

**Studies on the Relation between  
Antigen Presentation and Mycobacterial Trafficking  
and on the Importance of Coronin 1  
in Mycobacterial Pathogenesis,  
Neutrophil- and B Lymphocyte Functions**

**Inauguraldissertation**

zur

Erlangung der Würde eines Doktors der Philosophie  
vorgelegt der  
Philosophisch-Naturwissenschaftlichen Fakultät  
der Universität Basel

von

Benoit Martial Combaluzier  
aus Saint Marcel d'Ardèche, Frankreich

Basel, 2008



Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof. Jean Pieters und Prof. Daniela Finke.

Basel, den 20. Mai 2008

Prof. Hans-Peter Hauri

The work described in this thesis has been performed from October 2004 to February 2008 at the Biozentrum, Department of Biochemistry, University of Basel, Switzerland, in the laboratory of Prof. Jean Pieters.

Parts of this thesis are described in the following manuscripts:

Inhibition of Phagosome Maturation by Mycobacteria does not Interfere with Presentation of Mycobacterial Antigens by MHC Molecules (The Journal of Immunology, 2007 August 1; 179(3):1825-33)

Laleh Majlessi\*, Benoit Combaluzier\*, Imke Albrecht, Jessica E. Garcia, Clemence Nouze, Jean Pieters and Claude Leclerc.

Survival of Mycobacteria in Macrophages is Mediated by Coronin 1 Dependent Activation of Calcineurin (Cell, 2007 July 13; 130(1):37-50)

Rajesh Jayachandran\*, Varadharajan Sundaramurthy\*, Benoit Combaluzier, Philipp Mueller, Hannelie Korf, Kris Huygen, Imke Albrecht, Jan Massner and Jean Pieters

Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-triphosphate and calcium mobilization after T cell receptor triggering (Nature Immunology, 2008 April; 9(4):424-31)

Philipp Müller\*, Jan Massner\*, Rajesh Jayachandran\*, Benoit Combaluzier, Imke Albrecht, John Gatfield, Carmen Blum, Rod Ceredig, Hans-Reimer Rodewald, Antonius Rolink and Jean Pieters

Chemotaxis and Phagocytosis in Neutrophils is Independent of Coronin 1 (manuscript in preparation)

Benoit Combaluzier and Jean Pieters

Coronin 1 is Essential for IgM Mediated Ca<sup>2+</sup> Mobilization in B Cells but Dispensable for *in vivo* B Cell Function (manuscript in preparation)

Benoit Combaluzier, Jan Massner, Daniela Finke and Jean Pieters

\* equal contributors

# TABLE OF CONTENTS

<b>1 INTRODUCTION</b>	<b>1</b>
<b>1.1 Overview of the immune system</b>	<b>1</b>
1.1.1 Cells and organs of the immune system	1
1.1.1.1 Cells of the immune system	1
1.1.1.1.1 Mononuclear phagocytes	3
1.1.1.1.2 Granulocytes	4
1.1.1.1.3 Lymphoid cells	4
1.1.1.1.4 Dendritic cells	5
1.1.1.2 Organs of the immune system	5
1.1.1.2.1 Primary lymphoid organs	5
1.1.1.2.2 Lymphatic system	6
1.1.1.2.3 Secondary lymphoid organs	6
1.1.2 Innate immunity and Toll-Like Receptors	7
1.1.3 Acquired immunity	10
<b>1.2 Antigen processing and presentation</b>	<b>11</b>
1.2.1 Antigen Presenting Cells	12
1.2.2 Antigen processing and presentation	13
1.2.2.1 Antigen processing and presentation on MHC class I	13
1.2.2.2 Antigen processing and presentation on MHC class II	14
<b>1.3 Mycobacteria and the host immune system</b>	<b>16</b>
1.3.1 Mycobacteria species	16
1.3.2 Mycobacteria and phagocytic cells	16
1.3.3 Pathogenic mycobacteria and intracellular survival	17
1.3.3.1 Mycobacterial virulence factors	17
1.3.3.2 Mycobacteria and host cell components	18
<b>1.4 Polymorphonuclear Leukocytes</b>	<b>19</b>
1.4.1 Immune response launched by neutrophils	19
1.4.2 Chemotaxis of neutrophils	21
1.4.3 Killing of microbes by neutrophils	21
1.4.4 Neutrophils and mycobacteria	22
<b>1.5 B lymphocytes</b>	<b>23</b>
1.5.1 B Cell Receptor (BCR) complex and signaling	23
1.5.2 Development and functions of B cells	25
<b>1.6 The coronin proteins</b>	<b>27</b>
1.6.1 Coronin in <i>Dictyostelium</i> and yeast	27

1.6.2	Coronins in mammals	28
1.6.2.1	The coronin family	28
1.6.2.2	Expression and functions of coronins in mammalian cells	29
1.6.2.3	Role of coronin 1 in leukocytes	30
<b>1.7</b>	<b>Aim of the thesis</b>	<b>32</b>
<b>2</b>	<b><i>MATERIALS AND METHODS</i></b>	<b>34</b>
<b>2.1</b>	<b>Chemicals and reagents</b>	<b>34</b>
<b>2.2</b>	<b>Buffers and solutions</b>	<b>37</b>
<b>2.3</b>	<b>Media and supplements</b>	<b>39</b>
<b>2.4</b>	<b>Oligonucleotides</b>	<b>40</b>
<b>2.5</b>	<b>Antibodies and dyes</b>	<b>41</b>
<b>2.6</b>	<b>Mycobacteria and mammalian cells</b>	<b>45</b>
<b>2.7</b>	<b>Cell culture methods</b>	<b>46</b>
2.7.1	Determination of cell numbers	46
2.7.2	Freezing and thawing of cells	46
2.7.3	Preparation of L929 medium	46
2.7.4	Preparation of anti-CD4 and anti-CD8 cell supernatant	47
2.7.5	Mycoplasma detection	47
<b>2.8</b>	<b>Animal experiments</b>	<b>48</b>
2.8.1	Genotyping of coronin 1 $-/-$ mice	48
2.8.2	Preparation of bone marrow derived macrophages	49
2.8.3	Preparation of bone marrow derived dendritic cells	49
2.8.4	Purification of splenic B cells by T cell depletion	50
2.8.5	Isolation of peripheral neutrophils	50
2.8.6	Isolation of bone marrow neutrophils	51
2.8.7	Blood collection and serum preparation	51
2.8.8	Thymus independent immunization of mice	51
2.8.9	Thymus dependent immunization of mice	52
2.8.10	Leukocyte recruitment into the peritoneum	52
2.8.11	Analysis of peripheral blood composition	52
<b>2.9</b>	<b>Flow cytometry</b>	<b>53</b>
2.9.1	Staining of cells for FACS analysis	53
2.9.2	Determination of phagocytosis efficiency of neutrophils	53
2.9.3	Analysis of NADPH oxidase activity	53
2.9.4	Calcium measurements	54

<b>2.10</b>	<b>Microscopy</b>	<b>54</b>
2.10.1	Staining of leukocytes for immunofluorescence analysis	54
2.10.2	Mycobacterial infection and confocal laser scanning microscopy	55
2.10.3	Analysis of the neutrophil and B cell cytoskeleton by immunofluorescence	56
2.10.4	Acid fast staining of mycobacteria	56
2.10.5	Analysis of neutrophil spreading	57
2.10.6	Opsonization of beads and phagocytosis by neutrophils	57
2.10.7	Immunohistology	57
2.10.8	Coating of the slides	58
<b>2.11</b>	<b>Biochemical techniques</b>	<b>58</b>
2.11.1	Preparation of cell lysates	58
2.11.2	Determination of protein concentration	58
2.11.3	Discontinuous SDS polyacrylamide gel electrophoresis.	59
2.11.4	Western blot analysis	60
2.11.4.1	Semi-dry protein transfer onto nitrocellulose membranes	60
2.11.4.2	Immunodetection	60
2.11.4.3	Stripping of membranes for reprobing	61
2.11.5	Separation of organelles by density gradient electrophoresis	61
2.11.6	Macrophage stimulation and lysate preparation	62
2.11.7	Adhesion assay	63
2.11.8	Transwell migration assay	63
2.11.9	Enzyme-Linked ImmunoSorbent Assay	63
2.11.10	B cell proliferation	64
<b>3</b>	<b>RESULTS</b>	<b>65</b>
<b>3.1</b>	<b>Phagosome maturation and mycobacterial peptide presentation</b>	<b>65</b>
3.1.1	Influence of protein kinase G on intracellular trafficking of mycobacteria in professional Antigen Presenting Cells	66
3.1.2	Presentation of mycobacterial antigens upon infection with wild type <i>M. bovis</i> BCG or <i>M. bovis</i> BCG $\Delta$ pkng	71
<b>3.2</b>	<b>Activation of signaling pathways and mycobacteria trafficking in coronin 1 deficient macrophages</b>	<b>75</b>
3.2.1	Activation of signaling pathways upon recognition of pathogen-associated molecular patterns in the absence of coronin 1	76
3.2.2	Activation of signaling pathways upon encounter with mycobacteria in the absence of coronin 1	79
3.2.3	Mycobacterial trafficking in coronin 1 $-/-$ macrophages	81
<b>3.3</b>	<b>Coronin 1 and polymorphonuclear leukocytes functions</b>	<b>87</b>
3.3.1	Neutrophil morphology and F-actin distribution in the presence and absence of coronin 1	88
3.3.2	Adhesion and spreading of neutrophils in the absence and presence of coronin 1	90

3.3.3	Phagocytosis of neutrophils lacking coronin 1	92
3.3.4	Oxidase activity of coronin 1 <sup>-/-</sup> neutrophils	95
3.3.5	Chemotaxis of neutrophils in the presence and absence of coronin 1	97
<b>3.4</b>	<b>Role of Coronin 1 in B lymphocytes functions</b>	<b>99</b>
3.4.1	Immune responses in mice lacking coronin 1	100
3.4.2	B cell proliferation in the presence and absence of coronin 1	104
3.4.3	B cell signaling in the presence and absence of coronin 1	108
<b>4</b>	<b><i>DISCUSSION</i></b>	<b>111</b>
4.1	<b>Inhibition of phagosome maturation by mycobacteria does not interfere with presentation of mycobacterial antigens by MHC molecules</b>	<b>111</b>
4.2	<b>Resistance of pathogenic mycobacteria to lysosomal delivery is mediated by Coronin 1 dependent activation of calcineurin</b>	<b>114</b>
4.3	<b>Chemotaxis, phagocytosis and oxidase activity in neutrophils are independent of Coronin 1</b>	<b>116</b>
4.4	<b>Coronin 1 is essential for IgM mediated Ca<sup>2+</sup> mobilization in B cells but dispensable for in vivo B cell functions</b>	<b>117</b>
<b>5</b>	<b><i>SUMMARY</i></b>	<b>119</b>
<b>6</b>	<b><i>LITERATURE</i></b>	<b>120</b>
<b>7</b>	<b><i>ABBREVIATIONS</i></b>	<b>148</b>
<b>8</b>	<b><i>ACKNOWLEDGEMENTS</i></b>	<b>152</b>

# 1 INTRODUCTION

## 1.1 Overview of the immune system

The immune system is a broad organization constituted of multiple effectors, cells, tissues and organs that work together to protect the body against attacks by pathogenic invaders or against cancer development. The mammalian body actually provides an ideal niche for many microbes. The immune system developed to recognize self from nonself and therefore specifically respond toward foreign organisms. After sensing the invading microbes, the immune system is recruiting specifically and efficiently a variety of cells in a well-organized manner to clear or neutralize the hazardous organism. When the body is reexposed to the same intruder, the immune system can induce a memory response that is faster and stronger allowing the host to efficaciously eliminate the foreign organism.

