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Role of small GTPase Rab32 in CD8$^+$ T cell cross-priming by conventional type 1 Dendritic Cells

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Title

Role of small GTPase Rab32 in the $CD8^+$ T cell cross-priming by conventional type 1 Dendritic Cells.

Abstract

The conventional type1 Dendritic Cells (cDC1s) efficiently cross-prime $CD8^+$ T cells against cellular antigens derived from necrotic cells, viral-infected or tumour cells *in-vivo*. This functional specialization results from a combination of cDC1s characteristics, such as the ability to internalise and transport cellular antigens from peripheral tissues to draining lymph nodes, and the unique cross-presentation machinery of cDC1s. Therefore, it is interesting to investigate how cDC1 specific proteins affect their functions. Rab GTPases are the master regulators of intracellular vesicular trafficking and have been shown to promote $CD8^+$ T cell cross-priming *in-vivo*. The aim of this thesis is to study the role of GTPase Rab32 in optimizing $CD8^+$ T cell cross-priming against cellular antigens by cDC1s. We identify Rab32 to be highly and differentially expressed in splenic cDC1s compared to cDC2s. Using Rab32 deficient mice, we demonstrate that Rab32 promotes the proliferation of effector $CD8^+$ T cells in response to challenge with cellular antigens *in-vivo*. Interestingly, Rab32 does not affect cross-presentation by cDC1s *ex-vivo*. We further demonstrate that Rab32 promotes tumour infiltration of antigen-specific $CD8^+$ T cells by promoting the presence of tumour migratory cDCs in the draining lymph nodes. Intracellularly, Rab32 regulates the positioning of lysosomes in cDC1s, which has previously been linked to lysosomal functions. Based on these observations, we hypothesise that Rab32 promotes DC migration towards lymph lodes by optimising lysosomal signalling in these cells, thus impacting the priming of $CD8^+$ T cells.

Keywords

dendritic cells, cDC1, cellular antigens, cross-presentation, cross-priming, $CD8^+$ T cells, Rab GTPases, Rab32, dendritic cell migration, lysosomes

Titre de la thèse

Rôle de la petite GTPase Rab32 dans l'activation des cellules T CD8 + par les cellules dendritiques de type 1 conventionnelles.

Résumé

Les cellules dendritiques classiques de type 1 (cDC1) activent efficacement les cellules T $CD8^+$ naïves pour reconnaitre des antigènes cellulaires dérivés de cellules nécrotiques, de cellules virales infectées ou de cellules tumorales *in-vivo*. Cette la spécialisation fonctionnelle résulte des caractéristiques spécifiques aux cDC1, telles que la capacité d'internaliser et de transporter les antigènes cellulaires des tissus périphériques aux ganglions lymphatiques drainants, et la capacité unique de présentation croisée des antigènes internalisés. En conséquence, il est intéressant d'étudier comment les protéines exprimées spécifiquement par les cDC1 affectent la fonction de ces cellules. Parmi ces protéines, les petites GTPases de type Rab sont des régulateurs clé du système endocytique qui favorisent l'activation via la présentation croisée des cellules T $CD8^+$ naïves *in-vivo*. Le but de cette thèse a été d'étudier dans les cDC1 le rôle de la GTPase Rab32 dans l'activation des cellules T $CD8^+$ naïves contre les antigènes cellulaires. Nous avons identifié Rab32 comme étant fortement et différentiellement exprimé dans les cDC1 par rapport aux cDC2. En utilisant des souris déficientes pour Rab32, nous avons démontré que Rab32 favorise la prolifération des cellules T $CD8^+$ effectrices contre des antigènes cellulaires *in-vivo*. D'une façon intéressante, Rab32 n'affecte pas la présentation croisée par les cDC1s *ex-vivo*. Nous avons démontré en outre que Rab32 favorise l'infiltration tumorale des cellules T $CD8^+$ spécifiques de l'antigène en favorisant la migration de cDC à partir de la tumeur vers les ganglions lymphatiques drainants. Au niveau intracellulaire, Rab32 régule le positionnement des lysosomes dans les cDC1, pouvant ainsi réguler les fonctions lysosomales. Sur la base de ces observations, nous émettons l'hypothèse que Rab32 favorise la migration des DC vers les ganglions lymphatiques en optimisant la signalisation lysosomale dans ces cellules et impactant ainsi l'activation des cellules T $CD8^+$ contre les antigènes tumoraux.

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dendritic cells, cDC1, cellular antigens, cross-presentation, cross-priming, $CD8^+$ T cells, Rab GTPases, Rab32, dendritic cell migration, lysosomes

List of Abbreviations

AKAP	A-kinase anchoring protein
APCs	Antigen-presenting cells
ARF6	ADP Ribosylation Factor 6
BATF3	Basic leucine zipper transcription factor ATF-like 3
BLOC	Biogenesis of lysosome-related organelles complex
BMDCs	Bone marrow-derived dendritic cells
BMMs	Bone marrow macrophages
cDC	Conventional dendritic cells
CDP	Common dendritic cell progenitor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTL	Cytotoxic T Lymphocytes
DCs	Dendritic cells
EEA1	Early endosome Antigen 1
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate complex
Esam	Endothelial Cell Adhesion Molecule
FLT3	fms-like tyrosine kinase receptor-3
GAP	GTPase activating protein
GDP	Guanine di-phosphate
GEF	Guanine nucleotide exchange factor
GGT-II	Rab geranylgeranyltransferase
GILT	Gamma-interferon-inducible lysosomal thiol reductase
GTP	Guanine tri-phosphate
HEV	High endothelial venules
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
IRF	Interferon Regulatory Factor
ITAM	Immunoreceptor tyrosine-based activation motif
KLF4	Kruppel Like Factor 4
LAMP1	Lysosome-associated membrane glycoprotein 1
LC	Langerhans cells
LMPP	Lymphoid primed multipotent progenitors
LPS	Lipopolysaccharide
LROs	Lysosome related Organelles
LSV	Listeria containing vacuoles
MAM	Mitochondria-associated membranes

MAP Kinase	Mitogen-activated protein kinases
MDP	Macrophage-dendritic cell progenitor
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph nodes
moDCs	Monocyte-derived dendritic cells
MPP	Multipotent Progenitors
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NK cells	Natural Killer cells
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid Dendritic cell
PKA	Protein Kinase A
polyI:C	poly(inosinic-cytidylic) acid
REP	Rab escort protein
ROS	Reactive oxygen species
ROS	Reactive oxygen species
SCV	Salmonella containing vacuoles
sdLN	Skin draining Lymph nodes
SNARE	SNAP REceptor
STAT3	Signal transducer and activator of transcription 3
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TFEB	Transcription factor EB
TGN	Trans-Golgi network
Th response	Helper CD4+ T cell response
TLR	Toll-Like Receptor
Treg	Regulatory T cells
UTR	Untranslated region

INTRODUCTION

CHAPTER 1 – Dendritic Cell Biology

1.1. Introduction to Dendritic cells

Dendritic cells (DCs) constitute a family of mononuclear phagocytes in the mammalian immune system, which is at the forefront of innate-adaptive immune cross-talk. They were identified by Ralph Steinman and Zanvil Cohn in the 1970s in mouse spleen[1,2], and Steinman received a Nobel Prize in Physiology or Medicine in 2011 for his contribution to this discovery. DCs are short-lived cells that are continuously replenished from the haematopoietic progenitors in the bone marrow. Fate-mapping[3] and barcoding experiments[4] have established that DCs differentiate into an independent lineage distinct from monocytes and macrophages. DCs are embedded in various lymphoid as well as non-lymphoid peripheral organs in the body, where they perform immune surveillance for extra- and intracellular pathogens, such as bacteria or viruses, as well as tumour cells. Upon such encounters, DCs recognise the 'eat-me' signals on infected or tumour cells and internalise them through a process named phagocytosis. DCs then mature and migrate to the T cell zones in secondary lymphoid organs and prime naïve T Lymphocytes to mount adaptive immune responses. Along with B lymphocytes, monocytes, macrophages, and Langerhans cells, DCs constitute the 'Professional antigen-presenting cells (APCs)' of the immune system. Professional APCs process exogenous antigens into peptides, and present them on MHC-I/MHC-II molecules to $CD8^+$/$CD4^+$ T cells, respectively. While phagocytes such as macrophages and neutrophils are specialised at the innate function of eliminating the internalised pathogens, DCs in general are known for their ability to conserve exogenously acquired antigens and present them to T cells to induce either adaptive immune responses[5], or central[6] and peripheral T cell tolerance[7,8] to self-antigens. Hence, DC-mediated T cell responses are well established to form the core of anti-viral and anti-tumour responses[9,10], and the potential therapeutic benefits of modulating DC function are now being actively pursued by researchers in the field. Beyond their role in inducing adaptive immunity, DCs also perform innate functions, such as the secretion of regulatory and inflammatory factors[11–15]. The vast progress made in the field in the past four decades has led to the identification of DCs populations different from Steinman's stellate conventional DCs, which may be morphologically and functionally distinct (plasmacytoid DCs which secrete IFNα upon viral stimulation)[16–18] or which appear only in the presence of inflammatory stress (inflammatory DCs derived from $Ly6C^+$ monocytes or moDCs)[19–22]. Therefore, the family of DCs requires further classification into subsets. This chapter talks about the development and classification of cDC subsets, with a focus on the cross-presentation specialised murine conventional type 1 DC (cDC1).

1.2. DCs subsets in mice and humans

Mammalian DCs have been classified into subsets based on their ontogeny, gene-signature, location, morphology and functions[23]. Based on these criteria, broadly 3 types of DCs may be defined – the conventional DCs (cDCs), plasmacytoid DCs (pDCs) and inflammatory monocyte-derived DCs (moDCs).

1.2.1. Conventional DCs (cDCs)

cDCs were initially described as a cell population constitutively expressing the integrin CD11c and highly expressing MHC-II, and lacking the surface markers of lymphocyte, granulocyte and erythrocyte linages[24,25]. However, later observations recognized the constitutive expression of CD11c and MHC-II on cDC-progenitors, moDCs and several tissue-resident macrophage populations. Advances in the understanding of the developmental biology of cDCs and their lineage relationship across tissues have helped to clarify that cDCs develop as an independent lineage distinct from pDCs, monocytes and macrophages[23]. cDCs differentiate from the blood circulating cDC-committed precursors known as pre-DCs, which themselves develop in the bone marrow after a sequential differentiation from the hematopoietic stem cells (HSC)[26,27]. While circulating in the blood, pre-DCs get embedded in the various lymphoid and non-lymphoid tissues where they differentiate into cDCs. The lymphoid organ resident cDCs exist in the spleen marginal zone and lymph nodes, and they pick up blood and lymph derived antigens. Another population of cDCs exists in the non-lymphoid peripheral tissues. These cDCs pick up the antigens from the peripheral tissues and migrate to T cell zones of the draining lymph nodes, where they are called migratory cDCs. The resident and migratory cDC populations in the lymph nodes may be distinguished by the different levels of CD11c and MHC-II expression on these cells- the resident cDCs are CD11c-high and MHC-II-intermediate whereas the migratory cDCs are CD11c-intermediate and MHC-II-high. Comparative analysis of the transcriptome amongst DC populations, monocytes, and macrophages have further contributed to the identification of cDC ontogeny and their specific gene signatures[28–30]. Based on these observations, cDCs are defined as follows-

- cDCs arise from the bone marrow residing Common Dendritic Cell Progenitors (CDP)[31,32] and survive in various tissues depending on the growth factor fms-like tyrosine kinase receptor-3–ligand (FLT3L)[33,34].
- cDCs constitutively express a specific gene signature distinct from monocyte and macrophages including the transcription factor (TF) ZBTB46[35,36].
- Immature cDCs internalise exogenous antigens in peripheral organs and migrate towards the T cells zones of secondary lymphoid organs in a CCR7-chemokine receptor-dependent manner[37–40].
- Upon maturation, cDCs process exogenous antigens and present them as complexes with MHC-I or MHC-II to prime $CD8^+$ and $CD4^+$ T cell responses, respectively[9,10,41–43].

cDCs are a heterogeneous group of cells, and most tissues consist of minimally two distinct cDC subsets- the $IRF8^+$ $XCR1^+$ type 1 cDCs (cDC1) and the $IRF4^+$ SIRPα $(CD172)^+$ type 2 cDCs (cDC2)[23], which perform distinct immune functions[44–47].

1.2.1.1. Type 1 DCs (cDC1s)

1.2.1.1.1. Identification

cDC1s in both mice and humans express the XC-chemokine receptor XCR1, the C-type lectin domain family 9 member A (CLEC9A, also known as CD370 or DNGR-1) and CD24 irrespective of the organ in which they reside[48–52]. Other cDC1 markers are tissue-specific. In mice, the spleen and lymph nodes resident cDC1s express the surface markers CD8a and CD24 (high expression). In non-lymphoid tissues, cDC1s express CD103 instead, and its expression is maintained upon their migration to lymph nodes. In the skin, the dermal cDC1s are identified as $CD103^+$ Langerin $(CD207)^+$ $CD24^+$ cells, and they can be distinguished from the ontogenically distinct epidermal Langerhans cells which are $CD103^-$ $Langerin^+$ $CD24^+$ [46,47,53,54]. In the epithelial section and the lamina propria of the small intestine, cDC1s are defined as $CD103^+$ $CD11b^-$ cells. Beyond cDC1, CD103 is exceptionally expressed on a population of $CD11b^+$ cDC2s in the small intestine[55,56]. $CD103^+$ cDC1s are also identified in the lungs[57].

In humans, ontological studies along with the recent transcriptomics results obtained using single-cell RNA sequencing on $Lineage^-$ $HLA\text{-}DR^+$ enriched PBMCs have identified cDC1s in the human blood as $CD141(BDCA3)^+$ $XCR1^+$ $CLEC9A^+$ cDCs[23,58,59]. These cells originate from blood

$CD100^{+}CD34^{int}$ cDC progenitors. The human lymphoid organ resident cDC1s are also considered to be derived from blood DC precursors[60–63]. In the skin, the $CD1a^{+}$ CD1c $(BDCA1)^{low}$ $XCR1^{+}$ $CLEC9A^{+}$ cDCs have been described as the human equivalents of the mice CD103 migratory cDC1s[62]. Table 1.1 summarises the identifying features of cDC1s in various mice and human tissues.

Species	Tissue	Cell surface markers	Transcription factors	References
Mice	Spleen	$CD8a^{+}$ $XCR1^{+}$ $CD24^{hi}$	IRF8, BATF3	[23]
	Lymph node resident	$CD8a^{+}$ $XCR1^{+}$ $CD11c^{hi}$ MHC-II^{int}	IRF8, BATF3	[23]
	Dermal/sdLN migratory	$CD103^{+}$ $XCR1^{+}$ $Langerin^{+}$ $CD11c^{int}$ MHC-II^{hi}	IRF8, BATF3	[46,47,53,54]
	Small intestine	$CD103^{+}$ $CD11b^{-}$	IRF8, BATF3	[23]
	Lung	$CD103^{+}$ $CD11b^{-}$ $CD24^{+}$	IRF8, BATF3	[57]
Human	Blood/lymphoid organ resident	$CD141^{+}$ $XCR1^{+}$ $CLEC9A^{+}$	IRF8, BATF3	[23,58,59]
	Dermal	$CD1a^{+}$ $CD1c^{low}$ $XCR1^{+}$ $CLEC9A^{+}$	IRF8, BATF3	[62]

Table 1.1 Identification of cDC1s in various mice and human tissues.

1.2.1.1.2. Functional specialization in cross-presentation

The development of cDC1s in both mice and humans depends mainly on the expression of two TFs, BATF3 and IRF8[9,64,65]. Mice models with genetic depletion of BATF3 or a loss of function point-mutation in IRF8 have a deficiency in cDC1 development in all tissues[9,64,66], and these models have helped to decipher the physiological functions of cDC1 *in-vivo*. In mice, cDC1s are well studied. They are functionally specialized at internalizing, processing and cross-presenting exogenous-antigen derived peptides to $CD8^{+}$ T cells. This is evidenced by their unique ability at cross-priming cytotoxic $CD8^{+}$ T cells to generate adaptive immune responses against viruses and tumours[9,10,57] as well as intracellular pathogens[67,68]. Several properties of murine cDC1s support their functional specialization.

- cDC1s are specifically enriched in proteins associated with MHC-I presentation such as the peptide transporter complex Tap1-Tap2, calnexin, calreticulin, ERp57 and cystatin B and C (cysteine protease inhibitors) amongst others[69,70].
- Several genes implicated in cross-presentation are highly and specifically expressed in cDC1s compared to cDC2s. These include the genes encoding the phagocytic receptor CLEC9A[71,72], lipid coat protein PLPN2[73], Rab GTPases Rab43[74] and Rab39[75], and BEACH domain-containing protein WDFY4[76]. Thus, cDC1s seem to possess a piece of molecular machinery that enables their functional specialisation in cross-presentation.
- The chemokine receptor XCR1 is specifically expressed on mice and human cDC1s. This receptor binds to its ligand XCL1, a chemoattractant secreted by newly activated $CD8^+$ T cells. The XCR1-XCL1 axis enhances cooperation between cDC1 and T cells and promotes the proliferation, survival and activity of antigen primed $CD8^+$ T cells[77]. Recently, intra-tumoural NK cells have been shown to recruit cDC1 in mice and human tumours by producing CCL5 and XCL1, and this NK cell-cDC1 axis has been linked to enhanced patient survival[78].
- cDC1s produce the chemokine CXCL9, which promotes the recruitment of effector cytotoxic T-lymphocytes (CTLs) and the recall of $CXCR3^+$ $CD8^+$ T cells to the site of injury[15,79].

Several reports indicate that the functional specialisation of cDC1 in cross-presentation is also conserved in humans. Studies have suggested that the blood isolated $XCR1^+$ $CLEC9A^+$ $CD141^+$ cDC1[80,81], skin isolated $XCR1^+$ $CD1a^+$ $CLEC9A^+$ cDC1s[62,82] as well as the *in-vitro* cord blood $CD34^+$ HSC progenitor derived $CLEC9A^+$ $CD141^+$ DC1 (CBDCs)[83,84] efficiently cross-present exogenous antigens to $CD8^+$ T cells. However, Segura et al. have shown that all three DC subsets isolated from human lymphoid organs, including the $CD141^+$ cDC1s, $CD1c^+$ cDC2 and $BDCA2^+BDCA4^+$ pDCs are equally efficient at cross-presentation to $CD8^+$ T cells[85]. Therefore, the functional specialization of human cDC1 populations remains an open question.

1.2.1.1.3. Priming of $CD4^+$ T cells and regulation of Th1 responses

Beyond the cross-priming of $CD8^+$ T cells, murine cDC1s are also efficient at priming $CD4^+$ T cells via MHC-II [57,86]. The generation of effective and long-lasting $CD8^+$ T cell responses against bacteria and viruses depends on signalling from antigen primed 'helper' $CD4^+$ T cells (Th1 responses)[87,88]. These signals help the cross-primed $CD8^+$ T cells to undergo clonal expansion and differentiation into effector CTLs as well as memory cells. The sequence of interactions amongst

DCs-$CD8^+$/$CD4^+$ T cells, and exact DC subsets involved are not well defined, but two recent studies indicate a role of cDC1s in the mediating Th1 signals to support efficient $CD8^+$ T cell responses. Using Vaccinia virus infection models, Eickhoff et al. observed that early after the infection, the initial priming of $CD4^+$ and $CD8^+$ T cells is spatially segregated within the spleen and is independent of cDC1s[89]. On similar lines, Hor et al. used a cutaneous herpes simplex virus (HSV) model and observed that $CD4^+$ T cells interact with skin migratory DCs in the lymph nodes and undergo an initial proliferation before the $CD8^+$ T cells, independently of the resident cDC1s[90]. Approximately 40h after the initial infection, both $CD4^+$ and $CD8^+$ T cells cluster around $XCR1^+$ cDC1s in the spleen[89] and resident cDC1 in the lymph nodes[90]. Thus, these studies demonstrate that cDC1s are not necessarily required for $CD4^+$ T cell priming, but they rather act as a platform for the delivery of $CD4^+$ T cell help to $CD8^+$ T cells. Accordingly, mice lacking cDC1s (BATF3 deficient or Langerin-DTR mice) can efficiently prime $CD4^+$ T cell responses against West-Nile virus infection[9] or *Leishmania major* infection[91].

1.2.1.1.4. Innate functions

Recent studies have demonstrated that beyond T cell priming, cDC1s also perform innate immune functions. cDC1 sense tissue damage through the receptor CLEC9A, which recognises the exposed F actin on necrotic cells. One study shows that the engagement of CLEC9A inhibits the secretion of the chemokine CXCL2 by cDC1s, which in turn inhibits the recruitment of neutrophils to the sites of sterile and infectious injury. This anti-inflammatory response of cDC1 has been observed to be in effect during systemic *Candida* infections in mice[11]. On the other hand, dermal $EpCAM^+$ $CD59^+$ $Ly\text{-}6D^+$ cDC1s have been shown to produce vascular endothelial growth factor α (VEGF-α), which promotes the recruitment and function of neutrophils in the skin infected with cutaneous bacteria[12]. Thus, the role of cDC1 in the regulation of neutrophil recruitment to the site of injection has emerged.

Moreover, cDC1s seem to possess unique molecular mechanisms to promote recognition and interactions with pathogens. TLR11 expressed on these cells is a target of profilin, a ligand expressed by the protozoan parasite *Toxoplasma Gondii*[92]. TLR11-profilin interaction activates cDC1 and promotes IL-12 cytokine secretion by these cells[92], which is essential for mounting immune responses against *Toxoplasma Gondii*[67]. Further, IL-12 secretion by cDC1 has also been shown to promote immunity against *Leishmania major infections*[93,94]. Interestingly, steady-state

IL-12 production by migratory CD103$^+$ cDC1, independent of any TLR signalling, has been shown to suppresses Th2 responses against helminthic infections[95]. This suggests that IL-12 production by cDC1 might control the Th1 vs Th2 response axis[96]. Furthermore, mouse, as well as human cDC1, are the major source of potent antiviral cytokines – the type III IFNλ (IL-28/29) upon stimulation with TLR3 ligand polyI:C.[13] *In-vitro* culture of human cDC1 with the hepatitis C virus (HCV) induces IFNλ secretion by these cells, suggesting a role of cDC1 in anti-HCV immunity[97].

1.2.1.2. Type 2 DCs (cDC2s)

1.2.1.2.1. Identification

cDC2s comprise the second major branch of cDCs. In mice, cDC2s express the surface markers SIRPa (CD172) and integrin CD11b irrespective of their activation status and location[52]. Spleen and lymph node resident cDC2s may be characterised as CD4$^+$ CD8a$^-$ cDCs. The migratory cDC2s do not express CD4 and are identified as SIRPa$^+$ CD11b$^+$ cDCs. In peripheral tissues, resident steady-state monocytes and macrophages also constitutively express SIRPa and CD11b, making it difficult to distinguish cDC2. This identification is further complicated during inflammatory conditions due to the migration of Ly6C$^+$ CCR2$^+$ monocytes in the inflamed tissues and their differentiation into two separate CD11c$^+$ MHC-II$^+$ CD11b$^+$ SIRPα$^+$ phagocyte populations [22,98,99]. Therefore, the best characterisation of cDC2 comes from their specific expression and dependence on the TF IFR4, which is essential for cDC2 development across all tissues in mice and humans[100]. In the small intestine, two populations of IRF4$^+$ cDC2 exist- CD103$^+$ CD11b$^+$ and CD103$^-$ DC11b$^+$ [101–105]. In lungs, cDC2 are defined as CD103$^-$ CD24$^+$ CD11b$^+$ cDCs[101]. In the skin, two IFR4 dependent migratory cDCs populations distinct from the dermal CD103$^+$ Langerin$^+$ cDC1s are defined – the CD301b (MGL-2)$^+$ CD11b$^+$ CCR7$^+$ cDC2s[106–108] and CD11b$^-$ CD24$^-$ SIRPa$^+$ cDC2s[53,109]. Moreover, the cDC2 in mice are a heterogeneous population and are further divided into two functionally distinct subsets based on the expression of specific TFs – the NOTCH2 expressing cDC2[110] and KLF4 expressing cDC2[109].

In humans, the blood circulating and lymphoid organ resident cDC2s were characterised as CD1c$^+$ cells[23,59]. Recent transcriptomics analysis of DCs isolated from PMBCs has further split the cDC2 into two CD1c$^+$ subsets- DC2 and DC3[58]. DC2 are identified by their surface expression of CLEC10A (CD301), CD32B and FcεR1A (the α-chain for a high-affinity receptor for IgE). DC3 are identified by the surface expression of VCAM (CD106), CD36 and CD163, along with their

unique expression of the tissue macrophage/DC related transcripts S100A8 and S100A9. Both DC2 and DC3 arise from the blood $CD100^+$ $CD34^{int}$ cDC progenitors[58]. In the human skin, cDC2s have been identified as $CD1c^+$ $CD1a^+$ $SIRPa^+$ $CD11b^+$ cDCs [62], whereas in the human lungs, they have been characterised as IRF4 dependent $CD1c^+$ $CD11b^+$ cDCs[101].

Table 1.2 summarises the identifying features of cDC2s in various mice and human tissues.

Species	Tissue	Cell surface markers	Transcription factors	References
Mice	Spleen	CD4+ $SIRPa^+$ $CD24^{int/low}$ $ESAM^+$	IRF4, NOTCH2	[110]
		$CD4^+$ $SIRPa^+$ $CD24^{int/low}$ $ESAM^-$	IRF4, KLF4	[109]
	Lymph node resident	$CD4^+$ $SIRPa^+$ $CD11c^{hi}$ $MHC\text{-}II^{int}$	IRF4	[23]
	Dermal/sdLN migratory	$CD301b^+$ $CD11b^+$	IRF4	[106–108]
		$CD11b^-$ $CD24^-$ $Langerin^-$ $SIRPa^+$	IRF4, KLF4	[109]
	Small intestine	$CD103^+$ $CD11b^+$	IRF4, NOTCH2	[101–105]
		$CD103^-$ $CD11b^+$	IRF4	[101–105]
	Lung	$CD103^-$ $CD11b^+$ $CD24^+$	IRF4	[101]
Human	Blood/lymphoid organ resident	DC2 = $CLEC10A^+$ $CD32B^+$ $Fc\varepsilon R1A^+$	IRF4	[58]
		DC3 = $VCAM^+$ $CD36^+$ $CD163^+$	IRF4	[58]
	Dermal	$CD1c^+$ $CD1a^+$ $SIRPa^+$ $CD11b^+$	IRF4	[62]
	Lung	$CD1c^+$ $CD11b^+$	IRF4	[101]

Table 1.2 Identification of cDC2s in various mice and human tissues.

1.2.1.2.2. Functional specialisation in MHC-II presentation to $CD4^+$ T cells

The cDC2s are highly efficient at antigen presentation to $CD4^+$ T cells via MHC-II, compared to cDC1s and moDCs[111]. This has also been proven *in-vivo* using a mice model with conditional

deletion of IRF4 in CD11c$^+$ DCs. These mice specifically lack cDC2s and lose their ability to mount T helper cell responses without compromising CD8$^+$ T cell responses[70]. On the other hand, cDC2s are less efficient at cross-presentation of exogenous antigen derived peptides on MHC-I compared to cDC1s. This is evidenced by the inability of cDC2 to functionally compensate for the absence of cDC1s in BATF3 deficient mice[9]. Dudziak et al. have shown that both cDC1 and cDC2 can similarly present the pre-processed peptides to CD4$^+$ as well as CD8$^+$ T cells, therefore their functional differences arise from the differential processing of the exogenous antigens by these cells[69]. Accordingly, cDC2 are enriched in proteins involved in MHC-II presentation including Cathepsins C, H and Z (lysosomal cysteine proteases), Gamma-interferon-inducible lysosomal thiol-reductase (GILT), asparagine endopeptidase (AEP) and H2-Mb 1[69]. An IRF4-dependent regulatory module potentially contributes to enhanced processing of antigens for peptide-MHC-II complex formations in cDC2[70]. cDC2 also express higher levels of the transcription factor EB (TFEB), which enhances the lysosomal activity and antigen degradation in these cells[112]. On similar lines, cDC2s have reduced phagosomal ROS production[113] and lower lipid droplet accumulation[73], two features proposed to facilitate cross-presentation by cDC1.

1.2.1.2.3. NOTCH2 dependent cDC2s in Th17 and innate immune responses

The NOTCH signalling pathway is an evolutionarily conserved system that allows the tissue microenvironment to dictate cell fate. The role of this pathway in the differentiation of cDC2 populations was first described by Lewis et al. using a mouse model with conditional knockout of NOTCH2 in CD11c$^+$ cDCs[110]. A population of NOTCH2 dependent cDC2s exists in the spleen, where they are characterised as ESAM$^+$ CD11b$^+$ cDC2s. These cells facilitate CD4 T cell priming in the spleen[110]. A recent study has also shown that the splenic NOTCH2 dependent cDC2s, and not the splenic BATF3 cDC1s or KLF4 dependent cDC2s, are essential for activating the CD4$^+$ T follicular helper cells, which in turn promote humoral immunity against *Listeria monocytogenes*[114]. Another population of NOTCH2 dependent cDC2 exists in the intestinal lamina propria and is characterized as CD11b$^+$ CD103$^+$ cDC2s[110]. This population plays an essential role in inducing Th17 cell differentiation and IL-17 production in the gut[110,115]. The NOTCH2 dependent CD103$^+$ CD11b$^+$ cDC2s have also been characterized as the non-redundant source of IL-23 in the intestine, which stimulates IL-22 secretion by innate lymphoid cells[55]. This cDC2-mediated secretion of IL-23 is important for maintaining gut barrier integrity and immunity against

Citrobacter rodentium. In another study, the intestinal $CD103^+$ $CD11b^+$ cDC2s have been implicated in enhancing IL-22-based innate mucosal immunity by producing IL-23 in response to TLR5 stimulation by the bacterial protein flagellin[116]. The intestinal $CD103^+$ $CD11b^+$ cDC2s also constitutively express CCR7 and are the first DCs to transport *Salmonella Typhimurium* to the mesenteric lymph nodes in the infected mice[56]. Moreover, the IRF4 dependent $CD24^+CD11b^+$ cDC2 in the murine lung, as well as their $CD1c^+CD11b^+$ human lung counterparts induce IL-17 T helper cell response and secrete IL-23 to support lung mucosal innate immunity in response to challenge by the fungus *Aspergillus fumigatus*[101].

1.2.1.2.4. KLF4 dependent cDC2s in Th2 immune responses

Apart from the role of NOTCH 2 dependent cDC2s in the induction of Th17 and innate responses, multiple studies implicated IRF4 dependent cDC2s in mounting Th2 immune responses against pathogens[106,107,117,118]. Park et al. showed that the deletion of the TF KLF4 in $CD11c^+$ cDCs significantly reduced the proportion of $IRF4^+CD11b^+$ splenic cDC2s in mice, suggesting the role of KLF4 in the development of a population of cDC2s[119]. Correspondingly, Tussiwand et al. demonstrated that KLF4 is essential for the development of several $IRF4^+$ cDC2 populations across murine lymphoid as well as peripheral tissues, excluding the NOTCH2 dependent $ESAM^+$ cDC2 population in the spleen[109]. Selective deletion of KLF4 in $CD11c^+$ cDCs impaired the development of $CD11b^-$ $CD24^-$ $Langerin^-$ $SIRPa^+$ cDCs in the skin draining lymph nodes, $CD11b^+$ cDCs in the dermis, $CD301^+$ $CD24^+$ $SIRPa^+$ cDCs in the lung and $CD301^+$ $CD24^-$ $SIRPa^+$ cDCs in the liver. The report further demonstrated that KLF4 dependent cDC2s are essential for the mounting of Th2 immune responses in mice. Consequently, the ablation of KLF4 dependent cDC2 populations severely compromises Th2 responses against *Schistosoma.mansoni* infection and impacts mice survival, without compromising the ability of knockout mice to mount Th1 or Th17 immune responses[109].

1.2.2. Plasmacytoid DCs (pDCs)

1.2.2.1. Identification

pDCs possess a plasma cell-like morphological, distinct from cDCs. In mice, pDCs can be identified as PDCA1 $(Bst2/CD317)^+$ $CD11c^{int}$ $MHC\text{-}II^{int}$ $CD11b^-$ $CD45R\ (B220)^+$ $SIGLECH^+$ $Ly6C^+$ $Ly6G^-$ cells. The development of pDCs depends on two TFs – IRF8 and E2-2 (also known as TCF4). Unlike cDCs, they only express the TLR7 and TLR9 and lack other TLRs[120].

In humans, pDCs are defined by their unique expression of the surface markers BDCA2 (CD303 or CLEC4C), BDCA4 (CD304), CD123 and CD85g (ILT-7). The recent transcriptomics study has characterised pDCs as DC6[58]. Human pDCs are further characterised into $CD2^{high}$ and $CD2^{low}$ subsets which are phenotypically and functionally distinct, including differential survival in stress conditions[121,122].

1.2.2.2. Development

In mice, myeloid as well as the lymphoid origin of pDCs have been described. The myeloid-derived pDCs arise from c-kit$^+$ FLT3$^+$ GM-CSFR$^-$ common DC progenitors[31], whereas the lymphoid derived pDCs arise from IL-7$^+$ SIGLECH$^+$ Ly6D$^+$ lymphoid progenitors[123]. Thus, pDCs comprise a heterogeneous population of cells expressing myeloid-like or lymphoid-like transcriptional signature, depending on their source of origin. Rodrigues et al. have proposed that lymphoid-derived pDCs constitute a major fraction of the pDC pool[123].

1.2.2.3. Functions

pDCs are functionally specialised at sensing viral infections through TLR7 and TLR9 and producing large quantities of type-I IFN (α and β) in response in mice as well as in humans[16–18]. Production of type-I IFN by these pDCs activates NK cells to produce IFNγ, thus activating B cell-mediated humoral responses[124]. Like cDCs, pDCs also possess the capability for antigen presentation to T cells. However, this capability seems to be dependent on the origin of the cell. The pDCs derived from the lymphoid progenitors are selectively efficient at MHC-II presentation to CD4 T cells[123]. Moreover, pDCs have been reported to possess some *ex-vivo* cross-presentation ability after TLR stimulation[125] and *in-vitro* with antigen targeting to PDCA1[126], albeit lower compared to cDC1s. However, *in-vivo* studies have indicated that CD8$^+$ T cell cross-priming by pDCs is dispensable for mounting local antiviral adaptive immune responses[127,128].

A role of pDCs in cDC based anti-tumour immunity has also emerged. Liu et al. reported that the administration of TLR9-activated pDCs directly into melanoma in mice induces the recruitment of NK cells in a CCR5- dependent manner, which in turn induces cDC recruitment and their cross-priming of effector CD8$^+$ T cells[129]. Moreover, pDC activation by TLR7/9 ligands induces their cytotoxic activity and direct targeting of tumour cells through TNF-related apoptosis-inducing ligand (TRAIL) and granzyme B [130–132]. Activated pDCs also induce the recruitment and activation

of NK cells and $CD8^+$ T cells to tumours through IFNα production, thus promoting tumour regression[132,133].

Figure 1.1 summarises the present knowledge on DC subsets and their distinct functions.

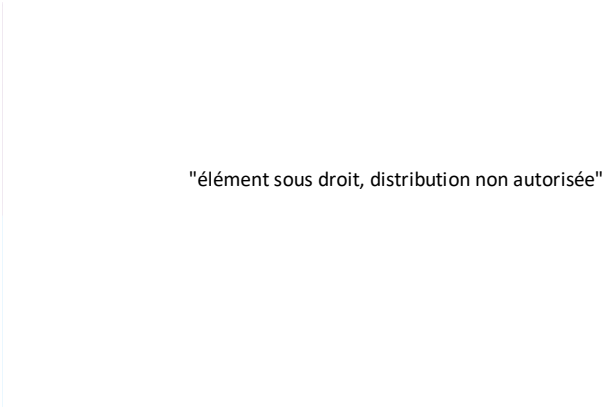

Figure 1.1 **Diversity of dendritic cell subsets**. DC subsets may be defined by their TFs dependency, surface marker expression and functions. The IRF8 dependency defines two types of DCs – the $XCR1^+$ cDC1s, which co-depend on BATF3 and are the premier cross-presenting DCs and the pDCs, which co-depend on E2-2 and are the major producers of anti-viral type I IFN. The IRF4 dependency defines a heterogeneous population of $SIRPa^+$ cDC2s – the NOTCH2 dependent cDC2s, which secrete IL-23 and drive TH17 responses and the KLF4 dependent cDC2s, which drive Th2 responses. The figure is taken from Murphy et al., Annual Review of Immunology 2016[134].

1.3. The organisation of the development pathway: cellular intermediates from HSCs to cDCs.

DCs develop as an independent cell lineage from the bone marrow residing HSCs, distinct from lymphocytes, granulocytes and monocytes. They are short-lived cells with a half-life of 3-6 days[135] and are continuously generated and replenished via haematopoiesis from their progenitors. The classical perspective of haematopoiesis suggests a series of sequential differentiation starting from the HSC, with a continuous loss of developmental potential in homogenous progenitor populations

until the functionally specialised cell progeny is reached. According to this model, a multipotent progenitor cell should be able to develop into any of the cells it is potent to form. Multiple studies of progenitors at the single-cell level have revealed this to not be the case[136–140]. Instead, these studies propose that progenitor populations are heterogeneous in nature and individual multipotent progenitors are imprinted at very early stages to develop a specific cell lineage.

