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UNDERSTANDING CELLULAR AND CYTOSKELETAL DYSREGULATION IN IMMUNODEFICIENCY DISEASES

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Understanding cellular and cytoskeletal dysregulation in immunodeficiency diseases

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The immune system is a complex network of cells and molecules interacting to preserve the integrity and survival of the host by distinguishing self from non-self, and dysregulation of immune cells can lead to immunodeficiency, autoimmunity, and cancer. The cytoskeleton is essential for several important immune cell functions, including proliferation, movement, signaling, and vesicle trafficking. The overall aim of this thesis was to investigate the importance of the cytoskeleton for lymphocytes in primary immunodeficiencies.

In **paper I**, we investigated the role of the actin-sensing protein MKL1 in B cells, by examining samples from three monozygotic triplets with an intronic deletion in *MKL1*, out of whom two had developed Hodgkin lymphoma. MKL1 deficiency has previously been described as causing primary immunodeficiency with dysregulation of actin. The intronic deletion in *MKL1* led to increased MKL1 expression, increased MKL1-dependent gene transcription, hyperproliferation, dysregulated cytoskeleton, genomic instability, and formation of tumors *in vivo*. Inhibition of MKL1 led to reversion of the phenotype. These results suggest a role of MKL1 in the development of Hodgkin lymphoma.

In **paper II**, our aim was to investigate the function of LRBA in lymphocytes from a patient with LRBA deficiency, presenting with immunodeficiency and autoimmunity. The function of LRBA has not been fully clarified previously. LRBA-deficient patient cells accumulated vesicles including endosomes, lysosomes, autophagosomes, and lipid bodies. Lipid metabolism was altered in patient cells in response to serum starvation. These results suggest that LRBA is involved in the response to cell stress, and that the accumulation of vesicles in LRBA deficiency may impair the survival and function of immune cells.

In **paper III**, we examined the anti-tumor responses of NK cells and T cells in X-linked neutropenia, a primary immunodeficiency caused by activating mutations in the actin-regulatory protein WASp, with increased risk of myelodysplasia. We studied samples from patients and mouse models and found that NK cells and T cells in X-linked neutropenia displayed normal to increased tumor killing, decreased expression of the exhaustion marker KLRG1, increased granzyme B expression, and polarization of actin without stimuli. These results suggest that tumor immunosurveillance is not deficient in X-linked neutropenia and that the increased risk of malignancy rather is caused by cell-intrinsic aberrations.

In **paper IV**, we investigated the development of the T cell receptor repertoire in mouse models for Wiskott-Aldrich syndrome, a primary immunodeficiency caused by loss-of-function mutations in the actin-regulating protein WASp. We examined thymocytes and spleen T cells from young and older mice and found that the thymocyte T cell receptor repertoire was intact in young mice but skewed in older mice. The spleen T cell receptor repertoire displayed signs of clonal expansions in older mice, suggesting development of an autoimmune response. These results suggest that the dependence on WASp for thymic output and the generation of a diverse T cell receptor repertoire increases with age.

In summary, the works presented in this thesis provide evidence for the role of dysregulated cytoskeletal responses in the development of primary immunodeficiency and cancer. Furthermore, potential drug targets are identified for diseases with increased actin responses or lipid dysregulation, and proteins identified as involved in cancer development may also be used as markers to screen for in the assessment of disease severity and prognosis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvaret är ett komplext nätverk av celler och molekyler som samverkar för att skydda människan från infektioner och cancer. För att göra detta krävs att immunförsvarets celler kan känna igen vad som är kroppsegna, friska celler, och vad som är främmande, sjukdomsframkallande celler eller mikroorganismer såsom bakterier och virus. Begreppet immunbrist innebär att immunförsvaret är så nedsatt att man får svåra infektioner man inte brukar få i normala fall. Detta kan bero på medfödda genetiska mutationer, och kallas då primär immunbrist, eller uppstå på grund av immundämpande behandlingar eller infektioner som HIV, och kallas då sekundär immunbrist. I denna avhandling har jag undersökt cellskelettets roll för en viss typ av immunceller i primära immunbristsjukdomar. Cellskelettet är cellernas inre struktur som hjälper dem att utföra många viktiga funktioner, såsom att växa och dela på sig, röra på sig, vidarebefordra signaler inne i cellen, och transportera saker inne i cellen.

I **artikel I** undersökte vi immunceller från tre enäggstrillingar födda 1945 varav två utvecklat en cancerform som heter Hodgkins lymfom. Alla tre trillingar hade en mutation i proteinet MKL1 som är viktigt för regleringen av cellskelettet, och brist av det här proteinet hade tidigare hittats i en primär immunbristsjukdom. Vi ville därmed undersöka om dessa trillingar också hade immunbrist och om det var orsaken till att de hade utvecklat cancer. Vi hittade istället att immunceller från trillingarna hade mer mängd än förväntat av proteinet MKL1 och att detta ledde till att cellerna växte snabbare, hade ett onormalt cellskelett, hade kromosomförändringar, och att cellerna bildade tumörer när vi injicerade dem i möss. Dessa fynd talar för att mutationen vi hittat i trillingarnas immunceller kan ha orsakat Hodgkins lymfom. Denna information skulle eventuellt kunna användas för att utveckla nya behandlingar och diagnostiska metoder för cancer.

I **artikel II** undersökte vi celler från en patient med primär immunbrist orsakad av brist på proteinet LRBA. Man har tidigare beskrivit andra patienter med denna immunbrist, men inte lyckats klargöra proteinets funktion i immunceller. Vi fann att patientceller som saknade proteinet LRBA samlade på sig mer än normalt av en typ av vätskeblåsor som finns inne i cellen och bland annat används för förvaring, transport eller nedbrytning av cellmaterial. Dessutom påverkades patientcellernas sammansättning av fettmolekyler annorlunda när de utsattes för svält. Dessa fynd talar för att proteinet LRBA är involverat i cellernas svar på stress och att brist på detta protein kan leda till försämrad överlevnad och funktion hos immuncellerna.

I **artikel III** undersökte vi immunceller i en primär immunbristsjukdom som orsakas av överaktivitet av ett protein som heter WASp och som är viktigt för regleringen av cellskelettet. Sjukdomen kallas ”X-bunden brist på neutrofiler” (neutrofiler är en viss typ av vita blodkroppar) eftersom genen som kodar för proteinet WASp finns på X-kromosomen, och leder till infektioner och ökad risk för cancer. Vi hade tillgång till prover från både patienter och musmodeller och vårt mål med projektet var att undersöka förmågan att bekämpa cancer hos en viss typ av immunceller i denna sjukdom. Vi hittade att immuncellerna hade bevarad förmåga att bekämpa cancer, och i vissa fall till och med ökad förmåga. Deras cellskelett var

dessutom överaktivt. Dessa fynd talar för att den ökade risken för cancer i denna immunbristsjukdom inte beror på nedsatt cancerbekämpningsförmåga hos immuncellerna, utan sannolikt på grund av orsaker inne i cancercellerna.

I **artikel IV** undersökte vi utvecklingen av en viss typ av immuncell i musmodeller för Wiskott-Aldrich syndrom, en primär immunbristsjukdom som orsakas av brist på proteinet WASp. Patienter med denna immunbristsjukdom får svåra infektioner, men immunförsvaret hos patienterna kan även vända sig mot frisk vävnad, vilket kallas för autoimmunitet. Vårt mål var att undersöka om en normalt bred repertoar av immunceller kunde bildas i Wiskott-Aldrich syndrom, eftersom en bred repertoar är viktigt för immunförsvarets förmåga att försvara sig mot många olika typer av infektioner. Vi fann att unga möss kunde bilda en normal cellrepertoar men att denna förmåga försämrades hos äldre möss med brist på proteinet WASp. Vi såg även tecken till autoimmunitet i cellrepertoaren hos de äldre mössen. Dessa fynd talar för att behovet av proteinet WASp för utvecklingen av en bred och normal repertoar ökar med åldern.

Sammanfattningsvis visar jag i denna avhandling ett antal exempel på vikten av ett normalt fungerande cellskelett i immunförsvaret och att felreglering av cellskelettet är involverat i både immunbrist och cancer. I framtiden kan man eventuellt vidareutveckla behandlingar för immunbrist och cancer med läkemedel som justerar cellskelettets funktion, eller använda cellskelettproteiner som markörer för cancerutveckling.

LIST OF SCIENTIFIC PAPERS

I. 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. 2019 Oct 3 (Epub ahead of print)
*shared first authorship

II. **Sendel A***, Albuquerque AS*, Brauner H, McLatchie A, Seitz C, Westerberg LS, Burns SO.
Altered intracellular vesicles and lipid content in patient cells devoid of lipopolysaccharide responsive beige-like anchor protein (LRBA)
Manuscript
*shared first authorship

III. Kritikou JS, Nigam SM, Oliveira MSS, Wagner AK, Keszei M, Rentouli S, Brauner H, **Sendel A**, Lain S, Lane D, Snapper SB, Kärre K, Vandenberghe P, Orange JS, Westerberg LS.
Human and murine NK cells and T cells expressing constitutively active WASp display hyperactivity
Manuscript

IV. Petersen SH*, **Sendel A***, van der Burg M, Westerberg LS.
Unraveling the repertoire in Wiskott-Aldrich syndrome
Front Immunol. 2014 Oct 27;5:539
*shared first authorship

CONTENTS

LIST OF ABBREVIATIONS

ABP	actin-binding protein
ADCC	antibody-dependent cell-mediated cytotoxicity
AID	activation-induced cytidine deaminase
Arp	actin-related protein
BCR	B cell receptor
CDR	complementarity-determining region
CRISPR	clustered regularly interspaced palindromic repeats
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DAMP	danger-associated molecular pattern
DN	double-negative
DP	double-positive
EBV	Epstein-Barr virus
F-actin	filamentous actin
G-actin	globular actin
GBD	GTPase-binding domain
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ITAM	immunoreceptor tyrosine-based activation motif
KIR	killer cell immunoglobulin-like receptor
KLRG1	killer cell lectin-like receptor subfamily G member 1
KO	knockout
LCL	lymphoblastoid cell line
LPS	lipopolysaccharide
LRBA	lipopolysaccharide-responsive beige-like anchor
MHC	major histocompatibility complex
MKL1	megakaryoblastic leukemia 1

NHEJ	non-homologous end joining
NK	natural killer
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PID	primary immunodeficiency
PRR	pattern recognition receptor
RAG	recombination-activating gene
SCID	severe combined immunodeficiency
SRF	serum response factor
Syk	spleen tyrosine kinase
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
T_H	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
T_{reg}	regulatory T
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WT	wildtype
VCA	verprolin homology, cofilin homology, acidic region
WIP	WASp-interacting protein
XLN	X-linked neutropenia

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

Our human bodies are highly complex organisms fine-tuned to keep us alive and produce offspring. For this matter, we have during the course of evolution developed sophisticated biological processes for e.g. the consumption of food and water, breathing oxygen, and interacting with other individuals. These basic needs require us to expose our bodies to the environment, constantly putting us at risk of harm from injuries and diseases both infectious and non-communicable, further influenced by varying genetic susceptibility to disease.

To prevent diseases and keep our complex system of tissues and organs healthy and operative, we have to preserve the integrity of the most fundamental structural and functional unit of the body: the cell. Cells are the smallest units of life and can differentiate to give rise to a wide variety of tissues. In humans and other organisms, the immune system works as a defense against both extrinsic and intrinsic threats to cellular health. Comprised of a wide range of cells and molecules, it constantly surveils the body, and depending on the nature of a potential threat, different types of immune responses can be mounted.

Around 1400 different pathogens, including bacteria, viruses, parasites, and fungi, are known to cause disease by infecting humans either extracellularly or intracellularly (1). Furthermore, healthy host cells can transform into cancerous cells, leading to different types of cancer depending on the cell origin. Over 100 different types of cancers have been described and as a disease group cancer acts as one of the leading causes of death worldwide (2). Immune cells can limit cancer cell development in a process called immunosurveillance. In its essence, the crucial task of the immune system is to distinguish self from non-self: to combat infections and cancer strongly and efficiently but leave healthy tissues unharmed.

The immune system of humans and other vertebrates consists of two major branches: innate immunity that responds swiftly and coarsely, and adaptive immunity that requires more stimulation cues but acts highly specific once activated. Although the cells involved in these respective branches are diverse, they originate from the same cell type residing in the bone marrow: hematopoietic stem cells, who give rise to *hematopoiesis* (from Greek αἷμα, "blood", and ποιεῖν, "to make"), the process of blood cell production.

1.1.1 Hematopoiesis and blood cell types

Blood is composed of blood plasma and blood cells, i.e. erythrocytes, thrombocytes, and leukocytes. All blood cells originate from hematopoietic stem cells in the bone marrow (Figure 1) but mature in different parts of the body depending on the cell type.

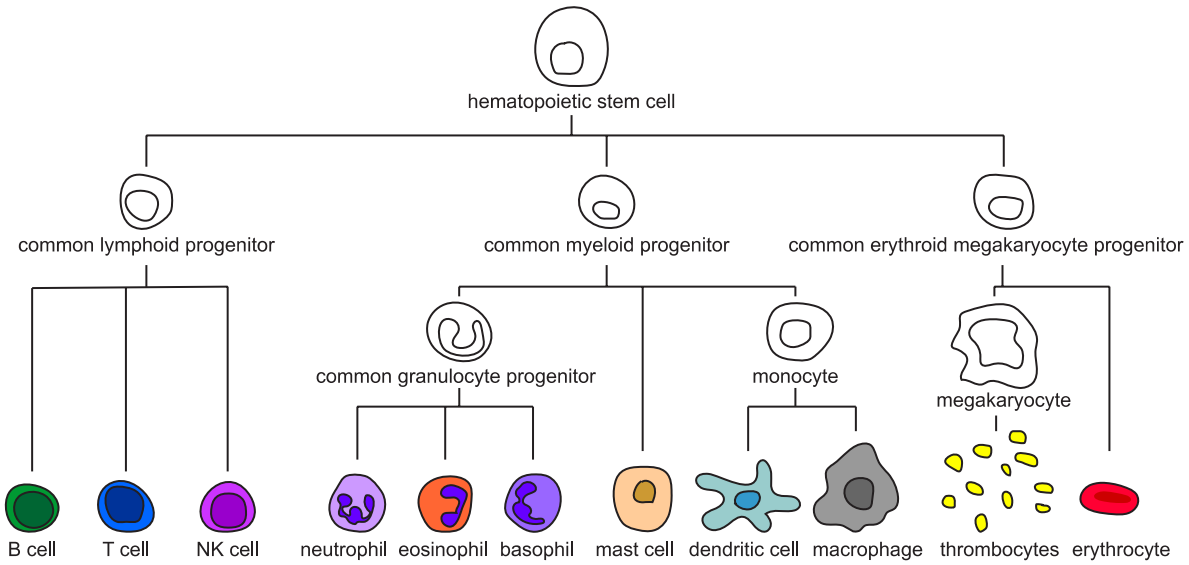

Figure 1. Hematopoietic cells and their lineages. All blood cells originate from hematopoietic stem cells residing in the bone marrow. B and T cells represent the adaptive immune system and myeloid cells together with NK cells represent the innate immune system. Thrombocytes are important for hemostasis and erythrocytes transport oxygen; however, these cells also have functions in the immune system. *N.B.* that this figure is simplified: several progenitors, alternate cell types and non-conventional cells have been omitted.

Erythrocytes are the main oxygen transporters in the body and make up 35–50 % of the blood volume, amounting to 3,9–5,7 x 10^{12} cells/L (3, 4). Apart from delivering oxygen to tissues, erythrocytes have immunological functions such as clearing immune complexes from the circulation and modulating the innate immune system (5, 6). Furthermore, the erythrocyte cell membrane contains several blood group antigens[A], highly important in the field of transfusion medicine and obstetrics as blood group incompatible transfusions or pregnancies can cause severe immune reactions.

Thrombocytes are important for hemostasis, i.e. the process of controlling bleeding, but also take part in immune responses by e.g. expressing Toll-like receptors (TLRs; see section 1.1.2.2) and secreting immunomodulatory substances (7).

Leukocytes are the main cell type of the immune system and comprise several cells of distinct functionalities, divisible into two major lineages: myeloid cells (largely corresponding to the innate immune system) and lymphocytes (where B and T cells represent the adaptive immune system). Myeloid cells develop from myeloid progenitor cells and include granulocytes, mast cells, dendritic cells, and macrophages. Lymphocytes develop from lymphoid progenitor cells and include B cells, T cells, and natural killer (NK) cells (Figure 1). Leukocytic cell counts in blood are displayed in Table 1 (8).

Non-conventional immune cells bearing properties of both innate and adaptive immunity include B1 cells, marginal zone B cells, γδ T cells, and natural killer T cells; however, these cells remain outside of the scope of this thesis and will not be described in-depth.

[A] Karl Landsteiner was awarded the Nobel Prize in 1930 for his discovery of blood groups.

	Cell count (x 10^9/L)	
	Males	**Females**
Total leukocytes	3,39–9,37	3,63–8,87
– Neutrophils	1,58–6,22	1,74–5,86
– Eosinophils	0,03–0,38	0,03–0,36
– Monocytes	0,11–0,55	0,11–0,44
– Lymphocytes	0,68–2,72	0,73–2,87
— B cells	0,07–0,44	0,08–0,46
— T cells	0,36–1,94	0,43–2,09
— NK cells	0,11–0,73	0,09–0,65

Table 1. Leukocyte blood counts in adult males and females. Numbers are based on 90 % confidence intervals with outliers removed, including adults of ages 40–79 years (8).

