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Marika Runsala

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ISBN 978-951-29-8965-2 (Print)
ISBN 978-951-29-8966-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2022

To the kids
taken into custody

UNIVERSITY OF TURKU
Faculty of Medicine
Pathology and Medical Biochemistry and Genetics
Turku Bioscience Centre
MARIKA RUNSALA: Antigen Processing In B Cells
Doctoral Dissertation, 143 pp.
Turku Doctoral Programme of Molecular Medicine
September 2022

ABSTRACT

B cells are responsible for specific antibody production. To produce antibodies, they recognize and internalize antigen (ag), cleave it into peptides, load the peptide-antigens (pAg:s) onto major histocompatibility complex II (MHCII) and traffic the pAg-MHCII to the plasma membrane, to receive proliferation-promoting signals from helper T cells. During the antigen processing, B cells have to process also the MHCII complex. Immature MHCII complexes are bound with an invariant chain (Ii) which protects the peptide-binding groove of the MHCII. On the endosomal pathway, Ii is clipped into shorter moieties with Cathepsin S (CatS). CatS leaves a short Ii tail to protect MHCII until a pAg, ready to replace Ii, is encountered. The Ii-pAg exchange is induced with a class II accessory molecule (DM). Despite the biochemical processes in the pAg-MHCII presentation being well described, compartments and proteins guiding the events are not reported in detail in B cells.

In this work, the endosomal compartments associated with antigen processing and MHCII maturation are described. This study reports B cells to possess a fast track for ag processing and pAg presentation. The fast track consists of previously unrecognized early peptide-loading compartments (eMIICs), which locate close to the plasma membrane and possess the proteins needed for antigen processing and pAg presentation in B cells, such as MHCII, CatS, DM and degrading activity.

This study also characterizes classical peptide loading compartments, MIICs. This work shows that MIICs possess several proteins of the endosomal pathways such as early, late, lysosomal and recycling-linked proteins. Interestingly, this study also suggests that (macro)autophagosomes, together with Rab7, might have an important role in ag processing and pAg presentation in B cells. Inhibitors of autophagy and Rab7 decrease pAg-MHCII presentation in B cells. Also, boosting Rab7 activity with constitutively active Rab7 mutation was found to increase pAg-MHCII presentation.

Together, these findings show that B cell antigen processing can occur in several pathways which might be coordinated together. These findings may provoke new insights and tools to modulate and tone antigen processing in B cells.

KEYWORDS: B cell, Endosomal pathway, Antigen processing, Rab-proteins, MHCII presentation, Rab7, macroautophagocytosis

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Patologia sekä Lääketieteellinen biokemia ja genetiikan laitos
Turun Biotiedekeskus
MARIKA RUNSALA: Vasta-aineen käsittely B-soluissa
Väitöskirja, 143 s.
Molekyylilääketieteen tohtoriohjelma
Syyskuu 2022

TIIVISTELMÄ

B-solut ja niistä erilaistuneet plasmasolut tuottavat vasta-aineita. Tätä varten B-solut käsittelevät taudinaiheuttajista lähtöisin olevia antigeeneja (ag), pitkän jatkumon läpi. B-solut tunnistavat antigeenin, ottavat antigeenin sisäänsä ja käsittelevät sen peptidi-antigeeniksi (pAg). pAg sidotaan major histocompatibility complex II (MHCII)-molekyyliin ja kuljetetaan takaisin solukalvolle, jossa B-solu esittelee pAg-MHCII kompleksin T-soluille saadakseen erilaistumiskäskyn. Antigeenin käsittelyn lisäksi B-solut käsittelevät myös MHCII:n. Erilaistumaton MHCII ei sido itseensä pAg:a vaan MHCII:ta suojaava molekyyli, invariant chain (Ii), täytyy ensin lyhentää katepsiini-S:n avulla. Kun Ii on pilkottu, tarvitaan class II accessory -molekyyli, DM, joka edesauttaa pAg sitoutumaan MHCII:een. Edelleen on jäänyt epäselväksi, missä solun sisäisissä rakentaissa antigeenin hajottaminen ja pAg:n sitominen MHCII:n tapahtuu, tai mitkä proteiinit näitä tapahtumia ohjaavat.

Kuvaamme tässä työssä antigeenin hajottamiseen ja MHCII:n kypsymiseen liittyviä kalvorakenteita. Osoitamme, että ag-käsittely ja pAg esittely voi olla hyvin nopeaa varhaisissa antigeenia käsittelevissä kalvorakkuloissa, eMIIC:ssa. eMIIC:t odottavat solukalvon alapuolella antigeenin sisäänottoa. eMIIC:ssa toimivia molekyylejä ovat muun muassa MHCII, katepsiini-S sekä DM. Nämä molekyylit pystyvät hajoittamaan materiaalia kalvorakkuloissa. Täten eMIIC:t sisältävät useita antigeenin hajottamiseen ja pAg:n esittelyyn tarvittavia molekyylejä. Kuvaamme myös pAg:n käsittelevän kalvorakkulan, MIIC:n piirteitä. Näytämme, että MIIC:ssä on useita proteiineja eri kalvorakkuloiden rakenteista. Mielenkiintoista on myös se, että autofagosomit vaikuttavat olevan tärkeässä roolissa. Tutkimuksessa esitetään, että autofagosomien tai Rab7:n toiminnan rajoittaminen vähentää pAg-MHCII esittelyä, kun taas Rab7 aktivointi vaikuttaa parantavan pAg-MHCII esittelyä.

Esitämme, että antigeenin käsittelyä tapahtuu useissa erilaisissa kalvorakkula-reiteissä, jotka saattavat toimia yhdessä. Löytömme voi myös tuoda uusia näkemyksiä antigeenin käsittelyreittien hallitsemiseksi B-soluissa.

AVAINSANAT: B-solu, solun sisäinen kalvorakkolakuljetus, vasta-aineen käsittely, Rab-proteiinit, MHCII esittely, Rab7 makroautofagosytoosi

Table of Contents

Abbreviations

AEP	asparagine endopeptidase
AID	activation-induced cytidine deaminase
APC	antigen presenting cell
BCR	B cell receptors
BSA	bovin serum albumin
CA	constitutively active
Cargo	internalized material
CatS	cathepsin S
CLIP	class II-associated invariant chain peptide
CLL	clathrin lattice
cSMAC	central supramolecular activation complex
CSR	class switch recombinant
DAMP	damage associated molecular pattern
DM	class II accessory molecule
DN	dominant negative
DQ-Ova	DQ Ovalbumin
EE	early endosome
EEA1	early endosomal antigen 1
ELISA	enzyme-linked immunosorbent assay
EM	electron microscope (EM)
eMIIC	early MIIC
ER	endoplasmic reticulum (ER)
$F(ab)_2$	BCR variable region
FACS	flow cytometer
Fc	BCR constant region
FCS	fetal calf serum
GAP	GTPase-activating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GTP	guanosine triphosphate
H	heavy chain

H2-M	human leukocyte antigen DM in murine
HEL	hen egg lysozyme
HLA-DM	human leukocyte antigen DM in human
HSC	hematopoietic stem cell
Ii	invariant chain
ILV	intraluminal vesicle
IM	internalized material
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
L	light chain
LAMP1	lysosomal associated membrane protein 1
LAMP2	lysosomal associated membrane protein 2
LAP	LC3-associated autophagocytosis
LC3	microtubule-associated protein 1 light chain 3
LE	late endosome
LIP	leupeptin-induced protein
LT	lysotracker
LYS	lysosome
M1, M2	Mander's colocalization coefficiency 1, 2
MHC	majod histocompatibility complex
MHCII	major histocompatibility complex class II
MIIC	MHCII containing endosome
MTOC	microtubule organisation centre
NOX2	NADPH oxidase 2
PAg	peptide antigen
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PI(3)P	phosphatidylinositol 3-phosphate
PI3K	phosphoinositide 3-kinase
PM	plasma membrane
PODXL	podocalyxin-like protein 1
PRR	pattern recognition receptors
RE	recycling endosome
RRX	rhodamine red x
RT	room temperature
SDCM	spinning disc confocal microscope
SHM	somatic hypermutation
SIM	structured illumination microscopy
SLIP	small leupeptin induced protein
TCR	T cell receptor

TLR	Toll like receptor
VDJ	variable diversity joining
WT	wild type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Sara Hernández-Pérez*, **Marika Vainio***, Elina Kuokkanen, Vid Šuštar, Petar Petrov, Sofia Forstén, Vilma Paavola, Johanna Rajala, Luqman O. Awoniyi, Alexey V. Sarapulov, Helena Vihinen, Eija Jokitalo, Andreas Bruckbauer, and Pieta K. Mattila.. "B Cells Rapidly Target Antigen and Surface-Derived MHCII into Peripheral Degradative Compartments." *Journal of Cell Science,* 2020; (133):1–15. **shared first authors*

II **Marika Runsala**, Elina Kuokkanen, Eveliina Uski, Vid Sustar, Meryem Özge Balci, Johanna Rajala, Vilma Paavola and Pieta K Mattila. The small GTPase Rab7 regulates antigen processing in B cells in a possible interplay with autophagosomal machinery. *Manuscript*

1 Introduction

All animals are constantly under a threat. The threat can lurk outside or inside of our bodies, and our bodies need to be constantly ready to battle for health and wellbeing. For instance, our skin and bones are ready to take on hits and shocks to protect vital inner organs from physical stress. On the other hand, our nasal cavities try to protect the respiratory tract from infections caused by polluted air, by filtering the air with nasal hairs. Despite all the precautions, quite often pathogens invade the host. Pathogens have developed ways to cheat all the blocks and firewalls in the body and get ready to cause chaos and disorder.

Equally often the threats are not coming from outside but are mistakenly created by the host himself. Such examples are malignant cells which develop rarely but constantly in the body. Luckily in most cases, malignant cells are spotted but occasionally, they become unstoppable and may turn into tumors. In the lucky cases, how are the threats then spotted by the body? Our body contains billions of protecting cells which are known as immune cells. Immune cells are constantly alert in our organs and hoover around to seek targets for destruction. They travel in the blood vessels, as well as in lymphatic vessels and lymphoid organs. When an immune cell encounters a pathogen or a malignant cell, it must destroy the target – either alone or by alerting more troops.

Immunity is divided into two categories: innate and adaptive immunity which consist of different types of cells. Innate immunity is the immune system which an individual is born with. Innate immune cells recognize common pathogen-associated structures, and some of them can destroy the target by engulfing the pathogenic invader. Macrophages are an example of these phagocytic leucocytes. Adaptive immunity, on the other hand, is trained throughout the lifetime of the host. Adaptive immunity is composed of B and T cells. Adaptive immune cells recognize specific antigenic structures. When they encounter one, they alert the whole immune system for an efficient immune response. An important part of adaptive immunity is antibodies that are produced against specific antigenic structures by B cells.

The antibody production is a result of a long cascade of events. At first, B cells, whose plasma membranes are enriched by antigen-specific B cell receptors (BCRs), encounter their cognate antigen. B cells recognize the antigen with their BCRs.

Typically, B cells spot the antigen on a membrane of another cell, such as antigen presenting cells of the innate arm of the immune system. Yet, B cells recognize also soluble antigens floating in the bloodstream, lymph or other tissues. Importantly, every B cell recognizes only one type of antigen which is specific for its BCR.

Upon antigen recognition, B cells bind to the antigen with their BCRs. Antigen-BCR bond is rigid and after binding the molecules are internalized as a complex. The internalized antigen is processed in intracellular compartments into shorter moieties called peptide-antigens (pAg). The antigen processing into peptides occurs in the endosomal-compartments, and the pAg:s are trafficked back to the plasma membrane from so the called peptide-antigen loading compartments, MIICs.

MIICs are characterized by their expression of Lysosomal Associated Membrane Protein 1 (LAMP1) and Major Histocompatibility Complex II (MHCII). Before the pAg trafficking back to the plasma membrane, pAg:s are loaded to MHCIIs. The pAg loading to MHCII is a joint effort of several proteins and molecules that are carefully inhibiting or prohibiting pAg loading to the MHCII. The joint effort acts also as a security step to prevent B cells from binding with wrong antigens, such as autoantigens. The security is guaranteed by enveloping the pAg binding groove in MHCII with a molecule called invariant chain (Ii). Ii conjugates with MHCII immediately after MHCII is released from the endoplasmic reticulum, and as long as MHCII is bound to the Ii, or its remaining, no peptides can bind to the pAg binding groove in MHCII. Along MHCII maturation, Ii is clipped shorter with different proteins and molecules until Ii remaining is replaceable with the correct pAg. After the exchange, the pAg-MHCII complex is sent to the plasma membrane to recruit T cells for an immunological response.

After processing and loading pAg to the MHCII, the pAg-MHCII complex is trafficked to the plasma membrane, to be presented for a cognate helper T cell. The cells form an immunological synapse and if the immunological synapse is strong enough, the T cell will also provide a stimulus for the B cell, after which the B cell can start proliferation processes. After several proliferation steps, B cells will differentiate into either memory B cells or antibody producing plasma cells. Again, it should be noted, that one memory B cell will remember only one type of antigen, and an antibody producing plasma cell will produce antibodies against only one type of antigen. Plasma cells can produce enormous amounts of antibodies. Freely floating antibodies bind to the pathogenic structures and guide pathogens for further, rapid destruction via marking them for the phagocytic cells, for instance. Memory B cells, on the other hand, create immunological memory. Memory B cells can live decades waiting for the pathogen to invade the host again, and when it does, memory B cells are ready to produce a fast and efficient immune response to destroy the pathogen.

Antibody production is widely utilized in vaccination therapies. With vaccination, the host is exposed to a small amount of the target antigen. The antigen will provoke the immune system, and vaccine antigen specific memory B cells will be formed. When the individual is exposed to the same antigen, in other words, to the same disease again, the immune system is ready to act immediately. The adaptive immune system will be alerted for a fast and efficient immune response, and thus the host might fully avoid the disease outbreak. Occasionally, the immune system can mistakenly targets non-toxic substances which can cause hypersensitivities, also known as allergies. Autoimmune diseases are also caused by dysfunction in the adaptive immune system as in autoimmune diseases, host own, healthy cells are seen as pathogenic and are targeted for destruction. Notably, if too few antibodies are produced, it can hint at severe diseases described as immunodeficiencies. Immunodeficiencies can be fatal if they are not diagnosed or treated properly.

Despite the fact that the adaptive immune responses and B cell activation have undisputed roles in autoimmune diseases and health, little is known about which molecular mechanisms are guiding the antigen processing phases initiating the cell proliferation and differentiation. In this doctoral thesis, antigen processing was studied over time and space. We utilized fascinating imaging modalities with sophisticated analysis tools to characterize intracellular compartments in antigen processing and identified a novel compartment, early MIIC (eMIIC) to participate in antigen processing and to provide a plausible site for fast antigen processing and pAg presentation. We characterized eMIICs to possess several proteins involved in endosomal trafficking and degradation. Also, macroautophagosomes were reported to participate in antigen processing and presentation together with Rab7. Indeed, our work proposes Rab7 to have an essential role in pAg presentation – with or without association with macroautophagosomes remains to be further investigated.

2 Literature review

2.1 Immune system

The purpose of the immune system is to protect individuals from diseases and it consists of innate and adaptive immunity. While innate immunity is something the individual is born with, adaptive immunity develops throughout life. The immune system is one of the best examples of homeostatic balance as its fast responses to changing environmental demands are essential for individual health. For instance, the immune system responds to threats by altering and tuning different immune cell subpopulations and their activation to always provide the best possible shield

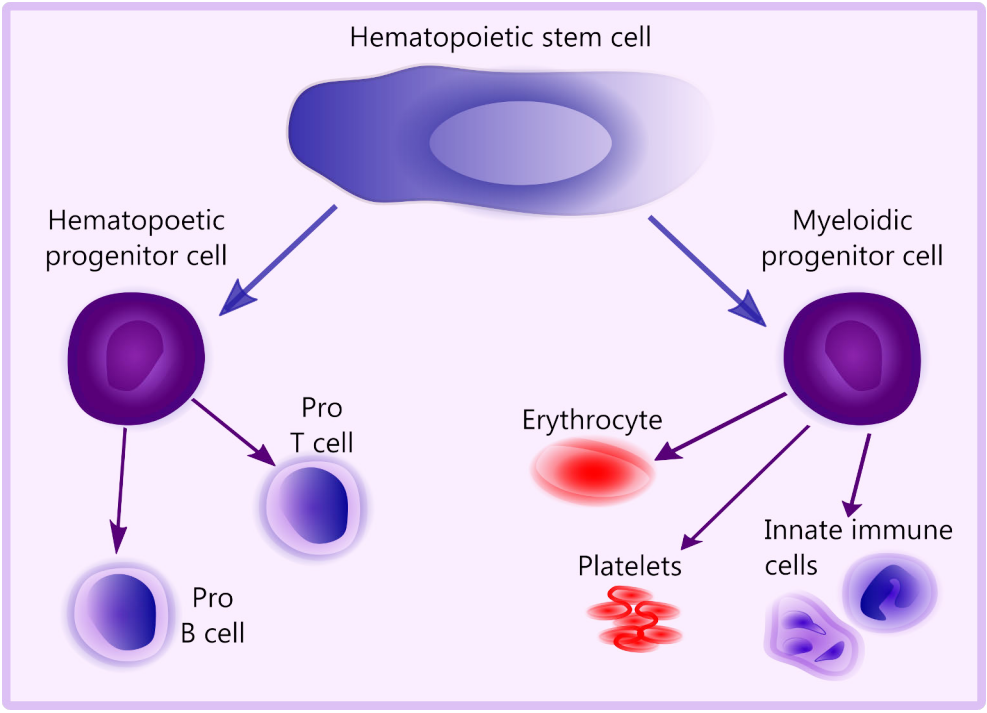

Figure 1. Hematopoietic and myeloid cell proliferate from hematopoietic stem cells. Hematopoietic progenitor cells further proliferate into adaptive immune cells known as progenitor T and B cells. Myeloid progenitor cells, on the other hand, proliferate into innate immune cells, platelets and erythrocytes.

(Garcillán et al. 2018). All the immune cells proliferate from hematopoietic stem cells. In the first proliferation steps, hematopoietic stem cells proliferate into either myeloid or hematopoietic progenitor cells, as described in **Figure 1**. Myeloid progenitor cells are created much more numerous than hematopoietic progenitor cells. This is greatly due the erythrocyte production, as hundreds of billions of erythrocytes are produced each day from the myeloid progenitor cells. (Jackson, Ling, and Roy 2021; Kato and Igarashi 2019; Zehentmeier and Pereira 2019)

2.1.1 The innate immune system

As described in **Figure 1**, innate immune cells are proliferated from myeloid progenitor cells. Innate immune cells include dendritic cells, mast cells, natural killer cells, granulocytes and macrophages. In health, the innate immune cell profile is typically constant but during a sickness and in elderly life, erythropoiesis is slower and more leucocytes are produced from the myeloid progenitor cells. (Jackson et al. 2021; Kato and Igarashi 2019; Zehentmeier and Pereira 2019)

The innate immune system is alarmed when innate immune cells recognize pathogenic motifs known as pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). DAMPs are generated passively by a dying cell, or actively by a living but damaged cell.. (Lee et al. 2021) PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs). PRRs are divided into four distinct groups from which the most common example are Toll-like – receptors that occupy the plasma membrane of immune cells. (Jentho and Weis 2021)

Innate immune cells aim to destruct the pathogen or damaged cell typically by phagocytosis. Macrophages are the most classical example of phagocyting cells, and they are referred to as professional phagocytic leucocytes. Macrophages seek pathogenic structures or damaged cells by migrating around the body. When they encounter a pathogen, they bind to it by PRRs and engulf the target. Endocytosed material is trafficked along the endosomal pathway towards its final degradation in acidic lysosomes. Along the endosomal pathway, useful material, such as receptors, are continuously recycled back to the plasma membrane while useless material will be fully destroyed. (Hartenstein and Martinez 2019; Kirchenbaum et al. 2019) The endosomal pathway and its compartments are described in detail in chapter 2.2.2.