Figure 1.2 provides an overview of the development of the DC lineage. The locations of different progenitors and the transcription factors involved at various stages are indicated, along with the growth factors dependency and immune functions of different subsets.

Figure 1.2 **Dendritic cells develop as an independent cell lineage.** The commitment towards the cDC lineage occurs from the MMPs in the early stage of haematopoiesis in the bone marrow.

Although multipotent, individual MPPs are imprinted to solely develop monocytes or DCs. A mono-potent MPP may directly develop into CDP without additional intermediates, whereas a multipotent MPP may develop into cMoP or CDP via the MDP intermediate which possesses monocyte-macrophage as well as DC potential. TFs such as IKAROS, GFI1 and PU.1 regulate DC commitment in early progenitor stages. The CDPs are identified by their unique gene signature, expression of TF ZBTB46 and dependence on growth factor FLT3L. They give rise to pre-cDC1s and pre-cDC2 precursors as well as fully developed pDCs in the bone marrow. Recent studies indicate that CDP may not be the primary source pDCs at steady state, and pDCs mainly arise from the lymphoid lineage via the CLP. The pre-cDCs circulate in the blood and embed various lymphoid and non-lymphoid tissues, where they mature into tissue-specific cDC1s or cDC2s. Across the tissues, cDC1 express TFs including IRF8 and BATF3 and are functionally specialised at antigen cross-presentation. The cDC2s instead express the TF IRF4 and comprise a heterogeneous population of NOTCH2 dependent, IL23 secreting cDC2s and KLF4 dependent, Th2 response driving cDC2s. The developmental pathways of monocyte-derived inflammatory DCs are ill-understood. The growth factor dependence and immunological functions of DC subsets are indicated. MMP – multipotent progenitors, MDP – macrophage DC progenitor, cMoP – common monocyte progenitor, CDP – common DC progenitor, pre-cDC – precursors of cDCs, CLP – common lymphoid progenitor. The figure is taken from Guermonprez et al., International Review of Cell and Molecular Biology 2019[96].

1.3.1. Bone marrow resident progenitors

The expression of lymphoid-associated markers CD8a and CD4 on cDC1 and cDC2 respectively[45] initially suggested the hypothesis that some subsets of DCs may arise from the lymphoid progenitors while others may arise from the myeloid progenitors. This hypothesis was invalidated by observations that adoptive transfer of both the common myeloid progenitors (CMPs) as well as the common lymphoid progenitors (CLPs, in high dose transplants) gives rise to $CD8a^+$ and $CD4^+$ cDCs in irradiated host mice[141,142]. On similar lines, it was observed that the stimulation of the FLT3 receptor, which is expressed on both CMPs and CLPs, induces their differentiation into cDCs[143,144]. A few subsequent studies also postulated a purely myeloid lineage-based origin of DCs. Genetic tracing based on the expression of lymphoid associated IL-7R showed that CLPs give rise to all lymphocytes (B, T and NK cells) but not cDCs in the thymus[145]. Moreover, it was

shown that the thymic CD8a$^+$ DCs arise from the intrathymic myeloid-type precursors[146]. More recent fate-mapping studies have established that early progenitor populations are heterogeneous and individual cells possess varied lineage commitment. Therefore, DCs can be derived from progenitors possessing both myeloid as well as lymphoid potential.

1.3.1.1. Lineage imprinting in early Multipotent Progenitors (MPPs)

Recent fate-mapping studies have demonstrated that commitment towards the DC lineage occurs at a very early stage of the developmental process before the CLP/CMP, at the lymphoid primed multipotent progenitors (LMPPs)[4]. In mice, LMPPs are characterised as Lin$^-$ Sca-1$^+$ c-kit (CD117)$^+$ CD34$^+$ FLT3(CD135)$^+$ cells in the HSC bone marrow compartment, and have had lost the capability to adopt erythrocyte and megakaryocyte lineage fates while sustaining myeloid and lymphoid potential[139]. Thus, these cells are thought to have branched off from the long term HSCs before the lymphoid-myeloid split. Naik et al. utilised a genetic barcoding technique to mark individual LMPPs and then adoptively transferred them into irradiated host mice to follow their development at the single-cell level[4]. They observed that only 3% of these cells are truly multipotent and could give rise to B cells, DCs and myeloid cells (monocytes and neutrophils). A majority of LMPPs, around 50%, are biased to develop DCs (splenic CD8a$^+$, CD11b$^+$ and pDCs)[4].

This lineage bias, especially in the context of cDC1s, is attributed to the expression of the TF IRF8 in a population of LMPPs[147–149]. IRF8 works synergistically with other TFs like PU.1 and RUNX1/Cbfb to induce 'epigenetic' chromatin rearrangements promoting the transcription of DC-related genes in downstream progenitors[148,150,151].

In humans, single-cell analysis has revealed that a large proportion of the multipotent common lymphoid progenitors (CLP) are efficient at generating cDCs CD1a$^+$ CLEC9A$^+$ cDC1s in particular)[152], similar to the LMPPs in mice.

1.3.1.2. Macrophage and DC progenitors (MDPs)

Fogg et al. have described the existence of bone marrow intermediates in mice which have lost their granulocyte potential but can develop into all mononuclear phagocytes. This population has been named as MDP and is characterised as the CX3CR1$^+$ CD115$^+$ CD135$^+$ fraction within the granulocyte-monocyte progenitors (GMPs)[153,154]. However, the origin and potential of MDPs have

been contested by a report from Sathe et al, in which clones of MDPs adoptively transferred into the host mice were found to mainly develop into macrophages and granulocytes, and very few cDCs and pDCs[155]. Notwithstanding the debatable existence of GMPs in mice, two reports by Lee et al. have helped to identify the progenitors in humans which lose their granulocyte potential and transition towards human DC progenitors[137,138]. Through *in-vitro* culturing of cord blood and bone marrow isolated $CD34^+$ HSC progenitors (supplemented with MS5 stromal cells and FLT3L+GM-CSF growth factors), they have described the existence of a heterogeneous population of granulocyte-monocyte-dendritic cell progenitors in humans (hGMDP). The hGMDPs further develop into the human MDPs, which are capable of developing into macrophages, cDCs as well as pDCs, but not granulocytes[137]. These progenitors exist in the human cord blood and bone marrow and are absent from the peripheral blood as well as lymphoid organs.

1.3.1.3. Transition to the dendritic cell- or monocyte- committed progenitors

All the three DC subsets (pDC, cDC1 and cDC2) can develop from the MDPs via the bone marrow residing intermediate cells known as the common DC progenitors (CDPs). The CDPs have been characterised in the bone marrow of mice as c-kit^{int} $FLT3^+$ M-CSFR $(CD115)^+$ cells[31,32], although the expression of M-CSFR has been linked to specifically to cDC-biased CDPs and not the pDC-biased progenitors[156]. CDPs lack the lymphoid associated marker IL7R, and upon adoptive transfer, they can develop into all three DC populations but not to monocytes and macrophages in host mice, in an FLT3L gradient dependent manner[31]. The CDPs also express CLEC9A, a marker that is selectively maintained on cDC1s and is absent from other lymphoid or myeloid lineages[3]. Thus, CLEC9A expression establishes cDCs as an independent haematopoietic lineage. Interestingly, the selective depletion of CDP by targeting of CLEC9A depletes the mice of CDP and cDC1, but cDC2 are still present in the spleen. These cDC2 are phenotypically similar to CDP derived cDC2 but show somatic Ig receptor rearrangements, typical of B cell, thus hinting at a possible alternate lymphoid origin of cDC2 in mice[157]. In humans, the CDPs are characterised as $CD123^{hi}CD115^-$ cells derived from the MDP and they give rise to pDCs as well as cDCs[137].

On the other hand, the MDP may commit to develop CCR2 dependent $Ly6C^+$ monocytes via the bone marrow-residing common monocyte progenitors (cMoP), which lack DC potential[158]. In mice, the cMoP have been characterised as $Ly6C^+FLT3^-$ $M\text{-}CSFR^+CD117^{hi}$ cells. The human counterparts of murine cMoPs have also been recently reported. Human cMoPs are derived from

the hGMDP progenitors present in the cord blood and bone marrow cultures and possess a restricted monocyte potential[159]. They are characterised as CLEC12A^{+} CD64^{+} progenitors. The proportion of MDPs committed to DC or monocyte commitment as well as the factors dictating these commitments remain to be characterised.

1.3.2. Blood circulating precursors

The CDPs give rise to cDC committed precursors (pre-cDCs) or fully developed pDCs in the bone marrow. The pre-cDCs then egress from the bone marrow and circulate in the blood to seed various lymphoid organs as well as peripheral tissues, where they terminally differentiate into cDC1 or cDC2. In mice, the pre-cDCs were initially identified in the blood as CD11c^{int}MHC-II$^{low/-}$ cells which had the potential to generate cDCs as well as pDCs but were devoid of lymphoid and monocytic differentiation potential[160]. Subsequent studies have revealed that the CD11c^{int}MHC-II$^{low/-}$ precursor population in the bone marrow and blood is heterogeneous. The pre-cDCs which act as immediate precursors of cDCs are characterised as Lin^{-} CD4^{-} CD8a^{-} CD11c$^{int/+}$ MHC-II$^{low/-}$ CD45RA^{low} FLT3^{high} CD43^{int} SIRPa^{int} cells in the bone marrow, blood and secondary lymphoid organs (spleen and lymph nodes). These cells lack other lymphoid or myeloid potential[161–163]. Moreover, the commitment of pre-DCs to generate cDC1 or cDC2 occurs in the bone marrow before the egress from the bone marrow into blood circulation[26,27,161]. The cDC1 committed pre-cDCs are characterised as SIGLECH^{-} Ly6C^{-} cells while the cDC2 committed cells are characterised as SIGLECH^{-} Ly6C^{+} cells[27]. The pDCs specifically develop from the CD11b^{-} CD45RA^{+} blood circulating precursors[164,165].

The circulating pre-cDC population has also been identified in humans, although it has been defined differently in different reports. Breton et al. have defined human pre-cDCs as CD34^{-} CD14^{-} CD1c^{-} CD117^{+} CD45RA^{+} FLT3^{+} M-CSFR^{-} population which expresses intermediate levels of CD123. Upon co-culture with DC stimulating factors and stromal cells, these cells give rise to both cDC subsets but not pDCs or monocytes[61,166]. Moreover, as in mice, the human pre-cDC population is heterogeneous and can be divided into cDC1 or cDC2 committed precursors based on their expression of SIRPa- cDC2 committed precursors are SIRPa^{+} while cDC1 committed precursors are SIRPa^{-}[61]. In another report, See et al. utilised high dimensional flow cytometry and single-cell RNA sequencing to identify early pre-cDCs as CD33^{+} CD45RA^{+} CD123^{+} FLT3^{+} cells co-expressing AXL (a tyrosine kinase receptor) and SIGLEC6, which gave rise to both the cDCs

but not pDCs[167]. The pre-DCs described in this report express classical pDCs markers such as CD303, CD304 and CD123, and thus they propose the use of CD33, CD2, CD5, CD327 and CX3CR1 to distinguish between pre-cDCs and pDCs. Finally, they divide the pre-DC population into uncommitted $CD123^+$ $CADM1^-$ $CD1c^-$ precursors, $CADM1^+$ $CD1c^-$ precursors committed to cDC1 and $CADM^-CD1c^+$ precursors committed to cDC2. The report by Villani et al. postulates that the AXL^+ $SIGLEC6^+$ cells observed in the pre-cDC population in the previous report are a committed DC subset which they term as AS-DCs[58]. Consequently, the AS-DCs show a low potential to develop cDC1 in *ex-vivo* cultures. Instead, they propose that a rare population of $CD45RA^+$ $CD34^{int}$ $CD100^+$ cells are endowed with the potential to develop cDC1s and cDC2s. Despite the differences in the interpretation of pre-DCs in these reports, a consensus exists on the capability of $CD45RA^+CD123^+$ subsets to develop cDCs. Moreover, the administration of FLT3L increases the numbers of pre-DCs as well as cDC1 and cDC2 in human trials[61,166].

1.3.3. pre-cDC tissue seeding and terminal differentiation of cDC1 in tissues

Pre-cDCs have a short half-life in the bloodstream[168] and enter the lymph nodes through CD62L (L-Selectin) dependent engagement with the surfaces of high endothelial venules (HEVs)[161], which are present in all secondary and tertiary lymphoid organs except the spleen. Pre-cDCs enter the spleen white pulp most likely through the marginal sinuses[169]. After the initial entry, these cells are gradually distributed throughout the lymph node paracortex and assume the typical behaviour of matured resident cDCs, including reduced migration and active probing[161]. In the human spleen, cDC1s have been observed to be mostly present in the white pulp region, along with the sinusoidal lining of the red pulp and some localisation at the T cell zones[170]. This localisation is thought to promote their ability to pick up blood and lymph derived antigens. cDC2s are known to exist at the marginal zones of the spleen[171].

The infiltration of pre-cDC1 and pre-cDC2 in the non-lymphoid organs has recently started to become clear. Fate mapping experiments have shown that upon seeding into peripheral tissues like the lung and small intestine, individual pre-DCs form a patchwork of closely positioned sister cells of the same cDC subset. Upon inflammation or tissue injury, new pre-cDCs seed the peripheral tissue and develop new cDC1s and cDC2s to meet the additional demands in the tissue, which dilutes the previous clones of cDCs[161]. Both pre-cDC1 and pre-cDC2 highly express the inflammation associated chemokine receptor CCR5 and nominally express the bone marrow

retention associated receptor CXCR4[172]. Moreover, pre-cDC1 and pre-cDC2 differentially express other chemokine receptors, which suggests a differential ability of these cells to respond to inflammatory signals and to perform distinct immune functions. Pre-cDC1 highly express the receptor CXCR3, while Pre-cDC2 instead express the receptor CX3CR1[172]. CXCR3 is associated with immune cell recruitment by CXCL9 and CXCL10 to peripheral tissues for generating Th1 responses. On similar lines, the expression of CXCR3 on pre-DC1s promotes their trafficking to melanoma tumours and their differentiation into cDC1[172], possibly promoting their involvement in anti-tumour responses.

Murine and human cDC1 are also unique in their surface expression of chemokine receptor XCR1. Böttcher et al. have shown that tumour resident NK cells secrete XCL1 as well as CCL5, the ligands for XCR1 and CCR5 respectively. This XCR1 and CCR5 based chemo-attraction facilitates the intra-tumoural recruitment and maintenance of cDC1s, which promotes anti-tumour immunity[78]. Moreover, intra-tumoural NK cells have also been shown to support cDC1 maintenance and function through their production of FLT3L[173].

1.4. DC proliferation in tissues

Although the differentiation of CDP to pre-cDCs marks a reduction in their proliferative capacity in mice[161,168] as well as in humans[166], the terminal differentiation of pre-cDCs to cDCs in lymphoid and non-lymphoid organs does not completely abrogate their proliferation potential. Mature cDCs can maintain a local tissue population through their residual proliferation capacity at steady state[174,175]. Several factors in the local environment seem to regulate this capacity. Splenic cDC1s and cDC2s express a receptor named Lymphotoxin-b receptor (LTbR), which has been shown to interact with its ligand LTa1b2, expressed on splenic B cells. This interaction acts as a survival and growth signal for splenic DCs ($CD4^+$ $CD8^-$ cDC2s in particular) under homeostatic conditions[174]. Moreover, the division and maintenance of cDCs in the lymph nodes has also been shown to be controlled in part by regulatory T cells and FLT3 signalling[161].

1.5. Migration of cDC1 towards T cell zones from tissues or within lymphoid organs.

When cDCs pick up an antigen in the peripheral tissue, they get activated and upregulate the surface expression of the chemokine receptor CCR7. Simultaneously, during inflammatory conditions, the endothelial cells of the terminal lymphoid vessels upregulate the secretion CCR7

ligands. Two ligands of CCR7 are known, homeostatic chemokines CCL19 and CCL21. CCL19 is secreted by fibroblastic reticular cells of the paracortex of lymph nodes, where T cells are found[176,177]. Migratory cDCs also secrete CCL19 on the luminal side of the HEVs[178]. CCL21 in mice has two functional variants – CCL21-Leu65 and CCL21-Ser65[179]. CCL21-Leu65 is secreted in lymphatic vessels in non–lymphoid tissues while CCL21-Ser65 is secreted by the fibroblastic reticular cells of the paracortex as well as endothelial cells of the HEV[179–182]. Thus, the interaction of CCR7 with its ligands facilitates the entry of migratory DCs into lymphatic vessels and their migration towards the T cell zones of the draining lymph nodes.

A recent study has identified that during viral infections, a re-organisation of the local DC network occurs in lymph nodes, which is mediated by $CD8^+$ T cells through the secretion of the chemokine XCL1[183]. In this process, the resident $XCR1^+$ cDC1s migrate to the $CD8^+$ T cell priming zones in an XCL1-XCR1 interaction dependent manner. This re-organisation promotes the interaction and cooperation between $XCR1^+$ cDC1 and pDCs to promote cDC1 maturation and cross-priming of $CD8^+$ T cells[183]. Thus, although resident cDC1s possess limited migratory behaviour in the steady-state, they can be induced to migrate to the T cell zones by chemoattraction during infections.

1.6. Hematopoietic growth factor requirements: FLT3L and GM-CSF

1.6.1. FLT3L

The development of dendritic cells (cDCs as well as pDCs) is dependent on cell-signalling via the Fms-like tyrosine kinase 3 (FLT3 or CD135) receptor. FLT3L serves as the ligand for this receptor. Upon ligation with FLT3L, a homo-dimerization of FLT3 occurs, which supports its auto-phosphorylation and promotes downstream signalling via STAT3 and STAT5 phosphorylation and the activation of PI3 kinase and MAP kinases.

The importance of growth factor FLT3L in DC development was highlighted by *in-vivo* observations in *Flt3*$^{-/-}$ as well as *Flt3l*$^{-/-}$ mice, both of which had a significant reduction in DCs populations to varying degrees[33,184]. While the *Flt3l*$^{-/-}$ mice lack lymphoid cDC1s, the *Flt3*$^{-/-}$ mice have a partial reduction in this population. Yet both these knockout mice have a large reduction cDC1s in non-lymphoid organs. This suggests that lymphoid organ resident and tissue-resident cDCs differ in their growth factor requirements. Moreover, the treatment of mice with recombinant FLT3L induces the expansion of DCs *in-vivo*[144,185]. The essential role of FLT3L in human DC

development has also been confirmed, and FLT3L administration in healthy human volunteers has been shown to induce a significant increase in pre-DCs, cDC1 and cDC2 in blood circulation[166,186].

The FLT3 receptor is expressed on several haematopoietic precursors in the bone marrow, starting at an early stage from the LMPPs in the HSC compartment, where it marks the loss of erythrocyte and megakaryocyte potential in these progenitors[139]. FLT3 is expressed on a majority of CLP which are efficient at developing DCs[143]. Within CMPs, FLT3 expression is limited to a small population that has precursor activity for DCs. FLT3 expression on CDP helps to differentiate these progenitors from cMoP, which lack FLT3 expression[31,32,158]. Towards the highly differentiated end of DCs lineage, pre-DCs, cDCs as well as pDCs highly express FLT3L[28].

1.6.2. GM-CSF

The Granulocyte-Macrophage Colony Stimulating factor (GM-SCF) is a soluble haematopoietic growth factor that engages the receptor GM-CSFR. This receptor is a heterodimer comprising a cytokine specific α-chain and common signalling β-chain which is shared with the receptors for IL-3 and IL-5[187]. Engagement of this receptor activates PI3 kinase, STAT5 and mTOR phosphorylation in the cell. GM-CSF is essential for the development of the myeloid lineage[188,189], as well as the development of moDCs in inflammatory conditions[22].

While GM-CSF is expressed on lymphoid organ resident as well as peripheral tissue-resident cDCs[99], it is not essential for their development in mice[190–192]. A couple of studies had initially reported that GM-CSFR knock mice possessed a deficiency in developing the $CD103^+$ cDC1s in non-lymphoid peripheral organs[43,99]. Later studies confirmed that GM-CSF is not required for development but rather for the acquisition of CD103 expression in the $CD11b^{low}$ DC1s residing in peripheral tissues[191,192]. In keeping with this role, GM-CSF stimulates the expression of CD103 on DCs in mice bone marrow cultures supplemented with FLT3L[193]. Moreover, Balan et al. have recently described a method to obtain human cDCs and pDCs from human cord blood $CD34^+$ progenitors cultures *in-vitro*, which are phenotypically and functionally similar to DC subsets in the human blood[194]. Using this method, they have discovered that a $CLEC9A^+$ $XCR1^-$ pre-cDC1 population exists in humans, and GM-CSF signalling is required to promote its terminal differentiation into $XCR1^+$ cDC1.

1.7. Transcription regulation of DC subsets

1.7.1. cDC lineage related TFs

1.7.1.1. STAT3

As mentioned above, the receptor for growth factor FLT3L is expressed on multipotent progenitors which possess DC, monocyte as well as lymphocyte (NK, T and B-cell) potential. However, the STAT3 activation and signalling downstream of FLT3-FLT3L engagement selectively promotes the transition of these early progenitors to common DC progenitors[195]. Consequently, STAT3 deficient mice have an accumulation of lymphoid and myeloid progenitors in the bone marrow. Moreover, they have an absence of common DC progenitors in the bone marrow and differentiated cDCs in the spleen. Moreover, STAT3 signalling also plays a cell-intrinsic regulatory role in maintaining the fully differentiated, immature as well as antigen primed cDCs in an inactivated state[196]. Hence, mice with conditional deletion of STAT3 in $CD11c^+$ cDCs have normal development of cDCs as wild-type mice, but display enhanced immune activity, inflammatory defects and impairment of mucosal tolerance.

1.7.1.2. IKAROS

The expression of the zinc finger DNA-binding TF IKAROS is restricted to the early hematopoietic and lymphoid progenitors, where it plays a major role in lymphoid differentiation[197] and pDC development[198]. Mice expressing a dominant-negative mutant of IKAROS have a complete loss of cDCs, whereas IKAROS knockout mice have a complete loss of cDC2 and pDCs and a reduction in cDC1 population[199]. These observations are in line with the reports that certain fractions of pDCs and cDC2 have a lymphoid origin[123,157]. Moreover, IKAROS is specifically expressed in a subset of early MMPs which also express FLT3, and it drives the myeloid commitment of the $FLT3^+$ MMPs[200]. Therefore, IKAROS might promote cDC development through both the myeloid as well as lymphoid progenitors.

1.7.1.3. PU.1

The TF PU.1 is expressed at intermediate levels in uncommitted LMPPs, and changes in subsequent levels become a determining factor for the fate of the progenitor – accumulation of PU.1 promotes myeloid commitment while its downregulation promotes lymphoid (B and T cell) commitment[201–204]. Kueh et al. studied the accumulation of PU.1 in fetal liver progenitors (FLPs,

Lin$^-$ cKit$^+$ CD27$^+$) at the single-cell level[205]. The introduction of exogenous PU.1 in uncommitted progenitors enhanced the expression of endogenous PU.1, promoting their myeloid commitment. The accumulation of PU.1 in these cells promotes the lengthening of the cell cycle and reduction in the rate of proliferation. This suggests the existence of a positive feedback pathway between PU.1 and cell cycle in myeloid-committed progenitors[205]. Lymphoid-committed progenitors avoid PU.1 accumulation by maintaining a rapid rate of proliferation, thus interrupting the positive feedback loop and inhibiting PU.1 activity. This suggests that the regulation of the cell cycle may be one factor which dictates the lineage fate of early multipotent progenitors.

More specifically for cDC commitment, PU.1 induces the expression of IRF8 in LMPPs through chromatin remodelling. This expression marks the imprinting of cDC commitment in this early progenitors[148]. PU.1 also induces the expression of FLT3 on early progenitor in a concentration-+dependent manner[204]. Moreover, the conditional deletion of PU.1 in CDP abrogates their FLT3L induced terminal differentiation into cDCs. A recent study by Chopin et al. has suggested that PU.1 promotes the terminal differentiation and survival of cDCs by inducing the transcriptional regulator[206] ZFP366/DC-SCRIPT, which promotes the transcription of cDC related genes (such as XCR1, CLEC9A, CD4, CD11c etc.) and repressing pDC related genes.

1.7.2. cDC1 related TFs

1.7.2.1. BATF3

The basic leucine zipper transcription factor ATF-like 3 (BATF3) is an activator protein1 (AP1) TF expressed in both cDC subsets and which specifically regulates the development of cDC1s during homeostasis[9]. Consequently, Batf3$^{-/-}$ mice are deficient in cDC1 compared to wild-type mice and are unable to mount anti-viral and anti-tumoural CD8$^+$ T cell responses, while maintaining pDC and cDC2 populations and their related immune functions. Later studies have established that BATF3 is required for cDC1 development across tissues[64,207]. The molecular mechanism of BATF3 function in cDC1s involves its interaction with IRF8 through the leucine zipper domain, which mediates a co-operative gene activation[208]. In the presence of intracellular pathogen infections or inflammatory conditions, two other members of the AP1 TF family, BATF and BATF2 can compensate for the function of BATF3 in cDC1s in Batf3$^{-/-}$ mice, and thus provide a BATF3 independent pathway for the development of functionally competent cDC1[208,209]. Similar

to its function in murine cDC1 development, BATF3 was shown to be essential for the development of human cDC1s in the *in-vitro* culture of $CD34^+$ HSCs[65].

1.7.2.2. IRF8

The IFN regulatory factor 8 (IRF8) is highly expressed in cDC1 across all tissues and is absent in cDC2[210,211]. It has been characterised as a 'terminal selector' for cDC1 and its expression is important for the survival of terminally differentiated cDC1s[212]. Moreover, IRF8 is also expressed highly in pDCs, although it is not required for the survival of these cells. Instead, IRF8 controls activation and type-I IFN production in pDCs[212]. A point mutation in IRF8 ($IRF8^{R294C}$) specifically disrupts its function in cDC1 development without affecting other lineages[66]. Moreover, biallelic mutations in the *Irf8* gene in humans (K108E/K108E and R83C/R291Q) result in severe immunodeficiency and the disruption of cDC as well as pDC development[213–215].

Apart from its expression in fully differentiated cDC1, IRF8 is also expressed in cDC progenitors at various levels of differentiation. Its expression in the early stage LMPPs defines their cDC lineage imprinting at the expense of other myeloid and lymphoid lineages[147–149,152,199]. In these cells, high levels of PU.1 activates IRF8 expression via chromatin modelling[148]. Moreover, the TF Runx1 and its co-factor Cbfb also enhance IRF8 expression in LMPPs, which induces their transition to MDPs and a loss in granulocyte potential [150]. Consequently, the LMPPs in $Cbfb^{-/-}$ mice have an enhanced granulocyte development potential compared to wild-type mice. Upon its expression, IRF8 induces epigenetic changes in LMPPs in the form of chromatin rearrangements, which enhances the proximity of chromatin enhancers to DC-specific genes and creates a bias in early progenitors to develop DCs[147]. Moreover, IRF8 is also expressed in CDPs, where it interacts with BATF3 to promote their development to pre-cDC1 and fully differentiated cDC1s[26]. Simultaneously, IRF8 also reduces granulocyte potential in CLP and CMP through an inhibitory interaction with C/EBPa, a TF required for granulocyte differentiation[151,216] Thus, IRF8 through its synergistic interactions with different TFs promotes the development and differentiation of cDCs.

1.7.2.3. ID2

The inhibitor of DNA protein 2 (ID2) is a helix-loop-helix TF which is expressed in all DC subsets, with the highest expression in lymphoid and non-lymphoid resident cDC1s[217]. Its expression is

induced in cDCs by TGFb signalling, and it is required for the development of cDC1s[207,218]. Thus, Id2$^{-/-}$ mice lack splenic cDC1. Jaiswal et al. have shown that IRF8 expression in cDC1s increases the ID2 and BATF3 expression in these cells, and the three TFs then work synergistically to promote cDC1 development[219]. Thus, IRF8 works upstream of ID2 and BATF3 in cDC1 development.

1.7.3. cDC2 related TFs

1.7.3.1. IRF4

The TF IFN regulatory factor 4 (IRF4) is selectively expressed in cDC2s across tissues and is essential or the development of mostly non-lymphoid tissue-resident cDC2s[101,115,210]. IRF4 has also been implicated in controlling the functional properties of cDC2, such as their CCR7-dependent migration[220] and antigen presentation by MHC-II[70].

1.7.3.2. NOTCH2 and KLF4

As mentioned earlier, the expression of TFs NOTCH2 and KLF4 defines mutually and functionally distinct populations within the cDC2 subset. The NOTCH2 dependent cDC2s drive Th17 immune responses[110,115] and IL-23 mediated innate immunity in the small intestine[55], whereas the KLF4 dependent drive Th2 responses[109].

CHAPTER 2 - Antigen cross-presentation

2.1. Antigen cross-presentation and CD8$^+$ T cross-priming

Antigen presentation is a cellular process through which protein antigens are processed into peptides, loaded onto MHC molecules and presented on the cell surface to naïve T cells. This presentation is restricted by MHC molecules – MHC-I-peptide complexes are recognised by TCRs on CD8$^+$ T cells while MHC-II-peptide complexes are recognised by TCRs on CD4$^+$ cells. The classical model of antigen presentation suggests that all nucleated cells expressing MHC-I can present cell-intrinsic peptides to CD8$^+$ T cells, whereas professional APCs expressing MHC-II present exogenous antigen-derived peptides to CD4$^+$ T cells. It is now established that this model is only partially correct. In 1976, MJ Bevan observed for the first time that CD8$^+$ T cells in mice were reactive against injected splenocytes which expressed an MHC-I haplotype different from that of the host[221,222]. This suggested that host APCs could internalise foreign antigens and 'cross-present' them on MHC-I to CD8$^+$ T cells. In another exception to the classical model, intracellular antigens can be processed via autophagy for MHC-II presentation to CD4$^+$ T cells [223–226].

When cross-presentation by APCs (signal 1) is accompanied by co-stimulatory and cytokine signals (signals 2 and 3 respectively), it leads to cross-priming of CD8$^+$ T cell and the induction adaptive immune responses. On the other hand, in the absence of signals 2 and 3, cross-presentation leads to the generation of immune tolerance. As described in the previous chapter, the cDC1s are functionally specialised in the cross-priming of CD8$^+$ T cells. Cross-priming by cDC1s is especially relevant for adaptive responses against viral infections which show a strict tissue tropism (such as papillomavirus infection in the skin epithelium[227]), or which do not infect APCs (such as Semliki forest virus[228]), or which infect APCs but impair direct presentation by MHC-I (such as measles, vaccinia and HSV[229–233]). Moreover, cross-priming is essential for combating intracellular parasitic infections such as *Toxoplasma gondii*[234], and intracellular bacterial infections such as *Listeria monocytogenes*[235] and *Mycobacterium tuberculosis*[236,237]. Last but not the least, cross-priming by cDC1s is crucial for mounting anti-tumour responses[9]. This chapter talks about the intracellular mechanisms which regulate cross-presentation by DCs.

2.2. Intracellular pathways of cross-presentation

Based on the intracellular location of the processing of antigen into peptides and their loading onto MHC-I molecules, broadly two pathways of cross-presentation have been described – vacuolar

and cytosolic pathways. Figure 2.1 summarises the present knowledge on these pathways and the molecules involved.

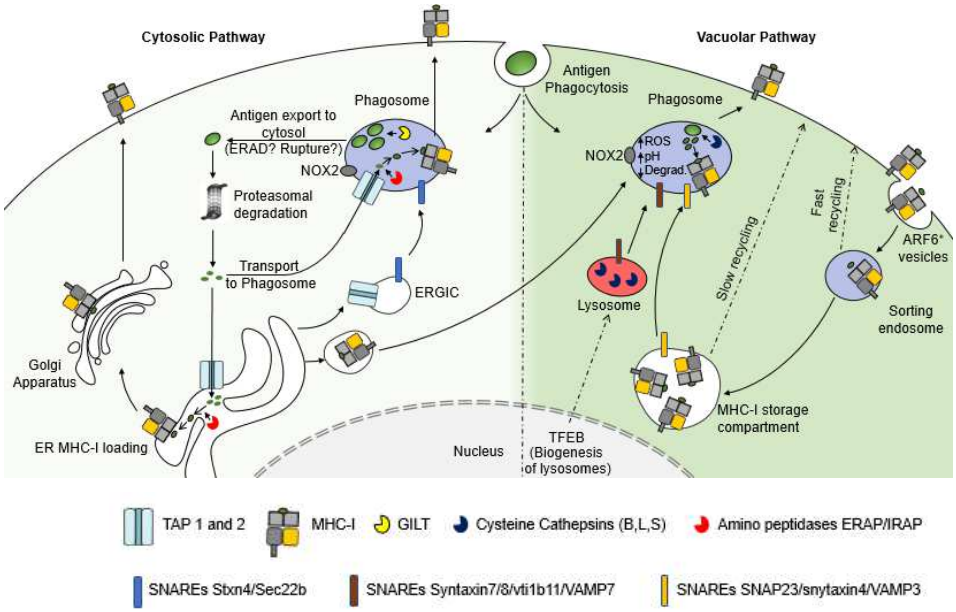

Figure 2.1 **Cellular pathways of cross-presentation.** Upon phagocytosis, the antigen can be degraded into MHC-I compatible peptides either through the cytosolic or the vacuolar pathway. In the cytosolic pathway, the antigen is unfolded and mildly degraded in the phagosome by the activity of thiol-reductase GILT and then transported to the cytosol for degradation by the ubiquitin-proteasome system. This transport may be carried out by the action of the ERAD transporter machinery present at the phagosome. Simultaneously, a rupture hypothesis is proposed, whereby the activity of NOX2 (NADPH oxidase complex) causes an accumulation of ROS in the phagosomal lumen. ROS cause lipid peroxidation, which may rupture the phagosomal membrane, thereby promoting antigen escape to the cytosol. The peptide products of proteasomal degradation may be transported to the ER in a TAP-dependent manner, where they are further trimmed by ERAP for MHC-I loading and subsequent surface presentation. The MHC-I loading machinery is also present at the phagosome, transported from the ERGIC by the activity of the SNARE complex

Syntaxin4-Sec22b. Therefore, TAP may also facilitate the transport of proteasomal products back into the phagosome. In the phagosomal, proteasomal products are trimmed by IRAP and loaded onto MHC-I molecules, followed by their surface presentation. In the vacuolar pathway, the antigen is degraded into MHC-I compatible peptides directly within phagosomes. Lysosomal cysteine proteases (such as Cathepsin B, L and S) are delivered to phagosomes by phagolysosomal fusion. Compared to macrophages and cDC2s, the phagosomal milieu in cDC1s is more alkaline, primarily due to ROS production by NOX2. ROS production neutralises the acidic pH of the maturing phagosome, thereby limiting the activity of cathepsins. Separately, ROS may also inactivate cathepsins through redox modulation. cDC1s also display reduced expression of TFEB, a transcription factor critical for lysosomal biogenesis and function. These features support a mild degradation of antigens in the phagosomes and the conservation of peptides for their loading on MHC-I. The MHC-I molecules may be transported to the phagosome from the ER, or recycled from the plasma membrane through an intracellular MHC-I storage compartment. GILT - γinterferon-inducible lysosomal thiol-reductase, ERAD - ER-associated degradation, ERGIC - ER-Golgi intermediate complex, ROS - reactive oxygen species, TAP - transporter associated with antigen processing, ERAP/IRAP - ER/Insulin regulated aminopeptidase, TFEB - Transcription factor EB. The figure is based on Joffre et al., Nature Reviews Immunology 2012[238].

2.2.1. Vacuolar versus Cytosolic pathway

Although cross-presentation was first described in 1976, the intracellular mechanisms at play only started to emerge in the 1990s. Multiple initial studies on cross-presentation were performed using macrophages and the model protein antigen - chicken Ovalbumin (OVA). In these studies, OVA was used in particulate forms (i.e. conjugated to latex beads or fused with intracellular bacteria *E. coli* or *S. typhimurium*). Cross-presentation was observed to be unresponsive to treatment with proteasome inhibitors, suggesting that instead of the classical proteasomal processing of antigens for MHC-I loading, the processing for cross-presentation occurred in the phagosome[239]. Moreover, cross-presentation was unresponsive to treatment with Brefeldin A[239–241] (an inhibitor of ER-Golgi transport) and occurred independently of TAP[239,242], suggesting that peptide loading onto MHC-I occurred in phagosomes instead of the ER. These observations gave rise to the vacuolar pathway of cross-presentation, wherein the antigen is processed and loading on MHC-I in the confines of the phagosome. In support of this pathway, it was observed that cross-presentation of particulate

antigens, as well as influenza virus, was disrupted upon treatment with the inhibitors of lysosomal proteases[243]. In the same study, the absence of the Cathepsin S in phagosomes led to a poor generation of MHC-I epitopes.

