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Sorbonne Université

École doctorale Physiologie, Physiopathologie et Thérapeutique

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The role of adenomatous polyposis coli in cytotoxic T cell functions

*Implication for familial adenomatous polyposis and anti-tumor
immune responses*

Par Marie Juzans

Thèse de doctorat en Immunologie

Dirigée par Vincenzo Di Bartolo et Andrés Alcover

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A Boule et Poulout,

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Summary

CD8 T cells form immunological synapses with antigen presenting cells to trigger their activation and differentiation into cytotoxic T cells (CTLs). Immunological synapses also form between CTLs and tumor cells, eliminating them.

Immunological synapse generation and functions are the result of T cell polarization toward the antigen presenting cell or the tumor target cell. This depends on the orchestrated action of the actin and microtubule cytoskeleton and of intracellular vesicle traffic. Actin cytoskeleton intensively polymerizes at the synapse, and then is excluded from the center to form a ring at the synapse periphery to stabilize it. Concomitantly, microtubules are repositioned at the synapse, allowing the polarization of the centrosome and its docking to the plasma membrane, defining a precise secretory domain in the synaptic cleft. Microtubules drive the polarized vesicular transport of TCR and several signaling molecules to the synapse, ensuring T cell activation. They also transport lytic granules in CTLs, ensuring their cytotoxic effector functions. Polarized vesicle traffic and fusion at the synapse therefore depends on the interplay between actin and microtubule cytoskeleton, but how this interplay is regulated is not completely understood.

In this thesis work, we have identified the polarity regulator and tumor suppressor adenomatous polyposis coli (Apc) as a key regulator of actin and microtubule cytoskeleton interplay in CTLs. Thus, Apc tunes actin cytoskeleton dynamics, allowing the actin ring formation at the synapse periphery. In addition, Apc regulates microtubule radial organization and centrosome polarization. As a likely consequence, Apc controls synapse shape symmetry and stability. Interestingly, Apc defects reduce early TCR signaling and nuclear translocation of the transcription factor NFAT, with no significant impact in CTL differentiation and cytokine production. Importantly, Apc modulates CTL cytotoxic activity, by allowing efficient lytic granule targeting, dynamics, and fusion at the synapse plasma membrane.

This work unveils a novel regulatory role of Apc in cytotoxic T cell effector functions, through its action as polarity regulator and cytoskeleton organizer. It provides further insight into the potential impact of Apc mutations in anti-tumor immune response in familial adenomatous polyposis. Apc mutations may thus cause a dual damage, primarily unbalancing epithelial

homeostasis, promoting the growth of epithelial adenomas, and, secondly, impairing CTL-mediated anti-tumor immunity, and thus tumor elimination.

Keywords: adenomatous polyposis coli, immunological synapse, cytotoxic T cells, T cell activation, cell polarity

Résumé

Les lymphocytes T CD8 forment des synapses immunologiques avec des cellules présentatrices d'antigène afin d'initier leur activation et différenciation en lymphocytes T cytotoxiques (CTLs). Les CTLs forment également des synapses avec les cellules tumorales afin de les éliminer.

La génération d'une synapse immunologique et ses fonctions sont le résultat de la polarisation du lymphocyte T vers la cellule présentatrice ou tumorale. Cette polarisation dépend de la réorganisation du cytosquelette d'actine et du réseau de microtubules, mais aussi du trafic vésiculaire intracellulaire. Le cytosquelette d'actine polymérise intensivement à la zone de contact avec la cellule présentatrice ou tumorale, puis est exclu du centre de la synapse pour former un anneau à sa périphérie, stabilisant ainsi la synapse. De façon concomitante, le réseau de microtubules est repositionné à la synapse immunologique, permettant la polarisation du centrosome et son positionnement très proche de la membrane, définissant un domaine de sécrétion précis à la synapse. La réorganisation des microtubules permet le transport polarisé à la synapse de vésicules contenant des TCR et des molécules de signalisation, assurant l'activation des lymphocytes T. Les microtubules facilitent également l'adressage et fusion des granules lytiques vers la synapse, assurant les fonctions effectrices cytotoxiques des CTLs. Les mécanismes moléculaires régulant ces différents processus, et plus particulièrement les interactions entre le cytosquelette d'actine et le réseau de microtubules, ne sont cependant pas bien caractérisés.

Dans cette étude, nous avons identifié adénomatus polyposis coli (Apc) comme un régulateur clé des interactions entre le cytosquelette d'actine et de microtubules dans les lymphocytes T cytotoxiques. Apc est un régulateur de la polarité cellulaire et un suppresseur de tumeurs dont la fonction dans les lymphocytes T CD8 n'était pas connue. Nous avons ainsi montré que Apc contrôle la dynamique du cytosquelette d'actine permettant la formation d'un anneau à la périphérie de la synapse. De plus, Apc contrôle l'organisation radiale des microtubules à la synapse. En conséquence, Apc module la forme, la symétrie et la stabilité de la synapse cytotoxique. De façon intéressante, des défauts de Apc réduisent la signalisation du TCR et empêchent la translocation efficace dans le noyau du facteur de transcription NFAT. Cependant, ces altérations n'induisent pas de défauts de différenciation ou de production de cytokines.

Néanmoins, Apc contrôle l'activité cytotoxique des lymphocytes T, facilitant l'adressage, la dynamique, et la fusion des granules lytiques à la membrane synaptique.

Ces résultats révèlent donc un nouveau rôle de Apc dans la régulation des fonctions cytotoxiques des lymphocytes T CD8, via son action de régulateur de polarité et d'organisateur du cytosquelette. Ils suggèrent une altération de la réponse immune anti-tumorale des patients atteints de polypose familiale. Les mutations de Apc pourraient donc d'une part, déséquilibrer l'homéostasie de l'épithélium dans le colon, favorisant le développement d'adénomes, et d'autre part, altérer la réponse cytotoxique des lymphocytes T CD8, diminuant leur capacité d'élimination des tumeurs.

Mots clés : adénomatos polyposis coli, synapse immunologique, lymphocytes T cytotoxiques, activation des lymphocytes T, polarité cellulaire

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Abbreviations

ALPS	Autoimmune lymphoproliferative syndrome
AP1	Activating protein 1
Apc	Adenomatous polyposis coli
Arp2/3	Actin related protein 2/3
Asef	Apc-stimulated guanine exchange factor
BCR	B cell receptor
CCL19	C-C motif chemokine ligand 19
CCL21	C-C motif chemokine ligand 21
CCR7	C-C motif chemokine receptor type 7
Cdc42	Cell division control protein 42 homolog
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAG	Diacylglycerol
Dlg1	Mammalian homologue of the Drosophila Disc large protein 1
EB1	End binding protein 1
ELISA	Enzyme-linked immunosorbent assay
Erk1/2	Extracellular signal-regulated kinase 1/2
F-actin	Actin filaments
FADD	Fas-associated death domain
FAP	Familial adenomatous polyposis
GAPs	GTPase-activating protein
GEFs	Guanine nucleotide exchange factors
GTPases	Guanosine triphosphatases
HEV	High endothelial venules
HLH	Hemophagocytic lymphohistiocytosis
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ikk β	I-kappa-B-kinase beta
ILC	Innate lymphoid cells
IL	Interleukin
IP ₃	Inositol trisphosphate
IQGAP-1	IQ motif containing GTPase activating protein 1
ITAMs	Immune receptor tyrosine-based activation motifs
JNK	Jun kinase
Lamp-1	Lysosomal-associated membrane protein 1
LAT	Linker for activation of T cells

Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
MAP kinase	Mitogen-activated protein kinase
MAPs	Microtubule-associated proteins
MHC	Major histocompatibility complex
MTOC	Microtubule-organizing center
NFAT	Nuclear factor of activated T cells
NF κ B	Nuclear factor kappa-B
NK cell	Natural killer cell
NPFs	Nucleation promoting factors
PD-1	Programmed cell death-1
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol (4,5)-bisphosphate
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PKC ζ	Protein kinase C ζ
PLC γ 1	Phospholipase C- γ 1
pMHC	Peptide bound to a major histocompatibility complex
Rac1	Ras-related C3 botulinum toxin substrate 1
SEB	Staphylococcal enterotoxin B
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
SMACs (c-, d-, p-)	Supramolecular activation complexes (central, distal, peripheral)
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TCR	T cell receptor
Th (1, 2, 17)	Helper T cells type 1, 2 or 17
TIRF	Total internal reflection fluorescence microscopy
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WASp	Wiskott Aldrich syndrome protein
WAVE	WASp-family verprolin-homologous protein
Zap70	ζ chain associated protein of 70 kDa

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Introduction

1. The immune system

The immune system is a highly developed network of organs (e.g. lymphoid organs), cells (e.g. lymphocytes, monocytes...) and humoral factors (e.g. antibodies, cytokines, chemokines...), which protects the organism against external or internal harm, while maintaining tolerance toward self and environmental common elements (e.g. food). It allows the host to detect and eliminate a diversity of pathogenic organisms, toxic substances, but also tumor cells.

Classically, the immune system has been divided in two categories: the innate immune system and the adaptive immune system, although the boundaries between the two systems are not very precise.

1.1. The innate immune system

Innate immune system acts as the first line of resistance against pathogen and is composed by anatomical and physiological barriers, numerous immune cells, cytokines and chemokines that they produce, and humoral components such as complement.

The innate immune system recognizes evolutionary conserved patterns (DNA, RNA, glycoproteins...) found on pathogens but not on any self structure. Therefore, the repertoire of innate cell receptors is limited, but still they can recognize a large variety of pathogens displaying molecular patterns binding those receptors. Furthermore, the innate immune system response is initiated really fast, as it starts to generate a protective inflammatory response within minutes after pathogen exposure (Turvey and Broide, 2010).

Anatomical and physiological barriers include epithelial cells layers, secreted mucus, mucociliary clearance mechanisms, low stomach pH and bacteriolytic lysozyme in tears or saliva. Additionally, innate immune cells increase anatomical and physiological barrier protection.

Innate immune cells, as all blood cells, derive from hematopoietic stem cells. Hematopoietic stem cells are found in bone marrow, peripheral blood and placenta and are capable of self-renewal and multilineage differentiation. They differentiate in multipotent progenitors, which

have lost their self-renewing capacity, and then further differentiate into common myeloid progenitors or common lymphoid progenitors. Common myeloid progenitors give rise to red blood cells, platelets, and innate immune cells including macrophages, dendritic cells, mast cell, neutrophils and eosinophils. On the other hand, natural killer (NK) cells and the most recently identified innate lymphoid cells (ILCs) differentiate from common lymphoid progenitors. Altogether, these cells are key for pathogen clearance but also for adaptive immune system activation (Seita and Weissman, 2010).

Indeed, macrophages, mast cells and neutrophils perform phagocytosis, i.e. they engulf and absorb pathogens and infected or tumor cells, destroying them. In addition, macrophages present phagocytosed antigens to adaptive immune cells. As macrophages, dendritic cells internalize antigens from their environment and process them for presentation to adaptive immune cells, thus triggering their activation (Guermonprez et al., 2002). ILCs produce a plethora of cytokines to tune other immune cell responses (Eberl et al., 2015). Finally, NK cells use combinations of activator and inhibitor receptors to target infected and tumor cells, leading to their killing through the release of granules containing lytic proteins that induce target cell apoptosis (Roda-Navarro, 2009).

1.2. The adaptive immune system

The adaptive immune system is only composed by cells, which as innate cells derive from hematopoietic stem cells, and cytokines and antibodies that they produce. However, they derive from common lymphoid progenitors that differentiate into T and B lymphocytes (Seita and Weissman, 2010).

In opposition to the innate immune system, the adaptive immune system has a more restricted recognition of pathogens. The repertoire of T cell receptors (TCRs) and B cell receptors (BCRs), as well as their soluble counterpart immunoglobulins, is highly diverse due to somatic gene rearrangements, making these proteins able to recognize very specific pathogen peptide fragments or antigens. The adaptive immune response thus needs longer time to be initiated. Indeed, T and B cells need first to recognize their cognate antigen presented by dendritic cells or macrophages, leading to their activation and differentiation into effector cells, before they generate a protective response (den Haan et al., 2014).

Once activated, B cells differentiate into plasma cells producing large amounts of antibodies, which recognize antigens on pathogens or infected cells and drive humoral immune responses (Nutt et al., 2015). On the other hand, T cells are divided into two main subsets, CD8 T cells that display cytotoxic effector functions similarly to NK cells, and CD4 T cells that display helper effector functions coordinating immune responses via cytokine secretion. CD4 T cells, which are divided in different subsets, can positively or negatively regulate other immune cells. Two major subsets have been identified: Th1 and Th2, which mostly induces cell-mediated immunity and humoral, including antibodies, responses, respectively. However, several other subsets have been characterized, such as Th17 which promotes inflammation and T regulator (Treg) which suppresses immune responses (Caza and Landas, 2015).

Besides the specificity, another characteristic of the adaptive immune system is the production of long-lived cells that have already encountered their specific antigen, allowing the maintenance of an immunological memory. Memory cells persist and can rapidly reactivate to achieve effector functions upon another encounter with their specific antigen, resulting in a faster and more efficient response upon re-infection by the same pathogen.

1.3. Link between innate and adaptive

While the innate and adaptive immune responses are fundamentally different in their mechanisms of action, and are often described separately, synergy between them is essential for an intact, fully efficient immune response. They act together with the innate response being the first line of host defense and the adaptive response becoming prominent after several days. Innate cells send activator signals to the adaptive cells, which in turn amplify their responses by recruiting innate cells, bringing about a complete immune response.

Furthermore, boundaries between the innate and adaptive immune systems are not fully strict, since some T cell subsets, as $\gamma\delta$ T cells display characteristics of both innate and adaptive immune cells (Hayday, 2019).

2. T lymphocytes

T lymphocytes are key cellular actors of adaptive immunity. Several T cell populations are involved in all aspects of the immune response due to their wide range of functions, from the positive or negative regulation and recruitment of other immune cells to the capacity to directly kill infected or tumor cells.

2.1. T cell development

Whereas the majority of hematopoietic lineages mature in the bone marrow, T cell development takes place in the thymus. This primary lymphoid organ is responsible for the generation and selection of T cells and their diverse TCR repertoire.

During their progress through the thymus, T cells differentiate into subpopulations, each with defined effector functions, the major ones being defined by their surface expression of CD4 or CD8 co-receptors.

Cells that are initially double negative (DN), i.e. express neither CD4 nor CD8, undergo somatic gene rearrangement of the genes coding for TCRs. This event marks their transition to double positive (DP) cells that express both CD4 and CD8. DP cells then undergo positive selection considering their recognition of the major histocompatibility complex (MHC), which present specific antigen to TCRs during immune responses, and negative selection considering their ability to recognize self-antigens (Germain, 2002).

Two types of MHCs have been described. Class I is expressed on all nucleated cells whereas class II is found only on certain cells of the immune system, including macrophages, dendritic cells and B cells. Class I MHC molecules present endogenous peptides while class II molecules present exogenous peptides. DP that are selected on class I MHC molecules become CD4⁻ CD8⁺, and those that are selected on class II MHC molecules become CD4⁺ CD8⁻ (Zuniga-Pflucker, 2004).

These processes of selection allow the generation of T cell subpopulations with different functions, but most importantly, ensure that only self-MHC-restricted and self-tolerant T cells survive and leave the thymus.

2.2. Homing into secondary lymphoid organs

Mature CD4 and CD8 T cells exit the thymus as naive cells, which need to encounter antigen-presenting cells displaying their specific antigen to be activated.

Naive T cells continuously circulate between the blood and lymph and the secondary lymphoid organs (lymph nodes, Peyer's patches and spleen) in search of cells presenting their specific antigen.

Migrating naive T cells enter secondary lymphoid organs through the process of homing, via high endothelial venules (HEV) through several steps of cellular migration and adhesion. Adhesion depends on the sequential activities of the L-selectin CD62L, the chemokine receptor CCR7 and the integrin LFA-1 (lymphocyte function-associated antigen 1). Mucin-like glycoproteins expressed on HEV endothelial cells interact with CD62L, allowing T cells to start the extravasation process through the endothelial cell layer lining the blood vessels (Hemmerich et al., 2001). Then, T cells undergo tethering and rolling steps, reducing their speed rate, allowing them to detect chemokines as CCL19 and CCL21 through their receptor, CCR7. Chemokine receptor signaling induces conformational changes in adhesion molecules like integrins LFA-1 and VLA-4, which binds to intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), respectively, and results in T cells arrest within HEVs. LFA-1 also triggers F-actin polymerization, leading to T cell flattening and crawling at the HEV surface, allowing them to cross the endothelial barrier and home to secondary lymphoid organ (von Andrian and Mempel, 2003).

Once in secondary lymphoid organs, T cells start scanning antigen presenting cell surface searching for their specific antigen. If they fail to encounter it, they egress secondary lymphoid organs and start again to circulate through the bloodstream or lymph.

2.3. T cell activation

In the rare cases where a naive T cell encounters an antigen presenting cell, generally dendritic cells, displaying its specific antigen bound to an MHC II at its surface, T cell stops. It polarizes toward the antigen presenting cell leading to the formation of a highly organized cell-cell contact zone called the immunological synapse, which ensures efficient antigen recognition and TCR signaling triggering leading to T cell activation, clonal expansion and differentiation.

Naive T cells require two essential signals for their activation and differentiation. Indeed, antigen recognition by the TCR alone can promote tolerance, whereas co-stimuli with a second signal help to develop an efficient immune response (Chen and Flies, 2013; Mueller et al., 1989).

Signal 1 relies on the TCR engaging with the appropriate antigen-MHC (pMHC) complex and provides the specificity to the response. The TCR is a glycosylated $\alpha\beta$ heterodimer expressed at the T cell surface. TCR α and β subunits have extracellular domains composed by variable and constant immunoglobulin-like domains. They are associated with the CD3 complex, formed by the γ , δ , ϵ , and ζ subunits, which are essential for the surface expression of the $\alpha\beta$ heterodimer and for TCR signaling, as they display signal transduction motifs named immune receptor tyrosine-based activation motifs (ITAMs). Furthermore, TCR-CD3 complexes form dynamic structures that are not stable at the plasma membrane but continuously traffic between the plasma membrane and endosomal compartments (Alcover et al., 2018).

The strength of signal 1 is influenced by the duration of the TCR-pMHC interaction, but also by the TCR affinity for the pMHC, the number of TCR-pMHC complexes formed, and the dose of antigen (Bullock et al., 2000; Henrickson et al., 2008).

In addition to specific pMHC complexes, T cells receive a 2nd signal through co-stimulatory molecules. Most of these proteins belong to the B7 family of immunoglobulin-like surface proteins, and are recognized by the CD28 family expressed by T cells. CD28 interacts with CD80 (B7.1) and CD86 (B7.2) expressed on the antigen-presenting cell surface. Another member of this family is ICOS (CD278) which, in contrast to constitutively expressed CD28, is upregulated upon T cell activation and interacts with ICOS ligand (ICOSL). Together, they allow the induction of the required signal for optimal T cell activation and proliferation, and for preventing T cell anergy (Acuto and Michel, 2003). However, the signal 2 also involves co-inhibitory molecules fundamental for T cell tolerance and autoimmunity regulation, as the

cytotoxic T-lymphocyte antigen-4 (CTLA-4), which is expressed after activation, and also interacts with CD80/86 but delivers inhibitory signals suppressing T cell proliferation (Rowshanravan et al., 2018).

Nevertheless, co-stimulation is not always sufficient to induce T cell complete differentiation into different subtypes, and an additional signal is often required. This signal corresponds to the cytokine combination presents in the environment, which strongly depends on the nature of the infection, and is key for the acquisition of effector functions (Mathieu et al., 2015; Raue et al., 2013; Read et al., 2016).

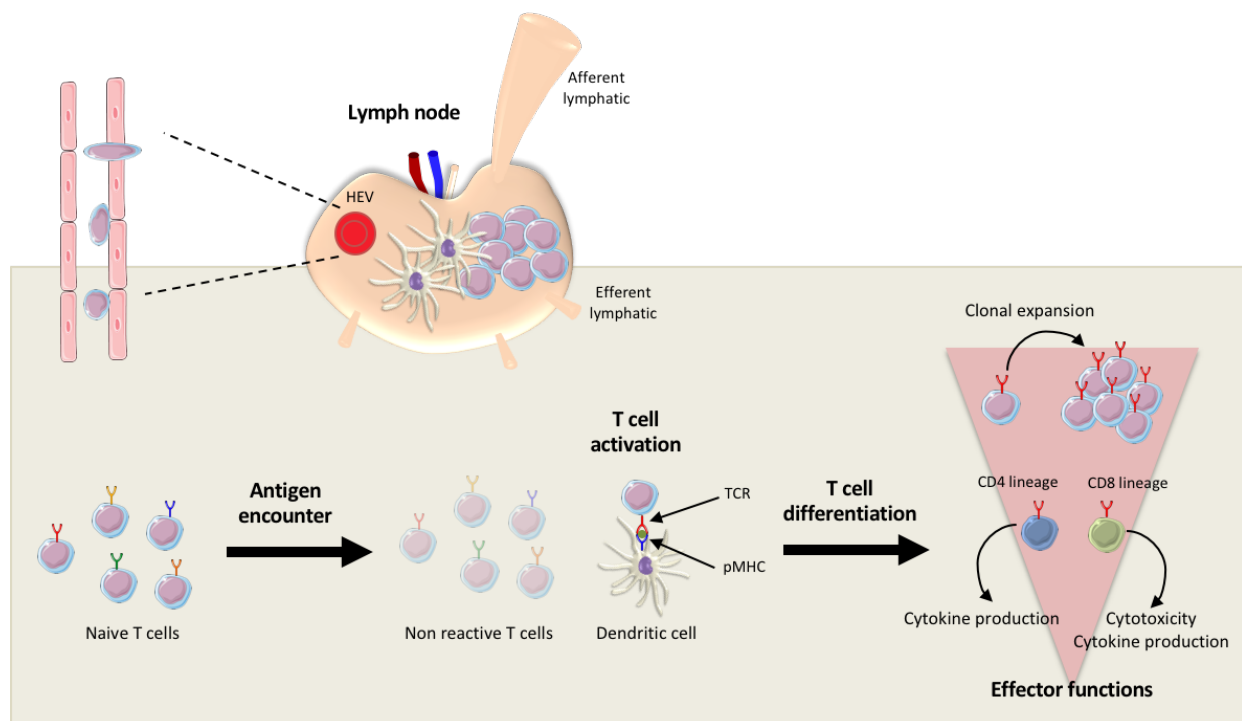


Figure 1. T cell journey from naive to effector cells

Simplified diagram of naive T cells entry into secondary lymphoid organs through high endothelial venules (HEV), the search and recognition of their cognate antigen on an antigen presenting dendritic cell, and the resulting T cell activation, clonal expansion and differentiation into effector cells. Based on (Courtney et al., 2018).

2.4. Naive T cell differentiation into effector cells

Upon activation, CD4 T cells differentiate into helper (Th) or T regulatory (Treg) T cells and CD8 T cells into cytotoxic T cells (CTLs). Their surface expression patterns of adhesion molecules and chemokine receptors change, allowing them to be guided to inflammatory sites where they achieve their effector functions. Several molecules are thus commonly used to study the journey of T cell through activation and differentiation.

- CD69 is a C-type lectin surface receptor. T cells rapidly express CD69 within a few hours upon TCR stimulation, therefore CD69 has been used as a very early activation marker (Cibrian and Sanchez-Madrid, 2017). CD69 regulates the differentiation of Treg cells as well as their secretion of cytokines (Yu et al., 2018). Additionally, CD69 tunes T cell migration by inducing the down-regulation of S1P1, a receptor required for lymphocyte egress from lymphoid organs. Consequently, CD69 controls the retention of activated T cells in secondary lymphoid organs (Shiow et al., 2006).

- CD25 is expressed within the first day upon TCR stimulation. CD25 is the alpha chain of the interleukin-2 (IL-2) receptor, its expression leads to the formation of high affinity IL-2 receptors, together with IL-2 β and γ_c subunits, that transduces the IL-2 signal required for T cell survival and proliferation (Letourneau et al., 2009). In addition, CD25 expression, associated with the expression of the FoxP3 transcription factor, allows to distinguish Tregs from others CD4 T cell subsets.

- CD44 is a surface receptor that binds to the extracellular matrix, promoting cell adhesion and migration. CD44 is up-regulated after TCR engagement and its expression is sustained on effector cells as well as on memory cells allowing their extravasation to inflammatory sites (DeGrendele et al., 1997; Nandi et al., 2004).

- CD62L, as previously mentioned (see 2.2.), is an L-selectin required for naive T cell homing in secondary lymphoid organs. CD62L ligands are highly expressed in the HEVs and their interaction initiates the extravasation process (Hemmerich et al., 2001). Upon T cell differentiation into effector or memory cell, CD62L expression decreases, preventing T cells from entering again into secondary lymphoid organs.

- CCR7 as presented previously is a chemokine receptor also involved in naive T cell homing in secondary lymphoid organs. After activation, as for CD62L, T cells lose CCR7 expression to prevent them from entering again in secondary lymphoid organs (Hauser and Legler, 2016).

- CD45 is a receptor protein tyrosine phosphatase key for TCR signaling (Desai et al., 1994). T cells display several CD45 isoforms, which vary depending on the stage of T cell activation and differentiation. Naive T cells express CD45RA. After activation, the extracellular domain of CD45RA undergoes alternative splicing and is replaced by CD45RO (Rheinlander et al., 2018).

The analysis of the expression of several of these markers is used to distinguish naive, effector and memory T cells. In human, CCR7 is often associated to CD45RA to make this distinction, and particularly to sub-divide T cell memory populations. Indeed, naive T cells are CCR7⁺ CD45RA⁺, whereas central memory (T_{CM}) cells are CCR7⁺ CD45RA⁻ and effector memory (T_{EM}) are CCR7⁻ CD45RA⁻, but can re-express CD45RA when they are highly differentiated during persistent viral infection or chronic disease. T_{CM} are mostly found in secondary lymphoid organs and proliferate extensively, whereas T_{EM}, which do not express CCR7, have the capacity to migrate to inflammatory sites and have increased effector functions (Sallusto et al., 2004).

In mice, CD62L is commonly associated with CD44. Naive T cells are CD62L⁺ CD44⁻ whereas T_{CM} are CD62L⁺ CD44⁺ and T_{EM} are CD62L⁻ CD44⁺. T_{EM} have thus the capacity to leave secondary lymphoid organs and migrate to inflammatory sites.

2.5. CD8 T cell effector functions

CD4 T cells play an important role in coordinating adaptive immune responses. Indeed, there are several T helper and regulatory subsets (see 1.2.), each one having specialized functions to control and regulate, positively and negatively, CD8 T cells and B lymphocytes differentiation, effector functions, and memory establishment (Yamane and Paul, 2013). Since this study has been conducted on CD8 T cells only, CD4 T cell effector functions will not be described here.

Once activated in secondary lymphoid organs, CD8 T cells migrate to inflamed tissues where they complete their differentiation into cytotoxic T cells (CTLs). There, they search for infected or tumor cells expressing their specific pMHC. Upon antigen recognition and immunological synapse formation, CTLs specifically kill target cells. The cytotoxic immune synapse requires the engagement of only three to ten pMHC, its duration is short, and cells detach rapidly after, allowing rapid elimination of pathogens (Purbhoo et al., 2004).

Three distinct mechanisms are involved in CTL effector functions. Two of them are complementary pathways leading to target cell lysis: the granule exocytosis pathway and the death-receptor pathway. The third one involves the release of different types of cytokines, as numerous others immune cells.

2.5.1. Lytic granule exocytosis

Lytic granule release is the most important mechanism for infected or tumor cell elimination. These granules are specialized secretory lysosomes released at the cytotoxic immune synapse cleft formed between the CTL and the target cell. The synaptic cleft has thus been assumed to optimize killing efficiency through high local concentration of lytic products and to prevent other non-targeted cells to be exposed to cytotoxic proteins.

Lytic granules share molecular markers and traffic regulation with late endosomes and lysosomes. They contain perforin, a pore forming protein, and granzymes, a family of serine proteases, in both T cells and NK cells (Peters et al., 1991). Both perforin and granzymes are poorly expressed in naive CD8 T cells, and are synthesized and stored in granules during CTL differentiation (Olsen et al., 1990; Sanchez-Ruiz et al., 2011).

Perforin inserts in the target cell plasma membrane, oligomerizes and forms pores (Thiery and Lieberman, 2014). Then, granzymes enter the target cell via these pores to trigger its apoptosis. Therefore, granzyme capacity to induce target cell death is dependent on perforin as highlighted in perforin knockout mice (Kagi et al., 1994; Walsh et al., 1994).

Several types of granzymes with different substrate specificity are expressed in CTLs. Granzyme B has the strongest pro-apoptotic function, and acts through two mechanisms. First, granzyme B can cleave its substrate after aspartate residues directly activating caspases, mostly caspase 3, which results in rapid apoptosis. Granzyme B can also facilitate caspase activation by cleaving the pro-apoptotic BH3 interacting death domain agonist Bid, inducing mitochondrial damage and cytochrome C release (Afonina et al., 2010). Granzyme A activates a slower mechanism of caspase-independent apoptosis by targeting the endoplasmic reticulum-associated complex SET. Granzyme A and B act independently to induce apoptosis, and the exact role of others granzymes is less known (Lieberman, 2003).

Defects in this cytotoxic pathway are associated with various disorders, particularly when perforin production is affected as in hemophagocytic lymphohistiocytosis (HLH), a disorder characterized by impaired granule-dependent cytotoxicity and uncontrolled T cell and macrophage activation, resulting in hyper-inflammation (Osinska et al., 2014; Stepp et al., 1999).

CTLs can kill multiple times in several hours, and thus need to be protected against their own cytotoxicity. Surface expression of the lysosomal protease cathepsin B locally at the synapse cleft could explain this self-protection (Balaji et al., 2002).

2.5.2. Death-receptor pathway

Upon CTL stimulation, Fas ligand (FasL) that is stored in intracellular granules is translocated to the CTL surface at the cytotoxic immune synapse, and recognizes the cell death surface receptor Fas expressed on target cells (Waring and Mullbacher, 1999). Fas, also known as CD95, is a member of the tumor necrosis factor (TNF) receptor family. After activation, the adapter molecule FADD (Fas-associated death domain) binds to Fas death domain recruiting pro-caspase-8. Caspase-8 then initiates the caspase cascade leading to target cell apoptosis (Micheau et al., 2002).

Defect in this cytotoxic pathway also lead to various disorders, as in autoimmune lymphoproliferative syndrome (ALPS), characterized by defective lymphocyte homeostasis and accumulation of autoreactive cells associated with *Fas* mutation (Rieux-Laucat et al., 2003).

