Université Fédérale
Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :
Roxana Khazen
le 29 Janvier 2016

Titre :

Dissecting early mechanism of melanoma cell resistance to cytotoxic T lymphocyte attack

---

École doctorale et discipline ou spécialité**:**
ED BSB : Immunologie

Unité de recherche :
INSERM U1043

Directeur/trice(s) de Thèse :

Prof. Salvatore Valitutti

Jury :

Prof. Roland Liblau	Président
Prof. Claire Hivroz	Rapporteur
Prof. Daniel Speiser	Rapporteur
Prof. Jérôme Delon	Rapporteur

Acknowledgement

This manuscript is a final work of 4 years of research and lab work and resumes most part of my scientific distribution to the field of tumor immunology that I hope would serve to a better understanding of tumor's dialogue with immune system. I have always dreamed of a peaceful and painless world where no one suffers. I chose science and particularly immunology as a mean of helping to construct such a place to live. Completing my PhD is just the beginning of my scientific journey. I am deeply thankful for all those people who thought me research and help me to advance.

I would first and for most like to thank my supervisor Prof. Salvatore Valitutti for his excellent supervision and support during my entire PhD, for the thoughtful discussion and guidance in my growth as a scientist. Thanks for giving me the opportunity to work with you in such a great collaborative environment of your team, for all you did for me as a researcher and as a human being, for understanding my complicated thoughts and coping with my surprises, for the late night corrections and for your encouragements. I couldn't have asked for a better supervisor.

I would also like to thank Prof. Roland Liblau, not only for his valuable insights and advices but also for his excellent leadership of the biomedical research center of physiopathology of toulouse-purpan "CPTP", which provides high rank facility and cooperative-scientific environment for researches to develop and advance their research projects.

I would also like to thank my jury members: Prof. Claire Hivroz, Prof. Daniel Speiser and Dr. Jérôme Delon, for their excellent and valuable advices and insights that helped me provide a better description of my thesis and my visions.

I am very much grateful to Sabina Muller, to whom I dedicate this manuscript. Sabina you were and you are the most kind-hearted person I have ever met. Although words cannot express my feelings and gratitude, I want to thank you for being a fantastic colleague and friend, for pushing me to believe in myself, for your constant support and for holding my hands to be strong. Thanks for teaching me how to do experiments and how to live. I will never forget a moment I spend with you from the chili peppers and champagne bottles on my hood to the anti-stress horse riding session.

I would like to thank Eric Espinosa and Loic Dupre for their valuable and thoughtful suggestions to improve my project. Thanks Eric for always supporting me, for great

discussions and suggestions, also for your patience with my FACS histograms. Loic your passion for science is contagious and you contaminated all of us. Thanks for your insights and your positive attitude.

I would like also to thank Marie-pierre Puissegur for helping me with molecular biology and to introduce me to the magic world of gene expression modification.

A big thank to Nicolas Gaudenzio for being my primary mentor, Nico I guess I have completed the “to do before PhD defense” list, thanks for all the great moments you made, for your helps and for sharing your ex-project and ideas.

Thanks to Fatima L’faqihi for her helps and guidance on Flow-cytometry experiments and her presence and support in my thesis follow up committee.

I would like to specially thank Astrid Canivet for being my personal bible of ImageJ. Astrid thanks a thousand times for answering my endless questions and finding solutions to ameliorate my work. I hope you keep advising me on microscopy when I’m gone.

I want to thank all my friends in the team:

Magda, thanks for welcoming me to the team from the very beginning and for teaching a foreign girl the French systems and for helping me whenever you could. I am happy to have been your colleague and your friend.

Camille, thanks for being there and supporting me no matter what, for sharing your experiences and knowledge as much as you could even when you were so busy.

Regis, you have been a great friend and a great officemate, thanks for sharing your knowledge and also your office stuff and for organizing the nice evenings.

Liza, your presence was precious, I enjoyed every moment of working with you and seeing you advancing, I still remember how excited I was the first time you made that beautiful actin staining on your microscopy slides. You made me find another person in me. I wish you all the best for your bright future.

Delphine, the cat-woman, you are as beautiful inside as you are outside. Thanks for being a good listener and a very kind person.

Raissa, how should I learn high resolution microscopy now that I’m leaving?!! Thanks for all

your kind gestures from the Vanilla Christmas gift till the little green lemon on the hard days of thesis submission. You were always there to comfort me and to cheer me up.

Javier, I should confess that I really regret not having enough time to develop a project with you. Thanks for accompanying me during the difficult moment of scarifying mice and for your talents in WB techniques. Also thanks a lot for your advice and encouragements for thesis completion.

Pauline, it's hard to write something brief for you, but thanks for your: antibodies, plasmid, advices on molecular biology and WB, English-French (and vice-versa) translations, cakes,… and most of all for your beautiful smile.

Romain, I hope there would be enough "choco bon" for you and I'm sorry that you have to compete for them with your other half during the ceremony. Yoan, I wish you a successful PhD and thanks for taking a good photo of me to submit to CPTP website!

I like also to thank ex members of our team: Pauline G., Camille F., Cedric M., Maxime B., Michael P., Rana M., Zilton V. Also all my friends around the world: Zohre, Neda, Mona, Bahareh, Amirali, Nicolas, Emiline, Amandine … thanks for your love and kindness during all these years.

At the end I want to thank my family, for their tremendous support, protection and understanding.

My Dear Yohan, I am so grateful for all the efforts you put in understanding my project, for trying to discuss it with me, for coping with my waves of stress and for the artistic technical support. Thanks for your patience and for your comforting talent of yours.

Khashayar and Sanaz, Thanks for being there for me in any manner and every way. Khashayar, I have always looked upon you to learn, you have been and you are a model for me. I hope that I can be as much a scientist as you are.

Finally my parents: Fereshte and Hossein, thanks for giving me all you could, for believing in me and for your patience during all these years of distance. Thanks for giving me the wings to fly and to achieve my dreams. You are the reason of everything I am and I have today. Thanks a million times for who you are and your unconditional love.

Roxana Khazen

I dedicate this manuscript to Sabina Muller. Thanks for everything…

Summary

Abbreviation

AIRE	Auto-Immune Regulator
AP-1	Activator Protein-1
AP-3	Adaptor Protein 3
APC	Antigen presenting cell
aPKC	atypical PKC
Arp 2/3	Actin-related protein-2/3
Bcl-2	B cell leukemia/lymphoma 2
B-EBV	Epstein Barr transformed B lymphocyte
CAR	Chimeric Antigen Receptors
CatB	Cathepsin B
CCE	Capacitative calcium entry
CD	Cluster of differentiation
CDR	Complementary determining region
CHS	Chediak-Higashi syndrome
CMA	Concanamycin A
cPKC	Conventional or Classical PKC
CR	Complete Response
CRAC	Calcium Release Activated calcium chanel
CRIB	Cdc-42/Rac interactive binding
Csk	C terminal Src Kinase
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated protein-4
DAG	1,2 Diacylglycerol
DC	Dendritic cell
DIA-1	Diaphanous-1
DN	Double Negative
DNA	Desoxyribonuclic Acid
DP	Double Positive
ECM	Extracellular matrix
ER	Endoplasmic Reticulum
Erk	Extra-cellular signal-Regulated protein Kinases
ERM	Ezrine-Radixine-Moesine
FACS	Fluorescence Activated Cell Sorting
FAMM	Familial atypical multiple mole melanoma syndrome
FHL	Familial hemophagocytic lymphohistiocytosis
FLIP	FLICE like inhibitory protein
Foxp3	Forkhead box P3
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating proteins
GEF	Guanine nucleotide Exchange Factor
GFP	Green fluorescent protein
GLUT-1	Glucose Transporter-1
GM-CSF	Granulo-Monocytes-Colony Stimulating Factor
GrzB	Granzyme B

GTP	Guanosine 5′-triphosphate
GvHD	graft versus host disease
Her2/neu	Human Epidermal Growth Factor Receptor 2
HIF1a	Hypoxia-inducible factor -1 alpha
HLA	Human Leukocyte Antigen
HPS2	Hermansky-Pudlak syndrome de type 2
HS1	Haematopoietic-cell-specific protein-1
ICAM-1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
Iκk	I kappa B kinase
IL	Interleukin
IP3	Inositol 1,4,5 trisphosphate
IQGAP1	IQ-motif-containing GTPase activating protein-1
IS	Immunological synapse
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JNK-c	Jun NH2-terminal Kinases
LAMP	Lysosome Associated Membrane Protein
LAT	Linker for Activation of T cells
LCK	Lymphocyte Cell-specific tyrosine Kinase
LFA-1	Lymphocyte function-associated antigen-1
LG	Lytic granule
LLE	Late lysosome endosome
LMP	Low Molecular mass Proteins
LS	Lytic synapse
MAPK	Mitogen activated protein kinase
MC	Microclusters
MCA	Methylcholanthrene
mDC	Myeloid dendtitic cells
MEK	Mitogen-activated or extracellular signal-regulated protein kinase
MHC	Major Histocompatibility Complex
MIP-1	Macrophage Inflammatory Protein-1
Mo-DC	Monocytes drived DC
MTOC	Microtubule organizing center
NFAT	Nuclear Factor of activated T cells
NFκB	Nuclear factor-kappa B
NK	Natural killer cells
NKT	Natural killer T cells
NOS2	Nitric Oxide Synthase 2
PBMC	Peripheral blood mononucleous cells
PD	Progressive Disease
PD-1	Programmed Cell Death
pDC	Plasmoctoide dendritic cells
PDK-1	Phosphoinositide-dependent protein kinase-1

PD-L1	PD-1 ligand
PI	propidiom iodide
PI3K	Phosphatidylinositol-3-OH kinase
PiP_2	Phosphatidylinositol -4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1
pMHC	peptide MHC
PR	Partial Response
pRb	Retinoblastoma protein
Prf	Perforin
PTEN	phosphatase and tensin homolog
RAG	Recombinaition Activating Genes
Ras	Rous avian sarcoma
RECIST	Response Evaluation Criteria In Solid Tumors
ROS	Reactive Oxygen Species
RSS	Recombinaition Signal Sequence
SAg	Super antigen
SH2	Src homology 2
SLP-76	SH2-domain-containing leucocyte protein of 76 kDa
SMAC	Supra Molecular Activation Cluster
Smurf-1	Smad ubiquitination regulatory factor-1
SNARE	Soluble N-ethylmaleimide±sensitive factor Accessory Protein Receptors
TAP	Transporter associated with antigen processing
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper
TIL	Tumor infiltrating lymphocytes
TIRF	Total Internal Reflection Fluorescence
TLR	Toll Like Receptors
TNF	Tumor Necrosis Factor
Tyrp1	Tyrosinase Related Protein 1
VEGF	Vascular Endothelial Growth Factor

List of Figures

Résumé

Roxana Khazen

Etude du mécanisme précoce de la résistance des cellules du mélanome à l'attaque des lymphocytes T cytotoxique.

Directeur de Thèse: Dr. Salvatore Valitutti

Soutenance de Thèse: 29 Jan 2016, Toulouse France

Les cellules de mélanome humain expriment différents antigènes tumoraux qui sont reconnus par les lymphocytes T cytotoxiques CD8 + (CTL) induisant des réponses spécifiques de la tumeur in vivo.

Cependant, chez les patients atteints de mélanome l'efficacité de la réponse naturelle des CTL ou stimulée par thérapie est limitée. Les mécanismes sous-jacents de l'échec de la phase effectrice des CTL contre les mélanomes sont encore largement méconnus.

Notre hypothèse est que l'efficacité limitée des CTL dans leur combat contre les tumeurs est le résultat d'une balance défavorable entre la capacité des CTL à tuer les tumeurs et une résistance tumorale intrinsèque à l'activité cytolytique des CTL.

Au cours de ma thèse je me suis concentrée sur la dynamique moléculaire qui se produit à la synapse lytique afin de pouvoir identifier un mécanisme précoce mis en place par les cellules de mélanome face à l'attaque des CTL.

En combinant l'utilisation d'approches de microscopie de pointe et des outils moléculaires, j'ai pu montrer que, lors de l'interaction avec les CTL, les cellules de mélanome humain subissent une activation de leur trafic vésiculaire endosomal et lysosomal, lequel est intensifié à la synapse lytique et corrèle avec la dégradation par la cathepsine de la perforine et un défaut de pénétration d'entrée du granzyme B. De plus, j'ai démontré que le blocage du trafic lysosomal dépendant de SNAP23, la modification du pH (intra-vésiculaire) et l'inhibition de l'activité lysosomale protéotlytique des cellules de mélanome permet de restaurer leur sensibilité à l'attaque des CTL.

Nos résultats révèlent une stratégie sans précédent d' " auto-défense " des cellules de mélanome à la synapse immunologique basée sur une sécrétion lysosomale massive et sur la dégradation de la perforine sécrétée par les CTL. Ainsi pouvoir interférer avec cette stratégie synaptique d'auto-défense des cellules de mélanome pourrait contribuer à potentialiser les réponses des CTL et les immunothérapies chez les patients atteints de mélanome.

Abstract

Roxana Khazen

Dissecting early mechanism of melanoma cell resistance to cytotoxic T lymphocyte attack

Thesis Director: Dr. Salvatore Valitutti

Thesis defense: 29 Jan 2016, Toulouse France

Human melanoma cells express various tumor antigens that are recognized by CD8+ cytotoxic T lymphocytes (CTL) and elicit tumor-specific responses in vivo. However, natural and therapeutically enhanced CTL responses in melanoma patients are of limited efficacy. The mechanisms underlying the failure of CTL effector phase against melanomas are still largely elusive. Our hypothesis is that the limited efficacy of CTL in their fight against tumors is the result of an unfavorable balance between CTL ability to kill tumors and an intrinsic tumor resistance to CTL cytolytic activity.

During my thesis I focused on the molecular dynamics occurring at the lytic synapse in order to identify possible "early response-mechanism" of melanoma cells to CTL attack.

Using a combination of cutting edge microscopy approaches and molecular tools, I showed that upon conjugation with CTL, human melanoma cells undergo an exacerbated late endosome/lysosome trafficking, which is intensified at the lytic synapse and is paralleled by cathepsin-mediated perforin degradation and deficient granzyme B penetration. Abortion of SNAP-23-dependent lysosomal trafficking, pH perturbation or impairment of lysosomal proteolytic activity restores susceptibility to CTL attack.

Our results reveal an unprecedented strategy of melanoma cell "self-defense" at the immunologic synapse based on a lysosome secretory burst and perforin degradation at the lytic synapse. Interfering with this synaptic self-defense strategy might be instrumental to potentiate CTL-mediated therapies in melanoma patients.

I- Introduction

General View of the Immune system:

Human immune response is based on an interactive network of lymphoid organs, cells, humoral factors[1], and cytokines that has evolved to simultaneously facilitate peaceful cohabitation with beneficial microorganisms that constitute the microbiota, to provide host defense against infectious agents, to eliminate cancerous cells and to initiate repair and remodeling processes that restore and maintain tissue homeostasis [2]. Groundbreaking studies over the past decades formally characterize immune system in to two main axes: innate immunity and adaptive immunity. Although, these two components of the immune response mainly differ in terms of specificity, rapidity, duration and effector functions, yet they are complementary as there is essential communication linking the cells of the innate and adaptive immune response. Development of immune system starts from a common pluripotent hematopoietic precursor cell that differentiates into more specialized progenitor cells in the bone marrow to form a heterogeneous group of immune cells called leukocytes, or white blood cells (**Figure I-1**).

Innate immunity plays an essential role in body's immediate defense allowing recognition of self/nonself by a system consisting of soluble proteins and relatively invariant cellular receptors. It involves a large number of cells such as inflammatory resident cells (mast cells, macrophages), endothelial cells, monocytes, dendritic cells, natural killer cells (NK), as well as subpopulations of lymphocytes (B-1, NKT cells and Tγδ cells), basophils and eosinophils.

Adaptive immunity is characterized by expansion and differentiation of specific lymphocytes for a given antigen. This type of immunity takes longer to get involved and constitutes the second line of body's defense. In contrast to innate response that is rapid but sometimes harmful to normal tissues due to lack of specificity, adoptive immunity requires several days or weeks to develop and finally allows efficient and specific elimination of antigens, infected cells and cancerous cells. It is noteworthy that adaptive response is capable of generating memory so that subsequent exposure to pathogens would lead to a more vigorous and rapid response and provide a long-lasting protection.

The adaptive response has two components: the cellular response, based on the action of T lymphocytes that coordinate the action of immune cells and destroy infected or cancerous cells, and the humoral response, based on the production of immunoglobulins by plasma cells.

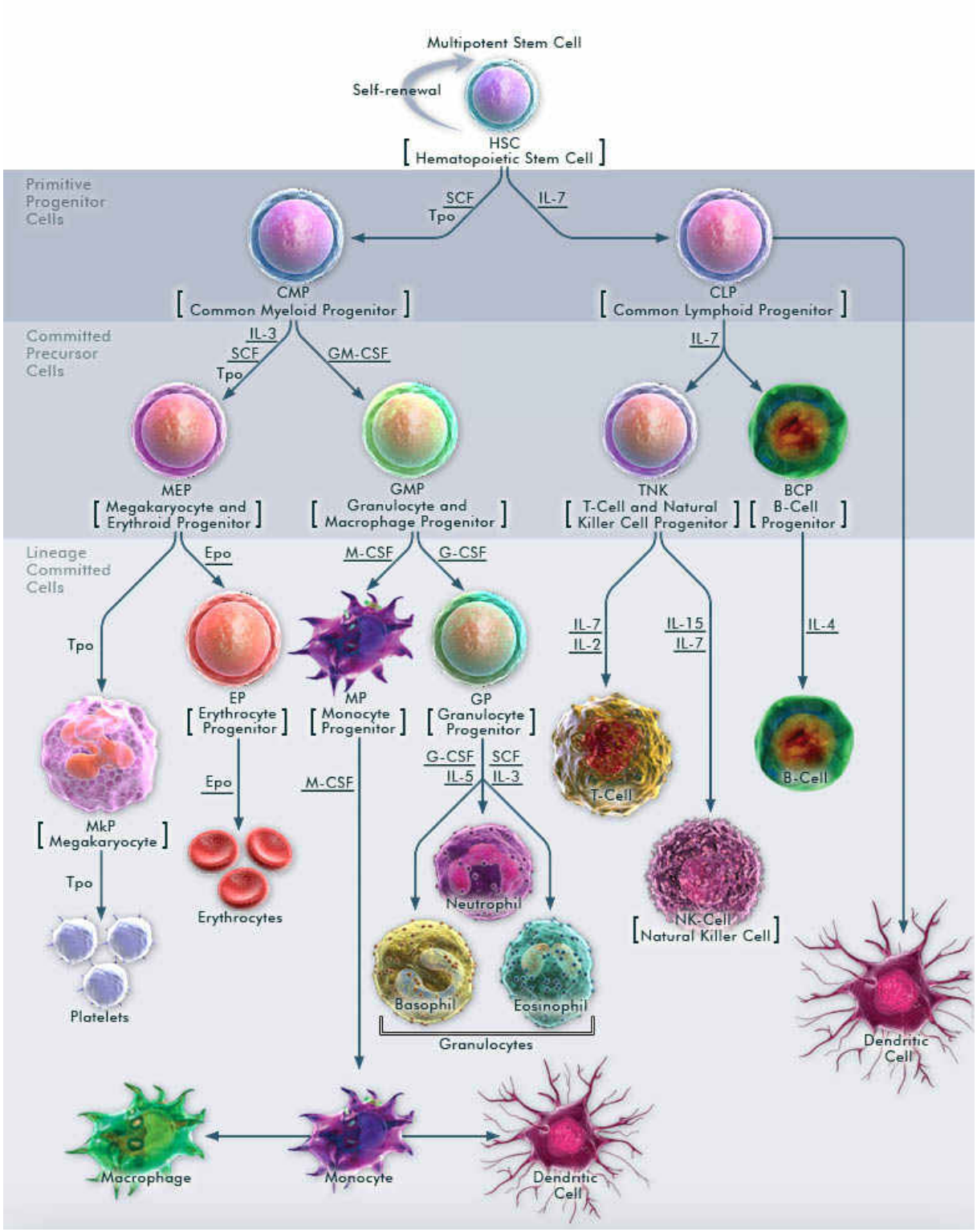


Figure I-1. **Development of cells of the immune system.** This development starts from a common pluripotent hematopoietic precursor cell that differentiates into more specialized progenitor cells in the bone marrow to form a heterogeneous group of immune cells called leukocytes, or white blood cells. Neutrophils, monocytes, macrophages and immature dendritic cells (DC) originate from common myeloid precursor cells, whereas B and T lymphocytes and natural killer (NK) cells arise from common lymphoid progenitors. These differentiated leukocytes circulate in the bloodstream or reside in peripheral tissues to scan the environment for danger or respond to danger through different effector mechanisms. (Adapted from eBiosciences).

The innate immune response

When epithelial barriers fail to block microorganism, pathogen entrance and replication activates innate immune system. The first line of defense is composed of tissue macrophages that mediate microorganism engulfment and destruction through recognition of pathogen associated patterns via their receptors. This pathogen clearance is either mediated through the fusion of the phagosome with lysosomes that contain antimicrobial compounds or through the generation of a "respiratory burst". Activated macrophages are also capable of secreting cytokines and chemokines, causing inflammation and recruitment of other immune cells. Such inflammatory responses increase the local blood flow, reduce blood flow velocity and induce up-regulation of adhesion molecules on activated endothelial cells with consequent extravasation of circulating leukocytes into the infected tissues. Neutrophils and monocytes are the first to arrive to the site of infection[3]. Monocytes differentiation will lead to the generation of additional macrophages in innate response. During viral or intracellular infection, NK cells play an important role in providing a primary defense by releasing lytic molecules when contacting target cells. Inflammatory responses also increase the flow of antigens from infected tissues to draining lymph nodes, a mechanism that will help mounting an adaptive immune response. Moreover complement system is activated by microbial products contributing to create an inflammatory environment. At this stage, innate immunity might succeed in infection removal. Alternatively, infection might be kept under control to prevent dissemination, while adaptive immune response develops to provide a stronger level of defense.

More recently, innate lymphoid cells (ILC) have been identified. They exhibit a broad range functions. The ILC are a family of innate effector cells characterized by a specific cytokine production repertoire. These cells share functional similarities with T cells but differ from T cells by the absence of TCR expression and of clonal deletion during their development in the bone marrow[4]. The ILCs are therefore defined as cells at the interface between innate and adaptive immunity and play a key role in the development of effective immune responses. They are implicated in immune responses to viruses, intracellular bacteria, parasites, and fungi (LC1s and ILC3) and in the induction of type 2 inflammation required for immunity to some extracellular parasites (ILC2)[2].

The adaptive immune response

The main feature of an adaptive immune response is the usage of antigen specific receptors expressed by T and B lymphocytes. The recognition of antigenic determinants by T or B lymphocytes leads to their activation, priming, and differentiation. These steps will result in the generation of an effector adaptive immune response that is composed of antibody secretion by plasma cells into blood and tissue fluids and by a T cell mediated cellular immune response.

- **Overview of B lymphocyte activation and function**

Specific antigen recognition by B lymphocytes is mediated by BCRs or membrane bound immunoglobulins. The BCR recognizes unprocessed antigens in soluble form or bound to cell surface (**Figure I-2**). BCRs can internalize antigens allowing B cells to process captured antigens within their lysosomal/late endosomal compartments and to express on their surface antigenic peptides bound to MHC molecules for antigen presentation to T cells. After activation, B-cells develop into plasma cells that release secretory forms of antibodies. BCRs do not possess an intrinsic signaling function. On B cell surface each BCR is associated with a heterodimer of protein belonging to the immunoglobulin superfamily: Igα and Igβ. These proteins bear a cytoplasmic tail containing the ITAM motifs (Immunoreceptor Tyrosine-based Activation Motifs, YxxL $(X)_{6\text{-}8}$YxxL) that are responsible for signal transduction. BCR/Antigen interaction causes BCR internalization and establishes a cascade of phosphorylation processes leading to the activation of B cells.

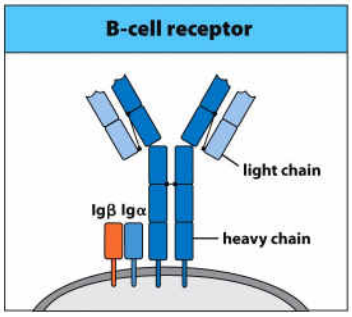


Figure I-2. **B cell receptor structure.** BCRs are composed of two identical light chains and two identical heavy chains. Each of these chains is composed of a variable region, determining the specificity of the receptor for the antigen, and of a constant region, defining the functional properties of the immunoglobulin. On B cell surface, each BCR is associated with a heterodimer of immunoglobulin superfamily proteins: Igα and Igβ. *Adapted from Murphy immunobiology 2012*[5].

- **Overview of T lymphocyte activation and function**

T lymphocytes comprise another type of adaptive immune cells. Among them αβ T cells constitute the majority of T lymphocytes. As result of gene rearrangements during development, most individual T lymphocytes express a unique TCR, responding to a specific Ag-derived peptide. T lymphocytes are characterized by simultaneous expression of CD3 lineage marker in combination with CD4 or CD8, molecules that are intimately tied to target recognition.

T lymphocytes are divided into several groups including: $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ Th1 and $CD4^+$ Th2 cells, Th17 cells, follicular Th cells and a heterogeneous group of regulatory T cells. In addition subsets of T lymphocytes that exhibit a limited diversity of their TCR repertoire also exist, such as γδ T cells, and invariant NKT cells. They somehow comprise an intermediary population at the interface between innate and adaptive immunity. The current picture of T cell biology postulates that T cells are a very diverse family of cell subsets that display some level of differentiation plasticity and carry either effector or regulatory functions depending on time and location during an immune response. **Figure I-3** summarizes some phenotypic and functional characteristics of these cell subsets.

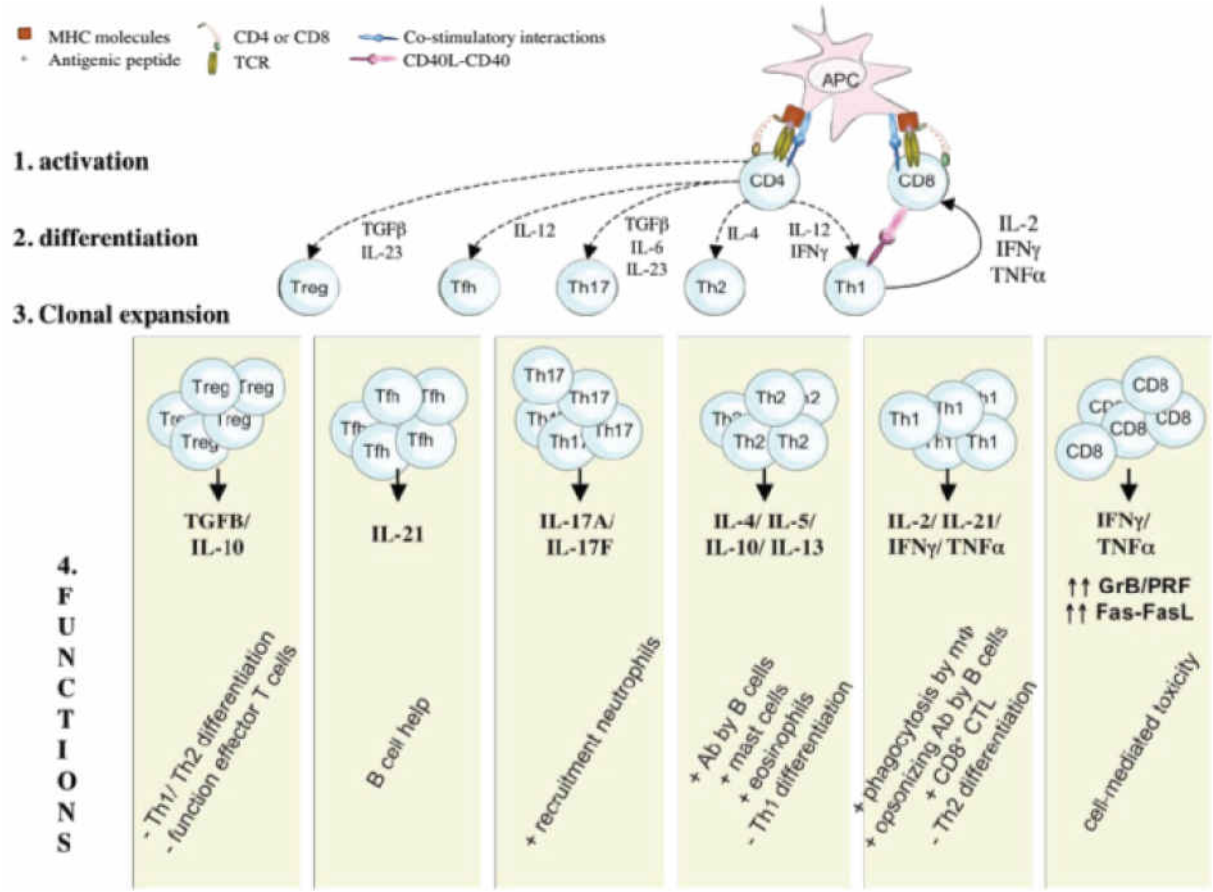


Figure I-3. **Schematic representation of T cell activation and differentiation in response to APC stimulation.** TCR stimulation by recognition of pMHC complexes in combination with costimulatory signals and cytokines provided by APC, triggers the activation and differentiation of naïve CD4+ and CD8+ T cells. CD4+ T cells can differentiate into conventional Th1, Th2, Th17 and Tfh or regulatory T cells, depending on the cytokine milieu in which activation occurs. After activation and differentiation, effector T cells undergo clonal expansion to assure host defense. *Adapted from Dee Meester J 2011 thesis* [6]

T lymphocyte/antigen presenting cells interaction:

1) Antigen Recognition

- **TCR gene organization:**

Organization and expression of genes encoding TCR chains are perfectly suited to their particular receptor function. A part of some exception[7], each T lymphocyte expresses a unique TCR type on its surface, with single antigen specificity, i.e. it is able to recognize a unique peptide-MHC complex type. In order to recognize the immense diversity of peptidic antigens, evolution has developed a system which permits formation of a large number of different TCR. This process randomly generates specificity of TCR for antigenic peptides. It takes place during thymic development and involves a process of gene recombination very similar to that used for rearrangements of the heavy and light chains of the Ig during B lymphopoiesis.

In its germline configuration, the locus encoding the α chain of the TCR is composed of numerous segments V for Variable and J for Joining and a C for Constant domain. In addition, two D domains for diversity are present in the locus encoding β chain of TCR. C domain encodes the constant, hinge, transmembrane region and cytoplasmic tail of TCR chains. Once rearranged the VJ and VDJ segments code variable regions of TCR chains.

These gene rearrangements are governed by RSS (Recombination Signal Sequence) sequences. These are palindromic sequences, heptameric and nonameric, separated by 12 or 23 pairs of bases and are serving as anchors to the corresponding machinery of RAG-1/RAG-1 recombinase[8,9]. The β chain genes rearrange first during thymic selection. First, there is the association of one of the two D segments with a J segment, and then a V segment will be added to previously obtained DJ segment producing a VDJ segment. At this point, all segments of genes located in the V-D-J complex intervals are eliminated and the synthesized primary transcript has the constant segment (Vβ-Dβ-Jβ-Cβ). Introns are eliminated and translation of messenger RNA generates TCR β-chain. This protein is then associated with a substitute of α chain of TCR to form pre-TCR and is expressed on the surface of the DN thymocytes (thymocytes expressing neither CD4 nor CD8 molecules on their surface). If rearrangement of TCR β chain is functional cells proliferate and subsequently, the rearrangement of genes encoding α chain takes place. A V segment will rearrange with a J segment and then, upon transcription and translation, α chain of TCR is expressed. It has been estimated that via such processes of somatic recombination more than 10^{15} TCR can be theoretically generated during thymic development. However a large number of thymocytes expressing a given TCR are eliminated during positive and negative thymic selection of developing T cells. Accordingly it has been estimated that the TCR repertoire of human T lymphocytes is of about 2 x 10^7 different TCR in the periphery[10].

- **TCR Structure**

αβ T lymphocytes recognize antigenic peptides in the context of MHC molecules via their TCR. The αβ TCR consists of two transmembrane glycosylated polypeptide chains α and β that are linked through a disulfide bond[11,12] (**Figure I-4**). These glycoproteins belong to the immunoglobulin (Ig) superfamily. They consist of an amino-terminal extracellular domain containing a variable region (V), a constant region (C) and a short hinge region with a cysteine residue necessary for the formation of disulfide bonds (for TCR generation refer to **Figure I-5**). These two chains contain also a short intracytoplasmic tail of 4 to 12 amino acids and a hydrophobic transmembrane domain composed of 20 positively charged amino acids allowing

TCR to stably associate with the CD3 molecules (endowed with a negatively charged transmembrane domain) (**Figure I-4**) [12-14].

While C domain is invariant for each chain of TCR independently of the T lymphocyte on which it is expressed, V domain varies considerably from one individual T cell to another. This variability mainly resides in 3 zones known as hyper-variable regions or CDR[15]. Crystallographic Studies have shown that CDR regions are projected outside of V domain to bind pMHC complexes. In TCR the variable regions of α and β chains are disposed side by side to form a unique site for antigen recognition by TCR. The CDR1 and CDR2 regions mainly bind to MHC molecules, while CDR3 (where most of TCR variability is concentrated) binds preferentially the antigenic peptide[16,17].

All three proteins of CD3, ε γ δ are encoded by adjacent genes belonging to the same region. They each have an extracellular domain that contains an Ig type domain, followed by a transmembrane region and a cytoplasmic domain of approximately 40 amino acids. CD3ζ chain has a very short extracellular domain thus it does not contain an Ig domain. On the other hand, it has the most important cytoplasmic region with 113 amino acids (**Figure I-4**).

The association of TCR with these six CD3 chains seems to have two main functions:

Firstly, since TCR lacks an intracellular domain, in order to initiate signal transduction, CD3 chains and ζζ dimer are requested for signal transduction via ITAM phosphorylation. Each CD3 complex consisting of 6 chains containing 10 ITAM motifs: 3 on each ζ chain, and 1 on each of the ε, γ, and δ chains. ζζ dimer has 6 ITAM motifs (**Figure I-4**). Secondly, TCR/CD3ζζ interaction allows stabilization of TCR expression on surface of T lymphocytes[18-21].

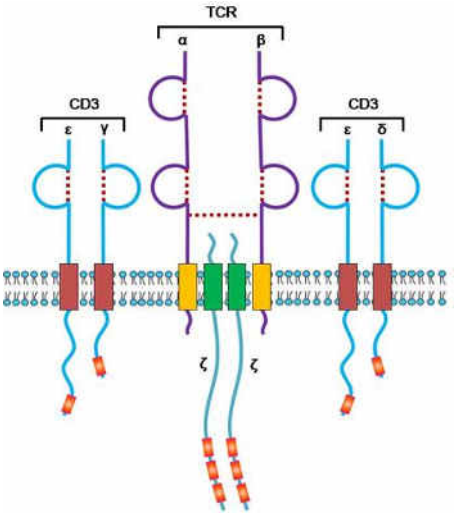


Figure I-4. αβ **T cell receptor structure.** TCR consists of two chains, α and β, and forms a complex with the CD3 signaling molecules that are composed of a γ and ε complex, a ε and δ and complex and a ζ-ζ complex (CD3γε, CD3δε, ζζ). These CD3 chains contain one or more ITAM motif of 14 to 16 amino acids in their cytoplasmic region, while each ζ chain that plays a central role in TCR signal transduction contains three ITAM motifs *Adapted from J. Gascoigne NR Nat Rev 2008*[22].

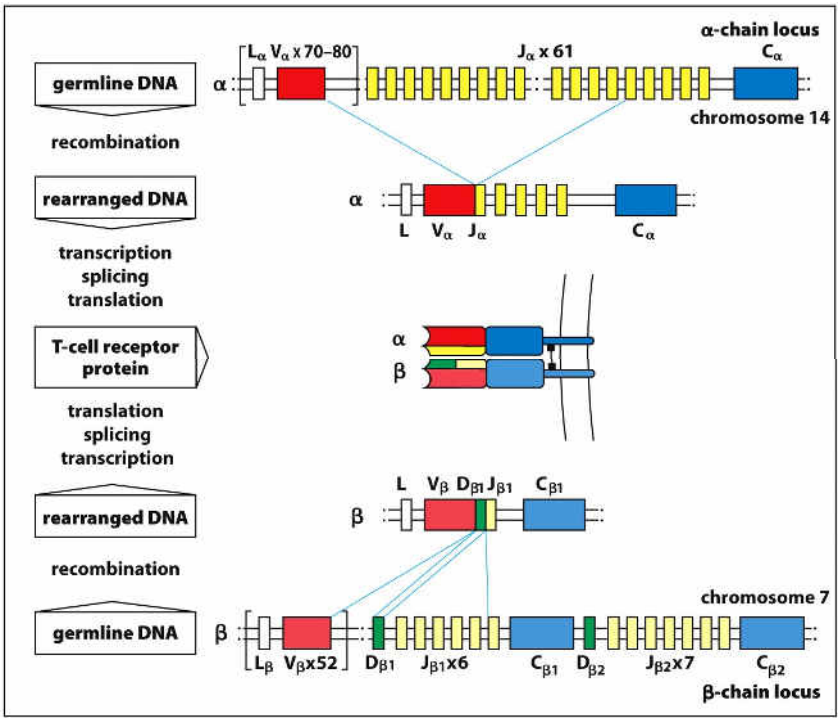


Figure I-5. **Gene re-arrangement for coding TCR.** The β chain genes rearrange first during thymic selection. First, there is association of one of the two D segments with a J segment, and then a V segment will be added to previously obtained DJ segment producing a VDJ segment. At this point, all segments of genes located in the V-D-J complex intervals are eliminated and the synthesized primary transcript has the constant segment (Vβ-Dβ-Jβ-Cβ). Introns are eliminated and translation of messenger RNA generates TCR β-chain. This protein is then associated with a substitute of α chain of TCR to form pre-TCR and is expressed on the surface of the DN thymocytes (Double Negative : thymocytes expressing neither CD4 nor CD8 molecules on their surface). If the rearrangement of TCR β chain is functional cells proliferate and subsequently, the rearrangement of genes encoding α chain takes place. A V segment will rearrange with a J segment and then, upon transcription and translation, α chain of TCR is expressed. *Adapted from Murphy immunobiology 2012*[5].

- **Antigen presentation to lymphocytes**

In T lymphocytes, the recognition of antigen fragments requires antigen processing and presentation by antigen presenting cells (APCs). Antigens are presented to T cells on the surface of the APCs in the form of peptides bound to the major histocompatibility complex (MHC) molecules. CD4 or CD8 co-receptors are also involved in recognition of these peptide MHC (pMHC) complexes. Whereas $CD4^+$ T lymphocytes recognize antigenic peptides associated with MHC Class II molecules, $CD8^+$ T lymphocytes recognize those associated with class I molecules.

Most of the cells are able to present antigen to $CD8^+$ T lymphocytes in the context of their MHC I molecules. Conversely, only cells expressing MHC-II can present antigens to the $CD4^+$ T cells, they are named professional APCs. Among these cells there are dendritic cells (DC), B lymphocytes and macrophages. There are also additional cells that can express MHC-II molecules and serve as unconventional APC for CD4+ T cells such as IFN-γ primed mast cells[23], endothelial or epithelial cells [24] and basophils[25-27].

- **MHC genes**

The MHC or HLA (Human Leukocyte Antigens) system in humans is a set of more than 200 genes, located on chromosome 6 and organized into regions encoding three classes of molecules: classes 1, 2 and 3. Class 3 codes for different proteins involved for instance in the complement cascade or cytokines expression such as TNF, and have no direct function in the antigenic presentation. Here we focus on the first two classes, involved in antigen presentation to T lymphocytes.

The molecules of MHC genes have three basic characteristics: polygenicity, co-dominance and the polymorphism. There are three classic genes encoding α chain of the MHC-I in humans. They are named HLA-A,-B and–C. There are also three genes encoding for human MHC-II. They are called DP, DQ and DR (polygenicity). The co-dominance refers to the expression of two alleles of a certain gene in a single cell. This property allows individual cells to express a higher number of MHC molecules with different ranges of specificity of bonded peptide.

In addition, MHC molecule genes are polymorphic genes referring to the presence of many alleles of each gene in the whole population. This feature supports recognition of a larger pattern of pathogens since different antigenic peptides are bound by different MHC alleles.

Even though molecules of MHC Class I and Class II all have the common property of binding to antigenic peptides, they differ in many other aspects:

a) The MHC Class I molecules are present on the surface of all nucleated cells in the body. The presentation of endogenous peptides by MHC Class I molecules leads to specific activation of $CD8^+$ T cells. These peptides are products of enzymatic degradation by proteasome inside the cell and are constituted of 8-10 amino acids[28] (**Figure I-6 and I-7**).

b) The MHC Class II molecules are present only on the surface of professional APCs. The association of the α1 domains with β1 domains of α and β chains forms a groove that can accommodates 12 to 24 amino acid long peptides to be presented to $CD4^+$ T lymphocytes[29]. These peptides are derived from degradation of extracellular proteins internalized via endocytosis (**Figure I-6 and I-7**).

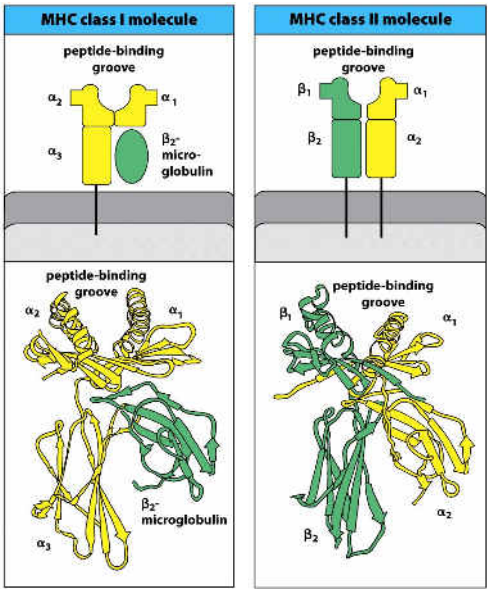


Figure I-6. **Structure of MHC molecules.** A) MHC class I are composed of a 45 kDa chain with three domain (α1, α2 and α3similar to those of immunoglobulins) associated to the β2-microglobulin. α1 and α2 domains form a pocket, which is the site where peptide antigens bind to MHC molecules and where the polymorphism of the molecule is mostly represented. B) MHC class II are heterodimeric molecules, composed of two transmembrane glycoproteins: a 30-32kDa α chain that is non-covalently associated to a 27-29 kDa β chain. The peptide binding pocket is formed by the α1 domain of the α chain and the β1 domain of the β chain. *Adapted from Murphy immunobiology 2012*[5].

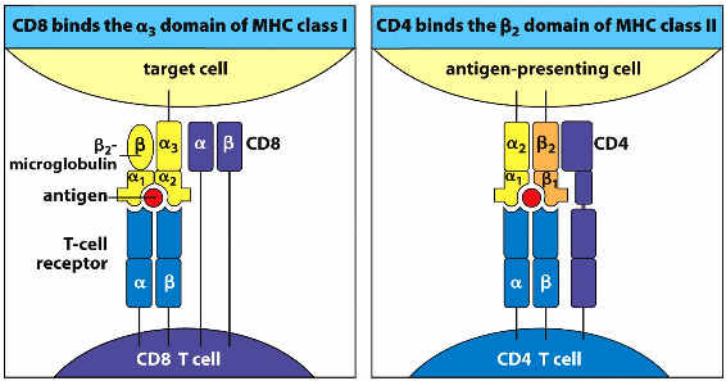


Figure I-7. **MHC molecules interaction with TCR.** The peptide complexed in the pocket of MHC interacts with CDR (Complementarity Determining Region) regions of the TCR and these interactions are stabilized through association of CD8 or CD4 co-receptor with MHC Class I and MHC Class II molecules respectively. *Adapted from Murphy immunobiology 2012*[5].

2) Molecular steps of T Lymphocytes and Antigen presenting cell interaction

- **TCR/pMHC interaction**

TCRs display remarkable ability to specifically recognize a wide array of structurally and chemically diverse antigens. Understanding of the molecular basis of TCR interactions with peptide-MHC has been greatly advanced by a number of studies that elucidated the interface sites, key contacts, and binding modes of several of these complexes.

Two important aspects of such interaction are mentioned here:

Affinity of contact:

Several cellular studies demonstrated that a full activation of T cells requires a su[30]stained signaling[31,32]. However, this concept was difficult to reconcile with several molecular studies aiming at dissecting pMHC/TCR interactions. These studies based on the measurement of TCR/pMHC binding parameters in solution revealed a low affinity of TCR/pMHC binding (Kd approximately between 1–100 μM) as well as a very short dwell-time of binding(seconds)[33,34]. This was a paradoxal finding considering the specificity and efficacy of TCR in recognition of as few as a single foreign antigen among a sea of self-pMHC complexes. The high specificity of TCR binding is also shown by the fact, that while a given pMHC may act as a strong agonist capable of triggering full activation of T cell through TCR engagement, other related peptides will be considered weak or null agonist and may have no or at best partial effects on T cell activation. Moreover, substitution of a single amino acid in the sequence of the antigenic peptide presented by MHC can render T cells unresponsive to subsequent stimulation with the

immunogenic peptide[35]. Moreover, using single-molecule microscopy and fluorescence resonance energy transfer (FRET) between fluorescently tagged TCRs and their cognate pMHC ligands for measuring kinetics of TCR-pMHC binding *in situ*, Huppa *et al.* reported that compared to solution measurements association and dissociation were even faster[36]. Complementary results were obtained using a different approach by J. Huang *et al.* [37]. Together these results underline the extreme rapidity of TCR/pMHC binding within the IS confines and that, for sustained signaling in T cells, multiple rounds of TCR/pMHC binding are required[36,37] .

Force of contact:

More recently, Zhu C. *et al.* introduce a new concept in TCR/pMHC interactions in which TCR uses mechanical contact (the forces involved in their binding to the antigens) for decision making. In this context, interactions between TCR and agonist pMHC form catch bonds that are strengthen with the application of additional force to initiate intracellular signaling. Less active MHC complexes form slip bonds that weaken with force and do not initiate signaling. Overall, they described how the signaling outcome of TCR/pMHC interaction depends on the magnitude, duration, frequency and timing of the force application, such that agonist peptides form catch bonds that trigger the T cell digitally, whereas antagonists form slip bonds that fail to activate[38].

Probability of interaction:

Interaction of TCR and its ligand has major constraints. First, even if a T cell expresses a significant number of TCR with the same affinity for a specific antigen on its surface, the APC will only present a few number of MHC molecules on their surface presenting the specific antigen for which those TCR are specific. In fact most of the pMHC express on an APC surface are self antigens, produced by processing intra and extracellular proteins. Demotz *et al* in 1990 measured that T cells are activated by APCs that display on their surface no more than 0.03% of specific pMHC complexes binding the specific peptide. Therefore, the probability of presence of specific pMHC complexes at the contact zone between two cells is rare. The second parameter that yet reduces the probability of TCR/pMHC interaction is the length of extracellular domains of TCR and their ligand comparing to other membrane molecules. Each of these two molecules is approximately 7.5 nm long. This length requires that the membrane of T cell and APC cells would be at a ~14 nm distance to allow productive TCR engagement, this scenario is difficult to envisage because of the expression on the surface of cells of a large number of glycosylated large proteins that might prevent such proximity.

In spite of those compelling factors, TCR/pMHC interaction is extremely sensitive. Recent studies, demonstrated that as few as single pMHC is able to induce a transient $[Ca^{2+}]i$ increase in both mouse $CD4^+$ and $CD8^+$ T cells and that by maintaining a sustained $[Ca^{2+}]i$ increase in T cells, TCR recognition of only 10-15 pMHC complexes is strong enough to induce T cell activation[39,40]. Likewise, CTLs were capable of killing target cells expressing 1-10 pMHC complexes on their surface[41,42] .

In the last decades many studies tried to investigate such intriguing paradox. Two models have been proposed for explaining the mechanism regulating antigen recognition by T cells: the kinetic proof reading model and the serial TCR engagement model.

Kinetic proofreading model:

In 1995 McKeithan *et al.* proposed the kinetic proofreading model in which a prolonged time of interaction of TCR with pMHC is considered as a crucial element for full activation of T cells. According to this model there is a lapse of time between initial TCR/MHC interaction and transduction of signal. The model proposes that TCR-coupled signaling is composed of a series of intermediate reversible steps leading to full activation of the signaling cascade (**Figure I-8**). The original model proposed three hypotheses; first, the inactive primary TCR/pMHC undergoes a sequence of N modifications leading to an activated state via a series of intermediaries. Even though these modifications haven't been described in details, it can include: phosphorylation or dephosphorylation in tyrosine within the receptor complex, conformational changes of proteins, generation of secondary messengers or even recruitment of scaffolding components to the receptor. Second, dissociation of the interaction leads to reversion of these modifications and, third, upon interaction of non-specific TCR/pMHC complexes the rate of dissociation is sufficiently high to avoid formation of productive interactions leading to reversion of such modifications and to a destructive signal. This model explains how T cells discriminate between ligands based on kinetic differences of intermediary interactions. In this manner, the modifications induced during TCR-pMHC interaction supposedly will be amplified in order to maximize minor differences and thus explaining the high specificity of such interaction.

The above theory revolves around the concept that short-term TCR engagement will induce proximal signaling events but not later T cell responses, due to the reversibility of early activation steps. Indirect support for these models has been obtained by the demonstration that there is a rough correlation between the duration of TCR/ligand contact and the biologic

efficacy of this interaction[43-45] but the mechanism by which these kinetic differences alter T cell responses is less clear.

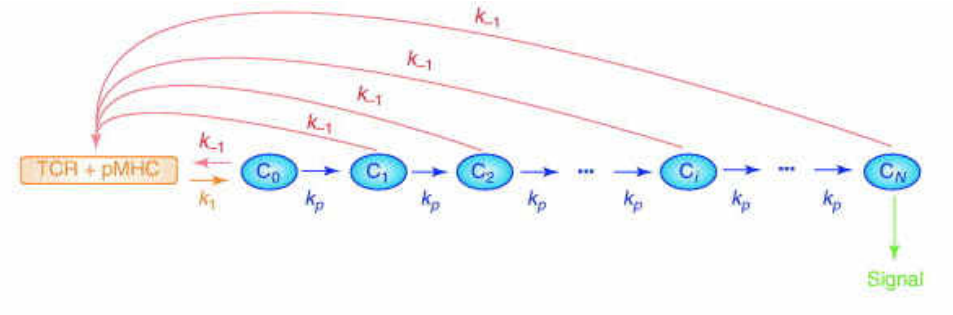


Figure I-8. **Kinetic proofreading model.** The kinetic proofreading model suggests that binding of a TCR to a pMHC to form a TCR–pMHC complex initiates a sequence of signaling events with intermediates (C_1, C_2 …). These culminate in the formation of the final signaling product (C_N), which signals to the cell. However, if the TCR–pMHC complex dissociates before the formation of C_N, then the signaling fails. The association (k_1) and dissociation (k_{-1}) rate constants for the TCR–pMHC interaction are shown, as are the rate constants of the intermediate pathway (k_p, is assumed to be the same for every step). *Adapted from George AJ Trends in immunology 2005* [30]

Serial TCR engagement:

Even though the kinetic proof reading model can explain the high specificity of T cell activation induced by TCR/pMHC interactions, it doesn't provide a model to describe the high sensitivity of such interactions with small number of specific pMHC. In other words, how such short interactions of TCR/pMHC permits T cells to recognize a very low number of specific pMHC complexes presented on the surface of APCs? It has been previously shown that a prolonged and uninterrupted TCR engagement with its pMHC ligand is required for a sustained signaling in T cells[46]. Considering the fact that TCRs have a low affinity of binding with their ligands, how these TCR remain engaged with the same individual pMHCs for such long time?

In 1995 S.Valitutti *et al.* proposed the serial engagement model in order to explain sustained TCR/pMHC interaction. They used TCR internalization as a parameter of TCR/pMHC binding and observed that a very few specific pMHC complexes can lead to high level of TCR internalization. They indeed estimated that on pMHC could engage trigger and internalize ~180 TCR, meaning that a very few pMHC complexes can trigger a considerable number of TCRs. The model proposes that low affinity TCRs by interacting in series with a small number of specific pMHC can lead to a sustained and amplified signaling. This model thus provides a plausible explanation of how a T cell can sensitively detect a limited number of pMHC through "sequential" high TCR occupancy (**Figure I-9**).

Recent publications reporting extremely fast TCR/pMHC binding on-rate and off-rates as well as other experimental and computational studies support serial triggering model for an optimal T cell responsiveness, however other observations do not support the model (reviewed in[47] Dushek O and van der Merwee PA *Nat Rev* 2011)

Kinetic proof reading and serial engagement models might be not mutually exclusive. In fact, on one hand the kinetic proof reading, mostly explains the specificity of interactions at the molecular level. On the other hand, serial engagement mostly explains the sensitivity of T cell for antigen stimulation at the cellular level. It is conceivable that in physiological conditions where the density of specific pMHC complexes on APC surface is low, in order to activate T cell, the half life of TCR/pMHC interactions should be long enough to allow signaling proofreading and short enough to allow a single pMHC complex to engage in a series multiple TCR.