Classically, it is thought that innate immunity does not form immunological memory. Nevertheless, in recent years some evidence of the immunological memory in innate immune cells has been reported (Jentho and Weis 2021). To draw a full picture of the memory capacity of innate immunity, more studies are still needed.

2.1.2 The adaptive immune system

Adaptive immune cells, B and T cells are small lymphocytes with a large nucleus. They are proliferated from hematopoietic progenitor cells as described in **Figure 1**. Adaptive immune cells are pathogen-specific, and they create sophisticated, specialized and efficient immune responses toward recognized antigens. T and B cells can cause efficient immune responses independently, but their cooperation is essential for the most efficient immune responses.

T cells consist of cytotoxic (CD8+) and helper (CD4+) T cells. Cytotoxic T cells can directly eliminate infected cells by releasing cytotoxic granules in the cell whereas helper T cells have an essential role in guiding and boosting B cell proliferation, for example. Both T cell types can also produce cytokines which lead to magnified immunological responses. Despite B cells being responsible for antibody production, B cell proliferation from the progenitor B cell to an antibody producing plasma cell is regulated by CD4+ T cells. T cells also coordinate immune responses and proliferation of the other professional antigen presenting cells (APC), which are dendritic cells and macrophages. After an antigen encounter, APCs form an immunological synapse with T cells to present the acquired antigen. If the antigen presentation is sufficient, the T cell provides a stimulus back to the APC, after which the APC can continue to proliferate. (Cruse, Lewis, and Wang 2004; Kirchenbaum et al. 2019; Shaw et al. 2018) Immunological synapse is described in greater detail in chapter 2.1.3.4.

2.1.2.1 B cell development

B cells develop from hematopoietic stem cells (HSCs) as described in **Figure 1** and their development begins already in utero. B lymphopoiesis, which is simplified in **Figure 2**, is a long proliferation cascade. During the Pro- and Pre-B cell maturation phases, cells undergo a random process of variable diversity joining (VDJ) recombination for the heavy (H) chain of the BCR to generate unique pre-BCRs expressing slightly but critically different IgH chains and surrogate light (L) chains. Pre-BCR signaling further promotes L chain rearrangement which, when successful, turns receptors into functional BCRs expressed in immature B cells. Immature B cells leave the bone marrow and travel to the secondary lymphoid organs. (Hystad et al. 2007; Jackson et al. 2021)

The final maturation step from immature B cells to memory B cells or antibody producing plasma cells occurs after the cell has encountered an antigen. The maturation is illustrated in **Figure 3.** During the maturation, B cells undergo somatic hypermutation (SHM) to create point mutations in the antigen binding sites of the

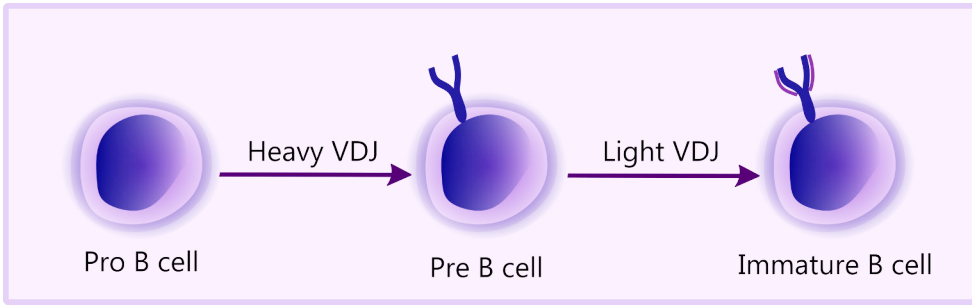

Figure 2. B lymphopoiesis. A B cell undergoes a random process of variable diversity joining (VDJ) recombination for the heavy (blue) and the light (magenta) chains of the B cell receptor (BCR) to generate unique BCRs. Pre-BCR signaling further promotes light chain rearrangement which turns receptors into functional BCRs expressed in immature B cells..

BCR. SHM is induced by a DNA mutator, activation-induced cytidine deaminase (AID) (Yu and Lieber 2019). AID expression is promoted by NF-kB binding to the Aicda gene and NF-kB on the other hand can be activated by receptor binding on the plasma membrane. Notably, NF-kB can be targeted via several downstream signaling proteins too, naming one of our key proteins, Rab7 (described in chapter 2.2.2.4) as one (Yan et al. 2020)

After the final maturation step, B cells can produce antibodies with improved, very high affinity. Importantly one should note that one B cell can recognize only one type of antigen but together all the B cells recognize billions of different antigens. Yet, for an even broader battlefront, B cells can also switch the class of the immunoglobulins they produce. This happens via class switch recombination (CSR) where the constant heavy chain proportion of the BCR is changed into IgG, IgE or IgA. Thus, the affinity and specificity of the produced antibody will remain the same but the molecules interacting with the released antibodies, change. CSR is guided by several factors, naming AID and cytokines as examples. Notably, defected CSR causes limited humoral immunity and thus lethal infections may occur. (Cruse et al. 2004; Stavnezer and Schrader 2015; Yu and Lieber 2019) The overall antibody variety is always influenced by both extrinsic and intrinsic signals. (Cruse et al. 2004; Hystad et al. 2007; Jackson et al. 2021; Nuñez et al. 1996).

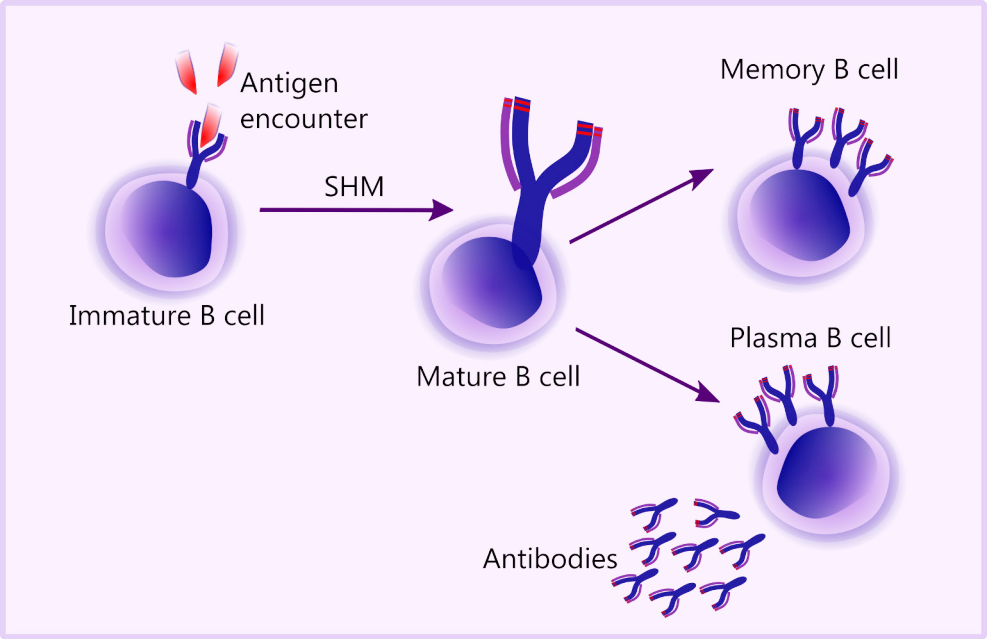

Figure 3. B cell maturation after an antigen encounter. An immature B cell encounters an antigen in the secondary lymphoid organs. The antigen encounter promotes the B cell to undergo somatic hypermutation (SHM) which creates point mutations in the antigen binding sites of the BCR (red stripes). Mature B cells create immunological memory with memory B cells and provide antibodies with plasma B cells.

2.1.3 Antigen processing in B cells

The goal of a B cell is to proliferate into an antibody producing plasma cell or a memory B cell. To achieve the goal, B cells have undergone complex maturation phases before and after an antigen encounter. To create antigen specific antibodies, B cells have initially processed the antigen. The antigen processing can be divided into four phases:

- antigen recognition and engagement with the BCR
- antigen internalization and processing into peptide-antigens (pAg)
- pAg loading into major histocompatibility complex (MHC) class II (MHCII)
- pAg-MHCII trafficking and presenting on the plasma membrane for cognate, helper T cells.

2.1.3.1 Antigen recognition and engagement with B cell receptors

2.1.3.1.1 The structure of a B cell receptor

B cell plasma membrane possesses BCRs of certain, unique characteristics. BCRs are antigen recognizing structures. BCRs consist of heavy and light chains. The heavy chain links the BCR to the plasma membrane and has short intracellular motifs. Light chains are fully extracellular, and they are bound to the heavy chain with disulfide bridges. BCR is closely associated with the secondary components Igα and Igβ. These secondary components have longer intracellular tails than BCR and the tails contain immunoreceptor tyrosine-based activation motifs (ITAMs). Igα and Igβ have a crucial role in the induction of intracellular signaling cascades as antigen binding to BCR phosphorylate ITAMs.

The antigen binding site in the BCR is at the tip of the BCR, on the variable region. Variable regions are located on the tip of y-shaped BCR regions called F(ab')2-region. F(ab')2-regions are identical to each other. The leg of the BCR is called Fc-region and it consists solely of a heavy chain. The constant heavy chain

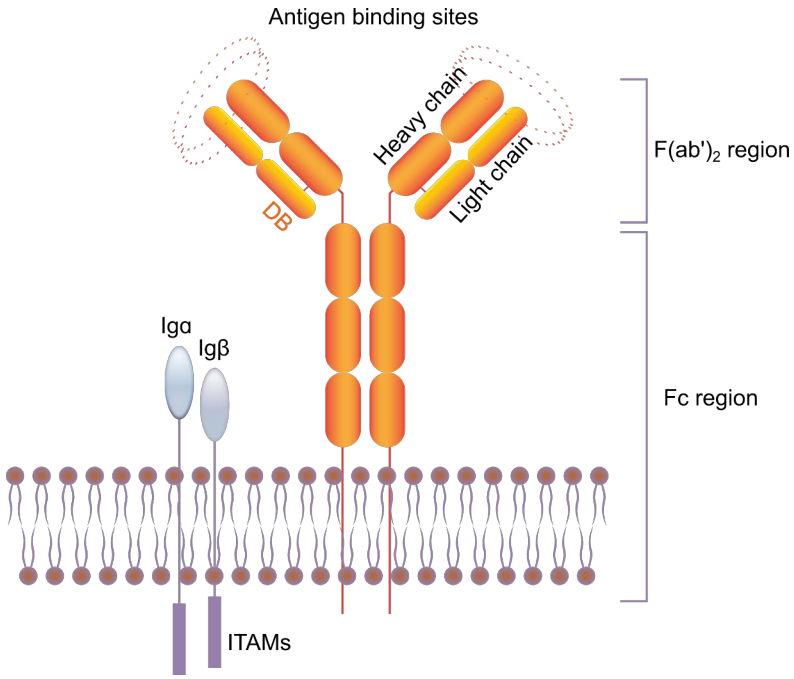

Figure 4. B cell receptor and its secondary components. A B cell receptor (BCR) recognizes the antigen with its variable regions F(ab')2-molecules. F(ab')2 region consists of heavy and light chains linked together with disulfide bonds (DB) whereas the Fc region, which is the body of the plasma membrane bounding immunoglobulin, consists of heavy chains only. The BCR activation induces intracellular phosphorylation in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the BCR secondary components Igα and Igβ.

region, which covers the Fc region and proportion of the F(ab')2 region, determines the immunoglobulin type. (Kuokkanen, Šuštar, and Mattila 2015; Kurosaki, Shinohara, and Baba 2010) BCR, with its secondary components, is illustrated in **Figure 4.**

2.1.3.1.2 BCR dependent activation

B cells extract antigen by pulling it from the presenting surface. The antigen-BCR binding causes actin cortex remodeling and actin then provide strength and support for the mechanical pulling of the antigen (Natkanski et al. 2013; Spillane and Tolar 2016). The actin cortex remodeling itself, on the other hand, can lead to intracellular signaling too (Mattila et al. 2013; Treanor et al. 2011).

The needed pulling force correlates with the rigidity of the surface where the antigen is presented. For instance, cells need more force when antigen is ripped from the glass bead than on the membrane of antigen presenting cell (APC). (Spillane and Tolar 2016; Tolar 2017) B cells can also use enzymatic degradation by secreting lysosomal substrates to the internalization site to facilitate antigen acquisition (Reversat et al. 2015; Sáez et al. 2019; Spillane and Tolar 2016; Yuseff et al. 2011). This is utilized especially when the pulling forces are not strong enough for the antigen ripping (Spillane and Tolar 2016).

When the antigen is presented from a surface, B cells form an immunological synapse (IS) with the antigen site, as described in **Figure 5**. The phenomenon consists of recognition (A), spreading (B) and contraction (C) phases. In the first

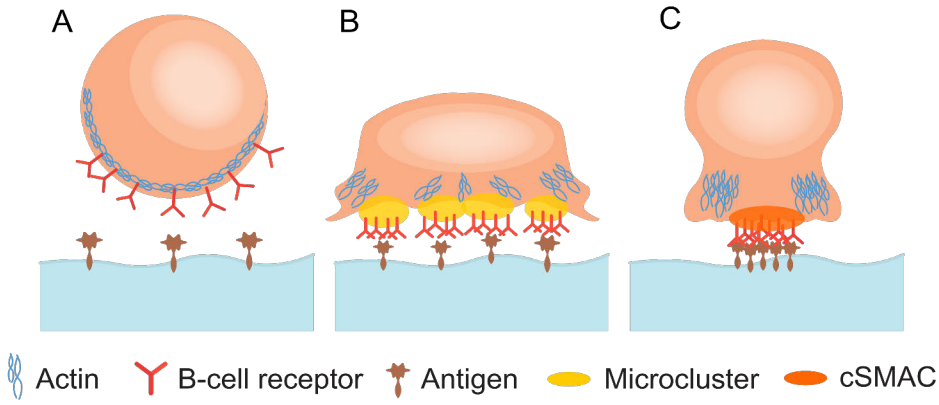

Figure 5. B cell immunological synapse fomation. (A) A B cell recognizes the membrane-tethered antigen with its B cell receptors (BCRs) and binds to the antigen. (B) The B cell spreads over the antigen presenting surface to gather as much antigen as possible. The B cell forms signaling antigen-BCR microclusters. (C) The B cell trafficks microclusters to a central position which is void of actin. Yet, actin supports the antigen pulling and internalization from the formed central SMAC (cSMAC). (Revised from Kuokkanen et al., 2015)

phase, the B cell recognizes the antigen on the surface and binds to the antigen with BCRs. After the initial recognition, B cells generate dynamic actin protrusions to find more antigen particles. The B cell spreads over the antigen presenting surface for gathering maximal antigen amount. After the maximal spreading, the B cell gathers so-called antigen-BCR microclusters to the central supramolecular activation complex (cSMAC) for internalization. cSMAC is void of larger actin structures and thus internalization is easier. Additional molecules, which are essential for further downstream signaling, are recruited to the cSMAC. This includes CD19, which functions as a positive co-receptor for B cell activation. (Bolger-Munro et al. 2019; Depoil et al. 2008; Kuokkanen et al. 2015; Mattila et al. 2013; Tolar 2017)

When the antigen is presented to B cells in soluble form, cells need the least force and actin cortex remodeling is not in a major role in the antigen-BCR internalization (Spillane and Tolar 2016). Yet, soluble activated cells trafficks antigen-BCR complexes into similar BCR clusters with CD19, as in an immunological synapse, and similar downstream signaling occurs. (Adler et al. 2017; Depoil et al. 2008). Nevertheless, in the secondary lymphoid organs, antigens presented on an APC, can induce strong B cell activation also at very low densities, whereas soluble antigens in a B cell zone need to be in higher concentration to induce equal B cell activation. Yet, soluble activation is commonly used in vitro assays as it is an easy approach and highly controllable. (Yuseff and Lennon-Duménil 2015)

2.1.3.2 Antigen internalization and processing into peptide-antigens

B cells internalize antigen-BCR complexes rapidly after the activation, mainly through clathrin-dependent endocytosis (Chaturvedi et al. 2013; Stoddart, Jackson, and Brodsky 2005). Clathrin-dependent endocytosis is a type of micropinocytosis and it is described in more detail in chapter 2.2.1.

Antigen-BCR complexes are internalized into endosomes, which are intraluminal vesicles described in more detail in chapter 2.2.2. In the endosomes, antigen-BCR complexes undergo biochemical changes, such as BCR ubiquitination. BCR ubiquitination drives antigen-BCR complexes to be delivered for further endosomal processing (Katkere, Rosa, and Drake 2012) and interestingly, BCR ubiquitination indeed, promotes endosomes to turn into more degradative compartments called late endosomes (Adler et al. 2017; Satpathy et al. 2015).

The endosomal compartments in antigen trafficking are not described in detail but it is well-established that antigen is ultimately trafficked into MIICs. MIICs are described as compartments where antigens could be degraded into peptides and where pAg is loaded to the MHCII. MIICs indeed contain many accessory proteins needed for peptide-antigen loading to MHCII such as Cathepsin-S (CatS) and

MHCII, class II accessory molecule (DM). These molecules will be described in more detail below.

MIICs possess lysosomal associated membrane protein 1 (LAMP1) and thus LAMP1, which is described in more detail in chapter 2.2.2, is often used as a marker for MIICs in B cells. Typically, MIICs are reported to localize in the perinuclear region, and they are seen as specialized endosomes. (Avalos and Ploegh 2014) Already 30 years ago Peters and Tulp found that MIICs were missing mannose 6-phosphate yet containing LAMP1, which made them distinct from early endosomes, late endosomes and lysosomes (Peters et al. 1991; Tulp et al. 1994). Nevertheless, a lot is still to be discovered in MIICs as the compartments are not thoroughly characterized, as many of the other endosomal structures are.

2.1.3.3 Major histocompatibility complex (MHC) class II in peptide-antigen presentation

MHCII, which is a heterodimer with a slightly bigger α subunit (33-35kD) and a smaller subunit (25-30 kD), has a key role in pAg presentation in B cells. MHCII is synthesized in the endoplasmic reticulum (ER) (Lindner 2017), as described in **Figure 6.** Immediately after the release from the ER, MHCII molecules form trimers and bind with an enveloping molecule, invariant chain, known also as CD74. Ii forms a surrounding triangular cage around the MHCII trimer and prevents MHCII from binding with antigens.. (Lindner 2017)

MHCII-Ii complexes are targeted to the plasma membrane or into different endosomal compartments. The destination is determined by MHCII-Ii sorting motifs. MHCII-Ii contains two sorting motifs which can act independently. Notably, the sorting motifs recognize clathrin adaptor proteins which promote the trafficking to the plasma membrane. From the plasma membrane, MHCII-Ii is targeted to early endosomes and gradually trafficked towards late endosomal compartments too. On the other hand, the invariant chain is not only a passive envelope for MHCII but also shapes the morphology of the endosomes. For instance, Ii can even delay MHCII transition through the endosomal compartments. (Lindner 2017; Pieters, Bakke, and Dobberstein 1993; Schröder 2016; De Silva and Klein 2015)

In B cells, MHCII-Ii is rich in MIICs as MIICs are the compartments where also pAg is loaded on the MHCII. MHCII-Ii processing is a long cascade of proteolytic events in which MHCII is released from the Ii cage by degrading the Ii. The Ii degradation is a joint effort of several molecules which function either simultaneously or in a cascade. Below, Ii degradation is described in a detail.

