From Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

LEARNING FROM PRIMARY IMMUNODEFICIENCIES TO TREAT CANCER

Mariana M.S. Oliveira

Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-285-2
Cover illustration: “Dendritic cell and cytotoxic T cells in tumor killing – a matter of communication” by Marta Sousa

Learning from primary immunodeficiencies to treat cancer
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Mariana M.S. Oliveira

The thesis will be defended in public at Ragnar Granit, Solnavägen 9, Karolinska Institutet, Friday October 1st 2021, 09.00

Principal Supervisor:
Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Associate Professor Lena Ström
Karolinska Institutet
Department of Cell and Molecular Biology

Professor Mikael Karlsson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Professor William Agace
Lunds Universitet
Department of Experimental Medical Science

Examination Board:
Associate Professor Peter Bergman
Karolinska Institutet
Department of Laboratory Medicine

Professor Maria Lerm
Linköping Universitet
Department of Biomedical and Clinical Sciences

Associate Professor Katharina Lahl
Lunds Universitet
Department of Experimental Medical Science

To my parents and brother,
for the unconditional support

Everything is worthwhile/
If the soul is not small

Fernando Pessoa

POPULAR SCIENCE SUMMARY OF THE THESIS

The immune system is a group of cells, tissues and organs all connected, working to defend our body against invaders, such as bacteria, virus, and parasites. We can imagine the immune system as an army in which their main purpose is to protect us against foreign bodies. In a healthy person, the army works in a coordinate way avoiding the person to get seriously ill; but sometimes the immune system does not work as it should. Immunodeficiency disorders occur when the body's immune response is reduced or absent. Immunodeficiencies can be primary or secondary depending how they are caused. When the cause of the deficiency is caused by other disease or environmental exposure to toxins, it is called secondary immunodeficiency disease but when the cause of this deficiency is genetic, it is called a primary immunodeficiency disease. Sometimes, a person carrying an immunodeficiency is also prone to develop other diseases, such as cancer and for that reason is very important to understand the mechanisms behind the disease.

In **paper I**, we work with a protein called Wiskott-Aldrich Syndrome protein (or WASp), named after the two people that described it first: Dr. Alfred Wiskott and Dr. Robert Aldrich. This protein is extremely important since it produces actin, a component of the cell skeleton. WAS patients do not have this protein at all, so the actin produced to maintain the cells structure is much decreased compared to a healthy person. Mutations on this protein can also induce this protein to produce much more actin than necessary leading to a disease named X-linked neutropenia (or XLN). In this study, we focus on a specific cell type of our immune system: dendritic cells (DCs). Their main function is to surveille our body for intruders and due to their structure with long protrusions-looking arms, they are able to do that in a very efficient way. Here we found that, compared to normal (wildtype; WT) DCs, XLN DCs migrate in a defective way in artificial systems that mimic our body but not in the body itself, using mice as experimental model. Moreover, WASp-deficient DCs migrate as well as WT DCs but when looking at microscopic structures such as the cell feet, named podosomes, WASp-deficient DCs differ from WT cells.

In **paper II**, we study how WASp-deficient DCs are able to process antigens. Antigens are small proteins released by pathogens after entering our body. Antigens are extremely important molecules since some of the cells in our immune system, such as DCs, can easily recognize them, starting an immune response to kill the pathogen. DCs cannot kill the pathogen themselves. Instead, they digest the antigen in a structure called phagosome (=cell stomach) and expose it to cytotoxic T cells, as in a relay race, if we imagine that the antigen is passed from DCs to cytotoxic T cells like a baton. After cytotoxic T cells have seen the antigen, they multiplicate/proliferate a lot and very quickly with the aim of fighting and kill the pathogen. Here we found that WASp-deficient DCs have their phagosome less acidic resulting in more cytotoxic T cell proliferation compared to WT DCs.

In **paper III**, we use WASp inhibitor CK666. CK666 is a small molecule that is used so far to inhibit a variety of cell functions. In this study, we use this molecule to modify the DC skeleton

to mimic the immune response seen on WASp-deficient DCs described in paper II as a strategy to kill cancer. Here, we show that CK666-treated DCs also have their phagosome less acidic resulting in more cytotoxic T cell proliferation compared to non-treated DCs. Moreover, mice that carry melanoma tumor and are later injected with CK666-treated DCs, survive longer, compared to mice that receive non-treated DCs or does not receive any immune cells. Mice survival is even prolonged when we combine CK666-treated DCs with another inhibitor molecule named anti-PD1.

In **paper IV**, we study how important are NK cells in tumor killing. NK cells are short for natural killer cells and their main function is the release of cytotoxic granules that will quick kill infected or cancer cells. In this study, we were lucky enough to get some material from the rare XLN patients but to confirm the findings we used XLN mouse models since they are more available. Here, we found that XLN NK cells have an increased capacity to respond and kill tumor cells.

ABSTRACT

The immune system is a fine-tuned network of cells, tissues, organs and biological processes working together in order to protect our body. Immunodeficiency disorders occur when the body's immune system is reduced or absent and can be characterized as primary, if the cause of disease is genetic; or secondary, if the cause of disease are extrinsic factors. Primary immunodeficiencies (PID) are in general rare, severe, and in many cases lethal. During my PhD, I aimed to investigate the role of dendritic cells (DCs) and natural killer (NK) cells in a primary immunodeficiency named Wiskott-Aldrich Syndrome (WAS). Moreover, I aimed to translate the findings from WAS to cancer treatment.

In **paper I**, we investigated how WAS protein (WASp) affects migration in DCs. In vitro assays, using microchannels and micropillars, showed that WASp-deficient DCs had increased migration speed while DCs expressing an overactive mutation in WASp, identified in X-linked neutropenia (XLN) patients, had similar average speed but increased speed fluctuations, reduced displacement, and atypical rounded morphology. Surprisingly, in vivo studies using an ear inflammation model, WT, WASp-deficient and XLN DCs migrated to the draining lymph nodes at the same extent, but deeper analysis by microscopy showed that WASp-deficient and XLN DCs localize differently in the draining lymph nodes, compared to WT DCs. Microscopy analysis revealed that XLN DCs had reduced cell area but formed larger podosome structures when compared to WT DCs. These results suggest that WASp activity regulates DC migration and that DCs have a remarkable adaptation for migration under inflammatory conditions in vivo.

In **paper II**, we examined how WASp affects DC cross-presentation to T cells. Using two different skin pathology models, challenge with Der p 2 or Leishmania major (L. major), we show that WASp-deficient mice had an accumulation of dendritic cells in the skin and increased expansion of interferon (IFN)γ-producing $CD8^+$ T cells in the draining lymph node and spleen. Specific deletion of WASp in dendritic cells led to expansion of $CD8^+$ T cells at the expense of $CD4^+$ T cells. WASp-deficient dendritic cells induced increased cross-presentation to $CD8^+$ T cells by activating Rac2 that maintains a near neutral pH of phagosomes. Our data reveal an intricate balance between activation of WASp and Rac2 signaling pathways in dendritic cells.

In **paper III**, we used the findings from paper II to develop a DC-based vaccine for cancer therapy. Here, we used the small molecule CK666 to inhibit signaling via WASp and Actin related protein 2/3 (Arp2/3) to enhance DC mediated killing of tumor cells. Treating DCs with CK666 in vitro during antigen uptake and processing of ovalbumin (OVA), murine and human DCs showed decreased phagosomal acidification that induced increased proliferation of OVA-specific OT-I CD8 T cells in vitro and in vivo. Furthermore, using the B16-mOVA melanoma tumor model, we show that mice injected with CK666-treated DCs and OVA-specific OT-I CD8 T cells showed higher rejection of tumor when compared to mice receiving non-treated DCs. This resulted in prolonged survival of tumor-bearing mice receiving CK666-treated DCs. Mice survival is extended when CK666-treated DCs are combined with checkpoint inhibitor

anti-PD1. Our data suggests that the small molecule inhibitor CK666 is a good candidate to enhance DC cross-presentation for cancer therapy.

In **paper IV**, we studied the anti-tumor responses of natural killer (NK) cells and T cells in XLN. Here, we examined NK and T cells from two XLN patients harboring the WASp L270P mutation. XLN patient NK and T cells had increased Granzyme B content and elevated degranulation and IFNγ production when compared to healthy control cells. Murine XLN T cells had normal degranulation and cytokine response whereas XLN NK cells showed an enhanced response. When compared to WT mice, XLN mice showed reduced growth of B16 melanoma and increased capacity to reject MHC class I-deficient cells. Together, our data suggests that cytotoxic cells with constitutively active WASp have an increased capacity to respond to and kill tumor cells.

In summary, the work presented in this thesis show that both DCs and NK cells are very important in starting an immune response and defects in their cytoskeleton dramatically affects their function. Still, cytoskeleton rearrangement and its outcome depend greatly on the context. Both WAS-deficiency and WASp-overactive patients suffer from negative effects however, we can learn from them and focus on the positive consequences in order to help cancer patients.

LIST OF SCIENTIFIC PAPERS

I. **Oliveira MMS**, Kung SY, Moreau HD, Maurin M, Record J, Sanséau D, Nylén S, Lennon-Duménil AM, Westerberg LS.
The WASp L272P gain-of-function mutation alters dendritic cell coordination of actin dynamics for migration and adhesion.
Journal of Leukocyte Biology (2021) Aug 25;1–11

II. Baptista MA, Keszei M, **Oliveira MMS**, Sunahara KK, Andersson J, Dahlberg CI, Worth AJ, Liedén A, Kuo IC, Wallin RP, Snapper SB, Eidsmo L, Scheynius A, Karlsson MC, Bouma G, Burns SO, Forsell MN, Thrasher AJ, Nylén S, Westerberg LS.
Deletion of Wiskott-Aldrich syndrome protein triggers Rac2 activity and increased cross-presentation by dendritic cells.
Nature communications (2016) Jul 18;7:12175

III. **Oliveira MMS**, He M, Baptista MAP, Nylén S, Westerberg LS.
Increased cross-presentation by dendritic cells and enhanced anti-tumor therapy using the Arp2/3 inhibitor CK666.
Manuscript

IV. Kritikou JS, **Oliveira MMS**, Record J, Saeed MB, Nigam SM, He MH, Keszei M, Wagner AK, Brauner H, Sendel A, Sedimbi SK, Rentouli S, Lane DP, Snapper SB, Karre K, Vandenberghe P, Orange JS, Westerberg LS.
Constitutive activation of WASp leads to abnormal cytotoxic cells with increased granzyme B and degranulation response to target cells.
JCI Insight (2021) Mar 22;6(6):e140273

PAPERS NOT INCLUDED IN THIS THESIS

I. He M, **Oliveira MMS**, Galvani RG, D'Aulerio R, Sedimbi S, Seitz C, Degn SE, Cotta-de-Almeida V, Ström L, Kitamura D, Kuznetsov NV, Westerberg LS.
Rescue of AID activity by uracil treatment resolve GC-mediated lymphadenopathy and B cell lymphoma into plasma cell differentiation.
Submitted

II. He M, Saeed MB, Record J, Keszei M, Pinho LG, Vasconcelos-Fontes L, D'Aulerio R, Vieira R, **Oliveira MMS**, Geyer C, Bohaumilitzky L, Thiemann M, Deordieva E, Buedts L, Lopes JPM, Pershin D, Hammarström L, Xia Y, Zhao X, Cunningham-Rundles C, Thrasher AJ, Burns SO, de Almeida VC, Liu C, Shcherbina A, Vandenberghe P, Westerberg LS.
Overactive WASp in XLN leads to aberrant B cell division and accelerated plasma cell generation.
Journal of Allergy and Clinical Immunology (2021) Aug 9; S0091-6749(21)01207-0. Online ahead of print

III. Zhou X, Singh M, Sanz Santos G, Guerlavais V, Carvajal LA, Aivado M, Zhan Y, **Oliveira MMS**, Westerberg LS, Annis DA, Johnsen JI, Selivanova G.
Pharmacological activation of p53 triggers viral mimicry response thereby abolishing tumor immune evasion and promoting anti-tumor immunity.
Cancer Discov. (2021) Jul 6; candisc.1741.2020. Epub ahead of print

IV. Record J, Sendel A, Kritikou JS, Kuznetsov NV, Brauner H, He M, Nagy N, **Oliveira MMS**, Griseti E, Haase CB, Dahlström J, Boddul S, Wermeling F, Thrasher AJ, Liu C, Andersson J, Claesson HE, Winqvist O, Burns SO, Björkholm M, Westerberg LS.
An intronic deletion in megakaryoblastic leukemia 1 is associated with hyperproliferation of B cells in triplets with Hodgkin lymphoma.
Haematologica (2020) May;105(5):1339-1350

V. **Oliveira MMS**, Westerberg LS.
Cytoskeletal regulation of dendritic cells: An intricate balance between migration and presentation for tumor therapy.
Review. Journal of Leukocyte Biology (2020) Oct;108(4):1051-1065

VI. Keszei M, Kritikou JS, Sandfort D, He MH, **Oliveira MMS**, Wurzer H, Kuiper RV, Westerberg LS.
Wiskott-Aldrich syndrome gene mutations modulate cancer susceptibility in the p53(+/-) murine model.
Oncoimmunology (2018) Jul 30;7(9):e1468954

VII. Kuznetsov NV, Almuzzaini B, Kritikou JS, Baptista MAP, **Oliveira MMS**, Keszei M, Snapper SB, Percipalle P, Westerberg LS.
Nuclear Wiskott-Aldrich syndrome protein co-regulates T cell factor 1-mediated transcription in T cells.
Genome medicine (2017) Oct 27;9(1):91

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AIRE	autoimmune regulator
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
Arp2/3	actin related protein 2/3
ATP	adenosine triphosphate
B16-mOVA	B16 containing ovalbumin in its membrane
BATF	basic leucine zipper ATF-like transcription factor
BCG	bacillus Calmette-Guérin
Bcl	B cell lymphoma
BHLH	basic helix-loop-helix family member
BM	bone-marrow
BMDC	bone-marrow derived DCs
BR	basic region
BST	bone marrow stromal cell antigen
C/EBPβ	CCAAT enhancer binding protein beta
CADM	cell adhesion molecule
Cat S	cathepsin S
CCL	chemoattractant C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
Cdc42	cell division cycle 42
CDP	common dendritic cell progenitor
Clec	c-type lectin domain
CLP	common lymphoid progenitor
CLR	C-type lectin receptor
cMoP	common monocyte progenitor
CMP	common myeloid progenitor
CTLA-4	cytotoxic t-lymphocyte associated protein 4

DAD	Dia autoregulatory domain
DAMP	damage-associated molecular patterns
DAP10	DNAX-activating protein of 10 kDa
DC	dendritic cell
DHR	dihydrorhodamine 123
dLN	draining lymph node
DNA	deoxyribonucleic acid
DNAM	DNAX accessory molecule
EE	early endosome
EpCAM	epithelial cell adhesion molecule
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
ESAM	endothelial cell specific adhesion marker
FH	formin homology
Flt3	Fms related receptor tyrosine kinase 3
Flt3L	Fms related receptor tyrosine kinase 3 ligand
GBD	GTPase binding domain
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-monocyte progenitor
HIGM	hyper IgM syndrome
HSPC	hematopoietic stem and progenitor cell
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
ID	inhibitor of DNA binding
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
InfDC	inflammatory dendritic cell
iNK	immature natural killer cell

IRAP	insulin-regulated aminopeptidase
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motifs
JMY	junction-mediating and regulatory protein
KLRG1	killer cell lectin-like receptor G1
KO	knockout
LC	Langerhans cell
LFA	leukocyte function-associated antigen
LPS	lipopolysaccharide
Mcl1	myeloid cell leukemia 1
MDA	melanoma differentiation-associated protein
mDia	mammalian homologous Diaphanous-related
MDP	monocyte-dendritic cell progenitor
MHC	major histocompatibility class
MIC	MHC class I polypeptide related sequence
MKL1	megakaryoblastic leukemia 1
mNK	mature natural killer cell
moDC	monocyte-derived dendritic cell
NADPH	nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NK	natural killer cell
NKG2D	natural killer group 2 member D
NKP	natural killer cell progenitor
NLR	NOD-like receptor
NLRX1	NLR family member X1
NOD	nucleotide binding oligomerization domain
NOX	NADPH oxidase
NSG	NOD-scid IL-2Rγ^{null}
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell

PD-1	programmed cell death protein 1
pDC	plasmacytoid dendritic cell
PDMS	polydimethylsiloxane
PID	primary immunodeficiencies
PIP2	phosphatidylinositol 4,5-bisphosphate
PLL	poly-L-lysine
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptors
RIG	retinoic acid inducible gene
RLR	RIG-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
Runx	runt-related transcription factor
SCAR	suppressor of the cyclic AMP receptor
SH	SRC homology domain
SiglecH	sialic acid binding Ig-like lectin H
SIRPα	signal regulatory protein α
TAP	transporter associated with antigen processing
TFEB	transcription factor EB
TGFβ	transforming growth factor
Th	T helper cell
TIL	tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	tumor microenvironment
TNFR	tumor necrosis factor receptor
TOCA1	transducer of Cdc42-dependent actin assembly 1
Treg	T regulatory cell
WAS	Wiskott-Aldrich syndrome
WASH	WASp and SCAR homolog
WASp	Wiskott-Aldrich syndrome protein
V-ATPase	vacuolar ATPase

WAVE	WASp-family verprolin-homologous protein
WDFY	WD repeat- and FYVE domain-containing protein
VEGF	vascular endothelial growth factor
WH	WASp homology domain
WHAMM	WASp homolog associated with actin, membranes, and microtubules
WIP	WASp interacting protein
WT	wildtype
XCL	X-C motif chemokine ligand
XCR	X-C motif chemokine receptor
XHIM	X-linked hyper IgM syndrome
XLN	X-linked neutropenia
Zeb	zinc finger E-box binding homeobox

1 INTRODUCTION

1.1 PRELUDE

In this thesis I will present the work that I have done during my PhD. But first, I would like to give a personal introduction. What moved me to study immunology in the first place was my mother being diagnosed with fibromyalgia syndrome. At the time, I was shocked and a little bit angry of how the human body, supposed to be "the perfect machine", could not work properly and inflict so much pain to someone. Fibromyalgia is a very poorly understood disease. Initially, it was thought that fibromyalgia was a brain pathology due to the patient's increased sensibility to pain but very recently, several universities, including Karolinska Institutet, found that it is actually an autoimmune disease with defective IgG [1]. Nowadays, after spending several years studying immunology, I have another vision of "the perfect machine". In fact, I do not think it is perfect, but I am extremely amazed how more people do not feel ill and more often. Having a system that includes so many components interconnected and working in syntony it is a reason to be thankful. We should also be thankful to science and all the researchers that contribute to better understanding of diseases, allowing the patients to have a life with any or minimal pain. With the work presented, I hope to have helped to expand the understanding of immune diseases and how this knowledge can be translated to cancer treatment.

1.2 THE IMMUNE SYSTEM

Our body is a very complex organism. In order to work properly as a whole, it is divided in biological systems where each system comprises several cells, tissues, organs and biological processes that work jointly with one common purpose. The immune system's main function is to identify and kill foreign bodies such as bacteria, virus and parasites that could cause us harm or even kill; but it can also defend us against own cell changes, as in the case of cancer.

According to Dr. Aaron Glatt, spokesperson for the Infectious Diseases Society of America, "the human body are exposed to approximately 60 000 types of germs on a daily basis, although only about 1 to 2 percent are potentially dangerous to normal people with normal immunity" [2]. From this statement, we can conclude that our immune system is extremely efficient. The human's immune system is divided in two main branches: the innate or natural immune system and adaptive or acquired immune system.

1.2.1 The innate immune system

The innate immune system is considered the first line of defense and develops when the human body is exposed to a new pathogen. It is not specific to a particular pathogen and, considering how fast a pathogen reproduces, it must be quickly activated to protect us from infection [3]. The innate immune system depends on pattern recognition receptors (PRRs) which recognize

conserved structures on pathogens, named pathogen-associated molecular patterns (PAMPs) or molecules released by damaged cells, named damage-associated molecular patterns (DAMPs) [4]. Classes of PPRs include Toll-like receptors (TLRs) [5,6], C-type lectin receptors (CLRs) [7], retinoic acid inducible gene 1 (RIG-1)-like receptors (RLRs) [8,9], nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) [10]. PAMP or DAMP recognition by the PPRs initiates signaling cascades that trigger the expression of pro-inflammatory molecules that ultimately leads to the pathogen annihilation. Innate immune system consists of different components: physical barriers such as skin and mucosa; antimicrobial peptides; soluble mediators such as cytokines and serum proteins; cellular receptors; and effector cells such as endothelial/epithelial cells, neutrophils, mast cells, granulocytes, natural killer (NK) cells, monocytes, macrophages, dendritic cells (DCs) [11]. Important for this thesis, I will focus on monocytes, DCs and NK cells.

1.2.1.1 Monocytes

Monocytes arise in the bone-marrow (BM) from hematopoietic stem and progenitor cells (HSPCs) – **Figure 1**. In both human and mouse, monocytes develop from oligopotent common myeloid progenitors (CMPs) [12,13], characterized by $Lin^{-}cKit^{+}Sca1^{-}(LKS^{-})CD34^{+}Fc\gamma R^{lo}Flt3^{+}CD115^{lo}$, that differentiate to granulocyte-monocyte progenitors (GMPs), characterized by $LKS^{-}CD34^{+}Fc\gamma R^{hi}Ly6C^{-}$; and to monocyte-DC progenitors (MDPs), characterized by $LKS^{-}CD34^{+}Fc\gamma R^{lo}Flt3^{+}CD115^{hi}$ [14]. Monocytes can be divided into two major classes: classical monocytes and non-classical monocytes. Classical monocytes are characterized by $Ly6C^{hi}CD43^{-}$ in mice to what corresponds to $CD14^{+}CD16^{-}$ in humans; and non-classical monocytes are characterized by $Ly6C^{lo}CD43^{+}$ in mice corresponding to $CD14^{lo}CD16^{+}$ monocytes in humans [15,16]. Both types of monocytes can differentiate from both GMPs and MDPs, though showing differences in function [17]. In case of an injury, classical monocytes that are stored in the BM and spleen, quickly differentiate into macrophages or monocyte-derived DCs [17,18]. Non-classical monocytes are less understood. However, it is thought to have the ability to be recruited to non-inflamed and have the function of intravascular housekeepers by repairing endothelium during homeostasis, and remove cell debris [16,19–21].

It was shown recently that mouse MDPs and GMPs differentiate from CMPs and both give rise to classical monocytes ($Ly6C^{hi}$), however, through distinct pathways. Moreover, GMPs generated "neutrophil-like" monocytes while MDPs generated moDCs and their balance modulates cell repertoire during homeostasis and following infection [17]. MDPs differentiate later into conventional DCs (cDCs) and plasmacytoid DCs (pDCs), via common DC progenitors (CDP); or into monocyte-derived DCs (moDCs), via common monocyte progenitors (cMoPs) [14,22]. Although, myeloid precursors are the main source of DCs, lymphoid precursors can also give rise to cDCs. Transfer experiments show that T-precursors from thymus expressing low CD4 can differentiate into T, B and cDCs but not myeloid cells [23–25]. Initial studies could only detect CLP-derived cDCs with high CD8α, however, using different transfer models, it was shown that CLP-derived cDCs can be $CD8\alpha^{+}$ or $CD8\alpha^{-}$[26].

