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REGULATION OF NON-RESPONSIVENESS AND DEATH IN CYTOTOXIC T CELLS BY THE AGONISTIC POTENCY OF MHC:PEPTIDE LIGANDS

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To the memory of my mother

ABSTRACT

CD8+ T lymphocytes are important for immunological control of infections and tumors. The key interaction required to initiate the process of T cell activation is the engagement of the T cell receptor (TCR) with a major histocompatibility complex (MHC) class I/peptide complex on an antigen presenting cell (APC). Depending on the affinity of the interaction between the TCR and MHC class I molecule, different arrays of signaling pathways can be activated in the T cell.

Molecular alterations in the peptide bound to the MHC class I can lead to a lower affinity of the MHC:TCR-interaction resulting in incomplete or qualitatively different T-cell responses. Altered peptide ligands (APLs) exhibiting such activity are referred to as partial agonists and often occur naturally through genetic instability, which affects T cell epitopes derived from rapidly mutating viruses or tumor-associated cellular antigens.

We studied the molecular basis of partial agonism using MHC class I/peptide tetramer complexes. By using tetramers assembled with a fully agonist peptide or its synthetic variant we could study the relationship between tetramer staining, cytokine production and different pathways of activation induced cell death (AICD). We found that positive tetramer staining correlated with at least two different activation programs in CD8+ T lymphocytes: full scale activation associated with Fas-dependent AICD and an incomplete activation followed by Fas-independent AICD.

Further, we used raft-disrupting agents to assess the role of lipid rafts in determining the agonistic potency of different peptide ligands. We showed that overall binding of specific tetramers to CTLs was reduced upon raft disruption, although the half-life of tetramer:TCR complexes formed under these conditions was not affected. These findings suggest that different TCR complexes on the surface of CTLs may have different requirements for cholesterol and CTLs may be heterogeneous in their raft structure.

In addition we analyzed programs of negative regulation of CD8+ T lymphocytes and the capacity of APLs to activate and modulate such programs. Upon specific triggering CD8+ T-cells become refractory to a secondary stimulus; a condition referred to Activation induced Non-Responsiveness (AINR). We have shown that TCR-triggering results in a novel degradation pathway of Lck, a kinase which plays a critical role in the initiation of T cell activation. Down-regulation of Lck through degradation correlated with AINR in CTLs. By blocking Lck degradation we could prevent the development of AINR. We further investigated how activation of CTLs with APLs affected Lck expression. The capacity of different peptide variants to induce Lck degradation correlated with their agonistic potency.

Inefficient recognition of APLs by specific T lymphocytes is believed to contribute to the failure of the immune system to control certain tumor types and progressive viral diseases. To better understand the regulation of APL activity by immunologic help, we analyzed the capacity of exogenous IL-2 and IL-15 to influence different aspects of

activation triggered in CTLs by either fully or partially agonistic peptide ligands. We showed that signals induced by the lymphokines synergize with weak TCR signaling induced by partially agonistic APL, converting many of these peptides from inhibitory to stimulatory ligands. We also demonstrated that IL-2 and IL-15 suppress induction of a death receptor-independent apoptotic program triggered by partially agonistic APL.

In conclusion, we have analyzed the molecular basis of partial agonism in CTL recognition of peptide epitopes and characterized molecular changes associated with death and AINR in specific CTLs. We have shown that structural changes in the sequence of CTL peptide epitopes may decrease the affinity of MHC/TCR interactions and generate APLs, which not only trigger incomplete activation programs but also induce and modulate negative regulation programs in CTLs. This APL induced signaling of suppressive nature appears to be more prominent in the absence of immunological help, suggesting that under conditions of immune deregulation APLs may actively suppress CTL responses against infectious agents or tumors.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. Wei C, **Uhlin M**, Masucci MG, Levitsky V
Tetramer binding and secretion of interferon-gamma in response to antigenic stimulation are compatible with a range of affinities of MHC:TCR interaction and distinct programs of cytotoxic T-lymphocyte activation.
Hum Immunol. 2002 Oct;63(10):821**.**

II. **Uhlin M**, Masucci MG, Levitsky V.
Pharmacological disintegration of lipid rafts decreases specific tetramer binding and disrupts the CD3 complex and CD8 heterodimer in human cytotoxic T-lymphocytes.
Scand J Immunol. 2003 Feb;57(2):99-106

III. **Uhlin M,** Masucci MG, Levitsky V
Regulation of Lck degradation and refractory state in CD8+ cytotoxic T-lymphocytes.
Proc Natl Acad Sci U S A. 2005 Jun 28;102(26):9264-9.

IV. **Uhlin M,** Sandalova E, Masucci MG, Levitsky V
The activity of partially agonistic peptides recognized by cytotoxic T-Lymphocytes is modulated by help signals triggered by exogenous lymphokines.
Eur J Immunol. 2005 Oct;35(10):2929-39.

CONTENTS

LIST OF ABBREVIATIONS

A8	IVTDFSVAK
AICD	Activation Induced Cell Death
AID	Activation Induced Degradation
AINR	Activation Induced Non Responsiveness
AP-1	Activation protein 1
APC	Antigen presenting cell
APL	Altered peptide ligand
CAD	Caspase activated DNAse
CD	Cluster of Differentiation
CTL	Cytotoxic T lymphocyte
EBNA	Epstein Barr virus nuclear antigen
EBV	Epstein Barr virus
ER	Endoplasmic Reticulum
FACS	Flourescence Activated Cell Sorter
FADD	Fas-Associated Death Domain
FasL	Fas ligand
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-based Activation Complex
IVT	IVTDFSVIK
JNK	Jun N-terminal kinase
LCL	Lymphoblastoid cell line
MAP kinase	Mitogen Activated Protein kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
NF-κB	Nuclear Factor κB
NFAT	Nuclear Factor of Activated T cells
PBMC	Peripheral blood mononuclear cells
PKC	Protein kinase C
PLC	Phospholipase C
SMAC	Supra Molecular Activation Cluster
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
W5	IVTDWSVIK
Y5	IVTDYSVIK

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is our defense against the outside world. This intricate system of cells, structures and substances is the barrier inhibiting the establishment of both infections and malignant outgrowth of cancers. During the co-evolution with the numerous microbes in our surroundings the immune system has developed into two major components: the innate and the adaptive part.
The role of the innate immune system is to be the first line of resistance against all kinds of foreign microbes. The protection is initiated already in the skin and mucosal epithelium with antimicrobial peptides and the complement system. If that barrier is broken, macrophages, granulocytes, dendritic cells and Natural killer cells steps in to continue the contest against the foreign microorganisms. These cells rely on receptors recognizing frequently encountered structures residing on the surface of a wide range of bacteria, viruses and protozoa. This part of the immune system is characterized by rapidity and the absence of memory. If the cells of the innate system are not able to eradicate the pathogen, the adaptive immune cells are later ready to assist. The cells of the adaptive immune system consist of immunoglobulin producing B-cells and two subsets of T lymphocytes. The response of the adaptive immunity is dependent on expansion of rare antigen-specific clones. This is likely to account for somewhat slower kinetics of the adaptive immune response compared to that of the innate immunity. The gain is the high specificity and subsequent protection from re-infection through the establishment of specific memory cells.

The two subsets of T cell, CD4+ helper cells and CD8+ cytotoxic cells, play an important role in the immune surveillance against extracellular and intracellular infections, respectively. The CD8+ Cytotoxic T lymphocytes are responsible for the scanning and destruction of cells infected by viruses, intracellular bacteria or malignant tumor cells.

1.2 MHC CLASS I PRESENTATION

MHC molecules are plasma membrane proteins expressed by almost all nucleated cells in the human body. They can bind peptides derived from endogenous, bacterial or viral proteins. The ability of MHC molecules to present a broad spectrum of peptide antigens for T cell recognition is based on a compromise between high affinity and broad specificity. The structural solution to this, as will be discussed in detail below, is a tight binding of the peptide backbone to the MHC molecule supplemented by a less restricted interaction with the peptide side chains allowing conformational as well as side chain variability in the formation of a unique MHC/peptide surface for the T cell receptor (TCR). To further widen the variability of peptides presented each individual is equipped with a number of different MHC molecules.

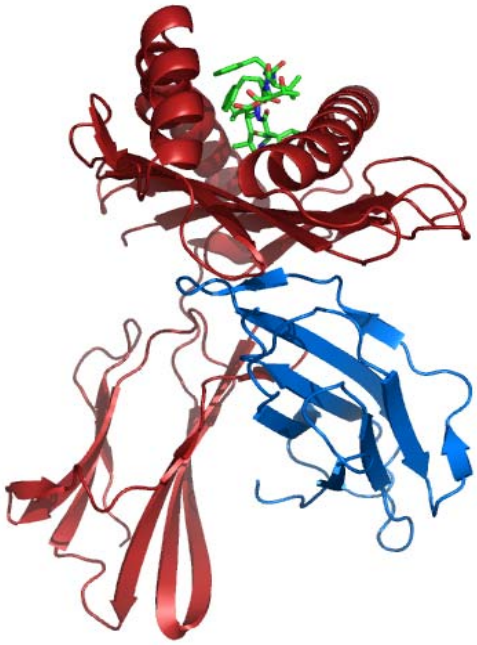
1.3 ANTIGEN PROCESSING AND MHC CLASS I PRESENTATION

The individual cell in the organism has to have an efficient way of showing the surrounding immune system whether it has been infected with an intracellular pathogen. The process starts with the degradation of the foreign proteins into smaller fragments. This is mediated by the proteasome complex, a large cylindrical multi-subunit complex consisting of several subunits with or without proteolytic activity [1]. In order to get recruited to the proteasome proteins need to be ubiquitinated [2]. The ubiquitin molecule is a small protein that gets attached to a lysine on the target protein through a covalent bound whose formation is promoted by a class of enzymes called ubiquitin ligases (reviewed in[3, 4]). Targeted proteins are then unfolded and processed in the proteasome to peptides. The proteolytic activities of this enzymatic machinery can be divided into three major classes, chymotrypsin like activity with preferred cleavage after hydrophobic residues, trypsin like activity with optimal cleavage after basic residues and peptidylglutamyl peptide hydrolyzing activity (PGPH) cleaving after acidic residues. In addition, activities cleaving after branched-chain amino acids and small neutral residues have also been identified [5, 6].
Proteasome degradation generates peptide fragments which have to be actively transported into the endoplasmic reticulum (ER) where they can associate with the relevant MHC I molecule. This process is mediated by the two transporter proteins TAP I and II located in the ER membrane [7]. Together they form a heterodimer, each contributing with one ATP binding domain to the complex. The transportation of peptides is energy dependent and requires hydrolysis of two ATP molecules resulting in a conformational change in the TAP complex that sends the peptide through the membrane into the lumen [8, 9]. The TAP complex has a preference for peptides of 8-16 amino acids in length, which is similar to the size of peptides binding to MHC class I molecules. There is also a selectivity of translocation that is determined by the peptide sequence and, in particular, the character of its C-terminus residue [10].

The MHC class I molecules are assembled in the ER with the help of chaperone proteins which stabilize MHC structure before binding of the peptide (reviewed in [11]). The heavy chain of the MHC class I is co-translationally inserted into the ER membrane and glycosylated. In the ER the heavy chain binds to the chaperone protein calnexin enabling the binding to the light chain β2microglobulin (β2m) [12]. After the conversion of heavy chain into a more mature state the calnexin is released enabling the binding of the next chaperone protein calreticulin [13]. The heavy chain-β2m complex is now able to interact with the TAP/peptide complex with the help of another chaperone protein called tapasin [14]. Upon binding of peptide, the MHC class I/peptide complex (pMHC) is released from TAP and transported through the Golgi apparatus to the cell surface. MHC molecules which can not bind an appropriate peptide are unstable and will eventually be translocated into the cytoplasm and degraded by the proteasome.

1.4 MHC STRUCTURE

All of the classical MHC class I and II molecules are heterodimeric cell surface receptors. The class I molecules are composed of a single membrane-spanning heavy chain divided into a α1, 2 and 3 domain paired with the soluble light-chain β2m. The heavy chain has a highly polymorphic region located in the α1 and α2 domains consisting of two α-helixes and 8 β-sheets. Together they form a cleft where a peptide of 8-10 amino acids in length can bind. The helixes form part of the floor and the walls of the groove while the β-sheets only help forming the floor. The most variable residues of the MHC point into this groove and up from the tops of both helices, conferring unique peptide-binding and TCR-binding specificity of each MHC-molecule (reviewed in [15]).



Structure of the human MHC class I molecule.

The majority of these variable residues are located in the middle of the peptide groove while the conserved ones are acting like anchors for the peptide in the ends of the cleft. These residues often have bulky aromatic side-chains, which seal off the ends of the peptide groove making it a closed section [15]. The majority of MHC class I molecules have 6 distinct pockets in their peptide binding groove. Residues that comprise each of the regions are approximately the same in all the defined MHC class I structures, so that each pocket is located in almost the same place in every MHC class I molecule. The first and the last pocket are conserved while the intermediates are more varying in size and character between different MHC class I alleles, thereby imposing different sequence requirements and constraints on the peptide that is bound. One consequence of this is that MHC class I binding peptides contain allele-specific sequence motifs [16].

The MHC class I molecules need to bind peptides of diverse sequence with both long half-life and high affinity. This is accomplished by using the total sum of the binding energy contributed from all the residues in the binding cleft. The conserved pockets flanking the peptide binding groove are responsible for the fact that the peptides are always oriented in a specific amino to carboxyl direction [17].

1.4.1 MHC class I tetramers

MHC class I tetramers are composed of recombinant MHC class I proteins produced in *E coli,* with a biotinylation site in the heavy chain [18]. Linking four of these biotinylated proteins to a flourochrome-tagged streptavidine creates an excellent tool for detecting the weak binding between antigen specific T cells and their cognate MHC class I molecule by flow cytometry or microscope. After addition of the peptide of interest the tetramer will bind very efficiently to specific T cells even if they are apparent in low frequencies [19-22]. This molecular tool has made a great impact on the knowledge of T cell biology.

1.5 CYTOTOXIC T CELLS

The immune system is a complex network of cells and molecules, witch functions to preserve the integrity of the organism by elimination of all elements being judged dangerous. The key players of adaptive cellular immune responses are T and B lymphocytes. T lymphocytes arise in the bone marrow and migrate to the thymus for maturation. During this process, T cells somatically rearrange gene segments, which combined together encode a unique antigen-binding molecule, the T-cell receptor (TCR). This receptor allows them to monitor almost all cells of the body and destroy cells posing a potential threat to the organism.

The TCR contacts the MHC complex through the TCR variable domains. The affinity between the specific TCR and peptide/MHC is orders of magnitude weaker than that of a specific antibody–antigen interaction [23]. However, because the MHC molecules serve not only as ligands for the TCR, but also as non-antigen-specific ligands for the TCR co-receptors [24]— for example, class I molecules for CD8 and class II molecules for CD4 — even these weak interactions are sufficient to initiate signal transduction in CTLs. Below I will discuss these as well as other unique features of MHC/TCR interactions in T cells which determines the high sensitivity and functional flexibility of this process.

