
CD25	mouse IgG	APC	BD Pharmingen	560133	FC	5µl/tube
CD31	mouse IgG	PE	BD Pharmingen	555446	FC	5µl/tube
CD40	mouse IgG	PE	BD Pharmingen	555589	FC	5µl/tube
CD45	mouse IgG	FITC	BD Pharmingen	345808	FC	5µl/tube
CD80	mouse IgG	PE	BD Pharmingen	557227	FC	5µl/tube
CD83	mouse IgG	FITC	BD Pharmingen	556910	FC	5µl/tube
CD86	mouse IgG	FITC	BD Pharmingen	555657	FC	5µl/tube
BB7.2	mouse IgG	purified	gift from P. Cresswell, Yale University Medical School, New Haven, USA	-	FC	1:100
FoxP3	mouse IgG	PE	BD Pharmingen	560133	FC	5µl/tube
-	mouse IgG	PE	BD Pharmingen	555748	FC	5µl/tube
-	mouse IgG	FITC	BD Pharmingen	555749	FC	5µl/tube
-	mouse IgG	APC	BD Pharmingen	554681	FC	5µl/tube
-	mouse IgG	purified	Serotec	mca928	FC	1:100
mouse IgG	goat antiserum	FITC	Jackson	115-095-003	FC	1:200

Table 3. Antibodies used for different experiments (continued).

Antigen	Species	Conjugate	Supplier	Cat. No.	Usage	Titer
Beta-1	mouse IgG	purified	Abcam	ab22672	WB	1:1000
β-Actin	mouse IgG	purified	Sigma-Aldrich	A5441	WB	1:80000
CD59	mouse IgG	purified	Serotec	MCA1054	WB	1:200
DDX5	goat IgG	purified	Abcam	ab10261	WB	1:750
LMP-2	rabbit IgG	purified	Abcam	ab3328	WB	1:1000
LMP-7	rabbit IgG	purified	Abcam	ab3329	WB	1:1000
PTRF	mouse IgG	purified	BD/Transd. Lab	P87520	WB	1:250
mouse IgG	goat antiserum	HRP	Jackson	115-035-071	WB	1:4000
rabbit IgG	goat antiserum	HRP	Jackson	P0448	WB	1:1000
goat IgG	rabbit antiserum	HRP	DAKO	P0449	WB	1:2000
Flag	mouse IgG	purified	Sigma	F1804	FC, IF,WB	1:50-250
vWF	rabbit IgG	purified	DAKO	A0082	IF	1:200
-	mouse IgG	biotin	BD Pharmingen	33812x	IF	1:80
-	rabbit IgG	biotin	Jackson	711-065-152	IF	1:200
-	Streptavidin	Cy2	Jackson	016-220-084	IF	1:100
PD-L1 (B7H1)	mouse IgG	purified	eBioscience	16-5983	Inhibition	10µg/ml

FC: flow cytometry, WB: western blot, IF: immunofluorescence

2.3 General methods

2.3.1 RNA isolation and RT-PCR

Total RNA was isolated from $5x10^6$ cells using TRIzol-Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. After RT (MMLV; Invitrogen Life Technologies) cDNA coding for Flu and Flag, respectively, was amplified using the following primers (all from Microsynth, Balgach, Switzerland): 5'-ATGGGGAAGGTGAAGGTCGG-3' and 5'-AGGGATGATGTTCTGGAGAG-3' for GAPDH, 5'-GGGAAGAACACCGATCTTGA-3' and 5'-CTCCGTTCCCATTAAGAGCA-3' for Flu, 5'-ATCCACGCTGTTTTGACCTC-3' and 5'-CCTGACGGGACGATA GAGAG-3' for Flag. The PCR amplification of cDNA fragments was performed as follows: 1 µl template cDNA, 1 µl dNTP mix (10 mM each, stock solution from

Sigma-Aldrich), 1 µl each of upper and lower primer (2.5 µM stock), 1 µl $MgCl_2$ (25 mM, Promega, Madison, WI, USA), 1 µl Promega 10xBuffer, 0.1 µl Taq Polymerase (Promega) and 4 µl dH_20 was added to a final volume of 10 µl. PCR for Flu, Flag and GAPDH cDNA was performed with an initial denaturation step at 96°C for 5 min, then 35 cycles with 30 sec denaturation at 96°C, 30 sec annealing at 58°C, 1 min elongation at 72°C, followed by the final extension for 10 min at 72°C. For agarose gel electrophoresis 3 µl of PCR samples were mixed with 1 µl 6x loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol) and put on a 1.5% agarose gel in 100 ml of 1xTAE running buffer (10 mM Tris-base, 1 mM EDTA, pH 8.0). 3 µl of a 100 bp ladder (Promega) was loaded into a separate slot as molecular weight standard. 80 Volts were applied for about 40 min and then the agarose gel was incubated for 15 minutes in dH_20 containing 2 µg/ml EtBr. Separated bands were visualized using UV light (ChemiIumager 5500, Alpha Innotech).

2.3.2 Western blot

Cells ($2.5x10^6$) were lysed in 200 µl lysis buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM DTT (Roche), 100 µM Na_3VO_4, 1 mM NaF and 1% Triton-X in dH_2O) containing the following protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin (both from Sigma-Aldrich) and 5 µg/ml pepstatin (AppliChem, Darmstadt, Germany)). Non-reducing conditions were used for CD59. PTRF (43.5kD) and DDX5 (68kD) were separated on a 9% and CD59 (19kD) LMP-2 (24kD), LMP-7 (23kD), and Beta1 (25kD) on a 15% SDS-PAGE, respectively. The proteins were transferred onto cellulose nitrate (Protran BA83, Whatman Schleicher&Schüll, Dassel, Germany) and blocked in TBS (25 mM Tris, 192 mM Glycin, 20% Methanol) containing 5% fat-free milk. The proteins were detected with mAbs specific for PTRF, DDX5, and CD59, LMP-2, LMP-7, and Beta-1 (see Table 3). β-Actin was used as a loading control. All antibody incubation steps were performed for 60 min at room temperature. After adding the peroxidase-conjugated Ab (peroxidase-conjugated goat anti-mouse antibody, peroxidase-conjugated rabbit anti-goat antibody), the chemiluminescence substrate (Supersignal West Pico, Pierce Chemical Company, Perbio Science, Sweden) was added according to the manufacturer's protocol, and a photographic film (Biomax, Eastman Kodak, Rochester, NY, USA) was exposed to the membrane and developed (Curix 60, Agfa-Gevaert, Mortsel, Belgium).

2.3.3 Immunofluorescence staining

Flag staining

Adherent cells were grown on gelatine-coated cover slips in 6-well plates (BD Falcon, San Jose, CA, USA) until the cells were confluent. For suspension cells, 200'000 cells per spot were spun down on gelatin/chromalaun-coated glass slides (Cytospin2, Shandon, Waltham, USA) at 800 rpm for 8 min [105]. The day before the staining, the cells were washed with ice-cold PBS (Gibco) and fixed with a mixture of cold acetone and methanol (1:1) for 4 min. Afterwards, the cover slips were air dried overnight. The next day, after washing in phosphate-buffered saline (PBS; pH 7.4), the cover slips and slides, respectively were incubated for 45 min at room temperature (RT) in medium 199 containing 20% FCS to block nonspecific protein-binding sites. Then, the slides were washed with RT PBS three times for 5 min. A mouse anti human Flag tag antibody (1:50) was used to detect endothelial cells. After one hour incubation at RT, slides were washed 3 times with PBS. Then, a secondary biotinilated anti mouse IgG antibody (1:80) was added for 1 hour at RT. After washing again 3 times with PBS for 5 minutes, the slides were incubated with a tertiary Streptavidin Cy2-conjugated Ab (1:100) for one hour at RT. Afterwards, the slides were washed 3 times with PBS, and then stained with Hoechst (1:3000, Nr. 33258, Sigma-Aldrich) for 10 min at RT. After washing again, the slides were mounted in fluorsave (Calbiochem, Merck Chemicals Ltd., Nottingham, UK) and stored at 4°C in the dark until fluorescence microscopy was performed.

von Willebrand factor staining

Cells were treated as described for Flag staining. As first antibody a rabbit anti human von Willebrand factor antibody (1:200) was used to detect endothelial cells. After washing, a secondary biotinilated anti rabbit IgG antibody (1:200) was added for one hour at RT. After washing again, the slides were incubated with a tertiary Streptavidin-Cy2 Ab (1:100) for one hour at RT. Afterwards, the slides were washed, and then stained with Hoechst (1:3000) for 10 min at RT and mounted in fluorsave as described before.

2.3.4 Peptide-MHC staining by FACS

For all pentamer stainings $1x10^6$ cells were used. Samples were stained with PE-labelled Pro5^{TM} MHC pentamers (ProImmune, 1:20, total volume 100µl) for 10 min at room temperature in the dark, then washed with PBS containing 2% FCS. In a second staining

step, the cells were incubated with saturating amounts of APC-conjugated anti-CD8 mAb (see Table 3) for 20 min on ice for subset identification. The cells were then washed two more times with PBS containing 2% FCS. Propidium iodide (PI, final conc. 5µg/ml, Sigma-Aldrich) was added as a vital dye prior to data acquisition. Analysis was performed on a CyAn™ ADP FACS instrument using Summit Software (Dako Cytomation, Glostrup, Denmark). The lymphocytes were gated and $5x10^5$ (CTL clones) aliving (PI negative) lymphocytes were acquired and analyzed.

*2.4 HLA-A*02 binding*

2.4.1 T2 peptide binding assay

A T2 binding assay was used as described [165]. The HLA-A*02 restricted peptides GILGFVFTL ($Flu_{(58-66)}$), FIDSYICQV ($SMCY_{(311-319)}$), SLLDKIIGA ($PTRF_{(56-64)}$), SLSEKTVLL ($CD59_{(106-114)}$) and YLLPAIVHI ($DDX5_{(148-156)}$) (generous gift from St. Stevanovic, University of Tübingen, Tübingen) were dissolved in DMSO at a concentration of 1mg/ml (Table 4). TAP-deficient T2 cells were incubated overnight at 37°C with $5x10^{-5}$M peptide in complete RPMI 1640 supplemented with 2.5% FCS. Cells were washed and surface expression of HLA-A*02 was then determined by flow cytometry using BB7.2 mAb (1:100, gift from Peter Cresswell, Yale Medical School, New Haven CT, USA) and an isotype control Ab, respectively, followed by incubation with FITC-labelled goat anti mouse IgG secondary Ab (1:100). All incubations were strictly performed at a temperature of 4°C. Data acquisition was performed on a Cyan™ ADP FACS instrument using Summit Software (Dako). Per sample, $1x10^5$ cells were acquired and analyzed. The Δ mean fluorescence intensity (ΔMFI) was calculated by subtracting the MFI with isotype control Ab from the MFI with BB7.2 mAb. The peptide binding index was calculated as ΔMFI with peptide/ΔMFI without peptide [166].

2.4.2 Peptide competition assay

Peptide concentrations were adjusted according to the results of a peptide competition assay. This experiment was performed with the help of C. S. Chennakesava in our lab. T2 cells were loaded with 10^{-8}M $Flu_{(58-66)}$ and different concentrations of $SMCY_{(311-319)}$, $DDX5_{(148-156)}$, $PTRF_{(56-64)}$, and $CD59_{(106-114)}$, respectively, ranging from 10^{-4}M to 10^{-12}M were added. Cells were incubated overnight at 37°C in complete RPMI 1640. The next day, a calcein-release cytotoxicity assay was performed as described below and the concentration of

peptide in the presence of 10^{-8}M Flu peptide was determined at which target cell killing by $Flu_{(58-66)}$-specific CTL was inhibited by 50%. Peptide concentrations of $DDX5_{(148-156)}$, $PTRF_{(56-64)}$, and $CD59_{(106-114)}$ were adjusted by normalization to the competing concentration of $SMCY_{(311-319)}$, which was set to be 1. Thus, for $DDX5_{(168-176)}$ a 2.4-fold higher concentration, for $PTRF_{(54-64)}$ a 4.4-fold higher concentration and for $CD59_{(311-319)}$ a 8-fold higher concentration was calculated (Table 4).

Table 4. Peptide concentrations.

peptide	MW	normal		adjusted	
		stock concentration	*molar concentration*	*stock concentration*	*molar concentration*
$Flu_{(58-66)}$	965.6	1 mg/ml	1.04 mM	-	-
$SMCY_{(311-319)}$	1087	1 mg/ml	0.92 mM	-	-
$DDX5_{(168-176)}$	1038	1 mg/ml	0.96 mM	2.4 mg/ml	2.3 mM
$PTRF_{(56-64)}$	928.6	1 mg/ml	1.08 mM	4.4 mg/ml	4.7 mM
$CD59_{(106-114)}$	988.6	1 mg/ml	1.01 mM	8 mg/ml	8.1 mM

2.4.3 Decay and half-life of the peptide-MHC class I-complex

For the determination of half-life of the HLA-A*02-peptide complexes, T2 cells were loaded with peptide in adjusted concentrations overnight at 37°C in complete RPMI 1640 supplemented with 2.5% FCS. The next day, peptide was washed away and the cells were incubated again at 37°C. At indicated time points (0h, 2h, 6h, 24h) the cells were stained for HLA-A*02 as described above and the fluorescence index was analyzed by flow cytometry.

2.5 Cell lines

2.5.1 Culture of EC, BLC, CC

All studies involving primary human cell lines and tissues were approved by the institutional ethical review board. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by enzymatic digestion as described previously [121]. EC were cultured in complete medium 199 containing 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen), supplemented with fibroblast growth factors (20 ng/ml human acidic fibroblast growth factor and 20 ng/ml human basic fibroblast growth

factor; both from PeproTech) and heparin (0.2 mg/ml; Sigma-Aldrich). The HLA-A*02-positive, male EBV-transformed B cell line JY, the female TAP-deficient T2 cell line (both gifts from A. Cerny, University of Bern, Bern, Switzerland), the HLA-I negative K562 cell line and the colon carcinoma cell line LS174 (provided by G. Spagnoli, University of Basel, Basel, Switzerland) were cultured in complete RPMI 1640 (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.5.2 Isolation of peripheral blood mononuclear cells (PBMC)

Buffy coats were obtained from healthy HLA-A*02 positive blood donors (Blutspendezentrum SRK, Basel, Switzerland). PBMC were isolated by Ficoll gradient separation (Lymphoprep, Axis-Shield, Dundee, UK) according to the manufacturer's instructions, washed twice with HBSS, suspended in ice-cold freezing medium (90% low endotoxin FCS (Invitrogen Life Technologies) containing 10% DMSO) and stored in liquid nitrogen. On average, $7.38x10^8 \pm 2.31x10^8$ cells were isolated per buffy coat. After thawing, viability of PBMC was >95% as judged by trypan blue staining and dye exclusion. In male donor PBMC, a 84 bp fragment of the male-specific DFFRY gene [167, 168] was amplified from genomic DNA using the following primers (all from Microsynth): 5'-AACTCACCTCCAACACATACTCCAC -3' and 5'-TTCATGATGAAATCTGCTTTTTGT TT -3'.

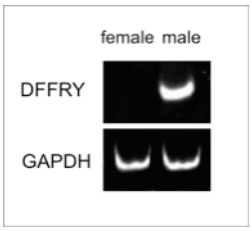


Figure 2. Gender confirmation of donors. Gender of male and female donors was confirmed by amplification of a 84 bp fragment of the male-specific DFFRY gene.

2.5.3 Monocyte-derived dendritic cell (DC) preparation

Monocytes were isolated from PBMC by negative selection using magnetic cell sorting by autoMACS® according to the manufacturer's protocol (Monocyte Isolation Kit II, Miltenyi Biotec, Bergisch-Gladbach, Germany). $2x10^6$ $CD14^+$ monocytes/well were seeded on 24-well plates (BD Falcon, Prod.-Nr. 353047) and cultured for 3 days in AIM-V medium (Invitrogen) supplemented with 500 U/ml GM-CSF (PeproTech) and 10^4 U/ml IFN-α (Intron A, Essex Chemie AG) [169, 170]. To induce final DC maturation, LPS (E. coli 026.B6, Sigma-Aldrich), TNF-α (Pepro-Tech, final concentration 50 ng/ml) and IFN-γ (Pepro-Tech, final concentration 100 ng/ml) were added for the last 24 hrs of culture. DC differentiation was confirmed by visual inspection (intense cluster formation) and by FACS.

2.5.4 Flow cytometry analysis of monocytes and dendritic cells

For flow cytometry staining the following antibodies were used: directly conjugated antibodies against CD14-PE, CD80-PE, CD86-FITC, CD40-PE, CD83-FITC and CD3-FITC (see Table 3). Mouse IgG-PE and IgG-FITC were used as control. $5x10^5$ monocytes or DC, respectively, were washed once with PBS containing 1% BSA (Fluka, Saint Louis, USA) and incubated with the antibodies (final concentration 1:20). For 30 min at 4°C. Cells were washed again twice and then fixed in 2% paraformaldehyde in PBS. Analysis was performed on a CyAn™ ADP FACS instrument using Summit Software (both from Dako). For dendritic cell differentiation, total leukocytes were gated according to forward (FSc) and sideward scatter (SSc). And for each staining condition, $1x10^5$ cells were acquired and analyzed.

2.5.5 Induction of peptide-specific CTL lines

Monocyte-derived DC were harvested with 1mM EDTA in HBSS (pH 7.5) for 30 min at 37°C, pooled and washed once with complete RPMI 1640 supplemented with 2.5% FCS. Cells were resuspended at $1x10^6$ cells/ml in complete RPMI 1640-AB containing 10% heat-inactivated human AB serum (Blutspendezentrum SRK, Basel, Switzerland) instead of FCS and were pulsed with peptide in adjusted concentrations (10^{-5}M for $Flu_{(58-66)}$, 10^{-5}M for $SMCY_{(311-319)}$, $2.4x10^{-5}$M for $DDX5_{(148-156)}$, $4.4x10^{-5}$M for $PTRF_{(56-64)}$ and $8x10^{-5}$M for $CD59_{(106-114)}$) for 2 hours at 37°C. The peptide-pulsed DC were washed twice with complete RPMI 1640 supplemented with 2.5% FCS, and $4x10^5$ DC were used at a ratio of 1:10 to stimulate $4x10^6$ $CD8^+$ T cells in 1 ml complete RPMI 1640-AB in 24-well plates. The responder cells were isolated from autologous PBMC ($CD8^+$ T Cell Isolation Kit II, Miltenyi Biotec). CD8 cell isolates were >85% pure. All cultures were maintained at 37°C in 5% CO_2 humidified air. IL-2 (Proleukin, gift from Roche, final concentration 20 U/ml) was added on day 3 of the induction culture. CTL lines were restimulated in weekly intervals with autologous irradiated (3000 rad) PBMC pulsed with peptide in adjusted concentrations and cultured in complete RPMI 1640-AB with 50 U/ml IL-2. After 2 weeks and then in weekly intervals, the CTL lines were tested for peptide-specific cytotoxicity using peptide-pulsed T2 cells as targets in a calcein-release assay (see below). For selected induction cultures, a blocking antibody against human PD-L1 (eBioscience, San Diego, USA, final concentration 10µg/ml) or the pancaspase inhibitor z-VAD (BD Bioscience Pharmingen, final concentration 50µM) were used at indicated concentrations to promote outgrowth of CTL [98, 171].

2.5.6 Induction of CTL clones by limiting dilution

Peptide-specific CTL lines that showed >70% of peptide-specific cytotoxic activity after 5 weeks were chosen for cloning by limiting dilution as published [103]. In brief, the $CD8^+$ T lymphocytes were counted and suspended in complete cloning medium (complete RPMI 1640 supplemented with 200 U/ml IL-2, and 1 µg/ml PHA-L (Oxoid AG, Pratteln, Switzerland)) containing 500,000/ml irradiated (3000 rad) PBMC and 25,000/ml mitomycin C-treated (Roche, final concentration 50 µg/ml) JY cells. These cell suspensions were distributed in 100 µl/well to round-bottom 96-well plates, resulting in input $CD8^+$ T cell numbers of 100, 10, and 1 per well. After 1 week, microcultures were fed by adding 100 µl/well incomplete cloning medium (without PHA and feeder cells). Expanding clones were identified by visual inspection. Microcultures that grew at the frequency corresponding to clonal growth and single hit kinetic were expanded and tested for peptide-specific lysis in a calcein-release assay [105]. The CTL clones were kept in culture in incomplete cloning medium until restimulation that was necessary on average every 3 weeks. Restimulation was performed by adding $5x10^6$ CTL into 20 ml complete cloning medium.

2.5.7 Induction of alloreactive CTL lines

PFA-fixed T2 cells loaded with $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ were used as stimulator cells for CTL differentiation from CD8 preCTL. T2 cells were taken from culture and loaded with $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ in adjusted concentrations (see 2.5.5). After 2 hrs incubation, cells were washed with complete RPMI 1640 supplemented with 2.5% FCS and then fixed with 1% paraformaldehyde for 10 min at room temperature. Afterwards, cells were extensively washed, and resuspended in complete RPMI 1640-AB in a 96-well round-bottom plate at 10^4 cells per well. Cocultures were initiated by addition of $CD8^+$ T cells. Purified $CD8^+$ T cells ($CD8^+$ T Cell Isolation Kit II, Miltenyi Biotec) were suspended in RPMI 1640-AB and added at 10^5 cells per well to the stimulator cells. The cocultures were fed with fresh medium containing IL-2 at a final concentration of 50 U/ml after 3 days and restimulated weekly. For restimulation, 150 µl of supernatant per well was removed from the microcultures. Fresh medium containing IL-2 (50 µl/well) was added, and the $CD8^+$ T cells were transferred to fresh PFA-fixed peptide loaded T2 cells. After 2 weeks, the cocultures were tested weekly for peptide-specific lysis in a calcein-release cytotoxicity assay (see below).

2.5.8 Calcein release-based cytotoxicity assay

CTL assay with B lymphoblastoid cells as targets

CTL mediated target cell lysis was measured by a calcein-release assay as described [172]. Target cells (T2 or JY cells) were incubated for 30 min at 37°C with 10 µM calcein-AM (Molecular Probes, Invitrogen). Cells were then washed and bleached for 30 min at 37°C in complete RPMI 1640. After washing twice, target cells were suspended in assay medium (medium 199, 2% FCS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin), counted and transferred to 96-well, round-bottom plates (Falcon, BD Biosciences) at $2x10^4$ cells/well. CTL were washed, counted, and added to the calcein-loaded target cells at a E:T ratio of 10:1 (final volume 200 µl/well). Spontaneous release (SR) was determined by adding assay medium to the target cells; maximum release (MR) was obtained by target cell lysis (lysis buffer: 50 mM sodium borate in 0.1% Triton X-100 (both from Sigma-Aldrich), pH 9.0). Then, the assay plates were centrifuged with 470g for 4 min at room temperature. After incubation for 2 hrs at 37°C, 75 µl of supernatant was carefully removed and transferred to a 96-well, flat-bottom plate. Released calcein was measured in a fluorescence multiwell plate reader (SpectraMax GeminiXS; Molecular Devices; excitation wavelength, 485nm; emission wavelength 538nm). Percent specific lysis was calculated as (sample release – SR)/(MR – SR) X 100%.

CTL assay with endothelial cells as targets

Adherent EC grown to confluency in 96-well flat-bottom plates (Falcon, BD Biosciences) were loaded for 30 min at 37°C with 20 µM calcein-AM (Molecular Probes, Invitrogen) in medium 199. Cells were then washed and bleached for 2 hrs at 37°C in complete medium 199. Cells were washed twice with complete medium 199, then assay medium was added. CTL were washed, counted, and added to the calcein-loaded target cells at a E:T ratio of 10:1 (final volume, 200 µl/well). For preparation of SR and MR wells and measurement see above.