A simultaneous study by Kovacsovics-Bankowski and Rock suggested that cross-presentation of bead-associated OVA by macrophages was indeed blocked by proteasome inhibitors as well as Brefeldin A, and was dependent on TAP activity[244]. Moreover, the authors observed that phagocytosis of a membrane-impermeant, ribosome-inactivating protein (gelonin) disrupted protein synthesis in cells, indicating that the antigen was exported from the phagosome to the cytosol. Additional studies also indicated that cross-presentation was disrupted by proteasome inhibitors[245–247]. These observations gave rise to the cytosolic pathway of cross-presentation, whereby the exogenous antigen undergoes export to the cytosol and degradation via the ubiquitin-proteasome system. The proteasomal peptides are transported by TAP to the ER, where they are further processed and loading onto MHC-I, followed by transport to the cell surface through the ER-Golgi network. Moreover, later studies identified that MHC-I, as well as the peptide loading machinery (TAP, tapasin, ERp57 and calreticulin), are also recruited to the antigen containing phagosomes[248,249]. Thus phagosomes are competent organelles to receive proteasomal peptides and to load them on MHC-I for cross-presentation.

Since TAP proteins are important for the stability of vacant MHC-I molecules, their deficiency can impact both the vacuolar as well as the cytosolic pathways of cross-presentation[250–253]. The relative contribution of each pathway may depend on the context and the nature of the antigen itself. The best evidence in support of the cytosolic pathway comes from a report by Palmowski et al. Using mice deficient in the immunoproteasome subunit LMP7, they showed that cross-priming of $CD8^+$ T cells against the immunoproteasome-dependent H-Y epitope is impaired *in-vivo*[254].

2.2.2. Mild degradation of antigen in the phagosome

Irrespective of the pathway, cross-presentation benefits from a mild phagosomal degradation of antigens, which helps to conserve peptides for MHC-I loading. cDC1s being specialised at cross-presentation possess mechanisms to limit antigen degradation. For example, small GTPase Rac2 mediates the assembly of an NADPH oxidase complex, NOX2, at the phagosomal membrane in cDC1s[255]. NOX2 constitutively produces ROS in the phagosomal lumen, thus maintaining an

alkaline phagosomal milieu[255,256]. The alkaline milieu limits the activity of cysteine proteases such as Cathepsin S. Interestingly, NOX2 has also been observed to limit the activity of cysteine proteases via redox modulation, without affecting the phagosomal pH[257]. In cDC2s on the other hand, Rac2 mediates NOX2 complex formation at the plasma membrane[255]. Moreover, Rab27a[258] has been implicated in the transport of NOX2 to the phagosome in BMDCs (obtained from GM-CSF supplemented bone marrow culture), and more recently, Rab39a has been implicated in this function specifically in cDC1[75]. cDCs in general also express much lower levels of lysosomal proteases such as Cathepsins S, B, D, H, O and E compared to macrophages[259]. Additionally, cDC1 express higher levels of cathepsin inhibitors compared to cDC2[69,70].

The transcription factor EB (TFEB) is a critical regulator of lysosomal biogenesis and function, and it has been shown to modulate cross-presentation by DCs[112]. The overexpression of TFEB in BMDCs significantly increased the number of lysosomes, acidification of phagosomes (by phagolysosomal fusion) and proteolysis of OVA, compared to BMDCs overexpressing a control construct. On similar lines, TFEB overexpressing BMDCs perform reduced cross-presentation of soluble and particulate antigens *in-vitro.* To check the effect of TFEB on cross-presentation *in-vivo*, the authors depleted cDCs in mice by administering Diphtheria toxin in CD11c-DTR mice, and then they injected TFEB/control construct overexpressing BMDCs in these mice. Mice injected with TFEB-overexpressing BMDCs showed a significant reduction in cross-presentation of soluble antigen compared to the control group. Moreover, using flow cytometry and western blot analysis, splenic cDC1 were found to express significantly lower levels of TFEB compared to cDC2s and macrophages[112].

A role of the ER-resident SNARE protein Sec22b in phagosomal antigen degradation has been debated. Cebrian et al. showed that the si-RNA mediated knockdown of Sec22b in the DC line JAWS-II, as well as BMDCs, significantly enhanced phagosomal antigen degradation and reduced cross-presentation compared to control cells[260]. Hence, they proposed a role of Sec22b in facilitating cross-presentation. This notion was further supported by a follow-up study, which showed that the selective deletion of Sec22b in cDCs significantly impaired the cross-priming of $CD8^+$ T cells for anti-tumour responses in mice *in-vivo*[261]. These observations have been challenged by a report by Wu et al. This report demonstrates that the si-RNA-based knockdown of Sec22b in BMDCs gives off-target effects, which are responsible for the cross-presentation

phenotype observed in these cells[262]. Moreover, the selective deletion of Sec22b in cDCs did not cause any defects in $CD8^+$ T cell cross-priming in mice *in-vivo*. Thus, the authors propose that Sec22b does not facilitate cross-presentation. Anyhow, Sec22b may contribute to phagosomal dynamics and cross-presentation in other ways, which are discussed in the following topics.

2.2.3. Antigen export to the cytosol

One of the initial reports on cross-presentation suggested that the export of antigens to the cytosol in macrophages requires their activation[245]. Subsequent studies with bone marrow-derived DCs (BMDCs) and a DC cell line (D1) revealed that DCs are specialised at cross-presentation, and can transport antigens to the cytosol without activation in the steady state[263,264]. Moreover, DCs are much more efficient at transporting antigens to the cytosol compared to macrophages[264]. Lin et al. developed an apoptosis assay to compare cytosolic export ability within DC subsets[265]. They intravenously injected Cytochrome C in mice, which is transported to the cytosol upon micropinocytosis and induces Apaf1-dependent apoptosis in cells. It was observed that apoptosis was induced in a specific population of splenic cDC1s. This population possessed the ability to efficiently cross-present antigens, whereas Cytochrome C-resistant cDC1s were less efficient at cross-presentation. Moreover, the depletion of this 'cytosol exporting' population of cDC1s by Cytochrome C-induced apoptosis resulted in the ablation of the CTL responses to exogenous and tumour antigens *in-vivo*. Thus, cytosolic export of antigens in cDC1 is important for *in-vivo* cross-priming by these cells.

Evidence suggests that cytosolic transport of antigen may depend on the nature of the antigen itself. Using microscopy, it has been observed that fluorescently labelled dextrans and soluble proteins (active horseradish peroxidase or HRP) are exported to the cytosol in DCs[263,264]. Smaller dextrans (3 to 40 kDa in weight) are more readily transported whereas larger dextrans (500 to 2000 kDa in weight) remain inside the vacuoles, suggesting that cytosolic export depends on the size of the antigen[264]. Particulate antigens, on the other hand, may form aggregates within the vacuoles, thus hampering their export to the cytosol. Therefore, the cytosolic export of particulate antigens is promoted by a mild degradation inside the vacuoles[264], a factor tightly regulated in DCs by NOX2 activity. Moreover, disulphide bond containing antigens, such as HSV glycoprotein B, require an initial unfolding in the phagosome by the thiol-reductase GILT for their export to the cytosol and subsequent cross-presentation by DCs[266]. Antigen unfolding is a critical step for

cytosolic export, and it has been shown that paraformaldehyde-fixed, inflexible OVA is weakly exported to the cytosol and cross-presented in comparison to non-fixed, flexible OVA in mouse DCs[267].

Two salient hypotheses have been proposed to explain the mechanism underlying the transport of antigens to the cytosol[268]. Figure 2.2 provides a brief description of these mechanisms.

Figure 2.2 **Proposed mechanisms for antigen export from phagosome to cytosol.** Two hypotheses have been proposed to explain the cytosolic transport of antigens for their proteasomal processing and cross-presentation. According to the transporter hypothesis (left), protein antigens are unfolded within the phagosome and transported to the cytosol via protein transporter channels. These channels may comprise ERAD components like Sec61 and p97, which are well known to mediate the ER-to-cytosol retro-translocation of misfolded proteins for proteasomal degradation.

Both these proteins are recruited to phagosomes, and have been shown to mediate cytosolic export and cross-presentation of antigens in DCs, although the role of Sec61 in this process is debatable. The cytosolic chaperon HSP90 has also been shown to facilitate cytosolic export and cross-presentation of antigens in DCs. HSP90 binds with transported polypeptides and co-operates with other chaperons to decide their fate in the cytosol. It may co-ordinate with a presently undefined transporter to form an accessory transport system on the phagosomal membrane. According to the membrane rupture hypothesis (right), rupture of phagosomal membranes leads to the leakage of antigens into the cytosol. (Top right) Recruitment of the NOX2 complex to the phagosomal membrane causes ROS production in the phagosomal lumen. ROS induce lipid peroxidation of the phagosomal membrane, which leads to the destabilisation and rupture of the membrane. (Bottom right) Sphingosine-based lipids have been proposed to induce membrane destabilisation through rigidification of membrane domains. Lipid bodies may promotes the enrichment of sphingosine-based lipids on phagosomal membranes. The figure is taken from Gros and Amigorena, Frontiers in Immunology 2019[268].

2.2.3.1. Transporter hypothesis

The ER-associated degradation machinery (ERAD) is a system which exists in cells to remove newly synthesised, misfolded proteins from the lumen of the ER. Components of ERAD recognise misfolded proteins and facilitate their retro-translocation to the cytosol, where they are targeted for degradation by the ubiquitin-proteasome system. Translocation across the ER membrane is carried out via transporter channels, and this step requires a prior unfolding of proteins due to conformational limitations of such channels. The Sec61 trimeric complex forms one such protein transporter channel in the ER. It is involved in the translocation of nascent polypeptides into the ER as well as retro-translocation of misfolded ER proteins to the cytosol for their degradation[269].

Two simultaneous reports demonstrated that following the phagocytosis of OVA-latex beads, partially unfolded OVA is retro-translocated to the cytosolic surface of the phagosome, which facilitates its degradation by the proteasome for cross-presenation[248,249]. These reports also demonstrated that following phagocytosis, the ER membranes fuse with phagosomes and deliver ER-resident proteins, including Sec61, to the phagosome. Hence, it was hypothesised that Sec61 transporter channel facilitates the cytosolic export of antigens from the phagosome. Consistent with this hypothesis, Imai et al. showed that the knockdown of Sec61 in a mouse DC line (DC2.4)

reduced proteasomal degradation and cross-presentation of OVA[270]. On similar lines, Zehner et al. have shown that the retention of Sec61 in the ER by expressing a Sec61-specific intrabody in BMDCs reduced its recruitment to OVA containing phagosomes, in turn reducing the cytosolic translocation and cross-presentation of OVA[271]. Moreover, the cDC1s express higher levels of Sec61α, β and γ subunits compared to cDC2s, consistent with their functional specialisation at cross-presentation[69].

However, the role of Sec61 in the cytosolic transport of antigens and cross-presentation remains controversial. Using a Sec61 inhibitor named 'mycolactone', Grotzke et al. have shown that Sec61 does not facilitate the cytosolic transport of antigens. Although the sustained treatment of MutuDCs (a splenic cDC1 line) with mycolactone reduced cross-presentation by these cells, this defect was independent of cytosolic export of antigen, and rather caused due to decreased expression of MHC-I[272]. Therefore, this study demonstrates that Sec61 affects cross-presentation indirectly, without affecting the transport or processing of the antigen. On similar lines, downregulation of Sec61 in human monocyte-derived DCs using si-Sec61α does not affect the cytosolic transport and cross-presentation of synthetic long peptides from the Melan-A/MAERT-1 tumour associated antigen in these cells.

Other ERAD components, including Hrd1 and Derlin1, have also been implicated in cross-presentation. Hrd1 is an ER-resident ubiquitin ligase which marks misfolded substrates in the ER lumen as well as within the ER membrane for degradation. It consists of six transmembrane domains which form a protein retro-translocation channel across the ER membrane[273]. Zehner et al. have investigated the role of Hrd1 in the cytosolic translocation of soluble OVA and its cross-presentation by BMDCs. Si-RNA mediated knockdown of Hrd1 does reduce cross-presentation of OVA by these cells without affecting the presentation of the pre-processed peptide, but it also reduces the MHC-II presentation of OVA[271]. Therefore, it remains to be seen whether the reduction of cross-presentation results from alterations in antigen processing or if it is an unspecific effect related to the knockdown of Hrd1. On the other hand, Derlin1 is a protease which associates which ER substrates as well as the Hrd1 complex. It contains four transmembrane proteins which are not enough to form a transmembrane channel, but through its association with Hrd1, it is thought to contribute to retro-translocation of ER proteins. Derlin1 was identified to exist in the phagosomes in association with ERAD components Sec61β and p97[274]. However, Derlin1 silencing in mouse

BMDCs[271] and human moDCs[275] does not affect cross-presentation, which excludes its role in antigen export to the cytosol.

The best evidence in support of ERAD dependent cytosolic translocation of antigens comes from studies on the ERAD component ATPase p97 (also known as Valosin containing protein or VCP). ATPase p97, in association with its cofactors Ufd1-Npl4 provides energy for retro-translocation of polyubiquitinated polypeptides from the ER to the cytosol[276]. P97 is recruited to the phagosome where it interacts with other ERAD components Sec61 and Derlin1[274]. Ackerman et al have shown that the ATPase activity of p97 is extremely essential for cytosolic export of antigens from phagosomes. The addition of purified, ATP bound p97 to isolated phagosomes loaded with luciferase induces the release of luciferase from the phagosomes, while the addition of a dominant-negative, functionally inactive form of p97 does not induce the release of luciferase[277]. Moreover, overexpression of the dominant-negative form of p97 in the human macrophage cell line KG.1 significantly reduces cross-presentation of exogenous OVA, without affecting the direct presentation of endogenously expressed OVA by these cells. On similar lines, silencing of p97 in CD2.4 as well as human moDCs has been shown to reduce their ability to cross-present OVA and Melan-A antigens, respectively[270,275].

Lastly, the cytosolic chaperone HSP90 (heat shock protein 90) co-operates with the ERAD machinery to bind transported proteins and determine their fate in the cytosol[278,279]. It has been shown that si-RNA mediated knockdown of HSP90 in DCs or treatment with HSP90 inhibitors reduces the ability of DCs to cross-present OVA[280,281]. This defect in cross-presentation is caused by the reduction of cytosolic export of antigens in the absence of HSP90 activity in DCs[267,281]. These studies suggest that HSP90 may itself form an accessory transporter complex to mediate the export of antigens from the phagosome to the cytosol, with implications in cross-presentation.

While these reports support the role of transporter channels in cytosolic export and cross-presentation of antigens, there still exist some unresolved questions. The transporter hypothesis is specific to the transport of protein antigens and requires the ubiquitination these antigens. It does not explain how non-protein entities like dextrans may be exported to the cytosol. Moreover, the existence of a singular transporter-mediated mechanism for the export of a wide variety of antigens seems unlikely. Therefore, there must exist supplementary mechanisms to mediate the process of cytosolic export.

2.2.3.2. Membrane disruption hypothesis

In the initial descriptions of the cytosolic pathway, Reis e Sousa and Germain proposed that overloading of phagosomes with OVA-beads leads to membrane rupture and export of OVA to the cytosol for proteasomal degradation and cross-presentation[247]. They termed this model of antigen export as the 'indigestion model'. Evidence in support of membrane rupture has only recently started to emerge. Two reports by Dingjan et al. have suggested that the recruitment of NOX2 to antigen containing phagosomes and subsequent ROS production cause lipid peroxidation of the phagosomal membrane[282,283]. Lipid peroxidation is a membrane disrupting chain reaction which destabilises the phagosomal membrane leading to antigen leakage in the cytosol. Moreover, these studies have implicated the ER-resident SNARE protein VAMP8, which interacts with the plasma membrane and phagosome resident SNARE syntaxin4, in the trafficking of NOX2 to the phagosomes. Subsequently, the knockdown of VAMP8 in mice, as well as human moDCs, impairs phagosomal ROS production, lipid peroxidation, antigen translocation and cross-presentation by these cells[282].

In addition to ROS mediated lipid peroxidation, Gros and Amigorena have argued for a role of sphingosine-based lipids (or sphingolipids) in phagosomal membrane destabilisation and antigen leakage for cross-presenation[268]. It has been shown that sphingolipids can promote membrane permeabilisation by inducing rigidification of membrane domains[284]. Sphingosine is synthesised through deacetylation of ceramides by two ceramidases, which are encoded by the genes Asah1 and Asah 2. These enzymes are functional in acidic and neutral pH respectively and have a higher expression in cDC1s compared to cDC2s (microarray data from immgen.org). These observations suggest that sphingolipid-based membrane rupture might promote cytosolic export of antigens for proteasomal degradation and cross-presentation by cDC1s. Moreover, it is proposed that cDC1 specific enrichment of sphingosine may be aided by lipid bodies (organelles for the storage of neutral lipids in the cells), Lipid bodies have been proposed to cause destabilisation of some ER – phagosomal membranes and leakage from these organelles[285]. Additionally, cDC1s contain significantly higher amounts of lipid bodies compared to cDC2s, and the accumulation of lipid bodies in BMDCs has been linked to increased cross-presentation ability by these cells[73].

2.2.4. Peptide-MHC-I loading

After the exogenous antigen is processed into peptides, they must be loaded on to MHC-I molecules for trafficking to the cell surface and cross-presentation to $CD8^+$ T cells. In the cytosolic pathway, proteasomal peptides may be loaded onto MHC-I in the ER or the phagosome. On the other hand, the vacuolar pathway requires MHC-I-peptide loading in the phagosome.

2.2.4.1. Peptide loading in the ER

Initial studies supporting the cytosolic pathway showed that cross-presentation was dependent on TAP activity and was sensitive to treatment with Brefeldin A[244,263,286,287]. These data suggest that upon proteasomal processing, antigenic peptides were transported to the ER by TAP activity and loaded onto the MHC-I. The ER-associated aminopeptidase 1 (ERAP1) is known to perform N-terminal trimming of peptides before they are loaded onto MHC-I molecules in the ER. ERAP1-dependent peptide trimming was shown to be essential for cross-presentation of immune-complexed and particulate OVA by BMDCs *in-vitro*, and of particulate OVA by splenic cDCs *in-vivo*[288,289]. Taken together, these observations support the cytosolic cross-presentation model, whereby the MHC-I-peptide loading occurs in the ER. The MHC-I-peptide complexes can be transported to the cell surface for cross-presentation via the Golgi network.

2.2.4.2. Peptide loading in the phagosome

As previously mentioned, components of the MHC-I peptide loading machinery, including TAP, tapasin, ERp57 and calreticulin are recruited to phagosomes[248,249]. This recruitment is mediated through temporary fusions of the ER membranes with antigen containing early phagosomes[249]. Moreover, Cebrian et al. have shown that the peptide loading machinery can be delivered to phagosomes through the ER-Golgi intermediate complex (ERGIC)[260]. The ERGIC resident SNARE protein Sec22b interacts with phagosome resident SNARE syntaxin4 to mediate this transport. Hence, proteasomal peptides can be transported to phagosomes in a TAP-dependent manner.

The loading of proteasomal peptides onto MHC-I in the phagosomes would require their final processing, akin to ERAP in the ER. Saveanu et al. have shown that an N-terminal trimming peptidase closely related to ERAP, known as the insulin-regulated aminopeptidase (IRAP), exists

in an endosomal storage compartment marked by Rab14[290]. Moreover, IRAP is strongly recruited to the phagosomes in cDCs, and its deficiency significantly reduces cross-presentation by these cells (more so in cDC1 compared to cDC2s)[291]. Thus, the presence of IRAP in phagosomes contributes to the superior cross-presentation ability of cDC1s.

Various pathways of MHC-I trafficking to the phagosome have been described. MHC-I molecules are recycled from the plasma membrane in clathrin-independent vesicles to a perinuclear MHC-I storage compartment in the cell, marked by Rab11a. Upon TLR signalling in the phagosome, the phagosomal SNARE protein-SNAP23 mediates fusion between phagosomes and the MHC-I storage compartment, which leads to the phagosomal recruitment of MHC-I[292]. Moreover, Rab22a[293] and Rab3b/3c[294] have also been implicated in the trafficking of cell surface-recycled MHC-I to phagosomes. Apart from recycled MHC-I molecules, ER-derived MHC-I molecules may also be recruited to phagosomes. A few studies have reported that CD74 (also known as the invariant chain), which stabilises MHC-II molecules and contributes to their trafficking to the endocytic pathway for antigen loading, can also associate with MHC-I molecules in the ER and mediate their trafficking to late endo-lysosomes[295–297]. This CD74 mediated MHC-I trafficking has been shown to promote cross-presentation of cell-associated as well as viral antigen by cDCs[295]. Moreover, a recent report has suggested that the ER-phagosome trafficking of MHC-I in cDC1s is mediated by Rab39a[75]. Additionally, Rab39a deficiency causes an accumulation of open conformation MHC-I molecules in phagosomes. Thus, Rab39a phagosomal MHC-I recruitment and promotes their loading with peptides in this compartment.

Taken together, these observations indicate that phagosomes can function as compartments for MHC-I peptide loading for cross-presentation.

2.3. Phagocytosis by cDC1s and receptor targeting for cross-presentation

den Haan et al. were the first to demonstrate in mice that cDC1s are the most efficient DC subset at internalising necrotic cell-associated antigens *in-vivo*[286]. Later studies confirmed that cDC1s perform higher phagocytosis of necrotic cell-associated antigens compared to cDC2s, while both these subsets performed comparable phagocytosis of bead-associated and soluble antigens[298–300]. Moreover, it has been reported that the lung migratory cDC1s ($CD103^+$) are the only DCs which internalise necrotic cell-associated antigens[301] as well as viral antigens from infected cells[57], and

transport them to draining lymph nodes for cross-presentation *in-vivo*. Therefore, in physiological conditions, murine cDC1s seem to be better at phagocytosis of antigens compared to cDC2s, which supports their superior ability to cross-present these antigens. On the other hand, both cDCs subsets in humans seem to possess comparable abilities to internalise necrotic cell-associated and soluble antigens[60,81,85].

Antigen targeting to several different phagocytic receptors has been studied in the context of cross-presentation. These studies are described as follows.

2.3.1. CLEC9A

The C-type lectin-like receptor CLEC9A/DNGR-1 is specifically expressed on cDC1s in mice as well as humans. Sancho et al. were the first to report the role of CLEC9A in the recognition of necrotic cells and cross-presentation of necrotic cell-derived antigens by cDC1s[72]. They observed that CLEC9A deficiency did not affect the internalisation of necrotic cells by BMDCs *in-vitro* as well as splenic cDC1s *in-vivo.* To study cross-presentation, the authors used murine embryonic fibroblasts immortalised from H-2^{kbm1} mice. These cells possess a loss of function mutation in H-2^{kb}, which rules out direct antigen presentation to $CD8^+$ T cells by these cells. Kbm1 fibroblasts were further transduced to express a non-secreted form of OVA antigen (Kbm1-OVA fibroblasts). CLEC9A deficient BMDCs cultured *in-vitro* with UV-treated Kbm1-OVA fibroblasts showed reduced cross-presentation to OT-I cells compared to CLEC9A expressing BMDCs. Similarly, CLEC9A deficient mice immunised with UV-treated Kbm1-OVA fibroblasts were less efficient at mounting CTL responses to compared to control mice *in-vivo*. Importantly, CLEC9A deficiency does not inhibit the cross-presentation of soluble or bead associated OVA, which indicates that CLEC9A engagement affects cell signalling in DCs rather than cross-presentation machinery itself[72]. The authors further explored the cell signalling downstream of CLEC9A which promoted cross-presentation. They observed that the cytosolic ITAM motif of CLEC9A contains a key tyrosine, which promotes binding with phosphorylated syk kinase upon CLEC9A engagement. Thus, CLEC9A/Syk kinase signalling is independent of phagocytosis and promotes cross-presentation in these DCs[72].

Later studies have shown that CLEC9A engages necrotic cells through interactions with the exposed cytoskeletal component 'F-actin' on the surface of necrotic cells[302,303]. Mutations in

CLEC9A residues which bind F-actin abolishes the cross-presentation of necrotic cell-derived antigens by DCs[302].

Zenelay et al. have extended the role of CLEC9A to cross-presentation during infectious settings. They found a non-redundant role of CLEC9A in generating anti-viral CTL responses in mice[304]. Moreover, they show that antigens internalised by CLEC9A are targeted to non-degradative early endosomal compartments and away from lysosomes, which promotes their cross-presentation.

2.3.2. Immunoglobulin-G Fc receptors (FcγRs)

Both cDC1s and cDC2s express low levels of FcγRI and significant levels of FcγRII and FcγRIII[305]. Immune-complexing of OVA with anti-OVA IgG allows targeting of OVA to FcγR on its DCs for uptake and cross-presentation. den Haan and Bevan have shown that both cDC1s and cDC2s isolated from mice injected with OVA immune-complexes can cross-present OVA to OT-I cells *ex-vivo*[305]. Upon similar immunisation in FcγRII /III deficient mice, cDC1s continue to cross-present OVA while cDC2s lose this function. Hence in mice, antigen targeting to the FcγRs does not affect cross-presentation by cDC1s.

On the other hand, cross-presentation by human cDC1 seems to be promoted by antigen targeting to FcγRs. Flinsenberg et al. have shown that while FcγR targeting stimulates antigen uptake by human cDC2s rather than cDC1s, cross-presentation of FcγR targeted antigens is specifically promoted in cDC1s and not cDC2s[306]. Thus, FcγR signalling may promote cross-presentation by human cDC1s independently of antigen internalisation.

2.3.3. Mannose receptor (MR)

Mannose receptor, also known as CD206, is a transmembrane C-type lectin which functions as a scavenger receptor in macrophages, and is also expressed on cDCs. Burgdorf et al. have shown that the internalisation and cross-presentation of soluble OVA by BMDCs *in-vitro* as well as DCs *in-vivo* is dependent on MR[307]. In a follow-up study, the same group has further demonstrated that DCs can take up soluble OVA dependently as well as independently of MR[308]. Soluble OVA internalised through MR is cross-presented to $CD8^+$ T cells while OVA internalised through micropinocytosis is presented to $CD4^+$ T cells. Moreover, MR drives cross-presentation of soluble OVA by diverting its trafficking to early endosomes and away from degradative lysosomes[308].

2.3.4. DEC-205 (CD205)

DEC-205 is a phagocytic receptor which recognises apoptotic and necrotic cells, and is highly expressed in cDC1s compared to cDC2s[309]. Iyoda et al. observed that cDC1s from DEC205 deficient mice can internalise cell-associated antigens as efficiently as cDC1s in wild-type mice[298]. At the same time, several studies have reported that targeting of antigens such as OVA, HIV gag, tumour antigen HER2/neu and others to DEC-205 enhances their cross-presentation by DCs[69,310–312].

2.3.5. T-cell immunoglobulin mucin-3 (TIM-3)

TIM-3 is another receptor involved in the phagocytosis of apoptotic cells. Nakayama et al. have shown TIM-3 expression is 3-fold higher in cDC1s compared to cDC2s in mice[313]. Moreover, the blockage of TIM-3 using a monoclonal antibody reduces the uptake and cross-presentation of necrotic cells derived antigens by cDC1s *ex-vivo* as well as *in-vivo.*

Based on these observations, it may be summarised that phagocytic receptors facilitate cross-presentation in cDC1s via downstream cell signalling and directing the antigen to the cross-presentation machinery in the cell.

2.4. Role of DC activation and maturation in cross-presentation

In infectious settings, the activation of DCs occurs upon the engagement of their pattern recognition receptors (PRRs) like TLRs with pathogenic molecules, or upon CD40-CD40L interactions with $CD4^+$ helper T cell. Activation results in the 'licensing' of DCs to cross-prime $CD8^+$ T cells, which involves the up-regulation of surface expression of peptide-MHC molecules (signal 1) as well as co-stimulatory molecules CD40, CD80, CD86 (signal 2), and secretion of T cell activating cytokines like IL-12 (signal 3). Engagement of all these signals together on $CD8^+$ T cells leads to efficient cross-priming of CTL responses.

The immunological outcome of cross-presentation depends on the activation status of DCs. Non-activated, immature DCs can cross-present internalised antigens, but the absence of signal 2 and 3 leads to the generation of tolerogenic $CD8^+$ T cell responses. On the other hand, activated DCs undergo maturation and can cross-present antigens to cross-prime effector and memory $CD8^+$ T cell responses. This is evidenced by *in-vivo* studies in which the co-administration of DC activation

factors like anti-CD40 or TLR ligands along with the antigen generates efficient CTL cross-priming in mice, which can be recalled upon a secondary antigenic challenge[228,314,315]. The administration of antigens alone, in the absence of DC activating factors, produces T cell tolerance against the antigens in these studies.

Upon activation, DCs in various stages of maturation differ in their ability to cross-present antigens. In the early and intermediate stages, DCs are efficiently at cross-presentation, while fully matured DCs have comparatively reduced ability to cross-present antigens. Activation of BMDCs i*n-vitro* and splenic cDC1 *in-vivo* with lipopolysaccharides (LPS, TLR4 ligand) shows that early and intermediate maturation states correspond to 0-5h and 16-20h post-activation respectively[316,317]. At these time points, BMDCs as well as mice-isolated splenic cDC1 are efficient at cross-presentation of soluble, immune complexed as well as bead-associated OVA. Approximately 24h post-activation, DCs are completely matured and are unable to efficiently cross-present these antigens, but maintain the ability to present pre-processed peptides[316,317]. Activation with ligands for other TLRs suggests a similar trend of functionality in maturing DCs. BMDCs stimulated for 24h with polyI:C (TLR3 ligand) or CpG (TLR9 ligand) have a reduced ability to cross-present soluble antigens[318]. *In-vivo*, the activation of splenic cDC1s by *the* administration of LPS, polyI:C or CpG in mice selectively abolishes their ability to cross-present cell-associated OVA after 9-12h of stimulation, but not the direct presentation of endogenous OVA[319]. Moreover, these mice also lose the ability to mount anti-viral responses to HSV1 infections. Thus, upon activation, maturing DCs reach a peak of cross-presentation in the early and intermediate stages, followed by the downregulation of this ability as they fully mature.

Moreover, it is interesting to note that upon innate signalling, cross-presentation is selectively enhanced for antigens that are present within the same phagosomes as the innate sensor. This has been shown to be true for TLRs[292,320] as well as IgG receptors[321,322]. The ability to distinguish between innate sensor-containing phagosomes and by-standing phagosomes may serve as a mechanism to prevent cross-priming against self-antigens (from host's dead cells for example), which may be internalised by DCs along with microbial antigens.

DC maturation may affect various aspects of cross-presentation -

2.4.1. Effect on antigen uptake

In the initial hours after exposure to TLR ligands, DCs upregulate antigen uptake through micropinocytosis and endocytosis *in-vitro*[316,323,324] as well as *in-vivo*[319]. This upregulation is mediated by an acute remodelling of the actin cytoskeleton via MAP kinase signalling downstream of TLR engagement[324]. Multiple studies have reported that DCs in the intermediate or late stages of maturation have a reduced endocytic capacity compared to early maturation stages[317,319,325]. Alloatti et al. propose that following the early peak of antigen uptake and cross-presentation by activated DCs, maturing DCs enter a 'surveillance state', during which they maintain the ability, albeit lower compared to early activation stages, to uptake and cross-present antigens. This allows DCs to internalise more antigens as the infection develops, thus enabling optimal cross-priming to neutralise the infection[317]. Interestingly, Drutman and Trombetta have reported that maturing DCs can efficiently internalise antigens after stimulation with LPS and CpG[326]. In this study, DCs in mice were systematically activated by intra-peritoneal injections of LPS or CpG. After 16h, splenic cDCs from non-injected control or injected mice were isolated and tested for their ability to capture and present soluble OVA to OT-I cells ($CD8^+$ T cells with a transgenic TCR to specifically recognize OVA peptide-MHC-I complex) *in-vitro*. The control immature cDCs as well as activated cDCs were equally able to capture and cross-present soluble OVA to OT-I cells[326]. Moreover, Platt et al. have shown that the targeting of antigens to endocytic receptors such as FcgR allows efficient and cross-presentation in mature DCs[327]. Therefore, efficiency of antigen uptake by maturing DCs depends on the method of antigen administration, and it is not a limiting factor through which DC maturation controls cross-presentation.

2.4.2. Effect on antigen export from phagosome to cytosol

Gil-Torregrosa et al. have shown that following a short activation with LPS (0-5h), BMDCs can efficiently export antigens to the cytosol, whereas a longer activation (24-40h) reduces their ability to export antigens[316]. This coincides with the high and low cross-presentation abilities of early and late matured DCs, respectively. The enhanced antigen export in the early stages of activation may be due to a reduction of phagosomal antigen degradation caused by TLR signalling.

2.4.3. Effect on phagosomal antigen degradation

Alloatti et al. have observed that a few hours after the activation of BMDCs with various TLR ligands, perinuclear clustering of the lysosomes is induced in these cells[317]. Lysosomal clustering causes the sequestration of lysosomes away from the antigen-containing phagosomes and inhibition of the fusion between the two organelles, thus limiting phagosomal maturation as well as antigen degradation. Lysosomes in resting-state BMDCs, which are not treated with TLR ligands, do not undergo perinuclear clustering, leading to phagolysosomal fusion and antigen degradation in these cells. Thus, while transitioning from immature to fully mature state, DCs enhance their ability to cross-present antigens by limiting antigen degradation in phagosomes. Further, it is reported that the GTPase Rab34 mediates the lysosomal clustering induced by TLR4 signalling[317]. Silencing of Rab34 reverts the lysosomal clustering and reduces cross-presentation selectively by maturing BMDCs, and not the resting state BMDCs. Thus, by controlling lysosomal positioning in maturing DCs, Rab34 facilitates cross-presentation in DCs.

Samie and Creswell have described the regulation of TFEB expression during DC maturation[112]. They found that the TFEB expression is relatively low in immature BMDCs. Upon activation with TLR2/4 ligands, but not TLR9 ligand, TFEB is upregulated in maturing BMDCs as a function of the duration of activation. Significant expression is observed in cells activated for more than 6h. Moreover, while control BMDCs lose their ability to cross-present antigens after 24h of activation with LPS, TFEB knockdown BMDCs can partially cross-present antigens even at this high stage of maturation[112].

2.4.4. Effect on phagosomal recruitment of MHC-I

As previously mentioned, MHC-I molecules can be recycled from the plasma membrane to an intracellular storage compartment, and then recruited to the phagosomes for peptide loading and cross-presentation. While the maintenance of MHC-I in the storage compartment depends on the GTPase Rab11a, and is independent of TLR signalling, the fusion of this compartment to the phagosomes for MHC-I delivery is induced by phagosomal TLR4 stimulation[292]. The storage compartment contains the R-SNAREs VAMP3 and VAMP8, whereas the phagosomes contain the Q-SNAREs SNAP23 and syntaxin4. Nair-Gupta et al. have shown that upon internalisation of *E.coli* or LPS-coated beads by BMDCs, a MyD88-dependent TLR signalling takes place at the phagosomes. This signalling leads to IKK2-dependent phosphorylation of SNAP23 on TLR-containing phagosomes. Phosphorylated SNAP23 then forms the SNAP23/Syntaxin4/Vamp-3

SNARE complex, which promotes the fusion between the MHC-I storage compartment and TLR-containing phagosomes, and delivery of MHC-I to phagosomes for peptide loading and cross-presentation. On these lines, BMDCs, as well as splenic cDCs isolated from MyD88-/- cells, have a significant reduction in cross-presentation of *E.coli*-associated OVA compared to wild-type controls[292].

In summary, cDC1s efficiently cross-present antigens through a combination of mechanisms. cDC1s possess the cellular machinery to optimise the processing of exogenous antigen for MHC-I loading. This involves a mild phagosomal degradation of the internalised antigens, as well as the export of unfolded antigens to the cytosol for their degradation into peptides by the ubiquitin-proteasome system. cDC1s are also efficient at internalising antigens and feeding them to the cross-presentation machinery. Moreover, in infectious settings, various aspects of the cross-presentation machinery are regulated, including intracellular trafficking and processing of antigens, as well as the delivery of MHC-I molecules to the peptide loading compartments. The mechanisms co-ordinate to upregulate cross-presentation at early time points of DC maturation.

CHAPTER 3 - Rab GTPases

3.1. The Rab GTPase family of proteins

Rab proteins (Ras-related proteins in the brain) were first described in the 1980s as evolutionarily conserved, guanine nucleotide-binding proteins essential for intracellular membrane trafficking in *S. cerevisiae*[328,329] and rat brain cDNA library[330]. They form the largest branch of the 'Ras (rat sarcoma) superfamily of small GTPases', also known as monomeric G-proteins. Small GTPases possess a conserved guanine tri-phosphatase (GTPase) domain which confers to them the ability to bind and independently hydrolyse GTP. The Ras superfamily is divided into 5 subfamilies – Arf, Rab, Ran, Ras and Rho, based on their structural sequences and functional similarities[331]. In general, Ras proteins are involved in the spatiotemporal regulation of gene expression, signal transduction, cytoskeleton organisation and vesicular trafficking within eukaryotic cells, with implications in cell growth, motility and differentiation[332,333].

Rab GTPases are characterised by their low molecular weight (20-25kDa) and their ability to localise to distinct intracellular membranes[334]. They are master regulators of all aspects of vesicular trafficking in the secretory, endosomal and transcytosis pathways – (i) vesicular budding and cargo sorting from the donor compartment, (ii) motor dependent transport to acceptor compartment, (iii) tethering and docking with acceptor membrane and (iv) fusion and transfer of cargo to the acceptor compartment. In humans, close to 70 proteins of Rab GTPases family have been described[335–337]. They have been named Rab1, Rab2 and so on, based on the sequence of their discovery.