The activation of CD8+ T lymphocytes can be divided into two phases, reflecting different aspects of the response. In the first phase, naïve cells become activated and differentiate into effector cells. In the second phase the effector cells recognize antigen on specific target cells at the site of antigen entry or replication resulting in the destruction of the target cells. Depending on the differentiation stage, CTLs may respond with different efficiencies to signals mediated by the TCR and, therefore they require different levels of co-stimulatory signals for activation. Naïve cells require the signal via the TCR as well as via CD8 and additional co-stimulatory signals, while

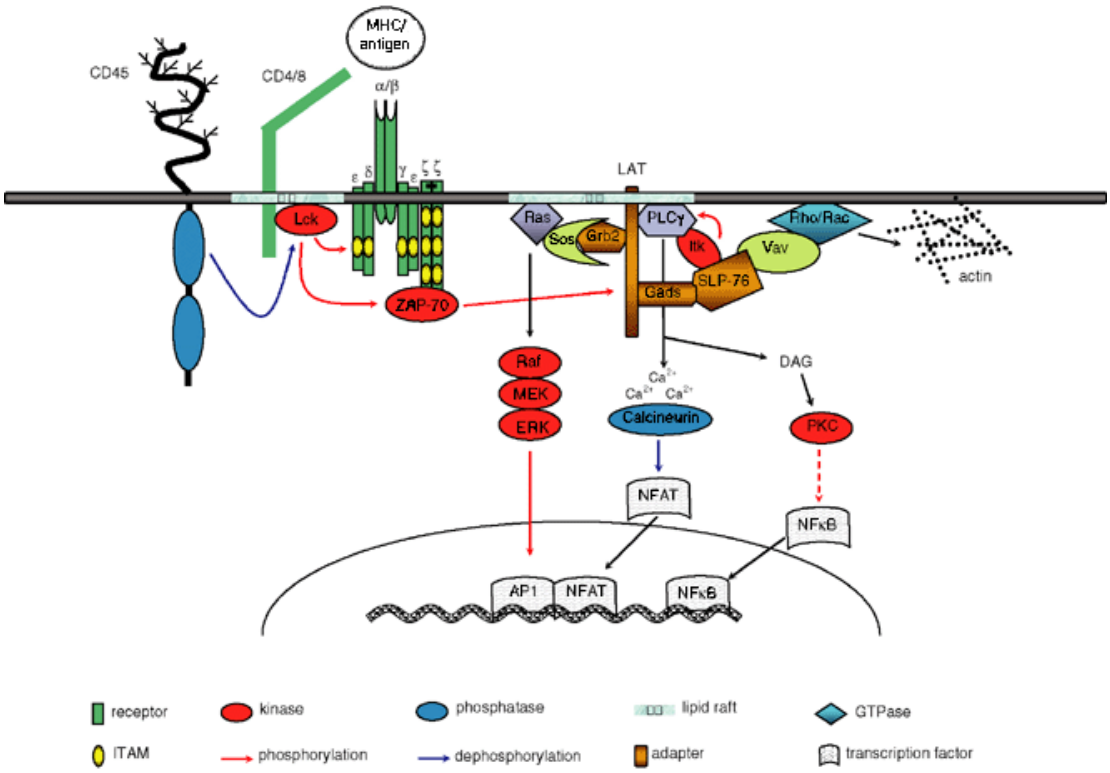
mature effector CTLs only needs stimulation through the TCR [25, 26]. CTLs can be divided into at least 4 different groups, naïve, effector, effector memory and central memory. The different states of T cell differentiation are associated with distinct functional and phenotypic characteristics. The naïve cells, which have yet to encounter their antigen are continuously recirculating between the secondary lymph nodes and the blood stream while the effector and memory cells modulate their expression of different homing molecules changing their paths of circulation. The effectors are found in inflamed tissues, the spleen, and the liver together with the effector memory cells. The circulating pattern of central memory cells more closely resembles that of naïve cells which circulate between the blood stream and the secondary lymph organs [27]. The question regarding the lineage differentiation of CTLs is still under debate [28, 29]. One of the models proposes that memory cells arise from effector cells which do not undergo AICD [30] while another model states that central memory cells arise from naïve cells and further differentiate into effector memory cells and at last end up as terminally differentiated effector CTLs [27, 31].

1.6 TCR MEDIATED SIGNALING

1.6.1 The early activation cascade

The T cell receptor and co-receptors CD4 and CD8 initiate a highly sophisticated network of signaling cascades with the purpose of activating or inhibiting both effector functions and gene transcription in T cells. The most common TCR consists of a α/β heterodimer. The cell surface expression of the TCR occurs in the association with the CD3 γε, δε, and ξξ signaling subunits ([32]and reviewed in [33]). A small subset expresses a variant TCR heterodimer consisting of γ/δ TCR chains. In contrast to the TCR α/β complex this receptor recognizes pathogen derived glycoproteins or nonclassical MHC molecules [34].

Upon encounter with the correct pMHC the α/β heterodimeric TCR probably dimerizes and promotes stabilization of the interaction with the pMHC, enabling the CD8 co receptor to bind non-covalently to an invariant part of the pMHC [24]. Engagement of CD8 has two important functions in T cell activation. It further increases the binding of the TCR to the pMHC and recruits the key src-kinase Lck into a position close to the TCR complex [35-37]. After co-ligation of the TCR with the co-receptor CD8 or CD4, active Lck is proximally positioned to phosphorylate tyrosine residues within the ITAMs located both in the CD3 molecules and in the TCRξ signaling chains [38, 39]. The ITAMs consist of conserved amino-acid sequence motifs D/ExYxxLx(6-8)YxxL. If the clustering of kinases is sufficient it leads to phosphorylation of ITAMs at both tyrosines on the TCRξ and a recruitment of the Syk-family kinase ZAP-70 which binds to the ξ-chain ITAMs via their tandem SH2 domains [40, 41]. ZAP-70 can then readily be phosphorylated and activated by Lck leading to further auto-phosphorylation to achieve its full activation [42]. ZAP-70 then phosphorylates the first wave of adaptor proteins, LAT, PLCγ1 and SLP-76 (reviewed in [43]).



Signaling cascades stimulated by the TCR. A Schematic description of key components of major signaling cascades that are stimulated following recognition of antigen by the TCR. (Adapted from ref [44])

1.6.2 The late activation cascade

The phosphorylated LAT and SLP-76 form a complex together with PLCγ1 that recruit additional adaptor proteins to the proximity of ZAP-70 enabling them to be phosphorylated by the kinase and by this initiating the MAP-kinase pathways [45, 46].
The phosphorylated SLP-76 recruits the adaptor protein Grb-2 that becomes activated by ZAP-70 [47]. This activation recruits the GTP/GDP exchange protein SoS that catalyzes the switch of a GDP on the Ras protein to a GTP rendering it active. The activation of Ras initiates the MAP kinase cascade, which involves at least three steps of sequential activation of kinases resulting in expression of the transcription factor fos. Fos is one of the two partners in the transcription factor complex AP-1 that regulates the transcription of many genes important for effector functions, proliferation and differentiation in T cells [48].
The phosphorylated LAT/SLP-76 also recruits and activates another GTP exchange protein called Vav [49]. The activated form of Vav activates a protein called rac that initiates another MAP kinase cascade including the JNK kinase ending with activation of the second member of the AP-1 transcription complex c-jun [50, 51].

1.6.3 Calcium signaling

The third member in the LAT/SLP-76/PLCγ1 complex PLCγ1 also becomes active upon phosphorylation and catalyzes the hydrolysis of a plasma membrane phospholipid

called phosphatidylinositol 4,5-biphosphate (PIP_2) into two products, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG)[52, 53]. These two products then activate two distinct signaling pathways. The IP_3 diffuses to the ER where it binds to specific receptors, opening Ca^{2+} channels releasing Ca^{2+} into the cytosol [54]. This results in a rapid transient rise in the cytosolic Ca^{2+} levels for a few minutes. This short burst of calcium flow from the ER is followed by a more sustained influx of calcium from the extracellular space. The late influx is mediated by the Ca^{2+} release activated Ca^{2+} channels (CRAC) that become activated following the emptying of intracellular ER Ca^{2+} stores [55]. The increased Ca^{2+} concentrations can in this way be kept in the cell for more than an hour [56]. The Ca^{2+} acts as a secondary signaling substance and binds to the regulatory protein calmodulin. The Ca^{2+}/calmodulin complex activates the serine/threonine phospatase calcineurin which dephosphorylates the transcription factor NFAT enabling it to translocate into the nucleus. There it helps switching on the IL-2, IL-4 and other cytokine genes important for the T cell response.
The other cleavage product of the PIP_2, DAG activates the transcription factor NF-κB through the activation of an isoform of PKC, which is transported into the immunological synapse upon its activation (reviewed in [57, 58]).

1.6.4 Co-stimulation

The ability of naïve T cells to expand and acquire effector functions depends not only on the strength of the stimulus through the TCR but also on an array of co-stimulatory receptors present on the T cells, such as CD28 (reviewed in [59]) as well as on soluble factors referred to as cytokines (reviewed in [60])

1.6.4.1 CD-28 Co-stimulation

The membrane receptor CD28 is a homodimer that has been shown to enhance TCR induced proliferation and differentiation of naive T cells [61]. These properties have given the receptor the reputation of being the molecule responsible for “signal two” in T cells. The two signal hypothesis [25, 26] postulates that an additional signal, besides the TCR, is needed for the naïve T cell activation. The two CD28 ligands CD80 and CD86 are expressed at high levels on professional APCs [62]. It was originally thought that the signal delivered by the CD28 receptor contributes qualitatively to the TCR signaling but this view is now under debate (reviewed in [59]) giving it a more quantitative role.

1.6.4.2 Cytokine co-stimulation

Cellular co-stimulation may play a less important role in the biology of mature effector and memory CTLs because they lack expression of co-stimulatory receptors, such as CD28 [63]. Instead, cytokines from the γ-common chain family have an important function in expansion and survival of both memory and effector subsets of T-cells [64-66].

1.7 MODELS OF TCR TRIGGERING

1.7.1 Serial triggering

The serial engagement theory of TCR triggering was formulated in the middle of the last decade by Valitutti and Lanzavecchia *et al* [67]. In their work the authors tried to explain how T cells can properly be activated by less than 100 specific peptide-MHC complexes presented by an APC [68]. According to the proposed hypothesis a single peptide-MHC complex can sequentially engage and trigger several TCRs as long as the dissociation rate of the MHC complex from the TCR is within the correct time frame. The simplified version of the model would imply that a TCR/CD3 complex can be triggered by a monovalent ligation for a minimum time (in the range between 1 and 5 seconds). This would be the minimum time required for inducing redistribution and clustering of the needed kinases and phosphatases surrounding the engaged TCR, resulting in phosphorylation of ITAMs and further recruitment of signaling components. Although premature dissociation would fail to activate the TCR, dissociation after activation of the signaling cascade would not interfere with signal transduction.
The number of TCRs that can be triggered by a single MHC depends on two factors: the time of ligation required for triggering and the stability of the complex. This implies that the optimal amount of TCRs triggered is when the two factors have equal values. Three assumptions have to be made in order to support this simplified model. The process of triggering must take place in a limited area where (i) adhesion molecules can facilitate the contact between the TCR and MHC/peptide complex, (ii) there is a saturated amount of components for the signaling machinery on the T cell side and (iii) new TCRs can be recruited to replace the ones being constantly downregulated. During the last years this model has gained support due to the increased resolution and power of imaging techniques [69-72].

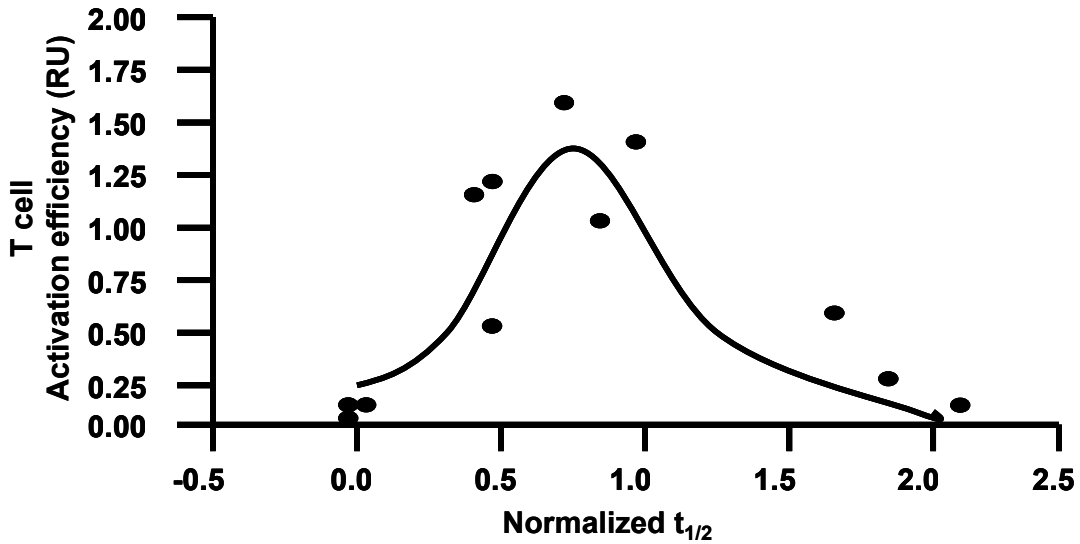
1.7.2 Kinetic proof reading

An additional model that has been proposed at the same time as the serial triggering model is referred to as the “kinetic proofreading” model[73, 74], which attempts to explain the ability of the TCR to discriminate between a foreign antigen and self antigens with the extremely minor difference in affinity that exists. A simple physical model was suggested to account for this sensitivity of T cell response to ligand structure. The kinetic discrimination model postulates that ligands, which bind to the TCR for an extended duration, activate positive T cell signaling, whereas ligands that only bind briefly activate negative signaling. In this way the most important factor for the signaling becomes the dissociation rate of the TCR from the pMHC.
This model is consistent with and elegantly explains reports on T cell receptor antagonism and partial agonism. A question that the model does not address is how the signal is sustained over a long period of time. Implementing the model of serial triggering in this context, the two models complement each other very nicely.

1.7.3 Problems with the models

The models have later been modified to incorporate the idea of temporal and spatial summation [75], where it was proposed that signaling emanating from serially triggered TCRs is not only sustained but incrementally builds up. Both of the models postulated that the activation must take place in a secluded area. This could not experimentally be addressed from the beginning due to the lack of appropriate imaging techniques. A few years later this notion has been experimentally supported by the characterization of the immunological synapse [69].

What these models still have not been able to incorporate was the observations that some peptide variants with a higher affinity and lower dissociation rate induce reduced responses [76-79]. This was elegantly explained by Kalergis *et al*, who proposed a model assuming that efficient T cell activation occurs only within a given half-life range for the TCR-pMHC interaction and that excessively long half-lives are detrimental to T cell function. This would explain previous observations which showed that lengthening half-life does not necessarily increase, but instead can decrease, the agonistic potency of pMHC variants [77, 80]. Furthermore some TCR/pMHC combinations seem to yield an inverse activation pattern stimulating proliferation but not cytotoxicity [77, 81, 82]. This is in total contradiction to what one would expect assuming that the stronger the interaction between the TCR and pMHC the more activation events are triggered in T-cells [83]. This issue still awaits the final resolution.

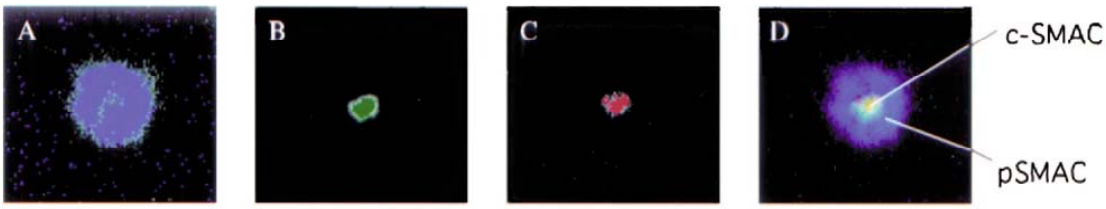


Correlation between the half-life of the TCR-pMHC interaction and TCR activation. Each point represents a TCR/pMHC pair. Parameters on both axes are expressed in relative units. (Adapted from [77])

1.8 IMMUNOLOGICAL SYNAPSE

Shortly after T cell activation is initiated by the engagement of the TCR with pMHC on the APC the T cell polarizes, remodeling the actin cytoskeleton and repositioning the Golgi apparatus and microtubule-organizing center (MTOC) between the nucleus and the T-cell/APC contact area. The contact area is termed the Supra Molecular Activation Cluster (SMAC) or the immunological synapse[69]. The contact area can be divided into the central SMAC and the peripheral SMAC compartment resembling a "bull´s eye" pattern [70]. The TCRs cluster in the central part while adhesion molecules such as ICAM-1 and LFA-1 are positioned in the outer ring-like structure. By numerous imaging studies most of the important signaling molecules involved in T cell activation, such as CD2, CD28, PKC-θ, Lck, CD4 and CD8, have now also been placed in the central SMAC (reviewed in [84, 85]). The precise mechanisms controlling c-SMAC and p-SMAC formation are still unknown although signals created by the TCR itself seem to be important.

