



Endophilin A3-mediated clathrin-independent endocytosis and retrograde transport in cancer cells promote cytotoxic CD8 T cell activation

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Abstract

Endocytosis, a fundamental cellular process, facilitates the internalization of extracellular substances and membrane components, maintaining cellular homeostasis and regulating signal transduction. While conventional clathrin-mediated endocytosis (CME) is well understood, the mechanisms of clathrin-independent endocytosis (CIE) remain less explored. Among CIE pathways, the Endophilin A3 (EndoA3)-mediated endocytic route has been identified as critical for the internalization of immunoglobulin-like proteins such as ALCAM in cancer cells. These proteins are involved in immune synapse formation, a polarized structure essential for T cell activation.

This thesis first investigates whether Intercellular Adhesion Molecule 1 (ICAM1), another immunoglobulin-like protein and crucial immune synapse component, is an EndoA3-dependent cargo. Then, we explore the role of EndoA3-mediated endocytosis and retrograde transport in immune synapse formation and its impact on anti-tumor CD8 T cell activation.

Our findings reveal that ICAM1 is indeed internalized through EndoA3-mediated endocytosis and, like ALCAM, undergoes retromer-dependent retrograde transport to the *trans*-Golgi network (TGN). Disruption of EndoA3-mediated endocytosis and subsequent retrograde transport in cancer cells impairs the recruitment of ICAM1 to the immune synapse and diminishes T cell activation. These results highlight the critical role of EndoA3-mediated endocytosis and retrograde transport in maintaining the polarized distribution of immune synapse components, providing new insights into potential therapeutic strategies for enhancing cancer immunotherapy.

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Abbreviations

ADBE	activity-dependent bulk endocytosis
ADC	antibody-drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
ALCAM	activated leukocyte cell adhesion molecule
ANK	Ankyrin
ANTH	AP180 N-terminal homology
AP2	adaptor protein 2
APC	antigen-presenting cell
APP	amyloid precursor protein
ARF	ADP-ribosylation factor
ARH	autosomal recessive hypercholesterolemia
BACE	β site APP cleaving enzyme
BAR	Bin/Amphiphysin/Rvs
BG	benzylguanine
BSE	bundle signaling element
C16orf62	chromosome 16 open reading frame 62
CAH	central amphipathic helix
CALM	clathrin assembly lymphoid myeloid leukemia
CCDC	coiled-coil domain-containing
CCP	clathrin-coated pit
Cdc42	cell division control protein 42 homolog
CHC	clathrin heavy chain
CI-MPR	cation-independent mannose-6-phosphate receptor
CIE	clathrin-independent endocytosis
CLIC	clathrin-independent carrier
CME	clathrin-mediated endocytosis
CMT2B	Charcot-Marie-Tooth Type 2B
COMMD	copper metabolism MURR1 domain
CPY	carboxypeptidase Y
CRIB-PR	Cdc42/Rac interactive binding-Proline-rich
CRMP4	Collapsin Response Mediator Protein 4
Csk	C-terminal Src kinase
CTL	cytotoxic T lymphocyte

CTxB	cholera toxin subunit B
DAG	diacylglycerol
DENND10	differentially expressed in normal and neoplastic cells-containing protein 10
DSCR3	Down syndrome critical region protein 3
EAE	experimental autoimmune encephalomyelitis
EAP	endocytic accessory protein
EGFR	epidermal growth factor receptor
EGFR-NCE	EGFR non-clathrin endocytosis
ELISA	enzyme-linked immunosorbent assay
EndoA	Endophilin A
ENTH	epsin N-terminal homology
Eps15	epidermal growth factor receptor substrate 15
Eps15R	Eps15-related protein
ER	endoplasmic reticulum
F-BAR	Fes/CIP4 homology-BAR
FCHo1/2	Fer/Cip4 homology domain-only protein 1/2
FCHSD2	FCH and double Src Homology 3 (SH3) domains protein 2
FDS	friction-driven scission
FEME	Fast Endophilin-Mediated Endocytosis
FERM	protein 4.1/ezrin/radixin/moesin
FH	familial hypercholesterolemia
GalT	Galactosyltransferase
GEEC	GPI-AP-enriched endocytic compartment
GGA	Golgi-localized, γ -adaptin-ear-homology-domain-containing, ADP-ribosylation-factor-binding protein
GL-Lect	Glycolipid-Lectin
GPCR	G-protein-coupled receptor
GPI-AP	glycosylphosphatidylinositol-anchored protein
GSK3β	glycogen synthase kinase 3 β
H1I	helix 1 insert
HER2	human epidermal growth factor receptor 2
HIP1R	Huntingtin-interacting protein 1-related protein
Hrb	HIV Rev-Binding
Hsc70	heat shock cognate 71 kDa protein
Hsp70	70 kDa heat shock protein

I-BAR	Inverse-BAR
ICAM1	intercellular adhesion molecule 1
ICOS	inducible T cell costimulator
IFNγ	interferon- γ
Ig	immunoglobulin
IL-2R	interleukin-2 receptor
IP3	inositol-1,3,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
L1CAM	L1 Cell Adhesion Molecule
LAMP-1	lysosomal-associated membrane protein 1
LAT	linker for activation of T cell
LDL	low density lipoprotein
LFA-1	lymphocyte function-associated antigen 1
Lpd	Lamellipodin
MAPK	mitogen activated protein kinase
MEND	massive endocytosis
MHC	major histocompatibility complex
N-WASP	neuronal Wiskott-Aldrich syndrome protein
NECAP	adaptin-ear-binding coat-associated protein
NF-κB	nuclear factor κ -light-chain enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NK	natural killer
OPHN1	Oligophrenin-1
PDZ	post-synaptic density 95/discs large/zonula occludens
PDZbm	PDZ-binding motif
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ1	phospholipase C γ 1
PRD	proline-rich domain
PTB	phosphotyrosine-binding
PTP	protein tyrosine phosphatase
PX	Phox homology
RasGRP	Ras guanyl-nucleotide releasing protein
REM-1	Rho effector motif class 1

RhoGAP	Rho GTPase-activating protein
RhoGEF	Rho Guanine-nucleotide exchange factor
RTK	receptor-tyrosine kinase
SH3	Src homology 3
SH3GL	SH3-containing Grb2-like protein
SKAP55	Src kinase-associated phosphoprotein of 55kDa
SMAC	supramolecular activation cluster soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein
SNARE	receptor
SNX	sorting nexin
SRCR	scavenger receptor cysteine-rich
STxB	Shiga toxin subunit B
TCR	T-cell receptor
TGN	trans-Golgi network
TIRF	Total Internal Reflection Fluorescence
TNFα	tumor necrosis factor α
UFE	ultrafast endocytosis
UIM	ubiquitin-interacting motif
Vps	vacuolar protein sorting receptor
WASH	Wiskott-Aldrich syndrome and scar homologue
WH2	WASP homology 2
Zap70	ζ -chain-associated protein kinase of 70 kDa
β1AR	β 1-adrenergic receptor
μHD	μ -homology domain

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Part I

Introduction

What is endocytosis?

Endocytosis is a physiological process that happens in almost all eukaryotic cells. Originally, it mainly refers to the uptake of extracellular substances into the cytoplasm of cells (9), but now it also includes the internalization of cell membrane components, which is important for many vital physiological activities of cells.

At the beginning, endocytosis was divided into phagocytosis (“eating”), which indicates the internalization of big particulate substances, and pinocytosis (“drinking”), which refers to the uptake of all the other extracellular materials (9). This was a quite rudimentary categorization. After decades of research, biologists now classify endocytosis into two categories according to the different mechanisms that are involved — clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE).

CME

Clathrin-mediated endocytosis (CME), as the name indicates, is a well-organized endocytic process that requires the presence of clathrin to help form the endocytic vesicles. CME is organized in several well-defined stages (10). It initiates with recruitment of adaptor protein 2 (AP2) complex and several pioneer proteins to the plasma membrane, where they further recruit clathrins, adaptor proteins and other endocytic accessory proteins to start the endocytic pit formation. Meanwhile, adaptor proteins recognize and bind to specific sorting signals in the cytoplasmic tails of the transmembrane proteins, thus leading to the enrichment of specific cargoes in the endocytic pits. When an endocytic vesicle is mature, it will get detached from the plasma membrane by the action of the GTPase dynamin. This step is called vesicle scission. Upon vesicle scission, the endocytic proteins will disassemble from the vesicles and then be reused for another round of CME. The released vesicles will then fuse with early endosomes, which terminates a typical CME event (Figure 1) (10).

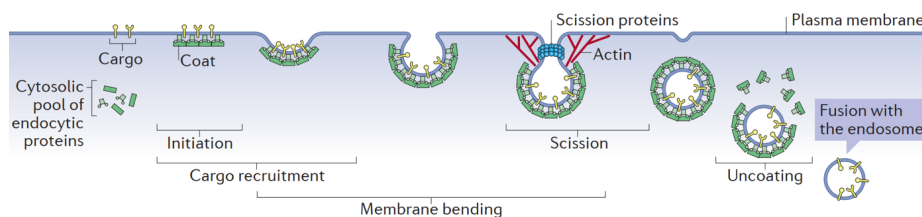


Figure 1. General illustration of CME. The complete process of CME consists of the following well-defined steps: initiation, cargo recruitment, membrane bending and pit formation, vesicle scission, and uncoating of clathrin. Image taken from (10).

A brief history of clathrin

All the stories of CME began with the discovery of clathrin, a coat protein that is commonly found on the surface of many constitutive endocytic vesicles.

In 1964, when studying the uptake of yolk protein in the oocytes of mosquitos, Thomas F. Roth and Keith R. Porter first reported the existence of coated pits and vesicles in oocytes. Under electron microscope, the coat is about 200 Å thick and seems to be made up of small bristles, so they referred to it as “bristle coat” (Figure 2a) (11). In 1969, Toku Kanaseki and Ken Kadota isolated these bristle-coated vesicles from Guinea pig brain and discovered that the coat has a basket-like structure which is composed of several regular pentagons and hexagons (Figure 2b) (12).

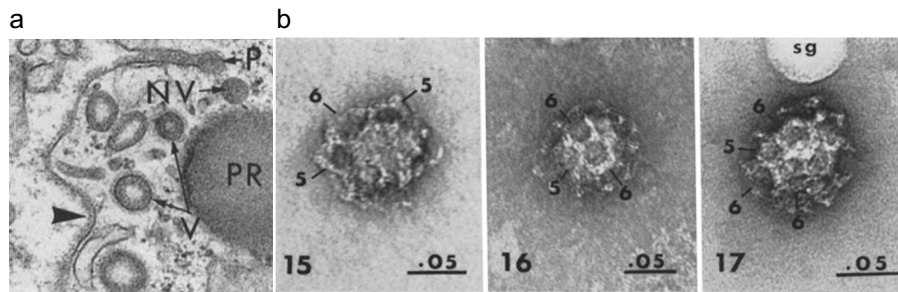


Figure 2. Coated pits and vesicles under electron microscope. **a**, coated pits (P) and vesicles (V) in an oocyte of mosquito. A naked vesicle (NV) and part of a proteid droplet (PR) are also shown. $\times 50,000$. Image taken from (11). **b**, the coat of the vesicles has a basket-like structure composed of several pentagons and hexagons. 5, regular pentagon. 6, regular hexagon. Image 15, $\times 260,000$; Image 16, $\times 220,000$; Image 17, $\times 250,000$. Images taken from (12).

In 1975, Barbara M. F. Pearse reported that the main component of the coat is a single protein with a molecular weight of ~ 180 kDa, which she termed “clathrin” (from Latin “*clatr*”, which means lattice) to indicate the lattice-like structure of the coat (13). Now, it is clear that what Pearse discovered in 1975 was the clathrin heavy chain (CHC, to be precise, CHC17), and a whole clathrin molecule consists of a heavy chain (~ 190 kDa) and a light chain (~ 25 kDa). Two isoforms of clathrin heavy chain exist, CHC17 and CHC22, which are encoded by genes in human chromosome 17 and chromosome 22, respectively (14-16). Even though sharing 85% of sequence identity, CHC17 and CHC22 have totally different biological functions (17-19). CHC17 is ubiquitously expressed in tissues and is important for receptor-mediated endocytosis, endosomal sorting, and *trans*-Golgi network (TGN) exiting of cargoes (20). By contrast, CHC22 is highly expressed in muscles, and is involved in targeting glucose transporter GLUT4 to the insulin-responsive GLUT4 storage compartment to regulate GLUT4 expression in response to glucose level in the blood (19). Hereafter in this thesis, clathrin refers to only CHC17.

The main function of clathrin is to assemble into coat structures around the endocytic pits, together referred to as clathrin-coated pits (CCPs). In non-assembled state, clathrin molecules exist in the form of trimers called triskelion with three “legs” protruding outward (Figure 3a) (21). When assembling into a coat structure, the triskelions interact with each other to form a polyhedral cage composed of several pentagonal or hexagonal faces (Figure 3b, clathrin heavy chains shown only), and they are the backbones of a coat structure (22). Clathrin coats impose their spherical shape onto the plasma membrane beneath them to help the formation of endocytic pits and vesicles.

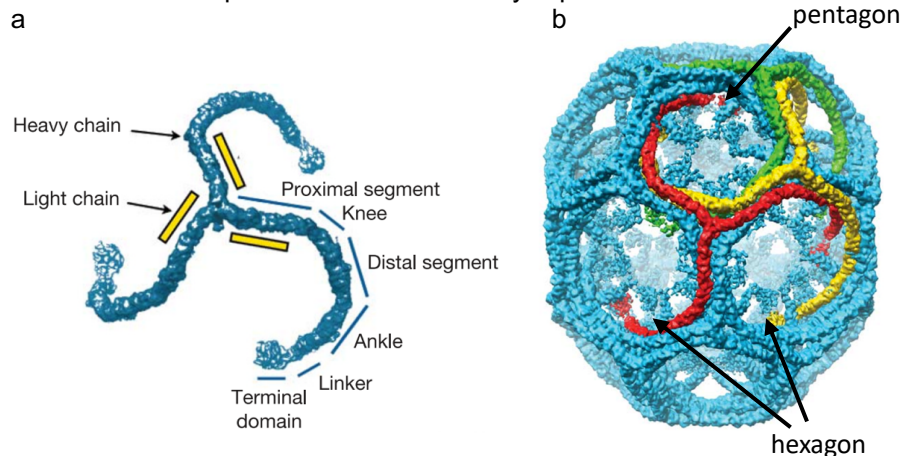


Figure 3. Structures of a clathrin triskelion and a clathrin lattice. **a**, A clathrin triskelion labelled with different segments of the heavy chain. The light chain is represented as a short yellow bar. The terminal domain is where the N terminus is, and the C terminus is at the center of the triskelion. **b**, A simple clathrin lattice image reconstructed at 7.9 Å resolution (clathrin heavy chains only). Three triskelions have been labelled out with colors. The interactions between the legs of triskelions form either a pentagonal structure or a hexagonal structure. At least 12 pentagons are needed to form a spherical structure, while the number of hexagonal structures varies. Images taken from (22).

CME initiation

Adaptor protein 2 (AP2) complex plays a central role in CME initiation. It is a heterotetramer composed of two ~100 kDa large adaptin subunits (α and β 2), one ~50 kDa medium adaptin subunit (μ 2), and one ~20 kDa small adaptin subunit (σ 2) (Figure 4). α - and β 2-adaptin each possess an appendage (ear) domain that is linked to a helical solenoid (trunk) domain by a flexible hinge domain (23, 24). The ear domains are able to bind several endocytic accessory proteins (EAPs), such as epidermal growth factor receptor substrate 15 (Eps15) (25). The hinge domain in β 2-adaptin contains a clathrin-binding site that recruits clathrin for coat formation (26). The two trunk domains, together with μ 2-adaptin and σ 2-adaptin, compose the core of AP2, which is responsible for endocytic cargo sorting by recognizing specific sorting

signals (amino acid motifs) present in the cytoplasmic tails of the proteins (23, 24).

When in cytosol, AP2 adopts a closed conformation with clathrin binding site on the hinge domain of β 2-adaptin and PI(4,5)P₂ and cargo binding sites on the μ 2-adaptin buried inside. Stochastic interactions between surface-exposed PI(4,5)P₂ binding sites on AP2 and PI(4,5)P₂ on the inner leaflet of plasma membrane trigger an allosteric conformational change of AP2 to an open conformation, with clathrin binding site as well as PI(4,5)P₂ and cargo binding sites on the μ 2-adaptin being exposed (27-29). AP2 will then recruit clathrin and EAPs to the plasma membrane to initiate CME.

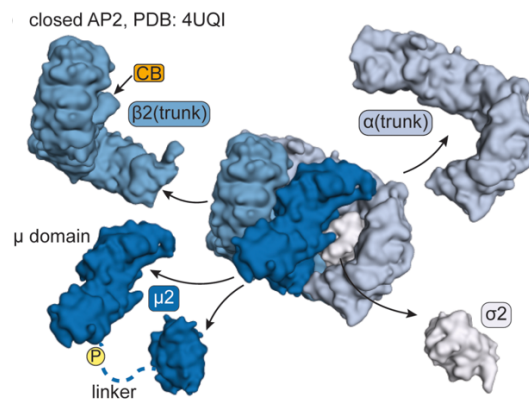


Figure 4. Structure of AP2 core split apart. The ear domains of α - and β 2-adaptins are not shown. The clathrin-binding site (CB) on the hinge domain of β 2-adaptin is depicted. The μ 2-adaptin consists of two subdomains connected by a flexible linker (dashed line), which contains the phosphorylation site (P). Image taken from (24).

Several early-recruited EAPs, including FCHo1/2 (Fer/Cip4 homology domain-only protein 1/2), Eps15/Eps15R (Eps15-related protein), intersectin, NECAP (adaptin-ear-binding coat-associated protein) and CALM (clathrin assembly lymphoid myeloid leukemia), which are collectively referred to as endocytic pioneers, can stabilize the open conformation of AP2, enhance clathrin recruitment, and promote cargo sorting and CCP formation (30-33). However, these different types of EAPs seem to be functionally redundant in CME initiation. In mammalian cells, the overexpression of FCHo1 can compensate the loss of NECAP (34), and in yeast, the deletion of several EAPs, including homologs of FCHo, Eps15, and CALM, did not stop CME even though partially impaired it (35). Consistent with this, one study from Kirchhausen's lab pointed out that AP2 and clathrin alone are sufficient to initiate CME, and FCHo1/2 are not required (36). All these observations indicate that the initiation of CME is quite flexible, which confers more robustness to this endocytic mechanism.

Cargo recruitment and membrane curvature generation

At the time of clathrin discovery, people didn't know how proteins, whether newly synthesized or endocytosed, could be accurately sorted and transported to their correct destinations within the cells. Studies on a specific case of familial hypercholesterolemia (FH) led to the discovery of sorting signals inside the proteins and eventually contributed to our understanding of cargo sorting in CME (37).

FH is a human genetic disease that is caused by defects in low density lipoprotein (LDL) receptors. People who suffer from FH have much higher concentration of cholesterol in their blood (hypercholesterolemia) and can have heart attacks at very early ages (38). In most homozygous FH patients, it is the impaired binding of LDL to the LDL receptor that caused hypercholesterolemia, because their receptors are either absent from the cell surface or defective for LDL binding (38, 39). However, in one homozygous FH patient termed J.D., LDL could bind to the LDL receptors properly. Instead, it is the impaired internalization of LDL receptor via CCPs that caused hypercholesterolemia (40). Later research revealed that there exists a "sorting signal" in the cytoplasmic tail of LDL receptor, the [FY]XNPX[YF] motif, where N is an asparagine, P is a proline, and Y is a tyrosine (41, 42). In J. D.'s LDL receptor, this [FY]XNPX[YF] motif is lost because of a point mutation at residue 807 (tyrosine to cystine), hence affecting the sorting and then endocytosis of LDL receptors via CCPs (41). However, it was still unclear which molecules in CCPs can recognize these sorting signals.

AP2 was the first confirmed adaptor protein that can recognize specific sorting signals in CME (43). On the μ 2-adaptin of AP2, there is the binding site for tyrosine motif YXX Φ , where Y is a tyrosine, X is any amino acid and Φ is a bulky, hydrophobic amino acid (43, 44). On the σ 2-adaptin, there is the binding site for dileucine motifs [DE]XXXL[LI], where D is aspartate, E is glutamate, L is leucine, and I is isoleucine (45). However, AP2 cannot recognize the [FY]XNPX[YF] motif of LDL receptor. The sorting of LDL receptor remained mysterious until the discovery of phosphotyrosine-binding (PTB) domain-containing adaptors Dab2 and ARH (46-48), which can specifically recognize [FY]XNPX[YF] motifs with their PTB domains and then sort these [FY]XNPX[YF] motif-containing cargoes into CCPs (49, 50). Many other adaptor proteins have been discovered, such as dimeric μ -homology domain (μ HD)-containing FCHo1 and Syp1p (32, 51), and monomeric PTB domain-containing adaptors, GGAs (Golgi-localized, γ -adaptin-ear-homology-domain-containing, ADP-ribosylation-factor-binding proteins), stonins, ENTH (epsin N-terminal homology) domain-containing proteins and ANTH (AP180 N-terminal homology) domain-containing proteins (49, 50, 52-58).

Table 1. CME sorting signals and adaptors (59)

Signals or domains	Adaptors	Adaptor subunits or domains
YXXØ	AP-2	µ2
[DE]XXXL[LI]	AP-2	α-σ2
Acidic clusters [YF]XNPX[YF]	AP-2? ARH; Dab2; Idol; SNX17, 27, and 31	α? PTB domain
NPF _{X(1,2)} D	Slalp	SLA1 homology domain
Ubiquitin	Eps15, Epsins 1 and 2	UIM domain
GPCR phosphorylation Synaptotagmin I C2A (C2B) domain	β-arrestins 1 and 2 Stonin 2	Amino terminus µHD domain
Mid2p cytosolic domain	Syp1p	µHD domain
Alk8 cytosolic domain	Fcho1	µHD domain
VAMP 7 longin domain	Hrb, AP-3	Carboxy-terminal unstructured domain
VAMP 2, VAMP 3, VAMP 8 SNARE motifs	CALM	ANTH domain

ARH, autosomal recessive hypercholesterolemia; PTB, phosphotyrosine-binding; UIM, ubiquitin-interacting motifs; GPCR, G-protein-coupled receptor; CALM, clathrin assembly lymphoid myeloid leukemia.

Up to date, the mechanism for cargo sorting has been well studied and reviewed (59-61). Different types of endocytic sorting signals and their corresponding adaptors have been identified (Table 1). In general, the endocytic sorting signals can be divided into three classes:

1. Linear motifs, such as YXXØ motif, [DE]XXXL[LI] motif, and [FY]XNPX[YF] motif.
2. Folded, structural motifs (or conformational determinants). For instance, clathrin adaptor Hrb (HIV Rev-Binding) sorts SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein VAMP7 into CCPs for endocytosis by recognizing the folded longin domain of VAMP7 with part of its unstructured C-terminal tail (62).
3. Ubiquitination and phosphorylation (or covalent modifications). Several adaptors can sort cargoes by recognizing specific ubiquitination sites or phosphorylation sites on the cargoes (63, 64).

With the arrival of AP2 and other EAPs to the plasma membrane to initiate

CME, cargoes with different sorting signals start to be recognized and then sorted out and packaged into CCPs by different adaptor proteins. Meanwhile, clathrin triskelions start to assemble into coat structures. Due to the cylindrical shape of most lipids, lipid bilayers are usually flat. Hence, to form a CCP, the endocytic machineries need to bend the plasma membrane by applying an external force on it. The clathrin coat is one of the structures that can apply this force.

In vitro, clathrin spontaneously forms a polyhedral clathrin lattice alone or with the presence of AP2 (65, 66). When liposomes are incubated with clathrin, clathrin-coated membrane invaginations and tubulations can be induced (67, 68). These observations indicate that clathrin can bend the plasma membrane to form endocytic pits by polymerizing into a polyhedral clathrin coat and then imposing the spherical shape of the coat structure onto the membrane.

The second structure that can bend plasma membrane during CME is actin cytoskeleton (69-72). FCH and double Src Homology 3 (SH3) domains protein 2 (FCHSD2) is a flat Bin/Amphiphysin/Rvs (BAR) domain protein that promotes actin polymerization at the base of CCPs (72). It is recruited by the scaffold protein intersectin to the endocytic pits, where it binds to the planar region of the plasma membrane surrounding the developing pit with its flat BAR domain to form an annulus. FCHSD2 then recruits N-WASP (neuronal Wiskott-Aldrich syndrome protein) to initiate actin polymerization (72). Actin polymerizes at the base of CCPs and generates a flow of actin filaments that pushes the actin network toward the tip of the endocytic pit. The actin network surrounding the CCP is coupled to the clathrin coat by epsins and proteins of Huntingtin-interacting protein 1-related protein (HIP1R; Sla2 in yeast) family (73, 74), and the pulling force generated by the movement of actin flow is transmitted to CCPs through clathrin molecules to help bend the plasma membrane. This mechanism is also involved in subsequent vesicle scission.

Vesicle scission

Once a CCP is mature, it will get detached from the plasma membrane and become a free clathrin-coated vesicle, ready to be transported to and fuse with early endosomes. The detachment process is called vesicle scission.

Vesicle scission in CME is mainly catalyzed by a 100 kDa GTPase dynamin, although other players, such as actin filaments and BAR domain proteins, may also participate. Dynamin is composed of a GTPase domain, a bundle signaling element (BSE), a PI(4,5)P₂-binding pleckstrin homology (PH) domain, and a stalk that links BSE and the PH domain (Figure 5a). From BSE to beyond the GTPase domain, there also exists an unstructured proline-rich domain (PRD) that can specifically bind SH3 domain-containing dynamin partners, such as some BAR domain proteins (10, 75, 76).

Dynamin can oligomerize into a helical collar around the neck of CCPs (Figure 5b) (77, 78). Without GTP hydrolysis, the dynamin helices have an outer

diameter of 50 nm and an inner diameter of 20 nm, which can already constrict the plasma membrane beneath them. Upon GTP hydrolysis, the dynamin helices can constrict to reach an inner diameter of only 3.7 nm (79, 80). This GTP hydrolysis-dependent super-constriction of dynamin can lead to a hemi-fission state of membrane by thermal fluctuation (81, 82), where the inner leaflet of the membrane tube beneath dynamins disappears, and then membrane fission happens.

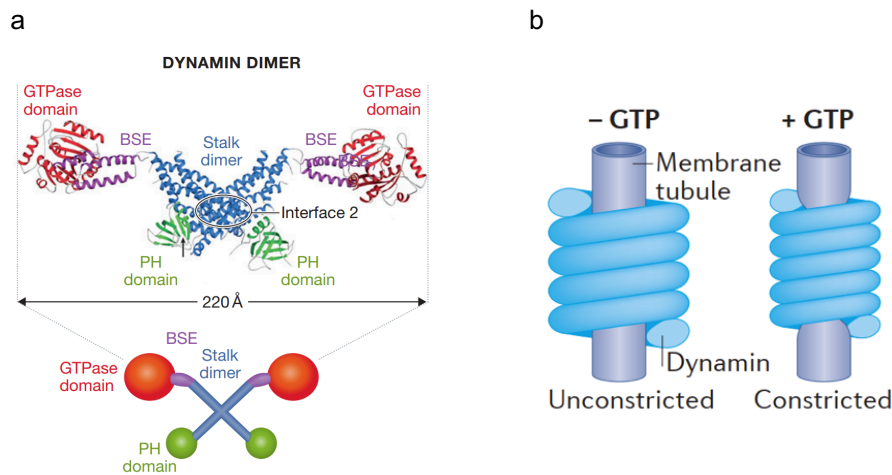


Figure 5. Dynamin structure and dynamin-mediated vesicle scission. **a**, crystal structure of the dynamin dimer, showing the interfaces required for assembly. **b**, dynamins oligomerize into helices around the neck of clathrin-coated pits. GTP hydrolysis induces the constriction of dynamins to catalyze vesicle scission. Image 5a taken from (75), image 5b taken from (10).

Other important players involved in CME vesicle scission are BAR domain proteins, which will be introduced in more details in CIE section. In general, BAR domain proteins all contain a crescent-shaped domain called BAR domain, which can sense or induce the curvature of the membrane (83, 84). Different BAR domain proteins have different curvatures in their BAR domains and are recruited to CCPs at different stages of CME. For example, shallow curved BAR domain proteins FCHo1/2 are recruited to plasma membrane at the initiation stage of CME and are proposed to induce or stabilize the shallow curvature of plasma membrane to form early CCPs (31). With CCPs maturing into the pre-scission stage, highly curved BAR domain proteins, such as amphiphysin and Endophilin A, are recruited to the neck of CCPs and may either promote or inhibit dynamin-mediated vesicle scission (76, 85), which is still under debate.

Indeed, it was originally suggested that BAR domain proteins promote vesicle scission. Several BAR domain proteins, in particular amphiphysins (86) and Endophilins A (87), contain SH3 domains that can recruit dynamins by binding to their PRDs. Since amphiphysins and Endophilins A are recruited to CCPs

at late stage, it is proposed that they are responsible for recruiting dynamins to the neck of CCPs to initiate vesicle scission. Indeed, disruption of the interactions between SH3 domains of Endophilins A and dynamins or depletion of amphiphysins and Endophilins A prevent the localization of dynamins at the neck of constricted CCPs and affect vesicle scission (88-90).

However, there is also evidence suggesting that BAR domain proteins actually inhibit vesicle scission. Co-assembly of dynamins with amphiphysins and Endophilins A on tubular lipid templates *in vitro* reduced the GTPase activity of dynamin 1 (91), which could be because these BAR domain proteins can intercalate between turns of dynamin helices around the membrane tubules (92), thus preventing the trans-dimerization of dynamin GTPase domains and subsequent membrane constriction and fission (93).

Uncoating

Once detached from the plasma membrane, the coat structure of CCPs will be quickly disassembled, and its components will be reused for other endocytic events, while the uncoated vesicles will fuse with early endosomes for intracellular trafficking. The uncoating process is mediated by a chaperon heat shock cognate 71 kDa protein (Hsc70) and a co-chaperon called auxilin (Figure 6) (94-97).