1.1.2 Innate immunity

The main purpose of the innate immune system is to quickly respond to threat, by identifying and removing foreign substances, recruiting other more specific cells, activating complement proteins, and presenting antigens to the adaptive immune system. This is accomplished by an elaborate system of barriers, molecules, and cells. A central process initiated by innate immune cells as a response to harm is called inflammation (from Latin *inflammare*, "to set on fire") and involves the cardinal signs heat (*calor*), pain (*dolor*), redness (*rubor*), swelling (*tumor*), and loss of function (*function laeasa*). Swelling, redness, and heat is the result of increased blood flow necessary for the delivery of cells and molecules to and from the affected area; pain and loss of function serves to alert the host and facilitate healing.

1.1.2.1 Barriers prevent pathogenic microbes from entering the body

In a highly simplified form, the human body can be likened to a tube with two orifices (the mouth and anus, openings of the gastrointestinal tract) and two cavities (the respiratory and urogenital tracts) (Figure 2). The external side of the body is covered with skin and the internal sides have a mucosal surface. As these sides are exposed to the environment, the first line of the body's defense are these physical barriers, preventing entry of pathogens to the body.

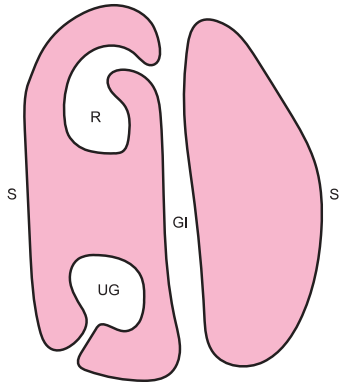

Figure 2. The human body. A schematic figure illustrating how the human body is exposed to the environment, displayed as a sagittal section. As the skin (S), respiratory tract (R), gastrointestinal tract (GI), and urogenital tract (UG) are potential entry points for pathogens, these barriers serve as the first line of defense of the body and have developed several features for this matter.

Mechanical processes, such as peristalsis of the gastrointestinal tract or movement of cilia in the respiratory tract, also aid in removing potential infectious threats. Mucosal surfaces produce mucus that contain antimicrobial compounds. Acidic compounds are created in the stomach, vagina, and skin, acting as chemical barriers capable of destroying pathogens.

Furthermore, several anatomical areas are populated by commensal microorganisms, forming the human microbiome. These normally non-pathogenic microorganisms inhibit pathogens either directly by secreted compounds or indirectly by competition for space and nutrients. The common gut bacterium *Escherichia coli* resides in the intestine, producing and secreting a cytotoxic compound called colicin, capable of killing other bacterial cells (9). A clinically relevant scenario illustrating the mechanisms of protective microbiota is when *E. coli* and other commensal gut bacteria are eradicated by broad-spectrum antibiotic treatments, which can lead to overgrowth of *Clostridium difficile*, a bacterium normally residing in the gut of 2–12 % of the population (10, 11), potentially causing mild to severe (even life-threatening) diarrhea.

1.1.2.2 Initiation of the innate immune response

When pathogens overcome the first line of defense and enter the body, they can be recognized by innate immune cells resident in the tissues infected. In most cases, the first cells to respond in the infected tissue are macrophages and dendritic cells (Figure 3). They do so by the expression of several different pattern recognition receptors (PRRs), able to detect molecules associated to harmful events and trigger an immune response based on this. The patterns recognized by PRRs can be grouped into pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), depending on their origin: PAMPs are associated with infectious microbes and DAMPs are molecules originating from damaged host cells. PRRs include TLRs[B], C-type lectin receptors, NOD-like receptors, and RIG-I-like receptors, and these receptors can either be membrane-bound or cytoplasmic (12). The classical prototype for PAMPs is the bacterial toxin lipopolysaccharide (LPS), a potent molecular trigger of the inflammatory response via TLR4 (13).

When macrophages encounter pathogens, they can engulf them by the process of phagocytosis[C] (from Greek φαγεῖν, "to eat", and κύτος, "cell") via several phagocytic receptors including TLRs. Furthermore, the sensing of pathogens by PRRs activate several intracellular pathways in macrophages, leading to the production of inflammatory cytokines including interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor (TNF), and CXCL8 (14). IL-1 and TNF activate the vascular endothelium, allowing for increased blood flow and permeability to the infected tissue, and induce fever. IL-6 induces the so-called acute-phase reaction in the liver, leading to the specialized production of proteins necessary in the inflammatory response. IL-12 stimulates NK cells and T cells (see sections 1.1.2.3 and 1.1.3.4, respectively). CXCL8 is a

[B] Bruce Beutler and Jules Hoffman were awarded one half of the Nobel Prize in 2011 for their work on TLRs (the other half was awarded to Ralph Steinman, see footnote D).
[C] First described by Ilya Ilyich Mechnikov, awarding him the Nobel Prize in 1908.

chemokine important for the recruitment of neutrophils to the site of infection. Neutrophils are the most abundant type of leukocyte in blood (Table 1) and of major importance in the acute inflammatory response by their potential to phagocytose and excrete radical oxygen species and other antimicrobial compounds.

Dendritic cells[D] function similarly to macrophages in the early immune response to pathogens, with phagocytosis as a central feature. However, instead of remaining in the infected tissue, activated dendritic cells can migrate to nearby lymph nodes where they present antigens from the ingested microbes to T cells in the adaptive immune system (Figure 3).

NK cells are lymphocytes, but unlike B and T cells, they belong to the innate immune system. Their main function is to eliminate tumor cells and virally infected cells. Constituting a central part of paper III, they will be described in depth in the following section.

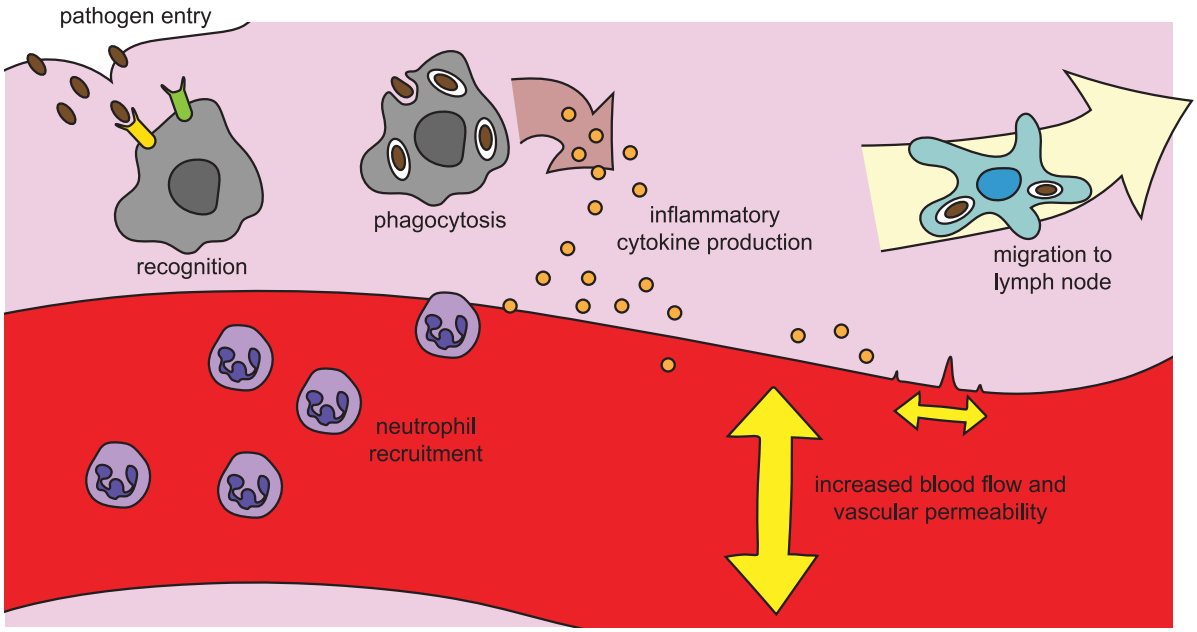

Figure 3. Initiation of the innate immune response. When pathogens enter the tissue, pathogen recognition receptors on resident macrophages and dendritic cells detect pathogen-associated molecular patterns, triggering phagocytosis and the production of inflammatory cytokines. These cytokines induce dilation of vessels and increase vascular permeability, facilitating the delivery of cells and plasma proteins to the infected tissues. Neutrophils are recruited to the tissue by chemokines also produced by macrophages and dendritic cells. Dendritic cells migrate to nearby lymph nodes to present antigens from the ingested microbes to T cells, initiating the adaptive immune response.

1.1.2.3 NK cells take part in the immune response against intracellular threats

NK cells belong to a group of lymphocytes called innate lymphoid cells (ILCs), also including ILC1s, ILC2s, and ILC3s. By their distinct cytokine responses when activated, they correspond to T cells of the adaptive immune system (cytotoxic T cells, T helper (T_H) 1, T_H2, and T_H17 cells, respectively; more on T cells in section 1.1.3.4), however, considered part of the innate immune system as they lack certain features of adaptive immunity cells, in that they do not express adaptive antigen receptors or undergo clonal expansion (15). NK cells in this way

[D] Ralph Steinman was awarded one half of the Nobel Prize in 2011 for his work on the central role of dendritic cells in the initiation of the adaptive immune response.

complement the response to intracellular pathogens and tumor cells by cytotoxic T cells. The study of NK cell immunodeficiencies has demonstrated that NK cells specifically are needed for infections with certain viruses that escape T cell cytotoxicity, in particular herpesviruses (16).

NK cells develop from common lymphoid progenitors in the bone marrow (Figure 1). NK cell precursors committed to the NK cell lineage are identified by their expression of the common β chain of the IL-2 and IL-15 receptors, these cytokines being of central importance for NK cell development (17-19). During the transition from immature to mature, NK cells typically acquire expression of CD16 (also known as FcγRIII, a receptor important for antibody-dependent cellular cytotoxicity (ADCC), see below), CD56, and several NK cell-specific receptors (17, 20, 21). However, the development of NK cell in humans is complex to study, and it remains unclear if the development is strictly linear or if many different precursor cells can develop into mature NK cells in a more branched manner (22).

NK cell receptors can be grouped into activating and inhibitory receptors. Activating receptors include CD16, NKG2D, and NKp46; inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs), killer cell lectin-like receptor subfamily G member 1 (KLRG1), and NKG2A (23) (Figure 4).

CD16 is a receptor for the constant region of immunoglobulin (Ig) G, and when binding antibody-coated cells, crosslinking of CD16 can initiate ADCC, a key function of NK cells in the killing of intracellular pathogens or tumor cells (24). NKG2D recognizes ligands homologous to major histocompatibility complex (MHC) class I molecules (corresponding to human leukocyte antigen (HLA)-A, -B, and -C; more on these molecules in section 1.1.3.4), thus activating NK cells when self-proteins are upregulated due to virus infection or cancer (25). NKp46 is an activating receptor recognizing ligands associated to a variety of intracellular pathogens, including influenza virus and cells infected by *Mycobacterium tuberculosis* (26).

KIRs are inhibitory NK cell receptors recognizing MHC/HLA molecules (27, 28); healthy nucleated cells expressing MHC class I will not activate NK cells. However, as both virally infected cells and tumor cells have been shown to downregulate MHC class I as a means of escaping T cell-driven immunity (29, 30), this so-called "missing self" recognition by NK cells is essential for the immune response in these cases (31, 32). KLRG1 is an inhibitory NK cell receptor that binds to cadherins, a class of cell adhesion molecules present on healthy tissue (33). Loss of KLRG1 inhibition in e.g. malignant cell transformation thus leads to MHC-independent "missing self" recognition and NK cell activation (34). KLRG1 is also expressed by antigen-experienced T cells and is associated to lack of proliferative capacity (35). NKG2A belongs to the same family of NK cell receptors as the activating receptor NKG2D, however, NKG2A initiates inhibitory pathways by recognition of its ligand, the non-classical MHC class I molecule HLA-E (36).

Activated NK cells have two types of effector function: cytotoxicity and cytokine secretion (Figure 4). NK cell cytotoxicity is mediated by secretion of lytic granules and signaling through

death receptor pathways. Lytic granules secreted by NK cells contain several cytotoxic and proteolytic proteins, including perforin and granzyme, forming pores in the membrane of target cells and inducing apoptosis (37, 38). Death receptor signaling induced by NK cells are mediated by members of the TNF family of cytokines, including Fas ligand and TNF-related apoptosis-inducing ligand, leading to apoptosis of target cells (39-41).

Cytokines secreted by NK cells include interferon (IFN)-γ and TNF, both important for immunity against intracellular infections and tumor cells (42-44). IFN-γ affects target cells in several ways, including upregulation of MHC/HLA expression, induction of phagocytosis, and stimulation of proinflammatory cytokine production (45). Macrophages and dendritic cells stimulated by IFN-γ increase their production of IL-12, further stimulating NK cells to secrete IFN-γ, resulting in a positive feedback loop (45).

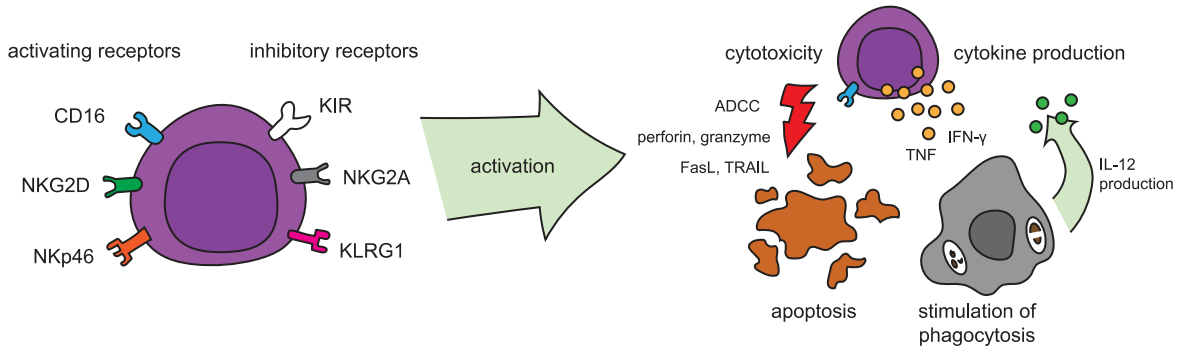

Figure 4. NK cell activation and effector functions. Activation of NK cells is the result of the balance of activating and inhibitory signals through a variety of receptors. The main functions of activated NK cells are cytotoxicity and cytokine production. A central feature of NK cells is antibody-dependent cellular cytotoxicity (ADCC), initiated by activation through CD16. Cytotoxicity is mediated by secretion of lytic granules containing perforin and granzyme, as well as by induction of death-receptor signaling by Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Cytokines produced by NK cells include IFN-γ and TNF. IFN-γ stimulates phagocytosis in macrophages and dendritic cells, as well as inducing IL-12 production that stimulates NK cells further, resulting in a positive feedback loop.

1.1.2.4 The complement system and antimicrobial peptides assist the innate response

Together with the cellular responses in the innate immune system, the so-called complement system[E] plays an important role in the clearing of pathogens and damaged cells. The complement system consists of several plasma proteins that are activated in three distinct but convergent pathways: the classical pathway, the alternative pathway, and the lectin pathway. The classical pathway requires antibody recognition and is initiated by complement proteins including C1, C2, and C4. The alternative pathway is initiated spontaneously on microbial surfaces and damaged host cells, and its initiation includes the complement proteins C3, B, and D. The lectin pathway is initiated by mannose-binding lectin, binding to carbohydrate structures on pathogens, and complement proteins C2 and C4. The three pathways converge in the formation of the C3 convertase, crucial for opsonization of pathogens and initiation of the

[E] Jules Bordet was awarded the Nobel Prize in 1919 for his work on the complement system. The term *complement* was coined in 1899 by Paul Ehrlich, a prominent immunologist of the late 19th century who was awarded one half of the Nobel Prize in 1908 for his work on immunity (the other half was awarded to Ilya Ilyich Mechnikov, see footnote C).

downstream pathway of C5 conversion and formation of the membrane attack complex capable of lysing target cells. Cleavage products of complement proteins, such as C3a, C4a, and C5a, trigger further innate immune responses including inflammation, recruitment of neutrophils, and degranulation of mast cells (46).

Other molecules involved in the innate immune response include antimicrobial peptides, such as defensins and cathelicidins. These peptides are typically amphipathic and cationic, facilitating their incorporation into membranes, and have a broad reactivity against bacteria, viruses, fungi, and parasites (47). Defensins and cathelicidins are constitutively produced by neutrophils and epithelial cells, and expression can be induced further by infectious or inflammatory triggers (48, 49). The main effect of antimicrobial peptides has been ascribed to membrane pore formation leading to lysis of target cells, however, they may also kill microbes by inhibiting their metabolism (50).

1.1.3 Adaptive immunity

The adaptive immune response is defined by its potential to produce highly specific responses to antigens, as well as creating immunological memory. The receptors of innate immune cells are encoded in the germline; receptors of adaptive immune cells are recombined from a variety of gene segments, creating vastly diverse receptor repertoires. Immunological memory ensures that repeated exposure to pathogens leads to increased capability to resist disease.

The main cells involved in the adaptive immune response are B and T cells, responsible for antibody-mediated and cell-mediated immunity, respectively. These processes largely take place in the lymphatic system, consisting of lymphatic vessels, primary (or central) lymphoid tissue i.e. the bone marrow and thymus, and secondary (or peripheral) lymphoid tissue i.e. lymph nodes and the spleen (Figure 5). Only a minority of circulating lymphocytes are present in blood: the absolute majority can be found in the lymphatic system.