2.5.3. Cytokine secretion

Upon TCR signaling, CTLs produce cytokines as IFN γ and TNF α , and secrete them at the synapse or in a multidirectional way (Huse et al., 2006; Kupfer et al., 1991).

IFN γ has multiple functions as it regulates more than 200 genes. IFN γ acts through a cell surface receptor whose ligation leads to Jak1 and Jak2 activation and subsequently to Stat1 phosphorylation which enters the nucleus and acts as a transcription factor (Schroder et al., 2004). IFN γ signaling in target cells ultimately promotes MHC I presentation pathway, amplifying their recognition by CTLs (Dolei et al., 1983). Furthermore, IFN γ is essential for pathogen clearance as it directly inhibits viral replication, and stimulates several immune cell types, as CD4 T cells, NK cells, or macrophages (Fensterl and Sen, 2009). This could also enhance anti-tumor responses.

TNF α functions are also diverse. TNF α acts through the TNFR1 or TNFR2 receptors that differ in their structure and in the signaling pathways they induce. Indeed, TNFR1 is almost ubiquitously expressed whereas TNFR2 is mainly expressed by immune and endothelial cells. TNF α binding to either receptors leads to the activation of the NF κ B transcription factor and MAP kinases (ERK, p38, JNK) (Hsu et al., 1995; Mehta et al., 2018; Rothe et al., 1995). However, TNFR2 engagement promotes cell survival, whereas TNFR1 engagement can induce either cell survival or cell death (Brenner et al., 2015). TNF α signaling can thus promote T cell activation and proliferation (Scheurich et al., 1987), and inflammatory response involving several cell types. However, it can also lead to their apoptosis via caspase 8 activation (Micheau and Tschopp, 2003).

2.6. Memory CD8 T cells

Upon pathogen clearance, most of the CTLs die within 1-2 weeks during the contraction phase of the immune response. Only 5-10% are left as long-lived memory cells that are able to rapidly induce a secondary response following re-exposure to the pathogen (Rocha and Tanchot, 2004; Youngblood et al., 2017).

Memory cells present common properties with effector and naive cells, including pluripotency and the ability to migrate in secondary lymphoid organs. In addition, cell expansion and re-acquisition of effector functions are faster in secondary responses, compared to primary responses, with much higher expansion.

In addition, several sub-populations of memory T cells displaying different properties have been unveiled. For example, as mentioned previously, T_{CM} are mostly found in secondary lymphoid organs and proliferate extensively, whereas T_{EM} migrate to inflammatory sites and have increased effector functions.

Therefore, T cell journey from hematopoietic stem cells to CTLs is long and highly regulated. Each step is crucial for efficient adaptive immune response. The impairment of anyone of them could indeed result in defective pathogen clearance, tumor escape, or autoimmunity.

3. The immunological synapse

As mentioned above, when T cells recognize pMHC on antigen-presenting cells or target cells, they form immunological synapses that allow the communication between the two cells and ensure fully efficient antigen recognition and the triggering of the TCR and co-signaling pathways, leading to T cell activation, proliferation, differentiation and the implementation of effector functions.

The immunological synapse is the result of a massive T cell polarization process that reorients the T cells toward the antigen-presenting cell or the target cell (see Figure 2). This process leads to the reorganization of a variety of T cell components, including cytoskeleton, several organelles, and the clustering of a plethora of receptors, signaling and adhesion molecules at the immunological synapse (Soares et al., 2013b).

3.1. The immunological synapse formation

Immunological synapse formation starts with TCR engagement by pMHC. Initial TCR signaling triggers T cell polarization toward the antigen presenting cell driven by cytoskeleton reorganizations, which in turn tunes TCR signaling pathways leading to T cell activation, differentiation, and cytokine production.

3.1.1 Cytoskeleton reorganization

Upon antigen recognition, T cells scanning their environment slow down and form stable interactions with antigen-presenting cells in the case of naive T cells, or with target cells in the case of CTLs. TCR engagement leads to a complete reorganization of T cell architecture in few minutes, driven by dynamic rearrangements and interplay of the actin and microtubule cytoskeleton.

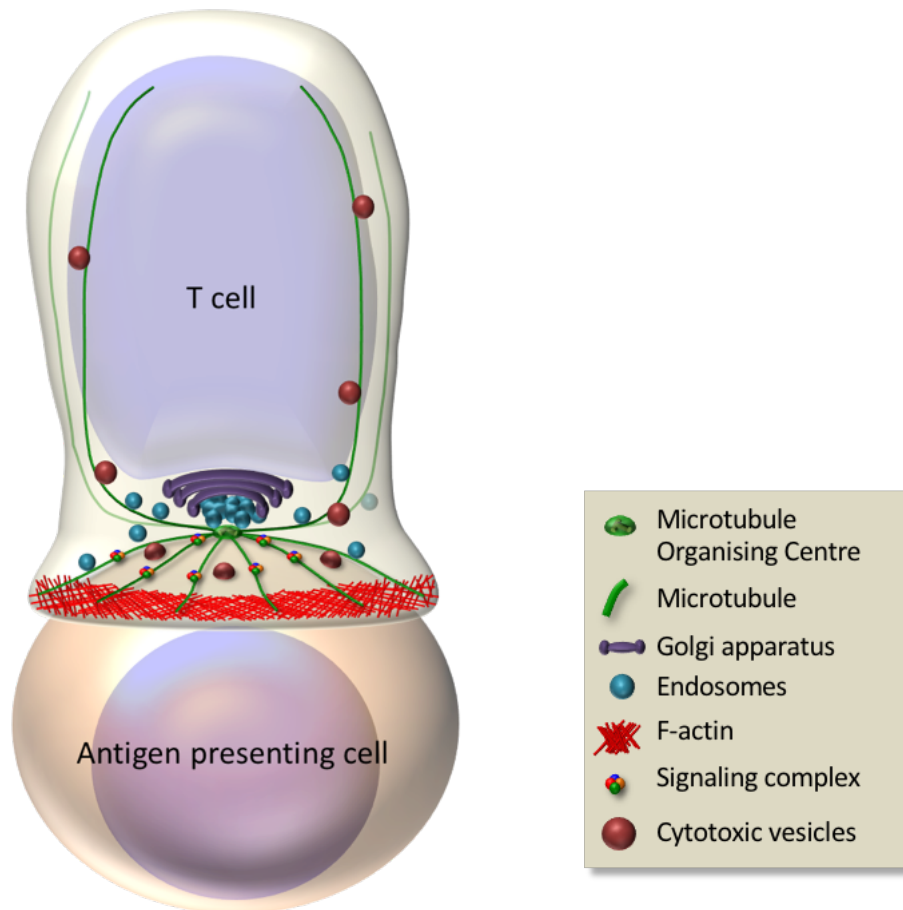


Figure 2. The immunological synapse

Simplified diagram of a T cell polarized toward an antigen presenting cell to form a synapse. Adapted from (Aguera-Gonzalez et al., 2015).

Actin cytoskeleton

Actin filaments, together with accessory and regulatory proteins, form the actin cytoskeleton, which modulates cellular morphology and plasticity. Actin dynamics are central for immunological synapse formation and stability.

Globular actin monomers are rapidly assembled in an ATP-dependent way at the barbed end of growing actin filaments (F-actin), which correspond to the extremity with high growth activity. ATP hydrolysis to ADP leads to the filament depolymerization at their pointed end, the less dynamic extremity. In addition, actin filaments are organized into a highly dynamic branched network, with new actin filaments elongating in a Y-branched orientation through the activity of nucleation promoting factors (Smith et al., 2013).

Actin reorganization is mainly regulated by the activation of Rho-family small GTPases whose best characterized members are Rac1, RhoA and Cdc42, and their downstream effector molecules (Etienne-Manneville and Hall, 2002; Pollard, 2016). Rho GTPases constantly cycle between an inactive, GDP-bound form, and an active, GTP-bound form, and bind effector proteins involved in actin remodeling. The switch between the GDP- and GTP-bound forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), such as Vav1 and the Apc-stimulated guanine exchange factor (Asef) (Tybulewicz and Henderson, 2009). When activated, Rho GTPases regulate the nucleation promoting factors WASp (Wiskott Aldrich syndrome protein) and WAVE (WASp-family verprolin-homologous protein), which in turn activate the Arp2/3 complex. The latter binds on the side of a pre-existing linear actin filament and allows the nucleation and elongation of a new actin filament in a Y-branch orientation (Goley and Welch, 2006; Rohatgi et al., 1999; Smith et al., 2013).

During immunological synapse formation, all these molecules are recruited at the cell contact area and are regulated by TCR signaling pathways. Therefore, F-actin actively polarizes at synapse. Once the synapse stabilized, F-actin clears from the center of the synapse leaving an actin-rich peripheral ring (see Figure 2) (Bunnell et al., 2001; Ritter et al., 2015). F-actin continuously pulls forces on the plasma membrane due to the molecular motor myosin II contraction. Additionally, actin polymerization pushes on the membrane and drives retrograde flow of the actin network. Together, these forces stabilize the actin cytoskeleton meshwork and maintain the radial symmetry of the immunological synapse (Babich et al., 2012; Comrie and Burkhardt, 2016).

Microtubule cytoskeleton

Microtubules contribute to cell shape determination, provide tracks for vesicle and organelle intracellular traffic, and control cell division by allowing chromosomal segregation. As the actin cytoskeleton, the microtubule network is highly reorganized at the immunological synapse.

Microtubules are formed by α/β tubulin heterodimers, which organize into tubular filaments with β -tubulin being exposed at the highly dynamic plus end and α -tubulin at the minus end. GTP-bound α/β heterodimers polymerize into microtubule filaments when hydrolyzed into GDP-bound subunits. Microtubules are nucleated from the centrosome, from where they radiate with their minus ends in and plus ends out, but also from the Golgi apparatus (Chabin-Brion et

al., 2001; Petry and Vale, 2015). Their nucleation, organization and stability are regulated by microtubule-associated proteins (MAPs) such as γ TuRC that serve as template for microtubule formation, by post-translational modifications such as acetylation, and by microtubule plus end tracking proteins including EB1, which recruits a range of different factors at the microtubule tips. Altogether, these regulatory features promote microtubule polymerization and stability (Nehlig et al., 2017; Roostalu and Surrey, 2017).

During synapse formation, microtubules are repositioned at the periphery of the T cell contact area through the action of several proteins (see Figure 2). These include the microfilament linker ezrin and the polarity regulators Dlg1 and adenomatous polyposis coli (Apc) that were all shown necessary for microtubule radial organization at the synapse (Aguera-Gonzalez et al., 2017; Bertrand et al., 2010; Lasserre et al., 2010). This process leads to the polarization of the centrosome and its translocation toward the cell contact area (Geiger et al., 1982; Stinchcombe et al., 2006). Numerous molecules have been shown necessary for centrosome polarization, such as the molecular motor dynein, Rac1, IQGAP-1, PKC ζ and formins (Combs et al., 2006; Gomez et al., 2007; Stinchcombe et al., 2006). However, the exact mechanisms involved are poorly understood.

Microtubules, by converging at the center of the immunological synapse, allow polarized transport of vesicular components and organelles, such as the Golgi apparatus, and several endosomes and secretory lysosomes as lytic granules (Bouchet et al., 2016; Soares et al., 2013b).

Actin and microtubule cytoskeleton dynamics are generally described separately. However, they interplay through various interacting proteins such as ezrin, Dlg1, IQGAP-1 or Apc (Fukata et al., 2002; Lasserre et al., 2010; Nelson and Näthke, 2013). Interestingly, centrosome translocation toward the center of the synapse occurs within a minute after F-actin clearance (Ritter et al., 2015; Stinchcombe et al., 2006). This observation, together with numerous other studies, suggests a critical interplay between the microtubule network and the actin cytoskeleton at immunological synapses.

Defects in actin or microtubule cytoskeleton reorganization during immunological synapse formation can lead to impaired T cell polarization, synapse architecture and/or T cell activation, associated with numerous disorders, e.g. ARPC1B mutation-associated immunodeficiency,

chronic lymphocytic leukemia, Wiskott-Aldrich syndrome, or familial adenomatous polyposis (Aguera-Gonzalez et al., 2017; Kallikourdis et al., 2015; Lasserre et al., 2010; Ramsay et al., 2008; Randzavola et al., 2019).

3.1.2 Polarized vesicular traffic to the immunological synapse

Centrosome repositioning close to the synapse plasma membrane drives the polarized transport along microtubules of several receptors and signaling molecules that are associated to endosomal and Golgi tubulovesicular structures (see Figure 2). Polarized vesicle traffic ensures their accumulation at the synapse, the formation of signaling complexes involved in TCR signaling and T cell activation.

Vesicles are associated with microtubules that ensure their directional transport by means of two molecular motors: toward the centrosome via dynein, and toward synapse periphery via kinesin (Daniele et al., 2011; Kurowska et al., 2012; Schroer et al., 1989; Vale et al., 1985). In addition, local actin polymerization generates forces for vesicle movement along microtubules (Derivery et al., 2009; Zhang et al., 2017). Vesicles then accumulate into a pericentrosomal compartment.

Trafficking involves several types of endosomal (e.g. early, recycling) and Golgi compartments, and vesicle traffic regulators, such as Rab GTPases, transport proteins, or vesicle fusion regulators (e.g. SNAREs) (see Figure 3). It concerns for instance TCR subunits, the tyrosine kinase Lck as well as the adapter LAT, downstream effectors of the TCR signaling pathway (Onnis et al., 2016). Those molecules travel back and forth between the plasma membrane and endosomes in non-stimulated T cells, but are reoriented to the synapse upon TCR engagement with pMHC (Blanchard et al., 2002; Bouchet et al., 2016; Bouchet et al., 2017; Das et al., 2004; Ehrlich et al., 2002; Finetti et al., 2009; Larghi et al., 2013; Purbhoo et al., 2010; Soares et al., 2013a; Zucchetti et al., 2019). In addition, polarized vesicular traffic is crucial for T cell effector functions by allowing secretion of cytokines and lytic granules in the synaptic cleft (Daniele et al., 2011; Kurowska et al., 2012; Sepulveda et al., 2015; Stinchcombe et al., 2006).

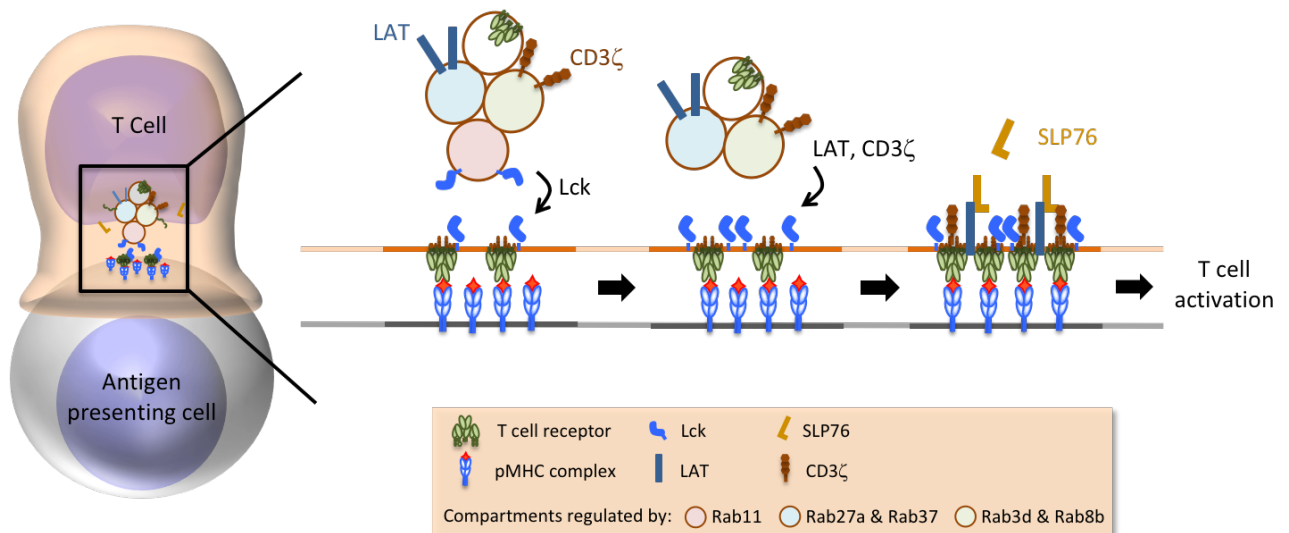


Figure 3. Polarized targeting of TCR and downstream signaling proteins at the immunological synapse.

After pMHC recognition by the TCR, Lck is targeted at the synapse by Rab11-dependant endosomes. CD3 ζ and LAT are then recruited, allowing efficient activation signaling. Adapted from (Bouchet and Alcover, 2014).

3.1.3. Molecular clustering at the immunological synapse

The immunological synapse structure was first described in 1998-99 by Monks, Kupfer and colleagues, and by Grakoui, Dustin and colleagues. They showed that TCRs, signaling and adhesion molecules, and cytoskeleton structures were not uniformly distributed at the plasma membrane, but were segregated into concentric domains called supramolecular activation clusters (SMACs) (see Figure 4) (Grakoui et al., 1999; Monks et al., 1998). The central-SMAC (c-SMAC) was shown to be enriched in TCR and associated proteins such as CD3 and CD28, and its downstream signaling proteins as the tyrosine kinases Lck and Zap70, and LAT. Surrounding the c-SMAC, the peripheral SMAC (p-SMAC) is a highly contractile zone composed of integrins, such as LFA-1, and the cytoskeleton linker talin. A third distal SMAC (d-SMAC), containing large proteins like the protein tyrosine phosphatases CD45 and CD148, was then added to this model (Cordoba et al., 2013; Lin and Weiss, 2003).

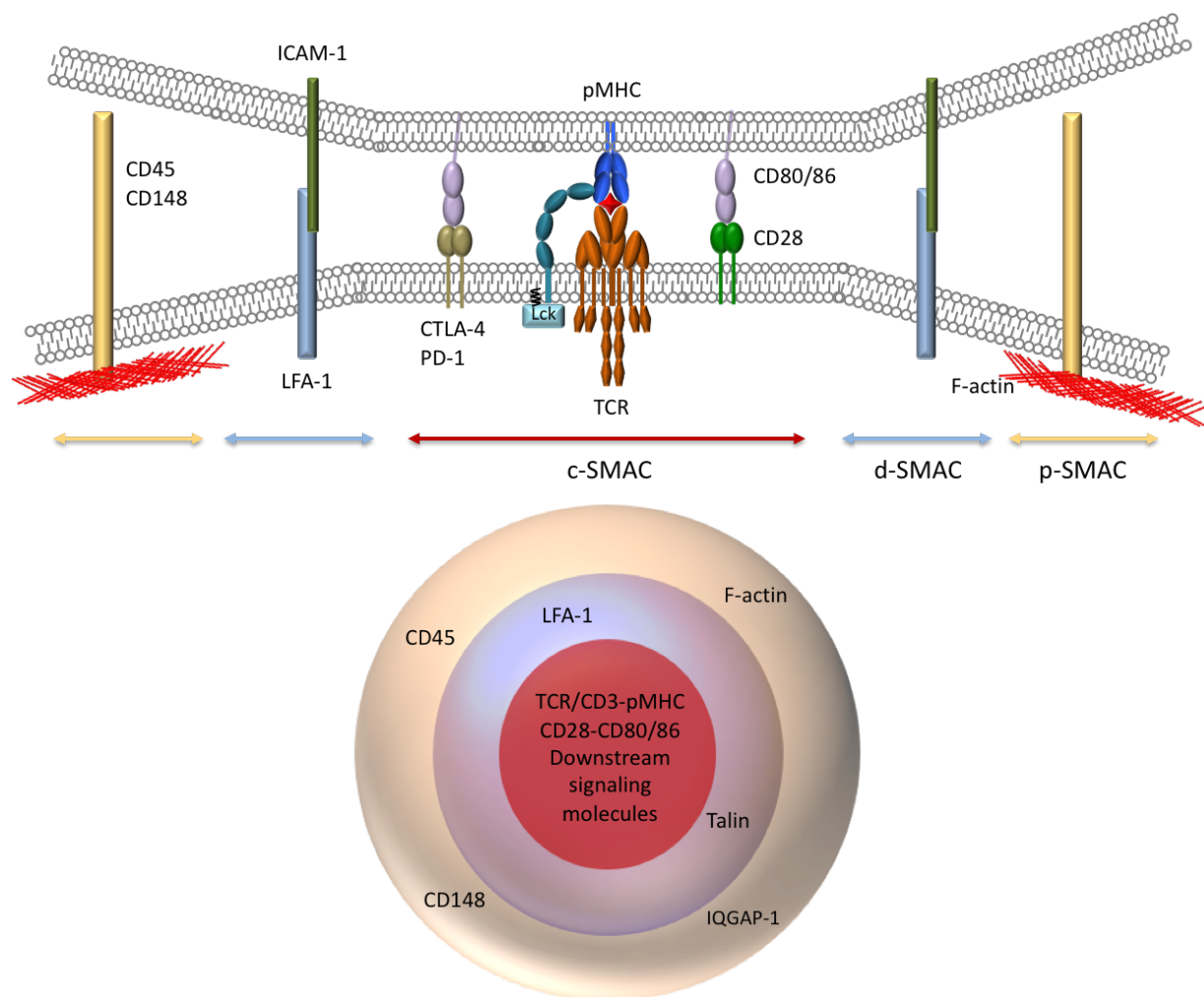


Figure 4. The supramolecular activation clusters.

Simplified diagram of mature synapse organization in SMACs, and the molecules/ligands enriched in each zone.

This organization in concentric domains was initially thought to allow an optimal synapse stability and thus T cell activation or effector functions. It is dictated, at least in part, by the size of the ectodomains of the different molecules involved. The c-SMAC, responsible for TCR signaling and the recycling of the molecules involved, contains molecules with short ectodomains, therefore their interaction with their ligands on the antigen-presenting cell brings the membrane of the two cells close to each other. The p-SMAC, which concentrates integrins and their ligands, forms a ring of thigh adhesion between the two cells to stabilize the immunological synapse. Conversely, molecules with larger ectodomains, such as the phosphatase CD45, are excluded into the d-SMAC thus avoiding an inhibitory effect on the TCR signaling pathway. Indeed, the artificial elongation of the p-MHC ectodomain, which

increases the distance between the two cells, prevents CD45 exclusion from the c-SMAC and leads to reduce TCR signaling (Choudhuri et al., 2005).

Further studies, using phospho-specific antibodies revealing active signaling molecules, showed that TCR signaling complexes appeared first in small clusters at the synapse periphery, while the c-SMAC contained dephosphorylated molecules and correlated with inhibitory signaling.

The use of TIRF live cell microscopy allowed to observe TCR and its downstream signaling proteins forming submicron-scale molecular complexes, or microclusters, appearing within the d-SMAC, then moving centripetally through the p-SMAC to the c-SMAC to form a mature (e.g. concentric) synapse. Signaling microclusters may disappear before reaching the c-SMAC or coalesce in the c-SMAC, colocalizing with some lysosomal markers. This led to propose that the immunological synapse p-SMAC and c-SMAC structures facilitate the balance between signaling and degradation, and depend on antigen stimulatory quality. In addition, microclusters containing the co-stimulatory molecule CD28 and the inhibitory receptors CTL-4 and PD-1 contribute to signaling regulation (Bunnell et al., 2002; Campi et al., 2005; Cemerski et al., 2008; Cemerski et al., 2007; Lasserre and Alcover, 2010; Lee et al., 2003; Varma et al., 2006; Yokosuka et al., 2008; Yokosuka et al., 2010; Yokosuka et al., 2005; Yokosuka et al., 2012). More recently, the c-SMAC has been associated with the production and accumulation of extracellular vesicles (ectosomes) containing TCR and CD40L that could represent a means of T cell signal downregulation while producing vesicles capable to stimulate antigen presenting cells (Choudhuri et al., 2014; Saliba et al., 2019).

The coordinated action of the actin cytoskeleton and the microtubule network is crucial for microclusters dynamics and SMAC formation. Noteworthy, the d-SMAC corresponds to the actin-rich peripheral ring and contains IQGAP-1 and ezrin that link the actin cytoskeleton and the microtubules (Lasserre et al., 2010; Stinchcombe et al., 2006; Watanabe et al., 2004). It is important to note that, concomitantly, TCR signaling regulates cytoskeleton dynamics, thus coordinating the formation of mature synapse. Furthermore, actin cytoskeleton is required to sustain TCR signaling (Valitutti et al., 1995).

As mentioned above, centrosome translocation toward the cell-cell contact area allows the polarized transport of vesicles containing TCR, Lck and LAT to the synapse and the formation of signaling complexes (Blanchard et al., 2002; Ehrlich et al., 2002; Finetti et al., 2009; Larghi et al., 2013; Soares et al., 2013b). These proteins assemble in signaling microclusters in the d-

SMAC. Actin-induced forces driving retrograde flow, through its polymerization and myosin II contraction, then allow the centripetal movement of the microclusters to the c-SMAC and of LFA-1 to the p-SMAC (see Figure 5) (Campi et al., 2005; Comrie et al., 2015; Hashimoto-Tane et al., 2011; Ilani et al., 2009; Nguyen et al., 2008). Microtubules support microcluster centripetal movement involving the molecular motor dynein (Hashimoto-Tane et al., 2011; Lasserre et al., 2010). Furthermore, actin clearance from the center of the synapse is the initiating event for the c-SMAC formation (Bunnell et al., 2002; Ritter et al., 2015). Therefore, blocking F-actin depolymerization reduces microcluster formation and impairs microcluster and LFA-1 centripetal movement. This slows down their translocation to the p-SMAC, decreasing TCR signaling and the adhesiveness of the synapse (Comrie et al., 2015; Comrie and Burkhardt, 2016; Varma et al., 2006). Collectively, these data indicate that polarized vesicular transport, actin and microtubule-mediated microcluster dynamics and force generation are thus essential for controlling synapse formation and T cell activation.

Importantly, the p-SMAC and c-SMAC structures have been mostly observed in T cell interacting with antigen presenting B cells or with lipid bilayer as surrogate synapses. In contrast T cells interacting with dendritic cells form multifocal synapses (Brossard et al., 2005). This indicates that depending on the quality of the antigen presenting cell, immunological synapse structure and signaling may be different.

Therefore, the immunological synapse formation results from a dynamic T cell reorganization, which allows a spatial and temporal molecular organization that tunes TCR signaling.

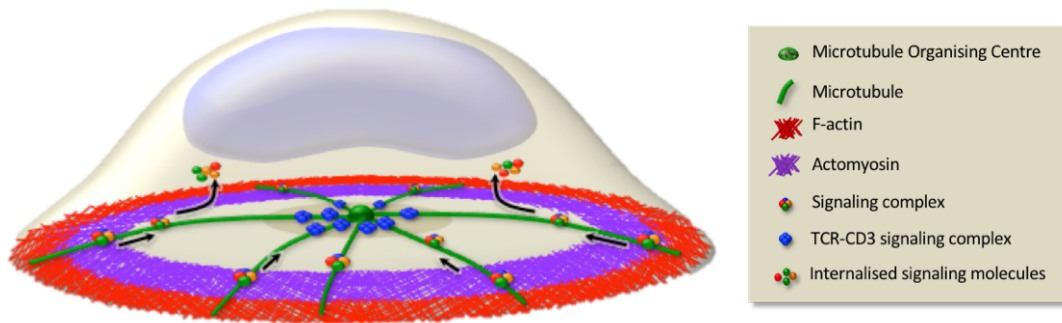


Figure 5. Cytoskeleton and signaling microcluster dynamics at the immunological synapse.

Signaling complexes form at the synapse periphery. Actin dynamics and myosin contraction then regulate their centripetal movement, represented here by arrows, along microtubules to reach the c-SMAC. Adapted from (Aguera-Gonzalez et al., 2015).

3.2. T cell receptor signaling

One major function of the immunological synapse formation is the regulation of the signal transmitted by the TCR (Courtney et al., 2018).

Upon TCR engagement, TCR/CD3 complexes form microclusters. The CD4/CD8 co-receptors recruit the Src family protein tyrosine kinases Lck and Fyn to the TCR-CD3 microclusters. Src family proteins then phosphorylate tyrosine-containing signaling motifs named ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tail CD3 chains (see Figure 6) (Barber et al., 1989). Phosphorylated ITAMs initiate TCR signal transduction by recruiting Zap70 via its SH2 domains (Iwashima et al., 1994). Activation of Zap70 then results in the phosphorylation of LAT, which in turn will recruit SLP76-Gads and PLC γ 1 (Finco et al., 1998; Zhang et al., 1998). LAT, SLP76 and PLC γ 1, together with others adaptor and effector molecules as the Rho GTPase exchange factor Vav1, form a signalosome complex driving downstream signaling events. Indeed, several signaling pathways involving kinases are triggered resulting in the activation of various transcription factors (e.g. NFAT, NF κ B and AP-1), which altogether control T cell proliferation, differentiation and effector functions, such as cytokine production.

Recruitment of SOS to LAT results in the activation of the Ras, which triggers the MAP kinase pathway involving Erk1/2 kinases. This results in the activation of Fos transcription factor. Additionally, SLP76 recruitment leads to Rac and Rho GTPase activation, which in turn activate JNK (Jun kinase) and the Jun transcription factor. Fos and Jun then form a heterodimer called AP1 (activating protein 1) (Courtney et al., 2018).

Furthermore, PLC γ 1 hydrolyzes the membrane phosphoinositide PIP₂ to generate IP₃ and diacylglycerol (DAG). IP₃ binds to its receptor localized in the endoplasmic reticulum, inducing the release of the calcium stored therein, which then leads to an extracellular calcium influx via calcium release-activated calcium channels (Zweifach and Lewis, 1993). The increase of cytosolic calcium will lead ultimately to the dephosphorylation of NFAT (nuclear factor of activated T cells) and allows its translocation to the nucleus. Concerning DAG, it activates serine threonine kinases PKC θ , responsible for the activation of the kinase Ikk β (I-kappa-B-kinase beta), which activates another key transcription factor NF κ B (nuclear factor kappa-B). Additionally, DAG activates a parallel pathway participating in Ras activation that involves the protein RasGRP (Courtney et al., 2018).

AP1, NFAT and NF κ B act together to optimally activate transcriptional programs leading to T cell differentiation, proliferation and cytokine production.

As seen previously, efficient T cell activation and differentiation require, in addition to the TCR signal, a secondary signal delivered by CD28 (Chen and Flies, 2013; Mueller et al., 1989). Upon CD28 engagement by CD80/CD86, the phosphoinositide-3-kinase (PI3K) is recruited at the plasma membrane where it converts PIP₂ into PIP₃, leading to the recruitment and activation of the protein kinase Akt. Ultimately, Akt activation enhances NF κ B nuclear translocation and provides multiple signals promoting cell survival and growth (Narayan et al., 2006).