Figure I-9. **Serial engagement model of TCR/pMHC interaction.** *Adapted from Valitutti 2012 Frontiers*

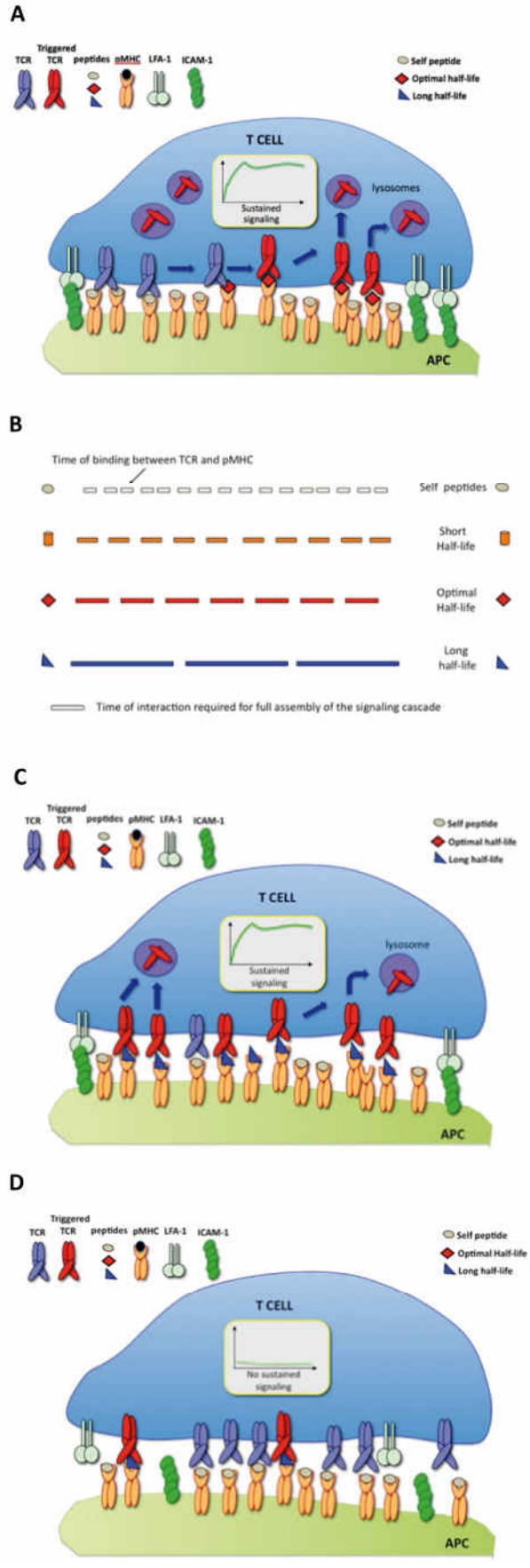


Figure 9A) The serial TCR engagement model. At the IS, a few specific pMHC (red) complexes sequentially trigger incoming TCR resulting in sustained signaling. Triggered TCR are internalized and targeted to lysosomes for degradation while unbound pMHC bind new TCR.

Figure 9B) The serial engagement model postulates that pMHC ligands exhibiting optimal binding half-lives to TCR behave as optimal agonists

Figure 9C) At high pMHC densities, pMHC exhibiting long binding half-lives are stimulatory

Figure 9D) At low pMHC densities, pMHC exhibiting long binding half-lives fail to trigger T cell responses.

- **Early steps of signal transduction in T cells:**

  TCR engagement:

Activation of T lymphocyte is a general term which includes signaling events leading to cell survival, proliferation, rearrangement of the cytoskeleton, metabolism increase and variation of gene expression profile. In naive T cells, activation requires the accomplishment of two distinct and synergistic signals: the TCR and the co-stimulatory molecule engagement.
TCR early signaling events can be divided into 4 steps (**Figure I-10**):

1) Rapid recruitment of Src family tyrosine kinases: LCK (major role player) and Fyn**.** Engagement of TCR will cause its translocation into membrane micro-domains, rich in cholesterol and sphingolipids, known as DRMs (detergent-resistant membranes), which facilitates phosphorylation of ITAMs motifs[48-50]. Following TCR/co-receptor/pMHC interaction, LCK is recruited into close proximity of ITAM motifs via its association with CD4 and CD8 co-receptors [51,52]. Early recruitment of CD45 facilitates LCK activity[53]. ITAM phosphorylation will cause recruitment of a set of enzymes (protein kinases, GTPases, phosphatases) and adaptor molecules in DRMs to forms a "TCR signalosome"[54,55] .

2) Phosphorylation of ITAM motifs present in the cytoplasmic domains of CD3 complex/ζζ dimer in their tyrosine residues by LCK and Fyn.

3) Recruitment of protein ZAP-70 (ζ-chain-associated protein kinase 70 kDa) to the phosphorylated ITAMs via its two SH2 (Src homology 2) domains and its activation by Src kinases-mediated phosphorylation in a regulatory tyrosine.

4) Activation of LAT protein (Linker for Activation of T cells) through phosphorylation by ZAP-70. LAT is associated with membrane micro-domains and becomes a platform for binding of additional signaling proteins.

In fact, another important substrate of ZAP-70 activity is SLP-76 (SH2-domain-containing leucocyte protein of 76 kDa) which is an adaptor protein implicated in signal transduction.

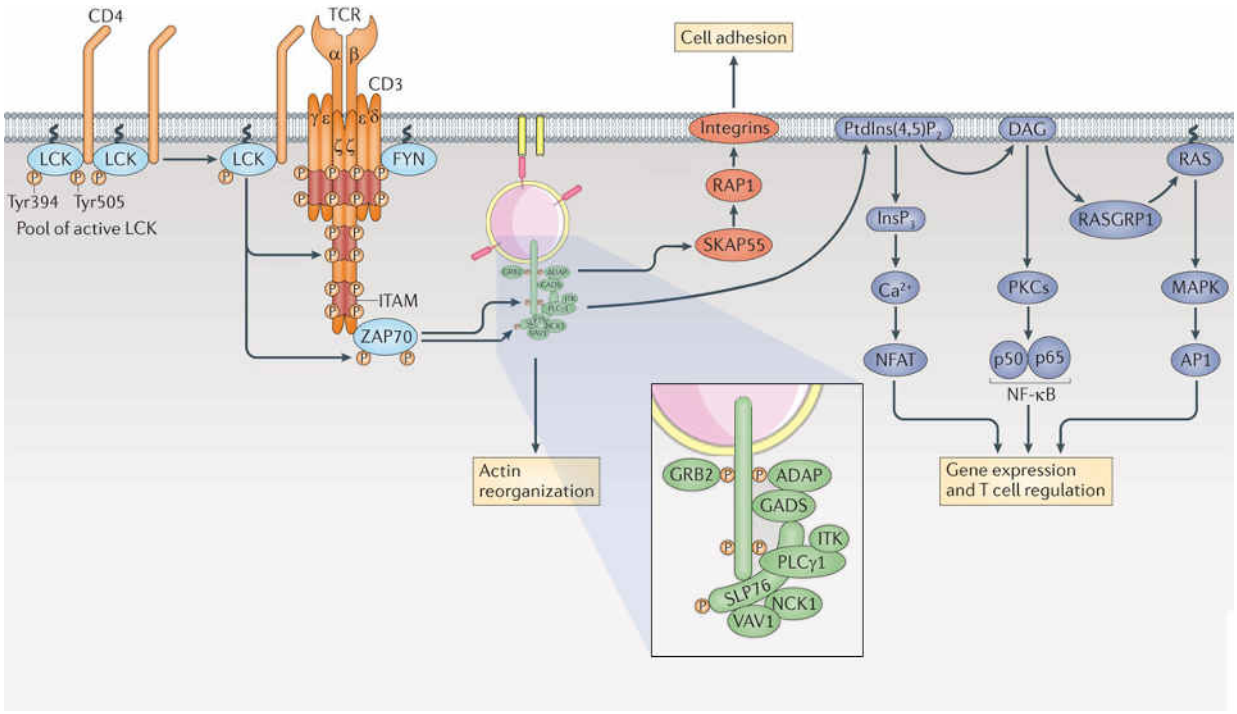


Figure I-10. **T cell receptor (TCR) signal transduction Pathways.** *Adapted from Brownlie 2013*[56]

Co-stimulatory molecules engagement:

As mentioned earlier, the second activation signal is generated by the engagement of several T cell's accessory molecules with their ligands on APCs including: co-stimulatory molecules such as CD28, CD4 and CD8 co-receptors and the adhesion molecules like LFA-1 and CD2. Such interactions not only influence and regulate the strength of TCR/pMHC binding, but also affect the magnitude and the quality of T cell signaling. We will briefly review some features these molecules and their contribution to downstream events of the TCR signaling process.

a) Role of CD80-CD86/CTLA-4 – CD28 interaction

The two member of B7 family the CD80 (B7-1) and CD86 (B7-2) can bind two receptors: CD28 and CTLA-4[57,58]. CD80 and CD86 are the monomeric proteins with two Ig-like extracellular domains and are expressed specifically by activated professional APCs[59,60]. These two molecules have particular kinetic of expression and affinity. While CD86 is expressed constitutively on APCs and is up-regulated upon interaction with CD28, expression of CD80 is only induced after APCs activation and is stable for a longer time frame of 4-5 days (compared to CD86 with a maximum of 48 hours)[61].

The CD28 molecule belongs to Ig family and is expressed constitutively on the surface of T cells. In contrast, CTLA-4 has a more complex kinetic of expression; in resting T cells, this transmembrane protein is present in intracellular vesicles and following TCR activation it

translocates to the immunological synapse. Moreover CD28 recycling rate is slower than that of the CTLA-4 whose membrane expression half-life is about 2 hours[61].

Differences in the expression of the two co-stimulatory molecules have been observed that could support a model where CD80 might be considered a preferential (but not exclusive) inhibitory ligand for CTLA-4[60]. Since CD86 and CD28 membrane expression is constitutive, their binding is more plausible as soon as T cell contacts APC. This interaction generates a positive signal to naive T cells for their activation and induces T cell proliferation, cytokine production while tolerance induction is inhibited[62,63]. On the other hand, interaction of CD80 and CTLA-4 is favored later, given the kinetic of expression of these two molecules and the higher affinity of CTLA-4 for CD80/CD86 molecules compared to CD28. This later binding generates a negative signal and is implicated in the termination of signaling and in anergy induction[64,65].

b) Role of CD40/CD40L interaction:

CD40 is a transmembrane receptor belonging to TNF family of receptors. Interaction of CD40 with its ligand CD40L is largely involved in T cell and DC dialogues as it induces many reciprocal effects in T cells and DCs[66,67]. CD40 engagement enhances DCs survival via up-regulating of Bcl-2 expression and results in DC maturation[68]. In addition, following CD40 engagement, DC increase expression of MHC class II molecules, of co-stimulatory molecules CD80 and CD86 and are activated to production of cytokines such as TNF-α, IL-8 and MIP-1α[69]. CD40 binding also induces production of IL-6 and IL-12 in response to IFN-γ [70,71].

- **Signal transduction to the nucleus:**

TCR signaling leads the activation of multiple intracellular effectors signaling pathways involved in expression of many genes:

1) Calcium pathway

2) Protein kinase C pathway

3) MAP Kinases pathway

All these pathways converge on the nucleus to regulate the expression of genes involved in cellular responses of T lymphocytes such as the proliferation, production of interleukins and differentiation.

Calcium Pathway:

Following TCR/pMHC interaction there is a rapid increase in $[Ca^{2+}]i$ concentration in T lymphocytes [72]. M.Davis and co-workers showed that as early as 6 seconds after specific TCR/pMHC engagement an elevation of $[Ca^{2+}]i$ can be detected[41]. Calcium signaling pathway and its role in inducing calcineurin dependent translocation of the transcription factor NFAT into the T cell nucleus is illustrated in **Figure I-11**.

In addition to its well described role in regulating T cell activation to cytokine production, $[Ca^{2+}]i$ increase appears to have an impact on the 'stop signal' of T cells. Indeed, while scanning the APC surface for antigen recognition, T cells are highly mobile cells with an elongated shape. T lymphocyte/APC interaction leads to an arrest and rounding of T cells known as 'stop signal'. T cell arrest is preceded by an increase in $[Ca^{2+}]i$. The artificial decrease in the intensity of calcium $[Ca^{2+}]i$ increase induced an inhibition of the 'stop signal'[73]. This T cell arrest requires activation of calpain, a calcium-dependent protease that frees LFA-1 from its cytoskeletal anchoring form thus permitting mobile LFA-1 to cluster at the cell-cell contact zone. Such LFA-1 clustering is fundamental for strengthening and stabilization of the intercellular contact[74,75].

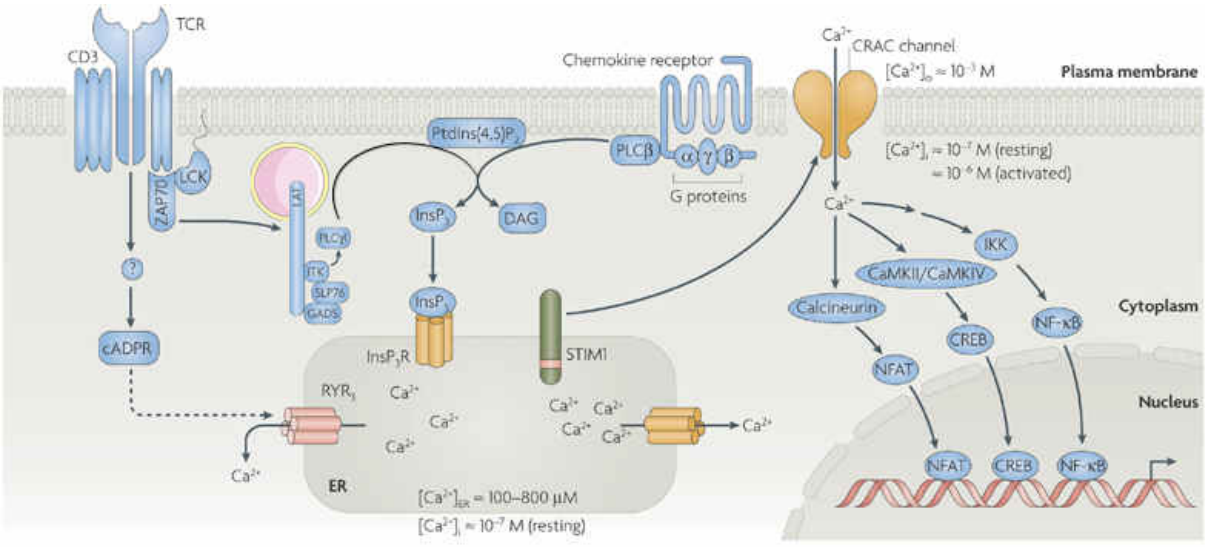


Figure I-11. **Calcium signaling pathway in immune cells.** Antigen recognition through the TCR results in the activation of protein tyrosine kinases, such as LCK and ZAP70, which initiate phosphorylation events of adaptor proteins, such as SLP76 and LAT. This leads to the recruitment and activation of the TEC kinase ITK (interleukin-2-inducible T-cell kinase) and phospholipase Cγ1 (PLCγ1). Similarly, binding of

G-protein-coupled chemokine receptors results in the activation of PLCβ. PLCβ and PLCγ1 catalyse the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to and opens $InsP_3$ receptors ($InsP_3Rs$) in the membrane of the ER, resulting in the release of Ca^{2+} from intracellular Ca^{2+} stores. A decrease in the Ca^{2+} content of the ER is 'sensed' by stromal interaction molecule 1 (STIM1), which in turn activates calcium-release-activated calcium (CRAC) channels in the plasma membrane. Ca^{2+} influx though CRAC channels and elevated intracellular Ca^{2+} concentration activate Ca^{2+}-dependent enzymes, such as calcineurin, and thereby transcription factors, such as NFAT (nuclear factor of activated T cells), NF-κB (nuclear factor-κB) and CREB (cyclic-AMP-responsive-element-binding protein). cADPR, cyclic ADP ribose; CaMK, calmodulin-dependent kinase; GADS, growth-factor-receptor-bound-protein-2-related adaptor protein; IKK, inhibitor of NF-κB kinase; RYR_3, ryanodine receptor 3. *Adapted from Feske S Nat Rev 2007*[76]

Moreover, A.Quintana et al. showed that mitochondria and ORAI channels are brought into close proximity (~200 nm) and PMCA (plasma membrane Ca^{2+}-ATPase) are re-organized into discrete regions of the plasma membrane where they are co-localized with mitochondria at the IS. This redistribution helps mitochondria to rapidly take up the inflowing Ca^{2+}, thereby avoiding high Ca2+ microdomains close to ORAI channels, thus preventing Ca^{2+}-dependent channel inactivation and reducing local Ca^{2+}-dependent PMCA modulation. This optimizes net Ca^{2+} influx at the IS. By slowly exporting Ca^{2+} further away from the PM, mitochondria sustain an elevated global Ca^{2+} signal that enhances the translocation of NFAT into the nucleus and the subsequent activation of Th cells[77,78].

PKC pathway:

A. Kupfer and co-workers, in their first description of an immunological synapse, showed that the PKC-θ is recruited very rapidly to the T cell/APC immunological synapse in an antigen specific manner[79]. TCR stimulation activates PLCγ1 that hydrolyses PIP2 into DAG and InsP3. DAG will recruit PKC-θ to the plasma membrane. GLK kinase phosphorylates PKC-θ on its catalytic site (Thr538) and activates it. Activated-PKC-θ will lead to the formation of a CARMA1/MALT/BCL10 complex and initiate a cascade of phosphorylation events, CARMA1 complex phosphorylation of IκB will result in its proteosomal degradation and release of the active form of NF-κB that can translocate to the nucleus and induce gene expression. This signaling pathway is important in Th1/Th2 differentiation process. Indeed, while absence of PKC-θ prevents development of Th2 lymphocytes differentiation, it does not influence Th1 phenotype[80] (**Figure I-12**).

The PKC family includes 10 structurally and functionally related isoforms that are grouped into three subfamilies based on the composition of their regulatory domains and their respective

cofactor requirements[81]. They are referred to as: conventional PKCs (cPKC: α, β, γ), novel PKCs (including: nPKC: δ, ε, η, θ) and atypical PKCs (aPKC: ζ, λ/ι). Novel PKCs are DAG-dependent, but Ca^{2+} and phospholipid independent for their activity[82]. Six of the PKC isoforms, including PKC α, δ, ε, η, θ, and ζ are expressed at varying amounts in T cells[83] and it has been shown that distinct PKC isoforms are required for different aspects of the activation and effector functions of T cells. PKC θ is one of the most well-known proteins recruited to the IS and accumulates at cSMAC upon TCR engagement in a CD28 dependent manner[66,79]. Recently, work from Hivroz' s lab and from Valitutti' lab identified atypical PKC ζ as a key molecular guide for Th cell polarization toward DC [84 85].

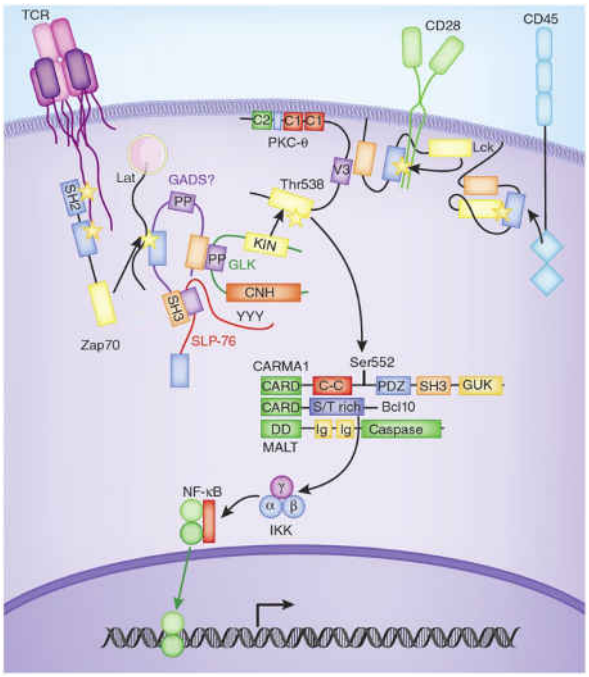


Figure I-12. **PKC θ signaling pathway.** TCR stimulation activates PLCγ1 that will hydrolyze PIP2 into DAG and InsP3. DAG will recruit PKC-θ to the plasma membrane. GLK kinase phosphorylates PKC-θ on its catalytic site (Thr538) and activates it. Activated-PKC-θ will lead to the formation of a CARMA1/MALT/BCL10 complex and initiate a cascade of phosphorylation events, CARMA1 complex phosphorylation of IκB will result in its proteosomal degradation and release of the active form of NF-κB that can translocate to the nucleus and induce gene expression. C1, DAG-binding domains of PKC; C2, calcium- and phosphatidylserine-binding domain; PP, polyproline motif; CNH, citron homology domain; YYY, Tyr-Tyr-Tyr; CARD, caspase-recruitment domain; PDZ, postsynaptic density 65–discs large–zonula occludens 1 domain; GUK; guanlylate kinase domain; DD, death domain; Ig, immunoglobulin domain; IKK, inhibitor of NF-κB kinase (γ, α, β are subtypes); KIN, kinase domain (tyrosine or serine-threonine) *Adapted from Dustin ML Nat Rev 2011* [86].

MAP kinases pathway:

The MAPK signaling pathway (mitogen activated protein kinase) is highly conserved in evolution and used ubiquitously. There are three groups of MAPK in mammals:

1) Erk 1 and 2 (Extra-cellular signal-Regulated protein Kinases)

2) p38 MAPK

3) JNK (c-Jun NH2-terminal kinase)

Each MAPK is activated by different upstream MAPK kinases (MEKs) and MAPK kinase kinases (MEKKs). MAPK are activated by dual phosphorylation on tyrosine and threonine residues. MAPK activation results in phosphorylation and activation of distinct downstream transcription factors, depending on the group of MAPK activated. Thus, ERK activates Elk-1 and c-Myc[87], p38 activates c-Fos[88] and ATF-2[89], and JNK activates c-Jun and ATF-2[90]. The phosphorylation and activation of these transcription factors permits the MAPK family to regulate gene expression and hence, cellular fate and effector function.

3) Cellular contacts in adaptive immune response:

T cell and APC cognate interaction is a crucial step in defining T cell destiny and thus a key element of initiation of an adaptive immune response. Depending on the type of presented antigen, duration and molecular organization of the T cell/APC interaction, T cells can be turned on or off.

These interactions contribute to several process of T cell biology including T cell differentiation, maturation, proliferation, the acquisition of effector or memory function. Alternatively these interactions can have tolerogenic effects leading to T cell anergy[91,92].

T cell/APC interaction can be schematically divided into 3 phases [93] (**Figure I-13**):

1 - Initiation of contact or searching for antigen

2 - Duration and stability of contact

3 – T cell detachment after a few minutes or a few hours followed by migration resumption.

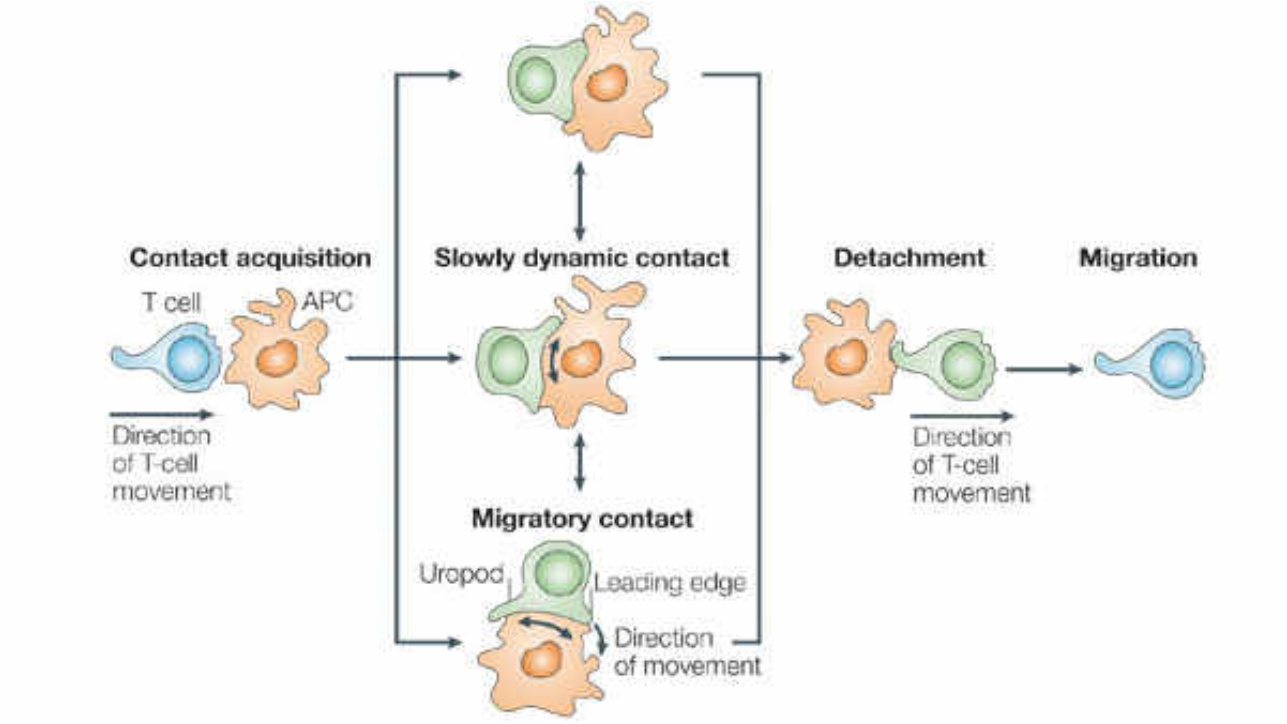


Figure I-13. **A schema of the phases of interaction between a T cell and an APC**. Contact acquisition is followed by initial scanning, contact, contact resolution (through detachment) and migration away from the APC. The interaction supports apposition of the membranes of the interacting cells and signaling, but stable and dynamic phases vary considerably in different experimental settings. T cells in which signal transduction is occurring are depicted in green. *Adapted from Friedl P 2005*[93]

- **Initiation of contact or searching for antigen**

First step in interaction between T cells and APC is their physical contact that may take place in lymph node for naive T cells or in tissues for effector T cells. however most studies performed using two-photon microscopy to monitor T cell/APC interaction in live tissues were performed in mouse lymph nodes[94]. This step is initially independent of the presence of antigen and of the type of the lately formed immunological synapse such random encounters are driven by T cell motility and by DC probing of the intercellular milieu using their highly motile dendrites. T cell scanning of the APC surface is a requisite element for detecting presence of an antigen and is finely controlled. Both $CD4^+$ and $CD8^+$ T lymphocytes migrate to T cell zone of amoeboid lymph nodes in a rapid fashion. Their average speed is approximately 10 µm/min with peaks of more than 25 µm/min[95-97]. APC, such as B cells or DC, are less mobile[98] and their average speed of migration is 6µm/min[95 96]. High migration capacity of T cells *in vivo* allows them to serially scan a large number of APC. Studies conducted by the team of M.Cahalan demonstrated that, in absence of antigen, approximately 5000 $CD4^+$ T cells per hour will

encounter a same DC[99] while E.Robey and co-workers estimated that about 500 $CD8^+$ T cells can scan a single DC in one hour[96], such rapid frequency of contact enables recognition of rare antigens by the pool of T cells in a lymph node within a reasonable timeframe. In addition there are other factors hastening antigen detection process. In vivo two-photon microscopy visualization of mice lymph nodes harboring florescent DCs revealed existence of a DC network (at sites of homing within high endothelial venules) within which mature DCs were more motile than steady-state DCs, facilitating their interaction with migrating T cells arriving in the lymph node. Moreover, chemokine release or the formation of tunneling nanotubes by mature DCs has also been proposed not only to globally optimize of antigen detecting process (guided priming)[100] but also to allow finding rare antigens. Proof of such highly effective organization is that less than 100 DCs in a lymph node are sufficient to initiate a response with very rare naive antigen-specific T cells[101].

During this first phase, LT are therefore highly mobile and move from one APC to another. The binding of their TCR with pMHC complexes initiates a process of activation and begins the second phase of specific interaction[102].

- **The stability and duration of contact:**
  Single vs successive encounter models

T cell response establishment requires signal integration via T cell-APC interaction, two models have been proposed.

The first model defined as: “single encounter model” emerged from the initial observation of firm interaction of T cells and APCs in early 1990s. This model describes T cell stop feature as a consequence of TCR engagement and a subsequent stabilization provided by adhesion molecules, such stable interaction forms an immunological synapse that may last for hours[103-105]. In this context several studies reported the necessity of prolonged T cell-APC contacts for T cell activation, indicating that full activation and initiation of proliferation requires stimulation over several hours (from 6-30 hours) depending on the strength of signal provided by APC[46,106].

Real time monitoring of T cell movements in 3D collagen during interaction with DCs provided support for a second model. This model proposed by P. Friedl and co-workers, is based on the observation that several successive transient interactions of about 3-20 minutes are formed between motile T cells and their cognate DCs. Even though these interactions were

rather dynamic, short lived and sequential, they have been shown to effectively induced T cell activation. Integration of signals from multiple contacts suggests existence of a short-memory that allows T cells to accumulate signals over few minutes[93]. The capacity of the T cells to add interrupted signals has been confirmed by S.Valitutti and coworkers[107] and has been recently well characterized *in vivo* by P.Bousso and Colleagues[108]. All in all, these observations leaded to a more precise description of the immunological synapse in which kinapses are referred as more dynamic synapses while synapses are T cell/APC junction of more stabilized interactions[109].

Given the difference of signaling and functionality in different types of synapses, the functional destiny of T cell depends on which kind of synapses are formed and on how these synapses contribute to T cell activation. Moreover, the quality of collected signals within individual contacts and signal sum up from serial encounters dictates the destiny of an activated T cell. In other words, it is tempting to speculate that there are 3 scenarios during T cells interaction with cognate APCs. First, APCs are presenting high affinity antigen that provides a STOP signal for T cells and prompt formation of synapses, in this context the T cells collect enough signal for full activation. Second, low affinity antigens will lead to formation of kinapses due to weak signal transmission and failure in T cell activation. Third, in conditions in which the strength of antigenic stimulation is intermediate, the signal accumulating capacity of T cells come in to account and depends on the density of APCs. In this condition, several kinapse formation and sum up of signals in the presence of high density of APCs can cause T cell activation.

In 2004 a 3-stage model of successive T cell-DC interaction was proposed based on two-photon microscope study of naive T cell dynamics in lymph node (LN). This study showed that newly arrived T cells in LN form transit interactions with DCs that last for about 3-11 minutes and results in up regulation of the activation marker CD69 on T cells. A second stage take place few hours later, when upon a progressive decrease of motility, contacts of longer duration (around 12-14h) are formed and T cells start to secrete IFN-γ and IL-2 and form dense clusters with DCs. Later on T cells recover their rapid migratory capacity and in parallel to proliferation T cells make transient interactions with DCs. This is referred to as 3rd stage. Several studies investigated formation and duration of such interactions *in vivo*, reporting somewhat controversial results most probably due to the variety of the utilized systems [110-112]. However, together these reports point out the influence of APC differentiation status as well as the quantity and quality of presented antigen in the control of the different stages of T cell/APC interaction. Such interaction will eventually determine T cell fate. Fully mature DCs with high

levels of adhesive and co stimulatory molecules more likely induce T cell arrest and priming, accordingly high affinity pMHC can more easily induce T cell arrest leading to formation of a productive immunological synapse, while low potency complexes ends up with T cell anergy and their retention in LN[113].

All in all, T cell/DC interaction can be described by both single and successive encounters depending of several factors including antigen presentation and environmental elements such as quantity and quality (potency) of antigens[113], nature and activation state of the APC[114], T cell phenotype and presence of bystander competitor[115], presence of Tregs[116,117] and even presence or absence of chemokine[118-121].

- **Termination of cell interaction:**

This final phase corresponds to dissolution of synapse, T cell detachment from its APC and its return to a migratory phenotype. This step is for the moment the less studied and, therefore, it is not fully understood. In particular, it is still not determined if termination of synapse is a consequence or a prerequisite for cell detachment[93]. Several mechanisms appear to be involved in this phenomenon such as: internalization and degradation of TCR[122] and adhesion molecules such as LFA-1[123], increasing of expression and recruitment of inhibitory receptors such as CTLA-4 at the synapse[124,125] or induction of the expression of chemokines and their receptors that can favor a migratory phenotype.

Dynamics of LT/APC interaction and synapse formation

As discussed earlier, αβ T cells are activated through engagement of their TCR with the cognate peptide-MHC complex expressed on the surface of their APC. This TCR/pMHC engagement in cooperation with duration of contact and recruitment of specific co-stimulatory molecules at the cell-cell interface provokes a complex of signaling cascade in T cells that dictates type and amount of information that is going to be exchanged between T cells and APCs. Such interactions between the two cells leads to the formation of a specialized signaling area referred as immunological synapse (IS), a term that was initially derived from neuronal synapses due to the analogy between T cell/APC contacts and neuronal contacts. This first observation of IS dates back to 1980 when microscopy-based studies revealed formation of a contact site between $CD4^+$, $CD8^+$ or NK cells with the APCs that was accompanied by the polarization of actin and microtubule cytoskeleton, the microtubule organizing center (MTOC) and the Golgi apparatus towards the contact site[126-129]. IS formation also results in the transport

of intracellular vesicles and cell-surface receptors (including TCRs and co-stimulatory molecules) to the contact zone[130]. All these rearrangements support the concept of formation of synapses for intercellular information exchange among immune cells. The main difference of IS and neuronal synapses, is the short duration of cell-cell contact of IS when compared to the ones of neurons or epithelial cells that can last for years[131]. This short interaction requires a very well organized dynamics of formation.

It was only twenty years after the term immunological synapse was coined that the development of lipid bilayers and advancement in microscopy, allowed the definition of molecular compartments of IS. In its original definition, the mature IS was described as a specialized signaling domain formed at the contact site between T cells and APCs, characterized by large-scale molecular segregation of surface receptors and signaling components[132]. Furthermore research lead to an expansion of this term, where the IS comprises a multitude of structures, with a common feature of having mediators of intercellular communication[75].

It is now well established that different types of contacts between immune cells and the subsequent type and amount of signal that is generated is determined by several factors:

1. Contact duration (short vs long termed interactions)
2. Physical dynamics (dynamic vs stable)
3. Strength of signals transmitted (weak vs strong)
4. Presence vs absence of secretion
5. Activation state of both T cell and the APC
6. Type of environment in which the interaction is taking place

1) Immunological synapse structure:

In a review published in 2004, P. Friedl's [133] team classified the different types of IS into 5 groups (**Figure I-14**): mono-centric (stable), secretory, multi-centric, non-segregated and dynamic synapse. Even if a specific type of synapse is observed in certain conditions, it is important to note that the kinetic observation of living cells points out variable molecular rearrangement that can result into different or incomplete structures.

Figure I-14. Five types of molecular arrangement at the immunological synapse. *Adapted from Friedl P Nat Rev 2005*

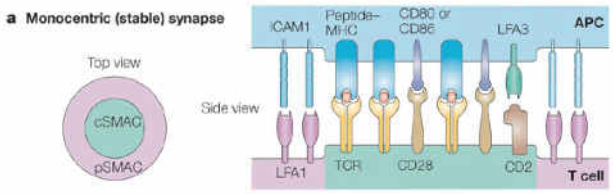


Figure a) A monocentric immunological synapse (also known as a stable immunological synapse) has a stably adhesive junction with a fully segregated central supramolecular activation cluster (cSMAC) and peripheral SMAC (pSMAC), which leads to T-cell priming, T-cell receptor (TCR) downregulation and sustained signalling. ICAM1, intercellular adhesion molecule 1; LFA, lymphocyte function-associated antigen.

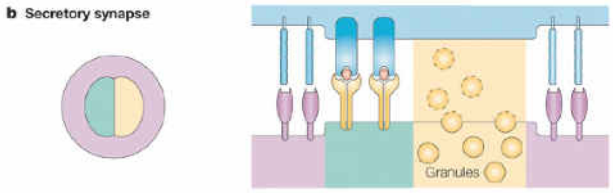


Figure b) A secretory immunological synapse has a fully segregated junction that includes a secretory domain for exocytosis of cytokines and/or perforin and granzymes.

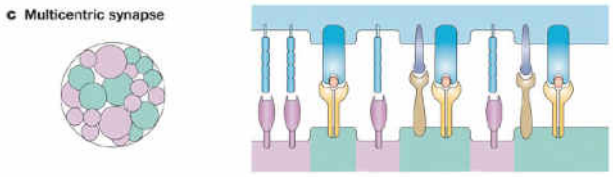


Figure c) A multicentric immunological synapse (also known as a partially segregated immunological synapse) is an assembly of partially mobile molecular clusters that lack complete segregation into a pSMAC and cSMAC.

Figure d) An unsegregated immunological synapse is enriched for TCR, co-stimulatory molecules and signalling molecules, yet it lacks pSMAC and cSMAC segregation.

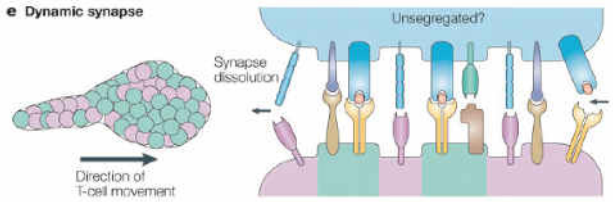


Figure e) A dynamic immunological synapse is an asymmetrical structure that is predicted to occur in T cells that are migrating across an antigen-presenting cell (APC); it is thought to involve receptor engagement at the leading edge of the cell (right arrow) and receptor exit at the rear of the cell (left arrow).

- **a. mono-centric or mature synapse: "bull eye"**

Spatial organization:

Since 1990's, T. Springer *et al*. proposed that molecular complexes of various sizes can be segregated into different domains located at the interface between T cell and APC. They proposed a model in which the contact site is enriched with TCR/pMHC complexes and CD2/CD58, while bigger size molecules, such as LFA-1 and CD45, are aggregating at their periphery[134]. Thanks to advances in microscopy studies, tri-dimensional molecular organization of the synapse has been revised through the detailed study of protein spatial segregation between CD4 T cell and antigen loaded B cells [132]. Obtained images confirmed and deepened T. Spriger's hypothesis. In its original description IS was composed of distinct areas called SMAC (Supra Molecular Activation Cluster). cSMAC (central SMAC) is synapse's heart, and correspond to TCR aggregation. Molecules known to be recruited to cSMAC, at least temporarily, includes: CD28[135], CD2[136], CD4 (transient)[137], CD45 (transient)[138] and CTLA-4[125]. CD28 co-stimulation molecule enrichment at cSMAC permits to facilitate its synergistic action with TCR. pSMAC (peripheral SMAC) is the area that directly surrounds the cSMAC. It is composed of accessory proteins, such as LFA-1 and talin (actin binding protein). Later, a third structure called dSMAC (distal SMAC) was described. It is the most peripheral area composing the synapse. It contains long and highly glycosylated molecules, that are excluded from the synapse core, such as the CD45 and CD148 phosphatase, and the CD43 molecules known to interact with actin cytoskeleton through ERM molecules (ezrin, radixin, moesin) [139,140] [141]. *In vivo* observation of T cells in lymph nodes showed TCR aggregation and CD43 exclusion from the IS[142] [102] (**Figure I-15**).

This surface protein segregation, is paralleled with an intracellular reorganization of intracellular signaling components. Molecules such as PKC-θ, LCK or Fyn, are aggregated at cSMAC level[132,143]. However, cSMAC formation is not required for productive TCR engagement, since TCR-mediated signal transduction precedes the formation of mature IS[143].

Mono-centric immunological synapse serves as a reference since it was the first synapse that was described. "Immunological synapse" and "immunological mature or mono-centric synapse" terms are often mixed up, however considering the following two facts may help avoiding confusions. First, if a mature synapse is possibly formed, preceding structure is also a synapse where information between two cells is already exchanged. Second, most synapses

rarely adopt a concentric structure, and rather form multifocal ones with several TCR aggregates[75].

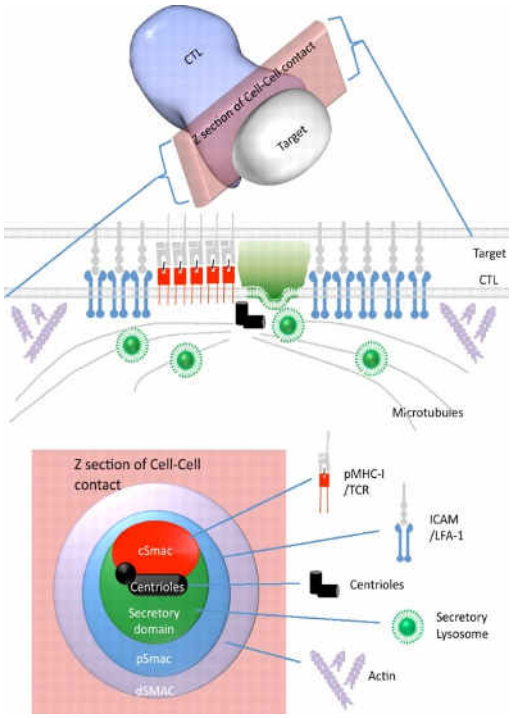


Figure I-15. **Immunological synapse.** Cartoon summary of the organization of receptors showing the relative positions of the cSMAC, pSMAC, dSMAC, centrioles, actin and microtubule cytoskeletons, and secretory lysosomes at the immunological synapse in cross section and across the area of cell contact. *Adapted from Griffiths GM J Cell Biol 2010*[144].

IS Formation dynamics:

Since 1999, the use of pMHC embedded into lipidic bilayers allowed the analysis of IS formation dynamics. During the first 30 seconds, the T cell stops its migration, and then a wide central area composed of ICAM-1 aggregation appears. At the same time, the engagement of pMHC and TCR is detected only in peripheral contact area[104]. This organization is therefore the inverse of the one previously described as mature immunologic synapse[132]. However, this engagement is clearly functional because an increase of $[Ca^{+2}]i$ is detected. Moreover, in 2002, J.Lee *et al.* observed ZAP-70 and LCK phosphorylation at the IS periphery before cSMAC formation[143]. During the following five minutes, engaged-TCRs in periphery migrate to the center of the contact area, while ICAM-1 moves to the periphery[104,143]. This spatial organization remains stable for hours. During this IS construction phase, several other receptors are submitted to dynamic changes between different areas of IS. For example, CD4 co-receptor rapidly aggregates at cSMAC just after cell-cell interaction, then relocates in the pSMAC after 5 to 10 minutes of interaction[137]. Same behavior is described for CD45 phosphatase[138,139].

Initial central enrichment of CD45 could explain how CD45 can activate LCK through its dephosphorylation before being exclude from the TCR signaling area[53]. TIRF microscopy provided new information on a smaller scale about molecular remodeling at the IS structure. In T cell interacting with lipid bilayer containing MHC and ICAM-1, the TCR and its associated molecules gather at the level of microclusters (MC) [145,146]. The number of detectable MC by T cell is between 100 and 300 and this number increases with the increase of antigen concentration[146,147]. TCR microclusters (MC) represent the main place of initiation and maintenance of TCR-induced signaling[147,148]. Indeed, in parallel with TCR signaling, ZAP-70, LAT molecules, Grb2 or SLP-76 MC are also forming[149]. These MC migrate from the periphery of the IS to the cSMAC, allowing the accumulation of TCR into the synaptic center. It is important to note that even after the formation of the cSMAC, MC continue to form on the peripheral area[148] and that they are the privileged place of the TCR signaling.

- **b. secretory synapse**

It has been described that the mono-centric IS is adapted to the secretion of soluble factors required for specific function of effector lymphocyte[93]. In this type of synapse, pSMAC is composed of LFA-1 and talin, which forms a ring of adhesion molecules, whereas cSMAC is divided into two parts: a TCR enrichment part and a secretory domain co-localizing with T cell secretion machinery[150,151]. This area is devoid of actin and it forms, a sort of gap between the two membranes[152]. The secretory synapse is therefore adapted to CTL intercellular granules release.

Th lymphocytes secrete vesicles containing cytokines such as IL-2, IL-4, IL-5 and IFN-γ towards the APC in a polarized fashion,[142,144,153-155]. Similarly, CTL and NK cells polarize their lytic granules, containing perforin and granzymes, to their target cell[144].

The definition of secretory synapse as a specialized mature synapse is controversial because several teams have shown that CTL cytotoxicity does not require formation of a mature IS[40,156 157]. In particular the team of S.Valitutti put forth the notion that CTL interacting with cognate target cells exhibit a dual activation threshold reflecting the formation of two distinct IS: the lytic synapse and the stimulatory synapse. The term lytic synapse was coined to refer to the polarization of the lytic machinery detectable both at low and at high antigen concentrations. The term stimulatory synapse was coined to refer to the large scale molecular segregation of surface molecules and signaling components characteristic of a mature IS and occurring only

with target cells providing the strong antigenic stimuli required for activation of cytokine production[107,156]. The team also demonstrated that the IS formed by CTL are endowed with a high degree of plasticity. Indeed CTL form multiple synapses with different target cells encountered simultaneously. Although CTL establish preferential "liaisons" with target cells offering a strong antigenic stimulus (stimulatory synapses) they simultaneously kill target cells offering a low antigenic stimulus (lytic synapses[158]). More recently, the team also showed that lethal hit deliver by CTL can occur extremely rapidly (within 30-40 seconds after CTL/target cell conjugate formation) thus it can precede mature IS formation and MTOC repolarization towards the target cell (both events requiring a few minutes to be accomplished,[159]). Yet, this work was done in $CD8^+$ T cells and does not exclude that control of cytokine release might be different in $CD4^+$ T cells.

- **c. multi-centric synapse**

During antigen recognition, there may be an interruption in the molecular mechanism leading to the formation of the mono-centric IS, resulting in the generation of a so-called immature or multi-centric IS. This synapse is characterized by formation of several aggregates of TCR/CD3 complexes next to area enriched with LFA-1. These molecular clusters are dynamic and can merge or split during the interaction. It would be simplistic to think that this multi-centric synapse results from a failure of a mono-centric synapse formation. In fact, it has been reported that concentric mature IS is not the prototypical structure; T cell even during prolonged contact with APC can establish synapses with a non-concentric structure. For instance, a multi-centric structure is observed at contact site between thymocytes and thymic epithelial cells[160,161] during thymic selection. A multi-centric IS also forms during the interaction between naive T cells and DC[162].

- **d. non segregating synapse**

In the case of immunological non segregating synapse, TCR and signaling molecules are enriched at contact zone but exhibit a diffuse pattern. This synapse is observed at least under 4 different physiological situations. First, this structure is formed when a T cell interacts with an APC displaying on its surface no specific antigens (for instance, T cell scanning of APC within a secondary lymphoid organ)[163]. This non-specific synapses have short-term and long-term consequences. They induce a moderate increase in tyrosine phosphorylation as well as a small increase in intracellular calcium concentration, that then turn into a modest proliferation and significantly prolonged survival. This phenomenon could therefore have an important role in the maintenance of naive T cells stock *in vivo*. The non-segregating IS is also observed during

the initial phase of contact before formation of segregating IS[102]. Thirdly, naive $CD8^+$ T cells can be activated and then proliferate after generation of a non-segregating synapse[164]. Finally, IS forming between naive T and DC can also be categorized in this type of synapse[163,165].

- **e. Dynamic synapse or kinapse**

In some cases, T cells are capable of forming dynamic synapses, i.e. to integrating activation signals while migrating (refer to page 32-34 [109])

Cell destinies related to type of formed-synapse have been summarized in **Figure I-16**.

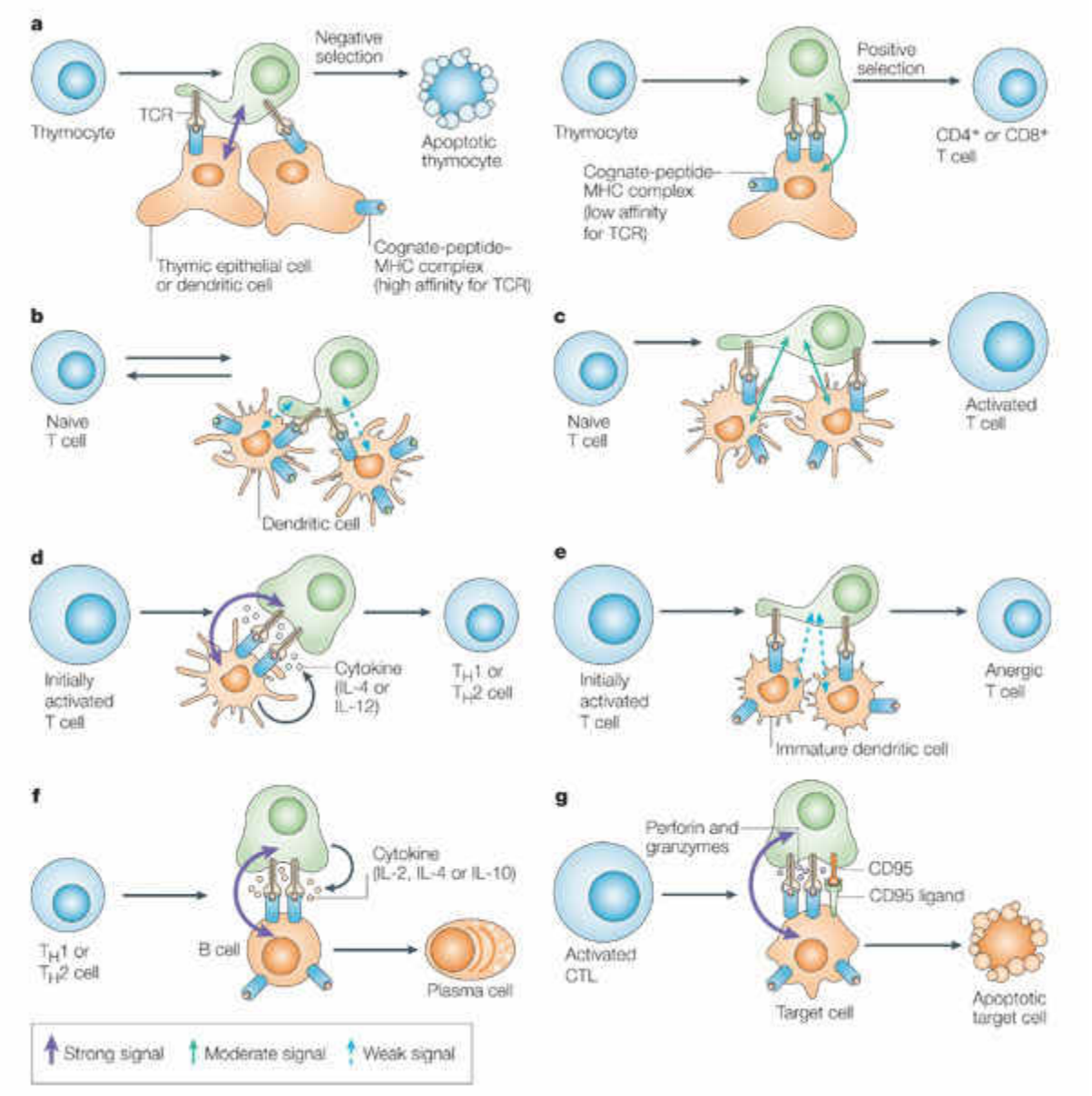


Figure I-16. **T cell/APC interaction define T cell destiny.** The interactions of T cells with different antigen-presenting cells (APCs) in the thymus (a), secondary lymphoid organs (b–f) and peripheral tissues (g) lead to T-cell development, maintenance, activation, and helper and effector function. The particular outcome depends on the contact mode and signal intensity. Stable interactions result from long-lived one-to-

one conjugation of a T cell and an APC, whereas short-lived contacts allow T cells to transit between different APCs. **a**) Different strengths of peptide–MHC–T-cell-receptor (TCR) binding might determine a range of stable and dynamic binding during positive selection (right panel), and mostly stable binding during negative selection (left panel). **b**) Serial encounters support T-cell homeostasis in the absence of agonist peptide. **c**) Serial encounters support the initial activation of naive T cells by agonist-peptide–MHC complexes. **d**) For initially activated T-cell blasts, contact stabilization supports cytokine release from the APC and maturation of T cells to effector T cells. **e**) Serial engagement that does not result in a stable phase has been implicated in tolerance induction. **f**) T helper (T_H) cells establish a stable immunological synapse with activated B cells and release cytokines that promote the differentiation of B cells into plasma cells. g) Cytotoxic T lymphocytes (CTLs) that receive a strong signal bind stably to target cells and induce target-cell killing by releasing the contents of lytic granules. T cells in which signal transduction is occurring are depicted in green. IL, interleukin. Adapted from Friedl P 2005[93]

2) Immunological synapse biological function

Since its discovery, mature immunological synapse and its various components (cSMAC, pSMAC and dSMAC) have been proposed to be involved in several biological processes.

- **Role of immunological synapse in initiating T cell signaling**

Microclusters, each composed of minimum 11 to 17 TCR molecules, are the smallest signaling unit known to date[148]. Interestingly, based on several studies it has been suggested that TCR microcluster formation do not necessarily require antigen recognition. High resolution microscopy techniques further enabled visualization of TCR pre aggregation at the surface of unstimulated effector cells[166-168] and of naive T cells[169]. Antigen stimulation did not change the size of the MCs and the number of TCR molecules present per MC. Moreover TCR MC at the surface of the T cell has been reported to slightly vary in terms of composition, independently of antigenic stimulation. While both non-phosphorylated form of GRB2 (growth factor receptor band 2) and LAT adapter molecules are present in preexisting TCR MCs, upon TCR engagement the number of GRB2 molecules, unlike LAT, increases in MC.

The above reported results indicate that the smaller signaling unit observed at the moment in T cells preexist signaling. Taken together with result discussed in page 38 (IS formation) in which I reported the observation by Lee *et al.* that signaling starts at the periphery synapses before molecular segregation[143], these results strongly suggest that molecular segregation at the IS is rather a consequence than the cause of TCR signaling.

- **Role of immunological synapse in tuning T cell signaling**

The proposed role of cSMAC in sustaining TCR-mediated signaling has been a controversial issue for prolonged time. Recently a model has been proposed suggesting that cSMAC formation can serve as a controller of T cell signaling by amplifying weak signals and by attenuating strong ones. Cemerski *et al* [170]reported that both signaling and TCR degradation can

take place in cSMAC and the balance between the two depends on the strength of antigenic stimulation and on antigen quality [170-172] (**Figure I-17**).