A central feature of the adaptive immune system is the ability to distinguish “self” from “non-self”: this is acquired through elaborate processes of so-called positive and negative selection during B and T cell development, ensuring the retainment of receptors capable of responding with enough strength to foreign antigens but not recognizing healthy host tissue. This has clinically important implications for immunodeficiency, autoimmunity, cancer, and transplantation.

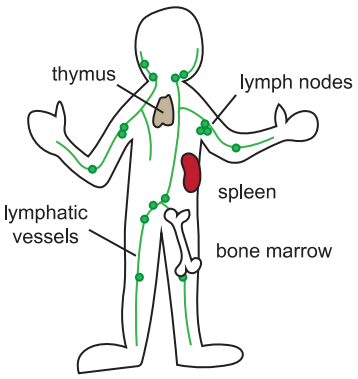

Figure 5. The lymphatic system. Lymphatic vessels return fluid (lymph) from peripheral tissues to the central circulation. The development of lymphocytes occurs in primary lymphoid tissues (thymus and bone marrow). Mature lymphocytes encounter antigen in secondary lymphoid tissues (lymph nodes and spleen).

1.1.3.1 The genetic process of V(D)J recombination yields extremely diverse B and T cell receptor repertoires

The ability of the adaptive immune system to produce highly specific antigen responses stems from the immensely diverse receptor repertoires of B and T cells. In contrast to the germline-encoded receptors of the innate immune system, B cell receptors (BCRs) and T cell receptors (TCRs) are recombined from separate gene segments in their respective gene loci, in a process called V(D)J recombination[F]. The resulting extreme diversity of genetic sequences leads to a capability of BCRs to potentially recognize 10^{13}–10^{18} different antigens and of TCRs to potentially recognize 10^{15}–10^{20} different antigens (51-54). *N.B.* that the genetic sequence diversity of BCRs and TCRs not necessarily corresponds to the actual number of different recognizable antigens; furthermore, the actual receptor repertoire in a given individual is restricted by the number of B and T cells present in the body, considerably lower than the theoretical number of receptor diversity (at least 10^{11} of both B and T cells, respectively (55, 56)).

V(D)J recombination takes place during the development of B and T cells in the bone marrow and thymus, respectively (Figure 5). The BCR and TCR gene loci are arranged according to the subunits of the receptors: Ig heavy chain and light chains κ and λ; and α and β chains (as well as γ and δ chains, not described further in the scope of this thesis), respectively. For BCR Ig heavy chains and TCR β chains, the genes are arranged in so-called variable (V), diversity (D), and joining (J) segments; for BCR light chains and TCR α chains, there are only V and J segments.

The recombination of VDJ segments is mediated by enzymes including recombination-activating genes (RAG) 1 and 2, terminal deoxynucleotidyl transferase (TdT), and Artemis. RAG1 and RAG2 bind to recombination signal sequences flanking each segment, creating DNA strand breaks that together with Artemis are mended by non-homologous end joining (NHEJ), simultaneously excising unused segments (Figure 6) (57, 58). Furthermore,

[F] Susumu Tonegawa was awarded the Nobel Prize in 1987 for his work on the genetic principle for generation of BCR/antibody diversity,

inaccuracies are introduced by TdT in the form of the addition of a random number of nucleotides between the joined segments, generating junctional diversity that dramatically increases the overall receptor diversity (58).

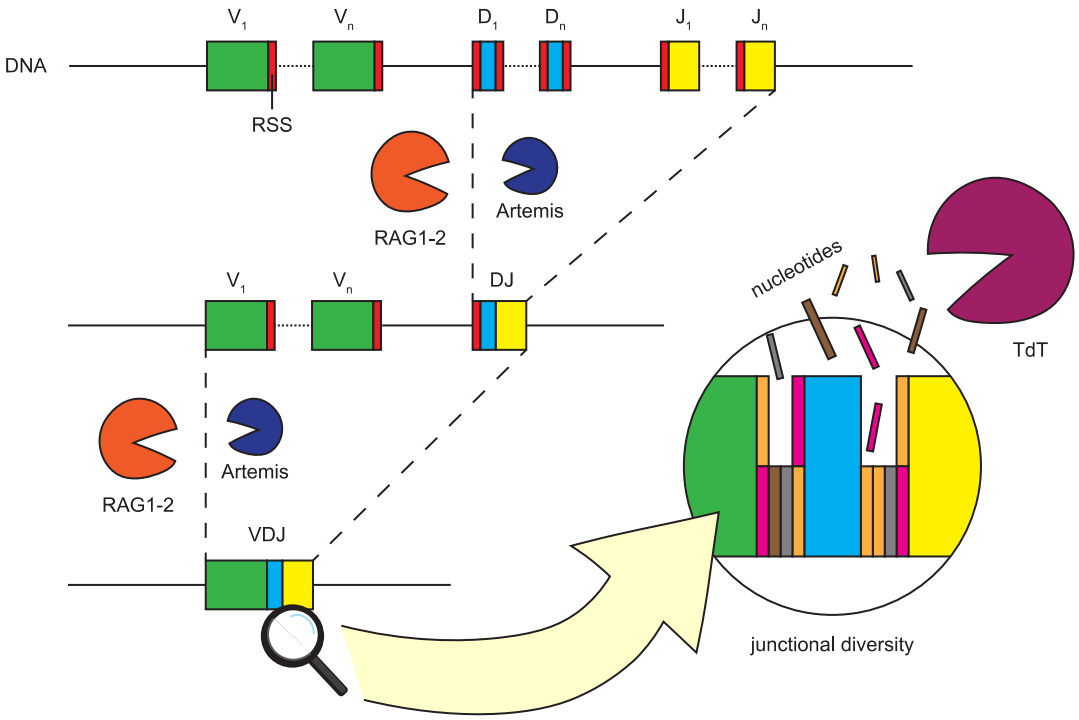

Figure 6. VDJ recombination. Functional B cell receptor (BCR) and T cell receptor (TCR) genes are recombined from gene segments in their respective loci. For BCR Ig heavy chains and TCR β chains, the genes are arranged in so-called variable (V), diversity (D), and joining (J) segments (their common principle of recombination shown in this figure); for BCR light chains and TCR α chains there are only V and J segments, but the mechanism of recombination is the same. Segments are flanked by recombination signal sequences (RSSs) targeted by recombination-activating genes (RAG) 1 and 2, inducing DNA strand breaks mended by non-homologous end joining by RAG1-2 and Artemis; segments not used are excised. D and J segments are recombined first, after which a V segment is joined. Further junctional diversity is added by the enzyme terminal deoxynucleotidyl transferase (TdT) by the addition of a random number of nucleotides.

1.1.3.2 B cells are the effector cells of humoral immunity

B cells are lymphocytes mainly developing in the bone marrow and spleen and constitute a major part of humoral immunity (from Latin *umor*, "fluid"), i.e. immunity originating from molecules in extracellular fluids (this term thus also involves the complement system and antimicrobial peptides; see section 1.1.2.4). Their main effector function is the production of antibodies[G], soluble forms of their BCR that take part in a wide variety of immune functions.

The development of B cells originates with common lymphoid progenitors in the bone marrow. A central process is the production of a functional BCR, why several developmental stages of B cells are tied to V(D)J recombination. Early B cells committed to the lineage start to express RAG1, RAG2, and TdT, needed at the subsequent stage when pro-B cells first recombine the Ig heavy chain D and J segments and later the heavy chain V segment to the joined DJ segments (Figure 7) (59, 60). During the pro-B cell stage, cells start to express the B cell marker CD19

[G] Nils Jerne was awarded the Nobel Prize in 1984 for his work on antibody immunity, sharing the prize with Georges Köhler and César Milstein for their work on the production of monoclonal antibodies.

(59). A successfully recombined heavy chain couples with the so-called surrogate light chain, assembling the pre-BCR complex also composed of the Igα and Igβ signaling subunits, and marks the transition to the pre-B cell stage (61, 62).

The assembly of and signaling through the pre-BCR complex is the first major checkpoint in B cell development and serves three main functions: allelic exclusion, i.e. inhibiting further heavy chain recombination to ensure only one heavy chain version per cell is expressed; proliferation of functional pre-B cells; and initiation of Ig light chain recombination (63). When light chain V and J segments are successfully recombined into a functional light chain (either κ or λ), the BCR is assembled and expressed as the membrane-bound antibody IgM (62).

A functional BCR is the second major checkpoint in B cell development and marks the transition to immature B cells. At this stage, several processes are engaged to limit the egress of autoreactive B cells from the bone marrow: if the assembled BCR binds with too much strength to self-antigens in the bone marrow, the B cell can undergo receptor editing (i.e. exchanging the Ig light chain), deletion, or become anergic (64).

Immature B cells that survive this so-called negative selection leave the bone marrow and proceed to become circulating transitional B cells, expressing IgD alongside IgM as well as the B-cell activating factor receptor, important for B cell maturation and survival in the periphery (51, 65-67). The final maturation stages occur in the spleen, where the majority of B cells differentiate into circulating so-called follicular B cells, and some differentiate into marginal zone B cells remaining in the spleen (51).

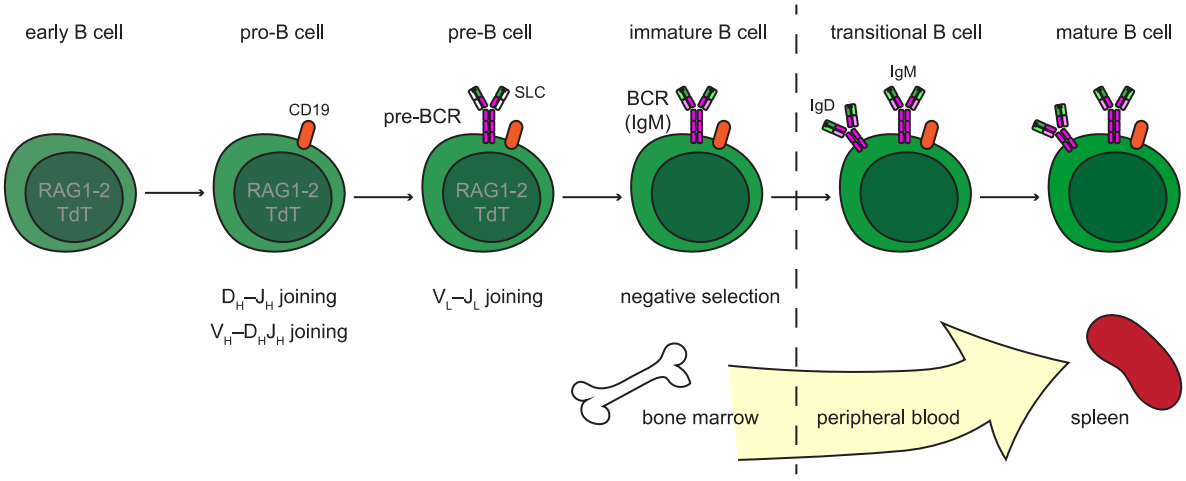

Figure 7. B cell development. Early B cells in the bone marrow start to express recombination-activating genes (RAG) 1 and 2 as well as terminal deoxynucleotidyl transferase (TdT). During the pro-B cell stage, the immunoglobulin heavy chain is recombined from V, D, and J gene segments, and co-receptor CD19 is expressed. A successfully recombined heavy chain couples with the surrogate light chain (SLC) to form the pre-BCR, marking the transition to the pre-B cell stage. When the immunoglobulin light chain is successfully recombined, the BCR is expressed (as membrane-bound IgM). These immature B cells undergo negative selection to minimize the amount of autoreactive B cells egressing from the bone marrow. Transitional B cells in the periphery express IgD and mature in the spleen.

As naïve follicular B cells circulate in the blood and the lymphatic system, they pass through follicles in lymph nodes and the spleen. B cells are capable of recognizing a wide variety of antigenic structures, including proteins, carbohydrates, and lipids (in contrast to T cells that only can recognize protein antigens presented on MHC/HLA molecules; see section 1.1.3.4).

If a B cell encounters its cognate antigen, it may become activated and initiate a humoral immune response involving the production of secreted antibodies. Antigen responses are classified as either T-dependent or T-independent based on if T cell help is needed to activate the antibody response of B cells.

When the BCR binds to its antigen, the BCR complex aggregates, with subunits Igα and Igβ transmitting signals and surface molecules CD19 and CD21 acting as co-receptors. CD19 lowers the threshold for BCR activation and CD21 is a complement receptor, further increasing stimulation in the presence of complement C3 fragments (68, 69). Activation-induced conformational changes in the BCR complex allow immunoreceptor tyrosine-based activation motifs (ITAMs) on Igα and Igβ to become phosphorylated by Src family kinases and bind spleen tyrosine kinase (Syk) (70-72). Syk initiates downstream signaling pathways, including the activation of Rho GTPases (73, 74), and ultimately activation of transcription factors necessary for B cell differentiation and proliferation, including Myc, NFAT and NF-κB (71, 72).

Activated B cells undergo clonal expansion and differentiate into plasma cells, able to secrete large quantities of antibodies (Figure 8). Depending on if T cell help has been provided, these plasma cells may be long-lived or short-lived. T-dependent antigen activation in lymphoid follicles leads to the development of so-called germinal centers, microanatomical structures where B and T cell interactions lead to the production of class-switched and highly specific antibodies. These interactions require follicular T_H cell recognition of internalized antigen presented on MHC/HLA molecules by B cells, and is mediated by CD40 on B cells binding to CD40 ligand on follicular T_H cells (75).

Class switching is the process of changing the constant region of antibodies from IgM to IgG, IgA, or IgE, enabling specialization of the immune response as these antibody classes have different effector functions. The outcome of this process depends on the cytokine environment in the germinal center, influenced by the type of immune response trigger. Class switching is mediated by the enzyme activation-induced cytidine deaminase (AID), expressed in germinal center B cells (76). AID introduces directed mutations in DNA by the deamination of a cytosine base, which becomes uracil, recognized and removed by uracil-DNA glycosylase (77). This leads to DNA double-strand breaks in class switch regions of the Ig heavy chain locus, whereby the constant region is rearranged and DNA strand breaks are mended by NHEJ (78).

To increase the antigen specificity of produced antibodies, B cells in the germinal center can undergo somatic hypermutation, a process where the antibodies with the highest antigen affinity are selected. In the germinal center, follicular dendritic cells present antigen to B cells, who require this antigenic stimulation to proliferate. As AID expression is upregulated for germinal center B cells, a multitude of random point mutations are introduced in the antibody V regions that bind antigen (79). The competition for antigen binding leads to a selective pressure on antibody specificity, ensuring that only B cells with high-affinity receptors survive the germinal center reaction.

B cells that emerge from the germinal centers differentiate into either plasma cells or memory B cells. Without T cell help, plasma cells are generally short-lived, however, T-dependent antigens generate long-lived plasma cells residing in the bone marrow and capable of producing antibodies for an extended period of time (80). Memory B cells remain dormant in the body, and rapidly respond to re-exposure to antigen with antibody production, forming a secondary response that generally is more efficient than the primary due to the antibodies produced already being class switched and affinity maturated (81).

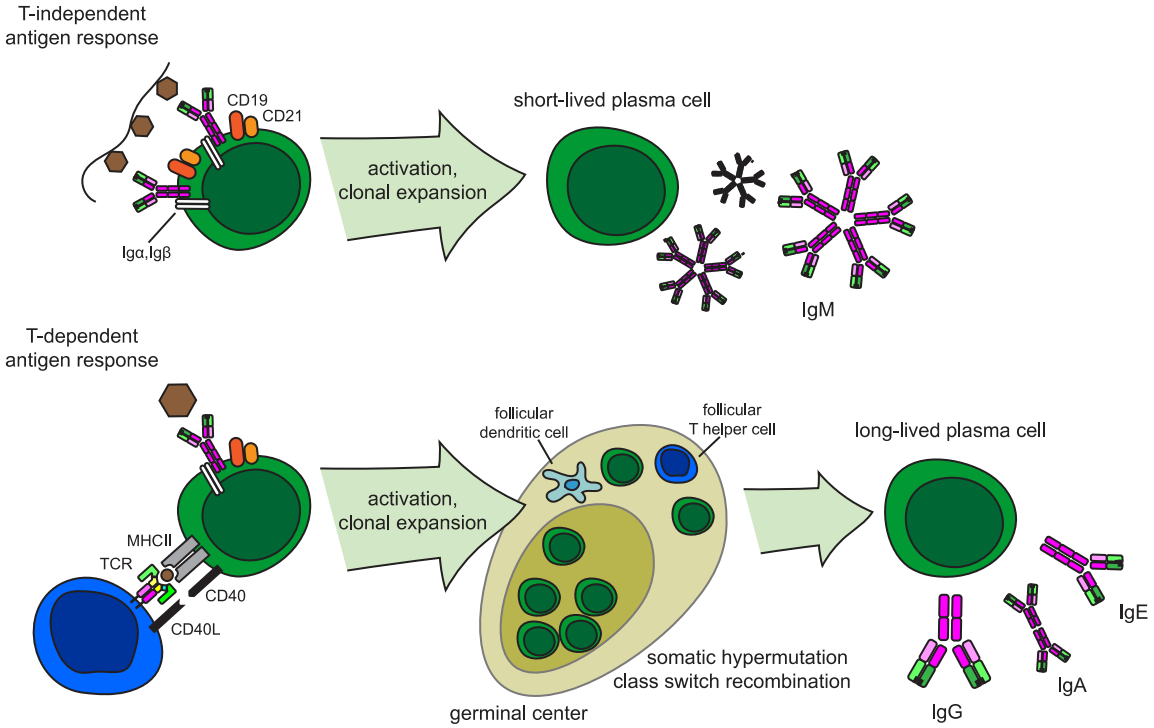

Figure 8. B cell activation and antibody production. When B cells encounter their cognate antigen, they are activated and undergo clonal expansion, becoming antibody-secreting plasma cells. T-independent antigen responses result in short-lived plasma cells producing IgM antibodies. T cells recognizing internalized antigen presented on B cell MHC/HLA molecules provide stimulation by CD40 ligand, resulting in a T-dependent antigen response where B cells undergo class switching and somatic hypermutation in the germinal center. The germinal center reaction results in long-lived plasma cells typically secreting IgG, IgA, or IgE antibodies. TCR, T-cell receptor; MHCII, major histocompatibility complex class II; CD40L, CD40 ligand.