Hsc70 was discovered in 1984 when Rothman's lab identified a 70 kDa ATPase that is required for clathrin uncoating (95), and it turned out that this ATPase is a member of the Hsp70 (70 kDa heat shock protein) stress-protein family (94). Auxilin was discovered later in 1990 (96) and was shown to be required for Hsc70-mediated clathrin coat disassembly (97). Auxilin harbors a clathrin-binding domain as well as a J domain that binds Hsc70. Immediately after vesicle scission, auxilins are recruited to cavities below clathrin coat vertexes with their clathrin-binding domains, where they further recruit Hsc70:ATP with their J domains. This positions Hsc70:ATP in the close vicinity of clathrin coats. Upon ATP hydrolysis, Hsc70:ADP is released from auxilin. The substrate-binding domain of Hsc70 clamps tightly onto the clathrin heavy chain to form a stable clathrin:Hsc70:ADP complex, which locks in place a transient fluctuation. Accumulation of local fluctuations under vertexes eventually lead to the disassembly of clathrin coat. The exact details of auxilin-Hsc70-mediated clathrin coat disassembly remain elusive.

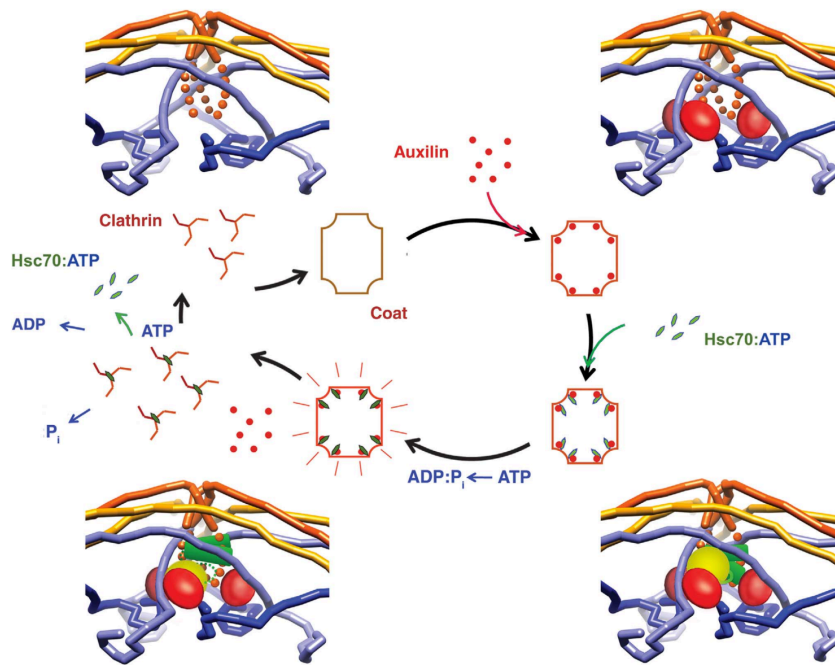


Figure 6. Model for auxilin-Hsc70-mediated clathrin uncoating. Clockwise, from upper left: clathrin coat recruits auxilin (red); auxilin recruits Hsc70:ATP (ATPase domain in yellow; substrate-binding domain in green); Hsc70 catalyzes ATP hydrolysis, and the substrate-binding domain clamps tightly onto the clathrin heavy chain; clathrin coat disassembles, releasing auxilin, clathrin:Hsc70:ADP and P_i. Image taken from (98).

CIE

Different types of CIE

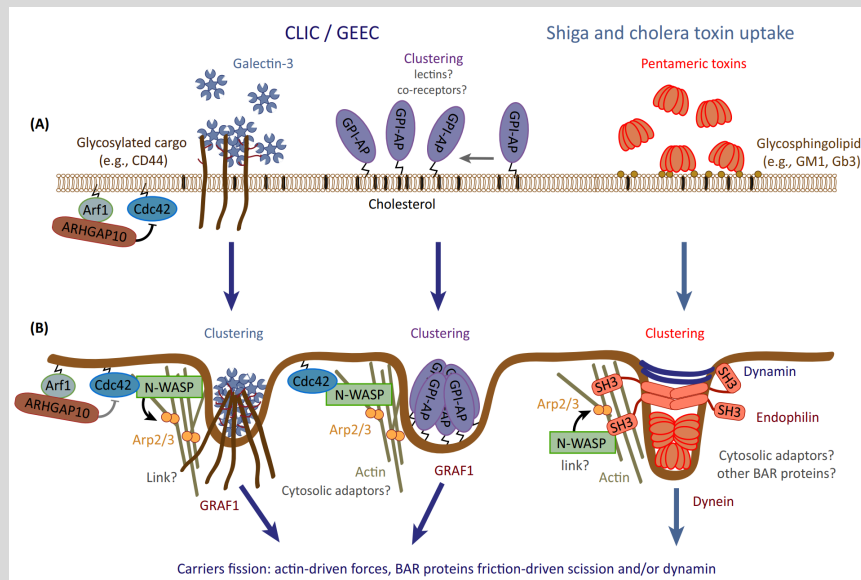
Unlike CME, which has been extensively studied and well characterized, CIE remains poorly understood. This is partly because of the diversity of mechanisms included in CIE.

There were several attempts to further classify CIE. According to the requirement of dynamin for vesicle scission, CIE can be broadly divided into dynamin-dependent and dynamin-independent CIE. Then, based on the different GTPases that are involved for endocytic regulation, CIE can also be classified as RhoA-, Cdc42- and ADP-ribosylation factor-6 (ARF6)-regulated endocytosis, to name a few (99). However, with the definition of new endocytic processes, such as Fast Endophilin-Mediated Endocytosis (FEME) (100), and the proposal of new endocytic mechanisms like the Glycolipid-Lectin (GL-Lect) hypothesis (see Box 1) (6), those classifications are not sufficient for modern research. Here, I adopt the new classification of CIE proposed by Antonio Ferreira and Emmanuel Boucrot, which is based on the different mechanisms that are used for endocytic carrier formation (8). According to this classification, CIE can be divided into three categories (Table 2) (4, 8).

The first type of CIE carriers is formed by cytosolic proteins capturing transmembrane cargoes and bending membranes, which is quite similar to the mechanism used by CME. FEME, interleukin-2 receptor (IL-2R) uptake, and ultrafast endocytosis (UFE) in neurons belong to this type. The second type of carriers is formed by membrane remodeling induced by acute signaling. When specific signals, such as Ca^{2+} influx and PI3K signaling, are received, cell membrane will form big protrusions and ruffles, and when they fold back to the cell surface, a big volume of extracellular materials as well as some membrane components will be engulfed by the cell. Macropinocytosis, activity-dependent bulk endocytosis (ADBE), massive endocytosis (MEND) and EGFR non-clathrin endocytosis (EGFR-NCE) belong to this type of CIE. The third type of carriers is formed by extracellular lipid or cargo clustering based on the GL-Lect hypothesis (6). These carriers are called **clathrin-independent carriers (CLICs)**. The recently discovered Endophilin A3-mediated ALCAM internalization (see below) also belongs to this group. ALCAM (activated leukocyte cell adhesion molecule, also known as CD166) is a glycosylated transmembrane protein and can be clustered by glycan-binding protein Galectin-8 (101). Galectin-8 will then drive the Endophilin A3-mediated endocytosis of ALCAM (101).

With further investigations, these classifications will probably be re-considered and revised, but for now, they facilitate our understanding of the complexity of CIE mechanisms. From here, I will focus on CIE mechanisms mediated by the BAR domain protein Endophilin A, *i.e.* FEME and Endophilin A3-mediated CIE.

Box 1. Glycolipid-Lectin (GL-Lect) hypothesis



Originally, it was found that bacterial Shiga and cholera toxins (1), the polyomavirus SV40 (2), and norovirus (3) can use glycosphingolipids to generate membrane curvature for cellular entry. For example, each B-subunit of Shiga toxin (STxB), a bacterial lectin, interacts with and clusters up to 15 glycosphingolipid Gb3, which can induce spontaneous membrane bending and endocytic carrier formation without the need of cytosolic clathrin machinery (1). Later, the cellular lectin Galectin-3 was reported to be able to bind to $\alpha_5\beta_1$ integrin and CD44, and cluster them by Galectin-3 oligomerization. Then, with the presence of glycosphingolipids, the oligomeric Galectin-3 drives the formation of clathrin-independent carriers (CLICs) (5). Based on these observations, *Johannes et al.* proposed the **GlycoLipid-Lectin (GL-Lect)** hypothesis (6). According to this hypothesis, the clustering of glycosphingolipids and glycosylated proteins by lectins can induce endocytic carrier formation without the need of clathrin, even though the potential involvement of clathrin is not necessarily ruled out (6). Besides, the endocytosis of glycosylphosphatidylinositol (GPI)-anchored proteins is also clathrin-independent (7). Lectins may bind to these GPI-anchored proteins and induce the formation of GPI-anchored protein-enriched endocytic compartments (GEECs) via the GL-Lect mechanism (6). After membrane invagination, other endocytic players, such as BAR domain proteins, actin, and dynamin, will be recruited to mediate the scission of CLICs and toxin containing carriers (8). Image taken from (8).

Table 2. Different mechanisms of CIE carrier formation

Cargo capture and local membrane bending by cytosolic proteins	FEME	IL-2R uptake	UFE	Acute signaling-induced membrane remodeling	ADBE	MEND	EGFR-NCE	Extracellular cargo clustering and GL-Lect hypothesis	EndoA2/STxB/CTxB	EndoA3/CD166/GaI8	CLIC/GEEC
Adaptors	Endophilin, Bin1	WAVE	?	Stimulus	Axon depolarization (high stimuli)	High Ca ²⁺ , PKC, PI3K	EGF (>2ng/mL)	Lectins	STxB, CTxB	Galectin-8	Galectin-3, SV40, CTxB
BAR domain proteins	Endophilin, Bin1, FBP17, CIP4, RICH1, SH3BP1, OPHN1	Endophilin	Endophilin	Markers	Syndapin-1	?	Rtn3	BAR domain proteins	Endophilin-A2	Endophilin-A3	GRAF1, IRSp53, PICK1
Cargoes	GPCRs (β1AR), RTKs (EGFR), PlexinA1	IL-2Rβ, IL-2Rγ, APP	SNAREs ?	Cargoes	VAMP4, other SNAREs?	Palmytoylated protein, Lipids	EGFR, CD147	Cargoes	STxB, CTxB, SNAREs (VAMP2/3/8)	CD166	GPI-AP, Glycosylated proteins
Rho GTPases	Cdc42, (Rac1)	RhoA, Rac1	?	Signaling	AKT, GSK3β, Calcineurin	Ca ²⁺ , PKC, PI3K	MAPK, AKT, Ca ²⁺	Rho GTPases	?	?	Cdc42, Arf1
Cytoskeleton & Scission factors	Actin Microtubules (FDS), Dynamin	Actin Dynamin	Actin Dynamin	Cytoskeleton & Scission factors	Actin Dynamin	no, Lipid phase separation?	Actin Dynamin	Cytoskeleton & Scission factors	Actin Microtubules (FDS), Dynamin	?	Actin

FEME, Fast Endophilin-Mediated Endocytosis; IL-2R, interleukin-2 receptor; β1AR, β1-adrenergic receptor; APP, amyloid precursor protein; GPCR, G-protein coupled receptor; EGFR, epidermal growth factor receptor; RTK, receptor-tyrosine kinase; FDS, friction-driven scission; UFE, ultrafast endocytosis; STxB, Shiga toxin subunit B; CTxB, cholera toxin subunit B; GPI-AP, glycosylphosphatidylinositol-anchored protein; CLIC/GEEC, clathrin-independent carriers/GPI-anchored protein enriched endocytic compartments; ADBE, activity-dependent bulk endocytosis; GSK3β, glycogen synthase kinase 3β; MEND, massive endocytosis; EGFR-NCE, EGFR non-clathrin endocytosis. Table adapted from (4).

BAR domain proteins

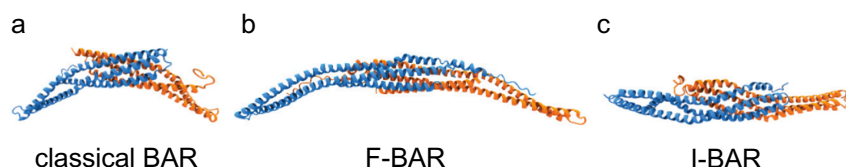


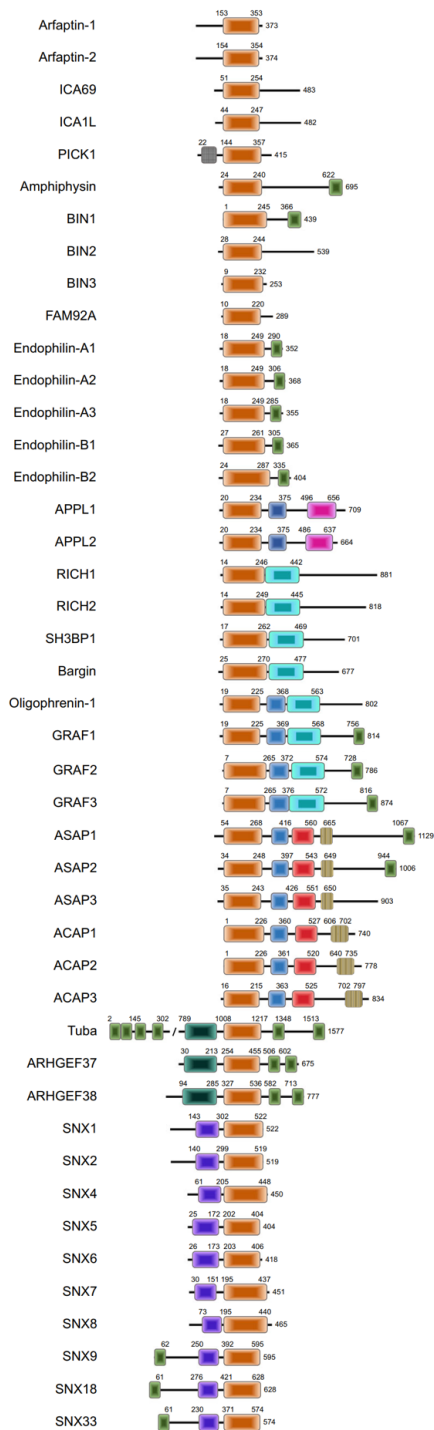
Figure 7. X-ray crystal structures of different BAR domains. The two dimerizing units are labelled with different colors. **a**, Amphiphysin 1 [PDB:1URU] of classical BAR subfamily. **b**, CIP4 [PDB:2EFK] of F-BAR subfamily. **c**, MIM [PDB: 2D1L] of I-BAR subfamily. The other domains of these BAR domain proteins are not shown. Images adapted from (102).

Endophilin A proteins are members of the BAR domain family of proteins. The BAR domain was named after the first three discovered members of this family of proteins: **B**in1, **A**mphiphysin, and **R**vs167 (**BAR**). All the BAR domain proteins contain a BAR domain that is composed of three antiparallel α -helices (H1, H2 and H3), which can dimerize with another BAR domain to form a crescent-shaped structure (103). BAR domain proteins use this crescent structure to sense or induce membrane curvature. In some BAR domain proteins, such as Endophilins A, H1 is split into H1a and H1b by an insert called helix 1 insert (H1I), which contains an amphipathic α -helix in it, the central amphipathic helix (CAH) (103). Generally, BAR domain proteins can be divided into three subfamilies according to the shapes of the BAR domains: the curved classical BAR, the more extended and less curved *Fes*/CIP4 homology-BAR (F-BAR), and inversely curved *Inverse*-BAR (I-BAR) (Figure 7a, b and c, respectively) (103). Some classical BAR domain proteins have another amphipathic α -helix (H0) to the N-terminus of their BAR domain, and they are termed N-BAR domain proteins. Apart from BAR domains, most of the BAR domain proteins contain auxiliary domains, including SH3, Phox homology (PX), pleckstrin homology (PH), Rho Guanine-nucleotide exchange factor (RhoGEF), and Rho GTPase-activating protein (RhoGAP) domains (Figure 8), which confer them abilities beyond sensing or inducing membrane curvature (103). For example, Endophilin A proteins have an SH3 domain that can recruit dynamins to promote vesicle scission (87), and BAR domain-containing sorting nexins (SNX-BARs) all have a typical PX domain (but not all the SNXs have a BAR domain) (104). Detailed information about auxiliary domains in BAR domain proteins are shown in Figure 8.

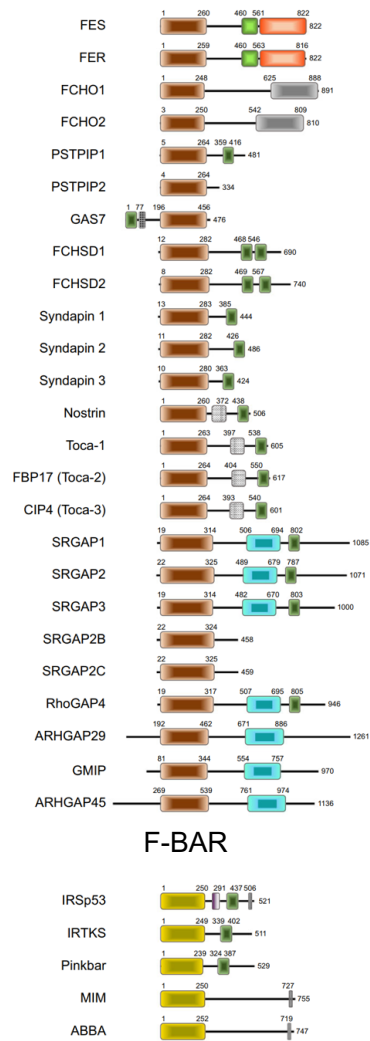
Both classical BAR and F-BAR domain proteins can induce positive membrane curvature. They bind (adhere) to the negatively charged inner leaflet of the membrane with positively charged concave face of their BAR domain, hence imposing their crescent shape onto the membrane and causing the invagination (Figure 9a) (103, 105). As F-BAR domain is less curved than classical BAR domain, the curvature radius of the membrane deformation caused by F-BAR domain proteins is generally larger than that caused by classical BAR domain proteins. I-BAR domain proteins, as indicated by their name, use the convex face of their BAR domain to bind the

membrane, thus leading to the negative curvature of the membrane, such as protrusions (103).

However, the curvature radius of a membrane deformation is very often not consistent with the one of the BAR domain that is bound to the membrane. There are two other mechanisms that are also involved in membrane deformation. One is the insertion of amphipathic α -helices (CAH or H0) into the membrane, which further pushes lipids apart, and amplifies the curvature of membrane (Figure 9b). This mechanism can be used by many N-BAR domain proteins, such as Endophilins (106). Apart from α -helices, some auxiliary domains can also participate in similar ways to curvature induction. For instance, β 1 centaurin (also known as ACAP1), which is a classical BAR domain protein (Figure 8), can wedge the amphipathic moiety of its pleckstrin homology (PH) domain into the lipid bilayer to induce membrane curvature (107). The other mechanism that is involved in membrane deformation is an entropy-driven mechanism, which indicates that crowded or clustered particles bound to the membrane will cause the deformation of the membrane to ensure the maximum distances between particles (Figure 9c) (108, 109). The final curvature of the membrane is a combined effect caused by all three mechanisms (102).

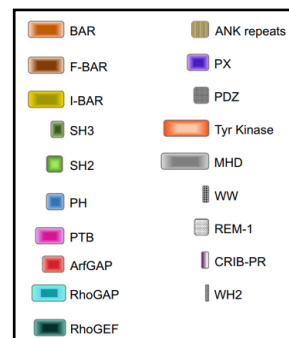


classical BAR



F-BAR

I-BAR



auxiliary domains

Figure 8. Domain organization of BAR domain proteins. SH3, Src homology 3; SH2, Src homology 2; PH, pleckstrin homology; PTB, phosphotyrosine binding; Arf, ADP-ribosylation factor; GAP, GTPase-activating protein; GEF, Guanine-nucleotide exchange factor; ANK, Ankyrin; PX, Phox homology; PDZ, PSD95, Dlg1 and Zo1; MHD, Mu homology domain; WW, rsp5 domain; REM-1, Rho effector motif class 1; CRIB-PR, Cdc42/Rac interactive binding-Proline-rich; WH2, WASP homology 2. Image adapted from (103).

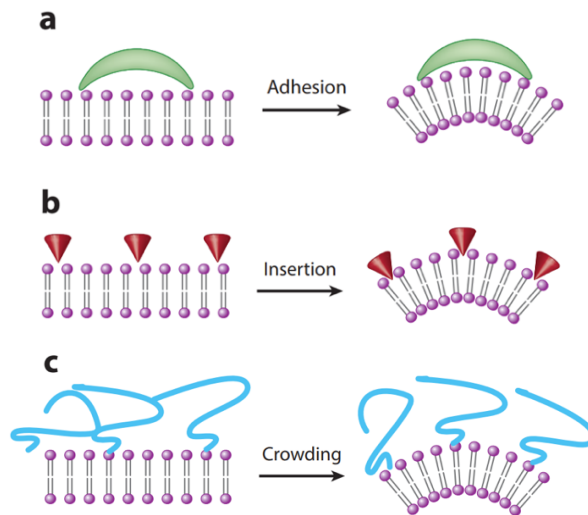


Figure 9. Mechanisms of membrane curvature induction by BAR domain proteins. **a**, BAR domain proteins impose the crescent shape of their BAR domain upon the membrane by adhesion to induce membrane curvature. **b**, BAR domain proteins insert amphipathic α -helices into the membrane to cause membrane deformation. **c**, BAR domain proteins clustered together drive the deformation of the membrane in an entropy-driven manner. Image taken from (102).

Endophilin A proteins

Endophilin A (hereafter referred to as EndoA) proteins were discovered through a screening for the identification of Src homology 3 (SH3) domain-containing proteins in an embryonic mouse cDNA expression library in 1997 (110). The three positive targets were then named SH3p4, SH3p8, and SH3p13, respectively (110). At the same time, an independent PCR-based screening of cDNA from normal human brain and brain tumor tissue also identified the same three isoforms of EndoA, which were termed SH3GL2 (SH3-containing Grb2-like protein 2), SH3GL1, and SH3GL3, respectively (111). Later, it was found that these SH3 domain-containing proteins have strong affinity for endocytic proteins, so they were renamed Endophilins (112), and now recognized as Endophilin A1 (EndoA1), A2 and A3 isoforms, respectively (113). EndoA1 is mainly expressed in brain, and EndoA3 is highly expressed in both brain and testes, while EndoA2 is widely expressed in all

tissues (111, 114). Another Endophilin subfamily, Endophilin B, was discovered in 2001, and two isoforms of this subfamily, Endophilin B1 (SH3GLB1) and Endophilin B2 (SH3GLB2), have been identified (115, 116). It seems that only EndoA subfamily is involved in endocytic processes, while Endophilin B subfamily, at least Endophilin B1, is important for apoptosis and autophagy (113, 117).

As BAR domain proteins, all Endophilins (both subfamily A and B) have an N-terminal BAR domain, a variable middle region and a C-terminal SH3 domain (Figure 10). Because of the presence of the α -helix H0 preceding the N-terminus of the BAR domain, Endophilins are classified into N-BAR domain proteins. The higher-order structure of the variable middle region is unclear, but it contains several phosphoresidues inside (Figure 10, red open or closed triangles on top), suggesting that it has a role in the post-translational regulation of Endophilin activities. The SH3 domain is well-known for its ability to bind PRDs, which is important for Endophilins to exert their functions (88-90).

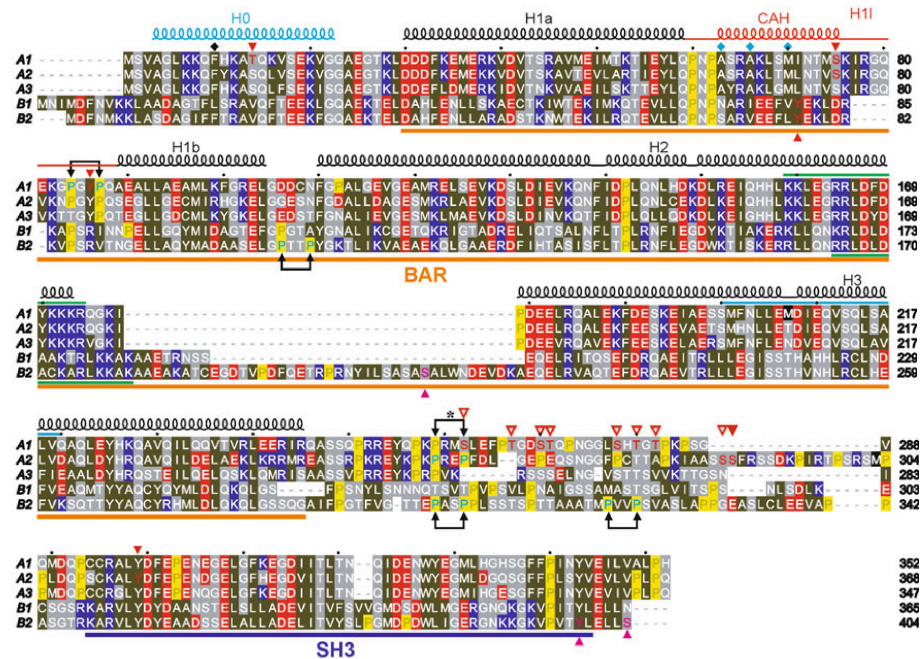


Figure 10. Alignment of human Endophilin A1 (accession EAW58661.1), A2 (EAW69229.1), A3 (EAW62422.1), B1 (EAW73181.1), and B2 (CAI12365.1). Hydrophobic (olive green), positively charged (dark blue), negatively charged (red), and proline (yellow) residues are indicated. Phosphorylation sites with high and low confidence are marked by closed and open triangles on the top, respectively. Image taken from (113).

At first, EndoAs were reported to be mainly involved in CME by recruiting dynamins and synaptojanins with their SH3 domains (88, 118), which has already been described in the previous section about CME. However, new studies pointed out that EndoAs may just play a peripheral role in CME and may actually have more important functions in CIE (90, 100, 119).

First, EndoAs can directly promote membrane fission without dynamin. In 2012, *Boucrot et al.* used a biophysical modelling approach and *in vitro* quantitative vesiculation assays to study the membrane fission ability of amphipathic helix insertions. They proved that their insertion into membranes is sufficient to drive membrane fission independently, possibly by destabilizing the membrane necks (106). However, the crescent BAR domain regions of EndoA3 can form scaffolds around tubulated membrane and stabilize it, limiting membrane fission driven by amphipathic insertions (106). In 2015, *Renard et al.* discovered that EndoA2 can form a scaffold layer surrounding endocytic tubules and limit the flow of lipids beneath the scaffold layer during the extension of endocytic tubules, thus leading to increased membrane tension and spontaneous squeezing of the tubules to facilitate vesicle scission (119). This idea was further developed by *Simunovic et al.* in 2017 into the so-called friction-driven scission (FDS) mechanism (120). In FDS mechanism, BAR domain containing proteins (here, the authors tested EndoA2) bind and stabilize the lipid tubules as scaffolds, which will not cut the tubules and mediate membrane fission in static state. Rather, these proteins limit the lipid flow beneath the scaffold layer by imposing a frictional force. However, when the tubules are elongated at a certain speed by a pulling force exerted *in vivo* by motor proteins moving along microtubules or by actin polymerization (119), the un-scaffolded part of membrane tubules will be thinner and the membrane tension will increase, while the scaffolded part will not. This will lead to, first, the formation of a pore between scaffolded and un-scaffolded part, and then subsequently, membrane fission (120). FDS does not necessitate the presence of dynamins (120), but can co-exist with dynamin- and actin-mediated membrane fission in an additive manner in CIE (119).

Besides, EndoAs are able to directly mediate CIE. In 2015, *Boucrot et al.* described an EndoA-dependent endocytosis of β 1-adrenergic receptor (β 1-AR), which is independent of neither clathrin nor AP2 (100), the markers of CME. Given that the endocytic vesicles were formed rapidly ($t_{1/2}=7s$) in this case, they termed this new endocytic route Fast Endophilin-Mediated Endocytosis (FEME) (100). FEME is a highly regulated endocytic mechanism and EndoAs have multiple roles in it (Figure 11) (119).

FEME

In resting cells, EndoAs are constantly pre-enriched into small discrete patches on the plasma membrane to ensure the prompt endocytosis of activated receptors via FEME. This step is called priming (121). GTP-loaded, membrane-bound GTPase cell division control protein 42 homolog (Cdc42) first recruits F-BAR proteins FBP17 and CIP4 to the plasma membrane.

FBP17 and CIP4 then further recruit SHIP1/2 phosphatases and Lamellipodin through their SH3 domains. SHIP1/2 can hydrolyze $PI(3,4,5)P_3$ to $PI(3,4)P_2$, which is then bound by the PH domain of Lamellipodin and can further stabilize it. Finally, Lamellipodin recruits several copies of EndoAs with its multiple PRDs to form the EndoA patches. If the receptors nearby are stimulated by ligands, EndoAs will initiate cargo sorting and FEME proceeds. Otherwise, GTP-loaded Cdc42 will be deactivated by GTPase-activating proteins (GAPs), such as RICH1, SH3BP1 and Oligophrenin, then the EndoA patches disassemble (121, 122). This priming cycle lasts 5-15s and constantly happens in resting cells (121).

In FEME, EndoAs can sort cargoes for endocytosis either directly or indirectly through the SH3 domains (122). For instance, $\beta 1$ -adrenergic receptor ($\beta 1$ -AR) is directly sorted out by SH3 domain binding to its PRD (100), while Plexin A1 is indirectly sorted out by EndoA binding to its adaptor Collapsin Response Mediator Protein 4 (CRMP4) through SH3 domain (123). Till now, many cargoes seem to be endocytosed through FEME, including a subset of the amine GPCRs ($\beta 1$ - and $\alpha 2a$ -adrenergic receptors, Dopamine receptors 3 and 4 and Muscarinic Acetylcholine receptor 4), RTKs (EGF, HGF, VEGF, PDGF, NGF and IGF-1 receptors), cytokine receptors (interleukin-2 receptor, IL-2R) and axon guidance receptors (Plexin A1 and ROBO1) (100, 123, 124). Some extracellular toxins, such as cholera and Shiga toxins, can also exploit FEME for cellular entry (119). EndoAs are also involved in FEME vesicle scission, likely through the aforementioned FDS mechanism (119, 120). But how FEME carriers are formed is still unclear.