Interestingly, TCR signaling promotes cytoskeleton reorganization necessary for immunological synapse formation, which in turn allows the regulation of TCR signaling. For instance, Lck/Fyn, Zap70, LAT, PLC γ 1 and DAG are required for centrosome translocation to the cell contact area (Blanchard et al., 2002; Kuhné et al., 2003; Quann et al., 2009; Tsun et al., 2011). Additionally, PIP₂ and PIP₃ promotes actin polymerization by regulating GEFs. GEFs then tune the activation of Rho GTPases that bind effector proteins involved in actin remodeling (Etienne-Manneville and Hall, 2002). F-actin depletion from the center of the synapse correlates with a reduction of PIP₂ at the plasma membrane (Ritter et al., 2015), whereas the organization

and maintenance of the actin-rich ring is controlled by the annular accumulation of PIP₃ (Le Floc'h et al., 2013).

In conclusion, interplay between T cell signaling events and cytoskeleton reorganization at the immunological synapse leads to the activation of transcription factors that control gene involved in T cell proliferation, differentiation, and effector function.

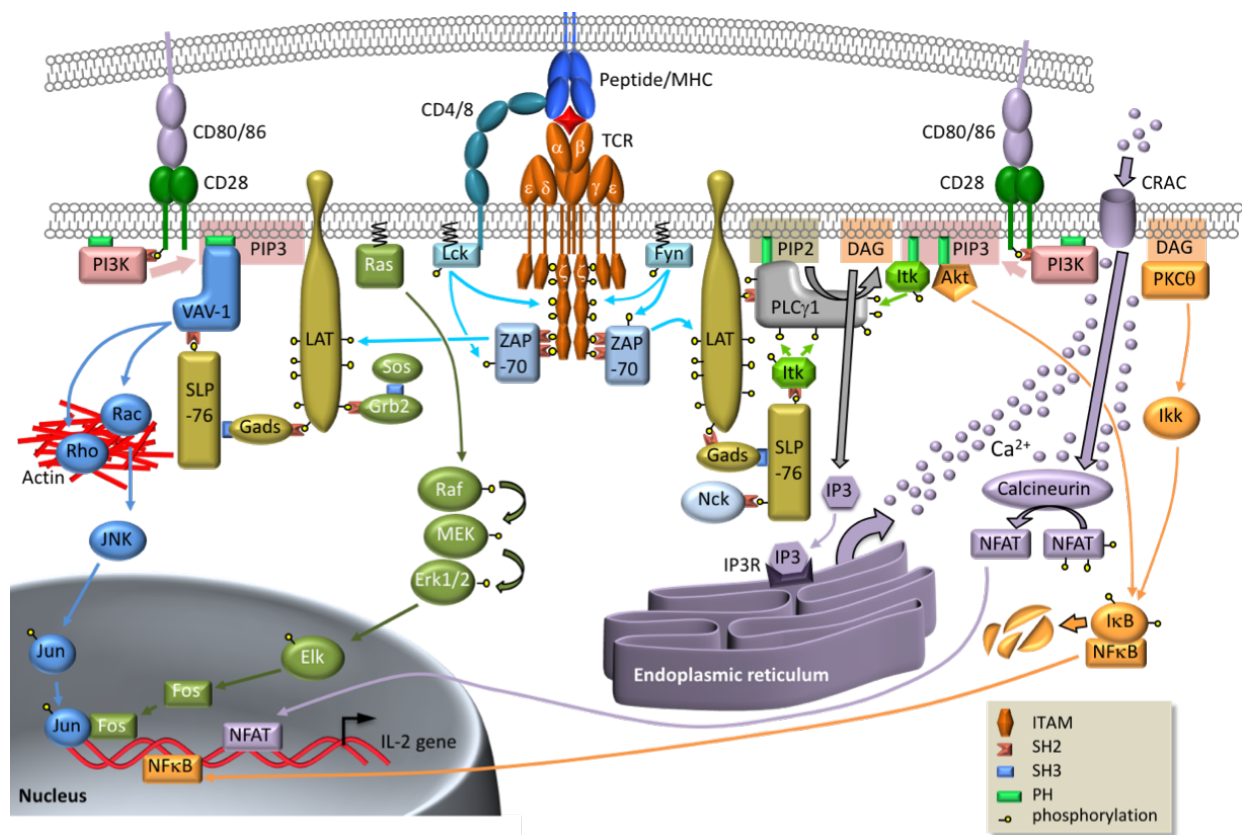


Figure 6. Main signaling pathways triggered by TCR-pMHC and CD28-CD80/86 engagement

Different signaling pathways activated upon TCR and co-receptor signalization, ultimately leading to transcription factor activation. From left to right, pathway color code is: blue for Rac/JNK pathway, green for Erk1/2/Fos pathway, violet for Calcium/NFAT pathway, and orange for PKCθ /NFκB pathway. (Aguera-Gonzalez et al., 2015).

3.3. The cytotoxic synapse

The immunological synapse formation and TCR signaling allow T cell activation, leading to their proliferation and differentiation, but also to the triggering of their effector functions. Indeed, the dynamic interplay between receptor signaling, polarized vesicle traffic and actin and microtubule cytoskeleton rearrangements results in production and polarized secretion of cytokines, membrane apposition allowing FasL-Fas interaction, and above all, lytic granule release in the synaptic cleft.

3.3.1. Cytoskeleton reorganization

The cytotoxic synapse organization is similar to the one described previously (see Figure 7), however the centrosome does not only translocate, but is docked at the plasma membrane. Cytotoxic granules move along the microtubules in a dynein-mediated manner toward their minus end to cluster around the moving centrosome, with which they polarized at the synapse (Ritter et al., 2015). The centrosome thus defines a secretory domain localized within the p-SMAC, next to the c-SMAC (Stinchcombe et al., 2001b; Stinchcombe et al., 2006). Its docking ensures a precise site for lytic granule secretion, concentrating granules in the synaptic cleft, but also promotes the delivery of FasL at the plasma membrane (Bossi and Griffiths, 1999). Furthermore, F-actin depletion from the c- and p-SMAC to form an actin-rich ring allows the centrosome docking and facilitates granules fusion at the plasma membrane. Conversely, actin recovery terminates cytotoxic granule release (Ritter et al., 2015; Ritter et al., 2017).

Several studies have also shown that, in opposition to the stimulatory synapse, which leads to T cell activation, the cytotoxic synapse does not require to be completely formed and stable to be efficient. Indeed, CTLs do not need a strong antigen stimulation to induce target cell death, compared to the one required for stimulatory synapse (Faroudi et al., 2003). Killing could occur with only three TCR-pMHC interactions, whereas stable synapse formation requires at least ten interactions (Purbhoo et al., 2004). The formation of a mature synapse with typical SMAC pattern may not always be necessary for efficient cytotoxic granules release (O'Keefe and Gajewski, 2005). Hence, CTLs could kill multiple targets simultaneously, only polarizing toward the target cell with the strongest antigen stimulus (Depoil et al., 2005). Lytic granule translocation to the T cell-target cell contact area could thus be independent from the centrosome repositioning (Bertrand et al., 2010; Wiedemann et al., 2006).

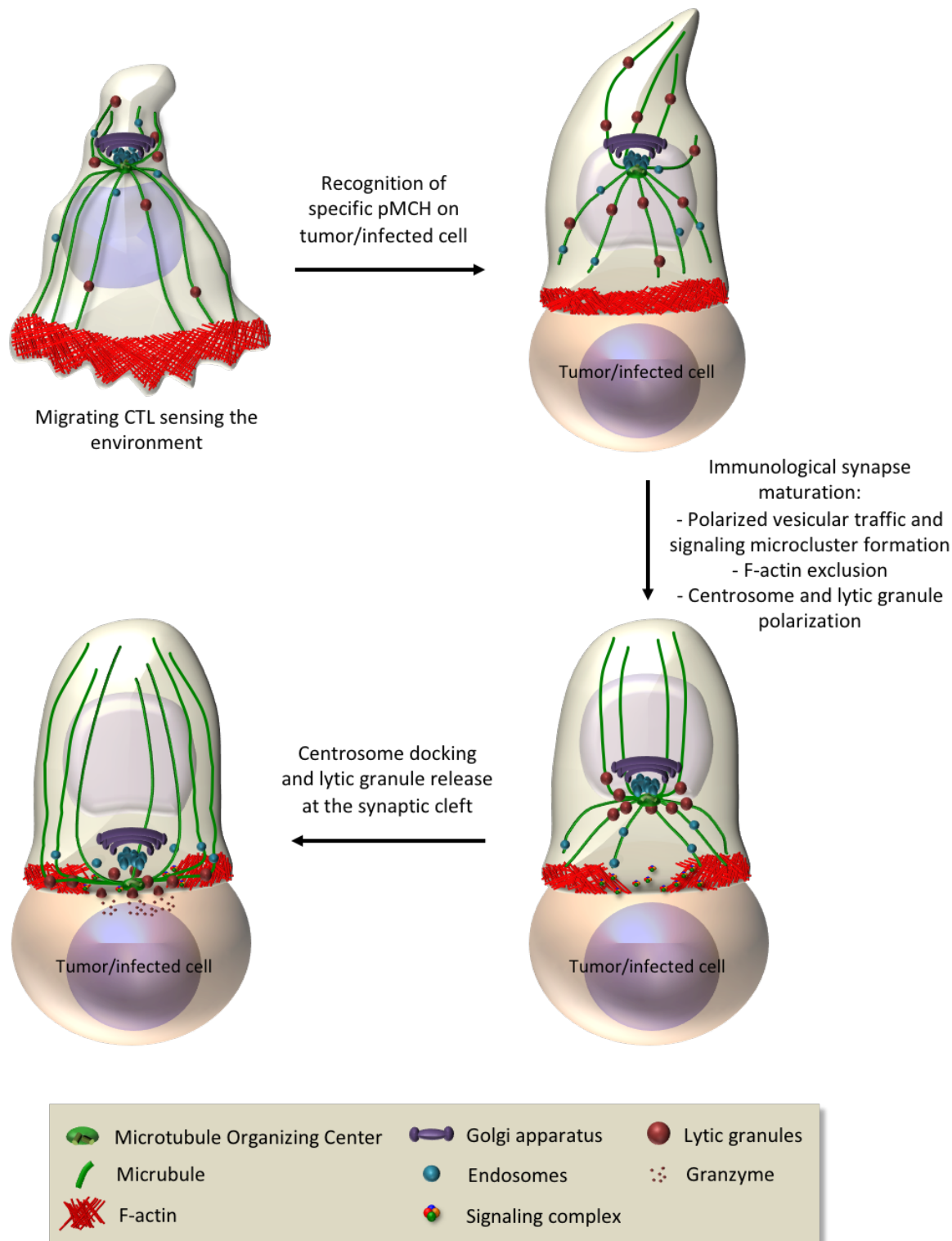


Figure 7. Formation of the cytotoxic synapse.

First the CTL recognizes a pMHC complex presented by a tumor or infected cell. Actin polymerizes at the synapse and rapidly clears the center, while lytic granules cluster around the centrosome and move toward the synapse. Granules fuse with the plasma membrane and perforin and granzymes are released in the synaptic cleft.

The importance of the F-actin dynamics for CTL effector functions has however not been questioned as impairment of numerous of its regulators, such as PIP₃, Arp2/3 or WASp, affects killing efficiency (De Meester et al., 2010; Le Floc'h et al., 2013; Ramsay et al., 2008).

3.3.2. Lytic granule transport and fusion

Once at the synapse, lytic granules fuse with the plasma membrane, and perforin and granzymes are released in the synaptic cleft. The study of human genetic disorders and mouse mutants allowed to identify numerous proteins involved. Indeed, characterization of the molecular defects leading to hemophagocytic lymphohistiocytosis (HLH) has contributed to understand the different steps and their regulators required for lytic granules maturation and exocytosis (de Saint Basile et al., 2010).

As highlighted above, HLH may result from defects in perforin (Stepp et al., 1999), but can also result from SNARE protein and SNARE accessory factor deficiency, both in human and mice. Therefore, deficient lysosomal trafficking regulator (LYST) alters granule maturation (Barbosa et al., 1996; Nagle et al., 1996; Sepulveda et al., 2015), deficient AP-3 adaptor alters lytic granule transport on microtubules to the synapse (Clark et al., 2003; Feng et al., 1999), deficient Rab27a their docking (Kurowska et al., 2012; Ménasché et al., 2000; Stinchcombe et al., 2001a), deficient Munc13-4 their priming (Feldmann et al., 2003; Ménager et al., 2007), and deficient syntaxin-11 and Munc18-2 their fusion at the plasma membrane (zur Stadt et al., 2009).

3.3.3. Importance of forces for killing

The interplay of pushing and pulling forces induced by the actin cytoskeleton described above are not only important for immunological synapse formation, but also for CTL effector functions. Indeed, the adaptation of single cell biophysical approaches (e.g. stimulatory deformable micropillar arrays, stimulatory beads attached to micropipettes) has shown that CTLs exert forces against antigen presenting surfaces with a spatiotemporal correlation between the force exertion and lytic granule release (Basu et al., 2016; Tamzalit et al., 2019). Forces applied to the synapse promote cytotoxicity by increasing the target cell membrane tension, which in turn enhances the perforin pore forming activity.

Hence, the immunological synapse formation is the result of a massive dynamic polarization of the T cell. This reorganization involves numerous cellular components as the actin cytoskeleton, microtubule network, vesicular traffic or receptors, but most importantly, is regulated by the interplay between them, coordinately regulated in space and time. Together they act to form a stable synapse ensuring T cell activation, differentiation and effector functions necessary for efficient adaptive immune response, and the elimination of pathogens and tumor cells.

4. Anti-tumor response

The involvement of the immune system in tumor development prevention and growth control have been extensively studied, both in human and mouse.

Tumor development and resulting immune response occur in three steps: elimination, equilibrium and escape (see Figure 8) (Dunn et al., 2004).

4.1. Control of tumor development

Tumors appear after cellular modifications such as mutations and chromosomal instability that alter normal cell growth and survival, resulting in premalignant lesions. These lesions are mostly eliminated through cell-intrinsic tumor suppression mechanisms activating apoptosis and cell-cycle arrest (reviewed in (Lowe et al., 2004)) or by immunosurveillance processes. Abnormal cells enter in a senescence state and secrete various cytokines inducing a pro-inflammatory environment (Kuilman et al., 2008). These cytokines mediate the recruitment of innate immune cells first, which leads to the killing of some senescent cells and consequent release of tumor antigens. These antigens are presented by antigen presenting cells to mature naive CD4 and CD8 T cells, driving their activation, proliferation and differentiation into effector cells. The development of tumor-specific adaptive immunity then provides the capacity to completely eliminate abnormal cells, in particular through CTL cytotoxic activity and IFN γ production (Kang et al., 2011; Ostroumov et al., 2018; Shankaran et al., 2001).

These mechanisms are remarkably efficient as on average cancers arise less than once in a human lifetime, despite trillions of potential target cells. However, it happens that some premalignant cells survive the elimination phase. If T cells and IFN γ actions can contain tumor development, an equilibrium state is achieved, sometime during several years (Dunn et al., 2004; Koebel et al., 2007).

Malignant cells can however escape the immune control, and grow to form clinically detectable tumors. Tumor escape is the result of immune-resistant cell selection over the time and the addition of further genetic alterations in abnormal cells during the equilibrium phase, such as

the mutation of tumor suppressors like the polarity regulator adenomatous polyposis coli in colorectal cancer or the transcription factor p53 that has been involved in 50% of cancers.

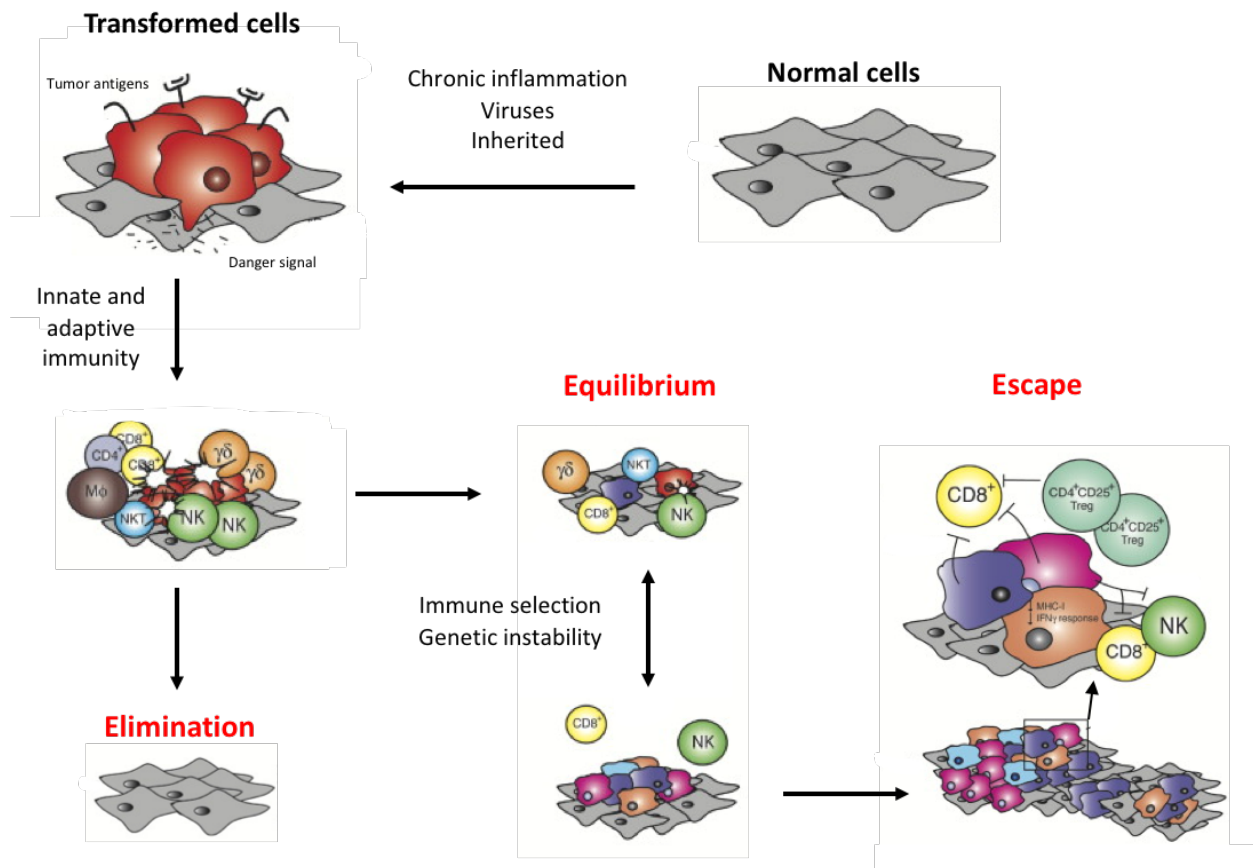


Figure 8. The three phases of tumor development.

Adapted from (Dunn et al., 2004).

4.2. Tumor escape

Escape and proliferation of tumor cells often is accompanied by the presence of an immunosuppressive environment, which is less permissive for T cell recruitment, persistence, and effector functions (Frey, 2015). Both T cell intrinsic and extrinsic factors have been shown to inhibit T cell functions in tumors. Indeed, CTLs that have a central role in anti-tumor immunity present impaired cytotoxic activity in the tumor bed.

Intrinsic factors mostly consist on the increased expression of checkpoint inhibitors at the T cell surface when they interact with tumor cells. Checkpoint inhibitors are signaling receptors, which during normal immune response control T cell over-activation to limit inflammation, but restrict anti-tumor functions in cancer. Checkpoint inhibitors include T cell co-stimulatory receptors such as CTLA-4, which is part of the CD28 family. It recognizes B7 family proteins as CD80/86 and suppresses T cell proliferation by competing for ligand binding with CD28 (Chen, 2004; Rowshanravan et al., 2018). In addition, tumor-infiltrating CTLs have been shown to form altered immunological synapses with tumor cells. The actin cytoskeleton does not polymerize at the cell contact area, TCR and CD3 do not colocalize in microclusters in the c-SMAC, TCR early signaling is inhibited, and the centrosome and cytotoxic granules are not translocated at the plasma membrane in CD8 T cells isolated from murine tumor models (Radoja et al., 2001). Intrinsic factors therefore induce hypo-responsive T cells that are exhausted, contributing to tumor progression.

Extrinsic factors occur via the recruitment of immunosuppressive cell populations such as Tregs. These cells are critical for autoimmunity protection and regulation of inflammation and immune responses. However, they may be an obstacle for anti-tumor CTL responses. Tregs are characterized by the expression of CD25 and of the transcription factor Foxp3, but also the inability to produce IL-2 (Hori et al., 2003). Tregs act through indirect and direct immunosuppressive mechanisms (Miyara and Sakaguchi, 2007). They produce anti-inflammatory cytokines such as IL-10 and TGF- β (Andersson et al., 2008), and consume IL-2 necessary for others T cell proliferation and survival (Pandiyani et al., 2007). They can also display similar cytotoxic functions to CTLs, and eliminate B and T lymphocytes through cytotoxic granule exocytosis and by inducing the death-receptor Fas pathway (Cao et al., 2007; Grossman et al., 2004; Strauss et al., 2009; Zhao et al., 2006). Extrinsic factors therefore induce anti-tumor effector cell apoptosis, also contributing to tumor progression.

Considering CTL and Treg functions, their number and ratio in tumors are crucial prognostic factors for patient survival. CTL infiltration is thus considered as favorable and associated with good prognosis, whereas Treg infiltration is generally associated with poor clinical outcome (Galon et al., 2006). However, the balance between CTLs and Tregs need to be equilibrated as inflammation can also promote tumor development particularly in colorectal cancers (Mantovani et al., 2008).

5. Adenomatous polyposis coli

As describe above, T cell immune responses are controlled by the ability of the cell to polarize. Indeed, the immunological synapse formation and thus the resulting T cell activation, differentiation and effector functions that are crucial to control tumor development, depend on the polarization of T cells toward antigen presenting cells and tumor target cells. Polarization is also key to control migration, and therefore allowing T cells to enter secondary lymphoid organs and to be recruited into tumors.

Cell polarity regulators are proteins controlling cellular asymmetric distribution of molecules, the asymmetric organization specific membrane domains, and the enrichment of organelles or the cytoskeleton at specific sites (Rodriguez-Boulán and Macara, 2014). Hence, polarity regulators may be essential to ensure fully efficient T cell anti-tumor immune responses. Several of them have been shown to control immunological synapse formation and T cell activation. For instance, Dlg1 silencing perturbs T cell shape, microcluster formation, actin polymerization, microtubule network organization and centrosome polarization, and NFAT-driven gene transcription (Lasserre et al., 2010; Round et al., 2007; Round et al., 2005). In addition, Scribble silencing perturbs synapse formation and TCR signaling molecule recruitment at the synapse (Ludford-Menting et al., 2005). Interestingly, both Dlg1 and Scribble interact with another polarity regulator called adenomatous polyposis coli (Apc), whose role has never been characterized in CD8 T cells (How et al., 2019; Matsumine et al., 1996).

5.1. Adenomatous polyposis coli structure and function

Apc is a large 310-kDa multi-domain cytoplasmic protein (see Figure 9). Its central region contains short peptide motifs that bind to the transcription factor regulator β -catenin, but contains also three set of SAMP (Ser-Ala-Met-Pro) domains binding Axin to regulate β -catenin (Rubinfeld et al., 1993). Apc amino-terminal portion contains a dimerization domain and an armadillo repeat domain. The armadillo domains interact with numerous cytoskeleton regulators as the actin and microtubule regulator IQGAP-1, the Rho GTPase Cdc42, the Rho GTPase regulator Asef, or kinesin regulators (Jimbo et al., 2002; Kawasaki et al., 2000; Sudhaharan et al., 2011; Watanabe et al., 2004). The carboxy-terminal portion also contains

domains binding cytoskeleton regulators as the microtubule plus end binding EB1 and Dlg1, but also a basic domain directly interacting with microtubules (Munemitsu et al., 1994; Nakamura et al., 2001). In addition, Apc has been shown to interact directly and indirectly with nuclear pore and nuclear transport proteins, and apoptosis- or mitosis-related proteins (Nelson and Näthke, 2013).

Therefore, due to its numerous interaction domains, Apc is a multi-functional protein involved in a large range of cellular mechanisms.

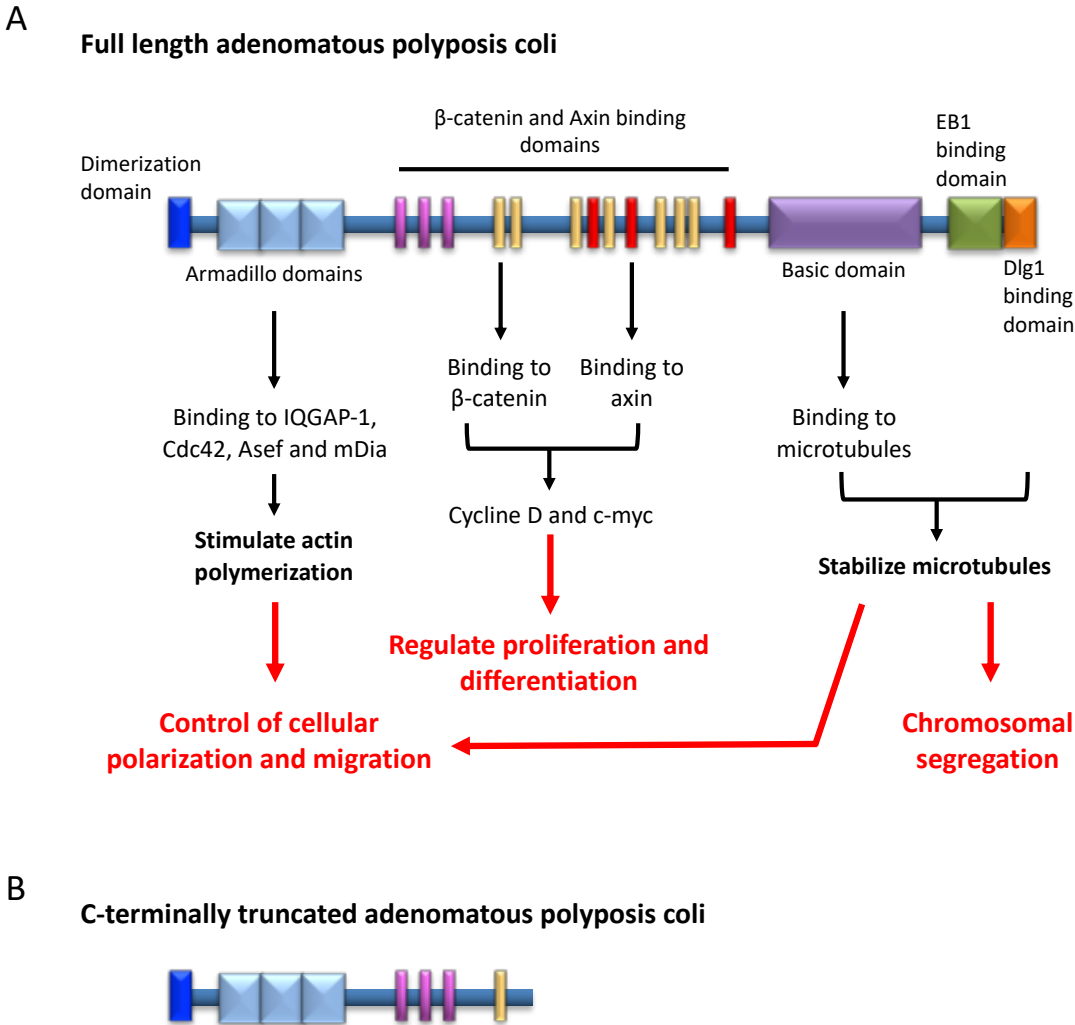


Figure 9. Adenomatous polyposis coli structure and functions

(A) Schematic domain structure of Apc describing binding sites and interacting partners, and cellular functions they are involved in. (B) Apc truncated form resulting from *Apc* mutation. Based on (Aoki and Taketo, 2007).

5.1.1. Apc as a Wnt signaling pathway regulator

Apc is mostly known for its role in the Wnt signaling pathway, which plays essential roles during embryonic development and in tissue homeostasis, by driving cell proliferation and survival.

The Wnt signaling pathway regulates the level of the transcription factor β -catenin via a specific destruction complex, which is composed by Apc, Axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). In absence of Wnt extracellular stimulus, this cytosolic destruction complex initiates β -catenin ubiquitination and ultimately its proteasomal degradation. In presence of Wnt stimulus, the destruction complex is inhibited via its recruitment to the plasma membrane, and β -catenin is stabilized and translocates to the nucleus, thus inducing the transcription of Wnt target genes, including c-myc, cyclin D and caspases. Increasing β -catenin availability thus results in increased proliferation, but also decrease cell differentiation (Munemitsu et al., 1995; Stamos and Weis, 2013).

5.1.2. Apc as a polarity regulator

Due to its numerous interaction domains with actin and microtubule cytoskeleton regulators (see Figure 7), Apc has been involved in numerous cellular processes that require their reorganization and cellular polarization, as cell directional migration and division, and is thus classified as a polarity regulator protein.

The importance of Apc in cytoskeleton regulation is reflected by its subcellular localization. Indeed, in migrating astrocytes and epithelial cells, Apc predominantly forms very small clusters mainly localized at microtubule plus ends and plasma membrane, which correlates with protrusive cell area and the leading edge (Etienne-Manneville et al., 2005; Näthke et al., 1996). In highly polarized cells as intestinal epithelial cells, Apc is mostly detected at the basal surface, where microtubule plus ends also are (Näthke et al., 1996). A more recent study from the Alcover lab has also unveiled a similar localization in CD4 T cells, where Apc clusters appeared enriched at the immunological synapse periphery, corresponding to microtubule plus end, but were also observed along microtubules (Aguera-Gonzalez et al., 2017). Furthermore, Apc has been observed in actin-rich leading edge of migrating epithelial cells (Watanabe et al., 2004).

Apc stabilizes the microtubule network in migrating fibroblasts and astrocytes, and ensures directional migration. Loss of Apc results in decrease of cellular protrusion and microtubule stability during cell migration (Etienne-Manneville, 2009; Etienne-Manneville and Hall, 2003). In addition, Apc ensures microtubule network organization and centrosome polarization at the immunological synapse of CD4 T cells (Aguera-Gonzalez et al., 2017). Furthermore, Apc loss has been associated with defective mitotic spindle structure during cell division (Dikovskaya et al., 2004). Finally, Apc truncation has been recently shown to induce the fragmentation of the Golgi apparatus, involved in protein trafficking and microtubule nucleation, in colorectal carcinoma cells (Kim et al., 2018).

Apc stimulates actin polymerization through its interactions with IQGAP-1 and Asef, but also with the formin mDia (Juanes et al., 2017; Okada et al., 2010). Loss of Apc results in impaired actin meshwork formation at the epithelial cell leading edge altering their directional migration (Watanabe et al., 2004).