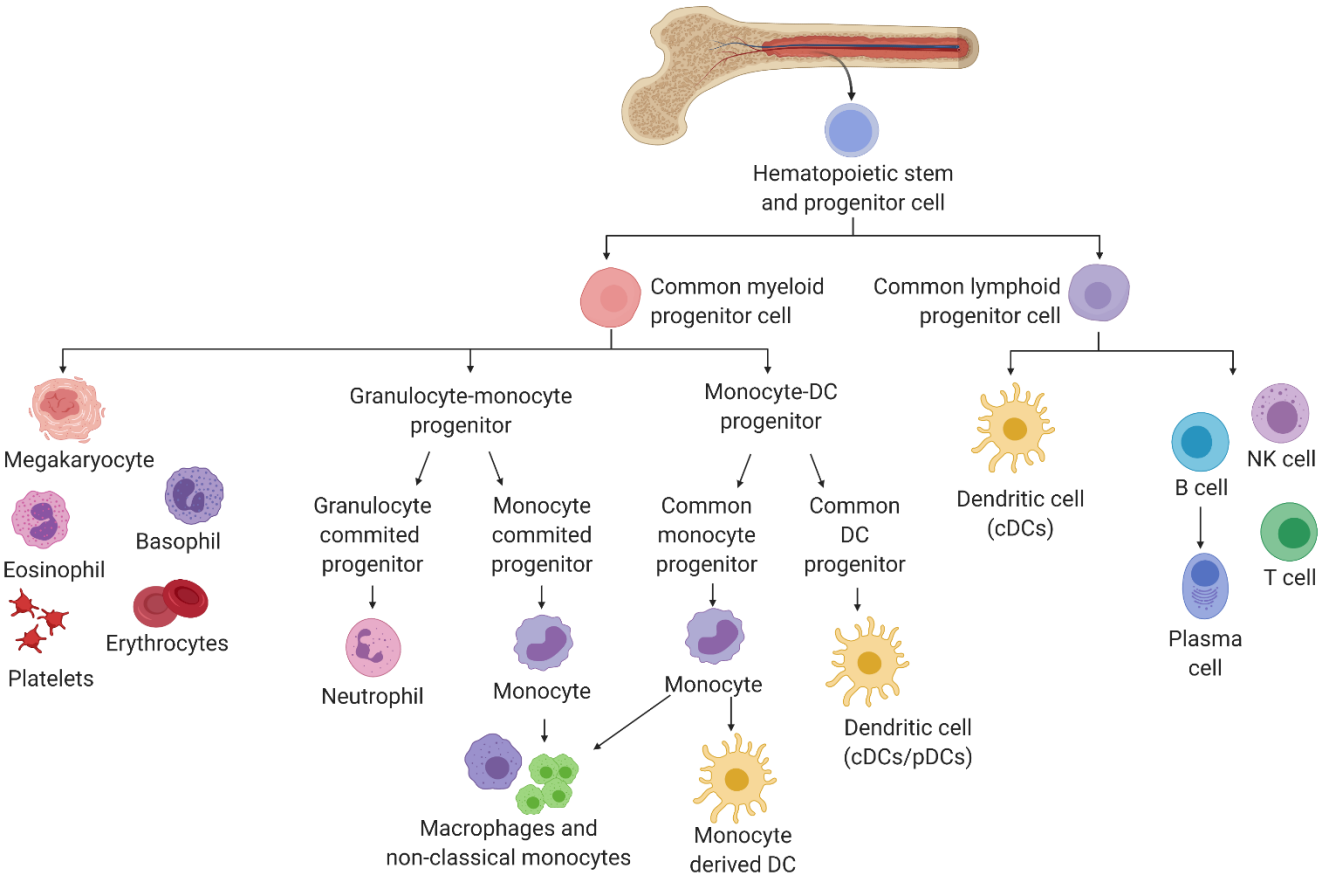

Figure 1. Dendritic cell differentiation pathways. Hematopoietic stem and progenitor cells (HSPCs) give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CLPs can differentiate into T cells, B cells, NK cells and conventional DCs (cDCs). CMPs differentiate into megakaryocyte, eosinophil, basophil, erythrocytes, granulocyte-monocyte progenitors (GMPs) and monocyte-DC progenitors (MDPs). MDPs differentiate into cDCs, plasmacytoid DCs (pDCs) and monocytes where monocyte-derived DCs (moDCs) arise from. GMP differentiate into neutrophils and monocytes. Macrophages and non-classical monocytes differentiate both from GMP and MDP, depending on the stimuli received. Image created with BioRender.com

1.2.1.2 Dendritic cells

Dendritic cells (DCs) are one of the most efficient antigen-presenting cells (APC) that patrol our body to capture antigen, process and present them to effector cells initiating an immune response [27]. DCs were identified by Steinman and Cohn in 1973 in the adherent cell populations derived from peripheral lymphoid organs such as spleen, lymph node and Peyer's patches. Initially, these cells were morphologically characterized as having a variety of branching forms, and continuously extend and retract cell processes. For this reason, "dendritic cell[a]" was the name suggested [28]. DCs were later characterized as cells that express high levels of major histocompatibility class (MHC) II molecules and integrin cluster of differentiation (CD)11c on their surface [29,30]. Finding that granulocyte-macrophage colony-stimulating factor (GM-CSF) can be produced by non-immune cells and mediate DC function and survival was a transforming step in the field, since it allows the differentiation of DCs in vitro [31]. This helped

[a] The word "dendritic" means "branched like a tree". It comes from the Greek " δένδρον/dendrón"=tree.

to find a suitable protocol for differentiation of human DCs by culturing $CD14^+$ monocytes present in human blood using GM-CSF and interleukin (IL)-4 [32,33].

DCs subsets are equivalent in mouse and human but differ on expression markers – **Figure 2**. For both, mouse and human, DCs can be divided in steady-state DCs, Langerhans cells and inflammatory DCs. Steady-state DCs can further be divided in conventional DC1 (cDC1), conventional (cDC2) and plasmacytoid DC (pDC). In this work, I will only discuss mouse DCs.

1.2.1.2.1 Mouse Dendritic Cell subtypes

Conventional DC1s (cDC1s) are identified based on expression of MHC II, CD11c and CD8α or CD103, and lack of CD11b and B220. CD8α is expressed on cDC1s in spleen and other lymphoid organs [34–36], while CD103 is expressed on cDC1s in skin and other non-lymphoid organs such as intestine, lung, liver and kidney [37–41]. cDC1s also express cell adhesion molecule 1 (CADM1), c-type lectin domain containing (Clec)9a and X-C motif chemokine receptor (XCR)1 [42–44]. CD26 could be used as an additional marker; or macrophages marker such as F4/80, CD64, CD11b and signal regulatory protein α (SIRPα; CD172α) can be used as exclusion markers for cDC1s [42].

cDC1s derive from HPSCs and its differentiation is modulated by interferon regulatory factor (IRF)8 that itself is regulated through nuclear factor regulated by IL-3 (Nfil3)/zinc finger E-box binding homeobox (Zeb)2 /inhibitor of DNA binding (ID)2 pathway; and basic leucine zipper ATF-like transcription factor (BATF)3 [45–47]. PU.1 has also been shown to be vital for cDC1 differentiation, since it directly regulates Fms related receptor tyrosine kinase 3 (Flt3) in a concentration dependent manner [48]. Intestinal IRF8-dependent cDC1s, characterized by $CD103^+CD11b^-$, are responsible to maintain T cell homeostasis. Additionally, mice lacking intestinal IRF8-dependent cDC1s, are not able to trigger T helper type (Th)1 responses to Trichuris muris infection [49]. B cell lymphoma (Bcl)-6 was shown to drive the transcriptional program of intestinal $CD103^+CD11b^-$ cDC1s [50]. cDC1s express several TLRs. Murine cDC1s from spleen express TLR3 that lead to IL-12 and interferon-lambda (IFNλ) production upon polyinosinic:polycytidylic acid (poly I:C) stimulation [51]; cDC1s express TLR4 upon lipopolysaccharide (LPS) activation [52,53]; and cDC1s also express TLR11 upon contact with profilin like molecules from the protozoan parasite Toxoplasma gondii resulting in high levels of IL-12 production [54,55]. Moreover, cDC1s express indoleamine 2,3-dioxygenase (IDO)1 and IDO2 that affect T cell responses [56]; several Rab GTPases Rab11A [57], Rab7B [58], Rab43 [59], SEPT3 [60]; and recently, WD repeat- and FYVE domain-containing protein (WDFY)4, was shown to be critical for cross-presentation in anti-viral and anti-tumor responses [61].

The main function of cDC1 is cross-presentation. The exact mechanism of cross-presentation is not completely understood however, some factors and key-molecules are known to affect cross-presentation. The small GTPase Rac2 control the phagosomal oxidation and pH, controlling this way cross-presentation [62]. Rab43-deficient DCs show normal viability but

defective in vivo and in vitro cross-presentation of cell-associated antigen [59]. Studies done in transcription factor Batf3 knockout mice show lack of virus-specific $CD8^+$ T cell responses to West Nile virus. Additionally, these mice showed impaired rejection of highly immunogenic syngeneic tumors. This was shown to be due to defective cross-presentation by dendritic cells [63]. Very importantly, cDC1s don't express the CD47-SIRPα, known as the "don't eat me" signal mainly expressed on cancer cells, avoiding a specific anti-tumor response. Besides DCs being professionals APCs, cDC1 can also induce strong humoral response and induce immunoglobulin (Ig)G class switching [64,65]. They also secrete IL-15 that activates and induces proliferation of NK cells [66,67]. Additionally, cDC1s can control the recruitment of neutrophils to the site of bacterial infection, including Staphylococcus aureus, bacillus Calmette-Guérin (BCG) and Escherichia coli. This mechanism is regulated by the cytokine vascular endothelial growth factor α (VEGF-α) [68]. cDC1s are also able to develop memory T cells in vaccinia cutaneous-infected mice, upon influenza infection, and upon secondary infections with Listeria monocytogenes [69,70]. In the intestine, cDC1s are required for $FoxP3^+CD8^+$ T_{regs} generation and suppressive function that is required for tolerance [71].

In summary, cDC1s have been shown to play critical roles in both innate and adaptive immune responses to prevent different pathogens infections.

Conventional DC2s (cDC2s) are identified based on expression of MHC II, CD11b, CD11c, CD1c and SIRPα. They can also express CD4 and CD26. cDC2s do not express CD8α, CD45RA, Clec9a, XCR1 and CD24. cDC2s from the spleen additionally express cell adhesion molecule (CADM)3 and cDC2s from the gut express specifically CD103, important to regulate T cells activity in the intestine [72]. cDC2s from the spleen can be divided in two subsets based on expression of Clec12A and endothelial cell specific adhesion marker (ESAM). $Clec12A^{lo}ESAM^{hi}$ differentiation is dependent on IRF4 and Zeb2 [73–75]. Intestinal $CD103^+CD11b^+$ cDC2s survival is dependent on IRF4 [76]. Moreover, cDC2s that express IRF4 promote the generation of colitogenic $CD4^+$ T cells, emphasizing the importance of cDC1 and cDC2 balance [77]. It has been identified several transcription factors present on cDC2s, including CCAAT enhancer binding protein beta (C/EBPβ) and PU.1 [78], the runt-related transcription factor (Runx)3 [79], nuclear factor (NF)κB1 [80,81], and basic helix-loop-helix family member (BHLH)E40 [82]. Differentiation of cDC2s is also Notch2-dependent [83]. Blimp-1 drives the transcriptional program of intestinal $CD103^+CD11b^+$ cDC2s [50]. cDC2s express immune receptors such as RIG-I and melanoma differentiation-associated protein (MDA)-5 that trigger inflammatory responses upon detection of viral double-stranded ribonucleic acid (RNA) in the cytoplasm [84]. cDC2s also express NLRs such as NOD1 and NLR family member X1 (NLRX1) [85,86]; and several TLRs including TLR3 [87], TLR5 [88–90], TLR7 [87,91,92], TLR9 [88] and TLR12 [88]. cDC2s secrete higher amounts of IL-6 and IL-8 starting inflammatory responses [93,94]; they also produce higher amounts of IL-23 upon fungal infections, promoting this way Th17 response [73]. IRF4 deletion on intestine cDC2s result in reduced amount of Th17 cells in the draining LNs

(dLNs), following immunization [73,76]. cDC2s are for this reason able of presenting antigen to activate $CD4^+$ T cells.

Plasmacytoid DCs (pDCs) are a unique type of DCs with important role in antiviral immunity due to their ability to produce very high amounts of IFN type I, mainly IFNα, upon activation with virus or methylated deoxyribonucleic acid (DNA). pDCs can also produce large amounts of IFN type III after stimulation with herpes simplex virus type 1 (HSV-1) or parapocvirus [53]. Morphologically, pDCs are different from cDCs due to the presence of higher amounts of rough endoplasmic reticulum (ER). pDCs are characterized by expression of CD11c and high amounts of MHCII. pDCs also express sialic acid binding Ig-like lectin H (SiglecH) [95] and bone marrow stromal cell antigen 2 (BST2; also known as HM1.24, tetherin, or CD317) [96]. BST2 is expressed specifically on IFN type I-producing cells but SiglecH can also be expressed on some macrophage's subsets. Additional pDCs markers, when in periphery (not in bone marrow), include CCR9 [97,98], SCA1 [99] and Ly49Q [100]. CCR9 is an important chemokine receptor since it mediates efficient pDCs homing and entry in the thymus. Additionally, pDCs take up antigen from the periphery and transport it to the thymus in CCR9-dependent way, contributing to immune tolerance [101]. pDCs express high levels of TLR1, TLR7 and TLR9, and respond to the specific agonists by producing IFN type I. pDCs also express high levels of TLR2 although being poor in responding to its ligand. pDC development depends on Flt3L [102,103] and some key genes are essential for its differentiation and function. Transcription factor Zeb2 regulates development of pDCs [104]; Spi-B is also shown to control to control pDC development by not allowing progenitor cells to develop into other lymphoid lineages [105]; deletion of IRF8 altered pDCs phenotype and decreased IFN type I production upon CpG-stimulation [45]; mice that express low levels of Ikaros lack peripheral pDCs [106]. pDCs are key cells in mucosal immunity and colitis models [107,108]; but they are also involved in malignancies. pDCs are known to be involved in various autoimmune diseases such as systemic lupus erythematosus [109,110]; psoriasis [111]; and type I diabetes [112].

Langerhans cells (LCs) are a subset of DCs that reside in the skin epidermis and are mainly characterized by the presence of Birbeck granules and the expression of langerin (or CD207) [113,114]. LCs also express MHC II, epithelial cell adhesion molecule (EpCAM; also known as CD326), CD24, CD11b, F4/80 and CD205. LCs differentiation is mainly regulated by the transforming growth factor-beta (TGF-β), but other transcription factors are equally important. These include pU.1, ID2 and RUNX3 [115–117]. The p14 adaptor molecule is critical in for LCs homeostasis and p14 conditional knockout mouse show a complete loss of LCs in the epidermis early after birth [118]. Activated LCs elongate their dendrites through keratinocyte tight junctions in order to have access to external antigen in the epidermis [119]. LC then migrate to the lymph nodes to initiate an immune response.

Inflammatory DCs (infDCs) are derived from monocyte precursors upon infection or injury that are recruited to lymphoid tissues in a C-C chemokine receptor type (CCR)2-dependent manner. InfDCs are very similar to inflammatory macrophages, so there is a need for caution when characterizing these cells. InfDCs are phenotypically similar to cDC2 expressing high levels of MHC II, CD11b and CD11c. InfDCs also express Ly6C, F4/80, CD206, CD115, CD107b, FCERI and CD64 [120]; and are able to produce cytokines such as IL1α, IL1β, IL-12 and IL-23. Although it can be difficult to distinguish infDCs from macrophages, they are functionally different. InfDCs are able to migrate to the lymph nodes in a CCR7-dependent manner and activate T cells, contrary to macrophages.

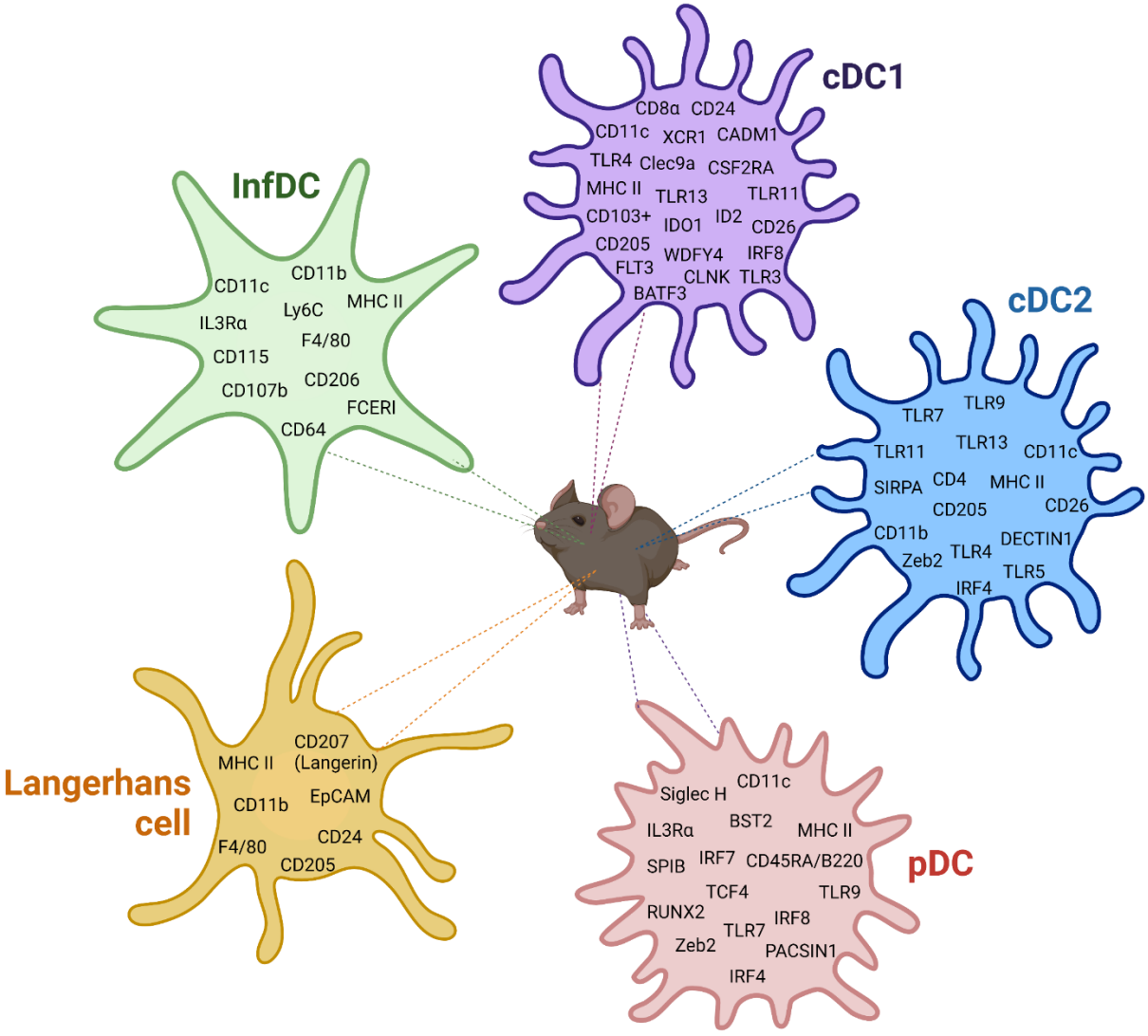

Figure 2. Expression markers on different DC subsets. Image created with BioRender.com

1.2.1.2.2 Cross-presentation

DCs are professional antigen-presenting cells and can process both endogenous and exogenous antigens to start an immune response. Most DCs process endogenous antigens and then present it in MHC class I molecules, and process exogenous antigens and then present it in MHC class

II molecules. However, specific DCs are able to shuttle an exogenous antigen to MHC class I molecules in a process called cross-presentation [121,122]. This phenomena is due to the ability of some DCs to keep their phagosomes at a neutral pH, limiting protein degradation by the proteosome [123–125]. Among murine DCs, $CD8\alpha^+$ DCs present in spleen and other lymphoid organs; and $CD103^+$ DCs present in skin and non-lymphoid organs, have higher capacity for cross-presentation. $CD8\alpha^-$ DCs can also cross-present but at lower extent [124,126,127]. There are two main cross-presentation pathways by DCs: the endosome-to-cytosol pathway and the vacuolar pathway – **Figure 3**.

The endosome-to-cytosolic pathway

The endosome-to-cytosolic pathway is sensitive to proteasome inhibitors and is dependent of transporters associated with antigen processing (TAP) [128]. Extracellular antigens are taken-up to the cytosol, where they are degraded by the proteasome. After being internalized by the DC, the antigen is stored in early endosomes (EE) or phagosomes, following by transportation by Sec61 to the cytosol. Sec61 is a trimeric translocator member of the ER associated degradation (ERAD) machinery [129]. This translocation is supported by energy produced by ATPase p97, another ERAD-member [130]. In the cytosol, the antigen is degraded into smaller immunogenic peptides by the proteasome before being translocated into the ER by TAP to be loaded on the newly formed MHC class I molecules [128]. For optimal MHC class I loading and therefore cross-presentation, the peptides are trimmed by the insulin-regulated aminopeptidase (IRAP) [131]. $Clec9A^+CD141^+$ and $CD1c^+$ pDCs have an inherent ability to cross-present exogenous antigens via the endosome-to-cytosolic pathway [132]. This pathway has also been shown to be the only used by $CD8\alpha^+$ DCs for cross-presentation [133].

The vacuolar pathway

The vacuolar pathway is insensitive to proteasome inhibitors and is independent of TAP. Here, extracellular antigens are degraded by lysosomal proteases, mainly cathepsin S (Cat S) [134], in endocytic compartments and then transported to the cell surface by EE-associated vesicular transport. Some antigens such as viral proteins, virus-like particles and Escherichia coli-derived proteins are cross-presented via the vacuolar pathway [135–137]. This is mainly due to activation of Cat S that is induced by IFNγ during viral infections [138].

The pathway which an antigen is cross-presented depends mainly on its properties (particulate or soluble), under which physiological conditions it occurs and the level of degradation of the antigen. It is believed that most antigens are cross-presented via the endosome-to-cytosolic pathway in vivo however the transcription factor EB (TFEB) can negatively regulate cross-presentation. TFEB regulates lysosomal activity promoting fast degradation of the antigen, shifting the presentation of an exogenous antigen on MHC class I molecules to presentation on MHC class II molecules [139]. It is known that the right amount of degradation is needed for the

antigen to preserve their epitopes that will be recognized by MHC class I molecules [140,141]. Endosomal pH is controlled by the vacuolar ATPase (V-ATPase) that transports protons into the cell lumen. This process results in activation of pH-dependent lysosomal proteases. Decreased V-ATPase activity prevents acidification of the internalized antigen [142]. In synergy with the proton pump, DCs also recruit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)2 to the endosomal membrane. NOX2 produces reactive oxygen species (ROS) that capture protons produced by V-ATPase forming hydrogen peroxide. This process causes endosomal alkalization preventing rapid antigen degradation and promoting cross-presentation [143,144].

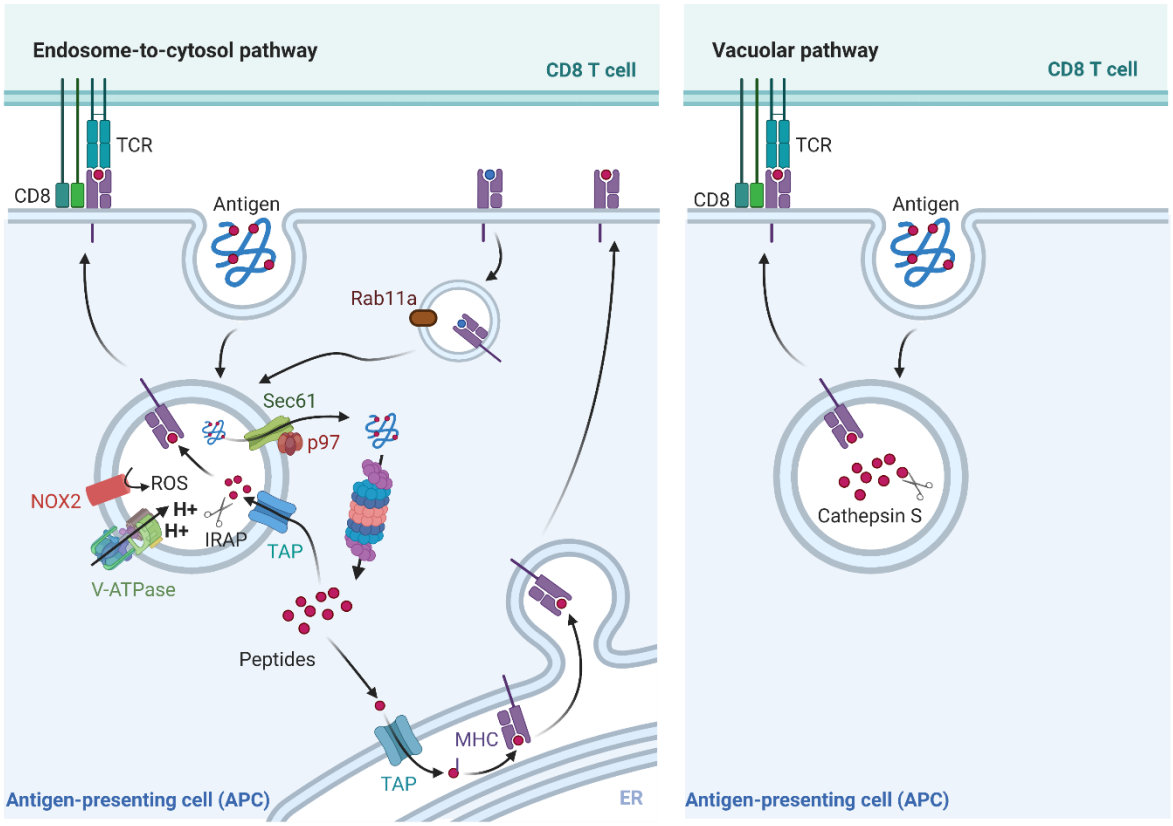

Figure 3. Different cross-presentation pathways. In the endosome-to-cytosol pathway, extracellular antigens are internalized by DCs to be processed in the phagosome. In the phagosome, the antigen is translocated to the cytosol via Sec61/p97, following by proteosome degradation. The degraded peptides are translocated to the ER by TAP and loaded into MHC class I molecules. In the vacuolar pathway, extracellular antigens are internalized by DCs and processed in the endocytic compartments by enzymes such as cat S. degraded peptides are directly loaded on MHC class I molecules and transported to the cell membrane. Image created with BioRender.com

Cross-presentation in tumor killing and DC-based cancer vaccines have become a hot topic in the past decades. The concept is based on the fact that DCs are efficient in activating tumor specific $CD8^+$ T cells to become tumor-infiltrating lymphocytes (TILs) that will in turn, kill the tumor. However, DCs are only efficient in this process if cross-presenting DCs are activated and tolerogenic DCs are inhibited. Tolerogenic DCs are unfavorable to tumor therapy due to their capacity of activating regulatory T cells [145,146]. Inducing DC cross-presentation and avoid tolerance was one of my thesis project's aims. We think this can be achieved by modifying

patient-derived DCs in vitro, during uptake and presentation, before injecting back into the patient.