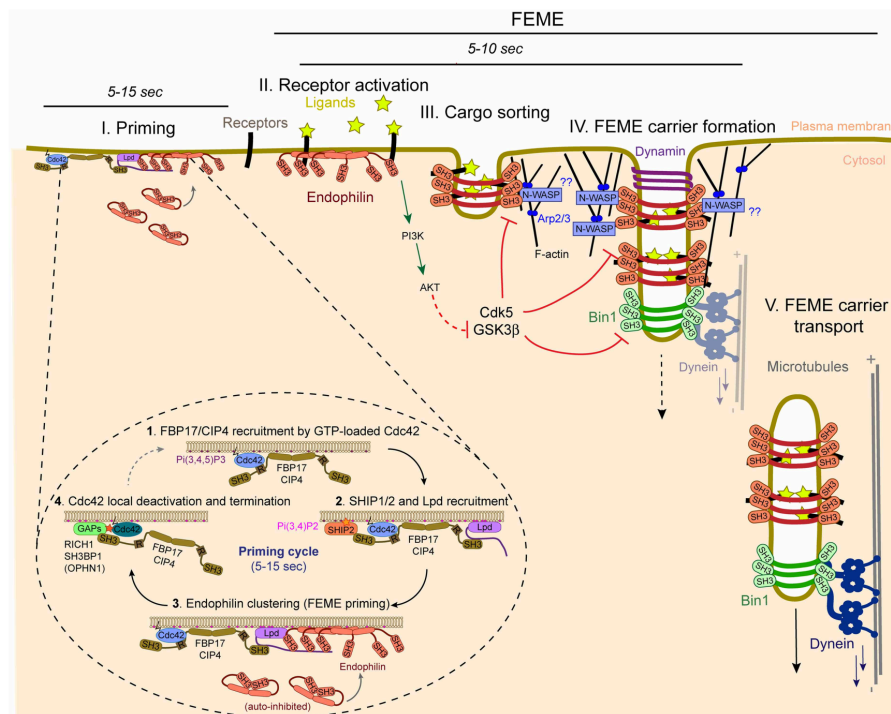


Figure 11. Molecular steps of FEME carrier formation. FEME is a highly regulated endocytic mechanism that includes several defined steps. It has a unique priming cycle that ensures the prompt initiation of FEME when it is needed. The cargo sorting is directly or indirectly mediated by the SH3 domains of EndoA, but the detailed mechanism for FEME carrier formation is yet unclear. The scission of FEME carrier is likely through the FDS mechanism, together with the help of dynamins and actin filaments. After scission, the vesicles are transported towards the inside of the cell by dynein, which are recruited by Bin1. FEME is negatively regulated by Cdk5 and GSK3 β . Lpd, Lamellipodin; OPHN1, Oligophrenin-1. Image taken from (122).

As local production of PI(3,4)P₂ in the plasma membrane is essential for the priming cycle of FEME (121), enzymes that boost the amount of PI(3,4)P₂ in the plasma membrane have an activating effect on FEME, such as class I PI3 kinases (convert PI(4,5)P₂ to PI(3,4,5)P₃, which is required for the production of PI(3,4)P₂) (125) and SHIP1/2 (convert PI(3,4,5)P₃ to PI(3,4)P₂) (121), while enzymes that reduce local levels of PI(3,4,5)P₃ and PI(3,4)P₂ suppress FEME, such as PTEN (converts PI(3,4,5)P₃ to PI(4,5)P₂) (125). Besides, FEME is also inhibited by Cdk5 and GSK3 β , as FEME is quickly initiated when their activities are low (123).

EndoA3-mediated endocytosis

At the beginning, all the three isoforms of EndoAs were considered to be involved in FEME (100). However, in 2020 *Renard et al.* discovered that only EndoA3, but neither A1 nor A2, can specifically mediate the endocytosis of an immunoglobulin (Ig)-like protein ALCAM (101), which suggests that the three EndoA isoforms may have different, non-redundant functions in different endocytic modalities.

ALCAM was the first identified EndoA3-dependent endocytic cargo. It is a highly glycosylated protein and can be clustered by Galectin-8 to promote its endocytosis according to the GL-Lect hypothesis (6, 101). In addition, pull-down experiments indicated a physical interaction between the cytosolic tail of ALCAM and EndoA3 (101), which is reminiscent of the cargo sorting by SH3 domains of EndoAs in FEME (122). A second EndoA3-dependent endocytic cargo, L1 Cell Adhesion Molecule (L1CAM), was identified later, but how galectins are involved in the uptake of this cargo is less clear (126). Interestingly, both ALCAM and L1CAM are Ig-like cell adhesion molecules, and in this current project, we identified a third EndoA3-dependent cargo, intercellular adhesion molecule 1 (ICAM1), which is also an Ig-like cell adhesion molecule (see results section). Hence, it is likely that there exists a common cargo recognition pattern for EndoA3-mediated endocytosis of Ig-like cell adhesion molecules.

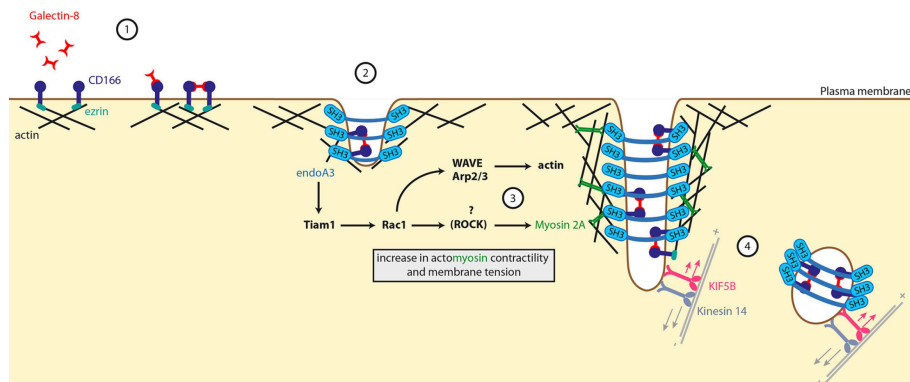


Figure 12. Working model for EndoA3-mediated endocytosis of ALCAM.

① Extracellular Galectin-8 clusters ALCAM (CD166) at the cell surface to induce membrane curvature. ② Recruitment of EndoA3 further increases membrane curvature. ③ EndoA3 initiate Rac1-mediated actin remodeling by recruiting RhoGEF Tiam1. ④ Kinesin motors (KIF5B and kinesin-14) pull endocytic carriers and nascent vesicles along microtubules.

Till now, the detailed mechanisms of EndoA3-mediated endocytosis remain unclear, but several key steps have been well-elucidated. Take EndoA3-mediated ALCAM endocytosis as an example (Figure 12) (127). ALCAM proteins are first clustered by extracellular Galectin-8 at the plasma membrane to induce membrane invaginations, which then recruit EndoA3 to further increase plasma membrane curvature. EndoA3 recruits Tiam1, a specific guanine-nucleotide exchange factor (GEF) of Rho GTPase Rac1, to initiate Rac1-mediated actin remodeling. Actin polymerization and contraction of actin filaments by myosin 2A locally increase membrane tension, and then, with the help from the BAR domains of EndoA3, mediate endocytic vesicle scission via FDS mechanism. The nascent endocytic carriers are then transported along the microtubules by kinesin motors (KIF5B and kinesin-14) (127).

Although sharing several similarities, such as the involvement of BAR domain proteins (EndoA) for endocytic carrier formation, EndoA3-mediated endocytosis and FEME differ in many aspects, which are summarized in Table 3.

Table 3. Comparison between FEME and Endophilin A3-mediated endocytosis

		FEME	EndoA3-mediated endocytosis
Initiation and carrier formation	Priming	Yes	?
	N-BAR protein	Mainly Endophilin A2	Endophilin A3
	F-BAR protein	FBP17, CIP4, Bin1	PSTPIP1
	GTPase	Cdc42	Rac1
	Cargoes	Some GPCRs and RTKs, IL-2R, Plexin A1, Shiga toxin, cholera toxin, etc.	ALCAM, L1CAM
Scission	Dynamin	Yes	No
	FDS	Yes	Most likely
Traffic	Motor protein	Dynein	Kinesins
Regulators	Positive	Class I PI3 kinases, SHIP1/2	?
	Negative	PTEN, Synaptojanin, Cdk5, GSK3 β	?

Cargo recycling

After being endocytosed, the cargoes will face two destinies: being degraded in lysosomes or being recycled back to the plasma membrane. Here, we focus on the recycling of cargoes. As to the fate decision between degradation and recycling, more information can be found in this review: (128).

Broadly speaking, cargoes can be recycled to the plasma membrane either directly from endosomes or indirectly by secretory pathway after being transported back to the *trans*-Golgi network (TGN). Transport between endosomes and Golgi apparatus is termed retrograde transport, as opposed to anterograde secretory transport. The endosomal system consists of early endosomes, late endosomes, and recycling endosomes. Both endosome-to-TGN and endosome-to-plasma membrane transport have been documented (128-130). For either pathway, the very first and pivotal step happens in the endosomes is to retrieve the right cargoes from degradative fate, which is mainly mediated by two protein complexes, retromer and Commander.

Retromer complex

Retromer was first identified in *Saccharomyces cerevisiae* as a heteropentamer that is responsible for retrieving the vacuolar protein sorting receptor Vps10 from the endosomes to the Golgi apparatus (131, 132). In yeast, Vps10 binds to hydrolases and targets them from the Golgi to the vacuole. Retromer in yeast is composed of two subcomplexes, the Vps26-Vps35-Vps29 heterotrimer and the Vps5-Vps17 heterodimer. Deficiency of either subcomplex can cause defective endosome-to-Golgi retrieval of Vps10, thus leading to the missorting and secretion of hydrolase carboxypeptidase Y (CPY) from the cell (131, 132). In mammals, the equivalent for Vps5-Vps17 heterodimer is the sorting nexin (SNX) 1/2 (homologs of Vps5)-SNX5/6 (homologs of Vps17) heterodimer (133, 134). Similarly, deficiency of retromer in mammalian cells can cause defective endosome-to-Golgi retrieval of cation-independent mannose-6-phosphate receptor (CI-MPR), which is a functional equivalent for yeast Vps10 (135), and subsequently cause enhanced secretion of hydrolase cathepsin D (136, 137). However, recent studies revealed that SNX1/2-SNX5/6 heterodimer does not form a stable heteropentamer with VPS26-VPS35-VPS29 heterotrimer as in yeasts, but can independently mediate the retrograde transport of CI-MPR (138-140). Hence, retromer is now accepted as a heterotrimer composed of only VPS26, VPS35 and VPS29 in mammals (Figure 13) (128, 141, 142).

VPS26 has two curved β -sandwich domains connected by a polar core and a flexible linker, and binds to the N-terminus of VPS35 through its C-terminal domain (143). Two paralogues of VPS26 exist, VPS26A and VPS26B, both of which can bind VPS35 and form functional retromer complexes but with different cargo specificities (144-146). For example, VPS26A-containing retromer mediates the retrograde transport of CI-MPR, whereas VPS26B-containing retromer does not (144). Besides, VPS26B-containing retromer

has a preferential plasma membrane localization instead of a characteristic perinuclear endosomal distribution (146), and is highly expressed in brain tissues (144). VPS35 is a helical solenoid protein with a total of 33 helices and binds VPS26 and VPS29 with its N- and C-terminal regions, respectively (147, 148). VPS29 has a metallophosphoesterase fold that can bind Mn^{2+} and Zn^{2+} ions weakly, but no detectable phosphoesterase activity is observed *in vitro* (149-151).

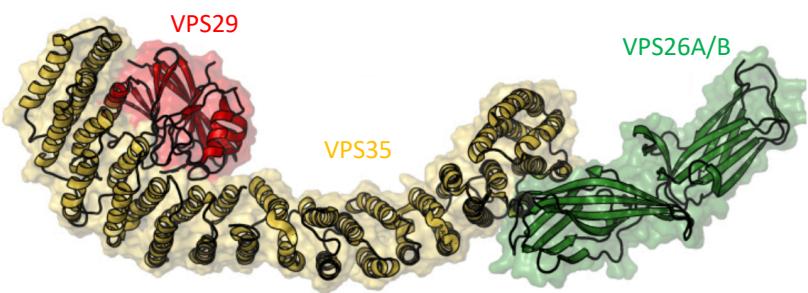
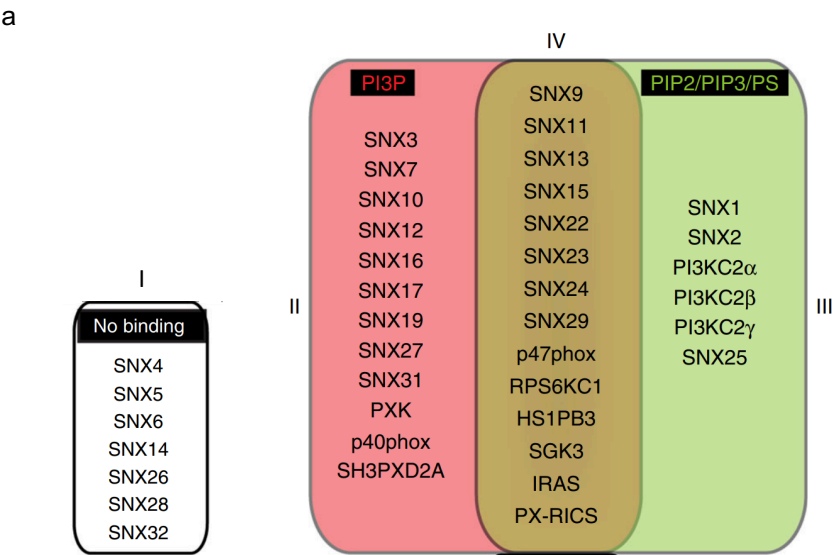


Figure 13. Structure of retromer from *C. thermophilum* determined by cryoEM. The three subunits of retromer are VPS35 (yellow), VPS29 (red) and VPS26 (green). Image adapted from (152).



b

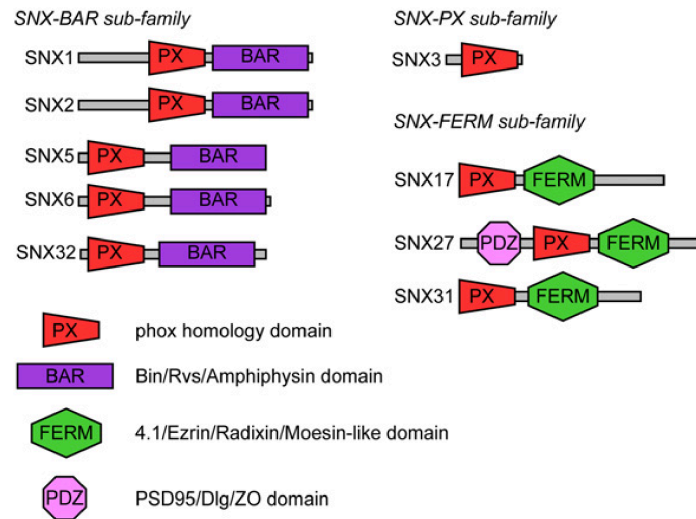


Figure 14. Classification of SNXs. **a**, PX (including SNX-PX) domains are classified into four groups based on their phosphoinositide-binding preferences. (I), PX domains do not bind to phosphoinositides; (II), PX domains specifically bind to PI(3)P; (III), PX domains bind to PIP2 (PI(3,4)P₂/PI(3,5)P₂/PI(4,5)P₂) and/or PIP3 (PI(3,4,5)P₃) and/or phosphatidylserine (PS); (IV), PX domains bind to both PI(3)P and PIP2/PIP3/PS. **b**, Domain architecture of SNXs that are involved in endosomal sorting, divided into three subfamilies based on their constituent domains. Image 13a taken from (153). Image 13b taken from (154).

SNX1/2 and SNX5/6 are members of the sorting nexin (SNX) family that is characterized by the presence of a specific type of Phox homology (PX) domain, the SNX-PX domain (104). There are 10 SNXs in yeast (Vps5 and Vps17 included) and 33 in mammals (155). SNX-PX domains can bind phosphoinositides, most commonly the canonical endosome-enriched PI(3)P, but can also bind alternative phosphoinositides. Recently, a study systematically screened all human PX domains—including SNX-PX domains—for their phospholipid preferences, and classified them into four groups: PX domains that bind (1) to no phosphoinositides, (2) specifically to PI(3)P, (3) non-specifically to various di- and triphosphorylated phosphoinositides, and (4) to both PI(3)P and other phosphoinositides (Figure 14a) (153). Apart from SNX-PX domains, many SNXs contain auxiliary domains, such as BAR domain and FERM (protein 4.1/ezrin/radixin/moesin) domain, hence SNXs can be divided into five subfamilies accordingly: SNX-PX (contains only SNX-PX domain), SNX-BAR, SNX-FERM, SNX-PXA-RGS-PXC and SNX-MIT subfamilies (104). Many SNXs are involved in endosomal cargo sorting, and are mainly from SNX-BAR, SNX-PX and SNX-FERM subfamilies (Figure 14b) (154). Retromer complex can associate with these SNXs to form higher order functional complexes, which are SNX-BAR

(SNX1/2 and SNX5/6)-, SNX3-, and SNX27-retromers (141, 156). SNX-BAR-retromer and SNX3-retromer mediate the endosome-to-Golgi retrograde transport pathway, while SNX27-retromer regulates the endosome-to-plasma membrane recycling pathway (141, 154, 157, 158).

SNX-BAR-retromer (Figure 15a) is composed of retromer complex and SNXs (Vps5-Vps17 heterodimer in yeast and SNX1/2-SNX5/6 heterodimer in metazoan) from the SNX-BAR subfamily, which all contain a C-terminal BAR domain in addition to the SNX-PX domain (159). As described previously, BAR domains can dimerize and form a crescent-shaped structure, which can sense or induce membrane curvature (103). Hence, SNX-BAR heterodimer of SNX-BAR-retromer is thought to bend the endosome membrane to form tubular carriers for retromer cargoes (160). Recent studies pointed out that SNX1/2-5/6 heterodimer, instead of retromer heterotrimer, can independently mediate the retrograde transport of CI-MPR, which has long been considered as a retromer cargo (136-139). This discrepancy calls into question our current understanding about SNX-BAR-retromer (161).

SNX3 is a member of SNX-PX subfamily that contains only one recognizable domain, the SNX-PX domain (Figure 14b). SNX3 was found to be involved in retromer-mediated endosome-to-Golgi trafficking when the retrograde transport of the Wnt sorting receptor Wntless was studied (162). It turned out that the retrograde transport of Wntless does not require SNX-BARs but SNX3, and SNX-BARs and SNX3 have different subcellular localizations with SNX3 primarily localizing to early endosomes while SNX-BARs to endosomes that are undergoing early-to-late endosomal transition (162, 163). This is consistent with the result from the recent systematic screening on human PX domains, which shows that SNX3 preferentially binds to PI(3)P, while SNX1/2 bind to PI(3,4)P₂ and SNX5/6 bind to no phosphoinositides (Figure 14a) (153). It is considered that SNX3 can recruit retromer to endosomes to form the SNX3-retromer (Figure 15b) carriers (164, 165), but it is still unclear how SNX3-retromer carriers can be formed because, unlike SNX-BARs, SNX3 has no BAR domain.

SNX27-retromer (Figure 15c) is unique because it mediates the endosome-to-plasma membrane recycling. SNX27 itself is a member of SNX-FERM subfamily but also contains an extra PDZ (post-synaptic density 95/discs large/zonula occludens) domain (Figure 14b) (154). SNX27 is able to mediate the recycling of transmembrane proteins by binding to the PDZ-binding motifs (PDZbm) present in the cytoplasmic tails of the proteins with the PDZ domain (157, 166). Meanwhile, it uses the same PDZ domain to bind retromer subunit VPS26 to form the functional SNX27-retromer complex (167). Unlike other members of SNX-FERM subfamily (SNX17 and SNX31), which can interact with cargo proteins by recognizing the NPXY motif with their FERM domain, the SNX27 FERM domain does not seem to bind any cargo proteins (166). Instead, SNX27 FERM domain recognizes a novel DLF (Asp-Leu-Phe) motif localized within the N-terminus of SNX1/2, which promotes the endosomal recruitment of SNX27 (168, 169). It is thus proposed that SNX27-retromer may first capture cargo proteins and then transfer them to SNX-BAR transport

carriers to promote endosome-to-plasma membrane recycling (168).

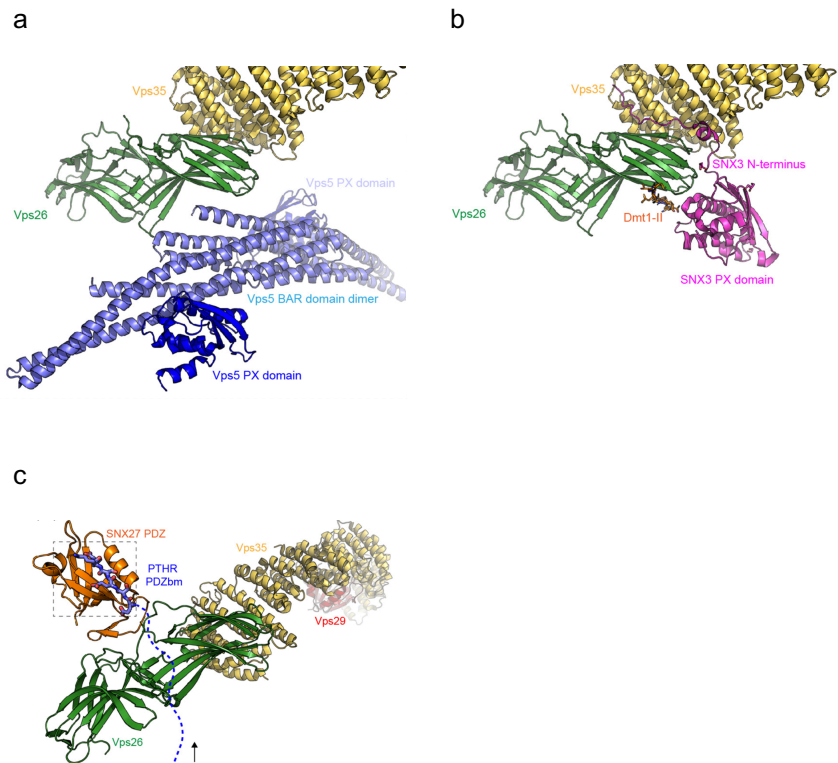


Figure 15. Different SNX-retromer complexes. **a**, The structure of *C. thermophilum* retromer in complex with the SNX-BAR protein Vps5 determined by cryoET and sub-tomogram averaging (170). **b**, A model of SNX3 bound to retromer, based on alignment of the crystal structure of human SNX3 bound to VPS35 and VPS26, with the cryoEM structure of *C. thermophilum* retromer shown in panel (a). **c**, A model of SNX27 bound to retromer and the PDZbm of cargo (here is the parathyroid hormone receptor PTHR (171)) with the same PDZ domain. Images taken from (152).

Apart from associating with SNXs, retromer complex also interacts with Wiskott-Aldrich syndrome and scar homologue (WASH) complex. WASH complex consists of WASH, FAM21, SWIP, strumpellin, and CCDC53. It nucleates branched chains of F-actin on the endosomal surface and coordinates endosomal tubule scission (172, 173). VPS35 directly binds to the unstructured tail of FAM21 to recruit WASH complex (174-176). Depletion of WASH, strumpellin or SWIP leads to uncontrolled endosome tubulation and CI-MPR mis-trafficking (172, 174).

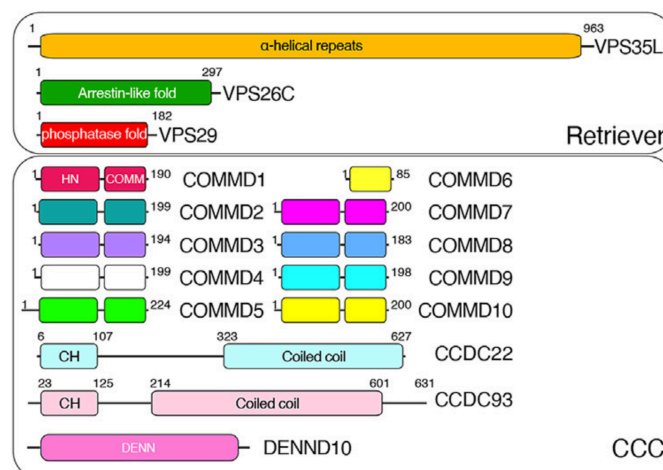
Lastly, the recruitment of retromer complex to endosomes is catalysed by the small GTPase Rab7 and inhibited by the Rab-GAP TBC1D5 (174, 177,

178). GTP-bound Rab7 directly binds to the N-terminal region of VPS35 (179, 180). Depletion of Rab7 impairs the endosome-to-Golgi trafficking of the CI-MPR (177, 178).

Commander complex

Commander is a macromolecular complex that comprises a retriever complex, a CCC complex, and DENND10 (differentially expressed in normal and neoplastic cells-containing protein 10, also called FAM45A) (Figure 16) (181-183). Retriever is a heterotrimer that is structurally similar to retromer (184). It is composed of VPS29, chromosome 16 open reading frame 62 (C16orf62), and Down syndrome critical region protein 3 (DSCR3) (184). C16orf62 has an α -helical solenoid structure similar to the one of VPS35, so it is renamed VPS35L. DSCR3 shares the structure of arrestin-like fold of VPS26, so it is renamed VPS26C, as two homologues of VPS26, VPS26A and VPS26B, have already been reported (144, 146). Apart from this structural similarity, retriever also associates with SNX17 and SNX31 to mediate endosomal cargo sorting with SNX-FERM domains (184-187). **CCC** complex contains twelve subunits: copper metabolism MURR1 domain (COMMD)1-COMMD10 and the coiled-coil domain-containing (CCDC) proteins CCDC22 and CCDC93 (181, 183). The COMMD proteins form a distinctive hetero-decameric ring stabilized by extensive interactions with CCDC22 and CCDC93, and CCDC22 and CCDC93 recruit DENND10 to form the complete Commander complex (183). CCDC22 and CCDC93 also interacts with WASH complex by binding to the tail of FAM21 (188). Generally, Commander is considered as a complex for retromer-independent endosomal cargo sorting, but some cargoes seem to use both complexes for endosomal sorting (184).

a



b

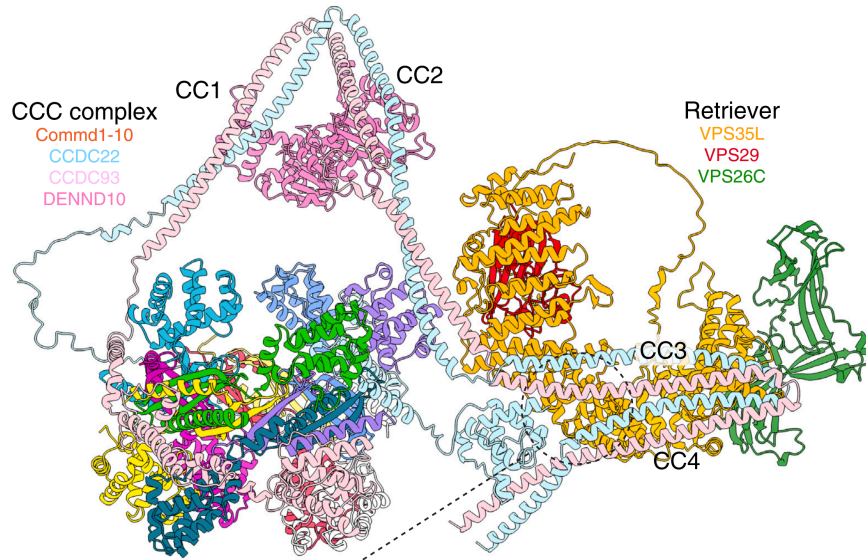


Figure 16. Structure of Commander complex. **a**, Schematic of Retriever and CCC sub-complexes that form the Commander complex. **b**, Model of Commander complex combining cryoEM and crystal structures of the CCC and Retriever sub-complexes and AlphaFold2 modeling of the coupling of CCC and Retriever via the C-terminal coiled-coil regions of CCDC22 and CCDC92 and the N-terminal CH domain of CCDC22. Images taken from (183).

Retrograde transport

Retrograde transport refers to the transport of cargo molecules from endosomes to the biosynthetic or secretory compartments, such as the Golgi apparatus and the endoplasmic reticulum (ER) (129, 189-191), which has significant pathophysiological implications.

Cells rely on retrograde transport to accomplish several housekeeping functions. One well-studied example is the retrieval of acid hydrolase sorting receptors from endosomes. Newly synthesized acid hydrolase precursors are transported from the TGN to endosomes by binding to the hydrolase sorting receptors, such as Vps10 in the budding yeast and mannose 6-phosphate receptors (MPRs) in mammals. Upon arriving at the endosomes, the hydrolase precursors dissociate from their receptors because of the acidic pH in the endosomal compartments (192). These hydrolase precursors will then be further transported to late endosomes or lysosomes where they will be cleaved and become mature enzymes, while their receptors will be retrieved from endosomes and transported back to the TGN for next rounds of hydrolase sorting (133, 136). Besides, some SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins mediating the fusion of endosome-derived carriers with the TGN are retrieved

from endosomes alongside other retrograde cargoes. Similarly, SNARE proteins responsible for Golgi-derived vesicle fusion with post-Golgi compartments also need to be returned to the TGN via retrograde transport to sustain anterograde trafficking (193, 194). In dendritic cells, internalized exogenous antigenic proteins are transported to the ER via retrograde transport, where they will be retro-translocated to the cytosol to be proteolytically processed by the proteasome. These processed antigenic peptides will then be loaded onto newly synthesized major histocompatibility complex (MHC) class I proteins in the ER lumen for antigen crosspresentation (195, 196).

Defective retrograde transport is responsible for several diseases. Rab7 is a small GTPase that recruits retromer complex to endosomes (179, 180). Mutations in Rab7 can increase the abundance of GTP-bound Rab7 (197), and cause the hereditary Charcot-Marie-Tooth Type 2B (CMT2B) disease likely through enhanced recruitment of retromer complexes to endosomes (198). Besides, reduced amounts of SNX17 and retromer subunits VPS35 and VPS26 are associated with enhanced production of amyloid β peptide ($A\beta$) (199-201), the predominant constituent of the amyloid plaques that are the pathological hallmark of Alzheimer's disease. This may be due to an increased retention time of amyloid precursor protein (APP) in maturing endosomes, caused by defective retrograde retrieval. This defect leads to enhanced cleavage of APP into $A\beta$ by β site APP cleaving enzyme (BACE, also known as β -secretase) and γ -secretase, both of which are localized in endosomes (202, 203).