Therefore, Apc controls both actin and microtubules, regulating cell protrusion and migration which require cellular polarization and the coordinated action of both cytoskeletons. As Dlg1 and IQGAP-1 (Fukata et al., 2002; Lasserre et al., 2010), Apc could tune the interplay between actin and microtubule dynamics to establish cell polarity.

Finally, Apc binds to intermediate filaments and controls their reorganization during cell migration (Sakamoto et al., 2013). Therefore, as a polarity regulator Apc is at the crossroads of the main three cytoskeleton structures actin, microtubules, and intermediate filaments playing key function in induced cell polarization.

5.1.3. Apc as a tumor suppressor

Due to the various cellular processes in which Apc is involved, Apc is also characterized as a tumor suppressor, meaning that its expression in cells prevent them to become cancerous. Indeed, its mutations, which usually lead to the carboxy-terminal region truncation, result in colorectal cancers.

The intestinal and colon epithelia are composed by crypts and villi. Crypts contain intestinal stem cells which differentiate into specialized epithelial cell types and migrate upward to the villi in the intestines, or to the crypt collar in the colon. The toxic environment of the intestinal

tract requires the rapid elimination and renewal of these specialized cells, their differentiation and migration are thus coordinated to ensure their exfoliation within 3-5 days.

Intestinal epithelium homeostasis is thus highly regulated by cellular differentiation, proliferation and migration, all processes regulated by β -catenin and cytoskeleton, and therefore Apc (Sansom et al., 2004). Loss of Apc results in increased cell proliferation, but reduced cell differentiation. Additionally, impaired cytoskeleton dynamics result in decreased cell migration (Kroboth et al., 2007; Watanabe et al., 2004). Stem cells cannot migrate to the villi or crypt collar (Mahmoud et al., 1997; Sansom et al., 2004), ultimately impairing their elimination from the toxic environment of the intestinal tract. In addition, loss of Apc leads to chromosome mis-segregation promoting genetic instability, and spending longer time in a toxic environment promotes additional mutation accumulation (Caldwell et al., 2007; Dikovskaya et al., 2007). Altogether, these defects lead to the growth of intestinal and colon polyps with highly tumorigenic risk (see Figure 10). Interestingly, restoration of Apc in established intestinal tumors in mice drives rapid cell differentiation, tumor regression, and reestablishment of epithelium homeostasis (Dow et al., 2015).

Hence, loss of Apc induces serious dysregulations in the processes regulating intestinal epithelium homeostasis and has been shown as an initiating event in colorectal cancer development.

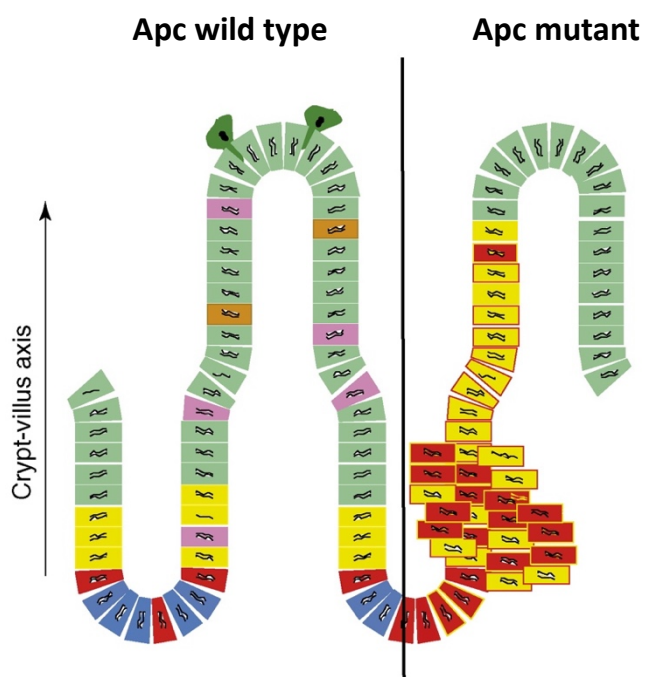


Figure 10. Apc in intestinal epithelium

In wild-type tissue, stem cells (red) give rise to differentiating cells (yellow). Cells differentiate in several specialized epithelial cell types (green, brown, blue, pink), and migrate along the crypt-villus axis. There, cells are rapidly eliminated. When Apc is mutated, cells do not differentiate and fail to migrate to the villi. Adapted from (McCartney and Näthke, 2008).

5.2. Familial adenomatous polyposis and colorectal cancers

More than 1 million people are diagnosed with colorectal cancer every year, and more than 600 000 die from this disease. Incidence strongly varies globally and is closely linked to elements of the so-called 'western lifestyle'. Incidence is higher in men than women and strongly increases with age. Furthermore, numerous risk factors have been identified such as heritable factors, smoking, alcohol consumption, high consumption of red meat, obesity, diabetes, but also family history of colorectal cancer (Brenner et al., 2014).

Most of colorectal cancer are sporadic and develop over long periods, often more than 10 years, through the formation of adenomas. These premalignant lesions then escape the equilibrium state of immune control and transform into tumors, most of the time because of *Apc* mutation and chromosomal instability which are seen in more than 70% of adenomas.

In addition to sporadic colorectal cancer, some hereditary forms have been observed, corresponding to approximately 5% of all colorectal cancers (Jasperson et al., 2010). These inherited forms of colorectal cancer are the result of autosomal dominant mutations of tumor suppressor genes. 20% of them are due to *Apc* mutations.

Apc gene is located on chromosome 5q21 and spans 22 exons (Kinzler et al., 1991). More than 1 000 different mutation have been described according to the Cancer Genome Atlas, leading to 95% of cases to missenses or premature stop codons resulting in the carboxyl-terminal region truncation of the Apc protein. The most common truncations occur approximately between the codons 1 200 and 1 500 (Rowan et al., 2000), generating Apc proteins lacking binding domains to β -catenin, microtubules and several cytoskeleton regulators such as EB1 and Dlg1 (Zhang and Shay, 2017).

Germ-line mutations of *Apc* induce the development of a syndrome called familial adenomatous polyposis (FAP) characterized by the growth of thousands of polyps throughout the colon and the rectum. The development of polyps is the result of the altered epithelial homeostasis described above, and begins during childhood with significant increase during adolescence. In half of the patients, polyps become adenomas by the age of 15 years, and in 95% by 35 years, ultimately becoming tumors in 100% cases if left untreated (Petersen et al., 1991). However, prophylactic surgery to remove the colon, and in some cases the rectum, is often performed around 20-25 years old. In addition, when *Apc* mutation has been identified in a family, all first-degree relatives are genetically tested, and prenatal or preimplantation testing is possible.

Most patients are asymptomatic for years until the adenomas are large and numerous, causing rectal bleeding. Nonspecific symptoms have also been observed as abdominal pain, but also osteomas, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium, soft tissue tumors, and extracolonic cancers (thyroid, liver, bile ducts and central nervous system) (Half et al., 2009).

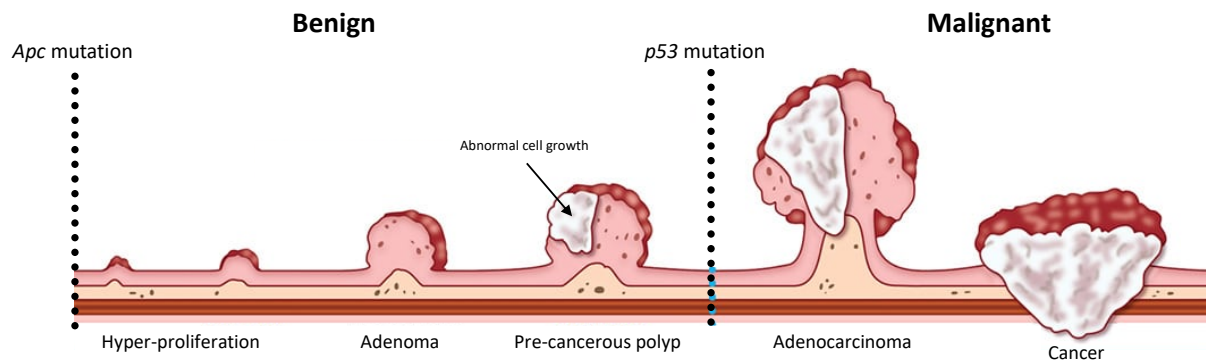


Figure 11. Progression from polyp to colorectal cancer

Adapted from www.hopkinscoloncancercenter.org.

5.3. *Apc* and the immune system

Apc involvement in familial adenomatous polyposis and colorectal cancers has been extensively studied. However, most studies only concern how and why the epithelium is altered to form premalignant lesions, without questioning the immune system involvement and if *Apc* mutation could also alter the immunosurveillance process.

Some studies conducted in *Apc* mutant mice have shown altered control of inflammation by Tregs. Indeed, Tregs have been shown to accumulate in lamina propria and adenomas at pre-cancer stages, reducing others anti-tumor effector CD4 and CD8 T cell frequency. The proportion of anti-inflammatory cells and pro-inflammatory cells is thus changed, unbalancing the intestinal immune homeostasis. These Tregs, however, present impaired expression of transcription factors key for their effector function regulation, as FoxP3 and Gata-3. Consequently, their differentiation and production of anti-inflammatory cytokines as IL-10 are decreased (Aguera-Gonzalez et al., 2017; Gounaris et al., 2009). Importantly, IL-10 production

by T cells or orally administered IL-10 reduces polyp formation in Apc mutant mice, while IL-10 deficiency increases intestinal polyp formation and carcinoma (Chung et al., 2014; Dennis et al., 2015).

Interestingly, some studies showed that Apc mutant Tregs start to produce the pro-inflammatory cytokine IL-17 (Chae and Bothwell, 2015; Gounaris et al., 2009), usually secreted by Th17 cells, which have been associated with several autoimmune diseases (Ogura et al., 2008). Furthermore, intestinal IL-17 production in Apc mutant mice has been shown to promote tumor progression (Chae and Bothwell, 2015; Chae et al., 2010). Therefore, Apc mutant Tregs could shift from a protective anti-inflammatory phenotype, required for colorectal tumor regression (Erdman et al., 2005), to a detrimental pro-inflammatory phenotype. However, such increase in IL-17 production was not observed by the Alcover lab (Aguera-Gonzalez et al., 2017).

In addition, loss of Apc results in reduction of mature naive T cells by rapidly inducing their apoptosis once they egress the thymus (Wong et al., 2015). Furthermore, thymocyte-specific Apc loss has been shown to result in an extensive thymic atrophy and interruption of T cell development at the double negative stage (Gounari et al., 2005).

However, really few studies have questioned if Apc loss or mutation directly affects T cell functions. Considering the altered inflammatory control in Apc mutant mice intestine, a recent study from the Alcover lab has unveiled a molecular mechanism involved. As said previously, Apc loss impairs microtubule organization at the immunological synapse and centrosome reorientation toward the cell contact area in human CD4 T cells. It was shown that the transcription factor NFAT, which is key in T cell activation, differentiation and cytokine production, forms microclusters along microtubules. Altering microtubule network reorganization impairs NFAT nuclear translocation and its transcriptional activity. Furthermore, analysis of Apc mutant mice revealed that intestinal Tregs undergo altered differentiation and produce lower amount of anti-inflammatory cytokine IL-10 (Aguera-Gonzalez et al., 2017).

Altogether, these data suggest that Apc mutations have a dual impact on familial adenomatous polyposis and colorectal cancer development, first, promoting polyp and adenoma formation by unbalancing epithelial cell differentiation and migration, thus epithelial homeostasis, and, second, impairing T cell anti-inflammatory responses which may favor development of pre-cancerous lesions and tumor growth.

Objectives

Since 1984 when Norcross described the ability of T cells to form specific junctions with antigen presenting cells, by analogy to the neuronal synapse (Norcross, 1984), numerous laboratories, including the Alcover lab, have described the importance of the regulation of the cellular remodeling for immunological synapse formation and T cell functions.

The Alcover lab has been investigating how the orchestrated actions of actin and microtubule cytoskeleton, and of intracellular vesicle traffic, control immunological synapse organization, T cell activation, proliferation, differentiation and effector functions, hallmarks of adaptive immune responses. The laboratory has unveiled the key interplay between the actin and microtubule cytoskeleton in regulating the synapse formation. Hence, a number of molecular regulators of these processes have been characterized, including the membrane-microfilament linker ezrin and the polarity regulator Dlg1 (Lasserre et al., 2010).

Apc could also regulate the interplay between actin and microtubule cytoskeleton, and therefore be a critical regulator of T cell polarization and immunological synapse formation. In addition, Apc has been identified for decades as responsible for colorectal cancers and familial adenomatous polyposis, but little is known about its role in immune responses.

Previous data from the Alcover lab have for the first time unveiled a direct involvement of Apc in T cell functions. Apc regulates microtubule repositioning at the synapse, altering TCR signaling molecule microclusters formation and nuclear translocation of the transcription factor NFAT (Aguera-Gonzalez et al., 2017). However, Apc involvement in actin dynamics have not been described.

In this thesis work, we made the hypothesis that Apc defects could also impaired microtubule, and probably actin cytoskeleton, reorganization at the synapse in CD8 T cells. Alteration of the cytoskeleton dynamics and cell polarization would thus result in deficient and non-mature immunological synapses. Consequently, we made the hypothesis that Apc defect could impaired CD8 T cell activation, their differentiation into CTLs, and effector function achievement. Studying Apc involvement in CD8 T cell functions could thus increase our knowledge on the synapse formation and regulation, but also shed light on the effects of Apc mutation in anti-tumor immunity exerted by CTLs. Our work may influence the current understanding of colorectal cancer development in familial adenomatous polyposis, by

understanding why premalignant lesion forming in the rectum and colon of FAP patients are not eliminated by the immune system.

The general objective of this thesis work was thus to investigate the impact of Apc deficiency on anti-tumor cytotoxic T cell functions. We addressed the following questions:

- I) Does Apc regulate CD8 T cell synapse formation?
- II) Are CD8 T cell activation and differentiation modulated by Apc?
- III) Is Apc involved in CTL effector functions?

Experimental procedures

To address these questions, two complementary models were used:

- Human CD8 T cells in which Apc expression was silenced using siRNA or shRNA,
- CD8 T cells from Apc mutant mice *Apc^{Min/+}*

Apc-silenced cells are a good model to assess fundamental Apc involvement in CD8 T cell morphology and functions. However, mice represent a closer physiopathological model to investigate Apc-mediated effects of familial adenomatous polyposis on CD8 T cells.

The *Apc^{Min/+}* mice present a nonsense mutation resulting in Apc truncation at the codon 851 (Moser et al., 1990). A homozygote mutation is embryonically lethal, but heterozygote mutation leads to the development of 20-100 polyps in the mouse intestinal tract. Therefore, these mutants have been largely used as animal model to investigate the molecular bases of Apc-mediated intestinal polyposis and carcinoma. However, in contrast to human familial adenomatous polyposis where polyps predominantly appear in the colon, in Apc mutant mice, polyp formation is favored in the small intestine (Zeineldin and Neufeld, 2013).

I) Does Apc regulate CD8 T cell synapse formation?

Apc tunes actin and microtubule cytoskeletons, and their reorganization is crucial for T cell polarization toward an antigen presenting cell and immunological synapse formation.

Therefore, we first assessed by confocal microscopy if Apc loss would impaired cytoskeleton polymerization and localization at the synapse. For this, we analyzed actin polymerization and pattern, and microtubule network organization at the immunological synapse formed between T cells and anti-CD3-coated coverslips (Figure 12). The coverslips act as surrogate antigen presenting cells, stimulating T cells that form a flat readily observable “pseudo-synapse” displaying higher spatial resolution.

Then, we analyzed synapse formation, morphology and T cell polarization after Apc loss in conventional synapses formed with antigen presenting cells (Figure 12).

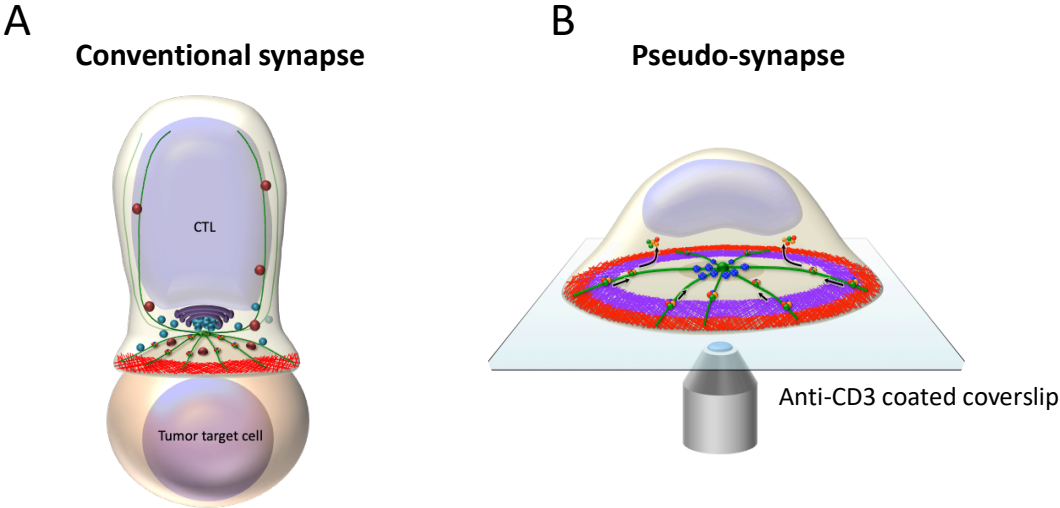


Figure 12. Systems to study immunological synapse formation

Diagrams of systems used to study immunological synapse by confocal microscopy. (A) Conventional synapse formed between a CTL and an anti-CD3 coated tumor cell. (B) Pseudo-synapse formed between a CD8 T cell or CTL and anti-CD3 coated glass surface. Adapted from (Aguera-Gonzalez et al., 2015).

Finally, we performed rupture force assays of cell-cell interactions. For this, we applied shear laminar flow on CTLs and antigen presenting cells conjugated in a laminar flow chamber to separate them (Figure 13). The lower the force used to detach conjugates is, the lower the strength between the two cells is. This assay is therefore a read out of synapse stability and adhesion.

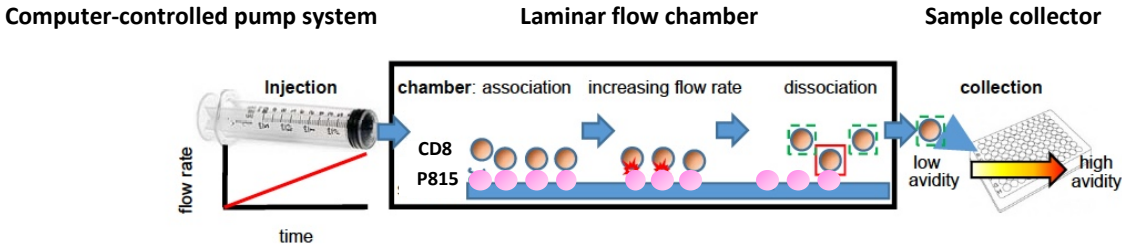


Figure 13. Rupture force assay of cell-cell interaction in laminar flow chamber

II) Are CD8 T cell activation and differentiation modulated by Apc?

The Alcover lab has unveiled Apc requirement for TCR downstream signaling molecule centripetal movement and NFAT nuclear translocation in CD4 T cells, but also in their differentiation into Tregs (Aguera-Gonzalez et al., 2017). We therefore investigated whether Apc could modulate CD8 T cells activation by quantifying by Western blot the activation of TCR partners after stimulation. In addition, *Apc*^{Min/+} mice CD8 T cell differentiation into CTLs was assessed by quantifying by flow cytometry at several days post-stimulation the expression of different makers commonly used to evaluate T cell differentiation, and described in the introduction (e.g. CD25, CD62L, CD44).

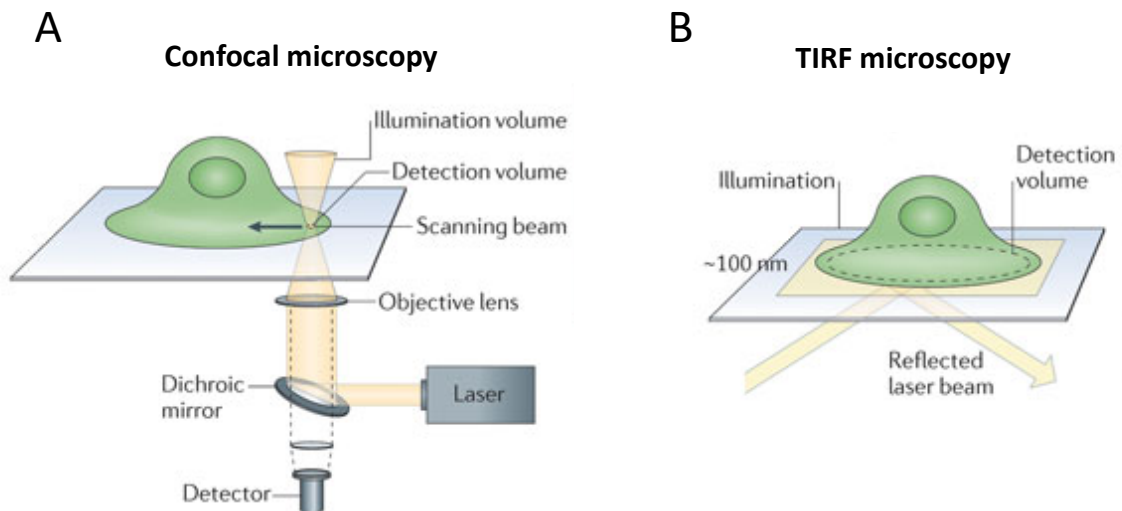


Figure 14. Physical basis of confocal and TIRF illumination

(A) Confocal microscopy. Lasers induce illumination focused onto a defined spot, specifically exciting fluorophores at the spot. A pinhole inside the optical pathway cuts off out of focus signal. (B) TIRF microscopy. Illumination enters with an incidence angle at the sample-coverslip interface displaying different refractive indices. The laser beam is reflected off the interface and an evanescent field is generated on the opposite side of the interface, in the sample. Only fluorophores in the evanescent field are excited. Adapted from (Balagopalan et al., 2011).

III) Is Apc involved in CTL effector functions?

Actin and microtubule reorganization are key for synapse formation, and therefore TCR signaling transduction leading to CD8 T cell acquisition of effectors functions. In addition, they are also key for the achievement of these effector functions.

Thus, we first assessed the killing efficiency of human Apc-silenced CTLs and *Apc*^{Min/+} CTLs measuring the lactate dehydrogenase released by tumor target cells after incubation with CTLs, which is a commonly used cytotoxicity assay.

Then, we evaluated the impact of Apc loss on the lytic granule exocytosis pathway. The CTL cell surface expression of the Lamp1 luminal epitope CD107a, a marker contained in endosome membrane and commonly used as a read out of the degranulation, was quantified by flow cytometry upon contact with tumor target cells. In addition, we studied the lytic granule dynamics, targeting and fusion at the pseudo-synapse formed with anti-CD3 coated coverslips by live cell total internal reflection fluorescent (TIRF) microscopy. TIRF microscopy uses a special mode of sample illumination to exclusively excite fluorophores near the adherent cell surface (~100 nm), corresponding to the plasma membrane at the synapse (Figure 14). This illumination mode is based on an evanescent field produced when light rays are totally internally reflected at the cover glass/substrate interface, and which does not propagate deeper into the sample (Poulter et al., 2015).

Finally, we evaluated the impact of Apc loss on CTL cytokine secretion by quantifying IFN γ and TNF α production and secretion by flow cytometry and ELISA.

The results of this thesis work are presented in the form of an article manuscript, which has been submitted to Journal of Immunology.

Results

Adenomatous polyposis coli modulates actin and microtubule cytoskeleton at the immunological synapse to tune cytotoxic T cell functions

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Abstract

Adenomatous polyposis coli (Apc) is a cell polarity regulator and a tumor suppressor associated with familial adenomatous polyposis and colorectal cancer. Apc involvement in T lymphocyte functions and anti-tumor immunity remains poorly understood. Investigating Apc-depleted human CD8 T cells and CD8 T cells from *Apc^{Min/+}* mutant mice, we found that Apc regulates actin and microtubule cytoskeleton remodeling at the immunological synapse, controlling synapse morphology and stability, and lytic granule dynamics, including targeting and fusion at the synapse. Ultimately, Apc tunes cytotoxic T cell activity leading to tumor cell killing. Furthermore, Apc modulates early T cell receptor signaling and nuclear translocation of the NFAT transcription factor with mild consequences on the expression of some differentiation markers. In contrast, no differences in the production of effector cytokines were observed. These results, together with our previous findings on Apc function in regulatory T cells, indicate that Apc mutations may cause a dual damage, first unbalancing epithelial cell differentiation and growth driving epithelial neoplasms, and, second, impairing T cell-mediated anti-tumor immunity at several levels.

Introduction

T cell antigen receptors (TCR) recognize peptide antigens associated with major histocompatibility complex molecules (MHC) on antigen-presenting cells. Antigen interaction induces early TCR signaling leading to the T cell polarization towards antigen-presenting cells and the formation of the immunological synapse at the T cell-antigen-presenting cell interface. Immunological synapses are key to control T cell activation leading to T cell growth, differentiation and cytokine production. They also control T cell effector functions like polarized secretion of cytokines by helper (Th) and regulatory (Treg) T cells, and lytic granules by cytotoxic T cells (CTLs) (1).

Immunological synapse generation and function depend on the orchestrated action of the actin and microtubule cytoskeleton, and of intracellular vesicle traffic that polarize at the T cell side of the immunological synapse. This drives the targeting and dynamic clustering of TCRs and signaling molecules to optimally control T cell activation (2). Furthermore, the Golgi and lytic granule intracellular traffic reorient towards the synapse, delivering directly T helper cytokines to antigen-presenting B cells (3-5), or lytic granules to infected or tumor target cells. Polarized secretion of lytic granules depends on the fine concomitant dynamic remodeling of actin and microtubule cytoskeleton at the immunological synapse (6-9).

Cell polarity complexes are key to ensure stable cell polarity (10), as well as induced polarization in migrating cells (11). Scribble, Dlg1 and PKC ζ polarity regulators were shown to control lymphocyte migration, immunological synapse formation and T cell activation (12-17). The polarity regulator and tumor suppressor Apc is known for its association with familial adenomatous polyposis (FAP), human colorectal tumors, and intestinal carcinomas in mice (18-21). Apc interacts with a variety of proteins, including transcription factor regulators as β -catenin, polarity regulators as Dlg1 or Scribble, cytoskeleton regulators as Cdc42 or EB1, nuclear pore and nuclear transport proteins, and apoptosis- or mitosis-related proteins (22, 23).

Apc mutations alter intestinal epithelium differentiation and induce tumor progression in colorectal cancer patients and in mouse models (18-22, 24). The role of Apc in immune responses is much less explored, in particular against tumors. However, altered intestinal immune homeostasis and control of inflammation by regulatory T lymphocytes (Tregs) was reported in Apc mutant mice (25-28). We have previously unveiled that Apc underpins various molecular mechanisms controlling CD4 T cell functions (29). These include microtubule network organization and centrosome polarization at the immunological synapse, and NFAT-driven cytokine gene activation. Interestingly, Apc regulates NFATc2 nuclear translocation

upon T cell activation. Furthermore, NFATc2 forms microclusters associated with microtubules and needs microtubules to translocate to the nucleus upon T cell activation. Finally, in *Apc^{Min/+}* mutant mice, lamina propria Tregs display lower capacity to differentiate and produce cytokines, mainly IL-10 (29). This cytokine is central to regulate intestinal inflammation and adenocarcinoma progression (30). These findings suggested that Apc mutations may also affect immune cell functions and particularly the anti-inflammatory capacity of Tregs, which could contribute to tumor development in patients carrying Apc mutations.

NFAT is involved in cytotoxic T cell differentiation and function (31). Furthermore, microtubule-mediated lytic granule polarization and release at cytotoxic T cell synapses is crucial for efficient tumor cell killing (7). Therefore, we hypothesized that Apc deficiency might also affect cytotoxic T cell functions reducing their capacity to eliminate tumor cells and contributing to tumor escape in Apc-dependent polyposis patients. To investigate this, we studied Apc-silenced human primary CD8 T cells and CD8 T cells from *Apc^{Min/+}* mutant mice. These heterozygous mice mutants have been largely used as an animal model to investigate the molecular bases of Apc-mediated intestinal polyposis and carcinoma (19), and represent a more suitable physio-pathological model to investigate Apc-mediated effects of inherited polyposis than Apc-silenced cells.

Here, we show that Apc is involved in microtubule and actin cytoskeleton remodeling at the immunological synapse of CD8 T cells. Furthermore, CD8 T cells from *Apc^{Min/+}* mice displayed reduced TCR-mediated Erk kinase activation and NFAT nuclear translocation. However mild or no effects were observed on some CD8 differentiation markers or production of cytokines. Importantly, synapse stability, lytic granule dynamics and fusion at the synapse, as well as cytotoxic activity against tumor cells were reduced in *ex vivo* differentiated CTLs from *Apc^{Min/+}* mice or from Apc-silenced human CD8 T cells. These results reveal a novel role of Apc in modulating cytotoxic T cell responses with potential consequences in anti-tumor immunity.

Results

Apc is expressed in human CD8 T cells

We first investigated the expression of Apc in primary human CD8 T cells. Immunoblotting on cell lysates revealed similar levels in CD4 and CD8 T cells of a protein band consistent with the 311 kDa full length protein previously described (24, 29) (Figure 1 A). This band disappeared upon siRNA silencing in CD8 T cells (Figure 1 B). Apc depletion was similarly detected by quantitative RT-PCR upon siRNA transfection in human resting CD8 T cells or shRNA retroviral transduction in differentiated CTLs (Figure 1 C). We next analyzed Apc subcellular localization. Both resting and differentiated CD8 T cells showed a punctate pattern of Apc associated with microtubules and aligned at the edges of immunological synapses formed on anti-CD3-coated coverslips (arrowheads) (Figure 1 D, E), consistent with our previous findings in CD4 T cells (29).

Apc regulates microtubule and actin cytoskeleton remodeling at the immunological synapse

Apc was shown to regulate microtubule network organization in polarized migrating cells (32-34), as well as actin polymerization (35). Furthermore, Apc coordinates actin polymerization and microtubules at focal adhesions in migrating cells (36). In both CD4 and CD8 T cells, actin and microtubule cytoskeletons cooperate to build functional immunological synapses (2, 8, 14). Indeed, in CD8 T cells, microtubule polarization towards target cells combined with actin polymerization, followed by rapid clearance from the center of the synapse, appear to be key for cytolytic granule release and target cell lysis (6, 9, 37). Importantly, we previously showed that Apc silencing in CD4 T cells results in microtubule network disorganization at the immunological synapse (29).