The immune system is tightly regulated. However, in abnormal situations, it cannot discern between self and nonself and thus launch an attack against the patient's own cells or tissues causing autoimmune disease. The immune system can also overreact against seemingly harmless substances and therefore induce an allergic reaction. Some pathogens have also evolved to avoid the immune system or to resist to their clearance.

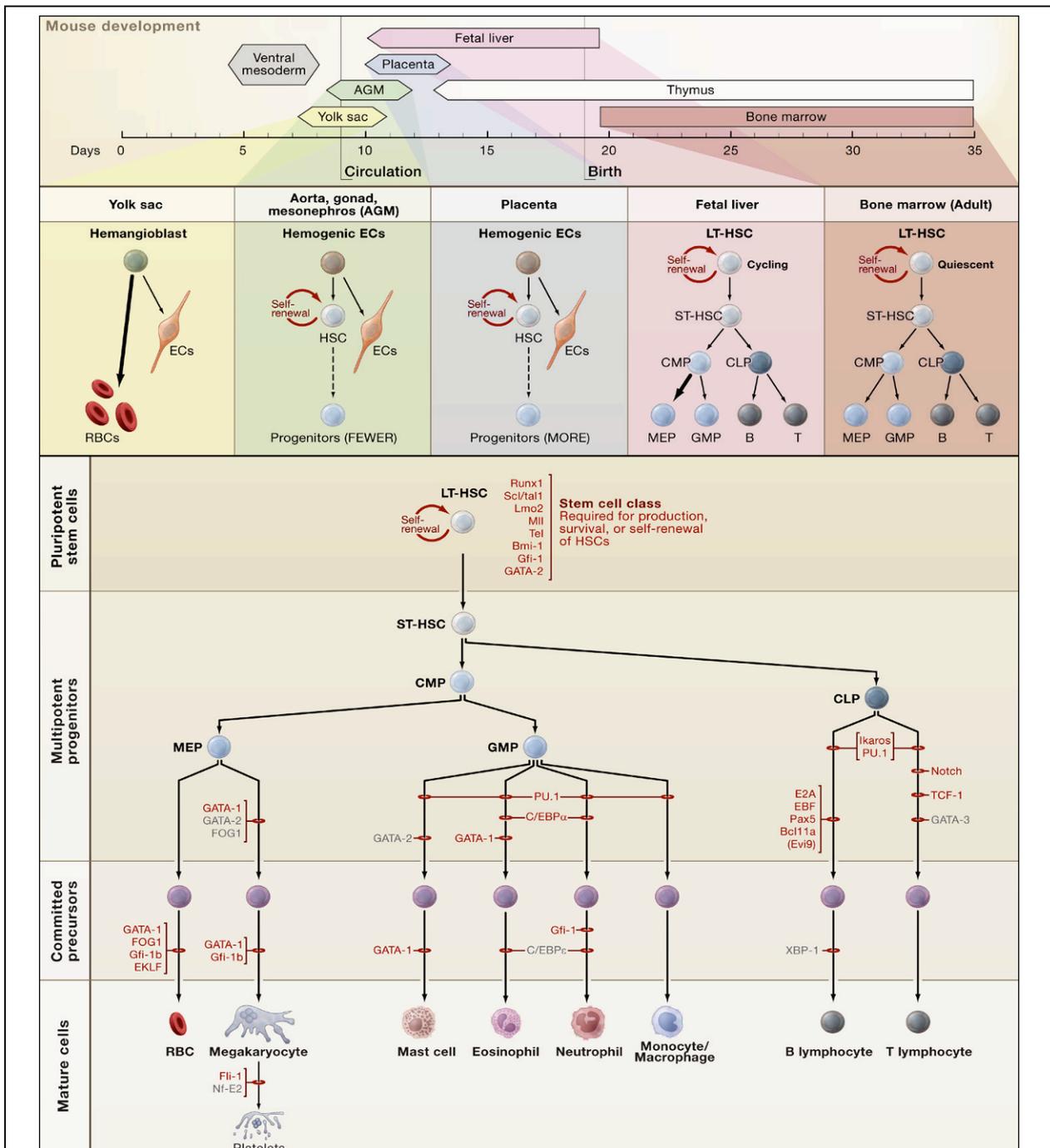
### 1.1.1 Cells and organs of the immune system

#### 1.1.1.1 Cells of the immune system

The blood is constituted by multiple cell populations originating from a common progenitor, the hematopoietic stem cell (HSC). The formation of the red and white blood cells, a process referred to as hematopoiesis, begins at the embryonic stage in the yolk sac. The differentiation of HSCs takes then place in the fetal liver and after birth predominantly in the bone marrow (figure 1).

Early in hematopoiesis, the multipotent HSCs differentiate into either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP) (Orkin, 2000; Orkin and Zon, 2008). The environment of the HSCs governs its differentiation and its further lineage commitment.

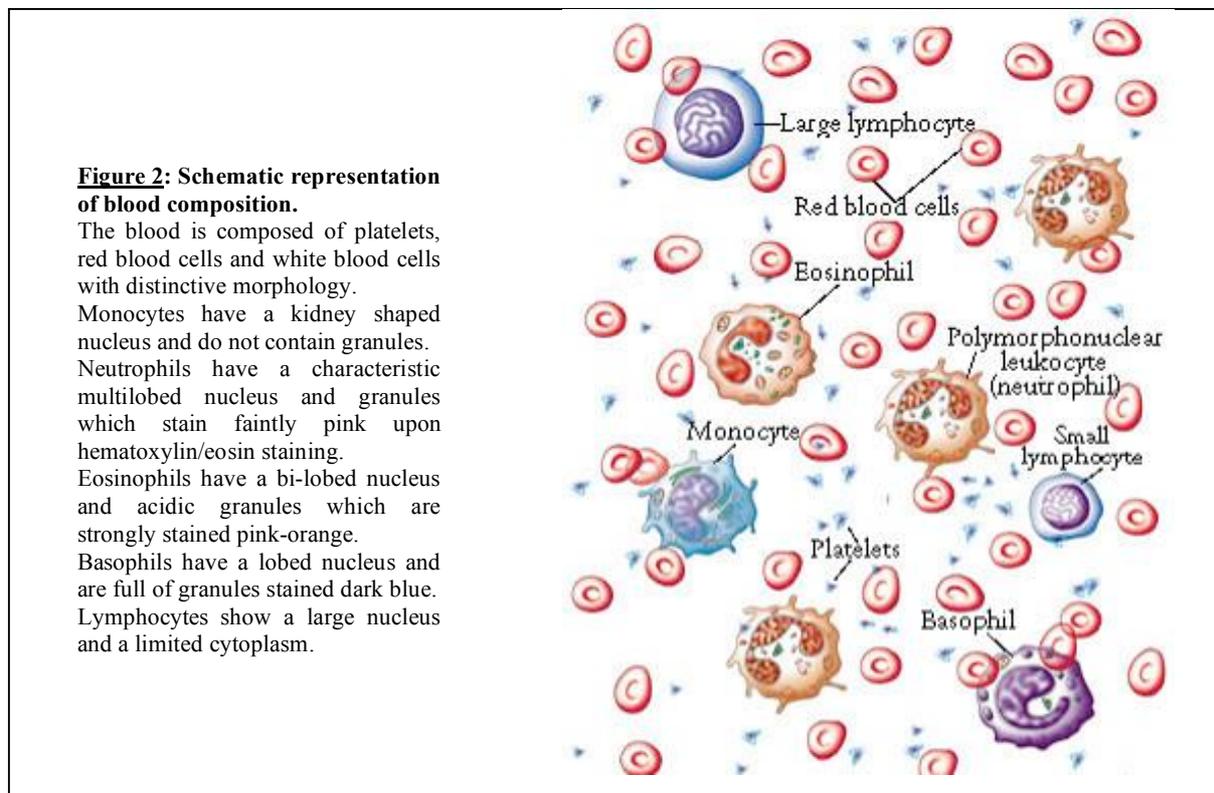
Common myeloid progenitors give rise to megakaryocyte/erythroid progenitors (MEP) and granulocyte/macrophage progenitors (GMP) (Iwasaki and Akashi, 2007; Kim and Bresnick, 2007). The MEPs will then mature into red blood cells or platelets while the GMPs will be at the origin of dendritic cells, mast cells, eosinophils, basophils, neutrophils, monocytes and macrophages. The CLPs are the source of committed precursors of B and T lymphocytes and also natural killer cells (Katsura, 2002).



**Figure 1 : Hematopoiesis.**

Pluripotent hematopoietic stem cells produce common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs are the source of megakaryocyte/erythroid progenitors (MEPs) which will then lead to red blood cells (RBC) and platelets, and of granulocyte/macrophage progenitors (GMPs) producing mast cells, eosinophils, neutrophils, monocytes and macrophages. CLPs will give rise to B and T lymphocytes. Adapted from Orkin and Zon, 2008.

The blood cells present different morphology and functions but are derived from the same progenitor (figure 2).



#### ***1.1.1.1 Mononuclear phagocytes***

The mononuclear phagocytic system is composed of monocytes in the blood stream, macrophages residing in the tissues and myeloid dendritic cells. These cells are derived from granulocyte/macrophage progenitors that developed in the bone marrow and differentiate into mature monocytes in the blood circulation. The mature monocytes can migrate into the tissues and become macrophages.

The main function of the macrophages is to phagocytose foreign organisms and dead or injured self tissues and cells. The phagosomes then fuse with lysosomes containing hydrolytic enzymes to degrade the ingested material. Mononuclear phagocytes can also produce powerful antimicrobial substances such as reactive oxygen species and reactive nitrogen intermediates. Macrophages are also efficient in antigen processing and presentation and they can secrete various cytokines upon activation to attract other leukocytes and to promote inflammation.

### ***1.1.1.1.2 Granulocytes***

The granulocytes include neutrophils, eosinophils and basophils and have received their name because of their characteristic cytoplasmic granules.

Neutrophils form the main population of the circulating leukocytes. Neutrophils have a granulated cytoplasm and a multilobed nucleus and are therefore often called polymorphonuclear leukocytes. They are produced in the bone marrow and circulate up to 10 hours in the blood before homing into the tissues. Neutrophils are the first cells recruited at the site of inflammation. They are efficient at chemotaxis, phagocytosis and oxidative burst (Nathan, 2006; Segal, 2005).

Eosinophils are motile and phagocytic cells that were thought for a long time to predominantly play a role in host protection against parasites. However, recent studies suggest that eosinophils are also key player in asthma (Rothenberg and Hogan, 2006).

The functions of the basophils are not well-known but these cells seem to be responsible for certain allergic responses due to the release of their cytoplasmic vacuole contents (Falcone et al., 2006).

Mast cells are those cells that are mainly responsible for allergy. Allergens are recognized by IgE and crosslink several FcεRI on the surface of mast cells inducing degranulation and therefore release of histamine (Robbie-Ryan and Brown, 2002).

### ***1.1.1.1.3 Lymphoid cells***

The lymphoid lineage is composed of B lymphocytes, T lymphocytes and natural killer cells that are all derived from common lymphoid progenitors.

B lymphocytes mainly develop in the bone marrow and then undergo further maturation in the secondary lymphoid organs. Different B cell subsets play distinctive roles that will be discussed later. Briefly, B cells can recognize foreign antigens via their B cell receptor (BCR), present foreign peptides on their MHC molecules, interact with T cells and finally develop into antibody-secreting plasma cells or memory B cells (Melchers and Rolink, 1998).