Retrograde transport can also be hijacked by exogenous toxins and pathogens. For instance, Shiga toxin, produced by bacteria *Shigella dysenteriae*, is a retrograde cargo. It possesses two subunits, the nontoxic B-subunit (STxB) and the catalytic A-subunit (STxA). STxB binds the host cell toxin receptor, the glycosphingolipid Gb3, for cellular entry, according to the GL-Lect hypothesis (6). After internalization, Shiga toxin is retrieved from early/recycling endosomes and transported to the TGN and further to the ER via retrograde transport (191). In the lumen of the ER, the catalytic A-subunit of Shiga toxin will be retro-translocated to the cytosol where it induces cleavage of ribosomal RNA and inhibits protein synthesis (204). Another example is HIV-1 protein nef, which can promote virus immune evasion by inducing retrograde transport of MHC-I molecules and costimulatory molecules from the host cell surface to the TGN (205, 206).

In recent years, retrograde transport has been increasingly linked to cell polarity. In migrating cells, $\alpha_5\beta_1$ integrins and the matrix metalloproteinase MT1-MMP are continuously transported to the TGN after endocytosis, from which they are secreted to the leading edge in a polarized manner (207, 208). In T cells, the protein linker for activation of T cells (LAT), a key organizer of signalosome formation, also undergoes retrograde transport to the Golgi before being secreted to the polarized immune synapse upon T-cell receptor (TCR) activation (209). These observations suggest that retrograde transport plays a critical role in maintaining or establishing cell polarity.

Immune synapse

Structure

The immune synapse is a polarized, close cell-cell contact that is important for direct communication between immune cells. In addition, immune cells use immune synapses to directly attack and eliminate infected or cancerous cells. The formation of immune synapses involves the reorganization of receptors that are involved in recognition and adhesion to form specialized functional domains called supramolecular activation cluster (SMAC) at the interface between two cells.

The first observation of SMACs came from work by Abraham Kupfer in CD4 T cells, where T cell receptor (TCR), together with protein kinase C (PKC)- θ and Src family kinase Lck cluster to the center of the immune synapse to form the central SMAC (cSMAC) (Figure 17a-b). The cSMAC is surrounded by a ring of adhesion molecules, such as lymphocyte function-associated antigen 1 (LFA-1) and its adaptor talin, to form the peripheral SMAC (pSMAC) (Figure 17b) (210). Later, a distal SMAC (dSMAC) composed of clustered CD45 and actin that surrounds the pSMAC was revealed (Figure 17c) (211, 212). These different SMACs create the now well-known “bull’s eye” model of immune synapses, which is widely adopted for both B and T cell activation, and even for phagocytosis of particles by macrophages (213-215). Besides, for CD8 T cells, an extra discrete secretory domain exists next to the cSMAC and within the pSMAC (Figure 17d) (216, 217).

Apart from classical “bull’s eye” immune synapses, there also exist non-classical immune synapses, such as multifocal immune synapses formed between T cells and dendritic cells, which do not have clearly segregated SMACs (218, 219). In fact, the structure of an immune synapse is determined by many factors including (but not restricted to) T cell differentiation states and antigen-presenting cell (APC) types. Even the structure of the same immune synapse can vary with time (220).

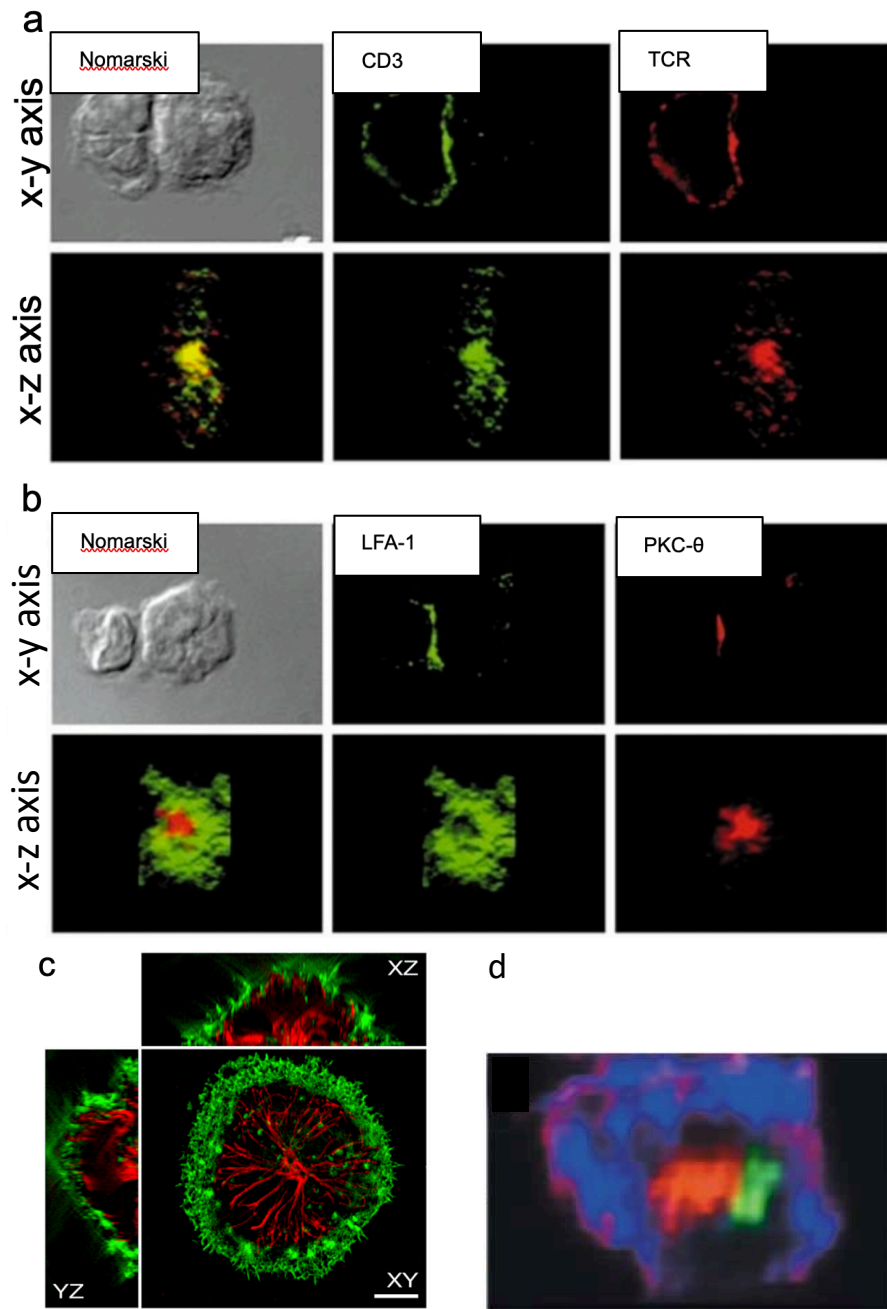


Figure 17. SMACs of immune synapse. **a**, The 3D redistribution of TCR-CD3 in antigen-specific T-APC conjugates. **b**, The 3D redistribution of PKC-θ and LFA-1 in antigen-specific T-APC conjugates. **c**, 3D structured-illumination images of a CD8 T cell on glass coated with anti-CD3ε antibody and stained with phalloidin (green) and anti-α-tubulin antibody (red). **d**, z axis image

reconstruction of an immune synapse formed between a CD8 T cell and a target cell, stained with antibodies against Lck (red), granzyme A (green) and actin (blue). The black ring lacking signal indicates pSMAC. Image **a-b** taken from (210); image **c** taken from (211); image **d** taken from (216).

TCR signaling

In T cells, the immune synapse is the place for T cell signaling generation (Figure 18). T cell signaling starts with TCRs recognizing antigen peptides presented by major histocompatibility complex (MHC) molecules (together referred to as pMHC complex) expressed on APCs. TCR recognizes either MHC class I (MHC-I)-presented peptide with the help of CD8 co-receptor (these T cells are termed CD8 T cells), or MHC class II (MHC-II)-presented peptide with the help of CD4 co-receptor (these T cells are termed CD4 T cells).

The cytoplasmic tails of co-receptor CD4 and CD8 are bound by Src family kinase Lck (221). During TCR engagement with pMHC, co-receptors deliver Lck to TCR complexes by binding to MHC molecules (222). TCR forms a noncovalent complex with CD3 ϵ -CD3 δ , CD3 ϵ -CD3 γ , and CD3 ζ -CD3 ζ dimers (223). The TCR complex has no intrinsic catalytic activity, but the cytoplasmic tails of the CD3 molecules contain 10 immunoreceptor tyrosine-based activation motifs (ITAMs) that can be phosphorylated by Lck. Each ITAM motif contains two tyrosine residues that, when phosphorylated, create binding sites for the tandem SH2 domains of kinase Zap70 (ζ -chain-associated protein kinase of 70 kDa) (224). ZAP70 phosphorylates the membrane-anchored linker for activation of T cells (LAT), which further recruits adaptor proteins GADS, SLP76 and other molecules to generate the LAT signalosome (225). LAT also recruits phospholipase C γ 1 (PLC γ 1), which hydrolyzes PI(4,5)P $_2$ to generate secondary messenger inositol-1,3,5-trisphosphate (IP $_3$) and diacylglycerol (DAG) (226).

IP $_3$ is free to diffuse in the cytosol, where it binds to its endoplasmic reticulum (ER)-associated receptor IP $_3$ R to induce the release of Ca $^{2+}$ stores from the ER and the influx of extracellular Ca $^{2+}$. Elevated levels of cytosolic Ca $^{2+}$ activate many proteins including the transcription factor NFAT (nuclear factor of activated T cells) (227). In contrast to IP $_3$, DAG remains in the plasma membrane where it activates protein kinase C (PKC) and Ras guanyl-nucleotide releasing protein (RasGRP). PKC signaling activates the transcription factor NF- κ B and eventually enhances the transcription of the key cytokines interferon- γ (IFN γ) and interleukin-2 (IL-2) (228, 229), while RasGRP activates Ras to initiate the mitogen activated protein kinase (MAPK) cascade (230, 231). All these signaling eventually contributes to the full activation of T cells.

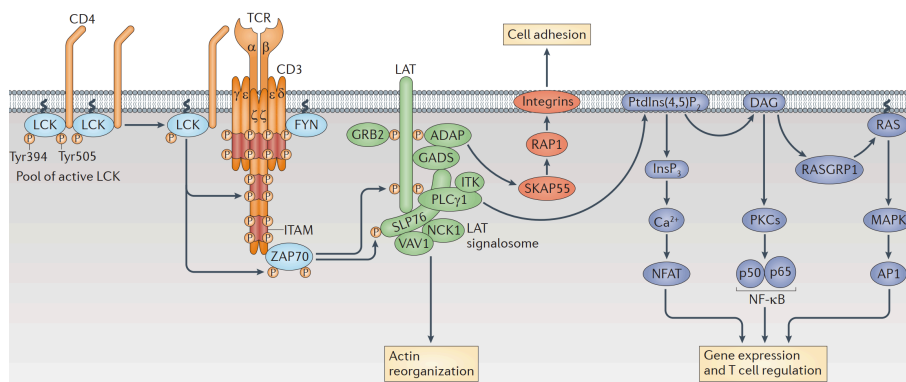


Figure 18. Overview of TCR signaling. TCR signal transduction is initiated by the recognition of cognate pMHC molecules. The first molecule to be recruited to the TCR-CD3 complex is the Src family kinase Lck, which phosphorylates ITAMs within the cytoplasmic tails of CD3 molecules. Phosphorylation of the ITAMs enables the recruitment of Zap70. Activated Zap70 phosphorylates LAT, which recruits numerous signaling molecules to form the LAT signalosome and propagates TCR signaling. AP1, activator protein 1; DAG, diacylglycerol; InsP₃, inositol-1,4,5-trisphosphate; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PtdIns(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; RASGRP1, RAS guanyl-nucleotide releasing protein 1; SKAP55, SRC kinase-associated phosphoprotein of 55kDa. Image taken from (225).

One critical regulator for TCR signaling is CD45. CD45 is a protein tyrosine phosphatase (PTP) that is expressed on all nucleated hematopoietic cells (232). The main function of CD45 in TCR signaling is to activate Src family kinase. The SH2 domain of Lck can bind to its own C-terminal tyrosine Y505 when phosphorylated by the C-terminal Src kinase (Csk), which leads to the autoinhibition of Lck. CD45 dephosphorylates Y505 so that Lck can be active. Deficiency of CD45 results in inhibition of TCR signaling (233). However, CD45 can also reverse activating phosphorylations mediated by Lck and Zap70 and inhibit T cell activation (234). Thereby, CD45 is necessary for the initiation of TCR signaling, but after that, it must be segregated from the TCRs to avoid the dephosphorylation of downstream signaling proteins (235).

T cell activation

The full activation of T cells requires several signals. The most important one is the TCR signal, which has already been described. Initially, it was believed that the cSMAC is the site for TCR signaling generation, since TCRs are enriched in this region. However, new discoveries suggested that TCR signaling actually starts at the sub-micron TCR microclusters generated in the dSMAC (236). TCR microclusters are the first structures to be observed at the interface of T cell-APC interaction, each containing approximately 50-300

CD3 ζ chains (236). Both Zap70 and SLP76 are detected inside the microclusters, suggesting that a microcluster is a functional signalosome for transducing T cell activation signals (236). Consistently, CD45 is excluded from the microclusters (237). The microclusters are continuously generated and move centripetally towards the center of the immune synapse to form the cSMAC, where the TCR signaling terminates (236, 237). Initially, it was reported that TCRs are ubiquitinated and internalized from cSMAC for degradation, thus terminating the TCR signaling (238). Recent discoveries show that TCR signaling can be terminated by both TCR endocytosis and ectocytosis from the cSMAC (239-242).

The second important signal for T cell activation comes from co-stimulatory receptors. CD28 is one of the co-stimulatory receptors. They are co-clustered with TCRs in the microclusters, and are transported with TCRs to the cSMAC area, where they form an annular cluster around the cSMAC, segregated from TCRs (243). CD28 engages with its ligands CD80 (B7.1) and CD86 (B7.2) to initiate the phosphoinositide 3-kinase (PI3K) signaling pathway and NF- κ B (nuclear factor κ -light-chain enhancer of activated B cells) pathway (244-246). In the absence of CD28-mediated co-stimulatory signals, T cells become unresponsive, entering a status termed “anergy” (247). Another example of the importance of co-stimulatory signals is the one mediated by inducible T cell costimulator (ICOS, CD278). ICOS is not expressed on naive T cells but is upregulated following T cell activation. Its engagement with ligand of ICOS (LICOS, CD275) is critical for the differentiation and expansion of human T_H17 cells, a subtype of CD4 T cells that have potent antitumor activity (248).

The third type of signals for T cell activation is mediated by adhesion molecules. The primary function of adhesion molecules is to tether the opposing membranes of T cells and APCs within tens of nanometers, facilitating TCR-pMHC interactions. There are many adhesion molecules on the surface of a T cell, and a very important one is lymphocyte function-associated antigen 1 (LFA-1) (249). LFA-1 is an integrin composed of an α subunit, CD11a, and a β subunit, CD18. The first identified ligand for LFA-1 is intercellular adhesion molecule 1 (ICAM1, CD54) (250). Upon ICAM1 engagement, LFA-1 quickly consolidates into an enriched pSMAC network, providing positional stability that significantly enhances T cell sensitivity to antigen by 100-fold compared to the absence of LFA-1 engagement (251). Adhesion molecules also generate signals to directly promote T cell activation. CD2 (initially termed LFA-2) is an adhesion molecule of the immunoglobulin (Ig) superfamily. Like LFA-1, the engagement of CD2 to its ligand CD58 facilitates T cell adhesion to APCs (252). In addition, CD2 engagement induces the phosphorylation of PLC γ 1 through another Src family kinase Fyn. This, together with TCR signaling-induced PLC γ 1 phosphorylation, increases cytosolic Ca²⁺ levels and promotes T cell activation in a synergistic manner (253).

One result of T cell activation is the generation of various cytokines, such as IFN γ and IL-2 (228, 229), which are important for T cell functions. For CD8 T cells, also known as cytotoxic T lymphocytes (CTLs), another key result of T

cell activation is T cell-mediated cytotoxicity. CD8 T cells, as well as natural killer (NK) cells, use this mechanism to directly kill infected or cancerous cells (254). The cytotoxicity is achieved by perforin-granzyme-based and FAS-based molecular mechanisms. Perforin and granzymes are stored in preformed lysosome-like structures called granules inside the CTLs, which are clustered around the centrosome (255). The centrosome is located in the uropod of migrating CTLs. Upon the CTL encountering the target cell and initiating TCR signaling, the centrosome starts moving towards the immune synapse and eventually docks at the plasma membrane next to the cSMAC, in a region where F-actin is depleted (Figure 17c) (211, 256). As granules are clustered around the centrosome, they will fuse with the plasma membrane after centrosome docking and release perforin and granzymes into the space between the CTL and the target cell (Figure 17d) (211). Perforin helps deliver granzymes into the cytosol of the target cell by forming pores in the membrane (257), while granzymes, which are serine esterases, cleave critical intracellular substrates controlling cell death and survival inside the target cell to induce apoptosis (258, 259). FAS-based molecular mechanism mediates another type of cytotoxicity. FAS (CD95) is a cell-death-transducing surface molecule that, when bound with its ligand FASL (also known as TNFSF6 and CD95L), triggers apoptosis of the cells (260). Activated CTLs can secrete FASL to mediate the apoptosis of target cells (261).

Objectives

My host lab recently reported a unique clathrin-independent, EndoA3-mediated endocytic mechanism in cancer cells, identifying ALCAM as its first cargo (101). ALCAM, a cell surface adhesion molecule of the Ig-like superfamily, is expressed across various tissues and is often overexpressed in cancers, such as bladder, prostate and colorectal carcinoma (262-264). Structurally, ALCAM contains five extracellular Ig domains, a transmembrane domain, and a short cytoplasmic tail (265). It interacts with CD6, a co-stimulatory molecule on T cells, to facilitate immune synapse formation, enhance T cell activation, and stabilize T cell-dendritic cell interactions to support T cell proliferation (266-268).

In addition to ALCAM, our lab identified L1CAM, another Ig-like protein, as a cargo of EndoA3-mediated endocytosis (126), suggesting that EndoA3-mediated endocytosis may preferentially internalize Ig-like proteins. Notably, ICAM1, a crucial component of the immune synapse that co-stimulates T cell activation by binding to its receptor LFA-1 on T cells, is also an Ig-like protein (269-271). This raises the possibility that ICAM1 could also be an EndoA3-dependent cargo in cancer cells.

Hence, the first objective of this thesis is to investigate whether ICAM1 is an EndoA3-mediated endocytic cargo in cancer cells and whether EndoA3-mediated endocytosis of these immune synapse components, such as ALCAM and ICAM1, impacts anti-tumor CD8 T cell activation.

The immune synapse is a highly polarized cellular structure that is critical for effective immune signaling and T cell activation. Several lines of evidence have now linked retrograde transport to the establishment of cell polarity (207, 208). In T cells, retrograde transport can participate in LAT recruitment to the immune synapse (209), where LAT is phosphorylated by the kinase Zap70 to generate the LAT signalosome (225). This highlights retrograde transport as a key mechanism for maintaining or establishing cell polarity in T cells. However, whether retrograde transport plays a role in polarity formation on the opposite side of the immune synapse, within APCs or cancer cells, remains underexplored.

Given the polarized nature of the immune synapse and the roles of ALCAM and ICAM1 in immune interactions, we hypothesize that ALCAM and ICAM1 may undergo retrograde transport following EndoA3-mediated endocytosis in cancer cells, thus contributing to immune synapse formation and modulation. The second objective of this thesis is to test this hypothesis.

To achieve these objectives, first, we used HeLa cells to determine whether ICAM1 serves as an additional EndoA3-dependent endocytic cargo. HeLa cell is a classical cell line for studying endocytosis and intracellular trafficking. They are easy to be genetically modified to generate stable cell lines for different purposes. Here, we generated a SNAP-tagged HeLa cell line that can facilitate our exploration of the intracellular fate of ALCAM and ICAM1

after EndoA3-dependent endocytosis, with a particular focus on retrograde transport.

Second, we employed the melanoma cell line LB33-MEL and an autologous CD8 T cell line that specifically recognizes LB33-MEL, to investigate whether EndoA3-dependent endocytosis and retromer-mediated retrograde transport in cancer cells influence CD8 T cell activation. The choice of these cell lines was deliberate and grounded in the desire for physiological relevance. Previous studies on immune synapse formation and T cell activation often relied on artificial systems, such as anti-CD3-coated lipid bilayers or CD4 T cells from OT-II transgenic mice, which may not accurately mimic real physiological conditions. In contrast, the LB33-MEL melanoma cell line was derived from a melanoma patient (LB33) in 1988 (272, 273), and the autologous CD8 T cell line was obtained from the same patient in 1990 (272). These cell lines can form authentic immune synapses in vitro, offering a more pathophysiologically relevant model.

Finally, we used another genetically modified HeLa cell line to examine how the disruption of EndoA3-mediated endocytosis can specifically impair the activation of CD8 T cells.

In summary, this project aims to elucidate how EndoA3-mediated endocytosis and retrograde trafficking of immune synapse components influence cancer cell and T cell interactions. By understanding these processes, we hope to identify new therapeutic strategies targeting endocytic mechanisms and retrograde transport to enhance anti-tumor immune responses, potentially improving the efficacy of cancer immunotherapy.

Part II

Results

This work has been uploaded to bioRxiv (<https://www.biorxiv.org/content/10.1101/2024.10.28.620627v2>) and accepted by *eLife*.

Supplementary movies can also be found online.

Clathrin-independent endocytosis and retrograde transport in cancer cells promote cytotoxic CD8 T cell activation

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One set of additional unpublished data is attached to the end of this work.

Abstract

Clathrin-independent endocytosis (CIE) mediates the cellular uptake of endogenous and exogenous cargoes, including bacterial toxins and viruses. Endophilin A3-mediated endocytosis is a specific CIE mechanism that differs from fast endophilin-mediated endocytosis (FEME), with ALCAM and L1CAM being the first confirmed Endophilin A3-specific cargoes. Here, we report ICAM1 as a new Endophilin A3-dependent endocytic cargo. ALCAM and ICAM1 are important components of immune synapses (IS), which are polarized structures formed between immune cells and target cells, such as cancer cells. These molecules transduce essential co-stimulatory signals to T cells to help their effective activation and proliferation. We find that both ALCAM and ICAM1 serve as cargoes for retromer-dependent retrograde transport to the *trans*-Golgi network (TGN) in cancer cells. Interestingly, disrupting Endophilin A3-mediated endocytosis or retromer-dependent retrograde transport machinery impairs activation of autologous cytotoxic CD8 T cells, likely by affecting the polarized redistribution of immune synapse components at the plasma membrane. Altogether, our findings demonstrate that CIE and retrograde transport are key pathways in cancer cells that promote the activation of cytotoxic CD8 T cells.

Introduction

Endocytosis is a physiological process that refers to the membrane bound carrier-mediated internalization of extracellular substances as well as cell membrane components into the cytoplasm of cells. This process is essential for maintaining the homeostasis of cell membrane and regulating signal transduction. Broadly, it can be divided into conventional clathrin-mediated endocytosis (CME) and unconventional clathrin-independent endocytosis (CIE). While CME has been extensively studied, CIE remains elusive as it includes several distinct mechanisms of endocytosis (4, 10). Upon endocytosis, internalized cargoes merge into early/sorting endosomes, from which they either follow the degradative route towards lysosomes or recycle back to the plasma membrane for a new round of activity. Among the recycled cargoes, some are directly recycled back to the plasma membrane via Rab4/Rab11 recycling endosomes (274, 275). Other cargoes are retrieved from early endosomes by retromer or Commander complexes and further transported to the *trans*-Golgi network (TGN) – a process termed retrograde transport – from where they are finally recycled back to the plasma membrane (131, 137, 183, 184). Recently, several lines of evidence have linked retrograde transport to cell polarity maintenance (276). For example, $\alpha_5\beta_1$ integrins are transported to the TGN via the retrograde transport route after being endocytosed (207). From there, they are re-secreted in a polarized manner to the leading edge of migrating cells, thus maintaining the front-rear polarity, crucial for persistent cell migration (207). In T cells, adaptor molecules Linker for Activation of T cells (LAT) are also transported to the TGN after endocytosis, from which they are re-distributed to the immune synapse (209).

The immune synapse is a polarized cellular structure formed between immune cells and target cells, *e.g.* between a cytotoxic CD8 T cell and a tumor cell (277). However, the link between specific unconventional CIE mechanisms and retrograde transport in polarized cellular contexts remains underexplored.

Among the numerous molecular players involved in endocytosis, one can find Endophilin A (EndoA) proteins. EndoA proteins are members of the BAR (Bin/Amphiphysin/Rvs) domain proteins, which are able to sense and/or induce membrane curvature (278, 279). There exist three different isoforms of EndoA proteins in mammalian cells, termed EndoA1, A2, and A3 (112, 280). EndoA1 is mainly expressed in brain, and EndoA3 is highly expressed in both brain and testes, while EndoA2 is widely expressed in all tissues (111, 114). Originally, it was reported that EndoAs are mainly involved in CME in neuronal cells, where they recruit dynamins and synaptojanins by binding to the proline-rich domains (PRDs) with their Src-homology 3 (SH3) domains to promote vesicle scission (118, 281) and clathrin uncoating after scission (88, 282). More recently, EndoAs were reported to play a central role in the clathrin-independent fast endophilin-mediated endocytosis, termed FEME (100). Meanwhile, EndoA2 was reported to function in membrane scission in the clathrin-independent endocytosis of Shiga and cholera toxins (119, 120). It was further reported that the three EndoAs seem to be not totally functionally redundant in CIE, as only EndoA3, but neither A1 nor A2, can specifically mediate the endocytosis of the immunoglobulin (Ig)-like protein ALCAM (Activated Leukocyte Cell Adhesion Molecule). ALCAM is the first identified cargo that is internalized in an EndoA3-dependent manner, which is driven by extracellular Galectin-8 and glycosphingolipids, in agreement with the glycolipid-lectin (GL-Lect) endocytosis mechanism (5, 6, 101). In addition to ALCAM, another Ig-like protein L1 Cell Adhesion Molecule (L1CAM, alternative name CD171) was also confirmed to be a cargo of EndoA3-mediated CIE (126).

ALCAM, also known as CD166, is an adhesion molecule that is widely expressed in many different types of tissues and overexpressed in several types of cancer, such as bladder, prostate and colorectal carcinomas (262-264). It is engaged in cell-cell interactions either by homophilic interactions with other ALCAM molecules (283) or by heterophilic interactions with CD6 (284). CD6 is a member of the scavenger receptor cysteine-rich (SRCR) family of proteins that is mainly expressed by immune cells, particularly T lymphocytes, and by some neuronal cells (285, 286). Evidence has been shown that heterophilic ALCAM-CD6 interaction is important for immune synapse formation and provides essential co-stimulatory signals for optimal T cell activation and proliferation (267, 268, 287). Considering that immune synapse is a polarized cell structure, we wondered whether EndoA3-mediated endocytosis in cancer cells participates in immune synapse formation via retrograde transport of its components, such as ALCAM. Also, given that both ALCAM and L1CAM are Ig-like proteins, we hypothesized that EndoA3-mediated endocytosis could serve as a preferred route for the uptake of Ig-like proteins. Interestingly, another well-known immune synapse component, Intercellular Adhesion Molecule 1 (ICAM1, alternative name CD54), which

plays a crucial role in co-stimulating T cell activation by binding to its receptor, Lymphocyte Function-associated Antigen 1 (LFA-1), on the T cell surface, is also an Ig-like protein (269-271). This led us to investigate whether ICAM1 could be another EndoA3-dependent CIE cargo in cancer cells, and whether its role in forming immune synapses relies on EndoA3-mediated CIE and retrograde transport.

Combining cell biology and immunology experiments, we uncover here that ICAM1 is an EndoA3-dependent endocytic cargo. Moreover, we show that both ALCAM and ICAM1 undergo retromer-dependent retrograde transport to the TGN upon EndoA3-mediated internalization. Disrupting EndoA3-mediated endocytosis or retromer-dependent retrograde transport machinery in cancer cells impairs the activation of autologous cytotoxic CD8 T cells, likely by affecting the polarized redistribution of immune synapse components from the Golgi to the plasma membrane after retrograde transport. Further supporting this hypothesis, disruption of EndoA3-mediated endocytosis in cancer cells impairs efficient recruitment of ICAM1 at the immune synapse and thereby its formation. Hence, our findings highlight the connection between a CIE mechanism and subsequent retrograde transport in a polarized context.

Results

EndoA3-dependent endocytosis mediates internalization of the immune synapse components ALCAM and ICAM1 in cancer cells

To investigate the impact of EndoA3-dependent CIE in cancer cells on the activation of cytotoxic CD8 T lymphocytes, we used the LB33-MEL melanoma cell line, derived from a melanoma patient (LB33) in 1988. This cell line harbours a mutated antigenic peptide encoded by the *MUM3* gene. The MUM-3 peptide is presented on the cell surface by HLA-A*68012 molecules, the HLA-A28 subtype of patient LB33 (272, 273). An anti-MUM-3-specific CD8 T cell line, obtained from the same patient in 1990 (272), was used to form immune synapses with LB33-MEL cells *in vitro*. However, LB33-MEL cells do not express detectable amounts of EndoA3 (101). For this reason, we generated a stable EndoA3-expressing LB33-MEL cell line. In this cell line, the exogenously expressed EndoA3-GFP protein can be efficiently knocked down by siRNAs targeting EndoA3 (decreased by 80%; Fig EV1A). First, we evaluated colocalization between EndoA3-GFP and ALCAM in this EndoA3-expressing LB33-MEL cell line (LB33-MEL EndoA3+) after a 15 min uptake in the continuous presence of anti-ALCAM antibody in culture medium (Fig 1A). High resolution Airyscan confocal images show a clear colocalization between ALCAM and EndoA3-GFP, confirming that LB33-MEL EndoA3+ cell line can recapitulate the EndoA3-mediated endocytosis of ALCAM (Fig 1A, white arrowheads). Moreover, we quantified the uptake of anti-ALCAM antibody and observed that it was significantly enhanced (3-fold higher) in EndoA3-expressing LB33-MEL cells than in the EndoA3-negative wild-type or empty plasmid transfected stable LB33-MEL cells (Fig 1B, EV1B). A reverse-rescue uptake assay was also conducted in LB33-MEL EndoA3+ cells transfected with EndoA3 siRNAs (Fig 1C, EV1C). siRNA efficiency was confirmed by Western blot analysis (Fig EV1A). ALCAM uptake was significantly decreased by 55% upon EndoA3 depletion (Fig 1C). Taken together, these data demonstrate that EndoA3-expressing LB33-MEL cells can be used to study EndoA3-dependent CIE.