1.1.3.3 The structure and function of antibodies

Antibodies[H] consist of two heavy chains and two light chains and have one variable antigen-binding region (Fab) and one constant region (Fc) (Figure 9A). The most crucial parts for antigen-binding of the Fab region are called complementarity-determining regions (CDRs) 1-3, where CDR1 and CDR2 correspond to sequences in the V gene segment and CDR3 spans the V(D)J junction of heavy and light chains, constituting the most variable region. AID has been shown to specifically concentrate its mutational activity on hotspots in these regions (82).

[H] Gerald Edelman and Rodney Porter were awarded the Nobel Prize in 1972 for their work on the structure of antibodies.

As the effector molecule of the B cell response, antibodies can perform a variety of functions, including neutralization of pathogens or toxins; complement activation; and recruitment of innate cells for phagocytosis, degranulation, and/or cytotoxicity (Figure 9B) (83). The functions of the different Ig classes are related to their constant regions and molecular structure.

Depending on the Ig class, the antibody constant regions bind to different Fc receptors expressed by a wide variety of cells; these receptors can be both activating and inhibitory. The co-expression of activating and inhibitory Fc receptors by individual immune cells sets a threshold for activation and thereby regulates downstream responses depending on the course of the immune reaction (84).

IgM is secreted as a pentamer, facilitating complement activation by providing several binding sites for the complement protein C1q (85). IgG can also activate complement, but as it is secreted as a monomer, at least two IgG molecules in proximity to each other are required for this activation. The main effector functions of IgG are instead neutralization, opsonization, and initiation of ADCC (83); IgG can also be transferred over the placenta in pregnancy, providing neonatal immunity. IgA is important for mucosal immunity and is secreted as a dimer. IgE is important in immunity against parasites, as it binds to Fcε receptors on mast cells and induces degranulation of histamine-containing granules, also a central mechanism in allergy (86).

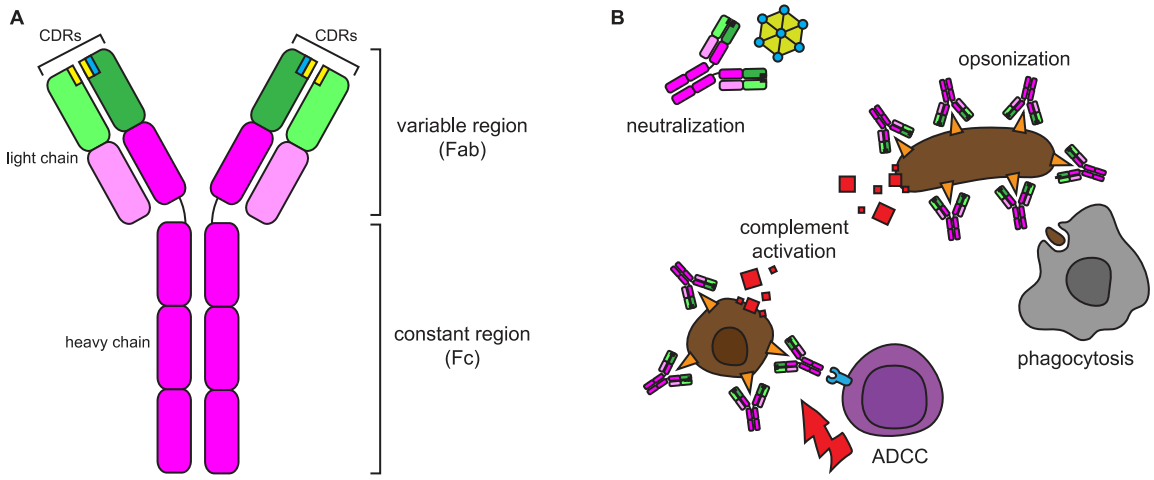

Figure 9. Antibody structure and function. (A) Antibodies consist of two heavy chains and two light chains and have one variable antigen-binding region (Fab) and one constant region (Fc). Complementarity-determining regions (CDRs) in the variable region are highly specific for antigen. (B) Antibody effector functions include the direct neutralization of pathogens or toxins; complement activation; and recruitment of innate cells for e.g. phagocytosis or antibody-dependent cellular cytotoxicity (ADCC).

1.1.3.4 T cells are the key mediators of cell-mediated immunity

T cells are lymphocytes central to the orchestration of a wide variety of immune responses, including cell-mediated immunity[I] against intracellular infections and providing help to e.g. B cells, neutrophils, and eosinophils in immunity against extracellular pathogens. The main types of T cells are cytotoxic T cells, T_H cells, and regulatory T (T_{reg}) cells. The antigen recognition

[I] Peter Doherty and Rolf Zinkernagel were awarded the Nobel Prize in 1996 for their description of cell-mediated immunity in the defense against viral infections.

of T cells is MHC-restricted, i.e. they can only recognize peptide antigens presented on MHC/HLA molecules[J].

The *T* in T cells comes from the *thymus*, the organ where they develop from common lymphoid progenitors that have migrated from the bone marrow. Early thymic progenitors enter the thymus in the corticomedullar junction and start migration towards the cortex, during which they interact with thymic epithelial cells providing signals for differentiation and survival (Figure 10) (87, 88); Notch and Wnt signaling pathways are essential for T cell development (89, 90). The different developmental stages of thymocytes are tied to V(D)J recombination of the TCR chains, as well as the expression of the T cell co-receptors CD4 and CD8: from double-negative (DN) through double-positive (DP) to single-positive T cells.

In humans, early thymic progenitors differentiate through three DN stages, based on their expression of surface markers CD38 and CD1a: DN1 identified as CD38-CD1a-; DN2 identified as CD38+CD1a-; and DN3 identified as CD38+CD1a+ (91). In mice, DN thymocyte differentiate through four DN stages, based on the expression of CD44 and CD25: DN1 as CD44+CD25-; DN2 as CD44+CD25+; DN3 as CD44-CD25+; and DN4 as CD44-CD25- (92).

Similar to early B cells, early thymocytes start to express RAG1-2 and TdT to initiate V(D)J recombination (89, 91). As thymocytes reach stage DN3, they are committed to the T cell lineage and start to recombine their TCR β chain, first by joining of D and J segments, and later by the joining of a V segment to the recombined DJ. The TCR β chain couples with the invariant pre-TCR α chain, leading to the assembly of the pre-TCR complex also involving the TCR co-receptor CD3 and the ζ chain (93, 94). Signaling through a functional pre-TCR stimulates proliferation and induces so-called β-selection, i.e. allelic exclusion preventing further recombination of TCR β chains, as well as initiates the recombination of the TCR α chain (95).

DN thymocytes become DP thymocytes by the expression of both CD4 and CD8 co-receptors, and at around this stage a successful recombination of the TCR α chain leads to the assembly of the TCR complex (89). Signaling through the TCR during T cell development is essential for the processes of positive and negative selection, whereby TCR recognition of antigen is tested on MHC/HLA molecules of thymic epithelial cells as the DP thymocytes migrate towards the thymic medulla. T cells with TCRs not recognizing antigen die by neglect (positive selection; resulting in MHC-restricted antigen recognition of surviving T cells), and T cells with TCRs with too high affinity to self-antigen are forced to undergo apoptosis (negative selection), limiting the egress of autoreactive T cells from the thymus (96, 97). As the thymic environment itself is not representative of the whole human body, thymic epithelial cells

[J] Baruj Benacerraf, Jean Dausset and George Snell were awarded the Nobel Prize in 1980 for their discovery of MHC molecules.

acquire the potential to display a wide variety of tissue-specific antigens to developing T cells by the expression of the autoimmune regulator gene (98).

The absolute majority of thymocytes fail positive and negative selection and thus undergo apoptosis: this overproduction reflects how important the sensitive calibration of TCR specificity is for the adaptive immune system. Surviving T cells proceed into either the CD4+ or CD8+ single-positive lineages, developing into T_H cells or cytotoxic T cells, respectively. Some T cells destined for negative selection may develop into T_{reg} cells, capable of inducing immunosuppressive responses and thus maintaining tolerance to self-antigens in the periphery (96). Furthermore, a separate lineage of non-conventional T cells, γδ T cells, may develop from common progenitors to αβ T cells in the thymus, bearing invariant antigen receptors and potentially taking part in a wide variety of innate-like responses (99).

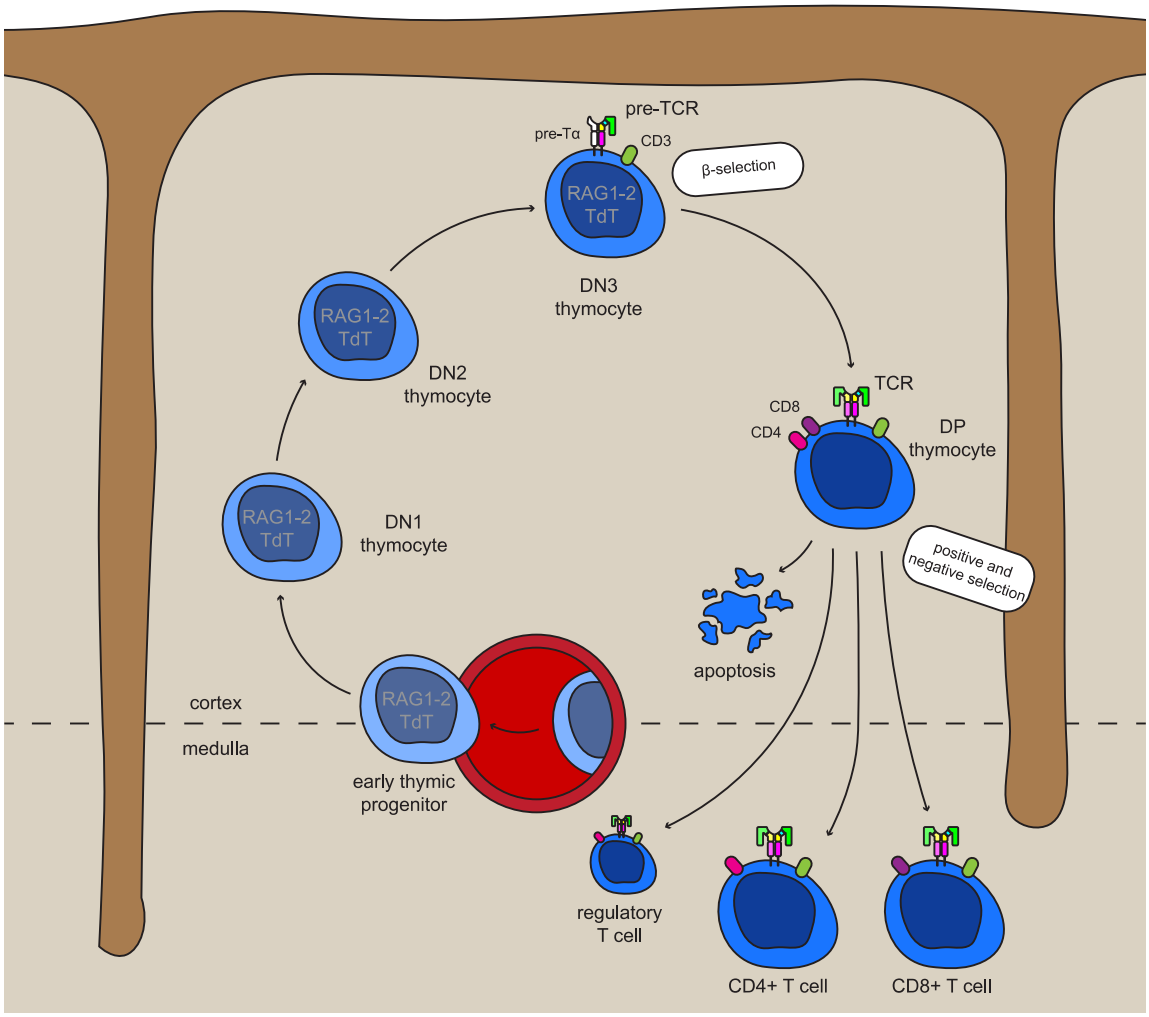

Figure 10. Human T cell development in the thymus. Early thymic progenitor cells enter through the blood into the corticomedullar junction of the thymus, expressing recombination-activating genes (RAG) 1 and 2 as well as terminal deoxynucleotidyl transferase (TdT) necessary for subsequent V(D)J recombination of TCR α and β chains. As thymocytes migrate through the cortex and medulla, they proceed through developmental stages linked to and named after the presence of CD4 and CD8 T cell co-receptors. At the end of the initial double-negative (DN) stages, thymocytes assemble a pre-TCR complex consisting of the TCR β chain, a pre-Tα chain, and the co-receptor CD3; signaling through the pre-TCR leads to so-called β-selection. Double-positive (DP) thymocytes that have functionally rearranged α and β chains form a TCR complex and undergo positive and negative selection. The majority of thymocytes undergo apoptosis; the surviving single-positive cells develop into either CD4+ T helper cells, CD8+ cytotoxic T cells, or regulatory T cells.

Naïve T_H cells and cytotoxic T cells circulate the blood and lymph, and being MHC-restricted, need to encounter antigen presented on the membrane proteins MHC/HLA class I and II molecules (100). MHC class I molecules are expressed on all nucleated cells and consist of an α chain and β_2 microglobulin, capable of presenting peptide antigens derived from cytosolic proteins. In humans, MHC class I corresponds to HLA-A, -B, and -C. MHC class II molecules are expressed on professional antigen-presenting cells such as macrophages, dendritic cells, and B cells, and consist of an α chain and a β chain. They are capable of presenting peptide antigens derived from extracellular internalized proteins and correspond to HLA-DP, -DQ, -DR, DM, and DO in humans. The MHC genes are the most polymorphic genes in humans (and mammals), reflecting evolutionary pressure from infectious diseases and has implications for the heritably differential risk of developing diseases related to the immune system (101-103).

As a naïve T cell recognizes its cognate antigen by its TCR α and β chains, its co-receptor CD4 (recognizing MHC class II) or CD8 (recognizing MHC class I) will provide additional stimulation and signal transduction. Activation-induced conformational changes in the TCR complex allow ITAMs on the co-receptor CD3 and the ζ chain to become phosphorylated by Lck, a Src family kinase that is non-covalently bound to CD4 and CD8 (104). The phosphorylated ITAMs recruit the adaptor protein ZAP-70 (105) (analogous to Syk in BCR signaling), initiating downstream signaling pathways, including the activation of Rho GTPases (106), and ultimately the activation of transcription factors AP-1, NFAT, and NF-κB, necessary for T cell activation and proliferation (107).

Further regulation of T cell activation comes from the effect of co-stimulatory and inhibiting receptors including CD28, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and programmed cell death protein 1 (PD-1)[K]. CD28 is a T cell co-stimulatory receptor capable of recognizing activation-induced molecules on antigen-presenting cells, including CD80 and CD86 (also known as B7-1 and B7-2, respectively), leading to amplification of the TCR signal (108, 109). CTLA-4 is a T cell inhibitory receptor also capable of recognizing CD80 and CD86, suppressing the TCR signal, and is also found in high numbers on T_{reg} cells (110, 111). PD-1 is an inhibitory receptor recognizing PD ligands on many different cell types, also suppressing the TCR signal upon ligation (112).

When CD8+ cytotoxic T cells are activated by antigen on MHC class I, they kill the antigen-presenting cell by the secretion of cytotoxic granules (Figure 11A). As in NK cells, the cytotoxicity is mediated by perforin and granzyme, forming pores in the membrane of target cells and inducing apoptosis (37, 38). In this way, cytotoxic T cells eliminate tumor cells or infected cells and limit the spread of disease.

CD4+ T_H cells that are activated by antigen on MHC class II may differentiate into different T_H subsets depending on the cytokine environment the activation occurs in: the main subsets

[K] James Allison and Tasuku Honjo were awarded the Nobel Prize in 2018 for their work on cancer therapy based on inhibition of CTLA-4 and PD-1 regulation, respectively.

are T_H1, T_H2, and T_H17 (other subsets, including T_H9 and T_H22 (113, 114), will not be discussed here).

T_H1 cells stimulate the phagocytic response of macrophages; IFN-γ secreted by T_H1 cells increases production of reactive oxygen species and nitrous oxide, facilitating the killing of phagocytosed bacteria (115, 116). Furthermore, T_H1-activated macrophages increase their secretion of inflammatory cytokines and the expression of MHC and co-stimulatory molecules (117). Differentiation of CD4+ T cells into T_H1 cells is induced by IL-12 and IFN-γ (118-120).

T_H2 cells participate in the immune response against parasites by the secretion of IL-4, IL-5, and IL-13, stimulating eosinophils, mast cells, IgE production by B cells, and alternative activation of macrophages (121); alternative macrophage activation is directed towards tissue repair and fibrosis rather than phagocytosis and inflammation (115). Differentiation of CD4+ T cells into T_H2 cells is induced by IL-4 (122, 123). Dysregulation of the T_H2 response has been shown in the development of allergic diseases, as cytokine secretion by allergen-activated T_H2 cells may drive the production of IgE by B cells and induce airway inflammation and fibrosis in asthma (124, 125).