T cells are generated and selected in the thymus (Ceredig and Rolink, 2002; Miller and Osoba, 1967). The T cells present on their surface a T cell receptor (TCR) that cannot recognize free antigens but peptides presented on MHC molecules. T cells expressing the coreceptor CD4, a membrane glycoprotein, are restricted to MHC class II molecules which are only present on antigen presenting cells whereas T cells expressing the coreceptor CD8 are restricted to MHC class I molecules that are present on every nucleated cells. CD4<sup>+</sup> T cells are also referred as T helper cells since they play a key role in mediating the immune system by cell to cell contact and by secreting

cytokines which nature will determine the type of immune response that will be triggered (Rincon and Flavell, 1997). CD8<sup>+</sup> T cells, also called cytotoxic T cells, mainly aim at cancer cells or virus-infected cells. To clear dysfunctional cells, they release perforin and granzyme after cell to cell contact and induce apoptosis of the target by binding to their Fas molecules (Wong and Pamer, 2003). Another T cell family, the regulatory T cells, has suppressive/regulatory properties to tightly control immune responses (Sakaguchi et al., 2007).

Natural killer cells are also lymphocytes but are part of the innate immune system and exert cytotoxic activity (Andoniou et al., 2006).

#### ***1.1.1.1.4 Dendritic cells***

Dendritic cells are phagocytic cells and the major professional antigen presenting cells (APCs). Dendritic cells are derived from either common myeloid progenitors or common lymphoid progenitor. Another type of dendritic cell, the follicular dendritic cells, does not function as APCs for T helper cells but are located in the germinal centers where somatic hypermutation and isotype switching occur (Park and Choi, 2005).

### **1.1.1.2 Organs of the immune system**

When foreign organisms enter the host body by crossing the skin barrier or via the mucous membrane they have to be recognized and transported to tissues or organs where the immune response can take place. Thus, leukocytes have to be well arranged within organs and the connections between the organs must be numerous to allow an efficient homing of the different cells (figure 3).

#### ***1.1.1.2.1 Primary lymphoid organs***

Precursors develop and mature mainly within primary lymphoid organs, which are the bone marrow and the thymus.

The bone marrow is the soft tissue found in the inside of bones where the production and the differentiation of hematopoietic stem cells takes place. Development of cells from the myeloid lineage and B cells happen in the bone marrow before they leave for further maturation.

The thymus is the organ where T cells develop and mature, and where positive and negative selection occurs.

### 1.1.1.2 Lymphatic system

Leukocytes circulate in the body inside the blood (plasma) or via lymphatic vessels that allow an efficient and quick homing of the cells to their located position.

### 1.1.1.3 Secondary lymphoid organs

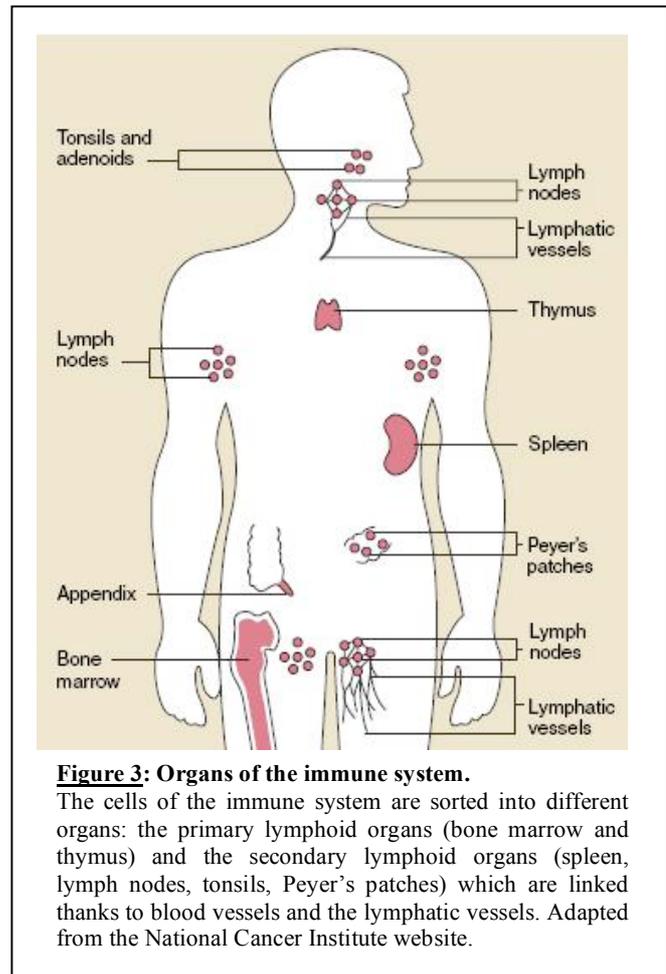
Further maturation and activation of the cells takes place inside the secondary lymphoid organs. They are also the sites where immune responses are efficiently mounted. Lymph nodes and spleen are the most highly organized of the secondary organs.

The lymph nodes are localized at the junctions

of the lymphatic vessels and are the place to which antigens that have entered the tissues are drained. The outer part of the lymph nodes is the cortex that mainly consists of B cells, macrophages and follicular dendritic cells. These cells are organized in primary follicles that will become secondary follicles containing a germinal center during immune response. The middle part of the lymph node is the paracortex, also called thymus dependent area, and is mainly composed of T lymphocytes and antigen presenting cells and is the area where T helper cells are mostly activated. The inner part of the lymph node is the medulla in which plasma cells are producing antibodies. The lymph nodes are connected both to lymphatic as well as to blood vessels.

The spleen is important to trap and respond to antigens present in the blood stream. It can be divided into a red pulp zone, mainly composed of erythrocytes, and the white pulp consisting of leukocytes arranged in the marginal zone and in follicles.

Some primordial lymphoid tissues are also found associated with the mucosa (the mucosa-associated lymphoid tissue or MALT). They are localized where many pathogens find their way to



**Figure 3: Organs of the immune system.**

The cells of the immune system are sorted into different organs: the primary lymphoid organs (bone marrow and thymus) and the secondary lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches) which are linked thanks to blood vessels and the lymphatic vessels. Adapted from the National Cancer Institute website.

enter the host organism. For example, the tonsils are situated inside the mouth, and the Peyer's patches along the gastrointestinal tract.

### **1.1.2 Innate immunity and Toll-Like Receptors**

Host organisms are constantly exposed to microbes. Most pathogenic microorganisms can be rapidly cleared following recognition of so-called pathogen-associated molecular patterns (PAMPs). PAMPs are small molecular motifs redundantly found on pathogens and sensed by cells from the innate immune system via pattern-recognition receptors (PRRs).

PRRs are composed of cytoplasmic receptors such as the NOD-like receptors important for regulation of inflammatory response and apoptosis (Strober et al., 2006), as well as membrane-bound receptors. These receptors include the mannose receptor (MR), the complement receptor type 3 (CR3), the scavenger receptor (SR) and the toll-like receptors (TLRs).

The mannose receptor is present at the surface of macrophages and dendritic cells and binds to carbohydrates present on the surface of pathogens that are subsequently phagocytosed. The mannose receptor is also important to link innate immunity to adaptive immunity (Apostolopoulos and McKenzie, 2001).

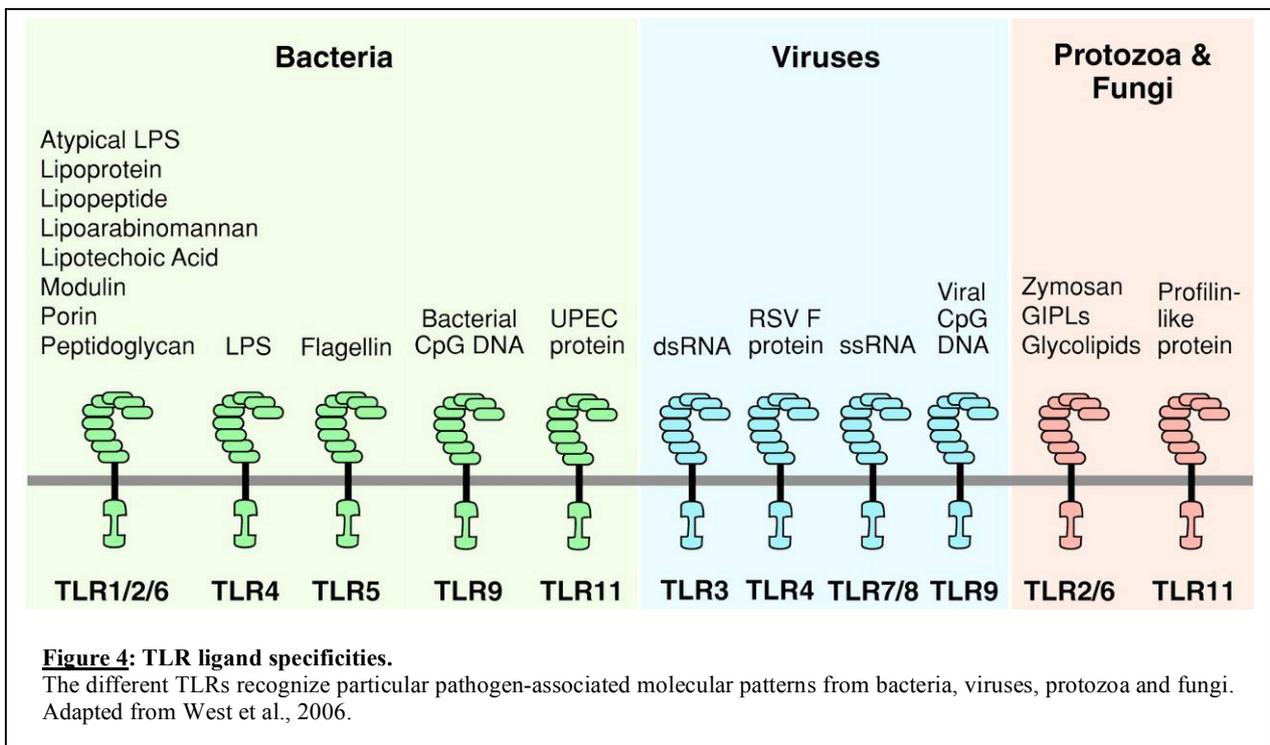
The complement receptor type 3 is mainly present on macrophages and neutrophils and binds to particles which have been opsonized with the complement component iC3b (Agramonte-Hevia et al., 2002). The complement system is composed of several small components triggered in a tightly regulated cascade, leading to target lysis, immune complex clearance, inflammation and opsonization (Rus et al., 2005). CR3 has been shown to be especially important during mycobacterial infections (Rooyackers and Stokes, 2005; Velasco-Velazquez et al., 2003).

The scavenger receptors have a relatively broad specificity and recognize low density lipoprotein which have been oxidized or acetylated. Scavenger receptors are used for the clearance of macromolecules and pathogens but are also involved in the development of atherosclerosis (Gough and Gordon, 2000; Mukhopadhyay and Gordon, 2004; van Berkel et al., 2005).

Toll-like receptors are essential sensors of the microbial infections and are type I transmembrane proteins from the Interleukin-1 receptor family. The recognition of pathogenic patterns via the TLRs leads to signal transduction resulting in activation and secretion of cytokines that promote

inflammation and leukocytes recruitment (Beutler et al., 2006; Kawai and Akira, 2007; West et al., 2006). TLRs possess an N-terminal leucine-rich repeat domain specific for the PAMPs, a transmembrane domain and a C-terminal intracellular signaling domain named Toll/Il-1 receptor (TIR) domain. Thus far, up to 13 TLRs have been discovered in mammals (Beutler, 2004).