To investigate whether ICAM1 is endocytosed in an EndoA3-dependent manner, similar continuous uptake assays were performed but failed due to the poor quality of commercially available anti-ICAM1 antibodies. Instead, Total Internal Reflection Fluorescence (TIRF) live-cell imaging was used to directly observe the dynamics of EndoA3 and ICAM1 at the plasma membrane of both EndoA3-GFP-expressing HeLa and LB33-MEL cells. Of note, HeLa cells endogenously express EndoA3, while the EndoA3-GFP-expressing HeLa cell line was previously generated and validated by our lab (101, 126). Both HeLa and LB33-MEL cells were transiently transfected with an ICAM1-mScarlet expression plasmid and observed by TIRF live-cell imaging. In both cell lines, EndoA3-GFP formed circular structures around ICAM1 patches (Fig 1D-E, white arrows; Mov EV1-2), and ICAM1 signals gradually disappeared from the plasma membrane (Fig 1D-E; Mov EV1-2). Besides, ICAM1-positive punctate structures could be observed, where EndoA3-GFP was recruited, after which both protein signals subsequently disappeared from the cell

surface (Fig 1E, kymograph; Mov EV3), suggesting endocytic events. This is similar to TIRF observations of the previously identified EndoA3-dependent endocytic cargoes, ALCAM and L1CAM (101, 126). Moreover, it is common to observe EndoA3-GFP positive punctate structures (Fig EV1D, white arrowheads; Mov EV4) or tubular structures (Fig EV1E, white arrowheads; Mov EV5) detaching from the tips or edges of continuously shrinking ICAM1 patches, also suggesting endocytic events. All these observations suggest that ICAM1 is an EndoA3-dependent endocytic cargo.

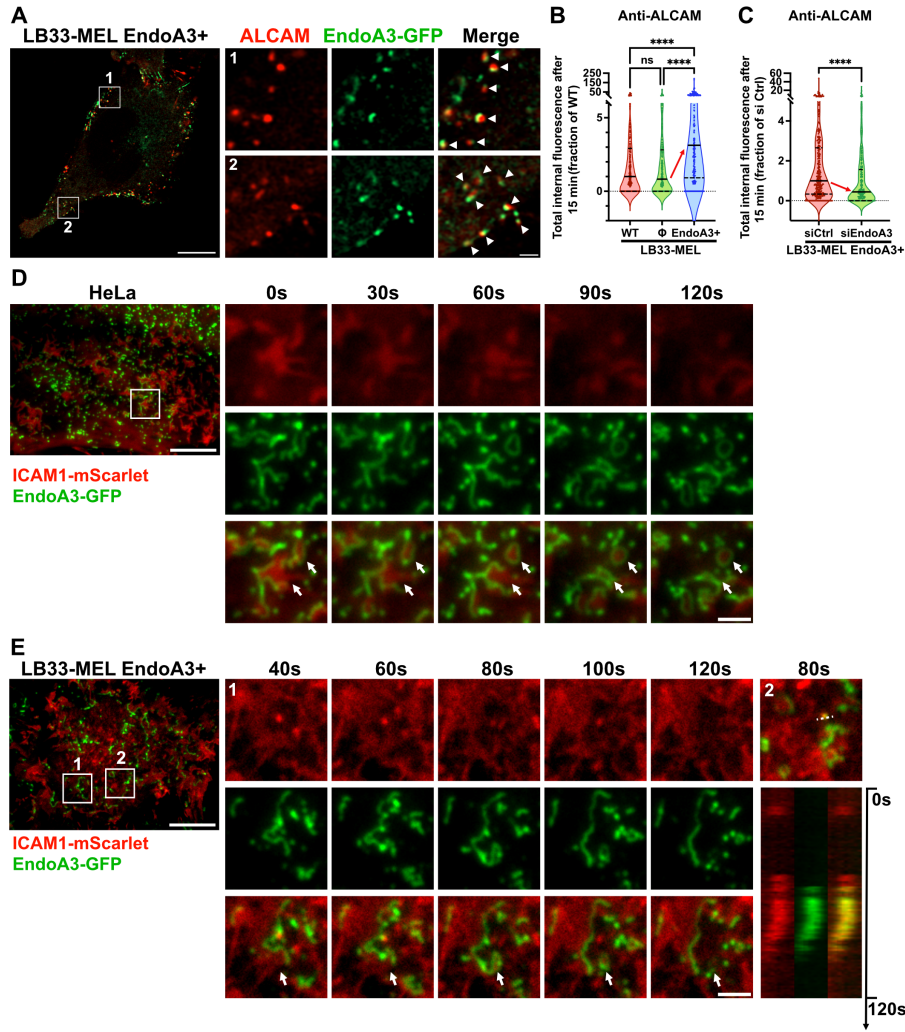


Figure 1. EndoA3-dependent CIE mediates the uptake of immune synapse components ALCAM and ICAM1 in cancer cells.

- A-C Continuous uptake of anti-ALCAM antibody for 15 min at 37°C in the following LB33-MEL cell lines: wild-type (WT, B), stably transfected with empty plasmid (Φ , B), or stably expressing EndoA3-GFP (LB33-MEL EndoA3+, A-C). In (C), cells were transfected with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3). Quantification of EndoA3 depletion by western blots in Fig EV1A. (A) Airyscan images of Anti-ALCAM (red) and EndoA3-GFP (green). White arrowheads show colocalization between ALCAM and EndoA3. Scale bars: 10 μ m (full size image), 1 μ m (enlarged cropped areas). (B-C) Quantifications of anti-ALCAM internalization, expressed as fractions of WT condition (B) or siCtrl condition (C). (B) n cells: WT, n = 270; Φ , n = 279; EndoA3+, n = 274. (C) n cells: siCtrl, n = 350; siEndoA3, n = 234. Representative image examples in Fig EV1B-C.
- D-E Live-cell TIRF images of EndoA3-GFP (stable) and ICAM1-mScarlet (transient) in HeLa (D) and LB33-MEL (E) cells. Time series show enlarged cropped areas extracted from Mov EV1 (D) and Mov EV2 (E). White arrows indicate dynamic co-distribution of both signals. Kymograph in (E) was made along dashed line region in the 2nd cropped area (D; Mov EV3). Scale bars: 10 μ m (full size image), 2 μ m (enlarged cropped areas).

Data information: All images (A, D and E) are representative of two independent experiments. In (B-C), data are pooled from three independent experiments. Data are presented as median and quartiles. ns, not significant. ****P < 0.0001 (B, Kruskal-Wallis test with Dunn's multiple comparison test; C, Mann-Whitney test). Source data are available online for this figure.

ALCAM and ICAM1 are cargo clients of retromer-dependent retrograde trafficking

Given the polarized structure of the immune synapse and the previously reported connections between retrograde transport and cell polarity (207, 209), we investigated whether ALCAM and ICAM1 are also cargoes for the retrograde transport. To address this question, we used a previously established SNAP-tag-based approach to detect retrograde transport of cargoes from the plasma membrane to the Golgi apparatus (288) (Fig 2A). Briefly, benzylguanine (BG)-labelled cargo-specific antibodies (IgG) are incubated with cells stably expressing the Golgi-resident Galactosyltransferase protein (GalT) fused to GFP and SNAP-tag (GalT-GFP-SNAP). If the cargo follows the retrograde route after endocytosis, it will be delivered to the TGN together with the BG-labelled antibody. Upon arrival in the TGN, BG covalently reacts with the SNAP-tag, generating a large complex (IgG-SNAP-GFP-GalT), trapping the antibody at the TGN. This complex can then be immunopurified and analyzed by Western blot.

Here, we used a HeLa cell line stably expressing the Golgi-resident SNAP-tag construct (HeLa GalT-GFP-SNAP), which has already been validated in previous studies (207, 289). We also generated a LB33-MEL cell line stably expressing the same Golgi-resident SNAP-tag construct (LB33-MEL GalT-GFP-SNAP, Fig EV2A-B). First, we validated the functionality of the SNAP-tag in both cell lines using the SNAP-Cell® TMR-Star reagent (Fig 2B for HeLa; Fig EV2A for LB33-MEL). We observed a strong and specific colocalization of TMR-Star with the GFP signal of the SNAP-tag constructs in both cell lines. Second, immunofluorescence experiments showed that the SNAP-tag construct co-distributes with the TGN-specific marker TGN46 in LB33-MEL cells, thus confirming the correct and specific localization of the construct (Fig EV2B).

Next, we used both cell lines in retrograde transport assays. BG-labelled anti-ALCAM or anti-ICAM1 antibodies were added to the cell culture medium for a 4-hour continuous uptake. If ALCAM or ICAM1 are retrograde cargoes, their specific BG-tagged antibodies (BG-IgGs) should also move along into the Golgi compartment and produce covalently bound IgG-SNAP-GFP-GalT complex species (simplified as SNAP signal) that should be observed in Western blot. Interestingly, we found that both ALCAM and ICAM1 are retrograde transport cargoes in HeLa cells, as the IgG-SNAP-GFP-GalT complexes were indeed detected by Western blotting when both BG-labelled antibodies were used (band ~ 210 kDa, Fig 2C-D, IB: anti-SNAP). As negative controls, we used unlabelled antibodies (IgGs), and no bands were observed (Fig 2C-D). In addition, we depleted EndoA3 by RNA interference in HeLa cells (Fig 2C-D, EV2E), and a significant decrease of the IgG-SNAP signal was observed (decreased by 60% for ALCAM and 64% for ICAM1, Fig 2C-D). We also carried out retrograde transport assays with BG-labelled anti-ALCAM antibodies in the SNAP-tag expressing LB33-MEL cell line (Fig EV2C). As this cell line was generated from the wild-type LB33-MEL background which does not express detectable levels of EndoA3, we transfected it transiently with an

EndoA3-GFP expression plasmid. Interestingly, we observed that the SNAP signal was ~50% higher upon EndoA3-GFP expression, as compared to the control condition where cells were transiently transfected with a plasmid allowing free GFP expression (Fig EV2C). Altogether, these data confirm that both ALCAM and ICAM1 are EndoA3-dependent endocytic cargoes, and further demonstrate that both proteins are retrograde transport cargoes, trafficking from the plasma membrane to the Golgi apparatus.

Subsequently, we wondered if these two retrograde transport cargoes need the canonical retromer complex machinery for endosomal sorting and retrograde transport to the TGN. The retromer complex is for now the best-studied retrograde transport sorting machinery and is composed of three subunits in mammalian cells, which are VPS26, VPS29 and VPS35 (137, 148). Two paralogues of VPS26 subunit exist: VPS26A, which is highly expressed in basically all tissues, and VPS26B, which is mainly enriched in the brain (144, 146). To address the role of retromer in the retrograde trafficking of ALCAM and ICAM1, we carried out SNAP-tag-based retrograde transport assays in HeLa cells where VPS35 and VPS26A were knocked down by RNA interference (Fig 2E-F, EV2F). In cells treated with negative control siRNA, unlabelled antibodies led to no detectable SNAP signal in Western blot (Fig 2E), while BG-labelled antibodies showed a clear one (Fig 2E-F), which is consistent with our previous experiments. Interestingly, depletion of either VPS35 or VPS26A caused a significant reduction of SNAP signal intensity for both cargoes (Fig 2E-F). Similar results were also obtained for ICAM1 in SNAP-tag expressing LB33-MEL cells, even though EndoA3 is not detectably expressed in these cells (Fig EV2D and EV2G). This suggests the existence of alternative endocytic mechanisms for this cargo, which result, at least partially, in its retrograde transport to the Golgi. Taken together, these data further demonstrate that the retrograde transport of significant fractions of ALCAM and ICAM1 to the Golgi is mediated by the retromer complex.

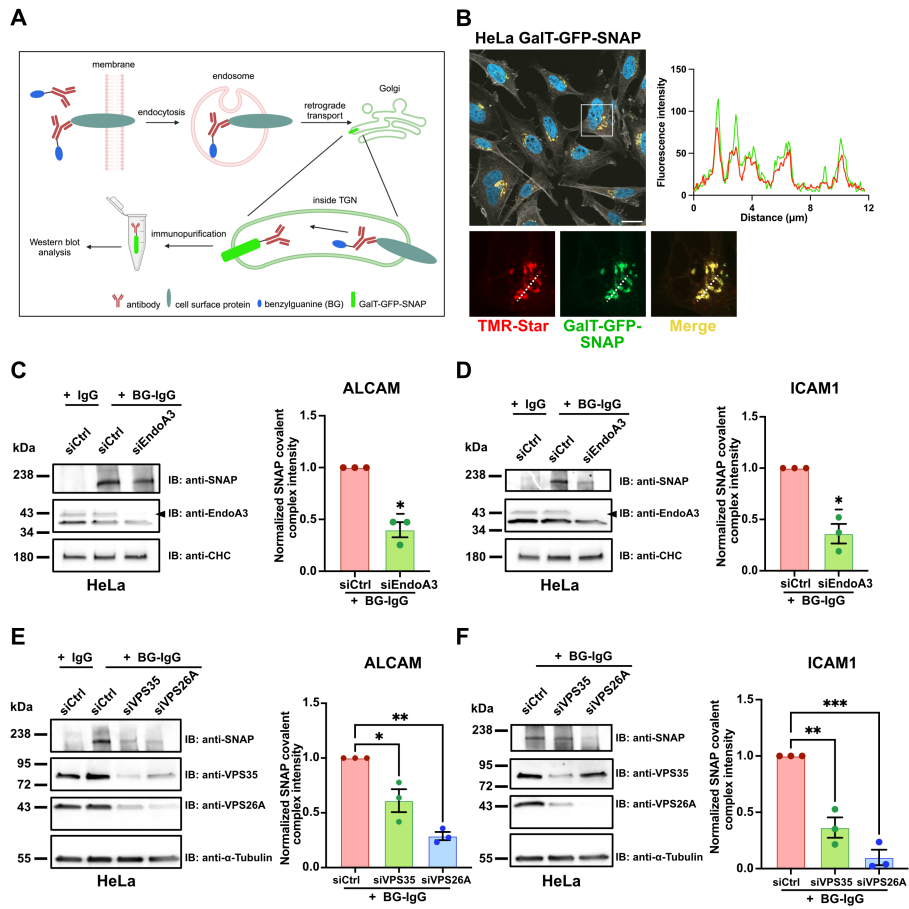


Figure 2. Immune synapse components ALCAM and ICAM1 are retrograde transport cargoes that rely on retromer

- A Illustration of the SNAP-tag-based BG-labelled antibody uptake assay to study membrane protein endocytosis and retrograde transport.
- B Confocal images of GalT-GFP-SNAP (green) and TMR-Star (red) in HeLa cells stably expressing the Golgi-resident GFP-fused SNAP-tag construct (HeLa GalT-GFP-SNAP). Actin (phalloidin, white) and nuclei (DAPI, blue) were also stained. Fluorescence intensity profile was made along the dashed line region in enlarged cropped area and shows the colocalization of both signals. Scale bar: 20 μ m.
- C-F Retrograde transport of ALCAM and ICAM-1. Continuous BG-labelled anti-ALCAM (C and E) and anti-ICAM1 (D and F) antibody uptake for 4h at 37°C in HeLa GalT-GFP-SNAP cells. (C-D) Western blot analysis of HeLa GalT-GFP-SNAP cells transfected for 72h with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3). Immunodetection made with anti-SNAP, anti-EndoA3 and anti-clathrin heavy chain (CHC, loading control) antibodies. Quantification of the covalent IgG-SNAP-GFP-GalT complex (IB:anti-SNAP) is shown as fractions of siCtrl condition (histogram). Quantification of EndoA3 depletion is shown in Fig EV2E. (E-F) Western blot analysis of HeLa GalT-GFP-SNAP cells transfected for 72h with siRNAs: negative control (siCtrl) or against retromer subunits (siVPS35 and siVPS26A). Immunodetection made with anti-SNAP, anti-VPS35, anti-VPS26A and anti- α -Tubulin (loading control) antibodies. Quantification of the covalent IgG-SNAP-GFP-GalT complex is shown as fractions of siCtrl condition (histogram). Quantification of VPS35 and VPS26A depletion is shown in Fig EV2F.

Data information: In (B), images are from a single experiment. Quantification data (C-F) are pooled from three independent experiments. Data are presented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (C-D, one sample *t* test; E-F, RM one-way ANOVA with Dunnett's multiple comparison test). Source data are available online for this figure.

Inhibition of both EndoA3-dependent endocytosis and retromer-mediated retrograde transport impairs cytotoxic CD8 T cell activation

Based on our results shown before and considering that ALCAM and ICAM1 are key components of the immune synapses, we hypothesized that their clathrin-independent endocytosis and subsequent retrograde transport might influence CD8 T cell activation. To test this, we conducted CD8 T cell stimulation assays using LB33-MEL cells, with and without EndoA3 expression. EndoA3-expressing LB33-MEL cells were treated with either control or EndoA3-targeting siRNAs before being co-cultured with CD8 T cells. The cells were then analyzed by flow cytometry to assess intracellular cytokine production in T cells (Fig 3A). Interestingly, when CD8 T cells were stimulated with EndoA3-depleted LB33-MEL cells, both the percentage of T cells producing interleukin-2 (IL-2) and tumor necrosis factor α (TNF α) (Fig 3B, upper and middle panels) and the absolute amounts of these cytokines (Fig 3C, upper and middle panels) significantly decreased compared to CD8 T cells stimulated with EndoA3-expressing LB33-MEL cells. Although the percentage of T cells producing interferon γ (IFN γ) remained unchanged (Fig 3B, bottom panel), the absolute amount of IFN γ produced was still significantly reduced (Fig 3C, bottom panel).

Next, we performed IFN γ secretion assays, where CD8 T lymphocytes were stimulated with differentially treated LB33-MEL cells, after which the supernatant was analyzed by enzyme-linked immunosorbent assay (ELISA) (Fig 3D). Wild-type, empty plasmid control, and EndoA3-GFP-expressing LB33-MEL cells all stimulated CD8 T cells to secrete IFN γ , as compared to the control condition where T cells were kept alone (Fig 3E). EndoA3-expressing LB33-MEL cells significantly enhanced CD8 T cell activation, with T cells secreting approximately 70% more IFN γ compared to wild-type or empty plasmid conditions (Fig 3E). This effect was lost when EndoA3 was depleted from LB33-MEL cells using RNA interference (Fig 3E). The efficiency of EndoA3-GFP depletion was confirmed by flow cytometry (Fig EV3A). We hypothesized that EndoA3-mediated CIE in cancer cells may influence retrograde transport and the subsequent polarized redistribution of immune synapse components, such as ALCAM and ICAM1, thereby affecting T cell activation. To explore this, we assessed the impact of disrupting the retromer complex in cancer cells on CD8 T cell activation. We first depleted the retromer subunits VPS26A and VPS35 using RNA interference in EndoA3-expressing LB33-MEL cells and then examined the effects on CD8 T cell activation by measuring IFN γ secretion. While VPS35 depletion showed no significant effect, depleting VPS26A clearly impaired CD8 T cell activation (Fig 3F, EV3B).

To conclude, these data suggest that inhibition of EndoA3-dependent endocytosis in cancer cells impairs CD8 T cell activation by affecting the retrograde transport of immune synapse components, such as ALCAM and ICAM1. This likely prevents the efficient recruitment of these components to participate in immune synapse formation through polarized re-distribution from the TGN.

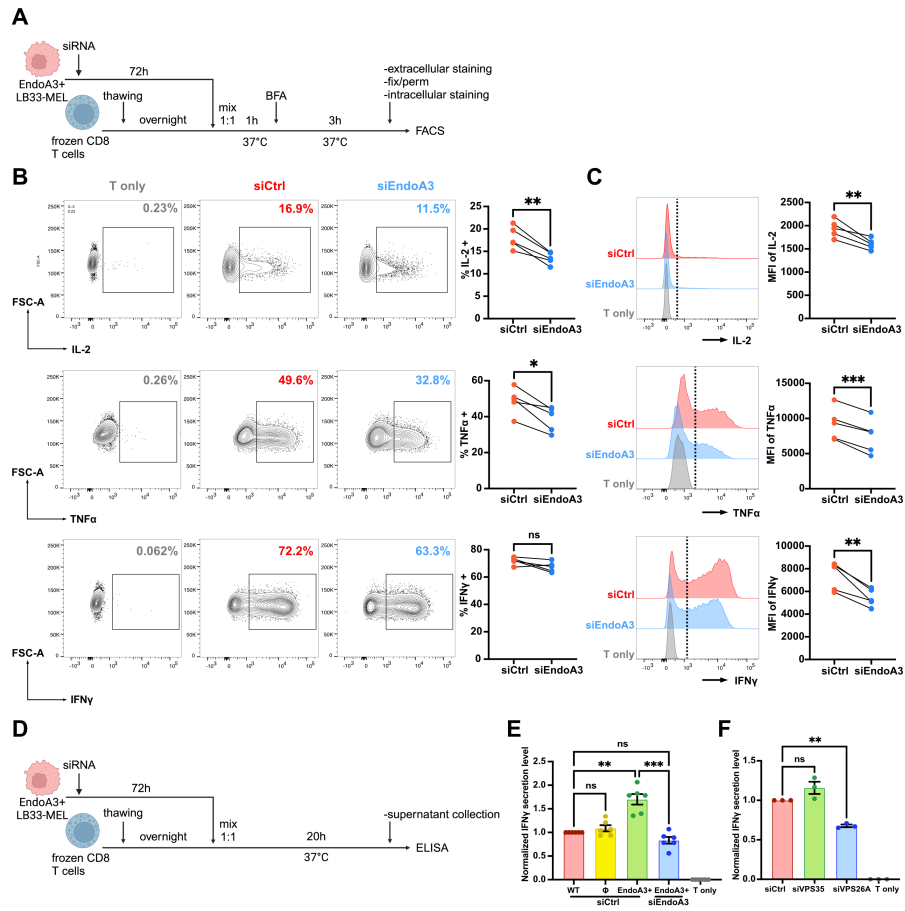


Figure 3. Inhibition of EndoA3-dependent endocytosis and retrograde transport impairs T cell activation

- A Scheme of flow cytometry analysis of cytokine production inside CD8 T cells stimulated by siRNA transfected EndoA3+ LB33-MEL cells.
- B-C Flow cytometry analysis of CD8 T cell intracellular cytokine production after co-culture for 4h at 37°C with: no LB33-MEL (T only), EndoA3+ LB33-MEL transfected with negative control siRNA (siCtrl) or with siRNA against EndoA3 (siEndoA3). Quantification of EndoA3 depletion is shown in Fig EV3A. (B) Representative examples and quantifications (scatter plots) of the percentages of cytokine producing CD8 T cells (up, IL-2; middle, TNF α ; bottom, IFN γ) after being stimulated by siCtrl or siEndoA3 treated LB33-MEL EndoA3+ cells. (C) Representative examples and quantifications (scatter plots) of the absolute amount of cytokines produced by CD8 T cells (up, IL-2; middle, TNF α ; bottom, IFN γ ; presented as median fluorescence intensity, MFI) after stimulation by siCtrl or siEndoA3 treated LB33-MEL EndoA3+ cells.
- D Scheme of ELISA analysis of cytokine secretion from CD8 T cells stimulated with siRNA transfected EndoA3+ LB33-MEL cells.
- E Quantification of IFN γ secretion (detected by ELISA) from CD8 T cells, cultured alone (T only) or co-cultured for 20h with the following LB33-MEL cell lines: wild-type LB33-MEL cells (WT), LB33-MEL cells stably transfected with empty (Φ) or EndoA3-GFP encoding plasmid (EndoA3+), treated with negative control siRNA (siCtrl) or with EndoA3-targeting siRNA (siEndoA3). Data are presented as fractions of WT siCtrl condition. The absolute concentration of secreted IFN γ in the supernatant of WT siCtrl condition is 2203 $\pm$ 201 (mean $\pm$ SEM) pg/mL.
- F Quantification of IFN γ secretion (detected by ELISA) from CD8 T cells, cultured alone (T only) or co-cultured for 20h at 37°C with EndoA3+ LB33-MEL cells transfected with siRNAs: negative control (siCtrl), or against retromer subunits (siVPS35 or siVPS26A). Data are presented as fractions of siCtrl condition. The absolute concentration of secreted IFN γ in the supernatant of siCtrl condition is 4077 $\pm$ 99.62 (mean $\pm$ SEM) pg/mL. Quantification of VPS35 and VPS26A depletion is shown in Fig EV3B.

Data information: In (B-C), data are pooled from five independent experiments. In (E), data are pooled from six independent experiments. In (F), data are pooled from three independent experiments. In (E-F), data are presented as mean $\pm$ SEM. ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001 (B-C, paired *t* test; E, RM one-way ANOVA with Tukey's multiple comparison test; F, RM one-way ANOVA with Dunnett's multiple comparison test). Source data are available online for this figure.

Inhibition of EndoA3-dependent endocytosis affects ICAM1 recruitment to the vicinity of CD8 T cells

The disruption of EndoA3-dependent endocytosis impairs CD8 T cell activation possibly through impaired polarized re-distribution of immune synapse components to the plasma membrane. To verify this possibility, we monitored the recruitment of ICAM1 to the immune synapse with high resolution Airyscan confocal microscopy, as the importance of ICAM1 for T cell activation is already well-established (269). Although we could successfully get LB33-MEL cells transiently transfected with ICAM1-expressing plasmid (Fig 1E), the transfection efficiency was very low, thereby limiting the possibility of using LB33-MEL cells for immune synapse imaging. To address this issue, we generated a stable HLA-A*68012-expressing HeLa cell line. Wild-type HeLa cells lack HLA-A*68012, making them unable to present MUM-3 antigenic peptide to stimulate CD8 T cell activation (Fig EV4A). When exogenous MUM-3 peptide was added to HLA-A*68012-expressing HeLa cells, they successfully stimulated the anti-MUM-3 CD8 T cells to secrete IFN γ (Fig EV4A), as well as to proliferate (Fig EV4B). Thereby, these data together confirmed the functionality of this HLA-A*68012-expressing HeLa cell line.

To monitor the recruitment of ICAM1 to the immune synapse, HLA-A*68012-expressing HeLa cells transiently expressing GFP-tagged ICAM1 were loaded with the MUM-3 peptide and co-cultured with CD8 T cells. Using Airyscan live-cell imaging, we were able to observe ICAM1-containing vesicular structures moving from inside the cells towards the contact area with CD8 T cells and then fusing with it (Fig 4A, white arrowheads; Mov EV6). Moreover, in immunofluorescence experiments using HeLa cells transiently expressing ICAM1-mScarlet, we observed a strong accumulation of ICAM1 signal in the contact zone with CD8 T cells (Fig 4B, EV4C, white arrowheads). Next, we treated ICAM1-mScarlet-expressing HeLa cells with control or EndoA3 siRNAs and incubated them with CD8 T cells (Fig 4C, EV4D). For quantification purposes, this experiment was conducted on cells in suspension where they adopt a regular spherical shape, which are easier to segment by quantification algorithms (Fig 4C, EV4D, phalloidin channel). The ICAM1 recruitment ratio in the vicinity of T cells was measured (see Materials and Methods). Surprisingly, on T cells, the ICAM1 signal was not limited to the contact region of the conjugates, but often expanded to surround the whole T cell (Fig 4C, EV4D). Quantifications revealed that depletion of EndoA3 in HLA-A*68012-expressing HeLa cells induced a slight but significant decrease of ICAM1 recruitment to the vicinity of anti-MUM-3 CD8 T cells (Fig 4D), which aligns with the previously observed impaired T cell activation (Fig 3).

Altogether, these observations suggest that EndoA3-mediated CIE in cancer cells promotes the recruitment of components to the immune synapse, likely by polarized trafficking from the Golgi to the plasma membrane in contact with the T cell.

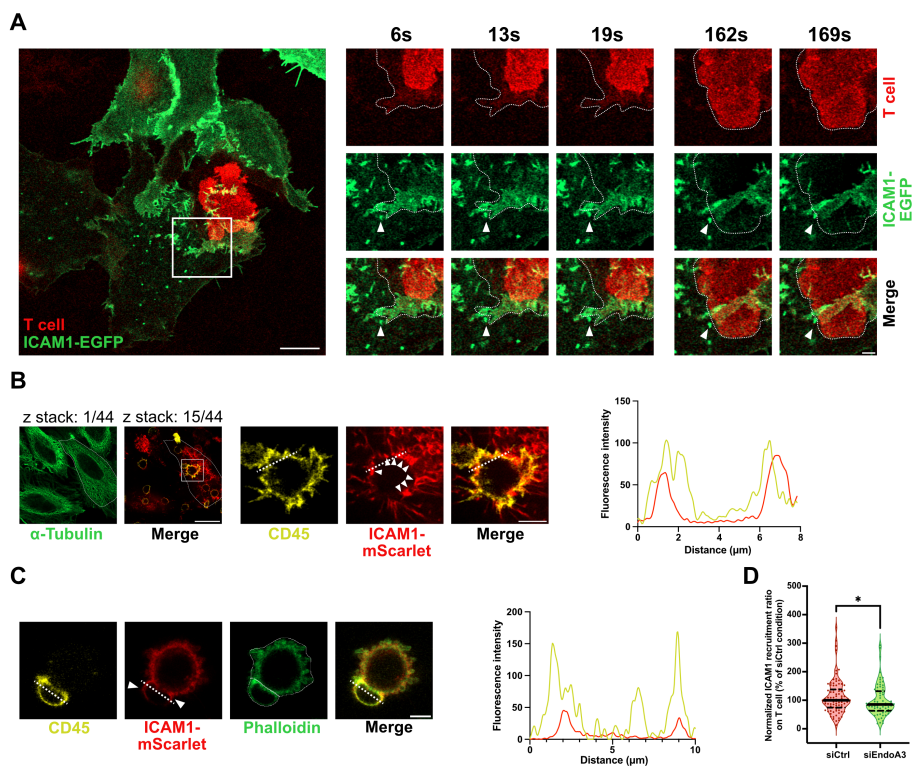


Figure 4. Inhibition of EndoA3-mediated CIE affects ICAM1 recruitment to immune synapses

- A Live-cell Airyscan images of an IS-like conjugate formed between a CD8 T cell (red) and an adherent stable HLA-A*68012-expressing HeLa cell line transiently expressing ICAM1-EGFP (green). Time series show enlarged cropped areas extracted from Mov EV6. The dashed line contours delineate the contact area between the HeLa cell and the CD8 T cell. White arrowheads show two ICAM1-positive vesicular structures moving towards the contact area. Scale bars: 10 μ m (full size image), 2 μ m (enlarged cropped areas).
- B Airyscan images of an IS-like conjugate formed between a CD8 T cell (stained for CD45, yellow) and an adherent stable HLA-A*68012-expressing HeLa cell (stained for microtubules, green) transiently expressing ICAM1-mScarlet (red). White arrowheads indicate recruitment of ICAM1 to the vicinity of CD8 T cell. The fluorescence intensity profile was made along the dashed line region in the enlarged cropped area, and shows enrichment of ICAM1 signal close to the CD8 T cell surface. Scale bars: 20 μ m (full size image), 5 μ m (enlarged cropped areas).
- C Airyscan images of an IS-like conjugate formed between a CD8 T cell (stained for CD45, yellow) and a stable HLA-A*68012-expressing HeLa cell (stained for actins, green) transiently expressing ICAM1-mScarlet (red) in suspension. The fluorescence intensity profile was made along the dashed line region. Cell segmentation (white contour) was based on the actin staining for further quantifications (D). Scale bar: 5 μ m.
- D Quantification of relative ICAM1 recruitment to the vicinity of CD8 T cell when an IS-like conjugate is formed between a CD8 T cell and a stable HLA-A*68012-expressing HeLa cell transiently expressing ICAM1-mScarlet in suspension. HLA-A*68012-expressing HeLa cells were transfected for 72h with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3). Western blot analysis of EndoA3 depletion is shown in Fig EV4E. n conjugates: siCtrl, n = 85; siEndoA3, n = 75.