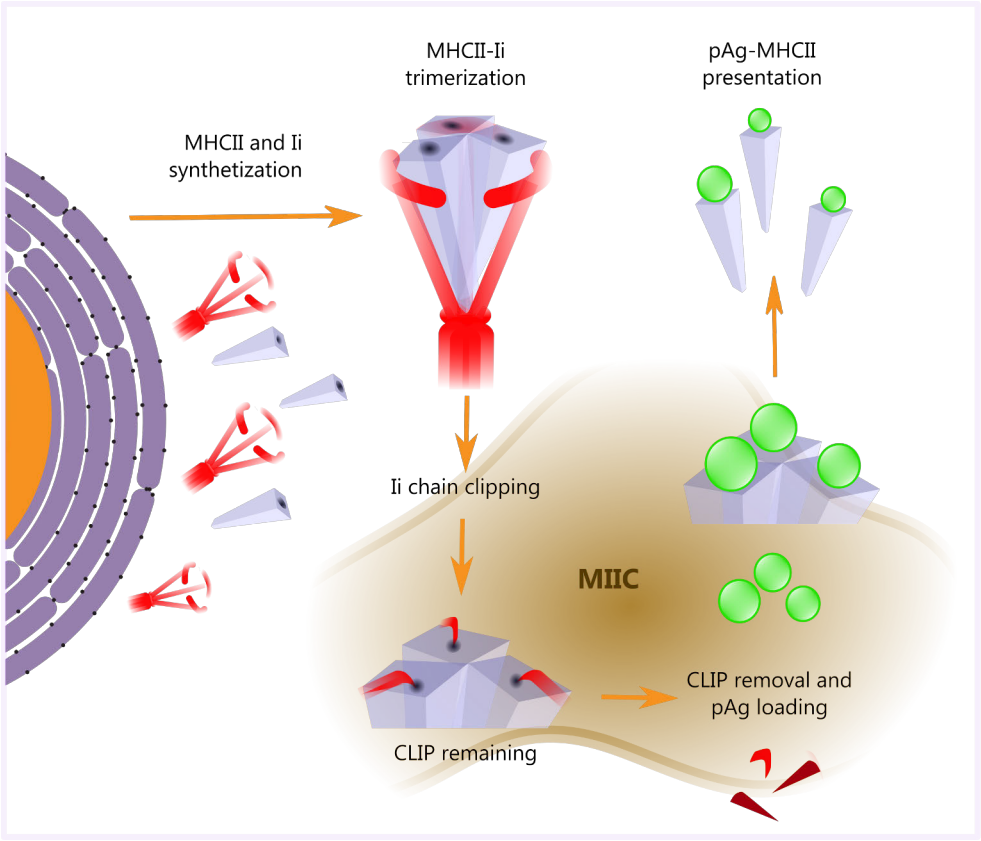

Figure 6. Major histocompatibility complex class II (MHCII) with its binding partners' invariant chain (Ii) and peptide-antigen (pAg). MHCII (gray) and Ii (red) are synthesized in the endoplasmic reticulum (ER). Immediately after MHCII has been synthesized, it forms dimers and binds with Ii. Ii is synthesized excessively compared to MHCII synthetization. Ii, or its remaining, CLIP, occupies the antigen binding groove (shaded dot in MHCII) until a correct pAg (green) has been processed. pAg replaces the CLIP and binds to MHCII. Finally, mature pAg-MHCII is trafficked to the plasma membrane to be presented for helper T cells. Ii cleaving to CLIP requires several molecules to collaborate and the process occurs in compartments called MHCII containing endosomes (MIICs). Notably, pAg loading to the MHCII molecules also occurs in MIICs.

Invariant chain degradation in B cells

Ii degradation begins from the Ii tail. Western blots have revealed that at the first, the full length, 33 kDa Ii is clipped into 22 kDa leupeptin-induced protein (LIP) fragment, by protease asparagine endopeptidase (AEP). Further, half of the LIP fragment is degraded and the 10 kDa Ii tail remaining, called small leupeptin induced protein (SLIP), is formed. The LIP – SLIP transformation happens with the help of

unspecified cysteine proteases. (van Kasteren and Overkleeft 2014; Lindner 2017; Watts 2004)

Nevertheless, the next clipping phase which produces only 23 amino acid long fragments, called class II-associated invariant chain peptide (CLIP), is well described in B cells. The degradation from SLIP to CLIP is induced by cysteine protease Cathepsin S (CatS). (van Kasteren and Overkleeft 2014; Lindner 2017; Watts 2004). CatS is highly expressed in B cells. CatS gets activated at low pH and its main task is the SLIP-CLIP degradation. CatS is also capable to promote other protein degradation in different endosomal compartments which may have a role in the antigen degradation, but for that, further studies are yet needed. (Verma, Dixit, and Pandey 2016; Wilkinson et al. 2015)

In the final MHCII-Ii maturation step, the CLIP is removed from the MHCII antigen binding groove. The CLIP removal is catalyzed by DM (human leukocyte antigen DM (HLA-DM) and murine H2-M). DM regulates self and foreign peptide antigen loading on MHCII molecules in all antigen presenting cells. In more detail, despite DM being a MHCII-like molecule, it does not bind peptides itself. DM induces transient conformational changes in MHCII which further catalyzes CLIP dissociation. Notably, DM also helps in the selection of the binding peptide as it can repeatedly induce conformational changes for the same MHCII molecule until the optimal peptide is found. Furthermore, DM is regulated by DO (human leucocyte antigen DO (HLA-DO) and murine H2-O). DO is a highly similar molecule to DM, but it targets DM itself instead of MHCII. Thus, DO can prevent DM from functioning and inhibit pAg-MHCII conjugation. (Demharter et al. 2019; Jiang et al. 2019; Mellins and Stern 2014; Pierre et al. 1996; Welsh and Sadegh-Nasseri 2020)

2.1.3.4 Targeting the peptide-antigen presentation on MHCII

As described above, pAg presentation results from the collaboration of several factors, and many more are yet to be distinguished. On the other hand, the process can also be tuned by even more numerous players. For instance, milder immune responses could be achieved by directly targeting MHCII, but also by decreasing the endosomal pH, which leads to Ii chain retention in the SLIP form. As a result, less mature pAg-MHCII complexes are formed. (Guo-Ping et al. 1999; Kuipers et al. 2005; Rupanagudi et al. 2015)

Similarly, targeting actin may alter pAg-MHCII presentation as the molecule docking to the plasma membrane is dependent on actin. One of the most puzzling players for pAg-MHCII presentation is DO. Increasing DO activity inhibits CLIP replacement when the pAg is DM sensitive but apparently, when the pAg is DM insensitive, DO increases the CLIP replacement. Overall, DO activity and the DM-DO relationship are yet under intensive study. Modulating DM/DO activity will alter

the pAg-MHCII presentation, but to understand the full picture, more studies are still needed. (Mellins and Stern 2014; Paul et al. 2011; Schröder 2016; Welsh, Song, and Sadegh-Nasseri 2019).

In a conclusion, MHCII and pAg-MHCII presentation have significant roles in health and disease. MHCII on B cells contributes to the development, differentiation and effector functions of helper T cells. MHCII on B cells is linked to the development of autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus. MHCII is also essential for B cell activation, proliferation and differentiation. (Giles et al. 2015; Molnarfi et al. 2013) MHCII and pAg-MHCII presentation can also act as therapeutic target sites but yet, but more detailed studies are needed.

2.2 Endosomal system

Organisms and cells self-regulate their internal conditions to adapt to changing external challenges. The self-regulation loop, called homeostasis, equilibrates numerous processes: cargo and nutrients transport, nutrients processing, receptor trafficking, and cellular signaling, too. To maintain normal, healthy physiology, homeostatic mechanisms need to be controlled and re-toned constantly. Notably, homeostatic imbalance might induce different diseases. (Billman 2020; Garcillán et al. 2018) To maintain healthy homeostasis, cells are constantly not only sensing but also internalizing, processing and degrading different cargo such as nutrients and receptors. These processes are carried out by the endosomal system.

2.2.1 Endocytosis

Endocytosis is a common term for the internalization of different molecules, particles, fluids, or receptors, for instance. In this chapter, any of the internalized material will be referred to as cargo. Endocytosis is essential for cell homeostasis and it provides the site for nutrient uptake. Cells can internalize cargo classically in three different ways: micropinocytosis, phagocytosis and macropinocytosis. Micropinocytosis is the most used internalization method for BCR and antigen in B cells.

Micropinocytosis (pinocytosis hereafter) describes endocytosis through small plasma membrane invaginations which produce small (0.1 – 0.2 μm in diameter) primary endocytic vesicles. Pinocytosis is a way for cells to endocytose fluids and dissolved small molecules. Clathrin-mediated endocytosis, which is described below, is an example of micropinocytosis. Other pinocytosis mechanisms are caveolin-dependent and clathrin/caveolin-independent endocytoses. (Hartenstein and Martinez 2019; Mayor and Pagano 2007; Mellman 1996) Pinocytosis and other internalization pathways are described in **Figure 7.**

2.2.1.1 Clathrin-mediated endocytosis

A clathrin unit is a triskelion and it can form up to hundreds of square nanometres of clathrin lattices (CLL) by joining with three other clathrin units. Clathrin-mediated endocytosis is based on the curving of the CLL. There are two distinct models on how CLL turns into invagination, the first model describes curvature to occur when the CLL area is large enough (constant area model) and the other one suggests that CLL invagination deepens at the same time as the CLL area grows (constant curvature model). Both models lead to the same result: a drop-like plasma membrane

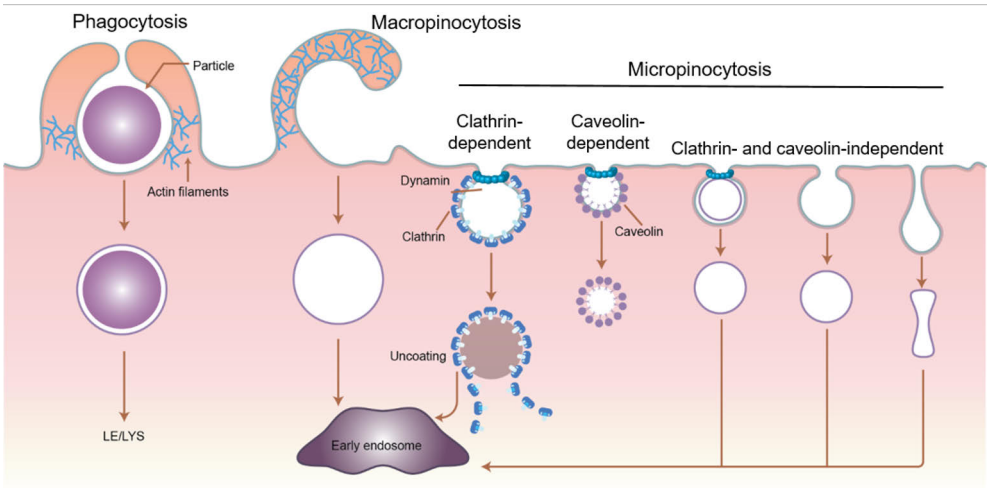

Figure 7. Internalization pathways in cells. Large particles are taken in by phagocytosis. Internalized material enters into late endosomes and lysosomes (LE/LYS). Fluids are engulfed for internalization via macropinocytosis. Phagocytosis and macropinocytosis are dependent on actin remodeling. Smaller cargo, such as receptors can be internalized via clathrin- and caveolin-dependent and independent ways. Both clathrin and caveolin pits are cut with dynamin and the internalized vesicles are rapidly uncoated and fused with early endosomes. Cargo, such as some integrins and Rab5, can be internalized also without clathrin or caveolin coating and the cut of the pit can happen with or without dynamin. Cargo can be also internalized into tubular structures. (Revised from Pagano 2007)

invagination that is supported by CLL. (Haucke and Kozlov 2018; Sochacki and Taraska 2019) The invagination can be notably deep and form tunnel-like structures up to one micrometer (Hartenstein and Martinez 2019). Finally, the neck of the invagination is cut with the help of dynamin (Cocucci, Gaudin, and Kirchhausen 2014) to form an endosome with neutral pH. Clathrin dissociates rapidly from the newly formed vesicle. The vesicle size is 60 – 200 nm external diameter, depending on the cell type. (Haucke and Kozlov 2018; Kirchhausen, Owen, and Harrison 2014; McMahon and Boucrot 2011). Clathrin-mediated endocytosis is illustrated in **Figure 7.**

2.2.1.2 Phagocytosis and macropinocytosis

Macromolecules and large (>0.5 μm in diameter) energy-rich particles are internalized via phagocytosis or macropinocytosis. Phagocytosis and macropinocytosis are schematically described in **Figure 7.**

Most, if not all, cells are capable to phagocytose and some immune cells, such as phagocytic leucocytes, are highly active in phagocytosis. Macrophages are one of the best examples of phagocytic cells as their target is to eliminate damaged cells or

pathogens by phagocytosis. In phagocytosis, cells engulf the target with plasma membrane (PM) protrusions and reseal the PM. After resealing, endocytosed cargo is captured in a newly formed vesicle. (Hartenstein and Martinez 2019; Mayor and Pagano 2007)

Macropinocytosis resembles phagocytosis. In macropinocytosis, the plasma membrane forms ruffles to "swallow" high volumes of extracellular fluids. The major difference between macropinocytosis and phagocytosis is in the induction of the internalization: phagocytosis is induced only by ligand-receptor interactions whereas macropinocytosis does not need a receptor interaction with ingestible cargo. Both phagocytosed and macropinocytosed cargo are trafficked through, maturating endosomal network until reaching the final destination in either phagolysosomes or endolysosomes for degradation. (Hartenstein and Martinez 2019; Mayor and Pagano 2007)

2.2.2 Endosomal trafficking

Endosomal trafficking lays the base for cell homeostasis. As all internalized cargo, including nutrients, plasma membrane proteins, viruses, and bacteria, as well as antigen-BCR complexes, is targeted to endosomes, endosomes can be seen as one of the master regulators in eukaryotic cells.

Immediately after the internalization, cargo is trafficked to an endosome to begin its journey along endosomal pathways. Typically, endosomes form long structural cascades, and pathways, where they constantly evolve, mature, or associate with other compartments. The classical endosomal pathway is simplified in **Figure 9**. Normal endosomal trafficking is essential for health, as several severe diseases, such as Parkinson's disease or Amyotrophic lateral sclerosis, are linked to altered endosomal trafficking. (Huotari and Helenius 2011; Schreij, Fon, and McPherson 2016; Scott, Vacca, and Gruenberg 2014) In B cells, the structural cascade for antigen and pAg trafficking has remained uncharacterized. Nevertheless, classical endosomal compartments and proteins could be postulated to have a role in antigen trafficking.

2.2.2.1 Small GTPases

Classical proteins functioning along endosomal pathways are Small GTPases. Rab-proteins are related to Arf, Ran, Ras and Rho protein families, and they are the largest Ras subfamily consisting of more than 70 proteins. Rab-proteins regulate endosomal trafficking, transportation and endosomal fusions. As with all small GTPases, Rab-proteins cycle between active and inactive states, this small GTPase cycle is illustrated in **Figure 8**. (MacKiewicz and Wyroba 2009) Activated Rab-proteins are

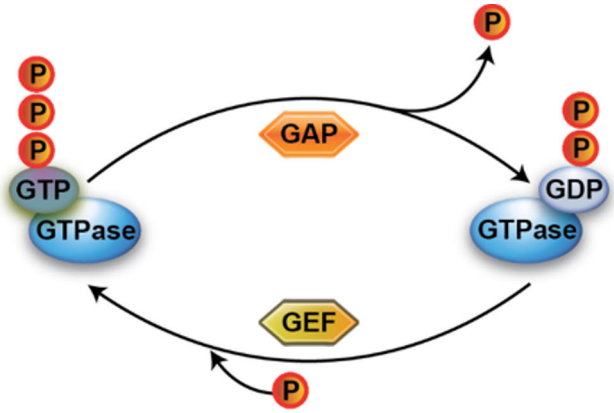

Figure 8. Small GTPases cycle. Inactive guanosine diphosphate (GDP) is switched into active guanosine triphosphate (GTP) form with the help of guanine nucleotide exchange factor (GEF). Active, GTP form is switched off by GTPase-activating proteins (GAPs).

anchored to the lipid bilayer by their C-terminus, and they recruit various effector proteins to the endosomes or organelles they are bound to. Inactive Rab-proteins, on the other hand, can be found in the cytosol. During the switch from active to inactive state, Rab-proteins undergo drastic conformational changes. The active state is called the guanosine triphosphate (GTP) state and the inactive state is known guanosine diphosphate (GDP) state. The activation, GDP-GTP switch, is promoted by various guanine nucleotide exchange factors (GEFs) and on the other hand inactivation, GTP-GDP conversion, is triggered by GTPase-activating proteins (GAPs). (Homma, Hiragi, and Fukuda 2021; Stenmark 2009; Wandinger-Ness and Zerial 2014)

Small GTPases contain 200-250 amino acids, and they are divided into different subfamilies that share high sequential identities. For instance, one of the well-studied Rab-proteins, Rab5 has three isotypes, Rab5A, B and C which share up to 95% sequence identity. The isoforms can have both overlapping and isoform-specific functions and responses. For instance, in our example, Rab5A delays growth factor receptor trafficking and regulates pinocytosis, whereas Rab5C is important in plasma membrane protrusions, for instance. (P. I. Chen et al. 2014; Homma et al. 2021)

2.2.2.2 Early endosomes and Rab5

Early endosomes are the first players along the long structural cascade of the endosomal pathway. Newly formed internalized vesicles and internalized cargo is guided to the early endosomes. Early endosomes are described as busy sorting stations with heterogenous composition, function and localization. A couple of minutes after the cargo has entered the EE, its further destination is already determined. The cargo is guided for further processing or it is recycled back to the plasma membrane. Receptors are the most typical example of the internalized

material that is recycled. (Huotari and Helenius 2011; Scott et al. 2014; Solinger et al. 2020)

The recycling-linked proteins, such as Rab4, Rab11 and retromer complexes are enriched in the tubular extensions of EEs multilobular structures. From the end of the tubules, recycling endosomes (RE) are pinched out while the EE body contains some vacuolar domains with neutral or slightly acidic pH of 6–7. EEs are 100–500 nm in diameter and most of them stay close to the plasma membrane. (Huotari and Helenius 2011; Scott et al. 2014; Solinger et al. 2020)

Early endosomes are surrounded by lipid bilayers. The lipid bilayer is enriched in phosphoinositide phosphatidylinositol 3-phosphate (PI(3)P). EEs also possess early endosomal antigen 1 (EEA1) that binds to PI(3)P via its C terminus and to Rab5 in its N-terminus. EEA1 is a transmembrane protein that has a long cytosolic tail. The long EEA1 tail senses the surrounding for encountering partners. When it finds Rab5 anchored to the membrane of another endosome, EEA1 reaches it and collapses. The collapse draws Rab5 containing endosome to the instant vicinity of the EEA1 containing vesicle and their fusion may begin. Endosomes can also fuse without the EEA1 collapse, by forming a direct bond between proteins in the neighboring endosomes. (Homma et al. 2021; Marat and Haucke 2016; Murray et al. 2016) Early endosomal fusions are essential for endosomal maturation as by the fusions, EEs become more acidic. (Hartenstein and Martinez 2019; Huotari and Helenius 2011; Scott et al. 2014)

Despite that Rab5 is classically described as functioning in early endosomal trafficking phases, it is having several effector proteins covering the whole endosomal range. Rab5 is essential for normal cargo internalization, endosomal fusions and trafficking of different proteins from Golgi to lysosomes. Notably, Rab5 is found also on phagosomes (described in detail in chapter 2.2.3) which are classically seen as degradation factories. (Wandinger-Ness and Zerial 2014)

2.2.2.3 Recycling endosomes and Rab11

To maintain plasma membrane surface homeostasis, nearly 80% of the endocytosed cargo is recycled back to the plasma membrane. Thus, the endosomal recycling system is greatly coordinating the cell surface composition. Proteins coordinating the recycling events are not found only from the tubular ends of the sorting endosomes, such as early endosomes, but they are found also in autophagosomes and even in the trans-Golgi network. (Ferro, Bosia, and Campa 2021; Puri et al. 2018; Redpath et al. 2019; Yeo et al. 2016)

The recycling system is highly dynamic and heterogenous and it can be coordinated by numerous different Rab-proteins naming the most classical ones, Rab4 and Rab11 as examples. Previously, Rab4 was reported as a marker for fast

recycling endosomes which would traffic cargo mainly from the early endosome. At the same time, Rab11 was reported as a protein involved in slow recycling endosomes which would recycle cargo from the perinuclear region and from late endosomal compartments. (Van Der Sluijs et al. 1991; Ullrich et al. 1996) Nowadays, the complexity of endosomal pathways is recognized and Rab4 and Rab11 are known to overlap on different endosomal pathways (Takahashi et al. 2012)

Defected recycling system can lead to various severe diseases, such as Alzheimer's disease (Buggia-prévot et al. 2014), Parkinson's disease (O'sullivan and Lindsay 2020) or cancer (Mellman and Yarden 2013). All the mentioned diseases have a link to Rab11, which is one of the main coordinators for endosomal recycling. It has in total three isoforms, Rab11a, Rab11b and Rab25. Rab11a and Rab11b share nearly 90% sequential identity, whereas with Rab25, the identity is less than 70%. (Kumar and Lukman 2018). Commonly, recycling-linked diseases are caused by the essential isoform of Rab11a (Ferro et al. 2021; Puri et al. 2018; Yeo et al. 2016).