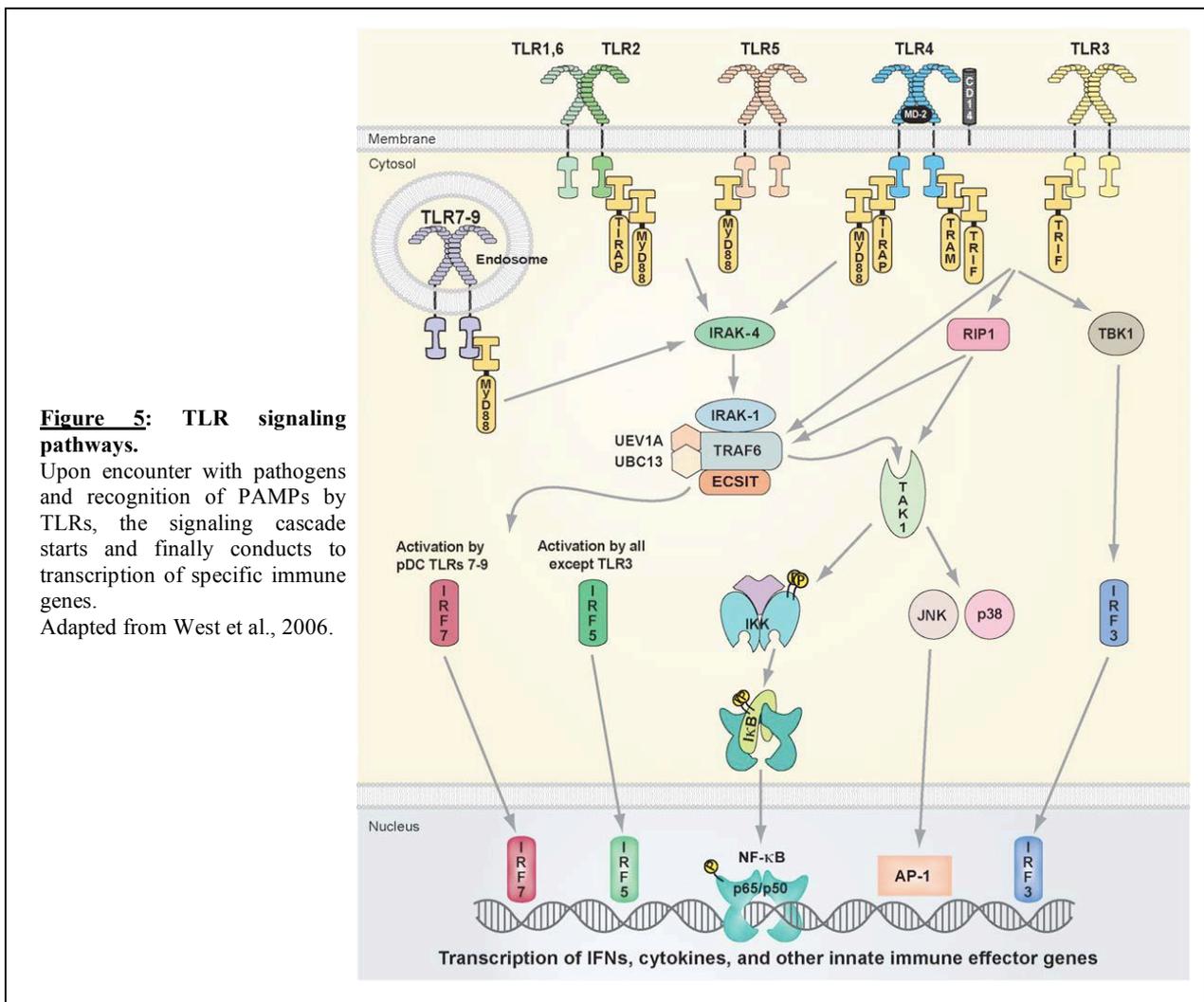
The different TLRs have defined specificities for bacteria, viruses, protozoa and fungi (see figure 4). TLR1 is specific for lipopeptides present on certain bacteria. TLR2 plays an important role in sensing Gram-positive bacteria via their peptidoglycan. TLR2 also recognizes multiple bacterial glycolipids, lipoproteins, lipoteichoic acid and zymosan from fungi. TLR3 identifies double-stranded RNA from viruses. TLR4 is one of the most studied TLRs because of its specificity for lipopolysaccharide (LPS) from Gram-negative bacteria. TLR5 recognizes flagellin from bacteria and TLR6 lipopeptides from mycoplasma. TLR7 and TLR8, which is only found in humans, are specific for viral single-stranded RNA. TLR9 is located in endosomal compartments and senses bacterial DNA containing unmethylated CpG motifs (CpG DNA). TLR11 recognizes profilin from uropathogenic bacteria.



Recognition of PAMPs by TLRs induces conformational changes and initiates signal transduction cascades (Kawai and Akira, 2007). The signaling is then propagated intracellularly via MyD88-dependent or TRIF-dependent pathways and leads to transcription of immune genes. Briefly, the

interaction of the cytoplasmic Toll/interleukin-1 receptor (TIR) domains with the myeloid differentiation primary response protein 88 (MyD88) and the TIR domain-containing adaptor protein (TIRAP) induces the recruitment of members of the interleukin-1 receptor-associated kinase (IRAK) family and of the tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 can activate numerous signaling molecules conducting to transcription of various immune effector genes. Alternatively, the TIR domains of the TLRs can interact with TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM) and therefore activate specific pathways (figure 5).

When pathogenic organisms are recognized by cells from the innate immune system, they are internalized for clearance and presentation to other leukocytes, as it will be discussed later.



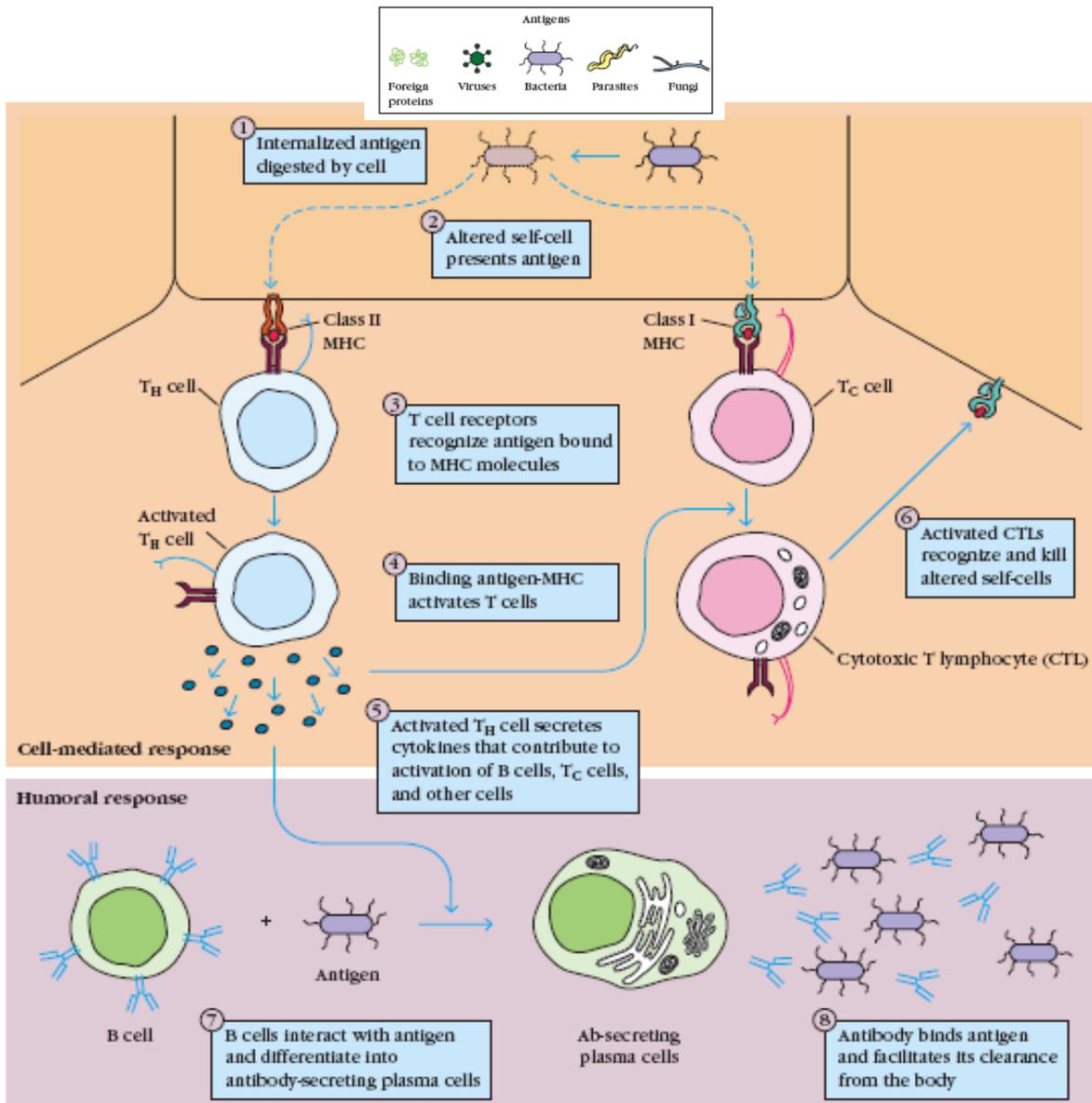
### 1.1.3 Acquired immunity

Most pathogens are cleared by the innate immune system. However some can evade immune surveillance and must be eradicated by a stronger and more specific response. The acquired or adaptive immune system is composed of highly specific and adaptable cells and is activated by cells and effectors from the innate immune system. The cooperation between these two systems is the key in eliminating foreign intruders or abnormal cells (Goldsby et al., 2003).

The central players of the acquired immune system are the B and T lymphocytes and the antigen presenting cells (APCs). B cells can recognize directly free antigens on their B cell receptor (BCR) whereas T cells can recognize antigens on their T cell receptor (TCR) when the peptides are presented in the context of Major Histocompatibility Complex (MHC) molecules at the surfaces of APCs (described later). The diversity of the lymphocytes receptors is remarkable and allows them to efficiently and specifically recognize a huge variety of antigens. This variety of cells is due to the somatic gene rearrangements of germline-encoded receptor segments, the V(D)J genes (Spicuglia et al., 2006; Tonegawa et al., 1974). The V(D)J recombination happens in the thymus for T cells and in the bone marrow for B cells which also further undergo somatic hypermutation in the germinal centers to obtain more specific antibodies (Di Noia and Neuberger, 2007).

Upon infection, antigen presenting cells internalize and process antigens prior to loading them onto MHC class II molecules.  $CD4^+$  T cells can sense these peptides, become activated and subsequently secrete cytokines to help other immune cells.  $CD8^+$  T cells can recognize infected or abnormal cells via their MHC class I molecules, become activated with the help of  $CD4^+$  T cells and can finally exert their cytotoxic activity, the cell-mediated response. B cells can identify antigens on their BCR, become activated due to T helper cells and then differentiate into plasma cells that will secrete specific antibodies, the humoral response (figure 6).

One hallmark of the acquired immune system is also to produce memory cells that will trigger a much quicker response when the body will be reexposed to the same pathogen.



**Figure 6: Schematic representation of the adaptive immune response.**

Cooperation between antigen presenting cells, T and B lymphocytes allow the host organism to mount an efficient acquired immune response upon pathogenic infection or against abnormal own cells. Adapted from Goldsby et al., 2003.

## 1.2 Antigen processing and presentation

Antigen processing and presentation is a mechanism that connects innate and adaptive immunity. T lymphocytes cannot directly detect pathogens or free antigens but these first have to be digested into peptides and loaded onto Major Histocompatibility Complex (MHC) molecules to be recognized by T cell receptor (Mellman, 2005). There are two main types of MHC molecules:

MHC class I and MHC class II. MHC class I molecules are expressed on every nucleated cells while MHC class II molecules are predominantly found on the surface of professional antigen presenting cells (APCs). The expression of MHC class II molecules is increased upon stimulation with inflammatory cytokines (Cella et al., 1997). MHC class I molecules present peptides to CD8<sup>+</sup> T cells whereas CD4<sup>+</sup> T cells are MHC class II restricted.

### **1.2.1 Antigen Presenting Cells**

While every nucleated cells express MHC class I molecules, only certain leukocytes present MHC class II molecules at their surface and are called professional antigen presenting cells (APCs). Dendritic cells, macrophages and B cells are able to take up antigens, to digest these and to load the resulting peptides on their MHC class II molecules for presentation to the T helper cells.