T_H17 cells participate in the response to extracellular bacteria and fungi. The secretion of IL-17 and IL-22 activates leukocytes and epithelial cells to produce antimicrobial peptides and cytokines, stimulating inflammation, neutrophils, as well as increasing the barrier integrity of epithelial cells (126-128). T_H17 differentiation is mainly induced by IL-1, IL-6, and IL-23 as well as transforming growth factor β (129, 130).

T_{reg} cells modulate the immune response by maintaining peripheral tolerance and suppressing the activity of other types of T cells. Their suppressive action is in part mediated by the secretion of inhibitory cytokines IL-10 and transforming growth factor β (131, 132), but other regulatory modes of action include suppression by cytolysis, metabolic disruption, or targeting dendritic cells (133).

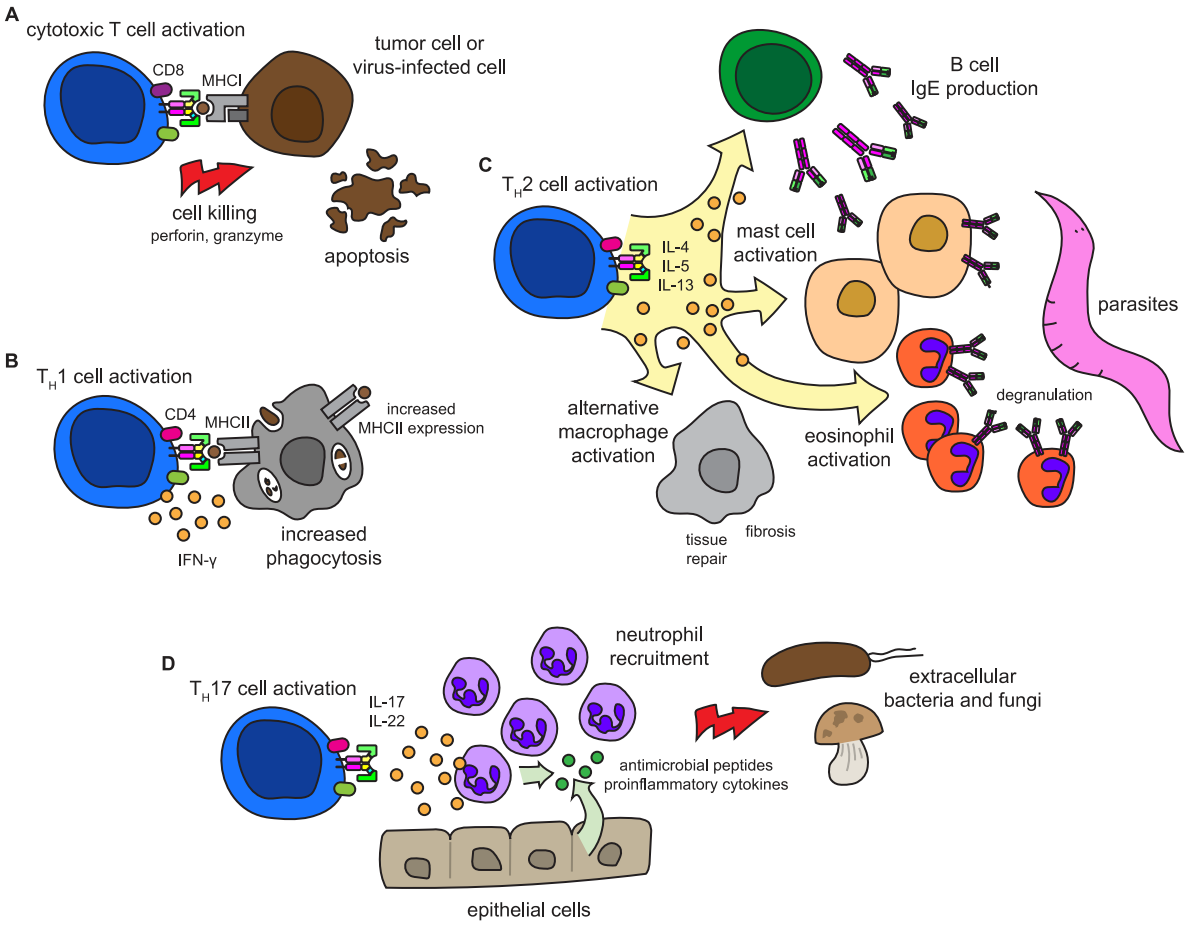

Figure 11. Activation and function of cytotoxic T cells and T_H cells. (A) CD8+ cytotoxic T cells recognize antigen presented on MHC class I molecules, leading to killing of the antigen-presenting cell by the secretion of cytotoxic granules containing perforin and granzyme. (B) CD4+ T_H1 cells recognize antigen presented on MHC class II molecules, leading to the secretion of IFN-γ that stimulates phagocytosis in the target cell and induces the expression of MHC class II and co-stimulatory molecules. (C) Activated T_H2 cells secrete cytokines IL-4, IL-5, and IL-13, important for the defense against parasites by the stimulation of B cell IgE production and activation of mast cells and eosinophils. IgE antibodies bound to the surface of mast cells and eosinophils mediate the release of histamine when binding to their antigen. T_H2 cells also stimulate alternative macrophage activation, promoting tissue repair and fibrosis. (D) T_H17 cells secrete IL-17 and IL-22 that stimulate leukocytes and epithelial cells to produce antimicrobial peptides and proinflammatory cytokines, recruiting neutrophils crucial for the defense against extracellular bacteria and fungi.

1.2 THE CYTOSKELETON

The cytoskeleton is the main structural component of the cell, composed of a network of proteins that are involved in a several essential functions, including preserving the integrity of the cell, mediating its connection to surrounding cells and tissues, controlling cell movement, and organizing a multitude of intracellular processes in space and time. The main cytoskeletal components are actin filaments (also known as microfilaments), microtubules, and intermediary filaments, where actin will be described in more depth as it constitutes a major part of the works included in this thesis.

1.2.1 Actin filaments

Actin filaments take part in a wide variety of cellular functions including movement, proliferation, signaling, endocytosis, and secretion, as well as providing a structural network for organelles and other intracellular structures. They consist of the protein actin, with a mass of 42 kDa, existing in free form as the monomeric globular (G)-actin or in polymeric filaments as filamentous (F)-actin (134). The turnover between these forms of actin is highly dynamic and regulated by several mechanisms.

1.2.1.1 Polymerization of actin

Each actin filament is made up by two parallel F-actin strands intertwined as a double helix; the structure is polar with a fast-growing barbed (+) end and a slow-growing pointed (-) end (Figure 12A). The first step in polymerization of actin is nucleation, i.e. the formation of actin nucleus consisting of three actin monomers, a process which is thermodynamically unfavorable; however, when nucleated, further polymerization is facilitated (135, 136). Polymerization of G-actin into F-actin is made possible by actin being an ATPase, hydrolyzing ATP into ADP at the barbed end as monomers are added; ADP and free actin monomers dissociate from the pointed end (137). Rapid turnover of F-actin is necessary for adaptive movement of the cell, and polymerization is regulated by several proteins. Actin filament treadmilling is the process where the polymerization at the barbed end matches depolymerization at the pointed end, a process keeping the filaments at the same length and generating force by the consumption of ATP (138).

1.2.1.2 Subcellular structures of actin filaments

Several subcellular structures can be formed by actin filaments, including lamellipodia, filopodia, stress fibers, focal adhesions, and cortical actin (Figure 12B). These structures consist of branched actin networks or cross-linked actin filaments (so-called bundles), contributing to different shapes and functions. Lamellipodia are broad cell protrusions on the leading-edge of cells, mediating migration, and are rich in branched actin filaments (139); they are the main site for actin filament formation in the cell (140). Filopodia (also known as microspikes) are thin antennae-like cell protrusions built up by bundles of parallel actin filaments, extending from the lamellipodium to sense the environment and mediate migration and cell-cell communication (141, 142). Stress fibers are made up of antiparallel actin filaments

coupled with myosin, rendering them contractible, and respond to mechanical stress; they can be categorized into dorsal and ventral stress fibers, transverse arcs, and the perinuclear actin cap (143, 144). Focal adhesions are actin-rich structures mediating binding to the extracellular matrix via integrins, attaching the cell to its surroundings and transmitting signals regulating migration, proliferation, and gene expression (145). Cortical actin is arranged in a network together with actin-binding proteins and myosin, connected to the cell membrane and providing shape as well as regulating several events at the cell surface (146).

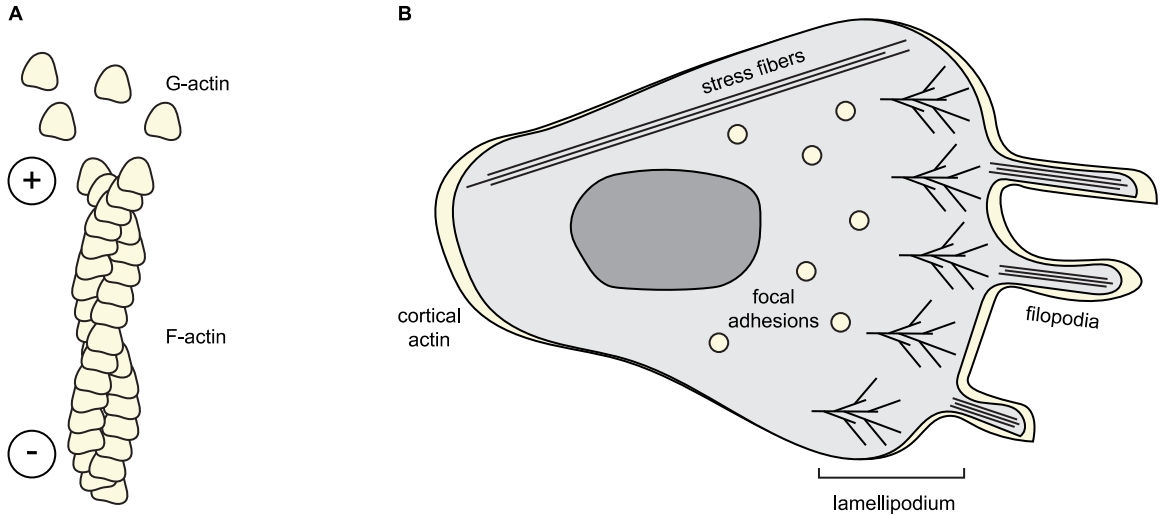

Figure 12. Actin polymerization and actin filament structures. (A) Globular (G)-actin polymerizes into filamentous (F)-actin. Two F-actin strands intertwine as a double-helical actin filament with a barbed (+) and pointed (-) end. (B) Actin filament bundles and branched networks form lamellipodia, filopodia, stress fibers, focal adhesions, and cortical actin.

1.2.1.3 Regulation of actin filament dynamics

As actin filaments are involved in many essential cell processes, its structural dynamics are tightly regulated by a group of proteins called actin-binding proteins (ABPs). These ABPs control several steps of actin filament assembly, including nucleation, monomer binding, branching, cross-linking, capping, severing, and depolymerization (Table 2) (147). Due to its connection to Wiskott-Aldrich syndrome protein (WASp), of central importance for papers III and IV, the actin-related protein (Arp) 2/3 complex will be described in detail in this section. WASp as well as the monomer-binding protein megakaryoblastic leukemia 1 (MKL1; of central importance for paper I) will be described in upcoming sections 1.2.2 and 1.2.3, respectively.

Type of regulation	**Actin-binding protein**
Nucleation	Arp2/3 complex, formin
Monomer binding	MKL1, profilin, ADF, cofilin
Branching	Arp2/3 complex
Cross-linking	Filamin, α-actinin, spectrin, transgelin
Capping	Capping protein, gelsolin, tensin
Severing	Gelsolin
Depolymerization	ADF, cofilin

Table 2. Actin-binding proteins. Examples of actin-binding proteins involved in the regulation of actin filament dynamics. Arp, actin-related protein; MKL1, megakaryoblastic leukemia 1; ADF, actin depolymerizing factor.

The Arp2/3 complex is a nucleating and branching ABP and consists of seven subunits: Arp2, Arp3, and ARPC1-5 (148, 149). Arp2 and Arp3 have structures similar to monomeric actin, why binding of the Arp2/3 complex to G-actin initiates nucleation by the formation of an actin trimer-like structure, facilitating further polymerization (150). The Arp2/3 complex binds to the side of already existing actin filaments, promoting the creation of a branch with a 70° angle (150, 151). However, the Arp2/3 complex is in itself not capable of initiating nucleation, and typically requires the presence of ATP and so-called nucleation-promoting factors, including WASp (152, 153). Arp2/3 is involved in several cellular functions including lamellipodia formation; adhesion and podosome formation; phagocytosis; endocytosis; vesicle trafficking; and secretion (149).

1.2.2 Wiskott-Aldrich syndrome protein

WASp is an actin nucleation-promoting factor uniquely expressed in hematopoietic cells, belonging to the WASp family of proteins (also including its analogous protein N-WASp, ubiquitously expressed) (154, 155). Its main function is to mediate signaling of Rho GTPases into Arp2/3 complex activation, leading to actin nucleation and branching (156, 157). Under steady-state conditions, it exists in an autoinhibited conformation in both the cytoplasm and the nucleus (158, 159).

WASp contains several protein domains, including the WASp homology domain 1; a basic region; the GTPase-binding domain (GBD); a proline-rich domain; and the verprolin homology domain, cofilin homology domain, acidic region (VCA) domain (Figure 13A) (154). GBD binds the VCA domain, resulting in the autoinhibited conformation (158). The WASp-homology domain 1 binds WASp-interacting protein (WIP), stabilizing the autoinhibited conformation and prevents WASp from degradation (160, 161). Upon binding of Rho GTPases to GBD, conformational changes are induced, releasing the binding of VCA whereby it can bind the Arp2/3 complex (Figure 13B) (158, 162). The structure of WASp is also regulated by phosphorylation of tyrosine residues in GBD, which may alter the stability of the autoinhibited conformation (163).

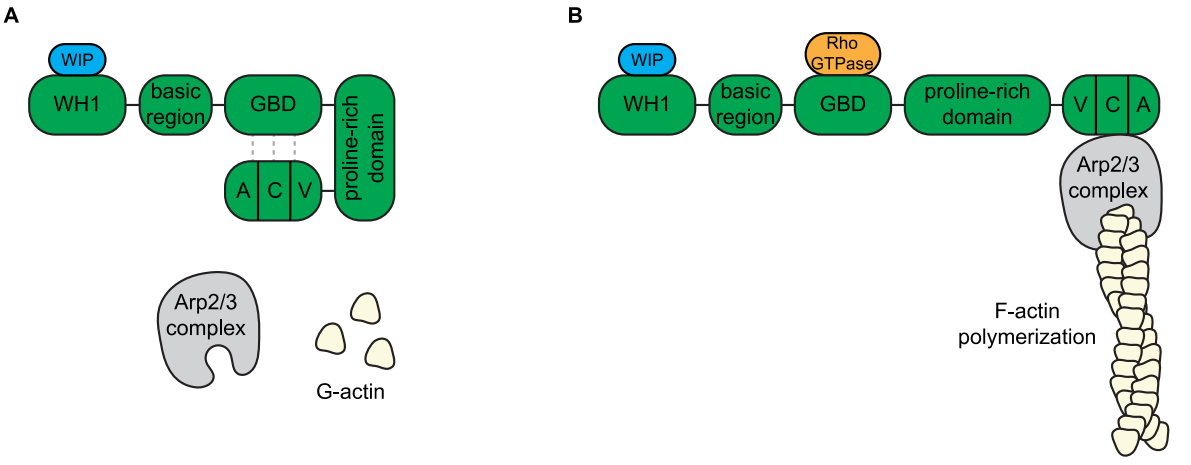

Figure 13. WASp function. (A) The inactive, autoinhibited conformation of WASp. (B) Binding of Rho GTPases to the GTP-ase binding domain (GBD) alters the conformation of WASp, exposing the verprolin homology domain, cofilin homology domain, acidic region (VCA) domain for binding of the Arp2/3 complex. This enables the polymerization of actin and branching of actin filaments. WH1, WASp homology domain 1; WIP, WASp-interacting protein; G, globular; F, filamentous.

WASp is of central importance in the primary immunodeficiencies Wiskott-Aldrich syndrome (WAS) and X-linked neutropenia (XLN) (see sections 1.3.1.1 and 1.3.1.2).

1.2.3 Megakaryoblastic leukemia 1

MKL1 is a ubiquitously expressed actin monomer-binding protein, mediating its binding via an RPEL motif (164, 165). The binding of G-actin to MKL1 inhibits the binding of importins to its nuclear localization signal, sequestering MKL1 in its actin-bound state in the cytoplasm (Figure 14A) (166). As actin polymerization consumes G-actin, it is released from MKL1, whereby MKL1 can translocate to the nucleus and bind serum response factor (SRF) (167); MKL1 acts as a transcriptional coactivator of SRF, inducing the transcription of several immediate early and cytoskeleton-related genes, including actin (Figure 14B) (168, 169). In this way, the dynamics of the cytoskeleton is linked to gene transcription, ensuring enough substrate for the rapid turnover of actin filaments and other cytoskeletal components (170).

MKL1 has been implicated in both cancer and primary immunodeficiency (see section 1.3.1.3).