Inside the cell, Rab GTPases act as molecular switches by cycle between two conformational states- GDP-bound 'inactive or OFF' state and 'GTP-bound 'active or ON' state. The nucleotide cycle of a Rab is regulated through its interactions with regulatory proteins. Rab proteins are linked reversibly to intracellular membranes by cysteine prenylation (lipid modification) at C-terminus and consequently targeted to specific membranes by escort proteins. Once recruited on their target membranes, Rab GTPases in their active state work as scaffolds to recruit downstream effectors on the vesicle. The Rab-effector complexes act as adaptors for vesicular coat proteins, cytoskeletal motors and SNARE complexes, thus guiding vesicular trafficking and fusion events. Multiple Rab proteins can be recruited to the same organelle at distinct membrane micro-domains called Rab domains, thus contributing to the functional compartmentalisation of a continuous membrane structure. Rab domains have been observed for Rab4, Rab5 and Rab11 on early/recycling

endosomes[338] as well as for Rab7 and Rab9 on late endosomes[339]. By recruiting specific effector proteins to restricted micro-domains, Rab GTPases specify intracellular membrane identity[340].

3.1.1. Structure of Rab GTPases

Small GTPases including Rab GTPases contain a universally conserved guanine-nucleotide binding and GTP-hydrolysing region called the G-domain beginning at the N-terminus, approximately 20-kD in weight. A general structural map of the Rab G-domains is depicted in Figure 3.1. The tertiary structure of this domain was first described in translational GTPase EFTu[341,342] and Human-Ras p21[343,344], using high-resolution crystallographic analyses. The domain comprises a six-stranded β-sheet, with five α-helices located on both sides. Hence small GTPases are classified as α,β proteins. In H-Ras p21, this domain has been determined to be approximately 160 amino acids in size. The guanine nucleotide-binding site is made up of conserved polypeptide loops, which interact with guanine base (G) or phosphate/Mg^{2+} (PM) [345,346].

- PM-1 or P loop (phosphate-binding loop) connects the β1 strand to the α1 helix. It has a consensus sequence GXXXXGKS/T[347], and interacts with the α, β-phosphates of GDP or GTP, which is the most important element for tight binding of nucleotide.
- PM-2 connects α1 helix and the β2 strand and contains a conserved threonine (position 35 in H-Ras p21) residue involved in Mg^{2+} (cofactor) dependent binding and stabilisation of γ-phosphate as well as GTP hydrolysis.
- PM-3 with the consensus sequence DXXG (WDTAGQE in Rab proteins by consensus) exists at the N terminus of the α2. The invariant aspartate (position 57 in H-Ras p21) binds the catalytic Mg2+ through an intervening water molecule, while the invariant glycine (position 60 in Ras p21) forms a hydrogen bond with the γ-phosphate of GTP. Conformations of amino acids 60-63 and the downstream α2 helix differ dramatically in GTP- and GDP-bound forms of the protein.
- G-1 with a conserved Phenylalanine-28 (replaced by tyrosine in certain Rab proteins) exists directly perpendicular to the guanine base and contributes to binding with it.
- G-2 with the consensus sequence N/TKXD links the β5 strand and the α4 helix and interacts with guanine ring of the nucleotide.

- G-3 is located between β6 and helix α5 with the consensus sequence (T/G)(C/S)A, supports the guanine base recognition site.

The ability of GTPases to cycle between GDP/GTP bound states is supported by a 'conformational switch' in their molecular structure induced by the presence or absence of γ-phosphate. A majority of these changes occur in two 'switch regions' – called switch I and switch II, initially identified in the G-domain of H-Ras p21[343,348]. Minor conformational changes in the flexible inter-switch regions have also been observed. The exact position of Switch regions varies in different Rabs[349–352], but by consensus, Switch I is present in the PM2 and β2 strand, while switch II is present in PM-3 and α2 helix. In the presence of GTP, the NH groups of the invariant threonine and glycine residues in switch I and II respectively form two hydrogen bonds with the γ-phosphate oxygens. Vetter and Wittingghofer have described this conformational change as a loaded spring mechanism, where the release of γ-phosphate after GTP hydrolysis allows the switch regions to relax into GDP-specific conformation[353] (figure 3.2).

Rab proteins possess 5 short conserved stretches of residues that distinguish them from other members of the Ras superfamily. These Rab family (RabF) motifs were identified by sequence analysis of mammalian Rab and Rab-like proteins and are proposed to contribute to a rigid structural conformation promoting Switch I/II interaction[354]. RabF1 (IGVDF) is localised to the effector-binding domain in the switch I, while RabF2 (KLQIW), RabF3 (RFRSIT), RabF4 (YYRGA) and RabF5 (LVYDIT) cluster around switch II. Rab proteins are further divided into subfamilies of related or isoform proteins. This classification is based on 4 conserved sequence motifs (RabSF) within the Rab family, along with variations of RabF motifs and G-domain loops[354,355]. Conformation regulating proteins of Rabs, such as GDP/GTP exchange factors and GTPase activating proteins, as well as downstream effector proteins bind to RabF and RabSF regions in Rab GTPases.

The C-terminus region in Rab proteins is hypervariable and possesses prenylation motifs which act as substrates for Rab geranylgeranyltransferase (GGT-II)[356]. These motifs are post-translationally linked to the Rab protein. This lipid modification is essential for the association of Rab proteins with their target membranes. Most Rab proteins present a motif consisting of two cysteine residues, found in one of the following combinations: *XXX*CC, *XX*CC*X*, *X*CC*XX*,

CC*XXX* or *XX*C*X*C. However, some Rabs such as Rab8 and Rab13 present a C*XXX* box, where only one cysteine residue is available for prenylation[357].

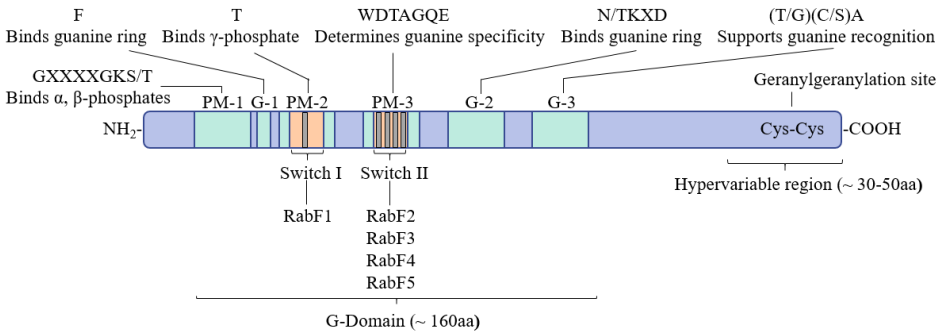

Figure 3.1 **A structural map of Rab GTPases.** The Guanine nucleotide-binding 'G-domain' is highly conserved in small GTPases. It comprises guanine base binding (G) as well as phosphate/Mg^{2+} binding (PM) motifs. The consensus sequences along with specific functions of these motifs are indicated. Conformational changes determining the GDP/GTP binding occur in switch I and II regions, which lie in PM-2 and PM-3 regions, respectively. Rab GTPases contain salient Rab family (RabF) motifs in the G-domain, which distinguish them from other families of small GTPases. The C-terminus hypervariable region contains two Cysteine residues (or one residue in some Rabs) which act as sites for geranylgeranylation. This lipid modification is essential to Rab-membrane binding. The figure is based on Valencia *et al*., Biochemistry 1991[346] and Pereira-Leal and Seabra, J. Mol. Biol. 2000[354].

Figure 3.2 **Schematic diagram of the universal switch mechanism.** Switch I and II domains are bound to the γ-phosphate via the main chain NH groups of the invariant threonine (position 35 in H-Ras p21) and glycine (position 60 in H-Ras p21) residues, in what might be called a loaded spring mechanism. The figure is taken from Ingrid R. Vetter and Alfred Wittinghofer, Science 2001[353].

3.1.2. Nucleotide cycling of Rab GTPases

The Rab GTPase cycling mechanism works as follows: a newly formed GDP-bound Rab is recognised by a Rab escort protein (REP), which targets it for prenylation by Rab geranylgeranyltransferase. The newly geranylgeranylated, GDP-bound Rab is delivered by REP to its target membrane[358,359]. A Guanine nucleotide exchange factor (GEF) then catalyses conformational changes in the Rab switch region by inserting residues in nucleotide and phosphate/$Mg2^{+}$ binding loop of the protein, thus inhibiting nucleotide-binding and 'kicking out' GDP[360–362]. The high concentration of GTP (approximately 1mM) in the cytoplasm ensures GTP binding to the vacant G-protein domain. The Rab is now GTP-bound and 'active' to engage downstream effector proteins, which mostly bind more tightly to the GTP bound protein compared to the GDP bound form. Rare exceptions exist where effectors prefer binding the inactive form[363–366]. The effector binding domain in Rab proteins is present in the switch and inter-switch region. In some cases, the effector-GTP-G domain tertiary complex is enough to activate effector function, while in others, effector proteins undergo conformational changes of their own. Different effectors in their activated state then modulate distinct membrane trafficking steps, and a given Rab protein can bind different effectors at separate locations[367]. The GDP-bound 'inactive' conformation is recovered with the hydrolysis of γ-phosphate of GTP by Rab GTPase activity, to give GDP and inorganic phosphate (Pi). This reaction is intrinsically very slow and hence requires acceleration by a GTPase-activating protein (GAP). For most Rab-GTPases, TBC (Tre-2, Bub2 and Cdc16) domain-containing proteins function as GAPs by contributing two conserved residues which catalyse GTP hydrolysis[368,369]. The GDP bound inactive Rab is now free to bind a guanine nucleotide dissociation inhibitor (GDI), which dissociates the intact GDP-Rab from the membrane, protects the hydrophobic C-terminus tail from the aqueous environment of the cytoplasm[370] and contributes to the subsequent cycling backing to the targeted membrane[371,372]. Membrane-bound proteins called GDI dissociation factor (GDF) play a key part in membrane trafficking by

recognising Rab-GDI complexes and promoting GDI release, and facilitating the association of geranylgeranylated Rab GTPase with the relevant membrane[373]. Figure 3.3 depicts the sequence of events in the Rab nucleotide cycle.

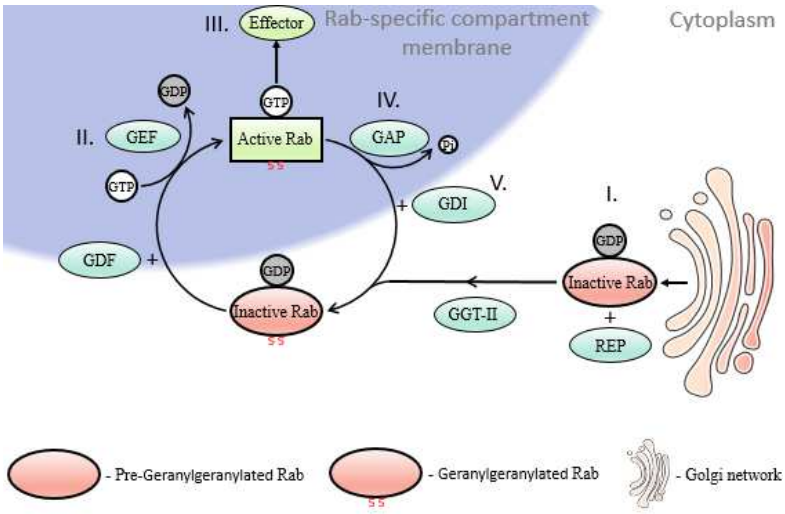

Figure 3.3 **The Rab nucleotide cycle.** I. Newly synthesised un- geranylgeranylated Rab protein is picked up and targeted by REP for geranylgeranylation to GGT-II. The geranylgeranylated Rab is then escorted by REP its target compartment membrane. II. At the membrane, GEF catalyses conformational changes in the G-domain of the Rab, which induces the release of GDP. The high concentration of GTP in the cytoplasm ensures its binding with vacant Rab G-domain. III. The GTP-bound active Rab interacts with downstream effector proteins to regulate vesicular trafficking. IV. The Rab GTPase activity is catalysed by its interaction with GAP and renders the Rab in GDP-bound inactive form with the release of Pi. V. GDI then detaches the intact geranylgeranylated GDP-Rab from the surface and chaperones it in the cytoplasm. The Rab is cycled back to the membrane upon encounter of the GDI-GDP-Rab complex with membrane-bound GDF, which conducts the transfer of GDP-Rab to the membrane. REP- Rab escort protein, GGT-II- Rab geranylgeranyltransferase, GEF- Guanine nucleotide exchange factor, GAP- GTPase-activating protein, Pi- inorganic phosphate, GDI- guanine nucleotide dissociation inhibitor, GDF- GDI dissociation factor.

3.1.3. Rab GTPases in vesicular trafficking

Vesicular trafficking forms an essential component to the interactions amongst various intracellular compartments. The trafficking of cargoes from the donor to the acceptor compartment comprises multiple steps, and Rab GTPases are well characterised to regulate these steps. Figure 3.4 summarises the intracellular localisation of various Rab GTPases and the vesicular trafficking pathways which they regulate.

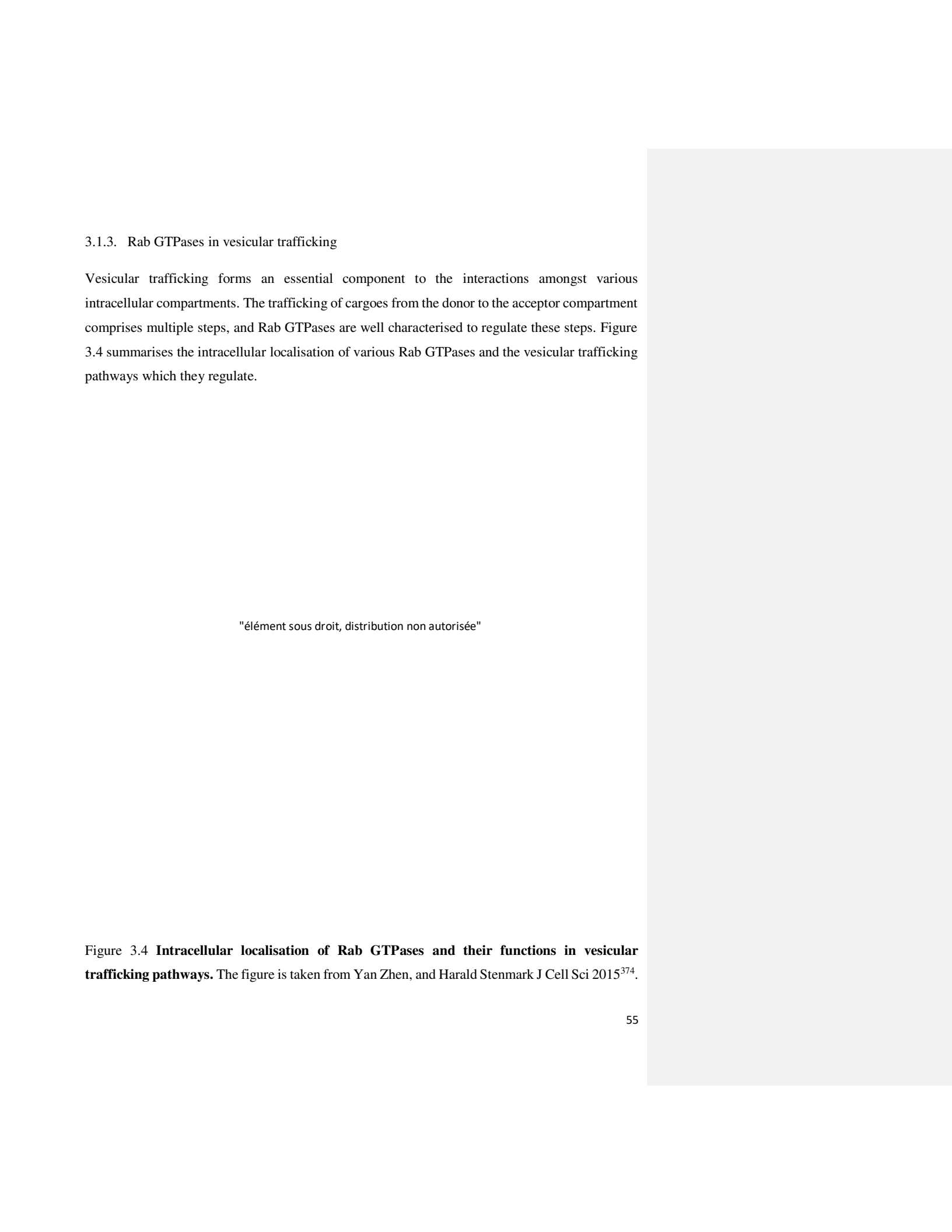

Figure 3.4 **Intracellular localisation of Rab GTPases and their functions in vesicular trafficking pathways.** The figure is taken from Yan Zhen, and Harald Stenmark J Cell Sci 2015[374].

3.1.3.1. Cargo sorting

The first step is the sorting of cargoes which are to be transported, inside the donor membrane. This involves the interaction of transmembrane regions of the cargo with cytosolic coat complexes. The assembly of coat complexes depends on membrane properties (curvature and lipid constitution) and Rab GTPases. For example, Rab9 which is present on late endosomes is involved in the sorting of mannose-6-phosphate receptors (M6PRs) to recycling buds and their subsequent recycling to the trans-Golgi network (TGN)[375]. It does so by recruiting its effector, sorting adaptor M6PR binding protein 1 (also known as TIP47), which recognises the cytosolic tail of M6PRs. Rab9 also enhances the affinity of TIP47 for M6PRs. Another example is that of Rab5, which is present on early endosomes. The GDI-Rab5 complex was identified to be essential for the assembly of clathrin-coated pits at the plasma membrane and clathrin-mediated endocytosis of transferrin receptors[376]. Rab7 is also recruited to early endosomes and is implicated in the sorting of multiple cargoes in microdomains for their transfer to late endosomes, including low-density lipoproteins (LDL), M6R, the IFNα receptor, Shiga toxin B-subunit and Semliki forest virus (SFV) particles[377,378]. Rab34 is known to control the sorting of lysosomal cargoes into phagosomes in a size-dependent manner[379].

3.1.3.2. Vesicle uncoating

The next step in the process is the shedding of coat proteins. This step is essential because coat proteins can hinder the fusion of transport vesicles with acceptor compartments. Clathrin-coated vesicles (CCVs) are a well-characterised system of vesicular trafficking in the TGN. For example, plasma membrane-derived endocytic vesicles are coated with clathrin and its cargo adaptor AP2 complex. The interaction of AP2 with these vesicles is stabilized by phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) and is enhanced by AP2-associated kinase 1 (AAK1), which mediates phosphorylation of the AP2 subunit μ2. Rab5 is present on CCVs and together with its GEFs, it coordinates AP2 uncoating by promoting dephosphorylation of μ2 and increasing PtdIns(4,5)P2 turnover[380].

3.1.3.3. Vesicle motility

Active movement of vesicles inside the cells occurs through motor protein-dependent linkage with cytoskeletal elements (actin filaments and microtubules). Rab GTPases mediate the recruitment of

motors to target vesicles either directly or via intermediate adaptors, thus contributing to the direction and specificity of transport. For example, Rab27a mediates the recruitment of the actin motor myosinVa on melanosomes (melanin filled pigment granules) via sequential interactions with two distinct 'myosin linker' effectors[381,382]. This mechanism is essential for correct distribution and function of melanosomes in melanocytes. In fact, loss of function mutations in Rab27a cause Griscelli syndrome in humans, and patients show not only pigmentation related but also immunological defects[383]. Rab32 and Rab38, two closely related proteins, also contribute to the biogenesis and secretion of melanosomes by recruiting MyosinVc directly as an effector to the organelle membrane[384]. Similarly, Rab11a recruits MyosinVb either directly[385] or via an adaptor (Rab11-FIP2)[386] on clathrin-dependent endocytic recycling compartments (ERCs), and is involved in the recycling of surface receptors such as transferrin. Rab8a recruits MyosinVb directly as an effector to clathrin-independent, Arf6 dependent tubular recycling networks[385] which are involved in MHC-I recycling to the plasma membrane.

Rab GTPases also mediate the recruitment of tubulin motors dynein (transport towards minus-end/ centrosome) and kinesin (transport towards plus-end/cell periphery) on target vesicles. For example, Rab6 recruits its effector Kinesin KIF20A (also known as RB6K) on Golgi and TGN and regulates transport within this organelle[387]. On the other hand, Rab6 links the dynein-dynactin complex to trans-Golgi via its effector Bicaudal-D1 to mediate Golgi to ER retrograde transport[388]. The role of Rab7 in bi-directional trafficking of late endosomes, lysosomes and autophagosomes has also been extensively studied. The plus-end transport is regulated through the interaction of Rab7 effector FYCO1 (FYVE and coiled-coil domain-containing 1) with kinesin[389], while the minus-end transport is carried out via dynein-dynactin complex interaction with two Rab7 effectors- RILP (Rab-interacting lysosomal protein) and a cholesterol sensor ORP1L (oxysterol-binding protein-related protein 1)[390–392]. By controlling the mobility of these vesicles, Rab7 plays a pivotal role in orchestrating late-endosome/phagosome/lysosome fusion[393] as well as autophagosome maturation in mammals[394]. Rab5 has been observed to regulate the motility of early endosomes on microtubules[395]. It is proposed to regulate retrograde transport through its effector - Huntingtin associated protein 40 (HAP40), which recruits Huntingtin (htt) - a known binding partner of the dynein-dynactin complex, on early endosomes [396]. The direct interaction of Rab5 with dynein using this mechanism remains to be seen.

3.1.3.4. Vesicle tethering

A key event in vesicular trafficking is the initial contact (tethering) and recognition between the transport vesicle and acceptor membrane. Using electro-microscopy, the distance between the tethered transport vesicle and target membrane has been determined to be approximately 75-150nm[397]. Tethering is required to induce the activation and proximity of SNARE complexes, which play a critical role in subsequent membrane fusion and are much more promiscuous in their binding. Rab GTPases mediate this step and determine the specificity of fusion with acceptor membranes. Tethering is mediated by the recruitment of elongated tethering complexes that form long-distance contacts between the two membranes. This function was first described in the yeast Rab Sec4. Sec15p, a subunit of the exocyst tethering complex, binds with GTP-Sec4, while other subunits of the tethering complex bind to Rho GTPases on the plasma membrane[398,399]. Sec15p has also been identified as a Rab11 effector in drosophila and mammals, indicating conserved interactions between Rabs and the exocyst complex[400,401]. Rab27a and its effector, the tethering protein granuphilin are also involved in the tethering of exocytic dense-core vesicles to the plasma membrane in neuroendocrine cells[402] and that of insulin granules to the plasma membrane in pancreatic b-cells[403]. Another example is of Rab1, which interacts with distinct effector tethering factors on the transporter and acceptor membranes, thus mediating intra-Golgi trafficking. Rab1 recruits the tethering protein p115 on coat protein complex II vesicles, which interacts with another Rab1 effector, the Cis-Golgi associated tethering protein GM130 to mediate their fusion[404]. Effectors of Rab5, rebenosyn5 and early endosome antigen1 (EEA1) act as tethering factors on early endosome membranes[405,406].

3.1.3.5. Vesicular fusion

Rab GTPases work in concert with SNARE proteins to mediate further recognition between the transport vesicle and acceptor membrane (i.e. the docking step) and their subsequent fusion. For example, Rab27 controls docking and fusion of exocytic vesicles to the plasma membrane through direct interaction of its effector granuphilin with the plasma membrane SNARE complex 'Munc18–1syntaxin1a'[402,403]. Another effector of Rab27a (and Rab3a), rabphilin is involved in the docking of dense-core vesicles to the plasma membrane through interaction with the SNARE synaptosomal-associated protein 25(SNAP25)[407]. Rab5 is well known to co-ordinate tethering, docking and fusion steps in the homotypic and heterotypic fusions of early endosomes. It does so

by regulating early endosome associated SNARE complexes via its effectors rebenosyn5 and EEA1. Rabenosyn5 bridges the Rab5-syntaxin7 (syntaxin Avalanche in Drosophila) interaction either directly or through the SNARE regulator VPS45 (vacuolar protein sorting-associated protein 45)[405,408], whereas EEA1 interacts directly with syntaxin6 and syntaxin13[409,410].

3.1.4. Rab GTPases in antigen cross-presentation by dendritic cells

Efficient cross-presentation by cDCs involves the optimisation of such events as phagolysosomal fusion, phagosomal maturation, antigen degradation and MHC-I trafficking to-and-from the peptide loading compartments. Rab GTPases being the master regulators of vesicular trafficking in cells, are involved in the spatiotemporal regulation of these events and have functional implications in cross-presentation and CTL mediated adaptive immunity. It is important to note that a majority of investigations into the roles of Rab GTPases in cross-presentation have been performed in BMDCs or DC cell lines. BMDCs in particular use a different transcriptional cross-presentation machinery compared to cDC1s[411]. The role of Rab GTPases in cDC1 specific cross-presentation machinery have only recently started to be investigated. Figure 3.5 summaries a general view of the functions of Rab GTPases implicated in cross-presentation by DCs.

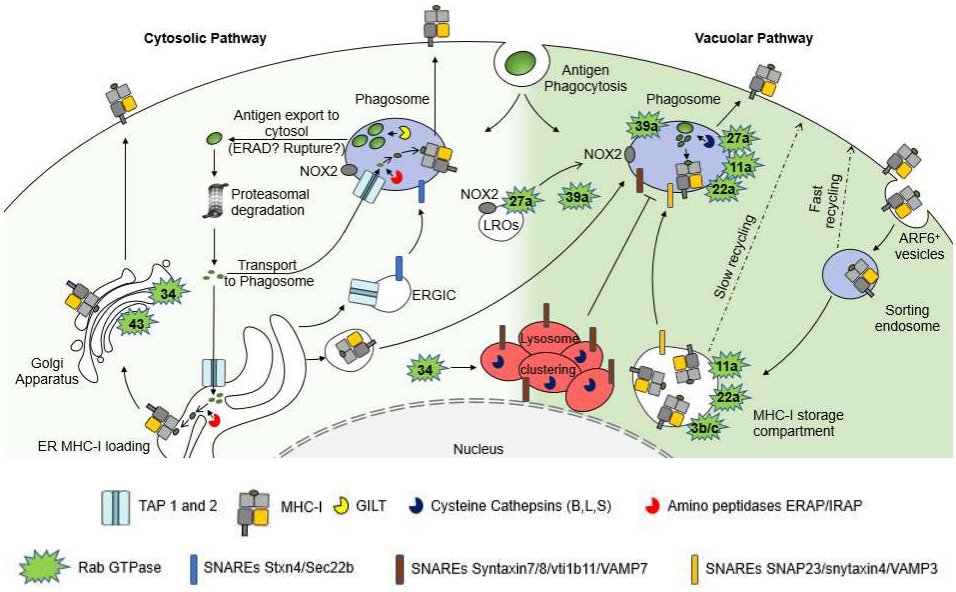

Figure 3.5 **Rab GTPases implicated in antigen cross-presentation by dendritic cells.** Rab GTPases being the master regulators of vesicular trafficking facilitate cross-presentation by (i) limiting phagosomal maturation and antigen degradation by controlling NOX2 trafficking to the phagosome and phagolysosomal fusion and (ii) facilitating the trafficking of recycled or ER-derived MHC-I for peptide loading to the phagosome. Intracellular localisation of various Rab GTPases is indicated. Rab27a and Rab39a are involved in NOX2 delivery to the phagosome. Moreover, Rab39a also promotes the delivery of ER-derived MHC-I to the phagosome. Rab34 localises at the Golgi apparatus and promotes lysosomal clustering to reduce phagolysosomal fusion upon DC activation by TLR signalling. Rab11a, Rab22a and Rab3b/c promote the stockage of MHC-I storage compartment with plasma membrane recycled MHC-I, and thus facilitate MHC-I delivery to the phagosome. Rab43 localises with cis-Golgi and specifically facilitates cross-presentation by cDC1s, although the mechanism of action is as yet unknown. The figure is based on Joffre et al., Nature reviews Immunology 2012.

3.1.4.1. Rab GTPases in Phagolysosomal fusion and antigen degradation

As described in previous chapters, cross-presentation benefits from the conservation of antigen inside phagosomes. Recruitment of the NADPH subunit 'NOX2' on phagosomes drives the production of superoxides and antigen conservation by limiting the activity of cysteine proteases such as Cathepsin B, L and S. Superoxides perform this function by neutralising the protease-activating acidic milieu in the phagosomes[256] and through oxidative inactivation of cysteine cathepsins[257]. Rab27a is present on lamp1$^+$ NOX2$^+$ lysosome-related vesicles in resting BMDCs, where it mediates the delivery of NOX2 to the phagosomes by promoting the fusion of NOX2$^+$ vesicles with phagosomes[258]. Correspondingly, BMDCs from Rab27a knockout mice showed higher phagosomal acidification, higher antigen degradation and a defect in cross-presentation of bead associated OVA, OVA-immune complexes, soluble OVA, as well as long peptide derived from male HY antigen compared to WT mice. This defect was due to a deferential antigen processing in the knockout mice since the presentation of pre-processed SIINFEKL peptide (OVA peptide recognised in context of H2-K^b) and uptake of antigens was unaffected.

In LPS stimulated BMDCs, Rab34 has been shown to modulate cross-presentation of bead associated and soluble OVA through the regulation of phagolysosomal fusion[317]. Upon TLR stimulation, Rab34 mediates the re-organisation of lysosomes around the nucleus. This perinuclear

clustering restrains phagolysosomal fusion and limits antigen degradation in phagosomes to optimise cross-presentation. The Rab34 mediated inhibition of phagolysosomal fusion is specific to TLR stimulation in DCs (and not macrophages), as treatment with the pro-inflammatory cytokine TNF or similar LPS stimulation in bone marrow macrophages (BMMs) does not induce lysosome clustering compared to steady-state cells. The exact mechanism of how the Golgi resident Rab34 controls lysosomal positioning in this context is not defined, although a possible explanation comes from the report that Golgi associated Rab34 interacts with its effector RILP to promote long-distance interorganellar regulation of lysosomal localisation to peri-Golgi regions[412]. Over-expression of WT or active Rab34 promotes peri-Golgi clustering of lysosomes, which is lost in mutant unable to bind RILP.

3.1.4.2. Rab GTPases in MHC-I delivery to exogenous peptide-loading compartments

Cross-presentation requires the delivery of MHC-I molecules to peptide loading compartments, which could be the ER, antigen containing phagosomes or ER-phagosome fusion compartments. As described in previous chapters, multiple sources of MHC-I for loading with exogenous peptide exist, including the ERGIC (trafficking via Sec22b), endolysosomal compartments (trafficking via CD74), and recycling from the plasma membrane. Multiple Rab GTPases are involved in MHC-I recycling from the plasma membrane, and this process is well characterised in non-professional APCs (like HeLa cells). MHC-I are internalised by Arf6 dependent (clathrin-independent) endocytic vesicles and reach $EEA1^+$ $Rab5^+$ early endosomes. From here, they can either be fast recycled back to the plasma membrane via $ARF6^+$ $EHD1^+$ tubular recycling network in a process mediated by Rab35[413] or transported to the perinuclear $Rab11a^+$ endocytic recycling compartments (ERC). At the ERC, Rab22a mediates the slow recycling of MHC-I to the plasma membrane via the tubular recycling network[414]. Thus, the $Rab11a^+$ ERC acts as an 'MHC-I storage compartment' in the perinuclear region of cells. The trafficking and role of recycled MHC-I in cross-presentation by professional APCs have been recently clarified.

In a pioneer study of MHC-I recycling in DCs by Nair-Gupta *et al.*, Rab11a was shown to stock the intracellular pool of MHC-I at the ERC, which contributes to TLR signalling induced phagosomal MHC-I recruitment and cross-presentation by BMDCs[292]. Similar to HeLa cells, Rab11a is localised on the MHC-I storage compartment and stocks the store with recycled MHC-I, most probably transported via early sorting endosomes. Upon TLR signalling in the phagosomes,

the Q-SNARE SNAP23 activity orchestrates fusion of the storage compartment with phagosomes and the delivery of MHC-I for peptide loading. Rab11a is also recruited to the phagosome upon phagosomal TLR signalling. Silencing of Rab11a in BMDCs by shRNA significantly decreases the intracellular pool of MHC-I, thus inhibiting the TLR orchestrated MHC-I recruitment on phagosomes. This effect translates to defects in cross-presentation of phagocytic cargo-derived peptides by siRab11a BMDCs without affecting surface MHC-I expression or presentation of pre-processed SIINFEKL. Interestingly, primary splenic $CD11c^+$ $CD8a^+$ DCs, the main cross-presenting population in mice, were also observed to harbour perinuclear $Rab11a^+$ MHC-I storage compartments in this study. This observation suggests that Rab11a is essential for facilitating MHC-I recycling for cross-presentation by cDC1 as well.

Rab22a was also shown to be a key regulator of MHC-I trafficking and cross-presentation by BMDCs[293]. In DCs, Rab22a is recruited to early endosomes ($EEA1^+$) and phagosomes, as well as to the vacuoles containing *T. gondii* parasite. It also partially localises with Rab11a and MHC-I positive storage compartments. Silencing of Rab22a by shRNA significantly reduces the intracellular pool and phagosomal recruitment of MHC-I, likely by regulating its recycling, while not affecting the surface MHC-I expression. The silencing of Rab22a also reduces the cross-presentation of soluble, particulate and *T. gondii*-associated antigens, but does not affect endogenous MHC-I antigen presentation through the classical secretory pathway.

To identify the Rab GTPases involved in cross-presentation, Zou *et al.* performed a functional screening of 57 mouse Rab proteins using shRNA based silencing in the murine DC cell line DC2.4[294]. *E. coli* expressing OVA was used as a model for particulate antigen and cross-presentation was measured as a function of IL-2 secretion by B3Z cells, T cell hybridoma expressing a TCR recognising SIINFEKL. Stable expression of siRNAs for 12 candidates were found to significantly decrease cross-presentation - Rab3b, Rab3c, Rab4a, Rab5b, Rab6, Rab8b, Rab10, Rab27a, Rab32, Rab34, Rab33a and Rab35. Promisingly, Rab27a and Rab34 have already been implicated in cross-presentation by regulating phagosomal antigen degradation, and act as technical controls in the study. The authors further pursued Rab3b/3c. In overexpression experiments in DC2.4, a colocation was observed between surface internalised β_2-microglobulin and Rab3b/3c in the perinuclear vesicles by confocal microscopy, hinting at a possible role of these Rabs in MHC-I recycling in DCs. The β_2-$microglobulin^+$ and $Rab3b/3c^+$ vesicles were further

found to be in juxtaposition with *E. coli* containing phagosomes. This observation the possibility that Rab3b/c mediates the delivery of recycled MHC-I to antigen containing phagosomes. Further investigation is required on the functional role of Rab3b/3c in MHC-I plasma membrane recycling and delivery to peptide loading compartments in DCs.

Rab39 has very recently been shown to optimise cross-presentation by DCs *in-vitro* and cross-priming of $CD8^+$ T cells *in-vivo*[75]. Rab39 is recruited to late endosomes and antigen containing phagosomes and supports the modification of phagosome into a peptide loading compartment. In support of this function, silencing of Rab39 in the DC cell line DC3.2 reduced the generation of peptide-MHC-I complexes in the phagosome. Rab39a performs this function by multiple means- (i) by promoting the delivery of peptide receptive MHC-I from the ER to the phagosome, (ii) by promoting the phagosomal recruitment of NOX2, in turn limiting phagosomal acidification and antigen degradation via ROS production (as previously described) (iii) by promoting phagosomal recruitment of Sec22b, which has debatably been implicated in cross-presentation (previously described in chapter II). In line with the established higher cross-presentation ability of splenic cDC1s over cDC2s and pDCs in mice, Rab39 was found to be highly expressed in $CD8a^+$ cDC1s and intermediately expressed in $CD11b^+$ cDC2s in the murine spleen. Interestingly, the absence of Rab39 specifically affected the phagosomal phenotype and cross-presentation in cDC2 and not in cDC1. MHC-II presentation by both cDC1 and cDC2 was not affected.

In addition to above mentioned Rab proteins, Rab43 is the first and only Rab GTPase described to optimise cross-presentation particularly by cDC1s in mice[74]. It is highly expressed in the $CD8a^+$ spleen resident as well as $CD103^+$ migratory cDC1s from skin-draining lymph nodes compared to their cDC2 and pDC counterparts. Rab43 localises to the Golgi apparatus and $Lamp1^-$ vesicles in the cytoplasm of steady-state and phagocytic cDC1s and is not recruited to the phagosomes. $CD8a^+$ cDC1 isolated from Rab43 knockout mice have a significant reduction in cross-presentation ability *ex-vivo* compared to the cells isolated from WT mice. This defect also translates into a reduction in cross-priming ability in Rab43 cDC1 conditional knockout mice *in-vivo*. This study also showed that Rab43 is expressed at low levels and does not affect cross-presenting ability in BMDCs. This observation is consistent with a previous report that BMDCs and cDC1s use different transcriptional programs to acquire cross-presentation ability[411]. The mechanism of how Rab43 facilitates cross-presentation by cDC1s is still an open question.

3.2. Small GTPase Rab32

The first description of small GTPase Rab32 comes from its cloning from human platelet mRNA by Bao *et al*[415]. The human Rab32 cDNA encodes a 225 residue protein of 25kDa which includes the guanine nucleotide-binding sequences and the two C-terminus cysteine-residues, characteristic of Rab proteins. One unusual feature of Rab32 is that the typical GTP-binding PM3 sequence WDTAGQE is replaced by WDIAGQE. Consistent with other Rab proteins, Rab32 contains the conserved glycine residue in the switch region and the two cysteine-residues in the C-terminus. The human and mice Rab32 orthologues have an 84% sequence identity. Other Rab proteins most closely resembling the human Rab32 sequence include human Rab38 (66% identity), human Rab7L/ Rab29 (53% identity) and *Dictyostelium* Rab E (57% identity). Together, they constitute the Rab32 subfamily of Rab GTPases. This subfamily has been shown to contain a unique and ultra-conserved FALK residue sequence present at the end of Switch I, but the functional importance of this sequence is as yet undefined[416].