1.2.1.3 NK cells

Natural killer (NK) cells were first identified by Rolf Kiessling, Eva Klein and Hans Wigzell in 1975. They were described as "killer cells with rapid cytolytic, specific activity against in vitro grown mouse Moloney leukemia cells" [147]. The hypothesis of "missing-self", in which NK cells recognize and eliminate cells that do not express MHC class I molecules, was later elaborated by Klas Kärre and Hans-Gustaf Ljunggren in 1985 [148]. NK cells correspond to 5–15% of circulating lymphocytes in humans [149] and 2-5% in the spleens and BMs of laboratory mice [150].

Human NK cells rise from a oligopotent CLP that derive from HSPCs [151]. Pre-NK cell precursors – stage 1 – start to express CD117 (c-Kit), CD7, CD127 IL-1R1 and CD122 – stage 2 – becoming a NK cell progenitor. NK cell progenitors evolve to become immature NK (iNK) cells – stage 3 – by upregulating the expression of IL-1R1 and by starting to express NKG2D, NKp30 (CD337), NKp46 (CD335) and NK1.1 (CD161). iNKs develop into mature NK (mNK) cells – stage 4 – characterized by high expression of all stage 3 markers and by expressing very bright CD56 (CD56bright). On stage 5, mNK cells decrease expression of CD56 (CD56dim) and start expressing FcγRIIIA (CD16) and killer immunoglobulin-like receptor (KIR; CD158). Stage 6 mNK cells express high levels of NKG2C, KIR and CD57 [152].

Classically, murine NK cells are defined as $CD3^{-}NK1.1^{+}NKp46^{+}$. As in human NK cells, murine NK cells rise from a oligopotent CLP that derive from HSPCs [151]. CLPs give rise to $CD122^{+}$ early NK cell progenitor (NKP) lineages [153]. $CD122^{+}$ NKPs will then express Natural killer group 2 member D (NKG2D)/DNAX-activating protein of 10kDa (DAP10) – stage A – and become iNK cells. This is followed by the expression of NK1.1 – stage B – and NCR1 – stage C. NK cells become mNK cells after expression of CD51 (Integrin αV) and CD49b (DX5, Integrin VLA-2α) – stage D. The final stage of mNK cells include the expression of CD43 (leukosialin), CD11b (Mac-1) and Ly49 receptors – stage E. After full maturation, NK cells migrate to secondary lymphoid organs where they express killer cell lectin-like receptor G1 (KLRG1) – stage F. Mature NK cells can also express CD27 and CD11b [152] – **Figure 4**.

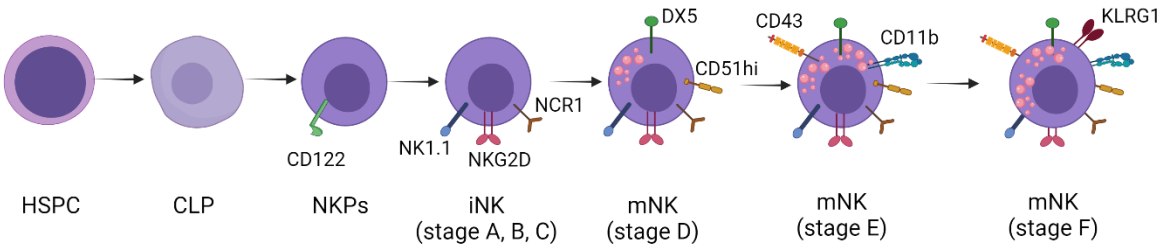

Figure 4. Murine NK cell maturation process. NK cells derive from CLPs that descend from HSPCs. NKPs only express CD122 that differentiate into iNKs by expressing NK1.1, NKG2D and NCR1 (stages A, B and C). iNK cells become mature after expression of CD51, DX5, CD43 and CD11b (stages D and E). After migrating to secondary lymphoid organs, mNK cells express KLRG1 (stage F). Image created with BioRender.com

NK cell biology is controlled in part by cytokines. Cytokine receptors start being expressed early in NK cell development and require signaling through common gamma (γc) chain for their function [154]. The family of cytokines that share the γc are IL-2 [155], IL-4 [156], IL-7 [157], IL-9, IL15 [158] and IL-21 [159,160]. IL-15 receptor starts being expressed by NKPs and is crucial for generation of NK cells and is extremely important for their survival and proliferation [150,153]. It also induces NK cell activation that results in production of pro-inflammatory cytokines and lytic granules. IL-15 binds to IL-15Rβ/γc on NK cell surface starting an activation cascade through JAK1/3 and STAT5 signaling pathways. Activation of these pathways induces STAT-5 target genes including pro-survival gene myeloid cell leukemia 1 (Mcl1) and the negative regulator cytokine-inducible SH2 (CIS)-containing protein encoded by Cish [161]. Both are essential for NK cell survival and homeostasis. Expression of other positive and negative regulators and dynamic balance between them allows NK cells sense changes in the environment – **Figure 5**. When needed, NK cells kill altered or infected cells through cytolytic mechanisms such as the release of granzymes and perforin, and activation of death receptors [150].

NK cells can be activated by several different types of cells, among those, DCs. DCs can activate NK cell through cell-cell contact between NKG2D-MICA and/or MICB (MHC class I polypeptide related sequence A/B); or by producing cytokines such as IFN-α, IFN-β, IL-2, IL-12, IL-15 and IL-18 [152,162].

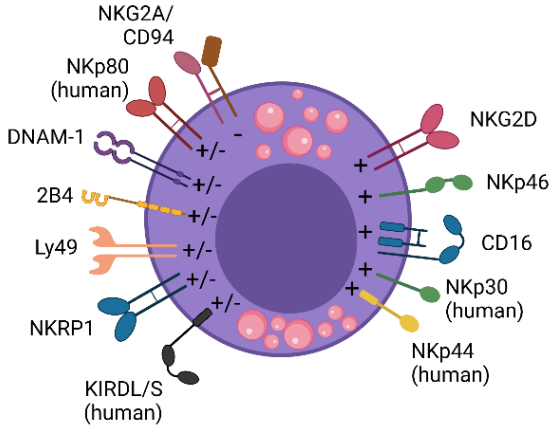

Figure 5. Receptors expression on mouse and human NK cells. Expression of activating receptors (+), inhibitory receptors (-) and co-activating receptors (+/-) on mouse and human NK cells. All receptors are expressed on mouse and human NK cells except NKp30, NKp44, NKp80 and KIRDL/S that are exclusively expressed on human NK cells. Image created with BioRender.com

1.2.2 The adaptive immune system

The adaptive immune system assumes control when the innate immune system is not able to destroy the pathogen. It is specific for each pathogen and although being more effective, it takes longer to act. One benefit of a pathogen being destroyed by the adaptive immune system is that it gives memory. If the same pathogen invades the human body, that person's immune system can respond immediately. This is the reason why some people only get ill from one

pathogen once in life. The adaptive immune system consists of: B lymphocytes, antibodies, and T lymphocytes [163]. Important for this thesis, I will focus on T lymphocytes.

1.2.2.1 T lymphocytes

T lymphocytes (or T cells) maturation occurs in the thymus. T cell progenitors migrate from the bone-marrow to the thymus where they start the maturation process. Each maturation step is characterized based on the expression of CD4 and CD8 co-receptors on the progenitor cell. The most immature progenitor does not express neither CD4 or CD8 and it is denominated pro-T cell. Pro-T cells undergo proliferation cycles induced by IL-7 produced in the thymus [164]. A pro-T cell can be divided by the expression of the adhesion molecule CD44 and IL-2 receptor α chain CD25 [165]. Cells that are positive to CD25 but are negative to CD44, undergo random rearranges in variable (V), joining (J), and diversity (D) – VDJ – gene segments to assemble a TCRβ chain locus [166]. The β chain couples with a surrogate α chain, pre-Tα, resulting in pre-T cell [167]. If a cell is not able to express either β or α chain, it dies. After successful expression of αβ TCR, the cells acquire both CD4 and CD8 co-receptors giving rise to double positive immature T cells. Double positive immature T cells undergo rounds of selection to form mature $CD4^+$ or mature $CD8^+$ T cells the following way:

- T cells whose TCRs recognize MHC class II-peptides complexes keep the CD4 co-receptor → Mature $CD4^+$ helper T cell;
- T cells whose TCRs recognize MHC class I-peptides complexes keep the CD8 co-receptor → Mature $CD8^+$ cytotoxic T cell;
- T cells whose TCRs do not recognize either complexes or strongly recognyze both complexes die by apoptosis.

The majority of T cells become activated upon recognition of antigens presented on MHC molecules of antigen-presenting cells such as DCs. Once a T cell is activated, it undergoes clonal expansion and differentiation to become either effector or memory T cell. For correct T cell activation, 3 different signals are necessary: signal 1, through the TCR [168]; signal 2, from co-stimulatory molecules [169,170]; and signal 3, from cytokines [171]. The TCR complex comprises the TCRα/β chains and the CD3 γ/δ/ε/ζ chains, linked through hydrophobic interactions [172]. In total, CD3 subunits, that can be arranged in CD3γε, CD3δε, and CD3ζζ, contain 10 immunoreceptor tyrosine-based activation motifs (ITAMs), that can be phosphorylated to start a signaling cascade in which T cells are activated [173,174]. The best known co-stimulatory molecules for T cells are B7-1 (CD80) and B7-2 (CD86), both expressed on APCs and increased in expression upon encounter to pathogens. The B7 proteins are recognized by the CD28 receptor, present on T cells. Another co-stimulatory molecule is CD40 present on APCs that is recognized by CD40 ligand (CD40L or CD154). CD70, OX40L and 4-1BBL are also stimulatory molecules that bind to the tumor necrosis factor receptor (TNFR) family members CD27, OX40 and 4-1BB, respectively [168,175]. APCs such DCs and macrophages, secrete cytokines that stimulate and induce differentiation of T cells. IFN type I, including IFNα and

IFNβ, IL-1, IL-2 and IL-12 are among these cytokines. IFN type I and IL-12 are of high importance for the formation of antigen-specific effector and memory $CD8^+$ T cells, since they fine-tune the antigen sensitivity of $CD8^+$ T cells by boosting T cell receptor signaling [176,177]. IL-12 is also important for $CD4^+$ T cells once it shifts the responses to Th1. IL-1 produced by DCs act on $CD4^+$ T cells to enhance their antigen-driven expansion and differentiation [178]. IL-2, that is widely used in in vitro T cell culture, was found to be important for survival of T regulatory (Treg) cells [179,180].

Important for this thesis is the notion of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) act as T cell inhibitory signals. High expression of PD-1 might mean T cell exhaustion from a chronic infection [181]. However, chronic viruses and tumors have the ability of decreasing the expression of co-stimulatory molecules and increasing the expression of co-inhibitory molecules, leading to anergic T cells [182]. This is one of the causes of poor anti-tumor immunity. For this reason, a big step in medicine was the discovery of CTLA-4 and PD-1 blockades in cancer therapy with a shared Nobel Prize in Physiology or Medicine for James P. Allison and Tasuku Honjo in 2018 [183–185].

1.2.3 Dendritic cells, NK cells and T cells – together to kill tumors

NK cells and DCs are considered the link between innate and adaptive immune system. NK cells and mainly cDC1s are important in modulating immune responses to cancer in both mice and humans. Tumors with infiltrated NK cells are positively correlated with survival of patients with different types of tumors, including melanoma [186], breast cancer [187], pulmonary adenocarcinoma [188], squamous cell lung cancer [189], non-small cell lung cancer [190], gastric carcinoma [191] and neuroblastoma [192]. The same applies to DCs, besides being a rare but efficient population, of patients with ovarian carcinoma [193], gastric cancer [194], hepatocellular carcinoma [195], breast cancer [196] and melanoma [197]. Studies suggest that NK cells and DCs work together in tumor killing. NK cells produce cDC1 chemoattractants C-C motif chemokine ligand (CCL)5, X-C motif chemokine ligand (XCL)1 and XCL2 leading to recruitment of DCs to tumors [198]. Recent work found that NK cells and cDC1s cluster together in the tumor microenvironment enhancing T cell tumor responses. Moreover, this NK cell–cDC1 axis can be used as a prognostic tool for T cell-directed immunotherapy. Additionally, melanoma patients with higher frequencies of NK cells-cDC1s have increased overall survival due to improved responsiveness to anti-PD1 [198]. NK cells produce Flt3L in the tumor microenvironment (TME), a chemokine that controls the development of DCs. For this reason, presence of NK cells in the tumor, leads to recruitment of cDC1s that increases patient responsiveness to anti-PD1 and improves overall survival. This results were shown for both metastatic melanoma and neuroblastoma [192,199]. On the other hand DCs also affect NK cell responses in tumors. DCs produce IL-12 upon activation, that in its turn stimulates NK cells and T cells for anti-tumor responses [200]. Adhesion molecules such as CD112 and CD155

upregulated upon DC activation, interact with receptors CD96 and CD226 (or DNAX accessory molecule – DNAM – 1) expressed on NK cells. This interaction activates NK cells in the TME [201,202]. All these data together suggest that NK cells, DCs and T cells work together to kill tumors and NK cell-cDC1 axis is an important mechanism in regulation immune responses in tumor.

1.3 ACTIN CYTOSKELETON

Actin is a structural protein present in eukaryotic cells. Its monomeric globular form (G-actin) can polymerize and assemble into actin filaments (F-actin). When actin structures organize into a dynamic network, is named cell actin cytoskeleton. Cell shape and mobility is defined by the actin cytoskeleton. G-actin polymerization into F-actin filaments relies on actin nucleator activity. Actin-related protein 2/3 (Arp2/3) – **Figure 6** – and Formins are the most studied actin nucleators in hematopoietic cells [203–208]. Together with Spire [209], Leiomodins [210,211] and Cordon-bleu (COBL) [212], these account for the five classes of actin nucleators described to date.

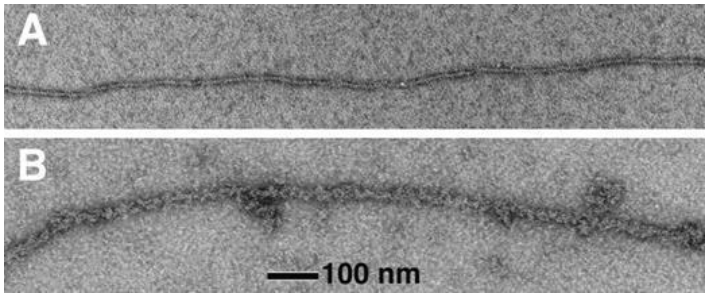

Figure 6. Electron micrographs of single, negatively stained actin filaments. Absence (A) and presence (B) of Arp2/3 complex. The diameter of actin filaments decorated with Arp2/3 complex is 24 ± 3 nm (n = 15), compared to 7.5 ± 0.8 nm (n = 15) for actin filaments alone. [204]

1.3.1 Formins

The role of formins in actin assembly was discovered in 2002 by Charles Boone's and David Pellman's groups [213,214]. Formins main function is to nucleate linear actin filaments from dimers of globular actin – **Figure 7**. Formins are a large family of proteins, being the first characterized formin is the yeast forming formin Bni1p [214]. Mammalian homologous Diaphanous-related (mDia)1-3 proteins were later discovered. Formin structure consists of a highly conserved carboxyl-terminal formin homology (FH)2 and a proline-rich FH1 domain. Most formins contain also a FH3 domain coupled with a GTPase binding domain (GBD) in the amino-terminal. Additionally, their structure can contain a WASp homology (WH)2 domain or a Dia autoregulatory domain (DAD). Functionally, the FH1 domain recruits profilin-actin complexes while FH2 initiates filament assembly. Profilin catalyzes the exchange of adenosine diphosphate (ADP) for adenosine triphosphate (ATP) in actin, increasing actin polymerization rate [215]. FH2 remains attached to the actin filament fast-growing barbed end, protecting it from

capping proteins and allowing rapid assembly of actin monomers [208,216]. Formins are activated by the binding of small Rho GTPases, such as RhoA/B/C and Cdc42 to the GBD [211]. Formins participate in the formation of several actin structures such as filopodia [217–220], lamellipodia [218], stress fibers [221,222], microtubules [223–225], adherens junctions [226,227] and phagocytic cup [228,229].

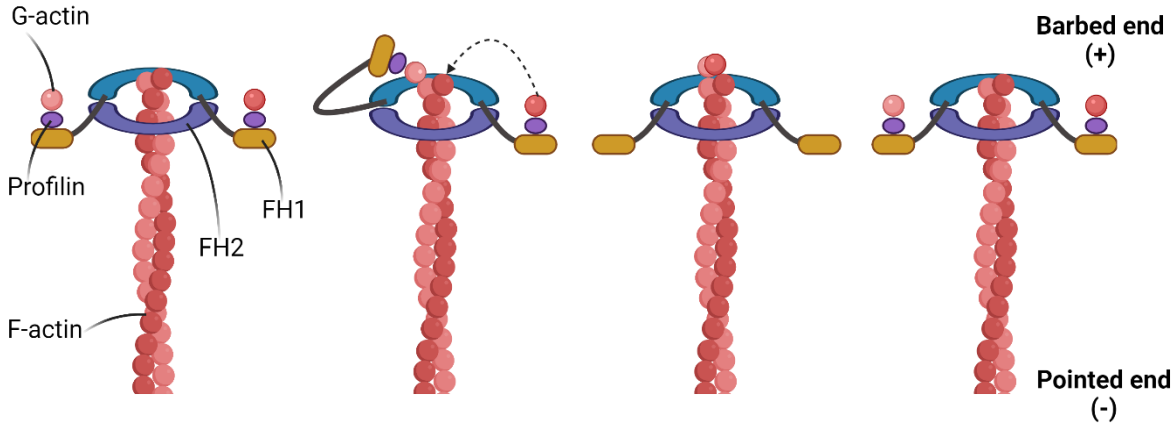

Figure 7. Formin-mediated actin polymerization. Image created with BioRender.com

1.3.2 Actin-related protein 2/3

Actin-related protein 2/3 (Arp2/3) complex structure and activity was first described in 1997 by Thomas Pollard. The Arp2/3 complex comprises seven subunits including the actin-related Arp2 and Arp3. The complex is also formed by five other polypeptides with molecular weight of 40, 35, 19, 18 and 14 kD named p40 (or ARPC1), p35 (or ARPC2), p19 (or ARPC3), p18 (or ARPC4) and p14 (or ARPC5) respectively. Both Arp2 and Arp3 subunits are similar to actin with sequence identity of 40-50% and 30-40%, respectively [204]. The Arp2/3 complex assembles G-actin into branched actin filaments and is activated by Wiskott-Aldrich Syndrome protein (WASp) family [211,230,231].

1.3.3 Wiskott-Aldrich Syndrome protein (WASp)

The Wiskott-Aldrich syndrome protein (WASp) is the first described member of an actin regulator family in hematopoietic cells implicated in actin polymerization, control of cell migration, adhesion, cell-cell interaction and intracellular signaling – **Figure 8**. WASp is a 501 amino acid protein, and its structure comprises a carboxyl-terminal with a verpolin homology (V) domain, a cofilin homology (C) domain and an acidic region (A) – named VCA [232]. Besides the VCA region, the WASp domain structure contains a polyproline rich region that binds to SRC Homology (SH)3 domain that contains the actin-binding protein profilin [233]. This domain is therefore responsible for helping the Arp2/Arp3 complex in G-actin recruitment to produce a new actin filament [234]. The new filament is synthesized with a 70 degrees angle from the already existing filament [235]. Upstream of the polyproline region, there is the GBD where it binds the rho family GTPase cell division cycle 42 (Cdc42) and the transducer of Cdc42-

dependent actin assembly 1 (TOCA1) [236]; a basic region (BR) that interacts with phosphatidylinositol 4,5-bisphosphate (PIP2); and WASp homology 1 (WH1) domain, that when bound to the WASp-interaction protein (WIP) stabilizes WASp auto-inhibited configuration. Cdc42-GTP is the main WASp activator. It binds to the GBD causing allosteric release of the VCA region from this domain. However, WASp activation is a synergy between Cdc42 and PIP2 [237,238].

Besides WASp, the WASp family includes neuronal (N)-WASp [239], suppressor of the cyclic AMP receptor (SCAR) 1–3, also known as WASp-family verprolin-homologous protein (WAVE) [240–243], WASp and SCAR homolog (WASH) [244], WASp homolog associated with actin, membranes, and microtubules (WHAMM) [245] and junction-mediating and regulatory protein (JMY) [246]. The WAVE/SCAR complex remains inactive in steady-state and can be activated by interaction with GTP-bound Rac1/Rac2 [247]. Important to mention that WASp and N-WASp are homologous proteins and the VCA domain is highly conserved among the WASp family members.

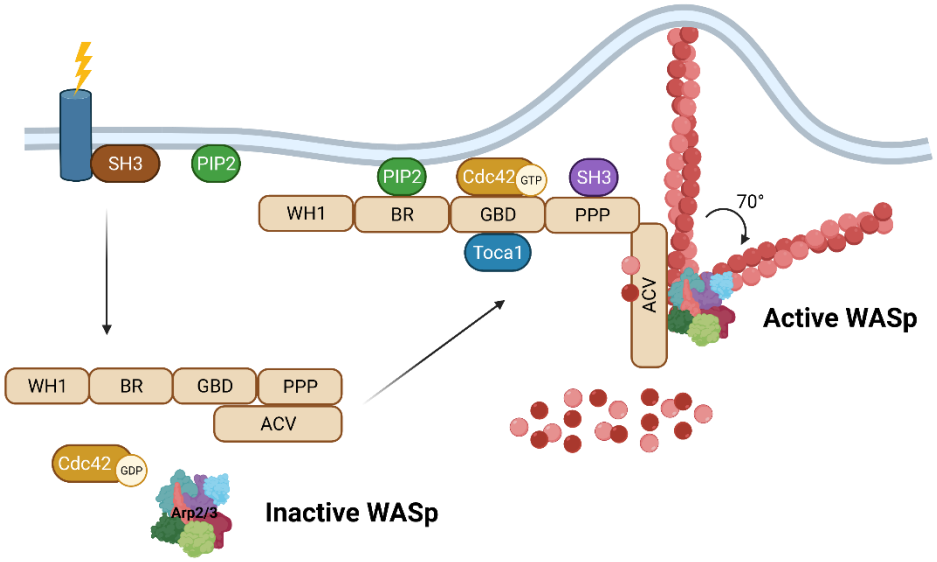

Figure 8. Domain structure of WASp. At steady-state, WASp remains in an inactive conformation. WASp is activated by the binding of Cdc42-GTP to the GBD domain, causing allosteric release of the VCA domain. VCA domain is now available to the Arp2/3 complex binding that recruits monomeric actin to assemble actin filaments. Image created with BioRender.com

1.3.4 Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) in an X-linked immunodeficiency characterized by loss-of-function mutations in WASp – **Figure 9**. Phenotypically, WAS patients are susceptible to develop eczema, autoimmune disease and malignancies of poor prognosis, and suffer from immunodeficiency with reduced ability to clear pathogens [248–255]. *WAS* gene is located on the short arm of the X chromosome at Xp11.22-p11.23 site [232]. Gain-of-function mutations in the GBD domain of WASp cause X-linked congenital neutropenia (XLN) – **Figure 9** – and are characterized by constitutively active protein expression [254,256–259]. XLN is considered an attenuated WAS-related syndrome, besides XLN patients suffer from severe neutropenia and

monocytopenia [259]. To date, it is reported four different mutations in WASp GBD domain that lead to XLN, including I290T, L270P, S272P and I294T reported in 18 patients from 5 families [256–258]. WASp is crucial for correct function of lymphocytes and, interestingly, WASp-deficient and XLN are often detected as opposing phenotypes. WASp-deficient B cells show reduced adhesion, migration, and homing, and a delayed humoral immune response in vitro culture [251]; WASp-deficient DCs and Langerhans cells show defects in redistribution to T-cell areas in the spleen after LPS challenge [260]; leukocyte directed migration is also impaired [261]. On the other hand, murine XLN neutrophils are hyperactive and with increased migration into tissues [259]; NK and T cells from XLN patients have increased granzyme B levels, as well as higher degranulation capacity and increased IFNγ production compared to control cells [262].