2.2.2.4 Late endosomes and late endosomal proteins Rab7, Rab9 and LAMP

Late endosomes are typically round or oval and their size is 250 – 1000 nm in diameter. The pH in late endosomes is more acidic than in early endosomes, approximately 5.5 and the endosomes contain several intraluminal vesicles which are 50 -100 nm in diameter. Late endosomes occupy the perinuclear area where they form larger late endosomal bodies. Often LEs possess Rab7, LAMPs and Rab9, among others. (Huotari and Helenius 2011; Mellman 1996)

Late endosomes are matured from the vacuolar domains of early endosomes. How the tubular domains, containing for example Rab11, are separated from the maturing late endosome, is not fully understood. Nevertheless, dynein-mediated pulling forces and vesicle fission are reported to occur during the endosomal separation. (Huotari and Helenius 2011; Wandinger-Ness and Zerial 2014)

During EE maturation into LE, Rab5 is replaced by Rab7. Similar maturation happens also in phagosomes. EE maturation into LE is seen as a sequential cascade. For instance, due to Rab5 GEF (RABGEF1/RABEX5) activity, the Rab5 level on the endosomal membrane reaches its peak. After high Rab5 concentration, Rab5 GEF is eliminated by SAND-1/Mon1 which also recruits Rab7 to the endosomal membrane. On the membrane, Rab7 interacts with CORVET/HOPS complex which is also a Rab7 GEF and Rab7 concentration is increasing in turn. (Huotari and Helenius 2011; Wandinger-Ness and Zerial 2014)

LEs increase the number of intraluminal vesicles (ILVs) which have several functions. At first, ILVs isolate signaling receptors from the cytosol and thus the receptors are silenced. ILVs are also more acidic as they contain phospholipid bisphosphate, and they are not protected by glycosylated proteins. Thus, ILVs are thought to promote lipid and protein hydrolyses and serve as a starting point for lysosome formation. (Huotari and Helenius 2011) LE cannot be distinguished from lysosomes at the molecular level since they both express the same characteristic proteins, but they can be distinguished by their physical properties and ultrastructure. In contrast to LEs, lysosomes are electron-dense structures featuring high gradient density. (Scott et al. 2014)

Rab7 and Rab9, which are both located in late endosomes, originate from the same Rab7 subfamily. In eucaryotes, the subfamily consists of two isoforms each: Rab7A/Rab7B and Rab9A/Rab9B. Yet, some lower species express more isoforms. Rab9 proteins diverged from Rab7 proteins hundreds of millions of years ago during the metazoan era. Even though Rab7 and Rab9 are sharing some overlapping functions, they have important individual functions as well. (MacKiewicz and Wyroba 2009)

LAMP1 is reported to localize in MIICs in B cells and it is a classical protein in LEs too. LAMP1 belongs to the family of lysosome-associated membrane proteins together with LAMP2, LAMP3, UNC-46 and CD68. LAMPs are important for late endosomal, lysosomal and autophagosomal acidification. LAMPs are also transporting hydrolases into endosomes, and they can regulate membrane fusions. Yet, LAMPs are not stable components in late endosomal membranes, but they are constantly newly synthesized. LAMPs also have another interesting role in natural killer and cytotoxic T cells, where it protects the T cell plasma membrane from the extracellular site while the cell is releasing lytic granules to destroy the target. (Cheng et al. 2018; Kuchitsu and Fukuda 2018; Wilke, Krausze, and Büssow 2012)

2.2.2.5 Lysosomes

Already more than a hundred years ago, Elie Mechnikoff found that endocytosed material was degraded in internal, acidic environment (Mellman 1996). Nowadays we know that this degradation occurs in lysosomes. Lysosomes are the endpoint for internalized cargo. Interestingly, studies have shown that Rab5 is essential for early and late endosome formation but lysosomes are formed normally also in Rab5 silenced cells. (Hatoyama et al. 2021)

Lysosomes (LYS) fuse with late endosomes or phagosomes. In LE, the cargo to be degraded is packed in ILVs which increase in number after lysosomal fusion. The trafficking to lysosomes is unidirectional and recycling is not occurring from the lysosomes as they do not possess recycling proteins. LYSs are commonly round

vesicles, but they can be also tubular as in dendritic cells and macrophages which professionally internalize and degrade a large amount of cargo, such as pathogens or malignant cells. pH in LYS is approximately 4.5, and they contain more than 70 different degradative enzymes. LYSs are highly dependent on nutrient availability. In fed cells, LYSs are significantly smaller in size (size in diameter 100–500 nm) but much more numerous compared with starved cells (size in diameter 0.5–1.5 μm). In starved conditions, LYSs are also localized in more peripheral areas than in fed cells. (Hipolito, Ospina-Escobar, and Botelho 2018; Huotari and Helenius 2011; Xu and Ren 2015)

The endosomal pH is decreased by V-ATPases, which are complex proton pumps adjusted on the endosomal membrane. V-ATPases generate positive membrane potential inside the endosome which further increases the influx of counter ions such as Cl- and cational (Ca2+, Na2+, etc) efflux. (Huotari and Helenius 2011) Degradation in lysosomes is not only caused by the low pH, but they also contain lysosomal cysteine proteases such as cathepsin C, L and S. Lysosomal cysteine proteases function most efficiently under low pH conditions. CatS has an important role in the MHCII mediated antigen presenting and processing, as described in detail in chapter 2.1.3.3. (Drobny et al. 2022; Turk, Turk, and Turk 2000) An important part of the lysosomal function is lysosomal exocytosis. In

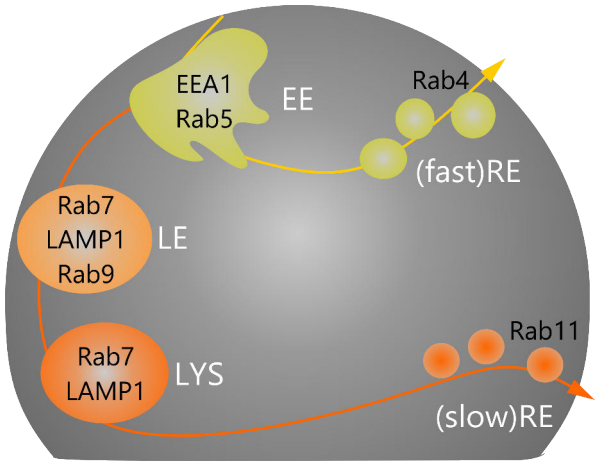

Figure 9. Classical cargo trafficking pathway. Classically cargo is described as trafficking via maturing endosomes. Internalized cargo is at first guided to early endosomes (EE) which can be marked with EEA1 or Rab5. From the EE cargo can be trafficked back to the plasma membrane via recycling endosomes (RE). Recycling endosomes possess classically Rab4 and/or Rab11. Classically Rab11 has been linked to slow recycling which would occur from the compartments along the later pathway, localizing close to the nucleus. Rab4, on the other hand, has been linked to fast recycling from the cell periphery. Cargo can be trafficked also to late endosomes (LE) which are expressing some or all of the next mentioned proteins: Rab7, LAMP1 and/or Rab9. LE matures into lysosomes (LYS) which are capable of degrading endosomal cargo. LYS contains Rab7 and LAMP1.

lysosomal exocytosis, the ILV content is released to the extracellular space. Natural killer and Cytotoxic T cells are active in lysosomal exocytosis as they eliminate pathogens or malignant cells by infecting them with lysosomal granules. (Casey, Meade, and Hewitt 2007; Van der Sluijs, Zibouche, and van Kerkhof 2013) The composition of exocytic granules was studied by Casey et al. and 9% of the identified proteins were hydrolases, such as cathepsins and lysosomal acids. Interestingly, nearly one-fourth of the identified proteins were small GTPases or other vesicular and trafficking-linked proteins. Thus, exocytic granules have remainings from the whole path of endosomal trafficking. (Casey et al. 2007)

2.2.3 Macroautophagy

Macroautophagy (autophagy hereafter) is a process where the cell engulfs cytoplasmic content for degradation. This "self-eating" is an evolutionarily conserved process and it is essential for cell survival. Autophagy helps cells to adapt to changing environments and to maintain homeostatic balance. Autophagy is also induced by different stressed conditions such as nutrient deprivation or DNA damage, for example. Defects in autophagy are linked to many pathological conditions such as cancer, heart diseases, different neurodegenerations and even aging.(Chang 2020). Macroautophagy is essential also for B cell proliferation, survival and humoral immune responses as described below in chapter 2.2.3.3.

2.2.3.1 Macroautophagosomal maturation

Autophagosomes are matured through a sequential process, beginning from omegasomes and ending to autolysosomes. The maturation cascade is described in **Figure 10**. Classically, autophagosome formation begins from the PI(3)P, which can conjugate autophagosomal protein microtubule-associated protein 1 light chain 3 (LC3), enriched ER subdomains. The ER subdomain forms and releases, an omega-shaped (Ω) membrane structure. This membrane structure is the autophagosome precursor called omegasome. As the omegasome further matures and bends into a cup shape, it is known as a phagophore. (Feng et al. 2014a; Hayashi-Nishino et al. 2009; Kucera, Borg Distefano, et al. 2016; Lamb, Yoshimori, and Tooze 2013; Ylä-Anttila et al. 2009). Interestingly, autophagosomal proteins have been shown to interact with clathrin heavy chain too, which suggests that also plasma membrane could act as a membrane source for autophagosome formation (Ravikumar et al. 2011).

Phagophores engulf cytosolic content during their elongation and maturation, until their closure. During the closure, the ends of the phagophore are joined together and a round double membraned structure, called the autophagosome, is formed.

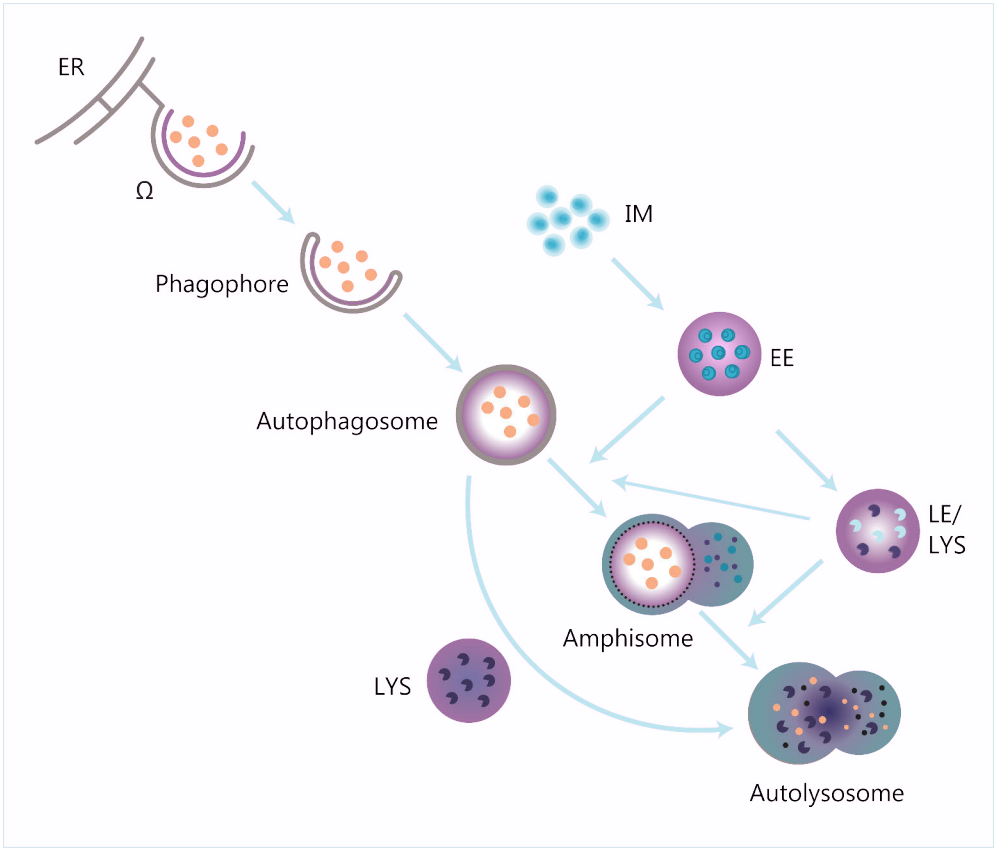

Figure 10. Autophagosomal maturation. Autophagosomal maturation is a sequential process. Classically, the autophagosomal precursor, omegasome (Ω) is formed from the extensions of the endoplasmic reticulum (ER). Omegasome elongates and curves at the same time as it engulfs cytoplasmic content. In this stage, they are called phagophores. When phagophores enclose, they become autophagosomes. Autophagosomes can fuse directly with the lysosomes (LYS) to form autolysosomes or fuse first with a late endosome (LE) to form an amphisome. In amphisomes, autophagosomal and late endosomal/lysosomal sites are separated by the inner membrane of the autophagosome. When amphisome becomes more acidic with the late endosomal/lysosomal fusions, the inner autophagosomal membrane is degraded and the lytic content reaches the autophagosytosed material. Autophagosomes receive also internalized material (IM) from the classical endosomal pathway as early endosomes (EE), late endosomes (LE) or lysosomes (LYS) fuse with the autophagosome or amphisome. Revised from Zhao & Zhang, 2019.

Autophagosomes continue maturation and fuse with freshly internalized material, early endosomes or late endosomes. The fusion event with a late endosome decreases the pH in the autophagosome and the fused structure is known as an amphisome. The autophagic site in the amphisome is partially isolated from the endosomal site. In practice, amphisomes contain two fused vesicles, one possessing autophagosomal characteristics and the other one possessing late endosomal characteristics. The

different sites in amphisomes are partially isolated by the inner autophagosomal membrane. Thus, amphisomes have special characteristics under EM too. (Shibutani and Yoshimori 2014; Zhao and Zhang 2019) In the final fusion phase, amphisomes fuse with lysosomes to form autolysosomes. In autolysosomes, the inner autophagic membrane is degraded by lysosomal hydrolases. Finally, the hydrolytic content of the lysosome reaches autophagocytosed, cytoplasmic content for total degradation(Feng et al. 2014b; Lamb et al. 2013; Nakatogawa et al. 2009) Notably, autophagosomes can also fuse with the plasma membrane (Zhang et al. 2015).

2.2.3.2 Cargo trafficking in autophagosomes

As described above, autophagosomal maturation is a multi-step process. Autophagosomes can fuse with many different endosomal compartments from lysosomes to freshly internalized cargo. Autophagosomes can indeed fuse with the internalized cargo immediately after internalization, even before the internalized vesicle expresses EEA1. Internalized cargo can end up in autophagosomes also from early endosomes after the fusion of the autophagosome and early endosome. Interestingly, cargo can be also loaded to autophagosomes from different endosomes without a stable fusion. (Cai et al. 2021; Pankiv et al. 2010) This is called a kiss-and-run –fusion and it has been described even more typical fusion type than a complete fusion between autophagosomes and lysosomes (Jahreiss, Menzies, and Rubinsztein 2008).

Notably, autophagosomes fuse with late endosomes and lysosomes which typically localize in the perinuclear region. Rab7, which is a typical late endosomal and lysosomal marker, has been shown to be essential for the perinuclear autophagosome gathering and the perinuclear localization of the autolysosomes. On the other hand, Rab7 has been suggested to be needed also for the final cargo degradation along the autophagosomal pathway (Eskelinen 2005). As LC3 expression correlates with the extent of autophagosomes (Kabeya et al. 2003; Shibutani and Yoshimori 2014) and it binds with a Rab7 effector, FYCO1, (Cai et al. 2021; Pankiv et al. 2010) it is interesting to speculate if Rab7 is promoting autophagosomal – late endosomal fusions.

2.2.3.3 Macroautophagosomes in immune cells

Not only does autophagy has important roles in external and internal cargo trafficking but it is also essential for immune cell proliferation, survival and humoral immune responses. For example, CD8+ helper T cells are not able to mature normally if a key autophagy-linked protein, Atg7, is deleted (Puleston et al. 2014). Also, B1a B cells which are found in peritoneal and pleural cavities and which are

the major source of IgM, require autophagy. B1 B cells are initially generated during fetal and neonatal life, and they are not under constant replenishment from hematopoietic stem cells as the other B cells. Thus, for B1 B cells self-renewing through autophagy, is essential for their survival. (Clarke et al. 2018) Yet, plasma cells, which have high autophagic flux, require autophagy for survival. Also, mice deficient in another autophagic protein, Atg5, have defective antibody responses and diminished numbers of long-lived antigen-specific plasma cells. (Pengo et al. 2013)

Despite defected autophagy being reported to cause severe immune cell failures, a comprehensive understanding of the root causes is not yet resolved.

2.2.3.4 Canonical and non-canonical autophagy

Classically, autophagy has been described as a conserved endosomal process in which a set of autophagy related (ATG) proteins are sequentially recruited to an autophagosomal precursor, to produce a functional, mature autophagosome. This hierarchical process is called canonical autophagy. Notably, non-canonical autophagy has recently been recognized as another form of autophagy and it has been suggested to have a role in B cell MHCII presentation too. In non-canonical autophagy, functional autophagosomes are formed in the absence of different ATG-proteins which were previously seen as essential proteins in autophagy. (Codogno, Mehrpour, and Proikas-Cezanne 2012)

The canonical autophagosome formation involves four stages: initiation, nucleation, elongation and closure and recycling. The different stages are orchestrated by different proteins, molecules and complexes. The initiation is dependent on ULK1. Nucleation is dependent on BECLIN1-complex. Elongation and closure are dependent on ATG5, ATG7 and LC3 and finally, recycling is dependent on ATG9. (Codogno et al. 2012)

In recent years, normal autophagy has been reported occurring also without some of the key proteins and molecules described in canonical autophagy. For instance, non-canonical autophagocytosis can bypass initiation machinery ULK1 and begin the initiation with ATG16L1. (Münz 2022)

On the other hand, Nishida et al. showed that mice lacking ATG5 and ATG7 (essential for canonical elongation and closure) can yet form normal autophagosomes and autolysosomes. In their study, Rab9 was suggested to induce autophagosome formation from the autophagosomal precursors. (Nishida et al. 2009). To support the suggestion, Rab9 was also reported conjugating autophagosomal key protein LC3 and ATG9 (essential in canonical recycling) to autophagosomal membranes (Urbańska and Orzechowski 2021). Rab9 is indeed an interesting candidate for a non-canonical autophagy coordinator as its

overexpression also increases non-canonical autophagocytosis. (Mareninova et al. 2022).