Rab32 is highly expressed in cells containing Lysosome related organelles (LROs) such as melanocytes, mast cell and platelets[417,418]. Moreover, data from The Human Protein Atlas Project indicates high Rab32 protein expression in various tissues such as lungs (pneumocytes), liver (bile duct cells), testis (Leydig cells), kidney, adrenal gland (glandular cells) and adipose tissues (adipocytes). Amongst lymphoid tissues, Rab32 mRNA is highly expressed in myelopoietic cells in the bone marrow[419]. The known functions of Rab32 are described as follows.

3.2.1. Rab32 in vesicular trafficking to lysosome-related organelles (LROs)

LROs are cell type-specific, specialised structures which share some features with lysosomes but are compositionally and functionally distinct from them. They contain certain lysosomal proteins and possess a low lumenal pH, but harbour unique cargoes that confer their distinctive properties. LROs perform wide-ranging physiological functions in different cell types. A few examples include- lytic granules secreted by CTL and NK cells which help to target virally infected or tumour cells; dense granules secreted by platelets which release ATP, ADP, serotonin and calcium for blood clotting; and melanosomes which synthesise, store and transport melanin pigments in melanocytes- the specialised pigment-producing cells in the skin and the eyes. In DCs, $Lamp1^+$ $Rab27a^+$ vesicles which transport NOX2 to the phagosome to boost cross-presentation are

proposed to be LROs[258]. The fact that lysosomes and LROs exist in the same cell indicates that distinct trafficking mechanisms exist in cells to distinguish between the two organelles and to ensure the correct transport of membrane and luminal content. While conventional lysosomes acquire their cargoes either directly from the Golgi network in clathrin-coated vesicles or by their trafficking through early sorting endosomes, the content of most LROs is derived by trafficking from the early sorting endosomes, in a direction distinct from the lysosomes. The ubiquitous sorting machinery consisting of clathrin adaptors (AP1 and AP3) and BLOC proteins (Biogenesis of lysosome-related organelles complex) is responsible for cargo sorting in early endosomes and its trafficking towards LROs and away from the degradative lysosomes[420–422]. As previously mentioned, Rab32 and its related protein Rab38 (66% sequence identity in humans, 75% in mice) are highly expressed in cells containing LROs. In particular, Rab32/38 are recruited to two LROs - melanosomes in melanocytes and secretory dense granules in platelets. In the past decade, Rab32/38 have been well characterised to co-ordinate with AP1, AP3 and BLOC proteins and facilitate the cargo trafficking from sorting endosomes for the formation of melanosomes[423–425]. Figure 3.6 summarises the role of Rab32/38 in the regulation of trafficking to melanosomes.

Melanosomes make an excellent model for studying LROs - they contain lysosomal proteins Lamp1-Lamp3 and have an acidic pH like lysosomes, but also possess specific cargoes to produce and store melanin. These cargoes include three melanogenic enzymes- tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (Dct/Tyrp2). After synthesis in the ER, these enzymes are transported from the Golgi network to sorting endosomes, where they are sorted and transported to the developing melanosomes. Rab32 and Rab38 were first observed to affect melanosome biogenesis in a coat colour mutant mice named 'Chocolate mice'[426]. These mice have a mutation in Rab38 (G19V) which renders it inactive, giving a mild pigmentation phenotype. The phenotype is partial because Rab32 can functionally compensate for the inactive Rab38. Consequently, melanocytes from chocolate mice depleted of Rab32 using siRNA treatment possess a severe hypopigmentation phenotype[427]. Rab32 /38 and their interacting partners have been shown to affect melanosomes biogenesis by regulating the trafficking of all three melanogenic enzymes- tyrosinase, Tyrp1 and Tyrp2 from the sorting endosomes to the melanosomes[384,423,428–431]. Recycling tubular transport vesicles play an important role in this process. These vesicles traffic the cargoes towards the melanosomes and fuse with them to deliver

the cargoes by the action of the v-SNARE VAMP7 (vesicle-associated membrane protein 7), and then recycle VAMP7 back to the sorting endosome for further rounds of transport.

A Rab32/38 effector, Myosin Vc localises on the early sorting endosomes. The interaction of Myosin Vc with Rab32/Rab38 on these endosomes is proposed to regulate trafficking of melanosomal cargoes Tyrp1, Tyrp2 and the fusion protein VAMP7 to the melanosome[384]. This is evidenced by the silencing of Myosin Vc in melanocytes, which causes the mistargeting of Tyrp1 and VAMP7 to the plasma membrane and a significant overall loss of Tyrp2 in the Myosin Vc silenced cells. Another Rab32/Rab38 effector, BLOC-2 is involved in the targeting of transport vesicles to the melanosome[423,428]. Silencing of BLOC-2 deviates the trafficking of Tyrp1 to the plasma membrane as well as the Golgi compartments. VARP is another Rab32/38 effector, and it is recruited by Rab32/38 on melanosomes. The Varp–Rab32/Rab38 complex has been implicated in two functions – (i) it promotes the trafficking of Tyr/Tyrp1/Dct from the sorting endosomes to the melanosomes[431], and (ii) it binds to and traps SNARE protein VAMP7 to inhibit its SNARE complex formation ability[432]. Dennis et al. have proposed that the binding and entrapment of VAMP7 by the Varp–Rab32/Rab38 complex is a mechanism to promote VAMP7 recycling to early endosomes where can be used for further rounds of the melanosomal cargo trafficking[433].

A few of the Rab32/38 regulating proteins in melanocytes have also been identified. The BLOC-3 protein complex, which comprises subunits HPS1-HPS4, acts as a GEF for Rab32/38[425]. This complex is important for the correct membrane localisation of Rab32/38 to Tyrp1 trafficking vesicles and subsequent pigmentation[424,425]. Consequently, the silencing of BLOC-3 subunits in melanocytes causes mislocalisation and cytoplasmic diffusion of Rab32/38, absence of Tyrp1 trafficking to melanosomes and loss of pigmentation[425]. In humans, the loss of function mutations in the BLOC-3 subunits HPS1-HPS4 cause a rare autosomal recessive disorder known as Hermansky-Pudlak syndrome (HPS)[434]. This syndrome affects various LROs including melanosomes, dense granules of platelets and lysosomal storage compartments, and is characterised by oculocutaneous albinism, bleeding tendency, excessive accumulation of lipopigments (ceroid-lipofuscin) in various tissues as well as immunodeficiencies. Similarly, in two mice models named as ‘light ear’ and ‘pale ear’, the absence of mouse HSP1 ortholog gives rise to defects in melanosome formation and functions[434].

The RPIP8/UNC-14/NESCA (RUN) and TBC domain-containing protein 1 (RUTBC1) has been identified as a GAP protein for Rab32/38 in melanocytes[430]. The overexpression of RUTBC1 in melanocytes leads to the inactivation of Rab32/Rab38, consequently affecting the trafficking of tyrosinase/Tyrp1/Dct to the melanosomes. Interestingly, the silencing of RUTBC1 in melanocytes inhibits cargo trafficking to melanosomes, even though active Rab32/38 accumulates on the melanosomes on these cells. This observation highlights the importance of spatiotemporal regulation of Rab32/Rab38 for the trafficking of cargoes to the melanosomes.

Interestingly, Rab32 has also been linked to the regulation of melanosome motility in *Xenopus* melanocytes[435]. This role is related to the A-kinase anchoring protein (AKAP) function of Rab32. Upon recruitment on the target membrane, Rab32 acts as a scaffold to recruit Protein Kinase A (PKA) to the melanosomes by binding the RIIa regulatory subunit of PKA. The PKA RIIa subunit can also bind to microtubule as well as actin motors, and mediates the aggregation/dispersion of melanosomes in response to the second messenger – cyclic AMP (cAMP).

Figure 3.6 **Role of Rab32/38 in the regulation of endosomal trafficking of melanosomal cargoes.** The nucleotide cycling of Rab32/38, regulated by their GAP (RUTBC1) and GEF (BLOC3) is hypothesised to dictate the trafficking of melanogenic enzymes from early endosomes to immature melanosomes for their biogenesis. I. The trafficking of melanosomal cargoes occurs through recycling tubular transport vesicles, which traffic the cargo from early endosomes to the

melanosomes. The sorting of cargos such as Tyr and Typr1 in early endosomes is facilitated by adaptor proteins (AP3 for Tyr) and BLOC proteins (BLOC1 for Tyrp1). BLOC1 is recruited to early endosomes as an effector of Rab5. II. An effector of Rab32/38, the BLOC2 complex is involved in the stabilisation of VAMP7 and syntaxin13 (stx13) containing transport vesicles and their targeting towards immature melanosomes. Another Rab32/38 effector, Myosin Vc is proposed to be recruited on these vesicles and mediate their transport towards the immature melanosomes. III. Upon contact with an immature melanosome, VAMP7/Syntaxin3/SNAP23 SNARE complex carries out the fusion events and the subsequent delivery of the cargoes to the immature melanosome. In a parallel model, the fusion event is carried out by the interaction of VAMP7 present on the melanosomes with Syntaxin13 present on the transport vesicles. During the fusion process, the inhibition of VAMP7 activity mediated Rab32/38-VARP complex must be ceased. This function is supposedly carried out by the GAP activity of RUTBC1, which inactivates Rab32/38. RUTBC1 is itself recruited on melanosomes as an effector of Rab9. IV. After carrying out the fusion, VAMP7 present on the melanosomes is now recycled back to the early endosomes for the next round of trafficking. This recycling occurs by the binding and entrapment of VAMP7 by the Rab32/38-VARP complex. For this process, the inactive Rab32/38 are first activated by their GEF BLOC3. Like RUTBC1, BLOC3 is also presumed to be recruited on melanosomes as an effector of Rab9. The figure is modified from Obhayashi et al., The Journal of Biochemistry 2017[436].

Apart from melanosomes, Rab32/38 have also been implicated in the biogenesis of another LRO- the dense granules in platelets. Dense granules have an acidic pH and possess some lysosomal markers like Lamp3. They also harbour molecules such as ADP, calcium and serotonin in platelets, which are released at the site of vascular injury. These molecules play an important role in stopping the loss of blood and maintaining haemostasis. On average, there are about three to eight dense granules in a platelet[437]. The transmembrane protein cargoes for dense granules originate in the TGN and follow a secretory pathway to the early endosomes. In the early endosomes, these cargoes are sorted by the function of AP3 and are transported in vesicles for fusion and delivery to immature dense granules. Rab32/38 partially colocalise with AP-3 as well as immature dense granules in platelets[438]. Moreover, these Rab proteins contribute to the biogenesis of dense granules by facilitating the tethering/fusion of sorted cargoes containing vesicles with the maturing dense granules[438].

3.2.2. Rab32 activity in mitochondrial dynamics

The ER forms contact sites with mitochondria at regions known as the mitochondria-associated membranes (MAMs) in cells. These membranes are the sites for the physical and biochemical communication between the two organelles and constitute a major cell signalling hub. One function of the MAM comprises the regulation of mitochondrial dynamics. MAM residing GTPases Mitofusin 1 and 2 promote mitochondrial fusion. On the other hand, the GTPase Dynamin-related protein 1 (DRP1) utilises ER tubules to promote mitochondrial constriction and fission[439]. The function of DRP1 is controlled in two ways- $Ca2^{+}$ release from the MAM promotes DRP1 activity to promote mitochondrial fission, whereas phosphorylation of a serine residue in DRP1 by cAMP-dependent PKA inhibits its activity. Consequently, DRP1 knockout cells have a collapse of the mitochondrial network around the nucleus[440,441]. Rab32 interacts with DRP1 in two ways. Firstly, DRP1 has been identified as an effector of Rab32, as evidenced by Co-IP experiments which show high interaction of DRP1 with active Rab32 compared with its inactive form[442]. Moreover, Rab32 localises at the mitochondria and MAM inside the cell[441–443], and in turn, recruits DRP1 to this membrane to promote mitochondrial fission[442]. Consequently, the overexpression of an inactive Rab32 mutant promotes a perinuclear collapse of elongated mitochondrial, similar to the DRP1 knockout cells[441–443]. Secondly, as previously described, Rab32 possesses the ability to function as a 'Protein Kinase A anchoring protein' (AKAP). In fact, it is the only human Rab GTPase which performs this function. By functioning as scaffolds for PKA recruitment, AKAPs allow localised phosphorylation of substrates by PKA. Rab32 has been shown to recruit PKA RIIa on the MAM and mitochondria by its AKAP activity. This recruitment results in increased serine phosphorylation and inactivation of DRP1[441]. Consequently, the knockdown of Rab32 in HeLa cells increases mitochondrial fission[441]. Therefore, Rab32 is able to dictate mitochondrial dynamics depending on its association with the ER and DRP1.

3.2.3. Rab32 in immunity against bacterial pathogens

Macrophages, DCs and neutrophils comprise the arm of the mammalian immune system specialised at internalizing and eliminating bacterial pathogens. The antimicrobial mechanisms involve complex membrane trafficking steps to target internalised bacteria with factors such as oxidative stress (ROS), antibacterial peptides and toxic metals (zinc and copper). However, successful bacterial pathogens have evolved to counter these trafficking steps and survive within

these phagocytic cells. Investigation of such counter mechanisms has led to the identification of Rab32-dependent pathways for bacterial restriction in host phagocytes. Figure 3.7 summarises the presently known antimicrobial functions of Rab32 against various bacterial pathogens.

3.2.3.1. Rab32/BLOC-3 dependent antimicrobial pathway against *Salmonella*

Salmonella enterica is a facultative intracellular pathogen which infects mammalian hosts. This species comprises over 2000 serovars and they make up a major cause of infectious diseases in a selective or broad range of hosts. Two human restricted serovars - *S. typhi* and *S. paratyphi* are responsible for a combined estimated 25 million cases of typhoid fever each year according to WHO figures, with severe cases ending in mortality. Ingested through contaminated food, *Salmonella* actively invades the epithelial lining of the intestine. It employs 'type III' secretion effectors to manipulate actin cytoskeleton dynamics and endocytic trafficking in the host cell, thereby inducing macropinocytosis and formation of *Salmonella*-containing vacuoles (SCVs). The bacteria then passes to macrophages in the lamina propria. *Salmonellae* which survive the initial anti-bacterial defences secrete a second 'type III effector' system, which enables them to replicate and spread systematically throughout the body. The broad-host serovar, *S. typhimurium* utilises the type III effectors to evade elimination in mouse macrophages.

A Rab32/BLOC-3 dependent pathway is critical for the elimination of vacuolar *Salmonella* in macrophages and for conferring systemic protection. This is evidenced by observations that Rab32 or BLOC-3 deficiency increases *S. typhimurium* infection in mice, and BLOC-3 deficient mice are even susceptible to the human restricted *S. typhi*[444]. In pioneer studies, Spano et al. have shown that Rab32 subfamily proteins (Rab32, Rab38 and Rab29) are recruited to the *S. typhi* SCVs in human epithelial cells as well as macrophages but not to *S. typhimurium* SCVs in permissive mice macrophages[445,446]. *S. typhimurium* evades this recruitment and subsequent elimination by secreting two 'type III' effector proteins - GtgE and SopD2. GtgE is a cysteine protease which proteolytically targets Rab32, Rab38 and Rab29[446] while SopD2 targets Rab32 activity by functioning as a GAP[444]. A *S. typhimurium* mutant with double functional mutations in GtgE and SopD2 exhibits a drastic reduction in mouse virulence, more so than individual functional mutants, and this effect is completely reversed in a mouse lacking BLOC-3[444]. Therefore, both effectors work in conjunction to block the Rab32/BLOC-3 dependent antimicrobial response. Separately, SopD2 also blocks the trafficking of endocytic cargoes towards the lysosomes for their

degradation[447]. It does so by interacting with Rab7 and inhibiting its GDP/GTP exchangeability. Consequently, this limits the interaction of Rab7 with its dynein and kinesin binding effectors-RILP and FYCO1, thus interrupting the host cell's ability to degrade the endocytosed *Salmonella*.

GtgE and SopD2 are not encoded by the human restricted *S. typhi*, which consequently does not infect mouse macrophages. The transient expression of GtgE in *S. typhi* allows it to overcome host-restriction and replicate in the non-permissive mouse BMMs[446]. Consequently, mice infected with *S. typhi* expressing GtgE develop significantly more CFUs (colony forming units) compared to the wild type infection. Importantly, GtgE specifically targets Rab32 and not Rab38 or Rab29 for *Salmonella* survival in mouse macrophages, and only Rab32 silenced mouse BMMs are infected by *S. typhi*[446]. This shows that Rab32 defines the host restriction of *S. typhi*.

The SCVs in macrophages appear similar to LROs observed in other cell types due to the presence of Lamp1, absence of lysosomal proteases and recruitment of Rab32 and Rab38 on their membrane. Given the importance of Rab32/Rab38/BLOC-3 complex in LROs biogenesis and the selective targeting of Rab32/BLOC-3 complexes by *S. typhimurium* effectors, it may be hypothesised that Rab32/BLOC-3 complex controls vesicular trafficking of anti-bacterial factors to the SCV[446]. A direct experimental proof implicating Rab32 in this function remains to be seen.

3.2.3.2. Rab32-PHB/PHB2 dependent antimicrobial pathway against *Listeria*

The intracellular pathogen *L. monocytogenes* causes a lethal foodborne infection named listeriosis. Upon receptor-mediated internalisation to early phagosomes in DCs and macrophages, this pathogen actively escapes to the cytoplasm and transmits to neighbouring cells using actin-based movements. $CD8a^+$ cDC1 are the primary site of entry and proliferation of *L. monocytogenes* in the spleen[448,449]. To obtain a global view of membrane dynamics during the infection, Li et al. studied the interactome of Rab GTPases in *L. monocytogenes* infected BMDCs[450]. To this goal, they applied a proteomics approach (tandem affinity protein purification followed by mass spectrometry), and further supplemented their studies with imaging tools. The Rab32 and its interacting partners were identified to be dispensable for initial phagocytosis but essential for the containment of *Listeria* infection in DCs. The authors further used a $CD11c^+$ DC conditional Rab32 knockout mice to study the Rab32-dependent *Listeria* containment in primary DCs and its physiological relevance in containing the infection. The conditional Rab32 knockout mice had a

significantly higher pathogen burden in the spleen and liver compared to the wild type controls. Mechanistically, Rab32 is recruited to *Listeria* containing phagosomes where it formed a persistent complex with two effector proteins, PHB and PHB2. This complex mediates the containment of *Listeria* in early phagosomes as well as sequestering of *Listeria* in multilaminar 'secondary phagosomes' after its escape to the cytoplasm. Moreover, silencing of these proteins in the cell line DC2.4 leads to (i) reduced acidification of listeria containing phagosomes and (ii) increased escape of Listeria from the acidified phagosomes. These observations suggest a role of Rab32-PHB/PHB2 complex in phagolysosomal fusion for pathogen elimination in DCs.

3.2.3.3. Rab32 in the maturation of *M. tuberculosis* phagosomes

M. tuberculosis is the main cause of tuberculosis, one of the deadliest infectious diseases in human history. Upon entry into the body, it is phagocytosed in the lower respiratory tracts primarily by macrophages, DCs and neutrophils. The host-pathogen interactions for *M. tuberculosis* have been well characterised at the cellular level. It primarily resides in the early state phagosomes of macrophages, where it promotes self-survival by arresting phagosomal acidification and maturation. It does so by promoting the recruitment of early endosome related Rab GTPases on the phagosome, such as Rab5, Rab14 and Rab22a[451]. Transferrin receptor, a marker for early endosomes remains in *M. tuberculosis* phagosomes as a result of fusion with early endosome markers. On the other hand, late endosomal GTPases such as Rab7, Rab10 and Rab20 are not recruited on these phagosomes. Seto et al. studied the relevance of 42 distinct Rab GTPases in the control of *M. tuberculosis* phagosome maturation in Raw264.7 macrophages using overexpression experiments[452]. In this study, Rab32 was identified to associate with *M. tuberculosis* phagosomes, albeit weekly, and was required for the recruitment of aspartic lysosomal protease Cathepsin D to the *M. tuberculosis* containing phagosomes. This function was dependent on the Rab32 GTPase activity since the overexpression of its dominant-negative T39N mutant inhibited phagosomal Cathepsin D recruitment. A Rab32/BLOC-3 dependent trafficking pathway might be at play here, and it would be interesting to further test the physiological relevance of Rab32-mediated Cathepsin D delivery in restricting *M.* tuberculosis infection in a Rab32/BLOC3 mice model.

3.2.3.4. Rab32 in the maturation of *Burkholderia pseudomallei* phagosomes

B. pseudomallei is a facultative bacterial pathogen which causes a fatal infectious disease named melioidosis. This bacteria can survive in phagocytes as well as non-phagocytic cells. Once internalised, *B. pseudomallei* can escape from the phagosome to the cytoplasm to avoid autophagy-dependent elimination. Hu et al. have recently shown that of RAW 264.7 macrophages with *B. pseudomallei* causes an upregulation of Rab32 expression in these cells[453]. This occurs as a consequence of miRNA-30b/30c inhibition upon infection, which normally inhibits the expression of Rab32 gene by interacting with its 3' UTRs. Subsequently, Rab32 is recruited to the *B. pseudomallei*-containing phagosomes and promotes the fusion of the phagosomes with lysosomes, resulting in phagosomal acidification and acquisition of Cathepsin D. Rab32 thus functions to restrict the intracellular growth of *B. pseudomallei* at an early phase of infection in macrophages.

3.2.3.5. Rab32 polymorphism in susceptibility to *M. leprae*

Leprosy remains a major global health problem with more than 200,000 cases reported every year, and a large burden of infection falls on the developing parts of the world. It is a granulomatous infectious disease caused by the obligate parasite *M. leprae*, which is adapted for infecting humans and replicates in phagosomes inside macrophages. Research into the antimicrobial response of host cells is limited by the narrow host range of this pathogen and the difficultly in culturing it *in-vitro*. Nonetheless, population epidemiological surveys have shown an important role of host genetics in the susceptibility of individuals to leprosy, with an estimated heritability of up to 57%[454]. In two reports by Zhang et al, genome-wide association studies were performed in leprosy patients and control individuals to identify the gene loci linked with leprosy susceptibility[455,456]. Both studies have implicated a single nucleotide polymorphism (rs2275606) at the Rab32 locus (6q24.3) in increased leprosy susceptibility in humans. The effect of this polymorphism on Rab32 structure or function was not defined in these genetic studies. These studies, along with the established anti-microbial cellular function of Rab32 strongly suggest a role of this protein in controlling *M. leprae* infections, although a piece of direct evidence in this regard remains to be seen.

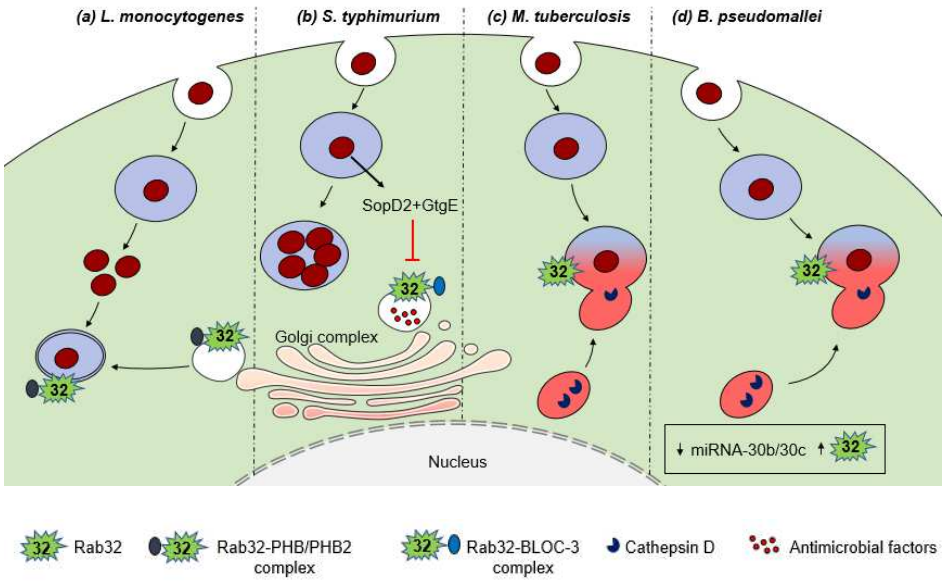

Figure 3.7 **Rab32 in immunity against bacterial pathogens.** (a) Upon internalisation by host DCs, *L. monocytogenes* actively escapes to the cytoplasm to infect neighbouring cells. Rab32-PHB/PHB2 complex encapsulates the escaped *Listeria* in multilaminar secondary phagosomes and contributes to phagolysosomal maturation. (b) Upon internalisation by host macrophages, *Salmonella* resides in SCVs. The Rab32/BLOC-3 complex is involved in *Salmonella* elimination possibly by delivering of antimicrobial factors to the SCV. *S. typhimurium* effectors- GtgE and SopD2 selectively target Rab32/BLOC-3 recruitment to the SCV. The human restricted *S. typhi* does not encode these effectors and is targeted by Rab32 for elimination. (c) Rab32 is recruited on *M. tuberculosis* containing phagosomes in macrophages and its activity is required for the recruitment of Cathepsin D to these phagosomes. (d) Upon internalisation of *B. pseudomallei* by macrophages, expression of miRNA-30b/30c is inhibited, which promotes the expression of Rab32 in the infect cells. Rab32 is recruited to *B. pseudomallei* containing phagosomes and promotes phagolysosomal fusion, phagosomal acidification and Cathepsin D recruitment. The figure is based on Solano-Collado et al., Small GTPases 2018[104].

Given the emerging role of Rab32 facilitating vacuolar trafficking, its implications in the biogenesis of specialised intracellular organelles and pathogen restriction in host phagocytes,

along with the initial report of Rab32 promoting cross-presentation of bacterial antigen by DC2.4[294], Rab32 makes an interesting candidate as a regulator of antigen processing and cross-presentation by cDC1s.

EXPERIMENTAL RESULTS
(Paper Manuscript)

Title

Small GTPase Rab32 promotes the proliferation of effector CD8$^+$ T cells in response to cross-presentation of cellular antigen by cDC1s *in-vivo*.

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Abstract

The conventional type1 Dendritic Cells (cDC1s) efficiently cross-prime CD8$^+$ T cells against cellular antigens derived from necrotic cells, viral-infected or tumour cells *in-vivo*. This functional specialization results from a combination of cDC1s characteristics, such as the ability to internalise and transport antigens from peripheral tissues to draining lymph nodes, and their unique cross-presentation machinery. Rab GTPases are the master regulators of intracellular vesicular trafficking and have been shown to promote CD8$^+$ T cell cross-priming against cellular antigens *in-vivo*. In this report, we identify the small GTPase Rab32 to be highly and differentially expressed in splenic cDC1s compared to cDC2s. Using Rab32 deficient mice, we demonstrate that Rab32 promotes the proliferation of effector CD8$^+$ T cells in response to challenge with cellular antigens *in-vivo*. Rab32 does not affect cross-presentation by cDC1s *ex-vivo*. We further demonstrate that Rab32 promotes tumour infiltration of antigen-specific CD8$^+$ T cell by promoting the presence of tumour-derived migratory cDCs in the draining lymph nodes. Intracellularly, Rab32 regulates the lysosomal positioning in cDC1s. We hypothesise that Rab32 promotes DC migration towards lymph lodes by optimising lysosomal signalling in these cells.

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Introduction

Conventional dendritic cells (cDCs) constantly survey lymphoid as well as peripheral tissues in the body for antigen-expressing cells such as necrotic cells, pathogen-infected or tumour cells. Upon such encounters, cDCs internalise and process the cell-associated protein antigens, and present them as peptides on MHC-I to naïve $CD8^+$ T cells in a process termed as cross-presentation (Bevan, 1976a, 1976b). When accompanied by co-stimulatory and cytokine signals, cross-presentation leads to activation and proliferation of antigen-specific $CD8^+$ T cells and generation of effector responses against antigen-expressing cells.

In mice as well as humans, cDCs are further divided into two subsets – $XCR1^+$ cDC1s and $SIRPa^+$ cDC2s. In mice, cDC1s are functionally specialized at cross-priming $CD8^+$ T cells against cellular antigens and are critical for mounting anti-viral and anti-tumoral adaptive immune responses *in-vivo* (Bedoui et al., 2009; Helft et al., 2012; Hildner et al., 2008). This specialisation seems to be conserved in humans since human blood-derived as well as dermal cDC1s can also efficiently cross-present cellular antigens *in-vitro* (Bachem et al., 2010; Balan et al., 2014; Henri et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010).

Several properties of cDC1s support this functional specialization. Lymphoid resident cDC1s seem to be more efficient at internalising necrotic cell-associated antigens compared to cDC2s *in-vivo* (den Haan et al., 2000; Iyoda et al., 2002; Pooley et al., 2001; Schnorrer et al., 2006). Similarly, migratory cDC1s in the lung are unique in their ability to pick up necrotic cell-associated antigens (Desch et al., 2011) as well as viral antigens from infected cells (Helft et al., 2012), and transport them to draining lymph nodes for $CD8^+$ T cell cross-priming *in-vivo*. cDC1 are also unique in their expression of necrotic cell-receptor CLEC9A, which targets the internalised antigens to the cross-presentation machinery in these cells (Sancho et al., 2009; Zelenay et al., 2012). cDC1s also possess specialised cross-presentation machinery, which promotes the conservation of internalised antigens in the phagosomes, thus allowing optimal processing of antigens into MHC-I compatible peptides (Samie & Cresswell, 2015; Savina et al., 2009; Kretzer et al., 2016; Theisen et al., 2018). Moreover, cDC1s show enhanced expression of the MHC-I peptide loading complex, which supports their superior cross-priming abilities (Dudziak et al., 2007; Vander Lugt et al., 2014).

While cDC1s are the primary cross-presenting cells in physiological settings, a significant fraction of studies investigating the cellular mechanism of cross-presentation have been conducted in murine bone marrow-derived DCs (BMDCs), generated *in-vitro* from GM-CSF supplemented bone marrow cultures (Cebrian et al., 2011; Nair-Gupta et al., 2014; Savina et al., 2006; Zehner et al., 2015, p. 61). BMDCs are a heterogeneous population resembling both DCs and macrophages, and they use a cross-presentation transcriptional program different from cDC1s (Briseño et al., 2016; Kretzer et al., 2016). Hence, a recent focus in the field has been to investigate the cross-presentation machinery utilised by cDC1s. Consequently, several cDC1 specific proteins have been implicated in facilitating $CD8^+$ T cell cross-priming *in-vivo*, including Rab43 (Kretzer et al., 2016), WDFY4 (Theisen et al., 2018) and Rab39a (Cruz et al., 2020).

Rab proteins are small GTPases which are well conserved in mammals. They make interesting candidates for cross-presentation studies in cDC1s. They exist in the cell as GTP-bound active or GDP-bound inactive forms, and function as the master regulators of intracellular vesicular trafficking (Stenmark, 2009). On these lines, Rab GTPases have been shown to facilitate cross-presentation by regulating the trafficking events involved in antigen processing and peptide-MHC-I loading (Alloatti et al., 2015; Cebrian et al., 2016; Cruz et al., 2020; Jancic et al., 2007; Kretzer et al., 2016; Nair-Gupta et al., 2014; Zou et al., 2009). The GTPase Rab32 plays a critical role in the restriction and clearance of intracellular bacterial pathogens such as *Listeria monocytogenes* (Li et al., 2016) and *Salmonella* (Spanò et al., 2016; Spanò & Galán, 2012) within DCs and macrophages, respectively. Rab32 deficiency in $CD11c^+$ cells has also been linked to increased colitis progression and bacterial invasion in colon tissues (Xie et al., 2018). Intracellular analysis in cell lines shows that Rab32 colocalises with the mitochondria-associated membranes of the ER (MAMs), which are marked by the ER chaperone calnexin (Alto et al., 2002; Bui et al., 2010; Ortiz-Sandoval et al., 2014). Moreover, Rab32 and its regulatory protein BLOC-3 (HPS1-4) play an essential role in the biogenesis of lysosome-related organelles (LROs) (Gerondopoulos et al., 2012; Ohishi et al., 2019). Disruptions in this machinery have been linked to Hermansky-Pudlak syndrome (HPS) in humans, which is characterised by oculocutaneous albinism, bleeding tendency and immunodeficiencies (Suzuki et al., 2002; Wei & Li, 2013). A role of Rab32 in adaptive immune responses has not yet been critically investigated.

In this report, we identify Rab32 to be highly expressed in murine cDC1 and demonstrate its role in the generation of effector $CD8^+$ T cells in response to cellular antigenic challenge *in-vivo*. We

show that Rab32 does not affect cross-presentation by cDC1s *ex-vivo.* We provide evidence that Rab32 promotes tumour infiltration of antigen-specific $CD8^+$ T cell by promoting the presence of tumour-derived migratory cDCs in the draining lymph nodes. Moreover, we observe that Rab32 promotes perinuclear clustering of lysosomes in cDC1s. Given the emerging role of lysosomal signalling in the regulation of DC migration (Bretou et al., 2017), we hypothesise that Rab32 promotes DC migration to the tumour draining lymph lodes (tdLNs) by optimising the lysosomal signalling in DCs.

Results

Rab32 is highly expressed in cDC1s but does not control their differentiation

We sought to identify cDC1 specific Rab GTPases by evaluating the expression profile of Rab proteins in different populations of murine DC subsets. To this end, we looked at the gene expression microarray data from Immgen database. Rab32 was amongst the GTPases most highly expressed in resident cDC1 populations within the spleen, the mesenteric lymph node (MLN) and skin draining lymph node (SLN), as compared to resident cDC2s or pDCs (Fig 1a). Rab32 is well expressed in a few other myeloid populations such as alveolar macrophages and is minimally expressed in lymphoid populations in mice (Fig S1a). In conclusion, Rab32 is mainly expressed in cDC1s amongst professional antigen-presenting cells.

To investigate the functional effects of Rab32 in a mice model, we used the constitutive Rab32 KO model named Rab32 tm1a (Fig 1b). These mice contain a promoter-driven cassette between the exons 1 and 2 of Rab32, which disrupts Rab32 protein expression in all cells. The absence of Rab32 in the tm1a mice was verified by western blot (Fig 2c, upper blot). In addition to the presence of the promoter-driven cassette, Rab32 exon 2 is floxed in the tm1a mice, which presents the opportunity to genetically delete Rab32 in mice. Hence, we crossed the Rab32 tm1a mice with a germ-line 'cre deleter' mice to generate Rab32 cre-deleted (cdel) mice, in which exon 2 was deleted in all the cells (Fig 1b). The absence of Rab32 in these mice was also verified by western blot (Fig 2c, lower blot). Hence, pre-cre and post-cre mice were similarly deficient for Rab32, and could serve as constitutive KO models for further investigations on Rab32.

Using the Rab32 KO mice, we sought to determine if Rab32 affected the differentiation of cDC1s and cDC2s *in-vivo*. MHC-II^+ $CD11c^+$ cDCs from the spleens of Rab32 WT or KO mice were identified by flow cytometry (Fig 1d). Within this population, $XCR1^+$ cDC1s and $SIRPa^+$ cDC2s were distinguishable. Rab32 WT and KO mice had similar percentages of cDC1s and cDC2s in

the spleen. Moreover, Rab32 also did not affect the percentages of other myeloid and lymphoid cell populations in the spleen (Fig S1b). Hence, although Rab32 is highly expressed in cDC1s, it does not affect their differentiation in mice.

We also examined the intracellular localisation of Rab32 in steady-state splenic cDC1s (Fig 1e). Rab proteins have previously been reported to colocalise with the Golgi complex in splenic cDC1s (Kretzer et al., 2016). Hence we checked if Rab32 shows colocalisation with this complex. We did not observe the colocalisation of Rab32 with the markers of cis- and trans-Golgi (Giantin and TNG38, respectively). Rab proteins have also been described to localise with the intracellular MHC-I storage compartments or lysosomal ($LAMP1^+$) vesicles in DCs (Cebrian et al., 2016; Nair-Gupta et al., 2014; Rybicka et al., 2012; Zou et al., 2009). Rab32 was not found to colocalise with these compartments in primary splenic cDC1s. Previous studies have reported that Rab32 co-localises with the ER and mitochondria in cell lines such as HeLa cells. We did not observe the colocalisation of Rab32 with the ER marker (calnexin) or Mitochondria (COX5B) in splenic cDC1s.