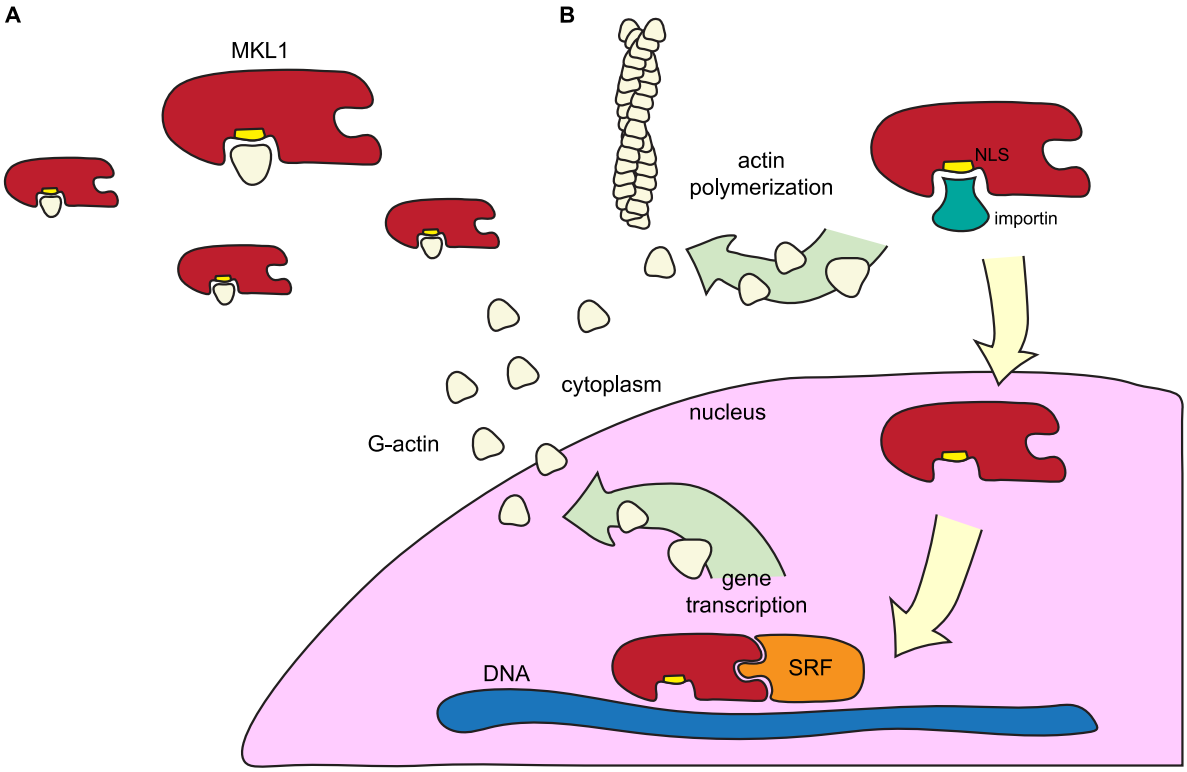

Figure 14. MKL1 function. (A) MKL1 is sequestered in the cytoplasm by the binding of globular (G)-actin. (B) Actin polymerization consumes G-actin, exposing nuclear localization signals (NLS) on MKL1 to the binding of importins, allowing MKL1 to translocate to the nucleus. In the nucleus, MKL1 binds serum response factor (SRF), acting as a transcriptional coactivator. MKL1-SRF induces the transcription of several immediate early and cytoskeleton-related genes, including actin.

1.3 PRIMARY IMMUNODEFICIENCY

Immunodeficiencies are disorders of the immune system where the immune system fails to combat infectious agents and/or tumor cells. As opposed to secondary (acquired) immunodeficiencies, such as AIDS (due to HIV infection) or immunosuppressive treatment, primary immunodeficiencies (PIDs) are inborn diseases due to mutations in genes important for normal immune cell function. Generally, PIDs as a group have been viewed as rare diseases,

although a recent epidemiological study suggests worldwide overall prevalence might be as high as 1 in 1,200 people (171); however, the incidence of each single disease is typically very rare, such as WAS (see section 1.3.1.1) with an incidence at the scale of 1–10 in 1,000,000 (172).

With recent advances in the research field of PID, it is increasingly clear that there often is an overlap in the disease spectrum between immunodeficiency and immune dysregulation: many PIDs also include features of autoinflammation, autoimmunity, and/or allergy (173). Furthermore, due to deficient immunosurveillance, PIDs carry an increased risk of cancer development, particularly lymphomas (174). PID symptomatology and severity differs greatly depending on the cell types being affected, ranging from e.g. lethal severe combined immunodeficiency (SCID; abolishing development of B and T cells) to IgA-deficiency (the most common immunodeficiency with a prevalence of 1 in 600, typically asymptomatic or presenting with recurrent mild mucosal infections) (175, 176).

PIDs may be categorized depending on the affected cell types and on the main pathological mechanism of disease. Most PIDs are monogenic and are caused by mutations in genes important for immune function; inheritance patterns vary from autosomal dominant, autosomal recessive, and X-linked recessive, where autosomal recessive inheritance is the most common among PIDs (173). Depending on what cells are affected, PIDs may be classified as combined (affecting both B and T cells); predominantly humoral (typically affecting B cells, involving impaired antibody responses); immune dysregulation (involving autoimmune features); and innate deficiencies (173).

Management of PID depends of disease phenotype and severity and may be divided into symptomatic and curative treatments. For milder PIDs, antibiotic treatments of recurrent infections may be sufficient, and some antibody deficiencies can be adequately treated with Ig replacement therapy (177). For severe PIDs like SCID, curative treatments involving hematopoietic stem cell transplantation (HSCT; typically in the first year of life) are currently the main option (178). However, HSCT success is to a large extent dependent on the HLA-matching between donor and recipient, which may complicate cases where no suitable donor is found as the risk for graft-versus-host disease is apparent (179). This risk may be avoided by gene therapy, i.e. delivery of healthy gene copies into autologous stem cells that are re-transfused into the host, essentially curing the disease. Gene therapy using retroviral vectors of gene delivery have been used successfully for PIDs such as adenosine deaminase-SCID, X-linked SCID, chronic granulomatous disorder, and WAS (180). A major pitfall for retrovirus-based gene therapy is the risk of developing leukemia or myelodysplasia, due to preferential genomic incorporation by retroviruses close to proto-oncogenes (181). This may be circumvented by the use of artificial endonucleases, a fast-growing field of research which is based on non-viral gene-editing, including zinc finger nucleases, transcription activator-like effector nucleases and the most recently discovered clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system. These forms of gene editing diminish the risk of off-target effects, but are not yet clinically available as treatment for PIDs (180).

1.3.1 Primary immunodeficiencies related to cytoskeletal dysregulation

The cytoskeleton is essential for a wide range of basal cell functions, including migration, adhesion, proliferation, and intercellular communication. Some cytoskeletal genes are specific to hematopoietic cells, why mutations therein will leave most organs intact except the immune system; loss-of-function mutations in ubiquitously expressed genes may be embryonic lethal due to the overall importance of the cytoskeleton during development (182). Cytoskeleton-deficiency PIDs typically affect a broad category of immune cells, thus classify as combined or severe combined PIDs (183). Examples of PIDs caused by cytoskeletal dysregulation are listed in table 3.

PID	Mutated protein	Protein function
WAS	Loss-of-function in WASp	Actin polymerization
XLN	Gain-of-function in WASp	Actin polymerization
WIP deficiency	Loss-of-function in WIP	Regulation of WASp activation and degradation
DOCK deficiencies	Loss-of-function in DOCK2 or 8	Regulating Rho GTPases
MKL1 deficiency	Loss-of-function in MKL1	Sensing actin levels

Table 3. Primary immunodeficiencies caused by mutations in genes important for cytoskeletal regulation. PID, primary immunodeficiency; WAS, Wiskott-Aldrich syndrome; WASp, Wiskott-Aldrich syndrome protein; XLN, X-linked neutropenia; WIP, WASp-interacting protein; DOCK, dedicator of cytokinesis; MKL1, megakaryoblastic leukemia-1.

1.3.1.1 Wiskott-Aldrich syndrome

WAS is a PID caused by loss-of-function mutations in the gene coding for WASp. Expressed uniquely in hematopoietic cells, WASp coordinates cellular signaling to actin polymerization and gene transcription, thereby controlling a wide range of cellular functions including migration, cell-to-cell communication and differentiation (see section 1.2.2) (154).

The incidence of WAS is estimated to 1–10 in 1,000,000 births and being an X-linked condition in practice almost only affects boys (172). Apart from immunodeficiency with recurrent infections, WAS patients typically exhibit other symptoms including hemorrhaging and eczema. Microthrombocytopenia (small platelets in low numbers) is present in virtually all patients, causing mild to severe hemorrhages in over 80 % of the patient population; 21 % of WAS patients die of hemorrhages (184). Eczema afflicts about 80 % of the patient population, also facilitating opportunistic infections (184, 185). Moreover, being a distinct sign of poor prognosis, malignancies such as leukemia, myelodysplasia and lymphoma develop in 13–22 % of the patients and result in up to 25 % of deaths (184, 185). In addition to this, WAS-associated autoimmune manifestations are present in 40–72 % of the patients: most commonly in the form of autoimmune hemolytic anemia, cutaneous vasculitis, arthritis, and nephropathy, and less commonly inflammatory bowel disease, idiopathic thrombocytopenic purpura, and neutropenia; all indicating significantly lower survival rates and also increased risk of developing tumors (186).

Diagnosis of WAS is based on clinical parameters, including hemorrhages, infections, and eczema, in combination with microthrombocytopenia and a potential family history of WAS (187). Currently, the standard treatment of WAS is allogeneic HSCT, which is considered curative: the survival rate is up to 80 % after five years of transplantation, the most common

complications being infections and graft-versus-host disease (188). Gene therapy for WAS has emerged as a potentially successful treatment option, however, correction of thrombocytopenia has not yet been sufficiently achieved (189).

Virtually all hematopoietic cells are affected in WAS (190). Macrophages and dendritic cells are unable to form podosomes and display impaired migration and phagocytosis (191-195). B cell subsets are affected differently, with a marked reduction of mature B cells (196); WAS patients also present with generally low IgM, high IgA and IgE, as well as a broad spectrum of autoantibodies (154, 197). T cells are present in low numbers, have altered morphology, and proliferate poorly in response to CD3-mediated stimulation (154, 198). Furthermore, the immunosuppressive function of T_{reg} cells is impaired in WAS (199).

1.3.1.2 X-linked neutropenia

XLN is a rare PID caused by gain-of-function mutations in the gene coding for WASp; disruption of the autoinhibited conformation of WASp leads to constitutive activation and a marked increase in polymerized actin (200-202). So far, only 20 patients have been described, with chronic neutropenia and monocytopenia constituting the major findings, resulting in recurrent infections (mainly pneumonia and otitis media) (200-204). Treatments suggested for XLN include granulocyte colony-stimulating factor and prophylactic antibiotics, as well as antibiotics for any ongoing infections (201, 204).

Neutrophils in XLN have been shown to be hyperactive and accumulate in tissues (205). NK cell numbers are reduced (201, 202). Ig production appears to be normal in patients (200, 202), while mouse model *in vitro* studies have shown impaired B cell proliferation and antibody secretion (206). One study found an increase of activated CD8+ T cells in peripheral blood (200), however, T cells have also been reported to proliferate normally in response to activation (206). Generally increased apoptosis and myelodysplasia have been reported (200, 201, 203, 206), and lymphocytes display decreased cell spreading and adhesion (206).

1.3.1.3 MKL1 deficiency

MKL1 regulates actin levels in the cell by sensing the amount of G-actin in the cytoplasm, linking actin dynamics to gene transcription. One case of MKL1 deficiency has been reported in a girl born to consanguineous parents, suffering from severe bacterial infections including *Pseudomonas* septic shock, meningitis, and recurring abscesses and ear infections, poorly responding to antibiotics (207). She was treated with Ig replacement and put on antibiotic prophylaxis (207). Blood tests showed normal lymphocyte numbers and Ig levels, however, T cell proliferation was deficient in response to CD3-mediated stimulation and Epstein Barr virus (EBV)-transformed B cells displayed impaired migration (207). Neutrophils displayed reduced phagocytosis and impaired migration, while dendritic cells displayed impaired cell spreading and podosome formation (207). F-actin levels were markedly reduced in both lymphoid and myeloid cells (207).

1.3.2 LRBA deficiency

PIDs caused by deficiencies in LPS-responsive beige-like anchor (LRBA) are not technically classified as PIDs related to cytosketelal dysregulation: the function of LRBA has not yet been fully clarified. However, as described in paper II, LRBA deficiency may present with accumulation of a number of different intracellular vesicles, the trafficking of which is known to involve the cytoskeleton.

To this date, 109 patients with LRBA deficiency have been described with varying symptomatology, most commonly including autoimmunity, enteropathy, splenomegaly, and hypogammaglobulinemia leading to recurrent infections (208, 209). Patients have been treated with antibiotics, Ig replacement, immunosuppression, and HSCT (208, 209).

Cellular deficiencies include reduced Ig production, low numbers and increased apoptosis of B cells (208-211); low numbers, increased apoptosis, and deficient activation and proliferation of T cells (208, 212); and impaired immunosuppression by T_{reg} cells linked to a decrease in CTLA-4 expression (212). Furthermore, LRBA has been shown to colocalize with CTLA-4 in endosomal vesicles, and LRBA deficiency leads to reduced surface expression of CTLA-4 due to decreased recycling (213).

2 AIMS

The overall aim of this thesis was to investigate the importance of the cytoskeleton for lymphocytes in primary immunodeficiencies.

Specific aims of the constituent papers:

Paper I — To investigate the immunological impact of a novel mutation in *MKL1* and assess its function in B cells from monozygotic triplets with Hodgkin lymphoma.

Paper II — To investigate the function of LRBA in lymphocytes from a patient with LRBA deficiency.

Paper III — To examine NK cell and T cell function in XLN patients and mouse models.

Paper IV — To examine the T cell receptor repertoire in WAS mouse models.

3 MATERIALS AND METHODS

The materials used in this thesis include primary cells and established cell lines from patients with mutations in *MKL1*, *LRBA*, and *WASP*, as well as immunodeficient NOD-SCID mice and mouse models for XLN and WAS.

Methods used include but are not limited to: flow cytometry; immunofluorescence microscopy; western blotting; quantitative reverse transcription polymerase chain reaction (PCR); live-cell imaging; transmission electron microscopy; ^{3}H-thymidine incorporation; fluorescent in situ hybridization of metaphase telomeres; *in vivo* tumor cell injection; EBV-transformation of B cells; gene targeting; mass spectrometry; imaging flow cytometry; and spectratyping.

Detailed descriptions of materials and methods used are provided in papers I–III. Materials and methods used in paper IV are described in the following section.

3.1 PAPER IV

3.1.1 The WASp knockout mouse model

WASp$^{-/-}$ mice were provided by Drs. Scott Snapper and Frederick Alt of the Department of Genetics and Medicine, Harvard Medical School, Boston. WASp$^{-/-}$ mice were bred with WASp$^{+/+}$ BALB/c strain mice (backcrossed 8 generations) to generate WASp$^{-/-}$ mice and wild type littermate controls. The mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Department of Microbiology, Tumor and Cell Biology at Karolinska Institutet, Solna.

Thymuses from 3–4 weeks old and 7–8 months old mice were excised after euthanasia. Spleens were excised from 7–8 months old mice. Three WASp knockout (KO) mice and three wildtype (WT) mice were used per experiment.

3.1.2 Spectratyping analysis of the T cell receptor repertoire

3.1.2.1 RNA preparation from thymocytes and spleen cells

Single-cell suspensions were generated from thymuses and spleens, respectively. Cells were lysed in 1 ml Trizol for 5 minutes in room temperature. 200 µl chloroform was added to bind nucleic acids, and after shaking and incubation in room temperature for 3 minutes, the mixture was centrifuged at 12,000 g for 15 minutes at 4 °C. 500 µl of the distinct upper phase, containing the nucleic acids, was transferred into a separate tube together with 500 µl of isopropanol to precipitate nucleic acids. After shaking and incubation in room temperature for 10 minutes, the mixture was spun at 12,000 g for 15 minutes at 4 °C, leading to the formation of a precipitated RNA pellet on the bottom of the tube. After discarding the supernatant, the pellet was washed with 75 % ethanol and spun at 7,500 g for 5 minutes at 4 °C. After discarding of the supernatant, the RNA pellet was air-dried for 10 minutes and resuspended in 20 µl nuclease-free H_2O. The concentration of obtained RNA was estimated using the NanoDrop

ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Nucleic acid purity was assessed by calculation of 260/280 nm and 260/230 nm wavelength absorbance ratios.

3.1.2.2 Preparation of complementary DNA from RNA

Using the Bio-Rad iScript cDNA Synthesis kit (Bio-Rad, Hercules, USA), the RNA template was mixed with 4 µl of 5x iScript reaction mix (Bio-Rad), 1 µl iScript reverse transcriptase (Bio-Rad) and nuclease-free water to a total volume of 20 µl. The reaction mix was subsequently put in a thermal cycler (Bio-Rad) and incubated for 5 minutes at 25 °C, 30 minutes at 42 °C and 5 minutes at 85 °C. Complementary DNA concentration was estimated using the NanoDrop spectrophotometer and the solution was diluted with PBS to acquire a concentration of 100 ng/µl. Nucleic acid purity was assessed by calculation of 260/280 nm and 260/230 nm wavelength absorbance ratios.