2.5.9 Treg marker analysis by flow cytometry

The induction cultures were analyzed for the presence of Tregs by staining for the Treg markers Foxp3, CD4 and CD25 (FoxP3 staining bundle kit, BD Pharmingen). Staining was performed according to the manufacturer's protocol. In brief, $1x10^6$ cells per staining were washed with PBS containing 2% FCS and stained with the conjugated surface markers CD4 and CD25 for 20 min at room temperature. Then, samples were washed with PBS containing

2% FCS and fixed with 1x Human FoxP3 Buffer A for 10 min at room temperature. Cells were washed again and permeabilized with 1x Human FoxP3 Buffer C for 30 min at room temperature. After permeabilization the cells were washed twice and incubated with conjugated FoxP3 antibody for 30 min at room temperature. Then, the samples were washed, resuspended in wash buffer and analyzed immediately.

2.5.10 Stimulation with proinflammatory cytokines

Cytokine stimulation for CTL assays or flow cytometry

EC or BLC were cultured in culture medium alone or in medium containing 100 ng/ml IFN-γ and 50 ng/ml TNF-α overnight. The next day, cells were washed to remove cytokines and CTL assay or flow cytometry experiments were performed as described above.

Induction of immunoproteasomes

EC, JY, T2 and CC cells were cultured in culture medium alone or in medium containing 100 ng/ml IFN-γ for 18 hrs. Cells were lysed and the lysates were analysed by western blot for the occurrence of the constitutive proteasome and the induction of immunoproteasomes as described in 2.3.2.

2.6 Transduction of EC and JY with FluM1

2.6.1 Transfection of GP-293 cells

The Retro-X Universal Packaging System (Clontech, Mountain View, CA, USA, Nr. 631530) was used for retrovirally transduction of primary EC and JY cells. The flag-tagged retroviral construct of the full length cDNA encoding influenza matrix protein M1 (kind gift from G. Kochs, Germany) was cloned into the bicistronic expression vector pQXIN (done by C. S. Chennakesava) (Figure 3). A neomycin resistance was included for selection. The host cell line GP-293 was seeded the day before transfection in collagen-coated 6-well plates at 200.000 cells per well and cultured in GP-2 medium (DMEM (high glucose from Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and 5mM sodium pyruvate) at 37°C. The next day, the medium was changed into 2 ml prewarmed GP-2 medium containing 25 µM chloroquine (Sigma-Aldrich) per well. The pantropic viruses were prepared as follows: 6 µg of the retroviral vector pVSV-G and 6

µg of the carrier vector pQM1IN (pQXIN vector containing the FluM1 sequence) per transfection were washed with sterile H_2O. Then, 2M $CaCl_2$ and 2xHBS (8.0 g NaCl, 0.37 g KCl, 201 mg $Na_2HPO_4 \bullet 7H_2O$, 5.0 g HEPES/500 ml H_2O, pH 7.0) were added quickly and mixed well with the DNA complexes. 1 ml of the DNA mixture was added to each well dropwise. The cells were incubated at 37°C. After 18 hrs, the medium was changed into fresh GP-2 medium and the cells were cultured at 32°C for 72 hrs.

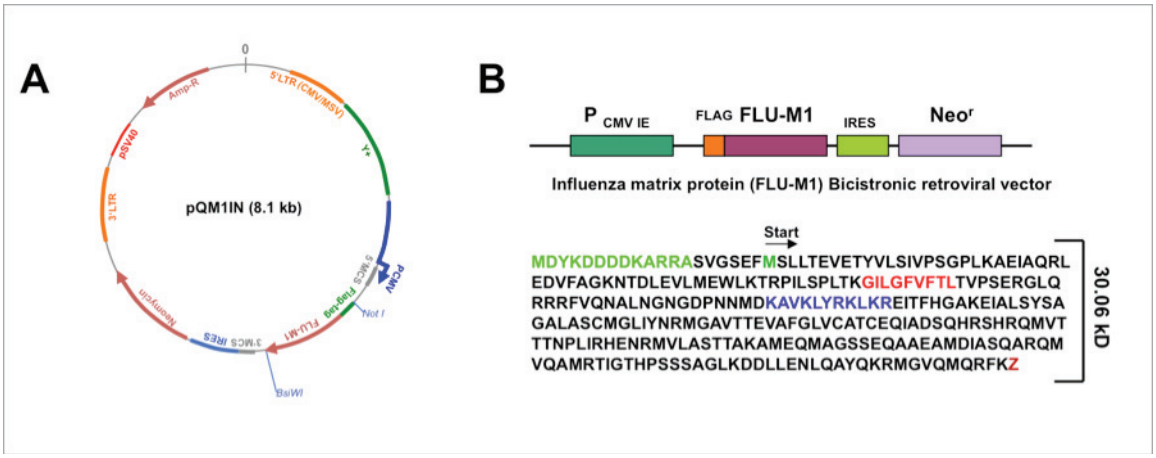


Figure 3. FluM1 vector map. (A) shows the pQM1IN carrier vector (pQXIN vector containing the FluM1 vector), (B) multiple cloning site with inserted FluM1 sequence under a CMV promoter flanked by a Flag-tag and Neomycin resistence.

2.6.2 Infection of endothelial cells

A day before infection very early passage EC were seeded in a 15x60 mm petri dish at 240.000 cells in complete medium 199. The viral supernatants from GP-293 cells were collected after 72 hrs, pooled and polybrene (8 µg/ml) was added to enhance the viral infection effectivity. Supernatants were centrifuged and filtered (Sartorius, Göttingen, Germany, 17598 K 0.45mm Minisart Nylon). The medium of the endothelial cells was replaced by 2.5 ml viral supernatant and cells were cultured at 37°C. After 24 hrs the viral supernatants were replaced by normal complete medium 199 and cells were left for 48-72 hrs. The process of infection was repeated twice by infecting the EC at a splitting ratio of 1:3. After three serial infections transduced cells were put under selection by adding selection medium (complete medium 199 with 600 µg/ml G418 (Gibco)). After selection, cells were splitted at a ratio of 1:2 and always kept in selection medium.

2.6.3 Infection of JY cells

The viral supernatants from GP-293 cells were collected after 72 hrs, pooled and polybrene (8 µg/ml) was added to enhance the viral infection effectivity. Supernatants were centrifuged and filtered (Sartorius, 17598 K 0.45mm Minisart Nylon). 200.000-250.000 JY cells were spun down and resuspended in 1ml of the viral supernatant. Cells were centrifuged for 90 min at 1258g at 32°C. Then, the supernatant was discarded and the cells were resuspended in 1 ml complete RPMI 1640 in a 24-well plate and cultured at 37°C. Infection of JY was accomplished by three serial infections and JY cells were selected with 1 mg/ml G418 per $2x10^6$ cells.

2.6.4 Proteasome inhibition

Transduced EC-FluM1 cells were grown confluent in 10x20 mm petri dishes in complete medium 199. Cells were washed with HBSS (Invitrogen) and 1 µM clasto-lactacystein β-lactone (Prod.-Nr. 426102, Calbiochem) was added as irreversible proteasome inhibitor in complete medium 199 for indicated time points (0h, 2h, 4h, 6h, 24h). For some experiments, 100 ng/ml IFN-γ and 50 ng/ml TNF-α were added at the same time points as the proteasome inhibitor. For cytotoxicity assay, EC were washed with HBSS once and then detached with trypsin/EDTA (Nr. 25300054, Invitrogen). After washing twice with EC washing medium, EC were resuspended in medium 199 and loaded with 20 µM calcein. Then, the cytotoxicity assay was performed as described above.

2.7 siRNA knockdown of abundant EC peptides

The following siRNAs (40 µM stock concentrations) from Microsynth were used: mock siRNA 5'-AGGUAGUGUAAUCGCCUUGtt-3'; CD59 siRNA 5'-GAAGUCUAAGAGUGAAGUAtt-3'; PTRF siRNA 5'-GAGGAAAGAUUGAAUCCUAT-3'; DDX5 siRNA 5'-GCAGAUAGAAUG CUUGAUAtt-3'. Early passage EC were seeded one day before transfection at 100.000 cells per well in medium 199-P/S (complete medium 199 without penicillin/streptomycin) in a gelatine-coated 6-well plate. The next day, the medium was replaced with 2.5 ml prewarmed medium 199-P/S. RNA complexes were prepared as follows (volumes for one well of a 6-well plate): 250 µl of room temperatured OptiMEM I Glutamax™ medium (Gibco) were mixed with 15 µl of 2 µM siRNA (final concentration 10 nM) in a RNAse-free eppendorf tube to prepare solution A. Then, for solution B 250 µl of OptiMEM were mixed with 5 µl of RNAiMAX

(Lipofectamine RNAiMAX transfection reagent, Invitrogen) in a second RNAse-free tube. Solutions A and B were slowly mixed together and incubated for 10-20 min at room temperature for complexing of siRNA. Then, the complexes were added dropwise to the cells without further pipetting and the cells were incubated at 37°C. The medium was changed after 16 hours and the cells incubated for indicated time points. Cells were either lysed as described in section 2.3.2 and analyzed by Western blot for knockdown of proteins or directly used for cytotoxicity assays and flow cytometry.

2.8 Statistics

Mean values between groups were compared using the student's t test. Tests were performed with SPSS 15.01 software (SPSS Inc, Chicago, USA). Unless indicated otherwise, mean ± standard deviation of replicates are shown.

3. Results

*3.1 Comparison of HLA-A*02-restricted peptide presentation between EC and BLC*

3.1.1 Characterization of the endothelial HLA-A*02-presented peptide repertoire

In a previous study in our lab (performed with the help of M. Keller in collaboration with S. Stevanovic, University of Tübingen) the endothelial and leukocyte-derived peptide profile from three healthy HLA-A*02 positive male individuals was examined. Endothelial cells (EC) and B-lymphoblastoid cells (BLC) were grown side-by-side under comparable conditions. The entire pool of peptides bound to endothelial or leukocyte MHC class I molecules was isolated, fractionated and the individual components were characterized by mass spectrometry. By this method, the most abundant MHC class I ligands of a cell can be identified. The amino acid sequences of the HLA-A*02 restricted peptides were used for sequence homology analysis and the source proteins from which these peptides derive were identified (Table 5). It is striking that endothelial cells and leukocytes shared only two out of the twenty peptides. Cyclin I [173] and DEAD box polypeptide 5 (DDX5) are the source proteins for the two shared peptides, and both are ubiquitously expressed in the body. Of the five peptides found on endothelial cells but not on leukocytes, none was novel but all were previously identified in at least one or more other tissues or cell lines (Table 5). Two of them were consistently identified in all the three EC donors analyzed in this study. The proteins from which these two peptides arise are polymerase I and transcript release factor (PTRF), and the complement inhibitor CD59. Both peptides have been isolated previously from other epithelial tissues, particularly renal cancer cell lines. Yet, the relative amounts of PTRF and CD59 derived peptides were remarkably higher in EC compared to other cell lines and tissues from which these peptides have been isolated previously. In EC, the two peptides were repetitively identified as the most abundant HLA-A*02 ligands. Therefore, $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ were chosen as representative endothelial-selective peptides and $DDX5_{(168-176)}$ as a ubiquitously expressed control.

Table 5. HLA-A*02-restricted peptides isolated from syngeneic EC and BLC.

A. Peptides isolated from human umbilical cord blood-derived B lymphoblastoid cells (BLC)

Sequence[1)]	**GeneID**	**Protein**	**3rd party BLC**
YLLPAIVHI	***1655 or 10521***	***DDX box 5 or 17***	***5***
VLIPKLPQL	94103	ORM1-like 3	4
ALSDHHIYL	226	aldolase A, fructose-bisphosphate	2
SIIGRLLEV	5499	protein phosphatase 1 alpha	3
LLDRFLATV	***10983***	***cyclin I***	***1***
FIIQGLRSV	3117 or 318	MHC-II, DQ alpha 1 or 2	2
FLDPRPLTV	1545	cytochrome P450, 1B1	n.d.

B. Peptides isolated from human umbilical vein endothelial cells (HUVEC)

Sequence[1)]	**GeneID**	**Protein**	**3rd party BLC**
SLLDKIIGA	284119	polymerase I and transcript release factor	n.d.
SLSEKTVLL	966	CD59 molecule complement regulatory protein	n.d.
TLADIIARL	57523	KIAA1305	n.d.
NLAEDIMRL	7431	vimentin	n.d.
YLPEDFIRV	3656	interleukin-1 receptor-associated kinase 2	n.d.
YLLPAIVHI	***1655 or 10521***	***DDX box 5 or 17***	***5***
NLLPKLHIV	1192 or 25932	chloride intracellular channel 1 or 4	3
LLIENVASL	2876	glutathione peroxidase 1	3
ILTDITKGV	1938	eukaryotic translation elongation factor 2	3
TLSDLRVYL	140809	sulforedoxin 1 homolog	2
LLGPRLVLA	10972	transmembrane emp24-like trafficking protein 10	2
QLIDKVWQL	6397	SEC14-like 1	1
LLDRFLATV	***10983***	***cyclin I***	***1***
SLLDRFLATV	10983	cyclin I	1
LLAAWTARA	351	amyloid beta precursor protein	1

[1)] peptides were identified by tandem mass spectrometry.

n.d. - not detectable. Samples where a peptide was absent respectively below the detection limit of the mass spectrometer.

3.1.2 Comparison of source protein expression in EC and BLC

The most obvious explanation for the preferential representation of MHC class I bound $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ on vascular endothelial cells would be the strong expression of PTRF and CD59 genes. Therefore, we compared levels of the source proteins DDX5, PTRF, and CD59 in EC and syngeneic EBV-immortalized BLC from the same individual as the EC, in the HLA-A*02 positive BLC line JY, in TAP deficient T2 cells and in LS174 colon cancer cells (Figure 4). PTRF was expressed exclusively in EC and was not found in any other cell line analyzed (Figure 4). CD59 was expressed in EC and in two BLC lines, but not in LS174 colon cancer cells. However, the expression level of CD59 in EC was extraordinary strong compared to the BLC (Figure 4). DDX5, the ubiquitously expressed gene that is the source of the allotypic reference peptide presented at large quantities on the HLA-A*02 molecules of most tissues [174], was present in all cell lines analyzed at similar levels (Figure 4). BLC and EC both expressed CD59 protein and therefore the vast abundance of $CD59_{(106-114)}$ peptide on EC compared to BLC cannot be explained by an endothelial cell-specific gene expression. Additional factors such as CD59 protein synthesis and/or degradation rate may be involved in efficient presentation of this peptide on EC.

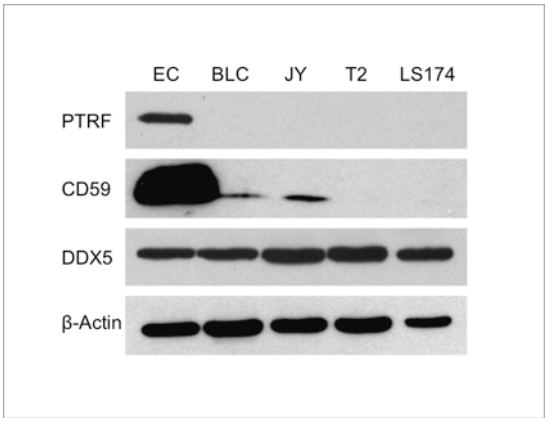


Figure 4. Cellular representation of source proteins that contain the HLA-A*02 presented endothelial peptide sequences. Whole cell lysates were used for western blot obtained from cultured human umbilical vein endothelial cells (EC), B lymphoblastoid cells (BLC), the B-cell line JY, T2 cells, and the colon carcinoma cell line LS174 and analyzed for the presence of the proteins PTRF, CD59 and DDX5. β-Actin was used as loading control.

3.1.3 Comparison of proteasome components in EC and BLC

Proteins are degraded by the proteasome into antigenic peptides. The constitutive proteasome consisting from two outer rings with seven α subunits and two inner rings with seven β subunits is expressed in most cells. In addition, there exists also a second proteasome type, the immunoproteasome. It has been shown that the immunoproteasome can be induced by IFN-γ in some cells which leads to the transcription and translation of the three immunoproteasome subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) [175]. The immunoproteasomes differ from constitutive proteasomes in qualitative and quantitative aspects of their proteolytic activity [142] and they have shown to be important for the

generation of $CD8^+$ T cell epitopes during viral infections and inflammation [144]. We wanted to compare the expression of the constitutive proteasome and the immunoproteasome in EC and BLC. Therefore, we established a western blot for the constitutive proteasome subunit beta-1 and the two immunoproteasome subunits LMP-2 and LMP-7 and compared EC with the BLC line JY, T2 cells and LS174 colon cancer cells (Figure 5). We found in resting EC only expression of the beta-1 subunit of the constitutive proteasome and not of the immunoproteasome subunits, whereas in resting JY cells also the immunoproteasome subunits LMP-2 and LMP-7 were expressed (Figure 5A). T2 cells and the colon carcinoma cell line LS174 expressed also only the constitutive proteasome. However, after stimulation with IFN-γ overnight, expression of the two immunoproteasome subunits LMP-2 and LMP-7 could be induced in EC (Figure 5B).

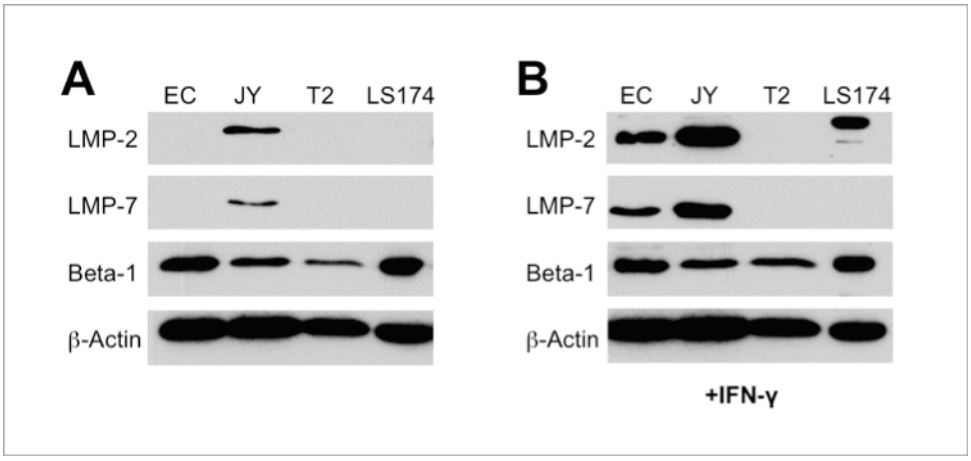


Figure 5. Expression of the constitutive and immunoproteasome. Whole cells lysates from EC, JY, T2 and LS174 cells were analyzed by western blot for the expression of the constitutive proteasome subunit beta-1 and the two immunoproteasome subunits LMP-2 and LMP-7, respectively. (A) Analysis of proteasome expression in resting cells, (B) expression in cells after stimulation with IFN-γ overnight.

3.1.4 Characterization of the HLA-A*02 binding properties of the abundant endothelial peptides

SYFPEITHI based algorithm (https://syfpeithi.de)

In a next step, the binding properties of the abundant endothelial peptides were examined. The assignment of $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ to the specific MHC class I allotype HLA-A*02 and the calculation of the HLA-A*02 binding strength was performed according to the software of the HLA ligand database SYFPEITHI 49 (https://syfpeithi.de) and compared with the known HLA-A*02 restricted peptides $Flu_{(58-66)}$ and $SMCY_{(311-319)}$ as positive controls and the ubiquitously expressed peptide $DDX5_{(168-176)}$ (Table 6). For the five peptides binding

scores between 24 and 30 were predicted (maximal binding score = 36) with $SMCY_{(311-319)}$ as the weakest binder and $Flu_{(58-66)}$ and $DDX5_{(168-176)}$, respectively, as the strongest. The binding of the endothelial peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ was predicted in between.

Table 6. SYFPEITHI binding score

$Flu_{(58-66}$	GILGFVFTL	30
$SMCY_{(311-319)}$	FIDSYICQV	24
$DDX5_{(168-176)}$	YLLPAIVHI	30
$PTRF_{(56-64)}$	SLLDKIIGA	25
$CD59_{(106-114)}$	SLSEKTVLL	29

*Peptide affinity for HLA-A*02: T2 binding and peptide competition assay*

To confirm the predicted values of the SYFPEITHI algorithm the peptides were subsequently tested for HLA-A*02 binding by an assay that is based on the stabilization of HLA-A*02 molecules by binding of exogenously added peptide, the T2 peptide binding assay [165]. TAP-deficient T2 cells were loaded with 10^{-5}M peptide overnight and surface expression of HLA-A*02 was then determined by flow cytometry. We analyzed the binding for the endothelial peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$, and for $DDX5_{(148-156)}$ as ubiquitously expressed peptide. Further, we used the low abundant $SMCY_{(311-319)}$ as control. The fluorescence index was calculated as ∆mean fluorescence intensity (∆MFI) with peptide / ∆MFI without peptide [166]. The fluorescence index was set to 1 for T2 cells without peptide. $SMCY_{(311-319)}$ was the strongest binder, increasing the fluorescence index about 3-fold (3.18±0.2) (Figure 6A). $DDX5_{(168-176)}$ showed a similar binding as $SMCY_{(311-319)}$ with a fluorescence index of 2.92±0.1. In contrast to the prediction of the SYFPEITHI algorithm, $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ both showed a very weak binding with only a small increase in HLA-A*02 expression (2.24±0.1 and 1.41±0.03, respectively).

To confirm the differences in binding a functional assay was performed. In the peptide competition assay, the concentration of peptide in the presence of 10^{-8}M $Flu_{(58-66)}$ peptide was determined at which target cell killing by $Flu_{(58-66)}$-specific CTL was inhibited by 50% Therefore, T2 cells loaded with 10^{-5}M $Flu_{(58-66)}$ were incubated with different concentrations of the peptide of interest ranging from 10^{-4} to 10^{-12}M. Correlating with the results of the T2 binding assay, we found that for $SMCY_{(311-319)}$ a 0.27±0.15, for $DDX5_{(148-156)}$ a 0.62±0.51, for $PTRF_{(56-64)}$ a 1.13±0.92 and for $CD59_{(106-114)}$ a 2.07±1.14 fold higher peptide concentration was necessary to reduce the $Flu_{(58-66)}$-specific lysis to 50% (Figure 6C). According to these results the peptide concentrations were normalized to the competing concentration of $SMCY_{(311-319)}$ which resulted in a 2.4-fold higher concentration for $DDX5_{(148-156)}$, 4.4-fold for

PTRF$_{(56-64)}$ and 8-fold for CD59$_{(106-114)}$. Then, the T2 binding assay was performed again with the adjusted peptide concentrations (Figure 6B) and a similar surface positivity of HLA-A*02 for all four peptides could be detected (fluorescence indexes: SMCY$_{(311-319)}$ 2.38±0.1, DDX5$_{(168-176)}$ 2.91±0.2, PTRF$_{(56-64)}$ 2.45±02, CD59$_{(106-114)}$ 2.58±0.1) (Figure 6B).