Rab32 promotes the proliferation of effector $CD8^+$ T cells against cell-associated antigens in-vivo

Given its high expression in cDC1s, we sought to determine if Rab32 contributes to the function of cDC1s in inducing $CD8^+$ T cell responses against cell-associated antigens. Rab32 WT or KO mice were immunised with UV irradiated Kbm1_OVA cells (mouse embryonic fibroblasts) along with the TRL3 agonist polyI:C. Kbm1_OVA cells possess a loss of function mutation in $H\text{-}2^{kb}$, which rules out direct antigen presentation to $CD8^+$ T cells by these cells. Day 10 post-injection, the spleen and blood of these mice were examined for OVA-specific ($Tetramer^+CD62L^{low}$) and effector ($CD62L^{low}CD44^{hi}$) endogenous $CD8^+$ T cells by flow cytometry (gating strategy – Fig S2). Rab32 KO mice showed significantly lower percentages of OVA-specific $CD8^+$ T cells in the spleen, compared to the WT mice (Fig 2a and b). Moreover, KO mice also had lower percentages of effector $CD8^+$ T cells in the spleen. Similar observations were made in the blood (Fig 2c and d). In both the genotypes, OVA-specific $CD8^+$ T cells had similar effector status (Fig S2). Hence, Rab32 promotes the generation of effector $CD8^+$ T cells against cell-associated antigens *in-vivo*.

To rule out $CD8^+$ T cell-intrinsic effects of Rab32 on their activation, we decided to test the response of adoptively transferred OT-I cells (transgenic $CD8^+$ T cells expressing SIINFEKL

specific TCR) to Kbm1_OVA challenge in the Rab32 WT and KO mice. CD45.1.2 OT-I cells were CTV labelled and injected into host CD45.2 mice, RAB32 WT or KO. 6-12h later, Kbm1_OVA cells were *i.v.* injected in the same mice. 3 days later, the spleens of these mice were examined by flow cytometry (Fig 2e). Rab32 KO mice showed consistently lower percentages of OT-I cells in the spleen for different numbers of Kbm1_OVA cells injected (Fig 2f and g). Moreover, Rab32 KO mice had significantly lower percentages of effector ($CTV^{low}Cd62L^{low}$) OT-I cells compared to the WT mice (Fig 2h and i). Hence, the observed effect of Rab32 on the proliferation of effector $CD8^+$ T cells is not T cell-intrinsic.

Taken together, these observations lead us to conclude that Rab32 promotes the proliferation of effector $CD8^+$ T cells in response to cross-presentation of cell-associated antigens by cDC1s *in-vivo*.

Rab32 does not control the cross-presentation of cell-associated antigens by cDC1s ex-vivo

Since Rab32 promoted the proliferation of effector $CD8^+$ T cells in response to cross-presentation by cDC1s *in-vivo,* we examined various aspects of cDC1-$CD8^+$ T cell interactions which could potentially be affected by Rab32. Firstly, the surface expression of MHC-I and II, as well as the co-stimulatory markers CD40 and CD80 in naïve cDC1s was not affected by the deficiency of Rab32 in these cells (Fig 3a). We then examined whether Rab32 affected the sensitivity of peptide-MHC-I: TCR interactions between cDC1 and $CD8^+$ T cells. To this end, splenic cDC1s were sorted from the Rab32 WT or KO, and co-cultured with CTV labelled OT-I cells in the presence of SIINFEKL peptide (normal OVA peptide presented on $H2^{kb}$ with high affinity to OT-I TCR) or SIIQFEKL peptide (mutated OVA peptide presented on $H2^{kb}$ with low affinity to OT-I TCR). Since OT-I cells are highly sensitive to the presentation of SIINFEKL, minor differences in OT-I activation by WT and KO could be better observed using SIIQFEKL. Rab32 WT and KO cDC1s induced similar OT-I proliferation in response to the presentation of both these peptides, as measured by OT-I CTV divisions (Fig 3b). Moreover, OT-I activation, measured as IL-2 and IFNγ secretions in the culture supernatant, was also not affected by the deficiency of Rab32 in cDC1s (Fig 3c). In conclusion, Rab32 did not affect the sensitivity of peptide-MHC-I: TCR interactions between cDC1 and $CD8^+$ T cells.

Next, we examined if Rab32 facilitated the process of antigen cross-presentation itself by splenic cDC1. Rab32 WT or KO splenic cDC1s were co-cultured with OT-I cells *ex-vivo*, in the presence

of different ratios of Kbm1_OVA:cDC1s (Fig 3d). IL-2 and IFNγ secretions by OT-I in the culture supernatant were measured as readouts for cross-presentation. OT-I cells co-cultured with WT or KO cDC1s secreted similar quantities of both cytokines (Fig 4e). Hence, Rab32 does not affect the cross-presentation of cell-associated antigens by cDC1s.

Based on these observations, we concluded that Rab32 promotes the proliferation of antigen-specific effector $CD8^+$ T cells without affecting peptide-MHCI:TCR interactions or antigen cross-presentation by cDC1s.

Rab32 does not control cDC1 maturation or survival upon activation with polyI:C

The maturation status of DCs is critical for determining their ability to induce $CD8^+$ T cell responses *in-vivo* as well as *in-vitro* (Alloatti et al., 2015; Bonifaz et al., 2002; Gil-Torregrosa et al., 2004; Hawiger et al., 2001; Schulz et al., 2005). Upon activation, DCs maintain the ability to activate $CD8^+$ T cells in the initial stages of maturation, but then gradually lose this ability as they reach complete maturation. Therefore, we examined if Rab32 regulated the activation and maturation of cDC1s, thus affecting their ability to activate $CD8^+$ T cells. Rab32 WT or KO mice were *i.p.* injected with 20ug polyI:C, and 14h later, their spleens were examined by flow cytometry. This time point was chosen so that the cDC1s could be examined while they are still maturing because complete maturation leads to cell death. Mice from both genotypes maintained similar numbers of splenic cDC1s at this time point after activation (Fig 4a). Moreover, blood serum from both the WT and KO mice contained similar levels of the cytokine IL-12 (Fig 4b). Rab32 WT and KO cDC1s also similarly upregulated MHC-I, MHC-II as well as the co-stimulatory markers (CD40 and CD80) upon polyI:C stimulation. Hence, we concluded that Rab32 does not affect the maturation, cell survival or production of co-stimulatory signals by cDC1s.

Rab32 promotes tumour infiltration of $CD8^+$ T cell and enhances the presence of tumour migratory cDCs in tdLNs

Tumour infiltration of $CD8^+$ T cells is poorly induced in cDC1 deficient mice (Hildner et al., 2008), as well as in the specific absence of cross-priming by cDC1s (Theisen et al., 2018). Therefore, we sought to determine whether Rab32 was essential for inducing $CD8^+$ T cell proliferation in a tumour model. B16_OVA melanomas were *s.c.* injected in $CD45.2^+$ Rab32 WT or KO mice. When the tumours grew to measurable sizes, $CD45.1.2^+$ OT-I cells were *i.v.* injected in the same mice, and a few days later, their pool was assessed in the tumour. (Fig 5a, gating strategy Fig S3a).

Rab32 KO mice showed a tendency to grow larger tumours compared to the WT mice (Fig 5b). Moreover, the KO mice had a significantly lower number of OT-I cells in the tumours compared to the WT mice (Fig 5c). The KO mice also had lower numbers of endogenous CD8 T cells in the tumours, although the difference was not significant compared to WT mice (Fig S3b). The total number of $CD8^+$ T cell (OT-I + endogenous) was significantly lower in the KO mice (Fig S3c). Hence, Rab32 significantly promoted the infiltration of antigen-specific as well as total $CD8^+$ T cells in B16 melanoma tumours.

In both mice and human melanomas, migratory cDC1s possess a robust ability to transport tumour antigens to the tdLNs for the priming of naïve $CD8^+$ T cells, in turn inducing their activation and infiltration in the tumour (Broz et al., 2014; Roberts et al., 2016). Migratory cDC2s also possess a substantial ability for the same, but to a lower extent compared to migratory cDC1s (Roberts et al., 2016). Since Rab32 promoted tumour infiltration of antigen-specific OT-I cells, we decided to evaluate if Rab32 affected the presence of tumour migratory cDCs in the tdLNs. Rab32 WT or KO mice were *s.c.* injected with B16_OVA, and the tumours were allowed to grow to measurable sizes. Tumours were then painted with fluorescent dye TRITC, which readily gets absorbed through the skin and fluorescently labels the tumour infiltrating leukocytes, including cDCs. 48h later, we evaluated various cDC populations within the tdLNs by flow cytometry. The MHC-$II^{hi}CD11c^{int}$ migratory cDC population was identified, within which the $XCR1^+$ cDC1 and $SIRPa^+$ cDC2s were distinguished (gating stategy Fig S3d). Rab32 KO mice were observed to possess lower numbers of $TRITC^+$ cDC1s as well as cDC2s in the tdLNs, with the difference in cDC2 being more prominent and significant (Fig 5d and e). TRITC was absent from the MHC-$II^{int}CD11c^{hi}$ resident cDC1s and cDC2s, suggesting the absence of dye leakage through the lymphatic system (Fig S3d). The total numbers of migratory and resident cDC1 and cDC2 in the LNs were not affected by the deficiency of Rab32 (fig S3e). These observations suggest that Rab32 affected the presence of migratory cDCs in the tdLNs which had specifically migrated from the tumour.

Taken together, these observations lead us to conclude that Rab32 promotes $CD8^+$ T cell-based anti-tumour immunity by promoting the presence of tumour migratory cDCs in the tdLNs.

Rab32 promotes perinuclear clustering of lysosomes in cDC1s through its GTPase activity

To further understand how Rab32 promotes the migration of cDCs from the tumour site to tdLN, we decided to investigate the impact of Rab32 deficiency on lysosome distribution. Lysosomes have recently emerged as major regulators of various cellular processes in maturing DCs, including DC migration to LNs, as well as the ability of DCs to cross-prime $CD8^+$ T-cells (Alloatti et al., 2015; Bretou et al., 2017). These functions are regulated by lysosomal signalling networks, which depend on the movement and position of lysosomes within the cytoplasm (Pu et al., 2016; Willett et al., 2017). To investigate if Rab32 regulated lysosomal positioning in cDC1s, we examined the distribution of lysosomes in splenic cDC1s sorted from Rab32 WT or KO mice using confocal microscopy (Fig 6a). Lysosomes ($LAMP1^+$) were visibly more dispersed in KO cDC1s, whereas in WT cDC1s they showed a perinuclear clustering. To quantify the lysosomal dispersion, we determined the LAMP1 centre of mass (CM) in each cell and calculated the distribution of LAMP1 intensity from the CM using radial profiling. Distance from the CM to reach 50% of the total LAMP1 intensity (I_{50}) was higher in KO cDC1s compared to WT cDC1s (Fig 6b). To further consolidate this finding, we studied the effect of Rab32 on lysosomal distribution in MutuDCs, a mouse splenic $CD8a^+$ DC-derived cell line (Fuertes Marraco et al., 2012). Rab32 was silenced in MutuDCs using siRNA, which led to the loss of Rab32 expression in these cells (Fig 6c). Similar to KO cDC1s, Rab32 silenced (pLKO-Rab32.sh1) MutuDCs had more dispersed lysosomes (Fig 6d) and showed a higher I_{50} compared to the control MutuDCs (pLKO-control) (Fig 6e). Hence, we concluded that Rab32 promoted the perinuclear clustering of lysosomes in cDC1s.

We next sought to examine whether Rab32 promoted lysosomal perinuclear clustering through its GTPase activity. To this end, we overexpressed either GFP-Rab32WT or its constitutively active Q85L mutant GFP-Rab32Q85L in HeLa cells. The cells were then fixed and stained with LAMP1 and examined by confocal microscopy. We observed that, similar to cDC1s, the overexpressed Rab32WT did not colocalise with LAMP1, while the overexpressed RAB32Q85L showed strong colocalisation with LAMP1 (Fig 6f and g). Moreover, this colocalisation led to a perinuclear clustering of $LAMP1^+$ $Q85L^+$ vesicles in these cells. Due to this clustering, the $LAMP1^+$ $Q85L^+$ vesicles were significantly bigger compared to $LAMP1^+$ (single positive) vesicles (Figure 6h). We further sought to determine if active Rab32 promoted lysosomal clustering at the perinuclear Microtubule Organising Centre (MTOC) within the cells. To this end, we stained the Rab32WT or Rab32Q85L overexpressing HeLa cells with the antibodies against lysosomal marker CD63

(LAMP3) and MTOC marker γ-tubulin. It was observed that LAMP1^{+}Q85L^{+} vesicles colocalised with γ-tubulin. Hence, the Rab32Q85L promoted lysosomal clustering at the perinuclear MTOC within the cells.

Taken together, these observations suggest that Rab32 promotes perinuclear clustering of lysosomes in cDC1s.

Discussion

In this report, we have evaluated the expression of various Rab GTPases in different resident DC subsets and have found Rab32 to be amongst the Rab GTPases highly expressed in resident cDC1 populations, as compared to cDC2s and pDCs (Fig 1a). Other Rab proteins found to be highly expressed in cDC1s include Rab43, Rab39a, Rab7a, Rab7b, Rab11a, Rab10 and Rab19. Few of these proteins have previously been implicated in CD8^{+} T cell cross-priming against cellular antigens *in-vivo*, but the associated molecular mechanisms are not always completely understood. Thus, the mechanism by which Rab43 facilitates cross-presentation of cell-associated antigens specifically by cDC1s *in-vivo* remains to be described (Kretzer et al., 2016). Rab39a has also been described to facilitate antigen cross-priming *in-vivo* by converting the phagosome into an antigen-loading compartment (Cruz et al., 2020). Similar to Rab32, neither Rab43 nor Rab39a was found to affect the differential of DC populations in mice (Fig 1d; (Cruz et al., 2020; Kretzer et al., 2016)).

Although well expressed in cDC1s, Rab11 and Rab10 have never been investigated particularly in this cell type. However, Rab11 and Rab10 were studied in bone marrow-derived DCs (BMDCs) and DC2.4 cell line respectively and are implicated in antigen cross-presentation by these cells. Rab11a is essential for the surface recycling of MHC-I molecules and the maintenance of the intracellular MHC-I storage compartment in DCs (Nair-Gupta et al., 2014). Upon phagosomal TLR signalling, MHC-I molecules are trafficked from this pool to the antigen containing phagosomes, and the absence of Rab11a hinders this pathway, thus limiting cross-presentation by BMDCs. The role of Rab11a in cDCs and *in-vivo* cross-priming remains to be seen. Rab10 is a late endosome/lysosome-related Rab GTPase that has been identified to promote cross-presentation in an shRNA-based screening performed in the DC line DC2.4 (Zou et al., 2009). The mechanism by which Rab10 participates in cross-presentation, its relevance for cDC1 function and *in-vivo* priming of T cells remain to be investigated. Rab7, which has two isoforms in

mammals-Rab7a and Rab7b, as well as Rab19 have never been investigated in antigen cross-presentation by DCs. However, Rab7, which controls lysosomal movement and regulates phagosome-lysosome fusion events within cells (Pu et al., 2016) might be an interesting candidate for cross-presentation study in cDC1s. Finally, Rab32, one of the most abundant Rab proteins in DCs1 was never investigated before in the context of antigen cross-presentation by cDC1s.

In this study, we have demonstrated that Rab32 promotes the proliferation of effector $CD8^+$ T cells in response to cross-presentation of cellular antigens *in-vivo* (Fig 2a-d). In mice as well as humans, the cross-presentation of cellular antigens relies on cDC1s that are functionally specialised for this task (den Haan et al., 2000; Desch et al., 2011; Helft et al., 2012; Iyoda et al., 2002; Pooley et al., 2001; Schnorrer et al., 2006). Considering the pivotal role of cDC1s in $CD8^+$ T cell priming and the high expression of Rab32 in these cells, it was likely that Rab32 affects $CD8^+$ T cell priming in a cDC1 intrinsic manner. However, since the micro-array data used in our report suggests that Rab32 has a relatively low express in lymphoid cells, including $CD8^+$ T cells (Fig S1a), we ruled out any $CD8^+$ T cell-intrinsic effects of Rab32 by adoptively transferring the OT-I cells in mice, which similarly to endogenous $CD8^+$ T cells, were also significantly less activated in Rab32 KO mice compared to the WT mice (Fig 2e-i). Hence, we verified that Rab32 indeed promotes $CD8^+$ T cell responses by affecting cDC1 function.

Interestingly, deficiency of Rab32 does not affect the cross-presentation of cellular antigens by cDC1s *ex-vivo* (Fig 3e). This observation is line with the recently published report on the cDC1-specific Rab39a, which shows that Rab39a deficiency also does not affect cross-presentation by cDC1 *ex-vivo*, yet it affects the cross-priming against cell-associated antigens *in-vivo* (Cruz et al., 2020). Several scenarios might explain why Rab32 deficiency did not affect *ex-vivo* cDC1 cross-presentation ability in our experiments. Since cDC1 are highly adapted for cross-presentation, compensatory mechanisms may exist in these cells which deal with the deficiency of specific proteins. Moreover, certain Rab proteins are also known to be redundant in their functions. In the case of Rab32, a possible candidate is the closely related protein Rab38 (75% sequence similarity in mice). Rab32 and Rab38 are known to be essential for the biogenesis of melanosomes, which are lysosome-related organelles responsible for the synthesis and storage of melanin pigment in the body (Bultema et al., 2012; Loftus et al., 2002; Wasmeier et al., 2006). In the absence of either Rab38 or Rab32 activity, one can functionally compensate for the other, and hence, only a mild

pigmentation phenotype is observed in single KO cells (Loftus et al., 2002). In Rab32 and Rab38 double KO cells, the hypopigmentation phenotype is much more severe (Wasmeier et al., 2006). The microarray data used in our report suggests that Rab38 is decently expressed in cDC1s (Fig S1a). Hence, such a functional redundancy may exist in cDC1s.

An alternative explanation for normal the cross-presentation ability of Rab32 deficient cDC1 *ex-vivo* is that the *ex-vivo* experiments test antigen processing abilities, but do not test other DCs characteristics, such as cell migration, which is crucial for *in-vivo* T cell priming. In cancer-immunity cycle, the *in-vivo* $CD8^+$ T cell priming involves tumour migratory DCs that pick up tumour antigens and transport them to the tdLNs. Within the T cell zones of the tdLNs, $CD8^+$ T cells are cross-primed by DCs against tumour antigens, which then egress from the tdLNs and infiltrate the tumour to perform anti-tumour cytotoxic functions (Chen & Mellman, 2013). Certain evidence also suggests a transfer of tumour antigens from the migratory to resident cDCs in the tdLNs, conferring resident cDCs the ability to also cross-prime $CD8^+$ T cells (Roberts et al., 2016). We have provided *in-vivo* evidence that Rab32 promotes the tumour infiltration of antigen-specific $CD8^+$ T cells (Fig 5c). We have further demonstrated that deficiency of Rab32 reduces the presence of tumour migratory cDCs within the tdLNs (Fig 5d and e). These results indicate that by controlling the migration of tumour DCs towards tdLN, Rab32 contributes to anti-tumour cellular immunity.

Our results further suggest that Rab32 regulates the migration of cDCs by controlling intracellular lysosome distribution. We showed that lysosomes in cDC1 form a perinuclear cluster, whereas in the absence of Rab32 they are redistributed towards the cell periphery (Fig 6a and b). Moreover, the overexpression of the constitutively active form of Rab32 in HeLa cells induced a perinuclear lysosomal clustering (Fig 6f and g), similar to what was observed in WT cDC1. These findings might be relevant for the migration of cDCs from the tumour, since lysosomal positioning has been linked with a variety of cellular processes, including cell migration (Pu et al., 2016). A major determinant of lysosomal function is the transcription factor-EB (TFEB), which is the master regulator of lysosomal genes (Sardiello et al., 2009). In the dephosphorylated (inactive) state, TFEB is present on the surface of the lysosome (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Upon perinuclear positioning of lysosomes (induced by starvation signals or lysosomal stress), TFEB gets dephosphorylated and translocates to the nucleus, thus

allowing expression of lysosomal proteins (Korolchuk et al., 2011; Napolitano et al., 2018). A seminal study by the team of Lennon-Duménil has recently reported the role of TFEB activation in the migration of DCs (Bretou et al., 2017). TFEB activity promotes the lysosomal signalling through Ca^{2+} channel TRPML1, which results in remodelling of the actin cytoskeleton to support direction movement of DCs. This mechanism is required for the migration of maturing migratory DCs from peripheral tissues to the LNs, since the absence of this pathway in TRPML1 KO DCs stops their fast and directional migration to the LNs once they are maturing and activated (Bretou et al., 2017). Further studies are required to establish whether Rab32, through its effect on lysosomal positioning, can regulate lysosomal signalling in DCs and subsequently DC migration from the tumour to the lymph nodes.

Materials and methods

Mice – All mice experimentation was performed under the authorisation from the Ministere de l'Education Nationale, de l'Enseignement Superieur et de la Recherche, APAFIS n. 15373 - 2018100811239028 v6. Heterozygous embryos of Rab32 carrying a tm1a (KOMP) Wtsi knockout first promoter-driven allele were purchased from the KOMP repository knockout mouse project (UC Davis, US) and homozygous knockout mice were generated in the laboratory. They possess a promoter-driven cassette insert between exon 1 and 2 of Rab32, which disrupts Rab32 translation in these mice. These mice have a $CD45.2^+$ C57BL/6 background. To generate littermates, Rab32 tm1a mice were crossed with WT C57BL/6 mice purchased from Charles River, France. WT/WT and tm1a/tm1a littermates were identified by genotyping in the second progeny generation and were used for experimentation. The Rab32 cdel mice have a constitutive deletion of Rab32 exon 2. They were generated at CNRS TAAMS at Orleans, France, by crossing the $CD45.2^+$ Rab32 tm1a mice (possessing floxed exon2) with $Cd45.2^+$ germ-line cre expressing 'Rosa26 Deleter CRE' mice. WT/WT and cdel/cdel littermates were identified by genotyping in the second progeny generation and were used for experimentation. The $CD45.1.2^+$ OT-I mice were generated in-house by crossing male $CD45.2^+$ OT-I Rag2KO mice (a kind gift from Dr Oliver Lantz, Institut Curie, France) with female $CD45.1^+$ OT-II mice (purchased from Charles River Laboratories). The first generation $CD45.1.2^+$ OT-I mice were then used for experimentation. The genotyping primers for Rab32 tm1a mice (cassette region) are – a. TGCAGGCAGTAGGCATTCTA, b. CCAACTGACCTTGGGCAAGAACAT, The genotyping primers for Rab32 cdel mice (exon 2 deletion) are – a. CACACCTCCCCCTGAACCTGAAA, b. TGTTTTCTTGGCCTCTTTCAA.

The genotyping primers for the cre gene are – a. CCTGGAAAATGCTTCTGTCCG, b. CAGGGTGTTATAAGCAATCCC.

Cell lines – Kbm1/Kbm1_OVA MEFs were a kind gift from Dr Caetano Reis e Sousa, The Francis Crick Institute, London and have been previously described (Sancho et al., 2009). They were cultured in complete DMEM-Glutamax medium (Gibco™ Life Technologies), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco™ Life Technologies), 1% penicillin-streptomycin (P/S) and 0.1 % 2-Mercaptoethanol. HeLa cells were also cultured in similar preparation of complete DMEM medium. The B16/ B16_OVA/ B16-FLT3L melanoma cells were a kind gift from Dr Julie Helft, Institut Curie, France. They were cultured in complete RPMI-Glutamax medium (Gibco™ Life Technologies), supplemented with 10% heat-inactivated FBS, 1% P/S and 0.1% 2-Mercaptoethanol. Mutu cells were created in the Lab of Dr Hans Acha-Orbea, University of Geneva, Switzerland (Fuertes Marraco et al., 2012). They were cultured in complete IMDM-Glutamax (Gibco™ Life Technologies), supplemented with 10% FBS (Gibco™ Life Technologies lot n. 42Q4467K OR 42F5471K), 1% P/S and 0.1% 2-Mercaptoethanol The cells were passaged every 2-3 days depending on the confluence using the 2.5% 10X Trypsin solution (Gibco™ Life Technologies).

Flow Cytometry- Flow cytometry was performed on the BD LSRFortessa™ FACS instrument. All data were analysed using FlowJo analysis software (Tree Star). Antibody staining was performed at on ice in home-made FACS buffer (PBS + 2% BSA + 2mM EDTA). The list of antibodies used for flow cytometry analysis is provided in supplementary materials.

Analysis of splenic cDC1 by flow cytometry – The spleens were harvested from the mice and digested in a 0.375 U/ ml solution of Collagenase D (ROCHE Ref 11-088-866-001, Lot n. 20350022) in the digestion medium (HBSS, 5 % FCS, b-me 50uM) for 30 min at 37^0C. RBCs were removed by treatment with home-made ACK lysis buffer (NH4Cl= 8290mg/L, $KHCO_3$= 5000mg/L, EDTA= 0.0995mM in 1L distilled water). Splenocytes were then passed through a 70um cell strainer, pelleted and stained with antibodies, including biotinylated lineage negative markers MHC-II, Gr-1, CD119, NK-1.1, TER-119 and TCRb for 30 min on ice (complete list of antibodies is provided in supplementary materials). Following two washes in cold FACS buffer by centrifugation, the cells were stained with Streptavidin BV605 for lineage negative cells (1:500 dilution, BD Biosciences 563260) for 30 min on ice. The cells were then washed in cold FACS

buffer by centrifugation and analysed by flow cytometry. Cell counting was performed by flow cytometry using Accucheck counting beads (Life Technologies PCB100).

Purification and sorting of splenic cDC1s – Mice were injected with B16-FLT3L to promote the numbers of cDC1s recovered. On day 9 after tumour injection, spleens were harvested from the mice digested with Collagenase D and RBC lysed as described above. Splenocytes were pelleted and stained with a home-made antibody cocktail for 30 min at 4^0C (including biotinylated lineage negative markers CD19, B220, PDCA1, NK-1.1., TER-119, TCRb, Gr-1, F4/80 and Ly6G; a complete list of antibodies is provided in supplementary materials). Anti-biotin microbeads (Miltenyi Biotec 130-090-485) were then added (10ul per 10^7 cells as per manufacturer's protocol), and the solution was incubated for 10 min at 4^0C. The solution was then passed through an LS MACS column (Miltenyi Biotec 130-042-401) placed in a magnetic field, and the flow-through enriched in XCR1$^+$ CD11c$^+$ cDC1s was collected. The XCR1$^+$ CD11c$^+$ cDC1s were further sorted using the BD FACSMelody™ Cell Sorter. Sorted cells were resuspended in complete IMDM (supplemented with 10% FBS, 1% penicillin-streptomycin and 0.1 % 2-Mercaptoethanol).

polyI:C preparation for injections- For annealing the polyI:C HMW (Invivogen ref. tlrl), required concentration was heated at 65^{o}C in the water bath for 10 min, then allowed to cool down at RT for 45 min. Annealed polyI:C was then used as required.

Assessment of endogenous CD8$^+$ T cells– Kbm1/Kbm1_OVA cells were passaged and a cell solution was prepared in PBS at 1x10^6 cells/180ul/injection. The cells were UV irradiated at 9999uJoules x 100 for 10 minutes. 10ug/20ul/injection annealed polyI:C was added to the cell solution. 200ul solution (Kbm1/Kb1m_OVA+10ug polyI:C) was *i.v.* injected in the Rab32 WT or KO mice. 10 days later, 500ul blood was collected from mice by cardiac puncture in the presence of heparin (anti-coagulant), following which the mice were sacrificed and their spleens were harvested. Both blood and spleen samples were treated with ACK buffer to lyse RBCs, counted by flow cytometry using Accucheck beads and stained with iTAg Tetramer/PE - H-2 Kb OVA (SIINFEKL) (1:20 dilution, MBL TB-5001-1) for 30 min at RT. Antibody mix was then added and the cells were stained for 30 min on ice (including biotinylated lineage negative markers; a complete list of antibodies is provided in supplementary materials). The samples were washed twice in cold FACS buffer by centrifugation and stained with Streptavidin BV605 for lineage

negative cells (1:500 dilution, BD Biosciences 563260) for 30 min on ice. The cells were then washed twice in cold FACS buffer by centrifugation, and analysed by flow cytometry.

Preparation of OT-I cells – Various lymph nodes were collected from OT-I mice in a 6 well plate on ice, including the inguinal, axillary, brachial and mesenteric lymph nodes. They were teased open to bring out the lymphocytes, and then gently smashed onto a 70um cell strainer within the well. The well and the cell strainer were properly washed with cold FACS buffer, and lymphocyte solution was were pelleted by centrifugation. The cells were labelled with 5uM CTV (Life technologies C34557) in PBS 0.1% BSA. The OT-I cells were then isolated using the $CD8a^+$ T cell isolation kit (Miltenyi Biotec 130-104-075) by following the manufacturer's protocol. For injections, the cells were responded in sterile PBS, whereas for culture the cells were resuspended in complete IMDM medium (supplemented with 10% FBS, 1% penicillin-streptomycin and 0.1 % 2-Mercaptoethanol).

OT-I adoptive transfer experiment – $CD45.1.2^+$ OT-I cells were CTV labelled and purified as described above. $1x10^6$ cells were *i.v.* injected in $CD45.2^+$ host Rab32 WT or KO mice. 6-12h later, different numbers of live Kbm1_OVA fibroblasts were *i.v.* injected in the same mice. On day 3 p.i., mice were sacrificed and their spleens were harvested, treated with ACK buffer, counted and stained for flow cytometry analysis. The complete list of antibodies is provided in supplementary materials.

Ex-vivo peptide presentation assay – 10,000 splenic $XCR1^+CD11c^+$ cDC1s were counted and plated per condition. The SIINFEKL and SIIQFEKL peptides (JPT peptide technology, Germany) were serially diluted in complete IMDM (supplemented with 10% FBS, 1% penicillin-streptomycin and 0.1 % 2-Mercaptoethanol), and different concentrations were added to the respective wells. 100,000 purified and CTV labelled OT-I cells were then added to all the wells. The plates were incubated at 37^0C, and 24h later the culture supernatant was collected and stored in -20^0C for IL-2 ELISA, and the culture medium was replenished in the wells. 48h later (day 3 of culture), the supernatant was collected for IFNγ ELISA. The cells were stained and analysed by flow cytometry.

Ex-vivo cross-presentation assay – $XCR1^+CD11c^+$ cDC1s were sorted from the spleens of Rab32 WT or KO mice as described above. 10,000 cells were counted and plated per condition. Different numbers of live Kbm1_OVA cells were added to the respective cells. 100,000 purified OT-I cells

were then added to all the wells. The plates were incubated at 37^0C for 24h, and culture supernatant was collected and stored in -20^0C. This supernatant was used to perform IL-2 and IFNγ ELISA using the manufacturer's protocol (Mouse IFNγ ELISA MAX™ Standard Set, Biolegend 430801 and Mouse IL-2 ELISA MAX™ Deluxe set, Biolegend 431004).

Tumour OT-I experiment- CD45.2$^+$ host Rab32 WT and KO mice were *s.c.* injected with $0.5x10^6$ B16-OVA cells. The tumours were allowed to grow to measurable sizes, and on day11-14, $1x10^6$ purified CD45.1.2$^+$ OT-I cells were injected i.v. in the same mice. On day 17-16, the mice were sacrificed, and the tumours were harvested. Tumours were then digested with Collagenase D (ROCHE Ref 11-088-866-001, Lot n. 20350022) in the digestion medium (HBSS, 5 % FCS, b-me 50uM) for 40 min at 37^0C, and RBC lysis was performed as described above. $5x10^6$ CD45$^+$ cells were then counted using Accucheck beads by flow cytometry, plated and stained for further flow cytometry analysis.

Tumour TRITC painting experiment- Rab32 WT and KO mice were *s.c.* injected with $0.5x10^6$ B16-OVA cells. The tumours were allowed to grow to measurable sizes, and on day11, the mice were anesthetised using CO_2 and the shaved at the region of tumour growth. For the preparation of TRITC solution, the protocol was modified from a previously described method(Hor & Mueller, 2016). Briefly, TRITC (Sigma Aldrich D2653806) was dissolved in DMSO at the concentration -0.1ug/1ul. Then, a 1:1 solution of DMSO-TRITC:Acetone was prepared. This solution was carefully applied, drop by drop to the tumour (10 µl dye/acetone mix for a ~1 cm2 diameter painting site is recommended). The mice were kept under anaesthesia for a few minutes until the solution was absorbed by the skin. 48h later (ie on day 13), the mice were sacrificed and the tdLNs were analysed by flow cytometry. The complete list of antibodies is provided in supplementary materials.

Confocal microscopy – For splenic sorted cDC1s and Mutu cells, glass coverslips were coated with purified I-A/I-E antibody (100 dil, Biolegend 107601) overnight at 4^0C to enhance cell adherence. For HeLa cells, the coverslips were coated with poly-D-lysine (1mg/ml). The next day, 30,000 sorted splenic XCR1$^+$ CD11c$^+$ cDC1s were added per coverslip and were allowed to spread for 2h at 37^0C. The cells were fixed in 2% paraformaldehyde (PFA) for 15 min at 37^0C, followed by two washes in PBS. The cells were then treated with the permeabilisation solution (PBS+0.2% Saponin+0.2% BSA) at RT for 20 min. Primary antibody staining was performed in the

permeabilisation solution at RT for 40 min. The cells were then washed twice in PBS, and stained for secondary antibodies along with 1X DAPI for nuclear staining, for 40 min at RT. Antibody references are provided in the supplementary materials. Following two washes, a final fixation was performed in 4% formaldehyde in PBS. The samples were then treated with 50mM NH_4Cl for 7 min at RT for quenching the residual formaldehyde, washed and mounted on glass slides with fluoromount mounting medium. Images were acquired on Leica SP8 confocal microscope (Leica Microsystems, Germany) using the 63x immersion lens, and deconvoluted using the Huygens Professional image processing software. Further processing was performed using the Fiji-ImageJ software.

BMDC culture- Bone marrow cells were collected from the tibias and femurs of mice, and approx. $5x10^6$ cells were cultured in a tissue culture treated dish in complete RPMI supplemented with 10% supernatant from J558 cells expressing granulocyte-macrophage colony-stimulating factor (GM-CSF). At day 4, 5mL of fresh complete RPMI were added to the cell cultures. At day 5 and 8 the complete media was replaced by 10mL of fresh RPMI. On day 10, loosely adherent cells were collected by gentle washing with PBS and used for further analysis.

Western blot – Approximately 500,000 cells were washed in PBS and pelleted by centrifugation for analysis. The pellet was resuspended in 40ul of cell lysis buffer comprising 0.5% NP40 and 1X protease inhibitor (Roche 05892988001) in PBS. Cell lysis was performed for 30 min on ice, accompanied by vortexing after every 10 min. The solution was centrifuged for 20 min at 15000 rpm at 4°C, following which the supernatant was carefully collected in a separate tube. Cell extract was denatured in Laemmli buffer at 95^0C for 5 min and then transferred on ice for 5 min. The tube was centrifuged at 2000rpm for 2 min, and the supernatant containing the denatured cell extract was loaded on precast polyacrylamide gels (4–15% Bio-Rad Criteron TGX Stain-Free gels, Cat n. 5678084), along with the molecular ladder. After running the gel, the blots were transferred to a nitrocellulose membrane. Non-specific reactions were blocked by incubating the membrane with Tris-buffered solution (TBS) containing 0.5% Tween-20 (TBS.T) and 5% milk at 4^0C overnight. The following day, the membrane was cut into two parts just below the 37KDa mark to perform separate stainings for Rab32 (25KDa) and Actin (42KDa). The membranes were stained with respective primary antibodies in TBS.T for 1.5h at RT - Rab32 (400 dil, host mouse, Santa Cruz 390178) or Actin (20,000 dil, host mouse, Sigma A1978). Following 3 washes in TBS.T with 10 min incubations each, the membranes were stained with secondary anti-Mouse HRP conjugated

IgG (H+L) (10000 dil, Thermofisher 62-6520) for 1h on the shaker at RT. Following 3 washes in TBS.T, the membranes were developed with the Clarity Western ECL Substrate (Bio-Rad 1705061) and analysed within 15 min using Bio-Rad ChemiDoc™ Imager. The blot images were processed in the ImageLab software.

Rab32 silencing in MutuDCs - Plasmids encoding the shRNA sequences for targeting the mouse Rab32 were generated in the laboratory. The forward and reverse strands of the Rab32 short hairpin RNA sequence (Rab32 shRNA1) are-

5' ccggGCCAAGTTTCTGTAGTGTAAActcgagTTTACACTACAGAAACTTGGCtttttg - 3' and 5' aattcaaaaaGCCAAGTTTCTGTAGTGTAAActcgagTTTACACTACAGAAACTTGGC 3'. The 19-nucleotide Rab32 target sequences are indicated in uppercase letters, whereas the hairpin and the sequences necessary for the directional cloning are depicted in lowercase letters. The hybridised oligos were cloned into the pLKO.1 lentivirus vector using the restriction enzymes *AgeI* and *EcoRI,* and were validated by sequencing. The lentivirus was produced as follows- HEK293T cells were seeded at $3*10^6$ cells in 100mm plates 24 hours before transfection with calcium Phosphate. Briefly, 3 hours before transfection the media in the culture plates was replaced by 10ml of warmed DMEM. In a microcentrifuge tube, 5µg of psPAX2 packaging plasmid, 2 µg of the pCMV-VSVG envelope plasmid and 10µg of the pLKO lentiviral vector encoding the short hairpin RNA sequences to knockdown Rab32 were mixed in a solution containing 36µL of 2M $CaCl_2$ in H_2O (the total volume of the mixture was brought to 300µL). The mixture was added dropwise to a solution containing 300µL of 2X Hepes Buffered Saline (HBS) while bubbling air through the solution. The resulting mixture was incubated for 30 minutes at room and added dropwise to the cell culture dishes. After 16 to 20 hours the complete media was replaced by 10mL of fresh DMEM. The next day the supernatant was collected, centrifuged at 1800 rpm for 5 minutes to pellet any floating cells, and passed through a 0.45µm filter. For the transduction of Mutu cells, $1*10^6$ cells were plated in wells of a 6-well plate. The next day 1mL of virus was added to the wells containing 1mL of complete IMDM supplemented with HEPES (20µg/mL) and polybrene (6µg/mL). Plates were centrifuged at 2000 rpm for 2 hours at room temperature and left in the incubator overnight. The next day the complete media was replaced by 2mL of fresh IMDM and the cells were left in culture for 2 days prior starting the selection with puromycin at 1µg/mL. Dying cells were removed by replacing the media 6-8 hours after the addition of puromycin,

repeating the same procedure the next day. Resistant cells were maintained in IMDM supplemented with 1µg/mL puromycin for a continuous selection.