Therefore, we investigated whether Apc controls cytoskeleton remodeling in CD8 T cell immunological synapses. Apc silencing altered microtubule network organization at the immunological “pseudo-synapses” formed by either resting or differentiated CD8 T cells on anti-CD3-coated coverslips (Figure 2 A, B). Microtubule patterns were more frequently radially organized in control cells, reaching synapse periphery and with visible microtubule organizing center. In contrast, Apc-silenced cells more often displayed randomly organized microtubules. In addition, we observed that actin remodeling at the synapse was also impaired. Actin could accumulate at the synapse (Fig. 2 C), and no differences between control and Apc-silenced cells were observed with regard to the total amount of F-actin at the contact site (Figure 2 D).

However, control cells often formed F-actin rings at the periphery of the synapse, excluding actin from the center (Figure 2 C, left panel, and 2 E). In contrast, Apc-silenced cells less frequently displayed this actin pattern, forming more often synapses with less defined actin ring and lower clearance from the center (Figure 2 C, center and right panel, and 2 E).

Apc conditions CTL centrosome polarization, immunological synapse shape, symmetry and stability

Our observations on cytoskeleton remodeling prompted us to investigate a larger number of features of immunological synapses formed between human CTLs and anti-CD3-coated P815 tumor target cells, which are a model of Ab-mediated T cell cytotoxicity. Following the examples of synapses shown in Figure 3 A, we analyzed i) CTL centrosome polarization, assessed by the distance of the centrosome (arrows) to the center of the synapse; ii) synapse symmetry, assessed by the relative aligned position of the centrosome and the T cell and target cell nuclei; iii) synapse shape, assessed by the presence of more or less large membrane extensions. Figure 3 A, left panel, provides an example of a symmetrical CTL-target cell conjugate, with polarized centrosome (arrow) and no extensions. The right panel is an example of an asymmetrical conjugate, with centrosome poorly polarized, and displaying large cell extensions (arrowhead) and more irregular shape. We observed that Apc silencing did not alter the number of conjugates (not shown), but impaired T cell centrosome polarization toward the synapse and T cell symmetry, and increased the presence of large cell extensions (Figure 3 B-D). These features may reflect the inability of Apc-silenced cells to make stable interactions with target cells.

To obtain further insight into the effect of Apc silencing on synapse stability, we measured the rupture force of cell-cell interactions between CTLs and anti-CD3-coated P815 cells immobilized in a microscope laminar flow chamber undergoing increasing flow rate. A laminar shear flow rate was applied generating dragging forces from 0 to 5 nN on individual cells, with an increasing slope of 34 pN/s. CTLs interacting with immobilized P815 were stretched by this dragging force until rupture of the contact and release of the T cells into the stream (Figure 3 E and Supplemental Movies 1 and 2). CTL-P815 cell rupture forces ranged from 0 to 4.5 nN with half of values between 0.5 and 2.5 nN suggesting large variety of synapse formation kinetics or complexity. Average rupture force was 1.52 nN and 0.67 nN for control and Apc-silenced cells respectively, indicating that the interacting force was decreased by loss of Apc expression (Figure 3 F). Interestingly, cumulative plots in Figure 3 G show linear distributions of cell-cell rupture events versus dragging force for control cells and hyperbolic

distributions for Apc-silenced cells. Of note is that although equal cellular input of shCtrl and shApc cells was applied to the chamber, the number of CTLs forming initial conjugates with P815 cells before applying flow pressure was lower in shAPc-treated cells in most experiments (n= # in figure 3 F legend). This further reflects the lower capacity of Apc-silenced cells to stabilize conjugates with P815 target cells.

Apc controls lytic granule dynamics, targeting and fusion at the immunological synapse

Perturbation of actin and microtubule remodeling at the immunological synapse, T cell centrosome polarization and the stability of CTL-target cell interactions of Apc-silenced cells (Figures 2 and 3) might result in reduced accessibility of lytic granules to the immunological synapse, reducing the capacity of CTLs to kill target cells (6, 7, 9).

To investigate this, we first analyzed lytic granule dynamics at the immunological synapse. Human control or Apc-silenced CTLs were labelled with Lyso-Tracker and set on anti-CD3-coated coverslips to obtain flat immunological synapses. Granule dynamics was then followed by live cell TIRF microscopy. We measured, i) the total number of bright granules appearing within the TIRF plan during the 4 min recorded time as a readout of granules targeted to the synapse; ii) the granule movement by tracking individual fluorescent particles at the synapse; iii) the number of granules generating a fluorescence burst at the TIRF zone, as a readout of granule fusion with the plasma membrane (6, 9), as the example provided in Figure 4 E, upper panels, and Supplemental Movie 3. We observed that Apc-silenced cells displayed reduced targeting events (Figure 4 A). Furthermore, lytic granules in Apc-silenced cells remained longer times at the synapse and their speed was lower than in control cells (Figure 4 B). However, the length of displacement of granules was similar in control and Apc-silenced CTLs (Figure 4 B, C). Importantly, Apc-silenced cells displayed fewer fusion events (Figure 4 D, E, Supplemental Movies 3 and 4). Therefore, Apc silencing, by impairing microtubule network organization and actin clearance, alters lytic granule targeting, dynamics and fusion at the immunological synapse.

We next studied immunological synapses formed between CTLs and anti-CD3-coated P815 tumor cells, upon 15 min of CTL-target cell contact. Subcellular localization of lytic granules was assessed by anti-perforin antibody staining. Interestingly, control cells displayed lower number of lytic granules and more randomly distributed (Figure 4 F, left and 4 G), whereas Apc-silenced cells displayed higher number of granules more grouped and polarized towards the synapse (Figure 4 F, right panel and 4 G).

These data suggest that, in *Apc*-silenced cells, lytic granules can group close to the centrosome and reorient towards the synapse, however, they are less efficiently targeted and fused to the synapse plasma membrane. As a consequence, a higher number of lytic granules is retained in CTLs during their interaction with target cells.

Apc regulates CTL killing of tumor target cells

We next asked whether the defects in cytotoxic granule dynamics observed in *Apc*-silenced cells could impair their capacity to kill tumor target cells. To this end, we used conventional longer incubation assays to measure degranulation and tumor target cell killing. Both human *Apc*-silenced CTLs and mouse *Apc*^{Min/+} CTLs were less efficient in killing anti-CD3-coated P815 tumor target cells, as assessed by an LDH-release colorimetric assay (Figure 5 A, B). However, no significant differences were observed in the capacity of these human and mouse CTLs to degranulate, as assessed by measuring cell surface expression of the Lamp1 luminal epitope CD107a upon CTL contact with anti-CD3-coated P815 tumor target cells for 3 hours (38) (Figure 5 C, D).

Altogether, the above described data indicate that *Apc* is necessary for efficient microtubule and actin cytoskeleton remodeling conditioning, lytic granule targeting and fusion at the immunological synapse and, ultimately, for efficient killing of tumor target cells.

Apc modulates TCR signaling and NFAT nuclear translocation

We had shown in CD4 T cells that the polarity regulators Dlg1 and *Apc* were involved in microtubule network organization at the immunological synapse conditioning NFAT transcriptional activation (14, 29, 39). We therefore investigated whether *Apc* modulation of cytoskeleton remodeling could affect CD8 T cell activation. To this end, we analyzed the capacity of *ex vivo* differentiated CTLs from control and *Apc*^{Min/+} mutant mice to respond to anti-CD3+anti-CD28 stimulation. *Apc*^{Min/+} CTLs exhibited a mild but significantly reduced capacity to activate Erk and Akt serine-threonine kinases in response to TCR stimulation, as assessed by the phosphorylation of regulatory residues of these kinases (Figure 6 A, B). In contrast, the upstream protein tyrosine kinase ZAP70 was more variably activated and no significant differences between control and *Apc*^{Min/+} cells could be appreciated (not shown). Furthermore, NFATc2 nuclear translocation in response to anti-CD3+anti-CD28 stimulation was reduced in *Apc*^{Min/+} CTLs (Figure 6 C, D).

Apc defects mildly alter ex vivo differentiation of CD8 T cells

The observed alteration in NFAT nuclear translocation prompted us to investigate whether Apc defects could be associated with impaired CD8 T cell differentiation and/or cytokine production. We therefore analyzed the capacity of *Apc*^{Min/+} CD8 T cells to differentiate into CTLs *ex vivo*. Splenocytes and lymph node cells were stimulated with concanavalin A (ConA) and IL-2, and the expression of the activation/differentiation molecules CD25, granzyme B, CD44 and CD62L was assessed at 2, 4 and 6 days upon activation. Expression of CD44 and CD62L adhesion molecules in CD8 T cells from *Apc*^{Min/+} mice appeared mildly affected mainly at day 2. CD62L expression remained lower but differences were statistically not significant, with high variability between animals (Figure 7 A-D). In contrast, no differences were observed in CD25 and granzyme B expression (Figure 7 E-H). Interestingly, differences in CD44 and CD62L expression were more readily observed in CD4 T cells with more pronounced and longer effects (Supplemental Figure 1). Interestingly, the level and kinetics of CD62L expression in CD4 T cells were different than in CD8 T cells.

These findings show that CD8 T cells from *Apc*^{Min/+} mice present reduced early TCR activation events, such as Erk and Akt activation, and NFAT nuclear translocation. Finally, minor differences in the expression of CD44 and CD62L differentiation markers in response to *ex vivo* stimulation in cells from *Apc*^{Min/+} mice were observed in CD8 T cells, with more significant differences in CD4 T cells.

Apc defects do not alter ex vivo CTL cytokine production

Since NFAT drives the transcription of a variety of T cell cytokines, we investigated the capacity of Apc-silenced human CTLs and *Apc*^{Min/+} CTLs to produce effector cytokines. In spite of the reduced capacity of *Apc*^{Min/+} CTLs to induce NFAT nuclear translocation, we did not observe significant differences in the production of IL-2, IFN γ or TNF α by differentiated CTLs re-stimulated with anti-CD3+anti-CD28, either by intracellular staining and FACS analysis, or by ELISA of cell culture supernatants (Figure 8 A, B). Similar results were found when assessing cytokine production in cell culture supernatants of control and Apc-silenced human CTLs by ELISA (Figure 8 C).

These findings indicate that Apc defects in both human and mouse CTLs do not affect their capacity to produce effector CD8 cytokines under our *ex vivo* stimulation conditions.

Discussion

Altogether the data shown here unveil a novel role of Apc in cytotoxic T cell effector functions. Apc defects in Apc-silenced human peripheral blood CD8 T cells or in *Apc*^{Min/+} mutant mice revealed the importance of Apc in cytoskeleton remodeling at the immunological synapse. Both microtubules and actin reorganization appeared affected. Thus, the radial organization of microtubules and centrosome polarization were perturbed, and F-actin ring formation at the periphery of the synapse and F-actin exclusion from the center of the synapse were impaired in Apc-defective T cells. Additionally, consistent with an impact on actin cytoskeleton dynamics (40), synapse stability, shape and symmetry were also altered in Apc-deficient cells.

Apc regulation of microtubule organization in CD8 T cells is consistent with the described role of Apc as polarity regulator in other cell systems (23). Apc performs this function in association with other regulators of cell polarity, including Cdc42, Scribble, Par6, PKC ζ and Dlg1, as shown for instance in migrating astrocytes (34, 41). Furthermore, we have shown before that Dlg1 and Apc control microtubule patterns, centrosome polarization and signaling microcluster dynamics at the immunological synapse of CD4 T cells (14, 29). Although less extensively studied, Apc may also regulate actin polymerization in synergy with its partner, the formin family protein mDia (35). In addition, Apc regulates actin-microtubule crosstalk at focal adhesions of migrating cells (36). Interestingly, molecular partners of Apc, as Cdc42 (34) and formins (35), were shown to control actin remodeling and centrosome polarization at the immunological synapse (42, 43). Therefore, Apc may collaborate with these cytoskeleton regulators to collectively reorganize both cytoskeleton components at the CTL immunological synapse, thus ensuring their interplay that is crucial for properly tuning T cell functions.

Concomitant centrosome polarization and F-actin exclusion from the center of the immunological synapse has been shown to be key for lytic granule targeting and fusion at the synapse, and efficient cytotoxic function against infected or tumor target cells (6, 7, 9, 37). Therefore, impairment of both microtubule organization and centrosome polarization, together with F-actin reorganization at the synapse in Apc-defective CTLs, may account for their altered lytic granule dynamics, targeting and fusion at the synapse observed by live cell TIRF microscopy. Apc-silenced cells could still concentrate lytic granules in the centrosomal area and polarize them to a certain extent towards the immunological synapse, suggesting that there is not a complete failure in granule transport on microtubules. Granules may be able to move towards the centrosome and microtubule minus end, a movement mediated by dynein motors.

In contrast, granules do not properly reach the immunological synapse plasma membrane. Disorganization of microtubule network at the immunological synapse likely prevents microtubules from reaching the plasma membrane and, as a consequence, precludes centrosome closing up to the synapse, which facilitates lytic granule docking and fusion (7). Kinesin-mediated transport to the microtubule plus ends may also help granules to reach the synapse plasma membrane (44, 45). As a likely consequence of inefficient lytic granule dynamics, targeting and fusion, *Apc* defective CTLs killed tumor target cells less efficiently than control cells. However, no difference in “degranulation” assessed by increase in Lamp1 (CD107a) cell surface expression was detected. The apparent contradiction between the live cell TIRF experiments and Lamp1 expression may be due to the different time of CTL-target cell contact in these two assays, and/or their distinct sensitivities to discriminate differences. Lack of direct correlation between degranulation and cytotoxicity has been reported in other experimental setups (46).

Although significant, the differences in tumor cell killing activity between *Apc*-defective CTLs and control cells were modest. This may be due to the fact that *Apc*^{Min/+} mice are heterozygous (homozygous mutation is embryonic lethal) (47) and may express residual levels of *Apc* protein. Likewise, shRNA silencing in human primary CTLs is not fully efficient. Hence, remaining *Apc* protein could account for the observed function. Furthermore, other polarity regulators acting together with *Apc* may also compensate *Apc* defects. Finally, the T cell activation conditions we used for CTL generation *in vitro* may compensate in part cytotoxicity defects due to *Apc*, by increasing the expression of other proteins. This effect was described for some forms of familial hemophagocytic lymphohistiocytosis due to syntaxin 11 or Munc18-2 mutations, where the addition of IL-2 used to induce T cell proliferation *in vitro* was partially compensating the loss of effector function (48, 49). Importantly, subtle differences in CTL cytotoxicity using *in vitro* assays may be predictive of more serious cytotoxic defects *in vivo* leading to poor immune responses (50). Therefore, the small differences in cytotoxicity we observed could have consequences for anti-tumor immunity in patients.

We have previously shown in CD4 T cells that *Apc* silencing leads to altered NFAT nuclear translocation and reduced NFAT-driven transcriptional activation leading to impaired IL-2 gene transcription. Furthermore, CD4 T cells from *Apc*^{Min/+} mice had reduced capacity to produce IL-2, IL-4 and IFN γ . Finally, regulatory T cells in these mutant mice showed impaired differentiation and production of the anti-inflammatory cytokine IL-10 (29). We show here that CTLs from *Apc*^{Min/+} mice also had an impaired capacity to translocate NFATc2 to the nucleus. Interestingly, NFATc2 formed microclusters associated to microtubules in CD8 T cells (data

not shown), as we previously showed in CD4 T cells (29). However, the analysis of the expression of a number of differentiation markers (e.g. CD25, granzyme B, CD44 and CD62L) in response to *ex vivo* activation with concanavalin A and IL-2 revealed only mildly altered CD44 and CD62L expression at day 2 in *Apc^{Min/+}* mice. Interestingly, under the same stimulation conditions, the differences in expression of CD44 and CD62L between *Apc^{Min/+}* and control T cells were more pronounced and significant in the CD4 T cell population. In addition, *Apc*-silenced human and *Apc^{Min/+}* mouse *ex vivo* differentiated CTLs produced equal amount of IL-2, TNF α and IFN γ after re-stimulation with CD3/CD28 antibodies. Therefore, CD8 T cells appear to be less sensitive to *Apc* defects than CD4 T cells when performing their activation programs leading to differentiation and production of cytokines.

Altogether the data presented here show that *Apc* is involved in the regulation of CTL effector functions driving target cell killing. Hence, *Apc* defects impair both actin and microtubule cytoskeleton remodeling at the immunological synapse, centrosome polarization and lytic granule dynamics, targeting and fusion at the synapse. However, CD8 T cell differentiation and cytokine production appear much less dependent on *Apc* compared to what has been observed in CD4 T cells (29). Thus, *Apc* defects may not significantly alter the acquisition of effector functions in CD8 T cells, but impair the CTL capacities to kill tumor cells and could therefore have an impact in anti-tumor immune responses in familial adenomatous polyposis patients allowing easier tumor escape.

Materials and Methods

Human cell isolation, siRNA transfection, lentiviral infection, CTL generation and tumor target cell culture

Human peripheral blood T cells from healthy volunteers were obtained from the French Blood Bank Organization (Etablissement Français du Sang, EFS), or through the Institut Pasteur Biological Resources Core Facility ICAReB (NSF 96-900 certified, from sampling to distribution, reference BB-0033-00062/ICAReB platform/ Institut Pasteur, Paris, France/BBMRI AO203/ 1 distribution/ access: 2016, May 19th, [BIORESOURCE]), under CoSImmGen protocol approved by the Committee of Protection of Persons, Ile de France-1 (No 2010-dec-12483). Informed consent was obtained from all donors.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation through Ficoll-Hypaque. CD8 T cells were isolated from PBMCs using the MACS CD8 T Cell Isolation Kit (Miltenyi Biotec) and maintained in human CD8 medium: RPMI 1640 + GlutaMAX-I (Gibco) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and nonessential amino acids, 10 mM HEPES, 1% penicillin-streptomycin (v/v).

To generate CTLs, freshly isolated CD8 T cells were stimulated 2 days with coated anti-CD3 (10 µg/mL; UCHT1 produced by A. Alcover), soluble anti-CD28 (7 µg/mL; Beckman Coulter), and recombinant human IL-2 (100 U/mL; PeproTech) in human CD8 medium. Cells were infected for 24 h with lentiviruses coding for control or Apc-specific shRNAs (10% v/v) in human CD8 medium with IL-2, where fetal bovine serum (FBS) was replaced by human serum, and then selected for 3 days with puromycin (3.9 µg/mL). Percentage of infected CTLs was 70-85%, as assessed by GFP expression by FACS.

For siRNA experiments, small double stranded RNA oligonucleotides sequences used were 5'-GAGAAUACGUCCACACCUU-3' (siApc; GE Healthcare) for Apc depletion, as we previously described (29). As control, 5'-UAGCGACUAAACACAUCAA-3' (siGENOME Non-Targeting siRNA #1; GE Healthcare) was used. 10⁷ freshly isolated CD8 T cells were transfected with 1 nmol of siCtrl or siApc using the Human T Cell Nucleofector kit and the program U-14 on an Amaxa Nucleofector II (Lonza). Cells were then harvested in human CD8 medium without penicillin-streptomycin and used 72 h after transfection.

For shRNA experiments, lentiviruses were produced by HEK293T cells transfected with the transient calcium phosphate DNA precipitation technique. Cells were transfected with pCMV-deltaR8-2 and pCMV-env-VSV, together with a pLKO.1-puro-CMV-tGFP lentiviral vector expressing or not (as negative control) a short hairpin (sh)RNA targeting Apc (5'-

GACTGTCCTTTCACCATATTT-3') (Sigma-Aldrich). Forty-eight hours later, supernatant was recovered and concentrated 40x by ultracentrifugation (26,000 rpm, 1.5 h, 4°C). Lentiviruses stocks were stored at -80°C.

The P815 mouse mastocytoma cell line was used as tumor target cell. P815 cells were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (v/v). To make stimulatory target cells, P815 were pulsed with anti-human or mouse CD3 antibodies, as described below.

Apc^{Min/+} and wild type mice lymphocyte isolation and cell culture

Heterozygous C57BL/6J-Apc^{Min} (*Apc^{Min/+}*) mice and wild-type controls were purchased from the Jackson Laboratory. Mice were housed and bred under pathogen-free conditions in the Central Animal Facility of the Institut Pasteur. Protocols used had been approved by the Ethical Committee for Animal Experimentation of the Institut Pasteur and by the French Ministry of Research. Males and females were sacrificed at 10-12 weeks of age. Spleen and lymph nodes were homogenized through a 70 µm filter. To generate CTLs, CD8 T cells were purified using the MACS CD8a T Cell Isolation Kit (Miltenyi Biotec), and stimulated 2 days with coated anti-CD3 (5 µg/mL; 145-2C11, eBiosciences), soluble anti-CD28 (2 µg/mL; eBiosciences) and IL-2 (10 ng/mL; Miltenyi Biotec). Cells were then cultured for 5-6 days in mouse CD8 medium: DMEM + GlutaMAX-I (Gibco) supplemented with 10% FBS, 1 mM sodium pyruvate and nonessential amino acids, 50 µM β-mercaptoethanol, 10 mM HEPES, 1% penicillin-streptomycin (v/v), in presence of IL-2 (10 ng/mL; Miltenyi Biotec).

Apc quantification

For Apc detection by Western blot, total cell extracts were obtained by lysing cells for 5 min on ice in lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 40 mM beta-glycerophosphate, 10 mM NaF, 10 mM Na₄P₂O₇, 2 mM ortho-vanadate and protease inhibitor cocktail). Cell lysates were cleared by spinning at 20,800 xg for 20 min at 4°C. Equal amount of protein content measured using the Bio-Rad protein assay (Bio-Rad Laboratories) was loaded in NuPAGE 3-8% Tris-Acetate gels (ThermoFisher Scientific). Proteins were transferred onto nitrocellulose membranes (LI-COR Biosciences) for 4 h using a Bio-Rad Mini Trans-Blot system in buffer containing 50 mM Tris, 380 mM Glycine, 20% ethanol, 0.1% SDS. Membranes were saturated with blocking buffer (Rockland Immunochemicals) and incubated with anti-Apc (2 µg/mL; Ali 12-28, Abcam) and anti-ZAP70

(50 ng/mL; BD Biosciences) or anti-PLC γ 1 (1/1000; Cell Signaling) overnight at 4°C. They were washed and then incubated with specific secondary antibodies conjugated with Alexa-Fluor680 or DyLight800 (ThermoFisher Scientific) for 45 min. Near-infrared fluorescence was imaged and quantified using the Odyssey Classic near-infrared imaging system (LI-COR Biosciences) and Apc band intensity was normalized to control ZAP70 or PLC γ 1 band.

For Apc detection by retrotranscription quantitative PCR, total RNA was extracted using the RNeasy Micro Plus Kit (Qiagen) following manufacturer's instructions. cDNA was obtained from 200 ng of total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). FastStart Universal SYBR Green PCR Master Mix (Roche) and ABI PRISM 7900HT Sequence Detection system (Applied Biosystems) were used to quantify gene products. Quantitative PCR were performed in triplicates, quantity values were calculated by the relative standard curve method and normalized to the mRNA expression of the *RLP13a* housekeeping gene. Primer sequences used were as follows:

Apc-F: 5'-CCAACAAGGCTACGCTATGC-3'/ R: 5'-TACATCTGCTCGCCAAGACA-3'
RLP13a-F: 5'-CATAGGAAGCTGGGAGCAAG-3'/
R: 5'-GCCCTCCAATCAGTCTTCTG-3'

Confocal microscopy, image post-treatment and quantitative image analysis

Coverslips were washed with HCl-ethanol 70% and coated with Poly-L-lysine 0.002% in water (Sigma-Aldrich). For pseudo-synapse formation, coverslips were further coated with anti-CD3 (10 μ g/mL; UCHT1, BioLegend) overnight at 4°C. Coverslips were washed and blocked for 30 min at 37°C with human CD8 medium. Human T cells were plated on coverslips for 5 min at 37°C, and fixed with 4% paraformaldehyde for 13 min at room temperature. For microtubule detection, cells were also incubated 20 min at -20°C in ice cold methanol. For CTL-target cell immunological synapse formation, P815 cells, previously coated with anti-CD3 (20 μ g/mL; OKT3 produced by A. Alcover) for 45 min at 4°C, were plated on poly-L-lysine-coated coverslips for 15 min at 37°C. Coverslips were carefully washed with PBS, and human CTLs were added at a 1:1 CTL-target ratio and incubated for 15 min at 37°C. Cells were then fixed with 4% paraformaldehyde for 13 min at room temperature. Coverslips were washed in PBS, and incubated 1 h in PBS with 1% bovine serum albumin (BSA) (v/v) to prevent unspecific binding. Cells were then incubated 1 h at room temperature with PBS, 1% BSA, 0.1% Triton X-100 and anti-Apc (1/300; gift of I. N athke, University of Dundee), anti- β -tubulin (6.6 μ g/mL; Merck Millipore), anti-pericentrin (1/100; Abcam), anti-perforin (10 μ g/mL; BD

Biosciences). Coverslips were then incubated with the corresponding fluorescent-coupled secondary Ab and Texas Red-X phalloidin (1/100; Invitrogen) for 45 min at room temperature. After three washes in PBS with 1% BSA, coverslips were mounted on microscope slides using ProLong Gold Antifade mounting medium with DAPI (Life Technologies).

Confocal images were acquired with an LSM 700 confocal microscope (Zeiss) using the Plan-Apochromat 63x/1.40 NA objective. Optical confocal sections were acquired with ZEN software (Zeiss) by intercalating green and red laser excitation to minimize channel cross talk. All analyses were performed using Fiji software (51). For F-actin analysis, optical sections were acquired at 1 μm intervals. Formation of the actin ring and phalloidin fluorescence intensity analyses were performed on one confocal section at the cell-coverslip contact. For microtubule pattern and synapses analyses, optical sections were acquired at 0.2 μm intervals and images were treated by deconvolution with the Huygens Pro Software (Scientific Volume Imaging). Microtubule pattern analysis was performed on projection of 4 confocal sections at the cell-coverslip contact, and phenotypes were classified by blinded image observation by three different investigators. Synapse morphology and cytotoxic granules localization were analyzed in 3D projections, and classified as described for microtubule patterns. Centrosome localization was estimated by measuring the distance between the anti-pericentrin staining and the CTL plasma membrane at the center area of the immunological synapse. Quantification of cytotoxic granules per cell was obtained using the TrackMate plugin for ImageJ developed by J.-Y. Tinevez (Photonic Bio Imaging UTechS, Institut Pasteur) (52).

Rupture force assay of cell-cell interactions in laminar flow chamber

Glass surface (RS, France) of the flow chamber (Slide I^{0.1}, Ibidi, Germany) was washed using 5 chamber volumes (CV) of sulfuric acid (2 M; Merck-Sigma) then 5 CV of hydrogen peroxide (33% Merck-Sigma), and rinsed with 10 CV of water then 10 CV of PBS. Bovine fibronectin (10 $\mu\text{g}/\text{mL}$ in PBS; Merck-Sigma) was incubated 1 h at room temperature and rinsed with 20 CV of PBS. The chamber was equilibrated with DMEM (Gibco) at 37°C then loaded with P815 cells for 15 min at 37°C. One CV of anti-CD3 (20 $\mu\text{g}/\text{mL}$ final; OKT3 produced by A. Alcover) in DMEM was carefully added, and cells were incubated for 15 min at 37°C. Unbound cells and antibodies were cleared by flowing 5 CV of RPMI (37°C; Lonza). One CV of human CTLs in RPMI 1% FBS (Lonza) was injected inside the chamber and incubated for 10 min at 37°C. A flow rate of PBS, increasing from 0 to 38.4 mL/min, was applied through the chamber for 115 s using a syringe pump (SP210iW, WPI) controlled by a computer, and

synchronized with image acquisition (3 image/s, 1100 x 840 μm) using an inverted transmission microscope (Observer D1, Zeiss) with a 10x/0.3 NA objective controlled by MicroManager (53).

Image series were analyzed using ImageJ software (51) and the plugin CellCounter. P815 and CTLs were distinguished from their size, shape and nucleus/cell ratio. The flow rate value at the cell-cell rupture event was used to compute the dragging force on the released cell according to its size (mean diameter of 8 μm), shape (roughly spherical) and density (mean value 1.20 kg/L). Calibration of dragging force was performed from sedimentation rate of cells in the chamber (measured PBS density 1.0034 kg/L and dynamic viscosity 0.6998 mPa.s at 37°C) (54) and the theoretical flow speed *versus* wall distance according to Poiseuille solution to Navier-Stokes formalism for a Reynolds' number below 10 characterizing a laminar flow.

Live cell total internal reflection fluorescence (TIRF) microscopy

Glass bottom microwell dishes (MatTek Corporation) were washed with HCl-ethanol 70%. They were coated with Poly-L-lysine 0.002% in water (Sigma-Aldrich) 30 min at room temperature, and then with anti-CD3 (10 $\mu\text{g}/\text{mL}$; UCHT1, BioLegend) overnight at 4°C. Human CTLs were incubated with LysoTracker Deep Red (0.1 μM ; Molecular Probes by Life Technologies) for 45 min at 37°C to label cytotoxic granules. Cells were washed and resuspended in human CD8 medium. 10^5 cells were dropped in a microwell, and once cells were seeded, images were acquired in TIRF plan every 150 ms for 4 min. TIRF images were acquired with an LSM 780-Elyra PS.1 confocal microscope (Zeiss) using an alpha Plan Apo 100x/1.46 N.A. oil immersion objective. Images were analyzed using the TrackMate plugin for ImageJ software (52). A fluorescence threshold above 2x the mean granule fluorescence was set, in order to select strong fluorescence events more likely closer to the plasma membrane.

Cytotoxicity Assay

At day 6-8 after initial stimulation, human or mouse CTLs were resuspended in RPMI 1640 + GlutaMAX-I (Gibco) 3% FCS, 10 mM HEPES, and mixed at the indicated ratios with P815 target cells previously coated with anti-human-CD3 (6 $\mu\text{g}/\text{mL}$; UCHT1, Biolegend) or anti-mouse-CD3 (6 $\mu\text{g}/\text{mL}$; 145-2C11, eBiosciences) for 45 min at 4°C. Mixed CTLs and target cells were incubated 4 h at 37°C. The percentage of target cell lysis was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following manufacturer's

instructions. Absorbance at 490 nm was measured using a PR2100 Microplate reader (Bio-Rad).