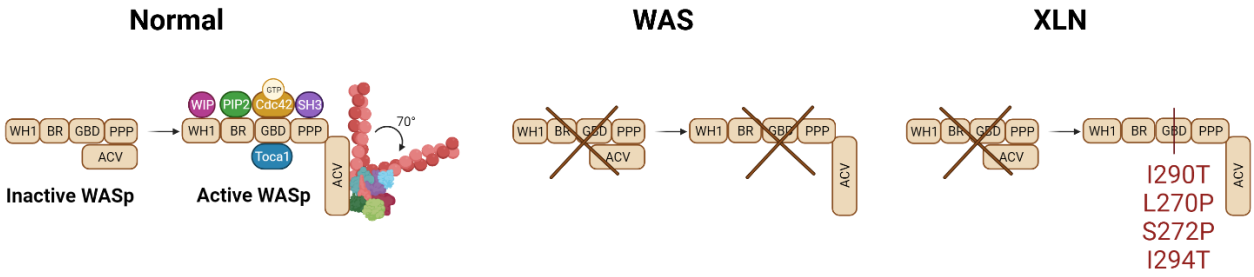

Figure 9. WASp-associated mutations. Cells from a normal patient have both inactive and active form of WASp; while WAS patients do not have neither form; XLN patients carry mutations in the WASp GBD domain leading to constitutively active protein. Mutations in GBD domain reported in XLN patients to date are I290T, L270P, S272P and I294T. Image created with BioRender.com

1.4 LESSONS FROM PRIMARY IMMUNODEFICIENCIES

Immunodeficiency disorders occur when the body's immune system is reduced or absent. Most cases of immunodeficiency are acquired due to extrinsic factors. In this case, it is called secondary immunodeficiency. When the cause of this deficiency is genetic, it is called primary immunodeficiency (PID). PIDs are a group of heterogeneous disorders characterized by defective immunity that leads to increased susceptibility to infections. These diseases are in general rare, severe, and in many cases lethal. The only curative treatment today is stem cell transplantation [263–266]. When it is not possible to find a match donor, other treatments can be done to alleviate the symptoms, including immunoglobulin therapy [267,268]; and cytokine therapy [269–271]. Determining the disease mechanism in patients and in animal models has revealed how the immune system functions in both health and disease. Besides learning more about rare and severe diseases, we can also translate this knowledge to other diseases. For instance, basic studies from autoimmune regulator (AIRE) protein animal models together with epidemiological studies from patients with monogenic disorder autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), significantly improved the understanding of immune mechanisms that avoid the development of autoimmune disease [272]. Studies from megakaryoblastic leukemia 1 (MKL1) deficiency or MKL(1/2)-dependent SRF-target genes allowed to better understand immune cell function, mainly on leukocyte adhesion and migration mechanisms [273]. Also, understanding the cause of one autoimmune disease can

help finding the mechanisms to treat other diseases with the same cause. Patients with autoimmunity in the hyper-IgM syndromes can be diagnosed with X-linked hyper IgM syndrome (XHIM) or hyper IgM syndrome type (HIGM). Besides all belonging to the same superfamily, patients show a wide range of symptoms [274]. Therefore, by studying one syndrome can give insights into other related syndromes.

2 RESEARCH AIMS

The overall aim of this study was to understand how WASp regulates DC and NK cell function and to apply findings for cancer therapy.

Specifically, the aims for each paper were:

Paper I – To examine WASp activity in DC migratory response and adhesion.

Paper II – To understand the implication of WASp in skin pathology and DC cross-presentation.

Paper III – To study inhibition of WASp function to promote cross-presentation and tumor killing.

Paper IV – To investigate the role of WASp in NK cell and T cell cytotoxic capacity in tumor killing.

3 MATERIALS AND METHODS

All methodology used in this study is described in the individual papers. However, I would like to better explain novel methods used.

3.1 MICROCHANNELS AND MICROPILLARS CHIP (PAPER I)

Microchannel chips were developed by Ana-Maria Lennon-Duménil and Matthieu Piel at the Curie Institute in order study immune cell migration in complex 3D environments that mimic a biological tissue. Seeing this as an opportunity to better study WASp-deficient and WASp overactive DCs, I did a 3-month internship in Dr Lennon-Duménil's laboratory. Our laboratory bred wildtype (WT), WASp knockout (KO) and WASp L272P mice (an XLN mouse model that corresponds to the human XLN WASP L270P) with LifeAct-green fluorescent protein (GFP) mice to be able to visualize the DCs under a fluorescent microscope; and DCs derived from these mice were used in these experiments.

A summary of the microchannel chips fabrication protocol is here explained [275]. The chip production has three major steps: 1. mask design; 2. wafer fabrication; and 3. chamber fabrication and assembly. The steps 1 and 2 require specialized machines and were therefore ordered to specific companies. For the chamber fabrication, we first prepared a mixture of uncured silicon rubber polydimethylsiloxane (PDMS) and curing agent at 10:1 ratio. This mixture was poured into an epoxy mold where the microchannels and micropillars are design (**Figure 10-A**). Each epoxy petri dish can contain more than one mold, so it is possible to produce several chips at the same time. Bubbles that formed during the mixing were removed using a vacuum bell jar (**Figure 10-B**). The molds were then placed in an oven at 65°C to allow PDMS curation. After this process, the chips are ready to be cut out from the mold and assembled (**Figure 10-C**). In each chip and using a core sample cutter, a hole where the cells were loaded was made (**Figure 10-D**). The chip is thoroughly cleaned using tape and ethanol before assembled on a glass-bottom petri dish. For the PDMS chip to adhere to the glass, both pieces were plasma activated (**Figure 10-E**). The chip is now ready to be coated with fibronectin and loaded with cells (**Figure 10-F**).

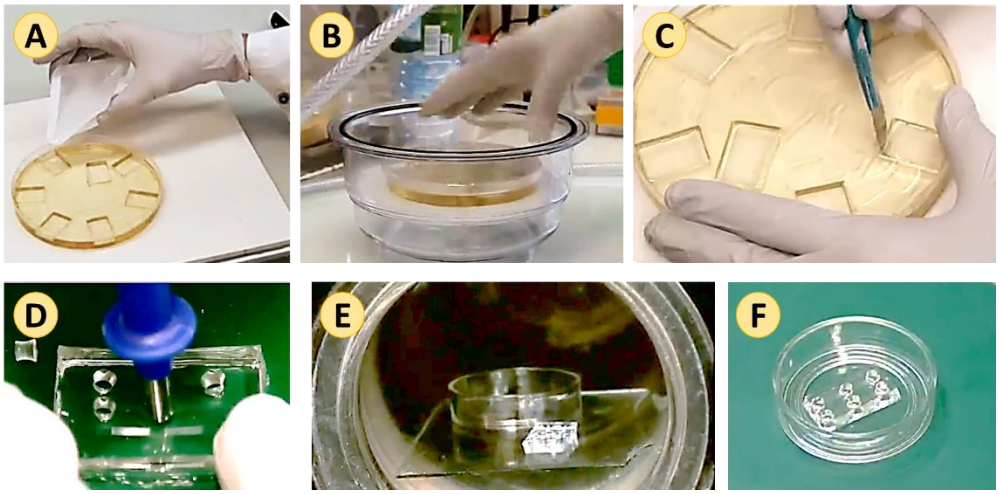

Figure 10. Microchannel and micropillar chip fabrication. A) PDMS polymer was poured into the customized epoxy mold; **B)** air bubbles are removed by incubation into a vacuum bell jar; **C)** chips are removed with the help of a surgical blade; **D)** holes for cell loading are made using a core sample cutter; **E)** chip and glass subtract are plasma activated and **F)** assembled. Images from [276].

Some technical issues are important to have in consideration when using the chips. One aspect is that the chip thickness needs to be just right. If the chip is too thick, it will be difficult to remain emerged in medium during the 16 hours of imaging acquisition; if it is too thin, the chip can collapse, preventing the cells to migrate. Collapse of the chips happens often, and this is not always noticed until we start the cell acquisition at the microscope. Due to the importance of comparing different genotypes or conditions in the same experiment, it is recommended to have extra chips with loaded cells ready to be used. In the chip-glass assembly step it is extremely important that both the chip and the glass are clean. Any dust will prevent chip adherence to the glass, resulting in cell leaking from the microchannel and random migration by the cell. This issue is also not always detected before cell acquisition. Cleaning the chip is especially important since dust or debris of PDMS can clog the channel causing the cell to migrate back. Not all these issues are easy to address when using this technique, however it is important to consider them in the data analysis.

3.2 DC-BEAD ASSAYS TO TRACK ANTIGEN PROCESSING (PAPER II AND PAPER III)

As mentioned before, DCs are professional phagocytes. This feature allows us to perform particulate assays on DCs to examine antigen fate. To better understand antigen processing and presentation, we have optimized assays in which aldehyde/sulfate 3 µm latex beads are coated with different probes. These assays were based on studies from Sebastian Amigorena's group [62,144]. To study phagosomal acidification, we coated ovalbumin-latex beads with pHrodo, a pH-sensitive probe that emits bright fluorescence at pH 5-3. To study ROS production, we coated latex beads with dihydrorhodamine 123 (DHR). DHR is non-fluorescent neutral indicator that senses free radicals. DHR becomes fluorescence in its cationic form after being oxidized by ROS. To investigate ovalbumin (OVA) degradation, we used the fluorogenic substrate for proteases DQ-OVA. DQ-OVA it is a self-quenched conjugate of OVA that emits fluorescence after proteolytic degradation. Increased DQ-ovalbumin mean fluorescence intensity indicates increased degradation. By using the 3 µm latex beads coupled sensor system, we can study a number of phagocytic characteristics. The results of acidification, ROS production and OVA degradation were assessed by flow cytometry. We consider this technique being robust since we can distinguish clearly, by flow cytometry, DCs containing different number of beads. According to FSC versus SSC parameters, we can distinguish DCs that did not phagocytized beads during the assay, DCs that phagocytized 1 bead, DCs that phagocytized 2 beads and onwards. We also used latex beads (not sensor coupled) to study Rac2 localization at the phagosome by microscopy. – **Figure 11**.

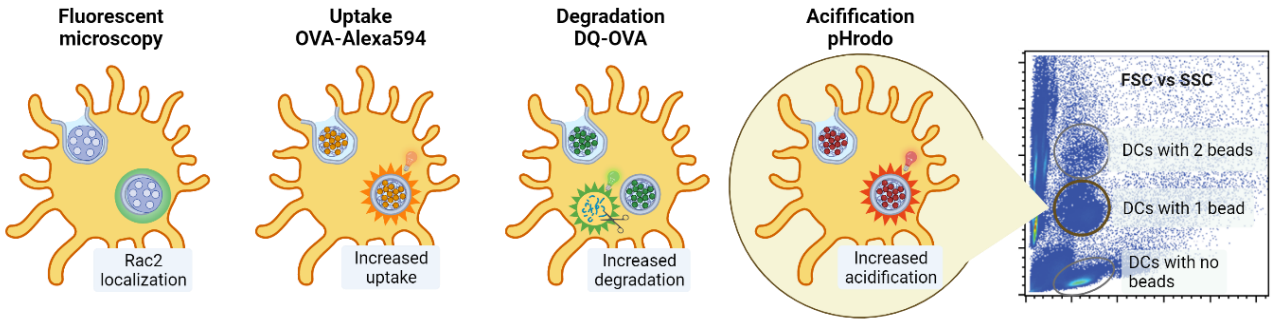

Figure 11. Latex-beads system coupled with different probes. Phagocytized beads reveal DC phagosome by microscopy allowing localization of phagosomal proteins; DC uptake can be quantified by OVA-Alexa594; OVA degradation by DC can be monitored by DQ-OVA quenching sensor; DC phagosomal acidification can be monitored by pHrodo probe. By flow cytometry, it is clearly seen different populations of DCs that phagocytized different amounts of beads. Image created with BioRender.com

3.3 TUMOR MODEL (PAPER III)

Tumor models often require ample optimization due to different growth rates of tumor cells, often having exponential growth rates such as the commonly used B16 melanoma tumor. Some tumors are more aggressive than others and that is a critical factor when choosing the working tumor model. The B16-mOVA tumor model that we set up in Paper III required some months of optimization. The protocol involves several steps, including subcutaneous tumor injection and subsequent tumor growth, OVA-primed CK666-treated or non-treated bone-marrow derived DCs (BMDCs) injection via footpad, and OT-I CD8 T cells injection intravenously. The idea behind this experiment was to mimic a real-life situation in which a patient goes to the hospital after realizing he/she has what can be a tumor, either because a lump can be palpated, or there are changes in the skin morphology; only after that, a treatment is applied. B16 melanoma is an aggressive tumor characterized by exponential growth, and for that reason, we aimed to inject the immune cells before the exponential phase of the curve. Considering that BMDCs were cultured for seven days, the tumor size needs to be predictable. I started by doing a B16-mOVA tumor titration in vivo. Different batches of tumors would grow at different rates in mouse. To be the most accurate when performing the experiment, I massively expanded several batches and used only one batch of tumor cells per testing. B16-mOVA tumor cells were titrated in at least 2 different experiments with 5 mice per group with concentrations ranging 50 000-300 000 cells. The right concentration was achieved when the tumors were approximately 0.1 cm^3 on day 14 of the protocol. Titration of DCs injected in the footpad was also done. Very low DC density or very high DC density injected into the footpad could not be detected in the draining LNs. We believe that high DC density creates a crowded area for the cells to be able to migrate. And last, anti-PD1 titration was also needed. Most protocols using anti-PD1 in cancer therapy, inject the anti-PD1 before or right after tumor injection. Since our aim is to treat the tumor after it is visible, the best time and concentration of anti-PD1 to be applied became a challenge. Anti-PD1 was titrated from 100-300 µg/mouse within the following settings: 1 anti-PD1 injection together with OT-I CD8 T cells; 2 injections with one week apart starting the day of OT-I CD8 T cell injection; 3 injections every 2 days starting the

day of OT-I CD8 T cell injection; and last 3 injections for 3 consecutive days starting the day of OT-I CD8 T cell injection. At least 5 mice/condition and 2 different experiments were performed. **Figure 12** represents the final optimized protocol to study tumor rejection using a DC-based vaccine combined with the Arp2/3 inhibitor CK666 and anti-PD1 as a checkpoint blockade inhibitor. In the future, other tumor models with a more stable growth and less complex aggressive can be used using the same protocol.

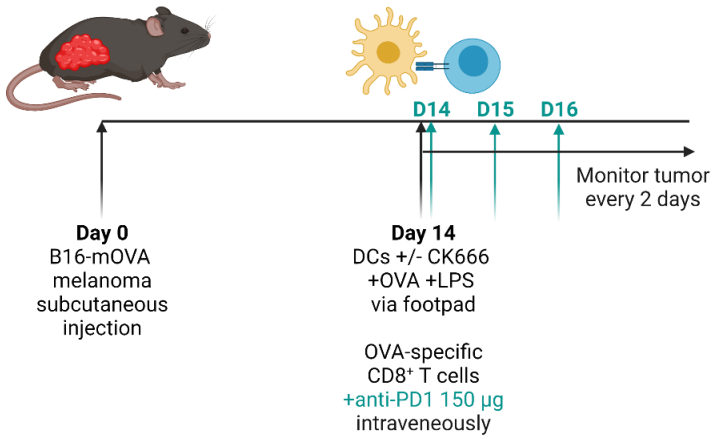

Figure 12. DC-based vaccination in B16-mOVA tumor model. Image created with BioRender.com

To be able to translate animal findings to human in a more physiological approach, we set up a model using the NOD-scid IL-2Rγ^{null} (NSG) mice – **Figure 13**. Preliminary data will be discussed in "Results and Discussion" section. The protocol used to humanize the mouse model is the following:

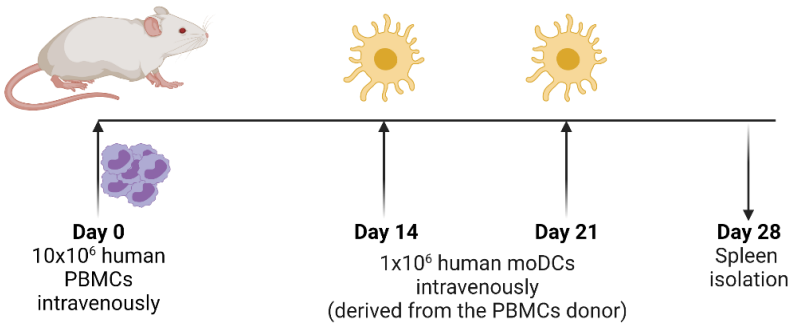

Figure 13. Humanized mouse model. Human peripheral blood mononuclear cells (PBMCs) were injected intravenously into NSG mice. MoDCs were differentiate for 7 days from the same donor PBMCs and injected on days 14 and 21 after PBMC engraftment. Mouse was sacrificed and spleen collected on day 28 for human cell reconstitution analysis. Image created with BioRender.com

3.4 ETHICAL CONSIDERATIONS

Research always raises ethical concerns. We try to find a balance between what is reasonable to do in research and the number of lives that we might save in the future. When working with animals, it is important to acknowledge that they are also living beings. Of course, if I do research, I am optimistic in finding some mechanism or treatment that will improve someone's

daily life. But it is important to reflect: “is it worth to sacrifice some lives to the benefit of other lives?” Maybe yes, maybe not. And that is the reason why a balance should be found.

Nowadays there are strict rules for animal research. To work with animals, it is needed an approved ethical permit from Jordbruksverket (the Swedish Agriculture Board) in which we specify several experimental parameters including purpose of the animal experiment, the choice of specie, breed and strain, severity of the experiment, endpoint of the experiment and a thoroughly description of the procedures. All parameters are written focusing on the animal welfare. Additional questions that we need to address are what benefits are expected from the experiment (what scientific progress will be achieved; how can people or animals benefit from the experiment?), what species should be used and the number of individuals? what are the expected adverse effects on the animals and what is the expected severity? The animals should be monitored by the researcher, animal facility staff and veterinary.

Apart from the ethical permit, it is important to consider the 3R’s policy: replace, reduce, refine. In fact, this rule should be applied in everything, even in our daily life in a laboratory. We should try to replace and reduce material that we use very often and refine our experiments to save time and money. According to the replacing rule, we should think if we can use other model than animals to perform our experiments. In my case, mouse is the most suitable species but, when possible, cell lines and in vitro experiments were used. However, it is currently impossible to recreate a complete immune response in vitro. To reduce the number of animals used, our group coordinate experiments. Very often it is possible to use control mice from one experiment for another experiment, use several organs from the same mouse and even split organs if not working with rare cell populations. Refine the methods and techniques that we use is extremely important so in vitro methods should be used when possible.

Mice are priceless for biomedical research due to the possibility of having many different genetically modified lines; also, animal facilities keep mice in pathogen-free environment, so the interfering effects of environmental impact on the immune response are minimal. Mice are priceless for biomedical research however, “is it worth to sacrifice some lives (mice) to save other lives (humans)?”

4 RESULTS AND DISCUSSION

4.1 WASP ACTIVITY REGULATES DC MIGRATORY BEHAVIOR AND PODOSOME FORMATION

The main function of a DC is to scan the environment for pathogens following migration to the draining LNs to start an immune response. DC migration is characterized by amoeboid type in which a cell leading edge is formed by actin polymerization followed by retraction of the cell rear due to myosin II-dependent contraction of the actin cytoskeleton. Different DC maturation status requires adjustments in the cytoskeleton; and impaired regulation of the actin cytoskeleton reflects in impaired DC migration to the draining LNs and consequently altered immune responses [277–280]. DCs that lack the actin regulator WASp display reduced directed migration and decreased podosomes formation. However, how different activation status of WASp influences DC migration and adhesion remains unknown. In **paper I** we aim to investigate how loss-of-function mutation and gain-of-function mutation in WAS affects DC migration behavior.

To understand how WASp regulates DC migration, we studied WT, WASp KO and WASp L272P LifeAct-GFP DC migration in microchannels for 16 hours. In this experimental setting, DCs are confined and can only migrate in one direction (1D); and due to channel size, only one DC at the time can enter the channel. DCs derived from WASp KO mice showed decreased F-actin front/back ratio. This can be interpreted as WASp KO DCs having higher content of F-actin in the cell rear or WASp KO DCs having less F-actin content at the front of the cell, compared to WT and WASp L272P DCs. Previous work shows that DCs patrolling the environment and searching for antigen decrease their migration speed at the same time that their actin pool is relocated to the front of the cell; on the contrary, when DCs migrate to the draining LNs, they increase migration speed and their actin content is relocated to the back of the cell [281]. We evaluated immature and LPS-activated DC migration average speed in microchannels. LPS-activated WASp KO DCs migrated at a higher average speed compared to WT and WASp L272P that migrated at similar average speed. Immature DCs migrated at similar average speed among phenotypes. Strikingly, WASp L272P DCs had an unusual migration pattern, compared to WT DCs. While DCs from WT and WASp KO mice migrated at a constant speed, WASp L272P DCs had moments of very high and moments of very low migration speed. Moreover, some DCs from WASp L272P mice would turn and migrate back, a behavior rarely seen in WT and WASp KO DCs. This behavior was quantified as migration speed fluctuations (Figure 1E and F).

To better study this phenomenon, we used the pillar area of the microchannel chip. In this region, cells can migrate in two directions (2D) through a pillar maze that mimics the intricate and complex geometry of interstitial matrices but controlled and accessed for imaging. Here, WASp KO DCs have increased average migration speed and path persistent compared to WT and WASp L272P DCs. Our lab recently showed that neutrophils from XLN mice have increased actin dynamics that results in hyperactivity and increased migration into tissues [259]. Surprisingly, in micropillar setting, DCs from WASp L272P mice had much reduced migration

displacement and increased cell roundness. We noticed that increased cell roundness is accompanied by other morphological changes in DCs. We observed that WT DCs and WASp KO DCs extend and retract their body during migration. On the contrary, DCs from WASp L272P mice seem to have higher number of small protrusions surrounding the whole cell body; and their migration behavior looks confused. Here we conclude that WASp-dependent actin structures are critical for DCs to perceive the environment and migrate in a directed way.

Due to all the migration defects observed in vitro from WASp L272P DCs, we tested whether DCs from WASp L272P mice also had impaired migration in vivo. We used an ear inflammation model where the TLR7 agonist imiquimod is applied epicutaneously on the mice ears [282]. Imiquimod induces Langerhans cells from the ear, characterized by $CD8^-$ $DEC205^+CCR7^+$, to migrate to the cervival LNs. Cell number was accessed 48 hours after imiquimod application and assessed by flow cytometry. Here, we wanted mainly to test the in vivo migration capacity of WASp L272P DCs. Using a similar model, we had previously showed that WASp KO DCs have impaired migration to the draining LNs [127]. Surprisingly, by flow cytometry, we found that WT, WASp KO and WASp L272P DCs were able to migrate in vivo upon inflammation at the same rate. As a complementary technique, we performed histology of the draining LNs from WT, WASp KO and WASp L272P mice after fluorescein isothiocyanate (FITC) painting. We found that besides DCs from all phenotypes being able to migrate in vivo, their localization within the LNs was different. While WT DCs entered the LNs, WASp KO and WASp L272P DCs were retained in the collagen capsule surrounding the LNs. As a result, quantification of the number of DCs inside the draining LNs was reduced in WASp KO and WASp L272P mice compared to WT mice. Due to different results in DC quantification using different techniques, we highlight the importance of histology as a complementary technique to flow cytometry. By flow cytometry, we were able to quantify total number of migratory DCs in the draining LNs due to the use of collagenase treatment. However, we lost the information that the migratory DCs from WASp KO and WASp L272P mice were not inside the draining LN.

To clarify the differences in DC migration in vitro and in vivo, we analyzed ultrastructural features of cell adhesion by interference reflection microscopy (IRM) and podosome formation by confocal microscopy after DC adhesion on fibronectin coated glass slides. WASp L272P DCs showed reduced cell area and reduced adhesion area compared to WT and WASp KO DCs. It is known that macrophages and DCs from WAS patients do not form podosomes and have impaired cell polarization [283,284]. Surprisingly, approximately half of the WASp KO DCs acquired were able to form podosomes. However, among the WASp KO DCs that form podosomes, only half formed normal podosomes whereas the other half formed ring-shaped podosomes or incomplete podosomes that resemble those in healthy immature osteoclasts [285]. Nearly all WT and WASp L272P DCs formed podosomes. Interestingly, WASp L272P DCs had enlarged podosome area. In conclusion, these data showed that WASp activity is critical for environment sensing and therefore directed migration. Moreover, we and others showed that WASp is essential in podosome formation of different types of immune cells. Reduction

in WASp L272P DCs cell area and adhesion area together with increased podosome area formation might be a compensation mechanism to support migration in vivo.