Data information: In (A) and (C), images are representative of three independent experiments. In (B), images are from a single experiment. In (D), data are pooled from three independent experiments. Data are presented as median and quartiles. *P < 0.05 (Kolmogorov-Smirnov test). Source data are available online for this figure.

CD8 T cells compensate the decreased recruitment of immune synapse components by increasing the size of immune synapses

Lastly, we explored the consequences of EndoA3 or VPS26A inhibition in cancer cells on the morphology of immune synapses. To study this, we imaged immune synapses formed between anti-MUM-3 CD8 T cells and EndoA3-expressing LB33-MEL cells in suspension with Airyscan confocal microscopy (Fig 5A and 5D, EV5A-B). We observed that immune synapses formed between CD8 T cells and EndoA3- or VPS26A-depleted LB33-MEL cells appeared to be larger than those formed between CD8 T cells and control LB33-MEL cells. Quantifications of the size of each immune synapse confirmed this observation, showing that the immune synapses are 24% and 12% larger following the depletion of EndoA3 and VPS26A, respectively (Fig 5B and 5E). The efficiency of siRNA was also validated by Western blot analyses (Fig 5C and 5F). Taken together, these data suggest that CD8 T cells try to compensate the decreased recruitment of the immune synapse components – due to inhibition of CIE or retrograde transport in cancer cells – by increasing the size of the immune synapses. However, despite their larger size, these immune synapses fail to promote CD8 T cell activation effectively (Fig 3, EV3).

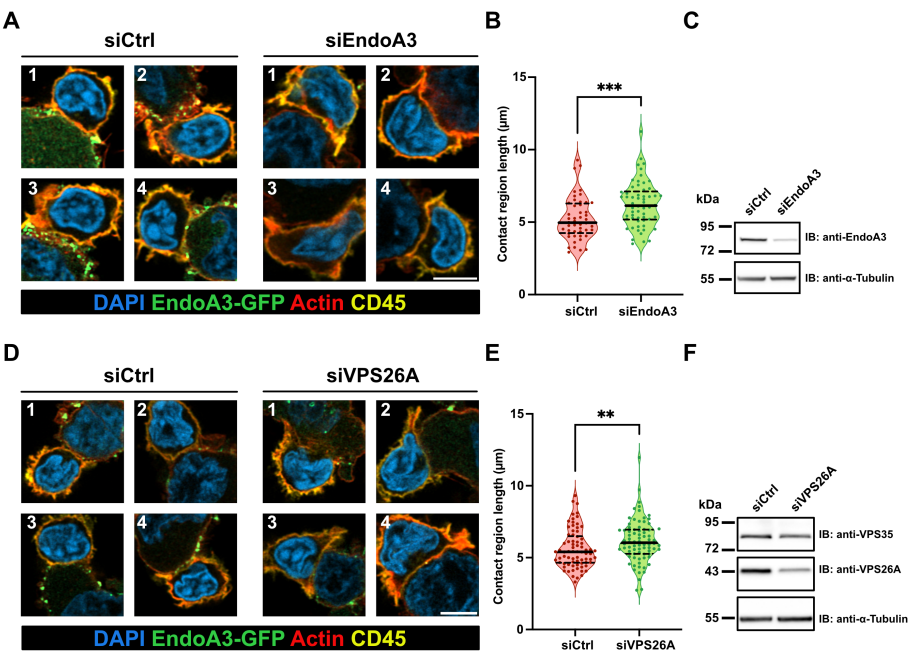


Figure 5. Inhibition of EndoA3-mediated CIE and retrograde transport affects immune synapse morphology

- A, D Airyscan images of IS-like conjugates formed between CD8 T cells (stained for CD45, yellow) and stable EndoA3-GFP-expressing LB33-MEL cells (EndoA3-GFP, green) transfected for 72h with different siRNAs: negative control (siCtrl), siRNAs against EndoA3 (siEndoA3, A) or against VPS26A (siVPS26A, D). Actin (phalloidin, red) and nuclei (DAPI, blue) were also stained. Four examples are displayed per condition. Scale bar: 5 μ m.
- B, E Quantifications of the sizes of IS-like conjugates formed between CD8 T cells and stable EndoA3-GFP-expressing LB33-MEL cells transfected with different siRNAs from images in panels A and D. (B) n conjugates: siCtrl, n = 48; siEndoA3, n = 58. (E) n conjugates: siCtrl, n = 74; siVPS26A, n = 78.
- C, F Western blot analyses of stable EndoA3-GFP-expressing LB33-MEL cells transfected with siRNAs: negative control (siCtrl), siRNAs against EndoA3 (siEndoA3, C) or against VPS26A (siVPS26A, F). In (C), immunodetection was made with an anti-EndoA3 antibody. In (F), immunodetection was made with anti-VPS35 and anti-VPS26A antibodies. In both panels, anti- α -Tubulin was used as a loading control.

Data information: In (A), (C-D) and (F), images are representative of three independent experiments. In (B) and (E), data are pooled from three independent experiments. Data are presented as median and quartiles. **P < 0.01, ***P < 0.001 (Mann-Whitney test). Source data are available online for this figure.

Conclusion

In summary, disruption of EndoA3-mediated CIE and subsequent retrograde transport in cancer cells hinders the recruitment of immune synapse components, such as ALCAM and ICAM1, likely through polarized re-distribution from the TGN to participate in immune synapse formation with autologous CD8 T cells. Consequently, this leads to decreased co-stimulatory signals necessary for effective CD8 T cell activation. Although T cells attempt to compensate for the decreased co-stimulatory signals by expanding their contact area with tumor cells, this compensation is insufficient, ultimately resulting in their impaired activation (Fig 6).

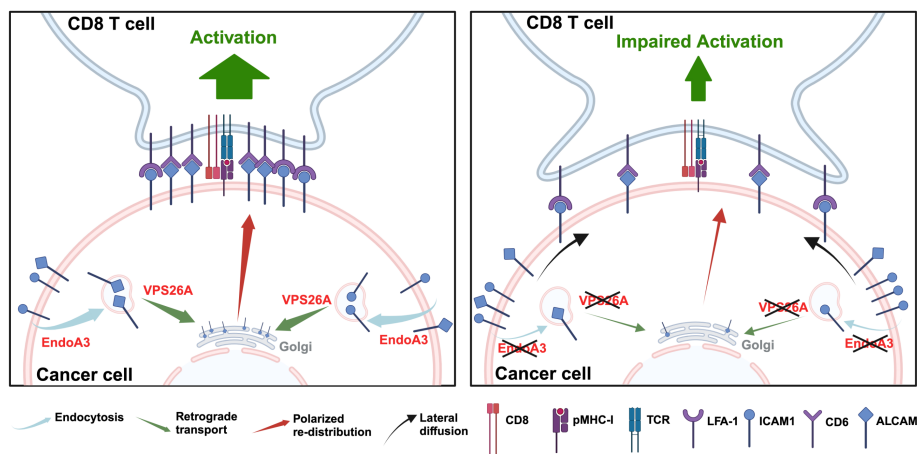
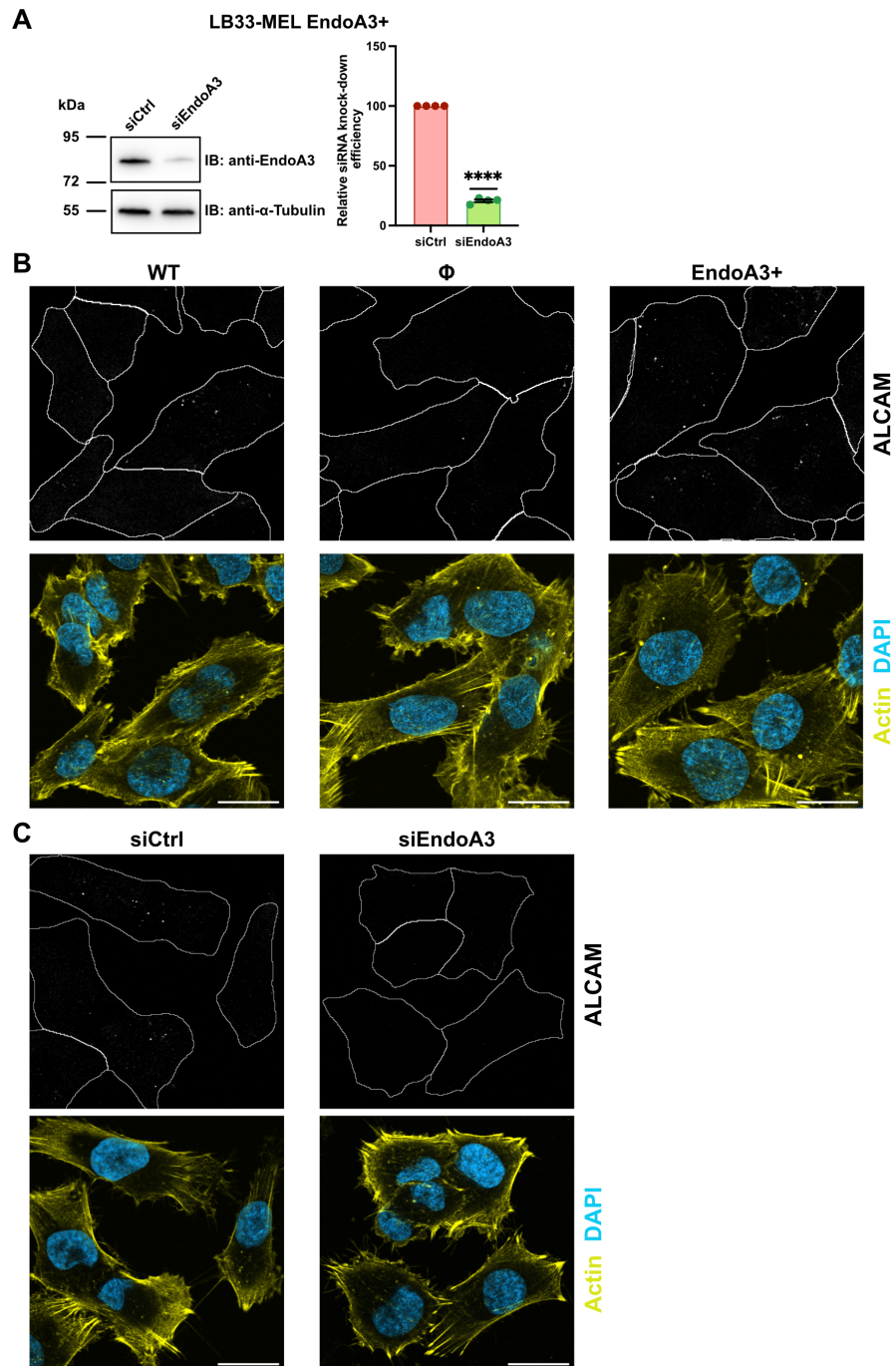


Figure 6. Model depicting how unconventional EndoA3-mediated endocytosis and retrograde transport in cancer cells promote CD8 T cell activation

Immune synapse components ALCAM and ICAM1 are endocytosed into cancer cells through EndoA3-mediated CIE and are subsequently transported to the TGN in a retromer-dependent retrograde manner. Upon immune synapse formation, immune synapse components, such as ALCAM and ICAM1, are efficiently recruited to the contact area with CD8 T cells, likely from the TGN by polarized re-distribution, which can stabilize the immune synapse and promote CD8 T cell activation (left). Consequently, disruption of EndoA3-mediated CIE or retromer-dependent retrograde transport in cancer cells impair the efficient recruitment of immune synapse components to the contact area with CD8 T cells. In this situation, CD8 T cells expand their contact areas with cancer cells to try to compensate the loss of immune synapse components. The compensation is insufficient, which ultimately results in impaired T cell activation (right).

Supplementary figures

Figure EV1



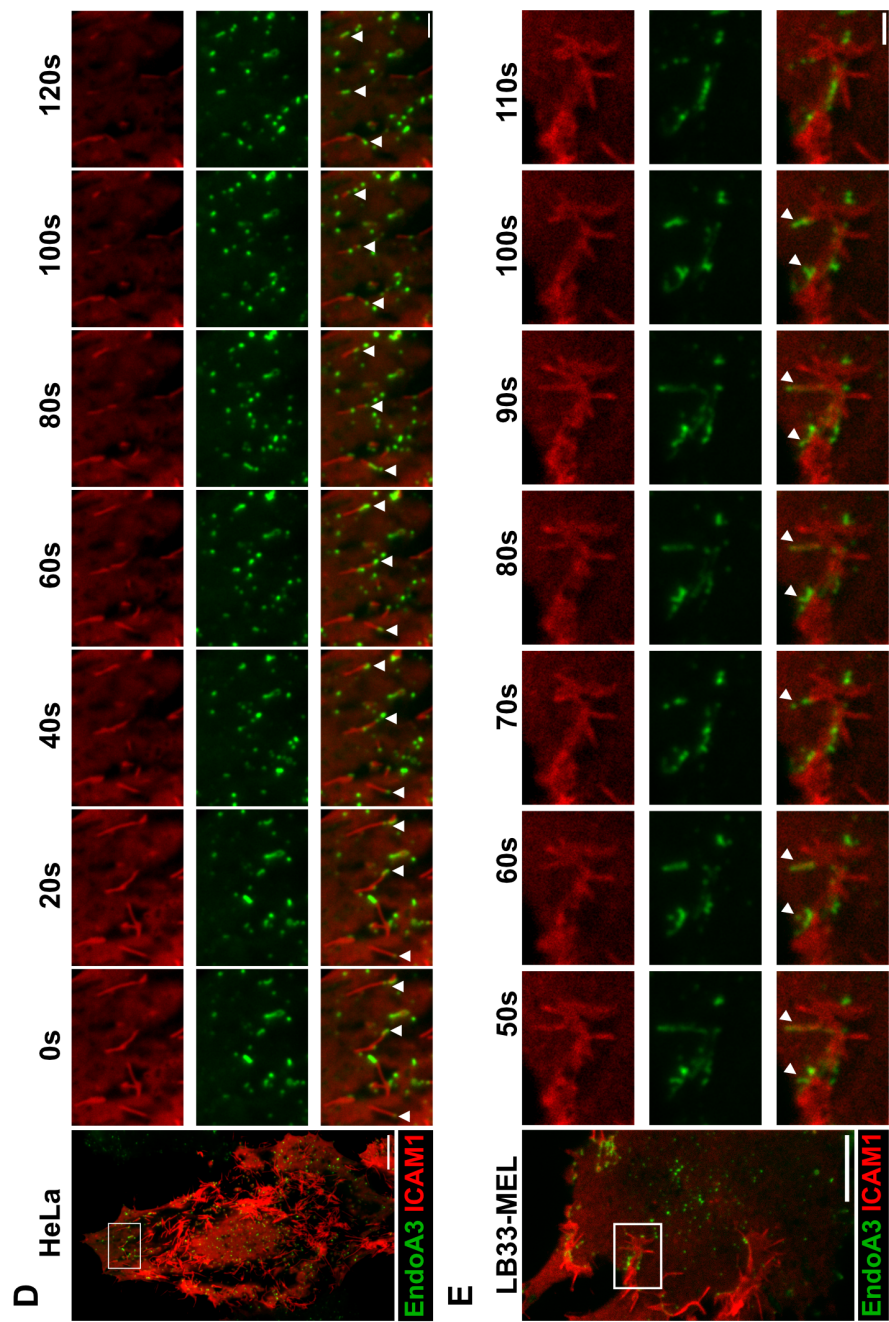


Figure EV1. EndoA3-dependent CIE mediates the uptake of immune synapse components ALCAM and ICAM1 in cancer cells

- A Western blot analysis of LB33-MEL cells stably expressing EndoA3-GFP (EndoA3+ LB33-MEL) transfected for 72h with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3). Immunodetection made with anti-EndoA3 and anti- α -Tubulin (loading control) antibodies. Quantification of immunoblots shows depletion efficiency of EndoA3 (histogram).
- B-C Confocal images of anti-ALCAM (white spots) internalization in LB33-MEL cells stained for actin (phalloidin, yellow) and nuclei (DAPI, blue). In (B), wild-type (WT), stably transfected with empty plasmid (Φ), or stably expressing EndoA3-GFP (EndoA3+) LB33-MEL cells were used. In (C), EndoA3-GFP-expressing LB33-MEL cells were transfected with negative control (siCtrl) siRNA or EndoA3 targeting (siEndoA3) siRNA. Quantification for anti-ALCAM internalization in Fig 1B-C. Scale bars: 20 μ m.
- D-E Other representative live-cell TIRF images of EndoA3-GFP (stable) and ICAM1-mScarlet (transient) in HeLa (D) and LB33-MEL (E) cells. Time series show enlarged cropped areas extracted from Mov EV4 (D) and Mov EV5 (E). White arrowheads indicate dynamic co-distribution of both signals. Scale bars: 10 μ m (full size image), 2 μ m (enlarged cropped area).

Data information: In (A), Western blot images are representative of four independent experiments, from which quantitative data are pooled. Data are presented as mean $\pm$ SEM. **** $P < 0.0001$ (One sample t and Wilcoxon test). In (B-C), images are representative of three independent experiments. In (D-E), images are representative of two independent experiments. Source data are available online for this figure.

Figure EV2

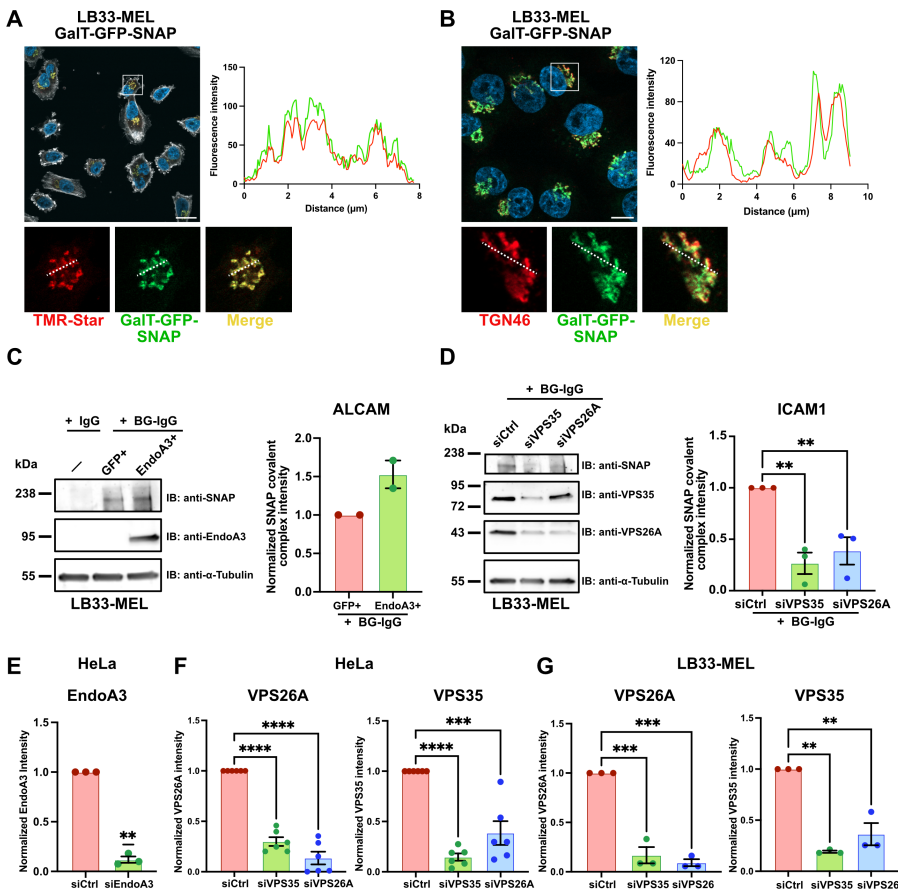


Figure EV2. Immune synapse components ALCAM and ICAM1 are retrograde transport cargoes that rely on retromer

- A Confocal images of GalT-GFP-SNAP (green) and TMR-Star (red) in LB33-MEL cells stably expressing the Golgi-resident GFP-fused SNAP-tag construct (LB33-MEL GalT-GFP-SNAP). Actin (phalloidin, white) and nuclei (DAPI, blue) were also stained. The fluorescence intensity profile was made along the dashed line region in enlarged cropped area, and shows the colocalization of both signals. Scale bar: 20 μ m.
- B Confocal images of GalT-GFP-SNAP (green) and TGN46 (red) in LB33-MEL GalT-GFP-SNAP cells. Nuclei (DAPI, blue) were also stained. The fluorescence intensity profile was made along the dashed line region in enlarged cropped area, and shows the colocalization of both signals, indicating that GalT-GFP-SNAP is correctly localized at the TGN. Scale bar: 10 μ m.
- C-D Retrograde transport of ALCAM and ICAM-1. Continuous BG-labelled anti-ALCAM (C) and anti-ICAM1 (D) antibody uptake for 4h at 37°C in LB33-MEL GalT-GFP-SNAP cells. (C) Western blot analysis of LB33-MEL GalT-GFP-SNAP cells transfected (or not) with plasmids encoding free GFP (GFP+) or EndoA3-GFP (EndoA3+). Immunodetection with anti-SNAP, anti-EndoA3 and anti- α -Tubulin (loading control) antibodies. Quantification of the covalent IgG-SNAP-GFP-GalT complex is shown as fractions of the GFP+ condition (histogram). (D) Western blot analysis of LB33-MEL GalT-GFP-SNAP cells transfected with siRNAs: negative control (siCtrl) or against retromer subunits (siVPS35 and siVPS26A). Immunodetection with anti-SNAP, anti-VPS35, anti-VPS26A and anti- α -Tubulin (loading control) antibodies. Quantification of the covalent IgG-SNAP complex is shown as fractions of siCtrl condition (histogram). Quantification of VPS35 and VPS26A depletion is shown in G.
- E Quantifications of the immunoblots shown in Fig 2C-D (IB: anti-EndoA3) confirm depletion efficiency of EndoA3 in HeLa GalT-GFP-SNAP cells.
- F Quantifications of the immunoblots shown in Fig 2E-F (IB: anti-VPS35 and anti-VPS26A) confirm depletion efficiency of VPS35 and VPS26A in HeLa GalT-GFP-SNAP cells.
- G Quantifications of the immunoblots shown in Fig EV2D confirm depletion efficiency of VPS35 and VPS26A in LB33-MEL GalT-GFP-SNAP cells.

Data information: In (A-B), images are from a single experiment. In (C), data are pooled from two independent experiments. In (D, E and G), data are pooled from three independent experiments. In (F), data are pooled from six independent experiments. Data are presented as mean $\pm$ SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001 (D, F and G, RM one-way ANOVA with Dunnett's multiple comparison test; E, One sample *t* and Wilcoxon test). Source data are available online for this figure.

Figure EV3

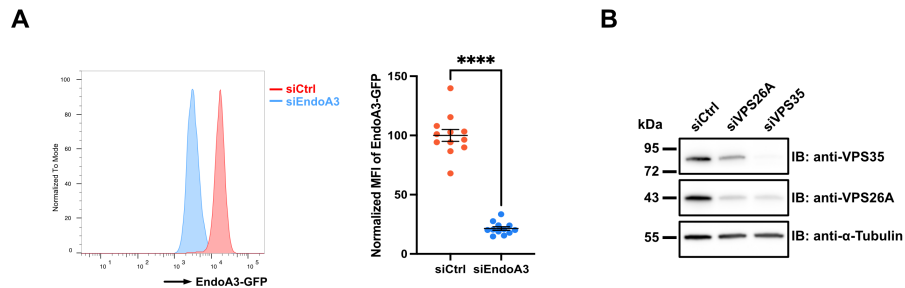


Figure EV3. Inhibition of endoA3-dependent endocytosis and retrograde transport impairs T cell activation

- A** Flow cytometry analysis of LB33-MEL cells stably expressing EndoA3-GFP (EndoA3+ LB33-MEL) transfected with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3) (related to Fig 3B-C). Quantification (scatter plot) based on GFP signal and presented as normalized MFI of GFP relative to siCtrl condition.
- B** Western blot analysis of EndoA3+ LB33-MEL cells transfected with siRNAs: negative control (siCtrl), or against the retromer subunits (siVPS35 or siVPS26A) (related to Fig 3F). Immunodetection with anti-VPS35, anti-VPS26A and anti-α-Tubulin (loading control) antibodies.

Data information: In (A), data are pooled from twelve independent experiments. Data are presented as mean $\pm$ SEM. ****P < 0.0001 (paired *t* test). In (B), the Western blot image is representative of three independent experiments. Source data are available online for this figure.

Figure EV4

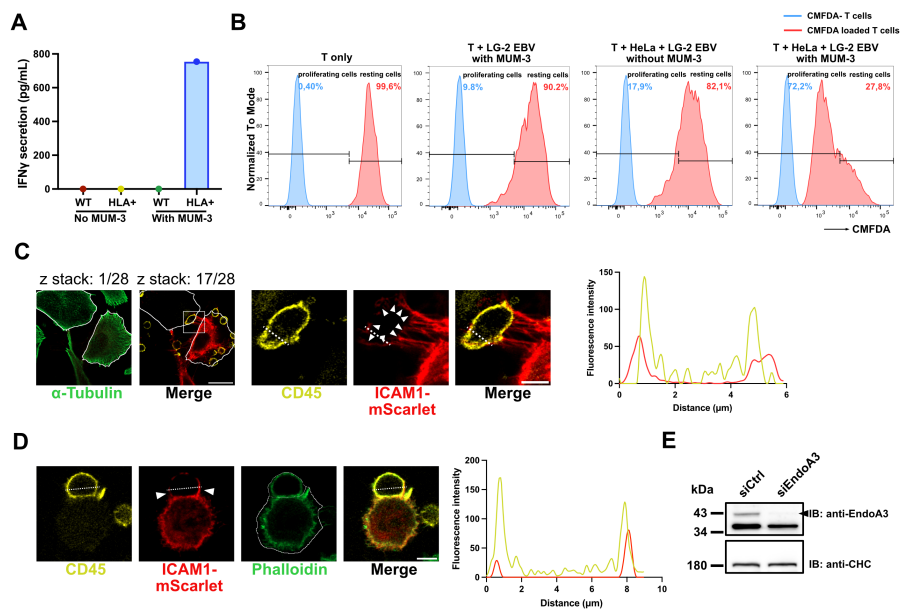


Figure EV4. Inhibition of EndoA3-mediated CIE affects ICAM1 recruitment to immune synapses

- A Quantification of IFN γ secretion (detected by ELISA) from anti-MUM-3 CD8 T cells, co-cultured for 20h with wild-type HeLa cells (WT) or stable HLA-A*68012-expressing HeLa cells (HLA+), with or without the presence of 300 ng/mL MUM-3 antigenic peptide. Data show absolute concentration of secreted IFN γ .
- B Flow cytometry analysis of anti-MUM-3 CD8 T cell proliferation after culture alone, or co-culture with LG-2 EBV feeder cells, or co-culture with both HLA-A*68012-expressing HeLa cells and LG-2 EBV feeder cells, with or without the presence of MUM-3 antigenic peptide, for 4 days. Proliferation was measured by the progressive dilution of CMFDA signal in T cells over generations.
- C Airyscan images of another IS-like conjugate formed between a CD8 T cell (stained for CD45, yellow) and an adherent stable HLA-A*68012-expressing HeLa cell (stained for microtubules, green) transiently expressing ICAM1-mScarlet (red). White arrowheads indicate recruitment of ICAM1 to the vicinity of CD8 T cell. The fluorescence intensity profile was made along the dashed line region in enlarged cropped area. Scale bars: 20 μ m (full size image), 5 μ m (enlarged cropped areas).
- D Airyscan images of another IS-like conjugate formed between a CD8 T cell (stained for CD45, yellow) and a stable HLA-A*68012-expressing HeLa cell (stained for actins, green) transiently expressing ICAM1-mScarlet (red) in suspension. The fluorescence intensity profile was made along the dashed line region. Cell segmentation (white contour) was made based on the actin staining for quantification (Fig 4D). Scale bar: 5 μ m.
- E Western blots analysis of stable HLA-A*68012-expressing HeLa cells transfected with siRNAs: negative control (siCtrl) or against EndoA3 (siEndoA3) (Fig 4D). Immunodetection made with anti-EndoA3 and anti-clathrin heavy chain (CHC, loading control) antibodies.

Data information: In (A-C), data and images are from single experiments. In (D-E), images are representative of three independent experiments.