Initially it was proposed that the function of the c-SMAC was to help initiating T cell activation by driving receptor aggregation [70]. However, this view has later been changed and it is now known that signaling by the TCR can occur before c-SMAC formation is completed [86, 87]. Also, some early readouts of TCR stimulation, such as Ca2+ mobilization and tyrosine phosphorylation, occur before c-SMAC formation [86, 88].
The characterization of the immunological synapse in many ways experimentally grounded the assumptions made in the framework of the serial triggering theory [89]. The synapse creates a limited area where (i) the adhesion molecules LFA-1 residing in the p-SMAC can facilitate the necessary contact between the cells, (ii) all the needed signaling components are clustered in the c-SMAC and (iii) the polarized MTOC making it possible to recruit more TCR needed for triggering after down-regulation.



The mature immunological synapse. Patterns of LFA-1, TCR and CD28 interaction in a functional synapse between a T cell and a supported planar bilayer containing I-CAM, MHC and CD80 (Adapted from [84])

1.9 LIPID RAFTS

Discoveries of the last decade have dramatically changed our view on the topography of eukaryotic cell membranes [90]. Being initially considered as a relatively uniform surface, today it is viewed as a highly complex arrangement of subdomains with multiple different structural and biochemical characteristics. The domain of interest for T cell activation is the cholesterol and sphingolipid enriched assembly called lipid rafts

[91]. Accumulating data attribute an important role for these surface assemblies in the activation and subsequent effector functions of T cells (reviewed in [92]). Furthermore it has been pointed out that there may be differences in the composition of these subdomains dependent on the differentiation status of the cells, suggesting the possibility for yet another dimension of activation control for different subsets of T cells [93].
Lipid rafts have been described as signal platforms on a vast number of different cell types, not only lymphocytes. The presence of the saturated sphingolipids enables the hydrophobic cholesterol to pack tightly together forming assemblies with less fluidity than the surrounding membrane. The increased rigidity together with the fact that certain proteins can be included or excluded from the rafts gives them an important regulatory role in signal transduction (reviewed in [91]). Proteins with higher raft affinity include GPI- anchored proteins, doubly acylated proteins, such as Src-kinases or cholesterol-linked and palmitoylated proteins [35, 94]. Although the mere fact that a protein is palmitoylated does not always necessarily mean that the protein is localized to rafts. Additional specific amino acid residues in the transmembrane region are often needed [95].
While some molecules are constitutively associated with rafts, several components of the TCR signal transduction cascade are only temporarily included into rafts upon T cell stimulation [96].
Conflicting data has been presented regarding the localization of important signaling molecules in lipid rafts in both resting and activated states of T cells, e.g the TCR [96-98]. These contradictions are probably largely dependent on that the sucrose density gradient-based isolation technique, used for separation of lipid rafts, is highly sensitive to the temperature and detergent composition used. This may introduce a great variability into results obtained by different research groups.
Methods used for analyzing lipid rafts function are mainly based on pharmacological disruption by treatment of cells with the membrane-impermeable cyclic oligosaccharide methyl-β-cyclodextrin (MβCD) or the antifungal polyene Filipin [99, 100]. They work by extracting or interchalating with cholesterol. The use of these methods has given rise to a number of concerns regarding relevance of data obtained with raft-disrupting reagents, because of their non-specific side effects on the treated cells.

1.10 NON-RESPONSIVENESS IN CTLS

The current model of T cell activation postulates that full activation requires the presence of at least two signals, the first provided by pMHC through the TCR and the second in the form of co-stimulation. The first signal ensures the specificity of the T cell response. The second signal involves a plethora of co-stimulatory molecules that regulate the proliferation, survival, and differentiation of T cells. CD28 and LFA-1 are the two most well-defined co-stimulatory receptors and the engagement of these receptors by their respective ligands, B7 and ICAM-1, is necessary for optimal IL-2 production and clonal expansion [26, 101].
In contrast to naïve cells or CD4+ cells, CD8+ cytotoxic T lymphocytes cells become unable to proliferate or produce IL-2 subsequent to the primary TCR triggering

combined with the engagement of co-stimulatory molecules, such as CD-28 [102, 103]. This functional condition of CTLs is often referred to as Activation-Induced Non-responsiveness[104]. In anergic CD4+ T cells, expression of phospholipase C-γ1 (PLC-γ), protein kinase C (PKC)-ϕ and RasGTPase-activating protein (RASGAP) is down-regulated [105]. Alterations in signal transduction were also reported in refractory CTLs [106] but the mechanisms responsible for the development and maintenance of AINR remain poorly defined. In mouse CTLs, AINR may be reverted by exogenous IL-2 [104] in contrast to human cells [107] where AINR seems to be reverted by IL-15 and IFN-α [108]. In contrast to CD4+ cells, the state of non-responsiveness to secondary stimulus seems to directly correlate with the strength of the primary stimulation [109] and (M.Uhlin, unpublished data).

1.10.1 TCR induced degradation of signaling molecules

Upon TCR triggering important signaling molecules in the signaling cascade are internalized and degraded [108, 110-112] The TCR/CD3 complex is degraded in the lysosome, ZAP-70 is degraded by calcium dependent proteases and Lck by other proteases. This activation-induced degradation is an effective way for T cells to efficiently control the activation process [86, 113].
The downregulation of the TCR and ZAP-70 has been shown to be transient [107, 111] in contrast to Lck whose expression was maintained at low levels correlating with non-responsiveness [108] in triggered CTLs. The level of Lck expression affects its net activity. It is noteworthy that Lck expression is often significantly decreased in T lymphocytes infiltrating tumors or circulating in the blood of patients with chronic infections or inflammation that may play a role in the functional incapacity of T cells observed during cancer progression or chronic infections[114-116]. Whether this is due to a constant TCR triggering or a stable down modulation is uncertain.

1.11 APOPTOSIS IN CTLS

Death of T lymphocytes in response to repeated antigen-specific stimulation or nonspecific TCR triggering is referred to as AICD[117]. AICD is believed to account for eventual elimination of the vast majority of specific lymphocytes expanding in response to a foreign antigen and to contribute to the development of transplant or peripheral tolerance [118, 119]. This is a very important homeostatic function keeping the T cell pool at a constant level. Thus, deregulation of death receptor signaling, either allowing too much or too little apoptosis, can lead to autoimmune disorders and also impacts on tumorogenesis and other diseases

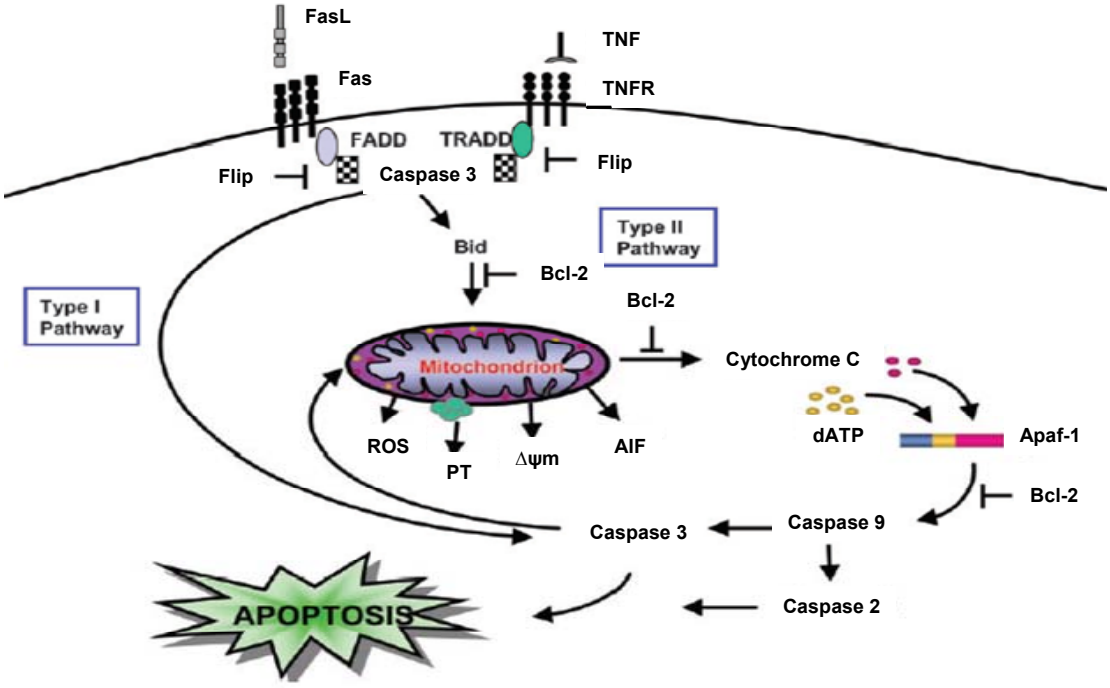
1.11.1 Death receptor mediated AICD

Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, and are able to trigger apoptosis through a caspase mediated pathway. Fas (CD95) has been shown to trigger rapid apoptosis when ligated in activated T cells. It was reported that

activation-induced Fas ligand upregulation and the engagement of Fas on activated mature T cells resulted in AICD *in vitro* [120-122]. However, Fas-FasL interactions make a relatively minor contribution to the peripheral deletion of T cells *in vivo* [123, 124]

Upon stimulation of activated CTLs, CD95 ligand mRNA and protein expression are rapidly induced [125, 126] and increased amount of FasL is exposed at the cell surface. CD95L can then bind to CD95 molecules on the same or neighboring cells and trigger CD95-dependent apoptosis.

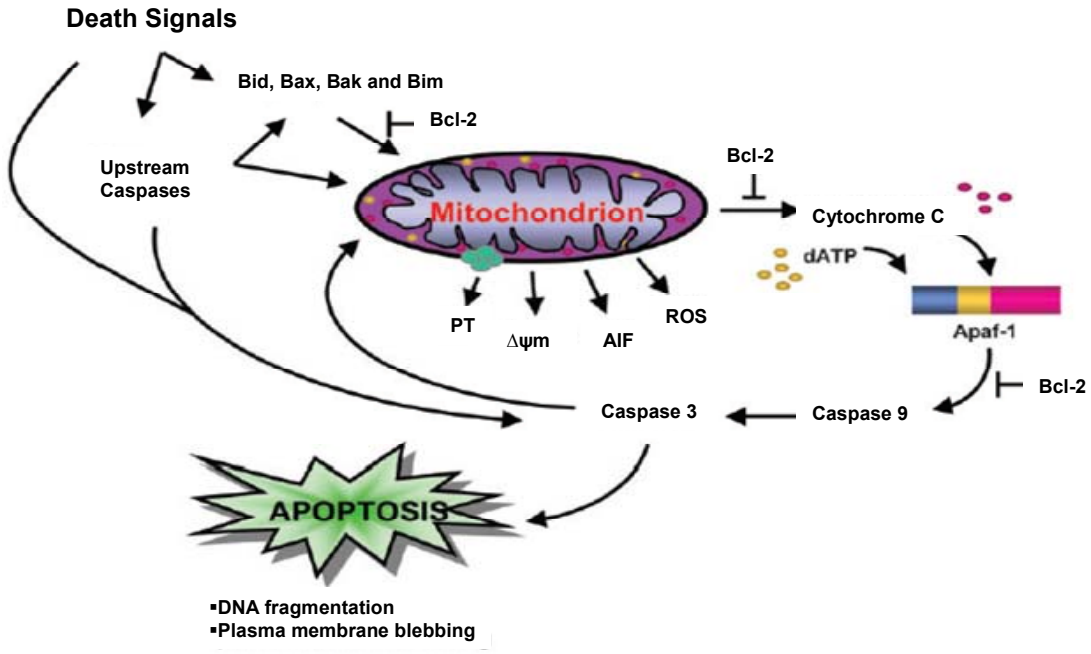
The pathway of Fas mediated apoptosis has been extensively studied (reviewed in [127, 128]. Fas-mediated apoptosis involves the activation of a cascade of cystein proteases, called caspases. FasL is always expressed on the surface in a trimeric form and upon binding of Fas on the target cell trimerization of the Fas is also induced. This multimerization enables an adaptor protein called FADD to bind to a specific death domain expressed in the cytoplasmic tail of the Fas molecule. FADD can then interact with the pro-caspase 8 enabling the caspase to autocatalyze and selfactivate. Activated caspase 8 further cleaves the procaspase 3 thereby activating it and starting the caspase cascade. The cascade culminates by entering of the caspase dependent DNAse into the nucleas of the cell which fragments the DNA into 200 base pair fragments.



The Fas cell death pathway. Fas-induced cell death requires the binding of either membrane-bound or soluble Fas ligand to the Fas receptor on the cell surface. This initiates the formation of the death-inducing signaling complex (DISC), including Fas, FADD and caspase 8. (adapted from [129])

1.11.2 Death receptor independent AICD in CTLs

Although Fas-FasL interaction has been shown to play an important role in AICD of mature T cells, the *in vivo* data clearly suggests that there are additional pathways involved in peripheral deletion of mature CTLs [123, 124, 130].
One important molecule that has been implicated in AICD is the pro-apoptotic bcl-2 protein family member BH3-only protein Bim [130-132]. Recent studies using gene knockout mice have identified Bim as a major regulator of apoptosis in the lymphoid system. Bim plays a critical role in both thymic and peripheral deletion of T lymphocytes [130, 133, 134] and is required for B cell death, in response to crosslinking of surface immunoglobulins [135] Bim is regulated both at the level of protein expression and by posttranslational modifications such as phosphorylation [134]. Three major forms of Bim protein, small (Bim_S), large (Bim_L), and extra-large (Bim_{EL}), can be generated by alternative mRNA splicing with the Bim_S variant having the most active pro-apoptotic function [136]. Exactly how Bim performs its pro-apoptotic function is still debated, but ectopic expression of Bim is sufficient to induce a drop of mitochondria membrane potential and initiate apoptosis[137]. The currently most accepted model suggest that Bim mediates apoptosis in mature T cells is by binding to anti-apoptotic proteins of the bcl-2 family, which reside in the mitochondria, thereby inhibiting their function [138].



Death receptor independent apoptotic pathway. Cells receive a non-receptor-mediated death signal to initiate the apoptotic pathway. Pro-apoptotic Bcl-2 family protein (i.e. Bid, Bax and Bim) are activated, resulting in a cascade of molecular events that act at the mitochondrion: loss of membrane potential, reactive oxygen species production, increased permeability and release of apoptosis-inducing factor. (adapted from [129])

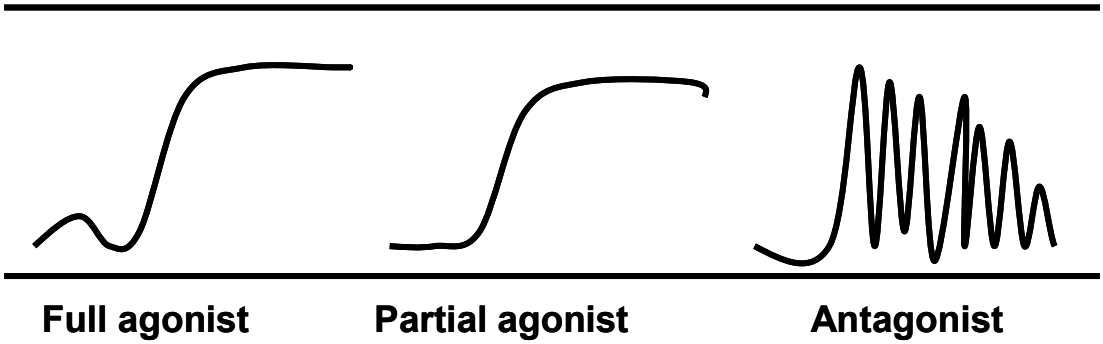
1.12 APLS AND TCR SIGNALING

TCR signaling does not act in a binary fashion described as a regulated transition from the on to off state in the system. Instead the TCR can be differently engaged by pMHC complexes to elicit a variety of functional outcomes [139, 140]. Depending on the capacity to activate different parameters of T cell responses, TCR ligands can be classified as agonists, partial agonists or antagonists. The agonistic ligands include full agonists, which are highly immunogenic and induce the full set of T cell effector functions, and weak agonists, which also induce the complete array of T cell effector functions but at decreased levels and only when significantly higher concentrations of the peptide antigen are used. Partial agonists are defined as peptides selectively inducing some effector functions while failing to induce others, e.g. proliferation [107, 141]. Antagonist peptides, when presented in combination with the native, immunogenic peptide, can inhibit T-cell effector functions.