3.1.2.3 Complementary DNA amplification by polymerase chain reaction

Primers specific for different V_β-gene segments (Table 4) were purchased from Invitrogen and diluted into a concentration of 10 µM. For PCR, a 20 µl mixture of 10 µl GoTaq DNA polymerase (Promega, Fitchburg, USA), 1 µl of 100 ng/µl complementary DNA, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer conjugated to fluorescein amidite (Invitrogen), and 7 µl of nuclease-free H_2O was prepared for each well of a 96-well PCR plate. Each primer was added to two wells to enable calculating the mean of the duplicates and correct for eventual pipetting errors. The plates were subsequently put in a thermal cycler (Bio-Rad); first incubated for 1 minute at 95 °C; secondly, incubated for 70 seconds at 95 °C, 60 seconds at 60 °C and 4 minutes at 72 °C, this second part repeated 40 times; finally, incubated for 10 minutes at 72 °C and held at 4 °C.

3.1.2.4 Size separation by electrophoresis

0.5 µl of each PCR product (the amplified transcript of the V region of the β-chain of the TCR) was added to 0.7 µl of 400HD Rox size standard (Applied Biosystems, Foster City, USA) and 12 µl of deionized formamide (Applied Biosystems). The mixture was subsequently put in a thermal cycler (Bio-Rad) at 95 °C for 5 minutes before being run in an ABI Prism 3730 DNA Sequencer (Applied Biosystems). DNA fragments were separated by electrophoresis according to their size and presented in a spectratype densitogram.

3.1.2.5 Statistical analysis

Fragment analysis data from the ABI Prism 3730 DNA Sequencer was analyzed using the PeakScanner software (Applied Biosystems). Duplicates means for each segment were calculated and plotted in GraphPad Prism (GraphPad Software, La Jolla, USA). Nucleotide size and area under curve for each segment were added and nonlinear regression analyses were performed in order to quantify goodness of fit, yielding an R^2 value ($0 \leq R^2 \leq 1$). WT and KO values were analyzed with Student's t-test for each segment. A p-value of < 0.05 was considered significant.

Primer sequence in nucleotides	Primer specificity	Notes
TCACTGATACGGAGCTGAGGC	TCR V_β1	
GCCTCAAGTCGCTTCCAACCTC	TCR V_β2	
CACTCTGAAAATCCAACCCAC	TCR V_β3	
ATCAAGTCTGTAGAGCCGGAGGA	TCR V_β4	
CTGAATGCCCAGACAGCTCCAAGC	TCR V_β5	
AAGGTGGAGAGAGACAAAGGATTC	TCR V_β12.1	
CATTATGATAAAATGGAGAGAGAT	TCR V_β12.2	Non-functional in BALB/c mice
AGAAAGGAAACCTGCCTGGTT	TCR V_β12.3	Non-functional in BALB/c mice
TGCTGGCAACCTTCGAATAGGA	TCR V_β13.1	
CATTATTCATATGGTGCTGGC	TCR V_β13.2	Non-functional in BALB/c mice
CATTACTCATATGTCGCTGAC	TCR V_β13.3	
AGGCCTAAAGGAACTAACTCCCAC	TCR V_β14	Non-functional in BALB/c mice
GATGGTGGGGCTTTCAAGGATC	TCR V_β15	
GCACTCAACTCTGAAGATCCAGAGC	TCR V_β16	
TCTCTCTACATTGGCTCTGCAGGC	TCR V_β17	
CTCTCACTGTGACATCTGCCC	TCR V_β19	
CCCATCAGTCATCCCAACTTATCC	TCR V_β20	
CTGCTAAGAAACCATGTACCA	TCR V_β21	Non-functional in BALB/c mice
TCTGCAGCCTGGGAATCAGAA	TCR V_β23	
AGTGTTCCTCGAACTCACAG	TCR V_β24	
CCTTGCAGCCTAGAAATTCAGT	TCR V_β26	
TACAGGGTCTCACGGAAGAAGC	TCR V_β29	
CAGCCGGCCAAACCTAACATTCTC	TCR V_β30	
ACGACCAATTCATCCTAAGCAC	TCR V_β31	
CTTGGGTGGAGTCACATTTCTC	TCR C_β	Conjugated to FAM

Table 4. Primer sequences used for the amplification of V_β segments. TCR, T cell receptor; FAM, fluorescein amidite.

4 RESULTS AND DISCUSSION

4.1 INCREASED MKL1 ACTIVITY PARTICIPATES IN B CELL TRANSFORMATION AND HODGKIN LYMPHOMA PATHOGENESIS

The actin-sensing protein MKL1 was initially described as part of a fusion protein in megakaryoblastic leukemia (214, 215). Dysregulated MKL1 expression and activity have been implicated in both primary immunodeficiency and cancer (207, 216-218). Recently, two monozygotic triplets with Hodgkin lymphoma were described, diagnosed in 1985 and 2008, respectively (219). Blood samples from these patients and their undiagnosed triplet sibling were analyzed using microarray comparative genomic hybridization, identifying a 15–31-kb deletion in intron 1 of *MKL1* (219). The immune status of the triplets and expression levels of MKL1 were not known. In **paper I**, we sought to investigate the impact of the *MKL1* mutation on B cell function and Hodgkin lymphoma pathogenesis and generated EBV-transformed lymphoblastoid cell lines (LCLs) from all three triplets and two matched controls.

MKL1 protein expression was increased in primary cells from the triplets with Hodgkin lymphoma (HL1 and HL2) and the undiagnosed triplet (HL0), as assessed by flow cytometry. HL0 LCLs were found to have higher *MKL1* mRNA and MKL1 protein expression than HL1-2 and controls, as assessed by quantitative reverse transcription PCR and western blotting, respectively. MKL1-SRF induces transcription of several genes, including *SRF* and *ACTB* (168, 169). To examine MKL1 activity in LCLs from the triplets and controls, we measured mRNA expression of MKL1-dependent genes *SRF* and *ACTB* by quantitative reverse transcription PCR and found that it correlated to *MKL1* mRNA expression: HL0 LCLs displayed the highest expression for both MKL1-dependent genes. Flow cytometry-assessed G-actin expression was increased in HL0 LCLs as compared to HL1-2 and controls. F-actin was increased in primary B cells from HL2, and in primary monocytes from all triplets.

To investigate morphological and functional differences associated to increased MKL1 expression, we examined cell spreading, aggregation, proliferation, genomic stability, and *in vivo* tumor formation. Actin-dependent cell spreading was increased in HL0 and HL1 LCLs, as assessed by immunofluorescence microscopy, and interference reflection microscopy showed larger adhesion areas for HL0 LCLs. HL0 LCLs in culture aggregated less, associated to a decrease in adhesion receptor CD11a. Primary B cells from all triplets proliferated more than control cells as measured by flow cytometry-assessed expression of proliferation marker Ki-67. ^{3}H-thymidine incorporation assessment showed higher DNA synthesis in HL0 LCLs as compared to controls, also having a larger proportion of cells in S or G2/M phase and cells with more than 4n DNA content. HL0 and HL1 had an increased proportion of cells with more than 46 chromosomes, as assessed by fluorescent in situ-hybridization on metaphase telomeres. When injected into immunodeficient NOD-SCID mice, control LCLs failed to form tumors while HL0 LCLs formed large tumors with distinct angiogenesis *in vivo*.

To investigate the causality between increased MKL1 activity and the observed phenotype of HL0 LCLs, we sought to revert its phenotype with a small molecule inhibitor of MKL1 (220, 221). CCG-1423-treated HL0 cells displayed a dose-dependent lowering of MKL1 and SRF expression, as well as decreased cell spreading, aggregation, and proliferation. *In vivo* tumors of HL0 LCLs were suppressed by repeated intratumoral CCG-1423 injections.

These results indicated that the intronic deletion in *MKL1* led to increased MKL1 expression and activity, as well as malignancy-associated features of cytoskeletal derangement, increased proliferation, genomic instability, and tumor growth *in vivo*. Curiously enough, these features were most pronounced in the undiagnosed (and thus untreated) triplet HL0, while triplets HL1 and HL2 mostly resembled controls. This led us to reason that HL0 may be in a premalignant state, acting as an untreated control compared to triplets HL1 and HL2. As the intronic deletion in *MKL1* was found to be heterozygous (Figure S2, paper I), increased MKL1 expression in cells may have been a stochastic process, providing selective advantage for cells expressing the allele bearing the intronic deletion. The low penetrance of the mutation may represent a slow build-up of the premalignant cell population, potentially reset by chemotherapy, which would explain the mild and varying phenotype of HL1 and HL2 cells.

As MKL1 has been implicated in PID (207), and PIDs convey a higher risk of cancer development (174), the cause of Hodgkin lymphoma development in the triplets with the intronic deletion in *MKL1* may have been both intrinsic to B cells and extrinsic as by deficient immunosurveillance. However, we found no signs of immunodeficiency in the triplets, as assessed by infection history, leukocyte subset numbers, or mitogen stimulation of lymphocytes, suggesting that the development of Hodgkin lymphoma may primarily have been cell-intrinsic. In breast cancer and melanoma cell lines, MKL1 has been shown to be required for cancer cell adhesion, spreading, invasion, and motility, and overexpression of MKL1 increased metastatic potential (216). In hepatic and breast cancer cell lines deficient of the tumor suppressor deleted in liver cancer 1, MKL1 mediated increased migration, proliferation, and cell growth (218). MKL1 also mediates the transformation of breast cancer cells into being independent of hormone receptor signaling, a sign of increased malignancy (217). We searched the *R2 Oncogenomics Platform* (222) for MKL1 and found increased expression in Hodgkin lymphoma and lymphomas in general (Figure S9, paper I). SRF has been shown to mediate signaling downstream of the BCR (223), suggesting that increased MKL1-SRF activity as seen in our patients drives B cell activation and maturation. Altogether, these findings by us and others support the notion that Hodgkin lymphoma developed as a result of increased MKL1 expression in the triplets with the intronic deletion in *MKL1*.

4.2 LRBA DEFICIENCY LEADS TO VESICLE ACCUMULATION AND ALTERED LIPID METABOLISM

Patients with LRBA deficiency suffer from a syndrome with features ranging from PID (similar to common variable immunodeficiency) to autoimmunity (209). The function of LRBA is yet to be clarified, but its protein domains are also found in other proteins involved in vesicle trafficking and membrane dynamics (224). We obtained blood and skin samples from a female

LRBA-deficient patient presenting at age 4 with lymphadenopathy, splenomegaly, neutropenia, and thrombocytopenia, as well as autoimmune enteropathy; over time she developed several deep infections (225). In **paper II**, we aimed to examine the role of LRBA in vesicular dynamics and generated cell lines of patient fibroblasts and EBV-transformed B cells (LCLs), as well as CRISPR/Cas9 LRBA KO Jurkat T cells.

As lack of LRBA expression previously was described in LCLs from this patient (225), we confirmed that patient fibroblasts also lacked LRBA expression, assessed by immunofluorescence microscopy and flow cytometry. Decreased LRBA expression was also confirmed in CRISPR/Cas9 LRBA-targeted Jurkat T cells generated by us. LRBA has previously been described to prevent degradation of CTLA-4 (213). $LRBA^{-/-}$ Jurkat T cells expressed lower CTLA-4 than control cells, and while control Jurkat T cells transduced with CTLA-4 recycled CTLA-4 efficiently to the cell surface, CTLA-4-transduced $LRBA^{-/-}$ Jurkat T cells failed. These results show that both patient B cells and fibroblasts indeed lack LRBA, and that the LRBA KO in Jurkat T cells was successful in generating a phenotype similar to previously described LRBA-deficient T cells.

In another previously described patient with LRBA deficiency, the number of autophagosomes was increased in B cells as measured in transmission electron micrographs (210). By flow cytometry, we found that LCLs from our patient had a higher expression of autophagosomes than control cells, as detected by a cationic amphiphilic tracer dye. Autophagic flux was increased in patient fibroblasts, as measured by western blotting. However, when examining patient and control fibroblasts by transmission electron microscopy, differences in numbers of autophagosomes and lysosomes in fibroblasts did not reach statistical significance. This may be related to the relatively small sample size or suggest that LRBA-deficient B cells were more prone than fibroblasts to accumulate autophagosomes.

Patient LCLs and fibroblasts accumulated endosomes and lysosomes, as assessed by immunofluorescence microscopy. The accumulation of lysosomes in patient fibroblasts was confirmed in several settings: in untreated cells; with stimulation with serum starvation, LPS, and bafilomycin A1 to induce autophagy; and with stimulation with oleic acid to induce lipid body formation (see below). Flow cytometric analysis also showed an increase in lysosomes in unstimulated patient fibroblasts. No apparent difference was detected between $LRBA^{-/-}$ and control Jurkat T cells.

When examining Jurkat T cells by transmission electron microscopy, we found that $LRBA^{-/-}$ cells had an increase in both size and number of lipid bodies, with and without serum starvation. This led us to investigate the lipid compartment in fibroblasts as well and we found an increase in lipid bodies in patient fibroblasts, corroborated by two different immunofluorescent markers for lipids. Oleic acid was added to the fibroblasts to induce lipid body formation. Patient fibroblasts did further not increase their amount of lipid bodies by oleic acid treatment; control fibroblasts increased their amount of lipid bodies to match patient levels. The finding that the difference between unstimulated and stimulated fibroblasts was significantly large for control

fibroblasts but not for patient fibroblasts indicates that lipid body formation in untreated patient fibroblasts was already induced to a high degree by lack of LRBA.

To examine the overall lipid composition in LRBA-deficient patient cells, we used liquid chromatography combined with mass spectrometry to identify 10 different lipid classes. Patient and control fibroblasts were either untreated, serum starved, or starved and refed with serum. By unsupervised multivariate statistical analysis, we found that while starved control fibroblasts clustered with untreated control and patient fibroblasts, starved patient fibroblasts clustered with refed starved control and patient fibroblasts. When analyzing individual lipid classes, we found that the latter cluster had an increase of several lipid classes, including phosphatidylcholine and sphingomyelin. Pairwise analysis showed that patient fibroblasts responded to starvation by the increase of several lipid metabolites, while control fibroblasts did not respond to this stimulation. When refeeding starved fibroblasts with serum, patient cells did not further increase their lipid content, however, starved control cells responded to refeeding with an increase in several lipid metabolites. These findings suggest that patient fibroblasts have an altered response to starvation-induced stress, increasing their biogenesis of several lipid metabolites.

In this study, we have examined LCLs and fibroblasts from a female patient with LRBA deficiency, and LRBA$^{-/-}$ Jurkat T cells. A limiting factor for has been the poor viability of patient LCLs in culture: they rapidly died, why the collection of the required number of cells for several experiments has been hampered. LRBA-deficient B cells have previously been described as more prone to undergo apoptosis (210), why this may have been the reason for poor LCL viability in our experiments. To combat this, we took the approach to use fibroblasts for a large part of the experiments: primary fibroblast cell lines are stable, easy to keep in culture and convey less confounding factors than transformed cell lines; furthermore, they are easily obtained from skin biopsies (226, 227). The drawback of this approach is that some phenotypes unique to immune cells may not be present in fibroblasts.

We found that several different intracellular vesicles accumulated in LRBA-deficient patient cells, including lysosomes. Dysregulation of lysosomes is found in a group of diseases called lysosomal storage disorders, some of which involve immunodeficiency features with recurrent infections (228): α-mannosidosis type II, caused by mutations in MAN2B1 (229); Griscelli syndrome type 2, caused by mutations in RAB27A (230); and Chédiak-Higashi syndrome, caused by mutations in LYST (231). LYST has a BEACH protein domain that may mediate vesicle dynamics, a domain also found in LRBA (224), supporting our findings of vesicle dysregulation in LRBA deficiency.

Lysosomes are important in the sensing of intracellular nutrients, and may play a role in lipid metabolism by the induction of lipophagy during cell stress such as serum starvation (232). This may explain our findings of lipid body accumulation and alterations of the lipid class composition in LRBA-deficient cells. We argue that the accumulation of endosomes, lysosomes, autophagosomes and lipid bodies may impair the survival and function of immune

cells, leading to the immunodeficiency and autoimmunity present in patients with LRBA deficiency.

4.3 NK CELLS AND T CELLS IN XLN PATIENTS AND MOUSE MODELS ARE HYPERACTIVE

XLN is caused by activating mutations in WASp, presenting with neutropenia and immunodeficiency as well as an increased risk of myelodysplasia (200-204). Cytogenetic analysis of both XLN patients and mouse models has shown increased genomic instability with increased DNA content, tetraploidy, and chromosomal breaks (206, 233). XLN T cells have been shown to proliferate normally, however, one study found an increase of CD8+ T cells in peripheral blood (200, 206). XLN NK cells are low in numbers (201, 202). NK cells and T cells are crucial to tumor immunosurveillance, and in WASp deficiency (WAS), NK cells and T cells are hyporesponsive and immune synapse formation is impaired (234, 235). NK cell and T cell responsiveness to tumors in XLN, however, remains unknown. To investigate this, in **paper III** we examined NK cells and T cells from XLN patients and two different mouse models with WASp mutations corresponding to those found in patients ($WASp^{L272P}$ and $WASp^{I296T)}$.

Using *in vivo* imaging, we found that the rejection of injected YAC-1 T cell lymphoma cells was similar in WT and $WASp^{L272P}$ mice. *In vivo* NK cell-mediated rejection of β_2 microglobulin-negative splenocytes (lacking MHC class I molecules) was similar in WT and $WASp^{L272P}$ mice at 24 hours, but higher in $WASp^{L272P}$ mice at 8 and 48 hours after injection. *In vitro* activation of NK cells with antibodies to the activating NK cell receptors NKp46 and NK1.1 led to IFN-γ production and degranulation in WT, $WASp^{L272P}$, and $WASp^{I296T}$ mice. WT, $WASp^{L272P}$, and $WASp^{I296T}$ responded similarly *in vitro* to activation by YAC-1 cells. These results show that NK cells in XLN mouse models were not hyporesponsive as in WAS, but rather hyperactive in their rejection of tumors.