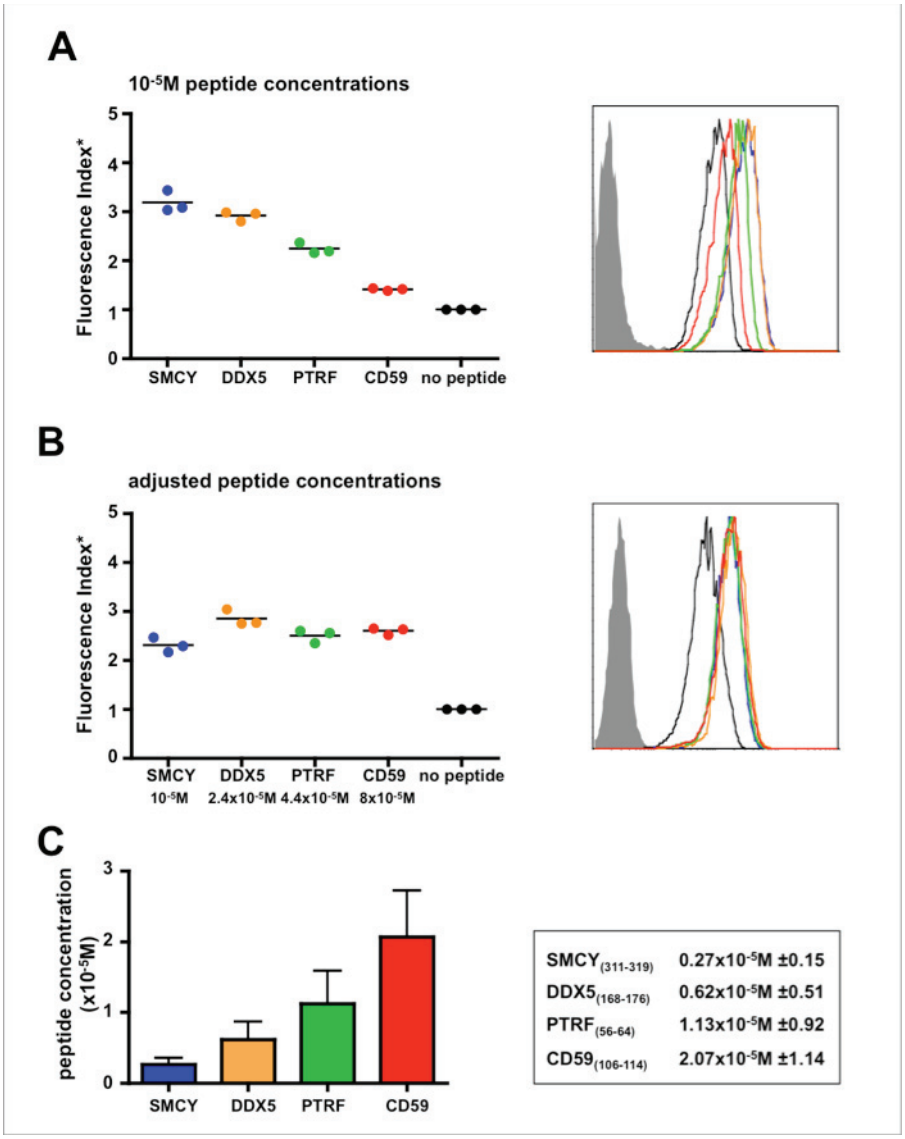


Figure 6. HLA-A*02 binding and peptide competition assay. The T2 peptide binding assay was performed for 4 different allo- and autopeptides (SMCY$_{(311-319)}$, DDX5$_{(148-156)}$, PTRF$_{(56-64)}$ and CD59$_{(106-114)}$) and analyzed by flow cytometry. The fluorescence index was calculated as Δ mean fluorescence intensity (ΔMFI) with peptide / ΔMFI without peptide. (A) shows the fluorescence index and typical histograms with 10^{-5}M peptide concentrations and (B) with peptide concentrations adjusted to binding differences calculated from peptide competition assays. (C) The peptide competition assay shows the peptide concentration (x10^{-5}M) in the presence of 10^{-8}M Flu$_{(58-66)}$ at which target killing by Flu$_{(58-66)}$-specific CTL was inhibited by 50%.

Decay and half-life of peptide-MHC complex

Next, the decay and half-life of the peptide-HLA-A*02 complexes were determined by measuring the HLA-A*02 binding at indicated time points. Therefore, T2 cells were loaded with peptide in adjusted concentrations for 24 hrs. Then, unbound peptide was washed away and HLA-A*02 binding was measured by flow cytometry at different time points (0h, 2h, 6h, 24h). $SMCY_{(311-319)}$, $DDX5_{(168-176)}$, and $PTRF_{(56-64)}$ showed similar kinetics with half-lives between 5.2 and 7 hrs (Figure 7). In contrast, the half-life of $CD59_{(106-114)}$ was only 2.8 hrs and very short compared to the other peptides indicating an unstable peptide-MHC class I complex (Figure 7).

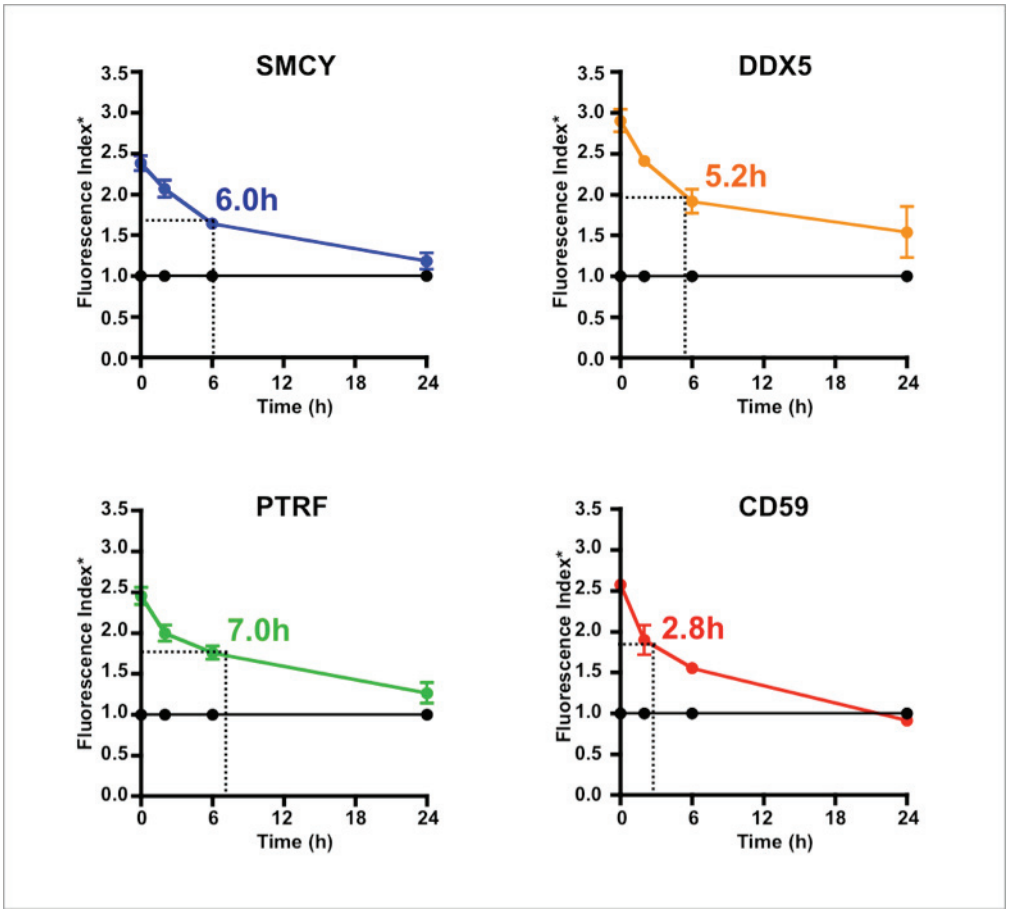


Figure 7. Half-life of HLA-A*02 bound peptides. T2 cells were loaded with the peptides ($SMCY_{(311-319)}$, $DDX5_{(148-156)}$, $PTRF_{(56-64)}$ and $CD59_{(106-114)}$) in adjusted concentrations according to the peptide competition assay and peptide binding was analyzed by flow cytometry at indicated time points (0h, 2h, 6h, 24h).

3.2 Immunogenicity of abundant endothelial cell-selective peptides

3.2.1 Induction of self-reactive CTL

Characterization of monocyte-derived DC as antigen-presenting cells

$CD14^+$ monocytes (purity 78±8.8%) were maturated in vitro into dendritic cell (DC) lines that were used as antigen-presenting cells. These cells were analyzed for the expression of monocyte- and DC-specific markers (Figure 8). They lost the LPS receptor CD14 (20-fold decrease in DC compared with monocytes) but expressed high levels of the costimulatory molecules CD80, CD86 and CD40 (41-, 4- and 7-fold increase, respectively, compared with monocytes), and were uniformly positive for the lineage marker CD83 (1.5-fold increase) (Figure 8B and Table 7). DC generated without final maturation with LPS, TNF-α and IFN-γ expressed lower levels of the costimulatory molecules CD80, CD86, CD40 and CD83 (13.5-, 2.5-, 1.75- and 1.2-fold increase, respectively, compared with monocytes) compared to fully maturated DC. In DC cultures that were used as antigen-presenting cells, a small subpopulation (<10% of total cells) of $CD14^-$, $CD80^-$, $CD86^-$, $CD83^-$ cells was always present. This subset localized in the lymphocyte region (Figure 8A, arrowhead) but contained <5% $CD3^+$ cells and was not further characterized. In order to avoid physical cell injury, DCs were not irradiated before use in cocultures.

Table 7. Characterization of dendritic cells

	Δ mean fluorescence [1]		**fold increase in dendritic cells [2]**
	monocytes	**dendritic cells**	
CD14	718±360	18±21	-20
CD80	0.6±1.2	114±73	41
CD86	18±9	57±38	3.4
CD40	0.4±1.3	18±11	7.0
CD83	1.3±1.1	4±1.9	1.5

[1] Δ mean fluorescence = mean fluorescence “sample” – mean fluorescence “negative control”

[2] fold increase of mean fluorescence

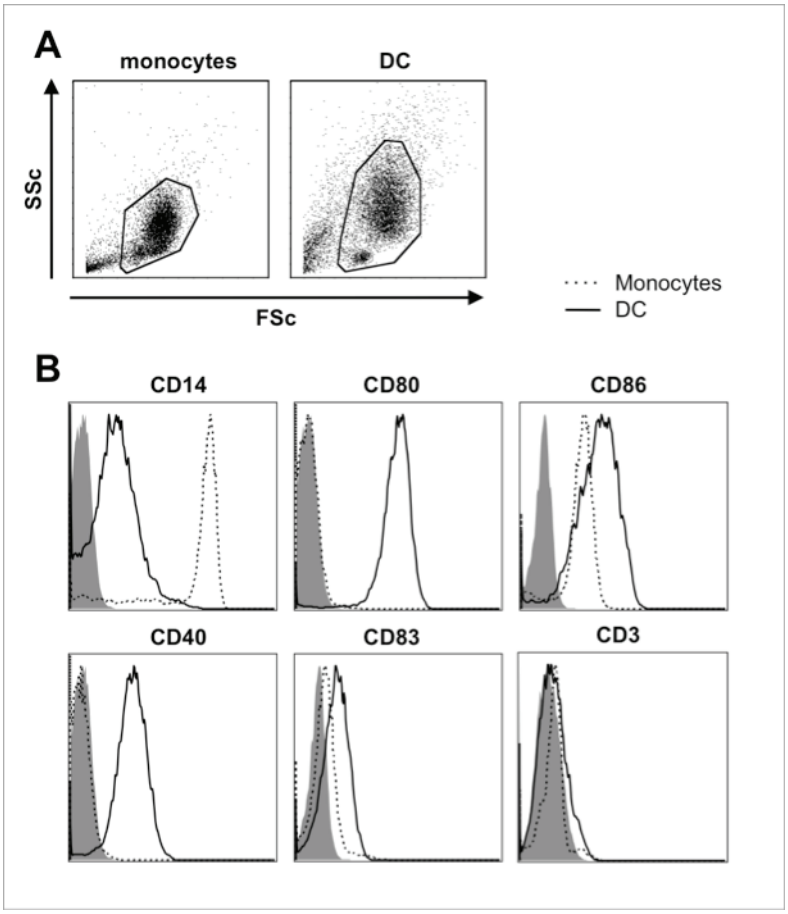


Figure 8. Characterization of monocyte-derived DC. (A) Leukocyte gate of CD14-isolated monocytes and IFN-α maturated DC. (B) Flow cytometry profiles of the staining of monocytes and IFN-α maturated DC for several DC markers. One of 10 experiments with similar outcome is shown.

Induction of peptide-specific CTL by monocyte-derived DC

After optimizing culture conditions, 10 different HLA-A*02 positive healthy male blood donors were tested for the presence of circulating, self-reactive CTL precursors using a coculture system of peptide-pulsed DC as stimulator and syngeneic $CD8^+$ cells as responder cells. We included the immunodominant foreign peptide $Flu_{(58-66)}$ [133, 176] as a positive control in the analysis. $SMCY_{(311-319)}$, [161, 162] and $DDX5_{(168-176)}$ [163] as well as the endothelial-selective peptides $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ were used as self-antigens for male PBMC donors.

The autologous $CD8^+$ responder cells were restimulated weekly with peptide-pulsed PBMC and the functional cytolytic activity of the peptide-specific CTL population was tested in a cytotoxicity assay (Figure 9A-C). For each peptide, 10 independent cultures with cells from 10 different donors were analyzed. For the viral peptide $Flu_{(58-66)}$, all ten induction cultures (100%) displayed potent peptide-specific target cell killing starting after three weeks (Figure 9A, left panel). Under the culture conditions described, self-reactive CTL specific for

SMCY$_{(311-319)}$ emerged in 7 of 10 independent coculture experiments, i.e. 70% of the cultures showed specific target cell lysis after 5 weeks (Figure 9A, right panel). For the endothelial-selective PTRF$_{(56-64)}$ 50% of the cultures showed specific cytotoxic activity after 5 weeks ranging from high killing to lower activity (Figure 9C, left panel). For the highly abundant DDX5$_{(168-176)}$ and CD59$_{(106-114)}$ only poor killing was observed (Figure 9B+C). Cloning of the CTL lines against the highly abundant self-peptides was not successful. These findings suggest that a hierarchy of tolerance against self-peptides exists in healthy blood donors.

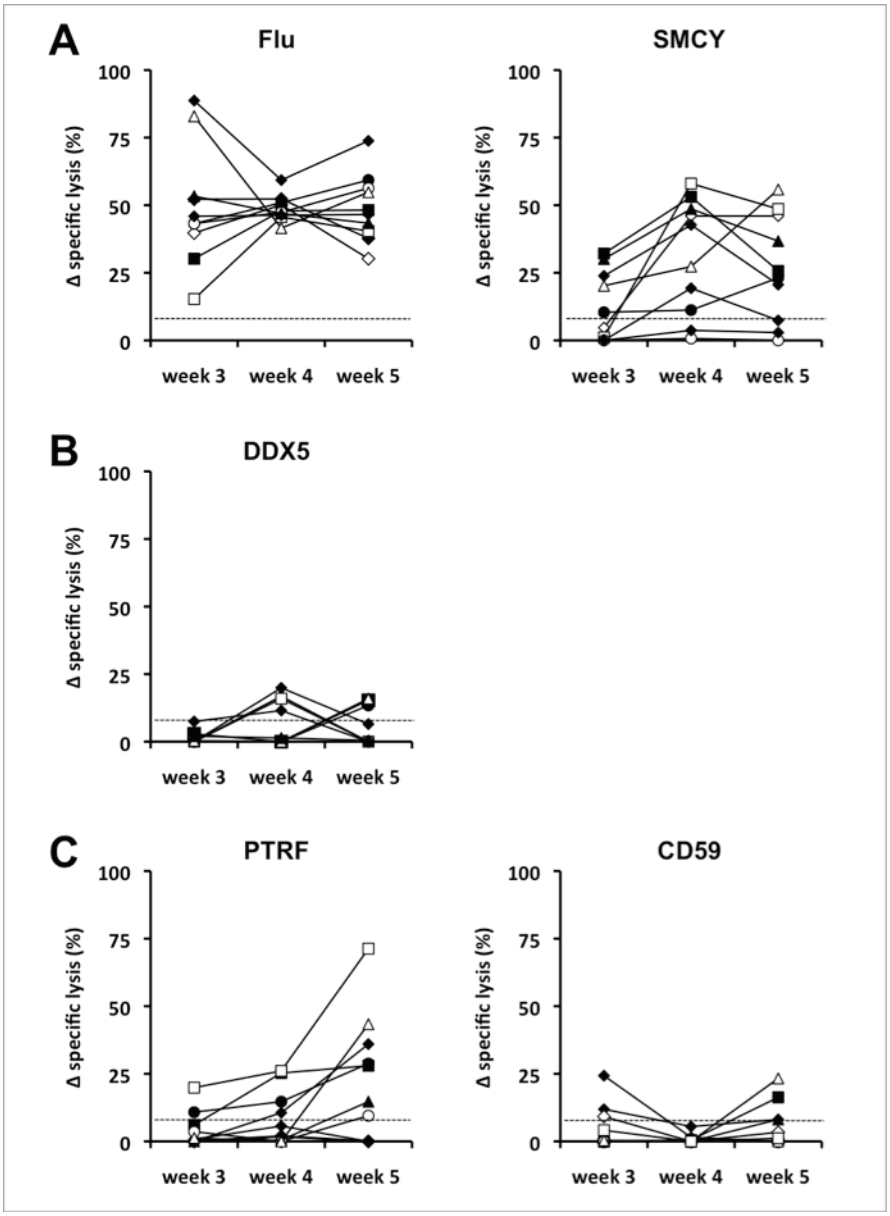


Figure 9. Induction of peptide-specific CTL against foreign and low abundant self-peptides. Cell lines were tested weekly from week 3 to 5 for cytotoxic activity in a calcein-release assay. Peptide specific lysis was determined using peptide-pulsed and non-pulsed T2 as target cells and CTL as effectors at an E:T ratio of 15:1. Shown are Δ specific lysis of 10 induction cultures for each peptide (A-C).

Induction of CTL clones recognizing foreign and self-peptides

CTL lines for $Flu_{(58-66)}$, $SMCY_{(311-319)}$, and $PTRF_{(56-64)}$, respectively, were cloned with limiting dilution at week 5 of the induction culture (Figure 10). All CTL clones were confirmed to express a CD8/CD3 double positive phenotype by flow cytometry (Figure 10D). CTL lines specific for $Flu_{(58-66)}$, and $SMCY_{(311-319)}$, respectively, showed good peptide-specific killing after week 5 of the induction culture and also maintained specificity after cloning with limiting dilution (Figure 10A and B). In contrast, although all selected CTL lines showed good peptide specificity, specific CTL clones against $PTRF_{(56-64)}$ did never emerge (Figure 10C). Either the CTL clones showed nearly no lysis or high unspecific killing. Some of the $PTRF_{(56-64)}$ CTL clones displaying high unspecific lysis were tested on the NK cell target K562. Interestingly, they all showed high killing of K562 cells consistent with NK-like activity (Figure 10C). Overall, 63% of all $PTRF_{(56-64)}$ CTL clones showed a "reprogramming" into a NK-like phenotype (Table 8). The NK-like clones maintained CD8 and CD3 positivity (Figure 11) and showed many features of NK-CTL as high positivity for CD56 and no expression of CD16 (Figure 11).

Table 8. Outcome cloning of $PTRF_{(56-64)}$ CTL lines.

Induction cultures	5
CTL clones	65
CTL clones with **no cytotoxic activity**	24
CTL clones with **NK-like activity**	41

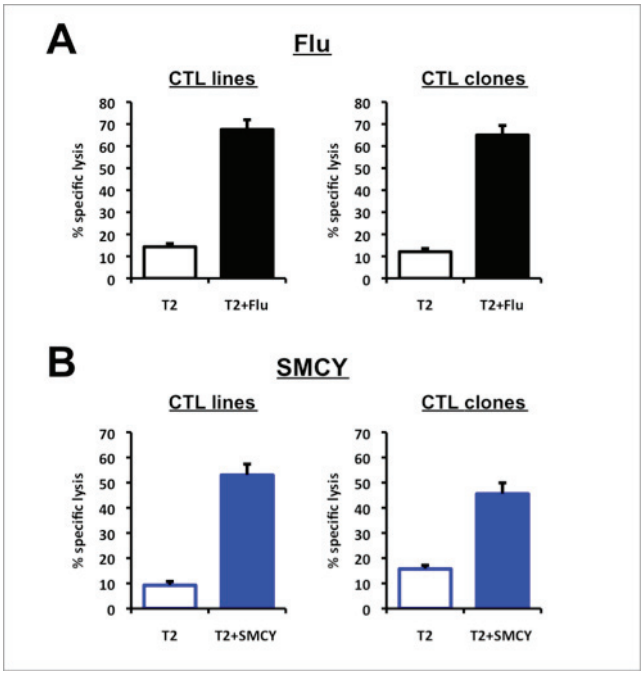
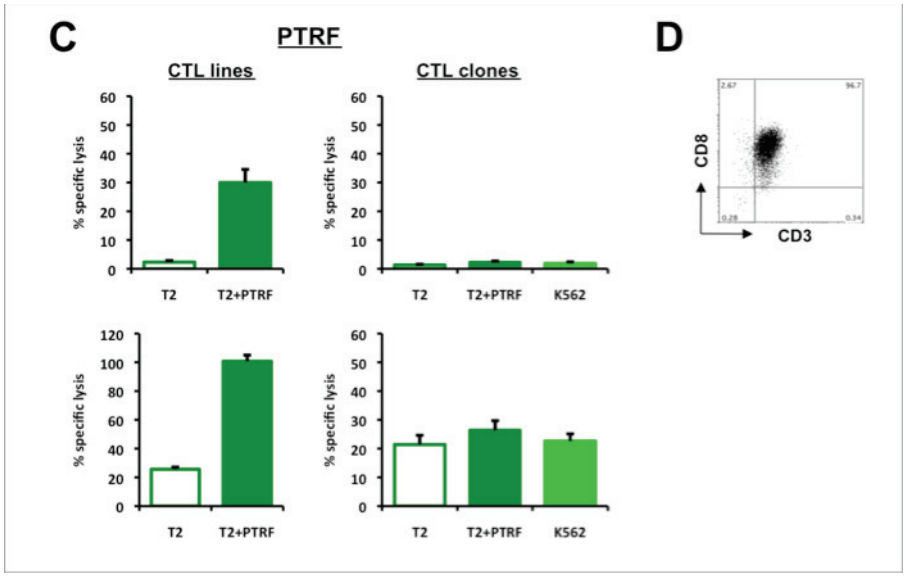


Figure 10. Induction of CTL clones recognizing foreign and self-peptides. (A+B) Specific and unspecific killing for $Flu_{(58-66)}$ and $SMCY_{(311-319)}$-specific CTL lines and the resulting CTL clones. (C) Specific and unspecific target cell killing and killing of K562 cells for $PTRF_{(56-64)}$-specific CTL lines and the resulting 2 phenotypes of CTL clones. E:T ratio was 15:1 for all experiments, shown are mean and standard deviations of one representative experiment in each group.