Notably, canonical and non-canonical pathways can function simultaneously too. Both of them can also direct cargo to the pAg-MHCII presentation through MIICs. (Urbańska and Orzechowski 2021) Interestingly, recent studies report non-canonical autophagocytosis to promote the pAg-MHCII presentation and on the other hand, attenuate MHCI presentation. Also, phagosomes created via non-canonical pathways maintain internalized antigens for a longer period. (Keller et al. 2017; Loi et al. 2016; Yamamoto et al. 2020) Yet, to gain a comprehensive understanding of the importance and even therapeutic potential, of non-canonical and canonical autophagocytosis in B cells, more studies are required.

3 Aims

The aim of this study was to understand antigen processing pathways in B cells and to build a comprehensive map of the structures and proteins involved in the process.

1. To follow antigen internalization, trafficking, degradation and to draw a comprehensive map of the compartments involved in the process, and to characterize peripheral, rapid antigen processing compartments in activated B cells.
2. To understand slow antigen processing in the perinuclear region of a B cell and to investigate the roles of macroautophagosomes and late endosomal proteins Rab7 and Rab9 in the process.

4 Materials and Methods

4.1 Cells

In the studies, mouse and human B cell lines and mouse T cell hybridoma cells were used. Some of the studies were conducted also with mouse primary B cells. All the cells were cultured in complete RPMI 1640 with 2.05 mM L-glutamine (cRPMI) supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 4 mM L-glutamine, 50 μM β-mercaptoethanol and 100 U/ml penicillin-streptomycin if not stated otherwise.

4.1.1 A20D1.3 B cells (I, II)

Most of the studies were done with A20D1.3 cells. A20D1.3 cells are A20 mouse lymphoma cells that stably express hen egg lysozyme (HEL) specific IgM BCR (D1.3) (Williams et al. 1994). Cells can be activated either with a real antigen (HEL) or by cross linking BCRs with mouse anti-IgM. A20D1.3 cells were a kind gift from Prof. Facundo Batista (the Ragon Institute of MGH, MIT and Harvard, USA).

4.1.2 A20D1.3 cells stably expressing Rab7CA, Rab9CA or LifeAct (II)

GFP-Rab7a-CA and GFP-Rab9a-CA stable cell lines were generated as described below (4.4.1). The cells were cultured in cRPMI supplemented with 0.55 mg/ml G418 (Geneticin 418 Sulphate, Calbiochem). GFP-LifeAct expressing A20D1.3 cells were a kind gift from Prof. Facundo Batista (the Ragon Institute of MGH, MIT and Harvard, USA).

4.1.3 1E5 T cells (II)

1E5 T cells stably express a $HEL^{108–116}$ peptide presented in MHCII $I\text{-}^{Ad}$ (Adorini et al. 1993). The cells were a kind gift from Prof. Facundo Batista (the Ragon Institute of MGH, MIT and Harvard, USA).

4.1.4 Primary B cells (I, II)

B cells were isolated from the spleens of 2–5 -month-old male and female MD4 mice (C57BL/6-Tg(IghelMD4)4Ccg/J, The Jackson Laboratory) using a negative selection kit (StemCell Technologies, 19854). Cells were left for recovery for 1 h before starting experiments. B cells originated from this mouse strain recognize HEL.

Experiments were approved by the Ethical Committee for Animal Experimentation in Finland. All the animal studies were performed according to the 3R-principle (animal license numbers: 7574/04.10.07/2014, KEK/2018-2504-Mattila, 10727/2018) under regulation of the Finnish Act on Animal Experimentation (62/2006).

4.2 Plasmids (I, II)

The plasmids used in the study were purchased from Addgene or they were kindly provided by Prof. Johanna Ivaska.

Table 1. Plasmids used in the experiments.

Plasmid	Mutation site	Origin	Catalogue nr
GFP Rab5a WT		Addgene	31733
GFP Rab5a CA	Q79L	A gift from prof. Ivaska	-
GFP Rab5 DN	N3S, N133I, S210N	Addgene	28048
GFP Rab7a WT		Addgene	12605
GFP Rab7a CA	Q67L	A gift from prof. Ivaska	-
EGFP Rab7 DN	T22N	Addgene	28048
GFP Rab9 WT		Addgene	12663
GFP Rab9 CA	Q66L	A gift from prof. Ivaska	-
GFP Rab9a DN	S21N	Addgene	12664
GFP Rab11 WT		Addgene	12674
EGFP Rab11 CA	Q70L	A gift from prof. Ivaska	49553
GFP Rab11 DN	S21N	Addgene	12678

4.3 Antibodies and other compounds (I, II)

Table 2. Antibodies and other compounds used in the experiments.

Antibody	Dilution	Provider	Catalogue nr
Anti-IgM-biotin	10 µg/ml	Southern Biotech	1021-08
6 nm Gold rat anti-mouse IgM	1:650	Jackson Immuno research	115-195-075
Donkey anti-mouse IgM RRX/AF647	10 µg/ml	Jackson Immuno research	715-295-140 715-605-140
Donkey anti-mouse IgM F(ab')$_2$ fragment 488/RRX/647	5-10 µg/ml	Jackson Immuno research	715-546-020 715-296-020 715-546-020
Hen Egg Lysozyme	5-10 µg/ml	Sigma-Aldrich	L6876
Biotin	1-10 µg/ml	Thermo Fisher Scientific	21338
Rabbit-anti-Rab5	1:150	CST	3547
Rabbit-anti-Rab6	1:200	CST	9625
Rat-anti-MHCII	1:50	Santa Cruz	Sc-59322
Mouse-anti-EEA1	1:50	Santa Cruz	Sc-6415
Mouse- anti-Rab7	1:100	Santa Cruz	Sc-376362
Rabbit-anti-Rab7	1:100	CST	9367
Rabbit-anti-Rab9	1:150	CST	5118
Rabbit-anti-Rab11	1:200	CST	5589
Rat-anti-LAMP1	1:100	DSHB	1D4B
Rabbit-anti-Cathepsin S	1:100	LSbio	LS-B16887
Rabbit-anti-Cathepsin S	1:50	Santa Cruz	Sc-271619
Rat-anti-H2-M	1:250	BD Biosciences	552405
Rat-anti-GFP-488	1:150	BioLegend	338002
Rabbit-anti-LC3a/b	1:100	CST	4108
Anti-PCM1-AF647	1:100	Santa Cruz	Sc-398365
Mouse-anti-IL-2	4µg/ml	Nordic Biosite	503804
Biotin mouse-anti-IL-2	1µg/ml	Nordic Biosite	503702
ExtrAvidin-AP (alkaline phosphatase)	1:5000	Sigma-Aldrich	E2636
FAST pNPP substrate tablet		Sigma-Aldrich	N2770
LysoTracker Deep Red	125nM	Sigma-Aldrich	L12492
DQ-Ova	1:10	Thermo Fisher Scientific	D12053
EZ-Ling Maleimide-PEG2-Biotin	1:10	Thermo Fisher Scientific	21901BID
Fibronectin	4 µg/ml	Sigma-Aldrich	F4759
Donkey-anti-rabbit IgG (H+L) AlexaFluor 488/555/647	1:500	Thermo Fisher Scientific	A21206 A31572 A31573
Donkey anti-goat IgG (H+L) AlexaFluor 488/555	1:500	Thermo Fisher Scientific	A11055 A21432
Mouse anti-rat IgG Fcy Fragment Specific AlexaFluor 488/RRx/647	1:500	Jackson Immuno Research	212-545-104 212-295-104 212-605-104
Goat-anti-mouse IgG Fcy subclass 1 AlexaFluor 488/RRx/647	1:500	Jackson Immuno Research	115-545-205 115-295-205 115-605-205

4.4 Transfections (I, II)

Transfections were done according to previously reported (Šuštar, Vainio, and Mattila 2018). In short, 4 x10^6 A20D1.3 cells were resuspended in 180 µl of 2S transfection buffer (pH 7.2) containing 180 mMNa_2HPO_4/ NaH_2PO_4, 50 mM sodium succinate, 15 mM $MgCl_2$, 15 mM HEPES and 5 mM KCl. 4 (or 2+2) µg of plasmid was added per 4 x10^6 cells, and they were transfected in 0.2 cm gap width electroporator cuvettes with an AMAXA Electroporator program X-005 (BioSystem).

After the transfection pulse, cells were immediately transferred in to 4 ml of +37ºC cRPMI supplemented with 10% of FCS and incubated (37ºC, 5% CO_2) for 4 hours before the experiments.

4.4.1 Stable clones (II)

For stable cell lines, A20D1.3 cells were transfected as mentioned above. The cells were left to recover over night in 5% CO_2 at 37°C after which they were seeded 2–5 000 cells per well on a 96-well plate, in 100 µl of cRPMI supplemented with 0.55 mg/ml G418.

GFP-expressing populations were enriched and sorted with Sony SH800 Cell Sorter.

4.5 anti-IgM and HEL activation (I, II)

Cells were activated with 10 µg/ml of surrogate antigen, donkey anti-mouse-IgM-$F(ab)_2$ (anti-IgM activation) or with 5 µg/ml of real antigen HEL conjugated with atto-633 (HEL activation). Donkey anti-mouse-IgM-$F(ab)_2$ was conjugated with Alexa Fluor 647, Rhodamine Red-X or Alexa Fluor 488. Activation solution contained 2% FCS-PBS in flow cytometer experiments and 10% FCS-PBS in SDCM microscopy experiments.

4.6 DQ-Ova activation (I, II)

A20D1.3 cells were incubated with 10 µg/ml Biotinylated HEL for 10 min on ice and washed with PBS. After the wash, the cells were incubated with either Alexa Fluor 633 conjugated Streptavidin (Streptavidin-633) or pure Streptavidin, on ice for 5 min. Biotin-streptavidin treated B cells were again washed with PBS and stained for the final time with biotinylated DQ-Ovalbumin on ice for 5 min and washed. As a final outcome, BCRs were linked with a sandwich of Biotin(HEL)-Streptavidin(633/pure)-Biotin(DQ-Ova). Cells were activated in 5% CO_2, 37°C for desired time points.

4.7 Microscopy

4.7.1 Spinning disk confocal microscopy (SDCM) (I, II)

Spinning disk confocal microscopy was used as a fast, low-phototoxic imaging modality for 3D imaging and live cell imaging. Cells were seeded for activation either on 12- wells PTFE diagnostic slides (Thermo Fisher Scientific, 10028210), 8-wells IBIDI uncoated µ-slides (IBIDI, 80806) or MatTek dishes (35 mm glass-bottom dishes, TIRFM quality, P35G-0.170-14-C, MatTek Corporation).

4.7.1.1 PFA fixation (I, II)

12-wells slides were coated with 1 µg/ml fibronectin for 30 min, at room temperature (RT) after which 50 000 cells in 20 µl, were seeded per well and activated in 5% CO_2, 37°C for desired time points. Activated cells were permeabilised and blocked with blocking buffer (5% donkey serum, 0.3% Triton X-100 in PBS) for 1 h at RT after which the cells were fixed with 4% PFA 10 min RT. After fixation, samples were stained with primary antibodies in staining buffer (1% BSA, 0.3% Triton X-100 in PBS) at +4°C, overnight.

Primary stainings were followed by PBS washes and incubation with the secondary antibodies for 1 h in PBS at RT. Samples were mounted with FluoroMount-G containing DAPI (Thermo Fisher Scientific, 00495952) and sealed with a microscope slide.

4.7.1.2 Methanol-Acetone fixation (II)

8-wells slides were coated with 22.4 µg/ml CellTak in the pH 8.0 for 1 h at RT, after which the slides were air-dried. 300 000 cells were seeded in 250 µl per well and activated in 5% CO_2, 37°C for desired time points. Activated cells were fixed with 50:50 acetone–methanol for 20 min at -20°C. Permeabilization was accomplished with acetone 5 min at -20°C, after which the cells were blocked with 5% donkey serum in PBS for 1 h at RT. After the blocking, samples were stained with primary antibodies in staining buffer (1% BSA in PBS) at 4°C, overnight

Primary staining was followed by PBS washes and incubation with the secondary antibodies for 1 h in PBS, at RT. Samples were mounted using 300 µl of FluoroMount-G containing DAPI (Thermo Fisher Scientific, 00495952).

4.7.1.3 Life cell imaging (I, II)

MatTek dishes were coated 30 min with 1 µg/ml fibronectin at RT after which cells were placed on the dishes for settle. Cells were placed in the pre-heated (37ºC) SDCM chamber and imaged with continuous capturing on Photometrics Evolve 10 MHz Back Illuminated EMCCD camera (512×512 pixels, 1×1 binning).

Lysotracker (I)

Cells (1×10^6/ml) were labeled with 125 nM LysoTracker Deep Red (Thermo Fisher Scientific, L12492) for 1 h in 5% CO_2, 37°C, washed with PBS and resuspended in cRPMI. Cells were then activated with 10 µg/ml of donkey anti-mouse-IgM antibody conjugated to Alexa Fluor 488 on ice for 10 min, washed with cold PBS and continued to imaging.

MHCII internalization (I)

Cells were labeled on ice with anti-MHCII conjugated to Alexa Fluor 488 and 10 µl/ml donkey-anti mouse-IgM conjugated to RRx for 5 min and washed with cold PBS. Cells were resuspended in cold imaging buffer and seeded on four-well MatTek dishes on ice. After seeding, cells were activated at 37°C inside the environmental chamber of the microscope and imaged immediately.

4.7.1.4 Image acquisition and processing (I, II)

The images were acquired using a 3i CSU-W1 spinning disk equipped with 405, 488, 561 and 640 nm laser lines and 510–540, 580–654 and 672–712 nm filters and 63× Zeiss Plan-Apochromat objective. Hamamatsu sCMOS Orca Flash4 v2 |C11440-22CU (2048×2048 pixels, 1×1 binning) was used to image fixed samples. Photometrics Evolve 10 MHz Back Illuminated EMCCD (512×512 pixels, 1×1 binning) camera was used in live cell imaging and in DQ-Ova imaging.

Images from the fixed samples were deconvoluted with Huygens Essential v16.10 (Scientific Volume Imaging, The Netherlands, http://svi.nl), using the CMLE algorithm, with a signal-to-noise ratio of 20 and 40 iterations. For SRRF, 20–50 images were acquired from one single plane using time-lapse mode and processed in Fiji ImageJ using the SRRF module

4.7.2 Transmission electron microscopy (EM) (I, II)

Transmission electron microscope was used to study antigen internalization and intracellular structures of activated A20D1.3 cells.

A20D1.3 cells were activated together with 20 µg/ml anti-IgM-F(ab')$_2$-647 and goat anti-mouse IgM conjugated with 6 nm colloidal-gold (1:650, Jackson ImmunoResearch, 115-195-075), in EM imaging buffer. The imaging buffer contained 10% FCS, 5.5 mM D-Glucose, 0.5 mM $CaCl_2$ and 0.2 mM $MgCl_2$. in PBS. Activated cells were placed on 4 µg/ml fibronectin coated glass coverslips for 15 or 75 min. The cells were fixed with 2% glutaraldehyde (EM grade, Sigma-Aldrich, G7651) in 0.1 M sodium cacodylate buffer with pH 7.4, for 30 min at room temperature. The fixed cells were washed twice for 3 min with 0.1 M sodium cacodylate buffer, with pH 7.4.

The samples were processed for EM as described in (Jokitalo et al. 2001). In short, 60-nm-thick sections parallel to the cover slip were cut using a Leica EM Ultracut UC7 ultramicrome (Leica Mikrosysteme GmbH, Austria). The electron micrographs were post stained with uranyl acetate and lead citrate and imaged with a Jeol JEM 1400 transmission electron microscope (Jeol Ltd., Tokyo, Japan) equipped with a bottom mounted CCD camera (Orius SC 1000B, Gatan Inc., Pleasanton, CA) and Jeol JEM-1400 Plus equipped with an OSIS Quemesa bottom-mounted CCD camera (EMSIS, Germany), both operating at 80 kV.

4.7.3 Structure illumination microscopy (SIM) (I, II)

Structure illumination microscopy was used to study endogenous proteins in activated primary B cells. The cells were seeded in 0.5% FCS-PBS on 4 µg/ml fibronectin coated MatTek dishes (35 mm glass-bottom dishes, TIRFM quality, P35G-0.170-14-C, MatTek Corporation) and activated for desired time points in 5% CO_2, +37ºC. After activation, the samples were mounted with Vectashield (Vector Laboratories) before imaging with DeltaVision OMX super-resolution system.

DeltaVision OMX V4 was equipped with 60x SIM Olympus Plan Apo N objective (NA 1.42) and 405, 445, 488, 514, 568, 642 nm lasers and corresponding filters for DAPI, CFP, Alexa488, YFP, Alexa 568 and Cy5 with optical sectioning of 0.125 µm steps. Images were captured with three PCO Edge Front illuminated sCMOS (1024×1024 pixels) and processed with OMX Acquisition v3.70 and softWoRx Deconvolution v7.0.0.

4.8 Flow cytometer (I, II)

Flow cytometer analysis on BD LSR Fortessa was used to study antigen internalization, pH assessment and GFP-Rab-expressions. BD LSR Fortessa was equipped with 405, 488, 561 and 640 lasers with corresponding filters and a software BD FACSDiva™ v8. The data was analyzed using FlowJo v10 (Tree Star).

4.8.1 Antigen internalization (I, II)

Cells were stained on ice with biotinylated anti-mouse IgM or house-made biotinylated HEL in PBS, for 10 min. Stained cells were incubated in +37°C with 5% CO_2, for desired time points. Activation was followed by streptavidin-633 staining on ice for 20 min. Streptavidin-stained cells were washed with PBS and kept on ice before analyzes with a flow cytometer. When GFP-expressing cells were studied, only GFP-expressing cells were selected for the analysis.

4.8.2 pH assessment (I)

Cells were stained with both anti-mouse-IgM-FITC and anti-mouse-IgM-F(ab')$_2$-647 (5 µg/ml each) for 10 min on ice and washed with ice cold PBS. Washed cells were incubated at 37°C with 5% CO_2 for desired time points. Cells were kept on ice until analyzing with flow cytometer.

4.9 Image analysis and statistical analysis (I, II)

Several different colocalization parameters were studied: Mander's colocalization coefficiences, Pearson correlation and Overlap coefficiences. Also, particle analysis was utilized to study several parameters: vesicle number, size, distance from the microtubule organisation center, MTOC, total and mean intensities.

4.9.1 Particle analysis with MATLAB R2018b (I, II)

Deconvoluted 3D stack SDCM images of activated and MTOC stained B cells were analyzed for the antigen particles in MATLAB R2018b (MathWorks). At the first, binary masks were created for the antigen signal from 3D images using Otsu-thresholding. Antigen vesicles were 3D segmented utilizing region props function and only objects inside the binary mask were kept for the particle analysis. MTOC channel was segmented in the same way detecting the brightest intensity value as the MTOC. Antigen vesicle distances were measured from the 3D centroid positions to the MTOC centroid position.

4.9.2 Particle analysis with Huygens Essential (II)

Rab7 and Rab9 particle analysis was done from deconvoluted SDCM images, with Huygens Essential version16.10 (Scientific Volume Imaging, The Netherlands, (http://svi.nl). The cells were thresholded for Rab7/Rab9 channel such that the dimmest 5% of the channels intensities were considered as background. Signal outside the thresholded particles were cleared from both channels of Rab7/Rab9 and

DQ-Ova. After clearing the outside intensities, the DQ-Ova intensity was studied inside the Rab7/Rab9 particles.