Initially, differences in the early TCR mediated signaling between agonists, partial agonists and antagonists were observed in the phosphorylation pattern of the CD3 ξ chain [139, 142]. Full agonists induce complete tyrosine phosphorylation of the ξ chain, giving rise to two ξ-forms p21 and p23, tyrosine phosphorylation of the other cd3 subunits, δ, ε, full activation of the Lck kinase which enables a complete recruitment, tyrosine phosphorylation and activation of the ZAP-70 kinase. In contrast, engagement of the TCR with partial agonist ligands causes differential tyrosine phosphorylation of the CD3 ξ chain and almost no phosphorylation of the CD3 ε and only recruitment but no phosphorylation of ZAP-70 [139, 143]. The changes in phosphorylation of CD3 subunits upon triggering with partial agonists be reproduced by stimulating T cells with weak agonists or low concentrations of full agonist [143].
It is not entirely clear, how exactly the phosphorylation of the components of the TCR signaling machinery is initiated and what accounts for the differences in the phosphorylation patterns induced by different peptides. Recently, it was reported that either ab-crosslinking of the TCR or engagement of TCR-CD3 with a cognate pMHC ligand induces a conformational change that exposes a proline-rich sequence in CD3 ε resulting in recruitment of the adaptor protein Nck. These events occurs prior to and independently of tyrosine kinase activation [144, 145]. Differences in the nature or kinetics of this conformational change might explain the differential CD3 phosphorylation pattern observed after T cell activation with different peptides, as well as the capacity of some MHC:peptide ligands to stimulate proliferation without cytotoxicity[81, 82].
Additional evidence for this conformational change has been provided in the study by Krogsgaard *et al* [146]. The observation that certain pMHC with a relatively high dissociation rate induce stronger T cells responses than their counterparts with a lower dissociation-rate, could be reconciled by the demonstration that functionally more potent MHC:peptide ligands induce a more profound conformational change in the TCR upon binding increasing the exposure of the phosphorylation sites in the CD3 subunits with a more rapid kinetics.

Calcium fluxes induced by partial agonists and antagonist differ from that induced by full agonists [147]. The most predominant pattern of a Ca2+ increase induced by a full

agonist is a small sinusoidal peak followed by a sustained high response. This differs from the most frequent pattern of calcium response induced by partial agonists which is characterized by a continuous high influx without the preceding sinusoidal peak. Triggering with antagonists induce yet another pattern with large frequent oscillations. By treating T cell clones with genistein, an inhibitor of protein tyrosine kinases, calcium flux induced by the full agonist could be blocked but not the differential fluxes that could be seen in cells stimulated with partial agonists or antagonists. These calcium fluxes on the other hand could be blocked by a PKC specific inhibitor. This suggests that APLs which are unable to activate calcium flux through the usual Lck, PLCγ pathway are able to do it by an alternative PKC dependent pathway.



Typical patterns of Ca2+ response in a T cell clone stimulated with various peptide ligands. (Adapted from [147])

1.13 APLS IN DISEASES

The role of altered peptide ligands in different pathologic conditions is dual. In the case of immune responses to antigens characterized by extensive antigenic variation e.g. HIV, HCV or certain tumors, the role of APLs might be everything else than beneficial while in other situations, such as vaccination or transplantation, physiological activity of APLs can be exploited for the development of new treatment modalities.

1.13.1 APLs in viral diseases

Some RNA viruses encode for RNA polymerases, which lack proofreading activity, and because of this they produce viral genomes containing random base mutations. In the presence of immune selection pressure, which is exerted by CTLs against the wild-type virus, this could facilitate preferential expansion of mutant progeny encoding altered epitopes that escape the recognition of effector CTLs. This phenomenon is especially well documented in the cases of HIV and HCV infections [148-152].

Selection of virus mutants in CTL epitopes always occur in the context of viral fitness when the optimal variants represents the result of adaptation to prevailing conditions within the host environment with minimal adverse effects on virus function. With a certain degree of simplification, all residues in a CTL peptide epitope can be divided into 2 types: the anchoring residues and TCR contact residues. Non-conservative amino

acid substitutions in the anchoring positions decrease the affinity of peptide binding to the relevant MHC molecule thereby preventing its presentation. Theoretically mutations of this kind should be the most efficient in abrogating the recognition of virus infected cells by specific CTLs. Therefore, it is surprising that selection of mutated HIV and HCV variants often favors viral strains that carry mutations in the TCR contact residues of CTL epitopes [150, 151]. It has clearly been proven that there is a positive selection of CTL escape virus variants during primary infection and during the progression to AIDS or chronic HCV [148, 149, 153, 154]. Because of the rapid replication rate even a very small survival advantage, can in this case, rapidly increase the proportion of a new variant within the host altering the efficiency of the original CTL response. Though, it is important to remember that even if one CTL can not recognize an escape mutant it does not necessarily mean that all CTLs are silent. This is all dependent on the flexibility of the specific TCR in a given clone [155].

APLs may play a role in these virus infections in multiple ways. They may act as decoys [156], as have been shown in cold target inhibition assays *in vitro*. Here target cells pulsed with APLs are added in excess to target cells pulsed with the original peptide that inhibits the killing of original targets. This decoy activity may be relevant *in vivo* particularly in lymph nodes where a high concentration of mutant virus can be achieved. Decoy activity seems to differ from antagonism where the agonist and antagonist must be presented on the same cell to observe the activity of the antagonistic ligand [155].

It has also been reported that APLs can induce proliferation of CTLs *in vitro* without acting as targets in short term killing assays [82]. This might reflect a slower activation of CTLs by the APL[157]. This inversed hierarchy may have a beneficial role for the virus skewing the CTL repertoire into proliferation in response to virus-infected cell without killing it. The opposite situation where the APLs trigger cytotoxicity but not proliferation in the CTLs is better documented (reviewed in [158]). In this scenario it is also easier to understand the benefit for the mutant virus progeny against the wild type. After T cell activation a high proportion of the specific CTLs undergoes AICD. While the proliferative response is not induced the T cells will slowly extinguish themselves and as a result the virus that has not been eradicated can multiply to higher virus titers.

In addition to eliciting no response or an altered response, APLs have been shown to inhibit the normal response of CTL when they are presented together with the original epitope on the same APC [155, 157, 159]. This phenomenon, termed antagonism, is not the result of competition for MHC class I binding sites with the antagonist ligands since the same effect is not seen when an excess of irrelevant peptides is added [155].

1.13.2 Antigenic sin

The term “original antigenic sin” was first used to describe the antibody response to influenza virus. After an initial infection, re-infection with a new virus strain increased the concentration of antibodies specific for the earlier infecting strain[160]. Although the antibodies generated during the first infection cross-reacted with the new virus they

still had higher affinity for the original strain. This phenomenon was adapted to CTL responses by Klenerman and Zinkernagel 1998 [161], when they described original sin in the CTL response against LCMV. They could show *in vivo* that mice primary infected with the wild type strain showed only a poor secondary response against a mutated variant reacting still mainly to the original strain.
Two possible explanations for this have been proposed. The first one suggests that after a very strong primary CD8 response a high number of the CTLs develop into memory phenotype. A weakly cross reacting virus variant might because of this more easily reactivate these memory cells than much less abundant naïve CTL precursors. High frequencies of specific memory CTLs can be readily observed in both HIV and chronic HCV virus infections [149, 162]. It should also be taken into account that lower numbers of CD4+ helper cells present at more advanced stages of HIV infection make it more difficult to elicit a naïve CTL response.
The second possibility is simply that memory CTLs from the primary infection remove APCs presenting the new viral epitope so that a new primary response is inhibited.
The possible implications for antigenic sin can be very important for setting up vaccination strategies against both viruses and tumors prone to antigenic variation

1.13.3 Vaccination and APLs

The phenomenon of "antigenic sin" might have major consequences for developing a good candidate vaccine against highly unstable viruses such as HIV and HCV. The reason being that monovalent vaccinations, in the form of one peptide, could worsen the disease by hindering the immune system from making any natural CTL response against the infection, because of the already existing memory against the peptide used in the vaccine. This has been described in mice where the vaccination with multiple peptides derived from HIV showed an enhanced CD4+ response than vaccination with single peptides [163].
In the human system several polyvalent vaccines are now in the phase I trials [164, 165].

2 RESULTS AND DISCUSSION

2.1 PAPER I:

Tetramer binding and secretion of interferon-gamma in response to antigenic stimulation are compatible with a range of affinities of MHC:TCR interaction and distinct programs of cytotoxic T-lymphocyte activation.

The low affinity of the interaction between the TCR and MHC class I does not allow detection of specific T cells with monomeric MHC class I molecules. This problem was overcome by the invention of MHC:peptide tetramers oligomerized through biotinylation and subsequent binding to streptavidin molecules carrying four biotin binding sites [18]. Later studies, which used tetramers for the analysis of specific T cell populations have revealed unexpectedly high frequencies of tetramer positive cells expanded in response to both natural and experimental infections [166, 167]. This raised concerns regarding the functional relevance of tetramer binding to T cells.
The plasticity in the interaction between MHC and TCR is large. This is reflected in the ability of a TCR to recognize and react with a set of peptides that can even lack any sequence similarity with the immunogenic peptide epitope (reviewed in [168]).
It has been shown that tetrameric complexes assembled with APL bind to specific T cells [169, 170] under certain experimental conditions. With this in mind, it could be surmised that at least a proportion of tetramer binding T cells detected during viral diseases or expanded in response to other self or non-self antigens might recognize the relevant peptide as an APL.

Binding of APL containing tetrameric complexes at 37 °C to EBV specific CTL clones

Wei et al have previously characterized an analogue of the highly immunogenic HLA A11 restricted peptide epitope IVTDFSVIK (IVT) derived from the EBNA-4 protein expressed by Epstein Barr Virus (EBV). This analogue contains the F to Y substitution in position 5 (designated Y5) of the IVT peptide and has been reported to act as a partially agonistic APL for the IVT specific CTL clone BK289 [107, 171], triggering only cytotoxicity and apoptosis but no proliferation or IL-2 production. To extend our analysis we tested the agonistic potency of the IVT and Y5 peptides on more T cell clones from different donors expressing different TCRs [172]. In all three clones tested, the Y5 peptide could induce cytotoxicity but no proliferation, as assessed by thymidine incorporation. In parallel we performed tetramer staining of the three clones with IVT and Y5 containing tetramers at 3 different temperatures: 4, 20, and 37 °C. We could demonstrate binding of the APL containing tetramer to all three clones at all temperatures. The staining with the Y5-containing tetramer was less bright than that observed with the full agonist and decreased upon increased temperature but was always above the MFI obtained with a tetramer associated with an irrelevant peptide. The difference in intensity of staining of the APL containing tetramer between the 3 different T cell clones also correlated with the agonistic potency of the peptides observed in functional experiments with these clones. The staining of an APL

containing tetramer at 37 °C is in contrast to what has been shown before by Whelan et al [170]. They have previously reported that HIV-specific HLA-A2 restricted cytotoxic T lymphocytes could efficiently bind APL containing tetramers but only at 4 °C. The concentrations of tetramers used in our study were lower then that used by Whelan and collogues, suggesting that the different observations made in the two studies could be explained by the higher agonistic potential of the APLs used in our model. It would be interesting to perform a wider screen of A2 restricted GAG specific T cell clones to find a T cell /APL combination that could mimic our results obtained in the EBV system.

The dissociation rate of the APL containing tetramer accounts for its decreased binding efficiency to IVT specific CTLs

In order to explain the decreased binding of the APL-containing tetramer we decided to test the on and off rate of the tetramer binding to the three CTL clones. The on-rates of the IVT and Y5 containing tetramers determined at 4 °C were comparable. The staining efficiency of specific T cells with either of the two tetramers decreased at a comparable rate as the amount of the tetramer was gradually titrated down.

The off rate of the interaction was tested by tetramer dissociation experiments. T cell clones were stained at 4 °C with the two tetramers and subsequently cultured at 37 °C in a two chamber well in a large volume to inhibit rebinding, prior to FACS analysis.
The estimated half-life of the Y5 containing tetramer was 5, 2 and less than 2 min with the three T cell clones tested that inversely correlated with their capacity to kill Y5 pulsed cells. The staining intensity with the IVT tetramer increased during the first 20 min of incubation by 25-45 % in all three clones. This was probably a result of two factors. It has been reported that high affinity binding tetramers can be internalized upon binding at 37 °C [170]. This was confirmed by stripping the tetramers from the surface with low pH buffer. This did not decrease tetramer staining intensity of the T cells suggesting that the tetramers were internalized. Another plausible reason for the increased MFI seen in early samples could be explained by a certain loss of tetramer binding from the time zero samples kept on ice for an hour.

***Ex vivo* isolated and *in vitro* expanded IVT-specific CTLs efficiently bind an APL containing tetramer.**

To exclude the possibility that the ability of binding an APL-containing tetramer is a unique characteristic of the three chosen clones, tetramer staining was performed on polyclonal EBV-specific CTL cultures obtained from four HLA A11 positive, EBV seropositive individuals. All four CTL cultures contained CTLs which bound both the IVT and Y5 tetramer although with different intensity. The MFI of IVT tetramer stained cells increased at 37 °C compared to staining intensity obtained at 4 °C in 3 out of 4 individuals. A great proportion of IVT positive cells from three of the donors also stained positive for the Y5 containing tetramers. By investigating the composition of the CTL bulk culture from the forth donor we found that the majority of T cells expressed the same TCR. This TCR was shown to be identical to the TCR expressed by

the T cell clone, described earlier in the study, showing the lowest reactivity against the APL.
Next we tested the ability of APL containing tetramers to stain IVT-specific T cells *ex vivo* from frozen or freshly isolated HLA A11 positive PBMCs. In one sample out of five we could find IVT specific CTLs with a frequency of 0.26 % of total PBMCs. Similar low frequencies of IVT specific T-cells in the peripheral blood of healthy virus carriers were observed by other investigators [173]. Approximately one fifth of the IVT specific cells also stained positive for the Y5 containing tetramer.
Our finding that IVT specific cells of different donors can be stained by Y5 containing tetramer in different donors both *in vitro* and *ex vivo* demonstrates that tetramer binding to specific CTLs does not require a fully agonistic type of interaction between the specific TCR and the correct pMHC. This is in contradiction to several other reports that suggested that only tetramers containing fully agonistic peptides can stain positively at 37 °C [170, 174].