HeLa cell transfection with GFP-Rab32WT/ Rab32Q85L – HeLa cells were seeded on coverslips as described. 24h later, HeLa cells were transfected with the plasmids pEGFP Rab32 or pEGFP Rab32 Q85L (kind gifts from Dr Yuko Hirota, Kyushu University, Japan) using X-tremeGene 9 DNA transfection reagent (Roche Diagnostics, Germany, ref. 06365787001). $0.1x10^6$ cells were plated in a 6 well plate and incubated overnight at 37^0C. The next day, 2ml complete DMEM was replaced in the wells. Transfection solution was prepared as follows- 100 ul of DMEM (without P/S or FBS), 6 ul transfection reagent and 2 ug of the plasmid. The solution was allowed to sit at RT for 10 min and then added dropwise to the cells. The transfected cells were incubated overnight at 37^0C and used for microscopy the next day.

Gene microarray data – The data was downloaded from the Immunological genome project database at Immgen.org. Heat maps were created using the web-based Morpheus tool (Broad Institute - https://software.broadinstitute.org/morpheus).

Statistical analysis – All statistical analyses were performed using the Prizm GraphPad software (Graphpad Software Inc., San Diego, CA, USA).

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Figures

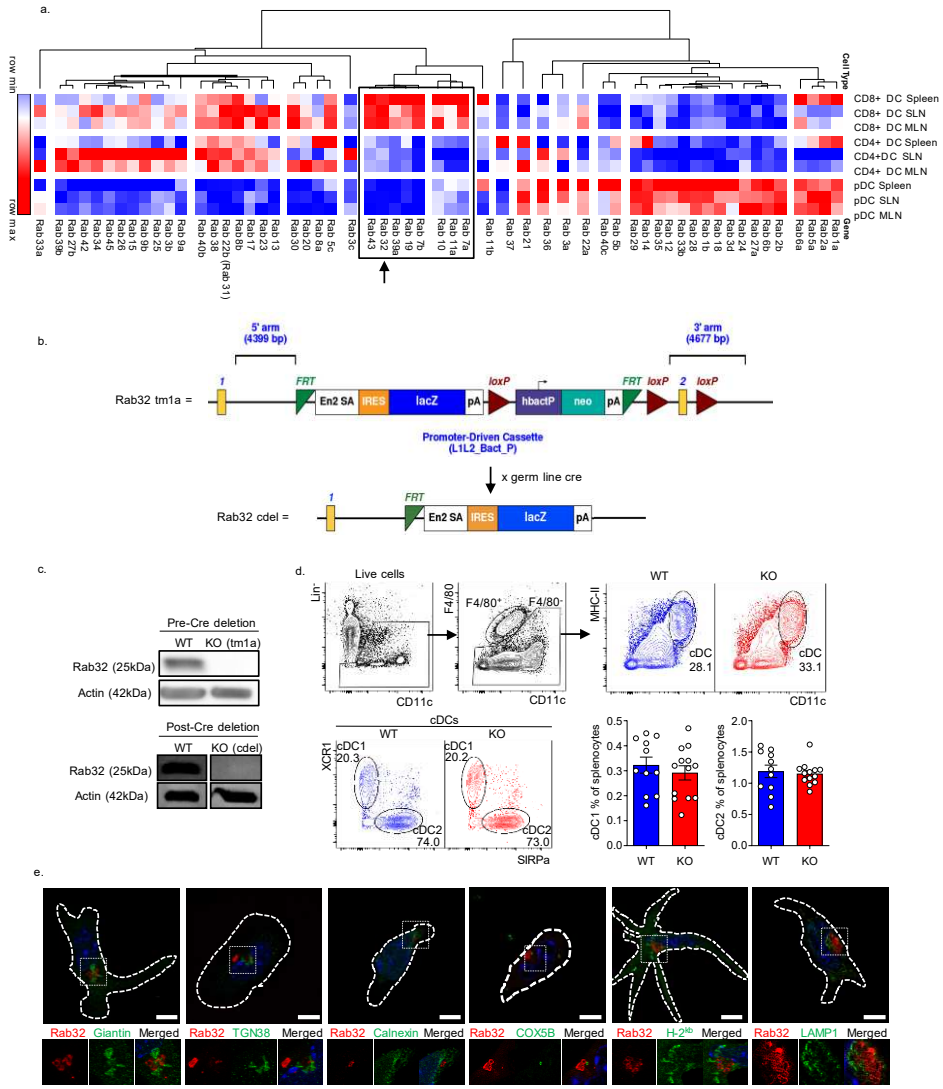
a.
row min
row max
Cell Type
CD8+ DC Spleen
CD8+ DC SLN
CD8+ DC MLN
CD4+ DC Spleen
CD4+DC SLN
CD4+ DC MLN
pDC Spleen
pDC SLN
pDC MLN
b.
5' arm (4399 bp)
3' arm (4677 bp)
Rab32 tm1a =
FRT
En2 SA
IRES
lacZ
pA
loxP
hbactP
neo
Promoter-Driven Cassette (L1L2_Bact_P)
x germ line cre
Rab32 cdel =
c.
Pre-Cre deletion
WT
KO (tm1a)
Rab32 (25kDa)
Actin (42kDa)
Post-Cre deletion
KO (cdel)
d.
Live cells
Lin-
F4/80
CD11c
MHC-II
cDC 28.1
cDC 33.1
cDCs
XCR1
cDC1 20.3
cDC2 74.0
cDC1 20.2
cDC2 73.0
SIRPa
cDC1 % of splenocytes
cDC2 % of splenocytes
e.
Rab32 Giantin Merged
Rab32 TGN38 Merged
Rab32 Calnexin Merged
Rab32 COX5B Merged
Rab32 H-2Kb Merged
Rab32 LAMP1 Merged

Figure 1. Rab32 is highly expressed in cDC1s but does not control their differentiation.

a. Expression of Rab GTPases in various DC subsets in mice (microarray data from Immgen database). A group of Rabs – 7b, 19, 39a, 32 and 43 is differentially and highly expressed in cDC1s.

b. The Rab32 tm1a have a promoter-driven cassette present between the exon 1 and 2 or Rab32, which disrupts Rab32 protein expression. The tm1a mice were crossed with a germline cre mice to create genetically deleted Rab32 KO mice (Rab32 cdel).

c. Verification of the absence of Rab32 in the pre-Cre deletion Rab32 KO (tm1a) and post-Cre deletion Rab32 KO (cdel) mice by western blot. Analysis on tma1 and cdel performed on BMDCs and sorted splenic $CD11c^{+}XCR1^{+}$ cDC1s, respectively, with corresponding littermate control and actin loading control.

d. FACS analysis of cDC populations in the spleens of naïve WT and KO mice. The Lin^{-} consists of non-cDC markers including TER119, NK1.1, TCRb, GR-1 and CD19. Bar graphs depict the Quantification of $XCR1^{+}$ cDC1s and $SIRPa^{+}$ cDC2s populations. Data from 4 independent experiments. Each point represents an individual mouse. (WT n=11, KO n=13).

e. Confocal microscopy projections of $CD11c^{+}$ $XCR1^{+}$ cDC1s sorted from the spleen of Rab32 WT mice, fixed and stained with antibodies against Rab32 along with various intracellular membrane markers- giatin (cis-Golgi), TGN38 (trans-Golgi), calnexin (Endoplasmic reticulum), COX5B (Mitochondria), $H\text{-}2^{Kb}$ and Lamp1 (Lysosomes). Scale bar - 7um.

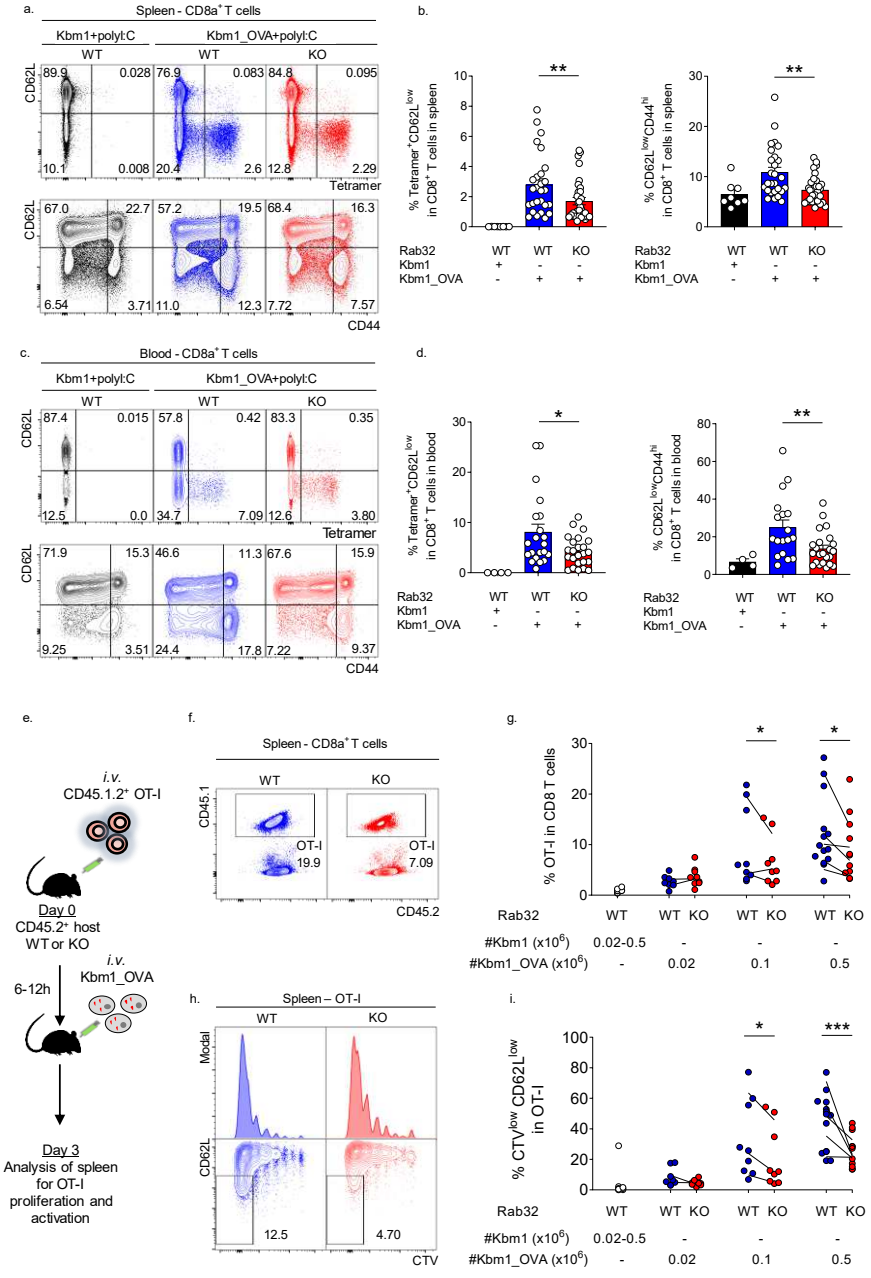
a.
Spleen - CD8a+ T cells
Kbm1+polyI:C
Kbm1_OVA+polyI:C
WT
WT
KO
CD62L
89.9
0.028
76.9
0.083
84.8
0.095
10.1
0.008
20.4
2.6
12.8
2.29
Tetramer
67.0
22.7
57.2
19.5
68.4
16.3
6.54
3.71
11.0
12.3
7.72
7.57
CD44
b.
% Tetramer+CD62Llow in CD8+ T cells in spleen
% CD62Llow CD44hi in CD8+ T cells in spleen
Rab32
Kbm1
Kbm1_OVA
c.
Blood - CD8a+ T cells
87.4
0.015
57.8
0.42
83.3
0.35
12.5
0.0
34.7
7.09
12.6
3.80
71.9
15.3
46.6
11.3
67.6
15.9
9.25
3.51
24.4
17.8
7.22
9.37
d.
% Tetramer+CD62Llow in CD8+ T cells in blood
% CD62Llow CD44hi in CD8+ T cells in blood
e.
i.v.
CD45.1.2+ OT-I
Day 0
CD45.2+ host
WT or KO
i.v.
Kbm1_OVA
6-12h
Day 3
Analysis of spleen
for OT-I
proliferation and
activation
f.
Spleen - CD8a+ T cells
CD45.1
OT-I
19.9
OT-I
7.09
CD45.2
g.
% OT-I in CD8 T cells
#Kbm1 (x10^6)
#Kbm1_OVA (x10^6)
h.
Spleen – OT-I
Modal
CD62L
12.5
4.70
CTV
i.
% CTVlow CD62Llow in OT-I

Figure 2. Rab32 promotes the proliferation of effector CD8$^+$ T cells against cell-associated antigens *in-vivo*.

a. FACS plots depict the profile of endogenous CD8$^+$ T cells in the spleens of Rab32 WT and KO mice, 10 days following immunisation with Kbm1_OVA+polyI:C. The upper panel depicts the OVA-specific (Tetramer$^+$CD62L^{low}) CD8$^+$ T cells, while lower panel depicts the effector (CD62L^{low}CD44^{hi}) CD8$^+$ T cells in the same spleen samples.

b. Quantification of the percentages of Tetramer$^+$ CD62L^{low} and CD44^{hi} CD62L^{low} endogenous CD8$^+$ T cells in the spleens of mice in each group. Data from 5 individual experiments. (Kbm1 control n=8, WT n=27, KO n=32, **p<0.01, Mann-Whitney test).

c. and d. Same as in a. and b. but in the blood. Data from 3 individual experiments. (Kbm1 control n=4, WT n=12, KO n=18, *p<0.05, **p<0.01, unpaired t-test).

e. Experimental design for the OT-I adoptive transfer experiment.

f. FACS panels depict a comparison of OT-I proliferation between WT and KO mice injected with 0.1x10^6 Kbm1_OVA.

g. Quantification of the percentage of OT-I in the CD8$^+$ T cell population in the spleens of WT and KO mice, for different numbers of injected Kbm1_OVA. Each point represents an individual mouse, and each line represents an individual experiment (*p<0.05, two-way ANOVA test).

h. FACS panels depict a comparison of OT-I proliferation (CTV dilutions) and gain of effector function (loss of CD62L) between WT and KO mice.

Commented [WU1]: Corrected, the whole point h was missing

i. Quantification of the percentage of CTV^{low}CD62L^{low} cells in OT-I population in the spleens of WT and KO mice, for different numbers of injected Kbm1_OVA (*p<0.05, ***p<0.001, two-way ANOVA test).

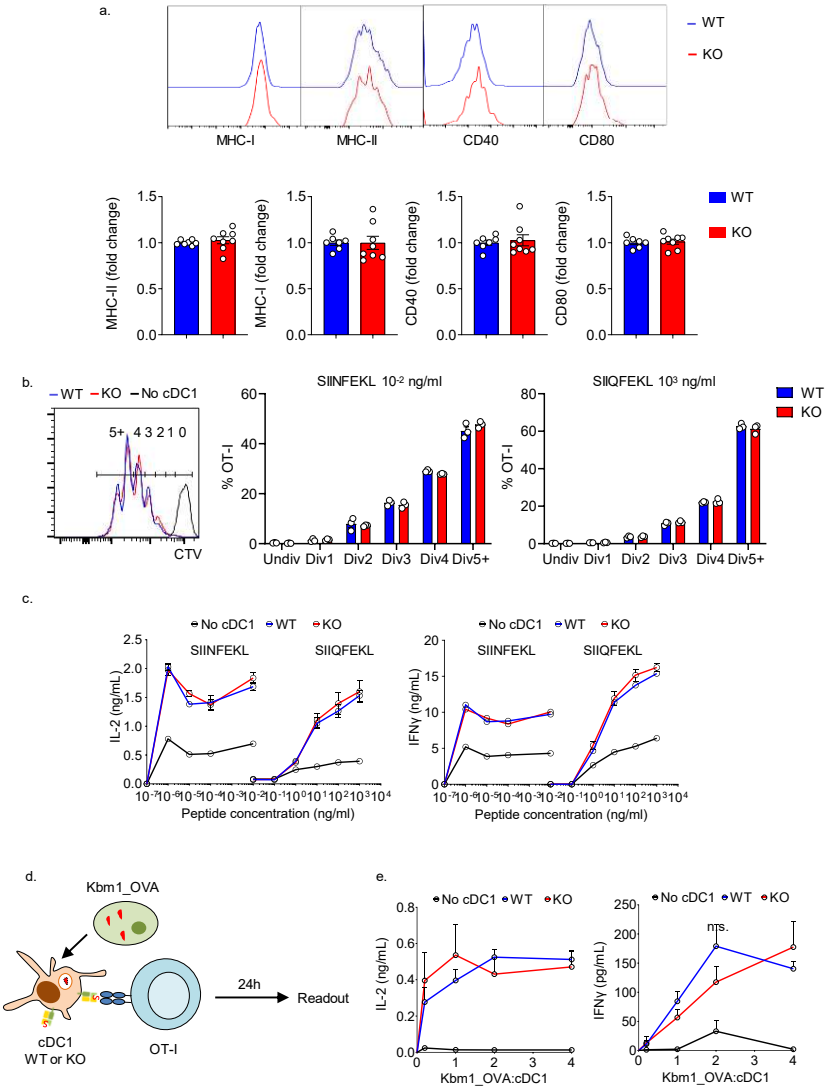
a.
WT
KO
MHC-I
MHC-II
CD40
CD80
MHC-II (fold change)
MHC-I (fold change)
CD40 (fold change)
CD80 (fold change)
b.
WT KO No cDC1
5+ 4 3 2 1 0
CTV
SIINFEKL 10-2 ng/ml
SIIQFEKL 10³ ng/ml
% OT-I
Undiv Div1 Div2 Div3 Div4 Div5+
c.
No cDC1 WT KO
SIINFEKL
SIIQFEKL
IL-2 (ng/mL)
IFNγ (ng/mL)
Peptide concentration (ng/ml)
d.
Kbm1_OVA
cDC1
WT or KO
OT-I
24h
Readout
e.
IL-2 (ng/mL)
IFNγ (pg/mL)
ns.
Kbm1_OVA:cDC1

Figure 3. Rab32 does not control the cross-presentation of cell-associated antigens by cDC1s *ex-vivo*.

a. FACS plots depict comparison MHC-I, MHC-II and activation marker (CD40 and CD80) expression on Rab32 WT and KO cDC1s. Bar graphs represent quantified data from multiple experiments. MHC-I, MHC-II and CD40 data from 2 independent experiments, CD80 data from 3 independent experiments.

b. Spleen sorted $CD11c^+$ $XCR1^+$ cDC1s were co-cultured *ex-vivo* with CTV labelled and purified OT-I cells in the presence of different concentrations SIINFEKL (high affinity for OT-I TCR) or SIIQFEKL (mutated peptide, low affinity for OT-I TCR). FACS plot depicts OT-I divisions (CTV dilutions) in response to SIINFEKL peptide presentation by WT or KO cDC1s. OT-I divisions in response to SIINFEKL or SIIQFEKL presentation by WT or KO cDC1s were quantified. Data from 1 experiment with 3 individual cDC1 sortings from WT or KO mice each.

c. In the same experiment, IL-2 (day1) and IFNγ (day3) secretions were quantified by ELISA.

d. Experimental design for the *ex-vivo* cross-presentation assay. IL-2 and IFNγ secretions were measured as readout for cross-presentation.

e. IL-2 and IFNγ secretions in presence of increasing Kbm1_OVA:cDC1 ration were quantified by ELISA. Data from 4 individual cDC1 sortings from WT or KO mice each.

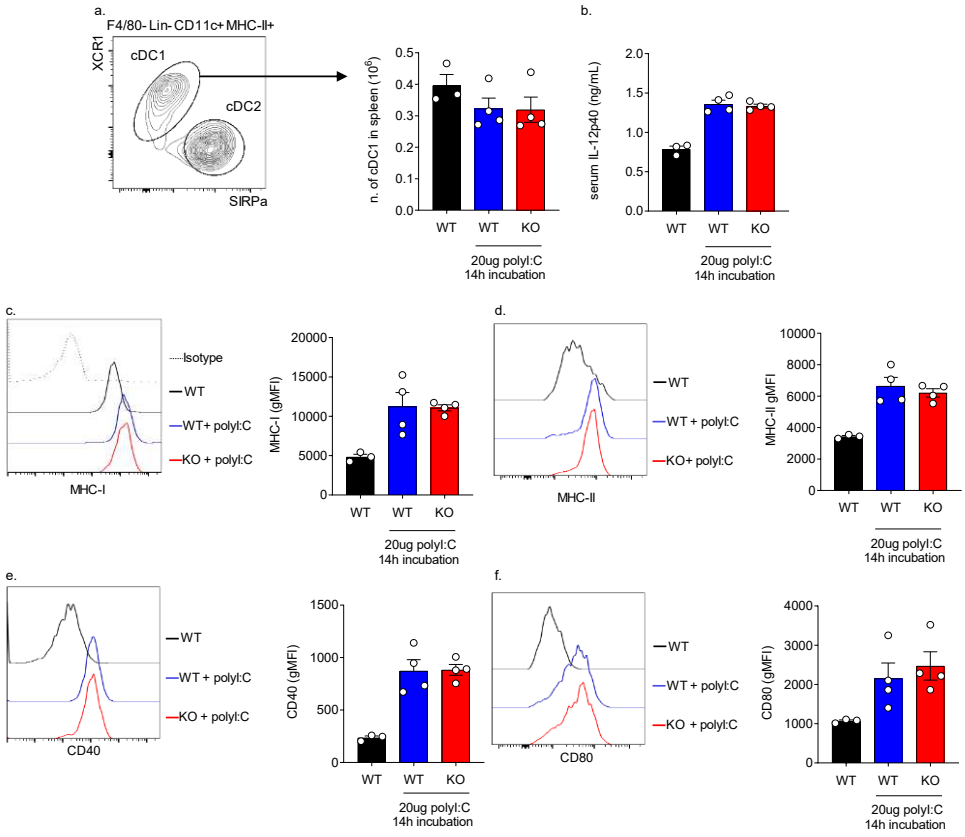

Figure 4. Rab32 does not control cDC1 maturation or survival upon activation with polyI:C.

a. Rab32 WT or KO mice were *i.p.* injected with 20ug polyI:C. 14h later, splenic cDC1s from the injected mice and non-injected WT controls were examined by FACS. cDC1 cell count was performed using Accucheck counting beads.

b. In the same experiment, secretion of IL-12p40 in the blood serum was analysed by ELISA.

c. To f. Surface expression of MHC-I and MHC-II (c. and d.) as well as activation markers CD40 and CD80 (e. and f.) was compared between the two genotypes. Data from one experiment.

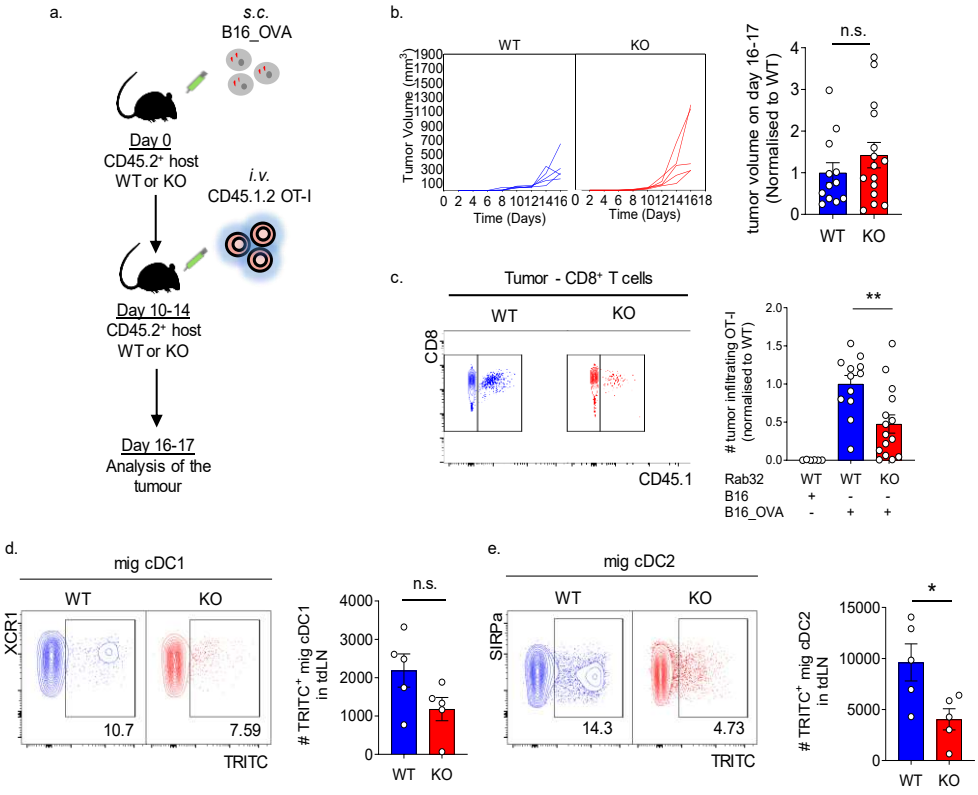

Figure 5. Rab32 promotes CD8⁺ T cell tumour infiltration by promoting the presence of tumour-associated cDCs in tdLNs.

a. Experimental design of the tumour-OT-I assay. CD45.2 host Rab32 WT and KO (tm1a) mice were injected with $0.5x10^6$ B16-OVA cells. Upon tumour growth on day 10-14, $1x10^6$ CD45.1.2⁺ OT-I cells were injected *i.v.* in the same mice.

b. The x-y graph depicts a comparison of tumour volume between the WT and KO mice till the day of sacrifice from 1 representative experiment. The bar graph depicts a comparison of tumour

volume on the day of sacrifice. Data from 3 independent experiments. (WT n=12, KO n=15, Mann-Whitney test).

c. The FACS plots depict a comparison of tumour OT-I infiltration between Rab32 WT and KO, and the bar graph depicts data from 3 independent experiments. (B16 control n=6, WT n=12, KO n=15, ** p < 0.01, Mann-Whitney test).

d. and e. Identification of TRITC^{+} cells, which had migrated from the tumour within the migratory cDC1 and cDC2 populations in tdLN. Data from one experiment. (n=5, *p<0.05, Mann-Whitney test).

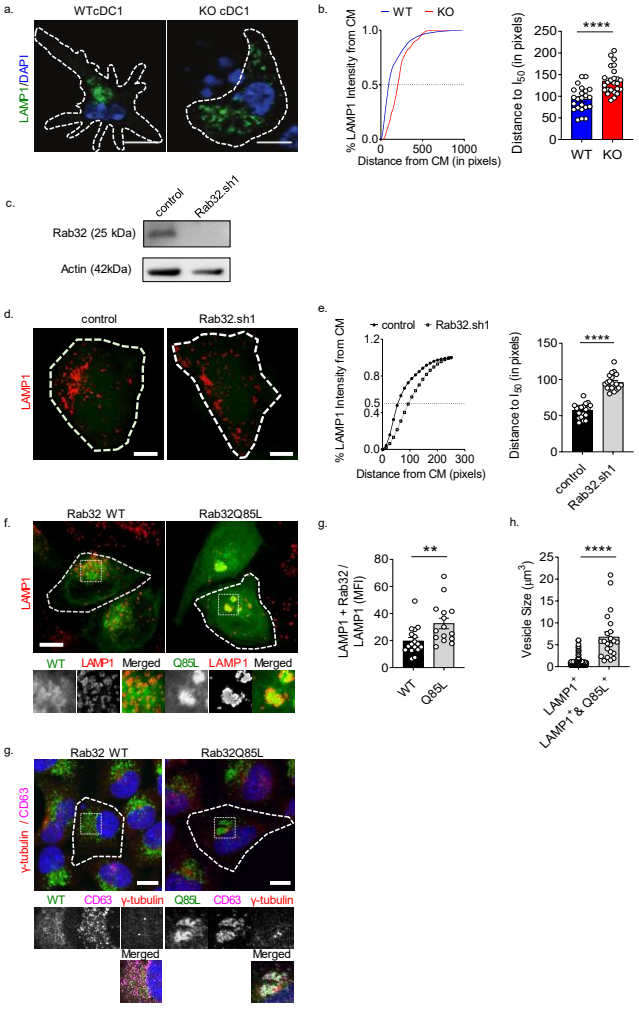

Figure 6. Rab32 promotes perinuclear clustering of lysosomes in cDC1s through its GTPase activity.

a. Confocal microscopy projections of $CD11c^+$ $XCR1^+$ cDC1s sorted from the spleen of Rab32 WT or KO mice, fixed and stained with anti-LAMP1 antibody and DAPI. Scale bar - 5um.

b. Lysosomal dispersion in cDC1s is depicted as distance of LAMP1 intensity for its centre of mass (CM) in the cell. The x-y graph depicts LAMP1 intensity distribution from its CM in the Rab32 WT and KO cDC1s represented. The bar graph depicts the distance from the lysosomal CM to reach 50% of the total LAMP1 intensity (I_{50}) in Rab32 WT or KO cDC1s. Data from one experiment in which at least 20 cells were quantified for each condition (****$p<0.0001$, Mann Whitney test).

c. MutuDCs (mouse splenic $CD8a^+$ DC derived cell line) were silenced for Rab32 using Rab32 siRNA. The absence of Rab32 in silenced MutuDCs (pLKO-Rab32.sh1) was verified by western blot.

d. Confocal microscopy projections of control (pLKO-control) and Rab32 silenced (pLKO-Rab32.sh1) MutuDCs, fixed and stained with anti-LAMP1 antibody. GFP is intrinsically expressed in the MutuDC line. Scale - 10um.

e. Lysosomal distribution in control (transduced with pLKO control) and Rab32.sh1 MutuDCs is quantified using the same method as in b. Data from one experiment in which at least 15 cells were quantified for each condition (****$p<0.0001$, Mann Whitney test).

f. Confocal microscopy projections of HeLa cells transfected with GFP-Rab32WT or the constitutively active Q85L mutant- GFP-Rab32Q85L, fixed and stained with anti-LAMP1 antibody. Scale bar – 10um.

g. Percentage of co-localisation of WT or Q85L Rab32 with LAMP1. Data from one experiment in which at least 15 cells expressing either GFP-Rab32WT or GFP-Rab32Q85L were analysed (**$p<0.01$, Mann Whitney test).

h. A size comparison of LAMP1^{+} (single positive) and Q85L^{+} LAMP1^{+} (double positive) lysosomal vesicles within the same cells (****p<0.0001 Mann Whitney test).

i. Confocal microscopy projections of HeLa cells overexpressing either Rab32WT or Rab32Q85L and co-stained with anti-CD63 and anti-γ-tubulin antibodies and DAPI. Scale bar - 10um.

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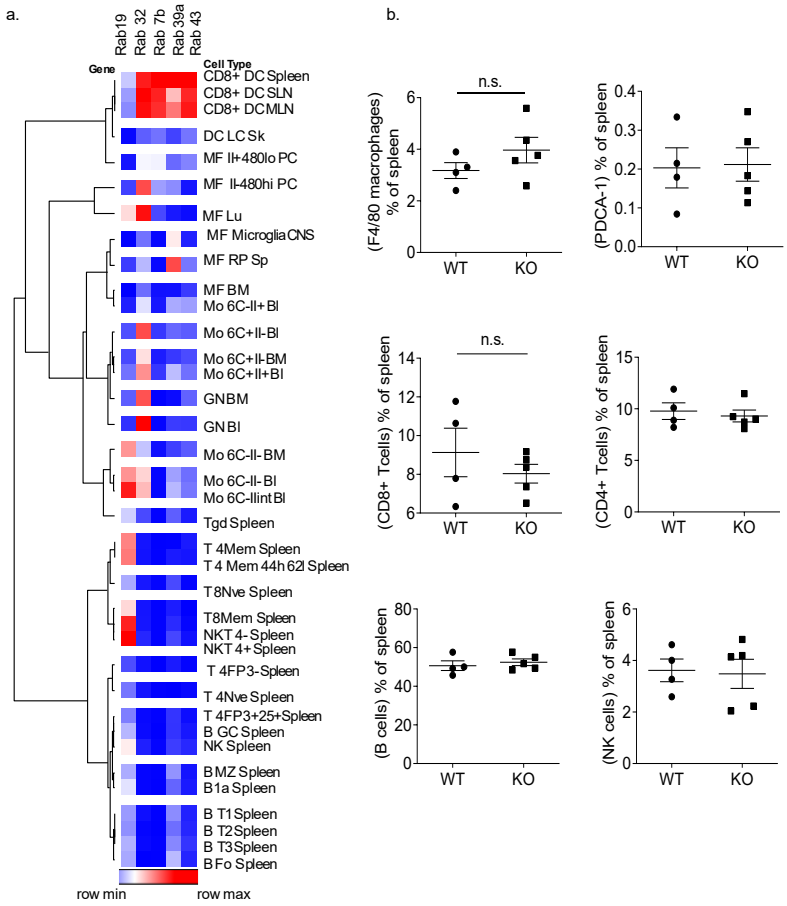
Supplementary information

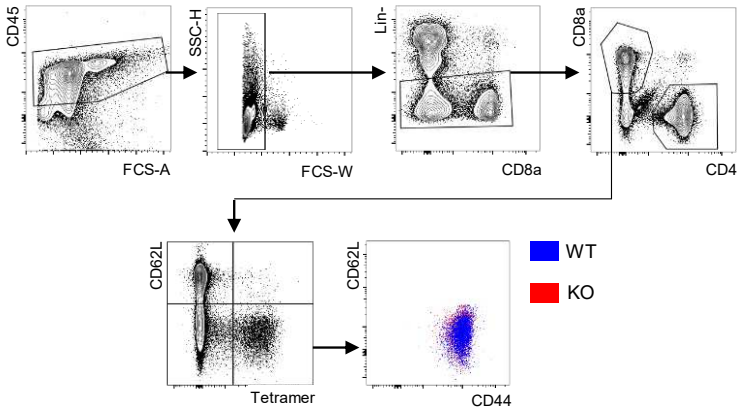
Supplementary figures



Supplementary figure 1.

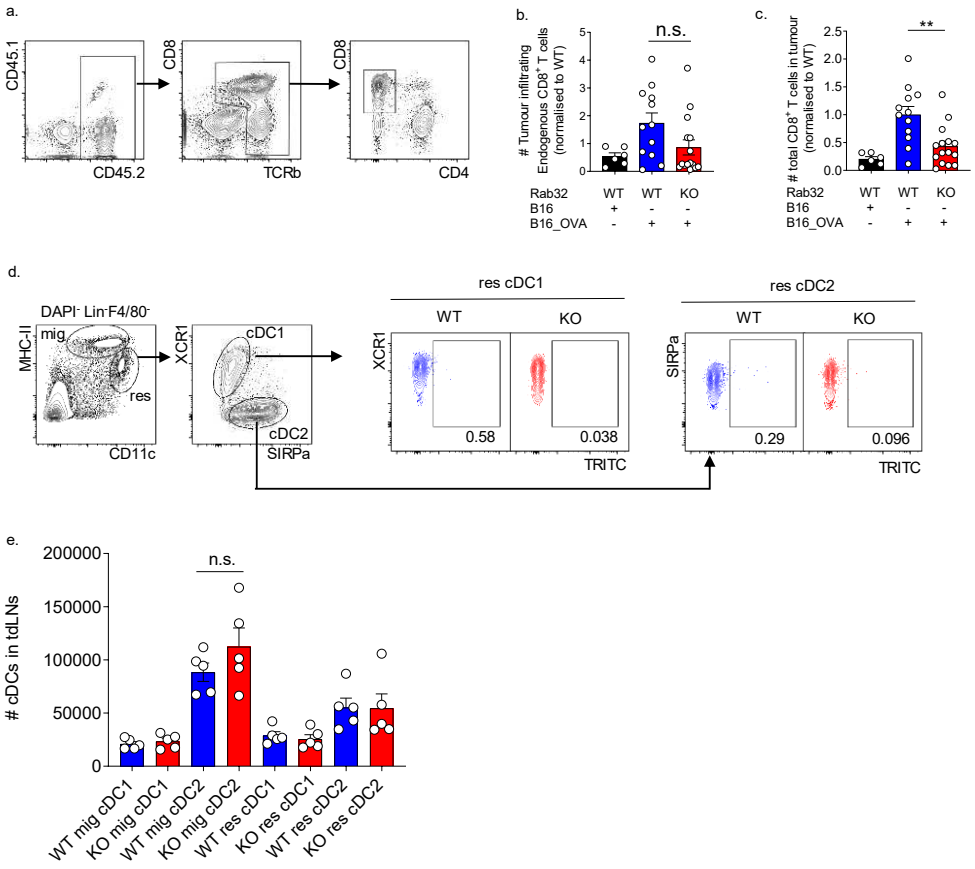
a. Rab32 is highly expressed in cDC1s, macrophages and neutrophils in comparison to T- and B-lymphocytes in mice (microarray data from Immgen database).

b. Percentages of various cell populations in the spleens of Rab32 WT and KO (tm1a) mice analysed by FACS. Data from 2 independent experiments (Mann-Whitney test).