4.9.3 Colocalization analysis with Huygens Essential (I, II)

Colocalization analysis were performed with Huygens Essential version 16.10 (Scientific Volume Imaging, The Netherlands, http://svi.nl), using optimised automatic thresholding.

4.9.4 Statistical analysis and illustrations (I, II)

Statistical significances were calculated using unpaired Student's t-test assuming normal distribution of the data or one way Anova. Statistical values are denoted as: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Graphs were created in GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Figure formatting was done on Inkscape v.092.2.

4.10 Antigen presentation measured by ELISA (II)

Secretion of IL-2 was measured as ELISA-system from 96-well plates which were pre-treated for the IL-2 capturing as described below: ELISA 96-wp (Microlon 600, Bio-One 675061) wells were coated with 25 µl of 4 µg/ml anti-IL-2 in PBS, for 1 h at 37°C. Coated wells were blocked with 150 µl of blocking buffer (1% BSA in PBS) for overnight at 4°C after which the IL-2 containing supernatant was transferred to the PBS washed IL-2-capturing wells.

For IL-2 secretion, B cells (A20D1.3 cells, or A20D1.3 cells stably expressing GFP-Rab7CA/GFP-Rab9CA/GFP-LifeAct, or A20D1.3 cells inhibited with 60 µM CID106770 or 1 µM Wortmannin) were at the first incubated with 5 µg/ml of HEL for 1 h at 37°C in cRPMI. After the activation, cells were washed with PBS and resuspend in cRPMI. Activated B cells were mixed with 1E5 T cells (ratio 2:1) on 96-wp and placed in 5% CO_2 at 37°C for 17 hours.

IL-2 secretion was measured fron the IL-2 capturing plates (described above) by placing 50 µl of the IL-2 supernatant per well. The plates were incubated for an hour (at 37°C), washed with 0.05% Tween-20-PBS and 50 µl of biotin-conjugated anti-IL-2 was added to the wells. The plates were incubated for 1 h at room temperature and washed with PBS and the IL2-sandwich formation was topped with blocking buffer containing ExtrAvidin Alkaline Phosphatase (1:5000, Sigma-Aldrich, 043M4771) and again the plate was incubated 1 h at RT. The plate was washed for the final time and the wells were supplemented with 50 µl of pNPP (SIGMAFAST p-Nitrophenyl phosphatase tablets, Sigma-Aldrich®, N2770). The optical density

was measured at 405 nm (Multiscan, Thermo Scientific, SkanIt Software, vs. 3.1.0.4) after 20–30 min. Samples were run in triplicates. All the samples were normalised to the control samples.

4.10.1 B cell preparation for the peptide-antigen presentation

To inhibit Rab7 activity, CID1067700 (Merck, #SML0545) was added to the A20D1.3 cells 1 h before the cells were activated with the antigen, at the concentration of 60 µM. Rab inhibitor was kept on the cells throughout the experiment because its reversible nature.

To inhibit PI(3)P activity with irreversible Wortmannin (Sigma-Aldrich®, M5273), the inhibitor was added to the A20D1.3 cells 1 h before the cells were activated with the antigen. Wortmannin was kept on the cells only during the B cell activations. Wortmannin was used in the concentration of 1 µM.

Table 3. Summary of the used inhibitors.

Inhibitor	Target	Conc.	Reversibility	During B cell activation	During B – T cell activation
CID1067700	Rab7	60 µM	Reversible	Yes	Yes
Wortmannin	PI(3)P activity	1 µM	Irreversible	Yes	No

5 Results

5.1 Rapid antigen processing in the B cell periphery (I)

To draw a comprehensive picture of antigen processing in B cells, we followed the intracellular migration of fluorescently labeled antigens with various endosomal markers in B cells. As antigens, we utilized either the surrogate antigen α-IgM $F(ab')_2$ fragments, or hen egg lysozyme (HEL), which was specifically recognized by a transgenic BCR, D1.3, in the A20 B cell line. These A20D1.3 cells were used as the model B cell in most of the experiments. The antigens were either conjugated with fluorescent probes, or the fluorescent probe was linked to the antigen via biotinylation-streptavidin after the activation, or the probe became fluorescent after degradation. As fluorescently tagged antigens can be tracked under a microscope or a flow cytometer (FACS), we utilized the setup to study antigen trafficking and localization in B cells.

5.1.1 Antigen traffics to a perinuclear pocket within an hour

First, we imaged activated B cells, which were processed for fluorescence imaging, under a spinning disc confocal microscope (SDCM). We found that the majority of the antigen was clustered to a perinuclear region within 60 min after the B cell activation (I, Fig. 1a). We noticed similar antigen gathering in MD4 mouse primary B cells within 30 min (I, Fig.1b). We were able to quantify antigen vesicle number and the mean distance from the Microtubule Organisation Centre (MTOC) with a MATLAB-based 3D analysis (I, Fig. 1c). We saw a clear decrease in the antigen vesicle number, and in the mean distance from the MTOC (I, Fig. 1d, e) revealing that antigen is gathered to a perinuclear region along the activation. The shift was also visible when we plotted the values of vesicle numbers and mean MTOC distances by their distribution (I, Fig. 1e). When we imaged B cells that were activated with a surrogate antigen for 75 min under an electron microscope (EM), we saw a drastic change in the nuclear appearance: the nucleus was indeed forming a nuclear pocket that was occupied with several types of multi-vesicular compartments resembling late endosomes (Adler et al. 2017; Lankar et al. 2002;

Unanue, Turk, and Neefjes 2016; Vascotto et al. 2007). Surprisingly, shortly after the activation (15 min), the antigen was again found in similar multilobular vesicles but close to the plasma membrane. (I, Fig. 1f, Fig. S1) The findings raise the question if antigen processing could start already shortly after the activation, in the perinuclear compartments.

5.1.2 Early and late endosomal Rab-proteins colocalizing with antigen

As Rab-proteins orchestrate numerous endosomal phenomena, including cargo trafficking and endosomal fusions (Huotari and Helenius 2011; Mellman 1996), we next studied early and late endosomal Rab-proteins in activated and resting B cells. Rab5 is typically decorating early endosomal (EE) compartments, whereas late endosomes (LE) are occupied by Rab7 and Rab9, and finally, Rab11 is a marker for recycling endosomes (RE) (Huotari and Helenius 2011; Zhao, Codogno, and Zhang 2021; Zhao and Zhang 2019). We decided to utilize these markers to map the endosomal maturation and processing route in B cells.

We activated B cells with a fluorescently tagged α-IgM for different time points after which fixed the cells and proceeded to immunostainings, with the above-mentioned endosomal markers. The samples were mounted for fluorescence imaging with SDCM, images were deconvoluted to gain better resolution, and colocalization with Rab-proteins and antigen was studied with the Huygens image analysis program (I, Fig. 2a,b; S3a,b). We saw that the Mander's colocalization coefficiency (M2) of the antigen and any of the markers, increased over time whereas the antigen colocalization with a control, a Golgi-specific transport protein Rab6, remained low (I, Fig. 2c).

10 min after the activation, antigen colocalization with the Rab-proteins was approximately 40–55%. Late endosomal and lysosomal Rab7 had the lowest colocalization level with the antigen, early endosomal marker Rab5 and late endosomal marker Rab9 possessed approximately 50% colocalization with the antigen, whereas the highest colocalization was seen with antigen and recycling endosomal marker Rab11. (I, Fig. 2c) As the Mander's colocalization coefficiency (M2) revealed, shortly after the activation, only approximately half of the antigen colocalized with the endosomal markers.

We next tested how large portion of the antigen is internalized within 10 min of the activation. Thus, we studied the antigen internalization with a microscope, and with Flow Cytometer (FACS) assay (I, Fig. S2). We saw with the SDCM that approximately 40% of the antigen was internalized 10 min after the antigen and on the other hand, nearly 80% of the antigen was internalized within 60 min (I, Fig. S2c). The finding strongly correlated with FACS analysis which showed similar

results on antigen internalization (I, Fig. S2d). Also, the EM analysis revealed that some antigen remained on the cell membrane after 15 min of the activation (I, Fig. S1a). The internalization assay suggested that some of the antigen signal in SDCM originates from the antigen trapped on the plasma membrane which decreases the Mander's colocalization coefficiency (M2, antigen overlap with the Rab-proteins). Thus, we were able to conclude that most of the internalized antigen colocalize with endosomal Rab-proteins already shortly after the activation. Notably, both antigen internalization and its colocalization with Rab-proteins increased over time (I, Fig 2, Fig. S2).

Next, we took an advantage of the super-resolution imaging tool called super-resolution radial fluctuation (SRRF) which is based on image post-processing analysis of signal fluctuation (Gustafsson et al. 2016). By SRRF imaging the axial resolution was improved significantly and we saw clear differences between the colocalization of different Rab-proteins with the antigen. Especially 45 min after the activation, late endosomal Rab7 and Rab9 showed more prominent colocalization with the antigen than Rab5 and Rab11 which were more scattered. (I, Fig 2d, Fig. S5) 10 min after the activation, the Mander's colocalization coefficiency (M2) was modest with the antigen and Rab5, Rab7 or Rab9 and low with Rab11. 45 min after the activation M2 was the highest with Rab7, Rab9 and Rab11 whereas colocalization with Rab5 remained low. (I, Fig 2e) The low colocalization of the antigen and the control (Rab6) confirmed the accuracy of the analysis used.

Together these SDCM and SRRF imaging, and the image analysis, led us to suggest that antigen is trafficked into a wide variety of endosomes possessing different characteristics.

5.1.3 Antigen trafficks to atypical compartments possessing both early and late endosomal markers

Early and late endosomal markers can also be distinguished by their EEA1 or LAMP1 expression, respectively (Huotari and Helenius 2011). Interestingly, LAMP1 is also described to be expressed in MHCII peptide-loading compartments (MIICs) in B cells (Aluvihare et al. 1997; Unanue et al. 2016). To complement the antigen trafficking map in B cells, we studied antigen colocalization with EEA1 and LAMP1, using SDCM (I, Fig. 3a,b; S3c,d; S6a). We saw a high Mander's colocalization coefficiency (M2) with both, LAMP1 and EEA1, in any of the studied time points. On the other hand, when we considered also the intensity of the labeled proteins and utilized the Pearson correlation of EEA1/LAMP1 with the antigen, we saw that the EEA1 colocalization with the antigen decreased over time whereas LAMP1 colocalization with the antigen increased. (I, Fig. 3c)

We again utilized SRRF imaging too (I, Fig. 3d). SRRF imaging revealed that EEA1 colocalization with the antigen was approximately 40% in both early and late activation time points whereas LAMP1 colocalization with the antigen increased from approximately 25% to 45% along the activation (I, Fig. 3e).

Interestingly, when we studied the colocalization of EEA1 and LAMP1 in resting cells and in short or late activation time points, we noticed a peak in their correlation 10 min after the activation (I, Fig. 3f). We also studied the colocalization of early and late endosomal markers in pairs: EEA1/Rab7, EEA1/Rab9 and Rab5/LAMP1 in resting cells and 10 min after the activation. This analysis revealed activation inducing a significant increase in the colocalization of the EE/LE markers (I, Fig. S6b). When we inspected EEA1, LAMP1 and antigen by 3D image rendering, we noticed triple colocalizations in vesicles with different structures (I, Fig. 3g).

Together these findings led us to speculate that the antigen could be trafficked in atypical endosomes containing both, early and late endosomal markers. To support the finding of the existence of atypical EE/LE compartments in B cell antigen trafficking, we transfected A20D1.3 cells with a green fluorescent protein (GFP) -fused Rab5a, loaded the cells with low pH-indicator LysoTracker and activated the cells. The cells were followed with SDCM from the activation, and we were indeed able to see that the internalized antigen was instantly trafficked to peripheral vesicles expressing Rab5, and containing LT signal too. (I, Fig. 3h; Movie 1; Fig. S6c).

5.1.4 Antigen is trafficked to degradative compartments shortly after the activation

To study if the atypical endosomes containing both, early and late endosomal markers, could also degrade received antigen, we linked biotinylated antigen, α-IgM and HEL, via streptavidin link with biotinylated DQ Ovalbumin (DQ-Ova) (I, Fig. 4a). DQ-Ova is a densely packed probe which is, by default, self-quenched and becomes fluorescent only when the probe is degraded. With this mean, we were able to study antigen degradation. We saw with flow cytometric analysis that DQ-Ova intensity was increased over time in the activated B cells, whereas the cells treated with DQ-Ova alone did not possess increased fluorescent signal (I, Fig. 4b). The DQ-Ova signal in activated A20D1.3 cells was visible already 20 min after the activation, yet the DQ-Ova intensity continued increasing throughout the activation, and was finally the highest in DQ-Ova-HEL activated A20D1.3 cells (I, Fig. 4b,c). DQ-Ova-HEL intensity was also visible under a SDCM 20 min after the activation (I; Fig 4c).

As CatS is responsible for modifying MHCII for pAg-MHCII binding (van Kasteren and Overkleeft 2014; Lindner 2017; Watts 2004), we studied antigen colocalization with CatS with SDCM and SRRF imaging (I, Fig. 4c,d). The imaging revealed that the colocalization of CatS and antigen was modest yet persisting in

both early and late activation time points (I, Fig. S6d). Next, we again took an advantage of life cell imaging to answer the question if antigen traffics to degradative compartments shortly after the activation. We loaded A20D1.3 cells with LT, activated the cells with α-IgM and followed the cells under SDCM. We indeed saw a very strong colocalization of LT and antigen already 1–5 min after the activation (I, Fig. 4e; Movie 2). We also saw that the antigen fused with LT vesicles immediately after the internalization (I, Fig. 4f; Movie 3). In addition, we studied the antigen degradation capacity utilizing FITC-conjugated antigen. FITC is a fluorophore unstable under acidic pH whereas AlexaFluor-fluorophores are highly stable in acidic environment. By following A20D1.3 cells activated with a mixture of α-IgM-FITC and α-IgM-AlexaFluor647 with FACS, we saw a clear decrease in FITC conjugated antigen already 5–15 min after the activation, yet 647-signal remained stable (I, Fig. S6e). Together these findings suggested that antigen is internalized shortly or even immediately after the activation into degradative compartments that could facilitate antigen processing into pAg.

5.1.5 Antigen colocalizes with MHCII fast after the internalization

To conclude whether the antigen could be processed into pAg and loaded to MHCII shortly after the activation, we studied MHCII colocalization with the antigen. We saw a punct colocalization with antigen and MHCII, in the cell periphery 10 min after the activation and they colocalized 30 min after the activation in the perinuclear region too. (I, Fig. S6f). However, the analysis was challenged by the density of the MHCII signal in the cytosol. To improve the image resolution, we utilized Structured Illumination Microscopy (SIM) and studied MHCII-antigen colocalization in A20D1.3 cells activated for 15 min. The SIM imaging verified the high colocalization of MHCII-antigen (I, Fig. 5a).

As the vesicular MHCII can be originated either from the plasma membrane or it can be received from the endoplasmic reticulum (ER), we decided to pre-label MHCII on the plasma membrane with anti-MHCII conjugated with AF488 (sMHCII) before cell activation. After the cells were activated and fixed, the cells were also labeled for total MHCII with MHCII-AF647 (I, Fig. 5b). We saw under SDCM that the internalized antigen colocalized with both surface and ER originated MHCII (I, Fig. 5c). Internalized antigen showed high colocalization with MHCII when we inspected them under a SDCM in live imaging (I, Fig. 5d; Movie 4).

H2-M promotes the release of the final shield of MHCII, CLIP fragment, and is thus also promoting pAg binding to MHCII (Demharter et al. 2019; Jiang et al. 2019). To support our hypothesis of pAg loading occurring in the same MHCII positive, atypical endosomes, we immunostained activated A20D1.3 cells for H2-M.

The samples were imaged with SCDM and SRRF. We found antigen colocalizing with H2-M in both 10 min and 45 min activation time points (I, Fig 5e; S6d). This suggested that the pAg loading could occur in the same atypical endosomes.

5.1.6 Rab-protein responses to B cell activation

As we wanted to draw a more detailed picture of the antigen processing and peptide-antigen (pAg) loading in B cell activation, we decided to study later antigen processing phases in activated B cells. First, we studied the localization of Rab7 and Rab9, among other Rab proteins, in resting and activated A20D1.3 cells. The cells were processed for immunofluorescence stainings with different Rab-proteins and Microtubule Organisation Centre (MTOC) (I, Fig. S4a). We studied the distribution of the Rab proteins from the MTOC and found interesting differences between late endosomal Rab7 and Rab9. We studied the relationship between the Rab7+ / Rab9+ endosomes close to the MTOC and in the cell periphery (I, Fig.S4b), and we saw that Rab7 was clustered towards the MTOC after the activation while being more dispersed in the resting state. Rab9, on the other hand, remained dispersed under any of the studied conditions. (I, Fig. S4c). Rab9 had also more endosomes than Rab7 (I, Fig. S4d), and the Rab9 endosome count did not fluctuate during the activation, whereas Rab7 endosomes did (Fig. S4d). This could suggest that the Rab7 vesicles get fused during the B cell activation, depending on the antigen processing phase.

5.2 Rab7 and autophagosomes in late phases of B cell antigen processing (II)

Inspired by these differences in the localization and behavior of Rab7 and Rab9, we next wanted to examine their potentially differential roles in ag-processing. To understand in more detail the later phases of antigen processing in B cells, we next concentrated on late endosomal proteins Rab7 and Rab9. Utilizing sophisticated image analysis, live cell imaging, cognate B and T cell lines and B cells expressing mutant versions of Rab7/Rab9, we suggest Rab7 to have an important role in the antigen processing and peptide-antigen presentation in B cells. Interestingly, also autophagosomal machinery seem to associate with these processes.

5.2.1 Late endosomal Rab7 and Rab9 responses to B cell activation

Next, to gain an understanding of the potential differential localization of Rab7 and Rab9, with respect to antigen, we inspected them in MD4 mouse primary B cells. A super-resolution microscopic approach was required, as the endosomal compartment

is very small in primary mouse B cells, which is challenging their visualization. The cells were activated for 10, 20/30, or 60 min with α-IgM, processed for immunostaining with Rab7 or Rab9 and imaged with Structural Illumination Microscope (SIM). We detected quite similar, yet partial, accumulation of Rab7 and Rab9. (II, Fig. 1a-d, S1a,b) To support the finding we proceeded to live cell imaging. We transiently expressed both, green fluorescent protein (GFP)-fused Rab7a and mCherry-fused Rab9a in A20D1.3 cells. We followed resting or activated A20D1.3 cells with Spinning Disk Confocal Microscope to evaluate if they occupy the same endosomes. We noticed that Rab7 and Rab9 were largely expressed in the same compartments and the colocalization modestly decreased after the activation. (II, Fig. S1c,d; Movie1,2)

Following live cell imaging we proceeded to systematic immunofluorescence analysis on Rab7 and Rab9 localization and colocalization with proteins linked to the antigen processing. Here, the A20D1.3 B cell line was again used, as it allowed imaging with SDCM greatly facilitating the data collection as compared to SIM. Cells were either resting or activated for 10, 30, or 60 min before proceeding for immunofluorescence sample preparation and imaging with SDCM. While the colocalization between Rab7 and Rab9 was the highest in resting cells and the lowest 60 min after the activation, it was at quite a high level in all time points (II; Fig. 2a,b), in concordance with the live cell imaging data. After studying the correlation of Rab7 and Rab9 during the B cell activation, we were curious to characterize Rab7 and LAMP1, which are described to be present in peptide-antigen loading compartments (Pierre et al. 1996) and CatS responsible for MHCII maturation (Honey and Rudensky 2003). We found LAMP1 to colocalize with Rab7 and Rab9 on the same, high level in any of the conditions (II, Fig. 2c,d). Interestingly, Rab7 colocalization with CatS was constantly relatively high whereas Rab9 colocalization with CatS significantly increased upon activation (II, Fig. 2e,f). The findings led us to speculate that Rab7 and Rab9 would have at least partially different roles in antigen processing.