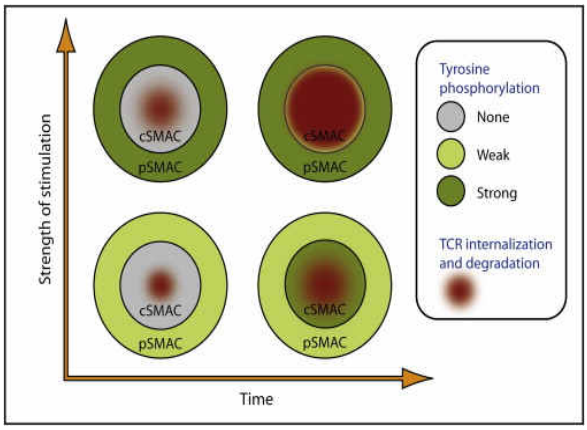


Figure I-17. **TCR Signaling can occur both at the pSMAC and the cSMAC.** After T cell-APC conjugation, signaling (shown as tyrosine phosphorylation, green) initiates in the periphery of the IS both at high and at low antigenic stimulation. TCRs are internalized and degraded in the IS center (red). With high antigen stimulation, signaling continues throughout the IS. Because TCR degradation rate in the cSMAC is high, tyrosine phosphorylations are not easily detected. However, signaling does occur in the cSMAC as detected by PI3K activation (see text). At low antigen densities, sustained tyrosine-phosphorylation events are more pronounced in the cSMAC where they coexist with a moderate rate of TCR degradation. *Adapted from Valittuti S 2008* [172].

- **Role of immunological synapse in the termination of T cell signaling**

Involvement of cSMAC in extinguishing signal has been confirmed by the use of lipid bilayers. Inserting physical barriers in these lipid bilayers generates spatially mutated synapses where cSMAC formation is inhibited[173]. T lymphocytes are forced to form multifocal synapses with TCR microclusters associated with patches of molecules phosphorylated in tyrosine. In these T cells, calcium signaling is significantly higher confirming the hypothesis that cSMAC is involved in inhibition of signaling via TCR.

- **Role of immunological synapse in privileged intracellular dialogues.**

It has been proposed that IS creates a sort of physical barrier that can prevent dispersion of secreted molecules in a polarized manner and would therefore participate in privileged intercellular communication by limiting the effect of these soluble factors on adjacent cells[109]. Accordingly, there is an inward movement of actin filaments towards cSMAC that guides molecules such as TCR and LFA-1 into the center of synapse[174]. Such a dynamic flux thus, could allow the entry of molecules more in the center of the synapse rather than the periphery[109]. The presence of sort of synaptic baskets avoiding the leakage of lytic molecules outside of the IS has been also reported at CTL/target cell lytic synapses[175].

3) Immunological synapse and secretory machinery

- **Microtubule organization center**

The discovery of T cell MTOC polarization towards APC occurred in the 1980s. First observations were made in CTL; it was shown that target cell killing by CTLs is a very specific and unidirectional phenomenon. G. Berke's team hypothesized that the unidirectional killing activity is at least partially related to the specific polarized re-arrangement of cytoskeleton. Their results suggested that CTL interact with their target cells via the membrane region close to the MTOC[127] CTL plasma membrane proximal to the MTOC is particularly active in forming stable intercellular contacts, resulting in CTL-target conjugation, and that subsequent modulation of the microtubular system of the CTL may be related to the cytolytic response and to detachment of the effector cell[127].

Two years later, A.Kupfer published a first of a long series of articles on the phenomenon of polarization of T lymphocytes. They demonstrated that T cells polarization is an active mechanism: the MTOC and the Golgi apparatus of CTLs and murine NK cells re-orient following specific contact with a target cell[126,128,176]. Two years later, the same team showed that $CD4^+$ T cells can, also, polarize their secretory machinery towards APC in an antigen-specific manner[129]. This phenomenon has then been widely confirmed in human T cells[177]. More recently, the study by J.Kuhn et al. showed that once located at the synapse, MTOC is not static but oscillates laterally over a distance not exceeding 2 µm. These oscillations may be caused by opposing forces and is probably generated by molecular motors anchored at pSMAC, such as dynein[178].

One major characteristic of MTOC polarization is its rapidity as it occurs as early as about 2-3 minutes after contact with APC and before formation of the synapse[41,179]. Therefore, IS formation is not necessary to this polarization. In fact, $CD4^+$ T lymphocytes are able to polarize their Golgi apparatus under conditions where the synapse formation is inhibited by anti-CD2 blocking antibodies[180]. In addition, this process is highly dynamic and selective allowing Th cells to polarize selectively towards the APC offering the strongest antigenic stimulation[179].

- **Lytic granules**

The polarized secretion of lytic granules by CTL was first observed during the investigation of their MTOC polarization[176]. This process of lytic granule polarization is highly dynamic[158]. The multi-polarization of the lytic granules to different targets was also observed *in vivo* in a mouse

model, during an interaction between CTLs and neurons[181]. This phenomenon of "multiple killing" is however questioned by other studies as discussed later.

There are major difference between secretion of lytic granules and secretion of cytokines because of their time kinetics. In fact, CTLs release their lytic granules as early as few minutes or seconds after interaction with the target cells[159]. The CTL can therefore undergo several cycles of antigenic recognition, polarization and cytotoxicity. In addition, a recent study showed that CTL can rapidly synthesize new stocks of perforin[182] and consequently kill several targets. For this reason CTLs are called serial killers. In contrast, cytokines are released a few hours after antigenic stimulation as their production requires, most often, gene transcription[46].

Lytic granules biogenesis:

Granules biogenesis process was unveiled in part through the study of two genetic diseases: Hermansky-Pudlak syndrome type 2 (HPS2) and Chediak-Higashi syndrome (CHS); patients with these diseases are mutated in the gene encoding AP-3 (adaptor protein 3) and in the gene encoding LYST respectively. LYST is a cytoplasmic protein that regulates the lysosomal trafficking and AP-3 is necessary for the transport of proteins from the trans-Golgi to lysosomes or endosomes. The CTL of these patients have a defect in polarization of granules to IS. Lytic granules are also larger than in normal[183]. Moreover, the deficient AP-3 T cell granules seem unable to migrate along microtubules[184]. This suggests that AP-3 may be required for the expression of one or more proteins involved in the dynamics of the granules along microtubules. LYST and AP-3 proteins are therefore involved in the early stages of the formation and transport of lytic granules.

During their biogenesis, lytic granules are loaded with lethal molecules namely perforin and granzymes. It has been suggested that the distal end of the C-terminal region of perforin encodes a highly efficient ER export motif that is required for protein delivery from the ER to the granules [185]. Deletion of the last 12 amino acids or even a mutation of the last C-terminal residue, tryptophan, results in the retention of functional and stable perforin in the ER and in the death of CTL owing to perforin pore-forming activity [185]. This notion is further supported by the observation that CTL that express an inactive perforin variant, which cannot properly bind to Ca^{2+} (required for perforin function) are protected from the toxic effects of mutations of the C-terminal region [185]. Accordingly, an explanation for cell survival during perforin synthesis is that it is rapidly exported from the ER, thus minimizing the concentration of active perforin in the ER at any given time[186]. Furthermore, it is notable that *N*-linked glycans are

essential to enable the delivery of perforin from the Golgi to secretory granules. The exact carrier (or carriers) of nascent perforin are unknown[185]; however, this process may involve members of the constitutive protein trafficking machinery (such as lysosome-associated membrane glycoprotein 1 (LAMP1)[187] or the mannose 6-phosphate receptor) [188].

Mechanism of lytic granule polarization:

Upon CTL/target cell interaction, the CTL quickly polarizes its MTOC and its Golgi apparatus[127,189] as well as its lytic granules towards the target cells (**Figure I-18**)[176]. Lytic granules contain proteins such as perforin, granzyme and Fas-L. This polarized granule secretion consists of several steps: polarization towards the synapse, followed by their anchorage and fusion with the plasma membrane allowing the release of the contents of the granules within the synaptic cleft.

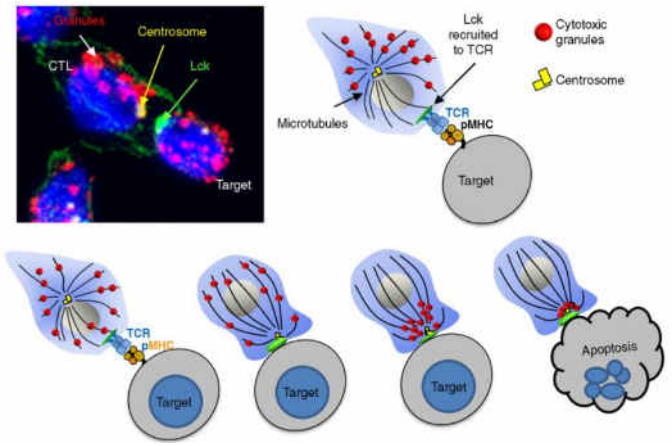


Figure I-18. **Mechanism of lytic granule polarization.** Recognition of the target MHC class I molecules bound to peptide antigen occurs via the TCR. Once the TCR is engaged, the most proximal signaling molecule to be recruited is LCK (shown in green). The centrosome (γ-tubulin, yellow), which is normally perinuclear, polarizes towards the synapse and docks at the plasma membrane. The granules (LAMP-1, red) migrate towards the centrosome, where they dock and fuse with the plasma membrane, releasing their contents into the synaptic cleft, causing target cell destruction. *Adapted from Griffiths and Jenkins 2014*[190].

MTOC and LG parallel polarization:

It is generally accepted that MTOC polarization governs granules secretion by CTL. Accordingly it was reported that, nocodazole-treated CTL (inducer of microtubules depolymerization) were unable to polarize and exhibited a reduced cytotoxicity[126]. Granules transit along the microtubules towards the negative end[191], to be released in the center of the synapse formed with target cells[192]. Thus according to this model, centrosome plasma membrane contact seems required[191].

Two ways of lytic granules secretion in a MTOC-dependent fashion have been reported: a fast way and a slow one. Both are regulated according to signaling kinetics. Strong signaling leads to a rapid enrichment of granules at MTOC location followed by a rapid MTOC re-positioning and granule secretion while weak signals induce a slow recruitment of granules via their migration along the microtubules from the periphery to the IS[175]. Recently, G. Griffiths's team has shown that polarization of the centrosome and lytic granules may be dissociated[193]. Indeed, through the use of OT-1 mouse and antigenic ligands of varying functional avidity for TCR, they showed that low and high avidity interactions induce polarization of the centrosome while only high avidity interactions allow the polarization of the lytic granules.

However, this one-way vision of the polarization of the lytic granules disagrees with studies showing that the CTL can simultaneously kill several cells target[158,181].

The study by J.Kuhn et al. provided a first explanation for this phenomenon of "multiple killing" [178]. They showed by time-lapse microscopy, that when a $CD8^+$ LT is in contact with two targets, its MTOC can oscillate from one cell to the other thus inducing lysis of both. However, recently S. Valitutti Lab clearly showed that lytic granules can be secreted independently of MTOC polarization. In a first approach by using TIRF microscopy, they have shown spatio-temporally dissociation of MTOC and lytic granules in CTLs stimulated with immobilized pMHC. This result was confirmed by studying by time lapse microscopy CTL/target cell conjugates in which lytic granule secretion preceded microtubule polarization and was detected during the first minutes or even second after initial cell-cell contact. Surprisingly, inhibition of microtubule organizing center and centrosome polarization impaired neither lytic granule release at the CTL synapse nor killing efficiency[159]. This MTOC-independent secretion of lytic granules of $CD8^+$ T lymphocytes is compatible with the multidirectionally secretion of cytokines (TNF and MIP-1α) exhibited by $CD4^+$ T cells as described by M.Huse et al.[154]. It is tempting to speculate that $CD8^+$ T cells are endowed of a first mechanism that allows them to secrete granules in a polarized manner towards the synapse and another allowing them to secrete to a second target cell without forming equally and simultaneously a stimulatory synapse with it. In this context, it is interesting to note that cytotoxic molecules such as perforin may also be secreted by a granule independent pathway[182] [194].

Lytic granules anchorage:

In eukaryotic cells, vesicular trafficking towards specific cellular locations is handled by families of protein highly conserved during evolution. In the center of this process are the SNARE proteins (soluble N-ethylmaleimide-sensitive factor accessory protein receptors). Typically, one R-SNARE protein residing on the vesicular membrane and three Q-SNAREs (Qa, Qb, and Qc) proteins residing on the target membrane can span the distance between two membranes, forming a parallel four-helical bundle that catalyzes membrane fusion[195]. When forming a SNARE complex, SNARE proteins demonstrate remarkable specificity for their cognate SNARE partners, resulting in highly specific combinations of SNAREs at different steps of vesicle fusion[196,197]. Different R and Q-SNARE have distinct cellular locations and their interactions are regulated by various accessory proteins that add a higher level of specificity. Many studies reported involvement of SNARE proteins in the mechanism of lytic granules fusion with the plasma membrane at synapse[198]. However, lytic proteins such as perforin, granzymes A and B and Fas-L, whose expression can be induced by TCR engagement can be also secreted directly from the Golgi apparatus at the IS[182]making it difficult to study the proteins involved in the vesicular pathway[194] .

Proteins involved in polarized granules trafficking into the IS and in secretion process have been discovered primarily in melanocytes due to genetic diseases causing immunodeficiency associated with an albinism[188,199,200]. However, it is now clear that even though there is a similar pattern of secretory gene expression shared among LT and melanocytes, the relative importance of each molecule is different within two cell types. Among other possibilities, these differences can be explained, by the fact that secretion occurs in opposite directions. In melanocytes, the MTOC is in the center of the cell and it is the positive ends extremity of microtubules that are connecting vesicles to the membrane via actin. In this case, the vesicles migrate to the positive end of microtubules. On the other hand, in T cells, the MTOC is polarized to the membrane and vesicles move towards the negative ends of microtubules. Melanocyte secretory machinery will be more detailed in page 68 (melanocyte vesicular trafficking).

Lytic granule Fusion:

Two studies have identified the PKC requirement for polarization and lytic granule exocytosis. PKC δ deficient murine CTLs have a defect in these 2 processes despite a correct MTOC polarization. This defect in lytic granules polarization results in a loss of the cytotoxic capacity of these CTLs[201,202]. The PKC δ kinase activity is therefore involved in the early steps of the lytic granule release.

Most of our knowledge on lytic granule trafficking came from the study of genetic diseases causing FHL (**Figure I-19**). Rab27 deficient CTLs can polarize their granules with the MTOC towards the synaptic zone but they cannot be anchored and fused to the plasma membrane [150] . Moreover, Rab27a is not constitutively associated with the lytic granules in human cytotoxic lymphocytes, but is recruited to them in response to T cell stimulation. Over-expression of Rab27a-GFP in CTLs did not show colocalization of Rab27a and perforin, until immediately before degranulation[203]. Rab27a may therefore be required for the dissociation of lytic granules from microtubules as was described for melanocytes [200]. While the function of Rab27a in CTLs appears to be mainly related to vesicle docking at the plasma membrane, the GTPase activity is also involved in the regulation of vesicle movement at the plasma membrane[204]. Decreased expression of Rab27a via siRNA treatment resulted in increased motility of granules in the cytoplasm of NK cells, and fewer and less mobile lytic granules reaching the plasma membrane, indicating that Rab27a is required for retention and cytoskeleton-dependent directional movement of lytic granules at the plasma membrane. Two studies show that the proteins Slp1 and Slp2 (synaptotagmin-like proteins) interact with Rab27a allowing granules to anchor to the membrane[205,206]. Rab27a complex with Kinesin1 and slp3 also mediates the terminal transport of lytic granules to the IS [207].

Protein Rab27a interacts with Munc13-4 protein that is known to regulate initial fusion steps. Munc13-4 deficient CTLs are indeed able to polarize their lytic granules and to anchor them to plasma membrane at IS but cannot fulfill fusion step[208]. Munc13-4 maintains the docking SNARE in an open conformation; without this molecule the fusion is impossible. Syntaxin 11 defect does not prevent polarization but the exocytosis of the granules. It can be counterbalanced by stimulation via IL-2 suggesting that this stimulation may induce the synthesis of proteins that overcome the defect, explaining thus the lesser severity of this form of deficiency[209]. It is assumed that Munc13-4 permits the conversion of syntaxine11 into its open conformation. Munc18-2 is the main partner of Syntaxin 11 (Stx11) in CTL. Complexes

Munc18-2/Syntaxin 11 at IS, probably regulated anchoring and formation of the SNARE complex required for the fusion step.

The vesicle-associated membrane protein (VAMP) family typically mediates fusion of vesicles with cognate, membrane-associated SNARE complexes. It is yet unclear how distinct VAMPs regulate cytotoxic granule trafficking and fusion for target cell killing. Accordingly, it has been reported that VAMP2 colocalizes with cytotoxic granules and that VAMP2 knockout mice display defective cytotoxic granule exocytosis210. VAMP4, and VAMP7 also co-localize with cytotoxic granule proteins and their siRNA-mediated knockdown in a human natural killer cell line resulted into deficit of perforin-mediated cytotoxicity211. Furthermore, in mice, VAMP8, an R-SNARE associated with exocytosis of secretory vesicles in multiple immune cell types, colocalized with granzyme B. *Vamp8* knockout mouse CTLs display reduced granule exocytosis and impaired target cell killing212 213. Recently, a study on human primary lymphocyte using SIM (Structured illumination microscopy) and TIRF microscopy, showed that VAMP8 is associated with recycling endosome compartments rather than with cytotoxic granules214. In addition the authors observed a rapid accumulation and fusion of recycling endosomes then fused at IS preceding lytic granule arrival. Inhibition of VAMP8 expression compromised both recycling endosome and cytotoxic granule fusion, revealing a VAMP8-dependent fusion step between recycling endosomes and the plasma membrane that brings Stx11 close to the immune synapse for cytotoxic granule exocytosis.

G. de Saint Basile proposed a model to describe lytic granules exocytosis at IS by gathering all the data acquired through the study of genetic diseases and the neuronal synapse (**Figure I-20**)[188] .

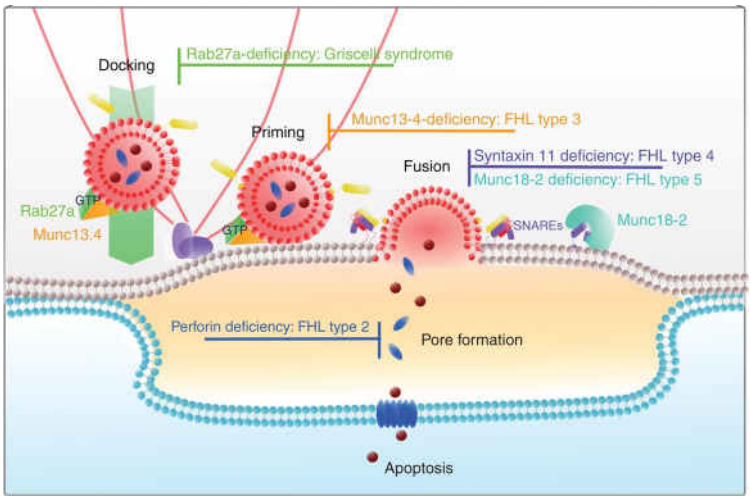


Figure I-19. **Genetic diseases giving rise to familial hemophagocytic lymphohistiocytosis (FHL).** At the IS, secretory lysosomes dock, prime, and undergo fusion at the plasma membrane into a small secretory cleft. Perforin (blue) forms a pore in the target cell allowing entry of granzymes (brown), which trigger apoptosis. Loss of perforin leads to FHL2. Rab27a (deficiency causes Griscelli syndrome [215]) and Munc13-4 (deficiency causes FHL3 [209]) associate with granules and are required for docking and priming, respectively. Syntaxin 11 (deficiency causes FHL4 [216,217]) and Munc18-2 (deficiency causes FHL5) are thought to be required for fusion by assisting formation of the final SNARE complex. *Adapted from Lieberman J 2013* [218].

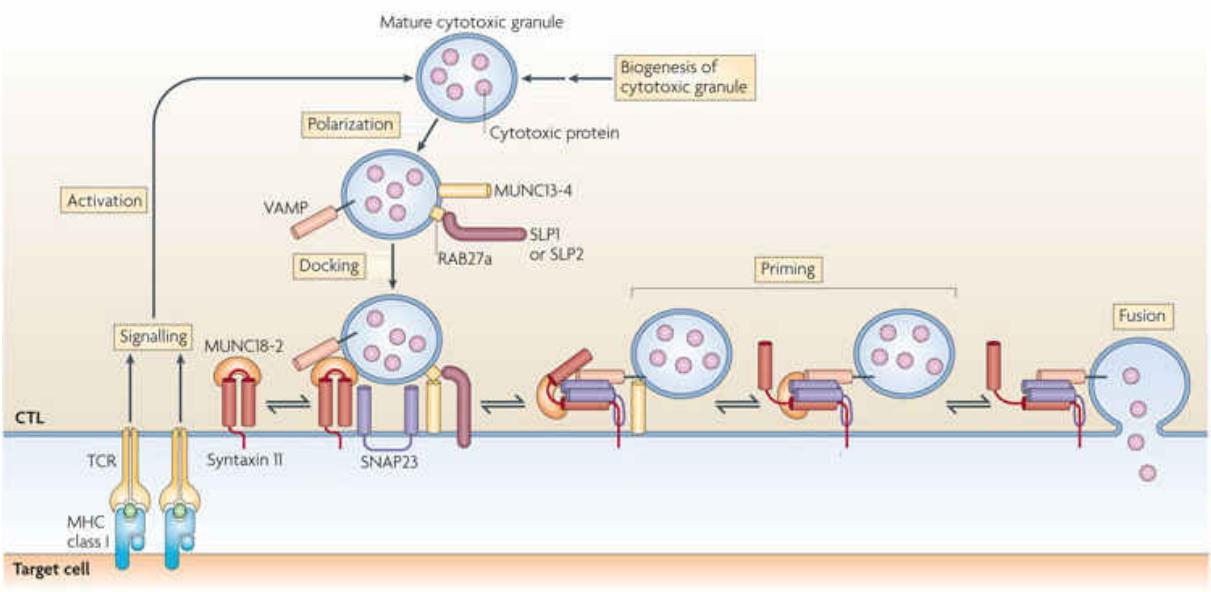


Figure I-20. **Lytic granule exocytosis.** Following antigen recognition and proximal signaling events, cytotoxic granules are polarized towards the CTL–target cell contact site where the immunological synapse forms. Actin filaments are cleared away from the site of secretion. Mature cytotoxic granules dock at the plasma membrane through the interaction of RAB27a with SLP1 or SLP2. In addition, cytotoxic granules interact with a docking complex formed of MUNC18-2 and Syntaxin 11 in the closed conformation. Docked vesicles are then primed by MUNC13-4, which probably triggers the switch of Syntaxin 11 from a closed to an open conformation. A SNARE complex then forms between a vesicle membrane SNARE, possibly VAMP7 and/or VAMP8, on one side and the target membrane SNAREs Syntaxin 11 and SNAP23 on the other side. Completion of the fusion reaction occurs when MUNC18-2 clasps across the zippering four-helix SNARE complex bundle. Formation of the SNARE complex precedes membrane fusion. *Adapted from de Saint basil and Fischer Nat Rev*[188]

- **Vesicular recycling at immunological synapse**

In addition to the polarization of lytic granules, cytokine containing vesicles and other types of vesicles such as recycling endosomal vesicles are also translocated to the IS. This pathway allows the internalization of proteins and their re-orientation to plasma membrane or to lysosomes in order to induce their degradation.

In 2004, M.Alonso *et al.* showed a polarization of transferring receptor and of recycling vesicle at the IS[219]. During the same year, A. Alcover's team demonstrated that TCR accumulation in cSMAC occurs through polarization of recycling endosomes containing TCR and that this phenomenon is guided by the polarization of the MTOC. Thus, colchicine treatment of T cells that induced inhibition of the polarization of recycling vesicles resulted in a defect in the accumulation of the TCR at the synapse. Proteins Rab35 and EP164C (a Rab GTPase activating protein (GAP) that activates Rab35) co-located with TCR in recycling endosomes and were involved in polarization of these vesicles at IS. Their inhibition induced alteration in IS formation[220].

As discussed earlier, CTL/target cell interaction induces lytic granule synaptic polarization. CTL degranulating is visualized through appearance of lysosomal associated membrane protein-1 (LAMP-1) at the cell surface. Study conducted by Liu *et al.* in 2009 using TIRF microscopy revealed that Lamp-1 is accumulated in a large and spatially stable cluster that overlapped with a site of membrane internalization. Perforin-containing granules were juxtaposed to an intracellular compartment where exocytosed LAMP-1 was retrieved. The authors thus, concluded that cytotoxic immune synapses include a central region of bidirectional vesicular traffic, which is controlled by integrin signaling[221].

Interestingly, in 2002, Henkart lab provided evidence that lysosomal exocytosis at the synaptic area is accompanied by the exposure of Cathepsin B (a cystein protease) that confers protection to CTL from their own-secreted perforin. This notion was later questioned by Baran et al in 2006[222]. These authors claimed that Cathepsin B deficient mice did not reveal a lack of protection of their CTLs upon target cell killing and that perforin is a poor substrate of Cathepsin B[223,224]).

Finally such a polarized lysosomal trafficking associated to MTOC re-polarization has been also described in B cell synapses during antigen extraction and processing. It depends on the function of small GTPase Cdc42 and aPKC ζ. These polarized secreted lysosomes create an

acidic extracellular microenvironment at the cell interface facilitating antigen extraction by lysosomal proteases[225] .

Cytotoxic T cells

1) Activation and Differentiation of Naïve CD8+ T cells into CTLs

Naive $CD8^+$ T cells that have not previously seen antigen do express neither granule effector molecules or nor receptors, and are incapable of cell-mediated cytotoxicity. The activation process of the $CD8^+$ T cells starts when naive T cells recognize their specific antigen presented by an APC in the context of MHC class I molecules in the secondary lymphoid organs. This recognition stimulates the TCR and provides the first signal of activation process and gives specificity to the response. The second signal is provided by the engagement of co-stimulatory molecules of $CD8^+$ T cells with their ligand molecules on APCs. Contrary to the first signal, this signal is nonspecific but is crucial to the development of an effective $CD8^+$ T cell mediated immune response. Absence of such interactions may lead to T cell anergy or death.

Moreover, there is evidence supporting the idea that $CD8^+$ T cell proliferation and differentiation, for establishment of an optimal response, can be influenced by the environment (third signal). The 3rd signal provided by inflammatory cytokines, act as a 'switch' that determines whether Ag and co-stimulatory signals lead to tolerance (deletion/anergy), or in their presence, to a productive response leading to strong effector function, $CD8^+$ T cell survival and memory T cell generation[226]. This signal is induced by exogenous inflammatory and antiviral cytokine[227] provides a means for a $CD8^+$ T cell that encounters Ag to determine if there is 'danger' present, and to respond accordingly. For instance *in vitro* and *in vivo* experiments identified IL-12 and IFN α/β as predominant sources of signal 3 for $CD8^+$ T cells responses to a variety of stimuli [226].

Combination of these 3 signals causes clonal expansion of a specific $CD8^+$ T cell population (up to 1000 fold increase) and induces their differentiation into cytotoxic T cells (CTL). While this clonal expansion guarantees presence of sufficient amount of antigen specific T cells for eradication of the invading pathogens or the cancerous cell, It is also controlled by a contraction phase during which the number of cells declines and only 5-10% of these antigen specific cells remains in order into develop the memory cells. These memory cells downregulate expression of cytotoxic effector proteins, but the kinetics of downregulation

varies with the molecule and depend on of the immunostimulatory environment[227]. Memory phase consist of long-lived antigen specific cells that are ready to respond in a faster and stronger manner upon second encounter with the same pathogen.

2) Effector functions of CTLs:

Upon activation, within about 5 days $CD8^+$ T cells differentiate into effector CTLs equipped with effector molecules. During this time period, they also down-regulated the expression of adhesive molecules and chemokine receptors (such as CD62L and CCR7) that retain them in the LN and in the mean time they acquire receptors that allow their traffic towards site of infection or of tumor invasion. For instance $CD8^+$ T cells that are activated within the skin-draining inguinal lymph nodes preferentially up-regulate functional E-selectin and P-selectin ligands as well as CCR4 and/or CCR10, which are associated with homing to skin[228].

CTLs are one of the most effective players of immune system in controlling the threats of intracellular infection and cellular transformation by destroying infected or cancerous cells. This elimination is targeted through contact dependent and independent pathways.

The main axis of CTL cytotoxicity is mediated by perforin/granzyme pathway and to lesser extent the Fas-FasL mediated pathway. These mechanisms are highly specific and require an intimate cell-cell contact.

- **Contact dependent target cell destruction:**
  Granzyme and perforin-mediated cytotoxicity

Lytic granules are specialized late endosome/lysosome, containing multiple proteins:

- Membrane-perturbing proteins: Pore-forming molecule “perforin” and “granulysin”.
- Granule serine proteases: in humans 5 “granzymes” have been identified: granzyme A, B, H, M and K.
- Proteoglycan matrix: serglycin
- Perforin-inhibitor: calreticulin
- Lysosomal enzymes or Cathepsin with roles in granzyme processing (Cathepsin C) and protecting CTLs against perforin (Cathepsin B)
- Stored T effector molecules: Fas Ligand

Upon $CD8^+$ T cell activation, the transcription of the genes encoding perforin and granzymes starts. *De novo* synthesized proteins are non-functional and need to undergo post-translational modifications in order to acquire their biological active form. Once modified, these molecules

are assembled in lytic granules. Lytic granules have a low pH (estimated around 5.5-6), that allows to keep granzymes in an inactive state and contain lysosomal-associated membrane glycoproteins (LAMPs) 1, 2 and 3[229]. These molecules are up-regulated upon $CD8^+$ T cell activation and are associated with exocytosis of lytic granules[230].

The perforin/granzyme dependent pathway is also shared by NK cells and is calcium dependent since perforin needs to be in a calcium-containing milieu to function[186]. Since lytic granules are among the lysosome related organelles[190], they are expressing CD107a and CD63 (LAMP-1 and LAMP-3 respectively); it has been shown that lytic granules upon opening towards the extracellular medium, will expose CD107a molecule on the surface of the T cells, this process is now being widely used as a readout of CTL granule release[159,231].

Perforin:

A clear indication of the role of perforin in anti-cancer response and in the clearance of intracellular pathogens has been shown in mice harboring a mutated form of perforin or perforin knock-out. The mice developed spontaneous lymphomas in paralleled with abolishment of granule-dependent target cell death[232-234]. Moreover, congenital perforin deficiency in fatal hyper inflammatory (FHL) patients, having inherited a mutated form of the perforin gene result in inefficient immune response against intracellular pathogens[188].
Structural studies performed using electron microscopy, have shown that perforin has a marked homology with pore-forming proteins such as the C9 protein of the complement complex[235,236]. Based on such observation, it has been suggested that perforin is capable of making pores (at neutral pH and in the presence of Ca^{+2}) on the surface of the target cells. Further detailed studies of perforin structure and pore formation revealed that perforin-formed pores have an internal diameter of 16-22nm[237-239].

Even though perforin mode of function has been widely investigated, so far there are 3 not fully agreeing models of perforin mediated cytotoxicity that are summarized below (**Figure I-21**):

1) The first model postulates that following Ca^{2+}-mediated interaction of perforin monomers with phosphorylcholine (specific Ca^{2+}-dependent receptor molecule for perforin[240]) on target cell membrane, perforin monomers assembles and produce pores that permits the entry of granzymes into the cytosol of the target cells, leading to initiation of the apoptotic process[241,242].

2) This model was later challenged by the view postulating that perforin and granzymes can be endocytosed within the endosomes in the target cells and it is within such endosomes that perforin monomers polymerization cause endosome lysis permitting granzyme access to cytosol. This hypothesis was also supported by the fact that granzyme B can be endocytosed by the cells in the absence of perforin[243-245]. However, perforin function requires neutral pH and high calcium concentration, given the acidic pH and low Ca^{+2} concentration of endosomal lumen [246], this way of perforin/granzyme uptake is rather unlikely[238,247,248].

3) Finally, there are studies in support of granzyme endocytosis via a process mediated by Mannose-6-phosphate receptors, heparin sulfates or even electrostatic forces. However, these mechanisms are less characterized[249,250].

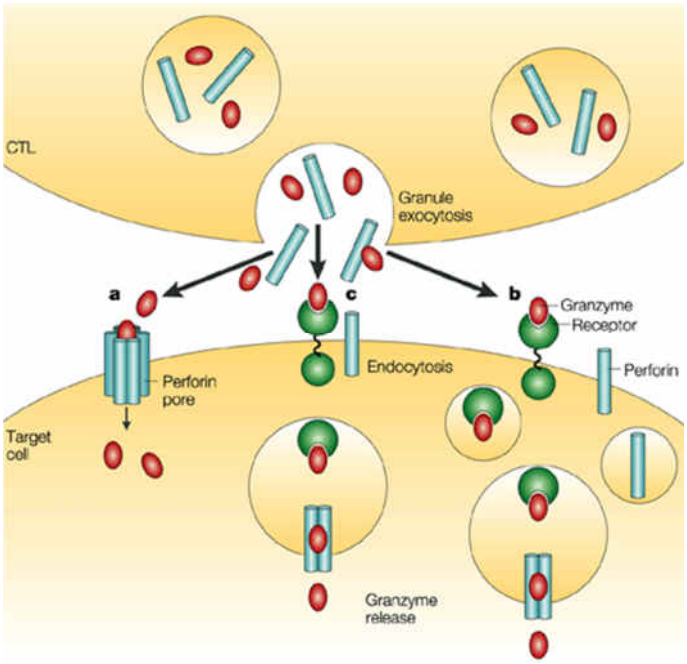


Figure I-21. **Perforin mediated granzyme B penetration.** On interaction of a CTL with a target cell, there is a directed exocytosis of the CTL granules into the extracellular space between the two cells. **a)** The original view was that perforin polymerized to form a pore in the target-cell membrane through which granzymes could pass. **b)** More recently, the discovery of a receptor for granzyme B has indicated that granzymes might be taken up by receptor-mediated endocytosis and that perforin acts to release granzymes that are sequestered in endosomes into the cytosol of the target cell. **c)** In addition, granzymes might bind to the cell surface such that granzyme uptake is stimulated by perforin-mediated damage to the membrane. *Adapted from Bleackley and Barry Nat Rev 2002*[251]

It has been previously described that any physical, chemical and biological damage to the membrane of the cells cause the re-localization of intracellular vesicles towards the damaged zone so that, by donation of a part of their membrane the integrity of the cell is preserved[252 253]. In this context, Keefe et al in 2005 tried to explore more in detail the step by step mechanism of

perforin mediated apoptosis using recombinant and purified mouse perforin and granzyme B. They have shown that indeed polymerized perforin forms small pores on the plasma membrane that initially cause a calcium influx in target cells. This calcium influx is followed up by triggering the membrane repair mechanism based on the recruitment of endosomes that are EEA^+ and $CD107a^-$. Endosomes uptake perforin and granzymes; fusion of such early endosomes results in the formation of gigantosomes that according to the authors is the organelle where perforin pores form channel large enough for granzyme B passage into the cytosol [227,245,254] .

More recently, Lopez JA. *et al* have developed a system by which perforin function can be visualized in real time by using propidium iodide as a read out. In fact, diffusion of solute through a pore is proportional to its concentration and radius, hence at high concentration of between 100 μM-200 μM, PI free diffusion inside the cells can be detected as PI will not only bind to the DNA but also to the cytosolic RNA by forming stable PI/RNA complexes, consequently any early pore formation activity can be followed using time-lapse microscopy. The authors showed that, within 60-80 seconds after $[Ca^{+2}]i$ increase in CTL (a read-out of degranulation), PI entry was detected, starting from the immunological synapse and moving towards to the distal area of the target cell, indicating that perforin acts by forming pores on the membrane. Rapidly after (within 30seconds) membrane repair mechanism are triggered to prevent osmotic lysis[238,247].

Granzymes:

Other important lethal molecules of CTLs' arsenal are granzymes, which are serine proteases implicated in the induction of target cell apoptosis. Once internalized via mechanisms described earlier, granzymes rapidly induce the cascade of apoptosis in target cells[255]. There are several granzymes in humans among them granzyme A and B are more important in CTL effector function.

Granzyme A:

Granzyme A is capable of inducing apoptosis process independently of caspases cascade[256]. This granzyme was the first discovered of its family and is the most widely expressed. Cells treated with granzyme A and perforin die rapidly within minutes, revealing typical apoptotic signs such as membrane blebbing, mitochondrial dysfunction, phosphatidyl serine externalization and nuclear fragmentation[257]. Human granzyme A has however, minimal

cytotoxicity even at high concentrations, reflecting evolutionary development of a customized range of granzymes that can initiate cell death pathways in infected or tumor cells.

Granzyme A functions through various mechanisms such as induction of cellular DNA break, interference with DNA repair mechanism or even by opening up the chromatin through cleaving the linker protein histone H1. Granzyme A function is mainly based on interfering with normal functions of SET complex (a complex associated to the reticulo-endothelial system.) by cleaving it.

Granzyme B:

Granzyme B is the most potent pro-apoptotic granzymes and its unique feature is that it cleaves after aspartic residues, like the caspases. Granzyme B functions through cleavage of Bid (a member of the pro-apoptotic molecules family). The truncated form of the Bid (t-Bid) will induce cytochrome c release from the mitochondria that will stimulate the activation of caspase signaling cascade for DNA fragmentation. Moreover, granzyme B can directly function on caspase-3 and caspase-8, inducing DNA fragmentation (**Figure I-22**)[258-263] .

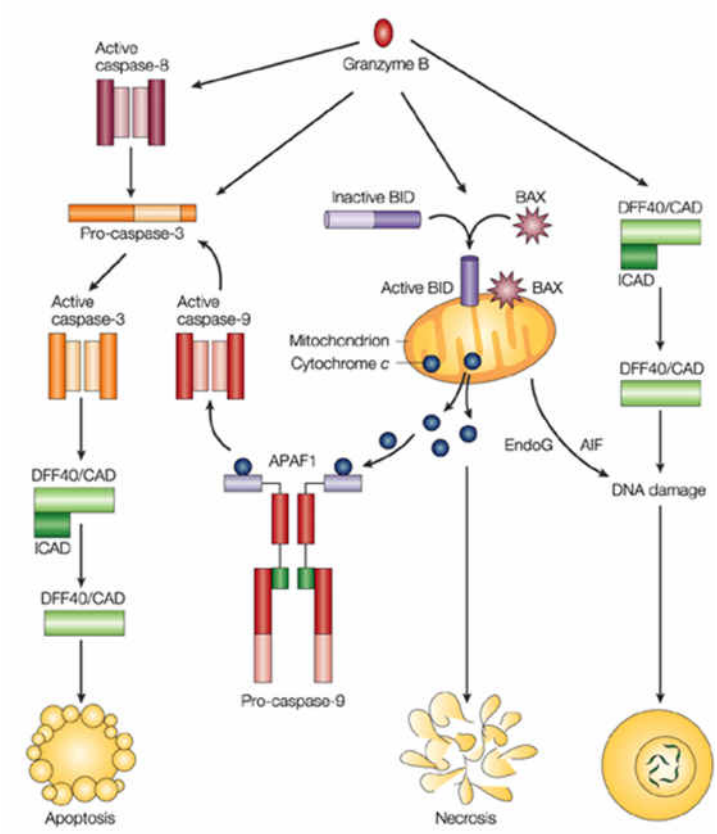


Figure I-22. **Apoptosis induction by Granzyme B.** Once released into the cytoplasm, granzyme B can initiate apoptotic cell death through the direct cleavage of pro-caspase-3 or, indirectly, through caspase-8. In addition, cleavage of BID results in its translocation, with other members of the pro-apoptotic BCL2-family such as BAX, to the mitochondria. This prompts cytochrome c release and the activation of caspase-9 through interaction with the adaptor molecule apoptotic protease-activating factor 1 (APAF1). Alternatively, mitochondrial dysfunction can lead to necrotic death and the release of factors such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which mediate caspase-independent cell death. Finally, studies have shown a direct activation of DFF40/CAD (DNA fragmentation 40/caspase-activated deoxynuclease) — which damages DNA and leads to cell death — by granzyme-B-mediated proteolysis of the inhibitor ICAD. *Adapted from Bleackley and Barry Nat Rev 2002*[251]

There are other types of granzymes in humans called orphan granzymes such as granzyme K, H and M. The mechanisms of function of these granzymes are yet to be understood, for instance granzyme K is a protease that can induce a Bid-dependent apoptosis[264], while granzyme M induce cell death via Ezrins (family of molecules linking proteins to plasma membrane) and a-tubulin cleavage[265]. It's noteworthy that although many of these granzymes are able to induce cell death their delivery via immunological synapse to the target cells hasn't been detected, thus it remained unclear whether other granzymes in addition to granzyme A and B have generic cytotoxic activity.

Once the "apoptotic switch" has been triggered the dying cell undergoes typical morphological transformation, characterized by:

- cell shrinkage and rounding because of the breakdown of the cytoskeleton by caspases
- dense cytoplasm with tightly packed organelles
- chromatin condensation (pyknosis) and fragmentation (karyorrhexis)
- the nucleus breaks into several nucleosomal units due to DNA degradation
- the cell membrane displays " blebbing"
- and finally the cell breaks apart into apoptotic bodies, which are then cleared by phagocytes

Granulysin:

Human cytotoxic granules also contain another effector molecule: granulysin which is a membrane perturbing saponin-like molecule [266]. This protein is expressed 3-5 days after T cell activation coincides with the expression and storage of other effector molecules in naïve T cells. Granulysin is synthesized and secreted by CTLs as a 15kDa protein that is later processed to 9KDa to be stored in lytic granules; the latter is only released during cytotoxic attack[267]. Main activity of granulysin is disruption of bacterial membrane but it has been shown *in vitro* that at high concentration granulysin can have anti-tumor activity[268]. Additional studies demonstrated the chemoattractant effect of secreted granulysin on dendritic cells and the induction in dendritic cells of the production pro-inflammatory cytokines[269]. Similar to granzymes, granulysin requires perforin pore to be delivered into the infected cell cytosol[270 271]).

A second contact-dependent mechanism of CTL to eliminate their cognate target cells involves ligation of death receptors expressed on the surface of target cells.

Death receptor Pathways

CTLS can trigger their target cell apoptosis by ligation and signaling via cell surface tumor necrosis factor (TNF) receptor family members containing cytoplasmic death domains. Among those is the Fas-FasL pathway (CD95-CD95L)[272]. FasL is a death receptor belonging to the super-family of TNF receptors[273]. This molecule is either expressed on the surface of the T cells or is secreted upon cleavage by matrilysine (a member of the zinc-dependent metallo-proteinase family)[274]. Interaction of Fas receptors expressed on the target cell surface with their ligand triggers an apoptosis cascade in target cell[275]. The FasL is expressed minimally on the surface of CTLs in resting conditions, however a few hours after TCR stimulation, FasL is upregulated on CTLs' surface. Initially it has been proposed that FasL is expressed and stored

in CTL and is delivered to target cells upon lytic granule release in parallel to perforin and granzyme, thus inducing cell death. However, as mentioned earlier, basal FasL expression in CTL is low, thus minimizing the effect of this pathway in inducing rapid cell death, since FAS-mediated cytotoxicity requires *de novo* synthesis of the protein. Fas-FasL is not only involved in cell-mediated cytotoxicity but is also involved in T cell homeostasis [276]. Defective Fas-mediated apoptosis could allow prolonged survival of lymphocytes, which could then become targets of transforming events, suggesting a role for Fas-FasL the control leukemia/lymphoma development[276]. Fas-FasL interactions are also implicated in graft versus host disease (GVDH) and autoimmune diseases[277]. Indeed, Fas-FasL-mediated killing would play a role in the elimination of cells chronically exposed to self-antigens, either by fratricide or by cell suicide processes [278,279].

- **Contact independent target cell destruction:**

Following their activation, CTLs secrete cytokines that can also contribute to their cytotoxicity function. In particular, they secrete IFN-γ and TNF-α two cytokines that can have both local and systemic effects.

Role of IFN-γ production

IFN-γ is produced predominantly by NK cells, NKT cells, $CD4^+$ Th1 cells and $CD8^+$ CTL. Defective production and/or secretion of IFN-γ lead to occurrence of tumors, inflammatory-and autoimmune phenomena, indicating its critical role in immunity. IFN-γ can directly inhibit viral replication and has antitumoral, immunostimulatory and immunomodulatory properties. Combined presence of IFN-γ and TNF-α leads to macrophage activation and recruitment to the site of infection. It is also noteworthy that IFN-γ induce MHC class I expression on cells, improving the possibility of target cell recognition by CTLs as well as up-regulation of adhesion and binding molecules required for leukocyte migration, NK cell activity and Th1 differentiation (by up-regulating the transcription factor Tbet)(reviewed in Schoenborn *et al* 2007[280] and Schroder *et al* 2004[281]) .

Role of TNF-α production:

TNF-α is an important regulator of the immune response and is also able to induce apoptotic cell death and inflammation, as well as inhibiting tumorigenesis and viral replication. TNF-α is produced mainly by macrophages, but also by other immune cells, in particular, T lymphocytes. Upon secretion, TNF-α binds to its receptor TNF-R presented on the surface of target cells. This receptor containing the death domain will recruit adapter molecules, Fas-associated death domain (FADD) that serves as a platform for recruiting signaling complexes. FADD recruits the apical caspases 8 and/or 10 to form the death-induced signaling complex (DISC) at the cell membrane. Within the DISC, these caspases are self-cleaved and activated. These activated caspases are then released into the cytosol to cleave the Bcl-2 family member Bid in order to activate mitochondrial damage and also cleave and activate the executioner caspases (3, 6, and 7) that ends up with cell death[236 282]. TNF-induced cell death plays only a minor role[283] compared to its overwhelming functions in the inflammatory process.

3) CTL effector function *in vivo*

Weninger and co-workers using multiphoton microscopy analyzed the behavior of tumor-infiltrating CTLs *in vivo* in an experimental context in which the CTLs efficiently rejected tumor cells, under these conditions, they observed a random migration of tumor-specific CTLs within the tumor microenvironment, and that sustained migration was dependent on specific antigen recognition by CTLs[18]. Following contact with tumor cells some CTLs physically engage long-lasting interactions (lasting longer than 30 min), while others established short-term and sequential contacts. Although reasons for such behavior haven't yet been nailed down, it is likely that lytic granules delivery possibly occurs only during long-lasting interactions.

Similarly, in another study conducted by S.Amigorena and co-workers CTLs accumulated initially in tumor periphery and then infiltrated towards the center[284]. Peripheral CTLs with typically high velocities were arrested on tumor cells as soon as recognizing their cognate antigen presented by tumor cell. CTL migration was observed typically in regions where neighboring tumor cells had been killed, this re-gaining migratory phenotype by CTLs were presumably in search of new tumor cells. The authors also observed that CTL infiltration in the center of the tumor required presence of cognate antigen. This is in agreement with previous studies in melanoma patients in which the authors, by using pMHC tetramers, showed that a substantial fraction of total CD8 T cells infiltrating the tumor was identified to be specific

individual melanoma antigens[285,286]. An additional recent *in vivo* imaging study confirmed that CTLs reach the center of the tumor (and control tumor growth) only when tumor cells express the cognate antigen[287].

P. Bousso's lab further investigated the kinetics of CTL cytotoxicity in vivo using a multiphoton microscopy-based real-time single-cell killing assay[288]. They utilized genetically modified tumor cell lines that expressed CFP and YFP molecules linked by a DEVD motif. Tumor cell apoptosis activates caspase 3, which cleaves DEVD and results in disruption of Forster resonance energy transfer (FRET) between the two fluorescent moieties. FRET can be measures by multiphoton-intravital microscopy (MP-IVM) in real-time and in individual cells. They reported that non-CTL-contacted tumor cells remained alive, whereas stable engagement of nearly all tumor cells with at least one CTL caused their apoptosis. They estimated that on average one CTL requires 6 hours to kill one tumor cell. In fact, CTLs often remained attached to apoptotic tumor cells for several hours; thus on average above 6 h was estimated for a CTL to get ready to attack a second target. In addition to such long procedure of *in vivo* killing, tumor cells killing by human CTLs has been also observed *in vitro* for highly resistant melanoma cells[231]. In addition, it has been reported that CTLs in lymph nodes can kill antigen-pulsed target cells and subsequently detach from them in only 15 min, and typically in <1 h [32]. The relatively low pace at which CTLs kill tumor cells may be influenced by several factors such as evolution of self defense mechanism, Treg inhibitory cytokines, etc that will be discussed in page 74-75 (tumor escape mechanism).

It is now known that CTLs are capable of killing multiple targets[289-291], either simultaneously or serially by bouncing from one target cell to another[158]. Although the underlying mechanism of such serial cytotoxicity is not yet elucidated, it has been proposed that target cell annihilation by CTLs may require exocytosis of small quanta of lytic molecules[144,156]. In parallel another series of experiment from Griffiths's lab proposed that *de novo* synthesis of lytic molecules is an event stimulated by specific TCR/pMHC engagement that will either refill the lytic granules or will be secreted directly via constitutive secretory pathway thus arming CTL for several rounds of cytotoxicity[194].

In addition, several studies currently revealed that both at polyclonal and intraclonal levels CTLs respond in a heterogeneous manner in terms of expansion and differentiation [292]. In line with these findings Vasconcelos *et al* recently established an assay to measure kinetics of target cell annihilation by individual human CTLs belonging to the same T cell clone specific for

single antigenic peptide. Using this experimental set up they described a heterogeneous killing capacity among individual cells belonging to the same T cell clones based on the onset of killing during sustained CTL target cell interaction of super-killer CTL killing target cells at high rate[293].

Cancer Biology and Tumor Immunology

1) Cancer development

Cancer is a complex process based on malignant transformation of a single cell that is initiated by a wide variety of events, mainly mutagenic such as ultraviolet (UV) or γ-irradiation or DNA intercalating agents. In other words, cancer is a multi step process of fatal deregulation of various cellular processes such as DNA repair, cell survival, proliferation and motility. These deregulations are often caused by accumulation of various genetic and epigenetic mutations. During oncogenesis, controlled cell proliferation is disrupted due to accumulation of mutations that results in formation of a tumor mass from which tumor cells can disseminate to form metastasis at distant sites in the body. The metastatic state is often fatal for the patient because it targets many organs of the body and complicates treatment strategies.

Tumorigenic mutations are typically sub-classified into driver and passenger mutations. Driver genes are mainly involved in cell fate, cell survival and genome maintenance processes, and impairment of any gene of this type cause loss of growth control. On the other hand, passenger mutations do not intrinsically confer cell proliferation advantages. Whole genome sequencing of the DNA of cancer patients estimate that a typical tumor contains 2-8 driver mutations accompanied by approximately 30-60 passenger mutations. This finding points out the complexity of malignant transformation. It has been shown that melanoma, is an extreme case in this context harboring approximately 200 mutations per tumor. It's noteworthy that every tumor accumulates unique mutations patterns and consequently will acquire distinctive immunogenicity[294].

Many tumorigenic mutations concern so-called oncogenes and tumor-suppressor genes. During tumorigenesis chronic activation of oncogenes and deactivation of tumor suppressors induce cancerous process in the given cell. Some prototypical oncogenes are myc, Her2/neu (human epidermal growth factor receptor 2) and Ras whereas typical tumor-suppressor genes are: pRb (retinoblastoma protein), p53 or PTEN (phosphatase and tensin homolog). As mentioned earlier, although tumors can comprise independent identities, many tumor entities share similar mutation patterns providing them with similar features which led to the classification of the hallmarks of cancer. For instance, mutation of a certain protein at various sites of several cancers indicates its importance in tumorigenesis.

Genetic instability acquisition by neoplastic cells grants ultimate tumor cell abnormal growth and disseminating behavior. D.Hanahan and RA.Weiberg in 2000[295] proposed an organizing

principle codifying the six traits that all cancers have in common, which was revised in 2011 by addition of emerging hallmarks[295,296].

- **Hallmarks of cancer**

1) Enabling replicative immortality

One characteristic of tumor cells is their immortalized state. In healthy cells, after many rounds of cell division, telomeres of the chromosomes get overly truncated and aggregate. This will cause cell death. Contrariwise, tumor cells continue to express a functional telomerase that constantly adds nucleotide repeats to the end of the chromosomes in tumor cells, avoiding cell death[297-299].

2) Activating invasion and metastases

Upon acquisition of certain mutation, primary tumors often initiate a sequence of certain steps, the invasion-metastasis cascade, leading to the dissemination of single tumor cells. These are transported via lymphatic or blood vessels to distant organs which they colonize. This process involves the de-regulation of adhesion molecules such as E-cadherin but also tumor-infiltrating bone-marrow derived cells like macrophages that can supply matrix-degrading enzymes can facilitate metastasis[300,301].

3) Inducing angiogenesis

Cancer cells are metabolically highly active needing proper nutrition with various metabolites as well as a well established gas exchange. Sustaining tumor growth requires establishment of an extensive blood vessel system. Generation of such blood vessels is called angiogenesis or neo-angiogenesis. In tumors the angiogenic switch is mainly triggered by vascular endothelial growth factor (VEGF)[301].

4) Resisting cell death

In healthy cells a sustained proliferative signaling will induce cell death. In order to keep malignancy, tumor cells often up-regulate or activate key elements of cell death signaling process. This is often achieved via: loss of p53[302,303], over-expression of anti-apoptotic factors such as B-cell leukaemia/lymphoma 2 (Bcl-2)[304] or down-regulation of pro-apoptotic factors such as Bax or Bim.

5) Sustaining proliferative signaling

In the process of oncogenic transformation, tumor cells constantly require receiving growth and/or survival signals. This is conveyed in large by autocrine stimulation with growth factors

or constant signaling of growth factor receptors (independent of ligand)[305]. One example is B-Raf mutation yielding a constitutively active version of the protein that can be detected in around 40% of melanoma samples[304].