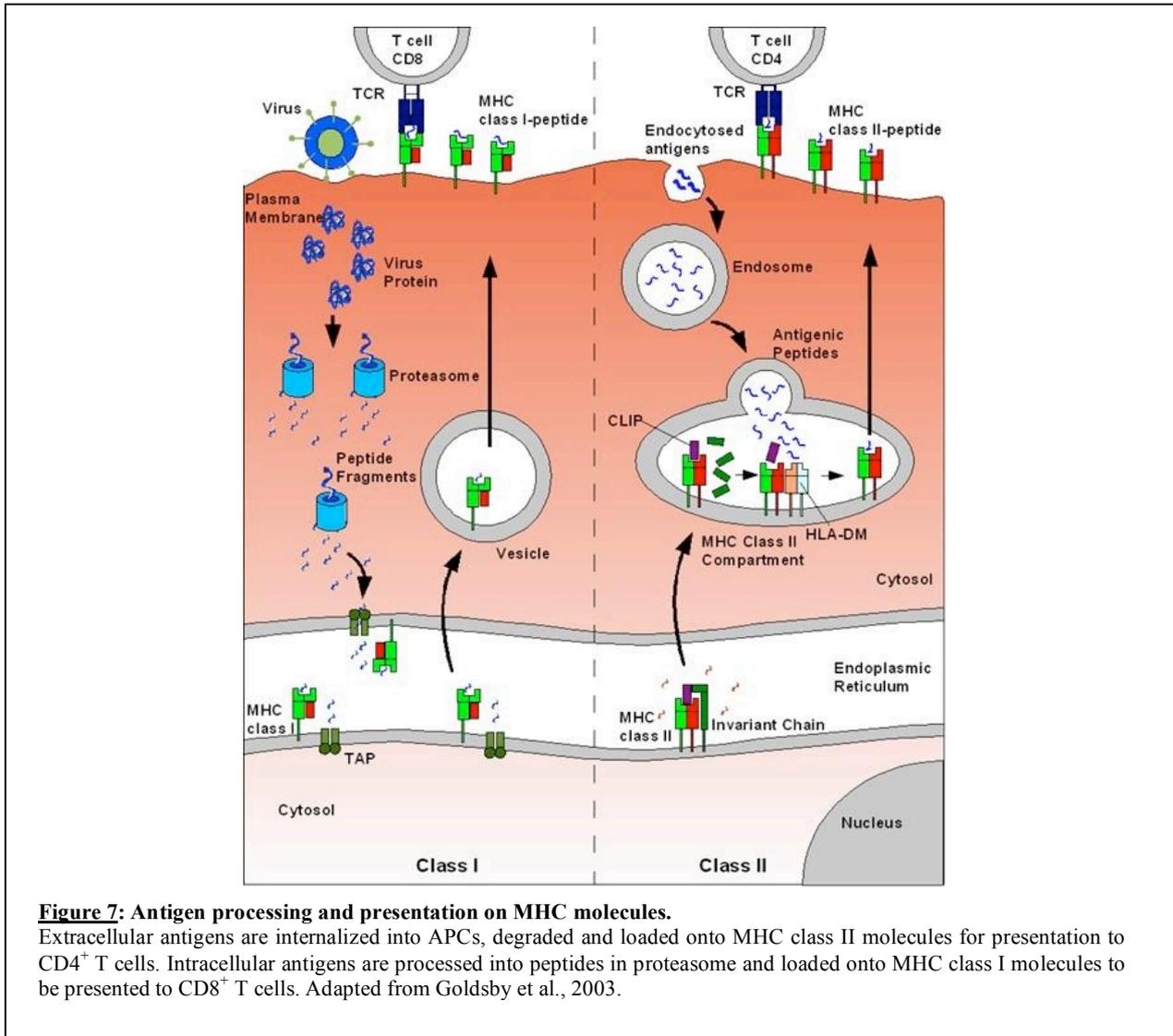
Dendritic cells are one of the most important APCs since its major role in the immune system is to capture and process antigens in the periphery to present these to T cells in the secondary lymphoid organs (Gatti and Pierre, 2003; Mellman, 2005; Mellman and Steinman, 2001). Dendritic are powerful endocytic cells seeking for foreign organisms and are also highly motile (Gunn, 2003). Immature dendritic cells are highly active in endocytosis but they express only few MHC molecules and costimulatory receptors on their surface. However, when immature dendritic cells sense microbial products or inflammatory cytokines they undergo a maturation process. Mature dendritic cells have a reduced antigen uptake capacity but upregulate at their surface the expression of MHC class II molecules, costimulatory molecules (such as CD80 and CD86) and T cell adhesion molecules (such as CD48 and CD58) (Cella et al., 1997; Inaba et al., 2000; Orabona et al., 2004; Turley et al., 2000). Dendritic cells, which have been activated and matured at the site of infection, are then homing to secondary lymphoid organs. Thus, the dendritic cells are efficient APCs since they trap antigens in the periphery and present them on their MHC class II molecules to activate T cells and to induce an efficient immune response in the lymphoid organs (Mempel et al., 2004; von Andrian and Mempel, 2003).

The function of macrophages is mainly to internalize and destroy pathogens to clear them during an early phase of innate immunity. In addition, macrophages express MHC class II molecules and therefore can function as APCs (Lanzavecchia, 1990).

B cells are APCs due to their intrinsic capacity of recognizing and binding antigens on their BCRs resulting into internalization, processing and presentation on MHC class II molecules (Lanzavecchia, 1987, 1990).

## 1.2.2 Antigen processing and presentation

Antigens have to be processed into peptides in order to load them onto either MHC class I or MHC class II molecules (figure 7) (DeFranco et al., 2007; Trombetta and Mellman, 2005).



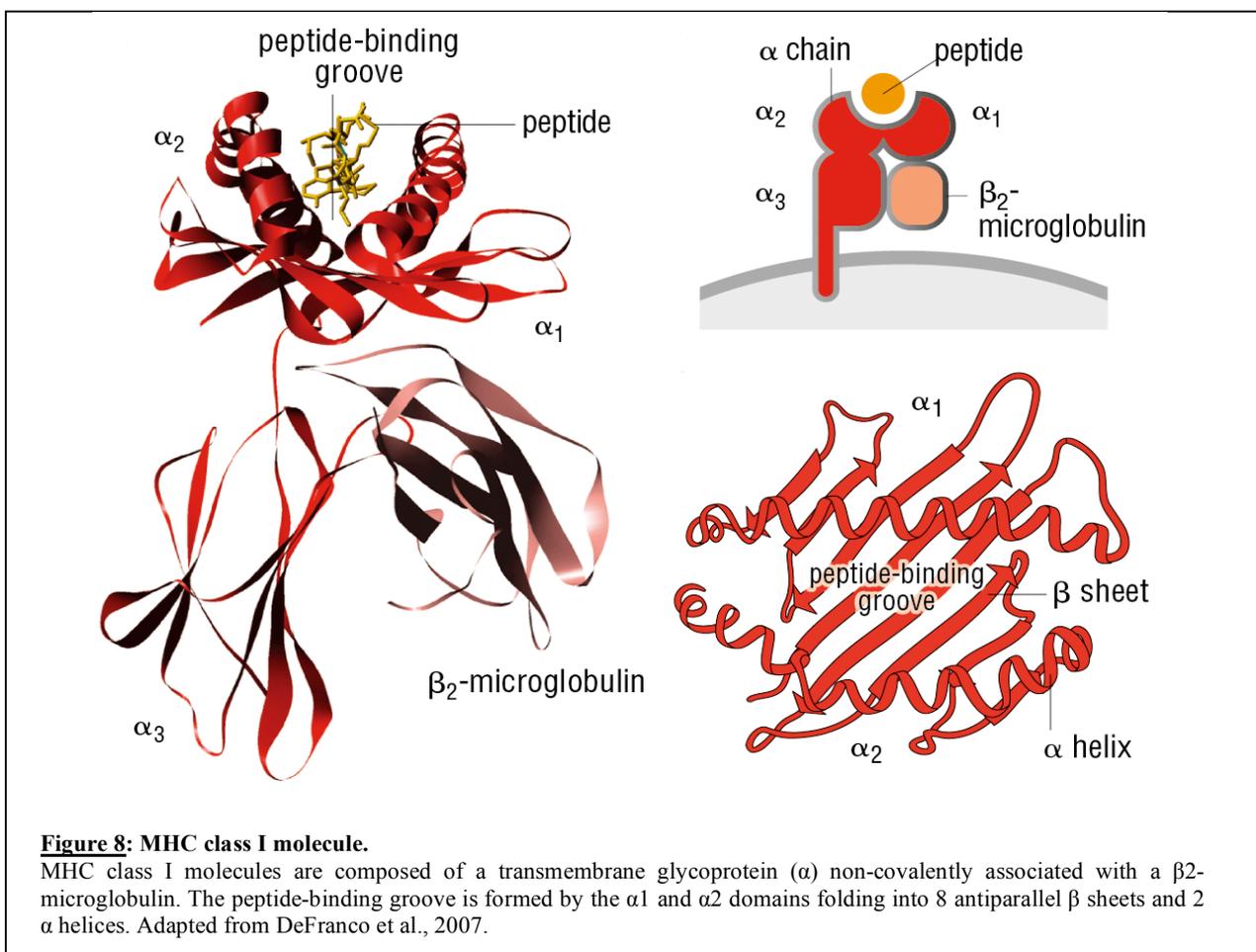
**Figure 7: Antigen processing and presentation on MHC molecules.**

Extracellular antigens are internalized into APCs, degraded and loaded onto MHC class II molecules for presentation to CD4<sup>+</sup> T cells. Intracellular antigens are processed into peptides in proteasome and loaded onto MHC class I molecules to be presented to CD8<sup>+</sup> T cells. Adapted from Goldsby et al., 2003.

### 1.2.2.1 Antigen processing and presentation on MHC class I

MHC class I molecules are heterodimers consisting of a transmembrane heavy chain non-covalently associated with  $\beta$ 2-microglobulin (see figure 8). The antigenic peptides bind inside a rigid groove formed by the folding of the  $\alpha$ 1 and  $\alpha$ 2 domains of the heavy chain into 8 antiparallel  $\beta$  strands spanned by 2 long  $\alpha$ -helical regions (Bjorkman et al., 1987). MHC class I molecule polymorphism is exhibited in this binding region.

Peptides presented on MHC class I molecules are typically derived from self or viral cytosolic proteins, alternative transcripts or defective ribosomal proteins, cytosolic proteins retrotranslocated from the endoplasmic reticulum (ER) or from endocytosed antigens transferred to the cytosol (cross-presentation) (Ackerman and Cresswell, 2004; Bevan, 1976). These cytosolic proteins are then ubiquitinated and processed in the proteasome into 10 to 20 amino acid long peptides (Baumeister et al., 1998; Kloetzel and Ossendorp, 2004). These peptides are next transferred into the ER by the TAP-1/TAP-2 heterodimer (TAP standing for transporter associated with antigen processing) (McCluskey et al., 2004). Inside the ER, the peptides are further proteolysed into 8-10 amino acid long peptides which can be loaded onto the MHC class I molecules that are then transported to the cell surface for presentation to the CD8<sup>+</sup> T cells (illustrated in figure 7).