5.2.2 Rab7$^+$ vesicles are more active in antigen processing than Rab9$^+$ vesicles

We next examined if Rab7- and Rab9-enriched compartments would have different degradation capacities by linking degradation-sensitive probe DQ-Ova to HEL (described in the first publication, Fig. 4a), which A20D1.3 cells recognize as a specific antigen. We activated A20D1.3 cells with DQ-Ova-HEL for 60 min, proceeded with endogenous immunostaining with Rab7 or Rab9, and imaged the samples with SDCM. We found a partial colocalization throughout the cells (II, Fig. 3a). Interestingly, when we inspected the central clusters where the DQ-Ova-antigen

was gathered, we noted that some DQ-Ova signal appeared to be excluded from the Rab7+ /Rab9+ compartments (II, Fig. 3b). When we measured with Huygens analysis program DQ-Ova intensity in Rab7 and Rab9 positive endosomes, we found nearly three times higher DQ-Ova signal in Rab7 possessing endosomes (II, Fig. 3c). Also, the overlap coefficiency of DQ-Ova was significantly higher with Rab7 than Rab9 compartments (II, Fig. 3d). This data suggested that antigen degradation occurs at the higher extent in Rab7 positive endosomes than in Rab9 positive endosomes, although a significant fraction of the antigen also appeared to localize to a compartment lacking these late endosomal Rab markers.

5.2.3 Antigen is trafficked to the perinuclear clusters with LC3 and H2-M

Rab7 and Rab9 are reported to possess divergent roles in the endosomal pathway (Dong et al. 2013; Huotari and Helenius 2011; Kucera, Borg Distefano, et al. 2016; Lombardi et al. 1993; Mellman 1996). One of the special roles of Rab7 is in macroautophagosome maturation (Jäger et al. 2004). Macroautophagosomes (autophagosomes hereafter) degrade cargo in the cytosol (Kawabata and Yoshimori 2020) and on the other hand, autophagy is recently described in antigen presentation (Münz 2021), thus we speculated if Rab7 would participate in antigen processing through autophagosomes. We inspected the perinuclear region in activated A20D1.3 cells 75 min after the activation with Electron Microscope (EM). Among other endosome types, we often also found antigen in phagophore and autophagosome-like compartments. (II, Fig. 4g,h, S5)

Next, we continued immunofluorescence studies and stained activated A20D1.3 cells with Autophagosome-linked protein LC3 (II, Fig. 4a). We found antigen colocalization with LC3 to increase over time and a very high colocalization was detected in the antigen clusters in the perinuclear region (II, Fig. 4b-c). We yet inspected whether the perinuclear LC3-antigen gathering could induce pAg-presentation and as H2-M promotes pAg loading to the mature MHCII (Demharter et al. 2019; Jiang et al. 2019), we studied antigen colocalization with H2-M. To study this, we activated A20D1.3 cells with α-IgM for 10 or 60 min and proceeded for immunofluorescence stainings with H2-M (II, Fig. 4d). We analyzed the Mander's colocalization coefficiences with H2-M and antigen (II, Fig. 4f). Importantly and similarly to LC3, we saw a strong H2-M–antigen colocalization in the large antigen clusters in the cell perinuclear region (II, Fig. 4e) and thus we were able to suggest that autophagosomes might have an interesting role in antigen processing and pAg loading in B cells.

5.2.4 Rab7 may coordinate perinuclear gathering of antigen

To study if Rab7 or Rab9 would coordinate antigen trafficking to the perinuclear region, we expressed GFP-Rab7 or GFP-Rab9, with mutations generating constitutively active (CA) and dominant negative (DN) forms, in A20D1.3 cells. First, we analyzed the mutated cells for their capability to internalize antigen and saw normal antigen internalization (II, Fig S4b). Next, we activated the GFP-expressing cells with fluorescently tagged α-IgM, proceeded the cells for immunostaining, and stained the cells for Microtubule Organisation Centre (MTOC) (II; Fig. S2a). We analyzed antigen vesicles with the particle analysis tool on MATLAB and found that Rab7CA cells had a higher antigen vesicle count than the control cells which expressed only EGFP (II, Fig. S2c). Interestingly, Rab7CA expressing cells gathered antigen vesicles closer to the MTOC than control or Rab9CA expressing cells (II, Fig. S2b). Curiously, Rab7DN cells showed a similar, more clustered antigen gathering phenotype as Rab7CA but notably, Rab7DN expressing cells also had fewer antigen vesicles and the antigen vesicles were larger whereas the antigen in the Rab9CA expressing cells was otherwise comparable to Rab7CA cells (II, Fig. S2c,d). Also, antigen intensity was the same in all the GFP-expressing cells (II, Fig. S2e).

As some of the antigen stays trapped in small clusters on the cell membrane (reported in the first publication, Fig. S2) and various small peripheral vesicles also persist in the later time points after activation, the particle analysis is complicated and sometimes challenging to interpret. To gain a better understanding of the antigen positioning in the mutants, we decided to analyze the largest 10% of the antigen vesicles. This analysis revealed that Rab7CA expressing cells had the vesicles carrying the bulk of the antigen nearly 0.5 μm closer to the MTOC than control cells (II; Fig. S3a,b). When we plotted the antigen distance distribution, we saw that Rab7CA had a higher proportion of antigen vesicles within the radius of 1.5 μm from the MTOC as compared to the control or Rab9CA cells (II, Fig. S3c).

5.2.5 Rab7 regulates peptide antigen presentation

Finally, we wanted to investigate if Rab7 has a direct impact on pAg presentation. This can be conveniently studied utilizing cognate B and T cell lines such as A20D1.3 and 1E5 T cells. 1E5 T cells recognize a HEL^{-}peptide presented in the MHCII molecule (Adorini et al. 1993) into which A20D1.3 cells can load the processed peptide after HEL-activation (Williams et al. 1994). We tried to create A20D1.3 cell lines that would stably express GFP-Rab7 or GFP-Rab9 with their WT, CA and DN mutants. Unfortunately, we succeeded to generate only cell lines expressing constitutively active mutants of them. We tried to create A20D1.3 cell lines that would stably express GFP-Rab7 or GFP-Rab9 with their WT, CA and DN

mutants. Unfortunately, we succeeded to generate only cell lines expressing constitutively active mutants of them. We performed pAg presentation assay with GFP-Rab7CA, GFP-Rab9CA and GFP-LifeAct (control) expressing A20D1.3 and studied the IL-2 secretion of 1E5 T cells. We saw a significant enhancement in the IL-2 secretion, indicative of stronger pAg-presentation, in cells expressing Rab7CA compared to Rab9CA and control cells expressing LifeAct-GFP (II, Fig. 4a). The antigen internalization was normal with all the mutants when inspected by FACS (II, Fig. S4b).

We also utilized a reversible Rab7-specific inhibitor, CID1067700 (CID), which binds to the nucleotide-binding pocket of the Rab7-GTP and thus prevents Rab7 to switch into the active state (Agola et al. 2012; Lam et al. 2016). We again noted that the antigen internalization was normal with CID (II, Fig. S4c) and performed an IL-2 secretion assay. CID treatment drastically decreased the IL-2 secretion in T cells (II, Fig 4b). Together this suggested that T cell activation is regulated by Rab7.

To further analyze if the decreased IL-2 secretion in CID-inhibited A20D1.3 cells was correlated with the role of Rab7 for the classical endosomal maturation, or if it was linked to the macroautophagosome maturation and macroautophagosomes role in the pAg-presentation, we utilized Wortmannin inhibition. Wortmannin is widely reported as an inhibitor for macroautophagocytosis as it inhibits phosphatidylinositol-3-phosphate (PI(3)P), which is a specific lipid molecule for autophagosomal membrane formation (Obara et al. 2008) and macroautophagosome precursor formation (Axe et al. 2008). Again, we found that the antigen internalization was normal with Wortmannin inhibition (II, Fig. S4c), and again, the inhibited A20D1.3 cells induced significantly decreased IL-2 secretion (II, Fig. 4c).

Together the findings suggest that both, Rab7 and macroautophagosomes have essential roles in antigen processing and pAg-presentation in A20D1.3 cells.

6 Discussion

B cells are an essential part of the immune system by producing all antibodies and by creating immunological memory. Erroneous antibody production can cause several different autoimmune diseases, such as systemic lupus erythematosus (Rupanagudi et al. 2015), multiple sclerosis (Stroupe 2018) and rheumatoid arthritis (Karlsson 2005). In autoimmune diseases, B cells are producing inappropriate antibodies which attack the host's own system and cause constant inflammation with abnormal cell death.

Before B cells can proliferate and differentiate into antibody producing plasma cells or memory B cells, they have undergone several changes. The first, B cells have recognized and internalized antigen which they process into peptide antigens (pAg) in complex endosomal pathways. pAg:s are conjugated with Major Histocompatibility Complex class II (MHCII) and the pAg-MHCII complex is transported to the plasma membrane, to request T cell help. (Adler et al. 2017; Avalos and Ploegh 2014; Lankar et al. 2002; Unanue et al. 2016; Watts 2004; West, Lucocq, and Watts 1994) In this work, we provided a comprehensive picture of the antigen processing routes in B cells. We utilized a broad range of microscopic and analysis tools to map endosomal compartments and their properties, involved in antigen processing.

B cells not only need to process antigen but they also need to modulate MHCII for the pAg-MHCII presentation. MHCII is never roaming freely in the endosomal system but it is protected from wrong and too low-affinity antigen binding with the Invariant chain (Ii) shield. Thus, B cells need to process the Ii at the same time as the antigen. Ii is cleaved with CathepsinS in B cells, CatS clips Ii into CLIP, which is a short tail of Ii. CLIP will protect MHCII from the inappropriate antigen binding until DM releases CLIP and promotes pAg to bind in the MHCII antigen binding groove. (Adler et al. 2017; Driessen et al. 1999; Karlsson 2005; Vascotto et al. 2007) We reported MHCII with its associate proteases and proteins to localize in the same endosomes as the antigen that is destinated for internal antigen processing.

Notably, we propose that B cells have an endosomal fast track for antigen processing. This fast track is composed of early antigen processing compartments which we called eMIICs. We see acidic vesicles constantly roaming beneath the

plasma membrane, ready to capture the internalized antigen immediately. eMIICs contain all the needed components for both, antigen processing and pAg loading to MHCII molecules.

Helper T cells produce stimuli, IL-2 secretion when they recognize successful pAg-MHCII compartments on the B cell plasma membrane. The stimuli act as permission for proliferation and further activation for B cells. The magnitude of IL-2 secretion correlates with the specificity and strength of the pAg-MHCII bond (Cruse et al. 2004). We showed, for the first time, that by tuning Rab7 or macroautophagosome activity in B cells, pAg-MHCII presentation efficiency can be modulated.

6.1 Characteristics of MIICs

LAMP1 and MHCII localization have been described as characteristics of peptide antigen loading compartment (MIIC) in antigen presenting cells (Adler et al. 2017; West et al. 1994). Our findings were in consensus with the earlier literature as we also reported significant colocalization of LAMP1, MHCII and antigen in later activation time points (I, Fig.3d,e; I, Fig.S6f). As expected, LAMP1 had a high colocalization with late endosome associated Small GTPases Rab7 and Rab9 too (II, Fig. 2c,d). Thus, Rab7 and Rab9 can be guiding the antigen trafficking and processing in the MIICs, in B cells.

6.2 Atypical early endosomes

As we wanted to characterize B cell antigen trafficking and processing pathway in detail, we mapped the colocalization of internalized antigen with different endosomal proteins that are classical characteristics for certain endosomal compartments reported in other cell types. For instance, Rab5 and early endosomal antigen 1 (EEA1) are classified as markers for early endosomes and LAMP1, Rab7 and Rab9 are present in late endosomes or lysosomes, and Rab11 marks recycling endosomes. (Huotari and Helenius 2011; Wandinger-Ness and Zerial 2014). As early antigen processing phases in B cells are not studied extensively, we were curious to learn how antigen is trafficked and processed in B cells over time.

Thus, we activated B cells with fluorescently labeled antigen, let B cells traffic and process antigen at different times and proceeded for immunofluorescence staining with the above-mentioned proteins. By this, we were able to map the antigen trafficking and processing pathway over time in B cells.

We saw that already 5 min after the activation, the antigen displayed a significant colocalization with early endosomal Rab5 and recycling Rab11 (I, Fig. 2c). The interesting finding of Rab11 acting in early receptor trafficking steps was previously

reported by Redpath. Redpath et al reported that endocytosed T cell receptors (TCRs) enter immediately after the internalization into Rab11a+ compartments which also possess Rab5. They found TCRs inside Rab11a positive endosomes within 3 min after the activation, and the same TCRs were found again back on the plasma membrane 3 min later. In B cells, BCR internalization and trafficking are not fully understood and it is yet under debate if some of the BCRs are recycled back to the plasma membrane and only part of them would end up in degradation after activation. Thus, in the early activation phases, Rab11 could function in BCR recycling in B cells too, as the total in-and-out –TCR trafficking time was only 6 min in T cells. (Redpath et al. 2019)

5 min after the activation, the antigen showed a significant colocalization with late endosomal Rab9 and Rab7 in peripheral antigen-containing endosomes, as well. This finding led us to speculate that peripheral antigen-containing endosomes would possess both, early and late endosomal characteristics. On the other hand, antigen internalization through an immunological synapse (IS) also recruits late endosomal compartments to the synapse site. LEs/LYSs in the IS site help B cell internalizes antigen as they secrete lysosomal content to the IS (Sáez et al. 2019; Yuseff et al. 2011). In the presented studies, the importance of microtubule organization center (MTOC) reorganization towards the IS, was highlighted. As described, microtubules would function as a route for lysosomes to traffic to the cell periphery. Nevertheless, in soluble activation MTOC reorganization is not reported as the antigenic stimulus is received homogenously on the plasma membrane. Notably, Rab7 positive endosomes can yet be trafficked bi-directionally: towards and outward the MTOC (Cai et al. 2021; Pankiv et al. 2010). Thus, Rab7 could be recruited and trafficked to the cell periphery to help in antigen internalization, also when activated with soluble antigens. It would be highly interesting to study if similar lysosomal exocytosis also occurs after soluble activation.

When we studied the colocalization of late and early endosomal markers (Rab7/Rab9/LAMP1 together with Rab5/EEA1) shortly after the activation, we did indeed saw them to be located in the same endsomes (I, Fig. 3f,g; I, Fig S6b). We also studied the colocalization of lysosomal and early endosomal markers in living cells, by visualising EEs with Rab5-GFP expression and LEs/LYSs with LysoTracker (LT). We indeed saw Rab5-GFP positive vesicles positive for LT which suggest that early endosomes in B cells can also have lysosomal activity as LT is fluorescent only in acidic organelles (I, Fig. S6c). Typically, $Rab5^+$ vesicles are converted into $Rab7^+$ at the same time as the early endosome matures into more acidic LE or LYS. Classically, Rab5 and Rab7 are seen in the same endosomes only transiently (Rink et al. 2005; Vonderheit and Helenius 2005). Notably, also EEA1 is not typically reported to colocalize with late endosomal Rab7 (Vonderheit and Helenius 2005). Yet, new insight on EE to LE maturation has been reported by

Skjeldal et al. They suggest that EEs are formed *de novo* from a maturing endosome. In their model, Rab5 is polarised on one side of the maturing endosome at the same time as Rab7 conquers larger membrane proportion on the endosome. Finally, the fully polarised Rab5 gathering is released from the endosome membrane, and it forms a new early endosome after the releasement. In the *de novo* -model, early and late endosomal proteins can localize in the same endosome for up to 10 min while the whole maturing time would be approximately 25 min. (Skjeldal et al. 2021) Thus, based on the *de novo* –theory, the endosomes possessing both early and late endosomal characteristics in B cells can also refer to slowly maturing endosomes. Yet, when reflected to the common literature, our findings suggest that B cells are possessing atypical endosomes, containing both early and late endosomal characteristic.

6.3 Novel antigen degradation compartments, eMIICs

We further characterized the atypical early endosomes for antigen degradation capability. We stimulated Rab5–LT expressing B cells (described above) with antigen and followed antigen trafficking in living cells. We saw indeed that Rab5-LT endosomes trafficked also antigen shortly after the activation (I, Fig. 3h, movie 1). When we followed antigen internalization, we were able to show that antigen was trafficked to LT+ endosomes immediately after the internalization (I, 4e,f; I, movies 2-3).

We further demonstrated that eMIICs can possess degradative nature, by analyzing DQ-Ova signaling in the activated and resting cells, with a flow cytometer (I, Fig. 4b) and with a microscope (I, Fig. 4c). DQ-Ova is a tightly packed probe that is fluorescent only when the compound is degraded into short moieties. While B cells subjected to DQ-Ova alone did not significantly degrade DQ-Ova, DQ-Ova conjugated with antigen, was efficiently degraded. We further showed that antigen ends in acidic organelles in B cells already 5 min after the activation by studying fluorescently unstable antigen and its diminishment with a flow cytometer (I, Fig. S6e). Together these findings suggest that antigen processing begins already in the atypical early endosomes which we decided to call early peptide antigen loading compartments, eMIICs.

We also saw clear punct antigen colocalization with macroautophagosomal (hereafter autophagosome) marker LC3, in eMIICs (II, Fig. 4a). LC3 localization in early endosomal compartments is expected to some extent as autophagosomes mature also gradually. They have early, late and lysosomal phases. In the early autophagosome phases, Rab5 is having an important role as it guides autophagosome maturation by recruiting lysosomal Rab7 to the compartment. Rab5 functions in the

phagophore closure too (M. Chen et al. 2014; Jäger et al. 2004; Zhou et al. 2017). Interestingly, autophagy has been recently reported as a new player to guide early events in B cell activation too. BCR activation is described to switch autophagocytosis on (Arbogast et al. 2019; Martinez-Martin et al. 2017). When we inspected electron microscope images from 15 min after activation (I, S1a), we were able to conclude that antigen was also found in doughnut-shaped endosomes that could resemble autophagosomes. Interestingly, also according to Martinez-Martin, the accumulation of autophagosomal proteins in B cells produces doughnut-shaped endosomes which are filled with internalized antigen (Martinez-Martin et al. 2017). Taking all together, we suggest that autophagosomes or autophagosome-related processes can have a role in early antigen processing phases.

6.4 MHCII maturation in eMIICs

We reported MHCII to localize in eMIICs (Fig. S6f). Moreover, we reported MHCII to be internalized together with the antigen from the plasma membrane (I, Fig. 5a-d). The simultaneous internalization of antigen and MHCII could lead to rapid pAg loading to the MHCII complex. Nevertheless, as the immature MHCII cannot bind with a pAg as such, we also studied proteins involved in the maturation of MHCII.

When MHCII is released from the endoplasmic reticulum it is immediately shielded with the Invariant chain (Ii). As it trafficks through Golgi, the Ii stays tightly on the molecule. (Roche and Furuta 2015) For pAg loading, Ii in immature MHCII molecule needs to be cut into a short Ii tail called CLIP. Ii clipping is done by CatS in B cells (Guo-Ping et al. 1999). Thus, we studied CatS localization in activated B cells, and we found it indeed to localize in the eMIICs (I, Fig.4d). This finding suggests that MHCII maturation can occur also in eMIICs.