Positive staining with an APL containing tetramer correlates with at least two distinct activation programs in CTLs

Activation of IVT specific CTLs cells with Y5-pulsed cells triggers an alternative activation program [107]. To correlate this with tetramer binding the cytotoxic capacities of the four polyclonal cultures was tested by chromium release assay. All four CTL cultures were able to lyse Y5-pulsed target cells, although less efficiently than cells pulsed with the full agonist. This was in contrast to proliferation measured by thymidine incorporation, as only, IVT pulsed cells were able to effectively trigger proliferation of the four CTL cultures. We further wanted to investigate the capacity of the Y5 peptide to induce lymphokine production in IVT specific clones. CTLs triggered with the Y5 peptide secreted significant amounts of IFN-γ and TNF-α although the amounts were two to three folds lower than that produced by CTLs when stimulated with full agonist. In contrast the partial agonist could not induce any measurable amounts of IL-2 or IL-4.

Our results demonstrate that the Y5-peptide acts as a partially agonistic analogue for IVT-specific T cells derived from different individuals and expressing structurally different TCRs. The program of Y5-mediated T-cell activation is characterized by induction of cytotoxic activity and selective triggering of IFN-γ and TNF-α secretion in the absence of IL-2 production and cell proliferation. We also show that this activation program is distinct from full activation correlated to a positive staining with APL containing tetramers both *in vitro* expanded cells and PBMCs *ex vivo* at 37 °C.

In previous studies involving analysis of tetramer binding to CTLs, the characterization of the agonistic potential of peptide ligands was restricted to the monitoring of cytotoxic activity [170, 174]. Our results stress the importance of a more detailed functional analysis of peptide ligands for assessing the biologic relevance of tetramer/TCR interactions including thymidine incorporation or IL-2 production. Based on our data we propose that at least a proportion of cells staining positive for tetramer may recognize the relevant peptide as an APL. This phenomenon may be relevant for the situation developing in the course of infections by rapidly mutating

viruses such as HIV or HCV, where different quasi-species of the virus coexist giving rise to differences in peptide epitopes [148-150].

2.2 PAPER II:

Pharmacological disintegration of lipid rafts decreases specific tetramer binding and disrupts the CD3 complex and CD8 heterodimer in human cytotoxic T lymphocytes.

Cholesterol extraction from T cells inhibits the capacity of TCR and lipid raft-associated molecules to redistribute following TCR triggering

Increasing amount of data supports an important role of cholesterol-enriched membrane sub-domains, lipid rafts in the regulation of T cell activation [96, 175-177].
However the difference in composition of lipid rafts in various subsets of T cells remains poorly investigated. Several studies have reported differences in the molecular composition of lipid rafts in different stages of T cell development and differentiation [93, 178]. In this study we wanted to investigate the role of lipid rafts in T cell activation of fully differentiated effector CTLs, using EBV-specific, HLA A11 restricted CTL clones [172, 179].

Disruption of lipid rafts was accomplished by treatment of CTLs with the two lipid raft disrupting agents MβCD and Filipin as has been previously described [100, 176]. In order to visualize the disruption of lipid rafts we stained T cell clones with FITC labeled Cholera toxin, which is known to bind to the raft-associated ganglisoside GM1 protein [92], together with CD3 specific antibody. In unstimulated cells both GM1 and CD3 appeared to be located in small patch–like clusters evenly distributed around the membrane, indicating a certain co-localization of the TCR/CD3 complex with lipid rafts exists already before activation. Whether or not the TCR localizes in lipid rafts has been debated, as some observations have placed it outside rafts [97], many other studies demonstrated that the receptors redistributes into rafts following its triggering [96] or is constantly associated with lipid rafts [98]. These observed differences might be explained by the use of T-cells representing different stages of differentiation.

Upon TCR triggering by cross-linking with CD3-specific antibody we observed aggregation of the CD3 complexes and GM protein and the formation of a cap structure facing the APC. Increasing amounts of the raft disrupting agent MβCD progressively blocked activation induced redistribution. These results indicated that cholesterol extraction or intercalation from the cell membrane inhibits the capacity of TCR molecules and lipid rafts to redistribute upon TCR triggering.

Lipid raft disruption inhibits the cytotoxic capacity of CTLs

To investigate the functional impact of lipid raft disruption on CTLs we tested the cytotoxic activity of the CTL clone BK289 against APCs pulsed with the relevant

peptide in a standard cytotoxic chromium release assay. The effector cells were pretreated with MβCD or Filipin or left untreated before addition of the peptide pulsed cells.
Lipid raft disruption induces morphological changes that can be monitored by flow cytometry. Treated cells have different forward/side scatter profile. Such analysis indicated that the effect of the pharmacological treatment was reversible within 1-2 hours. Hence we decided to add EDTA to the cytotoxicity assay in kinetics, in order to block possible cytotoxic activity of the CTLs after raft reconstitution. The results clearly showed that the cytotoxic capacity of the CTLs was initially totally abolished. After 1-2 hours the effect of the treated cells started to fade off correlating with the reversion of the lipid rafts seen by FACS analysis.

It is natural to assume that raft disrupting agents act by abrogating signaling through a significant proportion of TCRs, either excluding TCRs from productive interactions with specific MHC:peptide complexes or preventing the recruitment of TCr signaling components. However other mechanisms can be at play It has been reported that raft disruption could activate Ras/Erk pathway implying that abnormal activation of selected signaling pathways may directly lead to inhibition of T-cell activation [99].

Extraction of cholesterol from the cell membrane decreases the binding of MHC peptide tetrameric complexes to CTLs and affects the integrity of the CD3 complex and the CD8 αβ heterodimer.

In order to investigate whether the functional suppression after lipid raft disruption in CTLs was dependent on the level of alterations of signaling molecules in the cell membrane, CTLs were stained with a number of relevant antibodies and specific IVT-tetramers after raft disruption. We showed that the binding of specific tetramer was reduced by 45 % upon treatment with MβCD or filipin. This was in line with previous observations by Drake et al [100] in a model of mouse influenza specific response. In contrast to their data, however, we observed reduced binding of a conformation-dependent CD3-specific antibody (SK7) as well as an antibody recognizing the fully assembled CD8 heterodimer. This could not be explained by a decrease in the surface expression of these structures as TCR α/β and CD8 α specific, conformation-independent antibodies did not show decreased staining intensity. The conflicting observations between our and the previous studies might be explained by the use of different antibodies, because Drake et al, used CD3ε-specific confirmation independent antibodies to analyze CD3 expression in CTLs after raft disruption. Drake et al [100] proposed that the decreased binding of specific tetramers to T cells after disruption of lipid rafts could either be attributed to an unfavorable change in TCR display on the T cell surface, or that the decreased raft structure could influence the interaction between CD8 and the TCR. Because CD8 has been described to have an important stabilizing function in the binding of certain HLA molecules to the TCR [180], this could explain the decreased tetramer binding.
Our data support a model where the observed decrease in tetramer binding is attributed to a disintegration of both the CD3 complex and the CD8 hetero-dimer.
The incapacity of MβCD or filipin treated CTLs to efficiently kill peptide pulsed target cells is most likely explained by the disintegration of the CD3 complex. What is

interesting is that the disruption of the CD3 complex does not alter the expression level of the TCR α/β complex on the surface. This is interesting also in relation to the fact that surface expression of the TCR α/β complex is dependent on the structural integrity of the CD3 complex, in particular the ζ-chain [181].

The stability of tetramer binding is not altered upon lipid rafts disruption

Even though the staining intensity of specific tetramer binding to T cells decreased by approximately 50 % upon lipid raft disruption, a significant proportion of TCR retained their capacity of associating with MHC:peptide tetramers. By performing tetramer dissociation experiments on MβCD treated or control cells we investigated the possible interference of lipid rafts disruption with tetramer binding stability.

CTLs were stained with either the IVT tetramer or the Y5-containing tetramer [107, 171] for 1 hour at 4 °C and then incubated at 37 °C for different time periods ranging between 10 min and 1 hour before FACS analysis. We detected no significant difference in the kinetics of tetramer dissociation between treated and untreated cells both in the case of IVT tetramer and a less stable binder, the Y5-containing tetramer. This was in contrast to what we had predicted, reasoning that the Y5 tetramer binding, which has a much higher dissociation rate than the IVT tetramer [182], would be more sensitive to the changes in TCR/CD3 expression on the surface.

The fact that the residual TCRs expressed on the surface bind the tetramers with the same stability as untreated cells, indicates that pharmacological disruption of lipid rafts results in the disaggregation of a proportion of TCR signaling complexes as well as their dissociation from CD8 heterodimer, while the rest of the TCR complexes retain their structure integrity, and capacity to associate with specific MHC molecules with relatively high stability. More recent reports have demonstrated a heterogeneity of the molecular composition of different subtypes of lipid rafts characterized by different resistance to cholesterol extraction [183, 184] This could be one explanation for our finding. Whether subsets of TCRs with different sensitivity to cholesterol extraction have different functional properties is another aspect that should be investigated in the future.

2.3 PAPER III:

Regulation of Lck degradation and refractory state in CD8+ cytotoxic lymphocytes.

After specific activation, CTLs enter a refractory state termed activation induced nonreponsiveness (AINR) characterized by their inability to respond efficiently to a secondary stimulus [104, 106]. Here we investigated the activation induced degradation (AID) of Lck and its impact on AINR in activated EBV specific cytotoxic T lymphocytes [107, 171].

TCR triggering induces a rapid and persistent Lck down regulation in activated human T cells

We showed that AID of Lck takes place in CTLs triggered with either an agonistic peptide ligand or TCR specific antibody. The phenomenon was observed in CTLs of different specificity isolated from the three different donors and was reproduced upon CTL triggering with an LCL which endogenously processed and presented physiological levels of the relevant MHC peptide complexes. As a control we used a LCL transformed by an EBV strain that carries mutations in the anchoring residues of the relevant peptide epitopes preventing their presentation [185].

To test if the activation status of the T cell affects AID of Lck we generated five T cell cultures by PHA activation of PBLs from five healthy donors. AID of Lck could only be seen in PHA blasts of all donors after two weeks of culturing in IL-2. After 1 week only 3 donors showed down regulation of Lck. In parallel we analyzed the percentages of CD4 and CD8 positive T cells in the cultures. Increased frequencies of CD8+ cells in the cultures positively correlated with the ability of cells to down regulate Lck upon triggering with CD3/28 beads (M. Uhlin unpublished observation). Stimulation of naïve and memory T cells isolated based on CD45 RO expression (reviewed in [29]) did not reveal any down regulation of Lck indicating that the phenomenon is dependent on the activation status of the T cells rather than on their state of differentiation. This conclusion was confirmed by experiments with TCR transgenic mice. Lck downregulation was observed after *in vitro* challenge with the specific peptide in splenocytes of animals which were immunized with the same peptide but not in splenocytes of naïve TCR transgenic controls.

To test the kinetics of Lck down regulation by TCR triggering we collected samples of T cells activated for different periods of time ranging from 30 min to 120 hours and measured Lck expression by western blot. We observed a rapid downregulation of Lck expression which dropped to about 50 % of initial levels already after 30 min post triggering. The decline continued until about 8 hours, after which point lck expression stabilized at around 20-30 % of initial levels. Culturing the cells in IL-2 containing medium did not restore lck levels after up to 120 hours of culturing. In parallel with this we also measured the capacity of the T cells to respond to a secondary stimulus. In correlation with the AID of Lck the cells remained in a non-responsive state.

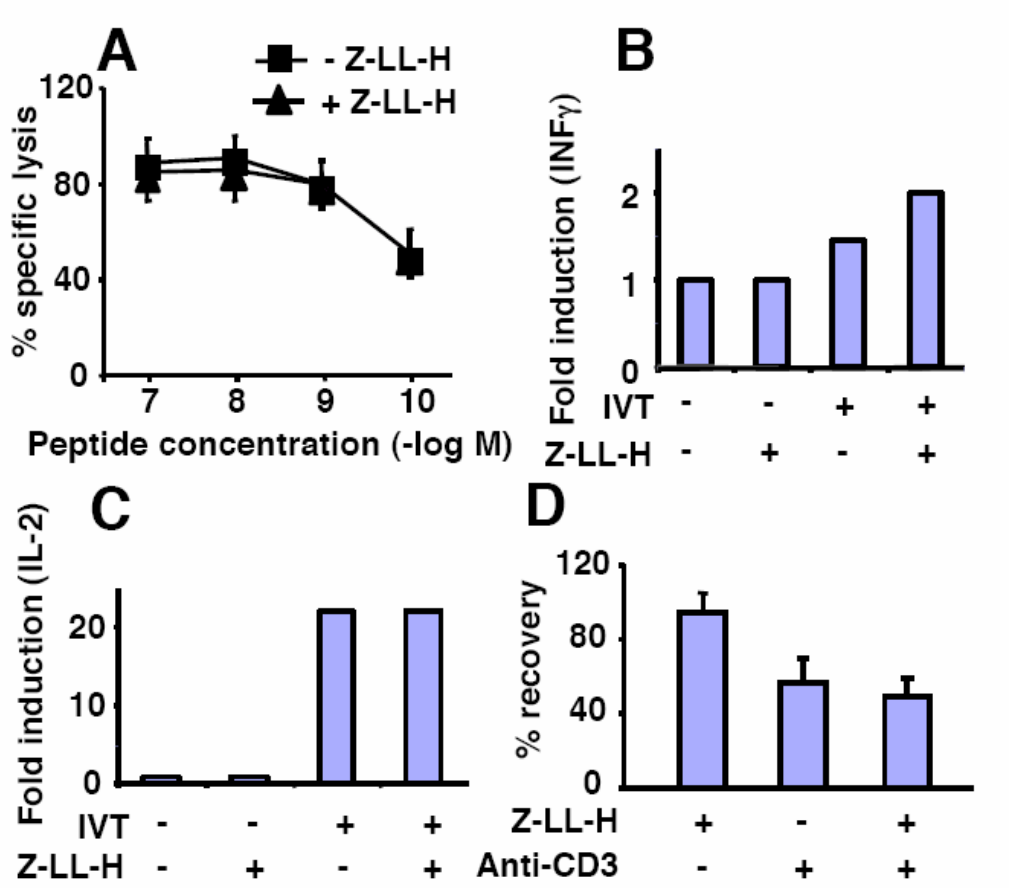
AID of Lck is blocked by cysteine protease inhibitors

The rapid down regulation of the enzyme Lck suggested that it is degraded upon triggering of the TCR. This has been shown before for other important signaling molecules in the TCR signaling cascade [111, 186, 187]. To find the responsible protease pathway we performed blocking experiments with a panel of protease, proteasome and lysosome inhibitors. Down-regulation of Lck was not affected by the presence of the proteasome specific inhibitors epoxomycin or lactacystin during T cell triggering. Nevertheless the inhibitors caused accumulation of ubiquitinated proteins in both activated and control CTLs, induced accumulation of p53 in unstimulated CTLs, and blocked AID of p53. The observation of AID of p53 upon TCR triggering is a

novel finding that could be worth investigating further as a phenomenon potentially linking cancer development and immunity. Interestingly, the steady-state levels of Lck were decreased in the presence of proteasome inhibitors, whereas AID of Lck was not affected. In contrast, MG132, a less selective inhibitor of the proteasome, blocked down regulation of Lck in a dose-dependent manner. The presence of NH_4Cl induced accumulation of the IL-15 receptor α-chain in nonactivated CTLs and further increased its up-regulation after CTL activation. However, NH_4Cl did not affect AID of Lck, indicating no rule of lysosomes in the process. The cysteine protease inhibitor Z-LL-H structurally resembles MG132 but does not inhibit the proteasome [188]. AID of Lck was blocked by this inhibitor in a dose dependent manner. We also observed a rise in the steady state levels of ZAP-70 and ζ-chain and p53 but no but no block in the AID of these molecules in the presence of Z-LL-H.

These results indicated that the degradation pathway of Lck is novel and distinct from the pathways described for the ZAP-70 and ζ-chain degradation [111, 186]. It is noteworthy that there appear to be at least three distinct degradation pathways for the proximal signaling components of the TCR signaling cascade.