Supplementary figure 2.

Gating strategy for the FACS analysis of endogenous $CD8^+$ T cells in spleens of mice injected with UV irradiated Kbm1_OVA+10ug polyI:C. The Lin^- consists of MHC-II, Gr-1, CD119, NK-1.1, TER-119, CD11b, B220, and F4/80. OVA specific ($Tetramer^+CD62L^{low}$) $CD8^+$ T cells in both Rab32 WT and KO mice had effector status ($CD62L^{low}CD44^{hi}$).



Supplementary figure 3.

a. Gating strategy for the analysis of tumour infiltrating OT-I cells. The Lin$^-$ consists of non-cDC markers including TER119, NK1.1, TCRb, GR-1 and CD19.

b. And c. Quantification of the absolute numbers of endogenous CD8$^+$ T cells and total CD8$^+$ T cells (endogenous + OT-I) in the tumour in the same experiments as in the Figure 5c.

d. Gating strategy for the identification of migratory cDCs (MHC-II^{hi} CD11c^{int}) and resident cDCs (MHC-II^{int} CD11c^{hi}) in the tdLNs of Rab32 WT and KO (cdel) mice. Within the resident cDC1 and cDC2s, TRITC$^+$ cells are absent, which verifies that there is no leakage of the dye from the tumour to the tdLNs.

e. Absolute numbers of migratory and resident cDC1 and cDC2 populations in the tdLNs B16-OVA tumour bearing mice on day 11 post injection.

Supplementary materials

Antibody used for the purification and sorting of primary cDC1s

Marker	Label	Antibody Clone	Dilution used	Reference
CD19 biotin	biotin	6D5	400	Biolegend 115503
B220	biotin	RA3-6B2	300	Biolegend 103204
PDCA1	biotin	927	500	Biolegend 127006
NK1.1	biotin	PK136	300	Biolegend 108703
TER119	biotin	TER-119	300	Biolegend 116203
TCRb	biotin	H57-597	400	Biolegend 109203
GR1	biotin	RB6-8C5	800	Biolegend 108403
F4/80	biotin	BM8	200	Biolegend 123106
Ly6G	biotin	1A8	400	Biolegend 127604
XCR1	FITC	ZET	200	Biolegend 148210
CD11c	PE	N418	200	Biolegend 117308

Antibodies used for the assessment of endogenous $CD8^+$ T cells by flow cytometry

Marker	Label	Antibody Clone	Dilution used	Reference
CD4	BV510	GK1.5	100	BD 743155
MHC-II	biotin	M5/114.15.2	175	Biolegend 107604
Gr-1	biotin	RB6-8C5	175	Biolegend 108403
CD19	biotin	6D5	125	Biolegend 115503

NK-1.1	biotin	PK136	100	Biolegend 108703
TER-119	biotin	TER-119	75	Biolegend 116203
CD11b	biotin	M1/70	200	Biolegend 101203
B220	biotin	RA3-6B2	125	Biolegend 103204
F4/80	biotin	BM8	100	Biolegend 123106
CD62L	APC	MEL-14	100	Biolegend 104412
CD8a	AF700	KT 15	100	Biorad MCA609A700
CD45	APC/Cy7	30-F11	200	Biolegend 103115
CD44	PE/Cy7	IM7	100	Biolegend 103029

Antibodies used for flow cytometry in OT-I adoptive transfer experiment

Marker	Label	Antibody Clone	Dilution used	Reference
CD4	BV786	GK1.5	400	BD Biosciences 563331
CD45.1	FITC	A20	150	Biolegend 110705
CD45.2	PerCP-Cy5.5	104	200	Biolegend 109827
CD62L	PE	MEL 14	200	Biolegend 104407
CD44	PE-Cy7	IM7	700	Biolegend 103029
TCRb	APC	H57-597	200	Biolegend 109211
CD8	APC-Cy7	53-6.7	500	Biolegend 104512
Fc block	purified	2.4G2	500	BD 553141

Antibodies used for flow cytometry in Tumour OT-I experiment

Marker	Label	Antibody Clone	Dilution used	Reference
CD8a	BV510	53-6.7	500	Biolegend 100752
CD4	BV785	GK1.5	400	BD Biosciences 563331
CD45.1	PE	A20	300	Biolegend 110708
TCRb	PE/Cy7	H57-597	200	Biolegend 109222
CD45.2	AF700	104	300	Biolegend 109822
Fc Block	purified	2.4G2	100	BD 553141

Antibodies used for analysing DCs in spleens or LNs by flow cytometry

Marker	Label	Antibody Clone	Dilution used	Reference
MHC-II	BV510	M5/114.15.2	600	Biolegend 107635
Gr-1	Biotin	RB6-8C5	700	Biolegend 108403
CD19	Biotin	6D5	300	Biolegend 115503
NK-1.1	Biotin	PK136	200	Biolegend 108703
TER-119	Biotin	TER-119	200	Biolegend 116203
TCR β chain	Biotin	H57-597	250	Biolegend 109203
SIRPa	FITC	P84	200	Biolegend 144006
XCR1	PerCP-Cy5.5	ZET	200	Biolegend 148207
F4/80	PE/Cy7	BM8	250	Biolegend 123113
CD11c	APC/Cy7	N418	300	Biolegend 117323
Fc block	purified	2.4G2	500	BD 553141
CD40	PE-CF594	3/23	500	Biolegend 124629

CD80	BV421	16-10A1	500	Biolegend 104725
MHC-I	APC	AF6-88.5.5.3	500	Thermo 17-5958-82
MHC-I Isotype	APC	eBM2a	500	Thermo 17-4724-81

Antibodies used for confocal microscopy of splenic cDC1s and MutuDCs

Type	Marker	Label	Host	Clone	Dilution	Reference
Primary	Rab32	Purified	Mouse	B-4	100	Santa Cruz 390178
	Calnexin	Purified	Rabbit	polyclonal	500	Sigma C4731
	TGN38	Purified	Rabbit	polyclonal	500	Abcam AB16059
	LAMP1	Purified	Rabbit	polyclonal	500	Sigma L1418
	$H2^{kb}$ P-8	Purified	Rabbit	P-8	300	kind gift from Dr H Ploegh, Harvard University, US
	COX5B	Purified	Rabbit	polyclonal	100	Proteintech 11418-2-AP
	Giantin	Purified	Rabbit	polyclonal	100	Biolegend 924301
Secondary	Anti-mouse IgG (H+L)	AF 594	Donkey	polyclonal	100	Thermofisher A21203
	Anti-rabbit IgG (H+L)	AF 488	Donkey	polyclonal	100	Thermofisher A21206

Antibodies used for confocal microscopy of HeLa cells

Type	Marker	Label	Host	Clone	Dilution	Reference
Primary	γ-tubulin	Purified	Rabbit	polyclonal	100	Abcam ab11317
	LAMP1	Purified	Mouse	H4A3	100	BD Bioscience 555798
	CD63	Purified	Mouse	H5C6	100	BD Bioscience 556019
Secondary	Anti-mouse IgG (H+L)	Cy-3	Goat	polyclonal	100	Jackson ImmunoResearch 115167003
	Anti-rabbit IgG (H+L)	APC	Goat	polyclonal	100	Jackson ImmunoResearch 111007003

DISCUSSION

cDC1 specific Rab GTPases-

In this report, the expression of various Rab GTPases in lymphoid resident DC populations in mice has been evaluated using gene microarray data from the Immgen database. According to this data, Rab32, along with Rab43, Rab39a, Rab7a, Rab7b, Rab11a, Rab10 and Rab19 were identified to be highly expressed in lymphoid tissue-resident $CD8a^+$ cDC1 populations compared to resident $CD4^+$ cDC2s and pDCs, as well as various other myeloid and lymphoid populations (Fig 1a and Fig S1b). Rab43 and Rab39a have been previously examined for their role in cross-presentation by cDC1s. Rab43 is presently the only Rab protein shown to promote $CD8^+$ T cell cross-priming by specifically promoting the process of cross-presentation by cDC1s[74]. Its mechanism of action in facilitating cross-presentation remains to be determined. Rab39a was also shown to promote $CD8^+$ T cell cross-priming against cellular antigens *in-vivo,* but *ex-vivo,* the deficiency of Rab39a affected cross-presentation of particulate antigens by cDC2s rather than cDC1s[75]. Mechanistically, Rab39a promotes cross-presentation in cDC2s by facilitating the conversion of phagosomes into MHC-I antigen-presenting compartment.

The role of Rab11a and Rab10 has been examined in cross-presentation in *in-vitro* generated BMDCs and DC2.4 cell line, respectively. Rab11a maintains the intracellular pool of surface recycling MHC-I molecules, which are transported to the phagosome for antigen loading and cross-presentation upon phagosomal TLR signalling[292]. The role of Rab11a in cDCs and in $CD8^+$ T cell cross-priming *in-vivo* needs further investigation. Rab10 was identified to promote cross-presentation by DC2.4 in an shRNA-based functional screening [294]. Its relevance in cross-presentation by cDC1s and in CD8+ T cell priming *in vivo*, as well as its mechanism of action remain to be determined.

Rab7, which has two isoforms in mammals - Rab7a and Rab7b, and Rab19 have not been studied in the context of cross-presentation. However, the well-established function of Rab7 in the control of lysosomal movement and regulation of phagosome-lysosome fusion events within cells[457] makes them interesting candidates for cross-presentation study in cDC1s.

One limitation of using the Immgen microarray data was that the data for non-lymphoid tissue-resident cDC1 populations such as $CD103^+$ $Langerin^+$ cells in the skin and $CD103^+$ $CD11b^-$ $CD24^+$ cells in the lungs was not available in this dataset, and hence could not be evaluated. These cDC1 populations play a major role in the internalising and transporting cell-associated antigens from necrotic cells, pathogen-infected or tumour cells from the peripheral organs to the respective

draining lymph nodes for their cross-presentation to $CD8^+$ T cells[10,57,301,458]. Therefore, it would be interesting to evaluate the expression of Rab32 in these populations of cells. This could be done by performing a FACS sorting of these cell populations from WT mice, followed by a qPCR analysis of Rab32 expression.

The functional specialisation of murine cDC1s seems to be conserved in humans as well since the lymphoid tissue-resident cDC1s ($CD141^+$), as well as dermal cDC1s ($CD1a^+$ $CD1c^{low}$), are also highly efficient at the internalization and cross-presentation of cell-associated antigens[53,80,81,83,84]. Therefore, it would be interesting to analyse the expression of Rab32 messenger RNA using gene expression data in human cDC subsets.

Role of Rab32 in $CD8^+$ T cell responses and cross-presentation-

We have demonstrated that Rab32 promotes the proliferation of endogenous effector $CD8^+$ T cells in response to cross-presentation of cellular antigens *in-vivo* (Fig 2a-d). For the same, we used UV irradiated Kbm1_OVA cells (mouse embryonic fibroblasts) as the cellular antigen model. These cells possess a mutation in H2kb, which renders them incapable to directly present OVA to $CD8^+$ T cells[72]. This ensures that $CD8^+$ T cell priming observed *in-vivo* is due to the cross-presentation of OVA by DCs. An alternative for the cellular antigen model in place for Kbm1_OVA would be to use OVA loaded splenocytes from MHC-I triple knockout mice ($Kb^{-/-}Db^{-/-}\beta2m^{-/-}$ TKO)[459]. These cells have a complete absence of functional H-2 and cannot thus directly present OVA to $CD8^+$ T cells. Nevertheless, the Kbm1 cells are probably more suitable as antigen source in *in vivo* cross-presentation assays, since *in vivo* elimination of MHC-I deficient cells by Natural Killer cells of the host mice can interfere with the result of cross-presentation assay.

Given the well-established specialisation of cDC1s in $CD8^+$ T cell priming as well as the high expression of Rab32 we observed in these cells, it was likely that Rab32 affects $CD8^+$ T cell priming in a cDC1 intrinsic manner. But at the same time, the micro-array data used in our report suggested that Rab32 has a relatively low express in $CD8^+$ T cells (Fig S1a). Moreover, we used a Rab32 constitutive KO mouse model, in which the $CD8^+$ T cells are deficient for Rab32. Therefore, we decided to rule out any $CD8^+$ T cell-intrinsic effects of Rab32 on their $CD8^+$ T cell proliferation. To this aim, we adoptively transferred naïve WT OT-I cells, which possess TCRs specific for recognising OVA in the context of H2kb, into the WT or Rab32 KO mice and evaluated their proliferation following immunisation with Kbm1_OVA cells. Adoptively transferred OT-I cells

showed less proliferation and activation in Rab32KO mice compared to the WT mice, which showed that Rab32 affected CD8 T cell proliferation in response to cross-presentation by cDC1s (Fig 2 e-g). Additionally, to further consolidate the role of Rab32 in cross-priming by cDC1s as presented in this report, we are momentarily working to create $XCR1^+$ cDC1-conditional Rab32 KO mice in the lab, in which Rab32 will be deleted only in cDC1.

We further show that *ex-vivo* cross-presentation is not affected by the deficiency of Rab32 (Fig 3c). This could be due to the existence of compensatory mechanisms in cDC1s which can deal with the absence of specific proteins. To this point, the results presented with Rab32 KO mice in this thesis (Fig 2), along with previously published reports with Rab43 KO[74] and Rab39a KO[75] mice, show a significant, but partial reduction in the cross-priming of $CD8^+$ T cells in these mice. On the other hand, in BATF3 KO mice[9] which have a complete absence of cDC1, or in WDFY4 KO mice[76] in which cross-presentation is completely inhibited by a presently unexplained mechanism, cross-priming is severely inhibited.

Moreover, functional redundancies are known to exist in the Rab family of proteins, which consists of more than 60 members in mammals. As previously mentioned, Rab38 is a protein closely related to Rab32 (75% sequence similarity in mice, 66% in humans). Both these proteins are well expressed in the similar cell types, and share common regulatory proteins are between them, including their GTP/GDP exchange factor BLOC3[425], as well as their GTPase activating protein RUTBC1[430]. A well-described function of Rab32/38 is their role in biogenesis of melanosomes, which are the organelles responsible for the production and storage of the pigment melanin in melanocytes[423,426,427]. Rab32/38 work together with BLOC proteins and sorting adaptors AP1 and AP3 to promote the trafficking of melanogenic enzymes from the Golgi compartment via early endosomes to the developing melanosomes[423]. The deficiency of either Rab38 or Rab32 produces a partial hypopigmentation phenotype, whereas a simultaneous deficiency of both the GTPases produces a more severe phenotype[426,427]. Since Rab38 is also decently expressed in cDC1s (Fig S1a), it may be hypothesised that Rab38 can functionally compensate for the role of Rab32 in cross-presentation in these cells. A simple way to test this hypothesis would be to create Ra32/38 single KO as well as double KO MutuDCs using the CRISPR/Cas9 system (which is well established in the lab) and to perform *in-vitro* cross-presentation assays with these cells. In case of the observation that the double KO cells have a more severe phenotype, this hypothesis would be validated.

The *ex-vivo* cross-presentation assays with sorted cDC1s described in this report were performed using live Kbm1_OVA cells. The use of a live cell line presented some challenges in the assay. When co-cultured with primary splenic cDC1s and OT-I cells, the live Kbm1_OVA grow and expand in the wells after 24h of culture. This can disrupt the pre-decided ratios amongst cDC1, OT-I and Kbm1_OVA cells in the culture. Moreover, the expanding Kbm1_OVA cells may take up the medium nutrient at a faster rate, which can be harmful to the viability of primary cDC1s and OT-I cells. Nutrient deprivation in the medium may also induce transcriptional changes in cDC1s, which can interfere with the functional responses of these cells. Thus, in our experiments, the analysis of cross-presentation was performed using only the supernatant after 24h of culture, at which time the Kmb1-OVA cells had not expanded in the culture. Perhaps an alternative method of performing this experiment would be to use UV irradiated, necrotic Kbm1_OVA cells which would not expand in the culture. Another possibility is to use heat-killed *Listeria monocytogenes* expressing OVA (HKLM-OVA) as the antigen model for *ex-vivo* cross-presentation assay with cDC1s, similar to what has been described in the Rab43 report[74]. In fact, DC1s are the primary site of entry and proliferation of *L. monocytogenes* in the spleen[448,449]. HKLM-OVA are efficiently internalised by cDC1s *ex-vivo*, and the OVA is processed and cross-presented to OT-I cells. At the same time, the use of a bacterial antigen model would activate other innate immune receptors than Kbm1 mammalian cells and will induce a different signalling in DCs. For example, the presence of PAMPs on the bacteria stimulates TLR signals in DCs. Thus, cross-presentation of bacterial and cell-associated antigens by cDC1s may be differently mediated. Since the focus of our study was cross-presentation of cell-associated antigens, perhaps the use of cellular antigen is more relevant.

Role of Rab32 in anti-tumour immunity-

In the cancer-immunity cycle, the tumour migratory cDCs pick up antigens from the dying tumour cells and transport them to the tdLNs. Within the T cell zones of the tdLNs, migratory cDCs cross-prime $CD8^+$ T cells against tumour antigens, which then egress from the tdLNs and infiltrate the tumour to perform anti-tumour cytotoxic functions[460]. While migratory cDCs can directly cross-prime $CD8^+$ T cells, some evidence also suggests that tumour antigens may be transferred from migratory to resident cDCs, allowing resident cDCs to also cross-prime $CD8^+$ T cells[458]. It has been shown that migratory cDC1s are highly efficient at transporting tumour antigens to the tdLNs,

and hence they are the primary DC population which cross-primes $CD8^+$ T cells[458,461]. At the same time, migratory cDC2s also possess this ability, albeit lower compared to cDC1s[458]. On these lines, the tumour infiltration of $CD8^+$ T cells is significantly reduced in cDC1 deficient BATF3 KO mice[9] as well as in cross-presentation deficient WDFY4 KO mice[76].

In this report, we have used B16_OVA melanoma cell line as the tumour model, which express OVA as a neo-tumour antigen in the B16 F10 cells. This model was used because these B16_OVA grow progressively in immune-competent mice and are easy to evaluate due to their subcutaneous localisation. We have provided evidence that Rab32 KO mice have a significant decrease in the infiltration of adoptively transferred OT-I in the B16_OVA tumours compared to the WT mice (Fig 5c). Moreover, we also observed a reduction in tumour infiltration of endogenous $CD8^+$ T cell in the Rab32 KO mice, although this difference is not significant compared to the WT mice (Fig S3b). This observation suggests that Rab32 promotes tumour infiltration in response to cross-presentation of tumour antigens. It would be interesting to evaluate if Rab32 affects the tumour infiltration by antigen-specific endogenous $CD8^+$ T cells. This could be tested using the SIINFEKL-H2kb-Tetramer, which recognises the OVA-primed $CD8^+$ T cells.

Our results further demonstrate that the deficiency of Rab32 reduces the presence of tumour migratory cDCs within the tdLNs (Fig 5d and e). For this experiment, we have used the 'skin painting' technique, in which the mice are shaved, and the tumour is painted with a solution of the fluorescent dye Tetramethylrhodamine (TRITC) in acetone. The TRITC solution is readily absorbed through the skin, and it marks the tumour infiltrating leukocytes, including the migratory cDCs. By tracking the presences of $TRITC^+$ migratory cDCs in the tdLNs in Rab32 WT and KO mice, we have assessed the role of Rab32 in DC migration. Based on our results, we could conclude that by controlling the migration of tumour DCs towards tdLN, Rab32 contributes to anti-tumour cellular immunity. One major characteristic of migratory cDCs is the dependence on the chemokine receptor CCR7 for this migration to the LNs. It would be interesting to test whether Rab32 affects the expression, the intracellular trafficking or the signaling of CCR7 on these cells.

In continuation of our results on the role of Rab32 in cDC migration, it would be further interesting to directly validate the role of Rab32 in tumour antigen transport by cDCs to the tdLNs. To this aim, we could inject Rab32 WT or KO mice with B78ChOVA melanoma cells, which are a modification of B16_OVA cells that express the fluorescent mCherry protein[461]. This would allow

the tracking of tumour antigens without the need for external TRITC painting. Another alternative would be to use the B16-Zsgreen cells, which express the highly stable fluorophore ZsGreen[461].
Another important aspect that remains to be investigated is whether Rab32 plays a role in the presence of DCs within the tumour. It could be possible that Rab32 decreases the presence of DCs within the tumour, which results in lower migration to the tdLNs, resulting in the reduced cross-priming of $CD8^+$ T cells. Although our results suggest that Rab32 does not affect the differentiation of splenic cDCs, we have not tested its role differentiation of peripheral cDCs populations, including dermal $CD103^+$ $Langrin^+$ cDC1s and $CD103^-$ $CD11b^+$ cDC2s. Evaluation of these cell populations in Rab32 WT and KO mice would provide a complete picture of the role of Rab32 in driving anti-tumour immunity.

Regulation of DC migration by Rab32 through optimization of intracellular lysosomal positioning-

Although lysosomes have classically been perceived as stationary, degradative organelles within cells, they are now emerging to be highly dynamic and multifunctional. Lysosomal positioning has been linked with a variety of cellular processes, including antigen presentation, autophagy and cell migration[457]. A major determinant of lysosomal function is the transcription factor EB (TFEB), a master regulator of lysosomal genes[462]. TFEB colocalises with the master growth regulator - the mechanistic target of rapamycin complex 1 (mTORC1) on the surface of lysosomes[463–465]. During this colocalisation at the lysosome, mTOR phosphorylates TFEB and keeps it in an inactive state. Upon perinuclear positioning of lysosomes (induced by starvation signals or lysosomal stress), mTORC1 activity is inhibited, which allow the dephosphorylation of TFEB[466,467]. Dephosphorylated TFEB then translocates to the nucleus, thus allowing expression of lysosomal proteins. A seminal study by the team of Lennon-Duménil has recently reported the role of TFEB activation in the migration of DCs[468]. TFEB activity promotes the lysosomal signalling through $Ca2^+$ channel TRPML1, which results in the re-modelling of the actin cytoskeleton to support direction movement of DCs. This mechanism is required for the migration of maturing migratory DCs from peripheral tissues to the LNs since the absence of this pathway in immature DCs or maturing TRPML1 KO DCs stops their fast and directional migration to the LNs[468].
In light of this literature, our results on the role of Rab32 in lysosomal positioning (Fig 6) might be relevant for the migration of cDCs from the tumour. To further investigate this possibility, we could compare the ratio of phosphorylated and dephosphorylated TFEB between the Rab32 WT

and KO cells, including migratory cDC1s and cDC2s sorted from tdLNs. Another method to quantify TFEB activity would be to compare the nuclear *versus* cytosolic presence of TFEB between Rab32 WT and KO cells.

Lastly, it would be interesting to examine the interacting protein partners of Rab32 in cDC1s using unbiased proteomics analysis. This can be realised using commercial anti-Rab32 antibodies raised in mice and a control isotype antibody as control for the background of the assay. Alternatively, we can express via lentiviruses in Mutu DCs the mCherry-Rab32 fusion protein that I produced and that can be immunoprecipitated with anti-mCherry alpaca antibodies (Chromotek). The identification of Rab32 interacting proteins could provide further clues into the intracellular mechanisms by which Rab32 facilitates the proliferation of effector $CD8^+$ T cells, whether it be by directly affecting cross-presentation *in-vivo* or the migration of cDCs to the tdLNs.

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Synthèse de thèse

Les cellules dendritiques conventionnelles (cDCs) examinent constamment les tissus lymphoïdes et périphériques de l'organisme à la recherche de cellules exprimant des antigènes, telles que les cellules nécrotiques, celles infectées par des agents pathogènes, ou les cellules tumorales. Lors de ces rencontres, les cDCs internalisent et apprêtent les antigènes protéiques associés aux cellules, pour les présenter ensuite sous forme de peptides sur le CMH-I (Complexe Majeur de Histocompatibilité de classe I) aux cellules T $CD8^+$ naïves. Ce processus est appelé présentation-croisée. Lorsqu'elle s'accompagne de signaux de co-stimulation et de la sécrétion de cytokines, la présentation-croisée entraîne l'activation et la prolifération des cellules T $CD8^+$ spécifiques de l'antigène et la génération de réponses cellulaires T effectrices contre les cellules exprimant l'antigène.

Chez la souris comme chez l'homme, les cDCs se divisent en deux populations : les cDC1 ($XCR1^+$) et les cDC2 ($SIRPa^+$). Chez la souris, les cDC1 sont fonctionnellement spécialisées dans l'activation via la présentation-croisée des cellules T $CD8^+$ contre des antigènes cellulaires. Ainsi, les cDC1 sont essentielles pour la mise en place de réponses immunitaires adaptatives antivirales et anti-tumorales *in-vivo*. Cette spécialisation semble être conservée chez l'homme puisque les cDC1 dérivées du sang humain, ainsi que les cDC1 dermiques, peuvent présenter d'une façon croisée des antigènes cellulaires *in-vitro*.

Plusieurs propriétés des cDC1 contribuent à cette spécialisation fonctionnelle. Les cDC1 résidentes lymphoïdes semblent être plus efficaces que les cDC2 pour internaliser les antigènes associés aux cellules nécrotiques *in-vivo*. De même, les cDC1 migratoires dans les poumons sont uniques dans leur capacité à capter les antigènes associés aux cellules nécrotiques, ainsi que les antigènes viraux à partir des cellules infectées, et à les transporter ensuite vers les ganglions lymphatiques drainants pour activer les cellules T $CD8^+$ *in-vivo*. Les cDC1 sont également uniques dans leur expression du récepteur CLEC9A qui reconnait les cellules nécrotiques et cible les antigènes internalisés vers la machinerie de présentation-croisée. Les cDC1 possèdent également une machinerie de présentation-croisée spécialisée, qui favorise la conservation des antigènes internalisés dans les phagosomes, permettant ainsi un apprêtement optimal des antigènes en

peptides compatibles avec le CMH-I. En outre, les cDC1 présentent une forte expression du complexe de chargement peptidique du CMH-I, qui contribue à leurs capacités supérieures de présentation croisée.

Alors que les cDC1 sont les principales cellules qui effectuent la présentation-croisée dans des contextes physiologiques, des nombreuses études portant sur les mécanismes cellulaires de la présentation-croisée ont été réalisées dans des cellules dérivées de la moelle osseuse murine (BMDC- « bone marrow derived dendritic cells »), générées *in-vitro* à partir de cultures de moelle osseuse en présence du GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor). Les BMDCs sont une population hétérogène ressemblant à la fois aux DCs et aux macrophages, qui utilise des mécanismes de présentation croisée différents des cDC1s. A cause de ces differences, aujourd'hui, de plus en plus des études se concentrent sur les mécanismes de présentation croisée utilisés spécifiquement par les cDC1. Par conséquent, plusieurs protéines spécifiques des cDC1 ont été découvertes comme impliquées dans l'activation de lymphocytes T $CD8^+$ via la présentation-croisée *in-vivo*, comme par exemple, Rab43, WDFY4 et Rab39a.

Les protéines Rab sont de petites GTPases qui sont bien conservées chez les mammifères. Elles constituent des candidats intéressants pour les études de la présentation-croisée dans les cDC1. Elles existent dans la cellule sous forme active liée au GTP, ou inactive liée au GDP, et fonctionnent comme des régulateurs clé du trafic vésiculaire intracellulaire. Ainsi, il a été démontré que les GTPases de type Rab facilitent la présentation-croisée en régulant le trafic intracellulaire impliqué dans l'apprêtement de l'antigène et le chargement des peptides sur le MHC-I. La GTPase Rab32 joue un rôle essentiel dans la restriction et l'élimination des pathogènes bactériens intracellulaires tels que *Listeria monocytogenes* et *Salmonella* dans les DCs et respectivement dans les macrophages. De plus, dans un modèle de colite murine, il a été démontré que la déficience de Rab32 exclusivement dans les cellules $CD11c^+$ détermine une progression accrue de la colite et une invasion bactérienne du côlon. L'analyse intracellulaire des lignées cellulaires montre que Rab32 colocalise avec les membranes des ER (réticulum endoplasmique) associées aux mitochondries (MAM), qui sont marquées par la calnexine, un chaperon du ER. De plus, Rab32 et sa protéine régulatrice BLOC-3 (HPS1-4) jouent un rôle

essentiel dans la biogenèse des organites liés aux lysosomes (LRO). Des perturbations de cette machinerie ont été liées au syndrome de Hermansky-Pudlak (HPS) chez l'homme, qui se caractérise par un albinisme oculo-cutané, une tendance aux saignements et des immunodéficiences.

L'objectif de cette thèse a été d'évaluer de manière critique le rôle de la GTPase Rab32 dans l'activation des cellules T $CD8^+$ naïves contre des antigènes cellulaires qui sont cross-présentés par les cDC1.

Nous avons identifié les Rab GTPases spécifiques aux cDC1 en évaluant le profil d'expression des protéines Rab dans différentes populations de DCs de souris. Par rapport aux cDC2 ou pDC résidentes, Rab32 était l'une des GTPases les plus fortement exprimées dans les populations de cDC1 résidentes dans la rate, le ganglion mésentérique (MLN) et les ganglions de drainage de la peau (SLN). Nous avons étudié *in-vivo* la fonction de cette protéine en utilisant des modèles de souris Rab32 KO (« knock-out ») constitutives.

Nous avons d'abord examiné l'effet de Rab32 dans la différenciation des sous-types de DC chez les souris, et n'avons trouvé aucune différence dans le développement de diverses populations de DC entre les souris Rab32 KO et WT (« wild-type »). Ensuite, nous avons exploré si Rab32 contribue à la fonction des cDC1 dans l'induction de réponses des cellules T $CD8^+$ contre les antigènes associés aux cellules. Nos résultats montrent qu'en effet, Rab32 favorise la génération de cellules T $CD8^+$ effectrices contre les antigènes associés aux cellules *in-vivo*. Compte tenu du rôle essentiel des cDC1 dans l'activation des cellules T $CD8^+$ naïves et de la forte expression de Rab32 dans les cDC1, il est probable que le défaut d'activation des cellules T $CD8^+$ naïves que nous avons observé dans les souris Rab32 KO est due à l'absence de Rab32 dans les cDC1 et non dans les lymphocytes T. Pour vérifier cette hypothèse, nous avons effectué des expériences de transfert adoptif des cellules T $CD8^+$ WT, spécifiques de l'antigène ovalbumine dans les souris Rab32 KO. Ces expériences ont confirmé que le défaut d'activation de cellules T $CD8^+$ naïves n'était pas du à l'absence de Rab32 dans les lymphocytes T, mais plutôt a son absence dans les cDC1.

Il est intéressant de noter que Rab32 a favorisé la prolifération des cellules T $CD8^+$ effectrices spécifiques à l'antigène sans affecter directement la présentation-croisée des antigènes cellulaires par les cDC1. Nous avons également examiné l'effet de la déficience de Rab32 sur d'autres aspects de l'activation des cellules T $CD8^+$ par les cDC1, tels que la capacité de cDC1s de présenter un peptide antigénique directement, sans besoin d'apprêtement antigénique ou leur capacité de maturation et co-stimulation. Nos résultats montrent que Rab32 n'affecte pas ces phénomènes.

L'infiltration des tumeurs par les cellules T $CD8^+$ est très faible en absence de cDC1, ainsi qu'en absence de la présentation-croisée des antigènes par les cDC1. Par conséquent, nous avons étudié si Rab32 était essentiel pour induire la prolifération des cellules T $CD8^+$ anti-tumorales dans un modèle de tumeur chez la souris. Nous avons constaté qu'en absence de Rab32 l'infiltration des tumeurs de type mélanome (B16) par des cellules T $CD8^+$ spécifiques de l'antigène est fortement réduite.

Dans les modèles de mélanome de souris, mais aussi dans les mélanomes humains, la sous-population de cDC1 migratoires possède une capacité robuste de transporter des antigènes tumoraux vers les ganglions lymphatiques drainants (tdLN) pour activer les cellules T $CD8^+$ naïves, qui, ensuite, vont infiltrer la tumeur. Les cDC2 migratoires possèdent également cette capacité, mais dans une moindre mesure, par rapport aux cDC1 migratoires. Comme Rab32 était nécessaire pour l'infiltration de la tumeur par les cellules OT-I spécifiques à l'antigène, nous avons décidé d'évaluer si Rab32 était aussi nécessaire pour la présence de cDC migratoires portant des antigènes tumoraux dans les tdLN. En utilisant un "test de peinture de la tumeur", nous avons découvert qu'en absence de Rab32 le nombre de cDC migratoires contenant des antigènes tumoraux était réduit dans les tdLN. Sur la base de ces observations, nous avons conclu que Rab32 participe à la génération de l'immunité anti-tumorale basée sur les lymphocytes T $CD8^+$ en favorisant la présence de cDC migratoires portant les antigènes tumoraux dans les tdLN.

Nos résultats suggèrent en outre que Rab32 régule la migration des cDC en contrôlant la distribution intracellulaire des lysosomes. Nous avons montré que les lysosomes des cDC1 forment un groupe périnucléaire, alors qu'en absence de Rab32, ils sont redistribués vers la

périphérie de la cellule. De plus, la surexpression de la forme constitutivement active de Rab32 dans les cellules HeLa a induit une agrégation périnucléaire de lysosomes, similaire à celle qui a été observée dans la cDC1 de souris WT. En conclusion, Rab32 favorise l'agrégation périnucléaire des lysosomes dans les cellules. Ces résultats pourraient être pertinents pour la migration des cDCs de la tumeur vers le ganglion lymphatique drainant, puisque le positionnement lysosomal est lié à plusieurs fonctions cellulaires, y compris la migration des cellules. Un facteur clé pour la fonction lysosomale est le facteur de transcription-EB (TFEB), qui est le régulateur principal des gènes lysosomaux. À l'état déphosphorylé (inactif), le TFEB est présent à la surface du lysosome. Lors du positionnement périnucléaire des lysosomes (qui est induit en absence de nutriments ou par le stress lysosomal), le TFEB est déphosphorylé et transporté dans le noyau, permettant ainsi l'expression des protéines lysosomales. Une étude fondatrice a récemment rapporté le rôle de l'activation du TFEB dans la migration des DCs. L'activité du TFEB favorise la signalisation lysosomale par le canal $Ca2^{+}$ TRPML1, ce qui entraîne un remodelage du cytosquelette de l'actine pour soutenir le mouvement directionnel des DCs. Ce mécanisme est nécessaire pour la migration des DCs en cours de maturation à partir des tissus périphériques vers les ganglions lymphatiques. L'absence de cette voie dans les DC déficientes pour TRPML1 arrête leur migration rapide et directionnelle vers les ganglions lymphatiques, une fois qu'elles sont matures et activées. D'autres études sont nécessaires pour établir si Rab32, par son effet sur le positionnement lysosomal, peut réguler la signalisation lysosomale dans les DC et, en conséquence, la migration des DC de la tumeur vers les ganglions lymphatiques.

Figure 1.1 **Diversity of dendritic cell subsets**, page n. 13, Introduction CHAPTER 1, section 1.2.2.3. – Functions. Reference – Figure 1 from Murphy, T. L. et al. Transcriptional Control of Dendritic Cell Development. Annu Rev Immunol 34, 93–119 (2016).

Figure 1.2 **Dendritic cells develop as an independent cell lineage,** page n. 14, Introduction CHAPTER 1, section 1.3. – The organisation of the development pathway: cellular intermediates from HSCs to cDCs. Reference – Figure 1 from Guermonprez, P., Gerber-Ferder, Y., Vaivode, K., Bourdely, P. & Helft, J. Chapter One - Origin and development of classical dendritic cells. in International Review of Cell and Molecular Biology (eds. Lhuillier, C. & Galluzzi, L.) vol. 349 1–54 (Academic Press, 2019)

Figure 2.2 **Proposed mechanisms for antigen export from phagosome to cytosol,** page n. 34, Introduction CHAPTER 2, section 2.2.3. – Antigen export to the cytosol. Reference – Figure 1 from Gros, M. & Amigorena, S. Regulation of Antigen Export to the Cytosol During Cross-Presentation. Front Immunol 10, (2019)

Figure 3.2 **Schematic diagram of the universal switch mechanism,** page n. 52, Introduction CHAPTER 3, section 3.1.1. – Structure of Rab GTPases. Reference – Figure 3 from Vetter, I. R. & Wittinghofer, A. The Guanine Nucleotide-Binding Switch in Three Dimensions. Science 294, 1299–1304 (2001).

Figure 3.4 **Intracellular localisation of Rab GTPases and their functions in vesicular trafficking pathways,** page n. 55, Introduction CHAPTER 3, section 3.1.3. - Rab GTPases in vesicular trafficking. Reference – Figure 1 from Zhen, Y. & Stenmark, H. Cellular functions of Rab GTPases at a glance. J Cell Sci 128, 3171–3176 (2015).

Figure 3.6 **Role of Rab32/38 in the regulation of endosomal trafficking of melanosomal cargoes,** page n. 67, Introduction CHAPTER 3, section 3.2.1. - Rab32 in vesicular trafficking to lysosome-related organelles (LROs). Reference – Figure 1 from Ohbayashi, N., Fukuda, M. & Kanaho, Y. Rab32 subfamily small GTPases: pleiotropic Rabs in endosomal trafficking. The Journal of Biochemistry 162, 65–71 (2017).