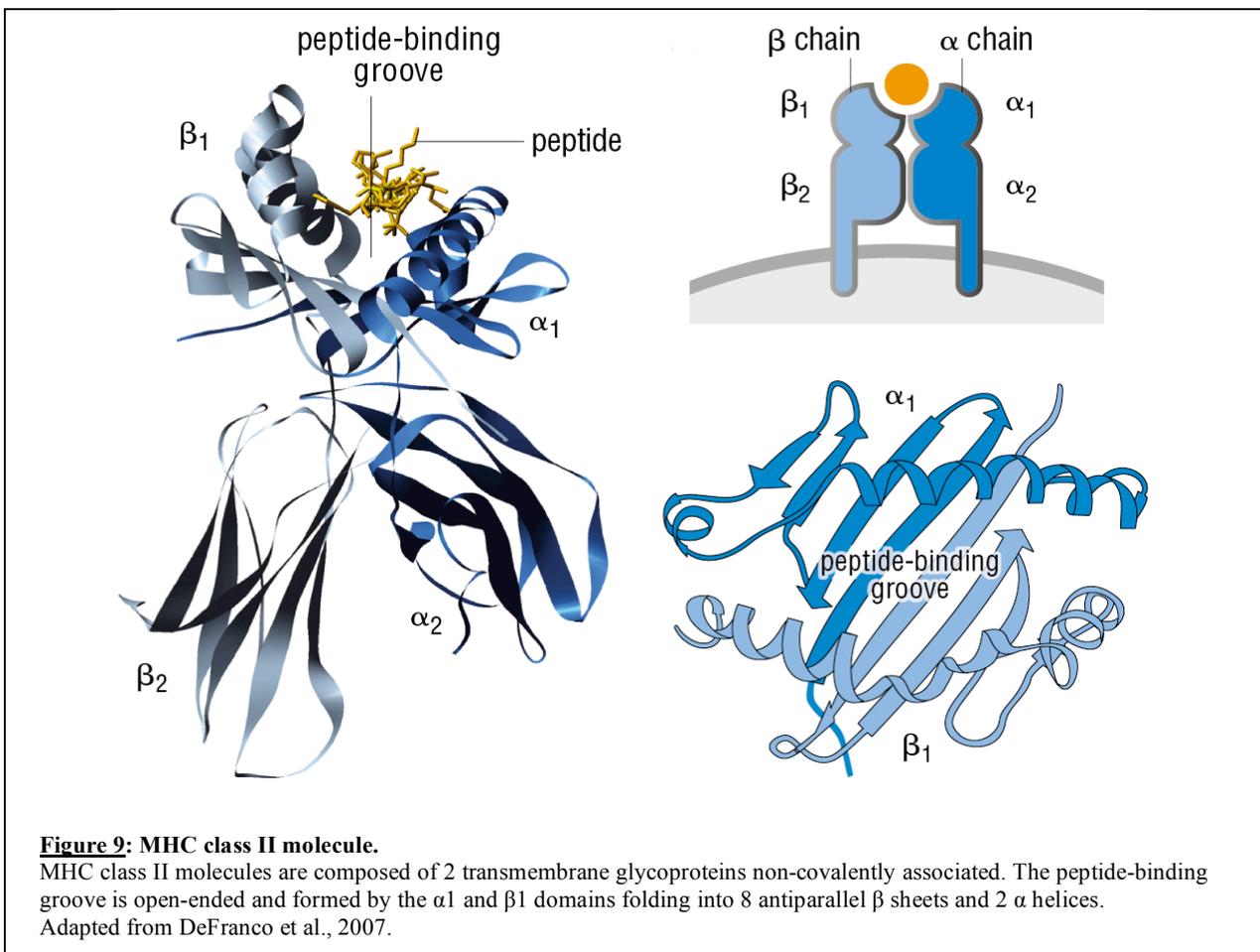


### 1.2.2.2 Antigen processing and presentation on MHC class II

MHC class II molecules consist of 2 different transmembrane glycoproteins ( $\alpha$  and  $\beta$  chain) non-covalently linked (figure 9). The antigenic peptides bind inside a cleft formed by the  $\alpha_1$  and  $\beta_1$

domains and composed by a floor of 8 antiparallel  $\beta$  strands and sides of  $\alpha$  helices (Brown et al., 1993). MHC class II molecule polymorphism is exhibited in this binding region and the fact that the binding groove is more open and flexible allows the MHC class II molecules to load larger peptides (13 to 24 amino acids) compared to the MHC class I molecules (8 to 10 amino acids). Interestingly, the MHC class I and II peptide-binding grooves are structurally homologous (Bjorkman et al., 1987; Brown et al., 1993).

Peptides presented on MHC class II molecules are originated from endogenous proteins found in endosomes or from internalized organisms. After proteolysis of the antigens, the suitable peptides are loaded in MHC class II compartment (MIIC) onto MHC class II molecules originating from the Golgi-ER (Amigorena et al., 1994; Bryant et al., 2002; Peters et al., 1991; Pieters, 1997b; Pieters et al., 1991; Tulp et al., 1994). The newly synthesized MHC class II molecules are associated with the invariant chain (Ii) sitting in the binding pocket with its class II linked invariant chain peptide (CLIP). The Ii is then degraded and the CLIP replaced by the antigenic peptide having a higher affinity. The loaded MHC class II molecule is then exported to the cell surface to interact with the CD4<sup>+</sup> T cells (figure 7).



## **1.3 Mycobacteria and the host immune system**

### **1.3.1 Mycobacteria species**

The genus *Mycobacterium* consists of at least 50 species which are widely spread and which have a characteristic thick cell wall that is hydrophobic and rich in mycolic acids. They lack an outer cell membrane and are therefore classified as acid-fast Gram-positive bacteria (Brennan and Nikaido, 1995; Karakousis et al., 2004). While some of the mycobacteria are highly pathogenic, most of the mycobacterial species are harmless.

The *Mycobacterium tuberculosis* complex is composed of *M. tuberculosis* (the main causative agent of tuberculosis disease), *M. africanum* (causing most of the tuberculosis cases in Africa), *M. canettii* (a smooth variant of *M. tuberculosis*), *M. bovis* (causing tuberculosis in cattle) and *M. microti* (pathogenic for voles, small rodents) (Cosma et al., 2003).

*M. marinum* is highly pathogenic for fish and amphibian and is the closest relative to the *M. tuberculosis* complex (Tonjum et al., 1998). It is also used to infect zebrafish as a model for tuberculosis (Davis et al., 2002). *M. ulcerans* is an evolutionary derivative of *M. marinum* and causes Buruli ulcers (George et al., 1999; Stinear et al., 2000).

*M. leprae* is the causative agent of leprosy and is thought to be composed of the essential pathogenic genes for mycobacteria (Cole et al., 2001). The *M. avium* complex, composed of *M. avium*, *paratuberculosis*, *silvaticum* and *intracellulare*, can cause disease in immunocompromised hosts (Inderlied et al., 1993).

### **1.3.2 Mycobacteria and phagocytic cells**

*Mycobacterium tuberculosis* is an intracellular pathogen which has to invade host organisms and to be recognized and internalized by cells of the innate immune system.

*M. tuberculosis* is spread to other persons by inhalation of droplets excreted by coughing persons having the active disease. Within lung, the bacilli are recognized by the macrophages and neutrophils and internalized. One of the major receptors playing a role in mycobacterial sensing is the complement receptor 3 (CR3). Mycobacteria are strongly opsonized by iC3b and mycobacteria even induce the activation of the alternative complement pathway (Schlesinger et al., 1990). The uptake of mycobacteria via CR3 is cholesterol-dependent and allows the bacteria to be phagocytosed without activation of the host cell (Gatfield and Pieters, 2000; Peyron et al., 2000; Pieters and Gatfield, 2002). Mycobacteria can also be phagocytosed by the mannose receptors

(Schlesinger, 1993, 1996), Fc receptors (with antibody-opsonized mycobacteria) (Armstrong and Hart, 1975) or scavenger receptors such as the CD36 molecule (Ernst, 1998; Philips et al., 2005). The internalization of the mycobacteria into phagosomes occurs in several steps: recognition and binding of the bacteria, invagination of the plasma membrane, closure and detachment of the newly formed vacuole.

### **1.3.3 Pathogenic mycobacteria and intracellular survival**

While bacteria are usually internalized and delivered to lysosomes for degradation, pathogenic mycobacteria can reside and survive inside phagosomes (Armstrong and Hart, 1971; Russell, 2001). Mycobacteria have developed different strategies to survive intracellularly. They express several virulence factors and also take advantage of certain host cell components to modulate intracellular trafficking (Houben et al., 2006; Nguyen and Pieters, 2005; Pieters and Gatfield, 2002; Sundaramurthy and Pieters, 2007).

#### **1.3.3.1 Mycobacterial virulence factors**

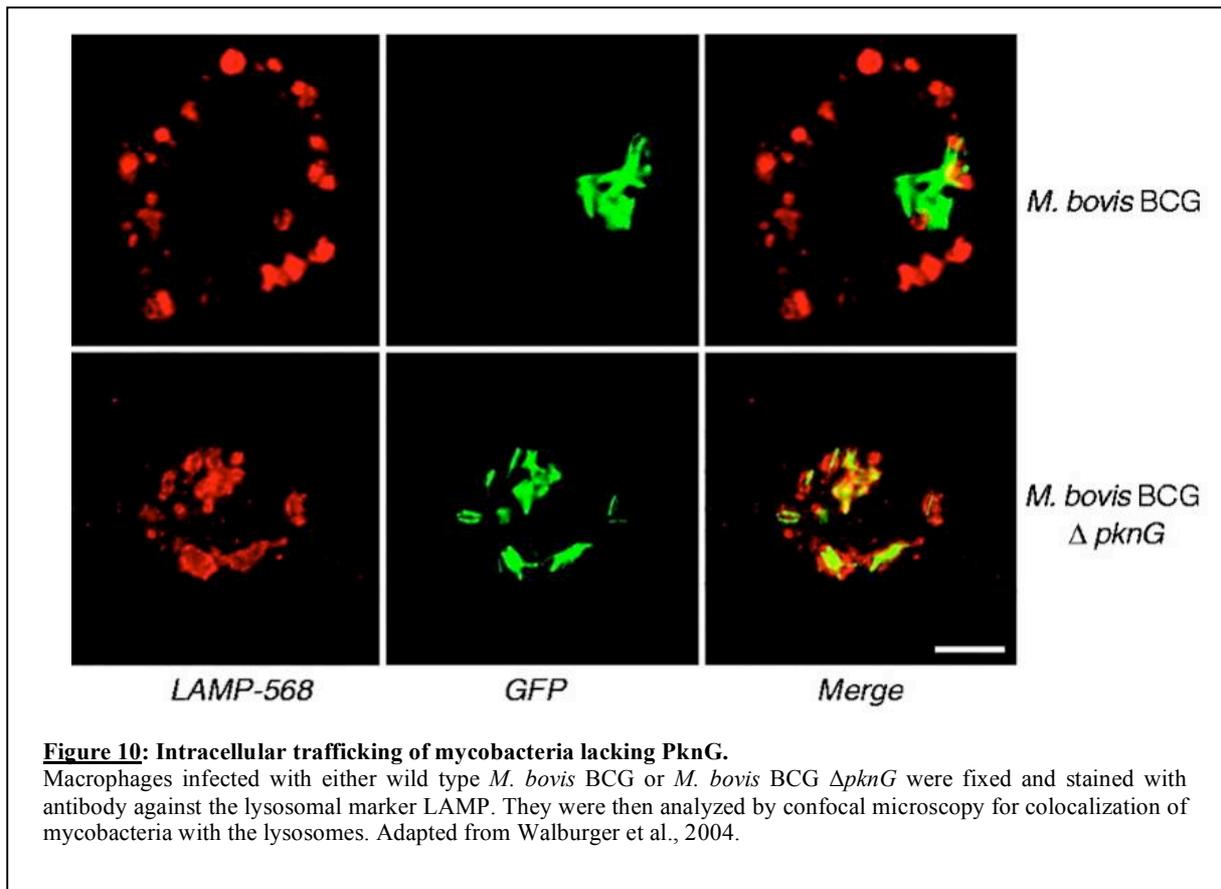
Mycobacteria produce several virulence factors that are necessary for their persistence inside infected macrophages.