4.2 WASP-DEFICIENT DCS HAVE ENHANCED CAPACITY FOR CROSS-PRESENTATION DUE TO ACTIVATION OF RAC2 PATHWAY

According to the Genetic and Rare Diseases Information Center (GARD) funded partially by the National Institutes of Health (NIH), 80% of WAS patients have eczema that can be mild to severe. This can be due to accumulation of DCs in the skin that can lead to inflammatory processes. The development of eczema in WAS patients is not understood. In **paper II**, we mimicked an eczema-like phenotype in mouse by patching Der p 2, a major dust mite allergen from Dermatophagoides pteronyssinus, for 3 × 4 days on the back of shaved animals on week 1, 4 and 7 [286]. Fifty days later, a punch biopsy from inflamed skin was taken and analyzed. Treated WT mice showed increased epidermal thickness, also known as epidermal hyperplasia, while WASp KO mice had less epidermal hyperplasia with levels almost to the unchallenged mice. Epidermal sheets were prepared for histology and with the aim of discriminating different skin DCs subsets, $CD11c^+EpCAM^+$, $CD11c^+EpCAM^-Langerin^-$, or $Langerin^+$ populations were quantified. Der p 2 challenged WASp KO mice showed accumulation of DCs and $CD8^+$ T cells in the dermis compared to WT mice. By flow cytometry, dermal DC number was increased after Der p 2 challenge but $CD4^+$ and $CD8^+$ T cell populations were similar in unchallenged and challenged WT and WASp KO mice. Analysis of draining LNs and spleen showed a decreased $CD4^+/CD8^+$ T cell ratio in WASp KO mice. WASp KO mice also had increased effector/memory $CD8^+$ T cells, characterized by $CD44^{high}/CD62L^-$, in the spleen after Der p 2 challenge, associated with increased IFNγ production.

We next used an infection model to address WASp KO responses. Leishmania major (L. major) infection triggers Th1 responses in dermal macrophages that result in IFNγ production by $CD4^+$ T cells [287,288]. L. major was injected intradermally in both ears and animals were sacrificed 2 or 6 weeks later. Ears and retromaxillar dLNs were collected for analysis. By the images on Figure 3a, it is possible to see that after 6 weeks of L. major infection, the ears of WT and WASp KO mice were inflamed and, in case of WASp KO mice, also bleeding. Ear analysis by flow cytometry showed MHC class II^{hi} DCs, $CD4^+$ T cells and $CD8^+$ T cells infiltration in WT and WASp KO mice, however, WASp KO mice had a significant lower number of cells compared to WT mice. The same tendency was found in the draining LNs. $CD4^+/CD8^+$ T cell ratio was much decreased in the draining LNs in both control and L. major infected mice. Surprisingly, after 6 weeks of infection, WASp KO mice had increased number of IFNγ-producing $CD4^+$ T and $CD8^+$ T cells compared to WT mice.

To understand why there was an increase of $CD8^+$ T cells in WASp KO mice, we used the Cre/loxP system to generate a mouse model where WASp was deleted in $CD11c^+$ cells, mostly corresponding to the DC population – referred to as DC/cWKO mice. Results showed that LN and spleen from DC/cWKO had decreased $CD4^+$ T cells number and increased $CD8^+$ T cells

number. Additionally, the effector/memory $CD8^+$ T cell population was increased in spleen and LN of DC/cWKO mice. Together, this data suggested that $CD11c^+$ WASp-deficient DCs induced increased number and activation of $CD8^+$ T cells.

To understand if WASp KO DCs had superior capacity of cross-presentation, we performed co-culture in vitro experiments with enriched $CD8\alpha^+$ and $CD8\alpha^-$ DCs from Flt3L tumor-injected mice and OT-I CD8 T cells that specifically recognize the SIINFEKL peptide, derived from OVA. First, we measured if increased $CD8^+$ T cells were directly related with SIINFEKL presentation on MHC class I molecules, but OT-I CD8 T cell proliferation induced by $CD8\alpha^+$ and $CD8\alpha^-$ DCs was similar among WASp KO and WT DCs. Second, we incubated $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs from WT and WASp KO mice with OT-I CD8 T cells and full-length OVA. Results showed an increased capacity of $CD8\alpha^-$ DCs from WASp KO mice to induce $CD8^+$ T cell proliferation, compared to WT DCs. As mentioned in the introduction, for efficient antigen cross-presentation, the phagosomal pH needs to be kept neutral [62]. To measure DC phagosomal pH, we coupled OVA with the pH sensor pHrodo (OVA-pHrodo), that emits fluorescence at low pH. By flow cytometry, we showed that $CD8\alpha^-$ DCs from WASp KO mice had decreased phagosomal acidification compared to WT DCs at different incubation time points. No difference was detected between WT and WASp KO $CD8\alpha^+$ DCs. Carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-I CD8 T cells also proliferated more in the presence of WASp KO $CD8\alpha^-$ DCs, compared to WT $CD8\alpha^-$ DCs.

It is known that the small GTPase Rac2 is critical for antigen cross-presentation capacity of $CD8^+$ DCs [62]. In the presence of Rac2, the production of ROS, more specifically superoxide anion radical ($O2\cdot^-$), in the DC phagosome increases and neutralizes the protons from the V-ATPase pump. This mechanism maintains a neutral phagosomal pH and favors cross-presentation [62]. By microscopy and flow cytometry, we showed that Rac2 levels were increased in $CD8\alpha^-$ WASp KO DCs. ROS production, measured by the probe DHR was also increased in $CD8\alpha^+$ and $CD8\alpha^-$ WASp KO DCs.

To confirm that decreased acidification, increased cross-presentation capacity, and $CD8^+$ T cell proliferation was due to WASp activity, we transfected WASp WT-GFP into WASp KO DCs. Successfully transfected DCs rescued the WT phenotype as seen by increased acidification and decreased OT-I CD8 T cell proliferation. The same results were showed with DCs transfected with WASp lacking the VCA domain (WASpΔVCA).

This study showed that DCs that lack WASp had decreased phagosomal acidification that resulted in increased capacity of cross-presentation which in turn increases $CD8^+$ T cell proliferation, compared to DCs that contain WASp. We also showed an accumulation of different DC subsets and $CD8^+$ T cells in the dermis of WASp KO mice. Studies show that Langerhans cells from WASp-deficient mice have compromised emigration from the skin after oxazolone-induced contact hypersensitivity [289]. Additionally, WASp-deficient DCs have impaired migration capacity and reduced $CD4^+$ and $CD8^+$ T cell priming ability [289]. For that reason, WASp is seen as the master regulator of these responses. In health, Langerhans cells and $CD103^+$ DCs in the skin had increased capacity of cross-presentation to cytotoxic T cells.

Together this could explain the eczema pathology in WAS patients where accumulation of skin DCs and $CD8^+$ T cells may induce skin pathology. Other mutations can regulate DC capacity for cross-presentation – **Figure 14**. Rac2 GTPase is needed for NADPH complex function. Rac2-deficient and gp91phox (a NADPH complex subunit)-deficient $CD8\alpha^+$ DCs have decreased ROS production that leads to increased phagosomal acidification, decreased antigen cross-presentation and lower $CD8^+$ T cell proliferation [62,144]. The same applies to BMDCs with mutated endoplasmic reticulum molecule UNC93B [290]. Our study suggested that WASp activity in healthy, downregulated cross-presentation to avoid $CD8^+$ T cells over proliferation. Together, higher phagosomal pH in DCs that lack WASp can also be interpreted as a compensatory mechanism by N-WASp or WAVE2. Compensatory mechanisms among WASp family members have been seen in B, T and NK cells lacking WASp [291–296].

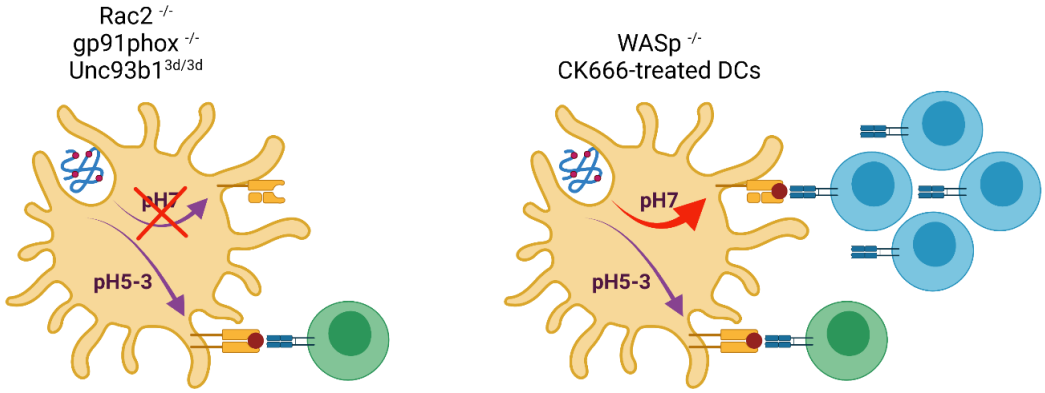

Figure 14. Mutations in actin cytoskeleton regulators affect DC cross-presentation. Rac2 -/-, gp91phox -/- and Unc93b1 3d/3d abolishes DC cross-presentation. WASp -/- and CK666-treated DCs favors DC cross-presentation pathway. Image adapted from [297]. Image created with BioRender.com

Increased cross-presentation and therefore increased cytotoxic T cell proliferation by DCs is the final goal in cancer immunotherapy. Hence, we saw this study as a concept to investigate a mechanism that could be applied in tumor therapy.

4.3 THE USE OF ARP2/3 INHIBITOR CK666 ACTIVATES CROSS-PRESENTATION PATHWAY IN DCS FOR CANCER THERAPY

To date, reversible actin inhibitors, such as CK666, Wiskostatin or ML141, are used only to inhibit or destroy cancer cells [298–303]. DC being a professional antigen presenting cell, one would expect that they are good candidates for cancer immunotherapy. Several research groups invested in DC studies with the aim of better understanding a mechanism that induced tumor regression. However, and besides promising results in vitro, DC vaccines have been disappointing with a success rate of approximately 3% [304]. This is believed to be due to activation of CD4 regulatory T cells by DCs in vivo that have the adverse effect of tumor killing [305]. Nowadays, one promising treatment in tumor therapy is the use of autologous tumor-specific $CD8^+$ T cells from the patient. In **paper III** we aim to promote antigens into the cross-

presentation pathway in vitro by treating patient-derived DCs with WASp small molecule inhibitors.

Since WASp inhibitors are mainly reported for cell death, we first did an inhibitor titration for 12 hours, that would be the maximum time of DC exposure to the drug. DCs tolerated CK666 and Wiskostatin inhibitor with concentrations up to 40 µM of inhibitor. ML141 inhibitor was toxic to DCs even at low concentrations so it was discarded for further experiments. F-actin was reduced on treated DCs, as expected. Since the cross-presentation pathway and phagosomal acidification are tightly related, we next performed an acidification assay in combination and $CD8^+$ T cell proliferation assays to compared non-treated and CK666-treated DCs. CK666-treated DCs had considerable decreased acidification compared to non-treated DCs. This was accompanied with increased OT-I CD8 T cell proliferation when using 0,5 mg/ml OVA.

Since the final goal is to apply the treatment in human cells, we also tested if monocyte-derived DCs (moDCs) isolated from healthy donors blood or buffycoat, could tolerate the same amounts of CK666 and Wiskostatin. Indeed, CK666-treated moDCs showed decreased phagosomal acidification when compared to non-treated moDCs.

Activation markers are an indication of $CD8^+$ T cell efficiency. For this reason, we co-cultured BMDCs with OT-I CD8 T cells for 48 hours and measured CD69, IFNγ and IL-2 levels. CD69 is a c-type lectin receptor and is expressed during early activated T cells, while IFNγ and IL-2 are secreted upon T cell activation. We detected similar levels of CD69 and IFNγ production from T cells cultured with CK666-treated and non-treated BMDCs. However, IL-2 secretion was increased on T cells activated by CK666-treated DCs compared with non-treated BMDCs.

Since we wanted to translate this approach to in vivo models, we tested weather CK666-treated BMDCs would have impaired migration to the draining LN. We treated or not ex-vivo BMDCs derived from CD45.1 mice with 10 µM of CK666 and injected them into the footpad of CD45.2 mice. CK666-treated DCs were extensively washed before injection to ensure that the inhibitor would not interfere with other immune cells and with DC migration. 48 hours later, we collected the popliteal LNs and measured DC population. Results showed that CK666-treated BMDCs migrated at a normal rate compared to non-treated BMDCs. Interestingly, CK666-treated OVA-primed BMDCs induced increased OT-I CD8 T cell activation and proliferation in vivo, compared to non-treated BMDCs.

Finally, we wanted to test if increased OT-I CD8 T cell proliferation resulted in B16 melanoma tumor rejection in vivo. We used B16 melanoma cells that express ovalbumin on their membrane (B16-mOVA) and are recognized specifically by OT-I CD8 T cells. In summary, we injected B16-mOVA subcutaneously into mice and let it grow for 14 days before injecting CK666-treated or non-treated BMDCs via footpad. OT-I CD8 T cells were isolated and injected intravenously into the same recipient mice. Tumor measurement was assessed every two days. As result, we saw higher B16-mOVA rejection and prolonged survival of mice-bearing tumors after CK666 DC treatment. Together, our data suggested that the small Arp2/3

inhibitor molecule CK666 is a good candidate to activate the DC cross-presentation pathway in cancer therapy.

Cancer therapy is usually a combination of treatments. Among treatments used nowadays are surgery, chemotherapy, radiotherapy and immunotherapy. Patient-specific cancer immunotherapies have been given special importance due to the reduction of side effects and enhanced efficacy in tumor killing. The use of immune checkpoint blockades such as anti-CTLA-4 and anti-PD-1/PD-L1 antibodies have been found useful in cancer therapies [183,306,307]. We have started a set of experiments where we combine, using the same tumor model, CK666-treated DC injection together with anti-PD1 injection in mice. So far, results showed that anti-PD1 synergized with the CK666 inhibitor and enhanced B16-mOVA tumor rejection. We think that the possibility of using reversible actin inhibitors to modify DC function may improve DC-based cancer immunotherapy. Besides, several inhibitors can be used in combination, for instance CK666 and Wiskostatin together.

Since the main aim of this approach is the translation of DC-based vaccination to human cancer therapy, we started to set up a humanized mouse model to test the feasibility of the approach. The following data is preliminary and is not included in the manuscript.

NSG mice are very immunodeficient animals that carry several mutation including scid mutation that impairs the DNA repair complex protein Prkdc resulting in B and T cell deficiency; it also has a mutated IL-2Rγ that results in abolished NK cell function [308]. These mice have NOD background that results in reduced function of DCs and macrophages. NSG mice have a Sirpα polymorphism that has high affinity for human CD47, which results in better engraftment. Mice engraftment was done as the protocol above (methods section) and tested by using healthy donor PBMCs isolated from buffy coats or by using the leukapheresis fraction of a recovered melanoma patient, obtained from the Karolinska University Hospital. Commercially available leukopaks consist of approximately 50% T cells, 20% monocytes, 10% B cells, 10% NK cells, 3% granulocytes, and 3% hematocrit. We are not sure which type of cells and how many were in the leukapheresis fraction that we obtained for this experiment, but we were able to reconstitute the NSG mouse with human cells as seen in the **Figure 15**.

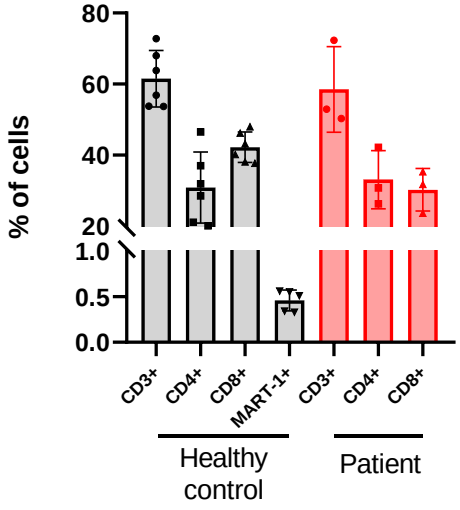

Figure 15. Human cell reconstitution after NSG engraftment. Total PBMCs from healthy donor (black bars) or leukapheresis fraction from a patient recovered from melanoma (red bars) were injected into NSG mice. Percentage of different immune cells in the spleen was assessed by flow cytometry. Results are pooled from 2 experiments with technical triplicates in healthy donor; and 1 experiment with technical triplicates in patient.

In our protocol, we also tested the priming of moDCs with MART-1 protein (melanoma-derived protein) and with the help of a custom-made tetramer, we could detect a percentage of MART-1-specific $CD8^+$ T cells. The protocol used was the following – **Figure 16**:

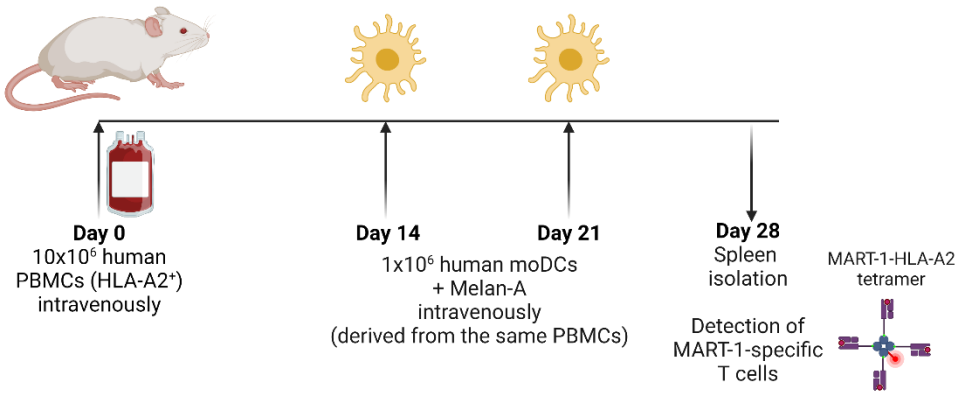

Figure 16. Melanoma specific T cells detected in humanized mouse model. Mice were reconstituted with total PBMCs positive for HLA-A2, followed by injection of MART-1-primed moDCs on days 14 and 21. On day 28 mice were sacrificed and spleen analyzed for the presence of MART-1 specific T cells. Image created with BioRender.com

4.4 XLN NK CELLS AND T CELLS HAVE INCREASED TUMOR KILLING CAPACITY

NK cells with WASp deficiency have reduced capacity of forming lytic immunological synapses and therefore impaired ability in tumor killing in vitro and in vivo [295,296,309–311]. However, tumor killing capacity of WAS patients NK cells can be corrected by IL-2 treatment [312,313]. XLN patients have low number of NK cells and the role of overactive WASp protein in tumor remains unknown [258]. In **paper IV** we aim to understand if XLN NK cells and cytotoxic T cells have altered tumor killing capacity.

We collected blood from a XLN family, including 2 brothers with the XLN mutation WASp L270P (corresponding to the mouse mutation WASp L272P), their mother and sister (mother/sister), and 2 healthy donors. PBMCs were isolated and analyzed by flow cytometry. The total NK cell population, defined by $CD3^-CD56^+$, was decreased in XLN patients, as well as $CD56^{dim}$ and $CD56^{bright}$, compared to the healthy donors. Total T cell percentage was increased in XLN patients, with $CD4^+$ T cell population being similar and $CD8^+$ T cell population being increased. A double positive cell population, characterized by $CD4^+CD8^{low}$ was seen in PBMCs from the XLN brothers. To examine the cytotoxic capacity of different cell populations, granzyme B content was measured. Granzyme B was increased in $CD56^{dim}$ NK cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+CD8^{low}$ T cells. ImageStream imaging flow cytometry on NK cells confirmed these data.

We studied the NK cell capacity to respond to stimulation, degranulation, and cytokine production from co-cultures of K562 myelogenous leukemia cells using isolated NK cells from the patients, their mother and sister and healthy controls. Patient NK cells stimulated with

phorbol 12-myristate 13-acetate (PMA)/ionomycin had increased IFNγ and CD107 production, compared to healthy controls. The same is showed for patient $CD4^+$ and $CD8^+$ T cells.

To investigate NK cell and T cells function in peripheral organs, we used the XLN mouse model carrying the WASp L272P mutation. Murine WASp L272P mutation corresponds to human L270P mutation [259]. WASp L272P NK cells had correct education in vivo compared to WT NK cells, according to inhibitory receptor expression. WASp L272P showed increased degranulation and IFNγ production upon activation of the receptors NKp46 and NK1.1, compared to WT NK cells. The lytic NK cell immunological synapse is characterized by actin reorganization with F-actin accumulation at the synapse interface and docking of granzyme B lytic granules that also accumulate at the synapse site [314]. To examine synapse formation, YAC-1 lymphoma cells were co-cultured with NK cell from WT, WASp KO and WASp L272P mice. YAC-1 cells:NK cells conjugates were analyzed by ImageStream imaging flow cytometry and results showed that WASp KO NK cells had impaired synapse formation while WASp L272P NK cells had normal synapse formation, compared to WT NK cells. Together the data showed that XLN NK cells are functional and show signs of hyperactivity, opposed to WASp-deficient NK cells.

We investigated how WASp L272P T cells respond to stimulus. T cells were stimulated either with plate-bound anti-CD3 and anti-CD28 antibodies or PMA/ionomycin and results showed similar degranulation and IFNγ production to WT T cells. To understand if WASp L272P T cells were able to accumulate F-actin at the synapse, we incubated $CD4^+$ and $CD8^+$ T cells with anti-CD3/anti-CD8-coated beads. Results showed that while $CD4^+$ and $CD8^+$ T cells from WASp KO mice had impaired synapse formation, $CD4^+$ and $CD8^+$ T cells from WASp L272P mice had normal synapse formation compared to WT cells. WASp L272P T cells also had similar capacity as WT T cells in TCR-mediated killing of A20 lymphoma cells.

To understand if WASp L272P NK and $CD8^+$ T cells are able to polarize F-actin and spread on coated glass slides, we used ligand-coated surfaces and quantitative confocal microscopy. We tested weather the cells would adhere to a simple positively charged glass surface, when coated with poly-L-lysine (PLL) or via intercellular adhesion molecule(ICAM)-1/leukocyte function-associated antigen (LFA)-1, important for efficient activation of T cells and NK cells [315,316]. Upon NKp46 activation, WASp KO NK cells showed reduced spreading while WASp L272P NK cells had similar spreading to WT NK cells. WASp L272P NK cells did not respond to ICAM-1 coated surface. Additionally, WASp L272P NK cells showed increased accumulation of adhesion and F-actin content towards glass lacking anti-NKp46. This suggested that WASp L272P NK cells had dysregulated actin dynamics during cell spreading and adhesion. Analyzing T cells responses, $CD8^+$ T cells from WASp KO mice spread less and accumulated less actin in response to ICAM-1 signaling. On the contrary, $CD8^+$ T cells from WASp L272P mice had increased spreading area and actin accumulation, compared to WT $CD8^+$ T cells, indicating altered actin dynamics in response to CD3/CD28 and LFA-1 signaling. In summary, the data suggested that constitutively activated WASp enhanced actin responses at lytic synapses formed by $CD8^+$ T cells but not NK cells.

Notably, WASp-deficient (WASp KO) and constitutively active WASp (WASp L272P) often have opposite phenotypes. Our group showed previously that mutations in WASp that result in constitutively active protein result, in vivo and in vitro, in increased actin dynamics and migratory capacity of neutrophils [259,317]. To study the competitive behavior of WASp L272P NK and T cells we used a bone marrow chimeric mouse model. In this model, CD45.1 WASp L272P/WASp KO cells and CD45.2 WT cells in a ratio 1:1 were injected into a lethally irradiated recipient mouse. NK cells and T cells from WASp L272P mice had a reconstitution advantage to WT cells while WASp KO cells showed a disadvantage. Spleenocytes of the recipient mice were analyzed. IFNγ production and granulation in response to NKp46 stimulation was accessed and results showed that WASp L272P spleenocytes had higher responding NK cells compared to WT spleenocytes. To better study the NK cell function in vivo, we did a competitive assay where a recipient mouse was injected with spleenocytes that express MHC class I molecule and spleenocytes that lack MHC class I molecule, named $\beta2m^{-/-}$ at a 1:1 ratio. $\beta2m^{-/-}$ mice have very little or any MHC class I molecules expression and very few $CD8^+$ T cells. By performing this competitive assay, we could study how the absence of MHC class I affects NK cells and their "missing-self" rejection capacity. To track the cells, WT and $\beta2m^{-/-}$ spleenocytes were labelled with different concentrations of CFSE. WASp L272P NK cells showed higher rejection capacity of $\beta2m^{-/-}$ cells, compared to WT NK cells, meaning they had higher capacity of "missing self" rejection.

To confirm the previous results in tumor-bearing mice, we used the B16 melanoma model, which cells have low expression of MHC class I molecules [318]. By examining B16 tumor growth among WT, WASp KO and WASp L272P mice, we showed that tumors injected in WASp KO mice grew much faster compared to tumors injected in WT mice, whereas tumors growing in WASp L272P mice grew slower. To address the role of NK cells in tumor control, we depleted NK cells from WT, WASp KO and WASp L272P mice by intraperitoneal injection of anti-NK1.1 antibodies. In WASp L272P mice that lack NK cells, the B16 tumor grew at the same rate as WT mice, suggesting that WASp L272P NK cells were important for tumor killing. $CD8^+$ T cells may be responsible for controlling tumor growth in WT mice, since WT mice had similar tumor size in non-depleted and NK cell depleted mice. By analyzing the tumors, we could detect a higher percentage of NK cells and $CD3^+CD8^+$ T cells in tumors from WASp L272P mice. Tumor-infiltrating WASp L272P NK cells showed higher CD69, indicating higher activation. Together, the data suggested that WASp L272P NK cells had high capacity to reject the aggressive B16 melanoma tumor.