6) Evading growth suppressors

In line with the sustained proliferative signaling, tumor cells should evade growth suppressing signaling. As genetic mutations are accumulating, control proteins (which can drive cells into senescence or apoptosis when DNA damage is overwhelming) need to be kept in check. Accordingly, p53 is one of the most frequently mutated control proteins in cancer showing point mutations in about 50% of all sequenced tumors[295,303].

Although during tumorigenesis the acquisition of these six important hallmarks is crucial, individual cancer cell may acquire them in varying orders or with different timings using distinct mechanisms. In addition, an important concept of tumorigenesis is the enabling characteristics that do not necessarily cause cancer but assist cells in a transition from normal to oncogenic state (see below).

- **Enabling characteristics**

1) Genome instability and mutation

Genetic instability enables cells or cancer cells to acquire mutations in a random fashion. The tumor promoting mutations are selected and support uncontrolled tumor proliferation. This selection gives rise to the most successful or tumorigenic sub-clones that dominate in the local tissue environment. To enhance the probability of clonal expansion and survival, cancer cells normally increase the rate of their mutations by either increased sensitivity towards mutagenic agents or by circumventing cell surveillance systems monitoring genomic integrity. These mutations may include protein coding regions of the genome or may affect the expression of regulatory elements.

2) Tumor-promoting inflammation

Various immune cell infiltrate in cancer microenvironment at different densities has been shown in many types of cancer. However, the effect of immune cell presense on tumor progression is a controversial issue.

In addition to the fact that many of these immune cells can help tumor evasion, tumor-associated inflammation may enhance tumorigenesis and tumor progression, helping incipient

neoplasias to acquire hallmark capabilities. In this context, inflammation can supply bioactive molecules to the tumor microenvironment such as growth, survival and pro-angiogenic factors. It can also modify the extracellular matrix (ECM) that may facilitate angiogenesis [306,307], invasion, and metastasis. Finally inflammation can release chemicals like reactive oxygen species (ROS) that are actively mutagenic for nearby cancer cells, inflammation has been shown to be able to promote tumorigenesis[308].

More recently thank to the immense research efforts in oncology another concept has been put forth that takes in consideration the influence of two new emerging hallmarks in tumor development and progression.

- **Emerging hallmarks:**

1) Deregulating cellular energetic

Tumor cells require many nutrients to fuel their excessive growth. Yet, the tumor microenvironment is often hypoxic, thus disabling the usage of the citric acid cycle that critically depends on normal oxygenation. O.Warburg first described this phenomenon in 1930s. Since then it became increasingly clear that hypoxia via Ras signaling can independently increase the hypoxia-inducible factor 1 alpha (HIF1α) which subsequently up-regulates glycolysis. Together with the over-expression of glucose transporters like the glucose transporter-1 (GLUT-1) this process provide enough energy to drive the uncontrolled proliferation of tumor cells in hypoxic tissues [309,310].

2) Avoiding immune destruction

It has been shown successfully in models of adoptive transfer and/or lymphocyte depletion that among other actors of the immune response, cytotoxic T lymphocytes (CTL) and NK cells can control tumor growth *in vivo* [311]. In line with this observation it has been shown that immune-suppressed individuals, for example organ recipients, also display a higher risk for developing cancer. Also, several studies reported that usually the level of infiltration of tumor tissue by T and/or NK cells correlated with better prognosis[312,313]. These data led to the addition of the hallmark “immune evasion” to the existing list of tumor progression arms.

2) Melanoma a prototype immunogenic solid tumor

- **Melanoma development and risk factors**

Melanocytes, originally derived from the neural crest, are situated just above the basal membrane between dermis and epidermis[314]. They are responsible for pigmentation in the skin. Common acquired melanocytic nevi are neoplasms arising mostly during the first 3 decades of life, whereas congenital nevi are present from birth[315]. Melanoma is the malignant transformation of melanocytic cells that express a large range of tumor associated antigens (TAA) and is considered a highly immunogenic tumor[316]. Morphological studies indicate that cutaneous melanomas are commonly pigmented and are recognized clinically as asymmetric lesions with irregular borders and variegated pigmentation as well as by the size and evolution over time of the lesion[314].

Four stages of melanoma have been described based on the spreading of the tumor to regional lymph nodes or distant site. At early stages of the disease melanoma cell proliferation takes place at its location which is between dermis and epidermis, while at later stages melanoma cell uncontrolled proliferation establish a radial growth and by reaching the blood vessels or lymphatics malignant cells start to metastasize throughout the body. This stage is termed stage IV of the disease (**Figure I-23**). Lung and liver are the common sites of melanoma metastasis. In fact, when compared to other skin cancers, melanoma has a high tendency of metastasis, even from small primary tumors.

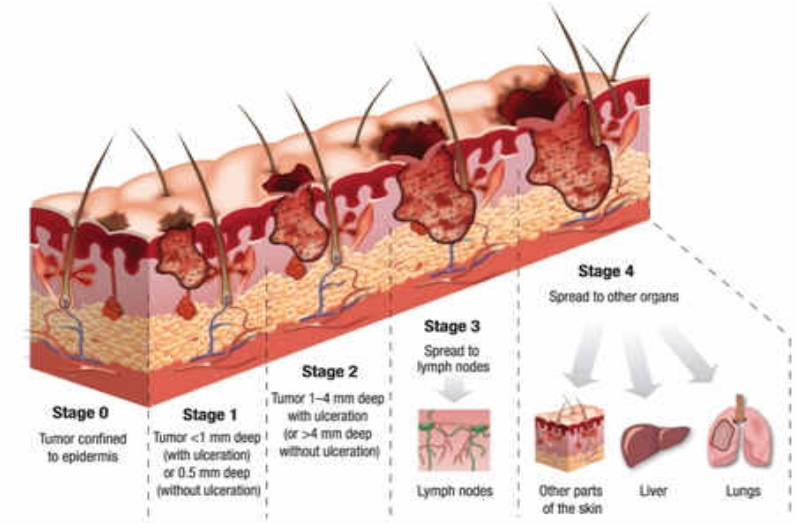


Figure I-23. **Progression of melanocytes to malignant melanoma.** There are 4 distinct states of melanocyte progression where each melanocyte clone gains advantage over surrounding tissue. Early benign nevus becomes dysplastic with morphologically atypical melanocytes. This is followed by a radial-growth-phase and a secondary vertical growth phase which is the first stage to have malignant potential. In the final

stage of malignant melanoma the tumor cells have infiltrated vascular and lymphatic systems and spread to distant organs. *Adapted from Gray-Schopfer et al, Nature 2007*[314]

Although melanoma is representing approximately 2% of skin cancer, the incidence of melanoma is increasing comparing to other common cancers, particularly in young generations [317]. Historically melanoma more commonly affected women than men, but the gender imbalance has recently leveled and melanoma is now the 6th most common cancer diagnosed for each sex. In Europe the highest cancer rate is found in France with 385 men and 267.7 women per 100,000 inhabitants diagnosed in 2012 (WCRF website, accessed 25/09/2015). Chance of melanoma risk, slightly increases in cases with a family history of melanoma incidence, although familiarity contributes to less than 10% of reported cases (Olsen et al., 2010). The most common familial syndrome is familial atypical multiple mole-melanoma syndrome (FAMM), other syndroms such as xeroderma pigmentosum and albinism can also lead to melanoma[318,319].

Although early-stage melanomas are commonly cured by surgical excision, locally advanced or metastatic melanoma has a dismal prognosis. The survival of patients with melanoma varies from 97% at five years for patients with small, easily resected tumors, through to 33% at one year for patients with the most advanced metastatic disease[320].

Furthermore, although the lifetime risk of developing cutaneous malignant melanoma (when compared to other tumors) is comparably low, melanoma incidence rate increases faster than for any other malignancy.

As already introduced, melanoma cells harbor usually more mutations than other cancer entities. This is due to the fact that melanocytes are frequently exposed to mutating UV irradiation which is part of the spectrum of sunlight. In fact, prominent mutations in melanoma samples are among critical genes controlling a range of important cellular processes that results in the proliferation and dissemination of melanoma throughout the body and are directly in line with issues that were discussed under “cancer development” chapter. As mentioned above the proto-oncogene B-Raf is mutated in about 40% of melanoma samples but also N-Ras, Bcl-2, PTEN or nuclear factor-κB (NF-κB) frequently exhibit mutations providing progenitors of melanoma cells with growth advantages leading to oncogenic transformation.

One of the reasons why, despite increasing incidence the melanoma-related death rate is virtually stable is that it is more often detected in its early stages where it can be removed with surgery. However, as melanocytes are derived from highly motile neural-crest progenitors they

are very prone of initiating metastatic spread either locally to lymph nodes or distally to other organs (preferentially lung[321], liver or brain[322,323]). Upon metastasis, surgical excision becomes problematic and survival rates are dramatically reduced, with a median life expectancy of 6 months and only 5% of patients surviving beyond 5 years. Also, melanoma cells are often refractory to most existing treatment strategies and can exhibit a pronounced apoptosis-resistance *in vitro* and *in vivo*. Thus, novel strategies targeting melanoma are desperately needed.

The high mortality rate of melanoma is essentially due to the lack of effective treatments, particularly for late stage or disseminated disease. For several decades researchers and clinicians have trialed several immunotherapeutic approaches in the treatment of melanoma, but without ever reliably improving on the existing standard of care except than during the last few years in which novel therapeutic strategies are under development. Improvements to existing therapies or the development of novel drug strategies are required in order to increase overall survival in patients with metastatic melanoma.

- **Vesicular trafficking in melanocytes**

Melanocytes contain two kinds of lysosome-related organelles: lysosomes (similar to those contained in many cells) and melanosomes (melanocytes specific organelles). These compartments have many common features such as presence of both soluble and transmembrane lysosomal proteins, an acidic luminal environment, accessibility by endocytic tracers, and the ability to fuse with phagosomes [324]. It has been suggested that melanosomes are specialized lysosomes[325]. Melanosomes and lysosomes provide different contribution to the cell homeostasis; melanosomes are involved in pigmentation and lysosomes are involved in degradation, therefore each compartment is equipped with specific protein sorting and transport systems while sharing some key elements.

A melanosome biogenesis model proposes that the membrane of melanosomes originates from smooth ER, and that this compartment receives melanin-synthesizing enzymes from the TGN-derived vesicles[326]. Immature melanosomes gradually mature into heavily melanized melanosomes (stage I-IV) and are finally transferred to adjacent cells, keratinocytes, by potentially one of the following mechanisms: cytophagocytosis, membrane fusion or shedding-phagocytosis or exo/endocytosis[327].

Melanosomes (vesicular) trafficking in melanocytes is accomplished by a combination activity of two cytoskeletal components: microtubules and actin filaments[328-330], before anchoring to the plasma membrane. These vesicles adhere to microtubules via kinesin or dynein molecules which move the melanosomes in an anterograde/retro grade direction respectively[331,332]. These motor proteins consume ATP to 'walk' along the microtubules [333] and are composed of globular domain with two heavy chain subunits for attaching to the microtubules, as well as several light chains for holding the vesicles[332]. Kinesin molecules perform the transfer of vesicles from perinuclear area to cell periphery where melanosomes are taken by actin filaments for vesicular distribution[334]. Actin filaments capture vesicles through a complex of proteins composing of several SNARE machinery and Rab-GTPase proteins. SNARE machinery composed of VAMP7 on vesicles and syntaxin-3 and SNAP23 on plasma membrane regulates this trafficking.

Several combinations of such vesicular trafficking molecules have been proposed to perform in melanocyte. For instance: a Rab27A–Slac2-a/melanophilin–myosinVa complex in actin-dependent melanosome transport[328], and a melanoregulin (or Rab36)–RILP–dynein motor complex in microtubule-dependent retrograde melanosome transport[329,330].

More recently, Yatsu and his colleagues[335] proposed that syntaxin-3 is a mediator of fusion between TRP-1 (tyrosinase-related protein 1)-containing vesicles and their target organelles by forming a complex with VAMP7 and SNAP23. In this manner, when the TRP-1-containing vesicles reach their target organelle, i.e., stage II/III melanosomes, VAMP7 is released from Varp (VPS9-ankyrin-repeat protein, a Rab32/38 effector) by an unknown mechanism, and a SNARE complex is formed between VAMP7 (on the TRP-1-containing vesicle) and two t-SNAREs, syntaxin-3 and SNAP23 (on the melanosome), leading to fusion between the two membranes. As a result, TRP-1 protein is transported to the melanosome, thereby enabling successful melanin synthesis (i.e., the formation of a stage IV melanosome).

Moreover, VAMP7 has at least two functions in melanocytes: regulation of LAMP-1 transport independently of syntaxin-3 and SNAP23, and regulation of TRP-1 transport in concert with syntaxin-3 and SNAP23[335,336]. In addition it has been shown that Syntaxin-13 regulates melanosomal cargo transport and biogenesis through its cycling between recycling endosomal domains and melanosomes in a mutual regulation with VAMP7 [337].

Rab27a provides the direct linkage between melanosomes and the transport complex (VAMP7/Syt3/SNAP23), and is independent of melanophilin and myosinVa [338]. Melanophilin is recruited to the complex by Rab27a-GTP, and binds to Rab27a by its N terminal domain and to actin via its C terminus. Interaction between melanophilin and myosinVa is via a coiled coil domain[339]. MyosinVa co-localizes with actin and is thought to be crucial for capturing the vesicles at the cell periphery[327,334].

These evidences suggest that both melanosomes and lysosomes utilize the same mechanism for membrane transport; however, there are specific features in melanosomes, melanin-synthesizing enzymes, and matrix filaments that can help us distinguish these two compartments.

More recently, it has been revealed by Immunofluorescence analysis that TRP--1 does not co-localize with lysosomal membrane proteins CD107a and LGP85 in melanocytes. In the same article the authors proposed a late sorting mechanism based on AP-3 complex function for targeting specific membrane proteins into melanosomes and lysosomes[340] (**Figure I-24**).

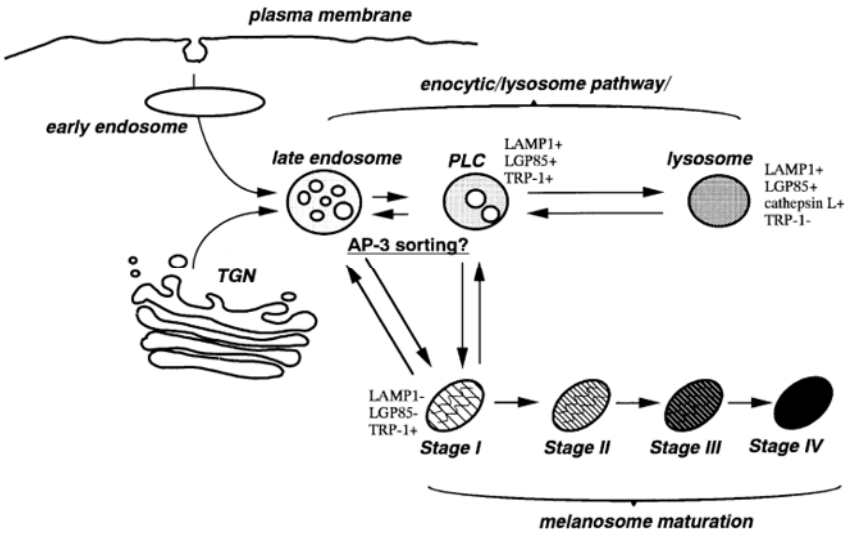


Figure I-24. **Model of the membrane traffic to melanosomes and lysosomes in melanocytes.** In biosynthetic pathways, the traffic route of melanosome membrane protein (tyrosinase-related protein 1: TRP-1) partially overlaps but is distinct from that of the endo/lysosomal one (LAMP1, LGP85). The melanosome pathway is separated from the lysosome one after the AP-3-dependent sorting steps, probably at late endosomes and/or PLC (prelysosmal compartment). Melanosomes gradually maturate into heavily melanised melanosomes (from stage I to stage II–IV) with the accumulation of melanin inside the membrane. In the presence of wortmannin, there is membrane retrieval from the lysosomes or immature melanosomes, but not from the heavily melanised melanosomes. The melanosome pathway is also segregated from the endocytic pathway (TR-dextran). *Adapted from Fujita H 2001*[340]

Table1. **Characteristic differences between lysosome related organelles (LRO)**

Characteristic	Lysosome	Melanosome	Lytic granule
Cell type	Most	Melanocytes	CTL, NK
Luminal pH	4.5–5.0	<5	5.1–5.4
Lysosome hydrolases	+	+	+
CD107a/LAMP-1	+	–	+
LAMP-2	+	+	+
CD63/LAMP-3	+	+	+
MPRs	–	–	–
Accessibility to endocytic tracers	+	+	+
TRP--1	-	+	-

3) Tumor /Immune system interface

- **Development of an anticancer immune response**

Besides fighting pathogenic invaders, the immune system also evolved to recognize altered self as target structures and to mount an immune reaction against transformed cells. However, the immune system effector cells and molecules are not always exerting beneficial effects, rendering tumor immunity a double-edged sword.

Paul Ehrlich has first predicted the concept of tumor suppression by immune cells more than 100 years ago. He was followed by F.M. Burnet who believed developing cancer would be eliminated by an effective immunologic reaction provoked via tumor cell-specific neo-antigens. Alternatively, L.Thomas theorized that there should be a barrier of protection against neoplastic diseases similar to that mediating homograft rejection. The results from studies in inbred mouse models strongly suggested the existence of tumor associated antigens and formed the renaissance of modern tumor immunology introducing “cancer immune surveillance hypothesis” which stated that thymus-dependent cells of the body constantly surveyed host tissues for recently transformed cells.

Yet, the similarity of tumor cells to healthy cells made the advancement of tumor immunology complicated, since danger signals like bacterial LPS for instance were recognized to be clearly missing in a tumor context. Also data were differing in various groups. The very first clear-cut result in favor of anti-tumor immune surveillance was achieved in the 1990s when IFN-$\gamma^{-/-}$ or IFN-γ receptor (IFN-γR)$^{-/-}$ mice were shown to exhibit defective tumor rejection compared to wild type (wt) mice upon carcinogen challenge or injection of tumor cell lines. Several important findings on this topic comprehensively reviewed by Vesely *et al.* and ultimately led to the general appreciation of tumor immune-surveillance[341]. More importantly, clinical results also support immune surveillance hypothesis. Immune-suppressed patients show higher incidence of tumors (both spontaneous tumors or virally induced). Moreover abundant lymphocytes infiltrate in some tumors correlates with a longer survival in cancer patients[342,343]. The most comprehensive of these studies were in colorectal cancer[341].

Moreover, several lines of evidence indicate that, although tumors at least induce transient immune responses, cancer still develops[344]. As discussed earlier, immune mediated recognition of tumors not only enhance tumor eradication but also cause a selective pressure favoring growth of less immunogenic tumor clones, that point out a continuous "sculpturing" of tumor phenotype by immune system in its environment. This comprehensive concept of immune surveillance was revised later by Schreiber and his colleagues and termed "immunoediting". The concept postulates three phases that describe tumor evolution in light of its interaction with the immune system: elimination, equilibrium and escape.

Elimination

Elimination phase refers to successful eradication of developing tumors who had escaped from intrinsic tumor suppressor mechanisms. Ideally, in this phase, both innate and adaptive immunity are involved. In this context, cells of the 1st immunity response wave including: Natural killer cells (NK), Natural killer T cells (NKT) and γδ T cells are alerted by new source of local "danger" such as pro-inflammatory cytokines produced by growing tumor cells, activated macrophages or the surrounding stromal cells. This cascade is followed by a positive feedback loop of an amplified cytokine production and immune cell recruitment that will produce additional pro-inflammatory cytokines, namely: IFN-γ and IL-12. NK cell tumor cytotoxicity through perforin, Fas-L and tumor necrosis factor receptor (TNFR) causes antigen release that will set off the adaptive immune system response. In fact, high levels of IFN-γ also exert direct anti-proliferative, anti-angiogenic and pro-apoptotic functions on tumor cells.

Hereby, NK cells will bridge innate and adoptive immunity through activation of dentritic cells (either in a contact dependent manner or indirectly by IFN-γ production). As mentioned earlier, the tumor microenvironment is also enriched of HSP and apoptotic cell fragments that harbor excessive amounts of tumor antigens. Activation of DCs triggers their maturation and migration towards the draining lymph nodes where they trigger adoptive immunity. This response ideally involves $CD4^+$ Th cell induction that subsequently supports the activation of cytotoxic $CD8^+$ CTLs via dentritic cell exerted cross-presentation of tumor antigens. $CD8^+$ CTLs and $CD4^+$ Th following activation and clonal expansion will infiltrate the tumor site. Accordingly, $CD8^+$ CTLs detect specifically antigen-expressing tumor cells and respond with cell cytotoxicity and/or IFN- γ secretion that in turn can activate macrophages, DCs, NK cells. In this context, $CD4^+$ Th produce IL-2 that, together with host cell production of IL-15, helps to maintain the function and viability of the tumor-specific $CD8^+$ T cells.

Equilibrium

Tumor elimination phase is not always fully successful in complete tumor clearance, in such cases the tumor cells and the effector cells of the immune system enter into a dynamic equilibrium, wherein the immune functions against the tumor cells are sufficient to avoid the tumor out-growth, but not sufficient for tumor annihilation, in other words tumor destruction and growth are matched. This conserved tumor bed gives rise to many genetically unstable and mutative emerging tumor cells. During the equilibrium phase, the immune-mediated selective pressure favors generation (sculpts) of a new population of tumor clones with reduced immunogenicity, compared to the parental population. Main factors for survival of such cancerous cells from the elimination phase can derive from the heterogeneity and high genetic instability of these cells that enable them to resist or escape the host's immune responses.

The various effector molecules of the immune system have different degree of influence in imposing the selection pressure. In fact, Koebel has identified a subset of animals treated with low dose of 3'-methylcholanthrene (MCA) that develop tumors, which do not progress. These tumors remain stable for months but the transient suppression of the adaptive immune system (by antibody-mediated depletion of IFN- γ and T cells) induces rapid tumor growth, indicating that these stable tumors were held in check by adaptive immunity [345]. This renders the equilibrium phase distinct from elimination phase that is based on both innate and adaptive immunity.

Equilibrium is believed to be the longest phase in the process of immunoediting. It has been estimated that it can take up to 20 years from the first contact with a carcinogen to clinically detected manifestation of malignant disease during which, most of the time, the tumor exists in an occult state in equilibrium phase. Solid organ transplantation has caused tumor transmission from a donor treated for melanoma 16 years earlier and considered tumor free. Indeed the pharmacologic suppression of the immune systems of transplant recipients facilitated the rapid and progressive outgrowth of occult tumors that had previously been maintained in the equilibrium phase by the donor's competent immune system.

A recent controversial work in a murine model of spontaneous melanoma, tumor cells were found to disseminate early in the development of the primary tumor and remain dormant depending on the tissue were they were localized. Dormant cells from lung showed low proliferation rate in comparison to primary tumors, which was partly mediated by cytostatic $CD8^+$ T cells. Therefore, immune strategies that favor the dormancy of disseminated cells are proposed to control the development of metastasis [346].

The end result of the equilibrium phase is variants that are more aggressive compared to the original tumor cells as they are less immunogenic and eventually cannot be killed by immune cells anymore and thus grow out leading to malignant disease.

Escape

When tumor cells are equipped with mutations that protect them from immune system they go towards the last step in the immunoediting process which is termed: escape phase. This escape from immune recognition is mainly achieved through three mechanisms: first, lack of recognition that can be due to loss or alteration of molecules important in recognition such as loss of MHC molecules, second, an alteration of activation processes and third, induction of immune dysfunction.

- **Mechanisms of tumor escape**

In response to selective pressure of immune system, tumors developed various strategies to hide from immune cell elimination process. One strategy shared by many tumor cells is down-regulation or loss of MHC class I expression or of molecules that are involved in antigen processing and presentation such as TAP or low molecular-mass proteins (LMPs). Loss of MHC I in melanoma have been associated with disease progression[347,348]. Melanoma cells are also able to express MHC II, but the costimulating molecules CD80 and CD86 are often absent,

for this reason they induce tolerogenic responses[349]. An additional escape strategy is the ectopic expression of HLA-G, a nonclassic MHC-I molecule, that can protect melanoma cells against natural killer (NK)-cells[350].

Moreover, to avoid CTL cytotoxicity tumor cells[351] can over-express anti-apoptotic molecules such as (Bcl-2) and perforin/granzymes inhibitors (PI-9)[352,353]. Down-regulation or epigenetic changes of death receptors and caspases [354] or over-expression of FLIP (caspase-8 like inhibitory protein)[355]are also linked to reduced efficacy of CTL-mediated tumor eradication.

In addition, many tumors, in particular melanoma can skew the immune system towards a more immunosuppressed state that protects them from immune response. Tumors can produce elements that prohibit maturation, differentiation and function of APC and effector cells. IDO (Indoleamine 2,3-dioxygenase) expression in melanoma blocks proliferation of $CD8^+$ cells and promotes apoptosis of $CD4^+$ cells at the tumor site [356]. CD95L expression and secretion by melanoma cells is proposed to induce apoptosis in CD95 expressing tumor-specific T cells[357] [358] [359], although this expression and its function has been questioned by other studies [360,361].

Tumors are also able to recruit and stimulate Tregs under the influence of certain, yet unknown factors that exert inhibitory effect on CTL cytotoxicity[362] [363]. Recently, it has been proposed that expression and secretion of the antioxidant protein thioredoxin in melanoma increases recruitment and function of Tregs[364]. Moreover, in tumor-microenvironment presence of IL-6, IL-10, TGFβ, NOS2 (nitric oxide synthase 2) not only induces the generation of tolerogenic APCs but also causes immune-suppressive effects. Inflammatory-cytokines can induce over-expression of immune-modulatory molecules such as PD-L1 and PD-L2 that can diminish the antigen-receptor signaling by binding to the receptors on T and B lymphocytes[365,366]. Such pattern of expression of PD-L1 has recently been linked to a worse prognosis in melanoma[366] [367].

4) Anti tumor immunotherapy for melanoma patients

Assessment of changes in tumor burden is fundamental for clinical evaluation of cancer therapeutics and both tumor shrinkage (objective response) and disease progression are important endpoints in clinical trials. Since the introduction of response evaluation criteria in solid tumors (RECIST) in 2000 and the revised RECIST 1 in 2009, many investigators, industry and government authorities have adopted these criteria in assessment of treatment outcomes. RECIST is based on radiological measurements of lesion size and can be summarized as: complete response (CR) – disappearance of all tumor foci; partial response (PR) – a reduction of at least 30 % in tumor diameters; stable disease (SD) – neither partial

response nor progressive disease; and progressive disease (PD) - at least a 20% increase in the sum of all tumor dimensions from the smallest tumor size or the apparence of a new tumor lesion. The term objective response refers to either CR or PR.

- **Surgery and chemotherapy**

Melanoma that is confined to the skin or lymph nodes corresponds to the early detected disease and is primarily treated by surgery. Some of these patients, particularly those with thin and nonulcerated lesions are cured by surgery only. Melanoma is notoriously resistant to chemotherapy and single-agent chemotherapy results in limited (< 25%) response rates[368,369].

Dacarbazine is the only chemotherapeutic drug approved by the US food and drug administration for treatment of melanoma. It induces produced response rates between 7 and 25 %, with median duration of 5 to 6 months and complete responses in < 5% of the patients. In order to improve response rates, combinatorial chemotherapies have been tested in clinical studies. The most effective multi agent combinations have included dacarbazine and other classes of chemotherapy agents (platinum compounds or alkaloids) and have been associated with 14 -37 % response rates. The most effective multidrug studies have included cisplatin, vinblastine and dacarbazine and have produced response rates of 40% with median duration of 9 months and a median survival of 12 months[370-372].

I have described above the interplay between Immune cells and, more importantly CTLs, with tumor cells. During the last decade many advances have been made in the understanding of cancer immunology that helped to make progresses in the amelioration of immune responses in cancer either by direct targeting of immune cells or by utilization of immune-modulatory drugs. All in all, these findings helped to build up the very promising field of immunotherapy of cancer that aims at stimulation, restoring, management and even completion of patient's own immune system for tumor control and annihilation. In this section I will introduce different relevant avenues of experimental anti-tumor immunotherapy with a focus on melanoma immunotherapy.

- **Cytokines**

Interleukin (IL-2)

IL-2 has been shown to have anti-tumor activity both *in vitro* and *in vivo*[373 374]. Treatment of metastatic melanoma patients with high doses of IL-2 as single agent demonstrated 7%

complete remission (CR) and 10% partial remission (PR)[375]. 8 year follow up of 270 patients under such treatment supported moderate beneficial outcome of IL-2 treatment as patient's CR median duration was estimated 59 months[370]. However, limited results in parallel with toxic side effects of high dose IL-2, restricts its general applicability. Combination of this treatment with chemotherapy had no statically significant response improvement [376,377].

Interferon (IFN)

IFNs are pleiotropic cytokines that induce synthesis of many proteins via activating the JAK-STAT pathway (cellular signaling pathway coupled to cytokine receptor). There are two types of IFNs based on their recognizing receptors. Type I, that binding to IFN-α receptors contains IFN-α, β and ω while type II includes IFN-γ and binds to IFN-γ receptor. Whereas Different IFNs may recognize different receptors and activate different pathways; they have some overlapping effects, particularly in their anti-proliferatory activity[378].

IFN treatment has been thoroughly evaluated and it is now the only approved adjuvant therapy for melanoma patients (at stage II/III of the disease) since it can significantly improve disease free survival and, in some cases, overall survival[320]. However IFN usage associates with a lot of side effects (including autoimmunity and thyroid dysfunction) and must be monitored carefully [379].

Yet, in addition to the systemic application of IL-2 and IFN-α local application of IL-2 and TNF-α have been reported to induce beneficial effects for patients with soft-tissue sarcomas or melanoma[380].

- **Vaccine-based strategies**

Several clinical cancer vaccination trials have been conducted, in particular with irradiated whole-tumor cells mixed with bacterial adjuvants that showed limited but significant effects in melanoma treatment [381,382].

More recently, with comprehensive molecular understanding of melanoma progression and immune recognition, the attention has turned towards specific melanoma antigens. The role of a variety of tumor-associated antigens (differentiation, cancer testis and viral antigens) in enhancement of anti-tumor responses has been investigated both in animal models and in clinical trials.

These antigen-based vaccines are divided into four categories:

1) Plasmid DNA-based vaccines, delivering the gene encoding the antigen

2) Recombinant viral and bacterial vaccines
3) Peptide or protein based vaccines, delivering antigen mixed with adjuvants
4) Antigen-loaded DC vaccines

DNA vaccines have the advantage of expressing the antigen over time as the expression relies on the cell ability to transcribe the gene from the delivered episomes. One example of this kind is Allovectin-7 that is designed to express allogeneic MHC class I antigen upon intralesional administration. However, this vaccine failed in providing encouraging results in phase III of clinical trials[383]. Viral vaccines are currently under investigation but they appear to be difficult to be employed in clinical use due to the generation of neutralizing antibodies. Peptide or protein based vaccines are designed to have a better binding to MHC molecules or to induce an enhanced T cell response[384]. A mixed newly emerging vaccine is MelQbG10. It contains an immunogenic virus-like nanoparticle to which the melanoma tumor antigen (MART-1) and immunomodulatory oligonucleotides for TLR-9 triggering are covalently coupled. This vaccine can be efficiently drained into LN for uptake and processing by DCs and macrophages. This treatment in phase II clinical trial showed quantitative and qualitative different T cell responses[385].

One important example of approved antigen-loaded DC vaccines is Sipuleucel-T against prostate cancer, in which mononuclear cells are collected from the peripheral blood of prostate cancer patients and cultured with prostate antigens and then coupled with GM-CSF before administration in patients[386].

- **Immune-targeted agents**

Anti – CTLA-4

As discussed earlier activated T cells play an important role in tumor eradication while cancer cells develop strategies to escape from immune system through local and regional immune-suppressive mechanisms. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a potent attenuator of immune responses expressed on many immune cells including $CD4^+$ and $CD8^+$ T cells. It is the CD28 homologue and competes with CD28 for binding to their mutual ligand (B7-family proteins) on APCs. CTLA-4 binding to its receptor will inhibit T cell activity. Blocking this receptor enhanced T cell responses both *in vitro* and *in vivo* and supports T cell proliferation of tumor specific T cells[387]. Ipilimumab is a fully human monoclonal antibody that binds to and blocks CTLA-4. Of note, in a large randomized trial ipilimumab was found to be the first treatment in 30 years to give a survival benefit in metastatic melanoma patients [388,389].

This treatment also requires specific managements as it's also associated with many immune-related disorders. It is a toxic therapy for many patients, and the response rate is relatively low, but its efficacy further asserts that the immune system can be coerced into yielding significant benefits for patients with melanoma. Combination therapies associating anti-CTLA-4 with other blocking agents are still under investigation[390].

Anti – PD-1

PD-1 is an inhibitory receptor on T cells that upon, interaction with PD-1 ligand on the tumor cells prevents activation of T cells by recruiting SHP-2 (a tyrosine phosphatase) that in turns inhibits ZAP-70 signaling. One of tumor escape strategies from immunological surveillance is the over expression of PD-1 ligand. Treatment with anti-PD-1 antibody in patients showed objective response rate in 41% in melanoma patients[391].

One of the main features of this treatment is that it targets the effector phase of the immune response. Combining this treatment with treatments acting in the T cell activation phase can provide favorable results in patients' protection. Indeed, a major problem in immune response against cancer is that T cells are mainly ineffective within the tumor microenvironment due to the strong inhibitory milieu. This hypothesis was confirmed in a mouse model[392].

Since this ligand is not expressed (or expressed minimally) by normal tissue, abrogation of PD-1 and PD-1 ligand interaction might have as less side effects in terms of auto-immnity[365,366].

- **Adoptive T cell transfer**

Immunotherapy strategies have also focused on a personalized treatment that in melanoma patients it has shown some success. Compared to the previously introduced methods adoptive cell therapy (ACT) is not relying on activating endogenous immune cells but rather supplies exogenously autologous or allogeneic effectors in high numbers. 50% of the patients who received high-dose infusions of *ex vivo* expanded and activated TIL showed an objective clinical response with 10% obtaining complete remissin[393].

This method was developed in the lab of SA.Rosenberg in 1988 and is subdivided in three strategies:

1) To obtain TILs from melanoma biopsies and expand them *ex vivo*. The expanded TILs are then transfused back into the patients.
2) To obtain lymphocytes from patient's peripheral blood, to modify them to express chimeric antigen receptors (CAR). To amplify them *ex vivo* and transfuse them back in

patients. CARs are chimeric molecules that are composed of an extracellular single chain antibody targeting a tumor antigen and an intracellular signaling domain[394]

3) To obtain lymphocytes from patient's peripheral blood, modifying them for expression engineered TCR that recognize melanoma associated antigen with very high affinity. This is followed by amplifying them *ex vivo* and by their transfusion back in patients.

In all three protocols the patients is immune-suppressed to gain stronger clinical response[395,396].

The first protocol has less risk but the main difficulty of is that in most cases it is impossible to isolate TILs from melanoma samples. It is also very time consuming and expensive as it is a kind of individualized therapy.

Strategy two is independent of melanoma antigen presenting machinery. This is an important aspect when considering that one escape mechanism is down-regulation of MHC class I. CARs will recognize antigen through the antibody portion of the molecule and the signaling domain will activate the T cells. The method is still under preclinical investigations.

The third strategy gives the opportunity of having sufficient amount of tumor specific T cells. However, this method is limited to a certain antigens in the context of restricted MHC molecules and can favor immunoediting based escape of cancer cells.

- **NK cell-based therapy**

For immunotherapy, activated autologous or allergenic NK cells from peripheral blood, stem cells or induced from pluripotent stem cells are also used. In this context, some NK cell lines that were genetically modified to secrete activating cytokines or to express chimeric antigen receptors, have been tested[397].

The use of NK cell lines as allergenic NK cell source has several advantages. They can be easily maintained and expanded *in vitro* permitting large-scale production of NK cells. Further, they allow the use of one NK cell product for various application or patients thus eliminating deviations of donor-to-donor variability. Of note, the NK-92 line was found to be safe and potentially exhibits beneficial effects in malignant melanoma and advanced lung cancer patients. Thus, so far it is the only cell line that was FDA approved for tumor therapy. Importantly, due to their virtual complete lack of KIR molecules NK-92 are potent mediators of allo-reactivity.

- **BRAF inhibitor**

Vemurafenib is an inhibitor of mutated forms of BRAF, present in around half of metastatic melanomas. Half the patients treated with vemurafenib benefited from objective responses, and early analysis suggests a 3 month improvement in progression-free survival[398]. It would be interesting to define whether BRAF inhibitors may synergize with different strategies of immunotherapy.

II- Aim of the study

Background:

Several lines of evidence indicate that an endogenous immune response against cancer develops in some patients. Tumor-specific CTL expand at relatively high frequency in cancer patients and tumor-infiltrating lymphocytes (TILs), can be isolated and expanded *in vitro* from a variety of human cancers. Some TILs have specific cytolytic activity for fresh autologous cancer cells. Yet, the effector function of these naturally occurring CTL is often insufficient to achieve clinical remission[285,344,356,399]. These data indicate that local specific immune responses may develop within tumors. Furthermore, tumor-associated antigen (TAA)-specific T cells have been detected in some tumor-infiltrated lymph nodes. These data lend strong support to the premise that the immune system can recognize and respond to endogenous cancer cells but is unable to eradicate it. Moreover, Therapeutic approaches based on adoptive transfer of ex vivo expanded tumor antigen-specific CTL emerged as a promising therapeutic strategies, in particular in melanoma[395,399]. However, these approaches still suffer limited efficacy and need to be improved before reaching high clinical benefits.

In this respect the main paradigm is to understand why efficient CTLs do not directly translate into an efficient status of immune surveillance against tumors.

Working Hypothesis and aim:

We assume that an important factor contributing to the limited efficacy of CTL is the unfavorable balance between, the global efficacy of CTL-mediated cytotoxicity and the intrinsic tumor resistance to CTL attack. Thus, we believe that a closer look at the molecular events occurring at the CTL/melanoma IS could allow a better understanding of the mechanisms determining the outcome of the CTL/melanoma interaction. While, so far main efforts of the research community has been given to potentiating immune responses, we feel it is necessary to extend our focus from the induction of an anti-tumor immune response towards the complexities of CTL/tumor interaction.

The aim of our project is therefore to shed light on the molecular and cellular mechanisms taking place at the IS formed between CTL and melanoma target cells and to define whether they may influence melanoma resistance. This project is conducted on isolated human cells and in future will be followed by *in vivo* experiments in mice and later on by examining patients' biopsies.

Our current research aims at identifying the molecular mechanisms allowing remarkable prolonged resistance of melanoma cells following initial CTL activation and lethal hit delivery.

Our experimental plan has been divided along the following three main lines:

I) Analysis of CTL attack/melanoma cell resistance at the single cell level: time-lapse monitoring of CTL/tumor cell dynamic interactions by applying new molecular probes and imaging techniques.

II) Identification of possible synaptic defects in lethal hit transmission at the CTL/melanoma cell contact site: investigate the IS from the tumor cell "point of view" in order to dissect early molecular steps of tumor cell resistance to CTL attack.

III) Define molecular mechanisms of tumor "self-defense" at the immunological synapse: attempt to define molecular pathways that might be targeted to improve melanoma cell susceptibility to CTL attack.

Targeting these synaptic self-defense mechanisms of melanoma cells could be a complementary approach to current strategies to stimulate the immune system to enhance tumor-specific CTL responses and could therefore provide a real clinical benefit.

The lysosome secretory burst of melanoma cells neutralizes CTL cytotoxicity at the lytic synapse

Roxana Khazen[1,2,3], Sabina Müller[1,2,3], Nicolas Gaudenzio[1,2,3,4], Eric Espinosa[1,2,3] , Marie-Pierre Puissegur[1,2,3] and Salvatore Valitutti[1,2,3,5*]

[1]INSERM, UMR1043, Centre de Physiopathologie de Toulouse Purpan, 31024 Toulouse, France; [2]Université Toulouse III Paul-Sabatier, 31062 Toulouse, France; [3]CNRS, UMR5282, 31024 Toulouse, France. [4]Present address: Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA;

[5]Departement of Pathology, Institut Universitaire du Cancer-Oncopole de Toulouse, 31059 Toulouse France

*To whom correspondence should be addressed:

Salvatore Valitutti, INSERM UMR 1043, CHU Purpan, 31024 Toulouse Cedex 3, France; telephone: +33562748366; e-mail: salvatore.valitutti@inserm.fr

Abstract

Human melanoma cells express various tumor antigens that are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) and elicit tumor-specific responses *in vivo*. However, natural and therapeutically enhanced CTL responses in melanoma patients are of limited efficacy. The mechanisms underlying CTL effector phase failure when facing melanomas are still largely elusive.

Here we show that, upon conjugation with CTL, human melanoma cells undergo an active late endosome/lysosome trafficking, which is intensified at the lytic synapse and is paralleled by chatepsin-mediated perforin degradation and deficient granzyme B penetration. Abortion of SNAP-23-dependent lysosomal trafficking, pH perturbation or impairment of lysosomal proteolytic activity restores susceptibility to CTL attack.

Inside the arsenal of melanoma cell strategies to escape immune surveillance, we disclose a self-defense mechanism based on exacerbated lysosome secretion and perforin degradation at the lytic synapse. Interfering with this synaptic self-defense mechanism might be instrumental to potentiate CTL-mediated therapies in melanoma patients.

Introduction

$CD8^+$ cytotoxic T lymphocytes (CTL) are major cellular effectors of the anti-tumor immune response[144,199]. They are, therefore, key components of therapeutic protocols aiming at potentiating immune response against cancer[400]. Clinical trials based on the induction of antigen-specific CTL responses against tumor cells have been reported in various types of cancer. Among those, melanoma is the most studied tumor type in terms of immune reactivity and experimental immunotherapy[401,402]. In melanoma patients, several strategies are currently being evaluated, including vaccination with dendritic cells carrying tumor antigens, adoptive transfer of tumor-specific CTL and treatment with immune-checkpoint inhibitors[356,402-405]. These strategies are mostly focused on strengthening CTL activation and effector function rather than on weakening tumor cell resistance to CTL attack. Although promising, those strategies are of limited efficacy and often present severe side effects[405]. Approaches aiming at dampening tumor cell resistance might therefore synergize with current therapeutic protocols and offer major benefits to patients.

A key pathway used by human CTL to kill their target cells is based on perforin/granzyme-mediated lethal hit delivery. Within minutes or seconds after productive TCR engagement, the

secretion of pore-forming protein perforin, granzyme B, and other proteases stored in CTL cytoplasmic granules (named lytic granules) takes place at the CTL/target cell lytic synapse[107,192,237,239,406,407]. Perforin-mediated penetration of granzyme B into target cells triggers an apoptotic cascade leading to target cell death[227,408]. Although CTL are equipped with other mechanisms of cytotoxicity, the perforin/granzyme pathway is key for human CTL and NK cell effector function, as indicated by the immunodeficiency status and the alteration of immune homeostasis in patients with genetic mutations of perforin or of molecules implicated in lytic granule docking and fusion to plasma membrane[188,409]. In spite of the major interest in potentiating CTL responses in melanoma patients, the molecular dynamics of perforin-mediated lethal hit delivery in this context remains to be elucidated. In particular, while it is well know that melanoma cells possess resistance mechanisms downstream of early pro-apoptotic granzyme B activity[352,353], whether melanoma cells are endowed with mechanisms interfering with the early steps of perforin-mediated pore formation at the lytic synapse is presently elusive.

In this work, we attempted to answer this outstanding question by monitoring the melanoma cell side of the lytic synapse during the encounter with cognate CTL, under conditions in which lethal hit delivery by CTL is efficiently triggered. Our results show that melanoma cells rapidly respond to CTL at the lytic synapse by a secretory burst of lysosome/late endosomes (LLE). Importantly, this leads to cathepsin-mediated degradation of perforin. Inhibition of this melanoma response by different means impairs melanoma cell resistance to perforin-mediated cytotoxicity. Our results reveal a previously unknown mechanism of perforin pathway inactivation, which might strongly contribute to melanoma cell immune resistance. They can inspire new therapeutic approaches that might be complementary to the immuno-modulatory strategies being currently used for the treatment of melanoma patients.

Results

Lethal hit deficiency at the CTL/melanoma cell lytic synapse

We have previously shown that human CTL interacting *in vitro* with antigen-pulsed melanoma cells are efficiently triggered to lytic granule secretion, yet melanoma cells can resist for prolonged time to CTL-mediated cytoxicity[231]. To dissect the molecular mechanisms of melanoma cell resistance to CTL attack at the lytic synapse, we used, as cellular model, melanoma cells that were either pulsed with strong antigenic ligands (peptides of the human cytomegalovirus protein pp65) or left unpulsed prior to conjugation with cognate CTL. This strategy was chosen to focus on the melanoma cell side of the lytic synapse in conditions in

which optimal CTL activation was ensured. Virus specific CTL are indeed fully activated to lethal hit delivery when interacting with peptide-pulsed melanoma cells[231].

As target cells we employed the metastatic melanoma cell line D10 that we have previously characterized for its sustained resistance to CTL-mediated cytotoxicity[231] and JY cells, an EBV-transformed B cell line largely employed as a conventional target cell for human CTL[410]. D10 cells have been recently characterized for their high clonogenic capacity and for their capacity to grow in spheroids[411].

To investigate whether melanoma cells might impair early steps of CTL mediated cytotoxicity, we assessed, in a first approach, perforin staining on target cell surface following short time interaction with CTL. As shown in **Fig. 1a** and **Supplementary Fig. 1**, melanoma cells exhibited a limited perforin staining when compared to conventional target cells, although CTL were similarly activated to lethal hit delivery during interaction with the two different target cell types, as revealed by the increase of surface CD107a expression (**Fig. 1b and Supplementary Fig. 2**). Under these experimental conditions melanoma cells exhibited resistance to CTL-mediated cytotoxicity when compared to conventional target cells, in line with our previously reported data (**Fig.1c**, and reference[231]). Deficient perforin staining was also observed in five additional metastatic melanoma cell lines (**Supplementary Fig. 3**).

To better characterize this phenomenon, we investigated whether the observed defective staining of perforin on melanoma cell surface would translate into an impaired pore formation. To this end, the efficacy and time kinetics of lethal hit delivery to individual melanoma cells were studied by time-lapse confocal laser scanning microscopy. Propidium Iodide (PI) was added at high concentration to the culture medium to monitor lethal hit transmission based on the entry of this probe via the pores formed upon perforin binding on target cell surface[412].

CTL/melanoma cell conjugates from four independent experiments were analyzed to define the intensity of PI staining and the time elapsed between the initial CTL/target cell contact and the appearance of the PI staining at the target cell synaptic area. Results were compared with those obtained with conventional target cells. This analysis showed that the initial entry of PI was delayed in melanoma cells when compared to conventional target cells **(Fig. 1d** and **1e** and **Supplementary Movie 1 and 2**). Moreover, melanoma cells exhibited an overall lower PI staining (**Fig. 1d** and **1f** and **Supplementary Movie 1 and 2**).

In a third approach aiming at defining whether a defective transmission of lytic enzymes might occur at the CTL/melanoma cell synapse, we visualized granzyme B (GrzB) staining in target cells 15 minutes after conjugation with CTL using confocal laser scanning microscopy. This analysis showed that following interaction with CTL, while GrzB staining was significantly detected in a large fraction of sensitive target cells (~73%), only a small fraction of melanoma cells were detected $GrzB^+$ (~8%) (**Fig. 2a**). Defective GrzB penetration in melanoma cells, when compared to sensitive target cells, was also measured by FACS analysis in fixed and permeabilized CTL/target cell conjugates (**Fig. 2b and 2c**). This analysis allowed us to show that GrzB release by CTL is similarly triggered following interaction with melanoma cells as compared to conventional target cells, thus ruling out the possibility that defective GrzB transfer would result from defective CTL activation (**Fig. 2b and 2c**).

Taken together, the above results point out a deficient lethal hit delivery at the CTL/melanoma cell lytic synapse characterized by altered perforin pore formation and GrzB internalization.

High-rate LLE vesicle trafficking in melanoma cells

It is well established that LLE play a key role in cell membrane repair following physical, chemical and biological assaults[413,414]. We thus investigated the dynamics of melanoma late LLE as compared to those of conventional target cells susceptible to CTL-mediated cytotoxicity. FACS analysis showed that melanoma cells exhibited higher constitutive CD107a and CD63 surface expression when compared to conventional target cells, suggesting that melanoma cells might exhibit a constitutively active secretion of LLE vesicles (**Fig. 3a**). We therefore attempted to monitor the constitutive exocytosis and recycling of LLE on melanoma cell surface using time-lapse microscopy.

To do so, we took advantage of a method we recently set up to visualize real-time granule exocytosis by human mast cells based on the addition of avidin-sulforhodamine (Av-SRho) to culture medium, which binds to the serglycin- proteoglycans exposed on cell surface upon granule exocytosis[415]. Although serglycin-proteoglycan is best known as a hematopoietic cell granule proteoglycan, it is also expressed in human endothelial cells and in some metastatic tumors[416,417]. Accordingly, our data show that it is expressed by both conventional target cells and melanoma cells (**Supplementary Fig. 4**). We thus monitored, by time-lapse microscopy the constitutive exocytosis of LLE vesicles in melanoma cells by adding Av-SRho to the culture

medium. This analysis revealed a constitutive exposure of lysosomal vesicles on melanoma cell surface. In comparison, the conventional target cells failed to display detectable constitutive exposure of lysosomal vesicles on their surface (**Fig. 3b**, **Supplementary Movies 3 and 4**).

In parallel experiments, overnight incubation of melanoma cells with Av-SRho resulted in its up-take. Intracellular Av-SRho partially co-localized with CD107a and CD63 within intracellular vesicles, validating the use of Av-SRho as a tool to study melanoma LLE exposure (**Fig. 3c**).

Taken together, the above results reveal that melanoma cells exhibit a constitutive high-rate LLE vesicle exocytosis at the cell surface.

Melanoma cell LLE vesicles exocytosis towards attacking CTL

We next investigated whether vesicular trafficking in melanoma cells might be exacerbated at lytic synapses formed with cognate CTL. In a first approach, we investigated, by confocal microscopy the localization of $CD63^+$ intracellular vesicles in fixed and permeabilized CTL/melanoma cell conjugates. As shown in **Fig. 3d**, peptide-pulsed melanoma cells engaged in cognate interactions with CTL, displayed a significant enrichment of CD63 towards the lytic synapse. Such enrichment was not observed in non-cognate interactions (unpulsed melanoma cells). Morphological data were quantified by drawing two equal regions, one on the melanoma cell side of the lytic synapse and the other on the opposite side of the cell (see scheme in **Fig. 3e**). Three-dimensional (3-D) measurements of the CD63 fluorescence intensity in the cell volume defined by the regions were performed (see material and methods). This analysis provided quantitative evidence of an antigen-dependent enrichment of melanoma cell $CD63^+$ endosomal compartment at the synaptic area in a significant number of CTL/melanoma cell conjugates (**Fig. 3f**).

To monitor the dynamics of the LLE in melanoma cells facing the attack of cognate CTL, we expressed CD107a/GFP in melanoma cells. CD107a/GFP^+ intracellular vesicles were visualized by time-lapse microscopy using a spinning-disk confocal microscope. Upon contact with CTL, peptide-pulsed melanoma cells displayed a rapid re-localization of the CD107a/GFP^+ compartment as measured by an accumulation of GFP fluorescence intensity at the synaptic area during the first minutes after conjugation with CTL (**Fig. 4a and 4b** and **Supplementary Movie 5**). In order to monitor CTL lytic granule polarization towards target

cells with the dynamics of melanoma LLE upon cognate cell-cell interaction in T cell/melanoma cell conjugates, we took advantage of the observation that pre-treatment of CTL with either Av-SRho or Av-Alexa488 allows to stain lytic granules (as shown by Av-SRho and perforin co-localization in fixed and permeabilized CTL, **Supplementary Fig. 5**). We could therefore monitor CTL lytic granule dynamics (as detected by the polarization of Av-SRho loaded lytic granules) together with melanoma cell LLE polarization by time-lapse microscopy as indicated in **Fig. 4a**.

Having observed that melanoma cells rapidly re-locate their LLE toward the contact site formed with cognate CTL, we asked whether the high-rate of constitutive lysosomal secretion observed in melanoma cells might be further enhanced upon contact with cognate CTL. We thus visualized the exposure of lysosomal vesicles on peptide-pulsed melanoma cell surface during interaction with CTL by adding Av-SRho to the culture medium. As shown in **Fig. 4c** and **4d** and **Supplementary Movie 6**, melanoma cells exhibited an increased staining with Av-SRho during the first 10 minutes after contact with CTL. In contrast, unpulsed melanoma cells did not undergo detectable increase of Av-SRho binding upon contact with CTL (**Supplementary Movie 7**). We also monitored CTL lytic granule polarization towards target cells in parallel with the secretory burst of melanoma LLE upon cognate cell-cell interaction. Dynamics of CTL lytic granules was visualized by monitoring the polarization of Av-Alexa488 loaded lytic granules whereas melanoma cell LLE exposure was detected by the addition of Av-SRho to the culture medium. This analysis extended the above results by showing that the melanoma cell LLE exocytosis occurs following CTL lytic granule re-polarization (**Supplementary Movie 8**).