To investigate whether this increased activity was related to any developmental aberrations, we examined the education, maturation, and receptor repertoire of NK cells in WT, $WASp^{L272P}$, and $WASp^{I296T}$ mice. There were no differences in NK cell numbers or in surface markers for education and maturation stages. The proportion of the main classes of NK cell inhibitory receptors and the amount of different receptor classes per cell was similar in WT, $WASp^{L272P}$, and $WASp^{I296T}$ mouse models. These results indicated that the development of NK cells in XLN proceeded normally. When examining the individual expression of several different inhibitory and activating receptors, levels were similar in all three models except for the inhibitory NK cell receptor KLRG1: $WASp^{L272P}$ and $WASp^{I296T}$ expressed significantly lower KLRG1 than WT; this result contrasts the increase of KLRG1 in WASp deficiency (236).

As mobilization of actin is important for immune synapse formation, we examined NK cell actin dynamics in the XLN mouse models. Total F-actin expression was normal in $WASp^{L272P}$ and $WASp^{I296T}$ as compared to WT. When incubated with YAC-1 cells, WT, $WASp^{L272P}$, and $WASp^{I296T}$ NK cells polarized actin and formed similar immune synapses to YAC-1 cells, as

assessed by imaging flow cytometry. When adhered to glass surfaces coated with antibodies to NKp46, NK cells polarized actin similarly in all three models, as assessed by stimulated emission depletion microscopy. However, $WASp^{L272P}$ and $WASp^{I296T}$ managed to polarize actin to the glass surface even without activation of NKp46. These results suggest that XLN NK cells are hyperactive in the way that they can polarize actin without activation signals.

Flow cytometric analysis of CD8+ T cells from WT, $WASp^{L272P}$, and $WASp^{I296T}$ mice showed similar degranulation and IFN-γ production in response to CD3 stimulation in all three models. However, while CD4+ T cells from $WASp^{I296T}$ and WT mice were similar in degranulation and IFN-γ production, these processes were impaired in $WASp^{L272P}$ CD4+ T cells. When analyzed by imaging flow cytometry, both CD8+ and CD4+ T cells in $WASp^{L272P}$ and $WASp^{I296T}$ polarized actin similarly to WT in response to CD3 and CD28 stimulation. However, when incubated with A20 B cell lymphoma cells coated with anti-CD3 and anti-CD28, $WASp^{L272P}$ T cells displayed a higher killing rate *in vitro* than WT T cells as assessed by live-cell imaging.

As opposed to XLN mouse models, XLN patients have lower numbers of NK cells (201, 202). To investigate whether these patient NK cells respond normally, we examined peripheral blood mononuclear cells from two XLN patients (bearing a L270P mutation corresponding to the L272P mutation in mice) and their mother and sister, heterozygotic carriers for the mutation, as well as two healthy controls. Flow cytometry analysis showed significantly smaller populations of $CD56^{bright}$ and $CD56^{dim}$ NK cells in the XLN patients. To examine tumor responsiveness, XLN patient NK cells were incubated with K562 myelogenous leukemia cells and analyzed by flow cytometry: degranulation was decreased in XLN NK cells but IFN-γ production was similar to controls and carriers. XLN patient NK cells responded normally to receptor-independent stimulation by phorbol myristate acetate and ionomycin. As in $WASp^{L272P}$ and $WASp^{I296T}$ mice, the XLN patients displayed lower KLRG1 expression. Granzyme B expression was higher in XLN patients.

When examining XLN patient T cells by flow cytometry, we found an increase in CD4+ $CD8^{low}$ cells as compared to controls and carriers. KLRG1 expression was higher in XLN patient T cells, as opposed to the lower expression found in NK cells. Granzyme B expression, however, was high in XLN patient T cells as well, similar to NK cells. As increased expression of KLRG1 and granzyme B may represent exhaustion in T cells (35), we examined the response of CD8+ and CD4+ T cells to phorbol myristate acetate and ionomycin but found that the proportional distribution of activated T cells in XLN patients was similar to controls and carriers.

These results show that the function of NK cells and T cells with activating mutations in WASp contrasts WASp-deficient NK cells and T cells. In summary, NK cells in XLN display normal to increased tumor killing (in mice), a decrease in the exhaustion marker KLRG1 (in patients and mice), increased granzyme B expression (in patients), and polarization of actin without stimuli (in mice). T cells in XLN show overall normal degranulation and IFN-γ production (in

mice), increased KLRG1 and granzyme B expression (in patients), and increased tumor killing (in mice).

Some results were inconsistent between different XLN models. The increase of YAC-1 killing and β_2 microglobulin-negative splenocyte rejection in XLN mice and concomitant decrease of degranulation in XLN patients in response to K562 cells may reflect different response pathways triggered by the different tumor models. To investigate this further, the tumor killing assays for XLN patients and mice should include the same tumor cells. Furthermore, the normal degranulation and IFN-γ production in CD8+ T cells in both XLN mouse models and in $WASp^{I296T}$ CD4+ T cells contrasted the decrease in $WASp^{L272P}$ CD4+ T cells. This may reflect T cell subset-specific modes of actions of the I269T and L272P mutations.

KLRG1 is an inhibitory NK cell receptor also associated to lack of proliferative capacity in T cells (33, 35), and increased KLRG1 expression has been described in WASp-deficient NK cells (236). Our findings of lower KLRG1 expression in XLN NK cells may indicate that these cells are hyperactive due to less inhibitory signals, although NK cells expressing KLRG1 have been linked to improved tumor immunosurveillance (237). The increase of KLRG1 in XLN patient T cells may indicate exhaustion, however, this is contradicted by our findings of normal receptor-independent responses. These findings illustrate the need for more studies on the connection between WASp, actin polymerization, and KLRG1 expression.

In summary, NK cells and T cells in XLN do not seem to be deficient in their tumor killing capacity, suggesting that the increased risk of myelodysplasia and malignancy observed in XLN patients is caused by cell-intrinsic aberrations rather than by deficient tumor immunosurveillance.

4.4 THE T CELL RECEPTOR REPERTOIRE IN WISKOTT-ALDRICH SYNDROME IS INCREASINGLY PERTURBED WITH AGE

Loss-of-function mutations in WASp result in WAS, presenting with hemorrhages, infections, and eczema (184, 185). T cells in WAS are fewer, have altered morphology, and proliferate poorly to stimulation (154, 198). Furthermore, T_{reg} function is impaired and B cells produce autoantibodies, potentially leading to autoimmune responses (197, 199). However, it has remained unknown if effector T cells in WAS can be autoreactive. Impaired distribution of the TCR repertoire has been correlated to the development of autoimmunity in PID and other diseases (238, 239). The aim in **paper IV** was to examine the naïve TCR repertoire in young mice (before the development of autoimmunity) and older mice to understand if WASp-deficient T cells undergo normal selection and differentiation. To investigate this, we obtained thymocytes and spleen T cells from WT and WASp KO mice.

In young (3–4 weeks old) mice, the thymocyte TCR repertoire in WASp KO mice was indistinguishable from WT mice (Figure 15), indicating normal thymic output in WASp-deficiency. When examining older (7–8 months old) mice, the thymocyte TCR repertoire displayed impaired diversity, most pronounced in WASp KO mice (Figure 1D-F, paper IV). Furthermore, the spleen T cell TCR repertoire showed signs of clonal expansion in WASp KO

mice (Figure 16), however, differences between WASp KO and WT in the overall spleen T cell TCR repertoire did not reach clinical significance (Figure 1F, paper IV).

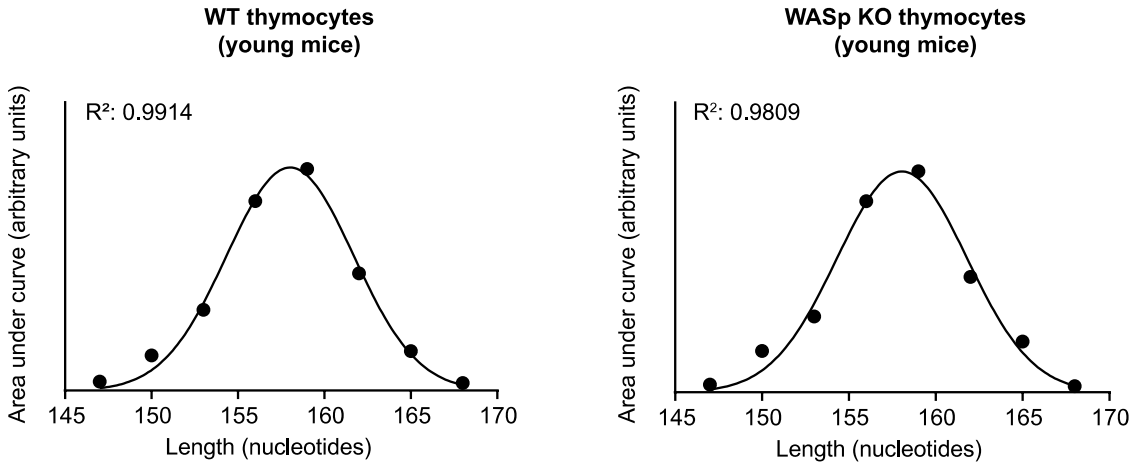

Figure 15. The thymocyte TCR receptor repertoire in young wildtype and WASp knockout mice. The distribution of thymocyte TCRs with the $V_\beta 1$ segment shown here (representative graphs). R^2 values indicate goodness of fit. WT, wildtype; KO, knockout.

Using the same technique of spectratyping, the first study of the TCR repertoire in WAS patients showed intact diversity in patients younger than 15 years of age and impaired diversity in older patients (240). However, later studies have shown oligoclonal repertoires also in young WAS patients, both using spectratyping and next generation sequencing (241-243). Furthermore, clonal expansions were identified in CD4+ memory T cells as well as in CD8+ naïve and memory T cells (243). The cause of the skewed TCR repertoire in WAS patients has been unknown, but it has been speculated to reflect impaired thymic output, decreased peripheral T cell survival, or chronic exposure to infections (243, 244).

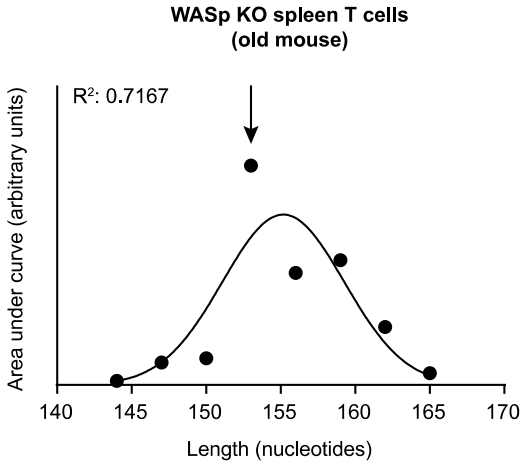

Figure 16. Clonal expansion of spleen T cells in an old WASp knockout mouse. The distribution of spleen T cells TCRs with the $V_\beta 1$ segment shown here. Arrowhead indicates clonal expansion, defined by an area under curve ≥ 60 % of the adjacent peaks. The R^2 value indicate goodness of fit. KO, knockout.

Our study based on WASp KO mouse models provided a unique advantage in that we were able to keep the animals free from infection, a condition not possible to maintain for patients, thus indicating that the clonal expansion observed in WASp KO spleen T cells from old mice may have been triggered by autoantigens rather than infections. Furthermore, thymic output could be assessed in our model by the selective examination of thymocytes in young and old

mice: our results show intact thymic output in young mice and suggest that the dependency on WASp to generate a diverse TCR repertoire increased with age. This contradicts the hypothesis raised by Wada et al. that skewed repertoires in older patients was not due to impaired thymic output (240). With increasing age, physiological involution of the thymus may indeed restrict the TCR repertoire by reduced thymic output and compensatory homeostatic proliferation (245). Increased homeostatic proliferation is seen in WAS (246), and has generally been associated to development of autoimmunity due to selective pressure on T cells expressing TCRs with high affinity to self-peptide-MHC molecules (247-249). These findings may explain the further increased skewing of the TCR repertoire in old WASp KO mice as compared to WT mice.

5 CONCLUSIONS AND FUTURE OUTLOOK

In this thesis, I have investigated the role of dysregulated actin and intracellular vesicles in lymphocytes and other cells in PID and cancer.

In **paper I**, we present a unique model to investigate the role of MKL1 in the development of Hodgkin lymphoma. We examined B cells from three monozygotic triplets with an intronic deletion in *MKL1*, leading to increased MKL1 expression. B cells from the two triplets diagnosed with and treated for Hodgkin lymphoma presented with variable phenotypes, while B cells from the third triplet presented with classical hallmarks of cancer cells: hyperproliferation, dysregulated cytoskeleton, genomic instability, and formation of tumors with induction of angiogenesis. This suggested that this triplet represented a premalignant phenotype, acting as an untreated control to the two other triplets. The correlation of MKL1 expression to increased MKL1-SRF-dependent gene transcription, reversion of the phenotype by the use of a small molecule inhibitor of MKL1, and evidence of MKL1 involvement in several other cancer models, suggest a role of MKL1 in the development of Hodgkin lymphoma.

In **paper II**, we examined cells from an LRBA-deficient patient presenting with immunodeficiency and autoimmunity. We show that patient cells accumulated vesicles including endosomes, lysosomes, autophagosomes, and lipid bodies. Furthermore, lipid metabolism was altered in patient cells in response to starvation. Our results suggest that LRBA deficiency conveys dysregulated responses to cell stress, and that the accumulation of vesicles may impair the survival and function of immune cells, leading to the immunodeficiency and autoimmunity present in patients with LRBA deficiency.

In **paper III**, we examined NK cells and T cells in XLN patients and mouse models. As opposed to the hyporeactive phenotype in WAS, XLN NK cells and T cells displayed normal to increased tumor killing, decreased expression of the exhaustion marker KLRG1, increased granzyme B expression, and polarization of actin without stimuli. These results suggest that the increased risk of myelodysplasia observed in XLN is caused by cell-intrinsic aberrations rather than by deficient tumor immunosurveillance.

In **paper IV**, we show that the distribution of the TCR repertoire is skewed in thymocytes and spleen T cells in old WASp KO mice. As young WASp KO mice showed a normal TCR repertoire distribution, these results suggest that the dependence on WASp for thymic output and the generation of a diverse TCR repertoire increases with age. Furthermore, clonal expansions in spleen T cells in the absence of infections suggested that autoantigens may trigger the development of a skewed TCR repertoire.

In summary, this thesis provides evidence for the role of dysregulated cytoskeletal responses in the development of PID and cancer. Increased actin by mutated *MKL1* and in XLN leads to aberrant activation of B cells, NK cells, and T cells. Decreased actin in WAS leads to disruption

of the TCR receptor repertoire. LRBA deficiency leads to vesicle dysregulation in B cells and fibroblasts.

Commercially available small molecular inhibitors of actin polymerization and MKL1 activity could potentially be used as therapeutic agents in diseases with increased actin responses. CK-666 and CK-689 are inhibitors of the Arp2/3 complex (250) and wiskostatin is an inhibitor of WASp and N-WASp (251). CCG-1423 as well as the more recently described CCG-100602, CCG-203971, and CCG-222740 inhibit MKL1 activity (220, 252, 253). However, cytotoxicity may be a prominent side-effect of these small molecular inhibitors, why further studies are needed to optimize their efficacy and safety before they can be used clinically.

Increased MKL1 expression has been shown by us and others to be involved in cancer development. However, at this point, it is unknown whether the level of MKL1 expression correlates to disease severity or prognosis, and if it is restricted to certain kinds of cancer cell types. Further studies are warranted to investigate MKL1 expression levels in different stages and types of cancer. Depending on the result of these studies, screening for MKL1 as a cancer marker could potentially be used in the clinic to determine which examinations or treatments to choose for individual patients.

Dysregulation of lipid metabolism has been shown for several diseases, including lysosomal storage diseases such as Niemann-Pick disease type C1 or Gaucher disease caused by enzymatic deficiencies in lipid metabolism (254, 255). For these diseases, enzyme replacement therapies have been proved successful (256, 257), why these readily available compounds may be used in studies on lipid dysregulation in LRBA deficiency. Enzymes involved in the metabolism of sphingomyelin and phosphatidylcholine would be of particular interest, as we observed accumulation of these in LRBA-deficient cells subjected to starvation-induced stress.

The advancement of DNA sequencing technologies has provided sophisticated means of identifying potential pathogenic mutations in the study of PIDs and other genetical diseases. However, regardless of how exact and readily available these techniques may become, the clinical relevance of a given mutation can not be determined without functional laboratory studies.

Studies of PIDs are highly important models to investigate the function of individual proteins in the immune system. And as PIDs generally are monogenic, in this aspect they are in practice comparable to knockout mouse models. However, a limiting factor in the study of human PIDs is the scarcity of material, due to the generally extremely low prevalence of each single PID. Furthermore, as HSCT early in life typically is required for severe PIDs, study material such as blood samples would have to be obtained before treatment, and there is a risk that the obtainment of large samples may be unethical and harmful to the often very young patients. This emphasizes the continued relevance of mouse models in research on PIDs and immunology in general, as a means of providing material and *in vivo* systems to understand the complexity of the immune system in health and disease. However, it should be noted that while mouse models have been essential for human immunological research, interspecies differences

may still be significant, and further increased by the artificial pathogen-free conditions most research mice are kept under (258-260). Altogether, future research on PID will likely require a well-balanced combination of mouse models, patient material, and genetically engineered human cells.

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