The degradation of Lck is not a prerequisite for T cell activation



Inhibition of lck degradation by Z-LL-H does not interfere with T cell activation and effector functions. The capacity of BK bulk CTLs to kill target cells (A), produce IFNγ (B), IL-2 (C) or undergo activation-induced cell death (D) has been tested in the absence or presence of 20 mM Z-LL-H

Lck down-regulation in CD4+ T cells is enhanced upon engagement of CD28 [186] A possible interpretation of this is that degradation of Lck is required for efficient activation of T cells. To address this question we stimulated CTL in the presence of the Z-LL-H inhibitor. We could not see any inhibitory effect of the compound on cytotoxic capacity, IFN-γ or IL-2 production of the drug-treated CTLs compared to untreated cells. We also observed a similar level of AICD after 24 hours in both treated and untreated cells. In the case of IFN-γ production it was even slightly increased.

AID of Lck plays an important role in the development of AINR in CTLs.

To analyze whether the levels of Lck down regulation correlate with both the agonistic potency of different pMHC ligands and the extent of nonresponsiveness induced by different ligands in CTLs, IVT specific T cells were activated using the wild type peptide or two partially agonistic variants, Y5 and A8, which contain an F to Y substitution in position 5 or an I to A substitution in position 8. Both peptides act as partial agonists stimulating only some activation events while being unable to induce proliferation in IVT specific cells [107, 132, 171, 182]. After 1 hour of triggering, cells stimulated with any of the three peptides showed similar downregulation of Lck. After 4 hours, the levels of Lck in T cells stimulated with the full agonist continued to fall whereas a significant reconstitution of the enzyme levels were seen in Y5 and A8 stimulated cells. This correlated with the capacity of the cells to respond by proliferation to a secondary stimulation provided 48 hours later. Cells stimulated with the partial agonists expressed Lck at intermediate levels at this time point (M.Uhlin unpublished observation), which correlated with the capacity of the cells to only partly respond to a secondary stimulation.

By treating CTLs with the Z-LL-H inhibitor we wanted to investigate whether or not non responsiveness against secondary stimulations can be reverted by blocking Lck degradation. CTL cultures or clones were stimulated by immobilized CD3-specific Abs in the absence or presence of Z-LL-H and transferred to new plates to prevent continuous CTL activation. The presence of Z-LL-H during the first CTL triggering inhibited Lck degradation as well as the development of AINR in these cells. Intermediate levels of Lck that could be seen after 48 hours in activated cells maintained in the presence of the inhibitor suggesting an additional mechanism of Lck downregulation that operates in addition to the degradation process, probably due to instability of Lck mRNA that has been shown previously [189].

By transfecting a CTL polyclonal culture with an Lck encoding plasmid we could directly assess the role of Lck in AINR. 24 hours after primary stimulation we transfected CTLs by electroporation with a Lck plasmid or control vector and kept the cells overnight before secondary stimulation. Because of considerable cell death due to the harsh transfection procedure we could not asses AINR by proliferation, instead we measured intracellular IFN-γ production by FACS. Transfection with the Lck encoding plasmid increased the percentage of cells able to produce IFN-γ upon secondary stimulation. The MFI of the IFN-γ positive cells also increased. This was in contrast to

cells transfected with the empty vector or EGFP encoding vector where no effect could be seen.

IFNα and IL-15 can reconstitute Lck expression and inhibit AINR in CTLs

To find physiological signals that could affect Lck levels after primary stimulation we screened a large panel of cytokines. None of the tested cytokines had any affect on the AID of Lck measured after 4 hours. However cells stimulated in the presence of IL-15 or IFN-α for 48 hours expressed levels of Lck comparable to that of unstimulated cells, which correlated with their capacity to respond to a secondary stimulation. We also checked the expression of a number of co-stimulatory molecules on CTLs stimulated in the presence of IL-15 or IFN-α but could not find any alterations.

That IL-2 could neither restore Lck levels nor inhibit AINR, is consistent with our previously published data demonstrating that addition of IL-2 had no significant effect on the production of endogenous IL-2 by refractory CTLs [107]. However, refractory cells in mice reconstitute their responsiveness in the presence of IL-2 [104, 106]. It is well known that IL-15 and IFN-α can promote primary and memory CTL responses. IL-15 has been shown to augment CTL responses against HIV antigens in experimental vaccination models and in PBMCs of AIDS patients ([190-192]). Because IL-15 and IFN-α are produced primarily by monocytes and subsets of dendritic cells (reviewed[193]), the upregulation of Lck by these lymphokines may represent one important mechanism responsible for the enhancing effect of innate immune activation on CTL responses. Decreased levels of Lck is observed in T cells isolated from patients with a number of pathologic conditions including tumors, chronic infections, or chronic systemic inflammation [115, 194, 195]. Our demonstration that low levels of Lck correlate with AINR in human CTLs and that reconstitution of Lck expression and reversion of AINR can be achieved by both pharmacologic agents and lymphokines opens up new possibilities for the development of new approaches to improve the immunologic control of tumors and infections.

2.4 PAPER IV.

Help signals provided by lymphokines modulate the activation and apoptotic programs induced by partially agonistic peptides in specific cytotoxic T lymphocytes.

It has been previously shown that peptides that bind to the MHC class I molecule with high affinity induce CTL activation in a help-independent manner, while suboptimal binders need additional signals in the form of exogenous lymphokines or CD40 triggering [196]. To study how different forms of help modulate the CTL response to APL, we analyzed the capacity of IL-2 and IL-15, which represent help factors produced by CD4+ T cells and cells of the innate immune system, respectively, to modulate the cytotoxic activity, proliferation and survival of human peptide-specific CTLs activated by fully or partially agonistic MHC peptide ligands represented by an

immunogeneic fully agonistic peptide-epitope derived from the EBV encoded EBNA 4 protein[107, 171].

Partial agonists acquire the capacity to induce proliferative response of IVT specific CTLs in the presence of exogenously provided IL-2 or IL-15

By using synthetic analogs of the immunogenic HLA-A11 restricted peptide epitope IVT, which act as partial agonists for the majority of IVT specific clones isolated from different HLA matched donors [107, 171, 182], we checked their capacity to induce cytotoxicity and proliferation in three IVT specific CTL clones. Different levels of cytotoxic activity against peptide pulsed target cells were observed in all of the peptide/ T cell clone combinations, but only the full agonist peptide was able to induce proliferation in the three clones. The only exception was the combination of th e clone BK289 CTLs and Y5-peptide where a small proliferative response could be seen, but it was several folds smaller than that induced by full agonist. The addition of exogenous IL-2 had a very prominent effect on Y5-stimulated BK250 and BK289 clones while in CAR13 the major effect could be seen in cells stimulated with the W5 analogue. The same effects could be seen after addition of exogenous IL-15. CAR 13 cells stimulated with the full agonists in the presence of the cytokines did not show any proliferation above background levels. This was accounted for by Fas-mediated AICD. Even though we could see this synergism between partially agonistic APL and lymphokines using a number of different clone-peptide combinations, it is clear, however, that to be able to stimulate CTL proliferation, a partially agonistic APL should possess a certain minimal agonistic activity. APLs that triggered lysis of target cells only at high peptide concentrations failed to stimulate proliferation even when exogenous IL-2 or IL-15 was present. The use of such weak partial agonists may explain the results of previous studies in which no synergism was observed between partially agonistic APL and IL-2 in the induction of proliferation of CD4+-specific T cells [197].

Triggering with partial agonists in the presence of IL-2 and Il-15 is not associated with alterations in the IL-2 and 15 expression or enhanced engagement of TCR or CD8 co-receptor

In the order to unravel the mechanism behind this synergistic effect of partial agonists and lymphokines we analyzed the expression levels of the receptors for IL-2 and IL-15 upon TCR triggering. There was no upregulation of CD25 on the surface upon APL triggering in the tested clones, and there were no alterations in expression levels upon treatment with either IL-2 or IL-15 compared to untreated cells. The IL-15 receptor expression was not altered in any way on the surface.

After this we wanted to check whether signaling induced by the cytokines affected the efficiency of TCR engagement by analyzing the activation induced redistribution of the CD3 complex on the surface of CTLs. We stained CTLs with both CD3 specific antibodies and cholera toxin β-subunit against lipid rafts. In unstimulated cells both lipid rafts and CD3 complex were uniformly distributed on the surface in small patches. Upon triggering with full agonist, lipid rafts and TCR were redistributed and co-

localized into the contact area between APC and T cells forming a cap structure. Cell stimulated with Y5 peptide did not induce distinct redistribution of rafts or the TCR in the contact area between APC and T cells. In a few cells a "semi-capping" could occur but nothing close to the cap-formations formed by full agonists. The addition of IL-2 or 15 did not alter this pattern.

The extent of TCR and CD8 down-regulation following specific TCR triggering is dependent on the number of peptide-MHC complexes presented by APC, the affinity of MHC-TCR interactions and the level of costimulation [67, 110, 186]. Stimulation with full agonist induced down-regulation of both CD3 and CD8 at the surface of T cells. In contrast, Y5-induced CD3 and CD8 down-regulation was barely detectable and was not enhanced or decreased upon stimulation of BK250 cells in the presence of IL-2 or 15.

Tyrosine-phosphorylation is enhanced by IL-2 and IL-15 in T cells after TCR triggering by both full agonists and partial agonists

We then assessed the repertoire of tyrosine-phosphorylation in CTLs before and after triggering with both IVT and Y5 in the presence or absence of IL-2 and 15. After 3 min of TCR triggering, the overall pattern of phosphorylated species did not significantly change in either IVT or Y5 stimulated samples, probably because of the pre-activated state of the expanding cells. However in both full and partial agonist triggered cells there was an increase in the intensity of many bands, and this effect was more pronounced after addition of exogenous IL-2 or IL-15. These results indicate a cross talk between the signaling pathways of the TCR and lymphokines receptors.

Cross-talk between lymphokine receptors and the TCR at the level of STAT5 activation has been suggested[198], but we could not detect any enhanced STAT5 phosphorylation when APL were combined with lymphokines in our system. The existence of STAT5-independent pathways leading to induction of proliferation of CD8+ T cells has been suggested by recent studies [199]. Therefore, the specific mechanisms accounting for the cooperation between APL-induced TCR triggering and lymphokine receptors remains to be characterized.

AINR induced by partial agonists is reverted by IL-2 or IL-15

It has previously been shown that TCR triggering with a full agonist results in AINR in our model system [107, 108]. We have also shown that triggering with altered peptide ligands results in partial inhibition of responsiveness, the extent of which correlated with the agonistic activity of these ligands [108]. What we could show in this study was that the APL-induced AINR appeared to be qualitatively different from AINR induced by the full agonist, because it could be completely reverted by culturing of the APL activated cells in IL-2 while full agonist activated cells remained non-responsive.

In agreement with our previous data AINR induced by the full agonist was partially reverted by IL-15 [108]. These data suggest that sustained high-affinity T cell response to chronic antigens may be more dependent on continued activation of cells from the

innate immune system producing IL-15 rather than on CD4 produced IL-2. Conceivably, different immunogenic peptides may elicit CTL responses of different affinity due to different levels of peptide presentation on APC or differences in the available TCR repertoires. This may be at least partly responsible for the discrepancy existing in the literature regarding the requirement for different help signals in the induction or maintenance of specific CTL responses.

Exogenous IL-2 or IL-15 suppress upregulation of the pro-apoptotic protein Bim and inhibit apoptosis in CTLs

Partially agonistic peptides can induce death receptor independent apoptosis, which is associated with the up regulation of the pro-apoptotic member of the Bcl-2 protein family Bim [132]. By addition of IL-2 or IL-15 about a third of the cells undergoing AICD upon triggering with the partial agonist W5 could be rescued. This correlated with the ability of the two cytokines to suppress the up regulation of Bim. In contrast the recovery of cells triggered with the full agonist was not affected by exogenous IL-2 or IL-15.

Our data would suggest the following model: In the presence of functional CD4+ help or ongoing activation of monocytes and dendritic cells, partially agonistic peptides will act as null peptides or weak stimulators as the Y5 or W5 peptide in this study. In the absence of help, the latter group of partial agonists is converted to inhibitory peptides that do not stimulate proliferation of specific CTL, suppress their responsiveness to repeated stimulation and actively induce apoptosis, which is predominantly death receptor independent. This change of biological activity of the partial agonist may contribute to the failure of CTL responses against viruses or tumors characterized by frequent mutations in CTL epitopes and dysfunctional CD4+-specific T cell responses. Incomplete or defective activation of monocytes and dendritic cells or loss of their numbers, frequently observed during tumor progression or chronic viral infections, may also contribute to the process, decreasing the levels of available IL-15. In support of this model, several recent studies demonstrated a temporal link between lack or inhibition of CD4 help and increased frequency of new viral quasi-species appearing in the course of HIV and hepatitis C virus infections [152, 200]. As mutations in CTL epitopes can be observed already at very early stages of HIV infection [201], comparison of the biological effects of mutations selected in the acute or late stage of the disease may be particularly interesting in light of our model. Escape from recognition by specific CTL with restricted TCR usage, antagonistic activity against immunogenic peptide ligands (reviewed in [202, 203]) and the phenomenon of antigenic sin [161] were suggested to explain the negative effect of epitope variants on specific CTL responses. It is possible that each given peptide variant can escape CTL recognition or affect CTL function through a combination of several mechanisms. In fact, some of the APL used in this study exhibited antagonistic activity against IVT-specific CTL. However, this effect was observed only for selected clones expressing particular types of IVT-specific TCR upon co-presentation of the IVT peptide and the relevant APL by the same APC (V.L., unpublished data). The results of this study suggest that active suppression of specific CTL responses in the absence of help signals is a common activity exhibited by a large proportion of APL. Detailed analysis of this

effect may result in new strategies for improving immune control over viruses and tumors.

	Proliferation	Fas mediated AICD	Bim mediated AICD	APL induced AINR
Impact on absolute number on specific CTLs	↑	↓	↓	↓
Exogenous IL-2 or IL-15 present	+	—/+	—	—/+
Exogenous IL-2 or IL-15 absent	—	—/+	+	+

The impact of exogenous IL-2 and IL-15 on functional parameters of CTL response induced by partial agonistic APLs.

3 CONCLUSIONS

In this thesis we have analyzed the molecular basis of partial agonism in CTL recognition of altered peptide ligands and characterized molecular changes associated with death and AINR in specific CTLs. From the studies that has been presented the following can be concluded:

We showed that specific staining of CTLs with tetrameric MHC:peptide complexes is compatible with at least two activation programs which can be induced in these cells following recognition of APCs presenting the corresponding peptides on their surface. Of of these programs includes full-scale CTL activation and death of a proportion of activated T-cells in a Fas dependent manner, while the alternative program is characterized by induction of IFN-γ and TNF-α, absence of proliferative response and Fas-independent cell death.

Pharmacological disruption of lipid rafts in human CTL clones disturbs the integrity of the CD3 complex and CD8 heterodimer, without affecting the reactivity with T-cell receptor (TCR)-specific antibodies.

The effect of raft disruption on CD3 and CD8 expression correlates with failure to bind specific tetrameric complexes of different agonistic potency by a proportion of surface TCR molecules.

Interaction of specific tetramer with the rest of surface TCR pools appears to be unaffected, demonstrating that TCR-signaling complexes may differ in their requirement for cholesterol to stably maintain their composition and to rearrange for efficient tetramer binding

T cell receptor triggering results in rapid degradation of the protein kinase lck through a mechanism that is distinct from the pathways involved in degradation of ZAP-70 kinase or ξ-chain of the CD3 complex.

Pharmacologic blockade of lck degradation, as well as transfection of refractory cells with an lck expression vector, increases responsiveness of CTLs to repeated antigenic challenge.

Exposure of exogenously added IL-15 or IFN-α restores both lck expression and responsiveness of preactivated CTLs.

AINR induced by APLs can be reverted by both IL-2 and IL-15.

Signals induced by the lymphokines IL-2 and IL-15 synergize with weak TCR signaling induced by partially agonistic APL, converting some of these peptides from inhibitory to stimulatory ligands.