To quantify this phenomenon in a large number of melanoma cells we analyzed fixed CTL/melanoma cell conjugates after 5 minutes co-culture in the presence of Av-SRho. As shown in **Fig. 4e** in unpulsed conditions perforin^{+} lytic granules of CTL did not polarize towards melanoma cells and melanoma cell exhibited non-polarized basal level of Av-SRho binding. Conversely, when melanoma cells were previously pulsed with the antigenic peptide, CTL polarized their lytic granules towards melanoma cells and melanoma cells exhibited a strong Av-SRho binding that was enriched at the synaptic area. Morphological data were quantified by drawing two equal regions, one on the melanoma cell side of the lytic synapse and the other on the opposite side of the cell (as indicated in **Fig. 3f**). 3-D measurements of the Av-SRho fluorescence intensity in the cell volume defined by the regions showed an increase of Av-SRho staining in the synaptic volume in the majority of peptide pulsed melanoma cells

(**Fig. 4f**). It should be noted that conventional target cells exhibited a barely detectable exposure of LLE vesicle when interacting with cognate CTL in the presence of Av-SRho (**Supplementary Movie 9 and 10**).

Taken together, the above results show that melanoma cells respond to CTL attack by adjusting the direction of their vesicular trafficking towards the lytic synapse and by enhancing global late-endosomal vesicle exocytosis on the cell surface.

Melanoma cells degrade perforin from CTL granules

Having observed that melanoma cells activate a late-endosomal vesicle exocytosis process towards cognate CTL, we asked whether this mechanism might neutralize CTL-mediated cytotoxicity by degrading critical lytic molecules. We investigated the possibility that perforin might be degraded by melanoma cells since this enzyme has been previously reported to be substrate of lysosomal hydrolyses cathepsin B (CatB)[222,223], and since human and mouse melanoma cells are known to express high levels of cathepsin B[418]. In line with this hypothesis, the level of exposure of the CatB on melanoma cell surface upon CTL attack was significantly higher than that on conventional target cell surface (**Supplementary Fig. 6**).

In a first approach, we employed FACS analysis to test whether CatB might inhibit perforin lytic activity. Perforin-sensitive Jurkat cells were incubated with purified human perforin either in the absence or in the presence of CatB or in the presence of CatB together with its inhibitor CA074. Incubation of Jurkat cells with purified perforin resulted in a strong cell permeabilization (as detected by PI entry) that was inhibited by CatB. Perforin lytic function was recovered in the presence of CA074 (**Fig. 5a** and **5b**).

In a second approach, we investigated cathepsin-mediated degradation of perforin contained in CTL lytic granules by Western blot analysis. Incubation of lytic granule extracts with CatB resulted in perforin degradation. This degradation was inhibited by the addition of CA074 (**Fig. 5c and Supplementary Fig. 7**). Interestingly, dose-dependent lytic granule perforin degradation was also observed when lytic granule extracts were co-incubated with melanoma cell lysates (**Fig. 5d and Supplementary Fig. 7**).

In a further approach, we incubated CTL lytic granule lysates with Jurkat cells in the presence of a purified vesicular fraction of melanoma cells. This analysis showed that melanoma cell

vesicular fraction was able to inhibit the lytic activity of CTL granules. Lytic activity was recovered in the presence of CA074 (**Fig. 5e and f**).

Taken together the above results show that melanoma cells are endowed of an intrinsic capacity to degrade perforin.

SNAP-23 silencing in melanoma cells enhances cytotoxicity

In order to establish whether a mechanistic link might exist between the polarized LLE exocytosis of melanoma cells and the defective perforin-mediated cytotoxicity, we devised strategies to interfere with melanoma cell lysosomal compartment dynamics and function. The expression of SNAP-23, a SNARE molecule known to mediate lysosomal vesicle exocytosis[419,420] was silenced in melanoma cells by transfection of a specific shRNA. This treatment resulted in the reduction of SNAP-23 expression in melanoma cells of 60-70%, as detected by polymerase chain reaction and by antibody staining (**Supplementary Fig. 8**). We tested whether the reduced SNAP-23 expression would interfere with the dynamics and secretion of LLE compartments in melanoma cells. The dynamics of CD107a/GFP$^+$ intracellular vesicles in cells treated with SNAP-23 shRNA was profoundly altered. Indeed, the rapid re-localization of the CD107a/GFP$^+$ compartment towards the CTL was abrogated and no enrichment of the GFP fluorescence intensity at the synaptic area could be measured (**Fig. 6a and 6b** and **Supplementary Movie 11**).

Accordingly, when we monitored the exposure of LLE vesicles (as detected by the addition of Av-SRho to the culture medium) on the surface of melanoma cells with reduced SNAP-23 expression, a profound inhibition of endosomal vesicle exocytosis was observed, as detected by a lower Av-SRho uptake (**Fig. 6c** and **6d** and **Supplementary Movie 12**). Transfection of melanoma cells with control shRNA did not affect CD107a/GFP$^+$ vesicle synaptic enrichment nor LLE exocytosis (**Fig. 6a–d** and **Supplementary Movies 13, 14 and 15**).

We next investigated the impact of SNAP-23 silencing on melanoma cell resistance to CTL attack. As shown in **Fig. 6e** and **6f**, reducing the expression of SNAP-23 resulted in increased susceptibility to CTL-mediated cytotoxicity.

Taken together, these results establish a mechanistic link between active lysosomal secretion in melanoma cells and defective perforin-mediated cytotoxicity.

Enhanced cytotoxicity by perturbing lysosome function

Having observed that interfering with LLE trafficking in melanoma cells weakened their resistance to perforin-mediated cytotoxicity, we investigated whether alteration of melanoma cell lysosome function would also increase their sensitivity to CTL attack. In a first approach, we perturbed melanoma cell LLE function by using drugs altering the pH of those compartments. Melanoma cells were pretreated with 40µM monensin, a carboxylic ionophore specific for monovalent cations that elevates vacuolar pH[421]. This treatment resulted in a profound alteration of melanoma cell LLE compartment as detected by impaired staining with the pH-dependent probe Lyso-Tracker Red (**Fig. 7a**). Monensin was thoroughly washed before co-culture with CTL. Monensin-treated melanoma cells exhibited enhanced cytotoxicity when compared to the untreated counterpart (**Fig. 7b**). Under the same experimental conditions, CTL activation was not affected, as detected by CD107a exposure on their surface (**Fig. 7c**).

Enhancement of CTL-mediated cytotoxicity following monensin treatment was confirmed in eight additional melanoma cell lines (**Supplementary Fig. 9**).

In further experiments, the impact of bafilomycin A1 and concanamycin A (two additional drugs altering acidic endosomal compartment pH[422]) was investigated. As shown in **Supplementary Fig. 10**, pre-treatment of melanoma cells with these drugs followed by washing before conjugation with CTL, significantly enhanced cytotoxicity.

In a second approach, we pre-treated melanoma cells with inhibitors of proteolytic enzymes. Pre-treatment of melanoma cells with E64d, a protease inhibitor targeting a limited number of protelytic enzymes including cathepsins (see material and methods) or specific inhibitor of cathepsins (CI3), followed by extensive washing before conjugation with CTL, significantly increased cytotoxicity (**Fig. 7d** and **7e**).

These results supported our finding that perforin can be degraded by melanoma cathepsins and further illustrated that the LLE pathway might serve as an important defense mechanism against CTL-mediated cytotoxicity in melanoma cells.

Finally, we investigated if it was possible to visualize, by confocal microscopy, synaptic quanta of perforin on melanoma cell surface. We stained therefore CTL/melanoma cell conjugates with anti-perforin antibodies followed by secondary antibodies labeled with QDots. This approach

allowed us to detect synaptic perforin and to quantify the amount of early exocytosed perforin bound to the melanoma cell side of the synapse. We performed 3-D confocal microscopy in conditions in which melanoma cells were either untreated or pre-treated with monensin and conjugated for 5 minutes with CTL. As shown in **Fig. 7f** and **Supplementary Movie 16**, perforin staining was barely detectable on untreated melanoma cells. Conversely, synaptic perforin quanta were detected on the surface of melanoma cells that had been pre-treated with monensin (**Fig. 7f and Supplementary Movie 17**). Measurement of perforin staining on a significant number of CTL/melanoma cell conjugates showed a significant increase in synaptic perforin staining in monensin treated melanoma cells (**Fig. 7g** and **7h**).

To further visualize this phenomenon, we performed 3-D reconstruction of images in which CTL/melanoma cell conjugates were stained with antibodies directed against CD107a and perforin. The 3-D images further show that in monensin-treated melanoma cells, perforin quanta decorate the synaptic side of the melanoma cell surface (**Supplementary Movies 18-20**).

Taken together, the above results illustrate that perforin, once released by CTL at the synaptic cleft, is rapidly destroyed by melanoma cells via a mechanism dependent on LLE proteolytic potential.

Discussion

In the present work, we investigated the dynamics of interaction between CTL and melanoma cells. We reveal a novel mechanism of melanoma cell defense from CTL attack, based on targeted trafficking of late LLE vesicles towards the synaptic area and on LLE burst exocytosis. This self-defense mechanism leads to synaptic degradation of perforin and failure in pore formation.

In this study, we took advantage of our previous observation that virus specific CTL are fully activated to lethal hit delivery when interacting with peptide-pulsed melanoma cells[231]. Using this cellular model we could therefore focus on melanoma cell mechanisms of resistance, with no concern for CTL activation. The fact that human CTL can be efficiently triggered to lethal hit delivery when interacting with peptide-pulsed melanoma cells (that might exhibit variable levels of HLA molecules) is not surprising. Others and we, indeed, put forth the notion that CTL are exquisitely sensitive to antigenic stimulation and exhibit saturating cytotoxicity

responses when interacting with target cells displaying on their surface an extremely low number of specific peptide-MHC complexes[40,107,410,423] .

Our results shed new light on the role played by lysosomal proteolytic enzymes in the regulation of CTL function. The interplay between cathepsins and various components of the CTL lytic cascade has been thoroughly investigated. It is well established that cathepsins contained within CTL lytic granules are instrumental for maturation of pro-caspases into caspases and for the efficient induction of target cell apoptosis [424,425]. Moreover, CTL granule CatB has been reported to restrain perforin function by degrading perforin bound on the CTL surface. This mechanism has been proposed to be instrumental in the protection of CTL from suicide or fratricide killing[223]. However, this function of CatB is controversial since it has been reported that in CatB knockout mice CTL can survive their own lytic granule secretion[222].

While the functional role of cathepsins contained in lytic granules has been studied, whether and how proteolytic enzymes released by target cells at the lytic synapse might enhance or dampen the efficacy of lethal hit delivery remains to be elucidated. We provide here experimental support to the hypothesis that perforin can indeed be a substrate of cathepsins and that perforin proteolysis is instrumental for the survival of melanoma target cells.

Our results are in apparent contrast with previous data showing that purified perforin is a relatively poor substrate for CatB *in vitro*[222]. A possible explanation of this apparent discrepancy might be that several proteolytic enzymes contained in melanoma LLE and in lytic granules themselves might concur to perforin degradation. An alternative explanation is that cathepsins might activate additional proteolytic enzymes contained in melanoma cell lysosomes that would be responsible for perforin degradation.

An interesting question raised by our work concerns where perforin degradation actually occurs in CTL/melanoma cell conjugates.

Our results are compatible with two scenarios. First, degradation might occur extracellularly on the melanoma cell side of the lytic synapse. Accordingly, melanoma cell lysosome secretion might allow synapse acidification thus creating a synaptic microenvironment facilitating hydrolases function. Such a scenario has been previously described for lysosomal exposure at the B cell synapse for antigen extraction and processing[225]. A second scenario also compatible with our data is that perforin degradation might rapidly occur intracellularly upon endocytosis.

We favor the hypothesis that perforin is degraded at the cell surface, since in our hands,

perforin quanta at the lytic synapse are barely visible in untreated melanoma cells while they are detected on the cell surface following alteration of lysosomal pH (Fig. 7 and Movies 16-20).

Taken together, our results underline the role played by the endosomal compartment of target cells in regulating the process of perforin-mediated cytotoxicity. It has been reported that perforin triggers in target cells an early endosomal compartment-based reparation mechanism that regulates cell death by favoring the induction of apoptosis as opposed to necrosis[245,254]. Here we extend this notion, by showing that, in CTL-resistant target cells, such as melanoma cells, the process of cell death is aborted by lysosomal proteases-mediated perforin degradation.

We also show that upon interaction with cognate CTL, melanoma cell LLE vesicles are enriched at the lytic synapse. These results indicate that melanoma cells are capable of dedicated lysosomal polarization responses to face CTL lytic assault. The molecular mechanisms triggering such dedicated responses are presently elusive. We speculate that they might be triggered by localized calcium entry due to perforin-mediated pore formation at the synaptic area[252].

An interesting aspect of our research is that we describe an important constitutive trafficking of lysosomal vesicles at the melanoma cell plasma membrane that is enhanced following contact with cognate CTL. Together, these results indicate that active lysosomal secretion might have been developed by melanoma cells as part of an immunoediting process occurring under the selective pressure of the immune system and might therefore serve to escape from CTL-mediated immune surveillance *in vivo*[426].

It is tempting to speculate that LLE-mediated defense of target cells from CTL attack is not a prerogative of melanoma cells but is shared by other tumor cells. A previous study reported a correlation between the resistance to natural killer cell–mediated killing and reduced perforin staining in a human leukemia cell line, however the molecular mechanisms of impaired perforin staining were not investigated[427]. Further research is required to define whether LLE secretion might be a general defense mechanism against the perforin/caspase pathway in cytotoxicity resistant cells. A first indication in favor of this possibility comes from our results showing that conventional target cells expressing high levels of CD63 and CD107a are more resistant to CTL-mediated cytotoxicity than their $CD63^{low}CD107a^{low}$ counterpart (**Supplementary Fig. 11**).

A number of studies have demonstrated that anti-apoptotic pathways and other escape mechanisms, operate in cancer cells to mediate resistance to CTL or NK cell attack[352,353,428-430]. Our observation of an early defect in lethal hit delivery does not exclude that additional downstream pathways might operate in melanoma cells to generate resistance to CTL mediated cytotoxicity. However, in our study, we show that the suppression of lysosome-based early defense mechanisms enhances cytotoxicity, indicating that this might be a relevant mechanism of defense among others.

It is well know that in melanoma patients the up-regulation of PD-L1 in response to inflammatory mediators released by infiltrating T lymphocytes and by other cells of the microenvironment plays a crucial role in reducing ongoing immune responses[365,431,432]. It is tempting to speculate that LLE secretory burst might work as an early "innate" mechanism of defense at the beginning of immune cell attack to give time to melanoma cells to deploy late "adaptive" defense mechanisms against infiltrating lymphocytes, including PD-L1 up-regulation. During late phases of the response, immune checkpoint receptors would serve as key mechanisms of melanoma cell escape from immune surveillance. Upon patient treatment with antibodies directed against immune checkpoint molecules, the LLE secretory burst might regain a primary role in melanoma cell defense from immune cell attack.

All in all, our results are compatible with a model in which LLE vesicle secretion at the lytic synapse by melanoma cells is a fundamental early molecular mechanism of cell resistance to CTL attack that acts during the first few minutes after the encounter with CTL and is per se sufficient to confer strong resistance to CTL attack. Nevertheless, this resistance mechanism is potentially complementary to other additional mechanisms of resistance that individual melanoma cells might develop, including up-regulation of PD-L1 and resistance to apoptosis induction.

Various strategies are currently employed to potentiate CTL-mediated immune responses in melanoma patients with the goal of impairing tumor progressions. However, current clinical results are overall unsatisfactory[356,405]. Although melanoma vaccines using peptides emulsified in incomplete Freund's adjuvant, irradiated whole cells (either untreated or genetically modified to release immuno-stimulating factors), cell lysates or autologous dendritic cells carrying melanoma antigens are currently being assessed, clinical benefits have been obtained only on a small number of patients[356,405]. Therapies based on adoptive transfer of autologous in

vitro expanded TILs are promising and can elicit clinical responses lasting for years in a fraction of treated patients, however these procedures are expensive and require lymphocyte depleting regimens that can expose patients to severe adverse effects[356,402,403,405]. Finally, therapies based on monoclonal antibodies targeting the CTLA-4/CD80-CD86 or the PD-1/PD-L1 axis are certainly very promising[356,405,433] however they need to be optimized to establish the best compromise between clinical benefits and adverse effects.

Our results point out a new possible therapeutic path worth following in order to tackle melanoma resistance to immune-surveillance from a new angle. Targeting lysosomal proteases-mediated defense of melanoma cells at the lytic synapse can be indeed complementary to any current immuno-stimulating strategy and can therefore provide a real clinical benefit. Protease inhibitors have been previously tested in the therapy of melanomas as well as of other tumors in an attempt to interfere with tumor invasion and metastasis[434]. It is tempting to speculate that the use of protease inhibitors might be re-considered in order to associate them to therapeutic strategies aiming at potentiating CTL responses.

In conclusion, our results underscore a previously unknown potential Achilles' heel of melanoma cells in respect to CTL mediated cytotoxicity that might be exploited in clinical trials. They can inspire the pioneering of new therapeutic strategies to enhance CTL-mediated activity in melanoma patients by targeting the melanoma cell side of the immunological synapse.

Methods

Cell lines, culture and transfection conditions

Human $CD8^+$ T cell lines were purified from healthy donor blood samples using the RosetteSep™ Human $CD8^+$ T Cell Enrichment Cocktail (StemCell Technologies). For cloning, HLA-A2–restricted $CD8^+$ T cells specific for the NLVPMVATV peptide or the VLAELVKQI peptide of the CMV protein pp65 were single cell sorted into 96-U-bottom plates using a BD FACSAria II cell sorter using tetramer staining. Cells were cultured in RPMI 1640 medium supplemented with 8% human AB serum (PAA), minimum essential amino acids, HEPES and sodium pyruvate (Invitrogen), 100 IU/ml human rIL-2 and 50ng/ml human rIL-15. $CD8^+$ T cell clones were stimulated in complete RPMI/HS medium containing 1µg/ml PHA with 1×10^6/ml 35Gy irradiated allogeneic peripheral blood mononuclear cells (PBMC isolated on Ficoll Paque Gradient from buffy coats of healthy donors) and 1×10^5/ml 70Gy irradiated EBV-transformed B cells. Re-stimulation of clones was performed every 2 weeks. Blood samples were collected and processed following standard ethical procedures (Helsinki protocol), after obtaining written informed consent from each donor and approval for this study by the local ethical committee (Comité de Protection des Personnes Sud-Ouest et Outremer II).

The following HLA-$A2^+$ cell lines were used as target cells: EBV-transformed B cells (JY); HBL and D10 cells (isolated from metastatic melanoma patients, kindly provided by Dr. G. Spagnoli, Basel, Switzerland); M17, M44 and M113 melanoma lines (isolated from metastatic melanoma patients, kindly provided by Dr. F. Jotereau, Nantes, France), EB81-MEL.B, LB3110-MEL and LB2259-MEL.A (isolated from metastatic melanoma patients, kindly provided by Dr. P. Coulie & N. V. Baren, Brussels, Belgium); ME275 (isolated from metastatic melanoma patients, kindly provided by Dr. Daniel Speiser Ludwig Institute for Cancer Research Lausanne, Switzerland). The human acute T cell leukemia cell line Jurkat was from the ATCC collection. Transfection of 1×10^6 melanoma D10 cells with 4µg plasmids coding for shRNA targeting SNAP-23 or a non-targeting shRNA plasmid (Sigma,SNAP-23: GTACCGGGAAACTCATTGACAGCTAAAGCTCGAGCTTTAGCTGTCAATGAGTTTCTT TTTTG and Control: no specific target) were performed using TransIT-X2 dynamic delivery system (Mirus), according to the manufacturers' recommendations.

The medium was replaced 8 hours after transfection. Alternatively, 1×10^5 melanoma D10 cells were transduced with 10 MOI lentiviral vector carrying the same plasmids over 6 hours in RPMI-1640 containing 5% FCS and polybrene (4µg/ml). Transfected cells were selected by

culturing them in the presence of 2µg/ml puromycin for 7 days. Puromycin was added 48 hours after transfection. Target cells were cultured in complete RPMI 1640 supplemented with 10% FCS. Transfection efficiency was evaluated by PCR and flow cytometry.

FACS analysis

The following mAbs were used: Alexa Fluor 488 or Alexa Fluor 647 anti-human perforin antibody (clone dG9; BioLegend), PE-Cy7 mouse anti-human CD107a (cloneH4A3; BD Biosciences), Pacific Blue anti-human CD8 antibody (clone RPA-T8; BioLegend) and FITC mouse anti-human CD63 (cloneH5C6; BD Biosciences).

To distinguish CTL from target cells in the analysis, CTL were loaded with 1µM CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate, (Molecular Probes, Invitrogen) in RPMI for 15 minutes at 37°C, prior conjugation with target cells. Alternatively, cells were not loaded with 0.1µM CMFDA before conjugation instead $CD8^+$ cells were identified by a staining with Pacific Blue anti-human CD8 antibody. Target cells were co-cultured with T cells in RPMI, 5% FCS/HEPES at 2 CTL versus 1 target cell ratio. At different time points, CTL/target cell co-culture cells were washed in ice-cold PBS containing 0.5mM EDTA. Nonspecific binding was prevented by 15 minutes incubation with 1% PBS containing 1% FCS which was used throughout the procedure as staining and washing buffer. Cells were stained with either specific antibodies or corresponding isotype controls. For cathepsin B expression on target cell surface cells were stained with a goat anti-human cathepsin B (sc-6493; Santa Cruz) followed by an Alexa Fluor 647 donkey anti-goat Ab (Invitrogen). Staining was performed on ice at 4°C for 30 minutes. Samples were acquired using a FACScalibur or LSR II (Becton and Dickinson). Results were analyzed using the FlowJo Pro software (Tree Star, Inc.).

Cytotoxicity assay

Target cells were left unpulsed or pulsed with 10µM antigenic peptide during 2 hours at 37°C/5% CO2, washed three times and subsequently transferred to a 96 well U-bottom plate at 25×10^3 cells/100µl RPMI, 5% FCS/HEPES. CTL were previously stained with 0.1µM CMFDA for 15 minutes at 37°C/5% CO2, washed and added to the target cells at 2 CTL versus 1 target cell ratio (unless indicated) in 100µl RPMI, 5% FCS/HEPES. Cells were pelleted for 1 minute,

1500 rpm, and incubated at 37°C/5% CO2 for 4 hours. In some experiments target cells were pre-treated with either 40µM monensin or 1µM bafylomycin A1 or 1µM concanamycin A and thoroughly washed before conjugation with CTL. In additional experiments, melanoma cells were pretreated for 16 hours with 10µM E64d (inhibitor of calpain, cathepsin B, H and L; Sigma) or 10µM Cathepsin inhibitor III (cathepsin B, H and L inhibitor; Merck-Millipore) in serum free medium. During the last 2 hours, cells were either unpulsed or pulsed with antigenic peptide. Cells were thoroughly washed before conjugation with CTL. Before FACS analysis, 0.25µg 7-Aminoactinomycin D (7-AAD; BD Biosciences) was added to each sample in order to measure the percentage of death targets. For the present study, a 4 hour 7-ADD uptake cytotoxicity assay has the advantage, over other possible tests, used to measure melanoma cell survival and growth (such as colony formation assays), since it allows to measure cell death during a time window compatible with perforin-mediated cytotoxicity.

Time-lapse microscopy monitoring of perforin pore formation

Target cells were pulsed with 10µM peptide, washed and seeded at $1.5x10^5$ cells/well on poly-D-lysine coated 8-well chambered slides (Ibidi, Munich, Germany) 5 minutes prior to imaging. Chambered slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C, and constant CO_2 concentrations (5 %). At the beginning of recording $2x10^5$ CTL, labeled with either 1µM CMFDA or with 5µM TubulinTracker™ Green (Molecular Probes, loading was performed for 30 minutes at 37°C/5% CO_2) were added to chambered slides in the presence of 200µM Propidium Iodide. Images of CTL/target cell conjugates were acquired for 1 hour using either a Zeiss LSM 510 or a Zeiss LSM 710 microscope (Zoom factor 63X).

Intracellular staining

Target cells were either unpulsed or pulsed with 10µM antigenic peptide for 2 hours at 37 °C in RPMI 5% FCS/HEPES and washed three times (in some experiments cells were pretreated with 40µM monensin for 2 hours). Conjugates of 2 CTL versus 1 target cell were then formed by 1 minute centrifugation at 1500 rpm. Upon 5 or 15 minutes of co-incubation at 37°C, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% saponin (in PBS/3% BSA/HEPES), and stained with the following primary antibodies: anti-human perforin mAb

(clone δG9; BD Pharminogen), anti-human CD63 mAb (ab1318; Abcam), anti-human GrzB mAb (clone G11; Santa Cruz Biotechnology), anti-human Serglycin mAb (ab76512; Abcam) anti-human CD107a rabbit Ab (ab24170; Abcam) and anti-human SNAP-23 (Ab4114; Abcam). Primary Abs were followed by goat anti-mouse isotype-specific Ab or goat anti-rabbit Ab labeled with Alexa 488, Alexa 555, Alexa 647, Alexa 700 or goat anti-Mouse IgG conjugate (H+L) labeled with either QDot 525 or QDot 585 (Molecular Probes). In some experiments target cells are identified by Cell Tracker Blue (life technologies) staining. The samples were mounted in 90% glycerol-PBS containing 2.5% DABCO (Fluka) and examined using either a LSM 710 (Zeiss) or SP8 (Leica) confocal microscope over a 63× Plan-Apochromat objective (1.4 oil). Electronic zoom 4 on LSM710 and 5 on SP8. Z-Stack images of optical sections were acquired throughout the cell volume. Alternatively, GrzB staining was evaluated by FACS analysis as described above.

Image quantification

Images were scored by evaluating, for each experimental condition, at least 45 CTL/target cell conjugates in randomly selected fields from at least three independent experiments. GrzB staining was evaluated by scoring the percentage of positive target cells. To quantify the polarization of $CD63^+$ vesicles towards the immunological synapse or synaptic enrichment of Av-SRho staining, unprocessed images were analyzed by dividing each target cell into three equal regions. Fluorescence was measured using the ROI statistics tool of the ImageJ software on image projections in two equally defined volumes (one at the synaptic area and the other at the distal area). In order to exclude CD63 or Av-SRho staining of CTL a region was carefully drawn on the melanoma cell side of the lytic synapse. Results were reported as the fold increase of the corresponding staining integrated fluorescence intensity at the synaptic region divided by the same fluorescence intensity measured at the distal region (see schemes drawn in Fig. 3). For quantification of perforin on melanoma cells, the lytic synapse was divided into two equal volumes, one on the CTL and one on the melanoma cell side. Total perforin fluorescence intensity at the lytic synapse of both volumes was acquired on image projections of CTL/melanoma conjugates. Perforin on melanoma cells was quantified as the percentage of perforin fluorescence intensity (FI) at the defined melanoma cell volume (red box) divided by the totality of perforin fluorescence intensity (FI, black box) at the lytic synapse (% of perforin FI = (perforin Fl on melanoma cell/Total perforin Fl) × 100; see schemes drawn in Fig. 7) using

the ImageJ software. The above-described measurements were confirmed by analyzing fluorescence intensity in melanoma cells z-stack projections using the Region Measurements of the Metamorph software (Universal Imaging). In further experiments z-stack images of CTL interacting with melanoma cells were reconstructed and analyzed using the Surface tool of the Imaris software.

Monitoring melanoma cell LLE trafficking and secretion

To quantify lysosomal localization at the lytic synapse, wild type D10 cells or SNAP-23 silenced D10 cells were infected with 40 particles per cell of baculovirus coding for CD107a-GFP (Cell Light Lysosomes; Invitrogen) overnight. Cells were either unpulsed or pulsed with 10µM peptide and monitored during interaction with CMFDA or Av-SRho loaded CTL. Time lapse video microscopy was performed for 1 hour using a Nikon inverted spinning disk confocal microscope equipped with a back-thinned charge-coupled device (CCD) camera (Evolve; Photometrics, Tucson, AZ, 512 X 512 pixels), equipped with a temperature-controlled chamber maintained at 37°C, and constant CO_2 concentration (5%), allowing acquisition of multi-position images using an NA1.3/40X oil-immersion objective piloted by the Metamorph7 software. The images were processed using ImageJ. CD107a-GFP integrated fluorescence intensity at the lytic synapse was quantified for several conjugates and reported as fold increase over the initial intensity at time 0 of conjugate formation.

To measure the exposure of the lysosomal compartments of melanoma cells, peptide-pulsed wild type D10 or SNAP-23 silenced D10 cells were seeded into poly-D-lysine coated chambered slides in 100□l medium containing 8µg/ml Av-SRho- Av-SRho (red) uptake in the presence or absence of CMFDA or Avidin-Alexa488 loaded CTL was monitored for 1 hour using a Nikon inverted spinning disk confocal microscope.

Measurement of perforin lytic activity

Human purified perforin (Enzo Life) permabilizing activity was determined by 1 hour incubation of $1x10^6$ Jurkat cells with 125ng of perforin following manufactures recommended conditions. Perforin was pre-treated or not for 2 hours at 37°C with 500ng/ml purified human liver cathepsin B (CatB, Merck-Millipore), or CatB that was previously co-incubated with

10µM CA074 (Merck-Millipore) for 30 minutes at 37°C. Cell permeabilization was measured in the presence of 25µg/ml Propidium iodide (PI) by FACS analysis. In some experiments, lytic granule lysates were pre-treated or not with melanoma cell vesicular fraction lysates for 2 hours at 37°C. Melanoma cell vesicular fraction lysates were previously co-incubated or not with 10 µM CA074 (Merck-Millipore) for 30 minutes at 37°C before being incubated with lytic granule lysates. Intracellular vesicles isolation from melanoma cells was performed following a published protocol[435], that we previously employed for lytic granule isolation with minor modifications (see below).

Western blot analysis of perforin cleavage

Granule isolation was performed following a published protocol[435] with minor modifications. Briefly, $CD8^+$ T cells were washed three times with ice cold PBS and reconstituted in 2×10^7cells/ml of relaxation buffer: 130mM KCl; 5mM NaCl; 2mM MgCl2; 1mM disodium-ATP/10mM Hepes pH 7.4. Cells were disrupted by N_2 cavitation at 400p.s.i and the cell suspension was collected in the presence of 4mM EGTA. The cell lysate was separated into a post-nuclear supernatant (SN1) and a pellet (P1) by centrifugation at 800×g for 10 minutes at 4°C. SN1 was submitted to another centrifugation step at 20.000×g for 30 minutes at 4°C, which resulted in a cytosolic supernatant (SN2) and a total vesicular extract enriched in the pellet P2. For melanoma cell lysates, 5×10^6 cells were diluted in cytobuster for protein extract following manufacture protocol (Merck-Millipore). The supernatant was harvested by centrifugation. Aliquots of 500ng of total protein from lytic granule (LG) extract lysates were either treated with 500ng/ml CatB or an increasing concentration of melanoma cell lysate (0.5, 1and 3µg/ml total protein) for 2 hours at 37°C, then mixed with SDS sample buffer. Reduced 10% SDS gels were run and blotted onto a nitrocellulose membrane. After blocking with 3% nonfat dry milk in TBST for 1 hour, the membranes were incubated with 10µg/ml anti-perforin (clone H-315; Santa Cruz Biotechnology) mAb overnight at 4°C, followed by 2 hours of incubation with an HRP-anti–rabbit IgG (SouthernBiotech). The blots were developed using ECL (GE Healthcare). Western Blot images were acquired using a ChemiDoc™ MP System (Biorad). In some experiments CatB was pre-treated with 10µM CA074 for 30 minutes at 37°C before addition of the mixture on LG lysates. Band intensity was quantified using ImageJ software as raw intensity over non-treated LG lysate.

- *cDNA synthesis, polymerase chain reaction*

RNA isolation was performed using the RNeasy Mini Kit (Roche Life sciences), and RNA concentration was assessed using the NanoDrop 1000 (Thermo Scientific) system RNA was converted to complementary DNA (cDNA) using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies). The gene expression of SNAP-23 (Hs001870775_m1) was evaluated by real time quantitative PCR (RT qPCR) using TaqMan gene expression assays (Applied Biosystems) according to the indicated protocol, using a LightCycler® 480 System (Roche Life sciences). All reactions were performed in triplicates and relative gene expression levels were evaluated using the comparative CT (threshold cycle) method (2-deltaCT). GAPDH (Hs03929097_g1) was used as endogenous controls for normalization.

- *Statistical analysis*
- Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups.

Author contributions

R.K. designed the research, performed the experiments, analyzed the results and wrote the paper; S.M, M.P.P, N.G, contributed cellular and molecular tools and performed experiments; E.E. contributed ideas and edited the paper; S.V. designed the research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Acknowledgements

We thank Loïc Dupré and Abdelhadi Saoudi for discussion and critical reading of the manuscript. We thank the "plateau technique de cytométrie et de microscopie" INSERM UMR 1043. We thank Astrid Canivet for help in image analysis. We thank Fatima-Ezzahra L'Faqihi-Olive and Manon Farce for expert help in flow cytometry data analysis. We thank Dr. G. Spagnoli, Dr. F. Jotereau, Dr P. Coulie, Dr. N. V.Baren and Dr. Daniel Speiser for kindly providing melanoma cell lines. This work was supported by the Fondation ARC pour la Recherche sur le Cancer (grant EML2012090493), the Institut National du Cancer (Grant INCa PBLIO11-130 and INCa/DGOS 2012-054) and the Laboratoire d'Excellence Toulouse Cancer (TOUCAN, grant ANR11-LABX). R.K. was supported by a fellowship by the Region Midi-Pyrénées and by Fondation ARC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Figures and Figure Legends

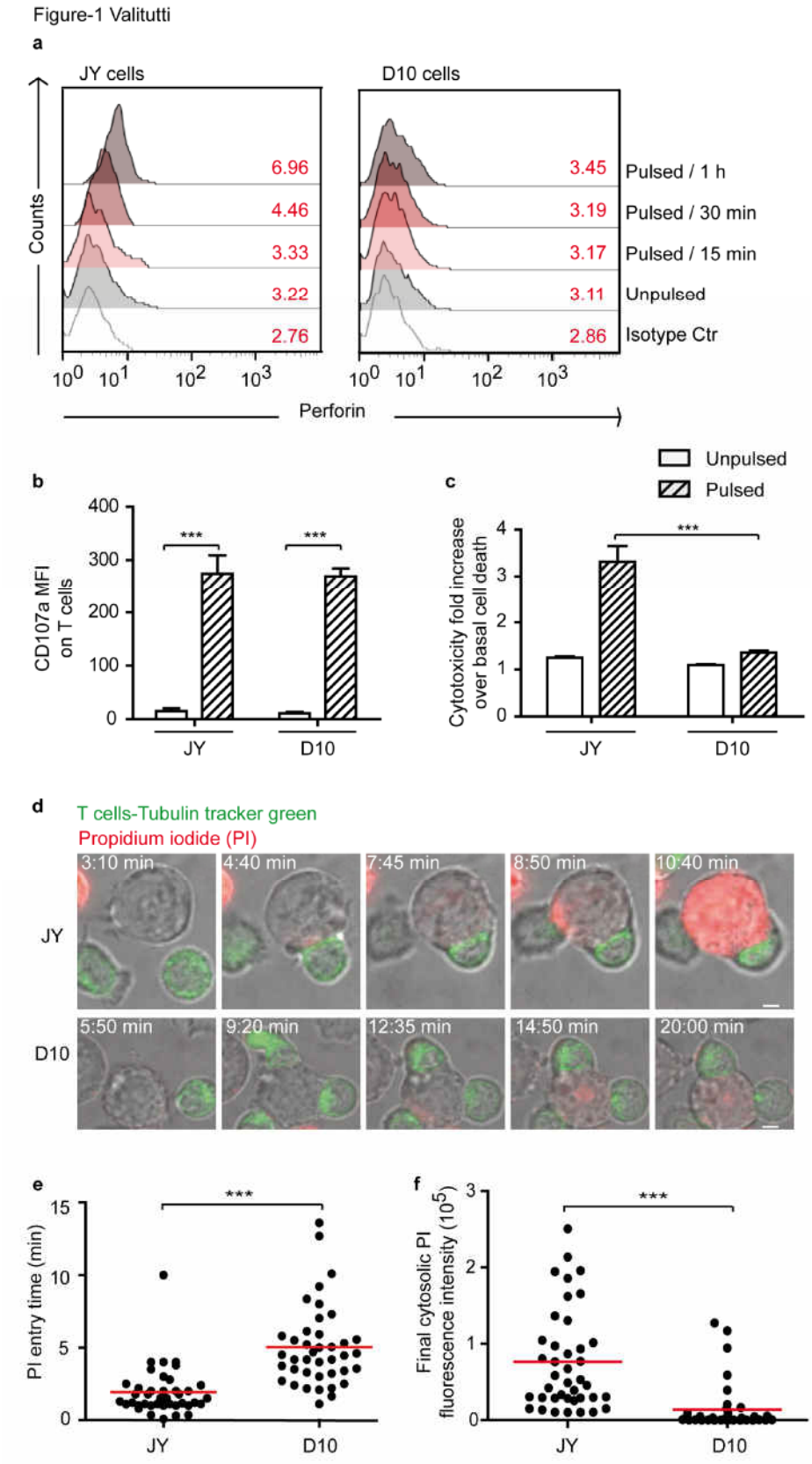


Fig.1. **Defective lethal hit delivery at the CTL/melanoma cell synapse. a)** Time kinetics of perforin staining on the surface of conventional target cells (JY) and melanoma cells (D10) following conjugation with CTL. Target cells were either unpulsed or pulsed with 10µM antigenic peptide. The MFI (Median Fluorescence Intensity) of perforin staining is indicated. **b)** Efficient activation of CTL to lethal hit delivery following encounter with peptide-pulsed melanoma cells. Target cells either pulsed or not with antigenic peptide were conjugated for 1 hour with CTL. Cells were stained for CD107a extracellular exposure at 4°C. **c)** Measurement of target cell killing. Cytotoxicity was evaluated by FACS analysis in target cells either pulsed or not with antigenic peptide following 4 hour incubation with CTL. Cytotoxicity is expressed as fold increase over corresponding basal death. **d)** Sequences of snapshots depicting either JY or D10 cells pulsed with the antigenic peptide interacting with tubulin tracker green-labeled CTL. 200µM PI was added to culture medium at time 0. Perforin pore formation on target cells was detected by PI internalization (red). Typical conjugates with the JY and D10 target cells are shown. Supplementary Movie 1 and 2 show the entire time-lapse recording. Bars, 5µm. **e)** Time required for detection of initial PI entry in target cells in JY or D10/CTL conjugates as detected by 1 hour time-lapse confocal microscopy. Red bars represent the mean time. **f)** Measurement of the cytosolic PI intensity in target cells at the end of the time-lapse video recording in CTL/target cell conjugates. Red bars represent the mean PI fluorescence intensity.

Analysis in **e** and **f** was performed only on cells exhibiting detectable PI entry. In **a,** results are representative of 3 independent experiments. In **b** and **c** results are expressed as mean ± SEM of 3 independent experiments (**b**) and 4 independent experiments (**c**). In **e** and **f** data are from 40 conjugates for each cell type. Data are from 3 independent experiments. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. ***$P < 0.001$, **$P<0.01$.

Figure-2 Valitutti

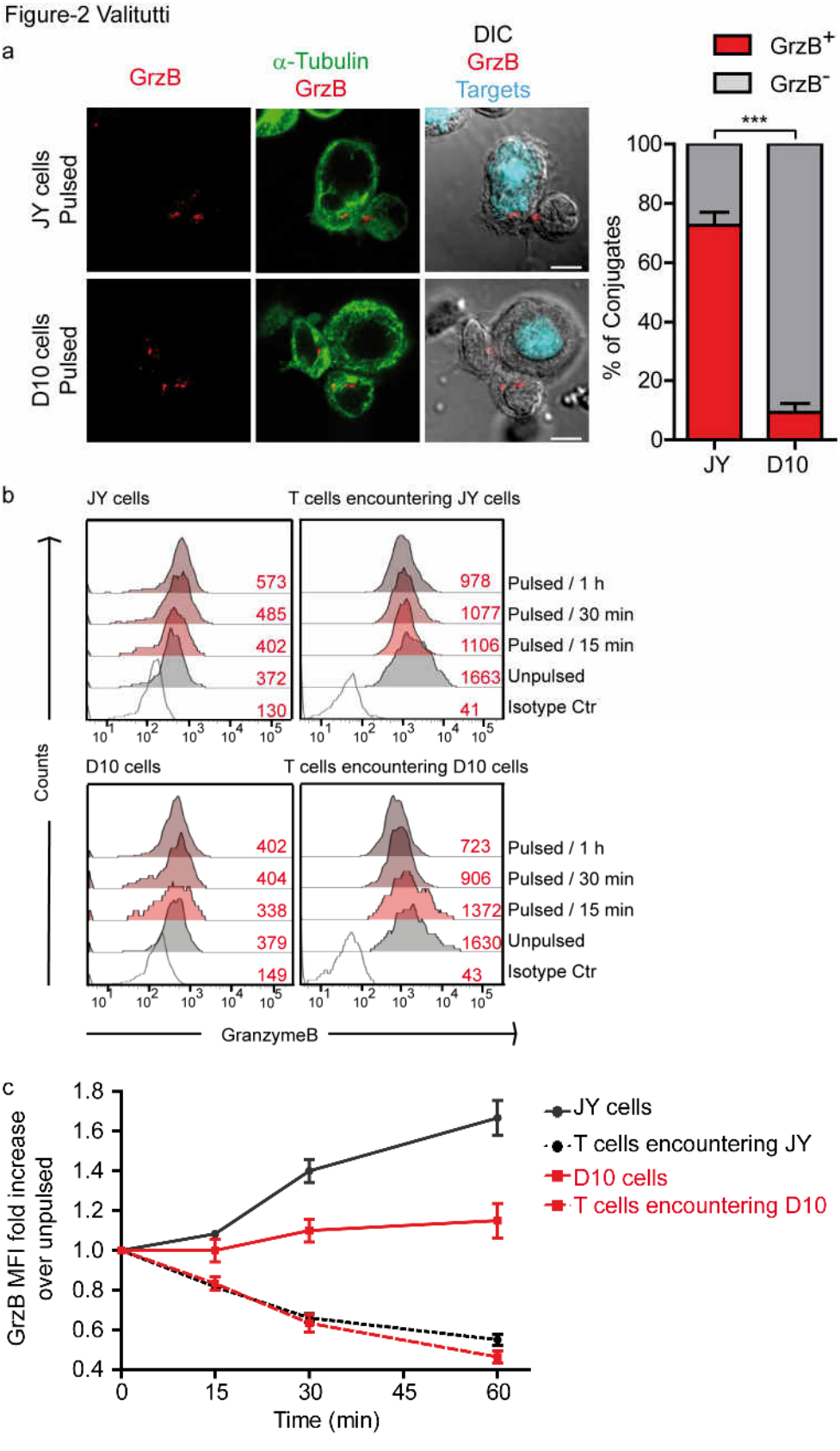
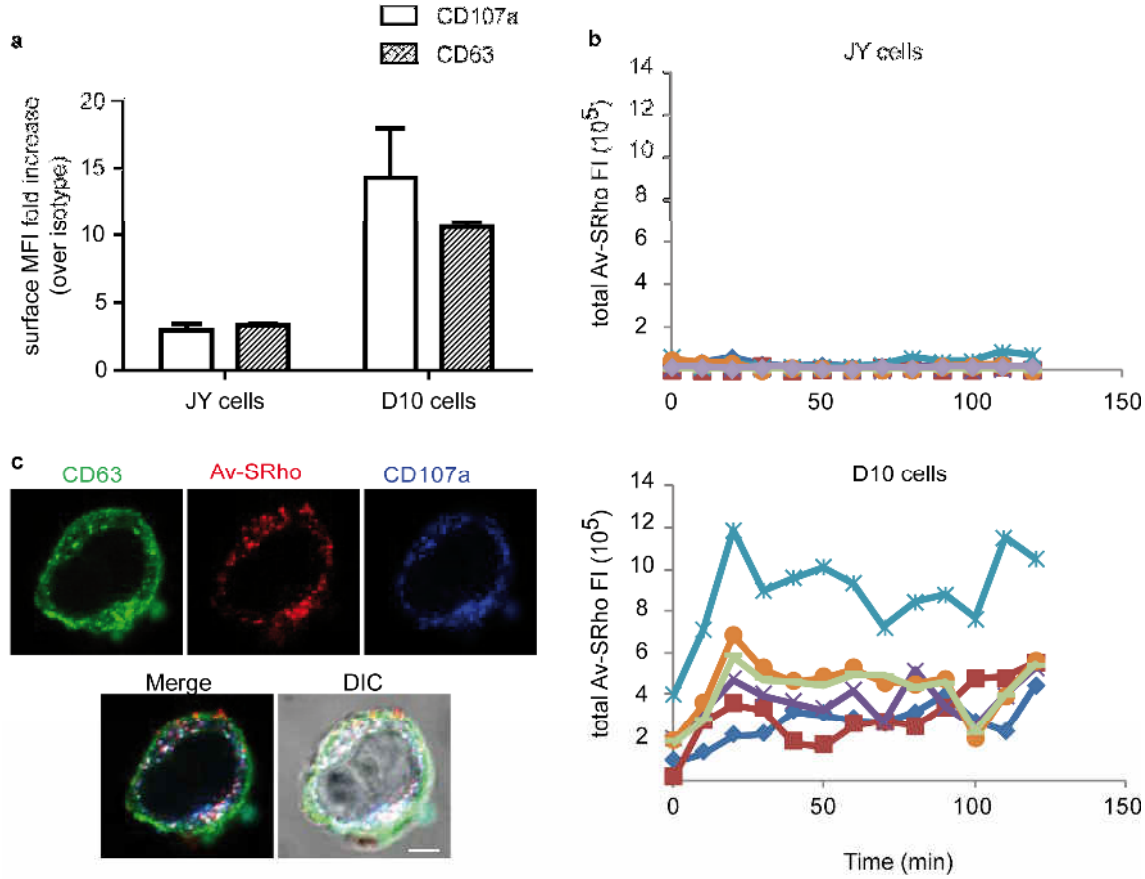
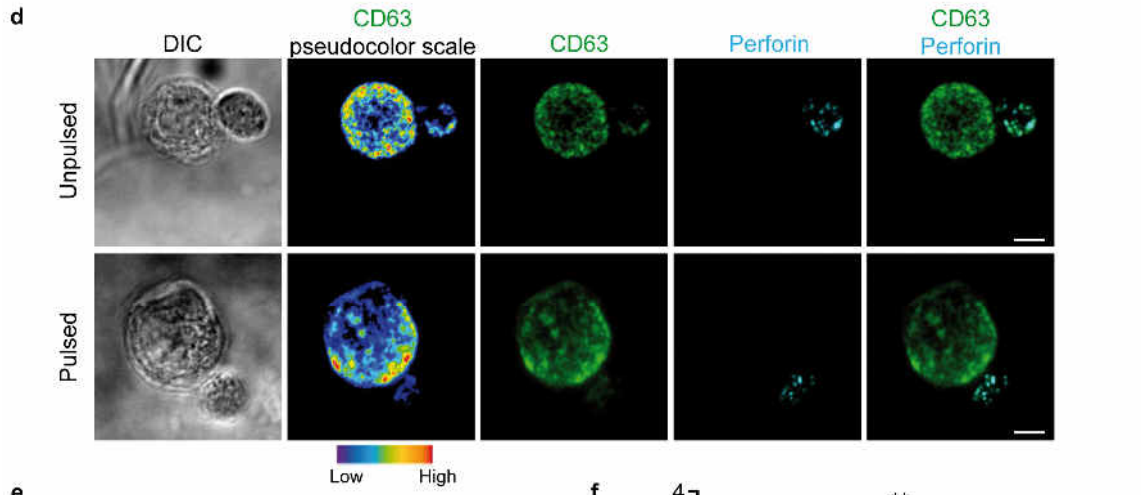


Fig. 2. **Defective granzyme B penetration in melanoma cells.** a) Visualization of GrzB staining in CTL/target cell conjugates by confocal laser scanning microscopy. Target cells were previously pulsed with 10µM peptide. Cells were stained with anti-α tubulin (green) and anti GrzB (red). Target cells were identified by loading them with Cell Tracker Blue before conjugation (cyan). Percentage of GrzB+ target cells in peptide pulsed JY and D10 cells are indicated. Sixty conjugates from three independent experiments were scored. Bars, 5µm. Results are expressed as mean ± SEM of 3 independent experiments. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. ***$P < 0.001$. b) Left panels: time kinetics of GrzB staining in conventional target cells (JY) and melanoma cells (D10), either unpulsed or pulsed with 10µM peptide concentration, following conjugation with CTL. Right panels: Time course of GrzB loss in CTL following interaction with target cells. Target cells were either unpulsed or pulsed with the antigenic peptide. The median fluorescence intensity of GrzB staining is indicated. Results are from one representative experiment out of three. c) Pooled results from three independent experiments showing the time-dependent loss of GrzB by CTL interacting with JY cells (black dotted lines) or with D10 cells (red dotted lines) and GrzB uptake by JY cells (black plain lines) or by D10 cells (red plain lines). MFI= Median Fluorescence Intensity. Results are expressed as mean ± SEM of 3 independent experiments.

Figure-3 Valittuti





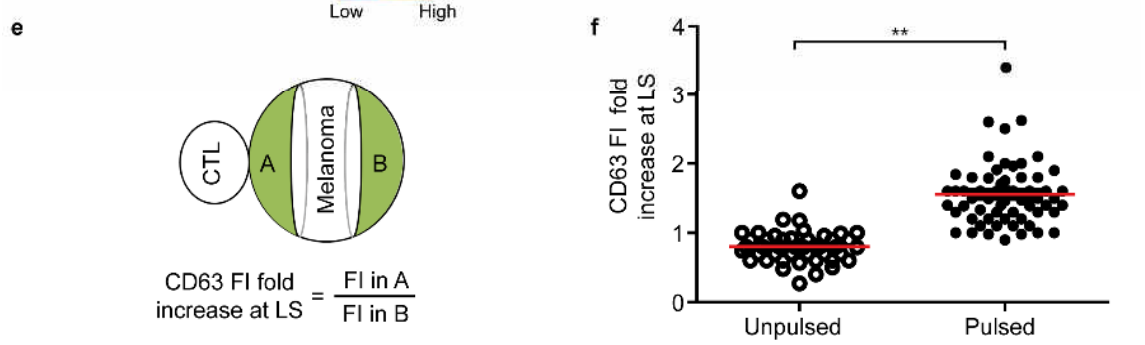


Fig. 3. **Melanoma cells exhibit a high rate vesicular trafficking and enrich LLE vesicles at the lytic synapse during conjugation with CTL.** a) Extracellular staining for CD107a and CD63 on JY and D10 cell surface as detected by FACS analysis. Results are shown as median fluorescence intensity (MFI) fold increase over isotype control. Results are expressed as mean ± SEM of 5 independent experiments. b) Basal lysosomal endo/exocytosis was quantified via Av-SRho uptake by JY (upper panel) or D10 cells (lower panel) in the presence of 8µg/ml Av-SRho in the culture medium. Panels show Av-SRho fluorescence intensity in cells during 2 hour acquisition, as measured by time-lapse confocal microscopy. Graphs show 6 cells representative of the two cell types from 3 independent experiments. c) Av-SRho accumulates in melanoma cells lysosomal compartment. Melanoma cells were incubated in the presence of 8µg/ml of Av-SRho (red) overnight. Cells were fixed, permeabilized and stained for CD107a (blue) and CD63 (green). d) Melanoma cells either unpulsed or pulsed with 10µM antigenic peptide were conjugated with peptide-specific CTL for 5 minutes at 37°C. Cells were then fixed, permeabilized and stained for CD63 (green and pseudo-color) and perforin (Cyan). Bars, 5µm. e) Scheme depicting the gating strategy used to measure CD63 fluorescence intensity in melanoma cells in a cell volume corresponding to the synaptic area and in an equal volume drawn at the opposite side of the cell. f) Re-localization of lysosomal compartments was quantified by measuring the CD63 fluorescence intensity fold increase at the lytic synapse in melanoma cells (as indicated in the scheme). 45 and 60 conjugates (for unpulsed and peptide pulsed cells respectively) formed by only 1 melanoma cell and 1 CTL were scored. Data are from 3 independent experiments. Bars indicate mean values. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. **$P<0.01$.