Degranulation assay

At day 6-8 after initial stimulation, human or mouse CTLs were mixed at a 1:1 ratio with P815 target cells previously coated with the indicated anti-CD3 concentration (OKT3 produced by A. Alcover for human; 145-2C11, eBiosciences for mice) in presence of anti-CD107a-PE (1/45, clone H4A3, Biolegend for human; 2.5 µg/mL, clone 1D4B, BD Biosciences for mice), and incubated for 3 h at 37°C. Cells were stained with either anti-human CD8a-APC-Cy7 (1/25; Biolegend) or anti-mouse CD8a-PerCP-Cy5.5 (1 µg/mL; BD Biosciences). Events were acquired on a MACSQuant Analyzer (Miltenyi Biotech), analysis was performed using FlowJo 10 software (FlowJo, LLC). All samples were gated on forward and side scatter and for singlets.

Analysis of protein phosphorylation

Mouse T cells were incubated with 20 µg/mL of biotin-conjugated anti-CD3 (145-2C11, eBioscience) and anti-CD28 (eBiosciences) antibodies for 30 min at 4°C. Cells were washed, resuspend in Opti-Mem + GlutaMAX-I (Gibco), and placed 1 min at 37°C. Streptavidin (10 µg/mL; Sigma-Aldrich) was added and cells were incubated at 37°C for the indicated times. Ice-cold PBS containing 2 mM ortho-vanadate and 0.05% sodium azide was added to stop cell stimulation. Cells were lysed in ice-cold LBM buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.25% lauryl-β-maltoside, 50 mM NaF, 10 mM Na₄P₂O₇, 1 mM EGTA, 2 mM ortho-vanadate, 1 mM MgCl₂ and protease inhibitor cocktail) for 10 min on ice. Cell lysates were cleared by spinning at 20,800 xg for 10 min at 4°C. Equal amount of protein content measured using the Bio-Rad protein assay (Bio-Rad Laboratories) was loaded in NuPAGE 4-12% Bis-Tris gels (ThermoFisher Scientific). Protein transfer was performed using the Trans-Blot Turbo system (Bio-Rad Laboratories). Membranes were saturated with blocking buffer (Rockland Immunochemicals) and incubated overnight at 4°C or 2 h at room temperature with anti-phospho-Akt (pSer473; 1/1000; Cell Signaling Technology), anti-phospho-Erk1/2 (pThr202/Tyr204; 1/1000; Cell Signaling Technology), anti-GAPDH (6.6 µg/mL; Calbiochem). Membranes were then incubated with specific secondary antibodies conjugated with Alexa-Fluor680 or DyLight800 (ThermoFisher Scientific) for 45 min at room temperature. Near-infrared fluorescence was recorded and quantified using an Odyssey Classic near-infrared scanner (LI-COR Biosciences). Band intensity was normalized to control GAPDH. For a pooled

analysis of several experiments, normalized intensities were then divided by the mean normalized intensity of the same experiment.

Nuclear NFAT detection

Nuclear NFAT was analyzed by Western blot analysis of cells fractionated for nucleus and cytoplasm separation. Mouse T cells were activated as for analysis of protein phosphorylation, for the indicated times. Cells were lysed in ice-cold low-salt lysis buffer (10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 50 mM NaF, 10 mM sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) and a protease inhibitor cocktail) for 15 min on ice. Detergent Nonidet P-40 (Sigma-Aldrich) was added (0.45% v/v final) and cells were vortexed for 10 s to break cytoplasmic membranes without altering nuclear membranes. Lysates were centrifuged at 20,800 xg for 30 min at 4°C. Supernatants, corresponding to cytosolic fractions, were recovered in ice-cold tubes. Pellets containing nuclei were washed once in PBS and resuspended in ice-cold high-salt buffer (20 mM Tris-HCl pH 8.0, 1% SDS, 2 mM EDTA and protease inhibitor cocktail), solubilized by sonication (3 x 10 s at 60% power) using a Vibracell 72434 (Bioblock Scientific), and then centrifuged at 20,800 xg for 30 min. Supernatants were recovered as nuclear fractions. Equal amount of protein content measured using the Bio-Rad protein assay (Bio-Rad Laboratories) was loaded in NuPAGE 3-8% Tris-Acetate gels (ThermoFisher Scientific). Protein transfer was performed in a Bio-Rad Mini Trans-Blot system. Membranes were saturated with blocking buffer (Rockland Immunochemicals) and incubated with anti-NFAT1 (NFATc2) (0.25 µg/mL; BD Biosciences), anti-SLP76 (1/1000; Thermo Fisher Scientific) or anti-Lamin B1 (1/2000; Abcam) overnight at 4°C. Then, they were incubated with specific secondary antibodies conjugated with Alexa-Fluor680 or DyLight800 (ThermoFisher Scientific) for 45 min. Near-infrared fluorescence was imaged and quantified using the Odyssey scanner as described above. NFAT band intensity was normalized to SLP76 cytoplasmic control or Lamin B1 nuclear control. For a pooled analysis of several experiments, normalized intensities were then divided by the mean normalized intensity of the same experiment.

Mouse T cell differentiation

Mouse cells freshly isolated from the spleen and lymph nodes were stimulated with concanavalin A (5 µg/mL; Sigma) in mouse CD8 medium with IL-2 (10 ng/mL; Miltenyi Biotec) for 2 days, and then cultured 4 additional days with IL-2. At day 0-2-4-6, cells were incubated with anti-CD16/32 (10 µg/mL; Biolegend) to block Fc receptors and then stained with Fixable Viability Stain 450 (25 ng/mL; BD Biosciences), anti-CD3-FITC (5 µg/mL; 145-2C11, BD Biosciences), anti-CD4-APC-Vio770 (1.5 µg/mL; Miltenyi Biotec), anti-CD8-PerCP-Cy5.5 (1 µg/mL; BD Biosciences), anti-CD25-PE-Cy7 (2 µg/mL; BD Biosciences), anti-granzymeB-A647 (1/50; Biolegend), anti-CD44-APC (2 µg/mL; eBiosciences) and anti-CD62L-RPE (2 µg/mL; eBiosciences). Events were acquired on a MACSQuant Analyzer (Miltenyi Biotec) and analysis was performed using FlowJo 10 software (FlowJo, LLC). All samples were gated on forward and side scatter, for singlets, and for live cells using Fixable Viability Stain 450 (250 ng/mL; BD Biosciences).

Detection of cytokine production

IL-2 was removed from CTL culture and 20 h later cytokine production was assessed by ELISA and flow cytometry, as follows:

For detection by ELISA, $3 \cdot 10^5$ human or mouse CTLs were restimulated at 37°C for 6 h with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL; Sigma-Aldrich) and calcium ionophore A23187 (500 ng/mL; Sigma-Aldrich), or 20 h with coated anti-CD3 (5 µg/mL, UCHT1, Biolegend for human; 145-2C11, 5 µg/mL, eBiosciences for mice) and soluble anti-CD28 (5 µg/mL, Beckman Coulter for human; 2 µg/mL, eBiosciences for mice). Secreted IL-2, TNFα and IFNγ levels were measured from culture supernatants with specific ELISA Duo-set kits (R&D Systems) following manufacturer's instructions.

For detection by flow cytometry, 10^5 mouse CTLs were restimulated as described above. Two hours after the beginning of restimulation, brefeldin A (10 µg/mL, Sigma-Aldrich) was added. Cells were fixed with 2% PFA for 15 min at room temperature, incubated with anti-CD16/32 (10 µg/mL; Biolegend), and subsequently stained with anti-IL-2-RPE (4 µg/mL; eBiosciences), anti-TNFα-FITC (1.2 µg/mL; Miltenyi biotech) and anti-IFNγ-PE-Cy7 (8 µg/mL; Biolegend) in presence of 0.05% saponin. Events were acquired on a MACSQuant Analyzer (Miltenyi biotech), analysis was performed using FlowJo 10 software (FlowJo, LLC). All samples were gated on forward and side scatter for singlets, and for live cells using Fixable Viability Stain 450 (250 ng/mL; BD Biosciences).

Statistics

Statistical analyses were carried out using Prism software (GraphPad). Error bars in plots represent the mean \pm SEM. The p values are represented as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS $p \geq 0.05$. The type of test used is mentioned in each figure legend.

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Disclosures

The authors declare no competing financial interests.

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Footnotes

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4 Authors contribution. M.J.: designed, performed and analyzed experiments, developed experimental models, and wrote the manuscript; C.C.: designed, performed and analyzed experiments, developed experimental models, and provided technical and lab management support; T.R.: designed, performed and analyzed experiments, and developed experimental models. M.M.: performed and analyzed some experiments, provided technical support and critical reading. P.B.: wrote software for rupture force flow experiments. V.D.B.: conceived the project, designed and performed experiments, and wrote the manuscript. A.A.: conceived the project, designed experiments, and wrote the manuscript.

5 Abbreviations used: Apc, Adenomatous polyposis coli. CV, chamber volume. INSERM, Institut National de la Santé et de la Recherche Médicale.

Figure Legends

Figure 1. Apc is expressed in CD8⁺ T cells.

(A) Expression of Apc in purified primary human CD8 and CD4 T cells. Cell lysates were analyzed by Western blot using anti-Apc and anti-PLC γ 1 antibodies. The band corresponding to Apc (~310 kDa) was quantified with respect to the PLC γ 1 band. Data are the mean \pm SEM of three donors.

(B) Expression of Apc detected by Western Blot in resting primary human CD8 T cells transfected with control or Apc siRNA. Apc band intensity was normalized with respect to ZAP70. Data are the mean \pm SEM of two experiments.

(C) Expression of Apc detected by qRT-PCR in resting primary human CD8 T cells transfected with control or Apc siRNA (top), or *ex vivo* differentiated CTLs infected with control or Apc shRNA expressing retroviruses (bottom). Data are the mean \pm SEM of three and five experiments respectively (Mann-Whitney U test).

(D, E) Human resting CD8 T cells (D), or *ex vivo* differentiated CTLs (E) were spread on anti-CD3-coated coverslips for 5 min to generate flat immunological synapses. Cells were then fixed and stained with anti- β -tubulin antibody to reveal microtubules and with anti-Apc antibody. Confocal optical sections were acquired at 0.2 μ m intervals. Images were post-treated by deconvolution. A 0.8 μ m section at the cell-coverslip contact is shown. Right panels are zoomed images of the framed cellular areas corresponding to the periphery of the synapse (1) and the center of the cell (2). Arrowheads point to Apc puncta. Scale bar 5 μ m.

Figure 2. Apc controls microtubule and actin cytoskeleton remodeling at the immunological synapse.

(A, D) Human resting CD8 T cells were transfected with control or Apc siRNA.

(B, C, E) Human *ex vivo* differentiated CTLs were infected with retroviruses expressing control or Apc shRNA and GFP.

Cells were stimulated on anti-CD3-coated coverslips for 5 min to generate flat immunological synapses. Cells were then fixed and stained with anti- β -tubulin antibody to label microtubules (A, B), or with phalloidin to label filamentous actin (F-actin) (C-E).

(A, B) Confocal optical sections were acquired at 0.2 μ m intervals. Images were post-treated by deconvolution. A 0.8 μ m section at the cell-coverslip contact is shown. Microtubule network organization patterns at the immunological synapse were ranked by visual observation of unlabeled images by three independent investigators in two categories: pattern 1 (P1), presence

of a radial plane of microtubules, or pattern 2 (P2), non-radial organization. Data are the mean \pm SEM of three siRNA experiments and two shRNA experiments in duplicates (Two-way ANOVA). Scale bar 5 μ m.

(C-E) Confocal optical sections were acquired at 1 μ m intervals, a section at the cell-coverslip contact is shown. F-actin organization patterns at the immunological synapse were ranked in three categories depending on the pixel intensity plot across the synapse (lower panels): F-actin ring + clearance, centrally depleted actin and accumulation at the periphery of the synapse; intermediate, uneven depletion of F-actin at the center of the synapse; low F-actin ring or clearance, F-actin all across the synapse. Analyses were performed on a 1 μ m section at the cell-coverslip contact. Data are the mean \pm SEM of two siRNA experiments and three shRNA experiments in duplicates (Two-way ANOVA). Scale bar 5 μ m.

(D, E) Quantification of total F-actin (D) and F-actin phenotype (E) at the immunological synapse in human resting CD8 T cells transfected with siCtrl or siApc (left panels) or in human CTLs expressing shCtrl or shApc (right panels).

Figure 3. Apc controls centrosome polarization, and immunological synapse shape, symmetry and stability.

(A) Control or Apc-silenced human *ex vivo* differentiated CTLs were incubated with anti-CD3 coated P815 for 15 min. Cells were then fixed and stained with phalloidin (red) to label F-actin, and anti-pericentrin antibody to label the centrosome (white, arrows). Control and Apc shRNA infected cells are GFP⁺ (green). Confocal optical sections were acquired at 0.2 μ m intervals. Images were post-treated by deconvolution. Conjugate 3D reconstructions are shown. Arrows point to centrosomes and arrowheads to membrane extensions. Scale bar 5 μ m.

(B) Centrosome distance relative to the synapse. Data are the mean \pm SEM of two experiments in duplicates (Unpaired t test).

(C, D) Morphological analysis of the synapses. Cell symmetry (C) and the presence of protrusions (A, arrowhead, and D) were analyzed by visual observation of unlabeled images by three independent investigators. Data are the mean \pm SEM of four experiments in duplicates (Unpaired t test).

(E-G) Control or Apc-silenced human CTLs were set to interact with anti-CD3-coated P815 tumor target cells previously adhered to a fibronectin-coated laminar flow chamber for 10 min at 37 °C. Then, a laminar flow of PBS, increasing from 0 to 38.4 mL/min, was applied through the chamber for 115 s, using a computer-driven syringe pump, and synchronized with image acquisition (3 images/s).

(E) Representative images of supplemental movies 1 and 2, showing the sequential steps of CTL detachment from the target cell. CTLs are distinguished from target cells by their size, cytoplasm and light refringency. Top line, shCtrl and bottom line, shApc-treated cells. Force in nN corresponding to each image is depicted.

(F, G) Quantification of rupture forces of interactions between CTLs and anti-CD3-coated P815 cells. Force was calculated as described in Methods. (F) four healthy donors (1-4), treated with shCtrl (n= 684, 671, 1189, 1201) or with shApc (n= 576, 239, 124, 152) were analyzed. Whiskers represents minimum and maximum values, while grey and black or red boxes represent second and third quartile framing the median. Plots on the right show the combined data from the four donors (Mann Whitney U test). The number of cells loaded in the chamber and located in the field of view may vary from one experiment to another. In order to give the same weight to each of the four experiments performed on different cell numbers in the overall whisker-boxes shCtrl (n=3745) and shApc (n=1091), cells released per force interval were scaled to a total number of 250 cells per experiment, 1000 cells after adding the four sets. (G) Cumulative plots of cell-cell rupture events *versus* dragging force combining data from the four donors. For each point, mean \pm SEM are shown. Graphs show linear distributions for control cells and hyperbolic distributions for Apc-silenced cells. See also supplemental movies 1 and 2.

Figure 4. Apc controls lytic granule dynamics, targeting and fusion at the immunological synapse

(A-E) Control or Apc-silenced human *ex vivo* differentiated CTLs were incubated with Lyso-Tracker to label lytic granules as part of the late endosomal-lysosomal compartment. Cells were then set on anti-CD3-coated coverslips to generate flat immunological synapses. Lyso-Tracker⁺ granules were tracked using live cell TIRF microscopy, which allows to follow vesicles within a 200 nm distance from the immunological synapse plasma membrane and therefore detects lytic granule targeting and fusion events (fluorescence burst, arrowhead), as well as granule dynamics at immunological synapse. TIRF images were acquired every 150 ms during 4 min. Data are the mean \pm SEM of three experiments.

(A) The number of lytic granules detected per synapse was used as a readout of granule targeting events. See also Supplemental Movies 3, 4.

(B, C) Granule dynamics was estimated by tracking individual granules (C). Tracking duration, average speed and displacement length were assessed (B).

(D, E) Among the detected lytic granules, some made a fluorescence burst, indicative of fusion with the plasma membrane. The number of bursts was used as a readout of granule fusion events. See also Supplemental Movies 3, 4.

(F) Control or Apc-silenced human *ex vivo* differentiated CTLs were incubated with anti-CD3 coated P815 for 15 min. Cells were then fixed and stained with phalloidin (red) and anti-perforin (white) to label cytotoxic granules. Control and Apc shRNA infected cells are GFP⁺. Confocal optical sections were acquired at 0.2 μ m intervals. Images were post-treated by deconvolution. Conjugate 3D reconstructions are shown. Scale bar 5 μ m.

(G) Lytic granule localization and quantification. Cells were ranked in three categories depending on granule patterns by blinded image analyses by three independent investigators. Polarized: granules are grouped and localized close to the immunological synapse; grouped: granules are grouped but far from the synapse; random: granules are randomly distributed throughout the cell. The total number of lytic granules was quantified using the TrackMate plugin on ImageJ. Data are mean \pm SEM of three experiments in duplicates (Unpaired t test).

Figure 5. Apc controls CTL killing of tumor target cells

(A, B) Control or Apc-silenced human *ex vivo* differentiated CTLs (A), or *ex vivo* differentiated CTLs from wild type or *Apc*^{Min/+} mice (B) were mixed with anti-human or mouse CD3-coated P815 tumor target cells at the depicted effector CTL-target cell ratios, and incubated for 4 h. Killing was assessed by a colorimetric assay, as described in Methods. Data are the mean \pm SEM of six shRNA experiments and twelve mice in duplicates (Two-way ANOVA).

(C, D) Human (C), or mouse (D) CTLs obtained as in A and B were incubated with P815 tumor target cells coated with anti-human or anti-mouse CD3 at the depicted concentration. Anti Lamp1-PE (CD107a) was added to the cells and incubated for 3 h. Lamp1 expression was analyzed by flow cytometry. Representative FACS plots are displayed in left panels and the % of cells overexpressing Lamp1 upon target cell incubation is shown in right panels. Data are mean \pm SEM of four shRNA experiments and six mice in duplicates (Two-way ANOVA).

Figure 6. Apc modulates early T cell activation and NFAT nuclear translocation.

Ex vivo differentiated CTLs from wild type or *Apc*^{Min/+} mutant mice were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times. Cells were subsequently lysed and the depicted signaling proteins were analyzed by immunoblotting.

(A, B) Quantification of Akt and Erk1/2 phosphorylation following CTLs stimulation. pAkt and pErk1/2 band intensities were normalized with respect to GAPDH used as loading control.

Normalized intensities were then divided by the mean normalized intensity of the same experiment before pooling data from multiple experiments. Data are mean \pm SEM of seven mice (Two-way ANOVA).

(C, D) Quantification of NFAT nuclear translocation following CTLs stimulation. Cell lysates were lysed, separated into nuclear and cytoplasmic fractions and analyzed by immunoblotting. NFAT band intensity was normalized with respect to lamin B1 used as loading control, and then divided by the mean normalized intensity of the same mouse to normalize between different experiments. Data are mean \pm SEM of three mice (Two-way ANOVA).

Figure 7. Apc defects slightly affect CD8 T cell differentiation *ex vivo*

Wild type and *Apc*^{Min/+} mouse cells from spleen and lymph nodes were stimulated for two days with concanavalin A. CD8 T cell differentiation was assessed at the depicted day after stimulation by quantifying by flow cytometry the expression of the following activation-differentiation markers: CD44, CD62L, CD25 and granzyme B (GzmB).

(A, C, E, G) Flow cytometry profiles of a representative experiment showing CD44, CD62L and CD25, cell surface expression and intracellular granzyme B gating on CD3⁺ CD8⁺ CD4⁺ population.

(B, D, F, H) Quantification of the expression of CD44, CD62L, CD25 and granzyme B at the indicated days. Each dot corresponds to one mouse (mean \pm SEM; Unpaired t test).

Figure 8. Apc defects do not alter CTL cytokine production

(A, B) *Ex vivo* differentiated CTLs from wild type or *Apc*^{Min/+} mice were stimulated with anti-CD3 and anti-CD28 antibodies for 20 h, or PMA and ionomycin for 6 h. The production of TNF α , IFN γ and IL-2 was analyzed by intracellular staining and FACS (A) or ELISA (B). Percentage of cytokine-positive cells quantified by FACS. Data are mean \pm SEM of 12 mice (A). Cytokine secretion assessed by ELISA. One representative experiment with four mice is shown out of three (B).

(C) Control or *Apc*-silenced human *ex vivo* differentiated CTLs were stimulated with anti-CD3 and anti-CD28 antibodies for 20 h, or PMA and ionomycin for 6 h. Cytokine production detected by ELISA. Data are mean \pm SEM of three experiments.

Supplemental figure 1. Apc defects slightly affect CD4 T cell differentiation *ex vivo*

Wild type and *Apc*^{Min/+} mouse cells from spleen and lymph nodes were stimulated for two days with concanavalin A. CD4 T cell differentiation was assessed at the depicted day after

stimulation by quantifying by flow cytometry the expression of the following activation-differentiation markers: CD25, CD44 and CD62L.

(A, C, E) Flow cytometry profiles of a representative experiment showing CD25, CD44 and CD62L surface expression gating on CD3⁺ CD8⁺ CD4⁺ population.

(B, D, F) Quantification of the expression of CD25, CD44 and CD62L at the indicated days. Each dot corresponds to one mouse (mean \pm SEM; Unpaired t test).

Supplement Video 1, related to Figure 3 E, top panel.

Supplement Video 2, related to Figure 3 E, bottom panel

Supplement Video 3, related to Figure 4 E, top panel.

Supplement Video 4, related to Figure 4 E, bottom panel.