In conclusion, given the question whether increased actin polymerization would permit T cells and NK cells in performing their cytotoxic functions, our data showed that this phenotype is beneficial for cytotoxic cell functionality.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, I have investigated the role of the actin cytoskeleton regulator WASp in DC migration and cross-presentation; Additionally, I have explored how WASp activity regulates cytotoxic DC and NK cell function in tumor killing. The main conclusions are here summarized.

In **paper I**, we used the innovative microchannel and micropillar system to study DC migration in vitro. By using DCs from LifeAct-GFPxWT, LifeAct-GFPxWASp KO and LifeAct-GFPxWASp L272P mice, we were able to study actin dynamics in vitro in a model resembling 3D migration in vivo. Here we found that WASp KO DCs migrated faster compared to WT DCs, however, WASp L272P DCs had a different behavior resembling of "confused" DCs. In vivo migration stimulated by an inflammatory response, WT, WASp KO and WASp L272P migrated to the draining LNs at the same extent. Deeper analysis by fluorescence microscopy on the draining LNs of TLR7 agonist-treated mice, revealed that while WT DCs entered the LNs, the WASp KO DCs and the WASp L272P DCs were stuck in the LN capsule and very little DCs managed to enter the LNs. By combining IRM with fluorescence microscopy on DCs attached to fibronectin coated slides, we found differences in podosome formation. While WT DCs formed normal podosomes, WASp KO formed ring-like podosomes resembling of an immature state, and WASp L272P DCs formed more podosomes resulting in enlarged podosome area. We think that differences in podosome formation by DCs might be a mechanism to compensate for impaired migration capacity.

In **paper II**, we aimed to understand the mechanisms of eczema formation in WAS patients. It is thought that the eczema formation is a result of immune cells that accumulate in the patients' skin and induce inflammatory responses, however the mechanism is not understood. Here, we found that WASp-deficient DCs characterized by CD11c expression induced proliferation of $CD8^+$ T cells. Moreover, we found that this activation is Rac2 dependent. Rac2 maintains a neutral phagosomal pH that promotes the antigen into the cross-presentation pathway of WASp KO DCs.

In **paper III**, we applied the WASp-deficiency phenotype of increased cytotoxic T cell proliferation to tumor immunotherapy. By using the reversible Arp2/3 inhibitor CK666, we showed that DCs increase their phagosomal pH, shuttling the antigen into the cross-presentation pathway, resulting in increased cytotoxic T cells proliferation. $CD8^+$ cytotoxic T cell proliferation was specific for the antigen given to the DC, in our case, OVA. Additionally, activated $CD8^+$ T cells were able to delay melanoma growth observed by increased survival of CK666-DCs-treated mice.

In **paper IV**, we studied NK cell cytotoxic capacity to kill tumors. It is known that WASp-deficient NK cells have reduced ability to kill tumors due to impaired capacity of forming lytic immunological synapses. How overactive activity of WASp in cytotoxic cells respond to tumor killing was not known. By studying NK cells and T cells from a family of patients carrying a mutation that leads to XLN and animal models, we found that XLN NK cells and T cells are

hyperactive in many cytotoxic functions. Furthermore, XLN NK cytotoxic cells showed better rejection of B16 tumors. As a result, XLN phenotype may be beneficial for cytotoxic cell function.

Although WAS patient's life expectancy is 3.5 years if not received any treatment and 20 years if treated [319], short time actin remodeling in specific immune cells might be useful in cancer therapy. Cytoskeleton inhibitors are already being used in tumor therapy. The microtubule-depolymerizing agents dolastatin 10 and ansamitocin P3 remodel DCs cytoskeleton and improve anti-tumor immunity [297,320,321]. Dolastatin 10-treated DCs results in increased capacity in antigen uptake and migration to the tumor draining lymph nodes. The efficacy of the treatment is exacerbated by combination with checkpoint inhibitors anti-CTLA4 and anti-PD1 treatment. Ansamitocin P3 induces DC maturation that result in anti-inflammatory cytokine production such as IL-1β, IL-6 and IL-12, in vitro and in vivo. Treatment of patients with ansamitocin P3 led to DC-mediated activation of tumor infiltrating lymphocyte and MC38 colon adenocarcinoma rejection. Combination of anti-PD1 and anti-CTLA4 treatment enhanced tumor rejection function. Not being a cytoskeleton regulator inhibitor, but being already studied in human pancreatic cancer, calcipotriol, a vitamin D analogue, promotes anti-tumorigenesis [322]. Low levels of vitamin D are common in palliative cancer patients and are associated with increased opioid consumption by the patients to alleviate pain [323,324]. Although calcipotriol upregulates PDL-1 and reduces T cell effector function, it could be combined with actin inhibitors and used in cancer therapy.

Actin cytoskeleton remodeling can have innumerous outcomes. By temporarily modifying the actin cytoskeleton of cells involved in tumor therapy, we might enhance their efficacy. The inhibitors can be combined and, from our own experience, being used at very low concentrations. The best benefit of using this type of inhibitors is that we can use it when wanted and then wash the inhibitor away, not to influence necessary cytoskeletal rearrangements for in vivo migration for example. Nowadays, by using live-cell imaging, it is also possible to do high-throughput whole-cell screening to find new drugs candidates and test them in a efficient and automated way [325]. **Table 1** contains a list of actin inhibitors that might enhance DC-based vaccine therapy in cancer.

Table 1. Actin inhibitors that can be used in DC-cancer therapy. Adapted from [297].

Compound	Target	Mechanism
CK-636	Arp2/3	Binds to a pocket between Arp2 and Arp3.
CK-666		Stabilizes the inactive form of the complex by blocking movement of the Arp2 and Arp3 subunits into short pitch conformation.
CK-548		Allosterically destabilizes the short pitch Arp3-Arp2 interface by binding to a hydrophobic cleft in subdomain 1 of Arp3. **CK-548 and CK-869 use the same inhibition mechanism.**
CK-869		
CDDO-Im		Docked to Arp2/3 in the same hydrophobic pocket as CK-869. **CDDO-Im and CDDO-Me use the same inhibition mechanism.**
CDDO-Me		
Wiskostatin	N-WASp WASp	Binds within a pocket in the GBD regulatory domain that maintains WASp and N-WASp in an autoinhibited conformation.
187-1	N-WASp	Inhibits allosteric actin assembly induced by PIP2.

However, DCs do not work alone in tumor killing. As mentioned in the introduction NK cell and DCs work together in the tumor environment and this notion is becoming more and more important when creating cancer vaccines. Additionally, the discovery of immune checkpoint blockades, improved tremendously the cancer field. Aside their proved efficacy, we faced a surprising issue when searching this subject for our own research project. Several studies report checkpoint inhibitors injection, such as anti-PD1 and anti-CTLA4, immediately after or even before tumor inoculation [326–328]. In my opinion, this cannot be used as a DC-based vaccine protocol because it cannot be applied in a real-life situation.

During my PhD I learned how important it is to study rare primary immunodeficiencies. I also learned that many of the features that characterize one disease, are present in other diseases. By the end of my studies, Lisa, my supervisor, asked what would I do if I get an unlimited research grant and/or start a biotech company? And my answer is that I would definitely invest in immunodeficiencies, both primary and secondary. Everyone, every single person that I met outside the medical field have asked me why do I study such rare diseases? Well, people with rare diseases also deserve to live and have quality of life. Apart from what people think, I believe that with more investment in immunodeficiencies, we could have different and improved protocols to study the diseases and making the patient's life better. One of the reasons why is so difficult to study primary immunodeficiencies is because, being them so rare, patient material is also rare. Patient material is the limiting factor of our work and although we have animal models that mimic the disease, they not always mimic the phenotype of the disease. WASp KO mice do not develop spontaneous eczema, for instance. In our lab we are trying to Epstein-Barr virus (EBV) transform patient cells, so we could perform more tests with them. This type of procedure takes time to be optimized and needs trained personal. Collaborations are the best way so far to get access to as much patient material as possible. And that we have. Because primary deficiencies matter and the kids deserve to enjoy life. And we can learn so much from them!

6 ACKNOWLEDGEMENTS

My PhD and therefore this thesis would not be possible without the contribution of many people. In one way or another you supported me and helped me during these years. Once Theodore Roosevelt said: “Believe you can, and you are halfway there”. Thank you all for making me believe that I can!

I would like to start by thanking my supervisor, **Lisa Westerberg**. Thank you for being the great supervisor that you are but also the great person that you are. Thank you so much for accepting me as your student during my master’s degree when my English was not that great. Thank you for believing and trusting me. Thank you for always supporting me and being positive even when experiments didn’t go exactly as planned. Thank you for turning me into an independent and critic scientist. But also thank you for seeing how value it is for a student to go to workshops and conferences both in Sweden and abroad. I did so many activities and met so many people because you gave me the opportunity to do it. And it made me grow so much as a scientist but also as a person. Doing a PhD is like running a marathon and you were my inspiration to keep smiling until the end. Thank you! And some words to **Aki, Linn** and **Jack**, because a supervisor can’t be that good if the family doesn’t help. Thank you for being great hosts during lab/outside lab events.

A big thank you to my co-supervisors **Mikael Karlsson** and **Lena Ström** for being there whenever I needed you. Micke, thank you for all the scientific input into my projects and thank you for all the activities both groups did together. Boule competition is “the” event of the year, and your enthusiasm makes it even better. Lena, thank you for always being interested in my projects and asking how things were going. Many thanks for attending all my yearly follow-up and halftime seminars. It was very important to have your support when I was more nervous.

Thank you to my mentor, **Eduardo Villablanca** for being the nice and fun person you are. When I chose you as a mentor, you sent me an e-mail immediately to book a meeting/fika and I knew right away that you were the right person for the job. Even though we don’t meet very often to discuss science you are always available when I need you, including to be the chairperson during my dissertation defense.

Thank you to all **co-authors and collaborators**: **Stina and Rolf Kiessling, Shi Yong and Andreas Lundqvist, Xiaolei and Galina Selivanova**. Collaborating with you was a pleasure and I learned a lot!

Thank you to **Ana-Maria Lennon-Duménil** and her group for finding my project interesting and hosting me as a lab member for 3 months. **Hélène, Judith, Odile, Paolo, Zahraa, Maria Graciela, Claudia, Doriane and Mathieu**, thank you for making me feel at home. You were all so kind and I learned so much with you. You all made me like Paris even more.

To all the **WASp group members**: **Nik**, your enthusiasm about Portugal and Portuguese culture is incredible. Believe it or not, I learned a lot about my own country with you. Thank you for your interest and knowledge about keeping plants alive… I promise that I’m doing the

best I can to keep mine in good shape. Thank you for being so kind to me and always remember to bring non-chocolate candies and snacks to the lab; **Ming**, thank you for teaching me everything I know about B cells. Your enthusiasm and hard work are inspiring and working with you is a piece of cake. I will never forget the dumpling class at your home and how fun and delicious it became; **Julien**, thank you for being such a great colleague and helping me whenever I needed, mainly with microscopy and ImageJ analysis. Thank you and **Hélène** for always joining the outside lab activities and bring **Coralie** for extra entertainment; **Mezida**, your stories+laugh as one package are phenomenal. You are such a gentle person, and I will never forget all the good moments we had. You are always up for a party, and I like that; **Roberta**, my loyal partner for mousini experiments. Your personality gives me extra energy when doing long experiments and your willingness to help others is extraordinary. Remember that hard work pays off and you will soon be a FACS expert. You are the best in making fresh pasta and maybe… only maybe, you will be a future pasta granny; **Zoe**, you always join our lab meetings even from the other side of the world. You are so knowledgeable in so many different types of cells and so many different techniques that I have the feeling that you have already worked with anything possible for an immunologist. Thank you for all the input and suggestions in my projects, they were really helpful; **Lia**, I know Brazilians are nice people, but you are extra nice. You are such a thoughtful and caring person and always remember important dates. Thank you for organizing the carnival parade "o bloquinho da Mariana" for my birthday and baking beijinhos specially for me; **Rhaissa**, thank you for all the good moments we had together. Your commitment and dedication to science is inspiring. Thank you for always asking if I need help with my experiments. Your only defect is that you don't gossip but maybe me and Lia can train you on that; **Rômulo**, the crossfit instructor, bartender and all-in-one person, thank you for keeping me active even in winter. You have so much energy both outside and inside the lab. You always have great suggestions of how to analyze data in different ways to make it more useful and understandable. To the Brazilian crew, please keep motivating Lisa for afterwork with caipirinha! **Pia**, your kindness and willingness to help others is incredible. Even though you are assistant professor, you are always open to listen to students' suggestions. I really admire your professional pathway and I'm very grateful to have met you; **Christian**, I think you did a great choice in joining the group and I'm sure you will like it. To all **former WASp group members**: **Marisa**, thank you for introducing me Sweden. I met you even before I moved here and after I arrived you really made sure that I felt welcome and comfortable. You were a great colleague and I'm happy to say that you are still a great friend. I learned so much from you and even received the DC project as heritage. We had so much fun together! Our burger dinners during long days/nights and our raporco experiment will always be remembered; **Carin**, thank you for being the nice person you are. Your commitment to science was incredible. I will never forget when we had so much fun at Roq bar and you let me stay at your place because I missed the last train home; **Jaime**, your straightforward personality is brilliant. Thank you for all the great moments we had and still have together. And my hair would not be the same if it was not braided by you; **Joanna**, the party girl, you are a very lovely and funny person. You taught me everything I know about flow cytometry and even thought we didn't manage to make B cells cross-present, we had a great time together. **Milind**, I always

saw you as a group member but working with Alzheimer disease instead of WASp. Thank you for all the fun moments and discussions we had. You cook amazing Indian food and the theme parties that you and Joanna organized were a must go; **Anton**, the person that heard all my health complains. Thank you for keeping everyone in the lab safe and sound. And because we were not tired of seeing each other all day, we often met at the supermarket by chance to talk about life with **Jenny** and Jonas; **Hanna**, you are such a sweet person and even thought you were busy taking care of your patients you found time to join my halftime dinner. It meant a lot to me so thank you; **Larissa**, my beauty queen. Your passion for science and enthusiasm to dive into new challenges is admirable. I miss our dinners together and I promise that whenever I travel to Brazil again, I will visit you. While this doesn't happen, videocalls work as good; **Marton**, the person that always plans five experiments at the time just in case one doesn't work. Your hyperactivity made me a little bit nervous when I first met you but knowing how much I learned with you, I'm only grateful for that. Thank you for keeping in touch and still having lunch together sometimes. To **Kathi and little Jonas**, one and half honorary group members that bring extra joy to Marton's life; **Adrià**, thank you for being a really nice person and be always willing to help others. I was sad that you left, but I hope you are doing what you like. To **all undergraduate and master students** with whom I learned so much.

To all the **Le Groupe members and former members**: **Chenfei**, you are such a nice and cheering person. Thank you for all the fun moments we still have together and for taking me to the hotpot Chinese restaurant; **Shan**, I love when you change hair color and that you are a fan of Mickey and Minnie – it gives a special vibe to the group. Besides being fun, you are super responsible and a real help in taking care of the Fortessa; **Polo**, your laugh just cheers my day. Thank you for being a positive and happy person**; Marit**, your sense of responsibility when we talk about serious things but also our funny conversations work just like a balance scale, and I really like it. Your hard work really inspires me; **Vanessa**, I will never forget all the fun moments and the trips we had. It was so fun when we went to Copenhagen but even funnier when we went together to Zurich. If we don't thrive in science, we will open a restaurant, right? **Silke**, we started approximately at the same time, I as a master student, you as a PhD student and I liked you immediately. You are a very easy-going person that is always up for dinner at Chili Masala or Asahi. I really miss all the things we used to do together. **Mitch**, it is super fun to be around you mainly because everything is fine for you. The parties that you and Silke organized were awesome and a great way of broking the routine; **Manasa**, although you were a postdoc, you always blend among the students because you are a very nice person. Thank you and **Arvind** for cooking delicious Indian food for us and for hosting us at your place. I bet **Arman** is so grown up right now; **Dhifaf**, thank you for all the expertise about monocytes, DCs and macrophages and for always helping me in translating Swedish letters and forms. Thank you **Mazin** for joining dinners at Bishop Arms; **Kiran**, thank you for being a really kind person; **Thomas**, thank you for being the more relaxed person in the lab, it kind of made me relaxed too (sometimes); **Amanda**, thank you for all the great moments at the MTC pubs. We had a lot of fun together.

To all the **members of National Clinical Research School in Chronic Inflammatory Diseases (NCRSCID): Helena Harris** and **Mia Olsson** for organizing an amazing school where I learned so much and had so much fun. To **Andy, Aida, Jonathan, Michael, Erik, Keying, Georgia, Emma, Jaime, Sara, Angeles, Yibo, Weiwei and Taotao**, thank you for two years of amazing moments and learning activities. I am so happy that we still keep in touch.

To **Gunilla Karlsson Hedestam** and **Jonathan Coquet** groups for being great neighbors: **Sharesta**, thank you for all the nice moments we had together and for always having something kind and smart to say; **Néstor**, for liking to dance as much as me and for always greeting me in Portuguese; **Leona**, for being a cool person and being a great dancer; **Ganesh**, for all the fun we had together when we had one-day teaching to 50+ students and all they could ask was where was the blood; **Móni**, thank you for keeping me company in late night experiments in the FACS room; **Marco**, thank you for doing a great job at MSA and for keeping me motivated at the gym in the morning; **Sanjana**, **Pradeepa** and **Uta**, thank you for always smiling and being nice to me; **Martin**, thank you for being my chairperson for yearly-follow up and for the interesting questions; **Junjie**, thank you for the help in finding antibodies in your fridge when I needed and for being a nice person; **Julian**, for always saying hi to me in the corridor and **Chris** for being a really nice person an easy to talk with.

To other friends that I got to know at Karolinska Institutet: **Vítor**, oh Vítor…thank you for all the fun moments we had together. We laugh so much about small and insignificant things until my belly starts to hurt; **Johanna**, thank you for being the best MSA president while I belong to it. I really like being around you and always have something nice to say. When you visit Stockholm, let's have a poke bowl; **Benedek**, thank you for being the nice and funny person you are. Keep your interest in cycling and kayaking to paradisiac places in Sweden; **Wesam**, thank you for all the great moments we shared at MSA; **Ali**, you were the very first person outside Lisa's group that I met. It was at Marisa's dissertation party and from that time on you always talk to me when we see each other. You are such a great person, and I was so happy that you even invited me to your dissertation party; **Christina**, thank you for being the nice person you are and for being so careful with everything. I really liked when you became part of Lisa's group, even if it was for a short time; **John**, thank you for all the fun things you say about Lisa and for allowing Christina to be part of our group; **Benedict**, thank you for all your funny jokes and for always being there to cheer us.

To all MTC administration, specially to **Åsa Belin**, **Gesan Arulampalam** and **Eva Noréns** for taking so good care of all PhD students and for helping with really anything. Thank you **Susanne Nylén** for both research input and help with my DC project and for taking care of the whole MTC department.

To all my **Portuguese friends** for always supporting me and finding time and space to meet me when I go to Portugal: **Ana** and **Diogo**, **Inês F., Inês A., Cláudia, João** – you are the best friends someone could have. To **Aline** that is not Portuguese, but it feels like that, thank you for hosting me when I visited Brazil and for making my 27th birthday very special.

To my **Swedish family**: **Anette, Staffan, Karin, Johanna, Julia** and **Kerstin**, a big, big thank you for receiving me with open arms and making me feel at home. **Jonas**, a huge thank for the 6 years we have been together. You are the best travel partner, the most understanding and calm person (someone needs to keep order when I become a little bit hysterical with small things) and a great friend. Thank you for feeding me during the time I was writing my thesis and forced me to take a 20 min daily walk to keep me sane.

Para a **minha família**: **mãe, pai, mano**, não tenho palavras para agradecer tudo o que vocês fizeram por mim e que me permitiu chegar onde cheguei. Obrigada por confiarem e apoiarem todas as minhas decisões, mesmo quando pareciam meio malucas, por exemplo quando eu disse com 3 meses de antecedência que ia viver na Suécia. **Marta**, obrigada por te juntares à família. O meu irmão é definitivamente uma pessoa melhor por te ter ao seu lado. Obrigada por desenhares a melhor capa de tese que alguém podia ter. Amo-vos do fundo do coracão!

This work was supported by a PhD fellowship from Fundação para a Ciência e a Tecnologia and European Social Fund.

7 REFERENCES

1. Goebel, A. *et al.* Passive transfer of fibromyalgia symptoms from patients to mice. *J. Clin. Invest.* **131**, e144201 (2021).

2. Brownstein, J. & Chitale, R. 10 Germy Surfaces You Touch Every Day. *ABC News Medical Unit* (2008).

3. Alberts, B. *et al.* Innate Immunity. in *Molecular Biology of the Cell* (Garland Science, 2002).

4. Janeway, C. A. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13 (1989).

5. Heguy, A., Baldari, C. T., Macchia, G., Telford, J. L. & Melli, M. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila Toll protein are essential for IL-1R signal transduction. *J. Biol. Chem.* **267**, 2605–9 (1992).

6. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. *Nature* **388**, 394–7 (1997).

7. Suzuki, T., Takagi, T., Furukohri, T., Kawamura, K. & Nakauchi, M. A calcium-dependent galactose-binding lectin from the tunicate Polyandrocarpa misakiensis. *J. Biol. Chem.* **265**, 1274–81 (1990).

8. Goubau, D., Deddouche, S. & Reis e Sousa, C. Cytosolic Sensing of Viruses. *Immunity* **38**, 855–69 (2013).

9. Yoneyama, M. *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**, 730–7 (2004).

10. Fritz, J. H., Ferrero, R. L., Philpott, D. J. & Girardin, S. E. Nod-like proteins in immunity, inflammation and disease. *Nat. Immunol.* **7**, 1250–7 (2006).

11. Aristizábal, B. & González, Á. Innate immune system. in *Autoimmunity: From Bench to Bedside* (El Rosario University Press, 2013).

12. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193–7 (2000).

13. Manz, M. G., Miyamoto, T., Akashi, K. & Weissman, I. L. Prospective isolation of human clonogenic common myeloid progenitors. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11872–7 (2002).

14. Wolf, A. A., Yáñez, A., Barman, P. K. & Goodridge, H. S. The ontogeny of monocyte subsets. *Front. Immunol.* **10**, 1642 (2019).

15. Geissmann, F., Jung, S. & Littman, D. R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71–82 (2003).

16. Auffray, C. *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science (80-.).* **317**, 666–70 (2007).

17. Yáñez, A. *et al.* Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity* **47**, 890–902 (2017).

18. Zigmond, E. *et al.* Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells. *Immunity* **37**, 1076–90 (2012).

19. Carlin, L. M. *et al.* Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. *Cell* **153**, 362–75 (2013).

20. Yona, S. *et al.* Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity* **38**, 79–91 (2013).

21. Hanna, R. N. *et al.* The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat. Immunol.* **12**, 778–85 (2011).

22. Mueller, U. G. *et al.* A clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic cells. *Science (80-.).* **311**, 83–7 (2006).

23. Wu, L., Li, C. L. & Shortman, K. Thymic dendritic cell precursors: Relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med.* **184**, 903–11 (1996).

24. Ardavin, C., Wu, L., Li, C. L. & Shortman, K. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362**, 761–3 (1993).

25. Wu, L. *et al.* Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood* **98**, 3376–82 (2001).

26. Martin, P. *et al.* Concept of lymphoid versus myeloid dendritic cell lineages revisited: Both CD8α- and CD8α+ dendritic cells are generated from CD4(low) lymphoid-committed precursors. *Blood* **96**, 2511–9 (2000).

27. Balan, S., Saxena, M. & Bhardwaj, N. Immunobiology of Dendritic Cells Part A. in *International Review of Cell and Molecular Biology* (eds. Lhuillier, C. & Galluzzi, L.) 1–68 (2019). doi:10.1016/s1937-6448(19)30015-2

28. Steinman, R. M. & Cohn, Z. A. Identification of a novel cell type in peripheral lymphoid organs of mice: I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137**, 1142–62 (1973).

29. Steinman, R. M. & Witmer, M. D. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5132–6 (1978).

30. Metlay, J. P. *et al.* The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* **171**, 1753–71 (1990).

31. Witmer-Pack, M. D., Olivier, W., Valinsky, J., Schuler, G. & Steinman, R. M. Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal langerhans cells. *J. Exp. Med.* **166**, 1484–98 (1987).

32. Bender, A., Sapp, M., Schuler, G., Steinman, R. M. & Bhardwaj, N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* **196**, 121–35 (1996).

33. Sallusto F & Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179**, 1109–18 (1994).

34. Vremec, D. *et al.* The surface phenotype of dendritic cells purified from mouse thymus and spleen: Investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med.* **176**, 47–58 (1992).

35. Vremec, D. & Shortman, K. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J. Immunol.* **159**, 565–73 (1997).

36. De Smedt, T. *et al.* CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo. *J. Leukoc. Biol.* **69**, 951–8 (2001).