IL-2 and IL-15 suppress both up-regulation of the Bim, and induction of a death receptor-independent apoptotic program triggered by partially agonistic APLs

The results of these studies improve our understanding of CTL biology and may be important in the development of new approaches to vaccination and immunotherapy.

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I will try to write these acknowledgements in chronological order, most to make it easier for myself, but also to hopefully make it a little bit more informative.

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5 REFERENCES

1. Seemuller, E., A. Lupas, and W. Baumeister, *Autocatalytic processing of the 20S proteasome.* Nature, 1996. **382**(6590): p. 468-71.
2. Michalek, M.T., et al., *A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation.* Nature, 1993. **363**(6429): p. 552-4.
3. Ciechanover, A., A. Orian, and A.L. Schwartz, *Ubiquitin-mediated proteolysis: biological regulation via destruction.* Bioessays, 2000. **22**(5): p. 442-51.
4. Glickman, M.H. and A. Ciechanover, *The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.* Physiol Rev, 2002. **82**(2): p. 373-428.
5. Orlowski, M., *The multicatalytic proteinase complex, a major extralysosomal proteolytic system.* Biochemistry, 1990. **29**(45): p. 10289-97.
6. Orlowski, M., C. Cardozo, and C. Michaud, *Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids.* Biochemistry, 1993. **32**(6): p. 1563-72.
7. Spies, T., et al., *A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway.* Nature, 1990. **348**(6303): p. 744-7.
8. Koopmann, J.O., et al., *Translocation of long peptides by transporters associated with antigen processing (TAP).* Eur J Immunol, 1996. **26**(8): p. 1720-8.
9. Momburg, F., et al., *Peptide size selection by the major histocompatibility complex-encoded peptide transporter.* J Exp Med, 1994. **179**(5): p. 1613-23.
10. Heemels, M.T. and H.L. Ploegh, *Substrate specificity of allelic variants of the TAP peptide transporter.* Immunity, 1994. **1**(9): p. 775-84.
11. Antoniou, A.N., S.J. Powis, and T. Elliott, *Assembly and export of MHC class I peptide ligands.* Curr Opin Immunol, 2003. **15**(1): p. 75-81.
12. Vassilakos, A., et al., *The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules.* Embo J, 1996. **15**(7): p. 1495-506.
13. Gao, B., et al., *Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin.* Immunity, 2002. **16**(1): p. 99-109.
14. Ortmann, B., M.J. Androlewicz, and P. Cresswell, *MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding.* Nature, 1994. **368**(6474): p. 864-7.
15. Maenaka, K. and E.Y. Jones, *MHC superfamily structure and the immune system.* Curr Opin Struct Biol, 1999. **9**(6): p. 745-53.
16. Rothbard, J.B. and W.R. Taylor, *A sequence pattern common to T cell epitopes.* Embo J, 1988. **7**(1): p. 93-100.
17. Bouvier, M. and D.C. Wiley, *Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules.* Science, 1994. **265**(5170): p. 398-402.
18. Altman, J.D., et al., *Phenotypic analysis of antigen-specific T lymphocytes.* Science, 1996. **274**(5284): p. 94-6.
19. Wu, Y., et al., *Frequencies of epitope-specific cytotoxic T lymphocytes in active chronic viral hepatitis B infection by using MHC class I peptide tetramers.* Immunol Lett, 2004. **92**(3): p. 253-8.
20. Loffredo, J.T., et al., *The high frequency Indian rhesus macaque MHC class I molecule, Mamu-B*01, does not appear to be involved in CD8+ T lymphocyte responses to SIVmac239.* J Immunol, 2005. **175**(9): p. 5986-97.
21. Migueles, S.A. and M. Connors, *Frequency and function of HIV-specific CD8(+) T cells.* Immunol Lett, 2001. **79**(1-2): p. 141-50.
22. Rubio, V., et al., *Ex vivo identification, isolation and analysis of tumor-cytolytic T cells.* Nat Med, 2003. **9**(11): p. 1377-82.

23. Matsui, K., et al., *Low affinity interaction of peptide-MHC complexes with T cell receptors.* Science, 1991. **254**(5039): p. 1788-91.
24. Gao, G.F., et al., *Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2.* Nature, 1997. **387**(6633): p. 630-4.
25. Lafferty, K.J., I.S. Misko, and M.A. Cooley, *Allogeneic stimulation modulates the in vitro response of T cells to transplantation antigen.* Nature, 1974. **249**(454): p. 275-6.
26. Schwartz, R.H., *A cell culture model for T lymphocyte clonal anergy.* Science, 1990. **248**(4961): p. 1349-56.
27. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
28. Kaech, S.M., E.J. Wherry, and R. Ahmed, *Effector and memory T-cell differentiation: implications for vaccine development.* Nat Rev Immunol, 2002. **2**(4): p. 251-62.
29. Sprent, J. and C.D. Surh, *T cell memory.* Annu Rev Immunol, 2002. **20**: p. 551-79.
30. Opferman, J.T., B.T. Ober, and P.G. Ashton-Rickardt, *Linear differentiation of cytotoxic effectors into memory T lymphocytes.* Science, 1999. **283**(5408): p. 1745-8.
31. Champagne, P., et al., *Skewed maturation of memory HIV-specific CD8 T lymphocytes.* Nature, 2001. **410**(6824): p. 106-11.
32. Brenner, M.B., I.S. Trowbridge, and J.L. Strominger, *Cross-linking of human T cell receptor proteins: association between the T cell idiotype beta subunit and the T3 glycoprotein heavy subunit.* Cell, 1985. **40**(1): p. 183-90.
33. Kuhns, M.S., M.M. Davis, and K.C. Garcia, *Deconstructing the form and function of the TCR/CD3 complex.* Immunity, 2006. **24**(2): p. 133-9.
34. Adams, E.J., Y.H. Chien, and K.C. Garcia, *Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22.* Science, 2005. **308**(5719): p. 227-31.
35. Kabouridis, P.S., A.I. Magee, and S.C. Ley, *S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes.* Embo J, 1997. **16**(16): p. 4983-98.
36. Shaw, A.S., et al., *Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56lck tyrosine protein kinase.* Mol Cell Biol, 1990. **10**(5): p. 1853-62.
37. Turner, J.M., et al., *Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs.* Cell, 1990. **60**(5): p. 755-65.
38. Straus, D.B. and A. Weiss, *Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor.* Cell, 1992. **70**(4): p. 585-93.
39. Molina, T.J., et al., *Peripheral T cells in mice lacking p56lck do not express significant antiviral effector functions.* J Immunol, 1993. **151**(2): p. 699-706.
40. Iwashima, M., et al., *Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases.* Science, 1994. **263**(5150): p. 1136-9.
41. Chan, A.C., et al., *ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain.* Cell, 1992. **71**(4): p. 649-62.
42. Duplay, P., et al., *p56lck interacts via its src homology 2 domain with the ZAP-70 kinase.* J Exp Med, 1994. **179**(4): p. 1163-72.
43. Chu, D.H., et al., *Pre-T cell receptor signals are responsible for the down-regulation of Syk protein tyrosine kinase expression.* J Immunol, 1999. **163**(5): p. 2610-20.
44. Razzaq, T.M., et al., *Regulation of T-cell receptor signalling by membrane microdomains.* Immunology, 2004. **113**(4): p. 413-26.
45. Zhang, W., et al., *LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation.* Cell, 1998. **92**(1): p. 83-92.
46. Yablonski, D., et al., *Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell.* Science, 1998. **281**(5375): p. 413-6.

47. Jackman, J.K., et al., *Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells.* J Biol Chem, 1995. **270**(13): p. 7029-32.
48. Jain, J., V.E. Valge-Archer, and A. Rao, *Analysis of the AP-1 sites in the IL-2 promoter.* J Immunol, 1992. **148**(4): p. 1240-50.
49. Salojin, K.V., J. Zhang, and T.L. Delovitch, *TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1-/PAK-1/p38 MAPK signaling pathway.* J Immunol, 1999. **163**(2): p. 844-53.
50. Li, W., et al., *Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells.* Science, 1996. **271**(5253): p. 1272-6.
51. Mondino, A., et al., *Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, FosB, and JunB in anergic T helper 1 cells.* J Immunol, 1996. **157**(5): p. 2048-57.
52. Mustelin, T., et al., *T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation.* Science, 1990. **247**(4950): p. 1584-7.
53. Berridge, M.J. and R.F. Irvine, *Inositol phosphates and cell signalling.* Nature, 1989. **341**(6239): p. 197-205.
54. Imboden, J.B. and J.D. Stobo, *Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores.* J Exp Med, 1985. **161**(3): p. 446-56.
55. Zweifach, A. and R.S. Lewis, *Mitogen-regulated Ca2+ current of T lymphocytes is activated by depletion of intracellular Ca2+ stores.* Proc Natl Acad Sci U S A, 1993. **90**(13): p. 6295-9.
56. Donnadieu, E., et al., *Imaging early steps of human T cell activation by antigen-presenting cells.* J Immunol, 1992. **148**(9): p. 2643-53.
57. Randriamampita, C. and A. Trautmann, *Ca2+ signals and T lymphocytes; "New mechanisms and functions in Ca2+ signalling".* Biol Cell, 2004. **96**(1): p. 69-78.
58. Lewis, R.S., *Calcium signaling mechanisms in T lymphocytes.* Annu Rev Immunol, 2001. **19**: p. 497-521.
59. Acuto, O. and F. Michel, *CD28-mediated co-stimulation: a quantitative support for TCR signalling.* Nat Rev Immunol, 2003. **3**(12): p. 939-51.
60. Ahlers, J.D., I.M. Belyakov, and J.A. Berzofsky, *Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines.* Curr Mol Med, 2003. **3**(3): p. 285-301.
61. Shahinian, A., et al., *Differential T cell costimulatory requirements in CD28-deficient mice.* Science, 1993. **261**(5121): p. 609-12.
62. Sharpe, A.H. and G.J. Freeman, *The B7-CD28 superfamily.* Nat Rev Immunol, 2002. **2**(2): p. 116-26.
63. Azuma, M., J.H. Phillips, and L.L. Lanier, *CD28- T lymphocytes. Antigenic and functional properties.* J Immunol, 1993. **150**(4): p. 1147-59.
64. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells.* J Exp Med, 2003. **198**(12): p. 1797-806.
65. Zhang, X., et al., *Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15.* Immunity, 1998. **8**(5): p. 591-9.
66. Marks-Konczalik, J., et al., *IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11445-50.
67. Valitutti, S., et al., *Serial triggering of many T-cell receptors by a few peptide-MHC complexes.* Nature, 1995. **375**(6527): p. 148-51.
68. Demotz, S., H.M. Grey, and A. Sette, *The minimal number of class II MHC-antigen complexes needed for T cell activation.* Science, 1990. **249**(4972): p. 1028-30.
69. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
70. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.

71. Wulfing, C. and M.M. Davis, *A receptor/cytoskeletal movement triggered by costimulation during T cell activation.* Science, 1998. **282**(5397): p. 2266-9.
72. Wulfing, C., M.D. Sjaastad, and M.M. Davis, *Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6302-7.
73. Rabinowitz, J.D., et al., *Kinetic discrimination in T-cell activation.* Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1401-5.
74. McKeithan, T.W., *Kinetic proofreading in T-cell receptor signal transduction.* Proc Natl Acad Sci U S A, 1995. **92**(11): p. 5042-6.
75. Rachmilewitz, J. and A. Lanzavecchia, *A temporal and spatial summation model for T-cell activation: signal integration and antigen decoding.* Trends Immunol, 2002. **23**(12): p. 592-5.
76. Ueno, T., et al., *Functionally impaired HIV-specific CD8 T cells show high affinity TCR-ligand interactions.* J Immunol, 2004. **173**(9): p. 5451-7.
77. Kalergis, A.M., et al., *Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex.* Nat Immunol, 2001. **2**(3): p. 229-34.
78. Sykulev, Y., et al., *Peptide antagonism and T cell receptor interactions with peptide-MHC complexes.* Immunity, 1998. **9**(4): p. 475-83.
79. Baker, B.M., et al., *Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling.* Immunity, 2000. **13**(4): p. 475-84.
80. Carreno, L.J., P.A. Gonzalez, and A.M. Kalergis, *Modulation of T cell function by TCR/pMHC binding kinetics.* Immunobiology, 2006. **211**(1-2): p. 47-64.
81. Lim, D.G., et al., *Allelic variation of MHC structure alters peptide ligands to induce atypical partial agonistic CD8+ T cell function.* J Exp Med, 2003. **198**(1): p. 99-109.
82. Klenerman, P., et al., *The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus.* Eur J Immunol, 1995. **25**(7): p. 1927-31.
83. Viola, A. and A. Lanzavecchia, *T cell activation determined by T cell receptor number and tunable thresholds.* Science, 1996. **273**(5271): p. 104-6.
84. Bromley, S.K., et al., *The immunological synapse.* Annu Rev Immunol, 2001. **19**: p. 375-96.
85. Friedl, P., A.T. den Boer, and M. Gunzer, *Tuning immune responses: diversity and adaptation of the immunological synapse.* Nat Rev Immunol, 2005. **5**(7): p. 532-45.
86. Lee, K.H., et al., *T cell receptor signaling precedes immunological synapse formation.* Science, 2002. **295**(5559): p. 1539-42.
87. Wulfing, C., et al., *Costimulation and endogenous MHC ligands contribute to T cell recognition.* Nat Immunol, 2002. **3**(1): p. 42-7.
88. Zaru, R., et al., *Cutting edge: TCR engagement and triggering in the absence of large-scale molecular segregation at the T cell-APC contact site.* J Immunol, 2002. **168**(9): p. 4287-91.
89. Lee, K.H., et al., *The immunological synapse balances T cell receptor signaling and degradation.* Science, 2003. **302**(5648): p. 1218-22.
90. Simons, K. and E. Ikonen, *Functional rafts in cell membranes.* Nature, 1997. **387**(6633): p. 569-72.
91. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.
92. Janes, P.W., et al., *The role of lipid rafts in T cell antigen receptor (TCR) signalling.* Semin Immunol, 2000. **12**(1): p. 23-34.
93. Balamuth, F., et al., *Distinct patterns of membrane microdomain partitioning in Th1 and th2 cells.* Immunity, 2001. **15**(5): p. 729-38.
94. Shenoy-Scaria, A.M., et al., *Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases p56lck and p59fyn mediates interaction with glycosyl-phosphatidylinositol-anchored proteins.* Mol Cell Biol, 1993. **13**(10): p. 6385-92.