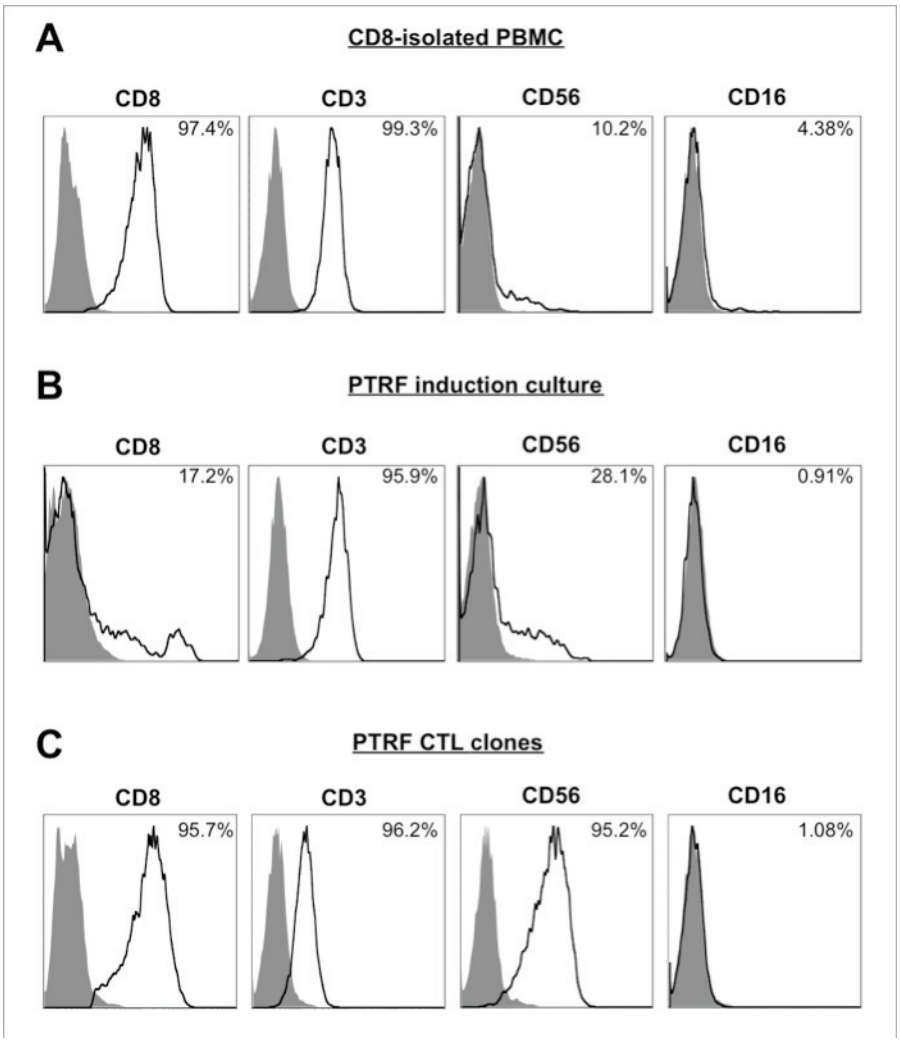


Figure 11. $PTRF_{(56-64)}$-specific CTL clones are NK-CTL. Analysis of NK-CTL marker of CTL clones specific for $PTRF_{(56-64)}$. (A) staining of CD8 cells directly after isolation from PBMC. (B) induction cultures specific for $PTRF_{(56-64)}$. (C) $PTRF_{(56-64)}$-specific CTL clones resulting from cloning with limiting dilution.

Induction of EC-peptide specific CTL with PFA-fixed T2 cells as target cells

A possible reason for the failing induction of peptide-specific CTL for $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ could be the weak HLA-A*02 binding and the unstable peptide-MHC complex. Further, the use of dendritic cells as antigen-presenting cells could provide too many other antigens that are more immunodominant than the endothelial peptides. To optimize both factors $CD8^+$ T cells were cocultured in an allogeneic system with peptide-loaded T2 cells as stimulators to reduce possible other antigenic sites [177]. Further, the peptide-MHC I complex was stabilized by mild paraformaldehyde fixation of stimulator cells [178, 179]. To verify the stabilization of the pMHC complex, HLA-A*02 binding over time was performed with fixed and non-fixed T2 cells. After 4 hrs the decay of the pMHC complex of fixed cells was abolished compared to non-fixed cells (Figure 12A). For the resulting CTL clones neither the reduction of possible antigenic sites nor the stabilization of the pMHC complex had any influence. Like in the autogenic system the $PTRF_{(56-64)}$- but also the resulting $CD59_{(106-114)}$-specific CTL clones showed either no more cytotoxic activity or a change into a NK-like phenotype (Figure 12B and C).

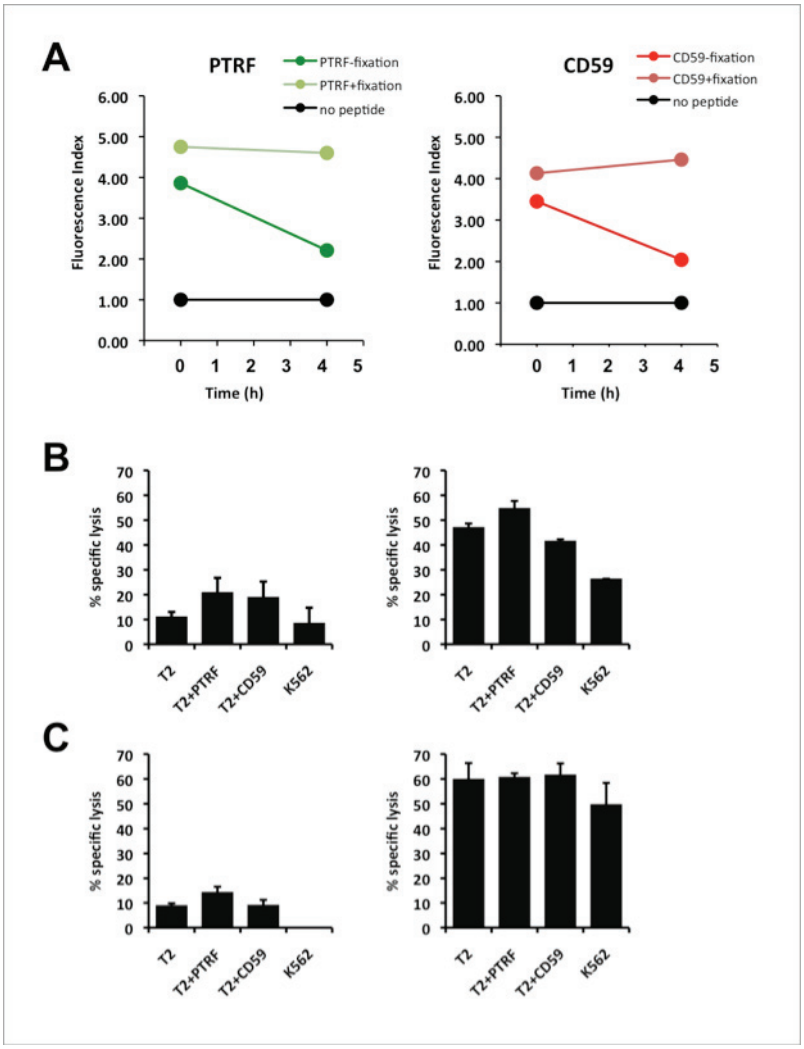


Figure 12. Induction of endothelial peptide-specific CTL with PFA-fixated T2 as target cells. (A) Decay of HLA-A*02 binding after fixation of the peptide-MHC I complex with PHA. (B+C) Δ specific lysis of CTL clones generated from allo-specific cultures either against $PTRF_{(56-64)}$ (B) or $CD59_{(106-114)}$ (C).

Improvement of self-reactive CTL induction by inhibition of PD-L1 and caspases

Programmed death-1 (PD-1)/PD-L1 interactions play an important role in regulating T cell activation [98] and members of the caspase family are involved in activation-induced cell death [171]. Both pathways participate in peripheral tolerance. Therefore, we tested whether inhibiting the two mechanisms in our autologous coculture system would allow the outgrowth of $DDX5_{(168-176)}$ and $PTRF_{(56-64)}$-specific CTL. A monoclonal antibody against PD-L1 and the pancaspase inhibitor z-VAD, respectively, were added to the early cocultures (Figure 13) and peptide-specific cytotoxic activity was analyzed again in weekly intervals.

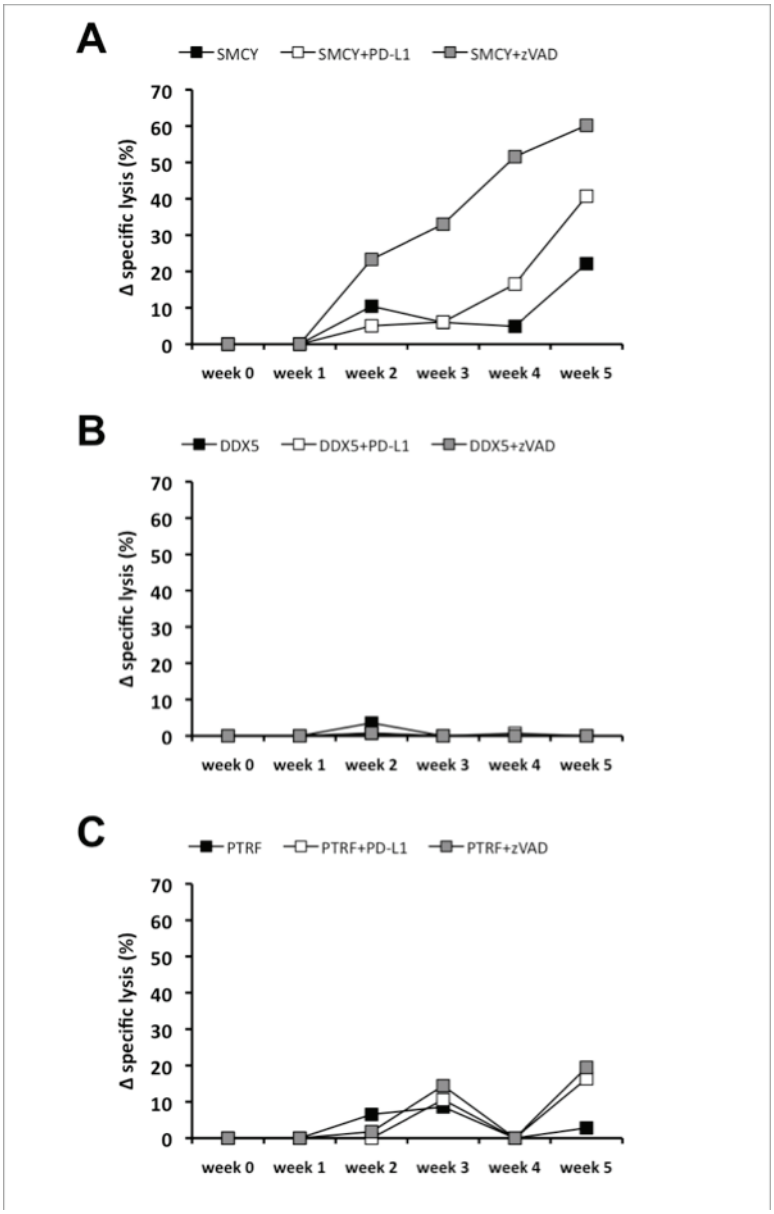


Figure 13. Improvement of self-reactive CTL induction by PD-L1 and caspase inhibition. Induction cultures were treated with an inhibitory anti-PD-L1 antibody and the pancaspase inhibitor z-VAD, respectively. Shown is the Δ specific lysis from week 1 until week 5 compared to untreated cultures for $SMCY_{(311-319)}$ (A), $DDX5_{(148-156)}$ (B) and $PTRF_{(56-64)}$ (C).

For $SMCY_{(311-319)}$-specific cultures inhibited with z-VAD, peptide-specific CTL emerged earlier and displayed more potent specific killing that was detectable after 2 weeks already, compared to 5 weeks in normal cultures without inhibition. The inhibition by anti PD-L1 antibody did mildly accelerate the outgrowth of specific CTL that were clearly detectable after 4 weeks (Figure 13A). For $DDX5_{(168-176)}$, neither anti-PD-L1 nor z-VAD had any influence on the outcome of the coculture and outgrowth of peptide-specific CTL failed under all circumstances tested (Figure 13B). Similar results were obtained for the other abundantly expressed self-peptide $PTRF_{(56-64)}$ (Figure 13C) suggesting that the precursor $CD8^+$ T cell number is substantially lower for $DDX5_{(168-176)}$ and $PTRF_{(56-64)}$ than for $SMCY_{(311-319)}$.

Characterization of T cell subsets

The induction of peptide-specific CTL was not only monitored by weekly analysis of the cytotoxic activity but also by the examination of the different T cell subsets during the time of culture (Figure 14). For $Flu_{(58-66)}$-specific CTL >85% of the cells were double positive for CD8 and CD3 after 6 weeks of culture correlating with the highly specific cytotoxic activity. For CTL lines against $SMCY_{(311-319)}$ about half of the population was CD8 positive whereas also a large part of CD4 positive cells were present. In the cultures induced for $PTRF_{(56-64)}$ we found after 6 weeks up to 75% of the population to be CD4 positive with only a small CD8 positive population (Figure 14, upper and middle panel). Further characterization of these CD4 positive cells showed that for $PTRF_{(56-64)}$ cultures, this population was consistent with $CD4^+CD25^+$ Treg cells (95.7% of $CD4^+$ cells). For $SMCY_{(311-319)}$, 52.6% of the $CD4^+$ cells were corresponding to Tregs (Figure 14, lower panel). A smaller number of $CD4^+CD25^+$ cells also expressed the Treg marker FoxP3 (36.8% in $SMCY_{(311-319)}$ and 14.1% in $PTRF_{(56-64)}$ cultures) (Table 9). From these findings we concluded that in cultures against highly abundant self-peptides Treg cells may play a role in inhibiting the outgrowth of peptide-specific CTL.

Table 9. $CD4^+$ T cells subsets.

	$Flu_{(58-66)}$	**$SMCY_{(311-319)}$**	**$PTRF_{(56-64)}$**
$CD4^+FoxP3^+$	6.54%	0.71%	0.31%
$CD4^+CD25^+$	8.28%	40.9%	72%
$CD4^+FoxP3^+CD25^+$	21.9%	36.8%	14.1%

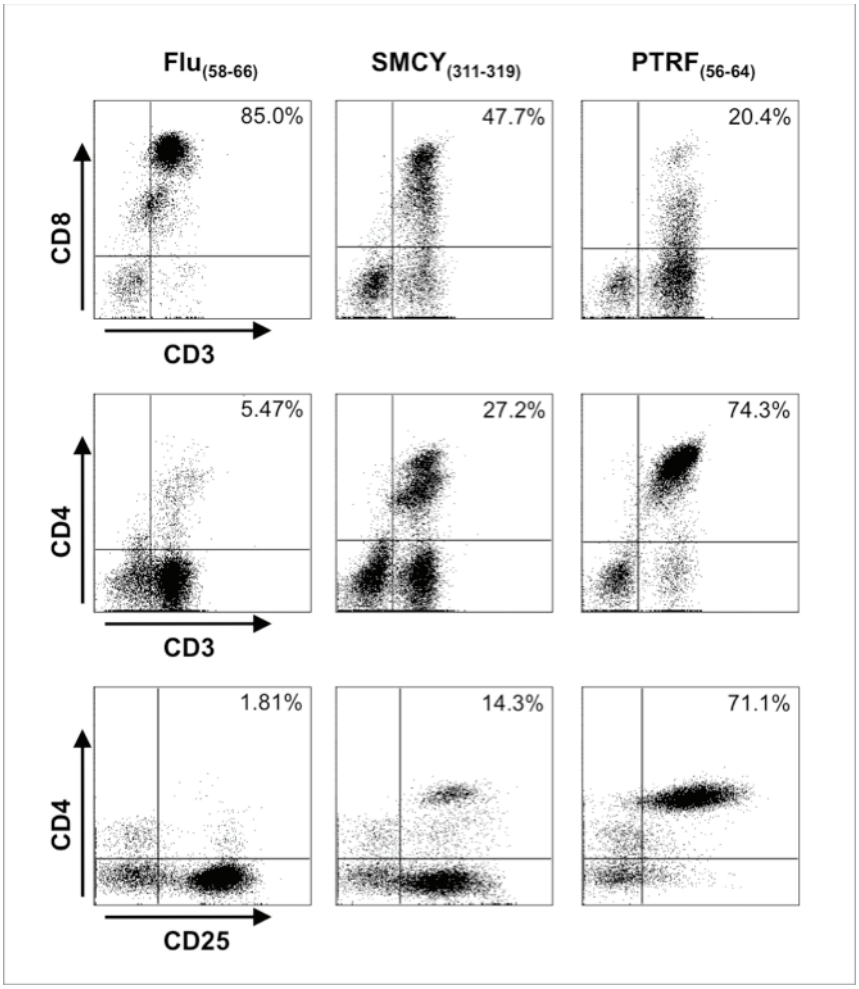


Figure 14. T cell subset analysis of induction cultures at week 6. FACS analysis of CD8/CD4 T cell subsets of $Flu_{(58-66)}$, $SMCY_{(311-319)}$ and $PTRF_{(56-64)}$-specific induction cultures after 6 weeks. The gate was set for CD3 positive cells and CD8 and CD4 positive populations were analyzed (one representative culture).

3.2.2 Characterization of self-reactive CTL

Functional characterization of self- versus alloreactive anti-$SMCY_{(311-319)}$ CTL

Since $SMCY_{(311-319)}$ can be used as self- and allopeptide in this experimental system, we were able to compare self- and alloreactive CTL clones functionally using the same HLA-A*02-peptide complex. Self-reactive CTL lines from induction cultures displaying high peptide-specific cytotoxic activity for $SMCY_{(311-319)}$ in the presence or absence of the pancaspase inhibitor z-VAD were cloned with limiting dilution. The resulting CTL clones specific for $SMCY_{(311-319)}$ as an autoantigen were compared to an alloreactive $SMCY_{(311-319)}$ clone derived from a patient after sex-mismatched stem cell transplantation [121]. First, the

allo- and autoreactive CTL clones were compared in a E:T titration on $SMCY_{(311-319)}$-loaded T2 cells (Figure 15A).

For the allo- and the autoreactive CTL clone two CTL clones and for the auto-zVAD CTL one clone were tested. All five CTL clones showed comparable killing at the same effector:target cell ratios and using the same peptide-saturated target cell. We next determined the peptide concentration at which half maximal target cell lysis was observed. It was $5x10^{-11}$M, $9x10^{-9}$M and 10^{-8}M for allo, auto, and auto-zVAD CTL (Figure 15B). Interestingly, self-reactive CTL clones that emerged in the presence of z-VAD showed a significantly higher plateau killing but similar half-maximal peptide concentration compared with CTL clones grown in the absence of the pancaspase inhibitor. This suggests that z-VAD during coculture affects cell differentiation and potency but not TCR-antigen interactions or affinity of the emerging CTL lines. The same pattern was observed in IFN-γ expression (Figure 15C) but as shown previously [121], more antigen was required to stimulate cytokine production by CTL than to trigger cytolysis. These findings suggest that self-reactive CTL display a lower affinity for the same $SMCY_{(311-319)}$ peptide-HLA-A*02 complex than alloreactive CTL.

This observation was confirmed by a staining with peptide-MHC pentamers (Figure 15D). 10 days after restimulation, alloreactive CTL clones showed uniform and strong staining for CD8 and SMCY-HLA-A*02 pentamer (Figure 15D). The self-reactive CTL clone showed less CD8 expression and virtually no pentamer binding. The high potent, self-reactive CTL clone grown in the presence of z-VAD showed weak but detectably more pentamer binding. However, it was not enough to influence affinity.

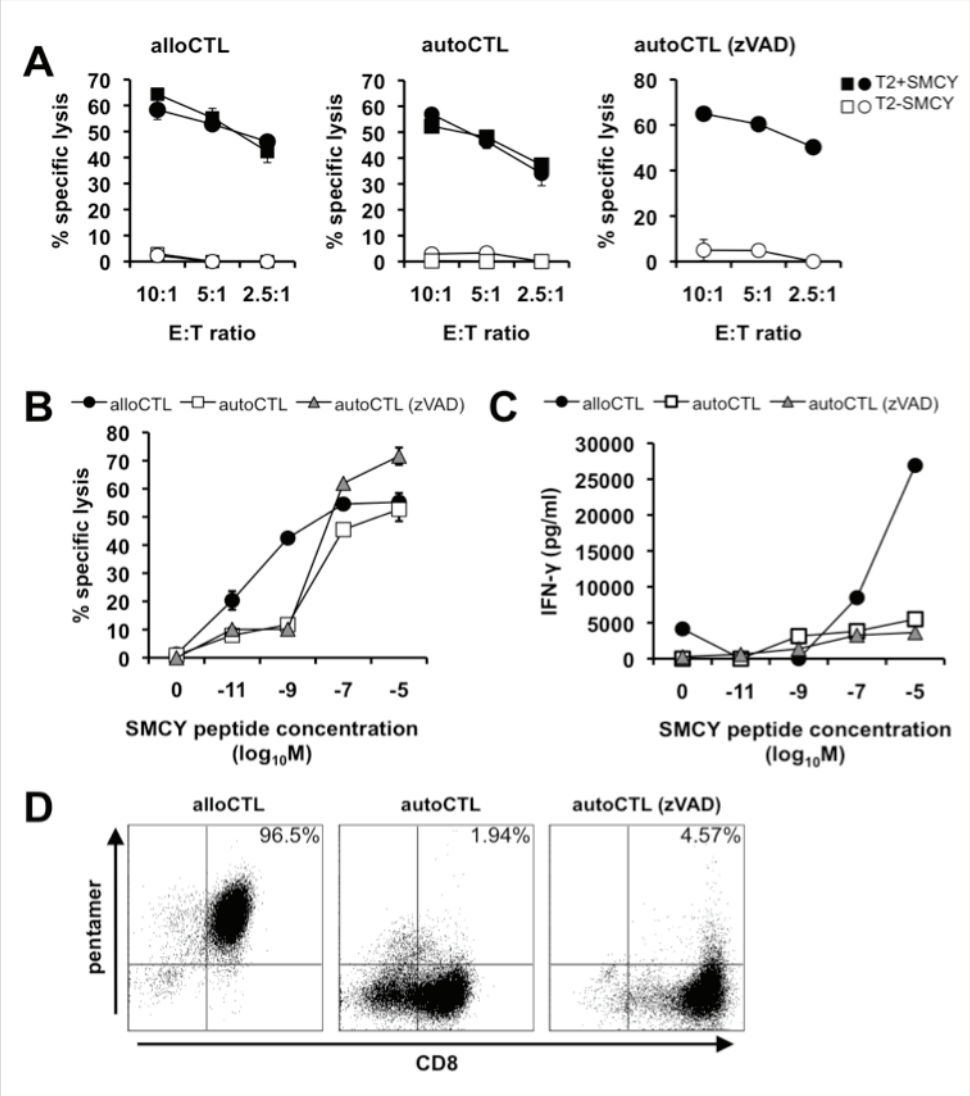


Figure 15. Functional characterization of self- and alloreactive anti-SMCY$_{(311-319)}$ CTL. (A) E:T titration and (B) peptide titration of an allo- and self-reactive CTL clone specific for SMCY$_{(311-319)}$. (C) comparison of IFN-γ expression of allo- and self-reactive SMCY$_{(311-319)}$ CTL after peptide titration. (D) Peptide-MHC multimer and CD8 double staining of allo- and self-reactive anti-SMCY$_{(311-319)}$ CTL.