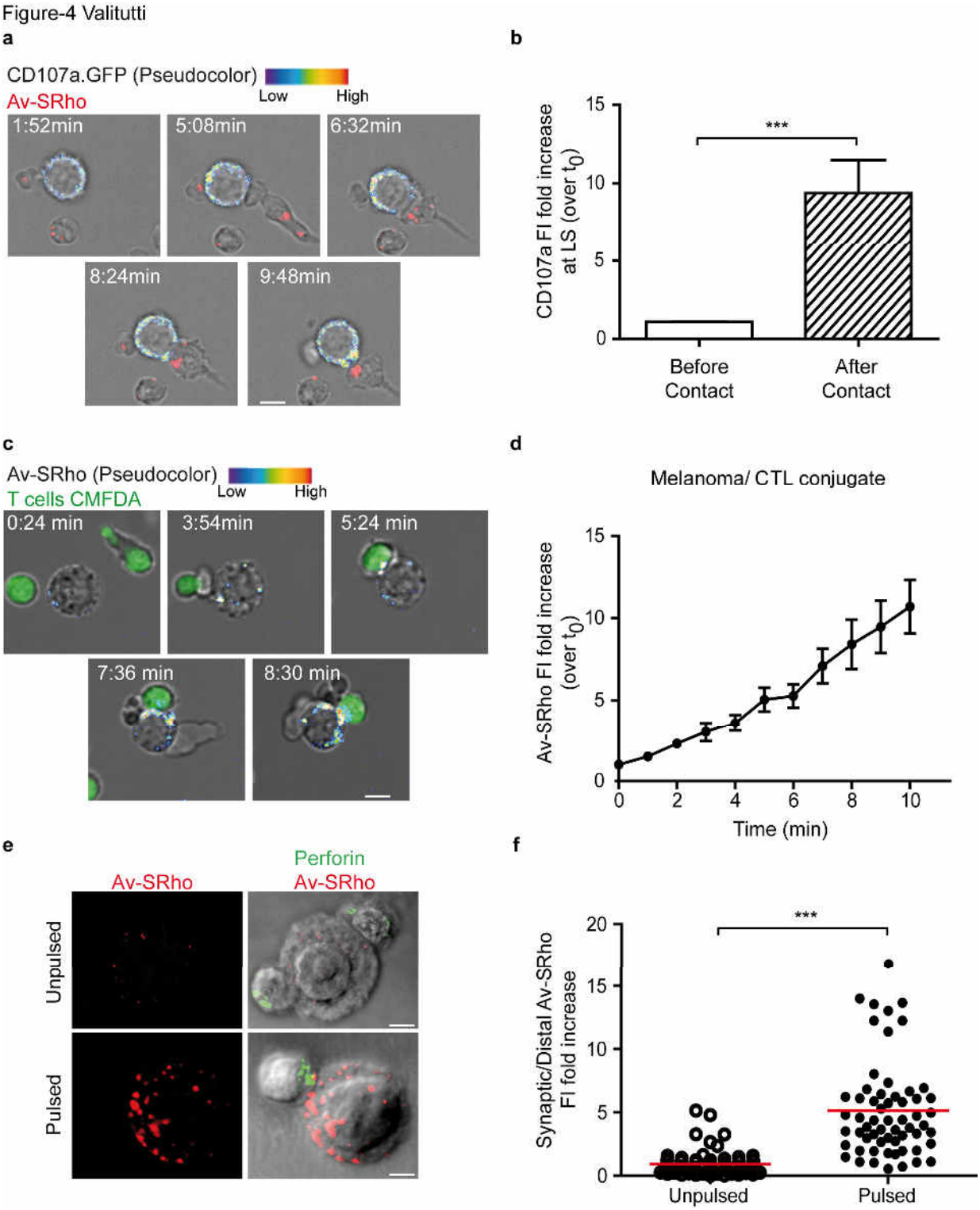


Fig. 4. **Recruitment of melanoma cell lysosomal compartment to the lytic synapse.** a) and b) Melanoma cells expressing CD107a-GFP and pulsed with 10µM antigenic peptide were monitored during interaction with CTL using a spinning-disk confocal microscope. a) Snap shots depicting the CD107a-GFP+ LLE localization before conjugation and after conjugation. The pseudo color scale indicates molecular enrichment. Snapshots are from Supplementary

Movie 5. b) Quantification of CD107a fluorescence intensity (FI) fold increase. Fluorescence intensity either before or 5 minutes after cell-cell conjugation in 13 melanoma cells conjugated with CTL was measured at the lytic synapse and normalized over fluorescence intensity measured in the region of interest at time 0. Data are from five independent experiments. c) and d) Peptide-pulsed melanoma cells were monitored during interaction with CTL in the presence of 8µg/ml Av-SRho by spinning-disk confocal microscopy. c) Sequences of snap shots depicting enhanced LLE vesicle exposure in peptide pulsed melanoma cells during conjugation with CMFDA loaded CTL (green). Lysosomal exposure is detected via Av-SRho uptake (pseudo color scale intensity). Snapshots are from Supplementary Movie 6. d) Measurement of Av-SRho fluorescence intensity fold increase in peptide pulsed melanoma cells during conjugation with CTL. Intensities were scored from 14 conjugates in nine independent experiments and normalized over Av-SRho fluorescence intensity of the cell of interest at time 0. Data are expressed as mean ± SEM of the scored cells. e) Melanoma cells either pulsed or not with antigenic peptide were conjugated with peptide-specific CTL for 5 minutes at 37°C in the presence of Av-SRho. Cells were fixed, permeabilized and stained for perforin (green). f) Exposure of LLE vesicles was quantified by measuring the Av-SRho fluorescence intensity fold increase at the lytic synapse in melanoma cells (as indicated in the scheme in Fig. 3e). 61 and 60 conjugates (for unpulsed and peptide pulsed cells respectively) formed by 1 melanoma cell and 1 CTL were scored. Data are from 3 independent experiments. Bars indicate mean values. Bars, 5µm. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. ***$P<0.001$.

Figure-5 Valitutti

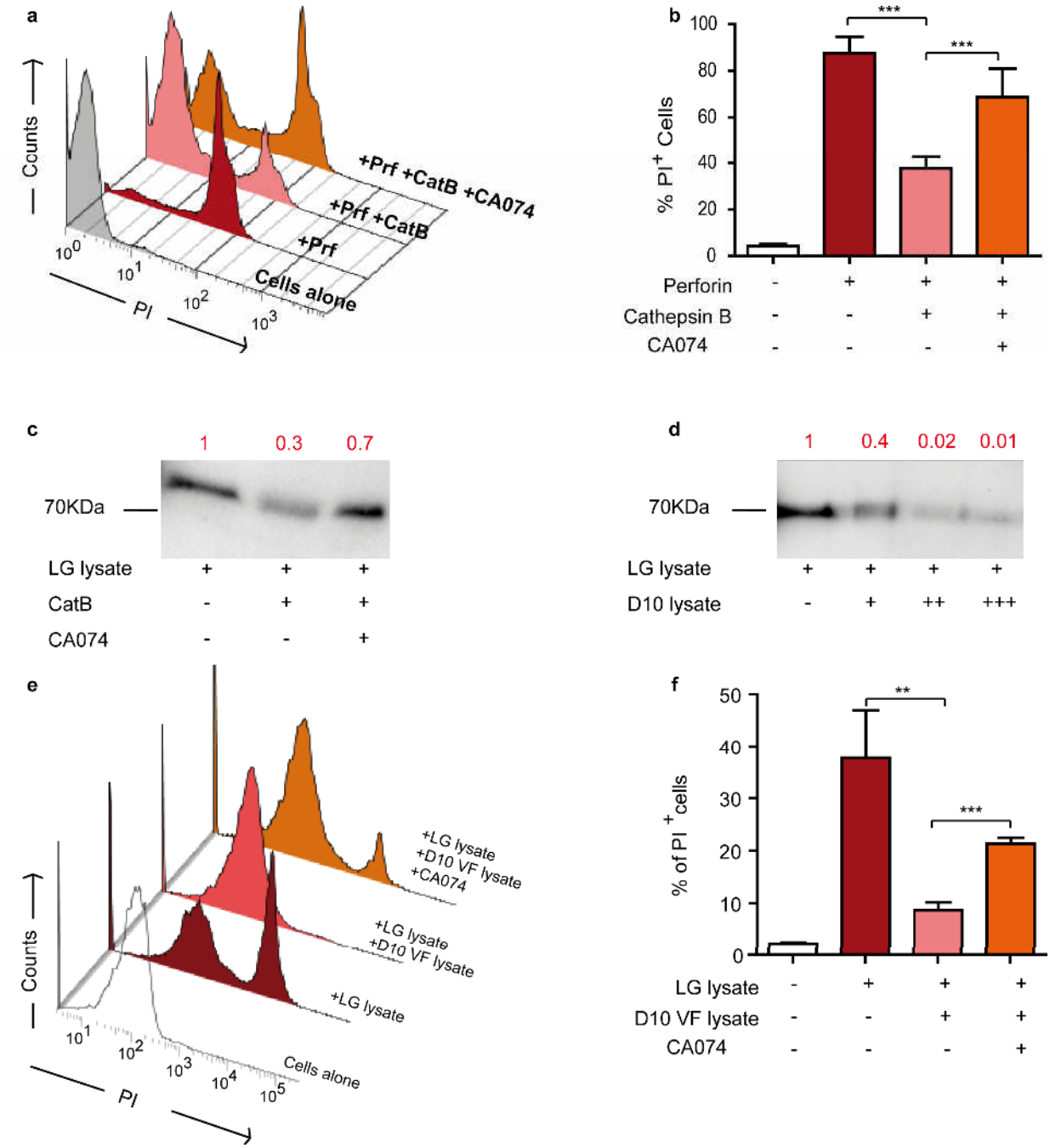


Fig. 5. **Perforin degradation by cathepsin and melanoma cell extracts.** a) and b) Measurement of perforin lytic activity on Jurkat cells. Jurkat cells were incubated with purified human perforin either in the absence or in the presence of CatB or of CatB plus CA074 for 1 hour. PI entry in cells was measured by flow cytometry. a) Plots show results from one representative experiment; b) data are expressed as mean ± SEM of four independent experiments. c) and d) Degradation of perforin in isolated CTL lytic granules. c) Western Blot analysis of lytic granule perforin either untreated or incubated with CatB or with CatB plus CA074. d) Western Blot analysis of lytic granule perforin either untreated or incubated with

increasing concentrations of melanoma cell lysates (+ 0.5µg/ml; ++ 1µg/ml; +++ 3µg/ml). Results are from one representative experiment out of three. Numbers indicate band intensity fold increase.

e) and f) Jurkat cells were incubated for 1 hour with purified human lytic granules lysate either in the absence or in the presence of melanoma cell vesicular fraction lysates or of melanoma cell vesicular fraction lysates plus CA074 for 1 hour. PI entry in cells was measured by flow cytometry. Data are from 3 independent experiments. Bars indicate mean values. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. ***P<0.001, **P<0.01. LG = lytic granules; VF = vesicular fraction.

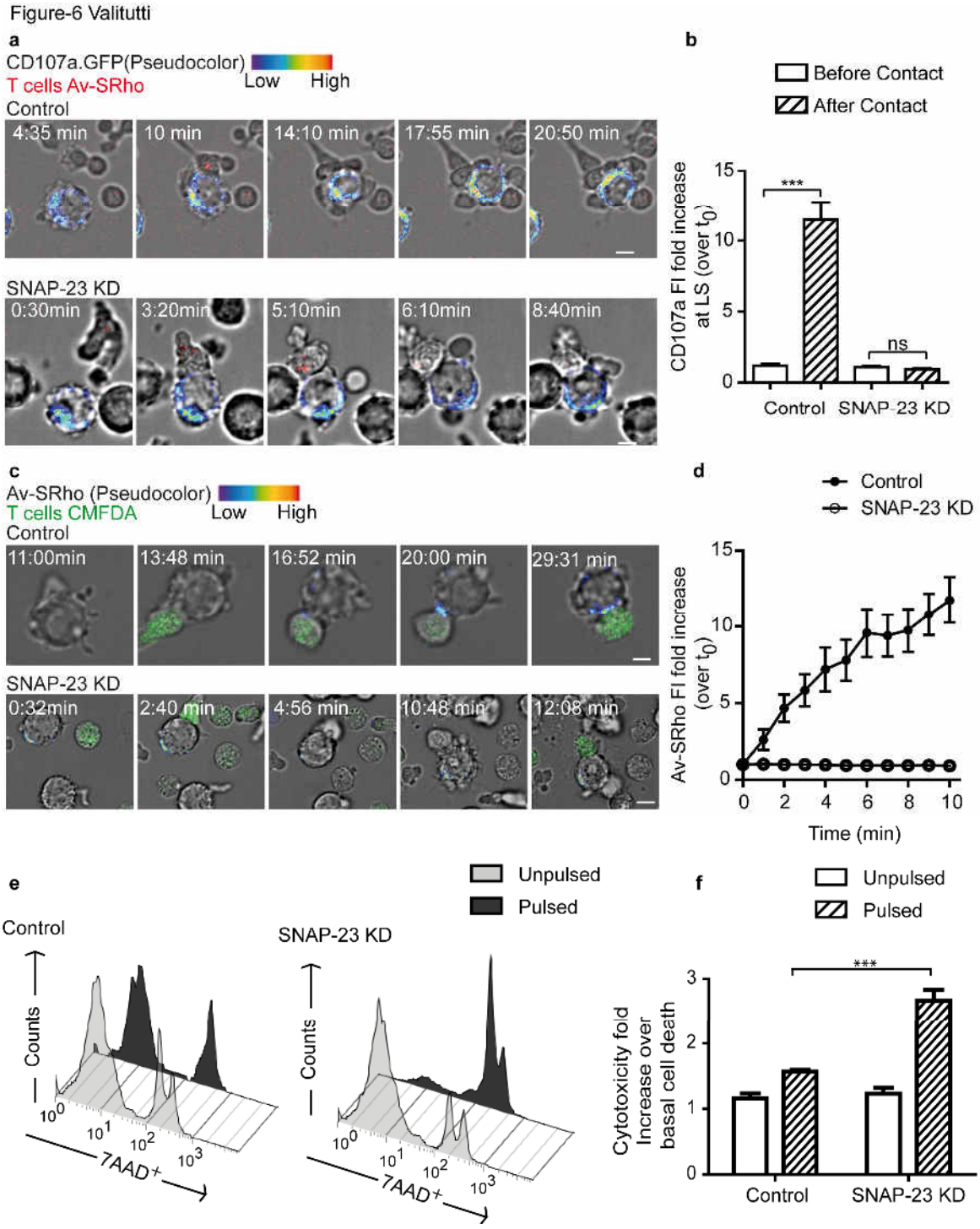


Fig. 6. **Interference with melanoma cell vesicular trafficking enhances their susceptibility to perforin-mediated cytotoxicity.** a) Upper panel: Sequence of snap shots depicting dynamics of CD107a/GFP+ intracellular vesicles (pseudo color scale intensity) in control shRNA transfected 10μM peptide-pulsed melanoma cell during conjugation with CTL. Snapshots are from Supplementary Movie 13. Lower panel: Sequence of snap shots depicting dynamics of CD107a/GFP+ intracellular vesicles (pseudo color scale intensity) in SNAP-23 silenced peptide-pulsed melanoma cell during its conjugation with a CTL. Snapshots are from

Supplementary Movie 11. CTL lytic granules are stained in red. b) Quantification of CD107a fluorescence intensity (FI) fold increase at the lytic synapse of 8 SNAP-23 silenced peptide-pulsed melanoma cells conjugated with CTL either before or 5 minutes after cell-cell conjugation. Data are from two independent experiments. c) Upper panel: sequence of snap shots depicting LLE vesicle exocytosis of control shRNA transfected peptide-pulsed melanoma cells interacting with CMFDA loaded CTL (green). Lysosomal exposure is detected via Av-SRho binding (pseudo color scale intensity). Snapshots are from Supplementary Movie 14. Lower panel: sequence of snap shots depicting LLE vesicle exocytosis of a SNAP-23 silenced peptide-pulsed melanoma cell interacting with CMFDA loaded CTL (green). Lysosomal exposure is detected via Av-SRho binding (pseudo color scale intensity). Snapshots are from Supplementary Movie 12. d) Measurement of Av-SRho fluorescence intensity fold increase in SNAP-23 silenced peptide pulsed melanoma cells during conjugation with CTL. Intensities were scored from 8 conjugates in three independent experiments. Data are expressed as mean ± SEM of the scored cells. e) and f) Cytotoxicity was evaluated in melanoma cells either transduced with control non-targeting shRNA (shCtr) or with shRNA targeting SNAP-23 (shSNAP-23) following 4 hour incubation with CTL. Melanoma cells were either unpulsed or peptide pulsed and cytotoxicity was quantified by measuring 7-AAD uptake by FACS analysis. e) A representative experiment is shown; f) pooled data from three independent experiments. Cytotoxicity is expressed as fold increase over corresponding basal death in transduced cells. Bars, 5μm. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. ***$P<0.001$.

Figure-7 Valitutti

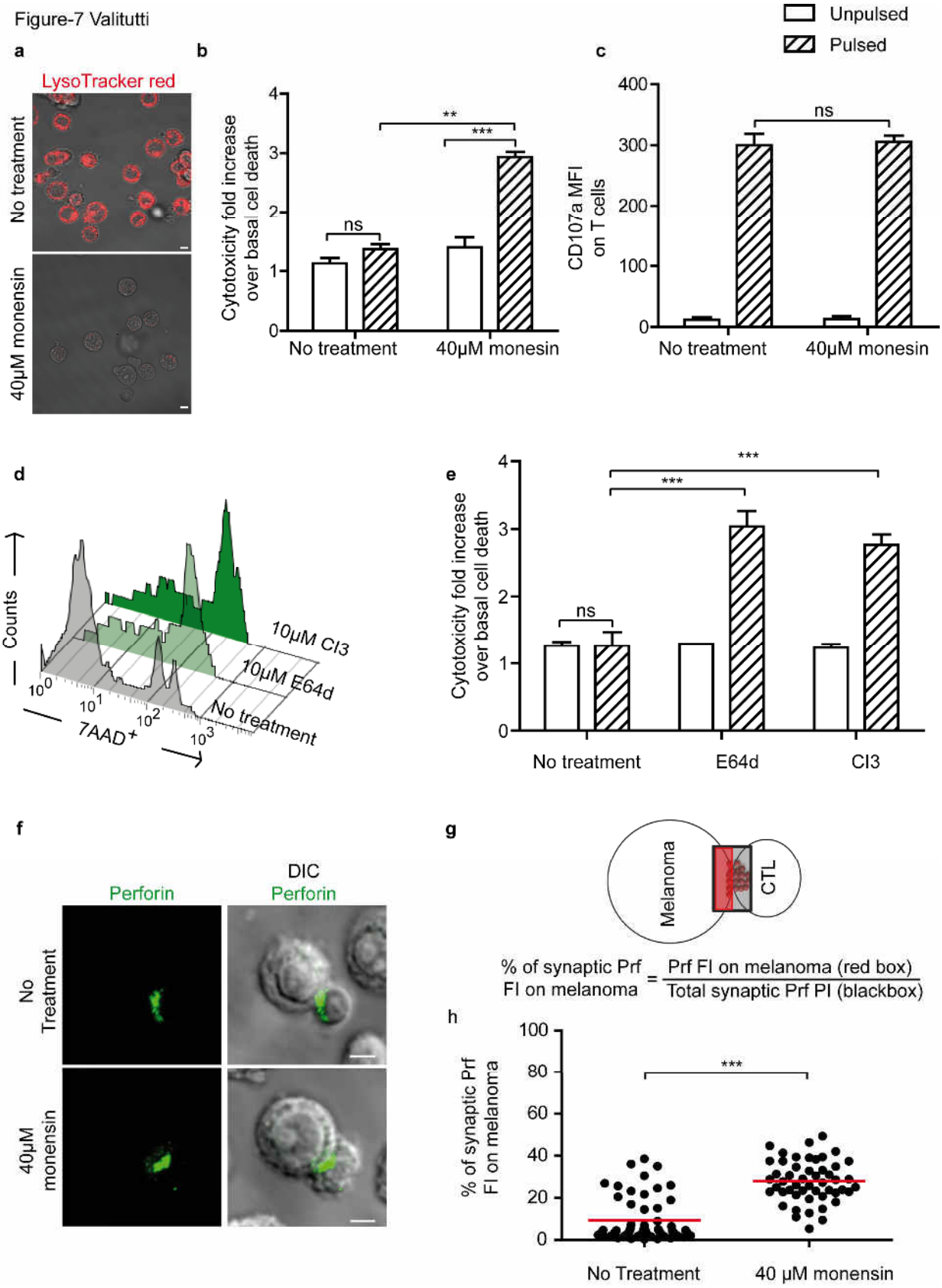
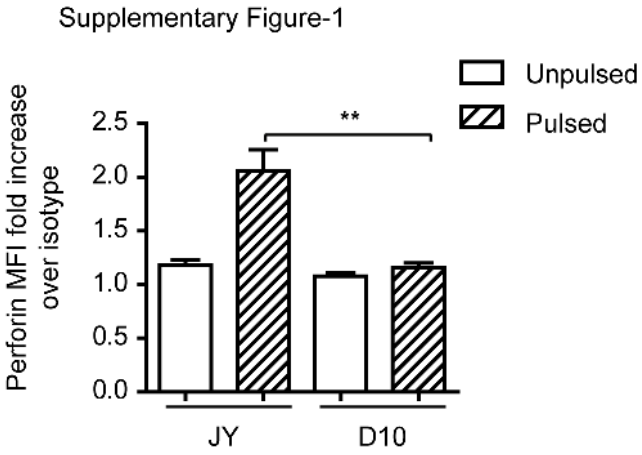


Fig. 7. **Impairment of melanoma cell lysosome function enhances their susceptibility to perforin mediated cytotoxicity.** a) Cells were either untreated or treated for 2 hours with 40μM monensin, washed and stained with lysotracker red. Panels show typical snap shots of treated and untreated melanoma cells. b) Cytotoxicity was evaluated by FACS analysis in melanoma cells following 4 hour incubation with CTL. Melanoma cells either pre-treated or not with 40μM monensin for 2 hours before conjugation were either unpulsed or pulsed with 10μM antigenic peptide. Cytotoxicity is expressed as % of positive 7-AAD cells. c) Efficient activation of CTL to lethal hit delivery after encountering melanoma cells treated or not with monensin. Target cells, either pretreated or not with 40μM monensin were conjugated for 1 hour with specific CTL. Cells were surface stained for CD107a. d) and e) Cytotoxicity was evaluated in melanoma cells following 4 hour incubation with CTL. Melanoma cells were either untreated or treated with E64d or CI3 protease inhibitors. Melanoma cells were either unpulsed or peptide pulsed and cytotoxicity was quantified by measuring 7-AAD uptake by FACS analysis. d) A representative experiment is shown; e) pooled data from three independent experiments. Cytotoxicity is expressed as % of positive 7-AAD cells. f) Perforin quanta (green) at the lytic synapse after 5 minutes conjugation with CTL of peptide-pulsed melanoma cells (either untreated or pre-treated with 40μM monensin). g) Scheme depicting the fluorescence intensity quantification strategy for perforin deposits at the lytic synapse of melanoma cell/CTL conjugates. h) Quantification of percentage of perforin fluorescence intensity on melanoma cell side of the lytic synapse in peptide-pulsed melanoma cells either pre-treated or not with 40μM monensin. Red bars indicate mean values of measured fluorescence. 54 and 55 conjugates (for untreated and monensin-treated cells respectively) formed by 1 melanoma cell and 1 CTL were scored. Data are from 3 independent experiments. In b, c and e results are expressed as mean ± SEM of 3 independent experiments. Bars, 5μm. Unpaired Student's t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups. ***P<0.001, **P<0.01, nsP>0.05.

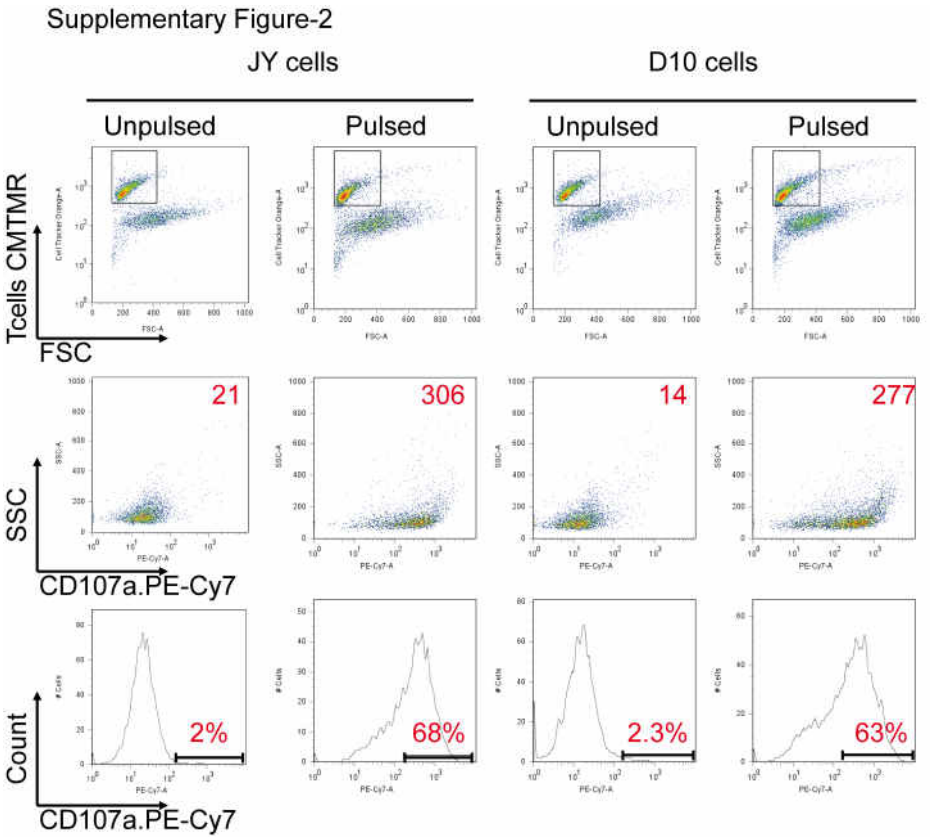
Supplementary Information

Melanoma cell lysosome secretory burst neutralizes CTL synaptic lethal hit delivery by cathepsin-mediated perforin degradation

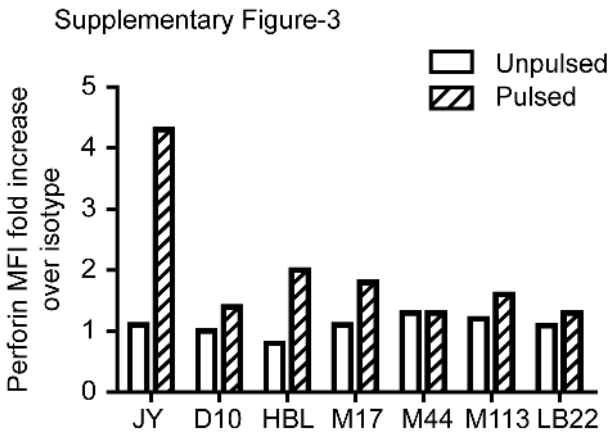
Roxana Khazen, Sabina Müller, Nicolas Gaudenzio, Eric Espinosa, Marie-Pierre Puissegur and Salvatore Valitutti



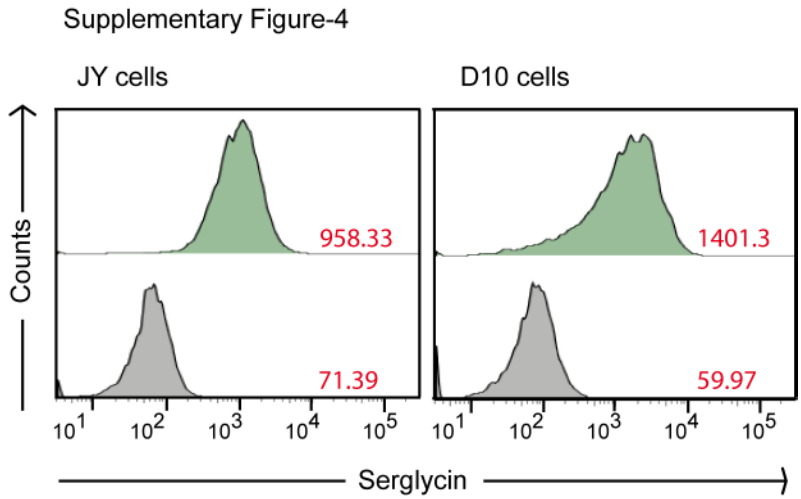
Supplementary Figure 1. **Limited perforin staining on melanoma cells.** Perforin staining on the surface of conventional target cells (JY) and melanoma cells (D10) following conjugation with CTL. Target cells were either unpulsed or pulsed with10µm antigenic peptide. The fold increase of median fluorescence intensity of the entire cell population over the isotype control is shown. Results are expressed as mean ± SEM of 6 independent experiments. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. **P<0.01.



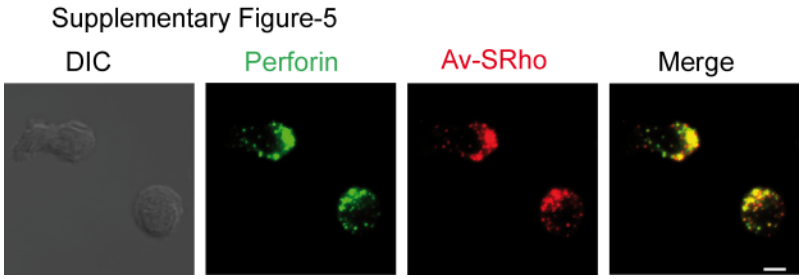
Supplementary Figure 2. **Expression of CD107a on the surface of CTL following interaction with melanoma cells or with conventional target cells.** The gating strategy and typical results of CD107a up-regulation on the surface of CTL interacting for 1 hour with either JY cells or with D10 cells either unpulsed or pulsed with 10μM peptide concentration is shown. Data are from one representative experiment out of three.



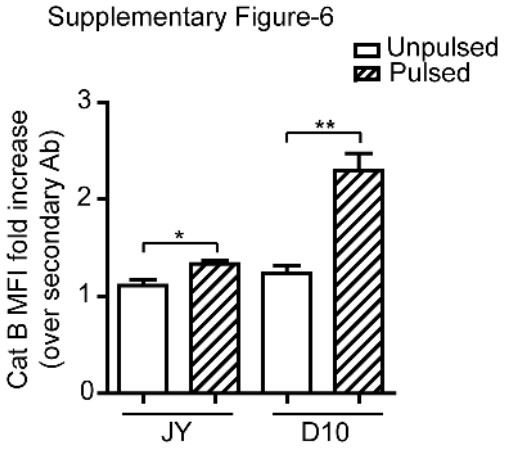
Supplementary Figure 3. **Perforin staining on several melanoma cell lines.** D10, HBL, M17, M44, M113, LB22 and JY cells were either unpulsed or pulsed with 10µM antigenic peptide. Cells were conjugated with CTL for 1 hour. Cells were assessed for perforin staining on their surface and analyzed by FACS. Data are from one representative experiment out of two.



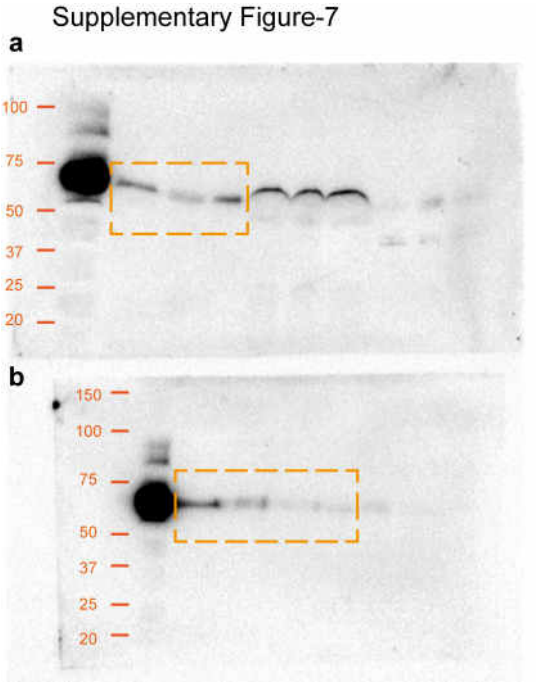
Supplementary Figure 4. **Serglycin expression in target cells.** Typical plots of intracellular staining for serglycin in D10 and JY cells are shown. Results are from one representative experiment out of two performed in triplicates.



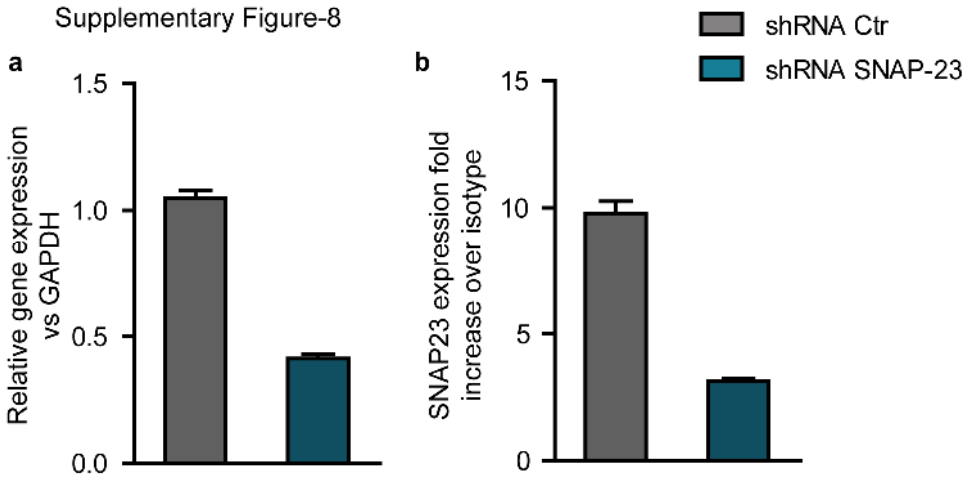
Supplementary Figure 5. **Av-SRho staining in CTL can be used as a marker of lytic granules.** CTL were cultured in the presence of 8µg/ml of Av-SRho (red) overnight. Cells were fixed, permeabilized and stained for perforin (green).



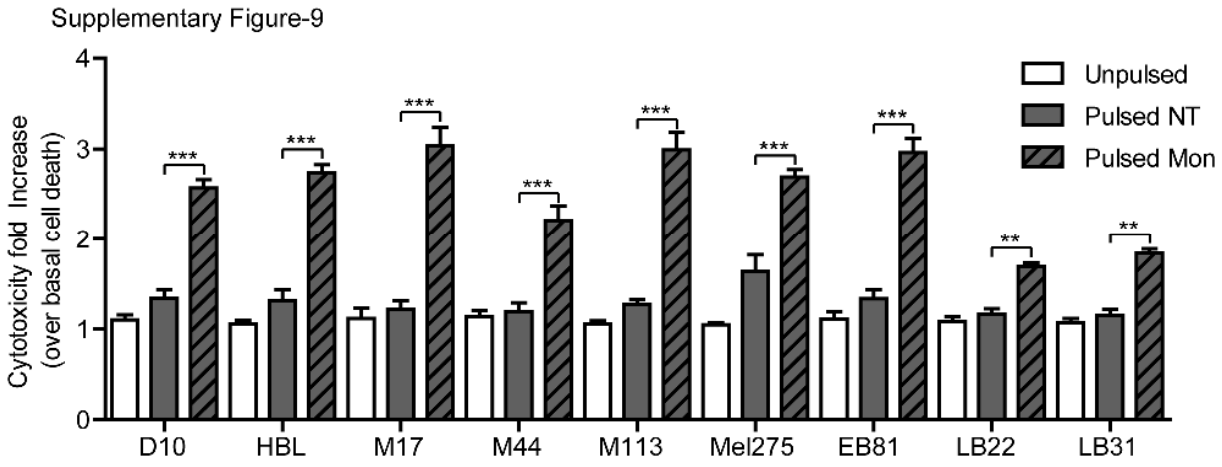
Supplementary Figure 6. **Cathepsin B exposure on the surface of target cells upon interaction with CTL.** JY cells and D10 cells either unpulsed or pulsed with 10μM peptide were conjugated for 15 minutes with CTL and stained for surface expression of cathepsisn B. The fold increase of median fluorescence intensity of the entire cell population over the secondary antibody is shown. Results are expressed as mean ± SEM of 3 independent experiments. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. **P<0.01; *P<0.05.



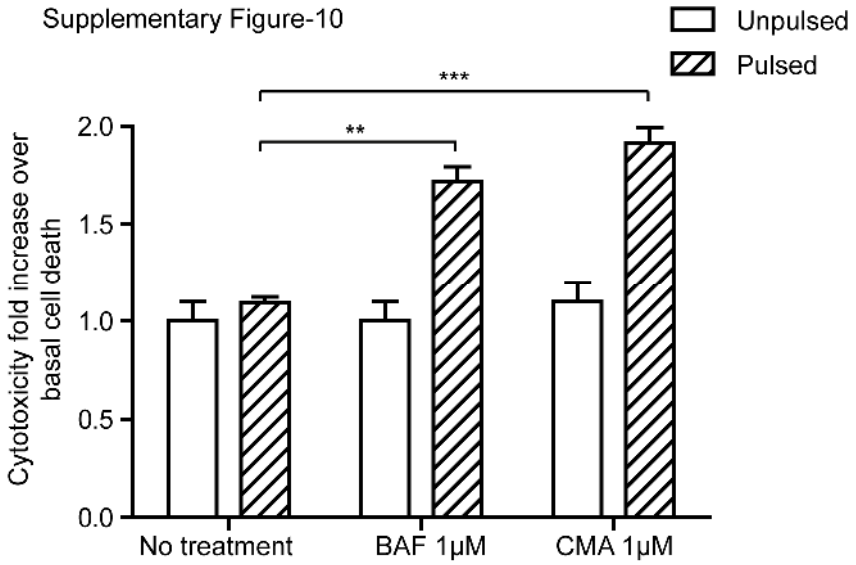
Supplementary Figure 7. **Uncropped Western Blots related to Figure 5.** The original Western Blots for results shown in Figure 5c and 5d are shown in panel a and b respectively.



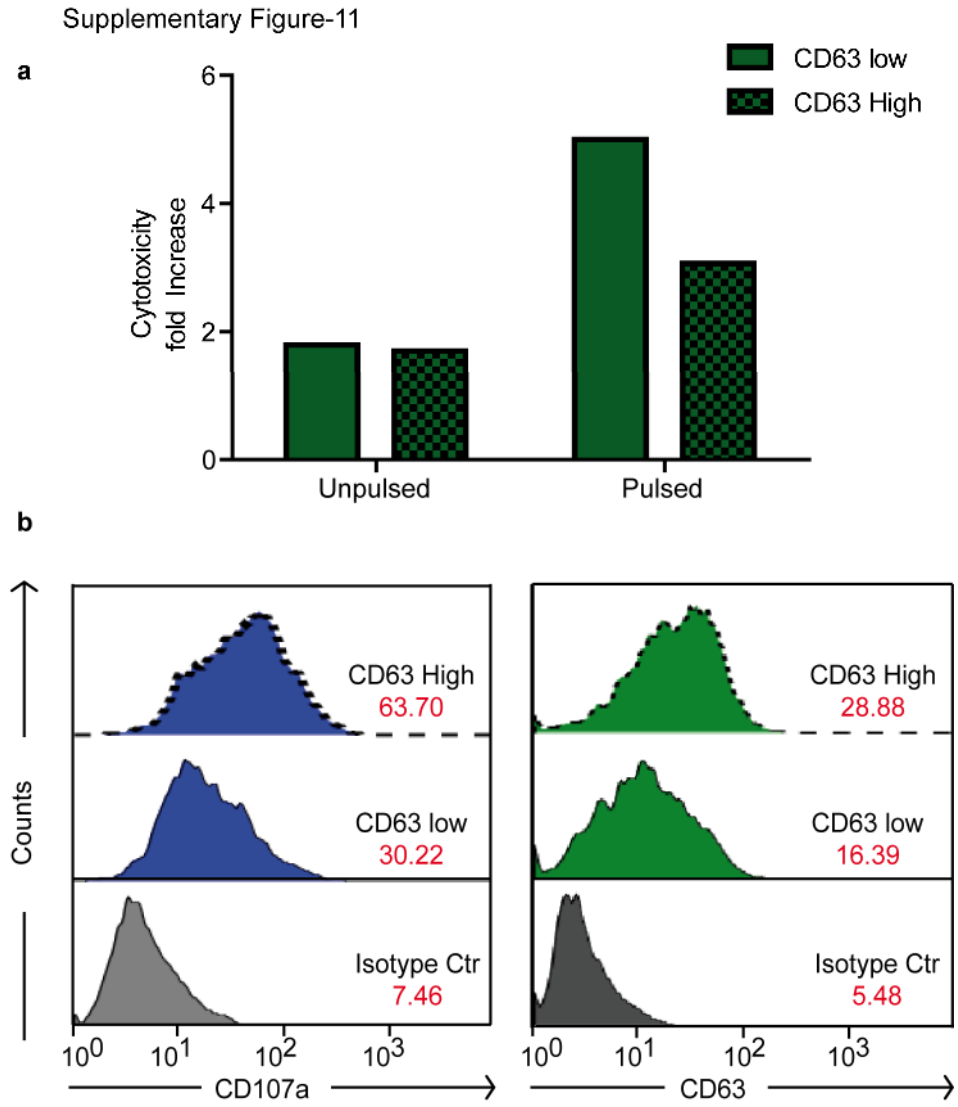
Supplementary Figure 8. **Reduction of SNAP-23 expression in melanoma cells transfected with SNAP-23 shRNA. a**) Reduced SNAP-23 gene expression as detected by RT-PCR. **b**) Reduced SNAP-23 protein expression as detectd by FACS analysis using an anti-SNAP-23 antibody. Results are from one representative experiment out of two performed in triplicates.



Supplementary Figure 9**. Treatment with monensin enhances CTL-mediated cytotoxicity in nine melanoma cell lines.** Melanoma either untreated (NT) or pretreated for 2 hours with monensin (Mon) were conjugated with specific CTL for 4 hours. Cells were either unpulsed of pulsed with 10µM antigenic peptide. Cytotoxicity is reported as fold increase over basal cell death. Results are expressed as mean ± SEM of 3 independent experiments. Two-Way Anova test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. ***P<0.001; **P<0.01.



Supplementary Figure 10**. Treatment of melanoma cells with bafilomycin A1 or concanamycin A enhances CTL-mediated cytotoxicity.** Melanoma cells either untreated or pretreated for 2 hours with bafilomycin A1 (BFA) or concanamycin A (CMA) were conjugated with specific CTL for 4 hours. Cells were either unpulsed of pulsed with 10µM antigenic peptide. Cytotoxicity is reported as fold increase over basal cell death. Results are expressed as mean ± SEM of 3 independent experiments. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. ***P<0.001; **P<0.01.



Supplementary Figure 11. **The resistance of conventional target cells to CTL-mediated cytotoxicity correlates with the level of CD63 surface expression. a)** JY cells previously sorted on the level of expression of CD63 were pulsed or not with antigenic peptide and co-incubated with CTL for 4 hours. Cytotoxicity was evaluated by FACS analysis using 7-AAD uptake and is reported as fold increase over basal cell death. **b)** To evaluate the level of LLE markers expression by cells used in a), the intracellular expression of CD107a and CD63 of sorted cells was measured by FACS analysis. Numbers indicate median fluorescent intensity (MFI). Data are from one representative experiment out of two.

Supplementary movie legends:

Movie 1. The video shows the interaction between a peptide pulsed JY cell and tubulin tracker green loaded CTL for 1 hour in the presence of PI (red). Cells were inspected using a confocal laser-scanning microscope (LSM 510; zoom factor 63X) to monitor PI entry. Recording time is indicated in minutes in upper left corner.

Movie 2. The video shows the interaction between a peptide pulsed D10 cell and tubulin tracker green loaded CTL for 1 hour in the presence of PI (red). Cells were inspected using a confocal laser-scanning microscope (LSM 510; zoom factor 63X) to monitor PI entry. Recording time is indicated in minutes in upper left corner.

Movie 3. JY cells were seeded in chambered wells in the presence of 8µg/ml Av-SRho (pseudo color) and monitored for 2 hours using a confocal spinning disk microscope to visualize constitutive vesicular exocytosis. A typical cell is shown; data are representative of three independent experiments.

Movie 4. D10 cells were seeded in chambered wells in the presence of 8µg/ml Av-SRho (pseudo color) and monitored for 2 hours using a confocal spinning disk microscope to visualize constitutive vesicular exocytosis. Three typical cells are shown; data are representative of three independent experiments.

Movie 5. The video shows interaction between a peptide pulsed D10 cell expressing CD107a-GFP (pseudo color) and two CTL (previously loaded with Av-SRho, red). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 6. The video shows interaction between a peptide pulsed D10 cell interacting with a CTL (previously loaded with CMFDA, green) in the presence of 8µg/ml Av-SRho (pseudo

color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 7. The video shows interaction between unpulsed D10 cells interacting with CTL (previously loaded with CMFDA, green) in the presence of 8µg/ml Av-SRho (pseudo color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 8. The video shows a peptide pulsed D10 cell interacting with a CTL (that was previously loaded with Av-Alexa488, green) in the presence of 8µg/ml Av-SRho (red). Cells were inspected using a confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 9 and 10. The videos show interactions between peptide pulsed JY cells with a CTL (previously loaded with CMFDA, green) in the presence of 8µg/ml Av-SRho (pseudo color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 11. The video shows interactions between SNAP-23 silenced peptide-pulsed D10 cells expressing CD107a-GFP (pseudo color) and a CTL (previously loaded with Av-SRho, red). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 12. The video shows interactions between SNAP-23 silenced peptide-pulsed D10 cells interacting with CTL (previously loaded with CMFDA, green) in the presence of 8µg/ml Av-SRho (pseudo color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 13. The video shows interactions between control shRNA transfected peptide-pulsed D10 cells expressing CD107a-GFP (pseudo color) and a CTL (previously loaded with Av-SRho, red). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 14. The video shows interactions between control shRNA transfected peptide-pulsed D10 cells interacting with CTL (previously loaded with CMFDA, green) in the presence of 8µg/ml Av-SRho (pseudo color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 15. The video shows interactions between control shRNA transfected peptide-pulsed D10 interacting with CTL (previously loaded with Av-SRho, red) in the presence of 8µg/ml Av-Alexa488 (pseudo color). Cells were inspected using confocal spinning disk microscope. Recording time is indicated in minutes in upper left corner.

Movie 16 and **17.** Movies show 3-D reconstructions of conjugates formed between CTL conjugated for 5 minutes with melanoma cells either untreated (Movie 16) or treated with monensin (Movie 17). Cells were stained for perforin (green).

Movie 18-20. Movies show 3-D reconstructions of conjugates formed between CTL conjugated for 5 minutes with melanoma cells either untreated (Movie 18) or pretreated with monensin (Movie 19 and 20). Cells were stained for perforin and CD107a (blue) The 3-D reconstructed images were processed using the Imaris software to show in green the perforin co-localizing with CD107a (mostly found intracellularly in CTL only), and in red the perforin not colocalizing with CD107a (detected extracellularly in conjugates in which melanoma cells were previously treated with monensin).

Movie 21. The videos show interactions between peptide pulsed JY cells (preloaded with Fluo-4) with CTLs (previously loaded with CMFDA (green) and Lysotracker red). Cells were

inspected using confocal microscope. Recording time is indicated in minutes in upper left corner.

Movie 21. The videos show interactions between peptide pulsed JY cells (preloaded with Fluo-4) with monensin pretreated CTLs (previously loaded with CMFDA (green) and Lysotracker red). Cells were inspected using confocal microscope. Recording time is indicated in minutes in upper left corner.

Movie 23. The videos show interactions between peptide pulsed D10 cells with CTLs (previously loaded with Tubulin tracker (green) in the presence of 8µg/ml Av-SRho (pseudo color). Cells were inspected using confocal microscope. Recording time is indicated in minutes in upper left corner.

IV- Related questions

Q.1) Is LLE exposure on melanoma cells a direct response to perforin action or is it related to cell-cell contact?

Having observed that specific interaction of CTL and melanoma cells induce LLE exposure, we were interested to know whether such self-defense response was triggered by cognate cell-cell contact or it was related to direct perforin action on melanoma cell surface.

A) LLE exposure from melanoma cell requires formation of a "lytic" synapse.

A.1 Technical approach for IS and LS dissociation.

We set up a method to dissociate stimulatory and lytic synapses (Refer to introduction chapter X; [156]).

It is well studied that intracellular organelles involved in endocytic and exocytic pathways are acidified through V-ATPase and that the internal acidification plays an important role in these pathways [436]. Monensin, is a monocarboxylic ionophore that exchanges Na^+/K^+ with proton and consequently neutralize the acidic pH of endocytic vesicles[437-439]. Moreover, it has been previously shown that monensin pretreatment inhibits NK cytotoxicity[440]. Later on, the importance of acidic environment of CTL lytic granules for their perforin mediated cytotoxicity was reported using CMA (a specific inhibitor of V-ATPase that is known to impair vesicular pH;[441]).Therefore we tested the effect of monensin pre-treatment on CTL cytotoxicity and synapse formation.

A.1.1 Monensin impairs CTL cytotoxicity.

In a first approach, we examined the effect of monensin treatment on cytotoxic responses of CTL encountering either unpulsed or peptide-pulsed conventional target cells. CTLs were pretreated with 40µM monensin for 2 hours and were thoroughly washed before conjugation with target cells. Cytotoxicity of CTL clones was evaluated in a classical 4 hours killing assay using 7-AAD. As shown in Figure IV-1a pretreatment of CTLs with monensin significantly reduced their cytotoxic activity.

A-1-2) Monensin does not alter activation induced CTL cytokine production and lytic granule exposure.

The observation of such strong inhibition of CTL-mediated cytotoxicity prompted us to investigate whether pre-treatment with monensin would affect CTL responses to antigenic stimulation. We observed that, neither IFN-γ production nor CD107a exposure was altered upon CTL pretreatment with monensin (Figure IV-1b and IV-1c). These results indicate that, although CTL cytotoxicity is strongly impaired, monensin treatment does not substantially influence CTL activation in response to recognition of specific targets.

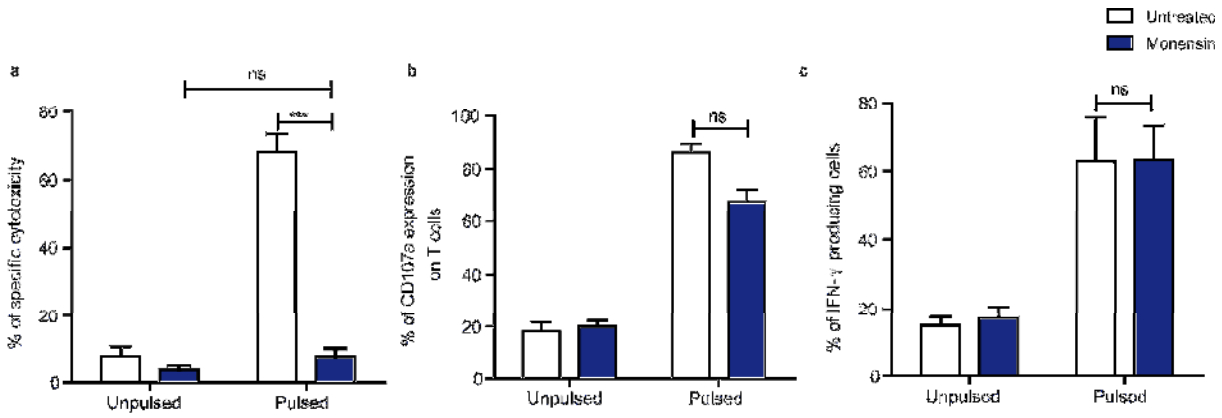


Figure IV-1. **Monensin CTL pre-treatment inhibits cytotoxicity of CTL but does not alter CTL activation. a**) Measurement of target cell killing. Cytotoxicity was evaluated by FACS analysis in target cells either pulsed or not with antigenic peptide following 4 hour incubation with CTL (CTLs were either untreated or pre-pretreated with 40μM monensin for 2h in 37°C, the drug was thoroughly washed before conjugation with target cells). Percentage of specific cytotoxicity is shown. **b**) Efficient degranulation of untreated and monensin pre-treated CTLs following encounter with peptide-pulsed target cells. Target cells either unpulsed or pulsed with antigenic peptide were conjugated for 1 hour with CTL. Cells were stained for CD107a extracellular exposure at 4°C. **c**) Efficient IFN-γ production in untreated and monensin treated CTLs. Target cells either unpulsed or pulsed with antigenic peptide were conjugated for 4 hours with CTL. Cells were stained for IFN-γ secretion. Results are expressed as mean ± SEM of 7 independent experiments. ***$P < 0.001$, $^{ns}P>0.05$

A-1-3) Monensin does not alter CTL polarization response at the IS.

We next investigated the polarization of tubulin cytoskeleton of monensin pretreated or untreated CTLs toward the IS formed with target cells. CTLs were co-cultured for 5 minutes

with peptide pulsed conventional target cells, fixed, permeabilized, and stained for α-tubulin and phosphotyrosine (pTyr). CTLs interacting with pulsed conventional target cells (independently of monensin treatment), indeed formed a signal inducing immunological synapse and polarized their secretory machinery toward target cells, as detected by pTyr staining at the CTL/target cell contact site and reorientation of the MTOC beneath the synaptic area respectively (Figure IV-2a and IV-2b).

Measurement of the distances between the center of the IS and the T cell MTOC in a large number of conjugates showed that the CTL MTOC polarization toward target cells occurred to a similar extent regardless of monensin pretreatment of CTLs (Figure IV-2b). The approach used for the measurement of the T cell MTOC distance from the IS was previously described[159].

A-1-4) Monensin treatment induce perforin conformational change via pH perturbation.

Having observed similar reorientation of microtubules of monensin treated and untreated CTLs toward target cells, we studied the content and integrity of lytic granules of CTLs under these conditions and during interaction with target cells. Monensin pretreated or untreated CTLs were co-cultured with peptide pulsed target cells. After fixation, permeabilization, and staining, lytic granule status was evaluated. As shown in Figure IV-2c, lytic granule polarization at the IS was similar as detected by CD107a enrichment towards target cells (Figure IV-2c and IV-2d). However, in CTLs pretreated with monensin we could not detect granzyme B penetration in target cell cytosol indicating that monensin treatment inhibited perforin-mediated granzyme B penetration (i.e. perforin pore formation) in target cells (Figure 2c and 2e). Under the same condition we failed to detect perforin staining using anti-perforin antibody clone dG. Interestingly, using another antibody for perforin detection (clone pf80) revealed perforin staining in these conjugates (Figure IV-2f), indicating that perforin is still present in CTL but under a different conformational status not detectable by the dG9 antibody.

Moreover, to confirm pH perturbation in CTLs by pretreatment with monensin, we investigated CTL/target cells conjugates using video microscopy. CTLs were pretreated or not for 2 hours with monensin, and their lytic granules were stained with lyso-tracker red (a pH dependent dye). As it is shown in Figure IV-2g lyso-tracker red failed to stain (or barely stained) the lytic granules of monensin pretreated CTLs, validating the pH perturbation action exerted the drug. Moreover, in agreement with the above results, independently of monensin pretreatment, both CTLs were capable or recognizing their

target cells and repolarize their MTOC towards the IS as detected by tubulin tracker staining (green) (Figure IV-2g).

All in all, the above results show that the acidic environment is essential for keeping the proper conformational state of perforin in the lytic granules before delivery to the synapse. It is tempting to speculate that such conformation is necessary to allow perforin to acquire an active form that is able to bind and polymerize on the surface of target cell.