When we studied CatS together with Rab7 and Rab9, we found it to colocalize strongly with both of the late endosomal markers (II, Fig.2f). Interestingly, only Rab7 showed a strong colocalization with CatS in resting cells, suggesting that Rab7 and CatS function constantly together in B cells whereas Rab9 would be recruited into the MIICs after the activation. Notably indeed, Rab9 has been reported as one of the proteins guiding MHCII-Ii trafficking from Golgi to endosomes (Kucera, Bakke, and Progida 2016). Thus, the sudden Rab9 appearance in MIICs after activation (II, Fig. 2f), can also hint at the duty of Rab9 in MHCII transportation.

We also studied DM expression in eMIICs. DM is a catalytic protein for CLIP replacement with pAg. As DM was also present in the eMIICs (I, Fig. 5e), we concluded that pAg loading to the mature MHCII molecules can also occur in the eMIICs.

We tried to create different functional experiments to stop antigen processing after different activation time points to further show degradation capacity in eMIICs

only. For instance, we tried several inhibitors and endosome ablation to target later endosomal pathway, but as inhibitors often target several sites, definitive conclusions were difficult to make. Nevertheless, taken together our results suggest that eMIICs possess all key components for antigen processing and pAg presentation in B cells. eMIICs may serve as an alternative fast track for antigen processing and pAg-MHCII loading in B cells, in addition to classical route via MIICs.

6.5 Structural changes in the late activation phases in B cells

We continued our studies on antigen processing in later phases and noted that the nucleus reforms to form a nuclear pocket possessing several different types of endosomes after the activation. The reason for nuclear deformation after the activation is not understood. Yet, fresh findings in our group have reported several nuclear proteins to be enriched in the vicinity of the plasma membrane after B cell activation (Awoniyi et al., submitted) and thus, nuclear reformation might serve a specific function and not only form due to the restricted cytoplasmic area in B cells.

The internalized antigen reaches the perinuclear region within an hour (I, Fig. 1a-e; II, Fig. S1a) and it is found in several types of endosomes (I, Fig. 1f). Antigen trafficking to a perinuclear region is often reported in B cells (Barroso et al. 2015; Pierre et al. 1996) and it can be seen as a typical phenomenon when B cells are activated with soluble antigens.

6.6 Late antigen processing phases and autophagosomes

We studied late antigen processing phases using the same endosomal markers as we used to study eMIICs. We saw increased colocalization with recycling marker Rab11 and late endosomal markers, Rab7 and Rab9, while the colocalization with early endosomal marker Rab5 was modest. (I, Fig. 2e). Nevertheless, we did have some challenges in imaging the perinuclear region, as the endosomes are extremely small and tightly organized. The difficulties in gaining high enough resolution caused challenges to determine the correct colocalization. We decided to utilize image post-processing with deconvolution or super-resolution radial fluctuation (SRRF), which led us to the conclusion that all the proteins present in eMIICs were also present in the perinuclear region. Despite we studied colocalizations from deconvoluted high-resolution images, we yet had moderate resolution. EM imaging revealed that the antigen-containing endosomes were typically 120–300 nm in diameter (I, Fig. 1f, S1a,c) being thus below the diffraction limit of light. Also, the SRRF imaging method revealed that the colocalization of the antigen with any of the studied

markers was decreased when the image resolution was increased (I, Fig. 2c vs. Fig 2e). This can lead to a situation where the real colocalization is lower than reported. Nevertheless, as we were especially interested in comparing colocalization in different time points and between different proteins, the possible increase in the colocalization would not change the conclusion that antigen colocalization with endosomal markers increases over time. The greatest increase in the colocalization was with autophagosomes (II, Fig. 3b), and as autophagocytosis is classically described as a way to clear useless material, the finding was surprising. Yet, Schmid et al. have described that LC3 decorates multivesicular compartments with MHCII and LAMP2, in dendritic cells. Schmid et al. reported that when antigens were targeted to autophagic degradation in dendritic cells, the dendritic cells induced enhanced T cell activation. (Schmid D 2007). Thus, we further speculated that autophagosomes can have a special role in antigen processing and pAg loading in B cells too.

We were yet curious to understand if autophagosomes are essential for antigen processing and pAg presentation in B cells. Thus, we decided to inhibit macroautophagosome formation with Wortmannin. Wortmannin is widely reported as an inhibitor for autophagocytosis as it inhibits Phosphatidylinositol-3-phosphate (PI(3)P) formation through PI3K (Arcaro and Wymann 1993). PI(3)P is on a specific lipid molecule for autophagosomal membranes (Obara et al. 2008) and it is essential for omegasome (precursor of autophagosome) formation (Axe et al. 2008). One should yet note, that Wortmannin inhibits the whole PI3K/Akt pathway, which on the other hand, controls some of the key aspects of B cell differentiation, for instance. Thus, as Akt has a complex role in B cells, inhibition of the Akt pathway may enhance some B cell responses while it silences others (Limon and Fruman 2012). Nevertheless, B cell inhibition with Wortmannin did indeed decrease pAg-presentation measured by IL-2 secretion by cognate T cells, in half. Thus, we conclude that autophagocytosis is one of the key players in antigen processing and pAg-presentation.

Autophagosomes can possess several different roles in B cell maturation and antigen processing. For instance, ATG5 seems to be essential for efficient antigen internalization from antigen presenting surfaces, yet soluble antigen internalization is independent on ATG5. On the other hand, ATG5 is suggested to guide late endosome trafficking toward PCM1 (Arbogast et al. 2019). PCM1 localizes in the perinuclear region in the later activation phases (I, Fig. 1), and in the synapse site when the antigen is presented from an antigen presenting membrane (Yuseff et al. 2011). Others have also reported that autophagosomes constantly deliver cytosolic and nuclear antigens onto MIICs and autophagosomes fuse with MIICs frequently in dendritic cells too (Schmid D 2007). Thus, despite we were able to suggest that autophagosomes have an important role in the antigen processing pathway, more

studies are needed to find a conclusion if the autophagosomal role is to regulate the classical endosomal pathway or if autophagosomes can also independently process antigens and load them onto MHCII.

6.7 Rab7 in B cell antigen processing and pAg presentation

Typically, late endosomes and lysosomes are more numerous in the perinuclear area whereas early endosomes are dispersed in the cell periphery (Huotari and Helenius 2011; Zhao et al. 2021; Zhao and Zhang 2019). Despite Rab7 and Rab9 being both described as late endosomal proteins, they had different patterns in the B cells. We saw Rab9 being more dispersed than Rab7, especially in the late activation phases (I, Fig. S4c; II, Fig. S2-S3). When we compared Rab7 and Rab9 in more detail, we learned that Rab7 vesicles degrade antigen more efficiently than Rab9 vesicles (II, Fig. 2). The more efficient degradation in $Rab7^+$ endosomes can be caused by lower intraluminal pH, higher presence of other degrading components in the endosome, or by being expressed in different organelles. To support the last-mentioned option, we did see that the Rab7-Rab9 colocalization was decreased significantly in the late phases of antigen processing (II, Fig. 1b) thus pointing toward partially different roles in antigen processing and pAg-loading in B cells.

To study in more detail the partially different roles of Rab7 and Rab9, we created B cell lines transiently expressing GFP-Rab7 or GFP-Rab9 with their wild type (WT), constitutively active (CA) and dominant negative (DN) mutants. By studying antigen trafficking in the mutants with particle analysis tools, we saw that Rab7CA expressing cells had internalized antigen vesicles closest to the MTOC after an hour, whereas GFP-Rab7DN and GFP-Rab7WT cells with all the Rab9 mutants had antigen as dispersed as the control cells (II, Fig.S2-S3). Thus, we postulated that Rab7 guides antigen trafficking to the perinuclear region.

When we took stable, and equally bright GFP-Rab7CA and GFP-Rab9CA expressing B cells under inspections, we found Rab7CA expressing cells induced clearly stronger pAg-presentation (II, Fig. S4a). Unfortunately, despite several trials, we were not able to create cell lines that would have expressed DN mutants (or WT) of Rab7 or Rab9. Interestingly, Rab9 is reported to promote alternative autophagocytosis which can create autophagosomes and autolysosomes without association with the core autophagosomal machinery (Urbańska and Orzechowski 2021). Despite Rab9 being an essential protein in alternative autophagocytosis, it is not needed in canonical autophagocytosis (Nishida et al. 2009). Thus, it is exciting to speculate if Rab7CA and Rab9CA differentially promote autophagosomal pathways and thus, the cells express different pAg-presentation levels too.

Further, we utilized a novel Rab7-specific inhibitor, CID106770 (Agola et al. 2012; Lam et al. 2016) to inhibit Rab7 activity in B cells. We saw a drastic decrease in pAg-presentation in Rab7 inhibited B cells (II, Fig. S4b). As the IL-2 secretion in Rab7 inhibited cells was approximately half of the IL-2 secretion in autophagosome inhibited cells, we concluded that Rab7 has several roles in B cell antigen processing and presentation, and is not important only for autophagosome maturation. Together, our findings strongly suggest that Rab7 plays an essential role in B cell antigen processing and presentation, with and without macroautophagosomes.

7 Conclusions

With expertise in immunology, a good understanding of high and super-resolution imaging and curiosity to combine these two fields, we decided to tackle the long-standing question of antigen processing pathways in B cells. In this thesis, we have been able to draw a comprehensive picture of the antigen processing pathways (illustrated in **Figure 11**) in B cells combining molecular biology, immunology and bioimaging.

7.1 Rapid antigen processing in the B cell periphery (I)

In this study we show, for the first time, a comprehensive map of endosomal compartments involved in antigen processing in B cells. We show antigen to traffic through early and late endosomes, and to associate with markers for recycling endosomes too. We noted cell activation to lead to nuclear deformation and antigen gathering to a nuclear pocket. We reported several endosomal proteins, especially late endosomal proteins, to occupy the perinuclear region, with the antigen. The colocalization of internalized antigen with late endosomal markers increased in the perinuclear peptide antigen loading compartments, MIICs over time. Interestingly, we found similar compartments also in the cell periphery waiting for the antigen to be internalized. At the same time, the peripheral MIICs were positive also for early endosomal proteins and thus, we named them as early MIICs, eMIICs.

We showed, with colocalization analysis and live cell imaging eMIICs to contain the key proteins and molecules needed for successful antigen processing and peptide-antigen loading to MHCII. Notably, we suggest that MHCII molecules for the peptide antigen loading could originate also from the plasma membrane, at the same time as the antigen is internalized. This could facilitate rapid pAg presentation and, perhaps, tune the peptide repertoire loaded on the MHCII.

7.2 Rab7 and autophagosomes in late phases of B cell antigen processing (II)

In this study, we inspected MIICs in detail. We concentrated especially on late endosomal proteins Rab7 and Rab9. We show that in the later activation phases Rab7 and Rab9 are partially in distinct compartments in B cells, and moreover, they seem to have divergent roles in B cell antigen processing. We show $Rab7^+$ vesicles to possess more efficient degradation activity and capacity than $Rab9^+$ vesicles. Interestingly, T cell responses were modulated via Rab7. Expressing constitutively active Rab7 in B cells, we were able to induce a stronger T cell response than with the corresponding Rab9 CA expressing B cells, or with the control cells. Notably, when we inhibited Rab7 activity in B cells, we induced drastically impaired T cell response too.

We also propose another, alternative pathway for antigen processing in B cells. We report antigen to localize in macroautophagosomal structures too, and to have very high colocalization with a macroautophagosomal marker LC3. Notably, LC3 localizes in the same perinuclear region as H2-M. H2-M on the other hand facilitates peptide antigen loading for MHCII presentation. Interestingly, inhibition of autophagosomes with Wortmannin induces impaired T cell response. Thus we suggest that both Rab7 and macroautophagy have important roles in antigen processing and peptide antigen presentation in B cells.

As both macroautophagocytosis and Rab7 can be targeted with inhibitors or activators (Agola et al. 2012; Lam et al. 2016; Thellung et al. 2019), the findings reported in this thesis may lead to further therapeutical investigations. By modulating the efficiency of peptide-antigen presentation in B cells, T cell-mediated immune responses can be tuned. Here, we propose Rab7 and macroautophagocytosis as two novel candidates that targeting could potentially allow to modulate immune responses.

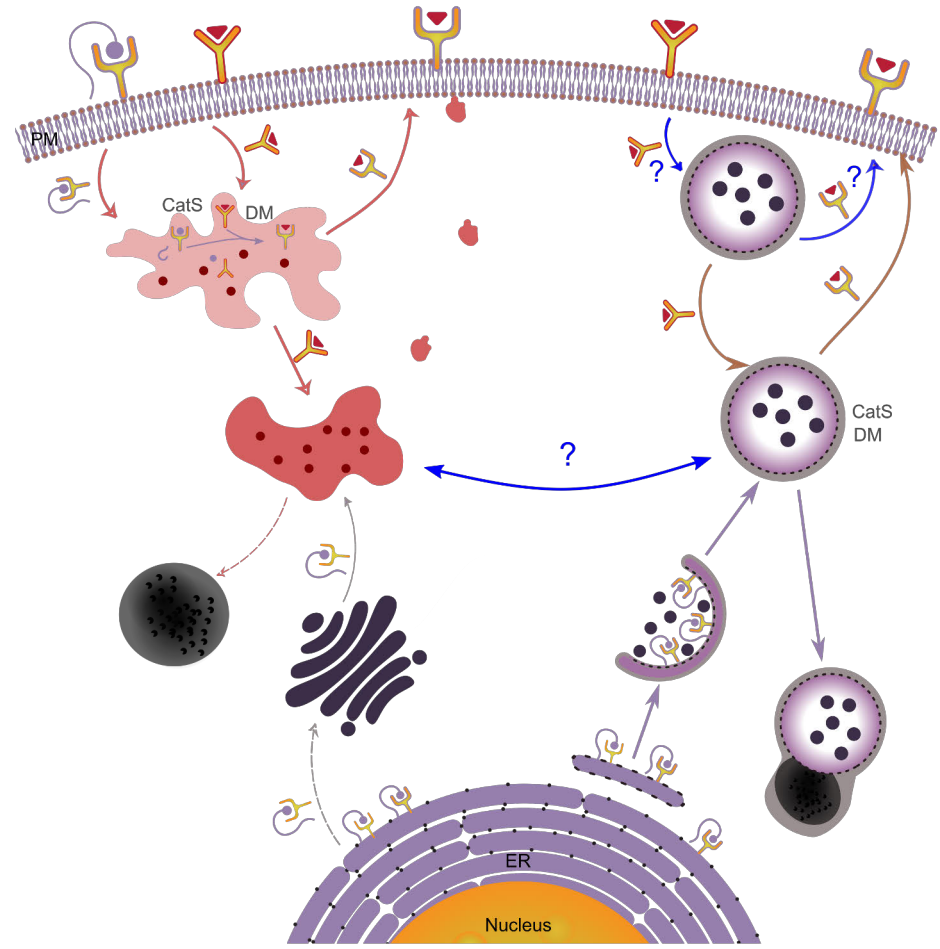

Figure 11 The antigen processing pathways proposed in this study. The path illustrated on the left side suggests an interesting role for early endosomal peptide antigen (pAg) loading compartments, eMIICs. eMIICs possess many key components for ag processing and pAg-loading onto immature Major histocompatibility complex II (Ii-MHCII). From eMIICs, pAg-MHCII can be trafficked fast to the plasma membrane (PM). In B cells, eMIICs mature into MIICs, from where pAg-MHCII can be trafficked to the PM with recycling endosomes (RE). The other pathway takes an advantage of (macro)autophagosomes (AP), which are constantly expressing autophagosomal protein LC3 (black dotted lines). Interestingly, APs have naturally Ii-MHCII on their membranes, as their precursors, omegasomes (Ω), are formed from the membrane of the endoplasmic reticulum (ER). Ω-matures into phagophores (PP) which may gather key components, such as CatS and DM, for the ag processing and pAg-MHCII. Both pathways may end up in the final degradation in either lysosomes (LYS) or autolysosomes (AL). Yet, it remains to be studied whether these two pathways co-operate or if their activities could be separately modulated.

Acknowledgements

This work was carried out at the University of Turku and Turku Biosciences. I am grateful that I have had the opportunity to become a member of the Medical Faculty and moreover, a member of Turku Biomedical Imaging team. I would like to thank my mentors in Biomedical imaging, Pekka Hänninen and John Eriksson: you introduced me to the fascinating world of imaging which never ceases to amaze me.

Moreover, I want to express my gratitude to the Central Imaging Core and especially to Markku Saari, Jouko Sandholm, and Ketlin Adele. You were always ready to help, to brainstorm, and you encouraged me to keep on going when obstacles seemed too high.

Dear emeritus professor Ari Helenius, I wish every young doctoral student could hear your motivational speech. You indeed inspired me with your wisdom, intelligence and experience. I will never forget your kindness and warmth.

Dear professor Johanna Ivaska, you are an exceptional scientist. You are one of the most respected scientists in Finland and yet, you always have time to confront young scientists with kind words and positivity. Dear follow-up committee members, I feel extremely lucky that I was able to share this journey with you.

I am forever grateful that associate professor Jane Zhi Chen accepted the invitation to be the opponent at the dissertation, thank you!

Dear professor Julia Jellusova and docent Annika Meinander, thank you for your help in improving the thesis!

I am grateful that I have had the privilege to work with the best research group in Turku! Dear group leader Pieta Mattila, you have succeeded in finding colorful B cell people, with a variety of expertise. You have created a solid research team, which is an amazing achievement! Congratulations! I want to thank all the previous and current colleagues in the Lymphocyte Cytoskeleton Group. Especial thanks I appoint to my previous supervisor doctor Elina Kuokkanen. Thank you for your kindness, guidance and words of wisdom.

I want to thank Turku Doctoral Programme of Molecular Medicine (TuDMM) for the financial support and help. Thank you, Eeva Valve and Outi Irjala for helping in the doctoral studies – from the very first day until the last one.

I want to thank my previous colleagues in the department of microbiology. The research we conducted years back was interesting, inspiring, multi-dimensional and novel. Catharina Alam, Raine Toivonen and Arno Hänninen, with you, I spent the first years of my scientific career. And to be honest, you are the triplet that drew me into biomedical science. Thank you!

I want to thank my previous colleagues in the DIPP/TEDDY/TrialNet research too. Family Simell, thank you for showing me how lovely, yet one of the world's leading Type 1 diabetes research communities is created with respect, appreciation and kindness. I will never forget the atmosphere you created. Thank you!

I want to thank my student fellows in Biomedical Imaging. Dudes, I will always remember you with warmth! Thank you for the happy days! I want to thank especially Henok Karvonen: thank you for the glorious master's years and thank you for your company, help and support during my doctoral studies too. I hope I do not have to ever miss you.

I want to thank all the fellow candidates whom I shared enjoyable days with, and had inspiring scientific and not-so-scientific discussions. I am also grateful for the never-ending source of novel reagents which our group had not yet purchased. These thanks go especially to Pranshu Sahgal and Martina Lerche!

I want to thank our working community at PerkinElmer Wallac Oy. I am extremely happy to work with you. You have encouraged me when combining these two jobs have felt like a mission impossible, and you have offered me another cup of coffee when you have seen that it is more than needed. You are exceptional team players and a great bunch of good friends!

Last but not least, I thank my family. During these years, we have climbed several mountains and the journey has not been easy. Mika, you have been walking beside me from the beginning and you are walking beside me in the end as well. It tells about true commitment and love. Thank you! I am also so blessed to have three kids, Jooa, Verneri and Ronja, who are made of steel but yet, have rousing hearts. We have been counting for the days when I can give you all the time in the world. You have been waiting for so long, with such resilience and patience, and I am happy to say: wait no longer!

22nd August 2022
Marika Runsala

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2022

ISBN 978-951-29-8965-2 (Print)
ISBN 978-951-29-8966-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)