Recognition of endogenously presented peptide by self- versus alloreactive CTL

We further analyzed the ability of the CTL to recognize endogenously presented SMCY$_{(311-319)}$ in male target cell lines (Figure 16). The HLA-A*02 positive B cell line JY and EC were compared. Target cells were used in killing assays before and after stimulation with IFN-γ (100ng/ml) and TNF-α (50ng/ml), two cytokines that upregulate HLA-A*02 expression synergistically in EC [125] (Figure 16). To assess maximal CTL-mediated cytolysis, T cells were activated by PHA, a lectin which activates the TCR pharmacologically [121]. Alloreactive CTL did effectively lyse male B cells and male EC after activation by proinflammatory cytokines (Figure 16A and B, left panel). In contrast, autoreactive CTL were unable to recognize and kill male B cells or EC even after activation by IFN-γ/TNF-α (Figure

16A and B, right panel). Both allo- and autoreactive $SMCY_{(311-319)}$-specific CTL failed to kill female target cells in the absence of PHA (Figure 16A and B).

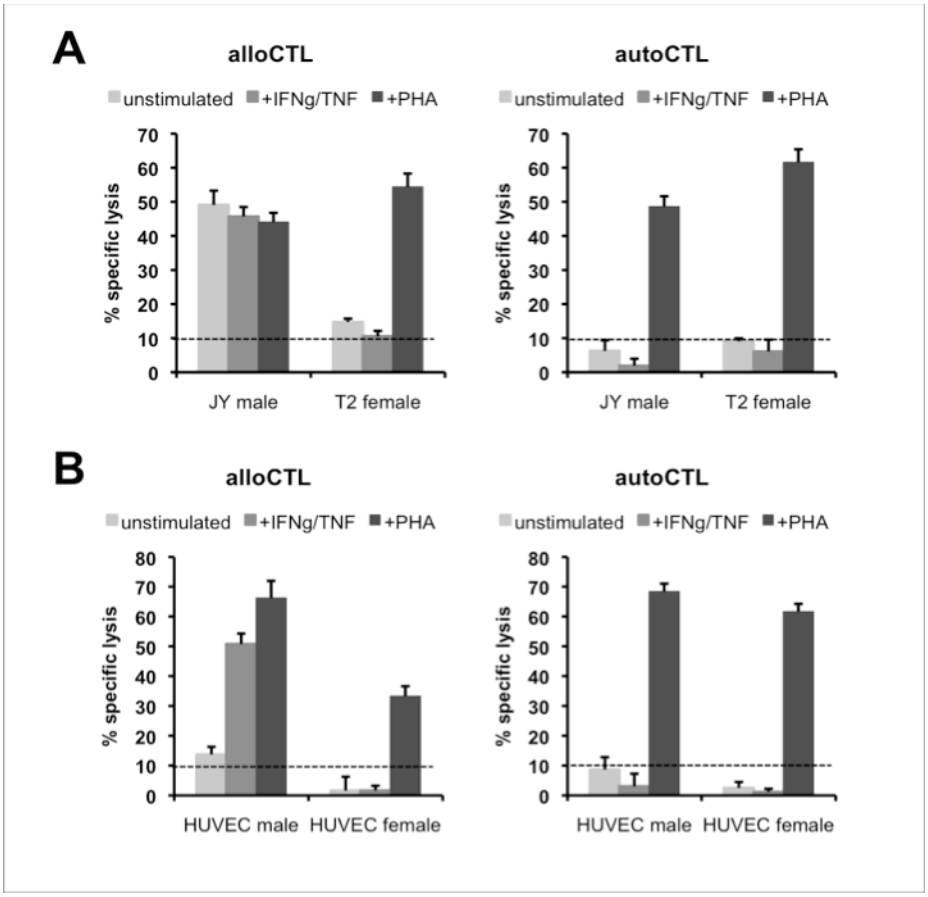


Figure 16. Recognition of endogenously presented $SMCY_{(311-319)}$. The specific lysis of male and female target cells is shown. As targets BLC (A) and EC (B) were compared. Target cells were either unstimulated or CTL were maximally activated by pharmalogical TCR stimulation with PHA-L.

3.3 Immunoprotection of EC by the abundant endothelial peptides

3.3.1 Retroviral transduction of EC and BLC with FluM1

In order to further explore the handling of source proteins for immunodominant viral peptides by EC and to improve their antigen-presenting abilities we wanted to establish a retroviral transduction system for influenza matrix protein (containing $Flu_{(58-66)}$). CTL specific for $Flu_{(58-66)}$ were used as a tool to determine and quantify the amount of $Flu_{(58-66)}$ on the cell surface of EC. The flag-tagged retroviral construct of the full length influenza matrix protein M1 was cloned into the bicistronic expression vector pQXIN (Figure 17). The transduction of EC was successful and stable expression of influenza matrix protein over >40 passages could be

obtained. Expression of the transduced construct could be confirmed both at the mRNA and at the protein levels (Figure 17A and B). Immunofluorescence staining for Flag showed >90% of the EC expressing the construct (Figure 17C). As a control the cells were stained for the endothelial cell marker von Willebrand-Factor (vWF).

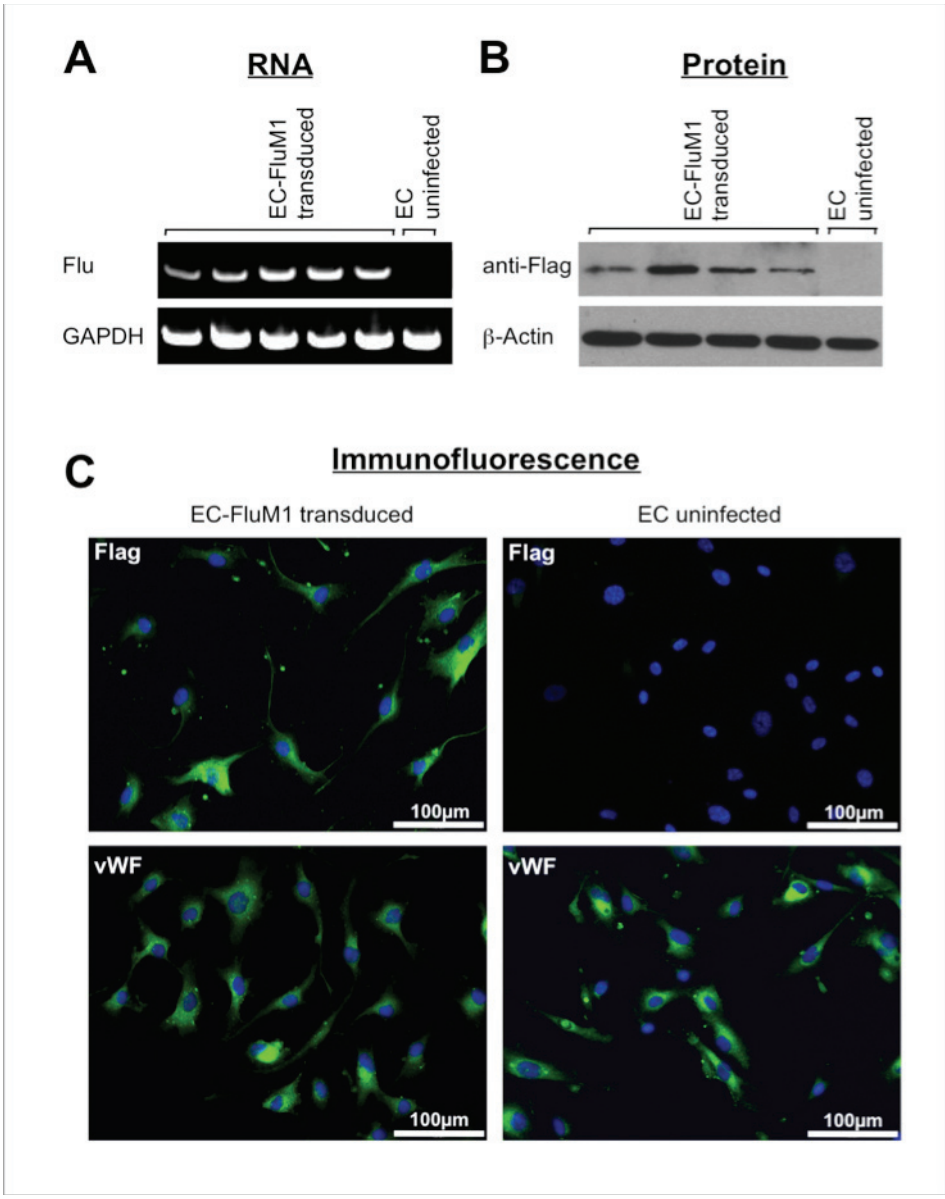


Figure 17. Characterization of influenza matrix protein M1 transduced EC. (A) mRNA expression of Flu in 5 transduced EC lines and in uninfected EC as control. (B) Western blot of FluM1 transduced and non-transduced EC. (C) Immunofluorescence staining of transduced EC for Flag and vWF showing >95% positivity for the transduced construct.

To compare the antigen processing and presentation of EC with another cell line we wanted to transduce also a B cell line. Therefore, we chose the male HLA-A*02 positive JY cell line, which was also retrovirally transduced for FluM1. The expression of the transduced construct

could be confirmed by mRNA expression and immunofluorescence (Figure 18 A and B). In contrast to the EC, FluM1 expression in JY cells was not stable and the transduced construct was lost after 4 to 5 passages. Therefore, transduced JY cells could not be used as a control cell line.

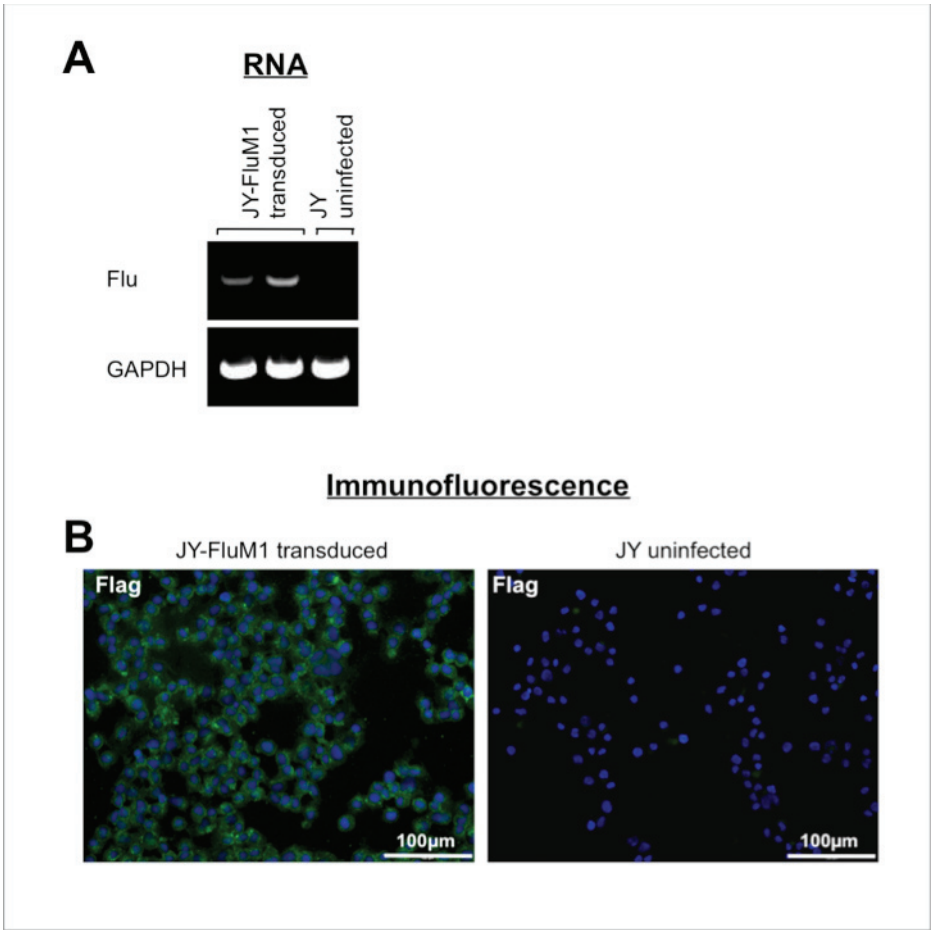


Figure 18. Characterization of influenza matrix protein M1 transduced JY cells. (A) mRNA expression of Flu in 2 transduced JY lines and in uninfected JY as control. (B) Immunofluorescence staining of transduced JY showing over 95% positivity for the transduced construct.

3.3.2 Quantification of $Flu_{(58-66)}$ presented by FluM1 transduced EC

We compared $Flu_{(58-66)}$-loaded EC and T2 cells in a peptide titration (Figure 19A). $Flu_{(58-66)}$-loaded EC are killed less efficiently by a $Flu_{(58-66)}$ specific CTL clone than T2 cells loaded with the same peptide. The peptide concentration at which half-maximal target cell lysis was observed was $5x10^{-9}$M for EC and $2x10^{-11}$M for T2 cells. The findings were confirmed regarding IFN-γ expression (Figure 19B), but as previously shown, more antigen was required to stimulate cytokine production by CTL than to trigger cytolysis.

We next analyzed the ability of the CTL to recognize endogenously presented $Flu_{(58-66)}$ in FluM1 transduced EC (Figure 19C). HLA-A*02 positive and negative EC-Flu were compared.

As a negative control, uninfected EC were used. Target cells were used in killing assays before and after stimulation with IFN-γ (100ng/ml) and TNF-α (50ng/ml). CTL were used at an effector:target cell ratio of 10:1. $Flu_{(58-66)}$ CTL did effectively lyse HLA-A*02 positive FluM1 transduced EC after activation by proinflammatory cytokines (Figure 19C). In contrast, the CTL showed only low or no killing of resting HLA-A*02 positive EC-Flu. (Figure 19C). As expected, $Flu_{(58-66)}$-specific CTL failed to kill HLA-A*02 negative or uninfected target cells. When the cytotoxicity assay was performed with higher effector cell numbers the specific lysis increased of both EC-Flu with and without stimulation with IFN-γ/TNF-α (Figure 19D).

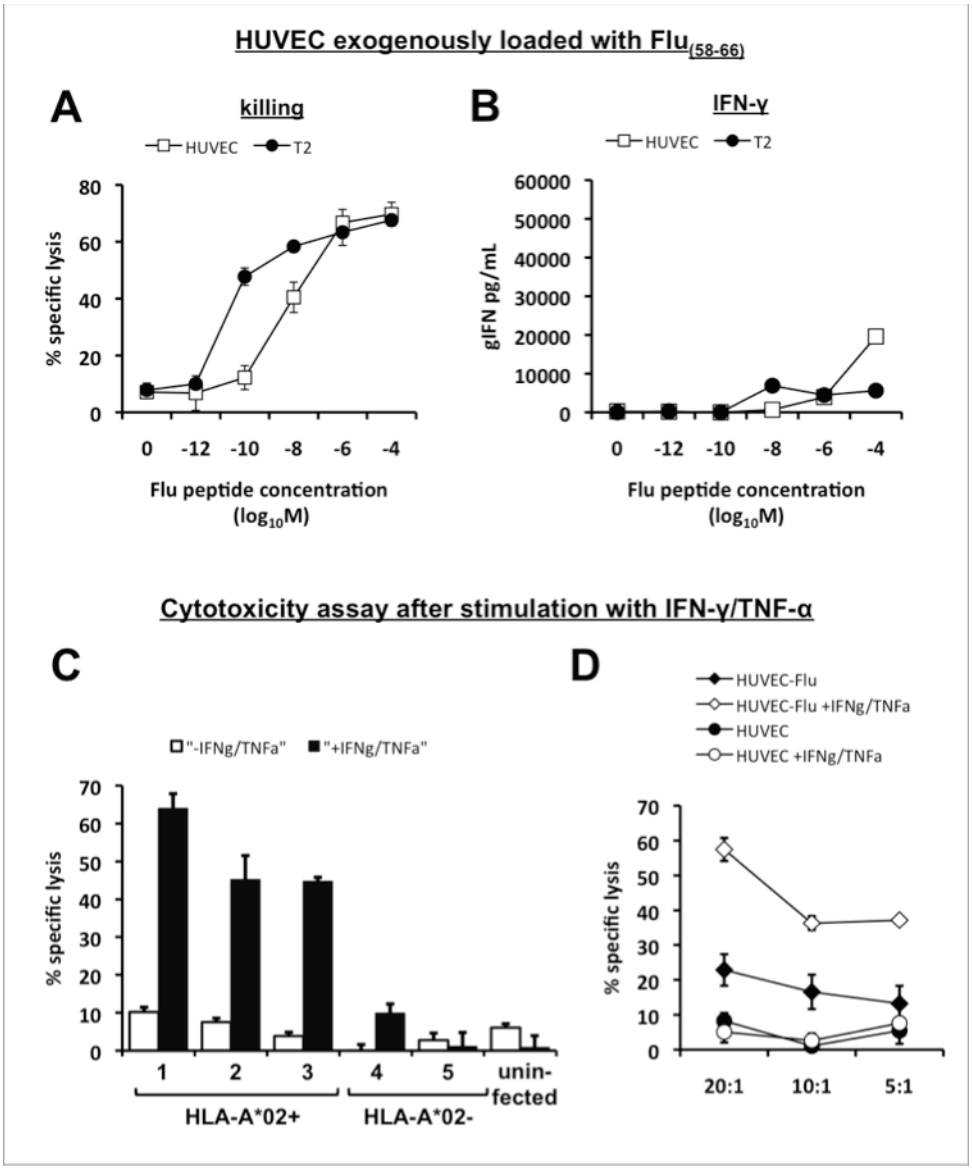


Figure 19. Lysis of FluM1 transduced EC. Comparison of peptide titration of $Flu_{(58-66)}$-loaded EC and T2 cells for specific lysis (A) and IFN-γ expression (B). (C) Specific lysis of FluM1 transduced EC by $Flu_{(58-66)}$-specific CTL. 3 transduced HLA-A*02 positive and 2 negative EC-Flu lines were tested. Uninfected EC were used as control. Cells were either resting or stimulated with IFN-γ/TNF-α overnight. (D) E:T titration of transduced and uninfected EC with or without cytokine stimulation.

As a second approach to quantify the amount of presented $Flu_{(58-66)}$ on EC and T2 cells, $Flu_{(58-66)}$/HLA-A*02-specific F(ab) M1-D12 fragments were used [180]. This recombinant humanized F(ab) monomer is specific for $Flu_{(58-66)}$ bound to HLA-A*02 and therefore can be considered a TCR-like protein. Compared with CTL-mediated target cell lysis, F(ab) binding to the pMHC complex has been shown to be less sensitive to detect antigenic peptides on the surface of target cells [121]. Despite this restriction, T2 cells exogenously loaded with $Flu_{(58-66)}$ could be detected by the monomeric recombinant F(ab). F(ab) binding was easily detectable at 10^{-4}M peptide concentrations and gradually reduced at decreasing peptide loading concentrations up to 10^{-7}M. (Figure 20A). However, the sensitivity of the F(ab) fragments was too weak to detect endogenously processed $Flu_{(58-66)}$ on FluM1 transduced EC (Figure 20B).

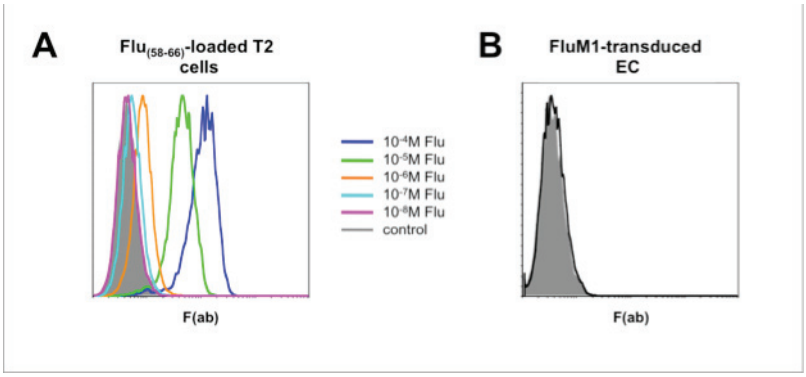


Figure 20. Quantification of presented $Flu_{(58-66)}$ by F(ab) fragments. The detection of presented $Flu_{(58-66)}$ by F(ab) fragments was tested on peptide-loaded T2 cells and transduced EC. (A) T2 cells loaded with $Flu_{(58-66)}$ in concentrations ranging from 10^{-4} to 10^{-8}M. (B) FluM1 transduced EC.

3.3.3 Effect of cytokines on Flag and $Flu_{(58-66)}$ expression on FluM1 transduced EC

IFN-γ and TNF-α are two cytokines that upregulate HLA-A*02 expression synergistically in EC (Figure 21). Therefore, we were interested to test the effect of both cytokines separately and in combination on FluM1 transduced EC. We compared Flag expression by flow cytometry on EC that were either unstimulated or stimulated with IFN-γ, TNF-α or both IFN-γ/TNF-α overnight (Figure 21A and B). As a control served uninfected EC. Stimulation with IFN-γ or TNF-α alone increased Flag expression compared to unstimulated EC, whereas stimulation with TNF-α led to a higher Flag expression than IFN-γ. However, the combination of both cytokines was more potent than stimulation with a single cytokine (Figure 21A and B). The same pattern was found when the specific lysis of FluM1-transduced EC was analyzed (Figure 21C). EC-Flu stimulated with TNF-α were killed to a higher percentage as targets stimulated with IFN-γ or unstimulated. Transduced EC stimulated with the combination of both cytokines led to a cytotoxic activity that reached nearly the levels of maximal CTL

activation by PHA. Stimulation with proinflammatory cytokines led further to an upregulation of several adhesion molecules like VCAM-1, E-Selectin and LFA-3 (Figure 21D). Therefore, the presentation of $Flu_{(58-66)}$ by transduced EC can be gradually enhanced after stimulation with IFN-γ or TNF-α or both. The increase in $Flu_{(58-66)}$ presentation is due to both upregulation of surface molecules and activation of transcription.