Figure EV5

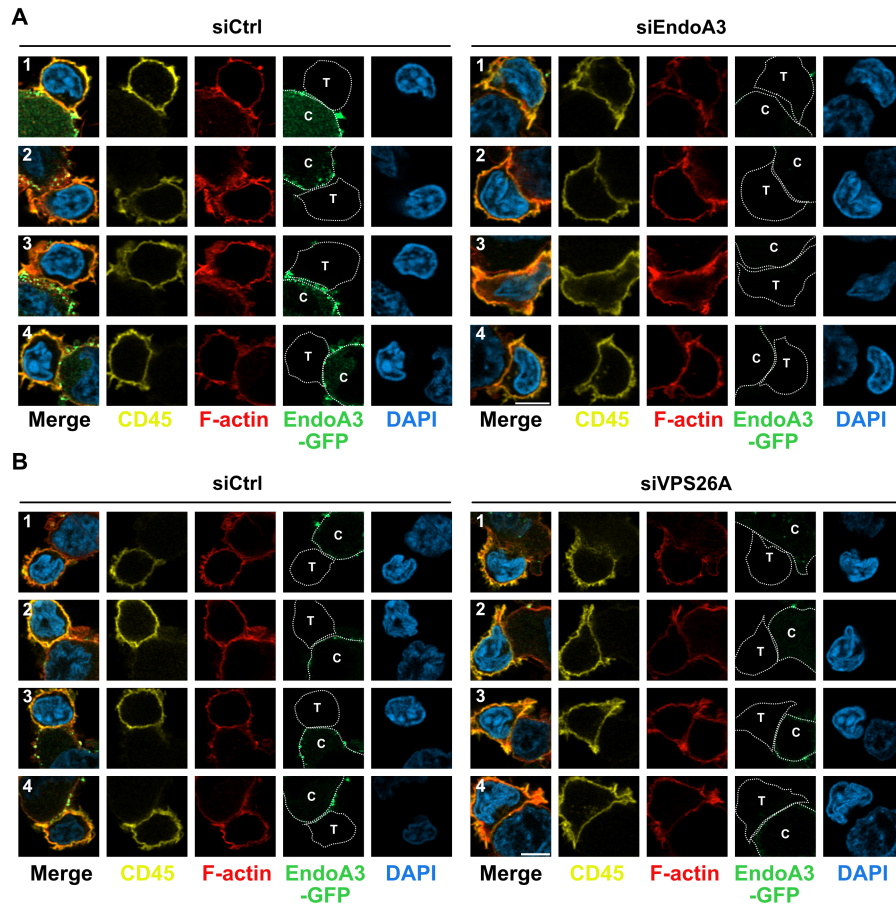


Figure EV5. Inhibition of EndoA3-mediated CIE and retrograde transport affects immune synapse morphology

A-B Separate channels of Airyscan images from Fig 5A and 5D, respectively. CD45 (yellow), EndoA3 (green), actin (red) and nucleus (blue) are shown. C, cancer cell; T, CD8 T cell. Scale bar: 5 μ m.

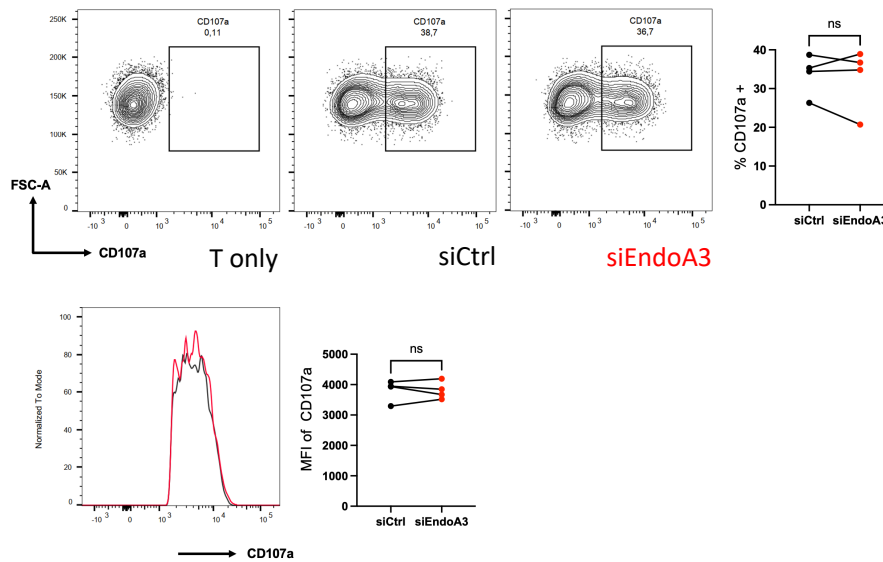
Data information: Images are representative of three independent experiments.

Unpublished results

Initially, when studying whether EndoA3-dependent endocytosis in LB33-MEL cells can affect CD8 T cell activation (see Results, Figure 3), we used degranulation ability of CD8 T cells as an indicator.

EndoA3-expressing LB33-MEL cells were first treated with either control or EndoA3-targeting siRNAs before being co-cultured with CD8 T cells. Cells were then collected and analyzed by flow cytometry to assess the degranulation of CD8 T cells by measuring the surface level of CD107a. CD107a is also known as lysosomal-associated membrane protein 1 (LAMP-1), which is a lysosome marker. When granules, essentially secretory lysosomes, are secreted from CD8 T cells by degranulation, CD107a will be re-located to the cell surface.

Clearly, disrupted EndoA3-mediated endocytosis in LB33-MEL cells did not affect the degranulation ability of CD8 T cells, as neither the percentage of CD8 T cells expressing CD107a on the plasma membrane (Unpublished Figure 1, upper panel) nor the absolute amounts of CD107a expressed on the cell surface (Unpublished Figure 1, bottom panel) changed.



Unpublished Figure 1. Inhibition of EndoA3-dependent endocytosis in LB33-MEL cells does not impair T cell degranulation

Flow cytometry analysis of CD8 T cell degranulation after coculture for 10 min at 37°C with: no LB33-MEL (T only), EndoA3+ LB33-MEL transfected with negative control siRNA (siCtrl) or with siRNA against EndoA3 (siEndoA3). Upper panel, representative examples and quantifications (scatter plots) of the percentages of surface CD107a-expressing CD8 T cells after being stimulated by siCtrl or siEndoA3 treated LB33-MEL EndoA3+ cells. Bottom panel, representative examples and quantifications (scatter plots) of the absolute amounts of CD107a expressed on the surface of CD8 T cells after being stimulated by siCtrl or siEndoA3 treated LB33-MEL EndoA3+ cells.

Data information: data are pooled from four independent experiments. ns, not significant (paired *t* test).

Part III

Discussion

The role of EndoA3 in EndoA3-mediated CIE

In this study, we discovered that ICAM1 is a new cargo of EndoA3-mediated CIE, thus extending the list of previously reported cargoes, the cell surface Ig-like proteins ALCAM and L1CAM (101, 126). Surprisingly, in the first paper of *Boucrot et al.* (2015) reporting the existence of FEME, the internalization of ICAM1 was not affected when all the three EndoA isoforms were depleted. This discrepancy could be explained by the different cell lines used: *Boucrot et al.* (2015) used a human T cell line (Kit255), where EndoA3 might be poorly expressed (111, 113), whereas we used human cancer cell lines (a HeLa cell line with endogenous EndoA3 expression and a melanoma cell line LB33-MEL engineered to stably express EndoA3). In addition, a recent consensus is that FEME is mostly mediated by EndoA2 isoform (4). Hence, our data further support the idea that the different EndoA isoforms are not totally functionally redundant and that EndoA3 mediates an unconventional endocytic modality distinct from FEME, as proposed in a previous study of our lab (101).

Interestingly, all three confirmed EndoA3-dependent endocytic cargoes – ALCAM, L1CAM and ICAM1 – are Ig-like cell adhesion molecules. This indicates that there might exist a cargo recognition pattern for EndoA3-mediated CIE. Unlike CME, where membrane cargoes are recognized by adaptor proteins binding to specific identified sorting signals present in the cytosolic tails of the cargoes (59), the mechanisms for cargo recognition in CIE are emerging only now (5, 290). In FEME, it is believed that EndoA proteins can either directly sort out G protein-coupled receptors (GPCRs) for endocytosis by binding to PRDs in GPCRs with their SH3 domains (100, 291), or indirectly sort out endocytic cargoes by binding to other proteins, such as binding to CIN85 for sorting out EGFR and HGFR (292, 293), and binding to srGAP1 for sorting out ROBO1 (124). However, how EndoA3-dependent endocytic cargoes are recognized and sorted remains unclear. Previous studies from our lab have shown that the cytosolic tails of ALCAM and L1CAM physically interact with EndoA3, but not EndoA2 (101, 126), but specific interaction motifs have not been identified in those two cargoes, yet. It also remains unknown whether the interaction with EndoA3 is direct or indirect, and, if direct, which part of EndoA3 protein is responsible for this interaction. Nevertheless, accumulating evidence showing that Ig-like cell adhesion molecules are preferential EndoA3-dependent cargoes suggest that a shared common recognition pattern might exist in this cargo superfamily, which deserves further investigations.

EndoAs are able to mediate vesicle scission by inserting their amphipathic α -helices into the necks of the endocytic pits or by imposing a frictional force onto the endocytic tubules surrounded by their BAR domains, based on the FDS mechanism (106, 119, 120). It has been confirmed that FDS mechanism is involved in vesicle scission in FEME (119), but whether EndoA3s can promote vesicle scission in EndoA3-mediated endocytosis is yet unclear. However, it is noteworthy that dynamin, which can be recruited by the SH3 domains of EndoAs, are involved in vesicle scission in FEME, but are not required for the EndoA3-mediated endocytosis of ALCAM (101, 119). This

might suggest that, in EndoA3-mediated endocytosis, dynamins are dispensable for vesicle scission, and the scission might be achieved by EndoAs-mediated FDS mechanism or by actin polymerization.

Besides, a striking feature of FEME is that it has a tightly regulated priming cycle to continuously form EndoA patches on the plasma membrane to ensure the prompt endocytosis of activated receptors (121). This priming cycle is initiated by the membrane-bound GTPase Cdc42. Instead, EndoA3-mediated endocytosis is regulated by another GTPase Rac1 (127), which indicates that the priming cycle of EndoA3-mediated endocytosis may be different. Nevertheless, other regulatory mechanisms, such as post-translational regulation, might exist in EndoA3-mediated endocytosis as EndoA3s have several phosphoresidues inside their variable middle regions (113). Future studies will be necessary to explore the regulation mechanisms of EndoA3-mediated CIE.

GL-Lect hypothesis and EndoA3-mediated CIE

According to GL-Lect hypothesis, glycosylated membrane proteins and glycosphingolipids can be clustered by extracellular lectins, such as galectins, to induce membrane invaginations and initiate CIE (see **Box 1**).

ALCAM is the first identified endocytic cargo of EndoA3-mediated endocytosis (101). It is highly glycosylated with a molecular mass of 69 kDa before glycosylation and 105 kDa after glycosylation (265). The previous study from my host lab confirmed that the EndoA3-mediated endocytosis of ALCAM is specifically driven by Galectin-8, which clusters cell surface ALCAM to induce membrane invaginations (101). This suggests that the EndoA3-mediated endocytosis may use GL-Lect mechanism to generate endocytic carriers. However, L1CAM, another cargo of EndoA3-mediated endocytosis, makes things more complicated (126). L1CAM contains 22 potential N-glycosylation sites and is heavily glycosylated. When global galectin binding was inhibited by N-Acetyllactosamine (LacNAc), the apparent endocytosis of L1CAM significantly increased (126). Hence, it seems that the presence of galectins inhibited the endocytosis of L1CAM. Interestingly, L1CAM colocalizes with several galectins, including Galectin-1, -3 and -8, in endocytic compartments (126). A hypothesis for that is, L1CAM, instead of being clustered by galectins, are sequestered by galectin lattices at the plasma membrane, as galectins are able to form lattices at the cell surface (294). When galectin binding is inhibited by LacNAc, the sequestered L1CAM molecules are released from the galectin lattices and are ready to be endocytosed via EndoA3-mediated endocytosis and even other endocytic pathways (126). In the current study, whether the endocytosis of ICAM1 relies on galectins is still an open question.

Retromer complex versus Commander complex

In this study, we confirm that two of the three identified EndoA3-dependent

endocytic cargoes, ALCAM and ICAM1, are retromer-dependent retrograde transport cargoes. This finding for ICAM1 is consistent with previously published proteomic analyses of retromer-dependent cargoes, in which ICAM1 was identified as a target (166).

The retromer complex is one of the two known protein complexes that can retrieve cargoes from endosomes for retrograde transport. The other one is Commander complex, which is composed of the CCC and retriever complexes. Generally, Commander is considered as a complex for retromer-independent endosomal cargo retrieval, though many cargoes can use both complexes (184).

In our study, we confirmed that both ALCAM and ICAM1 are retromer-dependent retrograde transport cargoes. However, even though the depletion of the retromer subunits VPS35 and VPS26A both impaired the retrograde transport of ALCAM and ICAM1 in HeLa cells, the effect of VPS26A depletion was systematically stronger than that caused by the loss of VPS35. Moreover, only the depletion of the retromer subunit VPS26A impaired the activation of CD8 T cells, while VPS35 depletion did not have any significant effect. Western blot analyses showed that the treatment with VPS35 siRNAs causes a significant loss of VPS26A, while the treatment with VPS26A siRNAs induces a relative milder loss of VPS35. Interestingly, it was previously reported that depletion of VPS35 or VPS29 accompanied the degradation of other subunits, whereas depletion of VPS26A had no effect on VPS29 and VPS35 levels. Hence, the authors suggested that VPS29 and VPS35 form a biologically stable sub-complex *in vivo* (295). Our results partially align with their discoveries as the depletion of VPS26A causes milder decrease of VPS35 signal.

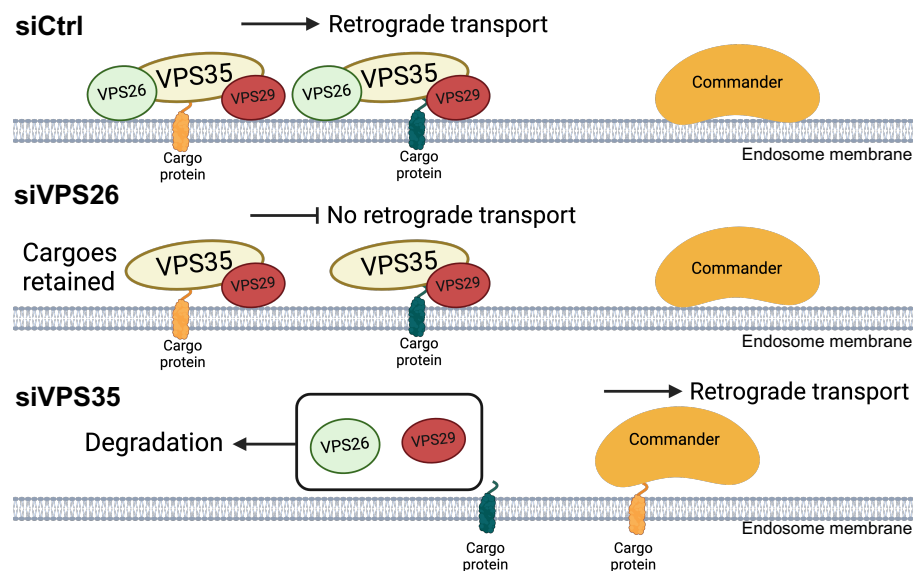


Figure 1. Potential compensatory effects between retromer and

Commander complexes. Some retrograde transport cargoes are preferentially recognized by retromer complex when retromer complex is intact but can also be recognized by Commander complex when retromer complex is gone. The depletion of VPS26A may preserve the VPS29-VPS35 intermediate of retromer complex. In this case, the immune synapse components, such as ALCAM and ICAM1, may remain captured by retromer intermediate but cannot undergo retrograde transport. Hence, Commander complex cannot exert compensatory effect. However, when VPS35 is depleted, the whole retromer complex is degraded and the cargoes are released from retromer. In this case, Commander complex may be able to recognize and capture these cargoes, and the cargoes can still enter the retrograde transport.

Based on these observations, we hypothesize that there may exist a mutual compensatory effect between retromer and Commander complexes for some cargoes (Figure 1). Depletion of VPS35 in HeLa cells caused almost complete loss of retromer complex, which might trigger the compensation by Commander complex for retrograde transport. However, depletion of VPS26A still preserves the VPS29-VPS35 intermediate of retromer complex (295), which might inhibit the compensatory effect by Commander complex via mechanisms remaining to be discovered. This could explain why, in our data, depletion of VPS35 caused a milder effect on the retrograde transport of ALCAM and ICAM1 in HeLa cells and why it did not significantly affect CD8 T cell activation.

As to why, in GalT-GFP-SNAP-expressing LB33-MEL cells, the depletion of VPS35 and VPS26A caused similar effects on the retrograde transport of ICAM1, we hypothesize that it could be because this LB33-MEL cell line does not express detectable levels of endogenous EndoA3, hence leading to much less efficient ICAM1 uptake and less significant compensatory effect from Commander complex. Overall, future investigations on potential mutual compensation between retromer and Commander complexes in the retrograde transport of Ig-like proteins are necessary.

Clathrin-independent endocytosis and retrograde transport

The retrograde transport and subsequent polarized re-distribution of specific molecules from the *Golgi* apparatus is important for cell polarity formation. For instance, in epithelial cells, the retrograde transport of the apical membrane determinant Crumbs regulates apicobasal polarity (296). In migrating cells, the retrograde transport of $\alpha_5\beta_1$ integrin helps localize it to the leading edge, thereby establishing front-rear polarity (207). In T cells, the retrograde transport of LAT, a key organizer of signalosome formation, to the *Golgi* apparatus facilitates its recruitment to the immune synapse, which is also a polarized cellular structure (209). In this study, we further report that in cancer cells, the retrograde transport of immune synapse components ALCAM and ICAM1 promote the activation of CD8 T cells.

While the link between retrograde transport and cell polarity formation is well established (207, 209, 276, 296), the relationship between specific endocytic pathways and retrograde transport within polarized cellular contexts is largely unclear. Here, we found that two of the three identified EndoA3-mediated endocytic cargoes undergo retrograde transport, while the third cargo, L1CAM, remains unexplored. Nevertheless, given that all three cargoes are Ig-like cell adhesion molecules, it is plausible that structural features of specific cargo types may influence their endocytic routes and intracellular trafficking pathways. The Ig-like structure of ALCAM, ICAM1 and L1CAM may predispose them to EndoA3-mediated endocytosis, although the precise mechanism remains to be elucidated. Similarly, this structural characteristic of these Ig-like proteins may make them suitable for retrograde transport via the retromer complex. Thus, the specific endocytic pathways and retrograde transport can be linked. Further experiments are needed to test this hypothesis.

EndoA3-mediated endocytosis and signals for T cell activation

A striking discovery in this study is that the disruption of EndoA3-mediated endocytosis or retromer-dependent retrograde transport in cancer cells significantly impairs the activation of CD8 T cells. Here, we assessed the activation of CD8 T cells by measuring the production and secretion of T cell cytokines. Clearly, signal transduction within these T cells was altered due to the disruptions of trafficking inside the cancer cells, thus leading to impaired T cell activation. However, which specific signal transduction pathway inside the T cells was affected is unclear. During preliminary experiments, we initially measured the degranulation ability of CD8 T cells instead of cytokine production to assess the activation status. Intriguingly, the degranulation ability of CD8 T cells was not affected when stimulated by LB33-MEL cells treated with EndoA3-targeting siRNAs. While it was challenging to interpret these data at that time, upon further scrutiny, we may uncover valuable insights into how disrupted EndoA3-mediated endocytosis and retrograde transport in cancer cells alter signal transduction in CD8 T cells.

The production of cytokines within T cells needs multiple signals. The most important one is the TCR signaling, which eventually activates downstream transcription factors NFAT, NF- κ B and AP-1 (activator protein 1) to initiate the expression of genes encoding for different cytokines (225, 297). Co-stimulatory signals are also important for cytokine production. CD28-mediated co-stimulatory signal amplifies the gene expression patterns initiated by TCR signaling (298), while the absence of this signal leads to T cell anergy (247). Besides, adhesion molecules are also involved in cytokine production. The engagement of LFA-1 with ICAM1 can not only increase T cell sensitivity to antigen and lower T cell activation threshold (251), but also directly promote IL-2 production (299, 300).

Unlike cytokines that need to be *de novo* synthesized within activated CD8 T cells, granules are preformed and are ready to be exocytosed. The

degranulation contains two steps. The first step is the polarization of microtubule-organizing center (MTOC) towards the immune synapse, so that granules can be transported along the microtubules to the immune synapse. MTOC is located in the uropod of migrating T cells. Upon the CD8 T cells encountering the target cells and initiating TCR signaling, the MTOC moves towards the immune synapse and eventually docks at the plasma membrane next to the cSMAC (211, 256). The second step of degranulation is exocytosis, where granules first tether to and then fuse with the plasma membrane. This fusion event releases the contents of the granules, including cytotoxic proteins like perforin and granzymes, into the narrow space between the CD8 T cell and the target cell. The two steps of degranulation require different signaling pathways. The polarization of MTOC requires the TCR-PLC γ 1-DAG-PKC signaling axis, which is Ca²⁺ independent, while the exocytosis of granules requires the TCR-PLC γ 1-IP₃ signaling axis, which is Ca²⁺ dependent (301, 302). However, both pathways originate from the TCR signaling.

As our observations showed that T cell degranulation was unchanged upon disruption of EndoA3-mediated endocytosis in cancer cells, it suggests that the TCR signaling pathway in T cells was not affected. Instead, it is the co-stimulatory and the adhesion molecule-mediated signaling pathways that might be affected. This hypothesis is consistent with our current data. In this project, we focused on two cell surface adhesion molecules, ALCAM and ICAM1, to study EndoA3-mediated endocytosis and retrograde transport. The receptors of these two molecules, CD6 and LFA-1, respectively, are both expressed on T cells, and both participate in immune synapse formation and T cell activation (251, 268, 287, 299, 300, 303-307). Thereby, it is plausible that disrupted EndoA3-mediated endocytosis and retrograde transport in cancer cells didn't impair the recruitment of MHC-I to the immune synapse. Instead, it impaired the recruitment of several adhesion molecules, such as ALCAM and ICAM1, and maybe also some ligands for co-stimulators, thus affecting the cytokine production within the CD8 T cells.

EndoA3 expression in cancer cells

In our study, we found that the expression of EndoA3 in LB33-MEL cells made them more vulnerable to CD8 T cell killing, as it promotes CD8 T cell activation. Hence, cancer cells may exploit this mechanism to avoid T cell killing by losing the expression of EndoA3. Several observations confirmed this possibility. For instance, the expression level of EndoA3 in glioma decreases as the grade of glioma increases (308). Comparing with World Health Organization (WHO) grade II and III gliomas, glioblastoma (a type of glioma, WHO grade IV) expresses the lowest level of EndoA3. Meanwhile, a high expression level of EndoA3 in glioma is correlated with a good prognosis and better survival of glioma patients (308).

However, many cancer cells still express high levels of EndoA3 in despite of possible enhanced T cell killing. In fact, when first reporting the existence of a unique EndoA3-mediated endocytosis, my host lab discovered that in U2OS

cells, the depletion of EndoA3 inhibited the migration of cancer cells. This inhibitory effect is related to the impaired endocytosis of ALCAM (101). Considering that ALCAM is a cell adhesion molecule, it is highly possible that EndoA3-mediated endocytosis promotes cancer cell migration by lowering the surface level of cell adhesion molecules. In this way, the expression of EndoA3 in cancer cells can be a beneficial factor. There are also observations supporting this possibility. Still in gliomas, EndoA3 expression is linked with cancer cell invasion, and the knockdown of EndoA3 inhibited the invasion (309). Similarly, compared with chemo-sensitive myeloma cells, chemo-resistant myeloma cells express much higher amounts of EndoA3 and display higher migration/invasion capability. Conversely, silencing SH3GL3 gene (encoding for EndoA3) using shRNA reduced myeloma cell migration/invasion (310). Hence, in general, the expression of EndoA3 in cancer cells can also be a marker of bad prognosis, which has been confirmed for melanoma patients (311).

To sum up, based on our findings, it looks like the expression of EndoA3 in cancer cells can be a double-sided sword. On one hand, EndoA3 expression increases cancer cell migration capacity. On the other hand, EndoA3 expression in cancer cells make them more immunogenetic. However, the real situation may be much more complicated, as discrepant data exist in literature. For instance, an analysis of gene expression profiles between primary tumors of oral squamous cell carcinoma with or without metastases in lymph nodes revealed that EndoA3 expression significantly decreased in the metastatic group compared to the non-metastatic group (312). Similarly, the overexpression of EndoA3 decreases the migration of lung cancer cells and glioblastoma cells (308, 313).

How can these contradictory observations be explained? According to the study from my host lab, the inhibitory effect on cancer cell migration caused by the depletion of EndoA3 in U2OS cells is tightly dependent on the presence of ALCAM (101). When ALCAM is absent, the depletion of EndoA3 has no significant effect on cancer cell migration (101). Thus, it seems that EndoA3 promotes the migration of cancer cells by lowering the surface level of cell adhesion molecule ALCAM via EndoA3-mediated endocytosis. If this hypothesis is correct, then evaluating the expression levels of EndoA3 in cancer cells alone may not be sufficient to correctly predict the prognosis of cancer patients, especially to predict the metastasis of the cancers. The surface expression levels of ALCAM and probably even other cell adhesion molecules, specifically the ones that are confirmed to be EndoA3-dependent endocytic cargoes, *i.e.* L1CAM and ICAM1, should also be taken into account. Similarly, several studies attempted to correlate ALCAM expression in cancer cells with patient prognosis, also yielding contradictory results. In melanoma, oral squamous cell carcinoma, and non-small-cell lung cancer, the overexpression of ALCAM is linked with poor prognosis and shorter survival time of patients (314-316), while in colorectal cancer and breast cancer, the loss of surface expression of ALCAM is associated with poor prognosis (317, 318). More consistent conclusions may be drawn if the expression levels of EndoA3 are considered.

Endophilin A and cancer therapy

Modulating endocytosis is vital to achieve desirable anti-tumor effects in targeted therapies. On one hand, inhibiting endocytosis can benefit anti-tumor therapy in some cases. For example, Rituximab is a monoclonal antibody that targets CD20 on B cells. It is used for treating B-cell malignancies by inducing antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells and subsequent clearance of malignant B cells. However, some types of B cell lymphomas are unresponsive to Rituximab treatment because of rapid internalization of Rituximab-CD20 complex from the membrane and weak ADCC-mediated killing of malignant B cells (319). Inhibiting Rituximab-CD20 complex endocytosis can overcome the resistance to Rituximab therapy (320). On the other hand, boosting endocytosis is necessary for ideal intracellular delivery of anti-tumor drugs with antibody-drug conjugates (ADCs) (321) and for downregulating overexpressed tumor markers that promote tumorigenesis (101, 126, 322-324).

Human epidermal growth factor receptor 2 (HER2) is a member of the EGFR family of receptor tyrosine kinases that is overexpressed in several types of breast cancers and promotes the proliferation of tumor cells (325). Trastuzumab is a monoclonal antibody that is used for anti-HER2+ breast cancer treatment. It can bind to HER2 to induce its internalization and/or degradation and can mediate ADCC for tumor clearance (325-327). Recently, it was found that EndoA2 is highly expressed in HER2+ breast cancers and is associated with poor prognosis (326). The depletion of EndoA2 decreases HER2+ cancer cell migration and invasion *in vitro*, but it also impairs HER2 internalization in response to Trastuzumab and reduces cytotoxicity response in HER2+ cancer cells treated with Trastuzumab-emtansine (T-DM1), an ADC (326). Hence, a temporary and reversible inhibition of EndoA2 might be useful in such dilemma. Indeed, temporary inhibition of endocytosis can enhance monoclonal antibody based anti-tumor therapy via increased ADCC, while upon the reversal of inhibition, ADC mediated cytotoxicity response will commence and will be enhanced because of increased ADC binding on the membrane surface during endocytosis inhibition (328). Here, in this case, temporary and reversible inhibition of EndoA2 may not only boost Trastuzumab mediated ADCC and T-DM1 mediated cytotoxicity, but also limit the metastasis of HER2+ cancer cells.

Another potential anti-tumor therapy directly targeting EndoAs may lie in immunotherapy. It is reported that EndoA2 deficiency can protect rodents from T cell-dependent autoimmune diseases, like autoimmune arthritis and experimental autoimmune encephalomyelitis (EAE), by reducing T cell activation (329). It is thus tempting to speculate that enhancing anti-tumor T cell activation by modulating EndoA2 expression may be achievable.

Anti-tumor therapies targeting EndoAs-mediated endocytic cargoes have also been explored. Efficient anti-tumor drug delivery has been achieved by using anti-ALCAM single-chain antibody fragment (scFv) in prostate cancer cells (330) and anti-HER2 and anti-EGFR scFv in breast cancer cells (331, 332).

In these cases, enhancing EndoAs-mediated endocytosis may be beneficial for anti-tumor therapy.

Part IV

Perspectives

In this PhD project, we discovered that disruption of clathrin-independent EndoA3-mediated endocytosis and subsequent retrograde transport in cancer cells impairs autologous CD8 T cell activation. This finding further supports the idea that EndoA3 expression in cancer cells may serve as a positive prognostic marker for cancer patients. However, given that EndoA3 expression may also facilitate cancer cell migration, how best to modulate EndoA3-mediated endocytosis to achieve optimal anti-tumor effects in targeted therapies remains an open question.

The exact mechanisms by which EndoA3-mediated endocytosis in cancer cells impacts CD8 T cell activation are not yet fully understood. Current data suggest that impaired EndoA3-mediated endocytosis and subsequent retrograde transport in cancer cells may hinder the recruitment of immune synapse components, such as ALCAM and ICAM1, which are essential for immune synapse formation. This may partially explain why CD8 T cell activation is affected. Notably, the disruption of EndoA3-mediated endocytosis impacted only cytokine production in CD8 T cells, while their degranulation ability remained intact. Thus, the extent to which EndoA3-mediated endocytosis in cancer cells contributes to CD8 T cell activation warrants further investigation.

Finally, it would be valuable to delve deeper into the mechanisms underlying EndoA3-mediated endocytosis and retrograde transport. Are there additional EndoA3-dependent endocytic cargoes? How are these cargoes selectively sorted into this pathway? Do they share structural characteristics, and what are their intracellular fates following endocytosis? Why is the retromer complex critical for EndoA3-mediated endocytosis and the redistribution of cargoes? Is the Commander complex also involved? Addressing these questions may enhance our understanding of EndoA3-mediated endocytosis in T cell activation and its potential implications for anti-tumor therapies.