95. August, A., et al., *Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11227-32.
96. Montixi, C., et al., *Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains.* Embo J, 1998. **17**(18): p. 5334-48.
97. Kosugi, A., et al., *Translocation of tyrosine-phosphorylated TCRzeta chain to glycolipid-enriched membrane domains upon T cell activation.* Int Immunol, 1999. **11**(9): p. 1395-401.
98. Xavier, R., et al., *Membrane compartmentation is required for efficient T cell activation.* Immunity, 1998. **8**(6): p. 723-32.
99. Kabouridis, P.S., et al., *Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes.* Eur J Immunol, 2000. **30**(3): p. 954-63.
100. Drake, D.R., 3rd and T.J. Braciale, *Cutting edge: lipid raft integrity affects the efficiency of MHC class I tetramer binding and cell surface TCR arrangement on CD8+ T cells.* J Immunol, 2001. **166**(12): p. 7009-13.
101. Jenkins, M.K., et al., *Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5409-13.
102. Hollsberg, P., et al., *Induction of anergy in CD8 T cells by B cell presentation of antigen.* J Immunol, 1996. **157**(12): p. 5269-76.
103. Deeths, M.J., R.M. Kedl, and M.F. Mescher, *CD8+ T cells become nonresponsive (anergic) following activation in the presence of costimulation.* J Immunol, 1999. **163**(1): p. 102-10.
104. Tham, E.L., P. Shrikant, and M.F. Mescher, *Activation-induced nonresponsiveness: a Th-dependent regulatory checkpoint in the CTL response.* J Immunol, 2002. **168**(3): p. 1190-7.
105. Heissmeyer, V., et al., *Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins.* Nat Immunol, 2004. **5**(3): p. 255-65.
106. Tham, E.L. and M.F. Mescher, *Signaling alterations in activation-induced nonresponsive CD8 T cells.* J Immunol, 2001. **167**(4): p. 2040-8.
107. Wei, C.H., et al., *A partially agonistic peptide acts as a selective inducer of apoptosis in CD8+ CTLs.* J Immunol, 1999. **163**(5): p. 2601-9.
108. Uhlin, M., M.G. Masucci, and V. Levitsky, *Regulation of lck degradation and refractory state in CD8+ cytotoxic T lymphocytes.* Proc Natl Acad Sci U S A, 2005. **102**(26): p. 9264-9.
109. Lim, D.G., P. Hollsberg, and D.A. Hafler, *Strength of prior stimuli determines the magnitude of secondary responsiveness in CD8+ T cells.* Cell Immunol, 2002. **217**(1-2): p. 36-46.
110. Valitutti, S., et al., *Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation.* J Exp Med, 1997. **185**(10): p. 1859-64.
111. Penna, D., et al., *Degradation of ZAP-70 following antigenic stimulation in human T lymphocytes: role of calpain proteolytic pathway.* J Immunol, 1999. **163**(1): p. 50-6.
112. Monjas, A., A. Alcover, and B. Alarcon, *Engaged and bystander T cell receptors are down-modulated by different endocytotic pathways.* J Biol Chem, 2004. **279**(53): p. 55376-84.
113. Valitutti, S. and A. Lanzavecchia, *Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition.* Immunol Today, 1997. **18**(6): p. 299-304.
114. Desbarats, J., K.E. You-Ten, and W.S. Lapp, *Levels of p56lck and p59fyn are reduced by a glucocorticoid-dependent mechanism in graft-versus-host reaction-induced T cell anergy.* Cell Immunol, 1995. **163**(1): p. 10-8.
115. Maccalli, C., et al., *Differential loss of T cell signaling molecules in metastatic melanoma patients' T lymphocyte subsets expressing distinct TCR variable regions.* J Immunol, 1999. **163**(12): p. 6912-23.
116. Matache, C., et al., *Dysregulation of p56lck kinase in patients with systemic lupus erythematosus.* Autoimmunity, 2001. **34**(1): p. 27-38.

117. Green, D.R., N. Droin, and M. Pinkoski, *Activation-induced cell death in T cells.* Immunol Rev, 2003. **193**: p. 70-81.
118. Li, X.C., et al., *T cell death and transplantation tolerance.* Immunity, 2001. **14**(4): p. 407-16.
119. Lenardo, M., et al., *Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment.* Annu Rev Immunol, 1999. **17**: p. 221-53.
120. Russell, J.H. and R. Wang, *Autoimmune gld mutation uncouples suicide and cytokine/proliferation pathways in activated, mature T cells.* Eur J Immunol, 1993. **23**(9): p. 2379-82.
121. Mixter, P.F., J.Q. Russell, and R.C. Budd, *Delayed kinetics of T lymphocyte anergy and deletion in lpr mice.* J Autoimmun, 1994. **7**(6): p. 697-710.
122. Alderson, M.R., et al., *Fas ligand mediates activation-induced cell death in human T lymphocytes.* J Exp Med, 1995. **181**(1): p. 71-7.
123. Mogil, R.J., et al., *Fas (CD95) participates in peripheral T cell deletion and associated apoptosis in vivo.* Int Immunol, 1995. **7**(9): p. 1451-8.
124. Renno, T., et al., *Peripheral T cells undergoing superantigen-induced apoptosis in vivo express B220 and upregulate Fas and Fas ligand.* J Exp Med, 1996. **183**(2): p. 431-7.
125. Westendorp, M.O., et al., *Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120.* Nature, 1995. **375**(6531): p. 497-500.
126. Ju, S.T., et al., *Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation.* Nature, 1995. **373**(6513): p. 444-8.
127. Fas, S.C., et al., *Death receptor signaling and its function in the immune system.* Curr Dir Autoimmun, 2006. **9**: p. 1-17.
128. Roberts, A.I., et al., *The role of activation-induced cell death in the differentiation of T-helper-cell subsets.* Immunol Res, 2003. **28**(3): p. 285-93.
129. Alimonti, J.B., T.B. Ball, and K.R. Fowke, *Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS.* J Gen Virol, 2003. **84**(Pt 7): p. 1649-61.
130. Hildeman, D.A., et al., *Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim.* Immunity, 2002. **16**(6): p. 759-67.
131. Davey, G.M., et al., *Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim.* J Exp Med, 2002. **196**(7): p. 947-55.
132. Sandalova, E., et al., *Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3011-6.
133. Bouillet, P., et al., *BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes.* Nature, 2002. **415**(6874): p. 922-6.
134. Bouillet, P., et al., *Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity.* Science, 1999. **286**(5445): p. 1735-8.
135. Enders, A., et al., *Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells.* J Exp Med, 2003. **198**(7): p. 1119-26.
136. O'Connor, L., et al., *Bim: a novel member of the Bcl-2 family that promotes apoptosis.* Embo J, 1998. **17**(2): p. 384-95.
137. Putcha, G.V., et al., *Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice.* J Cell Biol, 2002. **157**(3): p. 441-53.
138. Deverman, B.E., et al., *Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage.* Cell, 2002. **111**(1): p. 51-62.
139. Sloan-Lancaster, J., et al., *Partial T cell signaling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy.* Cell, 1994. **79**(5): p. 913-22.
140. Kersh, G.J. and P.M. Allen, *Essential flexibility in the T-cell recognition of antigen.* Nature, 1996. **380**(6574): p. 495-8.

141. Uhlin, M., et al., *Help signals provided by lymphokines modulate the activation and apoptotic programs induced by partially agonistic peptides in specific cytotoxic T lymphocytes.* Eur J Immunol, 2005. **35**(10): p. 2929-39.
142. Madrenas, J., et al., *Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists.* Science, 1995. **267**(5197): p. 515-8.
143. Reis e Sousa, C., E.H. Levine, and R.N. Germain, *Partial signaling by CD8+ T cells in response to antagonist ligands.* J Exp Med, 1996. **184**(1): p. 149-57.
144. Gil, D., et al., *Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation.* Cell, 2002. **109**(7): p. 901-12.
145. Gil, D., et al., *T cell receptor engagement by peptide-MHC ligands induces a conformational change in the CD3 complex of thymocytes.* J Exp Med, 2005. **201**(4): p. 517-22.
146. Krogsgaard, M., et al., *Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation.* Mol Cell, 2003. **12**(6): p. 1367-78.
147. Chen, Y.Z., et al., *Modulation of calcium responses by altered peptide ligands in a human T cell clone.* Eur J Immunol, 1998. **28**(12): p. 3929-39.
148. Timm, J., et al., *CD8 epitope escape and reversion in acute HCV infection.* J Exp Med, 2004. **200**(12): p. 1593-604.
149. Tester, I., et al., *Immune evasion versus recovery after acute hepatitis C virus infection from a shared source.* J Exp Med, 2005. **201**(11): p. 1725-31.
150. Allen, T.M., et al., *De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection.* J Virol, 2005. **79**(20): p. 12952-60.
151. Lee, J.K., et al., *T cell cross-reactivity and conformational changes during TCR engagement.* J Exp Med, 2004. **200**(11): p. 1455-66.
152. Grakoui, A., et al., *HCV persistence and immune evasion in the absence of memory T cell help.* Science, 2003. **302**(5645): p. 659-62.
153. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.
154. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.
155. Sewell, A.K., et al., *Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides.* Eur J Immunol, 1997. **27**(9): p. 2323-9.
156. Meier, U.C., et al., *Cytotoxic T lymphocyte lysis inhibited by viable HIV mutants.* Science, 1995. **270**(5240): p. 1360-2.
157. Klenerman, P., et al., *Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants.* Nature, 1994. **369**(6479): p. 403-7.
158. Valitutti, S., et al., *Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy.* J Exp Med, 1996. **183**(4): p. 1917-21.
159. De Magistris, M.T., et al., *Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor.* Cell, 1992. **68**(4): p. 625-34.
160. Francis, T., Jr., *Influenza: the new acquayantance.* Ann Intern Med, 1953. **39**(2): p. 203-21.
161. Klenerman, P. and R.M. Zinkernagel, *Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes.* Nature, 1998. **394**(6692): p. 482-5.
162. Ogg, G.S., et al., *Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA.* Science, 1998. **279**(5359): p. 2103-6.
163. Anderson, D.E., et al., *Overcoming original (antigenic) sin.* Clin Immunol, 2001. **101**(2): p. 152-7.
164. Sha, B.E., et al., *Safety and immunogenicity of a polyvalent peptide C4-V3 HIV vaccine in conjunction with IL-12.* Aids, 2004. **18**(8): p. 1203-6.
165. Zhan, X., et al., *Minor components of a multi-envelope HIV vaccine are recognized by type-specific T-helper cells.* Vaccine, 2004. **22**(9-10): p. 1206-13.

166. Murali-Krishna, K., et al., *Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection.* Immunity, 1998. **8**(2): p. 177-87.
167. Callan, M.F., et al., *Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo.* J Exp Med, 1998. **187**(9): p. 1395-402.
168. Eisen, H.N., *Specificity and degeneracy in antigen recognition: yin and yang in the immune system.* Annu Rev Immunol, 2001. **19**: p. 1-21.
169. Daniels, M.A. and S.C. Jameson, *Critical role for CD8 in T cell receptor binding and activation by peptide/major histocompatibility complex multimers.* J Exp Med, 2000. **191**(2): p. 335-46.
170. Whelan, J.A., et al., *Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent.* J Immunol, 1999. **163**(8): p. 4342-8.
171. Wei, C.H., et al., *Different programs of activation-induced cell death are triggered in mature activated CTL by immunogenic and partially agonistic peptide ligands.* J Immunol, 2001. **166**(2): p. 989-95.
172. Campos-Lima, P.O., et al., *Epitope-dependent selection of highly restricted or diverse T cell receptor repertoires in response to persistent infection by Epstein-Barr virus.* J Exp Med, 1997. **186**(1): p. 83-9.
173. de Campos-Lima, P.O., et al., *T cell responses and virus evolution: loss of HLA A11-restricted CTL epitopes in Epstein-Barr virus isolates from highly A11-positive populations by selective mutation of anchor residues.* J Exp Med, 1994. **179**(4): p. 1297-305.
174. Burrows, S.R., et al., *Peptide-MHC class I tetrameric complexes display exquisite ligand specificity.* J Immunol, 2000. **165**(11): p. 6229-34.
175. Miceli, M.C., et al., *Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes.* Semin Immunol, 2001. **13**(2): p. 115-28.
176. Bi, K. and A. Altman, *Membrane lipid microdomains and the role of PKCtheta in T cell activation.* Semin Immunol, 2001. **13**(2): p. 139-46.
177. Magee, A.I., J. Adler, and I. Parmryd, *Cold-induced coalescence of T-cell plasma membrane microdomains activates signalling pathways.* J Cell Sci, 2005. **118**(Pt 14): p. 3141-51.
178. Leitenberg, D., F. Balamuth, and K. Bottomly, *Changes in the T cell receptor macromolecular signaling complex and membrane microdomains during T cell development and activation.* Semin Immunol, 2001. **13**(2): p. 129-38.
179. Levitsky, V., et al., *The clonal composition of a peptide-specific oligoclonal CTL repertoire selected in response to persistent EBV infection is stable over time.* J Immunol, 1998. **161**(2): p. 594-601.
180. Arcaro, A., et al., *Essential role of CD8 palmitoylation in CD8 coreceptor function.* J Immunol, 2000. **165**(4): p. 2068-76.
181. D'Oro, U., et al., *Regulation of constitutive TCR internalization by the zeta-chain.* J Immunol, 2002. **169**(11): p. 6269-78.
182. Wei, C.H., et al., *Tetramer binding and secretion of interferon-gamma in response to antigenic stimulation are compatible with a range of affinities of MHC:TCR interaction and distinct programs of cytotoxic T-lymphocyte activation.* Hum Immunol, 2002. **63**(10): p. 821-33.
183. Schade, A.E. and A.D. Levine, *Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction.* J Immunol, 2002. **168**(5): p. 2233-9.
184. Shimada, Y., et al., *Separation of a cholesterol-enriched microdomain involved in T-cell signal transduction.* Febs J, 2005. **272**(21): p. 5454-63.
185. Levitsky, V., et al., *Natural variants of the immunodominant HLA A11-restricted CTL epitope of the EBV nuclear antigen-4 are nonimmunogenic due to intracellular dissociation from MHC class I:peptide complexes.* J Immunol, 1997. **159**(11): p. 5383-90.
186. Viola, A., et al., *T lymphocyte costimulation mediated by reorganization of membrane microdomains.* Science, 1999. **283**(5402): p. 680-2.

187. Naramura, M., et al., *c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation.* Nat Immunol, 2002. **3**(12): p. 1192-9.
188. Donkor, I.O., *A survey of calpain inhibitors.* Curr Med Chem, 2000. **7**(12): p. 1171-88.
189. Vanhee-Brossollet, C. and C. Vaquero, *Regulation of p56(lck) messenger turnover upon T cell activation: involvement of the 3' untranslated region in stability as determined in cell-free extracts.* Biochem Biophys Res Commun, 1997. **233**(2): p. 419-24.
190. Kanai, T., et al., *IL-15 stimulates the expansion of AIDS virus-specific CTL.* J Immunol, 1996. **157**(8): p. 3681-7.
191. Castelli, J., et al., *Mature dendritic cells can enhance CD8+ cell noncytotoxic anti-HIV responses: the role of IL-15.* Blood, 2004. **103**(7): p. 2699-704.
192. Oh, S., et al., *Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3392-7.
193. Theofilopoulos, A.N., et al., *Type I interferons (alpha/beta) in immunity and autoimmunity.* Annu Rev Immunol, 2005. **23**: p. 307-36.
194. Stefanova, I., et al., *HIV infection--induced posttranslational modification of T cell signaling molecules associated with disease progression.* J Clin Invest, 1996. **98**(6): p. 1290-7.
195. Romagnoli, P., et al., *A potential role for protein tyrosine kinase p56(lck) in rheumatoid arthritis synovial fluid T lymphocyte hyporesponsiveness.* Int Immunol, 2001. **13**(3): p. 305-12.
196. Franco, A., et al., *Epitope affinity for MHC class I determines helper requirement for CTL priming.* Nat Immunol, 2000. **1**(2): p. 145-50.
197. Grakoui, A., et al., *Molecular basis for the lack of T cell proliferation induced by an altered peptide ligand.* Int Immunol, 1998. **10**(7): p. 969-79.
198. Welte, T., et al., *STAT5 interaction with the T cell receptor complex and stimulation of T cell proliferation.* Science, 1999. **283**(5399): p. 222-5.
199. Duthoit, C.T., et al., *Uncoupling of IL-2 signaling from cell cycle progression in naive CD4+ T cells by regulatory CD4+CD25+ T lymphocytes.* J Immunol, 2005. **174**(1): p. 155-63.
200. Oxenius, A., et al., *Loss of viral control in early HIV-1 infection is temporally associated with sequential escape from CD8+ T cell responses and decrease in HIV-1-specific CD4+ and CD8+ T cell frequencies.* J Infect Dis, 2004. **190**(4): p. 713-21.
201. Jones, N.A., et al., *Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response.* J Exp Med, 2004. **200**(10): p. 1243-56.
202. McMichael, A.J. and R.E. Phillips, *Escape of human immunodeficiency virus from immune control.* Annu Rev Immunol, 1997. **15**: p. 271-96.
203. Collins, K.L., *How HIV evades CTL recognition.* Curr HIV Res, 2003. **1**(1): p. 31-40.