37. Bedoui, S. *et al.* Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat. Immunol.* **10**, 488–95 (2009).

38. del Rio, M.-L., Rodriguez-Barbosa, J.-I., Kremmer, E. & Förster, R. CD103 − and CD103 + Bronchial Lymph Node Dendritic Cells Are Specialized in Presenting and Cross-Presenting Innocuous Antigen to CD4 + and CD8 + T Cells. *J. Immunol.* **178**, 6861–6 (2007).

39. Edelson, B. T. *et al.* Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8α+ conventional dendritic cells. *J. Exp. Med.* **207**, 823–36 (2010).

40. Ginhoux, F. *et al.* The origin and development of nonlymphoid tissue CD103+ DCs. *J. Exp. Med.* **206**, 3115–30 (2009).

41. Sung, S.-S. J. *et al.* A major lung CD103 (α E)-β 7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J. Immunol.* **176**, 2161–72 (2006).

42. Guilliams, M. *et al.* Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and

Species. *Immunity* **45**, 669–684 (2016).

43. Sancho, D. *et al.* Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* **458**, 899–903 (2009).

44. Dorner, B. G. *et al.* Selective Expression of the Chemokine Receptor XCR1 on Cross-presenting Dendritic Cells Determines Cooperation with CD8+ T Cells. *Immunity* **31**, 823–33 (2009).

45. Sichien, D. *et al.* IRF8 Transcription Factor Controls Survival and Function of Terminally Differentiated Conventional and Plasmacytoid Dendritic Cells, Respectively. *Immunity* **45**, 626–640 (2016).

46. Bagadia, P. *et al.* An Nfil3–Zeb2–Id2 pathway imposes Irf8 enhancer switching during cDC1 development. *Nat. Immunol.* **20**, 1174–1185 (2019).

47. Chandra, J., Kuo, P. T. Y., Hahn, A. M., Belz, G. T. & Frazer, I. H. Batf3 selectively determines acquisition of CD8 + dendritic cell phenotype and function. *Immunol. Cell Biol.* **95**, 215–223 (2017).

48. Carotta, S. *et al.* The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity* **32**, 628–41 (2010).

49. Luda, K. M. *et al.* IRF8 Transcription-Factor-Dependent Classical Dendritic Cells Are Essential for Intestinal T Cell Homeostasis. *Immunity* **44**, 860–74 (2016).

50. Watchmaker, P. B. *et al.* Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nat. Immunol.* **15**, 98–108 (2014).

51. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–8 (2001).

52. Akira, S., Hoshino, K. & Kaisho, T. The role of Toll-like receptors and MyD88 in innate immune responses. *J. Endotoxin Res.* **6**, 383–7 (2000).

53. Lauterbach, H. *et al.* Mouse CD8α+ DCs and human BDCA3+ DCs are major producers of IFN-λ in response to poly IC. *J. Exp. Med.* **207**, 2703–17 (2010).

54. Reis E Sousa, C. *et al.* In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J. Exp. Med.* **186**, 1819–29 (1997).

55. Yarovinsky, F. *et al.* TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science (80-.).* **308**, 1626–9 (2005).

56. Grohmann, U. *et al.* IL-6 Inhibits the Tolerogenic Function of CD8α + Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase. *J. Immunol.* **167**, 708–14 (2001).

57. Nair-Gupta, P. *et al.* TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation. *Cell* **158**, 506–21 (2014).

58. Yang, M. *et al.* Rab7b, a novel lysosome-associated small GTPase, is involved in monocytic differentiation of human acute promyelocytic leukemia cells. *Biochem. Biophys. Res. Commun.* **318**, 792–9 (2004).

59. Kretzer, N. M. *et al.* RAB43 facilitates cross-presentation of cell-associated antigens by CD8α+ dendritic cells. *J. Exp. Med.* **213**, 2871–2883 (2016).

60. Miller, J. C. *et al.* Deciphering the transcriptional network of the dendritic cell lineage. *Nat. Immunol.* **13**, 888–99 (2012).

61. Theisen, D. J. *et al.* WDFY4 is required for cross-presentation in response to viral and tumor antigens. *Science (80-.).* **362**, 694–699 (2018).

62. Savina, A. *et al.* The Small GTPase Rac2 Controls Phagosomal Alkalinization and Antigen Crosspresentation Selectively in CD8+ Dendritic Cells. *Immunity* **30**, 544–55 (2009).

63. Hildner, K. *et al.* Batf3 deficiency reveals a critical role for CD8α+ dendritic cells in cytotoxic T cell immunity. *Science (80-.).* **322**, 1097–100 (2008).

64. Tesfaye, D. Y., Gudjonsson, A., Bogen, B. & Fossum, E. Targeting conventional dendritic cells to fine-tune antibody responses. *Front. Immunol.* **10**, 1529 (2019).

65. Wang, H., Griffiths, M. N., Burton, D. R. & Ghazal, P. Rapid antibody responses by low-dose, single-step, dendritic cell-targeted immunization. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 847–52 (2000).

66. Bergamaschi, C. *et al.* Heterodimeric IL-15 delays tumor growth and promotes intratumoral CTL and dendritic cell accumulation by a cytokine network involving XCL1, IFN-γ, CXCL9 and CXCL10. *J. Immunother. Cancer* **8**, e000599 (2020).

67. Pilones, K. A., Charpentier, M., Garcia-Martinez, E. & Demaria, S. IL15 synergizes with radiotherapy to reprogram the tumor immune contexture through a dendritic cell connection. *Oncoimmunology* **9**, 1790716 (2020).

68. Janela, B. *et al.* A Subset of Type I Conventional Dendritic Cells Controls Cutaneous Bacterial Infections through VEGFα-Mediated Recruitment of Neutrophils. *Immunity* **50**, 1069–1083 (2019).

69. Iborra, S. *et al.* Optimal Generation of Tissue-Resident but Not Circulating Memory T Cells during Viral Infection Requires Crosspriming by DNGR-1+ Dendritic Cells. *Immunity* **45**, 847–860 (2016).

70. Alexandre, Y. O. *et al.* XCR1+ dendritic cells promote memory CD8+ T cell recall upon secondary infections with Listeria monocytogenes or certain viruses. *J. Exp. Med.* **213**, 75–92 (2016).

71. Joeris, T. *et al.* Intestinal cDC1 drive cross-tolerance to epithelial-derived antigen via induction of FoxP3+CD8+ Tregs. *Sci. Immunol.* **6**, eabd3774 (2021).

72. Annacker, O. *et al.* Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J. Exp. Med.* **202**, 1051–61 (2005).

73. Schlitzer, A. *et al.* IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. *Immunity* **38**, 970–83 (2013).

74. Gao, Y. *et al.* Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* **39**, 722–32 (2013).

75. Scott, C. L. *et al.* The transcription factor Zeb2 regulates development of conventional and plasmacytoid DCs by repressing Id2. *J. Exp. Med.* **213**, 897–911 (2016).

76. Persson, E. K. *et al.* IRF4 Transcription-Factor-Dependent CD103+CD11b+ Dendritic Cells Drive Mucosal T Helper 17 Cell Differentiation. *Immunity* **38**, 958–69 (2013).

77. Pool, L., Rivollier, A. & Agace, W. W. Deletion of IRF4 in Dendritic Cells Leads to Delayed Onset of T Cell–Dependent Colitis. *J. Immunol.* **204**, 1047–1055 (2020).

78. Laiosa, C. V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L. & Graf, T. Reprogramming of Committed T Cell Progenitors to Macrophages and Dendritic Cells by C/EBPα and PU.1 Transcription Factors. *Immunity* **25**, 731–44 (2006).

79. Fainaru, O. *et al.* Runx3 regulates mouse TGF-β-mediated dendritic cell function and its absence results in airway inflammation. *EMBO J.* **23**, 969–79 (2004).

80. Artis, D. *et al.* Dendritic Cell-Intrinsic Expression of NF-κB1 Is Required to Promote Optimal Th2 Cell Differentiation. *J. Immunol.* **174**, 7154–9 (2005).

81. O'Keeffe, M. *et al.* Distinct roles for the NF-κB1 and c-Rel transcription factors in the differentiation and survival of plasmacytoid and conventional dendritic cells activated by TLR-9 signals. *Blood* **106**, 3457–64 (2005).

82. Piper, C. *et al.* Pathogenic Bhlhe40+ GM-CSF+ CD4+ T cells promote indirect alloantigen presentation in the GI tract during GVHD. *Blood* **135**, 568–581 (2020).

83. Lewis, K. L. *et al.* Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* **35**, 780–91 (2011).

84. Pichlmair, A. *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates.

Science (80-.). **314**, 997–1001 (2006).

85. Papaioannou, N. E. *et al.* Environmental signals rather than layered ontogeny imprint the function of type 2 conventional dendritic cells in young and adult mice. *Nat. Commun.* **12**, 464 (2021).

86. Fekete, T. *et al.* Regulatory NLRs control the RLR-mediated type i interferon and inflammatory responses in human dendritic cells. *Front. Immunol.* **9**, 2314 (2018).

87. Garcias López, A. *et al.* Migration of murine intestinal dendritic cell subsets upon intrinsic and extrinsic TLR3 stimulation. *Eur. J. Immunol.* **50**, 1525–1536 (2020).

88. Wylie, B. *et al.* CD8+XCR1neg dendritic cells express high levels of toll-like receptor 5 and a unique complement of endocytic receptors. *Front. Immunol.* **9**, 2990 (2019).

89. Sharma, P., Levy, O. & Dowling, D. J. The TLR5 Agonist Flagellin Shapes Phenotypical and Functional Activation of Lung Mucosal Antigen Presenting Cells in Neonatal Mice. *Front. Immunol.* **11**, 171 (2020).

90. Flores-Langarica, A. *et al.* Intestinal CD103+CD11b+ cDC2 conventional dendritic cells are required for primary CD4+ T and B cell responses to soluble flagellin. *Front. Immunol.* **9**, 2409 (2018).

91. Marsman, C. *et al.* Plasmacytoid dendritic cell heterogeneity is defined by CXCL10 expression following TLR7 stimulation. *Immunol. Cell Biol.* **96**, 1083–1094 (2018).

92. Brown, C. C. *et al.* Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. *Cell* **179**, 846 (2019).

93. Heink, S. *et al.* Trans-presentation of IL-6 by dendritic cells is required for the priming of pathogenic T H 17 cells. *Nat. Immunol.* **18**, 74–85 (2017).

94. Hatscher, L. *et al.* Select hyperactivating NLRP3 ligands enhance the TH1-and TH17-inducing potential of human type 2 conventional dendritic cells. *Sci. Signal.* **14**, eabe1757 (2021).

95. Zhang, J. *et al.* Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood* **107**, 3600–8 (2006).

96. Blasius, A. L. *et al.* Bone Marrow Stromal Cell Antigen 2 Is a Specific Marker of Type I IFN-Producing Cells in the Naive Mouse, but a Promiscuous Cell Surface Antigen following IFN Stimulation. *J. Immunol.* **177**, 3260–5 (2006).

97. Wendland, M. *et al.* CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 6347–52 (2007).

98. Hadeiba, H. *et al.* CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. *Nat. Immunol.* **9**, 1253–60 (2008).

99. Niederquell, M. *et al.* Sca-1 expression defines developmental stages of mouse pDCs that show functional heterogeneity in the endosomal but not lysosomal TLR9 response. *Eur. J. Immunol.* **43**, 2993–3005 (2013).

100. Toyama-Sorimachi, N. *et al.* Inhibitory NK Receptor Ly49Q Is Expressed on Subsets of Dendritic Cells in a Cellular Maturation- and Cytokine Stimulation-Dependent Manner. *J. Immunol.* **174**, 4621–9 (2005).

101. Hadeiba, H. *et al.* Plasmacytoid Dendritic Cells Transport Peripheral Antigens to the Thymus to Promote Central Tolerance. *Immunity* **36**, 438–50 (2012).

102. Björck, P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* **98**, 3520–6 (2001).

103. Gilliet, M. *et al.* The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **195**, 953–8 (2002).

104. Scott, C. L. *et al.* The transcription factor Zeb2 regulates development of conventional and plasmacytoid DCs by repressing Id2. *J. Exp. Med.* **213**, 897–911 (2016).

105. Schotte, R. *et al.* The transcription factor Spi-B is expressed in plasmacytoid DC precursors and inhibits

T-, B-, and NK-cell development. *Blood* **101**, 1015–23 (2003).

106. Allman, D. *et al.* Ikaros is required for plasmacytoid dendritic cell differentiation. *Blood* **108**, 4025–34 (2006).

107. Dasgupta, S., Erturk-Hasdemir, D., Ochoa-Reparaz, J., Reinecker, H. C. & Kasper, D. L. Plasmacytoid dendritic cells mediate anti-inflammatory responses to a gut commensal molecule via both innate and adaptive mechanisms. *Cell Host Microbe* **15**, 413–23 (2014).

108. Arimura, K. *et al.* Crucial role of plasmacytoid dendritic cells in the development of acute colitis through the regulation of intestinal inflammation. *Mucosal Immunol.* **10**, 957–970 (2017).

109. Jin, O. *et al.* Abnormalities in circulating plasmacytoid dendritic cells in patients with systemic lupus erythematosus. *Arthritis Res. Ther.* **12**, R137 (2010).

110. Scott, J. L., Wirth, J. R., EuDaly, J. G., Gilkeson, G. S. & Cunningham, M. A. Plasmacytoid dendritic cell distribution and maturation are altered in lupus prone mice prior to the onset of clinical disease. *Clin. Immunol.* **175**, 109–114 (2017).

111. Nestle, F. O. *et al.* Plasmacytoid predendritic cells initiate psoriasis through interferon-α production. *J. Exp. Med.* **202**, 135–43 (2005).

112. Diana, J. *et al.* Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. *Nat. Med.* **19**, 65–73 (2013).

113. Birbeck, M. S., Breathnach, A. S. & Everall, J. D. An Electron Microscope Study of Basal Melanocytes and High-Level Clear Cells (Langerhans Cells) in Vitiligo**From the Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3, and the Departments of Anatomy, and Dermatology, St. Mary's Hos. *J. Invest. Dermatol.* **37**, 51–64 (1961).

114. Valladeau, J. *et al.* Langerin, a novel C-type lectin specific to langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12**, 71–81 (2000).

115. Iwama, A. *et al.* Reciprocal roles for CCAAT/enhancer binding protein (C/EBP) and PU.1 transcription factors in Langerhans cell commitment. *J. Exp. Med.* **195**, 547–58 (2002).

116. Hacker, C. *et al.* Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol.* **4**, 380–6 (2003).

117. Chopin, M. *et al.* Langerhans cells are generated by two distinct PU.1-dependent transcriptional networks. *J. Exp. Med.* **210**, 2967–80 (2013).

118. Sparber, F. *et al.* The Late Endosomal Adaptor Molecule p14 (LAMTOR2) Regulates TGFβ1-Mediated Homeostasis of Langerhans Cells. *J. Invest. Dermatol.* **123**, 217–27 (2015).

119. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H. & Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.* **206**, 2937–46 (2009).

120. Segura, E. & Amigorena, S. Inflammatory dendritic cells in mice and humans. *Trends Immunol.* **34**, 440–5 (2013).

121. Embgenbroich, M. & Burgdorf, S. Current Concepts of Antigen Cross-Presentation. *Front. Immunol.* **9**, 1643 (2018).

122. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12**, 557–69 (2012).

123. Delamarre, L., Pack, M., Chang, H., Mellman, I. & Trombetta, S. Differential Lysosomal Proteolysis in Antigen-Presenting Cells Determines Antigen Fate. *Science (80-.).* **307**, 1630–4 (2005).

124. Savina, A. *et al.* NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* **126**, 205–18 (2006).

125. Lennon-Duménil, A. M. *et al.* Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* **196**, 529–

40 (2002).

126. Savina, A. *et al.* The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* **30**, 544–55 (2009).

127. Baptista, M. A. P. *et al.* Deletion of Wiskott–Aldrich syndrome protein triggers Rac2 activity and increased cross-presentation by dendritic cells. *Nat. Commun.* **7**, 12175 (2016).

128. Kovacsovics-Bankowski, M. & Rock, K. L. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science (80-.).* **267**, 243–6 (1995).

129. Zehner, M. *et al.* The Translocon Protein Sec61 Mediates Antigen Transport from Endosomes in the Cytosol for Cross-Presentation to CD8+ T Cells. *Immunity* **42**, 850–63 (2015).

130. Ackerman, A. L., Giodini, A. & Cresswell, P. A Role for the Endoplasmic Reticulum Protein Retrotranslocation Machinery during Crosspresentation by Dendritic Cells. *Immunity* **25**, 607–17 (2006).

131. Saveanu, L. *et al.* IRAP identifies an endosomal compartment required for MHC class i cross-presentation. *Science (80-.).* **325**, 213–7 (2009).

132. Segura, E., Durand, M. & Amigorena, S. Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. *J. Exp. Med.* **210**, 1035–47 (2013).

133. Segura, E., Albiston, A. L., Wicks, I. P., Chai, S. Y. & Villadangos, J. A. Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 20377–81 (2009).

134. Shen, L., Sigal, L. J., Boes, M. & Rock, K. L. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* **21**, 155–65 (2004).

135. Bachmann, M. F. *et al.* TAP1-independent loading of class I molecules by exogenous viral proteins. *Eur. J. Immunol.* **25**, 1739–43 (1995).

136. Ruedl, C., Storni, T., Lechner, F., Bächi, T. & Bachmann, M. F. Cross-presentation of virus-like particles by skin-derived CD8- dendritic cells: A dispensable role for TAP. *Eur. J. Immunol.* **32**, 818–25 (2002).

137. Campbell, D. J., Serwold, T. & Shastri, N. Bacterial Proteins Can Be Processed by Macrophages in a Transporter Associated with Antigen Processing-Independent, Cysteine Protease-Dependent Manner for Presentation by MHC Class I Molecules. *J. Immunol.* **164**, 168–75 (2000).

138. Beers, C., Honey, K., Fink, S., Forbush, K. & Rudensky, A. Differential regulation of cathepsin S and cathepsin L in interferon γ-treated macrophages. *J. Exp. Med.* **197**, 169–79 (2003).

139. Samie, M. & Cresswell, P. The transcription factor TFEB acts as a molecular switch that regulates exogenous antigen-presentation pathways. *Nat. Immunol.* **16**, 729–36 (2015).

140. Alloatti, A. *et al.* Toll-like Receptor 4 Engagement on Dendritic Cells Restrains Phago-Lysosome Fusion and Promotes Cross-Presentation of Antigens. *Immunity* **43**, 1087–100 (2015).

141. Chatterjee, B. *et al.* Internalization and endosomal degradation of receptor-bound antigens regulate the efficiency of cross presentation by human dendritic cells. *Blood* **120**, 2011–20 (2012).

142. Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M. & Mellman, I. Activation of lysosomal function during dendritic cell maturation. *Science (80-.).* **299**, 1400–3 (2003).

143. Mantegazza, A. R. *et al.* NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood* **12**, 4712–22 (2008).

144. Savina, A. *et al.* NOX2 Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. *Cell* **126**, 205–18 (2006).

145. Idoyaga, J. *et al.* Specialized role of migratory dendritic cells in peripheral tolerance induction. *J. Clin. Invest.* **123**, 844–54 (2013).

146. Steinman, R. M., Hawiger, D. & Nussenzweig, M. C. Tolerogenic dendritic cells. *Annu. Rev. Immunol.*

21, 685–711 (2003).

147. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5**, 112–117 (1975).

148. Ljunggren, H. G. & Kärre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11**, 237–44 (1990).

149. Angelo, L. S. *et al.* Practical NK cell phenotyping and variability in healthy adults. *Immunol. Res.* **62**, 341–356 (2015).

150. Jiao, Y., Huntington, N. D., Belz, G. T. & Seillet, C. Type 1 innate lymphoid cell biology: Lessons learnt from natural killer cells. *Front. Immunol.* **7**, (2016).

151. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661–72 (1997).

152. Abel, A. M., Yang, C., Thakar, M. S. & Malarkannan, S. Natural killer cells: Development, maturation, and clinical utilization. *Front. Immunol.* **9**, 1869 (2018).

153. Rosmaraki, E. E. *et al.* Identification of committed NK cell progenitors in adult murine bone marrow. *Eur. J. Immunol.* **31**, 1900–9 (2001).

154. Cao, X. *et al.* Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity* **2**, 223–38 (1995).

155. Takeshita, T. *et al.* Cloning of the γ chain of the human IL-2 receptor. *Science (80-.).* **257**, 379–82 (1992).

156. Russell, S. M. *et al.* Interleukin-2 receptor γ chain: A functional component of the interleukin-4 receptor. *Science (80-.).* **262**, 1880–3 (1993).

157. Noguchi, M. *et al.* Interleukin-2 receptor γ chain: A functional component of the interleukin-7 receptor. *Science (80-.).* **262**, 1877–80 (1993).

158. Giri, J. G. *et al.* Identification and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor. *EMBO J.* **14**, 3654–3663 (1995).

159. Asao, H. *et al.* Cutting Edge: The Common γ-Chain Is an Indispensable Subunit of the IL-21 Receptor Complex. *J. Immunol.* **167**, 1–5 (2001).

160. Ferrini, S., Meazza, R., Azzarone, B. & Orengo, A. M. Role of common-gamma chain cytokines in NK cell development and function: Perspectives for immunotherapy. *J. Biomed. Biotechnol.* **2011**, (2011).

161. Delconte, R. B. *et al.* CIS is a potent checkpoint in NK cell-mediated tumor immunity. *Nat. Immunol.* **17**, 816–24 (2016).

162. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic Cells Prime Natural Killer Cells by trans-Presenting Interleukin 15. *Immunity* **26**, 503–17 (2007).

163. Alberts, B. *et al.* The adaptive immune system. in *Molecular Biology of the Cell* (2002).

164. Takeda, S., Gillis, S. & Palacios, R. In vitro effects of recombinant interleukin 7 on growth and differentiation of bone marrow pro-B and pro-T-lymphocyte clones and fetal thymocyte clones. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1634–8 (1989).

165. Godfrey, D. I., Kennedy, J., Suda, T. & Zlotnik, A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol.* **150**, 4244–52 (1993).

166. Godfrey, D. I., Kennedy, J., Mombaerts, P., Tonegawa, S. & Zlotnik, A. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8- thymocyte differentiation. *J. Immunol.* **152**, 4783–92 (1994).

167. Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* **308**, 149–53 (1984).

168. den Haan, J. M. M., Arens, R. & van Zelm, M. C. The activation of the adaptive immune system: Cross-talk between antigen-presenting cells, T cells and B cells. *Immunol. Lett.* **162**, 103–12 (2014).

169. Hintzen, R. Q. *et al.* Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J. Immunol.* **154**, 2612–23 (1995).

170. Chirathaworn, C. *et al.* Stimulation Through Intercellular Adhesion Molecule-1 Provides a Second Signal for T Cell Activation. *J. Immunol.* **168**, 5530–7 (2002).

171. Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* **22**, 333–40 (2010).

172. Guy, C. S. & Vignali, D. A. A. Organization of proximal signal initiation at the TCR:CD3 complex. *Immunol. Rev.* **232**, 7–21 (2009).

173. Shlnkal, Y., Ma, A., Cheng, H. L. & Alt, F. W. CD3 epsilon and CD3 zeta cytoplasmic domains can independently generate signals for T cell development and function. *Immunity* **2**, 401–11 (1995).

174. Bettini, M. L. *et al.* Cutting Edge: CD3 ITAM Diversity Is Required for Optimal TCR Signaling and Thymocyte Development. *J. Immunol.* **199**, 1555–1560 (2017).

175. Croft, M. Co-stimulatory members of the TNFR family: Keys to effective T-cell immunity? *Nat. Rev. Immunol.* **3**, 609–20 (2003).

176. Wiesel, M. *et al.* Type-I IFN drives the differentiation of short-lived effector CD8 + T cells in vivo. *Eur. J. Immunol.* **42**, 320–9 (2012).

177. Richer, M. J., Nolz, J. C. & Harty, J. T. Pathogen-Specific Inflammatory Milieux Tune the Antigen Sensitivity of CD8+ T Cells by Enhancing T Cell Receptor Signaling. *Immunity* **38**, 140–52 (2013).

178. Ben-Sasson, S. Z. *et al.* IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 7119–24 (2009).

179. Williams, M. A., Tyznik, A. J. & Bevan, M. J. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* **441**, 890–3 (2006).

180. de la Rosa, M., Rutz, S., Dorninger, H. & Scheffold, A. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur. J. Immunol.* **34**, 2480–8 (2004).

181. Wherry, E. J. *et al.* Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection. *Immunity* **27**, 670–84 (2007).

182. Driessens, G., Kline, J. & Gajewski, T. F. Costimulatory and coinhibitory receptors in anti-tumor immunity. *Immunol. Rev.* **229**, 126–44 (2009).

183. Leach, D. R., Krummel, M. F. & Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science (80-.).* **271**, 1734–6 (1996).

184. Iwai, Y. *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12293–7 (2002).

185. Iwai, Y., Terawaki, S., Ikegawa, M., Okazaki, T. & Honjo, T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J. Exp. Med.* **198**, 39–50 (2003).

186. Ali, T. H. *et al.* Enrichment of CD56dimKIR+CD57+ highly cytotoxic NK cells in tumour-infiltrated lymph nodes of melanoma patients. *Nat. Commun.* **5**, 5639 (2014).