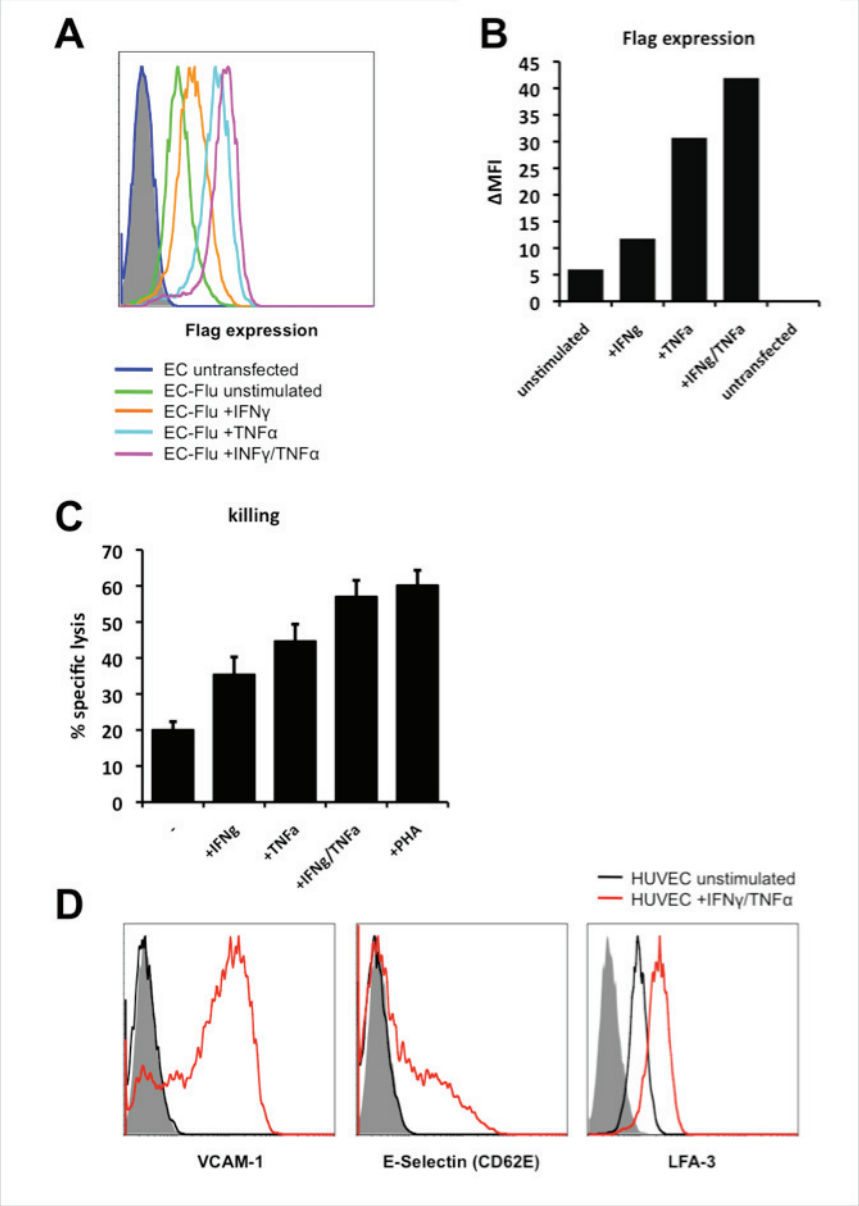


Figure 21. Effect of cytokines on Flag expression and lysis of transduced EC. Flag expression of transduced EC was analyzed by flow cytometry after stimulation with either IFN-γ, TNF-α or both IFN-γ/TNF-α. As control, uninfected or transduced unstimulated EC were used. (A) FACS profiles and (B) ΔMFI of Flag expression. (C) Specific lysis of transduced EC with different stimulation. (D) Upregulation of adhesion molecules VCAM-1, E-selectin and LFA-3 after IFN-γ/TNF-α stimulation.

3.3.4 Proteasome inhibition experiments

The supply of peptide into the MHC class I pathway is amongst other factors regulated by the degradation of proteins by the proteasome. Therefore, we wanted to examine whether peptide presentation on EC can be influenced by inhibiting proteasomic degradation and determine the time span up to the disappearing of peptide on the cell surface. To inhibit proteasomic degradation an irreversible proteasome inhibitor, clasto-lactacystein β-lactone, was used. Addition of 1 µM clasto-lactacystein β-lactone to cultured male EC-Flu led to a transient down-regulation of both $Flu_{(58-66)}$ and $SMCY_{(311-319)}$ expression (Figure 22A and B). The effect started already after 2 hours with maximal inhibition after 4 hours and lasted up to 24 hours. The inhibition of $Flu_{(58-66)}$ expression reached higher levels than of $SMCY_{(311-319)}$. Addition of the proteasome inhibitor led specifically to a decrease of presented peptide and had no effect on HLA-A*02 expression as was confirmed by flow cytometry (Figure 22C+D).

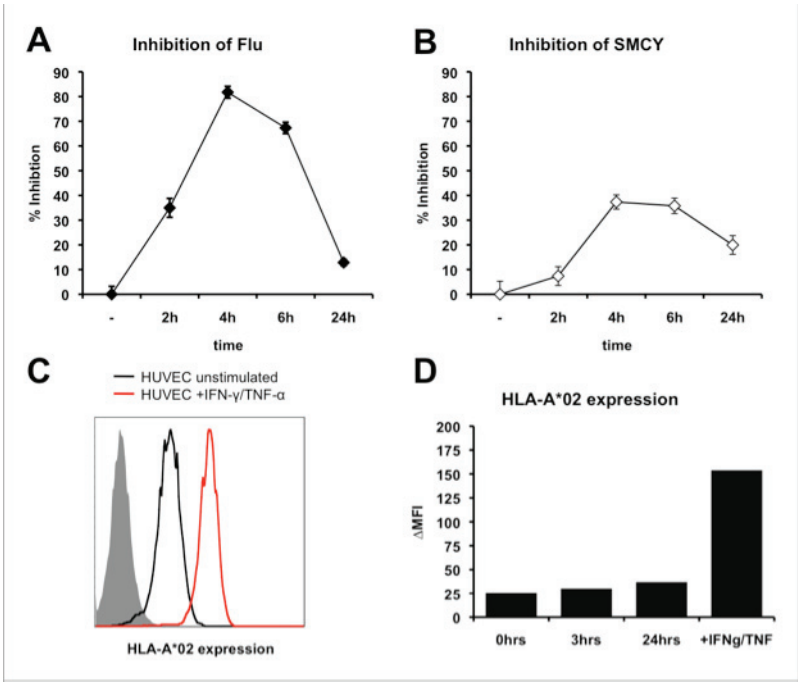


Figure 22. Effect of proteasome inhibition on peptide and HLA-A*02 expression of EC. Male FluM1 transduced EC were incubated with clasto-lactacystein β-lactone for different time points (0, 2, 4, 6, 24h). % inhibiton of $Flu_{(58-66)}$-specific (A) or $SMCY_{(311-319)}$-specific lysis (B) was analyzed. (C) Upregulation of HLA-A*02 expression upon stimulation with IFN-γ/TNF-α. (D) After addition of the proteasome inhibitor HLA-A*02 expression was monitored for different time points (0, 3, 24h).

3.3.5 siRNA knockdown of abundant EC peptides

RNA interference (RNAi) is a powerful tool that can be used to manipulate gene expression [181, 182]. One technique of expressing the short double-stranded RNA intermediates required for interference in mammalian systems is the introduction of short-interfering (si)

RNAs. The proteasome inhibition experiments showed that after inhibiting the supply of peptide into the MHC class I presentation pathway the presented peptide on the surface dissociated rapidly. Therefore, it was feasible to use as a second approach to modify the endothelial peptide repertoire siRNA knockdown of the abundant endothelial peptides. First, siRNA for $DDX5_{(168-176)}$, $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ was designed using a online software tool provided by Microsynth. Transduced EC were transiently transfected with siRNA using a Lipofectamine-based system. Knockdown of all proteins was confirmed by western blot (Figure 23). As a negative control, EC transfected with mock siRNA were used. For $DDX5_{(168-176)}$ a nearly complete knockdown was accomplished after 24 hours and lasted up to 96 hours. For $CD59_{(106-114)}$, protein levels started to decrease after 48 hours and were completely knocked down after 72 hours. $PTRF_{(56-64)}$ knockdown started after 48 hours with a maximum at 72 hours. Protein levels increased again after 96 hours. For $PTRF_{(56-64)}$ no complete knockdown was accomplished but protein levels could be reduced to <20%. Further experiments were performed at 72 hours after siRNA knockdown since at this time point all proteins reached minimal expression levels.

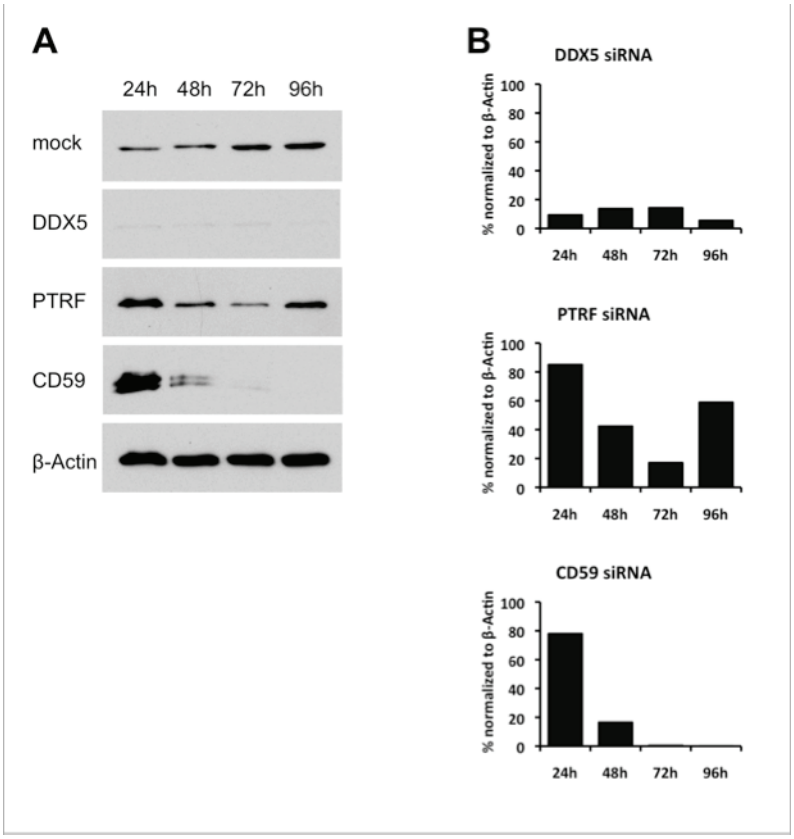


Figure 23. siRNA knockdown of endothelial-specific proteins. (A) Full protein lysates of EC after different time points (24h, 48h, 72h, 96h) after siRNA knockdown were analyzed by western blot. (B) Expression of the protein normalized to β-Actin expression.

3.3.6 Improved presentation of immunogenic peptides after knockdown of abundant EC-specific peptides

We hypothesized that EC may be protected by the presentation of the abundant endothelial peptides from CTL-mediated lysis. Therefore, simultaneous knockdown of $PTRF_{(56-64)}$, $CD59_{(106-114)}$ and $DDX5_{(168-176)}$ on uninfected and FluM1 transduced EC was performed and differences in $Flu_{(58-66)}$ as well as $SMCY_{(311-319)}$ expression were quantified by $Flu_{(58-66)}$ / $SMCY_{(311-319)}$-specific CTL after 72 hours. As a control, mock siRNA transduced EC were used. We found that after complete knockdown the presentation of both endogenously presented $Flu_{(58-66)}$ (Figure 24A and B) and $SMCY_{(311-319)}$ (Figure 24C and D) was increased (Figure 24), whereas after stimulation of EC with IFN-γ and TNF-α the presentation of both peptides decreased. For uninfected EC exogenously loaded with $Flu_{(58-66)}$ the presentation of peptide was slightly increased (1.35±0.36-fold) after knockdown whereas for $SMCY_{(311-319)}$ nearly no change was seen (1.04±0.18-fold increase). In contrast, endogenous $Flu_{(58-66)}$ was expressed at significantly higher levels after knockdown (1.79±0.37-fold, *p=0.0335) (Figure 24B). For $SMCY_{(311-319)}$ the increase in expression was slightly lower but still significant with 1.50±0.32-fold higher levels (*p=0.0300) (Figure 24D).

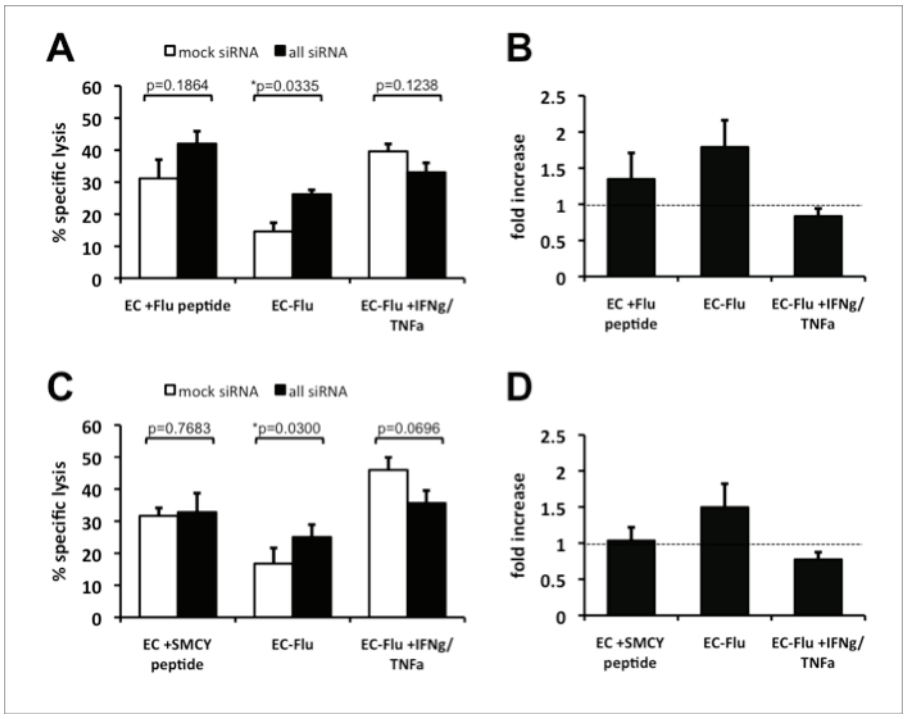


Figure 24. Increased presentation of $Flu_{(58-66)}$ and $SMCY_{(311-319)}$ after knockdown of endothelial-specific proteins. Uninfected and transduced EC that were unstimulated or stimulated with IFN-γ/TNF-α overnight were treated either with siRNA for $PTRF_{(56-64)}$/$CD59_{(106-114)}$/$DDX5_{(168-176)}$ or with mock siRNA. After 72 hours, specific lysis by $Flu_{(58-66)}$- (A+B) or $SMCY_{(311-319)}$-specific (C+D) CTL was analyzed. (A+C) shows the % specific lysis for $Flu_{(58-66)}$ and $SMCY_{(311-319)}$, respectively, (B+D) the fold increase.

Although RNA interference strategies are reliant on a high degree of specificity, there might also potential non-specific effects be induced. It has been shown that transfection of siRNAs leads in IFN-mediated activation of the JAK–STAT pathway and global upregulation of IFN-stimulated genes that may result in a general upregulation of adhesion and MHC class I molecules on EC [183]. To exclude the possibility of an increased peptide presentation due to upregulation of HLA-A*02 expression by siRNA we analyzed HLA-A*02 and MHC class I levels on untreated EC and cells treated with mock or knockdown siRNA. We found that there was no difference in HLA-A*02 (Figure 25A) and MHC class I (Figure 25B) expression between all conditions, thus, induction of peptide levels due to an increase in HLA-A*02 expression could be excluded.

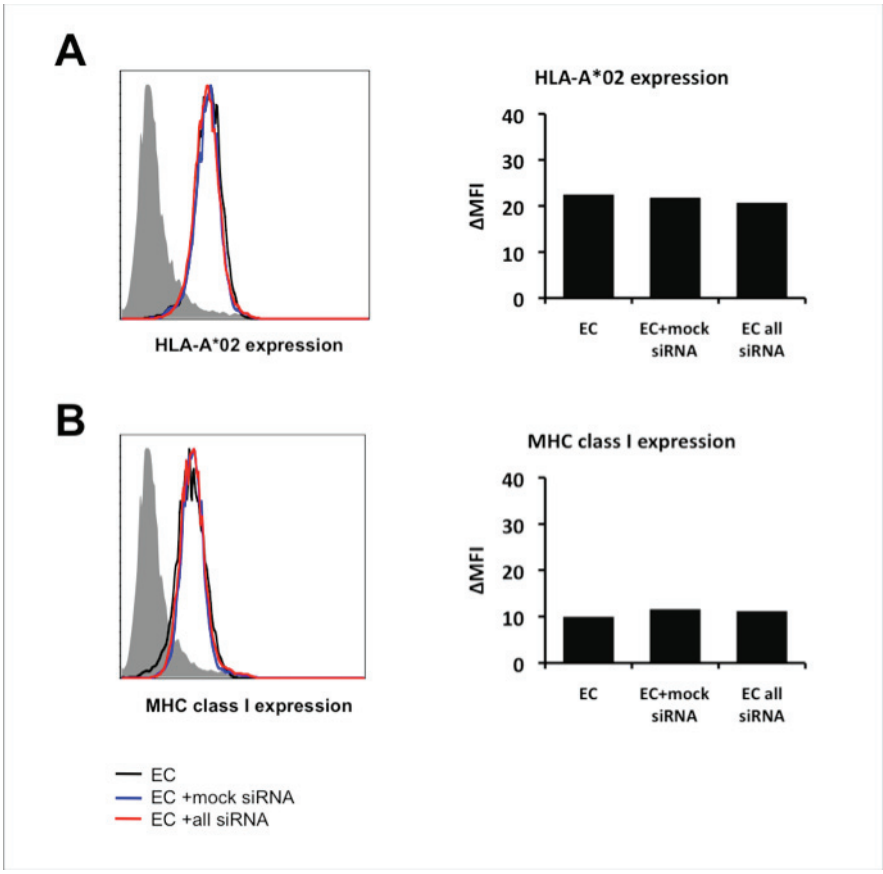


Figure 25. HLA-A*02 and MHC class I expression levels after siRNA knockdown. Staining for HLA-A*02 and MHC class I was performed on EC that were untreated or treated either with mock siRNA or siRNA for $PTRF_{(56-64)}$/$CD59_{(106-114)}$/$DDX5_{(168-176)}$. HLA-A*02 (A) and MHC class I (B) expression were analyzed by flow cytometry and the ΔMFI for each condition was calculated.

4. Discussion

In this study we have examined endothelial cells as targets for antigen-specific cytotoxic T lymphocytes. Several findings supported the accumulating evidence that EC present a substantially different repertoire of MHC class I ligands compared with syngeneic leukocyte-derived cells and that they are protected from CTL-mediated lysis by this mechanism. Here, we could show for the first time that EC present a quantitatively different peptide repertoire with abundance of $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ that protects EC from CTL-mediated lysis by competition with immunogenic peptides for the MHC class I binding site. The abundance of the endothelial peptides is mainly caused by the preferential expression of the source proteins. Under conditions that were sufficient to generate CTL specific for low abundant self-peptides induction of CTL against $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ failed. Improvement of culture conditions by stabilization of the pMHC I complex or interference with inhibitory pathways affected again only the induction of low abundant self-peptides but not EC-specific peptides indicating that these peptides are particularly tolerogenic. After modification of the endothelial peptide repertoire and knockdown of the abundant peptides, EC became better targets for CTL by increased presentation of immunogenic peptides indicating that $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ can protect EC by hiding them from CTL-mediated lysis.

4.1 The functional role and significance of cell-specifically different peptide repertoires

Compared to leukocyte-derived cells, EC present a quantitatively different peptide repertoire with abundance of two peptides, $PTRF_{(56-64)}$ and $CD59_{(106-114)}$. We found the abundance of the endothelial peptides to be mainly caused on the side of processing with a preferential expression of the source proteins. The presentation of $PTRF_{(56-64)}$ exclusively on EC was explained by the endothelium-specific expression of the source protein. This finding is consistent with previous studies that revealed PTRF as a major caveolae-associated protein [153] as caveolae are particularly abundant in adipocytes and endothelial cells. In contrast, CD59 was found in different cell lines although at a quantitatively different level with a much higher expression in EC. The strong expression of the precursor proteins is even able to compensate the low binding affinity of the endothelial peptides to HLA-A*02 and their short half-life. It has been showed that one important factor for the immunogenicity of a peptide is the stability of the pMHC complex [146, 184]. Peptides binding with lower affinity to the MHC

class I molecule may form less stable pMHC complexes leading to decreased immunogenicity. The presentation of those weak binding peptides may therefore be one of the mechanisms to protect EC from CTL mediated lysis.

There have been several studies about the importance of cell type-specific peptide presentation. In the context of allogeneic bone marrow transplantation (BMT) it has been shown that CTL directed against minor histocompatibility antigens (mHA) of the patient generally cause GVHD, which although a complication in allogeneic BMT, is strongly associated with graft-versus-leukemia (GVL) reactivity [185, 186]. The expression of some mHAs is restricted to hemopoietic cells, including leukemic cells, others are expressed by cells of all tissues [187, 188]. Since mHAs expressed by all host tissues induce GVHD and GVL, it is of great importance to identify leukemic restricted Ags because they exert antileukemia reactivity without GVHD. Dolstra et al. identified a CTL clone against a mHA with restricted tissue distribution, the B cell leukemia-associated mHA HB-1 [189]. HB-1-specific CTL exhibited specific cytotoxicity toward leukemic as well as EBV-transformed B cells, but not against untransformed B cells or other cell lines. These antigens are of potential use in immunotherapy against leukemia because they generate antileukemia reactivity that is not associated with GVHD.

Cell type-specific antigen expression plays also an important role in organ- or cell-specific T cell-mediated autoimmune diseases like type 1 diabetes mellitus or multiple sclerosis (MS). In type 1 diabetes, the insulin-producing β cells of the pancreas are selectively targeted by autoreactive $CD8^+$ T cells and CTL are involved in both initiation of β cell-directed autoimmunity and ultimate destruction of β cells preceding manifest disease [2]. In MS, autoreactive $CD8^+$ T cells responsive to myelin-derived peptides have been reported that have the potential to kill selectively HLA class I-matched oligodendrocytes [3-5].

Cell type-specific antigens recognized by self-reactive T cells may also be important in the course of autoimmune disorders of the blood vessels. Recently, *in vitro* studies suggested that $CD8^+$ T cell-mediated lysis of autologous platelets may contribute to platelet destruction in thrombotic thrombocytopenic purpura [190, 191]. In vasculitis, the blood vessel wall is the primary site of inflammation. Detailed studies have explored whether the T cells that accumulate in the arterial wall during giant cell arteritis are a random representation of the peripheral blood repertoire or whether they are selected, as one would expect in the presence of a stimulating antigen. It has been found that a small proportion of tissue-infiltrating T cells isolated from temporal artery biopsy tissues have undergone clonal expansion. Notably, T cells with identical TCR sequences were detected in independent and nonadjacent segments of the inflamed blood vessel [192]. This observations are compatible with antigen-dependent selection of T cells. If it would be possible to generate endothelial peptide-specific CTL they should target selectively endothelial cells and lead to autoimmune

disorders with blood vessels as targets, e.g. giant cell arteritis. However, our work is the first of its kind to prove a endothelial cell-specific peptide repertoire and to discuss cell type-specific peptide presentation in the context of tolerance.

4.2 Hierarchy of self-tolerance

We successfully developed a method to reproducibly generate self-reactive CTL against low abundant self-peptides. Under the same culture conditions, the induction of CTL against the highly abundant endothelial peptides failed. Factors that determine the outcome of a CTL response against an antigenic peptide include the strength of costimulatory signals delivered by the APC [38, 39], the binding of the antigen to MHC class I of an APC [193, 194] and the avidity of the TCR for the pMHC complex [195, 196]. The dendritic cells that were used as APC for CTL induction proved to be highly potent stimulators, even for the generation of self-reactive CTL lines. IL-2 was added exogenously to mimic signal 3 that is usually provided by T helper cells. Therefore, lack of costimulatory signals provided by the APC was unlikely to limit the induction of CTL. With the adjustment of the peptide concentrations the low HLA-A*02 binding was compensated and could be excluded as a reason for the failing outgrowth.
The failure of induction of self-reactive CTL against abundant endothelium-specific peptides indicates a complete absence of circulating precursor CTL that recognize their cognate pMHC complex with enough avidity. According to this, we found that preCTL for the endothelial peptides in the blood of healthy controls as well as of patients with atherosclerosis could not be identified (data not shown). These findings imply that there are different levels of immunological tolerance against low abundant and high abundant self-peptides. Thymic negative selection most effectively deletes those T cell precursors that express TCR having high avidity for self-pMHC complexes expressed on medullary DC and mTEC. Thus, peripheral immune tolerance mechanisms are most important for controlling mature T cells that bear a TCR of relatively low avidity for self pMHC and that escape to the periphery [197]. As the generation of CTL specific for the self-peptide $SMCY_{(311\text{-}319)}$ is feasible, thymic negative selection of naïve CTL seems not to be the reason for the tolerance. Thus, this may rather occur on the side of peripheral tolerance mechanisms as costimulatory molecules (like the PD-1/PD-L1 pathway), regulatory T cells, anergy induction by caspases, or exhaustion of T cells after continous contact with abundant peptides [96].
Negative or inhibitory costimulatory molecules like PD-1 are modulators of autoimmunity that regulate T cell activation and play a role in peripheral tolerance. PD-1 and its ligands regulate both the induction and maintenance of peripheral $CD8^+$ T cell tolerance [92] and are involved

in the restoring of exhausted T cells [94]. Members of the caspase family are involved in activation-induced cell death [171] which is known to have a tolerogenic effect by leading to a self-limiting CTL response and deletion of autoreactive T cells [198]. However, the inhibition of costimulatory pathways like the PD-1/PD-L1 pathway or of caspases during the induction of CTL led only to an earlier outgrowth of self-reactive CTL against low abundant self-peptides but had no effect on CTL against abundant peptides. Therefore, these mechanisms seem not to be involved in the tolerance against the endothelial peptides.