Part V

Materials and Methods

Cell culture

Wild-type HeLa cell line was from ATCC®. GalT-GFP-SNAP-expressing HeLa cell line was generated in a previous study (333). EndoA3-GFP-expressing HeLa cell line was previously generated by our lab (101). HLA-A*68012-expressing HeLa cell line was generated for the current study. Wild-type LB33-MEL cell line was derived from a melanoma patient in 1988 in de Duve Institute (272). EndoA3-GFP-expressing and GalT-GFP-SNAP-expressing LB33-MEL cell lines were generated for the current study. Anti-MUM-3 CD8 T cells were derived from the same melanoma patient in 1990 in de Duve Institute (272). Epstein-Barr virus transformed B cells from cancer patient LG-2 (LG-2 EBV) were obtained as described (334).

Wild-type HeLa cells were cultured at 37°C under 5% CO₂ in high glucose DMEM (Gibco, 41966-029) supplemented with 10% FBS (Dulis, 500105N1N), 100 U/mL penicillin and 100 µg/mL streptomycin (Penicillin-Streptomycin, Gibco, 15140-122). EndoA3-GFP-expressing, GalT-GFP-SNAP-expressing, and HLA-A*68012-expressing HeLa cells were cultured at 37°C under 5% CO₂ in high glucose DMEM (Gibco, 41966-029) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.5 mg/mL G418 (Roche, 108321-42-2). WT LB33-MEL cells were cultured at 37°C under 5% CO₂ in IMDM (Gibco, 12440-053) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.5 mM GlutaMAX I (Gibco, 35050-038), and 0.05 mM β-mercaptoethanol (Gibco, 21985-023). Empty plasmid stably transfected (Φ), EndoA3-GFP-expressing, and GalT-GFP-SNAP-expressing LB33-MEL cells were cultured in the same medium as WT LB33-MEL cells but supplemented with 0.7 mg/mL G418 (Roche, 108321-42-2). Anti-MUM-3 CD8 T cells were freshly thawed from cryovials and cultured at 37°C under 5% CO₂ in IMDM (Gibco, 12440-053) supplemented with 10% HS (Ludwig Institute for Cancer Research (LICR), Brussels Branch), 100 U/mL penicillin, 100 µg/mL streptomycin, 1.5 mM GlutaMAX I, and extra 5 U/mL DNase I (STEMCELL Technologies, 07469) and 50 U/mL IL-2 (PROLEUKIN®, CNK 1185-958) for at least 4 h before usage. For culture, T cells were maintained with the same medium, but without DNase I. LG-2 EBV cells were cultured at 37°C under 8% CO₂ in IMDM (Gibco, 21980-032) supplemented with 10% FBS (Gibco, A5256701), 100 U/mL penicillin, 100 µg/mL streptomycin and 1.5 mM GlutaMAX I (Gibco, 35050-038).

DNA constructs and transfection

Plasmids used in this study are listed below:

Protein	Backbone	Tag	Source
EndoA3	pIRESneo2	GFP	F. Tyckaert, LIBST, BE
/	pIRESneo3	/	Clontech
ICAM1	pLV-mScarlet	mScarlet	J. D. Van Buul, Amsterdam UMC, NL
ICAM1	pEGFP-N1	EGFP	Generated in the lab for this study
GalT-GFP-SNAP	pSNAP-tag	GFP	L. Johannes, Institut Curie, FR
/	pEGFP-N1	EGFP	Clontech
HLA-A*68012	pcDNA3	/	P. van der Bruggen, de Duve Institute, BE

C-terminally EGFP-tagged ICAM1 expression plasmid was generated in the current study for live-cell imaging. ICAM1 sequence was first amplified by PCR from the aforementioned mScarlet-tagged ICAM1 expression plasmid, and then inserted into the pEGFP-N1 vector by Gibson Assembly® (New England Biolabs). To do so, the vector was linearized with the restriction enzyme EcoR I, and corresponding overlapping sequences were added to both ends of ICAM1 inserts by PCR (forward primer: 5'-CGAGCTCAAGCTTCGATGGCTCCAGCAGC-3'; reverse primer: 5'-TACCGTCGACTGCAGGGGAGGCGTGGCTTG-3'). The Gibson assembly product was then amplified in *E. coli* DH10B strain and validated by sequencing.

For immunofluorescence and live-cell imaging experiments, plasmids were transfected with FuGene HD (Promega), according to the manufacturer's instructions. Cells were used for experiments 16–24 h after transfection.

To generate the stable HLA-A*68012-expressing HeLa cell line, the pcDNA3-HLA-A*68012 plasmid was transfected into wild-type HeLa cells by nucleofection (Amaxa Nucleofector 2b, Lonza) according to the built-in protocol. Transfected cells were cultured for 2 days. A selection pressure was then imposed by supplementing the culture medium with 1 mg/mL G418 for 7 days. Cells were then harvested and maintained with 0.5 mg/mL G418 in the culture medium.

For the generation of different stable LB33-MEL cell lines, 300,000 wild-type LB33-MEL cells were seeded in 2 mL culture medium. 4 µg of corresponding plasmid (pIRESneo3 for LB33-MEL Φ generation, pIRESneo2-EndoA3-GFP-FKBP for EndoA3-GFP-expressing LB33-MEL generation, and pGalT-GFP-SNAP-tag for GalT-GFP-SNAP-expressing LB33-MEL generation) were added to 200 µL of Opti-MEM (Gibco), followed by addition of 24 µL of FuGENE HD (Promega) and gentle mixing. The mix was incubated for 15 min at room temperature, and then added to the cells. After 2 days of culture, transfected cells were selected by supplementing the culture medium with 0.7 mg/mL G418 for 7–15 days. Cell sorting based on GFP fluorescence was then conducted with the FACS Aria flow cytometer (BD Biosciences) to yield a 100%

positive stable EndoA3-GFP-expressing LB33-MEL cell line. For LB33-MEL cells transfected with GalT-GFP-SNAP-expressing plasmid, the homogeneity of cells was checked on a FACSVerse at the end of selection, and all cells were GFP-positive. Stable LB33-MEL Φ , EndoA3-GFP-expressing LB33-MEL and GalT-GFP-SNAP-expressing LB33-MEL cell lines were then maintained with continuous presence of 0.7 mg/mL G418 in the culture medium.

RNA interference

siRNAs were used to knock down target molecules in HeLa and LB33-MEL cell lines at a total final concentration of 40nM. For HeLa cells, 150,000 cells were seeded per well of a 6-well plate for each siRNA condition, and siRNA transfection was conducted with 1 μ L Lipofectamine RNAiMAX (ThermoFisher). For LB33-MEL cells, 200,000 cells were seeded per well of a 6-well plate for each siRNA condition, and siRNA transfection was conducted with 2 μ L Lipofectamine RNAiMAX (ThermoFisher). All experiments were done 72 h after siRNA transfection. All siRNAs were purchased from QIAGEN, and their sequences are listed as follows:

Name	Reference	Sequence
Hs SH3GL3_5	SI04170376	CCAGACGAGAATACAAGCCAA
Hs SH3GL3_6	SI04176529	CCAGACGAAGAAGTCAGACAA
Hs VPS26A_1	SI03122273	AAAGGTAAACCTAGCCTTTAA
Hs VPS26A_2	SI04197914	TGCCACCTATCCTGATGTTAA
Hs VPS35_7	SI04287605	CAGAATTGCCCTTAAGACTTT
Hs VPS35_8	SI04316914	TTGCTGCATCCAAACTTCTAA
AllStars Negative Control	SI03650318	NA

T cell proliferation assay

HLA-A*68012 expressing HeLa cells were detached with PBS-EDTA 2 mM and resuspended to 1,000,000 cells/mL in the culture medium supplemented with or without MUM-3 peptide (EAFIQPITR) at 300 ng/mL. Then, HeLa cells and LG-2 EBV feeder cells were γ -irradiated for 45min (100Gy), washed, and maintained in T cell culture medium until the initiation of co-culture with T cells. The anti-MUM-3 CD8 T cells were thawed the day before and were harvested, washed, and resuspended in complete T cell culture medium with 0.5 μ M CMFDA (Invitrogen™, C2925) for 20 min at 37°C. T cells were then washed and resuspended to 300,000 cells/mL in T cell medium supplemented with 50 U/mL IL-2. In an ultra-low attachment round bottom 96-well plate (Corning® Costar®, CLS7007), 30,000 anti-MUM-3 CD8 T cells were seeded with 10,000 HeLa cells and 90,000 γ -irradiated LG-2 EBV feeder cells. The plate was quickly centrifuged and then incubated for 4 days at 37°C with 5% CO₂. Cells were then transferred on ice for antibody staining and flow cytometry analysis.

Flow cytometry data were acquired with a LSR Fortessa (BD Biosciences) and were analyzed using FlowJo (v10.8.1) software.

IFN γ secretion assay

CD8 T cells were thawed and cultured as described above. siRNA transfected LB33-MEL cells were harvested 72 h after the siRNA transfection and were resuspended to 300,000 cells/mL in T cell medium. CD8 T cells were then washed and resuspended to 300,000 cells/mL in fresh T cell medium supplemented with 50 U/mL IL-2. 100 μ L of LB33-MEL cells and 100 μ L of T cells were pooled together in a 96-well plate and were co-cultured for 20 h at 37°C under 5% CO₂. A technical triplicate was made for each siRNA condition. The supernatant was collected the next day for ELISA analysis.

ELISA plate was coated with 50 μ L IFN γ monoclonal coating antibody solution (Invitrogen, AHC4432; 1:250 dilution in PBS) in each well and was incubated at 4°C overnight. On the next day, two standard IFN γ concentration ladders were prepared: 1:2 dilutions for 7 successive times with T cell medium in 96-well plate (original concentration of 10,000 pg/mL; the last well contains only medium). The ELISA plate was washed with washing buffer (0.154 M NaCl solution with 0.1% Tween-20) twice and with water once to remove unbound antibody. 50 μ L of supernatant and standard IFN γ ladders were then added to the ELISA plate, followed by the addition of 50 μ L IFN γ monoclonal detecting antibody solution (biotin conjugated; Invitrogen, AHC4539; 1:1000 dilution in T cell medium) into each well. The plate was incubated on a shaker at 37°C for 2 h, and then was washed again. 50 μ L of Streptavidin-Peroxydase solution (Sigma, S5512; 1:1000 dilution in PBS containing 0.5% bovine serum albumin) was added to each well and the plate was again incubated at 37°C for 30 min. Then, the plate was washed and 100 μ L of 1-Step Ultra TMB-ELISA Substrate Solution (ThermoFisher, 34028) was added to each well. When the blue color of TMB stopped getting darker, 20 μ L of sulfuric acid (1 M) was added to each well to stop the reaction (the blue color turned yellow). The absorbance of substrate solution at 450 nm was then measured with an ELISA Analyzer (Bio-Rad Microplate Reader) and the IFN γ concentration was determined.

Degranulation assay

CD8 T cells were thawed and cultured as described above. LB33-MEL cells were harvested 72 h after siRNA transfection and resuspended to 2,000,000 cells/mL in T cell medium. T cells were then washed and resuspended to 1,000,000 cells/mL in fresh T cell medium supplemented with 50 U/mL IL-2. 50 μ L LB33-MEL cells and 100 μ L T cells were pooled together in a 96-well “U” bottom plate. 50 μ L PE-Cy7-conjugated anti-CD107a antibody solution (10 μ L antibody plus 40 μ L T cell culture medium) was added to each well and the plate was incubated at 37°C under 5% CO₂ for 10 min. Cells were then transferred to a 96-well “V” bottom plate and washed with 150 μ L/well of staining buffer (PBS with 1mM EDTA and 1% HS). The plate was quickly spun at 400 $\times$ g for 3 min and supernatant was removed. 50 μ L of extracellular staining antibody solution for CD8 and viability marker prepared with staining buffer were added to each well and mixed. The plate was then maintained at

4°C for 20 min in the dark. Afterwards, cells were washed with 150 µL/well PBS and resuspended in 250 µL staining buffer for flow cytometry analysis.

Flow cytometry data were acquired with a FACSVerse (BD Biosciences) and were analyzed using FlowJo (v10.8.1) software.

Quantification of intracellular cytokines in CD8 T cells

CD8 T cells were thawed and cultured as described above. LB33-MEL cells were harvested 72 h after siRNA transfection and resuspended to 1,000,000 cells/mL in T cell medium. T cells were then washed and resuspended to 1,000,000 cells/mL in fresh T cell medium supplemented with 50 U/mL IL-2. 100 µL LB33-MEL cells and 100 µL T cells were pooled together in a 96-well “U” bottom plate and were co-cultured at 37°C under 5% CO₂ for 1 h. Then, Brefeldin A (Sigma, B7651-5MG) was added to each well at a final concentration of 5 µg/mL. Co-culture continued for another 3 h, after which cells were transferred to a 96-well “V” bottom plate and washed with 150 µL/well of staining buffer (PBS with 1mM EDTA and 1% HS). The plate was quickly spun at 400×g for 3 min and supernatant was removed. 50 µL of extracellular staining antibody solution for CD8 and viability marker prepared with staining buffer were added to each well and mixed. The plate was then maintained at 4°C for 20 min in the dark. Afterwards, cells were washed with 150 µL/well PBS and resuspended in 100 µL/well of 1% paraformaldehyde (PFA) for 10 min at room temperature. Cells were then washed with PBS again and resuspended in 100 µL PBS containing 1 mM EDTA, 1% HS, and 0.1% saponin. After 10 min incubation at room temperature, cells were centrifuged, and supernatant was removed. 50 µL of intracellular staining antibody solution for cytokines prepared with the same buffer was added to each well, and the plate was incubated in the dark for 30 min at room temperature. Finally, cells were washed and resuspended in 250 µL 1% PFA for flow cytometry analysis.

Flow cytometry data were acquired with a FACSVerse (BD Biosciences) and were analyzed using FlowJo (v10.8.1) software.

Western blotting

Cell lysates were prepared with 200,000 cells and 50 µL of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, 2.5% β-mercaptoethanol, 0.005% blue bromophenol). Lysate samples were boiled at 95°C for 10 min before loading onto 4–15% Mini-PROTEAN® TGX™ precast protein gels (Bio-Rad, 4561084), as well as prestained protein standard. For the SNAP-Tag-based retrograde transport assay (see below), HiMark™ Prestained Protein Standard (Invitrogen, LC5699) was used; for all the other western blots, Broad Range (10-250 kDa) Color Prestained Protein Standard (New England Biolabs, P7719S) was used. After electrophoresis, proteins were transferred onto a PVDF membrane (pre-activated with methanol for 1 min; Millipore, IPFL85R) using a wet transferring system. The membrane was

then blocked in 5%-milk PBS-T (phosphate-buffered saline, 0.1% Tween-20) for 1h at room temperature, then stirred overnight at 4°C with primary antibody in the same solution. The next day, the membrane was incubated with corresponding horseradish peroxidase (HRP) conjugated secondary antibodies for 1h at room temperature. Chemiluminescent (ECL) substrate (SuperSignal™ West Femto Maximum Sensitivity Substrate, ThermoFisher, 34096, or SuperSignal™ West Pico PLUS Chemiluminescent Substrate, ThermoFisher, 34580) was used for image exposure with an Amersham ImageQuant™ 800 machine (Cytiva). Images were processed and quantified with ImageJ v2.14.0/1.54f (NIH) software.

SNAP-Tag-based retrograde transport assay

First, anti-ALCAM (Bio-Rad, MCA1926) and anti-ICAM1 (Bio-Rad, MCA1615) antibodies were labeled with benzylguanine (BG) by incubating them overnight at 4°C with a threefold molar excess of BG-GLA-NHS reagent (New England Biolabs, S9151S; prepared in anhydrous DMSO). The reaction was neutralized with 20 mM Tris (pH 7.4) for 10 min at 21°C. Free BG-GLA-NHS was eliminated by applying labelled antibodies on spin desalting columns with a 7 kDa cutoff (ThermoFisher, 89849), according to manufacturer's instructions. Then, retrograde transport assays were conducted according to previously published protocols (289).

Colocalization and uptake experiments

LB33-MEL cells were cultured on coverslips in 4-well plates to reach sub-confluence on the day of the experiment. Cells were pre-incubated in fresh serum-containing medium for 30 min at 37°C just before the experiment, then were incubated with anti-ALCAM antibody (Biolegend, 343902)-containing pre-warmed medium (5 µg/mL) for 15 min at 37°C to allow for endocytosis. For colocalization experiments, cells were quickly washed at 37°C with pre-warmed PBS and were subsequently fixed with pre-warmed 4% PFA for 20 min at 37°C in order to preserve the integrity of cellular structures and protein distribution. For uptake experiments, endocytosis was stopped on ice, and unbound antibodies were removed by extensive washes with ice-cold PBS++ (PBS supplemented with 0.4 mM MgCl₂ and 0.9 mM CaCl₂). Residual cell surface-accessible anti-ALCAM antibodies were stripped by two 5 s acid washes on ice (200 mM acetic acid, pH 2.5, 300 mM NaCl, 5 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂). After neutralization by extensive washes with ice-cold PBS++, cells were fixed with 4% PFA for 10 min on ice, followed by another 10 min at room temperature. For both experiments, after quenching with 50 mM NH₄Cl for at least 5 min, cells were permeabilized with 0.02% saponin in PBS containing 0.2% bovine serum albumin for 30 min at room temperature. After incubation with secondary antibodies in the same permeabilization solution for 30 min, samples were mounted with Fluoromount G (Invitrogen). Images were taken with a Zeiss LSM900 microscope equipped with an Airyscan detector and a Plan Apo 63× numerical aperture (NA) 1.4 oil

immersion objective. For colocalization experiments, Airyscan mode was applied. Pixel size: 0.04 micron. For uptake experiments, confocal mode was used. Pixel size: 0.07 micron. Microscope software: Zen blue (v3.3; Zeiss).

Conjugate formation for confocal fixed sample imaging

For conjugate formation between adherent HLA-A*68012-expressing HeLa cells and anti-MUM-3 CD8 T cells (Fig 4B, EV4C), HeLa cells were cultured on coverslips and transiently transfected with ICAM1-mScarlet expression plasmid 16-24h before the experiment. HeLa cells were first incubated in 300 ng/mL MUM-3 peptide-containing culture medium for 45 min at 37°C and were then washed extensively with T cell culture medium to remove residual unbound peptide. T cells were freshly thawed as described above and resuspended to 500,000 cells/mL with T cell culture medium supplemented with 50 U/mL IL-2. Then, 250,000 T cells were added to HeLa cells and the pool was incubated for 20 min at 37°C. Cells were then fixed at room temperature for 20 min by adding PFA directly to the culture medium to reach a final concentration of 4%.

For conjugate formation between suspended HLA-A*68012-expressing HeLa cells and anti-MUM-3 CD8 T cells (Fig 4C, EV4D), HeLa cells were cultured in 6-well plates and transfected with negative control siRNAs or siRNAs targeting EndoA3 72h in advance and with plasmid expressing ICAM1-mScarlet 16-24h in advance. HeLa cells were detached with PBS-EDTA 4 mM, resuspended to 1,000,000 cells/mL in culture medium containing 300 ng/mL MUM-3 peptide and incubated for 45 min at room temperature. Then, HeLa cells were extensively washed with T cell culture medium, and finally resuspended to 1,000,000 cells/mL with T cell culture medium supplemented with 50 U/mL IL-2. T cells were freshly thawed as described above and resuspended to 1,000,000 cells/mL with T cell culture medium supplemented with 50 U/mL IL-2. Subsequently, 250,000 HeLa cells and 250,000 T cells were pooled together and further co-cultured on coverslips for 20 min at 37°C. Of note, coverslips were pre-coated with poly-L-lysine (Sigma-Aldrich, P4832) according to manufacturer's instructions to stabilize conjugates. Cells were then fixed at room temperature for 20 min by adding PFA directly to the culture medium to reach a final concentration of 4%.

For conjugate formation between suspended EndoA3-GFP-expressing LB33-MEL cells and anti-MUM-3 CD8 T cells (Fig 5, EV5), LB33-MEL cells were transfected with negative control siRNAs or siRNAs targeting EndoA3 or VPS26A 72h in advance. LB33-MEL cells were detached with 0.05% Trypsin-EDTA (Gibco, 25300-054) and resuspended to 1,000,000 cells/mL with T cell culture medium supplemented with 50 U/mL IL-2. T cells were freshly thawed as described above and resuspended to 1,000,000 cells/mL with T cell culture medium supplemented with 50 U/mL IL-2. Then, 250,000 LB33-MEL cells and 250,000 T cells were pooled together and were co-cultured on poly-L-lysine pre-coated coverslips for 20 min at 37°C. Cells were then fixed at room temperature for 20 min by adding PFA directly to the culture medium to reach

a final concentration of 4%.

After fixation with PFA and quenching with 50 mM NH₄Cl for at least 5 min, cells were permeabilized with 0.02% saponin in PBS containing 0.2% bovine serum albumin for 30 min, both at room temperature. Then, cells were successively incubated with primary and secondary antibodies in the same PBS-saponin solution for 30 min and mounted with Fluoromount G (Invitrogen). Images were taken with Zeiss LSM900 microscope equipped with an Airyscan detector and a Plan Apo 63× numerical aperture (NA) 1.4 oil immersion objective. For the ICAM1 recruitment experiment (Fig 4B, EV4C) and immune synapse morphology experiment (Fig 5, EV5), z-stack was used. z-stack interval: 0.15 micron. For all images, Airyscan mode was applied. Pixel size: 0.04 micron. Microscope software: Zen blue (v3.3; Zeiss).

Conjugate formation for confocal live-cell imaging

HLA-A*68012-expressing HeLa cells were seeded in 24-well glass bottom plates (Greiner, 662892) and transfected with ICAM1-EGFP expression plasmid 16-24 h in advance. HeLa cells were first incubated in MUM-3 peptide-containing (300 ng/mL) culture medium for 45 min at 37°C and then washed extensively with T cell culture medium to remove residual unbound peptide. Anti-MUM-3 CD8 T cells were freshly thawed as described above and stained with Orange CellMask™ Plasma Membrane Stain (Invitrogen, C10045; 1:1000 dilution in T cell culture medium) for 10 min at 37°C, washed with culture medium, and resuspended to 500,000 cells/mL in T cell culture medium supplemented with 50 U/mL IL-2. 250,000 T cells were added to each well containing HeLa cells and live-cell imaging was started immediately after. Live-cell imaging was performed at 5% CO₂ and 37°C on a Zeiss LSM900 confocal microscope equipped with an Airyscan detector, an α Plan Apo 63× numerical aperture (NA) 1.4 oil immersion objective and a stage-top incubator. Microscope was controlled by software Zen blue (v3.3; Zeiss). Laser focus was maintained with definite focus 2. Airyscan mode was applied. Time interval between each frame: 6.5 s. Pixel size: 0.04 micron.

TIRF live-cell imaging

EndoA3-GFP-expressing HeLa/LB33-MEL cells were cultured on a 35 mm imaging dish with a glass bottom and low walls (ibidi, μ-Dish 35 mm, low, 80137) and transfected with ICAM1-mScarlet expression plasmid 16-24 h in advance. Live-cell imaging was conducted under 5% CO₂ and 37°C with Zeiss Axio Observer 7 microscope equipped with an iLas azimuthal TIRF module (Gattaca Systems), an α Plan-Apo 100× NA 1.46 oil immersion objective, an sCMOS Prime BSI Express camera (Photometrics) and a stage-top incubator (Tokai Hit). Microscope was controlled by Metamorph® software (v7.10.4.407; Molecular Devices). Laser focus was maintained with definite focus 3. Time interval between each frame: 1 s. Pixel size: 0.065 micron.

Microscope image analyses and quantifications

Airyscan image processing was performed with Zen blue (v3.3; Zeiss). Images were quantified with (Fiji Is Just) ImageJ v2.14.0/1.54f (NIH) software as follows.

For CD166 uptake experiments, images were quantified using Icy v2.4.2.0 (Institut Pasteur) software. Briefly, regions of interest (ROIs) were drawn manually around each cell. Within each ROI, bright spots corresponding to endocytic structures were automatically detected over dark background using the “Spot Detector” plugin. The plugin performs image denoising by computing wavelet adaptive threshold (WAT) on the union of all ROIs present in an image. This automatic thresholding was then manually adjusted, depending on the size of the spots. For each independent experiment, scale 2 was chosen and sensitivity was adjusted empirically. In addition, a size filter was added to discard spots smaller than 2 pixels. After processing, an excel file containing the fluorescence intensity of each endocytic structure was generated. The sum intensity of endocytic structures in each cell was calculated, then data were first adjusted according to the surface level of CD166 detected by flow cytometry in each experimental condition and were finally normalized to the control condition (set as 100%) for statistical analysis.

For conjugate formation experiments with HeLa cells to detect ICAM1 recruitment to the vicinity of CD8 T cells, masks were automatically generated for each image according to phalloidin staining by using Cellpose v3.0.7 software (335). First, an RGB format 512 × 512-pixel image of the phalloidin channel was generated from Fiji (ImageJ v2.14.0/1.54f) for each image and was imported to Cellpose. Segmentation of each cell was automatically performed using “cyto3” model with diameter (pixels) set as 40 for all independent repeats and was then exported as a mask. The mask was then imported into Fiji and re-scaled to the real size of the original image. Wand tool was used to select ROI corresponding to single cells. In each ROI, the raw integrated density (RawIntDen) of ICAM1 signal was measured. Then, the ICAM1 recruitment ratio in the vicinity of the T cell was calculated as the ratio between the ICAM1 signal in the T cell ROI and the total ICAM1 signal in the ROIs of the T cell and the adjacent HeLa cell: “RawIntDen of ICAM1 signal in T cell ROI/(RawIntDen of ICAM1 signal in T cell ROI + RawIntDen of ICAM1 signal in HeLa cell ROI)”. Data were normalized to control condition (set as 100%) for each independent repeat and were then pooled together for statistical analysis.

For conjugate formation experiments to measure the size of immune synapses, z-stack images (z-stack interval: 0.15 micron) for each conjugate were imported to Fiji. The contact region between a CD8 T cell and an LB33-MEL cell was delineated by “freehand line” tool manually, and then the length of the line was measured for each z-stack. The largest value was picked out from all z-stacks and was considered as the size of this immune synapse. All data from three independent repeats were then pooled together for statistical analysis.

Statistical analyses

Statistical analyses were performed with GraphPad Prism v10.1.0. D'Agostino–Pearson omnibus normality test was used to check the normality of datasets. If data passed the normality test, parametric tests were used, and data were plotted on graphs as mean $\pm$ SEM as error bars. If data did not pass the normality test, nonparametric tests were used, and data were plotted on graphs as median and quartiles. Details on the parametric and nonparametric tests used for each analysis are indicated in figure legends. Significance of comparison is represented on the graphs by asterisks.

Antibodies and reagents

Product	Reference	Application	Dilution
PE anti-human CD166 antibody	Biolegend, 343904	FACS	1:1000
APC anti-human CD54 antibody	BD Pharmingen, 561899	FACS	1:60
APC anti-HLA-A2, A28 antibody	Miltenyi Biotec, 130-099-582	FACS	1:300
Viability Dye eFluor 780	Invitrogen, 65-0865-14	FACS	1:1000
PerCP-Cy5.5 anti-human CD8 antibody	BD Pharmingen, 624060	FACS	1:100
APC anti-human IL-2 antibody	Biolegend, 500310	FACS	1:25
BV421 anti-human TNF- α antibody	Biolegend, 502932	FACS	1:100
PE anti-human IFN γ antibody	BD Pharmingen, 554701	FACS	1:200
Biotin anti-human HLA-A2 Antibody	Biolegend, 343322	FACS	1:400
CMFDA	Invitrogen, C2925	FACS	0.5 μ M
Anti-IFN γ coating antibody	Invitrogen, AHC4432	ELISA	1:250
Biotin anti-IFN γ detecting antibody	Invitrogen, AHC4539	ELISA	1:1000
Streptavidin–Peroxidase	Sigma-Aldrich, S5512	ELISA	1:1000
1-Step Ultra TMB-ELISA Substrate	ThermoFisher, 34028	ELISA	100 μ L
Anti-SH3GL3/Endophilin-A3 antibody	Sigma-Aldrich, HPA039381	WB	1:1000
Anti- α -tubulin antibody	Sigma-Aldrich, T5168	WB	1:10000
Anti-VPS26A antibody	abcam, ab23892	WB	1:1000
Anti-VPS35 antibody	abcam, ab10099	WB	1:500
HRP conjugated anti-mouse antibody	Agilent Technologies, P044701-2	WB	1:5000
HRP conjugated anti-rabbit antibody	Agilent Technologies, P044801-2	WB	1:5000
HRP conjugated anti-goat antibody	Santa Cruz Biotechnology, sc-2354	WB	1:1000
Anti-SNAP-tag [®] antibody	New England Biolabs,	WB	1:1000

	P9310S		
Anti-CD166 antibody	Bio-Rad, MCA1926	SNAP-Tag	10 µg/mL
Anti-CD54 antibody	Bio-Rad, MCA1615	SNAP-Tag	10 µg/mL
SNAP-Cell® Block	New England Biolabs, S9106S	SNAP-Tag	1:200
Anti-CD166 antibody	Biolegend, 343902	IF	1:100
Anti-CD45 antibody	Santa Cruz Biotechnology, sc-1178	IF	1:100
SNAP-Cell® TMR-Star	New England Biolabs, S9105S	IF	1:200
Anti-GM130 antibody	Biolegend, 937002	IF	1:200
Alexa Fluor™ 488 Phalloidin	Invitrogen, A12379	IF	1:200
Alexa Fluor™ 568 Phalloidin	Invitrogen, A12380	IF	1:200
Alexa Fluor™ 488 anti-Rabbit IgG	Invitrogen, A11008	IF	1:200
Alexa Fluor™ 568 anti-Mouse IgG	Invitrogen, A11031	IF	1:200
Alexa Fluor™ 633 anti-Mouse IgG	Invitrogen, A21050	IF	1:200
CellMask™ Plasma Membrane Stain	Invitrogen, C10045	IF	1:1000
DAPI	Sigma-Aldrich, D9542	IF	1:10000
Poly-L-lysine	Sigma-Aldrich, P4832	IF	NA

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