Mycobacteria secrete into the host cytosol a lipid phosphatase, SapM, that is necessary to block phagosome maturation and therefore mycobacterial clearance (Saleh and Belisle, 2000; Vergne et al., 2005). SapM is responsible for the exclusion of PtdIns-3P from the mycobacterial phagosome which blocks the association of proteins with a FYVE domain that are required for fusion with late endosomes.

The mycobacterial phosphotyrosine phosphatase MptpA is upregulated and secreted inside the host cytosol after phagocytosis (Castandet et al., 2005). This protein increases actin polymerization inside infected macrophages and inhibits subsequent phagocytosis allowing the mycobacteria to secure their own niche from other intruders.

The eukaryotic-like serine/threonine protein kinase G (PknG) is an important mycobacterial virulence factor that is secreted by an unknown mechanism inside host cells upon infection. PknG is necessary for blocking the fusion of the mycobacterial phagosome with lysosomes (figure 10), allowing the bacteria to survive intracellularly (Walburger et al., 2004). Mycobacteria lacking PknG are viable outside macrophages but are readily transferred to lysosomes and killed upon

infection in the absence of PknG or when PknG is inhibited (Nguyen et al., 2005; Scherr et al., 2007; Walburger et al., 2004).



### 1.3.3.2 Mycobacteria and host cell components

Mycobacteria can also use certain host components to facilitate their entry into the phagocytic cells or to modulate their trafficking. It was demonstrated that the entry of mycobacteria inside the macrophages via the CR3 requires the presence of cholesterol, a plasma membrane steroid (Gatfield and Pieters, 2000).

The leukocyte-specific protein coronin 1 was discovered as a protein that is exclusively associated with phagosomes containing live mycobacteria (Ferrari et al., 1999). Coronin 1 is recruited upon phagosome formation but only retained by pathogenic mycobacteria that are then blocking lysosomal delivery (Deghmane et al., 2007; Ferrari et al., 1999; Itoh et al., 2002; Schuller et al., 2001). Pathogenic mycobacteria are actually not able to inhibit phagosome maturation and to survive when infecting macrophages that are either naturally deficient in coronin 1 such a Kupffer cells (Ferrari et al., 1999), or where coronin 1 expression has been knocked down with siRNA (Jayachandran et al., 2008) or macrophages obtained from coronin 1  $-/-$  mice (Jayachandran et al., 2007).

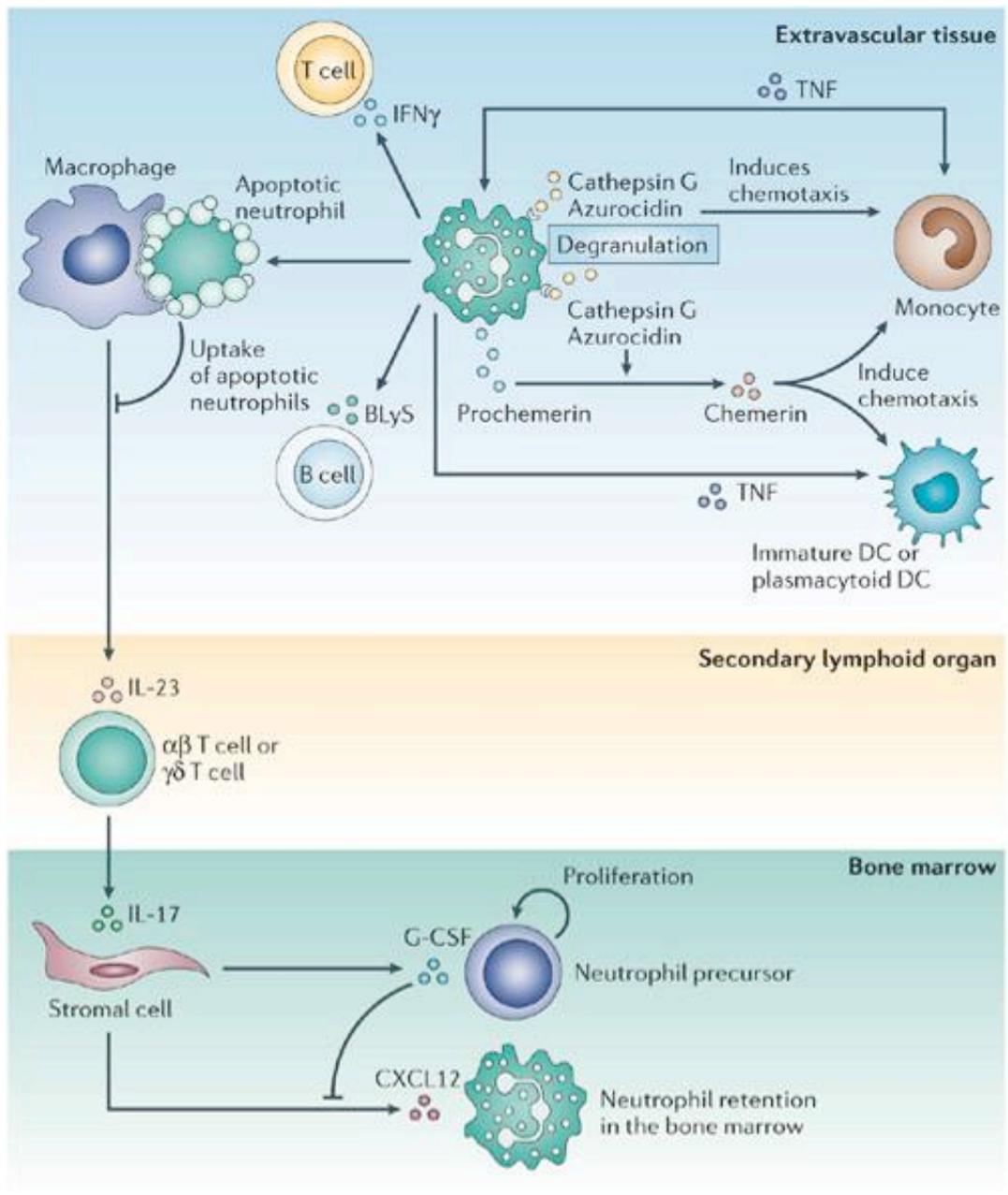
## **1.4 Polymorphonuclear Leukocytes**

The polymorphonuclear leukocytes, also called neutrophils, are highly motile cells that provide the first line of defense of the innate immune system by phagocytosing, killing and digesting intruders and that are also efficient in triggering cells of the adaptive immune system (Mayer-Scholl et al., 2004; Nathan, 2006; Nauseef, 2007; Segal, 2005).

### **1.4.1 Immune response launched by neutrophils**

Neutrophils are abundantly found in the different tissues and are often the first cells to detect the presence of a foreign intruder. In addition, upon an infection, neutrophils are actively recruited to the site of infection. Neutrophils internalize the microbes for degradation and secrete inflammatory cytokines to recruit and activate other immune cells. They produce high amount of cathepsin G and azurocidin that will induce chemotaxis of monocytes and other neutrophils (Chertov et al., 1997; Chertov et al., 2000; Sun et al., 2004). Activated neutrophils also process prochemerin into chemerin that is a powerful chemoattractant of immature and plasmacytoid dendritic cells (Bennouna et al., 2003; Wittamer et al., 2005). Neutrophils also drive dendritic cells into their mature stage by secreting TNF- $\alpha$  and by ligating DC-SIGN through Mac-1 interactions (van Gisbergen et al., 2005). Release of IFN- $\gamma$  improves the activation of T cells and macrophages while BAFF (B cell activating factor) enhances B cell proliferation (Ethuin et al., 2004; Scapini et al., 2008). The production of these diverse cytokines is low per neutrophil compared to other immune cells; however, neutrophils compensate by their high numbers at the site of inflammation.

Macrophages and dendritic cells, which are activated by microbial products or inflammatory cytokines produced by neutrophils, secrete IL-23 that supports the expansion of IL-17 secreting T cells (Aggarwal et al., 2003). This cytokine promotes the production of granulocyte colony-stimulating factor (G-CSF) by stromal cells in the bone marrow that consequently induces the proliferation of neutrophil precursors and the release into the blood stream of mature neutrophils that will home to the site of inflammation (Smith et al., 2007; Stark et al., 2005). However, this system has to be tightly regulated to avoid overproduction of neutrophils and an uncontrolled immune response. To do so, neutrophils become apoptotic at the late phase of the inflammatory response and are then ingested by macrophages. This phenomenon suppresses the secretion of IL-23 and as a consequence stops the production and the release into the circulation of neutrophils and helps the termination of the inflammation (summarized in figure 11).



**Figure 11: Interaction of neutrophils with other leukocytes to initiate immune responses.**

Neutrophils recognize the presence of pathogens in tissues, attract and activate monocytes, dendritic cells, B and T cells. They also regulate the production and release of neutrophils in the bone marrow. Adapted from Nathan, 2006.

Dendritic cells are considered to be the cell type responsible for the uptake of antigens in the different tissues and their transport to the lymphoid organs, while neutrophils are believed to be only in charge of clearing intruders and activating other cell types at the site of infection. However, some studies highlighted the fact that in some infection models neutrophils were more potent than dendritic cells to carry pathogens such as mycobacteria and salmonella to lymphoid organs (Abadie et al., 2005; Bonneau et al., 2006).

### **1.4.2 Chemotaxis of neutrophils**

Neutrophils are highly motile leukocytes that are strongly recruited to the site of inflammation through the process of chemotaxis. Neutrophils can move in a directional way along chemical gradients. They are especially responsive towards chemoattractants such as bacterial formylated peptides (fMLP), the complement molecule C5a and chemokines such as IL-8 (Zachariae, 1993). When neutrophils recognize these stimuli through their surface receptors, the cytoskeletal machinery becomes activated and leads to F-actin rearrangements resulting in migration towards the chemoattractant (Benard et al., 1999).

The recognition of formylated peptides by the G protein-coupled receptor (GPCR) drives cell motility of the neutrophils (Glogauer et al., 2000; Radel et al., 1994). This neutrophil migration is due to signaling cascades initiated upon fMLP stimulation. Several isoforms of phospholipase C, especially  $\beta 2$  and  $\beta 3$ , were shown to be activated upon addition of chemoattractant to neutrophils (Jiang et al., 1996; Li et al., 2000; Wu et al., 2000). The PI-3 kinases and their lipid products are also essential for neutrophils chemotaxis; PI(3,4,5)P<sub>3</sub> is transiently produced by neutrophils upon fMLP stimulation and associated to actin polymerization (Eberle et al., 1990; Rickert et al., 2000; Servant et al., 2000; Traynor-Kaplan et al., 1988). Thus, the signaling elements linking the chemotactic receptors and the actin cytoskeleton are essential for the chemotaxis of the neutrophils and therefore their recruitment to the site of inflammation (Cicchetti et al., 2002).

### **1.4.3 Killing of microbes by neutrophils**

Neutrophils are degrading pathogens by secreting the contents of their diverse granules into the phagosome containing the bacteria or into the extracellular milieu where bacteria are residing (Segal, 2005).

Neutrophils can starve bacteria from iron by producing lactoferrin, which will bind to iron, and lipocalin-2, which will act on bacterial siderophores (Flo et al., 2004). They also release calprotectin, a heterodimer of 2 calcium-binding proteins, and phospholipase A<sub>2</sub> to damage bacteria (Striz and Trebichavsky, 2004; Wright et al., 1990).