Tolerance induction against self-peptides depends further on the quantitative level [196], the localization and the persistence of antigen presentation [199]. CTL against antigenic peptides with persistent and high presentation on professional APC in the lymphohematopoietic system will eventually be eliminated by exhaustive induction or clonal deletion [199] contributing to the self-limitation of tissue-specific autoimmunity. The exposed position of EC and their intimate contact with lymphocytes in the microcirculation could increase tolerance against endothelium-specific peptides, either by inducing long-lasting anergy, peripheral deletion of $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ specific CTL or exhaustion as it is seen for chronic infections [95-98]. $SMCY_{(311-319)}$-specific CTL on the other hand which survived central tolerance are not completely tolerized in the periphery due to the relatively low amount of antigen presented [121].

Preliminary data in our lab showed that it might be possible to generate peptide-specific CTL for the highly abundant leukocyte-derived antigen $PP1A_{(11-19)}$. If this proves to be the case then not only a hierarchy in tolerance may be involved in the failure of anti-endothelial peptide-specific CTL induction but also cell type-specific antigen expression. On argument against this theory may be the impossibility to induce CTL specific for the highly abundant $DDX5_{(168-176)}$. However, $DDX5_{(168-176)}$ is no leukocyte-derived peptide but abundantly expressed on EC as well. Therefore, peptides expressed only on cells that are able to provide enough costimulation may not lead to activation-induced cell death whereas preCTL specific for peptides presented also on endothelial cells that provide poor costimulation may be eliminated peripherally.

In a few cultures CTL lines specific for $PTRF_{(56-64)}$ could be induced. Interestingly, those CTL lines showed a ‘reprogramming’ into cells with NK-like activity after clonal expansion. NK-CTL have previously described in literature as $CD3^+CD4^+$ or $CD8^+CD56^+CD16^-$ cells that possess one single TCRVβ. They are able to lyse certain NK targets and express HLA class I-specific NK receptors (NKR) [200]. The NKR on $CD8^+$ T cells are in most situations dependent on TCR signaling for their function and work as utensils modulating T cell responses [201, 202]. It has been shown that these reprogrammed CTL have always undergone significant oligoclonal or monoclonal T cell expansions [200]. The $PTRF_{(56-64)}$-specific CTL clones expressed several features of NK-CTL like a $CD8^+CD56^+CD16^-$

phenotype and lysis of the NK target K562 whereas the CTL lines specific for $PTRF_{(56-64)}$ before cloning did not express a NK-like phenotype. Therefore, the reprogramming is likely to occur during the clonal expansion phase of the CTL culture. The transformation of CTL into more “innate-like” cells with NK cell properties has been found previously under different conditions. Bjorkstrom et al. showed elevated number of CTL with NK-like properties in chronic HCV infection, which showed evidence for clonal expansion as well as TCR-independent effector functions [203]. $CD8^+$ cells expressing NK associated receptors were found to be increased in melanoma patients and to display an effector phenotype [204]. The reprogramming of CTL into NK-like cells in celiac disease led to a stepwise transformation of CTL to more ‘innate-like’ cells with the properties of NK cells and upregulation of multiple activating NKR on oligoclonally expanded IFN-γ-producing effector T cells [205]. From these studies there is accumulating evidence that NK receptors can modulate antigen-driven T cell responses. The interaction between inhibitory NKR expressed on NK and NK-like cells and MHC molecules expressed on normal cells leads to the inhibition of NK-cell function. By contrast, failure to express MHC molecules may render tumor or virus-infected cells susceptible to NK-mediated lysis [206, 207]. Understanding of the mechanism regulating the surface expression of inhibitory NKR may be particularly important for the implications in the negative regulation of T cell responses. The expression of inhibitory NKR may be detrimental to the host in case of CTL responses to cytopathic viruses or tumor cells. For example, in the case of chronic viral infections, this inhibitory phenomenon may lead to a less efficient control of virus replication. On the other hand, the expression of inhibitory NKR by autoreactive T lymphocytes could be potentially useful in certain autoimmune diseases. Moreover, a similar favorable situation can be envisaged if NKR expression occurs *in vivo* in alloreactive CTL during transplantation. It has been suggested that the stepwise transformation of CTL into more ‘innate-like’ cells with NK cell properties is one underlying mechanism for immunopathology in chronic inflammatory diseases [205] and may result from prolonged CTL stimulation due to chronic infection. However, the underlying mechanism and significance of the reprogramming transformation of $PTRF_{(56-64)}$-specific CTL has yet to be examined.

Regulatory T cells (Treg) have been shown to be crucial for the maintenance of self-tolerance and to control $CD8^+$ T cell effector functions. Recently, it has been reported that self-reactive Treg are able to control CTL priming in an antigen-specific manner at the level of T cell expansion and prevent the induction of autoimmune responses through selective blockade of autoreactive T cell proliferation [208]. We found that in CTL cultures induced against self-peptides also a large population of CD4 cells emerged and increased during culture time. Further characterization showed that the population was $CD4^+CD25^+$ corresponding to Tregs. In cultures against low abundant self-peptides about a quarter of the cells accounted for the Treg population. In contrast, in cultures against the highly abundant

endothelial peptides, up to 75% of the cells were $CD4^+CD25^+$. It is imaginable that this population is able to influence and even control the outgrowth of self-reactive $CD8^+$ T cells in an antigen-specific manner. Further, early elimination of this population might enable T cell expansion and allow endothelial peptide-specific CTL to emerge.

From all these findings, we conclude that there exists a hierarchy of tolerance dependent on the abundance of the antigen. T lymphocytes specific for low abundant peptides can escape central tolerance mechanisms and are ignored in the periphery whereas highly abundant peptides lead to the efficient deletion of precursor CTL probably by using mechanisms of peripheral tolerance like clonal exhaustion of T cells, induction of inhibitory antigen-specific Tregs or the reprogramming into NK-CTL

4.3 Stealth effect of endothelial peptides

In this study, we provide evidence for the first time that the predominant presentation of endothelium-specific antigens can protect EC from CTL-mediated lysis by competing with immunogenic peptides for the binding site on MHC class I molecules. Since $CD8^+$ T cells with high avidity for self-peptides are deleted by negative selection in the thymus [209], self-reactive CTL usually have low avidity for self-peptides and require a higher amount of pMHC complexes per target cell for efficient recognition and killing [196]. By the predominant presentation of $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ against which no circulating CTL are detectable, these peptides could outnumber immunodominant peptides and thereby hide EC from an immune attack. With the transduction of the immunodominant FluM1 gene into male EC we got a system that allows not only to analyze two endogenously processed antigens at the same time but where antigen expression can also be induced and modulated by proinflammatory cytokines. Inhibition of the proteasome in this system led to a decrease in both $Flu_{(58-66)}$ and $SMCY_{(311-319)}$ expression with unchanged levels of HLA-A*02. These findings confirmed that first, the presentation of the peptides can be influenced by inhibition of proteasomic degradation, second the pMHC complex dissociates at a reasonable time span and third, the proteasome inhibitor did not influence HLA-A*02 levels but decreases the amount of peptide on the surface by specifically diminishing the supply of peptide into the MHC class I pathway. Knockdown of the abundant endothelial peptides $PTRF_{(56-64)}$, $CD59_{(106-114)}$ and $DDX5_{(168-176)}$ by RNA interference led to an increased presentation of other endogenously processed peptides proving that without the abundant endothelial peptides EC become better targets for both $Flu_{(58-66)}$ and $SMCY_{(311-319)}$-specific CTL.

In previous studies, several observations have been made that support our findings. Murine lung EC have been shown to negatively regulate $CD8^+$ T cell function [106]. Bolinger et al. showed in a mouse model that vascular EC expressing minor histocompatibility antigens are immunologically ignored by specific $CD8^+$ T cells unless CTL are primed by DC cross-presenting the endothelial antigens [210]. Further, autoreactive CTL against two endothelium-specific peptides have been identified [211, 212]. However, those peptides are derived from KDR (Kinase insert domain-containing receptor) and CD105 (Endoglin) but are expressed on angiogenic, proliferating EC only. Therefore, the presentation of these immunogenic peptides is temporally and spatially restricted. This is a sharp contrast to the constitutive and ubiquitous expression of $CD59_{(106-114)}$ and $PTRF_{(56-64)}$ on EC and may be a possible condition to break the balance between EC-induced active peripheral tolerance and ignorance by disguise. However, our work is the first of its kind providing evidence for a endothelium-specific repertoire that protects EC from CTL-mediated lysis.

4.4 Limitations, importance and summary of the study

One of the limitations of this study may be that the experiments were performed with cells from human donors only, therefore our data result from cell culture models but cannot be compared with a compatible *in vivo* model. On the other hand, our model provides advantages as the antigen expression can be modulated by cytokines, proteasome inhibition or RNA interference. The analysis of the human CTL repertoire is usually limited to the peripheral blood. The fact that we could not generate CTL against $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ from circulating peripheral blood cells does not rule out that such autoreactive CTL might be found in other tissues. However, CTL lines grown out from arterial tissue samples from patients with atherosclerosis were tested for specific lysis of $PTRF_{(56-64)}$/$CD59_{(106-114)}$-loaded cells but also failed to recognize specifically the endothelial peptides (data not shown). Furthermore, it remains open whether $PTRF_{(56-64)}$ and $CD59_{(106-114)}$ could be recognized during auto- or alloimmune vascular diseases as the PBMC used in this study were taken from healthy donors. However, precursor CTL for the endothelial peptides could not be identified in both healthy donors and patients with atherosclerosis (data not shown). We tried to improve the culture conditions by blockade of inhibiting costimulatory pathways that are known to play a role in tolerance but this affected only the induction of CTL against low abundant self-peptides. However, the blockade of the PD-1/PD-L1 pathway may not be enough to break the tolerance as it has been shown that PD-1 and CTLA-4 have distinct, yet

possible synergistic roles [213]. Therefore, blockade of both pathways may be inevitable to affect also the outgrowth of endothelial-peptide specific CTL.

The importance of self-reactive $CD8^+$ effector T cells in autoimmune diseases has become more and more clearer. Yet, there is still lack of measures to detect self-reactive T cells, especially in the human setting. First, the relevant (disease-associated) self-reactive T cells act in the tissue lesion, and only circulate at very low precursor frequencies in the peripheral blood. Second, the autoantigens are mostly unknown as the identification of autoantigens is extremely difficult. Therefore, methods that enable and improve the induction and examination of self-reactive CTL may have implications for the diagnostic and therapy of autoimmune diseases.

The integrity of the endothelium is crucial for the maintenance of organ homeostasis and endothelial dysfunction has devastating effects on the organism. It can be caused by inflammation and immune-mediated injury and is the first step in the formation of atherosclerosis. Here, we show that EC have an immune privilege due to presentation of a quantitatively different peptide repertoire. Our findings have several implications for the diagnostic and therapy of immune-mediated endothelial diseases. First, the mechanism that endothelial cell-specific surface expression of MHC class I-bound peptides leads to efficient tolerance could be explored in other contexts of cell-specific antigen expression such as desensitizing individuals who suffer from CTL driven, organ-specific autoimmune disease (e.g. diabetes mellitus). This would also point to novel targets for organ-specific immunosuppression. Our findings have as well further implications for the pathogenesis of alloimmune diseases such as GVHD. They may explain why mature, fully differentiated effector CTL that were activated by dendritic cells in secondary lymphoid organs tend to ignore vascular EC on their way to the epithelial compartment of the affected organ, e.g. skin, gut, or liver [117]. Unless the CTL manage to express a sufficiently high amount of the immunogenic antigen against which specific CTL are directed, CTL are not triggered to release cytolytic granules and therefore do not kill these cells.

In this study, we showed for the first time that EC are protected from CTL-mediated lysis by the presentation of an endothelial cell-specific peptide repertoire. The abundance of the endothelial peptides is mainly caused by the preferential expression of the source proteins. The mechanism of protection consists from different layers of tolerance induction. First, the endothelial peptides compete with immunogenic peptides for the MHC class I binding site. Second, the endothelial peptides have distinct properties like weak binding to the MHC I molecule and unstable pMHC complexes that may participate in the protection by reducing the immunogenicity of the peptides. Third, the selective and abundant presentation of $PTRF_{(56\text{-}64)}$ and $CD59_{(106\text{-}114)}$ on EC leads to the complete elimination of self-reactive CTL specific for these peptides. Fourth, even the improvement of culture conditions like the

blockade of inhibitory pathways did not enable the induction of CTL against EC-specific peptides. And fifth, after cloning of $PTRF_{(56-64)}$-specific CTL lines, cells with NK-CTL phenotype did emerge that might be able to modulate antigen-driven T cell responses. Thus, by these mechanisms, EC avoid being targeted by CTL despite their continuous intimate contact and become an immune privileged site supporting their crucial role in organ homeostasis.

5. References

1. Puliaeva, I., R. Puliaev, and C.S. Via, *Therapeutic potential of CD8+ cytotoxic T lymphocytes in SLE.* Autoimmun Rev, 2009. **8**(3): p. 219-23.
2. Enee, E., et al., *Equivalent specificity of peripheral blood and islet-infiltrating CD8+ T lymphocytes in spontaneously diabetic HLA-A2 transgenic NOD mice.* J Immunol, 2008. **180**(8): p. 5430-8.
3. Babbe, H., et al., *Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction.* J Exp Med, 2000. **192**(3): p. 393-404.
4. Sun, D., et al., *Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice.* J Immunol, 2001. **166**(12): p. 7579-87.
5. Huseby, E.S., et al., *A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis.* J Exp Med, 2001. **194**(5): p. 669-76.
6. Green, E.A. and R.A. Flavell, *The initiation of autoimmune diabetes.* Curr Opin Immunol, 1999. **11**(6): p. 663-9.
7. Nakamura, A., et al., *Fcgamma receptor IIB-deficient mice develop Goodpasture's syndrome upon immunization with type IV collagen: a novel murine model for autoimmune glomerular basement membrane disease.* J Exp Med, 2000. **191**(5): p. 899-906.
8. Feldmann, M., et al., *Mechanism of Graves thyroiditis: implications for concepts and therapy of autoimmunity.* Int Rev Immunol, 1992. **9**(2): p. 91-106.
9. Kong, Y.C., et al., *HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene.* J Exp Med, 1996. **184**(3): p. 1167-72.
10. Vincent, A., O. Lily, and J. Palace, *Pathogenic autoantibodies to neuronal proteins in neurological disorders.* J Neuroimmunol, 1999. **100**(1-2): p. 169-80.
11. Domen, R.E., *An overview of immune hemolytic anemias.* Cleve Clin J Med, 1998. **65**(2): p. 89-99.
12. Clark, S.H., *Animal models in scleroderma.* Curr Rheumatol Rep, 2005. **7**(2): p. 150-5.
13. Weyand, C.M. and J.J. Goronzy, *Arterial wall injury in giant cell arteritis.* Arthritis Rheum, 1999. **42**(5): p. 844-53.
14. Lhote, F., P. Cohen, and L. Guillevin, *Polyarteritis nodosa, microscopic polyangiitis and Churg-Strauss syndrome.* Lupus, 1998. **7**(4): p. 238-58.
15. Savage, C.O., L. Harper, and D. Adu, *Primary systemic vasculitis.* Lancet, 1997. **349**(9051): p. 553-8.
16. Kotzin, B.L., *Systemic lupus erythematosus.* Cell, 1996. **85**(3): p. 303-6.
17. Casciola-Rosen, L.A., G. Anhalt, and A. Rosen, *Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes.* J Exp Med, 1994. **179**(4): p. 1317-30.
18. Hardin, J.A. and J.E. Craft, *Patterns of autoimmunity to nucleoproteins in patients with systemic lupus erythematosus.* Rheum Dis Clin North Am, 1987. **13**(1): p. 37-46.
19. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. **423**(6937): p. 356-61.
20. Singer, G.G., et al., *Apoptosis, Fas and systemic autoimmunity: the MRL-lpr/lpr model.* Curr Opin Immunol, 1994. **6**(6): p. 913-20.
21. Fink, P.J. and M.J. Bevan, *H-2 antigens of the thymus determine lymphocyte specificity.* J Exp Med, 1978. **148**(3): p. 766-75.
22. Hogquist, K.A., et al., *T cell receptor antagonist peptides induce positive selection.* Cell, 1994. **76**(1): p. 17-27.
23. Kappler, J.W., N. Roehm, and P. Marrack, *T cell tolerance by clonal elimination in the thymus.* Cell, 1987. **49**(2): p. 273-80.
24. Kisielow, P., et al., *Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes.* Nature, 1988. **333**(6175): p. 742-6.

25. Zinkernagel, R.M. and P.C. Doherty, *Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis.* Nature, 1974. **251**(5475): p. 547-8.
26. Zinkernagel, R.M. and P.C. Doherty, *Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system.* Nature, 1974. **248**(450): p. 701-2.
27. Mescher, M.F., et al., *Signals required for programming effector and memory development by CD8+ T cells.* Immunol Rev, 2006. **211**: p. 81-92.
28. Bachmann, M.F., et al., *Cutting edge: distinct roles for T help and CD40/CD40 ligand in regulating differentiation of proliferation-competent memory CD8+ T cells.* J Immunol, 2004. **173**(4): p. 2217-21.
29. Badovinac, V.P. and J.T. Harty, *Programming, demarcating, and manipulating CD8+ T-cell memory.* Immunol Rev, 2006. **211**: p. 67-80.
30. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-34.
31. Naeher, D., et al., *A constant affinity threshold for T cell tolerance.* J Exp Med, 2007. **204**(11): p. 2553-9.
32. Matzinger, P. and S. Guerder, *Does T-cell tolerance require a dedicated antigen-presenting cell?* Nature, 1989. **338**(6210): p. 74-6.
33. Zal, T., A. Volkmann, and B. Stockinger, *Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen.* J Exp Med, 1994. **180**(6): p. 2089-99.
34. Bouneaud, C., P. Kourilsky, and P. Bousso, *Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion.* Immunity, 2000. **13**(6): p. 829-40.
35. Lo, D., et al., *Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells.* J Exp Med, 1989. **170**(1): p. 87-104.
36. Stockinger, B., *T lymphocyte tolerance: from thymic deletion to peripheral control mechanisms.* Adv Immunol, 1999. **71**: p. 229-65.
37. Walker, L.S. and A.K. Abbas, *The enemy within: keeping self-reactive T cells at bay in the periphery.* Nat Rev Immunol, 2002. **2**(1): p. 11-9.
38. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone, *CD28/B7 system of T cell costimulation.* Annu Rev Immunol, 1996. **14**: p. 233-58.
39. Bour-Jordan, H. and J.A. Blueston, *CD28 function: a balance of costimulatory and regulatory signals.* J Clin Immunol, 2002. **22**(1): p. 1-7.
40. Ohashi, P.S., et al., *Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice.* Cell, 1991. **65**(2): p. 305-17.
41. Oldstone, M.B., et al., *Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response.* Cell, 1991. **65**(2): p. 319-31.
42. Redmond, W.L., J. Hernandez, and L.A. Sherman, *Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC.* J Immunol, 2003. **171**(12): p. 6349-54.
43. Moskophidis, D., et al., *Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells.* Nature, 1993. **362**(6422): p. 758-61.
44. Starzl, T.E. and R.M. Zinkernagel, *Antigen localization and migration in immunity and tolerance.* N Engl J Med, 1998. **339**(26): p. 1905-13.
45. Effros, R.B. and G. Pawelec, *Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion?* Immunol Today, 1997. **18**(9): p. 450-4.
46. Qian, S., et al., *Apoptosis within spontaneously accepted mouse liver allografts: evidence for deletion of cytotoxic T cells and implications for tolerance induction.* J Immunol, 1997. **158**(10): p. 4654-61.
47. Chai, J.G. and R.I. Lechler, *Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4+ T cells in vitro.* Int Immunol, 1997. **9**(7): p. 935-44.

48. Macian, F., et al., *Transcriptional mechanisms underlying lymphocyte tolerance.* Cell, 2002. **109**(6): p. 719-31.
49. Schwartz, R.H., *T cell anergy.* Annu Rev Immunol, 2003. **21**: p. 305-34.
50. Choi, S. and R.H. Schwartz, *Molecular mechanisms for adaptive tolerance and other T cell anergy models.* Semin Immunol, 2007. **19**(3): p. 140-52.
51. Zinkernagel, R.M., *Antiinfection immunity and autoimmunity.* Ann N Y Acad Sci, 2002. **958**: p. 3-6.
52. Nabholz, M. and H.R. MacDonald, *Cytolytic T lymphocytes.* Annu Rev Immunol, 1983. **1**: p. 273-306.
53. Zinkernagel, R., *On observing and analyzing disease versus signals.* Nat Immunol, 2007. **8**(1): p. 8-10.
54. Barry, M. and R.C. Bleackley, *Cytotoxic T lymphocytes: all roads lead to death.* Nat Rev Immunol, 2002. **2**(6): p. 401-9.
55. Trambas, C.M. and G.M. Griffiths, *Delivering the kiss of death.* Nat Immunol, 2003. **4**(5): p. 399-403.
56. Jabbari, A. and J.T. Harty, *The generation and modulation of antigen-specific memory CD8 T cell responses.* J Leukoc Biol, 2006. **80**(1): p. 16-23.
57. Kurts, C., et al., *Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells.* J Exp Med, 1997. **186**(2): p. 239-45.
58. Jondal, M., R. Schirmbeck, and J. Reimann, *MHC class I-restricted CTL responses to exogenous antigens.* Immunity, 1996. **5**(4): p. 295-302.
59. Heath, W.R. and F.R. Carbone, *Cross-presentation, dendritic cells, tolerance and immunity.* Annu Rev Immunol, 2001. **19**: p. 47-64.
60. Kita, H., et al., *Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells.* J Exp Med, 2002. **195**(1): p. 113-23.
61. Fujinami, R.S. and M.B. Oldstone, *Molecular mimicry as a mechanism for virus-induced autoimmunity.* Immunol Res, 1989. **8**(1): p. 3-15.
62. Olson, J.K., et al., *A virus-induced molecular mimicry model of multiple sclerosis.* J Clin Invest, 2001. **108**(2): p. 311-8.
63. Panoutsakopoulou, V., et al., *Analysis of the relationship between viral infection and autoimmune disease.* Immunity, 2001. **15**(1): p. 137-47.
64. Mason, R.D., et al., *Cross-reactive cytotoxic T lymphocytes against human immunodeficiency virus type 1 protease and gamma interferon-inducible protein 30.* J Virol, 2005. **79**(9): p. 5529-36.
65. Anderton, S.M., *Avoiding autoimmune disease--T cells know their limits.* Trends Immunol, 2006. **27**(5): p. 208-14.
66. Kap, Y.S., et al., *Fast progression of recombinant human myelin/oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in marmosets is associated with the activation of MOG34-56-specific cytotoxic T cells.* J Immunol, 2008. **180**(3): p. 1326-37.
67. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annu Rev Immunol, 2004. **22**: p. 531-62.
68. Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky, *Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice.* Nat Immunol, 2007. **8**(2): p. 191-7.
69. Waldmann, H. and S. Cobbold, *Exploiting tolerance processes in transplantation.* Science, 2004. **305**(5681): p. 209-12.
70. Walsh, P.T., D.K. Taylor, and L.A. Turka, *Tregs and transplantation tolerance.* J Clin Invest, 2004. **114**(10): p. 1398-403.
71. Cohen, J.L., et al., *CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease.* J Exp Med, 2002. **196**(3): p. 401-6.
72. Hoffmann, P., et al., *Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation.* J Exp Med, 2002. **196**(3): p. 389-99.