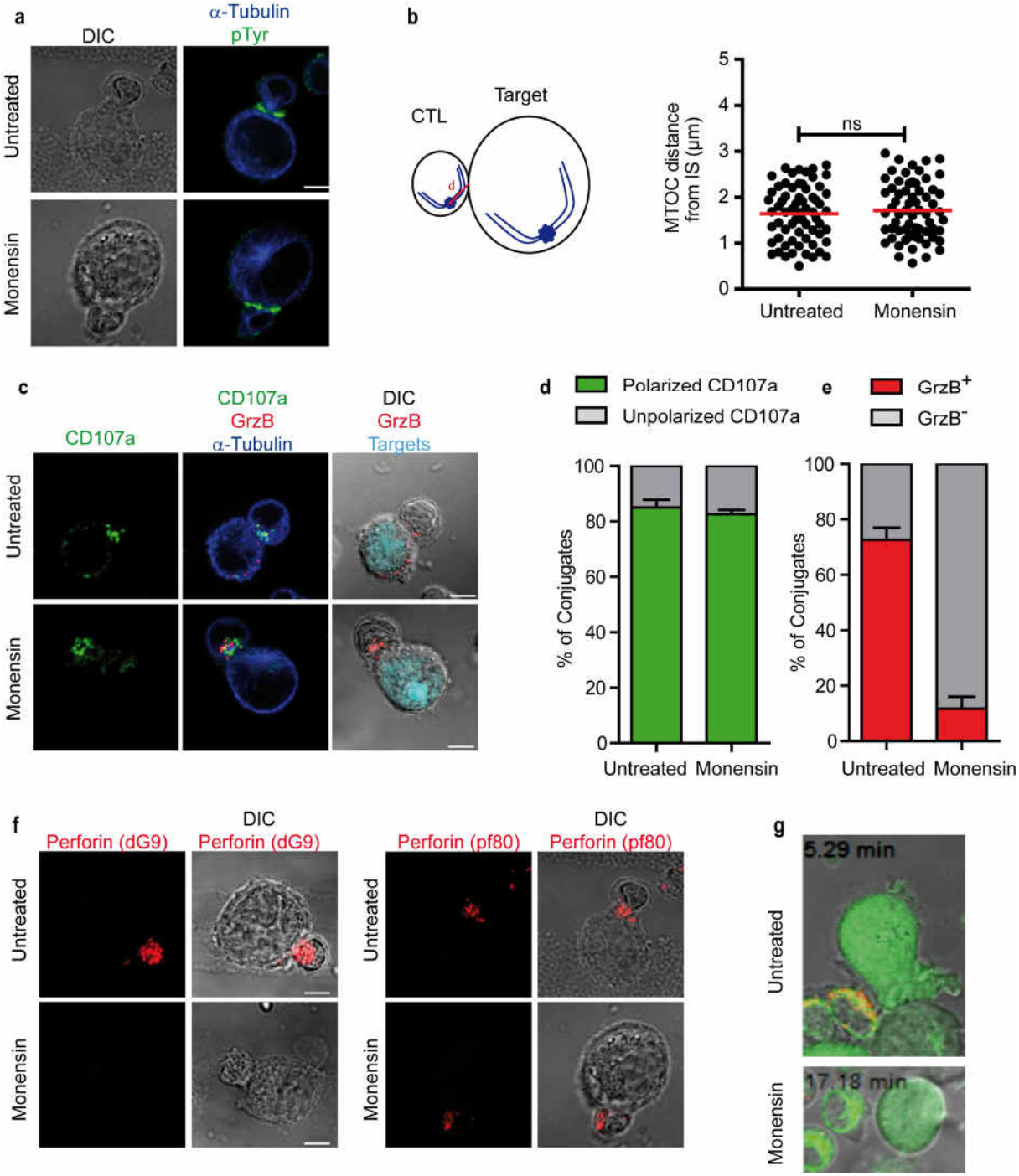


Figure IV-2.**Monensin alters perforin conformation without impairing T cell polarization responses at the IS.** a) Peptide-pulsed target cells were conjugated for 5 minutes with CTLs (either untreated or pretreated with monensin). Cells were fixed, permeabilized, and stained for α-tubulin (blue) and pTyr (green). Bars, 5μm. b) Synaptic re-localization of MTOC was quantified by measuring the distances between the center of the IS and the T cell MTOC (as shown by red line in the scheme) using ImageJ software. Bars indicate mean values. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. C) Under similar conditions cells were stained for CD107a (green), α-tubule (blue) and granzyme B (red). Target cells' were identified by loading them with Cell Tracker Blue before conjugation (cyan). d) Percentage of CD107a polarized CTLs (untreated or monensin treated) in conjugate with peptide pulsed target cells are indicated. e) Percentage of granzyme B positive ($GrzB^+$) peptide pulsed target cells are in conjugate with either untreated or monensin treated CTLs are indicated. f) Similarly to a and c, cells were stained for perforin (red) using two different clones of anti-human perforin antibody (*Right panel:* dG9; *Left panel:* pf80). g) CTLs were stained with tubulin tracker green and lysotracker red (pH dependent) and conjugated with peptide pulse target cells. Snap shots are from supplementary movie 21 and 22. In a, d and e 60 conjugates were scored. Data are from three independent experiments.

A.2) Melanoma cells interacting with monensin treated CTLs do not expose their LLE compartments.

Using the above described technique, we have investigated whether monensin treated CTLs (containing non functional perforin molecules) could trigger LLE secretory burst in melanoma cell. Using time-lapse video microscopy we studied conjugates between monensin-pretreated CTLs and peptide pulsed melanoma cells for 1hour in the presence of Avi-SRho (for LLE exposure). As shown in figure IV-3, specific recognition of target cell results in formation of an effective IS between the two cells that as detected by polarization of CTL MTOC towards melanoma cells (Figure IV-3 and supplementary Movie 23). Melanoma cells did not expose their LLE compartments (since we did not detect any Avi-SRho staining), probably as a consequence of the absence of a functional perforin in CTL (compare to Figure 4 and Supplementary Movie 6 and 15).

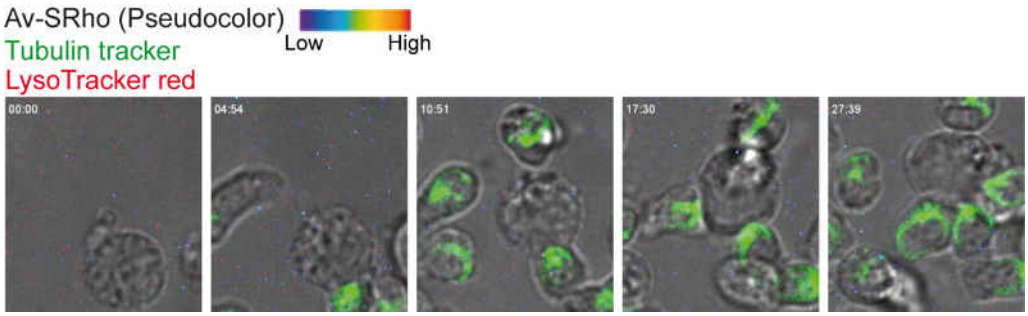


Figure IV-3.: **Melanoma cells do not expose their LLE compartments during interaction with monensin pre-treated CTLs.** Sequences of snap shots depicting monensin treated CTLs (loaded with tubulin tracker green) and peptide pulse melanoma cells in the presence Av-SRho. No lysosomal exposure was detected via Av-SRho staining (pseudo color scale intensity). Snapshots are from supplementary movie 23.

B) LLE exposure on melanoma cells is triggered by perforin binding.

B.1) Melanoma cells expose their LLE compartments in the presence of purified perforin.

To confirm the relationship between LLE secretory burst and perforin binding on melanoma cell surface, we thus monitored, by time-lapse microscopy the exocytosis of LLE vesicles in melanoma cells by adding purified perforin to the medium in the presence of Av-SRho. This analysis revealed that perforin addition enhanced exposure of lysosomal vesicles on melanoma cell surface that was paralleled by a rapid $[Ca^{+2}]_i$ increase when compared to constitutive LLE exposure on melanoma cells (no perforin in culture medium).

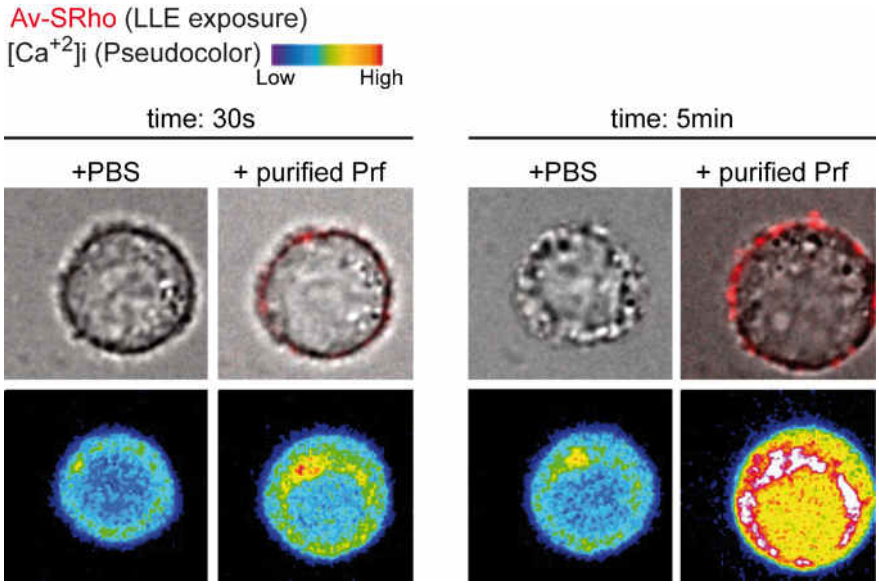


Figure IV-4. **Purified perforin induces LLE exposure in melanoma cells.** *Upper panels:* LLE exocytosis was visualized via Av-SRho uptake by melanoma cells in the presence of either PBS or 125ng purified perforin (prf) in the culture medium. *Lower panels:* Detection of $[Ca^{+2}]_i$ increase in melanoma cells loaded with Fluo-4 AM (in pseudo color scale).

We are presently finalizing the above results that we are planning to submit in the future in a short paper aiming at better characterize the link between CTL lytic activity and melanoma cell defense.

Q.4) Are there other molecules implicated in vesicular trafficking of melanoma cells involved in their self defense mechanism?

Given the importance of vesicular trafficking in melanoma cell self defense against CTL attack, it is important to identify other effector molecules that are contributing to this self defense mechanism. Using a large panel of shRNA plasmids targeting various key elements of melanoma vesicular trafficking, we evaluated their influence in melanoma cell resistance to CTL attack. To this aim, CTLs were conjugated with peptide pulsed melanoma cells and cytotoxicity was assessed in a 4 hours classical killing assay. As shown in figure IV-5, downregulation of CD107a, VAMP-7 as well as SNAP-23 expressions in melanoma cells enhanced their susceptibility to CTL attack. Further investigations may identify other molecules implicated solely in melanoma cells LLE trafficking.

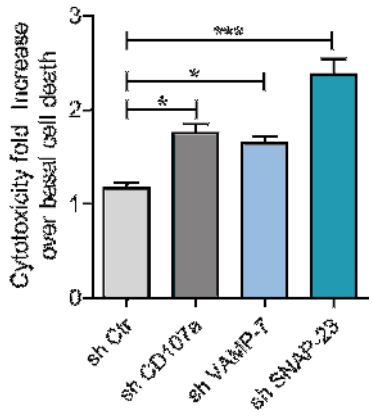


Figure IV-5. **Interference with melanoma cell vesicular trafficking enhances their susceptibility to perforin-mediated cytotoxicity.** Cytotoxicity was evaluated in peptide pulsed melanoma cells either transduced with control non-targeting shRNA (shCtr) or with shRNA targeting SNAP-23 (shSNAP-23), CD107a (sh CD107a) or VAMP-7 (sh VAMP-7) following 4 hour incubation with CTL. Cytotoxicity was quantified by measuring 7-AAD uptake by FACS analysis and is expressed as fold increase over corresponding basal death in transduced cells. Unpaired Student's t-test using the GraphPad Prism software (version 6; GraphPad) was used to determine the statistical significance of differences between the groups. ***P<0.001; *P<0.05.

V- Discussion and Perspective:

The interest of our experimental model:

In this study we have investigated the molecular crosstalk occurring during early CTL/melanoma cell interaction. To this aim, we set up a cellular model allowing *in vitro* evaluation of the response that human metastatic melanoma cell lines might provide when encountering fully activated antigen specific CTL clones. *In vitro* experiments using human cells have the advantage to allow a detailed observation of cell contact site as well as to enable precise quantification of molecular expression and localization in parallel to their interactions by employing reagents and readouts that can be directly transferred to clinical research and practice. In other words, such studies provide high resolution outcomes that are thoroughly reproducible and consistent. However, this approach has the obvious limitation of simplifying the scenario of a physiological immune response based on multiple and heterogeneous cellular interactions (detailed in chapter "Cancer biology and tumor immunology").

The Objective of this thesis was to focus mainly on the melanoma cell side of the lytic synapse, and study the responses of individual melanoma cells to CTL attack outside the context of tumor microenvironment that may influence T cell activity. Given the complexity of CTL/tumor cell interaction, we focused on a system in which CTLs would be capable of, first, recognizing melanoma cells as specific targets and, second, being fully activated upon contact with melanoma cells. We thus used T clones specific for viral antigen that we previously characterized for their extremely high efficacy in responding to peptide-pulsed melanoma cells. The use of cells pulsed with antigenic peptide is a useful method to bypass the necessity for antigen processing and exposure on the cell surface and is a method that is largely used in the laboratory of Dr. Valitutti since his initial work at the Basel Institute in the laboratory of Dr. Lanzavecchia[7,442] .

As discussed in the introduction, activation of CTLs is a very efficient phenomenon with high degree of sensitivity and rapidity that is initiated upon recognition of as few as 2-3 pMHC complexes at the cell-cell interface. TCR-mediated signaling is followed by a lethal hit delivery that occurs extremely rapid [159,238,247] [443]and that has an extraordinary low threshold (pico/nano molar concentration of specific peptide used for pulsing). The efficacy of cytotoxic activity of CTLs is reinforced by their rapidly detachment and recycling behavior that endows them with

simultaneous/serial killing capacity [159,293]. In our cellular model we used a saturating concentration of an antigenic peptide from CMV pp65 protein that, during the 2 hours of incubation, is loaded into the MHC class I molecules of target cells, due to its high affinity for the peptide groove of the HLA-A2 molecules[442,444]. Thus, in our system target cells are displaying on their surface high densities of specific pMHC recognized by our CTL clones. It's generally accepted that one key processes of cancer cells escape from immune surveillance is MHC class I molecules downregulation [351,445]. Nevertheless, results I obtained during my research show that although HLA-A2 expression by melanoma cells is relatively low, under optimal pulsing conditions with antigenic peptide they can efficiently stimulate CTL to lethal hit delivery and to IFN-γ production (Supplementary figure 12 and 13). My observations are in agreement with previously published data by members of Valitutti's team who investigated in details the extent of CTL responses (in terms of MTOC polarization, lytic granule exposure, perforin discharge and $[Ca^{+2}]_i$ increase) during interaction with melanoma cells as compared to conventional target cells. They showed that melanoma cells nullify the rapidity and sensitivity of CTL responses and concluded that melanoma cell annihilation requires multiple hits during a longer cell/cell interaction time[231]. My results extend this view by revealing one key molecular mechanism of such a sustained resistance of melanoma cells to the attack of fully activated CTL. Thus, the use of an "artificial system" to fully activate CTL against melanoma cells allowed us to identify a previously non-described melanoma self defense barrier that CTLs should overcome.

A limitation of our study was that we had limited access to melanoma cells at different stages of differentiation, to be able to conclude whether the observed defense mechanism might be related to a defined stage of melanoma cell differentiation [446].

Moreover we still do not know whether the abundant lysosomal reserve and the capacity of intense LLE vesicle exposure in response to perforin action is an intrinsic characteristic of melanocytes or if it evolved during tumorigenesis in melanoma patients. To address this question it would be important to compare response of untransformed HLA-$A2^+$ melanocytes and melanoma cells to CTL attack. The team is currently setting up procedure to obtain human untransformed melanocytes. Although we did not directly answer to this question, several lines of evidence support a tumor-related acquisition of such a behavior. First, there is a high level of $CD63^+$ exosomes in the serum of patients at the late stage of melanoma [447]. Second, other teams

have published that non-metastatic melanoma cells are less resistant to CTL cytotoxicity when compared to metastatic melanoma cells [448;449]. Third, several molecules involved in the regulation of vesicular trafficking or belonging to LLE are disregulated during melanoma tumorgenesis[450 451-453].

It is also interesting to speculate whether our described self-defense mechanism is a general cell defense mechanism or is only related to melanoma. Our preliminary data indicates that even conventional target cells expressing on their surface high level of lysosomal markers show higher resistance to cytotoxicity when compared to low level expressing conventional target cells (Supplementary Figure 11). In addition, cells with abundant secretory potentials such as CTL, NK and mast cells, have been reported by others and us to be more resistant to perforin mediated cytotoxicity ([222,223,454] and R. Khazen unpublished observation).

Detailed investigation of intercellular communication:

1) Lethal hit delivery by CTL and its reception by target cells:

One interesting aspect of our work is that we contribute to ameliorate the concept of lethal hit delivery by CTL and consequent lethal molecules uptake by target cells.

In this context, several studies have been conducted (detailed in chapter "effector function of CTLs" of the introduction, page 53-59) to study perforin-mediate granzyme B delivery to target cells. CTL lethal hit delivery is a finely regulated process in which small quanta of toxic molecules, triggers membrane repair mechanism in target cells that modulates target cell destiny towards apoptosis rather than necrosis, thus target cells actively participate in determining their own fate during cell-mediated death. This target cell participation is mediated by mechanisms of plasma membrane resealing. Currently, the perforin mode of action is controversial. It is not established whether perforin pore formation takes place on cell surface[186,247] or it rather functions within newly internalized endosomes[227,245,254].

In our study we add to the present knowledge. We applied time-lapse microscopy and flow cytometry analysis to investigate spatiotemporal response of melanoma cells to lethal hit delivery in comparison to conventional targets both at single cell level and at whole population level. We show that majority of melanoma cells are resistant to perforin mediated cytotoxicity as they exhibit limited and delayed membrane perforation (paper Figure 1) under condition in

which they are receiving several lethal hits as indicated by $[Ca^{+2}]_i$ increase in their cytosol ([231] and R.Khazen data not shown). Interestingly, my preliminary results indicates that $[Ca^{+2}]_i$ was not delayed in melanoma cells encountering CTL when compared to conventional targets cells, indicating that lethal molecules efficiently achieve melanoma cell membrane. However, the duration of the $[Ca^{+2}]_i$ differed between the two cell types. Conventional target cells exhibited a transient $[Ca^{+2}]_i$ that was followed by their rapid death while a majority of melanoma cells exhibited a sustained and repetitive pattern of $[Ca^{+2}]_i$. Rapid $[Ca^{+2}]_i$ in target cells subjected to CTL attack[158,159,455] or treated with purified Perforin [456] has been previously reported. Our previous and recent data differentiate $[Ca^{+2}]_i$ pattern in sensitive and resistant target cells. The sustained calcium signaling in melanoma cells might influence melanoma cell gene expression and even trigger secondary self defense mechanism that needs to be elucidated.

2) The parameter time in the dynamics of CTL/melanoma cell interaction.

The time parameter in CTL cytotoxicity has been previously investigated. Previous studies reported that a prolonged time is required for tumor cell annihilation both *in vitro* and *in vivo* [110,231,288]. A preliminary observation I made during my thesis is that CTL polarize for prolonged time their lytic compartment towards melanoma cells (>1h) while CTL polarization towards conventional target cells appears to be more dynamic and transient. Indeed I observed that CTLs in contact with conventional target cells tend to rapidly relocate their lytic granules towards different targets. In addition, CTLs treated with drugs affecting lysosomal pH stayed attached to their targets for a prolonged time with no cytotoxicity. This preliminary observation is in agreement with data recently reported by J. Trapani and co-workers who showed that the absence of either perforin or granzymes dramatically increases synapse dwell time. They proposed that the signal for synapse breakage is generated in a caspase-dependent fashion and is mediated by target cell. Our preliminary observations together with these recently published data points out a dynamic communication between CTL and their target cells and raise the possibility that target cell death per se provides signals for CTL killing termination.

Together, these findings points out that CTL/target cell interaction might be viewed as a platform for information exchange between the two cells that are mutually regulating signaling and function processes of each other. On one hand, to optimize the outcome of cytotoxicity in terms of inducing apoptosis rather than necrosis[254] and on the other hand to economize CTL lethal potential for their multiple/serial killing activity[293]. Via this bidirectional exchange of

information, CTLs send small quanta of toxic molecules repetitively that will be translated into death signals in target cells until a threshold reached. Once the threshold is reached, target cells send, still undetermined, signals for cytotoxicity termination. This mechanism might be favorable to the efficacy of CTL multiple killing capacity, but might become detrimental in conditions in which melanoma cells resist for prolonged time, since repetitive lytic granule release might conduct CTL to waste of their lytic potential.

3) Melanoma cell synaptic defense against CTL attack

A central finding of my study is that that the LLE compartment enrichment and exposure at the synaptic area accounts for melanoma cell self defense to perforin mediated CTL attack, since impairment of vesicular trafficking (fusion and docking to the plasma membrane) and pH perturbation enhanced perforin binding on the surface of melanoma cells (paper Figure 6 and 7 and supplementary movies 6, 12,14 and 15).

This observation can be explained by two possibilities. First, such a synaptic oriented intense lysosomal exposure will cause the localization and clustering of several heavily glycosylated lysosomal molecules at LS that can create a shield at the site of perforin release that might reduce the possibility of perforin binding ("space related hypothesis"). In line with this hypothesis, Cohen et al recently proposed that increased exposure of CD107a during degranulation of NK cells might be involved in their protection from self-annihilation. During my thesis work I observed that silencing of CD107a expression reduce melanoma cell resistance to CTL attack (refer to chapter: Related questions, Q4), a finding in agreement with the "space related hypothesis".

Second, the exposure on melanoma cell surface of lysosome resident perforin inhibitory molecule(s) upon vesicular exocytosis at LS would block or cleave perforin molecule ("cleavage related hypothesis"). Of note, In the process of forming membrane channels, perforin goes through an intermediate membrane-associated stage in which it is highly susceptible to proteolysis before polymerization into a more resistant pore-forming complex[224,457,458]. In our submitted article, we proposed that cathepsin B exposure on the surface of melanoma cells, in response to perforin might contribute to proteolytic cleavage of perforin at the lytic synapse. Such extracellular activity of membrane bound cathepsin B has been reported for extracellular matrix degradation during tumor metastasis in melanoma, or for

antigen extraction and processing by B cells[225]. This protease was not (or minimally) expressed on melanoma cell surface at steady state, but rapidly detectable on the surface of melanoma cells after interaction with CTL. The presence of both molecules in the synaptic cleft and the acidic environment of the lytic synapse by analogy to what has been described in B cells[225] might favor cathepsin B mediated proteolytic cleavage of perforin monomers.

Both scenarios can take place simultaneously, we do not have enough proof to eliminate one of them since our current data supports both hypothesis. To further dissect the specific ongoing molecular interactions at the interface between CTL and melanoma cells, it would be helpful to investigate with super resolution microscopy: 1) If SNAP-23 and VAMP-7 molecules play a role in focalizing CD107a clustering at the synaptic area and if perforin is trapped in such compartments, rapidly endocytosed and degrades inside the melanoma cell polarized lysosomes that are rich in lytic enzymes. 2) The localization of Cathepsin B and perforin at LS and the kinetic of their exposure, insertion and cleavage.

Moreover, while we have shown rapid exposure of LLE cathepsin B on melanoma cell surface and proved that it can indeed block perforin pore formation, we did not quantify the activity of cathepsin B and specificity of such cleavage. Furthermore, such cleavage can be also mediated by other proteolytic enzymes resident in the lysosomes. Further experimental support is required.

An alternative not mutually exclusive mechanism that may contribute to melanoma cell defense is that since melanoma cells are highly efficient in exosome release [447], one can imagine that lethal molecules are prohibited from target cell entry by a rapid uptake and release in the medium, quantify the content of perforin and granzyme in the released vesicles of extracellular medium of short duration CTL/melanoma interaction can give hints on the existence of such a phenomenon.

Another possibility to explain limited perforin binding could be expression of additional inhibitory molecules. Fraser *et al.* reported complete blockade of perforin activity by Calreticulin at 2.2 x 10^{-7} M, they proposed that calreticulin indirectly prevents perforin polymerization by stabilizing the membrane. In 1991 Matsudka identified a perforin inhibitor protein (ApoB100) that not only inhibits the membrane binding activity of perforin but also the pore insertion activity of membrane-bound perforin[459]. In our model, melanoma cells did not

over express these molecules at their surface when compared to conventional target cells[231]. However, we cannot exclude the exposure of such inhibitors on the melanoma cells surface during CTL attack. These inhibitors might therefore contribute to melanoma self-protection by mechanisms complementary to cathepsin B-mediated cell protection.

4) How CTL resist to their own perforin?

CTLs are well known to resist to perforin action. CTLs resist to lysis not only when attacked by other killer cells, but also when soluble effector proteins are used [224,460,461]. In 1980s several articles reported resistance of CTL clones to perforin mediated cytotoxicity when they served as target cells and described the CTL "self-sparing concept"[460-463]. It is noteworthy that in all of these articles some clear examples of lysis, even rare, were shown. In the 1990s, additional fratricidal processes among CTL clones were described[464-466]. Therefore CTLs are in principle able to kill other CTLs, and such mechanism can be sometime of great importance, for instance in viral infection of CTLs[223].

One study reported that perforin binding to CTL membranes was defective when compared to binding to sensitive targets cell membranes[467], whereas another study could not detect a correlation between perforin binding and susceptibility to lysis, but provided evidence for a different conformation of membrane-bound perforin on CTLs probably mediated by cleavage of an inhibitory molecule present on CTL membrane[224].

All in all, published data indicate that CTL are not completely refractory to cytotoxicity and that they might need local self-defense mechanisms to avoid self-annihilation. In 2002 Henkart and co-workers provided evidence that upon degranulation CTLs expose Cathepsin B on their surface. Cathepsin B stays bound to the membrane and is proteolyticaly active[223]. Exposed Cathepsin B is capable of membrane bound perforin cleavage at its susceptible intermediate stage before polymerization into the resistant pore-forming complex. In agreement with this finding, our results also suggest that cathepsin B might be indeed a valuable self defense mechanism. Further research would be require to demonstrate this phenomenon in our cellular model and I am presently investigating whether, in agreement with Henkart's findings, treatment of T cell with cathepsin inhibitor might render them more prone to self-annihilation.

5) Vesicular trafficking in melanoncytes/melanoma cells.

Our cytotoxicity assays also suggested a various range of importance of several key elements of vesicular transport (Myosin Va, Bloc1) or vesicular fusion (SNAP23, VAMP7) in protecting melanoma cells against perforin mediated cytotoxicity (refer to chapter "Related questions": Q4). Among those mechanisms SNAP-23 silencing had the highest impact on breaking melanoma cell resistance barrier, pointing out the crucial role of this molecule in melanoma lysosomal secretion.

These observations can be explained by two possibilities.

First, it is possible that the role played by SNAP-23 in lysosomal secretion in melanoma cells, is not redundant with other homologues of this molecule. Predominant expression of SNAP-23 was observed in melanocytes[335]. The relationship between the levels of SNAP-23 expression and tumorigenesis in melanoma cells is presently unknown. Also, whether other tSNARE molecules such as SNAP-25 and SNAP-24 can play a functional role although they are expressed to a much lower level remain to be elucidated.

Second, SNAP-23 might be the core component of the vesicular trafficking complexes in melanoma cells. Several studies aimed at describing melanocytes/melanoma secretory pathways (as detailed in the introduction) and reported the necessity of formation of molecular complexes between v and t SNAREs for secretion. One can imagine that such complexes are solely formed in the presence of SNAP-23. According to this view, staining of CTL/melanoma conjugates for different molecules involved in several steps of vesicular fusion may provide information on the possible co-localization of these molecules in each cell type, either in the presence or in the absence of SANP-23. The enrichment of these complexes towards the synaptic area, as well as, possible differences of vesicular trafficking in melanoma cell versus melanocytes should be also investigated.

Furthermore, we have tried to describe more in details molecular mechanism of LLE based self-defense during CTL/melanoma interaction. We have shown that conjugation of melanoma cells with CTLs harboring non functional form of perforin molecules did not initiate LLE secretory burst whereas incubation of purified perforin with melanoma cells induced LLE exposure on melanoma cells (refer to chapter "Related questions": Q1 and Q2). Thus, we concluded that such polarized LLE secretory burst is a specific response to initial perforin binding on

melanoma cell membrane. Moreover we observed that LLE secretory burst was not accompanied by a polarization of MTOC in melanoma cells, indicating that a complete re-polarization of tubulin is not necessary[159] (data not shown). However, it is yet to define whether microtubule integrity is required for LLE dynamics in melanoma cells. In addition, studying the exact signaling pathway responsible for LLE recruitment in melanoma cells can provide molecular targets for turning off this auto-defense mechanism. Accordingly, synaptotagmin (Syt) VII[413,468], dysferlin [469,470], and apoptosis-linked gene-2 (ALG-2)[471,472], are reported to be involved in plasma membrane resealing. Depending on injury size and the type of injury, multiple sources of Ca^{2+} may be recruited to trigger and orchestrate repair processes [473].

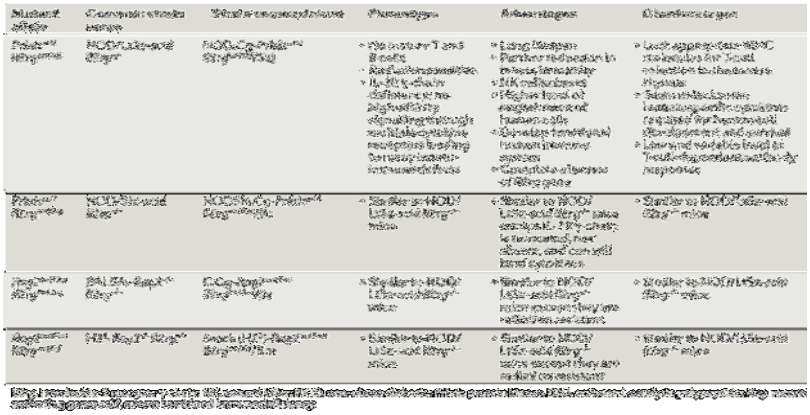
6) Heterogeneity of response to CTL attack

Intratumoral heterogeneity is one of the obstacles of current therapies[474,475]. Targeted immunotherapies, while targeting a specific subset of melanoma cells, might indirectly apply a selection pressure that gives rise to a population resistant to such a treatment, thus treatments which appeared to be beneficial in the beginning could be followed by recurrence of a form of disease no longer susceptible to the given treatment. Using a propidium iodide pore formation assay I observed a heterogeneity of response among melanoma cells and we categorized them in 3 sub groups termed (paper Figure 1): sensitive, persistent and resistant among which 80% of the cells (persistent and resistant) showed a delayed and limited perforin-mediated pore formation (while the other 20% were easily killed by CTLs). The absence of PI staining in a large fraction of cells might be explained by the fact that melanoma cells rapidly ignite a LLE secretion defense mechanism that does not allow the stable formation of pores in plasma membrane. Hence, our described strategy of melanoma cell resistance reversion might be of great interest for the future melanoma immunotherapy treatments since they might aim to the large fraction of resistant cells.

7) In vivo confirmation of LLE secretion-based melanoma cell resistance to CTL attack

It is now well described how cancer cell interaction with the tumor microenvironment may influence their gene expression as well as their function[476], therefore tumor microenvironment is a critical determinant of the response of cancer cells to therapeutic agents. To verify my finding in a model better mimicking *in vivo* immune responses we are starting to use

humanized mice. Humanized mice models provide a powerful tool in pre-clinical testing of various *in vitro* finding, by creating a scenario mimicking, as close as possible, the features of the tumor micro-environmental context[477]. Humanized mice are immunodeficient mice engrafted with human hematopoietic cells (either stem cells or PBMCs) or tissues, or mice that transgenically express human genes [478]. Several research teams tried to establish a proper model by using strains that are often used as recipients of human xenografts such as Severe Combined Immunodeficiency (SCID) mice that being immunodepressed can accept xenografts. Improved tumor engraftment rates have been reported in the NOD-*scid* strain, where introduction of the scid mutation into the non-obese diabetic (NOD) background results in reduced macrophage and NK function, as well as, an absence of complement-dependent hemolytic activity [479,480]. Recently, two additional immune-deficient strains have been described: NOD-*scid* $\beta 2m^{null}$, and NOD-*scid IL2R* γ^{null} ([477]). The NOD-*scid IL2R* γ^{null} strain (NSG) greatly increased engraftment of human tissue; HSCs and PBMCs compared with all previously developed immunodeficient humanized mouse models. IL-2R γ-chain leads to severe impairments in T and B cell development and function, and completely prevents NK-cell development. Features of such strains are summarized in table below.



(Modified from Christianson, S. W. *et al*. 1997 and Lapidot, T.2001)

Up to now our observations were limited to in vitro studies. Observation of the same trend of response to CTL attack in several highly metastatic melanoma cell lines (Supplementary figure 9) prompted us to establish *in vivo* experiments for studying the relevance of such defense mechanism in mice. In particular it is interesting to study the potential role that LLE secretory burst might play during tumor progression *in vivo*.

Intracutaneous injection of Melan A (an immune dominant antigenic peptide) expressing tumor cell lines in NSG mice provides an experimental model in which one can study tumor nodule attack by Melan A specific T cell clones. In this model several aspects of melanoma vesicular trafficking can be studied: a) The impact that silencing of molecules implicated in vesicular transport and/or vesicular docking and fusion to plasma membrane might have on melanoma cell resistance to CTL-mediated cytotoxicity. b) Which kind of Cathepsin B inhibitor pretreatment and at what concentration and duration can enhance CTL tumor annihilation? c) The impact of lysosome pH altering drugs such as chloroquine, monensin, etc. can be evaluated.
For such a study our team has established collaboration with the team of Dr. J.E Sarry at the CRCT in Toulouse.

8) Human tissues a vital source of information

Currently, the gold standard for melanoma diagnosis is the histopathological assessment of tissue sections stained with haematoxylin and eosin, combined with knowledge of the clinical context of the lesion and the patient. Immunohistochemical staining is an important and frequently used adjunct to the histopathological diagnosis of melanoma. For example, immunohistochemistry using the S100 marker can more clearly delineate 'subtle' melanoma cells present in the epidermis or help to identify rare subtypes of disease, such as desmoplastic melanoma[481]. Yet there is no diagnostic biomarker that can allow prediction in the early stages of the melanoma disease progression. In this manner, improved diagnostic and prognostic biomarkers are of increasing importance in the treatment of melanoma. Tumor biomarkers might help to identify patients with early-stage melanoma who are likely to develop advanced disease and would benefit from additional therapies. Analysis of tissue specimens provides important information of mutations and modification of melanoma cell compartments during tumor development that can guide future researches towards improved immunotherapy strategies.

Concerning our *in vitro* findings, it is interesting to investigate how abundance of LLE vesicle expression may correlate with tumor aggressiveness. Staining human tissue samples of different stages for lysosomal compartment markers such as LAMP-1 and LAMP-3 as well as vesicular docking and fusion proteins such as SNAP23 or VAMP-7, may provide some new

prognostic markers that may also be related to patient responsiveness to various immunotherapy strategies.

Moreover, such experimental approach might not only reveal useful biomarkers, but might also provide key information on how melanoma cell secretory alteration may influence the recruitment of immune cells and the profile of response (type, positioning, activation state and reciprocal distance/interaction among infiltrated immune cells) in melanoma microenvironment and help us better correlate ongoing immune responses to disease progression.

VI- Conclusion

In the last decades many studies were conducted to better understand the biochemical nature of tumor antigens and to identify cellular and molecular mechanisms leading to activation of innate and adaptive immune cells. Obtained results have been of paramount importance in the progress of tumor immunology. Studies on the intricate network of interactions between tumor and immune cells have revealed novel regulatory signals, including cell surface inhibitory receptors and costimulatory molecules, intracellular regulatory pathways, immunosuppressive cytokines and pro-apoptotic mediators, which may operate in concert to orchestrate tumor-immune escape[351].

Enforcing effector function of innate and adaptive immune cells, manipulation of their subsequent interaction with tumors as well as tumor intrinsic self protection mechanism, either alone or in combination, could be exploited for therapeutic purposes in cancer patients.

In this respect our data suggest an early synaptic level of melanoma cell resistance to CTL attack, in which melanoma cell hijacked LLE machinery as a tool of protection. This mechanism is based on targeted trafficking of LLE vesicles towards the synaptic area and on LLE burst exocytosis. This self-defense mechanism leads to synaptic degradation of perforin and failure in pore formation due to exposure of proteolytic activity of cathepsin B enzyme. A better understanding of this phenomenon would open the possibility of enhancing CTL activity via alteration of cancer cell resistance and it can pave the road to immunotherapy strategies aiming at ameliorating CTL potential.

VII- Bibliography

1. Parkin, J. & Cohen, B. An overview of the immune system. *Lancet* 357, 1777-89 (2001).
2. Spits, H. et al. Innate lymphoid cells--a proposal for uniform nomenclature. *Nat Rev Immunol* 13, 145-9 (2013).
3. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 13, 159-75 (2013).
4. Diefenbach, A., Colonna, M. & Koyasu, S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity* 41, 354-65 (2014).
5. Murphy KM, J.C., Travers P, Walport M. *Janeway's immunobiology*, (Garland Science, London, 2012).
6. Meester, J.D. Paul Sabatier University (2011).
7. Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375, 148-51 (1995).
8. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68, 869-77 (1992).
9. Shinkai, Y. et al. RestoMOM. *Science* 259, 822-5 (1993).
10. Nikolich-Zugich, J., Slifka, M.K. & Messaoudi, I. The many important facets of T-cell repertoire diversity. *Nat Rev Immunol* 4, 123-32 (2004).
11. Garcia, K.C. et al. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science* 274, 209-19 (1996).
12. Schumacher, T.N. T-cell-receptor gene therapy. *Nat Rev Immunol* 2, 512-9 (2002).
13. Rudolph, M.G., Stanfield, R.L. & Wilson, I.A. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* 24, 419-66 (2006).
14. Alarcon, B., Berkhout, B., Breitmeyer, J. & Terhorst, C. Assembly of the human T cell receptor-CD3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the CD3-gamma.delta.epsilon core and single T cell receptor alpha or beta chains. *J Biol Chem* 263, 2953-61 (1988).
15. Chothia, C., Boswell, D.R. & Lesk, A.M. The outline structure of the T-cell alpha beta receptor. *Embo J* 7, 3745-55 (1988).
16. Jorgensen, J.L., Esser, U., Fazekas de St Groth, B., Reay, P.A. & Davis, M.M. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature* 355, 224-30 (1992).
17. Sant'Angelo, D.B. et al. The specificity and orientation of a TCR to its peptide-MHC class II ligands. *Immunity* 4, 367-76 (1996).
18. Koning, F., Maloy, W.L. & Coligan, J.E. The implications of subunit interactions for the structure of the T cell receptor-CD3 complex. *Eur J Immunol* 20, 299-305 (1990).
19. Kuhns, M.S., Davis, M.M. & Garcia, K.C. Deconstructing the form and function of the TCR/CD3 complex. *Immunity* 24, 133-9 (2006).
20. Manolios, N., Letourneur, F., Bonifacino, J.S. & Klausner, R.D. Pairwise, cooperative and inhibitory interactions describe the assembly and probable structure of the T-cell antigen receptor. *Embo J* 10, 1643-51 (1991).
21. Sommers, C.L., Samelson, L.E. & Love, P.E. LAT: a T lymphocyte adapter protein that couples the antigen receptor to downstream signaling pathways. *Bioessays* 26, 61-7 (2004).

22. Gascoigne, N.R.J. Do T cells need endogenous peptides for activation? *Nature Reviews Immunology* 8, 895-900 (2008).
23. Gaudenzio, N. et al. Cell-cell cooperation at the T helper cell/mast cell immunological synapse. *Blood* 114, 4979-88 (2009).
24. Collins, T. et al. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. *Proc Natl Acad Sci U S A* 81, 4917-21 (1984).
25. Perrigoue, J.G. et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* 10, 697-705 (2009).
26. Sokol, C.L. et al. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol* 10, 713-20 (2009).
27. Yoshimoto, T. et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol* 10, 706-12 (2009).
28. Bouvier, M. Accessory proteins and the assembly of human class I MHC molecules: a molecular and structural perspective. *Mol Immunol* 39, 697-706 (2003).
29. Pieters, J. MHC class II-restricted antigen processing and presentation. *Adv Immunol* 75, 159-208 (2000).
30. George, A.J., Stark, J. & Chan, C. Understanding specificity and sensitivity of T-cell recognition. *Trends Immunol* 26, 653-9 (2005).
31. Huppa, J.B., Gleimer, M., Sumen, C. & Davis, M.M. Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nat Immunol* 4, 749-55 (2003).
32. Mempel, T.R., Henrickson, S.E. & Von Andrian, U.H. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427, 154-9 (2004).
33. Matsui, K. et al. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* 254, 1788-91 (1991).
34. Weber, S., Traunecker, A., Oliveri, F., Gerhard, W. & Karjalainen, K. Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature* 356, 793-6 (1992).
35. 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* 2, 229-34 (2001).
36. Huppa, J.B. et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature* 463, 963-7 (2010).
37. Huang, J. et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature* 464, 932-6 (2010).
38. Liu, B.Y., Chen, W., Evavold, B.D. & Zhu, C. Accumulation of Dynamic Catch Bonds between TCR and Agonist Peptide-MHC Triggers T Cell Signaling. *Cell* 157, 357-368 (2014).
39. Irvine, D.J., Purbhoo, M.A., Krogsgaard, M. & Davis, M.M. Direct observation of ligand recognition by T cells. *Nature* 419, 845-9 (2002).
40. Purbhoo, M.A., Irvine, D.J., Huppa, J.B. & Davis, M.M. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol* 5, 524-30 (2004).
41. Huse, M. et al. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. *Immunity* 27, 76-88 (2007).

42. **Jiang, N. et al. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. *Immunity* 34, 13-23 (2011).**
43. **Davis, M.M. et al. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 16, 523-44 (1998).**
44. **Germain, R.N. & Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu Rev Immunol* 17, 467-522 (1999).**
45. **Gascoigne, N.R., Zal, T. & Alam, S.M. T-cell receptor binding kinetics in T-cell development and activation. *Expert Rev Mol Med* 2001, 1-17 (2001).**
46. **Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med* 181, 577-84 (1995).**
47. **van der Merwe, P.A. & Dushek, O. Mechanisms for T cell receptor triggering. *Nat Rev Immunol* 11, 47-55 (2011).**
48. **He, J.S., Gong, D.E. & Ostergaard, H.L. Stored Fas ligand, a mediator of rapid CTL-mediated killing, has a lower threshold for response than degranulation or newly synthesized Fas ligand. *J Immunol* 184, 555-63 (2005).**
49. **Harder, T. Lipid raft domains and protein networks in T-cell receptor signal transduction. *Curr Opin Immunol* 16, 353-9 (2004).**
50. **Dykstra, M., Cherukuri, A. & Pierce, S.K. Rafts and synapses in the spatial organization of immune cell signaling receptors. *J Leukoc Biol* 70, 699-707 (2001).**
51. **Veillette, A., Bookman, M.A., Horak, E.M. & Bolen, J.B. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55, 301-8 (1988).**
52. **Artyomov, M.N., Lis, M., Devadas, S., Davis, M.M. & Chakraborty, A.K. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc Natl Acad Sci U S A* 107, 16916-21 (2010).**
53. **Johansson, A., Driessens, M. & Aspenstrom, P. The mammalian homologue of the Caenorhabditis elegans polarity protein PAR-6 is a binding partner for the Rho GTPases Cdc42 and Rac1. *J Cell Sci* 113 (Pt 18), 3267-75 (2000).**
54. **Werlen, G. & Palmer, E. The T-cell receptor signalosome: a dynamic structure with expanding complexity. *Curr Opin Immunol* 14, 299-305 (2002).**
55. **Drevot, P. et al. TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts. *Embo J* 21, 1899-908 (2002).**
56. **Brownlie, R.J. & Zamoyska, R. T cell receptor signalling networks: branched, diversified and bounded. *Nature Reviews Immunology* 13, 257-269 (2013).**
57. **Freeman, G.J. et al. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol* 143, 2714-22 (1989).**
58. **Azuma, M. et al. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 366, 76-9 (1993).**
59. **Acuto, O. & Michel, F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol* 3, 939-51 (2003).**
60. **Sansom, D.M. CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* 101, 169-77 (2000).**
61. **Bhatia, S., Edidin, M., Almo, S.C. & Nathenson, S.G. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. *Immunol Lett* 104, 70-5 (2006).**

62. **Thompson, C.B. et al. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A* 86, 1333-7 (1989).**
63. **Linsley, P.S. et al. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* 173, 721-30 (1991).**
64. **Walunas, T.L. et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1, 405-13 (1994).**
65. **Tivol, E.A. et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3, 541-7 (1995).**
66. **Ma, D.Y. & Clark, E.A. The role of CD40 and CD154/CD40L in dendritic cells. *Semin Immunol* 21, 265-72 (2009).**
67. **Bevan, M.J. Helping the CD8(+) T-cell response. *Nat Rev Immunol* 4, 595-602 (2004).**
68. **Elgueta, R. et al. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 229, 152-72 (2009).**
69. **Caux, C. et al. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* 180, 1263-72 (1994).**
70. **Cella, M. et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* 184, 747-52 (1996).**
71. **Koch, F. et al. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* 184, 741-6 (1996).**
72. **Donnadieu, E. et al. Imaging early steps of human T cell activation by antigen-presenting cells. *J Immunol* 148, 2643-53 (1992).**
73. **Donnadieu, E., Bismuth, G. & Trautmann, A. Antigen recognition by helper T cells elicits a sequence of distinct changes of their shape and intracellular calcium. *Curr Biol* 4, 584-95 (1994).**
74. **Randriamampita, C. & Trautmann, A. Ca2+ signals and T lymphocytes; "New mechanisms and functions in Ca2+ signalling". *Biol Cell* 96, 69-78 (2004).**
75. **Trautmann, A. & Valitutti, S. The diversity of immunological synapses. *Curr Opin Immunol* 15, 249-54 (2003).**
76. **Feske, S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol* 7, 690-702 (2007).**
77. **Quintana, A. et al. T cell activation requires mitochondrial translocation to the immunological synapse. *Proc Natl Acad Sci U S A* 104, 14418-23 (2007).**
78. **Quintana, A. et al. Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. *Embo J* 30, 3895-912 (2011).**
79. **Monks, C.R., Kupfer, H., Tamir, I., Barlow, A. & Kupfer, A. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* 385, 83-6 (1997).**
80. **Marsland, B.J., Soos, T.J., Spath, G., Littman, D.R. & Kopf, M. Protein kinase C theta is critical for the development of in vivo T helper (Th)2 cell but not Th1 cell responses. *J Exp Med* 200, 181-9 (2004).**
81. **Pfeifhofer-Obermair, C., Thuille, N. & Baier, G. Involvement of distinct PKC gene products in T cell functions. *Front Immunol* 3, 220.**
82. **Rosse, C. et al. PKC and the control of localized signal dynamics. *Nat Rev Mol Cell Biol* 11, 103-12 (2010).**

83. **Meller, N., Elitzur, Y. & Isakov, N. Protein kinase C-theta (PKCtheta) distribution analysis in hematopoietic cells: proliferating T cells exhibit high proportions of PKCtheta in the particulate fraction. *Cell Immunol* 193, 185-93 (1999).**
84. **Bertrand, F. et al. Activation of the Ancestral Polarity Regulator Protein Kinase C{zeta} at the Immunological Synapse Drives Polarization of Th Cell Secretory Machinery toward APCs. *J Immunol* (2010).**
85. **Tourret, M. et al. T cell polarity at the immunological synapse is required for CD154-dependent IL-12 secretion by dendritic cells. *J Immunol* 185, 6809-18 (2010).**
86. **Dustin, M.L. PKC-theta: hitting the bull's eye. *Nat Immunol* 12, 1031-2.**
87. **Ward, Y. et al. Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature* 367, 651-4 (1994).**
88. **Gille, H., Sharrocks, A.D. & Shaw, P.E. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* 358, 414-7 (1992).**
89. **Price, M.A., Cruzalegui, F.H. & Treisman, R. The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light. *Embo J* 15, 6552-63 (1996).**
90. **Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. & Davis, R.J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* 16, 1247-55 (1996).**
91. **Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179, 1109-18 (1994).**
92. **Benvenuti, F. et al. Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. *J Immunol* 172, 292-301 (2004).**
93. **Friedl, P., den Boer, A.T. & Gunzer, M. Tuning immune responses: diversity and adaptation of the immunological synapse. *Nat Rev Immunol* 5, 532-45 (2005).**
94. **von Andrian, U.H. & Mempel, T.R. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol* 3, 867-78 (2003).**
95. **Miller, M.J., Wei, S.H., Parker, I. & Cahalan, M.D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 296, 1869-73 (2002).**
96. **Bousso, P. & Robey, E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol* 4, 579-85 (2003).**
97. **Miller, M.J., Wei, S.H., Cahalan, M.D. & Parker, I. Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy. *Proc Natl Acad Sci U S A* 100, 2604-9 (2003).**
98. **Lindquist, R.L. et al. Visualizing dendritic cell networks in vivo. *Nat Immunol* 5, 1243-50 (2004).**
99. **Miller, M.J., Hejazi, A.S., Wei, S.H., Cahalan, M.D. & Parker, I. T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. *Proc Natl Acad Sci U S A* 101, 998-1003 (2004).**
100. **Watkins, S.C. & Salter, R.D. Functional connectivity between immune cells mediated by tunneling nanotubules. *Immunity* 23, 309-18 (2005).**
101. **Celli, S. et al. How many dendritic cells are required to initiate a T-cell response? *Blood* 120, 3945-8 (2012).**

102. Stoll, S., Delon, J., Brotz, T.M. & Germain, R.N. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296, 1873-6 (2002).
103. Dustin, M.L., Bromley, S.K., Kan, Z., Peterson, D.A. & Unanue, E.R. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc Natl Acad Sci U S A* 94, 3909-13 (1997).
104. Grakoui, A. et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285, 221-7 (1999).
105. Hauss, P., Selz, F., Cavazzana-Calvo, M. & Fischer, A. Characteristics of antigen-independent and antigen-dependent interaction of dendritic cells with CD4+ T cells. *Eur J Immunol* 25, 2285-94 (1995).
106. Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8, 89-95 (1998).
107. Faroudi, M., Zaru, R., Paulet, P., Muller, S. & Valitutti, S. Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling. *J Immunol* 171, 1128-32 (2003).
108. Clark, C.E., Hasan, M. & Bousso, P. A role for the immediate early gene product c-fos in imprinting T cells with short-term memory for signal summation. *PLoS One* 6, e18916 (2011).
109. Dustin, M.L. Insights into function of the immunological synapse from studies with supported planar bilayers. *Curr Top Microbiol Immunol* 340, 1-24 (2009).
110. Celli, S., Lemaitre, F. & Bousso, P. Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4+ T cell activation. *Immunity* 27, 625-34 (2007).
111. Shakhar, G. et al. Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. *Nat Immunol* 6, 707-14 (2005).
112. Wei, S.H. et al. Ca2+ signals in CD4+ T cells during early contacts with antigen-bearing dendritic cells in lymph node. *J Immunol* 179, 1586-94 (2007).
113. Skokos, D. et al. Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. *Nat Immunol* 8, 835-44 (2007).
114. Hugues, S. et al. Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* 5, 1235-42 (2004).
115. Friedl, P. & Brocker, E.B. TCR triggering on the move: diversity of T-cell interactions with antigen-presenting cells. *Immunol Rev* 186, 83-9 (2002).
116. Tadokoro, C.E. et al. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 203, 505-11 (2006).
117. Tang, Q. & Bluestone, J.A. Regulatory T-cell physiology and application to treat autoimmunity. *Immunol Rev* 212, 217-37 (2006).
118. Okada, T. & Cyster, J.G. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. *J Immunol* 178, 2973-8 (2007).
119. Asperti-Boursin, F., Real, E., Bismuth, G., Trautmann, A. & Donnadieu, E. CCR7 ligands control basal T cell motility within lymph node slices in a phosphoinositide 3-kinase-independent manner. *J Exp Med* 204, 1167-79 (2007).
120. Worbs, T., Mempel, T.R., Bolter, J., von Andrian, U.H. & Forster, R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med* 204, 489-95 (2007).
121. Woolf, E. et al. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol* 8, 1076-85 (2007).