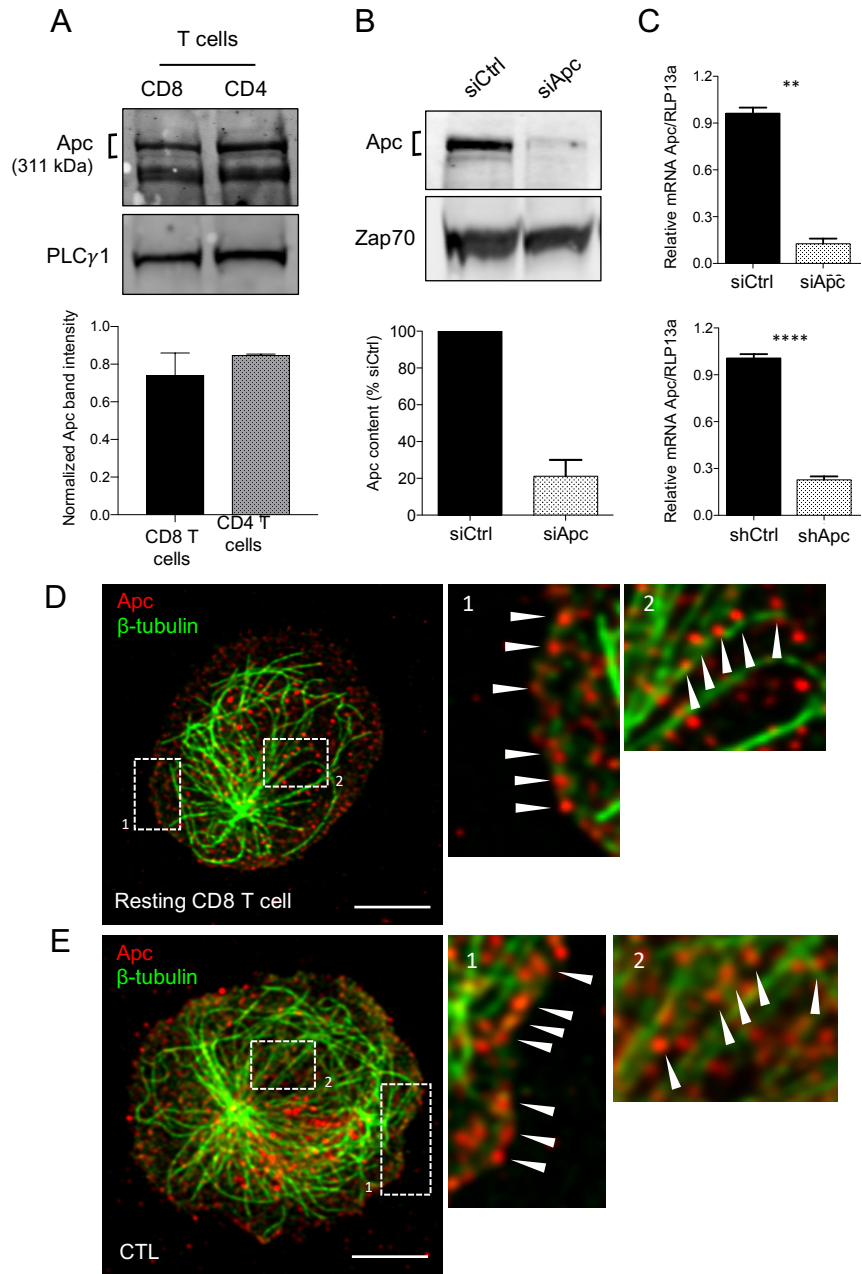


Figure 1

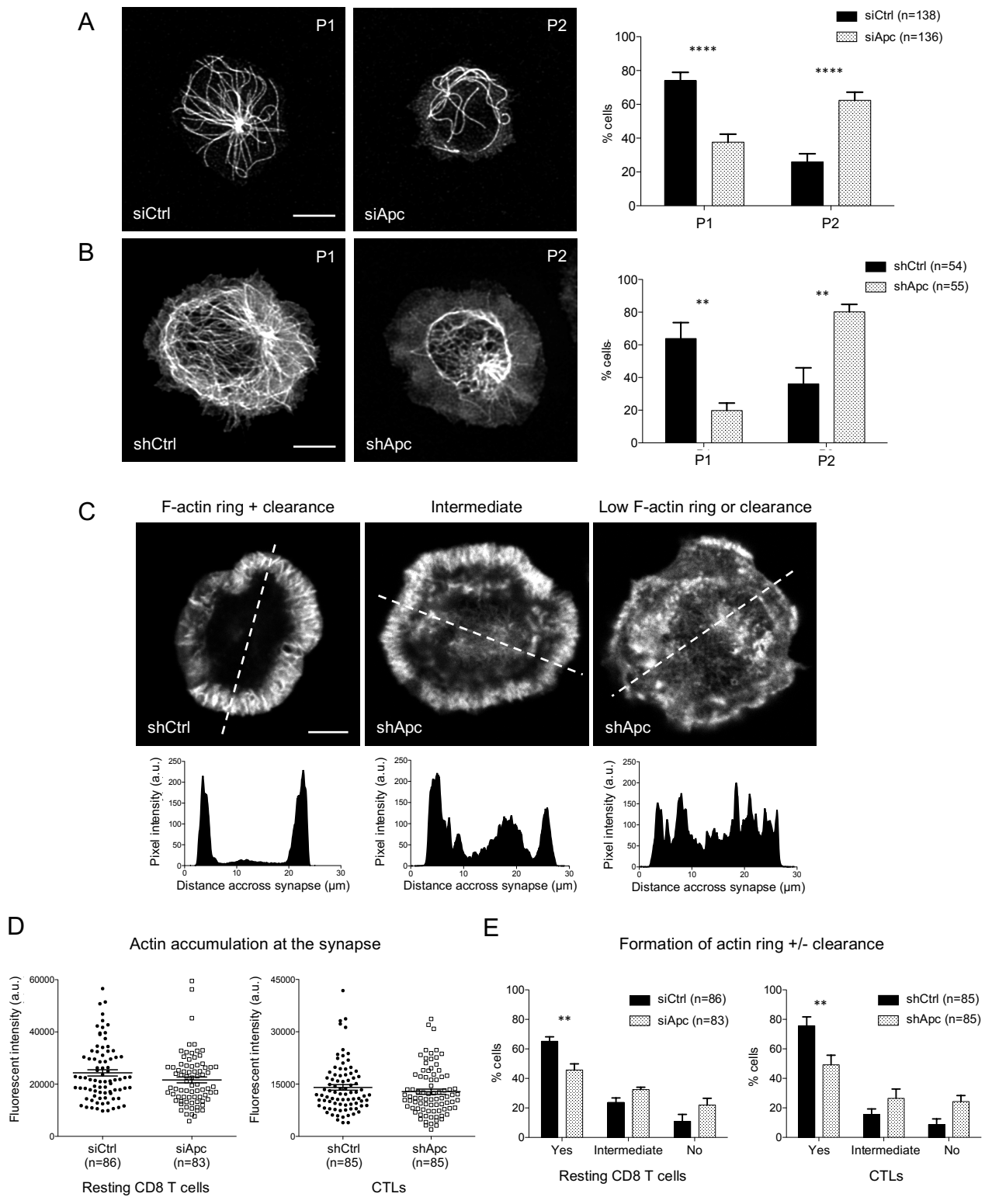


Figure 2

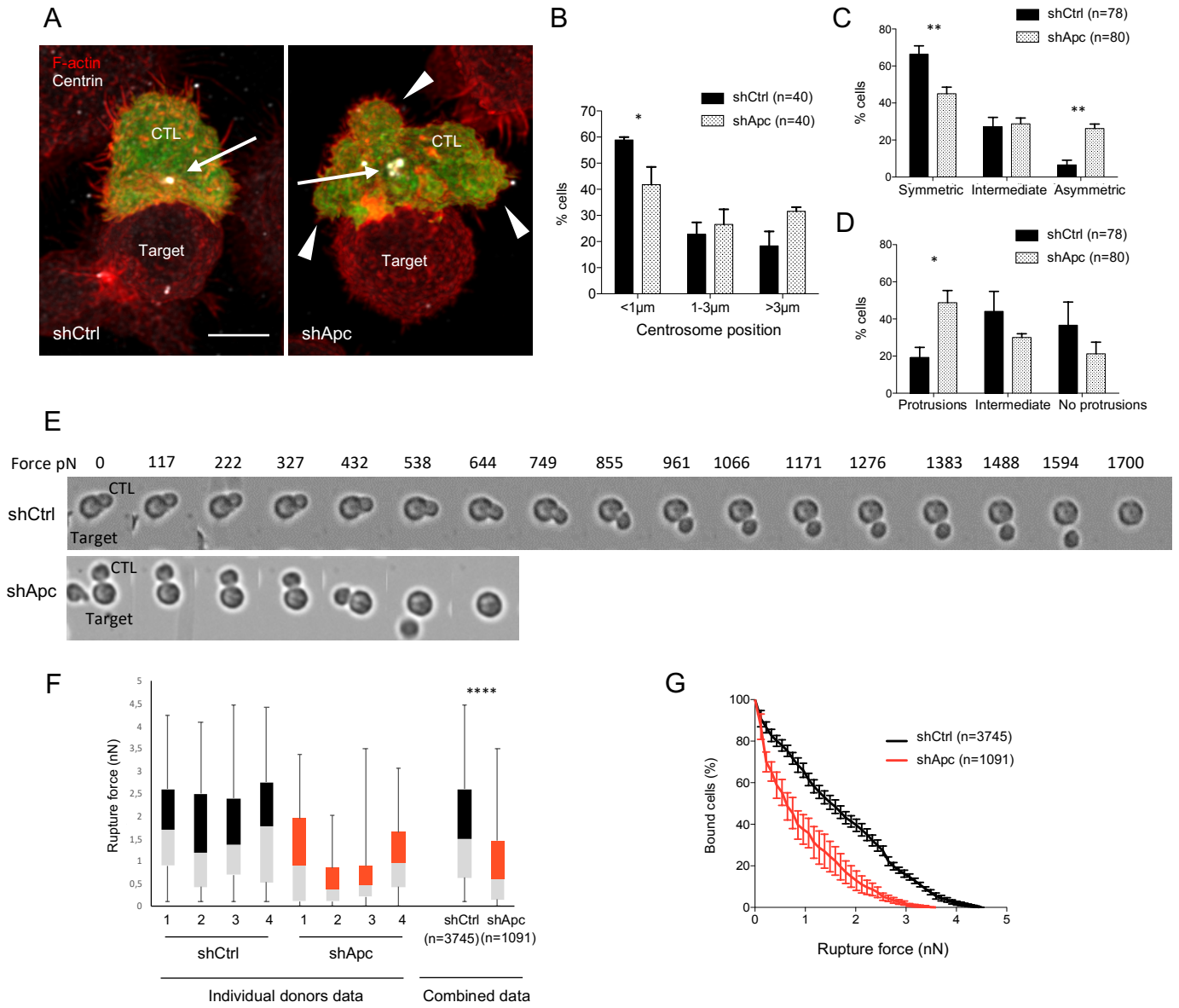


Figure 3

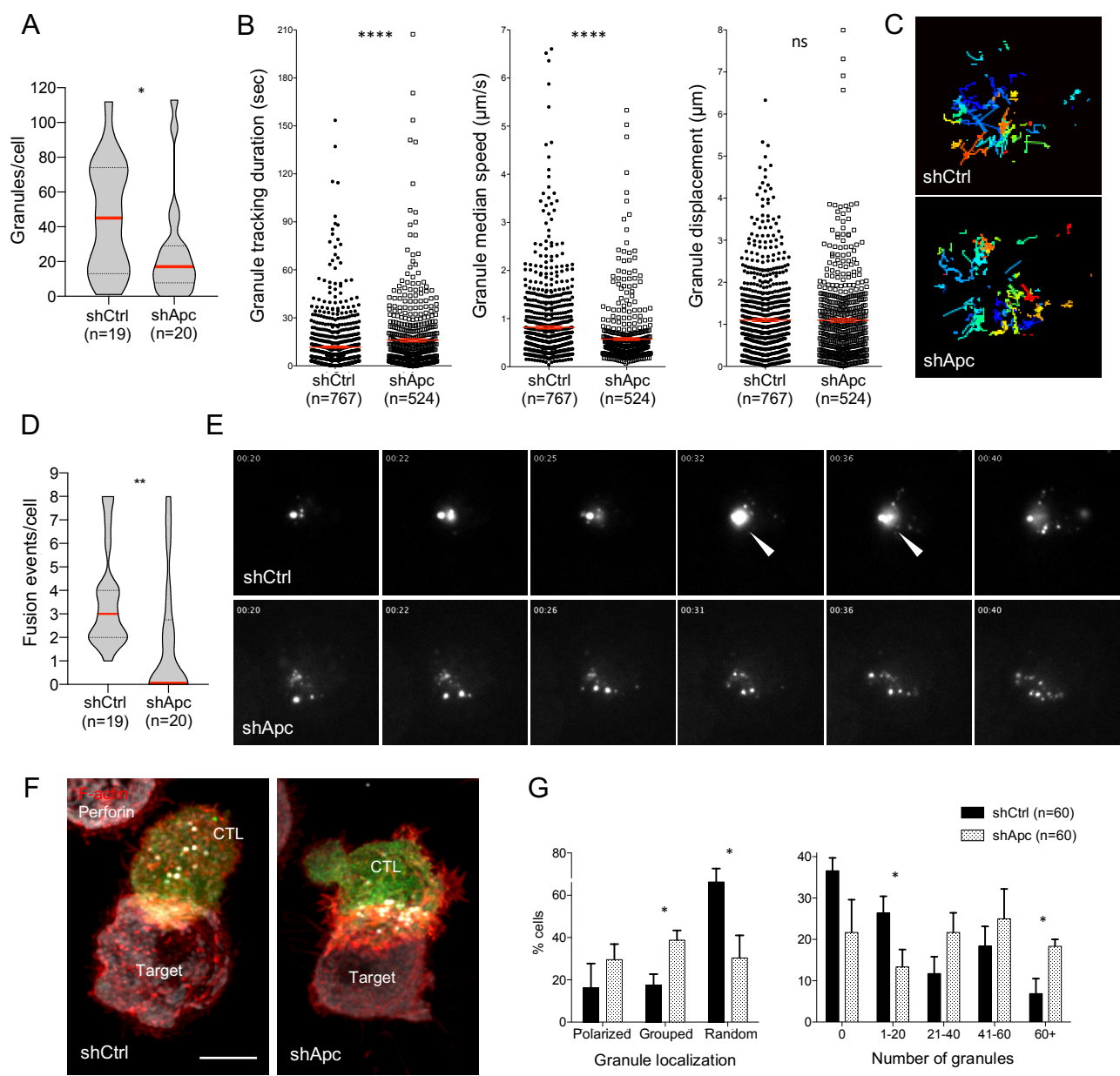


Figure 4

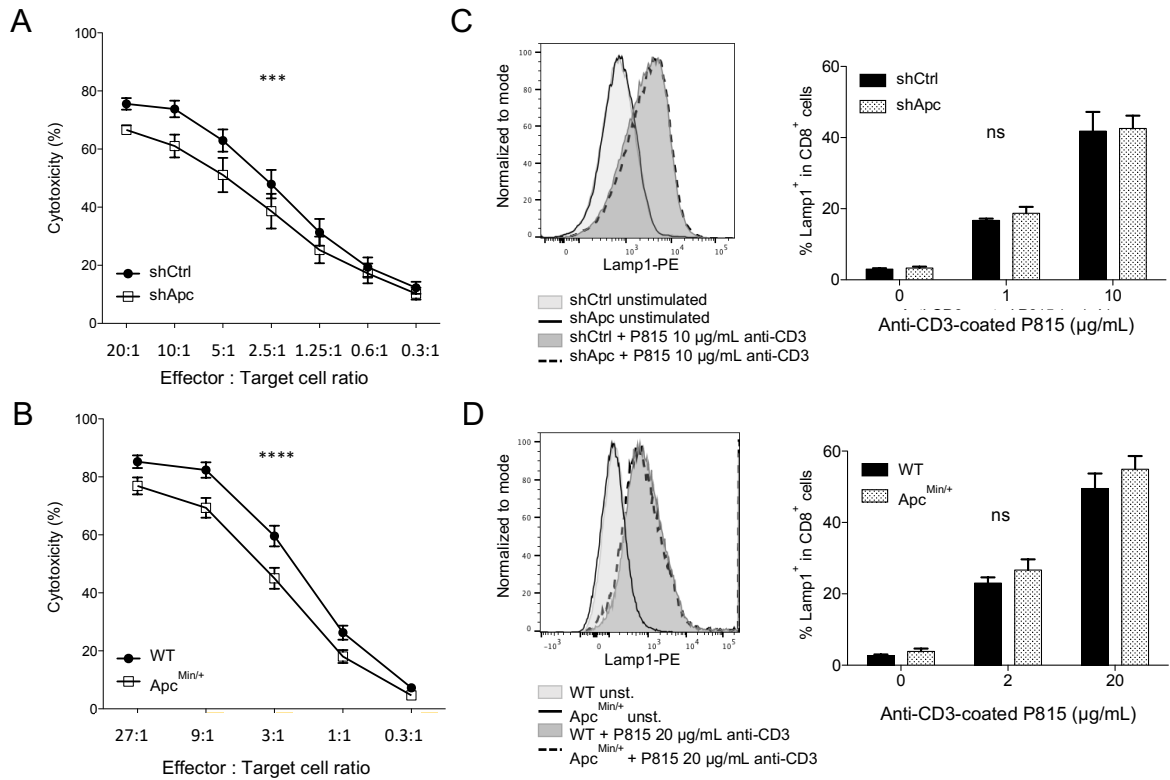


Figure 5

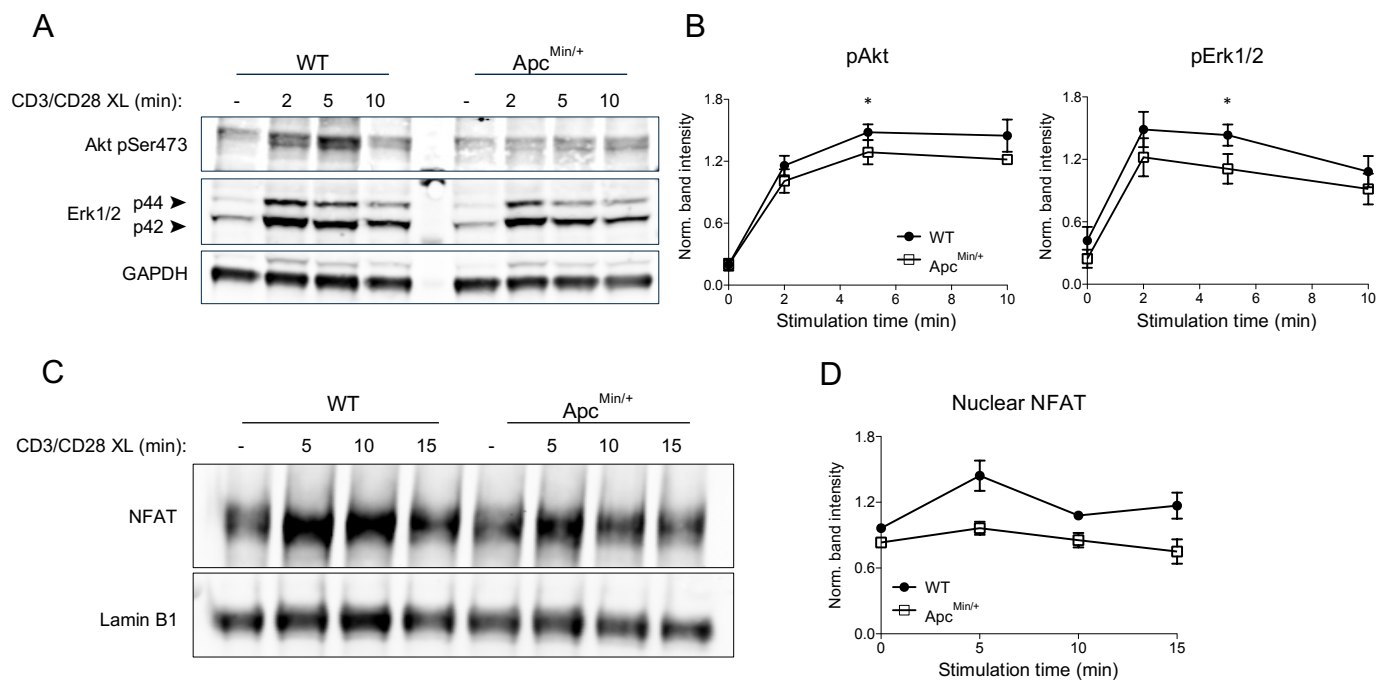


Figure 6

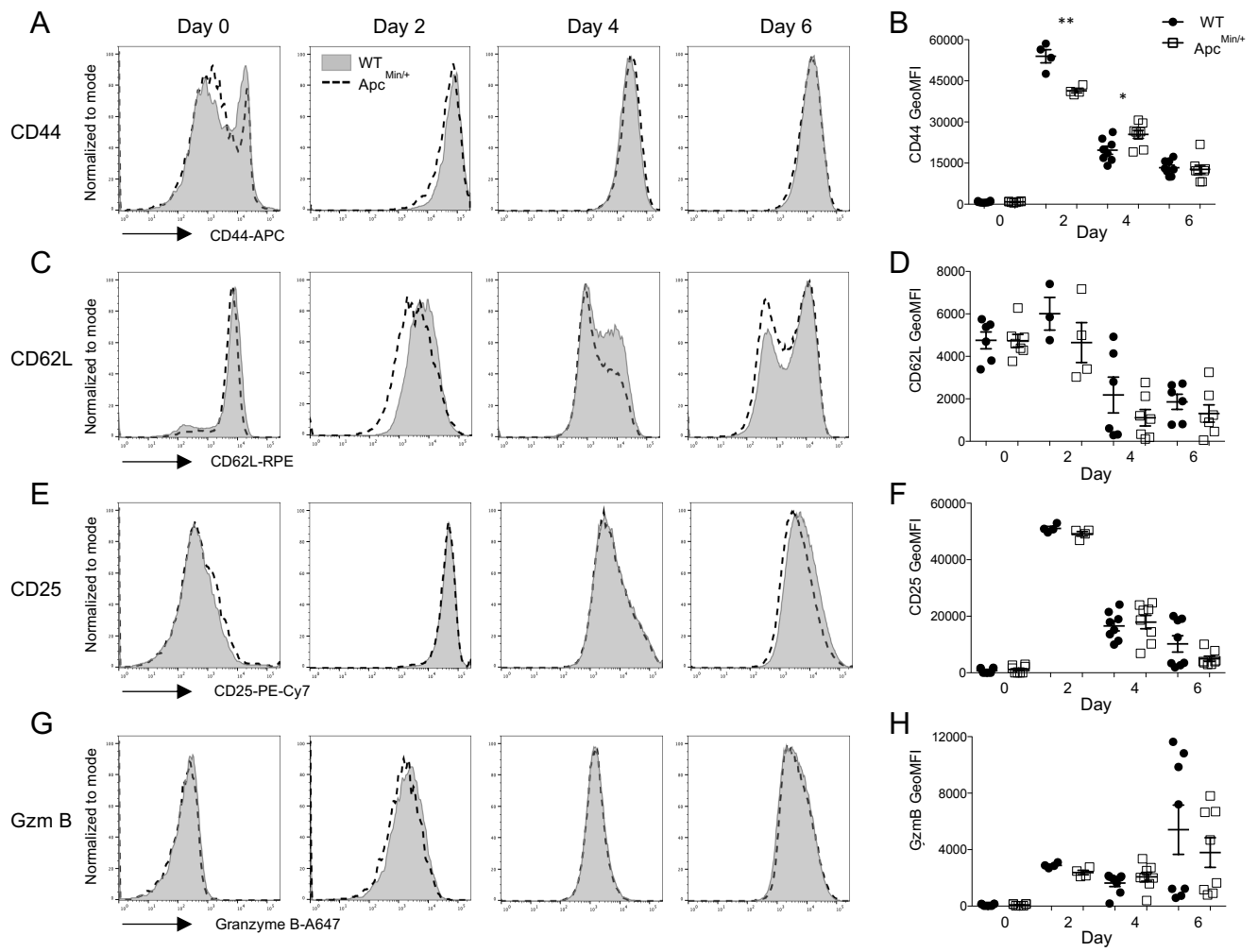


Figure 7

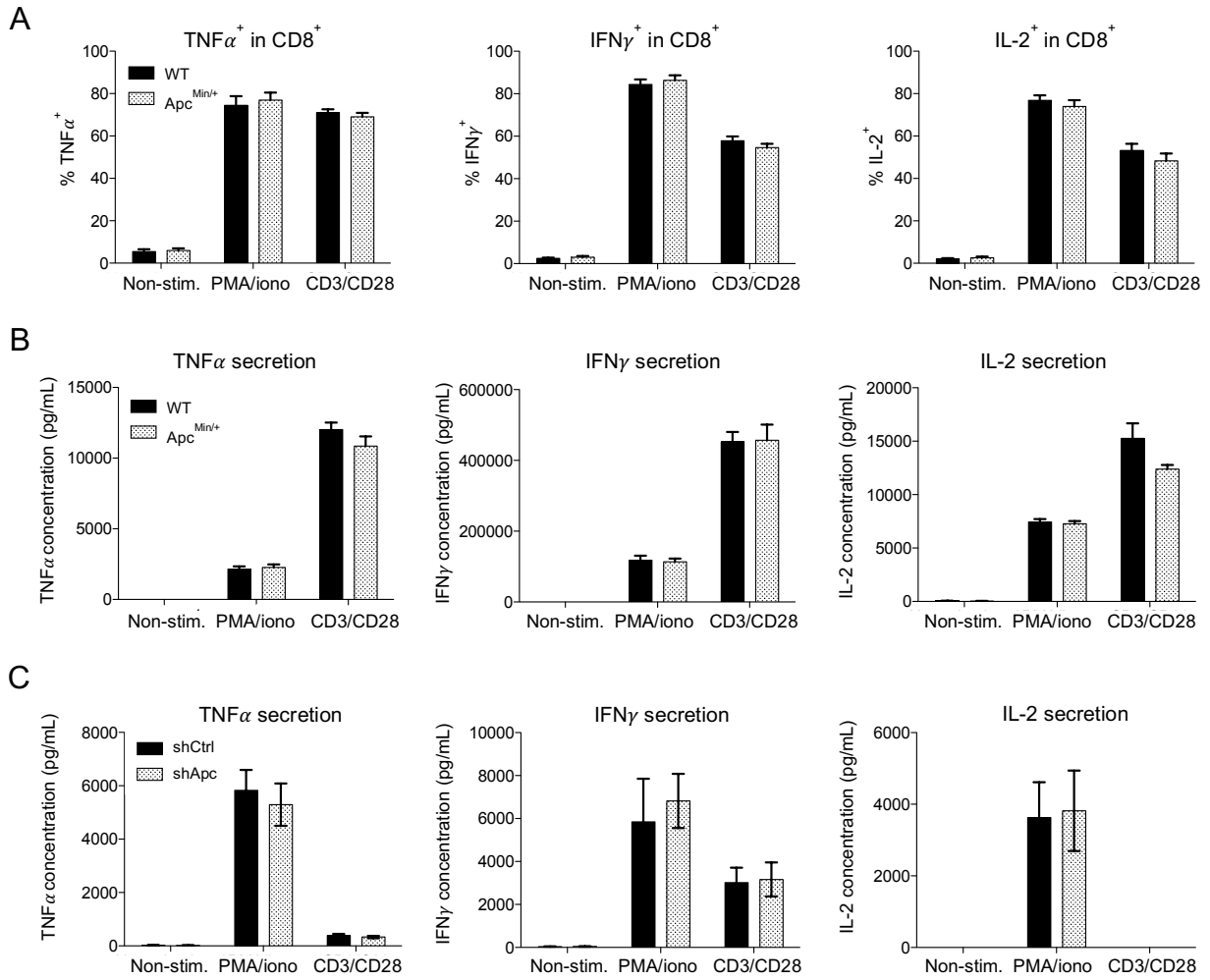
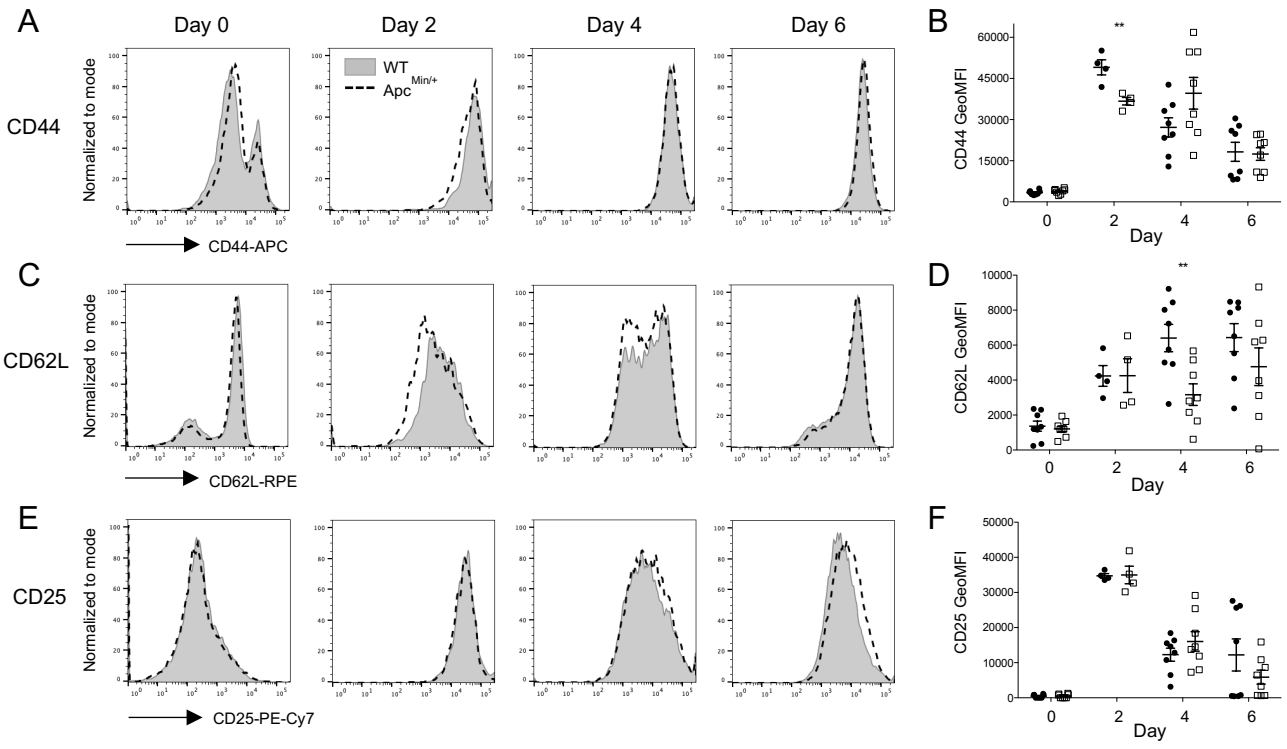


Figure 8



Supplemental figure 1

Discussion

The mechanisms regulating T cell polarization and the immunological synapse formation and functions have been intensively investigated since the characterization of its molecular composition and spatial organization 20 years ago (Grakoui et al., 1999; Monks et al., 1998). While the involvement of the actin cytoskeleton, microtubule network, and polarized vesicular traffic is well established, the interplay between them remained much less considered.

Numerous data pointed out Apc as a candidate regulator of the crosstalk between actin and microtubules. Indeed, this polarity regulator interacts with several cytoskeleton interacting-proteins, stabilizes microtubules and stimulates actin polymerization promoting polarization in migrating cell (Kroboth et al., 2007; Okada et al., 2010; Watanabe et al., 2004).

Using Apc-silenced human CD8 T cells and T cells from a mouse model of familial adenomatous polyposis (the *Apc*^{Min/+} strain), we have characterized the involvement of Apc in CD8 T cells functions and anti-tumor immunity. We have unveiled the regulatory role of Apc on CD8 T cell actin and microtubule cytoskeleton organization, and therefore synapse formation and architecture. Hence, Apc defects resulted in reduce immunological synapse stability and CTL killing activity, but interestingly only mildly impairs CD8 T cells activation and differentiation into CTLs.

1. Does Apc regulate CD8 T cell synapse formation?

1.1. Apc is expressed in CD8 T cells and tunes actin and microtubules cytoskeleton organization

To our knowledge, no studies have been conducted on the role of Apc in CD8 T cells. Thus, we first investigated Apc expression and localization in primary human CD8 T cells. Apc appears to have a similar localization in several cell types (e.g. T cells, fibroblasts, astrocytes). Apc is associated with microtubules and localized in cellular areas with highly dynamic cytoskeleton reorganization, such as the leading edge of migrating cells and the immunological synapse periphery in T cells (Etienne-Manneville et al., 2005; Näthke et al., 1996; Watanabe et al., 2004). Therefore, Apc subcellular localization in CD8 T cells is consistent with its conserved role as cytoskeleton and cell polarity regulator (Nelson and Näthke, 2013).

Actin cytoskeleton and microtubule network remodeling induce T cell polarization and immunological synapse formation, ultimately driving T cells through activation and differentiation (Soares et al., 2013b). It also drives T cells to accomplish their effector functions, like polarized secretion of cytokines and cytotoxic granules. Under normal conditions, microtubules are repositioned with their plus ends toward the synapse periphery (Bertrand et al., 2010; Lasserre et al., 2010). The centrosome is therefore translocated toward the cell contact area, and microtubules, which radiate from the centrosome, converge at the center of the immunological synapse allowing polarized transport of several molecular and vesicular components, as signaling microclusters, the Golgi apparatus, endosomal compartments and lytic granules (Geiger et al., 1982; Soares et al., 2013b; Stinchcombe et al., 2006). In line with a previous study of the Alcover lab conducted in primary CD4 T cells and in the Jurkat T cell line (Aguera-Gonzalez et al., 2017), Apc silencing in human CD8 T cells leads to a disorganized microtubule network, with microtubules less radially organized, and reaching the synapse periphery less frequently. Both, resting CD8 T cells and differentiated CTLs displayed similar patterns.

Unfortunately, we were not able to confirm this defect in Apc mutant mice due to the smaller size of the cells and spreading surface, and the high number and less structured microtubules, that did not allow us to conclude on the microtubule network phenotype.

Several others proteins including the microfilament liker ezrin and the polarity regulator Dlg1 have been shown to induce the same defects when silenced (Lasserre et al., 2010). In addition, Dlg1 interacts with both ezrin and Apc (Lasserre et al., 2010; Matsumine et al., 1996). Therefore, we can hypothesize that they act together to regulate microtubule network translocation at the synapse and promoting its stability. In addition, since ezrin is a cortical actin-associated protein, the complex ezrin-Dlg1-Apc may control the interplay between cortical actin and microtubules, which is crucial for immunological synapse architecture and function (Lasserre and Alcover, 2010).

Actin dynamics is also crucial for synapse formation and its stability. Under normal conditions, actin filaments actively polymerize in response to TCR signaling at the T cell-antigen presenting cell contact area, and then depolymerize from the center of the synapse to form a F-actin-rich peripheral ring (Ritter et al., 2015). Additionally, F-actin is continuously exerting pulling and pushing forces on the plasma membrane stabilizing the actin network and maintaining the radial symmetry of the synapse (Babich et al., 2012; Comrie and Burkhardt,

2016). *Apc* silencing in human CD8 T cells did not affect actin accumulation and polymerization at the synapse, as the total quantity of F-actin at the synapse was similar than in control cells. However, actin clearance from the center of the synapse appeared to be impaired as cells displayed less frequently an actin-rich ring at the synapse periphery.

Analyses conducted on mouse T cells revealed high variability in control and *Apc* mutant mice, showing no clear tendency concerning F-actin accumulation at the synapse. In addition, no difference was detected on actin ring formation (data not shown). This might be due to the fact that *Apc*^{Min/+} mice are heterozygous and might express higher levels of *Apc*, displaying a less clear phenotype.

Actin and microtubule cytoskeleton cooperate to build functional immunological synapses in T cells (Soares et al., 2013b). Indeed, the microtubules and centrosome translocation toward the synapse is occurring concomitantly to actin cytoskeleton remodeling. The interplay between the two types of cytoskeleton is crucial for synapse formation and T cell functions, however, the mechanisms underlying their crosstalk remain poorly understood. *Apc* interacts with both actin and microtubule regulators (Nelson and Näthke, 2013). In addition, actin cytoskeleton and microtubules share numerous others partners such as ezrin, Dlg1 or IQGAP-1, but also Cdc42 and formins that were shown to control actin remodeling and centrosome polarization at the synapse (Fukata et al., 2002; Gomez et al., 2007; Lasserre et al., 2010; Matsumine et al., 1996; Stowers et al., 1995). Interestingly, all these molecules are interacting partners of *Apc* (Nelson and Näthke, 2013). Therefore, *Apc* and those molecules could form a complex regulating actin and microtubule interplay during T cell polarization and synapse formation. This hypothesis is also consistent with a recent study unveiling the role of *Apc* in coordinating microtubules and actin polymerization at focal adhesions to control osteosarcoma cell migration (Juanes et al., 2017).

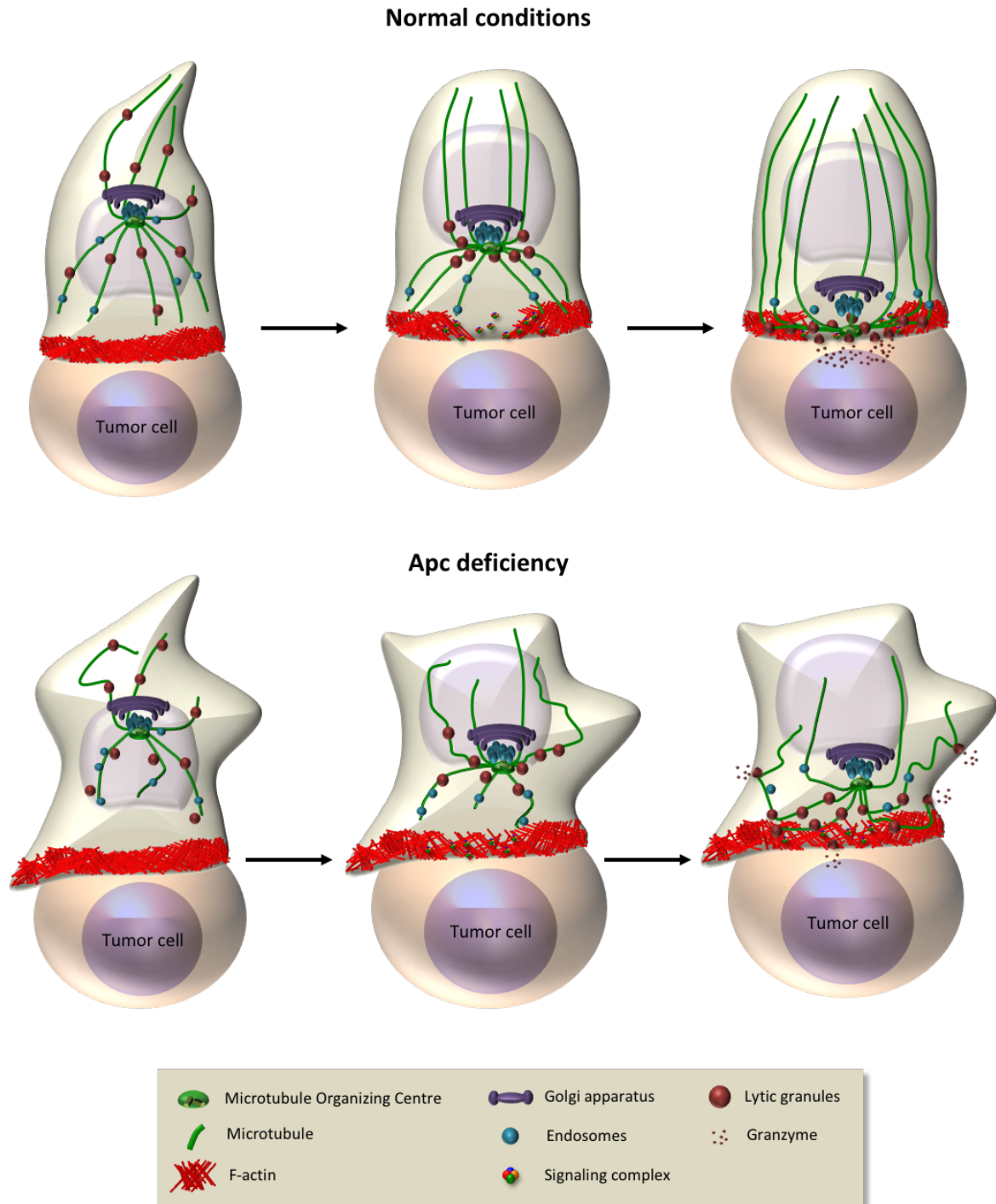


Figure 15. Apc deficiency alters CD8 T cell immunological synapse formation.