73. Gross, D.A., et al., *CD4+CD25+ regulatory T cells inhibit immune-mediated transgene rejection.* Blood, 2003. **102**(13): p. 4326-8.
74. Gross, D.A., et al., *Simple conditioning with monospecific CD4+CD25+ regulatory T cells for bone marrow engraftment and tolerance to multiple gene products.* Blood, 2006. **108**(6): p. 1841-8.
75. Cao, O., et al., *Induction and role of regulatory CD4+CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer.* Blood, 2007. **110**(4): p. 1132-40.
76. Dobrzynski, E., et al., *Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells.* Proc Natl Acad Sci U S A, 2006. **103**(12): p. 4592-7.
77. June, C.H., et al., *Role of the CD28 receptor in T-cell activation.* Immunol Today, 1990. **11**(6): p. 211-6.
78. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.* Nature, 1992. **356**(6370): p. 607-9.
79. Ansari, M.J. and M.H. Sayegh, *Costimulation couture: a designer approach to regulating autoimmunity.* J Clin Invest, 2006. **116**(8): p. 2080-3.
80. Walunas, T.L., et al., *CTLA-4 can function as a negative regulator of T cell activation.* Immunity, 1994. **1**(5): p. 405-13.
81. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation.* J Exp Med, 1995. **182**(2): p. 459-65.
82. Eggena, M.P., et al., *Cooperative roles of CTLA-4 and regulatory T cells in tolerance to an islet cell antigen.* J Exp Med, 2004. **199**(12): p. 1725-30.
83. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation.* J Exp Med, 2000. **192**(2): p. 295-302.
84. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.* J Exp Med, 2000. **192**(2): p. 303-10.
85. Tang, Q., et al., *Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function.* Eur J Immunol, 2004. **34**(11): p. 2996-3005.
86. Perez, V.L., et al., *Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement.* Immunity, 1997. **6**(4): p. 411-7.
87. Bluestone, J.A., *Is CTLA-4 a master switch for peripheral T cell tolerance?* J Immunol, 1997. **158**(5): p. 1989-93.
88. Judge, T.A., et al., *The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival.* J Immunol, 1999. **162**(4): p. 1947-51.
89. Fife, B.T., et al., *Inhibition of T cell activation and autoimmune diabetes using a B cell surface-linked CTLA-4 agonist.* J Clin Invest, 2006. **116**(8): p. 2252-61.
90. Khoury, S.J. and M.H. Sayegh, *The roles of the new negative T cell costimulatory pathways in regulating autoimmunity.* Immunity, 2004. **20**(5): p. 529-38.
91. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu Rev Immunol, 2005. **23**: p. 515-48.
92. Keir, M.E., G.J. Freeman, and A.H. Sharpe, *PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues.* J Immunol, 2007. **179**(8): p. 5064-70.
93. Sharpe, A.H., et al., *The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection.* Nat Immunol, 2007. **8**(3): p. 239-45.
94. Zajac, A.J., et al., *Viral immune evasion due to persistence of activated T cells without effector function.* J Exp Med, 1998. **188**(12): p. 2205-13.
95. Wherry, E.J. and R. Ahmed, *Memory CD8 T-cell differentiation during viral infection.* J Virol, 2004. **78**(11): p. 5535-45.
96. Klenerman, P. and A. Hill, *T cells and viral persistence: lessons from diverse infections.* Nat Immunol, 2005. **6**(9): p. 873-9.

97. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
98. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.
99. Daar, A.S., et al., *The detailed distribution of HLA-A, B, C antigens in normal human organs.* Transplantation, 1984. **38**(3): p. 287-92.
100. Briscoe, D.M., S.I. Alexander, and A.H. Lichtman, *Interactions between T lymphocytes and endothelial cells in allograft rejection.* Curr Opin Immunol, 1998. **10**(5): p. 525-31.
101. Libby, P. and J.S. Pober, *Chronic rejection.* Immunity, 2001. **14**(4): p. 387-97.
102. Choi, J., et al., *T lymphocyte-endothelial cell interactions.* Annu Rev Immunol, 2004. **22**: p. 683-709.
103. Biedermann, B.C. and J.S. Pober, *Human vascular endothelial cells favor clonal expansion of unusual alloreactive CTL.* J Immunol, 1999. **162**(12): p. 7022-30.
104. Dengler, T.J. and J.S. Pober, *Human vascular endothelial cells stimulate memory but not naive CD8+ T cells to differentiate into CTL retaining an early activation phenotype.* J Immunol, 2000. **164**(10): p. 5146-55.
105. Biedermann, B.C. and J.S. Pober, *Human endothelial cells induce and regulate cytolytic T cell differentiation.* J Immunol, 1998. **161**(9): p. 4679-87.
106. Marelli-Berg, F.M., et al., *Activated murine endothelial cells have reduced immunogenicity for CD8+ T cells: a mechanism of immunoregulation?* J Immunol, 2000. **165**(8): p. 4182-9.
107. Kreisel, D., et al., *Mouse vascular endothelium activates CD8+ T lymphocytes in a B7-dependent fashion.* J Immunol, 2002. **169**(11): p. 6154-61.
108. Rose, M.L., *Endothelial cells as antigen-presenting cells: role in human transplant rejection.* Cell Mol Life Sci, 1998. **54**(9): p. 965-78.
109. Valujskikh, A., et al., *Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway.* Nat Immunol, 2002. **3**(9): p. 844-51.
110. Limmer, A., et al., *Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance.* Nat Med, 2000. **6**(12): p. 1348-54.
111. Meehan, S.M., et al., *Cytotoxicity and apoptosis in human renal allografts: identification, distribution, and quantitation of cells with a cytotoxic granule protein GMP-17 (TIA-1) and cells with fragmented nuclear DNA.* Lab Invest, 1997. **76**(5): p. 639-49.
112. Sumitran-Karuppan, S., et al., *Hyperacute rejections of two consecutive renal allografts and early loss of the third transplant caused by non-HLA antibodies specific for endothelial cells.* Transpl Immunol, 1997. **5**(4): p. 321-7.
113. Suntharalingam, G., et al., *Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412.* N Engl J Med, 2006. **355**(10): p. 1018-28.
114. Ruegg, C., et al., *Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma.* Nat Med, 1998. **4**(4): p. 408-14.
115. van den Brink, M.R. and S.J. Burakoff, *Cytolytic pathways in haematopoietic stem-cell transplantation.* Nat Rev Immunol, 2002. **2**(4): p. 273-81.
116. Dickinson, A.M., et al., *In situ dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens.* Nat Med, 2002. **8**(4): p. 410-4.
117. Biedermann, B.C., et al., *Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease.* Lancet, 2002. **359**(9323): p. 2078-83.
118. Hausermann, P., et al., *Cutaneous graft-versus-host disease: a guide for the dermatologist.* Dermatology, 2008. **216**(4): p. 287-304.
119. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.

120. Weyand, C.M. and J.J. Goronzy, *Medium- and large-vessel vasculitis.* N Engl J Med, 2003. **349**(2): p. 160-9.
121. Kummer, M., et al., *Vascular endothelial cells have impaired capacity to present immunodominant, antigenic peptides: a mechanism of cell type-specific immune escape.* J Immunol, 2005. **174**(4): p. 1947-53.
122. Daar, A.S., et al., *The detailed distribution of MHC Class II antigens in normal human organs.* Transplantation, 1984. **38**(3): p. 293-8.
123. Gakamsky, D.M., et al., *Assembly and dissociation of human leukocyte antigen (HLA)-A2 studied by real-time fluorescence resonance energy transfer.* Biochemistry, 2000. **39**(36): p. 11163-9.
124. Johnson, D.R., *Locus-specific constitutive and cytokine-induced HLA class I gene expression.* J Immunol, 2003. **170**(4): p. 1894-902.
125. Johnson, D.R. and J.S. Pober, *HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons.* Mol Cell Biol, 1994. **14**(2): p. 1322-32.
126. Ackerman, A.L., A. Giodini, and P. Cresswell, *A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells.* Immunity, 2006. **25**(4): p. 607-17.
127. Goldberg, A.L., *Functions of the proteasome: from protein degradation and immune surveillance to cancer therapy.* Biochem Soc Trans, 2007. **35**(Pt 1): p. 12-7.
128. Abele, R. and R. Tampe, *The ABCs of immunology: structure and function of TAP, the transporter associated with antigen processing.* Physiology (Bethesda), 2004. **19**: p. 216-24.
129. van Hall, T., et al., *Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants.* Nat Med, 2006. **12**(4): p. 417-24.
130. Santos, S.G., et al., *Major histocompatibility complex class I-ERp57-tapasin interactions within the peptide-loading complex.* J Biol Chem, 2007. **282**(24): p. 17587-93.
131. Lehner, P.J. and P. Cresswell, *Recent developments in MHC-class-I-mediated antigen presentation.* Curr Opin Immunol, 2004. **16**(1): p. 82-9.
132. Yewdell, J.W., *The seven dirty little secrets of major histocompatibility complex class I antigen processing.* Immunol Rev, 2005. **207**: p. 8-18.
133. Gotch, F., et al., *Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2.* Nature, 1987. **326**(6116): p. 881-2.
134. Nayersina, R., et al., *HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection.* J Immunol, 1993. **150**(10): p. 4659-71.
135. Yewdell, J.W., E. Reits, and J. Neefjes, *Making sense of mass destruction: quantitating MHC class I antigen presentation.* Nat Rev Immunol, 2003. **3**(12): p. 952-61.
136. Tellam, J., et al., *Translation efficiency of EBNA1 encoded by lymphocryptoviruses influences endogenous presentation of CD8+ T cell epitopes.* Eur J Immunol, 2007. **37**(2): p. 328-37.
137. Khan, S., et al., *Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein.* J Immunol, 2001. **167**(9): p. 4801-4.
138. Reits, E., et al., *Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I.* Immunity, 2003. **18**(1): p. 97-108.
139. Fruci, D., et al., *Quantifying recruitment of cytosolic peptides for HLA class I presentation: impact of TAP transport.* J Immunol, 2003. **170**(6): p. 2977-84.
140. van Endert, P.M., et al., *The peptide-binding motif for the human transporter associated with antigen processing.* J Exp Med, 1995. **182**(6): p. 1883-95.
141. Basler, M., et al., *Immunoproteasomes down-regulate presentation of a subdominant T cell epitope from lymphocytic choriomeningitis virus.* J Immunol, 2004. **173**(6): p. 3925-34.

142. Van Kaer, L., et al., *Altered peptidase and viral-specific T cell response in LMP2 mutant mice.* Immunity, 1994. **1**(7): p. 533-41.
143. Pang, K.C., et al., *Immunoproteasome subunit deficiencies impact differentially on two immunodominant influenza virus-specific CD8+ T cell responses.* J Immunol, 2006. **177**(11): p. 7680-8.
144. Sijts, A.J., et al., *Efficient generation of a hepatitis B virus cytotoxic T lymphocyte epitope requires the structural features of immunoproteasomes.* J Exp Med, 2000. **191**(3): p. 503-14.
145. Chen, W., et al., *Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses.* Immunity, 2000. **12**(1): p. 83-93.
146. van der Burg, S.H., et al., *Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability.* J Immunol, 1996. **156**(9): p. 3308-14.
147. Micheletti, F., et al., *The lifespan of major histocompatibility complex class I/peptide complexes determines the efficiency of cytotoxic T-lymphocyte responses.* Immunology, 1999. **96**(3): p. 411-5.
148. Tse, D.B. and B. Pernis, *Spontaneous internalization of Class I major histocompatibility complex molecules in T lymphoid cells.* J Exp Med, 1984. **159**(1): p. 193-207.
149. Gromme, M., et al., *Recycling MHC class I molecules and endosomal peptide loading.* Proc Natl Acad Sci U S A, 1999. **96**(18): p. 10326-31.
150. Jansa, P., et al., *Cloning and functional characterization of PTRF, a novel protein which induces dissociation of paused ternary transcription complexes.* EMBO J, 1998. **17**(10): p. 2855-64.
151. Jansa, P., et al., *The transcript release factor PTRF augments ribosomal gene transcription by facilitating reinitiation of RNA polymerase I.* Nucleic Acids Res, 2001. **29**(2): p. 423-9.
152. Hasegawa, T., et al., *PTRF (polymerase I and transcript-release factor) is tissue-specific and interacts with the BFCOL1 (binding factor of a type-I collagen promoter) zinc-finger transcription factor which binds to the two mouse type-I collagen gene promoters.* Biochem J, 2000. **347 Pt 1**: p. 55-9.
153. Aboulaich, N., et al., *Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes.* Biochem J, 2004. **383**(Pt 2): p. 237-48.
154. Vinten, J., et al., *Identification of a major protein on the cytosolic face of caveolae.* Biochim Biophys Acta, 2005. **1717**(1): p. 34-40.
155. Holguin, M.H., et al., *Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria.* J Clin Invest, 1989. **84**(1): p. 7-17.
156. Davies, A., et al., *CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells.* J Exp Med, 1989. **170**(3): p. 637-54.
157. Kimberley, F.C., B. Sivasankar, and B. Paul Morgan, *Alternative roles for CD59.* Mol Immunol, 2007. **44**(1-3): p. 73-81.
158. Brooimans, R.A., et al., *CD59 expressed by human endothelial cells functions as a protective molecule against complement-mediated lysis.* Eur J Immunol, 1992. **22**(3): p. 791-7.
159. Wu, G., et al., *Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex.* Circ Res, 2009. **104**(4): p. 550-8.
160. Bednarek, M.A., et al., *The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2.* J Immunol, 1991. **147**(12): p. 4047-53.

161. Rufer, N., et al., *HA-1 and the SMCY-derived peptide FIDSYICQV (H-Y) are immunodominant minor histocompatibility antigens after bone marrow transplantation.* Transplantation, 1998. **66**(7): p. 910-6.
162. Meadows, L., et al., *The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition.* Immunity, 1997. **6**(3): p. 273-81.
163. Hirling, H., et al., *RNA helicase activity associated with the human p68 protein.* Nature, 1989. **339**(6225): p. 562-4.
164. Schirle, M., et al., *Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach.* Eur J Immunol, 2000. **30**(8): p. 2216-25.
165. Sun, Y., et al., *Identification of a new HLA-A(*)0201-restricted T-cell epitope from the tyrosinase-related protein 2 (TRP2) melanoma antigen.* Int J Cancer, 2000. **87**(3): p. 399-404.
166. Pascolo, S., et al., *A MAGE-A1 HLA-A A*0201 epitope identified by mass spectrometry.* Cancer Res, 2001. **61**(10): p. 4072-7.
167. Brown, G.M., et al., *Characterisation of the coding sequence and fine mapping of the human DFFRY gene and comparative expression analysis and mapping to the Sxrb interval of the mouse Y chromosome of the Dffry gene.* Hum Mol Genet, 1998. **7**(1): p. 97-107.
168. Fehse, B., et al., *Real-time quantitative Y chromosome-specific PCR (QYCS-PCR) for monitoring hematopoietic chimerism after sex-mismatched allogeneic stem cell transplantation.* J Hematother Stem Cell Res, 2001. **10**(3): p. 419-25.
169. Gatti, E., et al., *Large-scale culture and selective maturation of human Langerhans cells from granulocyte colony-stimulating factor-mobilized CD34+ progenitors.* J Immunol, 2000. **164**(7): p. 3600-7.
170. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha.* J Exp Med, 1994. **179**(4): p. 1109-18.
171. Lopes, A.R., et al., *Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection.* J Clin Invest, 2008. **118**(5): p. 1835-45.
172. Lichtenfels, R., et al., *CARE-LASS (calcein-release-assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity.* J Immunol Methods, 1994. **172**(2): p. 227-39.
173. Nakamura, T., et al., *Cyclin I: a new cyclin encoded by a gene isolated from human brain.* Exp Cell Res, 1995. **221**(2): p. 534-42.
174. Hillen, N. and S. Stevanovic, *Contribution of mass spectrometry-based proteomics to immunology.* Expert Rev Proteomics, 2006. **3**(6): p. 653-64.
175. Fruh, K. and Y. Yang, *Antigen presentation by MHC class I and its regulation by interferon gamma.* Curr Opin Immunol, 1999. **11**(1): p. 76-81.
176. Bednarek, M.A., et al., *Soluble HLA-A2.1 restricted peptides that are recognized by influenza virus specific cytotoxic T lymphocytes.* J Immunol Methods, 1991. **139**(1): p. 41-7.
177. Munz, C., et al., *Alloreactivity as a source of high avidity peptide-specific human CTL.* J Immunol, 1999. **162**(1): p. 25-34.
178. Falo, L.D., Jr., et al., *Analysis of antigen presentation by metabolically inactive accessory cells and their isolated membranes.* Proc Natl Acad Sci U S A, 1985. **82**(19): p. 6647-51.
179. Rock, K.L., et al., *Chemical cross-linking of class I molecules on cells creates receptive peptide binding sites.* J Immunol, 1992. **148**(5): p. 1451-7.
180. Biddison, W.E., et al., *Tax and M1 peptide/HLA-A2-specific Fabs and T cell receptors recognize nonidentical structural features on peptide/HLA-A2 complexes.* J Immunol, 2003. **171**(6): p. 3064-74.
181. Zamore, P.D., et al., *RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals.* Cell, 2000. **101**(1): p. 25-33.

182. Hannon, G.J., *RNA interference.* Nature, 2002. **418**(6894): p. 244-51.
183. Sledz, C.A., et al., *Activation of the interferon system by short-interfering RNAs.* Nat Cell Biol, 2003. **5**(9): p. 834-9.
184. Feltkamp, M.C., et al., *Efficient MHC class I-peptide binding is required but does not ensure MHC class I-restricted immunogenicity.* Mol Immunol, 1994. **31**(18): p. 1391-401.
185. Butturini, A., M.M. Bortin, and R.P. Gale, *Graft-versus-leukemia following bone marrow transplantation.* Bone Marrow Transplant, 1987. **2**(3): p. 233-42.
186. Horowitz, M.M., et al., *Graft-versus-leukemia reactions after bone marrow transplantation.* Blood, 1990. **75**(3): p. 555-62.
187. Niederwieser, D., et al., *Correlation of minor histocompatibility antigen-specific cytotoxic T lymphocytes with graft-versus-host disease status and analyses of tissue distribution of their target antigens.* Blood, 1993. **81**(8): p. 2200-8.
188. de Bueger, M., et al., *Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens.* J Immunol, 1992. **149**(5): p. 1788-94.
189. Dolstra, H., et al., *Recognition of a B cell leukemia-associated minor histocompatibility antigen by CTL.* J Immunol, 1997. **158**(2): p. 560-5.
190. Olsson, B., et al., *T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura.* Nat Med, 2003. **9**(9): p. 1123-4.
191. Zhang, F., et al., *Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura.* Eur J Haematol, 2006. **76**(5): p. 427-31.
192. Weyand, C.M., et al., *Distinct vascular lesions in giant cell arteritis share identical T cell clonotypes.* J Exp Med, 1994. **179**(3): p. 951-60.
193. Anderton, S.M., et al., *Negative selection during the peripheral immune response to antigen.* J Exp Med, 2001. **193**(1): p. 1-11.
194. Yu, Z., et al., *Poor immunogenicity of a self/tumor antigen derives from peptide-MHC-I instability and is independent of tolerance.* J Clin Invest, 2004. **114**(4): p. 551-9.
195. Hemmer, B., et al., *Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells.* J Immunol, 1998. **160**(12): p. 5807-14.
196. Anderton, S.M. and D.C. Wraith, *Selection and fine-tuning of the autoimmune T-cell repertoire.* Nat Rev Immunol, 2002. **2**(7): p. 487-98.
197. Mueller, D.L., *Mechanisms maintaining peripheral tolerance.* Nat Immunol, 2010. **11**(1): p. 21-7.
198. Parish, I.A., et al., *Tissue destruction caused by cytotoxic T lymphocytes induces deletional tolerance.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3901-6.
199. Zinkernagel, R.M. and H. Hengartner, *Regulation of the immune response by antigen.* Science, 2001. **293**(5528): p. 251-3.
200. Mingari, M.C., et al., *Human CD8+ T lymphocyte subsets that express HLA class I-specific inhibitory receptors represent oligoclonally or monoclonally expanded cell populations.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12433-8.
201. McMahon, C.W. and D.H. Raulet, *Expression and function of NK cell receptors in CD8+ T cells.* Curr Opin Immunol, 2001. **13**(4): p. 465-70.
202. Ugolini, S. and E. Vivier, *Regulation of T cell function by NK cell receptors for classical MHC class I molecules.* Curr Opin Immunol, 2000. **12**(3): p. 295-300.
203. Bjorkstrom, N.K., et al., *Elevated numbers of Fc gamma RIIIA+ (CD16+) effector CD8 T cells with NK cell-like function in chronic hepatitis C virus infection.* J Immunol, 2008. **181**(6): p. 4219-28.
204. Casado, J.G., et al., *CD8 T cells expressing NK associated receptors are increased in melanoma patients and display an effector phenotype.* Cancer Immunol Immunother, 2005. **54**(12): p. 1162-71.
205. Meresse, B., et al., *Reprogramming of CTLs into natural killer-like cells in celiac disease.* J Exp Med, 2006. **203**(5): p. 1343-55.

206. Ljunggren, H.G. and K. Karre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunol Today, 1990. **11**(7): p. 237-44.
207. Moretta, A., et al., *Receptors for HLA class-I molecules in human natural killer cells.* Annu Rev Immunol, 1996. **14**: p. 619-48.
208. Chappert, P., et al., *Antigen-specific Treg impair CD8(+) T-cell priming by blocking early T-cell expansion.* Eur J Immunol, 2010. **40**(2): p. 339-50.
209. Redmond, W.L. and L.A. Sherman, *Peripheral tolerance of CD8 T lymphocytes.* Immunity, 2005. **22**(3): p. 275-84.
210. Bolinger, B., et al., *Immunologic ignorance of vascular endothelial cells expressing minor histocompatibility antigen.* Blood, 2008. **111**(9): p. 4588-95.
211. Sun, Y., et al., *The kinase insert domain-containing receptor is an angiogenesis-associated antigen recognized by human cytotoxic T lymphocytes.* Blood, 2006. **107**(4): p. 1476-83.
212. Lee, S.H., et al., *Endoglin (CD105) is a target for an oral DNA vaccine against breast cancer.* Cancer Immunol Immunother, 2006. **55**(12): p. 1565-74.
213. Srinivasan, M. and K.A. Frauwirth, *Peripheral tolerance in CD8+ T cells.* Cytokine, 2009. **46**(2): p. 147-59.

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