187. Muntasell, A. *et al.* NK cell infiltrates and HLA class I expression in primary HER2 þ breast cancer predict and uncouple pathological response and disease-free survival. *Clin. Cancer Res.* **25**, 1535–1545 (2019).

188. Takanami, I., Takeuchi, K. & Giga, M. The prognostic value of natural killer cell infiltration in resected pulmonary adenocarcinoma. *J. Thorac. Cardiovasc. Surg.* **121**, 1058–63 (2001).

189. Villegas, F. R. *et al.* Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer* **5**, 23–8 (2002).

190. Soo, R. A. *et al.* Prognostic significance of immune cells in non-small cell lung cancer: Meta-analysis. *Oncotarget* **9**, 24801–24820 (2018).

191. Ishigami, S. *et al.* Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* **88**, 577–83 (2000).

192. Melaiu, O. *et al.* Cellular and gene signatures of tumor-infiltrating dendritic cells and natural-killer cells predict prognosis of neuroblastoma. *Nat. Commun.* **11**, 5992 (2020).

193. Truxova, I. *et al.* Mature dendritic cells correlate with favorable immune infiltrate and improved prognosis in ovarian carcinoma patients. *J. Immunother. Cancer* **6**, 139 (2018).

194. Chen, F. *et al.* Expression of the Chemokine Receptor CXCR3 Correlates with Dendritic Cell Recruitment and Prognosis in Gastric Cancer. *Genet. Test. Mol. Biomarkers* **22**, 35–42 (2018).

195. Cai, X. Y. *et al.* Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. *J. Cancer Res. Clin. Oncol.* **132**, 293–301 (2006).

196. Treilleux, I. *et al.* Dendritic cell infiltration and prognosis of early stage breast cancer. *Clin. Cancer Res.* **10**, 7466–74 (2004).

197. Albershardt, T. C. *et al.* Therapeutic efficacy of PD1/PDL1 blockade in B16 melanoma is greatly enhanced by immunization with dendritic cell-targeting lentiviral vector and protein vaccine. *Vaccine* **38**, 3369–3377 (2020).

198. Böttcher, J. P. *et al.* NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* **172**, 1022–1037 (2018).

199. Barry, K. C. *et al.* A natural killer–dendritic cell axis defines checkpoint therapy–responsive tumor microenvironments. *Nat. Med.* **24**, 1178–1191 (2018).

200. Garris, C. S. *et al.* Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-γ and IL-12. *Immunity* **49**, 1148–1161 (2018).

201. Bottino, C. *et al.* Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* **198**, 557–67 (2003).

202. Pende, D. *et al.* Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: Relevance for natural killer-dendritic cell interaction. *Blood* **107**, 2030–6 (2006).

203. Machesky, L. M., Atkinson, S. J., Ampe, C., Vandekerckhove, J. & Pollard, T. D. Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. *J. Cell Biol.* **127**, 107–15 (1994).

204. Mullins, R. D., Stafford, W. F. & Pollard, T. D. Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from Acanthamoeba. *J. Cell Biol.* **136**, 331–43 (1997).

205. Welch, M. D., Iwamatsu, A. & Mitchison, T. J. Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. *Nature* **385**, 265–9 (1997).

206. Evangelista, M. *et al.* Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science (80-.).* **276**, 118–22 (1997).

207. Faix, J. & Grosse, R. Staying in Shape with Formins. *Dev. Cell* **10**, 693–706 (2006).

208. Evangelista, M., Zigmond, S. & Boone, C. Formins: Signaling effectors for assembly and polarization of actin filaments. *J. Cell Sci.* **116**, 2603–11 (2003).

209. Quinlan, M. E., Heuser, J. E., Kerkhoff, E. & Mullins, R. D. Drosophila Spire is an actin nucleation factor. *Nature* **433**, 382–8 (2005).

210. Chereau, D. *et al.* Leiomodin is an actin filament nucleator in muscle cells. *Science (80-.).* **320**, 239–43 (2008).

211. Campellone, K. G. & Welch, M. D. A nucleator arms race: Cellular control of actin assembly. *Nat. Rev. Mol. Cell Biol.* **11**, 237–51 (2010).

212. Ahuja, R. *et al.* Cordon-Bleu Is an Actin Nucleation Factor and Controls Neuronal Morphology. *Cell* **131**, 337–50 (2007).

213. Pruyne, D. *et al.* Role of formins in actin assembly: Nucleation and barbed-end association. *Science (80-.).* **297**, 612–5 (2002).

214. Sagot, I., Rodal, A. A., Moseley, J., Goode, B. L. & Pellman, D. An actin nucleation mechanism mediated by Bni1 and profilin. *Nat. Cell Biol.* **4**, 626–31 (2002).

215. Pollard, T. D., Blanchoin, L. & Mullins, R. D. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 545–76 (2000).

216. Paul, A. & Pollard, T. The Role of the FH1 Domain and Profilin in Formin-Mediated Actin-Filament Elongation and Nucleation. *Curr. Biol.* **18**, 9–19 (2008).

217. Schirenbeck, A., Bretschneider, T., Arasada, R., Schleicher, M. & Faix, J. The Diaphanous-related formin dDia2 is required for the formation and maintenance of filopodia. *Nat. Cell Biol.* **7**, 619–25 (2005).

218. Yang, C. *et al.* Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *PLoS Biol.* **5**, e317 (2007).

219. Stastna, J. *et al.* Differing and isoform-specific roles for the formin DIAPH3 in plasma membrane blebbing and filopodia formation. *Cell Res.* **22**, 728–45 (2012).

220. Young, L. E., Heimsath, E. G. & Higgs, H. N. Cell type-dependent mechanisms for formin-mediated assembly of filopodia. *Mol. Biol. Cell* **26**, 4646–59 (2015).

221. Takeya, R., Taniguchi, K., Narumiya, S. & Sumimoto, H. The mammalian formin FHOD1 is activated through phosphorylation by ROCK and mediates thrombin-induced stress fibre formation in endothelial cells. *EMBO J.* **27**, 618–28 (2008).

222. Tominaga, T. *et al.* Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Mol. Cell* **5**, 13–25 (2000).

223. Eng, C. H., Huckaba, T. M. & Gundersen, G. G. The formin mDia regulates GSK3β through novel PKCs to promote microtubule stabilization but not MTOC reorientation in migrating fibroblasts. *Mol. Biol. Cell* **17**, 5004–16 (2006).

224. Young, K. G., Thurston, S. F., Copeland, S., Smallwood, C. & Copeland, J. W. INF1 is a novel microtubule-associated formin. *Mol. Biol. Cell* **19**, 5168–80 (2008).

225. Palazzo, A. F., Cook, T. A., Alberts, A. S. & Gundersen, G. G. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat. Cell Biol.* **3**, 723–9 (2001).

226. Kobielak, A., Pasolli, H. A. & Fuchs, E. Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. *Nat. Cell Biol.* **6**, 21–30 (2004).

227. Carramusa, L., Ballestrem, C., Zilberman, Y. & Bershadsky, A. D. Mammalian diaphanous-related formin Dia1 controls the organization of E-cadherin-mediated cell-cell junctions. *J. Cell Sci.* **120**, 3870–82 (2007).

228. Junemann, A. *et al.* A Diaphanous-related formin links Ras signaling directly to actin assembly in macropinocytosis and phagocytosis. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E7464–E7473 (2016).

229. Rengarajan, M., Hayer, A. & Theriot, J. A. Endothelial Cells Use a Formin-Dependent Phagocytosis-Like Process to Internalize the Bacterium Listeria monocytogenes. *PLoS Pathog.* **12**, e1005603 (2016).

230. Moulding, D. A., Record, J., Malinova, D. & Thrasher, A. J. Actin cytoskeletal defects in immunodeficiency. *Immunol. Rev.* **256**, 282–99 (2013).

231. Alekhina, O., Burstein, E. & Billadeau, D. D. Cellular functions of WASP family proteins at a glance. *J. Cell Sci.* **130**, 2235–2241 (2017).

232. Derry, J. M. J., Ochs, H. D. & Francke, U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* **78**, 635–44 (1994).

233. Yang, C. *et al.* Profilin enhances Cdc42-induced nucleation of actin polymerization. *J. Cell Biol.* **150**, 1001–12 (2000).

234. Banin, S. *et al.* Wiskott-Aldrich syndrome protein (WASp) is a binding partner for c-Src family protein-tyrosine kinases. *Curr. Biol.* **6**, 981–8 (1996).

235. Amann, K. J. & Pollard, T. D. The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. *Nat. Cell Biol.* **3**, 306–10 (2001).

236. Aspenström, P., Lindberg, U. & Hall, A. Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldrich syndrome. *Curr. Biol.* **6**, 70–5 (1996).

237. Rohatgi, R. *et al.* The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221–31 (1999).

238. Rohatgi, R., Nollau, P., Henry Ho, H. Y., Kirschner, M. W. & Mayer, B. J. Nck and Phosphatidylinositol 4,5-Bisphosphate Synergistically Activate Actin Polymerization through the N-WASP-Arp2/3 Pathway. *J. Biol. Chem.* **276**, 26448–52 (2001).

239. Miki, H., Miura, K. & Takenawa, T. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *EMBO J.* **15**, 5326–35 (1996).

240. Machesky, L. M. & Insall, R. H. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8**, 1347–56 (1998).

241. Miki, H., Suetsugu, S. & Takenawa, T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* **17**, 6932–41 (1998).

242. Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M. & Kirschner, M. W. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* **418**, 790–3 (2002).

243. Suetsugu, S., Miki, H. & Takenawa, T. Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem. Biophys. Res. Commun.* **260**, 296–302 (1999).

244. Linardopoulou, E. V. *et al.* Human subtelomeric WASH genes encode a new subclass of the WASP family. *PLoS Genet.* **3**, e237 (2007).

245. Campellone, K. G., Webb, N. J., Znameroski, E. A. & Welch, M. D. WHAMM Is an Arp2/3 Complex Activator That Binds Microtubules and Functions in ER to Golgi Transport. *Cell* **134**, 148–61 (2008).

246. Zuchero, J. B., Coutts, A. S., Quinlan, M. E., La Thangue, N. B. & Mullins, R. D. p53-cofactor JMY is a multifunctional actin nucleation factor. *Nat. Cell Biol.* **11**, 451–9 (2009).

247. Chen, Z. *et al.* Structure and control of the actin regulatory WAVE complex. *Nature* **468**, 533–8 (2010).

248. Snapper, S. B. *et al.* Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**, 81–91 (1998).

249. Zhang, J. *et al.* Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J. Exp. Med.* **190**, 1329–1342 (1999).

250. Cotta-de-Almeida, V. *et al.* Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15424–15429 (2007).

251. Westerberg, L. *et al.* Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response. *Blood* **105**, 1144–1152 (2005).

252. Westerberg, L., Greicius, G., Snapper, S. B., Aspenström, P. & Severinson, E. Cdc42, Rac1, and the Wiskott-Aldrich syndrome protein are involved in the cytoskeletal regulation of B lymphocytes. *Blood* **98**, 1086–1094 (2001).

253. Westerberg, L. S. *et al.* WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. *Blood* **112**, 4139–4147 (2008).

254. Westerberg, L. S. *et al.* Activating WASP mutations associated with X-linked neutropenia result in enhanced actin polymerization, altered cytoskeletal responses, and genomic instability in lymphocytes. *J Exp Med* **207**, 1145–1152 (2010).

255. Bosticardo, M., Marangoni, F., Aiuti, A., Villa, A. & Roncarolo, M. G. Review article Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome. *Blood* **113**, 6288–6295 (2009).

256. Devriendt, K. *et al.* Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat. Genet.* **27**, 313–7 (2001).

257. Ancliff, P. J. *et al.* Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. *Blood* **108**, 2182–2189 (2006).

258. Beel, K. *et al.* A large kindred with X-linked neutropenia with an I294T mutation of the Wiskott-Aldrich syndrome gene. *Br. J. Haematol.* **144**, 120–6 (2009).

259. Keszei, M. *et al.* Constitutive activation of WASp in X-linked neutropenia renders neutrophils hyperactive. *J. Clin. Invest.* **128**, 4115–4131 (2018).

260. De Noronha, S. *et al.* Impaired dendritic-cell homing in vivo in the absence of Wiskott-Aldrich syndrome protein. *Blood* **105**, 1590–7 (2005).

261. Snapper, S. B. *et al.* WASP deficiency leads to global defects of directed leukocyte migration in vitro and in vivo. *J. Leukoc. Biol.* **77**, 993–8 (2005).

262. Kritikou, J. S. *et al.* Constitutive activation of WASp leads to abnormal cytotoxic cells with increased granzyme B and degranulation response to target cells. *JCI Insight* **6**, e140273 (2021).

263. Buckley, R. H. *et al.* Development of immunity in human severe primary T cell deficiency following haploidentical bone marrow stem cell transplantation. *J. Immunol.* **136**, 2398–407 (1986).

264. Lenarsky, C. & Parkman, R. Bone marrow transplantation for the treatment of immune deficiency states. *Bone Marrow Transplant.* **6**, 361–9 (1990).

265. Bildik, H. N. *et al.* Clinical, Laboratory Features and Clinical Courses of Patients with Wiskott Aldrich Syndrome and X–linked Thrombocytopenia–A single center study. *Immunol. Invest.* 1–12 (2021). doi:10.1080/08820139.2021.1933516

266. Thrasher, A. J. & Williams, D. A. Evolving Gene Therapy in Primary Immunodeficiency. *Mol. Ther.* **25**, 1132–1141 (2017).

267. Bauhofer, A., Schimo, S. & Klausmann, M. Benefits of immunoglobulin substitution in primary and secondary immunodeficiencies: Interim analysis of a prospective, long-term non-interventional study. *Int. J. Clin. Pharmacol. Ther.* **59**, 417–427 (2021).

268. Li, Z., McCoy, B., Engl, W. & Yel, L. Steady-State Serum IgG Trough Levels Are Adequate for Pharmacokinetic Assessment in Patients with Immunodeficiencies Receiving Subcutaneous Immune Globulin. *J. Clin. Immunol.* **41**, 1331–1338 (2021).

269. Marciano, B. E. & Holland, S. M. Primary immunodeficiency diseases: Current and emerging therapeutics. *Front. Immunol.* **8**, 937 (2017).

270. Kruskal, B. A. & Ezekowitz, R. A. B. Cytokines in the treatment of primary immunodeficiency. *Biotherapy* **7**, 249–59 (1994).

271. Roy-Ghanta, S. & Orange, J. S. Use of cytokine therapy in primary immunodeficiency. *Clin. Rev. Allergy Immunol.* **38**, 39–53 (2010).

272. Constantine, G. M. & Lionakis, M. S. Lessons from primary immunodeficiencies: Autoimmune regulator and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *Immunol. Rev.* **287**, 103–120 (2019).

273. Sprenkeler, E. G. G., Guenther, C., Faisal, I., Kuijpers, T. W. & Fagerholm, S. C. Molecular Mechanisms of Leukocyte Migration and Its Potential Targeting—Lessons Learned From MKL1/SRF-Related Primary Immunodeficiency Diseases. *Front. Immunol.* **12**, 615477 (2021).

274. C., C.-R. Autoimmunity in primary immune deficiency: Taking lessons from our patients. *Clin. Exp. Immunol.* **164 Suppl**, 6–11 (2011).

275. Heuzé, M. L., Collin, O., Terriac, E., Lennon-Duménil, A. M. & Piel, M. Cell migration in confinement: A micro-channel-based assay. *Methods Mol. Biol.* **769**, 415–34 (2011).

276. Vargas, P., Terriac, E., Lennon-Duménil, A. M. & Piel, M. Study of cell migration in microfabricated channels. *J. Vis. Exp.* e51099 (2014). doi:10.3791/51099

277. Benvenuti, F. *et al.* Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. *Science (80-.).* **305**, 1150–3 (2004).

278. Harada, Y. *et al.* DOCK8 is a Cdc42 activator critical for interstitial dendritic cell migration during immune responses. *Blood* **119**, 4451–61 (2012).

279. Lämmermann, T. *et al.* Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration. *Blood* **113**, 5703–10 (2009).

280. Frittoli, E. *et al.* The Signaling Adaptor Eps8 Is an Essential Actin Capping Protein for Dendritic Cell Migration. *Immunity* **35**, 388–99 (2011).

281. Vargas, P. *et al.* Innate control of actin nucleation determines two distinct migration behaviours in dendritic cells. *Nat. Cell Biol.* **18**, 43–53 (2016).

282. Suzuki, H. *et al.* Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. *J. Invest. Dermatol.* **114**, 135–41 (2000).

283. Linder, S., Nelson, D., Weiss, M. & Aepfelbacher, M. Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9648–53 (1999).

284. Burns, S., Thrasher, A. J., Blundell, M. P., Machesky, L. & Jones, G. E. Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. *Blood* **98**, 1142–9 (2001).

285. Calle, Y. *et al.* WASp deficiency in mice results in failure to form osteoclast sealing zones and defects in bone resorption. *Blood* **103**, 3552–61 (2004).

286. Darsow, U. *et al.* The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: A European multicenter study. *Allergy Eur. J. Allergy Clin. Immunol.* **59**, 1318–25 (2004).

287. Nylén, S. *et al.* Splenic accumulation of IL-10 mRNA in T cells distinct from CD4 +CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J. Exp. Med.* **204**, 805–17 (2007).

288. Nylén, S. & Eidsmo, L. Tissue damage and immunity in cutaneous leishmaniasis. *Parasite Immunol.* **34**, 551–61 (2012).

289. Bouma, G., Burns, S. & Thrasher, A. J. Impaired T-cell priming in vivo resulting from dysfunction of WASp-deficient dendritic cells. *Blood* **110**, 4278–84 (2007).

290. Tabeta, K. *et al.* The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat. Immunol.* **7**, 156–64 (2006).

291. Volpi, S. *et al.* N-WASP is required for B-cell-mediated autoimmunity in Wiskott-Aldrich syndrome. *Blood* **127**, 216–20 (2016).

292. Dahlberg, C. I. M. *et al.* Deletion of WASp and N-WASp in B cells cripples the germinal center response and results in production of IgM autoantibodies. *J. Autoimmun.* **62**, 81–92 (2015).

293. Westerberg, L. S. *et al.* Wiskott-Aldrich syndrome protein (WASP) and N-WASP are critical for peripheral B-cell development and function. *Blood* **119**, 3966–74 (2012).

294. Cotta-de-Almeida, V. *et al.* Wiskott-Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15424–9 (2007).

295. Orange, J. S. *et al.* IL-2 induces a WAVE2-dependent pathway for actin reorganization that enables WASp-independent human NK cell function. *J. Clin. Invest.* **121**, 1535–48 (2011).

296. Kritikou, J. S. *et al.* IL-2 in the tumor microenvironment is necessary for Wiskott-Aldrich syndrome protein deficient NK cells to respond to tumors in vivo article. *Sci. Rep.* **6**, 30636 (2016).

297. Oliveira, M. M. S. & Westerberg, L. S. Cytoskeletal regulation of dendritic cells: An intricate balance between migration and presentation for tumor therapy. *J. Leukoc. Biol.* **108**, 1051–1065 (2020).

298. Hong, L. *et al.* Characterization of a Cdc42 protein inhibitor and its use as a molecular probe. *J. Biol. Chem.* **288**, 8531–8543 (2013).

299. Liu, Z. *et al.* Expression of the Arp2/3 complex in human gliomas and its role in the migration and invasion of glioma cells. *Oncol. Rep.* **30**, 2127–36 (2013).

300. Kazazian, K. *et al.* Plk4 promotes cancer invasion and metastasis through Arp2/3 complex regulation of the actin cytoskeleton. *Cancer Res.* **77**, 434–447 (2017).

301. Frugtniet, B. A., Martin, T. A., Zhang, L. & Jiang, W. G. Neural Wiskott-Aldrich syndrome protein (nWASP) is implicated in human lung cancer invasion. *BMC Cancer* **17**, 224 (2017).

302. Escudero-Esparza, A., Jiang, W. G. & Martin, T. A. Claudin-5 is involved in breast cancer cell motility through the N-WASP and ROCK signalling pathways. *J. Exp. Clin. Cancer Res.* **31**, 43 (2012).

303. Henson, J. H. *et al.* Arp2/3 complex inhibition radically alters lamellipodial actin architecture, suspended cell shape, and the cell spreading process. *Mol. Biol. Cell* **26**, 887–900 (2015).

304. Rosenberg, S. A., Yang, J. C. & Restifo, N. P. Cancer immunotherapy: Moving beyond current vaccines. *Nat. Med.* **10**, 909–15 (2004).

305. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* **6**, 295–307 (2006).

306. Murciano-Goroff, Y. R., Warner, A. B. & Wolchok, J. D. The future of cancer immunotherapy: microenvironment-targeting combinations. *Cell Res.* **30**, 507–519 (2020).

307. Iwai, Y., Terawaki, S. & Honjo, T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int. Immunol.* **17**, 133–44 (2005).

308. Shultz, L. D. *et al.* Human Lymphoid and Myeloid Cell Development in NOD/LtSz- scid IL2R γ null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. *J. Immunol.* **174**, 6477–89 (2005).

309. Keszei, M. *et al.* Wiskott-Aldrich syndrome gene mutations modulate cancer susceptibility in the p53± murine model. *Oncoimmunology* **7**, e1468954 (2018).

310. Orange, J. S. *et al.* Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11351–6 (2002).

311. Catucci, M. *et al.* Wiskott-Aldrich syndrome protein deficiency in natural killer and dendritic cells affects antitumor immunity. *Eur. J. Immunol.* **44**, 1039–45 (2014).

312. Gismondi, A. *et al.* Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: Ability of IL-2 to correct NK cell functional defect. *Blood* **104**, 436–43 (2004).

313. Jyonouchi, S. *et al.* Phase I trial of low-dose interleukin 2 therapy in patients with Wiskott-Aldrich syndrome. *Clin. Immunol.* **179**, 47–53 (2017).

314. Orange, J. S. Formation and function of the lytic NK-cell immunological synapse. *Nat. Rev. Immunol.* **8**, 713–25 (2008).

315. Urlaub, D., Höfer, K., Müller, M.-L. & Watzl, C. LFA-1 Activation in NK Cells and Their Subsets: Influence of Receptors, Maturation, and Cytokine Stimulation. *J. Immunol.* **198**, 1944–1951 (2017).

316. Ni, H. T., Deeths, M. J., Li, W., Mueller, D. L. & Mescher, M. F. Signaling pathways activated by leukocyte function-associated Ag-1-dependent costimulation. *J. Immunol.* **162**, 5183–9 (1999).

317. Oliveira, M. *et al.* The WASp L272P gain-of-function mutation alters dendritic cell coordination of actin dynamics for migration and adhesion. *J. Leukoc. Biol.* 1-11 (2021).

318. Lennicke, C. *et al.* Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid. *Oncoimmunology* **6**, e1259049 (2017).

319. Blancas-Galicia, L., Escamilla-Quiroz, C. & Yamazaki-Nakashimada, M. A. Wiskott-Aldrich syndrome: An updated review. *Rev. Alerg. Mex.* **58**, 213–8 (2011).

320. Müller, P. *et al.* Microtubule-depolymerizing agents used in antibody-drug conjugates induce antitumor immunity by stimulation of dendritic cells. *Cancer Immunol. Res.* **2**, 741–55 (2014).

321. Martin, K. *et al.* The microtubule-depolymerizing agent ansamitocin P3 programs dendritic cells toward enhanced anti-tumor immunity. *Cancer Immunol. Immunother.* **63**, 925–38 (2014).

322. Gorchs, L. *et al.* The vitamin D analogue calcipotriol promotes an anti-tumorigenic phenotype of human pancreatic CAFs but reduces T cell mediated immunity. *Sci. Rep.* **10**, 17444 (2020).

323. Bergman, P., Sperneder, S., Höijer, J., Bergqvist, J. & Björkhem-Bergman, L. Low Vitamin D levels are associated with higher opioid dose in palliative cancer patients-results from an observational study in Sweden. *PLoS One* **10**, e0128223 (2015).

324. Björkhem-Bergman, L. & Bergman, P. Vitamin D and patients with palliative cancer. *BMJ Support. Palliat. Care* **6**, 287–91 (2016).

325. Kalsum, S., Andersson, B., Das, J., Schön, T. & Lerm, M. A high-throughput screening assay based on automated microscopy for monitoring antibiotic susceptibility of Mycobacterium tuberculosis phenotypes. *BMC Microbiol.* **21**, 167 (2021).

326. Hu, Z., Ye, L., Xing, Y., Hu, J. & Xi, T. Combined SEP and anti-PD-L1 antibody produces a synergistic antitumor effect in B16-F10 melanoma-bearing mice. *Sci. Rep.* **8**, 217 (2018).

327. Kleffel, S. *et al.* Melanoma Cell-Intrinsic PD-1 Receptor Functions Promote Tumor Growth. *Cell* **162**, 1242–1256 (2015).

328. Pilon-Thomas, S., Mackay, A., Vohra, N. & Mulé, J. J. Blockade of Programmed Death Ligand 1 Enhances the Therapeutic Efficacy of Combination Immunotherapy against Melanoma. *J. Immunol.* **184**, 3442–9 (2010).