Apc tunes CD8 T cell actin cytoskeleton and microtubules during synapse formation by promoting actin dynamics, microtubule stability, and centrosome polarization. Apc would thus stabilize the synapse, allowing its maturation and efficient lytic granule release in the synaptic cleft. However, if Apc deficiency induces granule release non-specifically at the synapse, as schematized on the left bottom panel, is still unknown.

1.2. Apc regulates the immunological synapse stability and its maturation

Alteration of the cytoskeleton dynamics can result in deficient or non-mature immunological synapses, this is observed in several severe pathologies such as chronic lymphocytic leukemia and Wiskott-Aldrich syndrome (Kallikourdis et al., 2015; Ramsay et al., 2008). As actin and microtubule reorganization are altered in human Apc-silenced CD8 T cells stimulated by anti-CD3 coated surfaces mimicking antigen presenting cells, we hypothesized that conventional synapses formed with antigen presenting cell could present defects.

Considering that FAP patients and *Apc*^{Min/+} mutant mice are not immunodeficient, we did not expect to observe a total impairment of synapse formation, and indeed, quantification of the proportion of Apc-silenced CD8 T cells conjugated with antigen presenting cell revealed no differences compared to control cells (data not shown). However, conjugated T cells displayed large membrane extensions altering their radial symmetry and regularity, suggesting cellular instability and defective cortical rigidity. In addition, the centrosome was more distant from the cell contact area, hinting at an impaired CD8 T cell polarization toward the antigen presenting cell (see Figure 15). It could also be interesting to study the Golgi apparatus polarization as it is translocated at the synapse with the centrosome and is required for the proper recycling of TCR signaling molecules and delivery of lytic granules (Kupfer et al., 1985; Roig-Martinez et al., 2019).

These results therefore reflect the inability of Apc-silenced cells to stabilize their interactions with target cells and form mature immunological synapses. Thus, we assessed T cell-target cell interaction by the resistance of conjugates to resist an incremental shear laminar flow. The measurement of the force range needed to detach CTLs from target cells using flow-induced forces unveiled the lower adhesion strength of Apc-silenced CTLs compared to control cells. Indeed, the rupture force for cell-cell interactions involving Apc-silenced cells was significantly decreased.

It would be now interesting to characterized in more detail the Apc-dependent mechanisms regulating CTL interactions. Indeed, lower adhesion forces could be the result of impaired actin cytoskeleton dynamics and therefore synapse stability, suggested by the large membrane protrusions and impaired formation of the actin-rich ring. An additional explanation could be the alteration of molecules directly responsible for adhesion. Therefore, to complete this study, it would be interesting to quantify the expression of several integrins such as LFA-1 and VLA-4 at the synapse and their capacity to adhere to their ligands. Studying their localization may

also unveiled Apc involvement in synapse maturation and stability. Indeed, impairing the microtubule network organization and the actin-rich ring formation at the synapse periphery would impair the centripetal movement of membrane proteins, which results from the pushing and pulling forces generated by actin polymerization and myosin II contraction. The SMAC segregation would then not be optimal and the p-SMAC may not provide a sufficiently tight adhesion between CTLs and antigen presenting cells (Comrie et al., 2015; Yi et al., 2012). Impaired centripetal flow would furthermore inhibit integrin activation, as their activation is regulated through conformational changes induced by the actin cytoskeleton-generated forces (Comrie et al., 2015; Martín-Cófreces et al., 2018). Therefore, it appears crucial to study Apc involvement in actin-induced centripetal flow regulation and p- and c-SMAC formation.

2. Are CD8 T cell activation and differentiation modulated by Apc?

2.1. Regulation of TCR signaling pathways by Apc

Immunological synapse architecture and stability are crucial to trigger TCR signaling pathways, ultimately resulting in the activation of transcription factors initiating CD8 T cell activation and differentiation into CTLs.

Apc deficiency in CD4 T cells, which also impairs microtubule network remodeling at the immunological synapse, alters nuclear translocation of the transcription factor NFATc2, as well as its transcriptional regulation, without impairing the activation of TCR signaling molecules and other transcription factor downstream of the TCR like NFκB (Aguera-Gonzalez et al., 2017). Here, we found in *Apc^{Min/+}* CD8 T cells that some signaling molecules (e.g. Akt and Erk1/2) were subtly but significantly less activated upon TCR engagement. In addition, lower NFATc2 nuclear translocation was also observed.

Hence, Apc mutation in mouse CD8 T cells could affect different TCR signaling pathways (Erk1/2/Fos, Akt/PKCθ, IP₃/Ca⁺) that participate to the activation of several crucial transcription factors (JNK/Fos, NFκB, NFAT) (see Figure 5). However, in CD4 T cells, NFκB and Jun/Fos activation as well as calcium flux were unaffected. Thus, decreased NFAT activity was likely the consequence of its defective microtubule-dependent nuclear translocation. Indeed, NFATc2 was found forming microclusters associated with microtubules, and

microtubule depolymerization was inhibiting NFATc2 nuclear translocation and transcriptional activity (Aguera-Gonzalez et al., 2017). These data therefore suggest that NFATc2 could be similarly affected in CD8 T cells. Conversely, Apc mutation may somewhat alter early TCR signaling in CD8 T cells but not in CD4. The reason of this apparent discrepancy is unknown but it may stem in a different sensitivity of CD4 versus CD8 T cells to Apc mutation or differences in the experimental model system used (Apc-silenced human CD4 T cells versus Apc-mutant murine CD8).

Further insight into this question may be gained by studying the activation of other transcription factors in CD8 T cells, or the transcription of their target genes. In addition, it could be interesting to study microcluster formation and localization at the synapse, which are the earliest events of TCR signaling pathways, and could reflect an alteration of the centripetal flow generated by the actin cytoskeleton remodeling.

2.2. Apc mutation is not sufficient to alter CD8 T cell differentiation into CTLs

The observed TCR signaling and NFAT nuclear translocation defects did not appear to be sufficient to significantly block or modify *Apc^{Min/+}* CD8 T cell differentiation into CTLs. In contrast, previous work of the Alcover lab unveiled decreased differentiation of *Apc^{Min/+}* CD4 T cells into Tregs.

Apc mutant CD8 T cells expressed similar level of CD25 and granzyme B compared to control cells along their *ex vivo* differentiation, suggesting that signal transduction and effector protein synthesis are correctly induced in mutant cells under the experimental conditions used. Nevertheless, mild changes in the expression of other maturation markers were observed in the early phase of the differentiation process. This was the case for CD62L and CD44, which are involved in cellular adhesion and regulate T cell entry and egress in secondary lymphoid organs (Hemmerich et al., 2001; Nandi et al., 2004). These data suggest no effect of Apc loss on terminal CD8 T cell differentiation, but some difference on the kinetics of this cellular program. Experiments performed on CD8 T isolated specifically from the intestinal lamina propria did not display more significant differences (data not shown), suggesting that this phenotype is not dependent on tissue localization.

Therefore, it would be interesting to take advantage of the *Apc* mutant mouse model to perform *in vivo* experiments to study naive T cell homing into secondary lymphoid organs, and the recruitment of differentiating cells to inflammatory sites and tumors.

Interestingly under the same experimental differentiation conditions, CD4 T cells displayed different kinetics of CD62L and more pronounced differences in CD44 and CD62L expression between control and *Apc*^{Min/+} cells.

Altogether, these data suggest that altered T cell polarization and synapse formation only mildly impact CD8 T cell activation and differentiation.

As described in the introduction of this thesis, immunological synapse formation induced by TCR stimulation regulates the spatiotemporal molecular organization that tunes TCR signaling and therefore T cell activation. Actin polymerization stabilizes the synapse to maintain TCR-pMHC engagement, centrosome translocation toward the cell contact area favors the polarized transport of vesicles containing TCR and its downstream signaling proteins (Blanchard et al., 2002; Ehrlich et al., 2002; Hashimoto-Tane et al., 2011; Soares et al., 2013a), while actin-induced retrograde flow and dynein drive the centripetal movement of microclusters along microtubules and SMAC formation (Aguera-Gonzalez et al., 2017; Campi et al., 2005; Hashimoto-Tane et al., 2011; Lasserre et al., 2010; Nguyen et al., 2008). *Apc* silencing in human CD8 T cells impairs synapse stability, actin cytoskeleton dynamics, centrosome polarization, and microtubule network repositioning at the synapse. Therefore, it is surprising to detect only a slight reduction in TCR signaling, and mild changes in the expression of differentiation markers that do not affect CD8 T cell activation and effector function acquisition.

However, it is important to note that microscopy analyses and activation/differentiation experiments have not been conducted in the same cellular models. Indeed, confocal microscopy analysis of cytoskeleton reorganization and synapse formation and stability were performed on human cells silenced with siRNA or shRNA, whereas functional experiments on activation and differentiation were conducted with *Apc*^{Min/+} mouse cells. As mentioned previously, we tried to investigate cytoskeleton defects in *Apc* mutant mice but the results on microtubule organization and actin polymerization at the synapse were not conclusive. Human and mouse CD8 T cells may have some functional differences, but the most likely explanation for these discrepancies is that *Apc* silencing and *Apc* heterozygous mutation do not induce the same defects. Although *Apc* silencing does not result in a complete knockdown (about 25% of residual protein may

persist in cells), the amount of wild-type protein left in murine mutant T cells may be higher, and the expression of a truncated form of Apc could be sufficient to perform physiological functions. Unfortunately, we were unable to measure the amount of residual protein in mouse cells due to the lack of reactivity of the available anti-Apc antibodies.

3. Is Apc involved in CTL effector functions?

3.1. CTL cytokine production and secretion are not regulated by Apc

TCR signaling pathways ultimately induce the activation of transcription factors such as NFAT, which is dephosphorylated following calcium entry in the cell, leading to its translocation to the nucleus (Im and Rao, 2004). There, NFAT binds promoter or enhancer regions of DNA, driving the expression of genes involved in T cell activation, differentiation, and cytokine production. Cytokines dependent on NFAT include TNF α and IFN γ produced by CTLs during immune responses (Campbell et al., 1996; Goldfeld et al., 1994).

The Alcover lab has previously shown the importance of Apc in CD4 T cell cytokine production. Indeed, Apc-silenced human CD4 T cells displayed lower NFAT activity and IL-2 gene transcription. In addition, CD4 T cells from *Apc*^{Min/+} mice produced less IL-2, IL-4 and IFN γ and lamina propria Tregs shown impaired differentiation and production of the anti-inflammatory cytokine IL-10 (Aguera-Gonzalez et al., 2017). However, neither human Apc-silenced nor mouse Apc-mutated CTLs presented any defect in IL-2, TNF α or IFN γ cytokine production or secretion. Therefore, decreased NFAT nuclear translocation may not be sufficient to impair CTL cytokine production under the experimental conditions used.

Considering the cytoskeleton organization defects we unveiled in CTL synapses, it is intriguing that cytokine transport and exocytosis, regulated by actin and microtubule cytoskeletons, were not affected. Indeed, upon TCR stimulation, they are released at the synapse (Kupfer et al., 1991). As lytic granules, vesicles containing cytokines are transported through the Golgi apparatus, which is associated with microtubules and localized in the pericentrosomal area, therefore co-polarizing toward the immunological synapse (Huse et al., 2006; Huse et al., 2008; Kupfer et al., 1994). In addition, the Hivroz laboratory reported the importance of actin clearance from the synapse center in CD4 T cells to allow cytokine secretion. Indeed, Cdc42

silencing which inhibits actin-rich ring formation also impaired concentration of the vesicles containing cytokines and their exocytosis (Chemin et al., 2012).

However, several studies have shown a second distinct secretion pathway from the one targeted toward the synapse, that required cytoskeleton reorganization and dynamics. Indeed, cytokine secretion can also be unpolarized and multidirectional, using different trafficking and secretory processes (Huse et al., 2006; Sanderson et al., 2012). Therefore, it could be interesting to study secretion localization in the experimental model we used to understand whether Apc may affect polarized and/or non-polarized secretion pathways.

Together with the results from the *ex vivo* study of CD8 T cell activation and differentiation presented above, this thesis work suggest that Apc plays a limited minor role in CD8 T cell activation, differentiation into effector cells, and in cytokine production driven by NFAT transcriptional activity. These observations appear at odds with previous finding of the laboratory in CD4 T cells (Aguera-Gonzalez et al., 2017).

Hence, despite their common origin and the high apparent degree of similarity in some processes, CD4 and CD8 T cells are still very different and their activation, differentiation, and effector function acquisition do not undergo the same gene transcription processes (Sabins et al., 2016; Seder and Ahmed, 2003; Whitmire et al., 2000). Therefore, our results indicate that CD8 T cells are less sensitive to Apc defects than CD4 T cells, at least in their programs of activation leading to differentiation and cytokine production.

3.2. Apc regulates CTL cytotoxic activity

Cytoskeleton reorganization at the cytotoxic synapse has been extensively studied. Actin filaments actively polymerize at the cell contact area. Once the synapse is stabilized, F-actin depolymerizes from the center of the synapse to form an actin-rich peripheral ring (Ritter et al., 2013(Ritter et al., 2013). Microtubules are repositioned at the periphery of the cell contact area leading to the centrosome polarization and docking at the plasma membrane (Aguera-Gonzalez et al., 2017; Bertrand et al., 2010; Geiger et al., 1982; Hashimoto-Tane et al., 2011; Lasserre et al., 2010; Stinchcombe et al., 2006). Lytic granules move along the microtubules to cluster around the moving centrosome which defines a precise secretion site in the actin-poor region

of the synapse (Stinchcombe et al., 2001b; Stinchcombe et al., 2006). Finally, actin recovery terminates lytic granule release (Ritter et al., 2015; Ritter et al., 2017). These cellular processes are schematized on Figure 15, top panel. By confocal microscopy, we saw that human Apc-silenced CTLs display impaired actin-rich ring formation, microtubule network radial organization, and centrosome docking at the membrane. Consistent with the importance of these cytoskeleton reorganization processes for CTL cytotoxicity, human Apc-silenced and Apc mutant mouse CTLs presented decreased target cell killing activity.

Actin polymerization at the cell contact area, the formation of the actin-rich ring, and, as recently shown, the formation of small actin-rich protrusions that extend into the target cell surface apply forces at the CTL plasma membrane. These forces stabilize the synapse and generate centripetal flow for SMAC formation, but also are exerted from the CTL to the target cells (Husson et al., 2011; Tamzalit et al., 2019). In cytotoxic synapses, force exertion has been correlated in space and time with lytic granule release. Applying tension on the target cell membrane potentiates killing by enhancing the insertion of hydrophobic perforin in the target cell plasma membrane, without changing the lytic granule exocytosis (Basu et al., 2016).

Here, the reduced actin-rich ring formation at the synapse periphery and the abnormal large protrusion formation suggest impaired actin cytoskeleton dynamics in Apc-silenced CTLs. In addition, the lower adhesion strength of CTLs to target cells indicates synapse instability and reduced force engagement between the two cells.

Therefore, the decreased ability of Apc deficient CTLs to eliminate target cell could be the result of lack of actin dynamics at the immunological synapse. Hence, it appears again necessary to study Apc involvement in actin-induced centripetal flow by live high-resolution microscopy, but also in the regulation of force exertion on target cells using, for instance, micropillar deformation assay (Schoen et al., 2010).

However, although significant, the reduction of tumor cell killing in Apc-deficient CTLs was surprisingly modest considering the importance of the cytoskeleton reorganization defects and synapse instability unveiled. This could be due to the activation conditions we used *in vitro* to generate CTLs. Indeed, IL-2 regulates a plethora of processes involved in T cell function regulation, including gene transcription and cytoskeleton organization (Ross and Cantrell, 2018). Therefore, the addition of IL-2 could partially restore cytotoxic functions, as it was described in some forms of hemophagocytic lymphohistiocytosis (Bryceson et al., 2007; Marcenaro et al., 2006).

Another possibility is that our results support studies that are challenging the commonly accepted synapse model described in the introduction. Indeed, several studies have shown that the cytotoxic synapse, in opposition to the stimulatory synapse leading to T cell activation, does not need to be completely formed and stable to be efficient. To induce target cell death, CTLs do not need a strong antigen stimulus compared to the one required for stimulatory synapse (Faroudi et al., 2003). Indeed, killing may be triggered by only three TCR-pMHC interactions, whereas stimulatory synapses require at least ten interactions (Purbhoo et al., 2004). In addition, lytic granule exocytosis may occur in absence of centrosome polarization toward the targeted cell, and CTLs could kill simultaneously several targets (Bertrand et al., 2013; Depoil et al., 2005; Wiedemann et al., 2006). This effect could be particularly exacerbated in *in vitro* assays where T cells and target cells are forced to be together. Therefore, despite Apc deficiency effects on immunological synapse stability and centrosome docking at the cell contact area, it would less severely affect CTL killing activity *in vitro*.

3.3. Apc tunes late events leading to cytotoxic granules release

In order to study granule exocytosis, we have used several approaches that gave somehow conflicting results, possibly due to difference in assay sensitivity and kinetics. Indeed, long and strong stimulation as in the commonly used degranulation assay, where CD107a surface expression is used as a readout of granule release, did not allow us to observe significant differences in both human Apc-silenced and Apc mutant mouse CTLs compared to controls. In contrast, shorter stimulation and analyses by real-time TIRF microscopy at single cell level revealed significant differences in lytic granule dynamics, targeting and fusion at the synapse.

Indeed, after 15 min of cell contact, most of the CTLs have released their lytic granules under our experimental conditions, whereas Apc-silenced cells still presented a high number of cytoplasmic granules. Interestingly, those granules were grouped and polarized in a majority of the cells, while the centrosome distance to the synapse remains longer. In addition, during the first four minutes of stimulation on an activatory surface, less granules were seen reaching the plasma membrane by TIRF microscopy. The speed of these granules was reduced, and fusion events were decreased. Altogether, data obtained by fixed confocal and live TIRF microscopy therefore suggest that defect of centrosome docking at the plasma membrane does not impact

granules ability to concentrate and polarize to a certain extent at the immunological synapse, suggesting that the potential defect occurs at the last steps of granule targeting, docking and fusion.

Alternatively, our data would support studies mentioned previously according to which CTLs may also transport lytic granule and kill target cells independently from centrosome polarization (Bertrand et al., 2013; Depoil et al., 2005; Wiedemann et al., 2006). Nevertheless, polarized release may still increase the efficiency.

Impaired microtubule network organization could still reduce lytic granule transport and movement close to the plasma membrane, since microtubules in Apc-silenced cells appear to reach the plasma membrane with more difficulty. Furthermore, the remaining F-actin at the center of the synapse may reduce the plasma membrane accessibility of lytic granules, acting as a barrier that prevents efficient secretion, as previously seen by Ritter and colleagues (Ritter et al., 2017). Lytic granule fusion at the synapse is therefore less efficiently achieved.

Furthermore, we made the hypothesis that an unstable synapse and cytoskeleton defects could lead to the exocytosis of granules in a non-polarized way or in a less restrictive synaptic cleft (see Figure 15). This could be an alternative explanation to the conflicting results obtained by Lamp1 surface expression quantification and TIRF microscopy, as granules would be released but not specifically at the synapse. We may therefore observe lytic protein release in the cellular environment and bystander killing. Unfortunately, we have not been able to confirm this hypothesis yet as we have not found the optimal experimental conditions to induce specific but not non-specific killing.

Hence, these data showed that Apc deficiency alter late steps of lytic granule targeting to the synapse, consequently decreasing CTL killing efficiency.

4. Involvement for familial adenomatous polyposis and colorectal cancers

This thesis work has unveiled for the first time the role of Apc in CD8 T cell functions, and more precisely on immunological synapse formation and cytotoxic function regulation. Furthermore, these data replaced in the context of FAP development can shed light on the potential effect of Apc mutation in CTL anti-tumor immunity.

Indeed, so far little is known about Apc involvement in anti-tumor immune responses. Its role in the epithelium and adenomas formation have been extensively studied, however why these adenomas are not eliminated and can escape anti-tumor control is unclear.

4.1. Anti-tumor immune responses in *Apc^{Min/+}* mice

The Alcover lab, and others, have shown impaired *ex* and *in vivo* anti-inflammatory immune response in Apc mutant mice due to Treg function alterations. Indeed, Tregs have been shown to accumulate in the lamina propria and in adenomas at pre-cancerous stages, unbalancing the intestinal immune homeostasis. However, intestinal Tregs have impaired expression of the transcription factor FoxP3. Consequently, their differentiation and production of anti-inflammatory cytokines as IL-10 are decreased (Aguera-Gonzalez et al., 2017; Gounaris et al., 2009). According to others, their production of pro-inflammatory cytokines as IL-17 increases (Aguera-Gonzalez et al., 2017; Chae and Bothwell, 2015; Gounaris et al., 2009). Therefore, *Apc^{Min/+}* intestinal Tregs shift from a protective anti-inflammatory phenotype, required for colorectal tumor regression, to a detrimental pro-inflammatory phenotype, unbalancing intestinal immune homeostasis.

Unlike Tregs, we did not observe significant changes in *Apc^{Min/+}* CD8 T cell differentiation and cytokine production. However, *ex vivo* differentiating CD8 T cells were displaying some subtle changes in the expression of molecules involved in migration and recruitment to tumor site, as CD62L and CD44. In addition, CTLs presented reduced ability to lyse tumor target cells.

Several *in vivo* experiments thus need to be performed to better characterize the altered CD8 T cell functions due to Apc mutation in a mouse model of polyposis:

- Study CD8 T cell migration and homing, by injecting naive control or *Apc^{Min/+}* cells in control mice, and then analyzing their localization in blood or secondary lymphoid organs.

- Study CTLs and others immune cell types recruitment and tumor infiltration, by implanting solid tumor in control and *Apc^{Min/+}* mice, and then quantifying immune populations in the tumor.
- Study CD8 T cell ability to mount an anti-tumor response, by implanting solid tumor in control and *Apc^{Min/+}* mice, then injecting tumor peptide to activate *in vivo* CD8 T cells, and finally measuring tumor growth, and its infiltration, at different time points.
- Study CD8 T cells control of adenoma and tumor formation and their progression, by injecting control or *Apc^{Min/+}* cells in *Apc^{Min/+}* mice, and then measuring adenoma and tumor number and size in the intestines.

These experiments could confirm our *ex vivo* results and unveiled impaired tumor infiltration and tumor cell elimination by CD8 T cells in anti-tumor immune response due to *Apc* mutation.

Together, studies conducted both on CD4 and CD8 T cells could therefore shed light on the impaired anti-tumor immune responses occurring during adenoma and tumor formation in FAP. Indeed, increased intestinal inflammation and decreased abnormal cell elimination would together promote premalignant lesion development and then tumor escape.

4.2. Characterization of immune responses in familial adenomatous polyposis patients

The *Apc^{Min/+}* mutant mouse is a very useful model to study familial adenomatous polyposis, allowing to conduct numerous studies that would not be possible, or very difficult, with patients such as isolation of different cell types from the lamina propria or *in vivo* homing analysis. However, some differences exist between the pathological features in mice and humans, as highlighted by the different localization of polyps in *Apc^{Min/+}* mice versus patients. Hence, it may be argued that potential effects of *Apc* on immune cells may also be different between human and mice.

Therefore, in addition to using human primary *Apc*-silenced cells and *Apc*-mutant mouse cells, our laboratory recently started a study aiming to analyze the phenotype and function of immune cells purified from blood of familial adenomatous polyposis patients carrying mutations in the *Apc* gene.

This project is conducted in collaboration with the ICAReB bio-bank core facility at the Institut Pasteur, managing the collection of blood samples from patients and healthy donors. FAP patients carrying mutations in the *Apc* gene are being recruited with the help of the patients' association *Association Polyposes Familiales*, as well as physicians specialists on polyposis. In addition, we developed some of our experimental protocols with the collaboration of *Milieu Intérieur LabEx* team, who set up standardized methods to investigate a large variety of parameters of immune responses of a cohort of 1 000 healthy individuals (Hasan, 2019) (<http://www.milieuinterieur.fr/en>).

With this clinical study, we first aim to corroborate and extend the results obtained in this thesis work with *Apc*-silenced human cells and *Apc*^{Min/+} cells. Therefore, we are analyzing microtubule and actin organization in immunological synapses formed by CD8 T cells on anti-CD3 coated stimulatory surfaces. In addition, we are assessing immunological synapse formation, stability, and strength of adhesion. We are studying CD8 and CD4 T cell activation, and differentiation, following a protocol similar to the one used for mouse cells. CD4 T cells differentiation into Tregs is also assessed, in order to compare with results previously obtained by our laboratory in mouse intestinal Tregs (Aguera-Gonzalez et al., 2017). Furthermore, CTL effector functions including cytokine production, tumor cell lysis, and lytic granule release are evaluated.

Additionally, we have extended our study to NK cells. Indeed, NK cells are innate cells that, similar to CD8 T cells, are able to directly eliminate infected and tumor cells via the exocytosis of lytic granules containing perforin and granzyme B. NK cell form immunological synapses with infected or tumor cells without prior activation. These synapses do not involve the same receptors and signaling pathways than T cells, however their architecture and organization are similar, displaying polarization toward the target cell, and microtubules and centrosome repositioning toward the cell contact area (Orange, 2008). In addition, F-actin polymerizes at the synapse and is excluded from the secretion site (Brown et al., 2011; Rak et al., 2011). We therefore hypothesized that *Apc* mutation could also alter NK cell cytotoxic functions, contributing to impaired abnormal cell elimination, adenoma progression, and finally tumor escape in FAP.

The second aim of this clinical study is to assess *Apc* involvement in T cell adhesion and migration. Indeed, this study was focused on CD8 T cell functions that are regulated by immunological synapse formation. However efficient T cell immune response also requires

recirculation of naive cells between the blood and lymph and secondary lymphoid organs in search of cells presenting their cognate antigen, and recruitment of activated cells to inflammation or tumor sites. Both of these processes are based on T cell ability to migrate.

Migration is a polarized cellular process that opposes a protrusive leading edge to a retracting trailing edge. The leading edge, at the advancing front of the cell, is enriched in chemokine receptors and F-actin, providing sensitivity to guidance cues and protrusive forces for directional migration. In contrast, the uropod, at the rear of the cell, contains the centrosome and microtubules, providing contraction and retraction (Vicente-Manzanares et al., 2005).

In migrating cells, Apc is localized at microtubule plus ends and in actin-rich region, which correlates with protrusive cell area and the leading edge (Etienne-Manneville et al., 2005; Näthke et al., 1996; Watanabe et al., 2004). Apc silencing slows down fibroblast and astrocytes during *in vitro* migration by preventing the actin meshwork formation in the leading edge, destabilizing microtubules, and decreasing centrosome reorientation (Etienne-Manneville and Hall, 2003; Kroboth et al., 2007; Watanabe et al., 2004). In addition, Apc mutation abrogates *in vivo* cell migration from the crypts to the villi in the intestinal tract (Mahmoud et al., 1997; Sansom et al., 2004).

Therefore, we hypothesized that Apc mutation could also alter T cell migration. To answer this question, we are studying their migration toward chemokine gradient in transwell assays or by live microscopy. Additionally, using the rupture force assay, we are comparing adhesion strength of healthy donor and patient cells on different substrates or extracellular matrix components (e.g. fibronectin or VCAM-1).

Furthermore, we have chosen to extend our clinical study to other cell types. Indeed, our third aim is to characterize the general landscape of immune responses in FAP patients. Therefore, we are performing multiparametric whole blood immune cell phenotyping by flow cytometry, and multiplex transcriptome and secreted proteome analyses after different types of innate and adaptive stimulation.

Importantly, mutations carried by each patient will be analyzed to establish a correlation between the type of mutation, Apc expression and functional alterations, as so far, no strong correlation between genotype and phenotype has been highlighted.

This clinical study will allow us to characterize CD8 T cells, but also other immune cell type responses in FAP patients. By unveiling alterations, we could shed light on impaired immune

processes, and therefore on why premalignant lesion forming in the rectum and colon of FAP patients are not eliminated by the immune system.

Conclusion

My thesis work aimed to better understand how immunological synapse formation, CD8 T cell activation and differentiation, and CTL effector functions could be regulated by the polarity regulator Apc, with the ultimate purpose of better understanding potentially altered CD8 T cell responses in familial adenomatous polyposis patients carrying *Apc* mutations.

Using Apc-silenced human CD8 T cells and T cells from a mouse model of familial adenomatous polyposis *Apc*^{Min/+}, we have first shown the regulatory role of Apc in CD8 T cytoskeleton dynamics, and synapse symmetry and stability. Our results therefore suggest that Apc reorganizes actin cytoskeleton and microtubule network at the CD8 T cell immunological synapse, ensuring their interplay. We have also investigated Apc involvement in different steps of CD8 T cell journey through immune response, as their activation, differentiation into CTLs, and their effector functions. Interestingly, we have shown that Apc modulates CTL cytotoxic activity, by allowing efficient lytic granule exocytosis, but is mildly involved in CD8 T cell activation, differentiation and cytokine production. Unexpectedly, we have shown that Apc deficiency affects differently CD4 and CD8 T cells.

Therefore, we propose that Apc regulates CD8 T cell cytotoxic functions through its function of polarity regulator and cytoskeleton organizer.

Although our findings indicate that Apc is not absolutely required for the different processes studied, it cannot be excluded that mild impairments of several processes results in defective anti-tumor responses *in vivo*. Our results open numerous questions but confirm the relevance of studying Apc regulatory role in CD8 T cell functions to better characterize FAP. In addition, they underline the importance of characterizing Apc role in NK cell cytotoxic activity, as well as in phagocytosis, which both depend on the orchestrated action of the actin and microtubule cytoskeleton (Niedergang et al., 2016; Orange, 2008).

Therefore, the extension of this study through future *in vivo* experiments in mice, the analysis of additional processes regulating T cell responses, such as their migration, and the ongoing characterization of immune cell function in FAP patients will allow us to clarify the role of Apc in anti-tumor responses. This might ultimately inspire future immunomodulatory approaches to fight cancers and/or provide alternative treatments for patients.

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