122. Lee, K.H. et al. The immunological synapse balances T cell receptor signaling and degradation. *Science* 302, 1218-22 (2003).
123. Mazerolles, F., Barbat, C., Hivroz, C. & Fischer, A. Phosphatidylinositol 3-kinase participates in p56(lck)/CD4-dependent down-regulation of LFA-1-mediated T cell adhesion. *J Immunol* 157, 4844-54 (1996).
124. Linsley, P.S. et al. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* 4, 535-43 (1996).
125. Egen, J.G. & Allison, J.P. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* 16, 23-35 (2002).
126. Kupfer, A. & Dennert, G. Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. *J Immunol* 133, 2762-6 (1984).
127. Geiger, B., Rosen, D. & Berke, G. Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J Cell Biol* 95, 137-43 (1982).
128. Kupfer, A., Dennert, G. & Singer, S.J. Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. *Proc Natl Acad Sci U S A* 80, 7224-8 (1983).
129. Kupfer, A., Singer, S.J. & Dennert, G. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. *J Exp Med* 163, 489-98 (1986).
130. Das, V. et al. Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. *Immunity* 20, 577-88 (2004).
131. Norcross, M.A. A synaptic basis for T-lymphocyte activation. *Ann Immunol (Paris)* 135D, 113-34 (1984).
132. Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395, 82-6 (1998).
133. Friedl, P. & Storim, J. Diversity in immune-cell interactions: states and functions of the immunological synapse. *Trends Cell Biol* 14, 557-67 (2004).
134. Springer, T.A. Adhesion receptors of the immune system. *Nature* 346, 425-34 (1990).
135. Andres, P.G. et al. CD28 signals in the immature immunological synapse. *J Immunol* 172, 5880-6 (2004).
136. Dustin, M.L. et al. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* 94, 667-77 (1998).
137. Krummel, M.F., Sjaastad, M.D., Wulfing, C. & Davis, M.M. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* 289, 1349-52 (2000).
138. Freiberg, B.A. et al. Staging and resetting T cell activation in SMACs. *Nat Immunol* 3, 911-7 (2002).
139. Leupin, O., Zaru, R., Laroche, T., Muller, S. & Valitutti, S. Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr Biol* 10, 277-80 (2000).
140. Delon, J., Kaibuchi, K. & Germain, R.N. Exclusion of CD43 from the immunological synapse is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. *Immunity* 15, 691-701 (2001).

141. Allenspach, E.J. et al. ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. *Immunity* 15, 739-50 (2001).
142. Reichert, P., Reinhardt, R.L., Ingulli, E. & Jenkins, M.K. Cutting edge: in vivo identification of TCR redistribution and polarized IL-2 production by naive CD4 T cells. *J Immunol* 166, 4278-81 (2001).
143. Lee, K.H. et al. T cell receptor signaling precedes immunological synapse formation. *Science* 295, 1539-42 (2002).
144. Griffiths, G.M., Tsun, A. & Stinchcombe, J.C. The immunological synapse: a focal point for endocytosis and exocytosis. *Journal of Cell Biology* 189, 397-406 (2010).
145. Campi, G., Varma, R. & Dustin, M.L. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med* 202, 1031-6 (2005).
146. Yokosuka, T. & Saito, T. The immunological synapse, TCR microclusters, and T cell activation. *Curr Top Microbiol Immunol* 340, 81-107 (2005).
147. Saito, T. & Yokosuka, T. Immunological synapse and microclusters: the site for recognition and activation of T cells. *Curr Opin Immunol* 18, 305-13 (2006).
148. Varma, R., Campi, G., Yokosuka, T., Saito, T. & Dustin, M.L. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* 25, 117-27 (2006).
149. Bunnell, S.C. et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* 158, 1263-75 (2002).
150. Stinchcombe, J.C. et al. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol* 152, 825-34 (2001).
151. Bossi, G. et al. The secretory synapse: the secrets of a serial killer. *Immunol Rev* 189, 152-60 (2002).
152. Stinchcombe, J.C. & Griffiths, G.M. The role of the secretory immunological synapse in killing by CD8+ CTL. *Semin Immunol* 15, 301-5 (2003).
153. Wulfing, C. & Davis, M.M. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* 282, 2266-9 (1998).
154. Huse, M., Lillemeier, B.F., Kuhns, M.S., Chen, D.S. & Davis, M.M. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* 7, 247-55 (2006).
155. Chemin, K. et al. Cytokine secretion by CD4+ T cells at the immunological synapse requires Cdc42-dependent local actin remodeling but not microtubule organizing center polarity. *J Immunol* 189, 2159-68.
156. Faroudi, M. et al. Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. *Proc Natl Acad Sci U S A* 100, 14145-50 (2003).
157. O'Keefe, J.P. & Gajewski, T.F. Cutting edge: cytotoxic granule polarization and cytolysis can occur without central supramolecular activation cluster formation in CD8+ effector T cells. *J Immunol* 175, 5581-5 (2005).
158. Wiedemann, A., Depoil, D., Faroudi, M. & Valitutti, S. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. *Proc Natl Acad Sci U S A* 103, 10985-90 (2006).
159. Bertrand, F. et al. An initial and rapid step of lytic granule secretion precedes microtubule organizing center polarization at the cytotoxic T lymphocyte/target cell synapse. *Proc Natl Acad Sci U S A* 110, 6073-8 (2013).
160. Richie, L.I. et al. Imaging synapse formation during thymocyte selection: inability of CD3zeta to form a stable central accumulation during negative selection. *Immunity* 16, 595-606 (2002).

161. Hailman, E., Burack, W.R., Shaw, A.S., Dustin, M.L. & Allen, P.M. Immature CD4(+)CD8(+) thymocytes form a multifocal immunological synapse with sustained tyrosine phosphorylation. *Immunity* 16, 839-48 (2002).
162. Brossard, C. et al. Multifocal structure of the T cell - dendritic cell synapse. *Eur J Immunol* 35, 1741-53 (2005).
163. Revy, P., Sospedra, M., Barbour, B. & Trautmann, A. Functional antigen-independent synapses formed between T cells and dendritic cells. *Nat Immunol* 2, 925-31 (2001).
164. O'Keefe, J.P., Blaine, K., Alegre, M.L. & Gajewski, T.F. Formation of a central supramolecular activation cluster is not required for activation of naive CD8+ T cells. *Proc Natl Acad Sci U S A* 101, 9351-6 (2004).
165. Blanchard, N. et al. Strong and durable TCR clustering at the T/dendritic cell immune synapse is not required for NFAT activation and IFN-gamma production in human CD4+ T cells. *J Immunol* 173, 3062-72 (2004).
166. Lillemeier, B.F. et al. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol* 11, 90-6 (2010).
167. Schamel, W.W. et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med* 202, 493-503 (2005).
168. Sherman, E. et al. Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* 35, 705-20 (2011).
169. Crites, T.J. et al. TCR Microclusters pre-exist and contain molecules necessary for TCR signal transduction. *J Immunol* 193, 56-67 (2014).
170. Cemerski, S. et al. The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* 29, 414-22 (2008).
171. Cemerski, S. et al. The stimulatory potency of T cell antigens is influenced by the formation of the immunological synapse. *Immunity* 26, 345-55 (2007).
172. Valitutti, S. Immunological synapse: center of attention again. *Immunity* 29, 384-6 (2008).
173. Irvine, D.J. & Doh, J. Synthetic surfaces as artificial antigen presenting cells in the study of T cell receptor triggering and immunological synapse formation. *Semin Immunol* 19, 245-54 (2007).
174. Kaizuka, Y., Douglass, A.D., Varma, R., Dustin, M.L. & Vale, R.D. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci U S A* 104, 20296-301 (2007).
175. Beal, A.M. et al. Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. *Immunity* 31, 632-42 (2009).
176. Yannelli, J.R., Sullivan, J.A., Mandell, G.L. & Engelhard, V.H. Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinemicrography. *J Immunol* 136, 377-82 (1986).
177. Valitutti, S., Muller, S., Dessing, M. & Lanzavecchia, A. Signal extinction and T cell repolarization in T helper cell-antigen-presenting cell conjugates. *Eur J Immunol* 26, 2012-6 (1996).
178. Kuhn, J.R. & Poenie, M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity* 16, 111-21 (2002).
179. Depoil, D. et al. Immunological synapses are versatile structures enabling selective T cell polarization. *Immunity* 22, 185-94 (2005).
180. Zaru, R., Cameron, T.O., Stern, L.J., Muller, S. & Valitutti, S. Cutting edge: TCR engagement and triggering in the absence of large-scale molecular segregation at the T cell-APC contact site. *J Immunol* 168, 4287-91 (2002).

181. McGavern, D.B., Christen, U. & Oldstone, M.B. Molecular anatomy of antigen-specific CD8(+) T cell engagement and synapse formation in vivo. *Nat Immunol* 3, 918-25 (2002).
182. Makedonas, G. et al. Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity. *J Immunol* 182, 5560-9 (2009).
183. Barrat, F.J. et al. Defective CTLA-4 cycling pathway in Chediak-Higashi syndrome: a possible mechanism for deregulation of T lymphocyte activation. *Proc Natl Acad Sci U S A* 96, 8645-50 (1999).
184. Clark, R.H. et al. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. *Nat Immunol* 4, 1111-20 (2003).
185. Brennan, A.J. et al. Protection from endogenous perforin: glycans and the C terminus regulate exocytic trafficking in cytotoxic lymphocytes. *Immunity* 34, 879-92 (2011).
186. Voskoboinik, I., Whisstock, J.C. & Trapani, J.A. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* 15, 388-400 (2015).
187. Krzewski, K., Gil-Krzewska, A., Nguyen, V., Peruzzi, G. & Coligan, J.E. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 121, 4672-83 (2013).
188. de Saint Basile, G., Menasche, G. & Fischer, A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nature Reviews Immunology* 10, 568-579 (2010).
189. Kupfer, A. & Singer, S.J. Cell biology of cytotoxic and helper T cell functions: immunofluorescence microscopic studies of single cells and cell couples. *Annu Rev Immunol* 7, 309-37 (1989).
190. Jenkins, M.R. & Griffiths, G.M. The synapse and cytolytic machinery of cytotoxic T cells. *Curr Opin Immunol* 22, 308-13 (2013).
191. Stinchcombe, J.C., Majorovits, E., Bossi, G., Fuller, S. & Griffiths, G.M. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* 443, 462-5 (2006).
192. Stinchcombe, J.C., Bossi, G., Booth, S. & Griffiths, G.M. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15, 751-761 (2001).
193. Jenkins, M.R., Tsun, A., Stinchcombe, J.C. & Griffiths, G.M. The strength of T cell receptor signal controls the polarization of cytotoxic machinery to the immunological synapse. *Immunity* 31, 621-31 (2009).
194. Isaaz, S., Baetz, K., Olsen, K., Podack, E. & Griffiths, G.M. Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. *Eur J Immunol* 25, 1071-9 (1995).
195. Bonifacino, J.S. & Glick, B.S. The mechanisms of vesicle budding and fusion. *Cell* 116, 153-66 (2004).
196. Chen, Y.A. & Scheller, R.H. SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol* 2, 98-106 (2001).
197. Ungermann, C. & Langosch, D. Functions of SNAREs in intracellular membrane fusion and lipid bilayer mixing. *J Cell Sci* 118, 3819-28 (2005).
198. Griffiths, G.M., Tsun, A. & Stinchcombe, J.C. The immunological synapse: a focal point for endocytosis and exocytosis. *J Cell Biol* 189, 399-406 (2010).
199. Huse, M., Quann, E.J. & Davis, M.M. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nature Immunology* 9, 1105-1111 (2008).

200. Stinchcombe, J.C. & Griffiths, G.M. Secretory mechanisms in cell-mediated cytotoxicity. *Annu Rev Cell Dev Biol* 23, 495-517 (2007).
201. Ma, J.S., Haydar, T.F. & Radoja, S. Protein kinase C delta localizes to secretory lysosomes in CD8+ CTL and directly mediates TCR signals leading to granule exocytosis-mediated cytotoxicity. *J Immunol* 181, 4716-22 (2008).
202. Ma, J.S. et al. Protein kinase Cdelta regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J Immunol* 178, 7814-21 (2007).
203. Menager, M.M. et al. Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. *Nature Immunology* 8, 257-267 (2007).
204. Krzewski, K. & Cullinane, A.R. Evidence for defective Rab GTPase-dependent cargo traffic in immune disorders. *Experimental Cell Research* 319, 2360-2367 (2013).
205. Holt, O. et al. Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse. *Traffic* 9, 446-57 (2008).
206. Menasche, G. et al. A newly identified isoform of Slp2a associates with Rab27a in cytotoxic T cells and participates to cytotoxic granule secretion. *Blood* 112, 5052-62 (2008).
207. Kurowska, M., Goudin, N. & Nehme, N.T. Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/Slp3/Rab27a complex (vol 119, pg 3879, 2012). *Blood* 123, 1432-1432 (2014).
208. Feldmann, J. et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 115, 461-73 (2003).
209. Bryceson, Y.T. et al. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. *Blood* 110, 1906-15 (2007).
210. Matti, U. et al. Synaptobrevin2 is the v-SNARE required for cytotoxic T-lymphocyte lytic granule fusion. *Nat Commun* 4, 1439 (2013).
211. Krzewski, K., Gil-Krzewska, A., Watts, J., Stern, J.N. & Strominger, J.L. VAMP4- and VAMP7-expressing vesicles are both required for cytotoxic granule exocytosis in NK cells. *Eur J Immunol* 41, 3323-9 (2011).
212. Loo, L.S. et al. A role for endobrevin/VAMP8 in CTL lytic granule exocytosis. *Eur J Immunol* 39, 3520-8 (2009).
213. Dressel, R., Elsner, L., Novota, P., Kanwar, N. & Fischer von Mollard, G. The exocytosis of lytic granules is impaired in Vti1b- or Vamp8-deficient CTL leading to a reduced cytotoxic activity following antigen-specific activation. *J Immunol* 185, 1005-14 (2010).
214. Marshall, M.R. et al. VAMP8-dependent fusion of recycling endosomes with the plasma membrane facilitates T lymphocyte cytotoxicity. *J Cell Biol* 210, 135-51 (2015).
215. Menasche, G. et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet* 25, 173-6 (2000).
216. Cote, M. et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest* 119, 3765-73 (2009).
217. zur Stadt, U. et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. *Am J Hum Genet* 85, 482-92 (2009).
218. Lieberman, J. cell-mediated cytotoxicity. in *Fundamental Immunology* (ed. WE, P.) 891-909 (Williams & Wilkins, 2013).

219. Batista, A., Millan, J., Mittelbrunn, M., Sanchez-Madrid, F. & Alonso, M.A. Recruitment of transferrin receptor to immunological synapse in response to TCR engagement. *J Immunol* 172, 6709-14 (2004).
220. Patino-Lopez, G. et al. Rab35 and its GAP EPI64C in T cells regulate receptor recycling and immunological synapse formation. *J Biol Chem* 283, 18323-30 (2008).
221. Liu, D. et al. Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. *Immunity* 31, 99-109 (2009).
222. Baran, K. et al. Cytotoxic T lymphocytes from cathepsin B-deficient mice survive normally in vitro and in vivo after encountering and killing target cells. *Journal of Biological Chemistry* 281, 30485-30491 (2006).
223. Balaji, K.N., Schaschke, N., Machleidt, W., Catalfamo, M. & Henkart, P.A. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *Journal of Experimental Medicine* 196, 493-503 (2002).
224. Muller, C. & Tschopp, J. Resistance of CTL to perforin-mediated lysis. Evidence for a lymphocyte membrane protein interacting with perforin. *J Immunol* 153, 2470-8 (1994).
225. Yuseff, M.I. et al. Polarized secretion of lysosomes at the B cell synapse couples antigen extraction to processing and presentation. *Immunity* 35, 361-74 (2011).
226. Curtsinger, J.M. & Mescher, M.F. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* 22, 333-40 (2010).
227. Thiery, J. et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nature Immunology* 12, 770-U146 (2011).
228. Masopust, D. & Schenkel, J.M. The integration of T cell migration, differentiation and function. *Nat Rev Immunol* 13, 309-20 (2013).
229. Matter, A. Microcinematographic and electron microscopic analysis of target cell lysis induced by cytotoxic T lymphocytes. *Immunology* 36, 179-90 (1979).
230. Betts, M.R. et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281, 65-78 (2003).
231. Caramalho, I., Faroudi, M., Padovan, E., Muller, S. & Valitutti, S. Visualizing CTL/melanoma cell interactions: multiple hits must be delivered for tumour cell annihilation. *Journal of Cellular and Molecular Medicine* 13, 3834-3846 (2009).
232. Voskoboinik, I., Smyth, M.J. & Trapani, J.A. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol* 6, 940-52 (2006).
233. Badovinac, V.P., Hamilton, S.E. & Harty, J.T. Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. *Immunity* 18, 463-74 (2003).
234. Bolitho, P., Voskoboinik, I., Trapani, J.A. & Smyth, M.J. Apoptosis induced by the lymphocyte effector molecule perforin. *Curr Opin Immunol* 19, 339-47 (2007).
235. Lowin-Kropf, B., Shapiro, V.S. & Weiss, A. Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism. *J Cell Biol* 140, 861-71 (1998).
236. Kagi, D. et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265, 528-30 (1994).
237. Law, R.H.P. et al. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature* 468, 447-U277 (2010).
238. Lopez, J.A. et al. Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. *J Immunol* 191, 2328-34 (2013).

239. Baran, K. et al. The Molecular Basis for Perforin Oligomerization and Transmembrane Pore Assembly. *Immunity* 30, 684-695 (2009).
240. Tschopp, J., Schafer, S., Masson, D., Peitsch, M.C. & Heusser, C. Phosphorylcholine acts as a Ca2+-dependent receptor molecule for lymphocyte perforin. *Nature* 337, 272-4 (1989).
241. Dennert, G. & Podack, E.R. Cytolysis by H-2-specific T killer cells. Assembly of tubular complexes on target membranes. *J Exp Med* 157, 1483-95 (1983).
242. Dourmashkin, R.R., Deteix, P., Simone, C.B. & Henkart, P. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin Exp Immunol* 42, 554-60 (1980).
243. Froelich, C.J. et al. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *J Biol Chem* 271, 29073-9 (1996).
244. Metkar, S.S. et al. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity* 16, 417-28 (2002).
245. Thiery, J. et al. Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. *Blood* 115, 1582-1593 (2010).
246. Gerasimenko, J.V., Tepikin, A.V., Petersen, O.H. & Gerasimenko, O.V. Calcium uptake via endocytosis with rapid release from acidifying endosomes. *Curr Biol* 8, 1335-8 (1998).
247. Lopez, J.A. et al. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* 121, 2659-68 (2013).
248. Voskoboinik, I. et al. Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: A critical role for aspartate residues 429, 435, 483, and 485 but not 491. *J Biol Chem* 280, 8426-34 (2005).
249. Pinkoski, M.J. et al. Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood* 92, 1044-54 (1998).
250. Motyka, B. et al. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell* 103, 491-500 (2000).
251. Barry, M. & Bleackley, R.C. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2, 401-9 (2002).
252. Reddy, A., Caler, E. & Andrews, N.W. Plasma membrane repair is mediated by Ca2+-regulated exocytosis of lysosomes. *Molecular Biology of the Cell* 12, 266A-266A (2001).
253. Idone, V., Tam, C. & Andrews, N.W. Two-way traffic on the road to plasma membrane repair. *Trends Cell Biol* 18, 552-9 (2008).
254. Keefe, D. et al. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23, 249-262 (2005).
255. Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H. & Ley, T.J. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* 76, 977-87 (1994).
256. Beresford, P.J. et al. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem* 276, 43285-93 (2001).

257. Lieberman, J. & Fan, Z. Nuclear war: the granzyme A-bomb. *Curr Opin Immunol* 15, 553-9 (2003).
258. Sutton, V.R. et al. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J Exp Med* 192, 1403-14 (2000).
259. Alimonti, J.B., Shi, L., Baijal, P.K. & Greenberg, A.H. Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem* 276, 6974-82 (2001).
260. Slee, E.A. et al. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 144, 281-92 (1999).
261. Medema, J.P. et al. Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *Eur J Immunol* 27, 3492-8 (1997).
262. Sutton, V.R., Wowk, M.E., Cancilla, M. & Trapani, J.A. Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. *Immunity* 18, 319-29 (2003).
263. Adrain, C., Murphy, B.M. & Martin, S.J. Molecular ordering of the caspase activation cascade initiated by the cytotoxic T lymphocyte/natural killer (CTL/NK) protease granzyme B. *J Biol Chem* 280, 4663-73 (2005).
264. Chavez-Galan, L., Arenas-Del Angel, M.C., Zenteno, E., Chavez, R. & Lascurain, R. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol* 6, 15-25 (2009).
265. Hoves, S., Trapani, J.A. & Voskoboinik, I. The battlefield of perforin/granzyme cell death pathways. *J Leukoc Biol* 87, 237-43 (2010).
266. Pena, S.V. & Krensky, A.M. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. *Semin Immunol* 9, 117-25 (1997).
267. Anderson, D.H. et al. Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol* 325, 355-65 (2003).
268. Stenger, S. et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 282, 121-5 (1998).
269. Deng, A. et al. Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol* 174, 5243-8 (2005).
270. Clayberger, C. & Krensky, A.M. Granulysin. *Curr Opin Immunol* 15, 560-5 (2003).
271. Walch, M. et al. Cytotoxic cells kill intracellular bacteria through granulysin-mediated delivery of granzymes. *Cell* 157, 1309-23 (2014).
272. Ostergaard, H.L., Kane, K.P., Mescher, M.F. & Clark, W.R. Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature* 330, 71-2 (1987).
273. Rouvier, E., Luciani, M.F. & Golstein, P. Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. *J Exp Med* 177, 195-200 (1993).
274. Powell, W.C., Fingleton, B., Wilson, C.L., Boothby, M. & Matrisian, L.M. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr Biol* 9, 1441-7 (1999).
275. Scaffidi, C. et al. Two CD95 (APO-1/Fas) signaling pathways. *Embo J* 17, 1675-87 (1998).
276. Clementi, R. et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. *Blood* 105, 4424-8 (2005).
277. Baker, M.B., Riley, R.L., Podack, E.R. & Levy, R.B. Graft-versus-host-disease-associated lymphoid hypoplasia and B cell dysfunction is dependent upon donor T

cell-mediated Fas-ligand function, but not perforin function. *Proc Natl Acad Sci U S A* 94, 1366-71 (1997).

278. Van Parijs, L. & Abbas, A.K. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 280, 243-8 (1998).

279. Russell, J.H. & Wang, R. Autoimmune gld mutation uncouples suicide and cytokine/proliferation pathways in activated, mature T cells. *Eur J Immunol* 23, 2379-82 (1993).

280. Schoenborn, J.R. & Wilson, C.B. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* 96, 41-101 (2007).

281. Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75, 163-89 (2004).

282. Zheng, L. et al. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* 377, 348-51 (1995).

283. Ratner, A. & Clark, W.R. Role of TNF-alpha in CD8+ cytotoxic T lymphocyte-mediated lysis. *J Immunol* 150, 4303-14 (1993).

284. Boissonnas, A., Fetler, L. & Amigorena, S. [Stepwise strategy adopted by cytotoxic T cells to eliminate solid tumors]. *Med Sci (Paris)* 23, 570-2 (2007).

285. Romero, P. et al. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol Rev* 188, 81-96 (2002).

286. Romero, P. et al. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med* 188, 1641-50 (1998).

287. Pittet, M.J. et al. In vivo imaging of T cell delivery to tumors after adoptive transfer therapy. *Proc Natl Acad Sci U S A* 104, 12457-61 (2007).

288. Breart, B., Lemaitre, F., Celli, S. & Bousso, P. Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. *J Clin Invest* 118, 1390-7 (2008).

289. Macken, C.A. & Perelson, A.S. A multistage model for the action of cytotoxic T lymphocytes in multicellular conjugates. *J Immunol* 132, 1614-24 (1984).

290. Perelson, A.S. & Bell, G.I. Delivery of lethal hits by cytotoxic T lymphocytes in multicellular conjugates occurs sequentially but at random times. *J Immunol* 129, 2796-801 (1982).

291. Rothstein, T.L., Mage, M., Jones, G. & McHugh, L.L. Cytotoxic T lymphocyte sequential killing of immobilized allogeneic tumor target cells measured by time-lapse microcinematography. *J Immunol* 121, 1652-6 (1978).

292. Buchholz, V.R. et al. Disparate individual fates compose robust CD8+ T cell immunity. *Science* 340, 630-5 (2013).

293. Vasconcelos, Z. et al. Individual Human Cytotoxic T Lymphocytes Exhibit Intraclonal Heterogeneity during Sustained Killing. *Cell Rep* 11, 1474-85 (2015).

294. Schumacher, T.N. & Schreiber, R.D. Neoantigens in cancer immunotherapy. *Science* 348, 69-74 (2015).

295. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* 100, 57-70 (2000).

296. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* 144, 646-74 (2011).

297. Shay, J.W. & Bacchetti, S. A survey of telomerase activity in human cancer. *Eur J Cancer* 33, 787-91 (1997).

298. Bryan, T.M. & Cech, T.R. Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol* 11, 318-24 (1999).

299. Counter, C.M. et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *Embo J* 11, 1921-9 (1992).
300. Christofori, G. & Semb, H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci* 24, 73-6 (1999).
301. Varner, J.A. & Cheresh, D.A. Integrins and cancer. *Curr Opin Cell Biol* 8, 724-30 (1996).
302. Symonds, H. et al. p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell* 78, 703-11 (1994).
303. Harris, C.C. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst* 88, 1442-55 (1996).
304. Vaux, D.L., Cory, S. & Adams, J.M. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335, 440-2 (1988).
305. Witsch, E., Sela, M. & Yarden, Y. Roles for growth factors in cancer progression. *Physiology (Bethesda)* 25, 85-101 (2010).
306. Qian, B.Z. & Pollard, J.W. Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39-51 (2010).
307. Murdoch, C., Muthana, M., Coffelt, S.B. & Lewis, C.E. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 8, 618-31 (2008).
308. Grivennikov, S.I., Greten, F.R. & Karin, M. Immunity, inflammation, and cancer. *Cell* 140, 883-99 (2010).
309. Semenza, G.L. Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta* 1813, 1263-8.
310. Kroemer, G. & Pouyssegur, J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell* 13, 472-82 (2008).
311. Kim, R., Emi, M. & Tanabe, K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* 121, 1-14 (2007).
312. Pages, F. et al. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* 29, 1093-102 (2010
).
313. Nelson, B.H. The impact of T-cell immunity on ovarian cancer outcomes. *Immunol Rev* 222, 101-16 (2008).
314. Gray-Schopfer, V., Wellbrock, C. & Marais, R. Melanoma biology and new targeted therapy. *Nature* 445, 851-7 (2007).
315. Gelfer, A. & Rivers, J.K. Long-term follow-up of a patient with eruptive melanocytic nevi after Stevens-Johnson syndrome. *Arch Dermatol* 143, 1555-7 (2007).
316. Speeckaert, R. et al. Immune reactions in benign and malignant melanocytic lesions: lessons for immunotherapy. *Pigment Cell Melanoma Res* 24, 334-44 (2010).
317. Lee, K.C. & Weinstock, M.A. Melanoma is up: are we up to this challenge? *J Invest Dermatol* 129, 1604-6 (2009).
318. Bonadies, D.C. & Bale, A.E. Hereditary melanoma. *Curr Probl Cancer* 35, 162-72 (2011).
319. Tsao, H., Chin, L., Garraway, L.A. & Fisher, D.E. Melanoma: from mutations to medicine. *Genes Dev* 26, 1131-55 (2012).
320. Raaijmakers, M.I. et al. Melanoma immunotherapy: historical precedents, recent successes and future prospects. *Immunotherapy* 5, 169-82 (2013).
321. Minn, A.J. et al. Genes that mediate breast cancer metastasis to lung. *Nature* 436, 518-24 (2005).

322. Huang, F.J. et al. Molecular basis for the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis. *Cancer Res* 68, 9634-42 (2008).
323. Xie, T.X. et al. Activation of stat3 in human melanoma promotes brain metastasis. *Cancer Res* 66, 3188-96 (2006).
324. Diment, S., Eidelman, M., Rodriguez, G.M. & Orlow, S.J. Lysosomal hydrolases are present in melanosomes and are elevated in melanizing cells. *J Biol Chem* 270, 4213-5 (1995).
325. Orlow, S.J. Melanosomes are specialized members of the lysosomal lineage of organelles. *J Invest Dermatol* 105, 3-7 (1995).
326. Jimbow, K. et al. Biological role of tyrosinase related protein and its biosynthesis and transport from TGN to stage I melanosome, late endosome, through gene transfection study. *Pigment Cell Res* 10, 206-13 (1997).
327. Wu, X. & Hammer, J.A. Melanosome transfer: it is best to give and receive. *Curr Opin Cell Biol* 29, 1-7 (2014
).
328. Kuroda, T.S. & Fukuda, M. Functional analysis of Slac2-c/MyRIP as a linker protein between melanosomes and myosin VIIa. *J Biol Chem* 280, 28015-22 (2005).
329. Matsui, T., Ohbayashi, N. & Fukuda, M. The Rab interacting lysosomal protein (RILP) homology domain functions as a novel effector domain for small GTPase Rab36: Rab36 regulates retrograde melanosome transport in melanocytes. *J Biol Chem* 287, 28619-31 (2012).
330. Ohbayashi, N. & Fukuda, M. Role of Rab family GTPases and their effectors in melanosomal logistics. *J Biochem* 151, 343-51 (2012).
331. Byers, H.R., Yaar, M., Eller, M.S., Jalbert, N.L. & Gilchrest, B.A. Role of cytoplasmic dynein in melanosome transport in human melanocytes. *J Invest Dermatol* 114, 990-7 (2000).
332. Hara, M. et al. Kinesin participates in melanosomal movement along melanocyte dendrites. *J Invest Dermatol* 114, 438-43 (2000).
333. Gennerich, A. & Vale, R.D. Walking the walk: how kinesin and dynein coordinate their steps. *Curr Opin Cell Biol* 21, 59-67 (2009).
334. Barral, D.C. & Seabra, M.C. The melanosome as a model to study organelle motility in mammals. *Pigment Cell Res* 17, 111-8 (2004).
335. Yatsu, A., Ohbayashi, N., Tamura, K. & Fukuda, M. Syntaxin-3 is required for melanosomal localization of Tyrp1 in melanocytes. *J Invest Dermatol* 133, 2237-46 (2013).
336. Pols, M.S. et al. hVps41 and VAMP7 function in direct TGN to late endosome transport of lysosomal membrane proteins. *Nat Commun* 4, 1361 (2013).
337. Jani, R.A., Purushothaman, L.K., Rani, S., Bergam, P. & Setty, S.R. STX13 regulates cargo delivery from recycling endosomes during melanosome biogenesis. *J Cell Sci* 128, 3263-76 (2015).
338. Hume, A.N. et al. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *J Cell Biol* 152, 795-808 (2001).
339. Strom, M., Hume, A.N., Tarafder, A.K., Barkagianni, E. & Seabra, M.C. A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. *J Biol Chem* 277, 25423-30 (2002).
340. Fujita, H. et al. Evidence for distinct membrane traffic pathways to melanosomes and lysosomes in melanocytes. *J Investig Dermatol Symp Proc* 6, 19-24 (2001).
341. Vesely, M.D., Kershaw, M.H., Schreiber, R.D. & Smyth, M.J. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 29, 235-71 (2011).

342. Clemente, C.G. et al. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 77, 1303-1310 (1996).
343. Al-Batran, S.E. et al. Intratumoral T-cell infiltrates and MHC class I expression in patients with stage IV melanoma. *Cancer Research* 65, 3937-3941 (2005).
344. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 6, 715-27 (2006).
345. Koebel, C.M. et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450, 903-7 (2007).
346. Eyles, J. et al. Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. *J Clin Invest* 120, 2030-9 (2010).
347. Chang, C.C., Campoli, M. & Ferrone, S. Classical and nonclassical HLA class I antigen and NK Cell-activating ligand changes in malignant cells: current challenges and future directions. *Adv Cancer Res* 93, 189-234 (2005).
348. Campoli, M. & Ferrone, S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* 27, 5869-85 (2008).
349. Denfeld, R.W. et al. In situ expression of B7 and CD28 receptor families in human malignant melanoma: relevance for T-cell-mediated anti-tumor immunity. *Int J Cancer* 62, 259-65 (1995).
350. Paul, P. et al. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc Natl Acad Sci U S A* 95, 4510-5 (1998).
351. Rabinovich, G.A., Gabrilovich, D. & Sotomayor, E.M. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25, 267-96 (2007).
352. Zhuang, L. et al. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. *Mod Pathol* 20, 416-26 (2007).
353. Lickliter, J.D. et al. Small-molecule Bcl-2 inhibitors sensitise tumour cells to immune-mediated destruction. *Br J Cancer* 96, 600-8 (2007).
354. Schinke, C. et al. Aberrant DNA methylation in malignant melanoma. *Melanoma Res* 20, 253-65 (2010).
355. Tian, F. et al. Expression of c-FLIP in malignant melanoma, and its relationship with the clinicopathological features of the disease. *Clin Exp Dermatol* 37, 259-65 (2012).
356. Gajewski, T.F. et al. Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. *Current Opinion in Immunology* 25, 268-276 (2013).
357. Hahne, M. et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 274, 1363-6 (1996).
358. Andreola, G. et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med* 195, 1303-16 (2002).
359. Peter, M.E. et al. The role of CD95 and CD95 ligand in cancer. *Cell Death Differ* 22, 885-6 (2015).
360. Chappell, D.B., Zaks, T.Z., Rosenberg, S.A. & Restifo, N.P. Human melanoma cells do not express Fas (Apo-1/CD95) ligand. *Cancer Res* 59, 59-62 (1999).
361. Eberle, J. et al. CD95/Fas signaling in human melanoma cells: conditional expression of CD95L/FasL overcomes the intrinsic apoptosis resistance of malignant melanoma and inhibits growth and progression of human melanoma xenotransplants. *Oncogene* 22, 9131-41 (2003).

362. Viguier, M. et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 173, 1444-53 (2004).
363. Oelkrug, C. & Ramage, J.M. Enhancement of T cell recruitment and infiltration into tumours. *Clin Exp Immunol* 178, 1-8 (2014).
364. Wang, X., Dong, H., Li, Q., Li, Y. & Hong, A. Thioredoxin induces Tregs to generate an immunotolerant tumor microenvironment in metastatic melanoma. *Oncoimmunology* 4, e1027471.
365. Dong, H. et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8, 793-800 (2002).
366. Taube, J.M. et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 4, 127ra37 (2012).
367. Hino, R. et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* 116, 1757-66 (2010).
368. Marquez-Rodas, I. et al. Immune checkpoint inhibitors: therapeutic advances in melanoma. *Ann Transl Med* 3, 267 (2015).
369. Eggermont, A.M. & Kirkwood, J.M. Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? *Eur J Cancer* 40, 1825-36 (2004).
370. Atkins, M.B. et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* 17, 2105-16 (1999).
371. Phan, G.Q., Attia, P., Steinberg, S.M., White, D.E. & Rosenberg, S.A. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. *Journal of Clinical Oncology* 19, 3477-3482 (2001).
372. Hill, G.J., Krementz, E.T. & Hill, H.Z. Dimethyl Triazeno Imidazole Carboxamide and Combination Therapy for Melanoma .4. Late Results after Complete Response to Chemotherapy (Central Oncology Group Protocols 7130, 7131, and 7131a). *Cancer* 53, 1299-1305 (1984).
373. Rosenberg, S.A. et al. Development of long-term cell lines and lymphoid clones reactive against murine and human tumors: a new approach to the adoptive immunotherapy of cancer. *Surgery* 92, 328-36 (1982).
374. Rosenberg, S.A. et al. Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* 210, 474-84; discussion 484-5 (1989).
375. Rosenberg, S.A. et al. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 86, 1159-66 (1994).
376. Ridolfi, R. et al. Cisplatin, dacarbazine with or without subcutaneous interleukin-2, and interferon alpha-2b in advanced melanoma outpatients: results from an Italian multicenter phase III randomized clinical trial. *J Clin Oncol* 20, 1600-7 (2002).
377. Keilholz, U. et al. Dacarbazine, cisplatin, and interferon-alfa-2b with or without interleukin-2 in metastatic melanoma: a randomized phase III trial (18951) of the European Organisation for Research and Treatment of Cancer Melanoma Group. *J Clin Oncol* 23, 6747-55 (2005).
378. Platanias, L.C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 5, 375-86 (2005).

379. Rozati, S., Naef, L., Levesque, M.P., French, L.E. & Dummer, R. Real-life experience with pegylated interferon and conventional interferon in adjuvant melanoma therapy. *J Immunother* 36, 52-6 (2013).
380. Grunhagen, D.J. et al. Outcome and prognostic factor analysis of 217 consecutive isolated limb perfusions with tumor necrosis factor-alpha and melphalan for limb-threatening soft tissue sarcoma. *Cancer* 106, 1776-84 (2006).
381. Berd, D., Maguire, H.C., Jr., McCue, P. & Mastrangelo, M.J. Treatment of metastatic melanoma with an autologous tumor-cell vaccine: clinical and immunologic results in 64 patients. *J Clin Oncol* 8, 1858-67 (1990).
382. Balch, C.M. et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 27, 6199-206 (2009).
383. Ogi, C. & Aruga, A. Clinical evaluation of therapeutic cancer vaccines. *Human Vaccines & Immunotherapeutics* 9, 1049-1057 (2013).
384. Speiser, D.E. et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 115, 739-46 (2005).
385. Goldinger, S.M. et al. Nano-particle vaccination combined with TLR-7 and -9 ligands triggers memory and effector CD8(+) T-cell responses in melanoma patients. *Eur J Immunol* 42, 3049-61 (2012).
386. Weber, J.S. Current perspectives on immunotherapy. *Semin Oncol* 41 Suppl 5, S14-29 (2014).
387. Walker, L.S. & Sansom, D.M. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* 11, 852-63 (2011).
**388. Hodi, F.S. et al. Ipilimumab plus sargramostim vs ipilimumab alone for treatment of metastatic melanoma: a randomized clinical trial. *Jama* 312, 1744-53 (2010
).**
389. Ribas, A. Clinical development of the anti-CTLA-4 antibody tremelimumab. *Semin Oncol* 37, 450-4 (2010).
390. Margolin, K. Ipilimumab in a Phase II trial of melanoma patients with brain metastases. *Oncoimmunology* 1, 1197-1199 (2012).
391. Topalian, S.L. et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol* 32, 1020-30 (2012).
392. Sierro, S.R. et al. Combination of lentivector immunization and low-dose chemotherapy or PD-1/PD-L1 blocking primes self-reactive T cells and induces anti-tumor immunity. *Eur J Immunol* 41, 2217-28 (2011).
393. Dudley, M.E. et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298, 850-4 (2002).
394. Kochenderfer, J.N. & Rosenberg, S.A. Treating B-cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nat Rev Clin Oncol* 10, 267-76 (2013).
395. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 348, 62-8 (2015).
396. Zang, Y.W., Gu, X.D., Xiang, J.B. & Chen, Z.Y. Clinical application of adoptive T cell therapy in solid tumors. *Medical Science Monitor* 20, 953-959 (2014).
397. Holsken, O., Miller, M. & Cerwenka, A. Exploiting natural killer cells for therapy of melanoma. *J Dtsch Dermatol Ges* 13, 23-9 (2015).
398. Chapman, P.B. et al. Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. *New England Journal of Medicine* 364, 2507-2516 (2011).

399. Romero, P., Cerottini, J.C. & Speiser, D.E. The human T cell response to melanoma antigens. *Adv Immunol* 92, 187-224 (2006).
400. Chen, D.S. & Mellman, I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* 39, 1-10 (2013).
401. Coulie, P.G., Van den Eynde, B.J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nature Reviews Cancer* 14, 135-146 (2014).
402. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 348, 62-8.
403. Kalos, M. & June, C.H. Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology. *Immunity* 39, 49-60 (2013).
404. Wimmers, F., Schreibelt, G., Skold, A.E., Figdor, C.G. & De Vries, I.J. Paradigm Shift in Dendritic Cell-Based Immunotherapy: From in vitro Generated Monocyte-Derived DCs to Naturally Circulating DC Subsets. *Front Immunol* 5, 165.
405. Aris, M. & Barrio, M.M. Combining immunotherapy with oncogene-targeted therapy: a new road for melanoma treatment. *Front Immunol* 6, 46.
406. Bertrand, F. et al. An initial and rapid step of lytic granule secretion precedes microtubule organizing center polarization at the cytotoxic T lymphocyte/target cell synapse. *Proc Natl Acad Sci U S A* 110, 6073-8.
407. Lopez, J.A., Brennan, A.J., Whisstock, J.C., Voskoboinik, I. & Trapani, J.A. Protecting a serial killer: pathways for perforin trafficking and self-defence ensure sequential target cell death. *Trends in Immunology* 33, 406-412 (2012).
408. Lopez, J.A. et al. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* 121, 2659-68.
409. Voskoboinik, I. & Trapani, J.A. Perforinopathy: a spectrum of human immune disease caused by defective perforin delivery or function. *Front Immunol* 4, 441.
410. Valitutti, S., Muller, S., Dessing, M. & Lanzavecchia, A. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J Exp Med* 183, 1917-21 (1996).
411. Zimmerer, R.M. et al. Functional features of cancer stem cells in melanoma cell lines. *Cancer Cell Int* 13, 78.
412. Lopez, J.A. et al. Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. *J Immunol* 191, 2328-34.
413. Reddy, A., Caler, E.V. & Andrews, N.W. Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. *Cell* 106, 157-69 (2001).
414. Jimenez, A.J. et al. ESCRT Machinery Is Required for Plasma Membrane Repair. *Science* 343, 986-+ (2014).
415. Joulia, R. et al. Mast cells form antibody-dependent degranulatory synapse for dedicated secretion and defence. *Nature Communications* 6(2015).
416. Korpetinou, A. et al. Serglycin: at the crossroad of inflammation and malignancy. *Front Oncol* 3, 327.
417. Meen, A.J. et al. Serglycin Is a Major Proteoglycan in Polarized Human Endothelial Cells and Is Implicated in the Secretion of the Chemokine GRO alpha/CXCL1. *Journal of Biological Chemistry* 286, 2636-2647 (2011).
418. Matarrese, P. et al. Cathepsin B inhibition interferes with metastatic potential of human melanoma: an in vitro and in vivo study. *Mol Cancer* 9, 207.

419. Rao, S.K., Huynh, C., Proux-Gillardeaux, V., Galli, T. & Andrews, N.W. Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. *Journal of Biological Chemistry* 279, 20471-20479 (2004).
420. Soares, H. et al. Regulated vesicle fusion generates signaling nanoterritories that control T cell activation at the immunological synapse. *Journal of Experimental Medicine* 210, 2415-2433 (2013).
421. Misinzo, G., Delputte, P.L. & Nauwynck, H.J. Inhibition of endosome-lysosome system acidification enhances porcine circovirus 2 infection of porcine epithelial cells. *Journal of Virology* 82, 1128-1135 (2008).
422. Valitutti, S., Muller, S., Salio, M. & Lanzavecchia, A. Degradation of T cell receptor (TCR)-CD3-xi complexes after antigenic stimulation. *Journal of Experimental Medicine* 185, 1859-1864 (1997).
423. Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J. & Eisen, H.N. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* 4, 565-71 (1996).
424. Pham, C.T.N. & Ley, T.J. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 96, 8627-8632 (1999).
425. D'Angelo, M.E. et al. Cathepsin H Is an Additional Convertase of Pro-granzyme B. *Journal of Biological Chemistry* 285, 20514-20519 (2010).
426. Schreiber, R.D., Old, L.J. & Smyth, M.J. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science* 331, 1565-1570 (2011).
427. Lehmann, C., Zeis, M., Schmitz, N. & Uharek, L. Impaired binding of perforin on the surface of tumor cells is a cause of target cell resistance against cytotoxic effector cells. *Blood* 96, 594-600 (2000).
428. Jazirehi, A.R., Nazarian, R., Torres-Collado, A.X. & Economou, J.S. Aberrant apoptotic machinery confers melanoma dual resistance to BRAF(V600E) inhibitor and immune effector cells: immunosensitization by a histone deacetylase inhibitor. *Am J Clin Exp Immunol* 3, 43-56.
429. Jazirehi, A.R., Baritaki, S., Koya, R.C., Bonavida, B. & Economou, J.S. Molecular mechanism of MART-1+/A*0201+ human melanoma resistance to specific CTL-killing despite functional tumor-CTL interaction. *Cancer Res* 71, 1406-17.
430. Murakami, T. et al. Immune evasion by murine melanoma mediated through CC chemokine receptor-10. *J Exp Med* 198, 1337-47 (2003).
431. Ribas, A. & Tumeh, P.C. The future of cancer therapy: selecting patients likely to respond to PD1/L1 blockade. *Clin Cancer Res* 20, 4982-4.
432. Taube, J.M. Emerging immunologic biomarkers: setting the (TNM-immune) stage. *Clin Cancer Res* 20, 2023-5.
433. Lindsay, C.R., Spiliopoulou, P. & Waterston, A. Blinded by the light: why the treatment of metastatic melanoma has created a new paradigm for the management of cancer. *Therapeutic Advances in Medical Oncology* 7, 107-121 (2015).
434. Frohlich, E. Proteases in cutaneous malignant melanoma: relevance as biomarker and therapeutic target. *Cellular and Molecular Life Sciences* 67, 3947-3960 (2010).
435. Sanchez-Ruiz, Y., Valitutti, S. & Dupre, L. Stepwise maturation of lytic granules during differentiation and activation of human CD8+ T lymphocytes. *PLoS One* 6, e27057.
436. Mellman, I., Fuchs, R. & Helenius, A. Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem* 55, 663-700 (1986).

437. Maxfield, F.R. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J Cell Biol* 95, 676-81 (1982).
438. Halaban, R. et al. Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway. *J Biol Chem* 277, 14821-8 (2002).
439. Ndolo, R.A., Luan, Y., Duan, S., Forrest, M.L. & Krise, J.P. Lysosomotropic properties of weakly basic anticancer agents promote cancer cell selectivity in vitro. *PLoS One* 7, e49366 (2012).
440. Acha-Orbea, H., Groscurth, P., Lang, R., Stitz, L. & Hengartner, H. Characterization of cloned cytotoxic lymphocytes with NK-like activity. *J Immunol* 130, 2952-9 (1983).
441. Kataoka, T. et al. Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H(+)-ATPase, on CTL-mediated cytotoxicity. *J Immunol* 153, 3938-47 (1994).
442. Lanzavecchia, A. & Watts, C. Peptide partners call the tune. *Nature* 371, 198-9 (1994).
443. Ritter, A.T. et al. Actin Depletion Initiates Events Leading to Granule Secretion at the Immunological Synapse. *Immunity* 42, 864-876 (2015).
444. Morita, Y. et al. Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A*02 or HLA-A*24 phenotype undergoing hematopoietic stem cell transplantation. *Bone Marrow Transplant* 36, 803-11 (2005).
445. Igney, F.H. & Krammer, P.H. Immune escape of tumors: apoptosis resistance and tumor counterattack. *J Leukoc Biol* 71, 907-20 (2002).
446. Maddodi, N. & Setaluri, V. Prognostic significance of melanoma differentiation and trans-differentiation. *Cancers (Basel)* 2, 989-99 (2010).
447. Logozzi, M. et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One* 4, e5219 (2009).
448. Hamai, A. et al. ICAM-1 has a critical role in the regulation of metastatic melanoma tumor susceptibility to CTL lysis by interfering with PI3K/AKT pathway. *Cancer Res* 68, 9854-64 (2008).
449. Takenoyama, M. et al. A point mutation in the NFYC gene generates an antigenic peptide recognized by autologous cytolytic T lymphocytes on a human squamous cell lung carcinoma. *Int J Cancer* 118, 1992-7 (2006).
450. Hoek, K. et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res* 64, 5270-82 (2004).
451. Wasmeier, C., Hume, A.N., Bolasco, G. & Seabra, M.C. Melanosomes at a glance. *J Cell Sci* 121, 3995-9 (2008).
452. Alves, C.P. et al. Myosin-Va contributes to manifestation of malignant-related properties in melanoma cells. *J Invest Dermatol* 133, 2809-12 (2013).
453. Barbarin, A. & Frade, R. Procathepsin L secretion, which triggers tumour progression, is regulated by Rab4a in human melanoma cells. *Biochem J* 437, 97-107 (2011).
454. Milstein, O. et al. CTLs respond with activation and granule secretion when serving as targets for T-cell recognition. *Blood* 117, 1042-52 (2011).
455. Poenie, M., Kuhn, J. & Combs, J. Real-time visualization of the cytoskeleton and effector functions in T cells. *Curr Opin Immunol* 16, 428-38 (2004).
456. Binah, O., Liu, C.C., Young, J.D. & Berke, G. Channel formation and [Ca2+]i accumulation induced by perforin N-terminus peptides: comparison with purified perforin and whole lytic granules. *Biochem Biophys Res Commun* 240, 647-50 (1997).

457. **Kuta, A.E., Reynolds, C.R. & Henkart, P.A. Mechanism of lysis by large granular lymphocyte granule cytolysin: generation of a stable cytolysin-RBC intermediate. *J Immunol* 142, 4378-84 (1989).**
458. **Kuta, A.E., Bashford, C.L., Pasternak, C.A., Reynolds, C.W. & Henkart, P.A. Characterization of non-lytic cytolysin-membrane intermediates. *Mol Immunol* 28, 1263-70 (1991).**
459. **Matsuda, K. et al. Apolipoprotein B is a major perforin inhibitor protein in human serum. *Mol Immunol* 28, 1211-6 (1991).**
460. **Liu, C.C. et al. Resistance of cytolytic lymphocytes to perforin-mediated killing. Induction of resistance correlates with increase in cytotoxicity. *J Exp Med* 169, 2211-25 (1989).**
461. **Verret, C.R., Firmenich, A.A., Kranz, D.M. & Eisen, H.N. Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. *J Exp Med* 166, 1536-47 (1987).**
462. **Blakely, A. et al. Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. *J Exp Med* 166, 1070-83 (1987).**
463. **Kranz, D.M. & Eisen, H.N. Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* 84, 3375-9 (1987).**
464. **Schick, B. & Berke, G. The lysis of cytotoxic T lymphocytes and their blasts by cytotoxic T lymphocytes. *Immunology* 71, 428-33 (1990).**
465. **Su, M.W., Walden, P.R., Golan, D.B. & Eisen, H.N. Cognate peptide-induced destruction of CD8+ cytotoxic T lymphocytes is due to fratricide. *J Immunol* 151, 658-67 (1993).**
466. **Dutz, J.P., Walden, P.R. & Eisen, H.N. Effects of cognate peptides on cytolytic and proliferative activities of cloned cytotoxic T lymphocytes. *Int Immunol* 4, 571-80 (1992).**
467. **Jiang, S.B., Ojcius, D.M., Persechini, P.M. & Young, J.D. Resistance of cytolytic lymphocytes to perforin-mediated killing. Inhibition of perforin binding activity by surface membrane proteins. *J Immunol* 144, 998-1003 (1990).**
468. **McNeil, P.L., Vogel, S.S., Miyake, K. & Terasaki, M. Patching plasma membrane disruptions with cytoplasmic membrane. *J Cell Sci* 113 (Pt 11), 1891-902 (2000).**
469. **Bansal, D. et al. Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature* 423, 168-72 (2003).**
470. **Redpath, G.M. et al. Calpain cleavage within dysferlin exon 40a releases a synaptotagmin-like module for membrane repair. *Mol Biol Cell* 25, 3037-48 (2014).**
471. **Andrews, N.W., Almeida, P.E. & Corrotte, M. Damage control: cellular mechanisms of plasma membrane repair. *Trends Cell Biol* 24, 734-42 (2014).**
472. **Jimenez, A.J. et al. ESCRT machinery is required for plasma membrane repair. *Science* 343, 1247136 (2014).**
473. **Cheng, X., Zhang, X., Yu, L. & Xu, H. Calcium signaling in membrane repair. *Semin Cell Dev Biol* (2015).**
474. **Bedard, P.L., Hansen, A.R., Ratain, M.J. & Siu, L.L. Tumour heterogeneity in the clinic. *Nature* 501, 355-64 (2013).**
475. **Somasundaram, R., Villanueva, J. & Herlyn, M. Intratumoral heterogeneity as a therapy resistance mechanism: role of melanoma subpopulations. *Adv Pharmacol* 65, 335-59 (2012).**
476. **Spranger, S. et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med* 5, 200ra116 (2013).**

477. Shultz, L.D., Ishikawa, F. & Greiner, D.L. Humanized mice in translational biomedical research. *Nat Rev Immunol* 7, 118-30 (2007).
478. Aspord, C., Yu, C.I., Banchereau, J. & Palucka, A.K. Humanized mice for the development and testing of human vaccines. *Expert Opin Drug Discov* 2, 949-60 (2007).
479. Greiner, D.L., Hesselton, R.A. & Shultz, L.D. SCID mouse models of human stem cell engraftment. *Stem Cells* 16, 166-77 (1998).
480. Hudson, W.A., Li, Q., Le, C. & Kersey, J.H. Xenotransplantation of human lymphoid malignancies is optimized in mice with multiple immunologic defects. *Leukemia* 12, 2029-33 (1998).
481. Ohsie, S.J., Sarantopoulos, G.P., Cochran, A.J. & Binder, S.W. Immunohistochemical characteristics of melanoma. *J Cutan Pathol* 35, 433-44 (2008).

Abstract

Roxana Khazen

Dissecting early mechanism of melanoma cell resistance to cytotoxic T lymphocyte attack

Thesis Director: Dr. Salvatore Valitutti

Thesis defense: 29 Jan 2016, Toulouse France

Human melanoma cells express various tumor antigens that are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) and elicit tumor-specific responses in vivo. However, natural and therapeutically enhanced CTL responses in melanoma patients are of limited efficacy. The mechanisms underlying the failure of CTL effector phase against melanomas are still largely elusive. Our hypothesis is that the limited efficacy of CTL in their fight against tumors is the result of an unfavorable balance between CTL ability to kill tumors and an intrinsic tumor resistance to CTL cytolytic activity.

During my thesis I focused on the molecular dynamics occurring at the lytic synapse in order to identify possible "early response-mechanism" of melanoma cells to CTL attack.

Using a combination of cutting edge microscopy approaches and molecular tools, I showed that upon conjugation with CTL, human melanoma cells undergo an exacerbated late endosome/lysosome trafficking, which is intensified at the lytic synapse and is paralleled by cathepsin-mediated perforin degradation and deficient granzyme B penetration. Abortion of SNAP-23-dependent lysosomal trafficking, pH perturbation or impairment of lysosomal proteolytic activity restores susceptibility to CTL attack.

Our results reveal an unprecedented strategy of melanoma cell "self-defense" at the immunologic synapse based on a lysosome secretory burst and perforin degradation at the lytic synapse. Interfering with this synaptic self-defense strategy might be instrumental to potentiate CTL-mediated therapies in melanoma patients.