Neutrophils possess the so-called peroxidase-negative granules, also known as specific (or secondary) and tertiary (or gelatinase) granules (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003). These are the first to be released and contain lipocalin, lysozyme and numerous

antimicrobial peptides. They are also filled with matrix metalloproteinases (MMPs) that will degrade laminin, collagen, proteoglycans and fibronectin and enhance neutrophils recruitment. Neutrophils also possess granules named azurophilic or peroxidase-positive granules. These primary granules contain azurocidin, cathepsin G, elastase and protease 3 that have several antibiotic activities. The bactericidal permeability increasing protein (BPI), as well present in the azurophilic granules, binds to lipopolysaccharide and is therefore a powerful antibiotic against Gram-negative bacteria (Ooi et al., 1991; Weiss et al., 1978). Defensins and myeloperoxidase are also found in azurophilic granules and have strong antimicrobial activities (Klebanoff, 2005; Selsted and Ouellette, 2005).

To degrade bacteria, neutrophils can release reactive oxygen species in a process called respiratory or oxidative burst. Upon infection, the NADPH oxidase assembles at the phagosomal membrane and becomes activated. NADPH oxidase produces superoxide ( $O_2^-$ ) that is then converted to hydrogen peroxide ( $H_2O_2$ ) by the superoxide dismutase. Hyperchlorous acid (HOCl) is also derived from  $H_2O_2$  with the help of the myeloperoxidase present in the azurophilic granules (Babior, 2004; Babior et al., 1973; Lambeth, 2004).

It has been also shown recently that neutrophils are able to release chromatin coated by granule proteases to form extracellular fibers (Brinkmann et al., 2004). These neutrophil extracellular traps (NETs) can bind to bacteria and kill them.

#### **1.4.4 Neutrophils and mycobacteria**

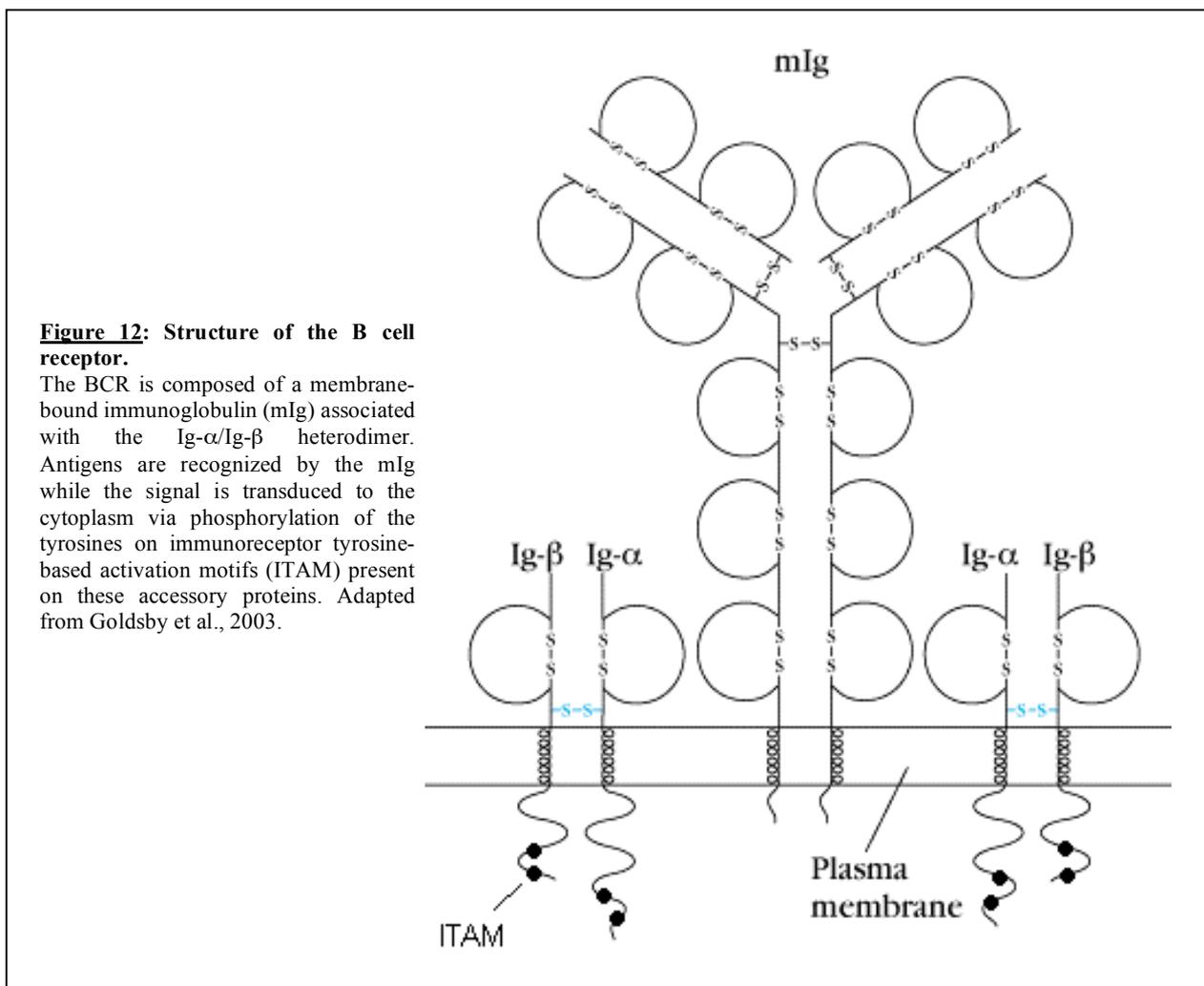
Neutrophils are implicated in clearing diverse pathogens and it was proposed that they could play a key role in defense against mycobacterial infections. It was demonstrated that beige mice or mice depleted of neutrophils were more susceptible to mycobacterial invasion (Appelberg et al., 1995). Neutrophils can efficiently phagocytose mycobacteria and trigger the secretion of specific granules and the production of reactive oxygen species (May and Spagnuolo, 1987; N'Diaye et al., 1998). They also help the recruitment and activation of macrophages at the site of infection. Apoptotic neutrophils are also internalized by macrophages and can therefore transfer their competent antimicrobial peptides to macrophages and allow them to clear more efficiently this intracellular pathogen (Tan et al., 2006). Defensins that are present in azurophilic granules are also potent antimicrobial agents against mycobacteria (Fu, 2003).

## 1.5 B lymphocytes

B lymphocytes are cells from the adaptive immune system whose development takes place in the bone marrow. Immature B cells leave the bone marrow to reach the spleen for further maturation. Mature B cells present on their surface a receptor with a single antigenic specificity.

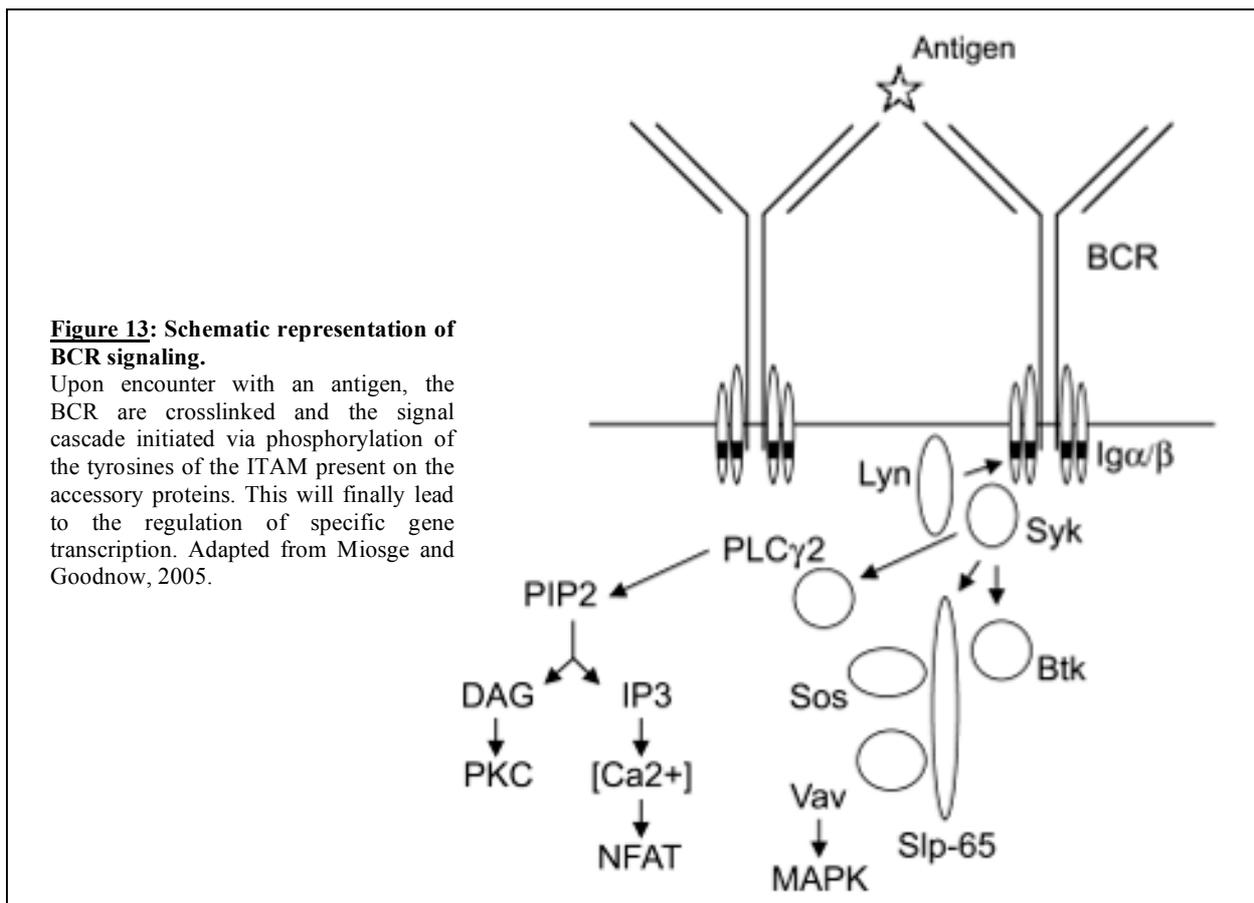
### 1.5.1 B Cell Receptor (BCR) complex and signaling

The B cell antigen receptor (BCR) plays a central role in the development and activation of B lymphocytes. The BCR is multisubunit complex consisting of a membrane-bound immunoglobulin (mIg) and the signaling transducing Ig- $\alpha$ /Ig- $\beta$  (CD79 a and b) heterodimer (Schamel and Reth, 2000). B cells recognize antigens on the variable domain of membrane-bound immunoglobulins and the signal is then intracellularly transduced via invariant accessory proteins (Reth and Wienands, 1997) (and see figure 12).



Upon BCR crosslinking, the 2 immunoreceptor tyrosine-based activation motifs (ITAM) tyrosines present in the long cytoplasmic tails of the Ig- $\alpha$ /Ig- $\beta$  heterodimer are phosphorylated by the protein tyrosine kinases Lyn and Syk (Johnson et al., 1995; Kulathu et al., 2008; Sada et al., 2001; Xu et al., 2005). The binding and activation of these kinases result in the rapid recruitment of the B cell linker molecule (BLNK or SLP65) to the signaling complex and its phosphorylation (Baba et al., 2001; Fu et al., 1998; Taguchi et al., 2004).

Phosphorylation of BLNK induces the recruitment and activation of the growth-factor-receptor-bound protein 2 (GRB2) (Sarmay et al., 2006), Sos and Vav (Miosge and Goodnow, 2005). Activated BLNK also recruits and activates phospholipase C $\gamma$ 2 (Ishiai et al., 1999; Taguchi et al., 2004) and the Bruton's tyrosine kinase (Btk) (Hashimoto et al., 1999). Btk interacts with PtdIns(3,4,5)P $_3$  and is as a result rapidly recruited to the plasma membrane (Saito et al., 2001). Consequently, Btk brings PLC $\gamma$ 2 closer to PtdIns(4,5)P $_2$  which is then hydrolysed into the soluble messenger InsP $_3$  and the lipid second messenger diacylglycerol (DAG) (Wang et al., 2000; Watanabe et al., 2001) (figure 13).



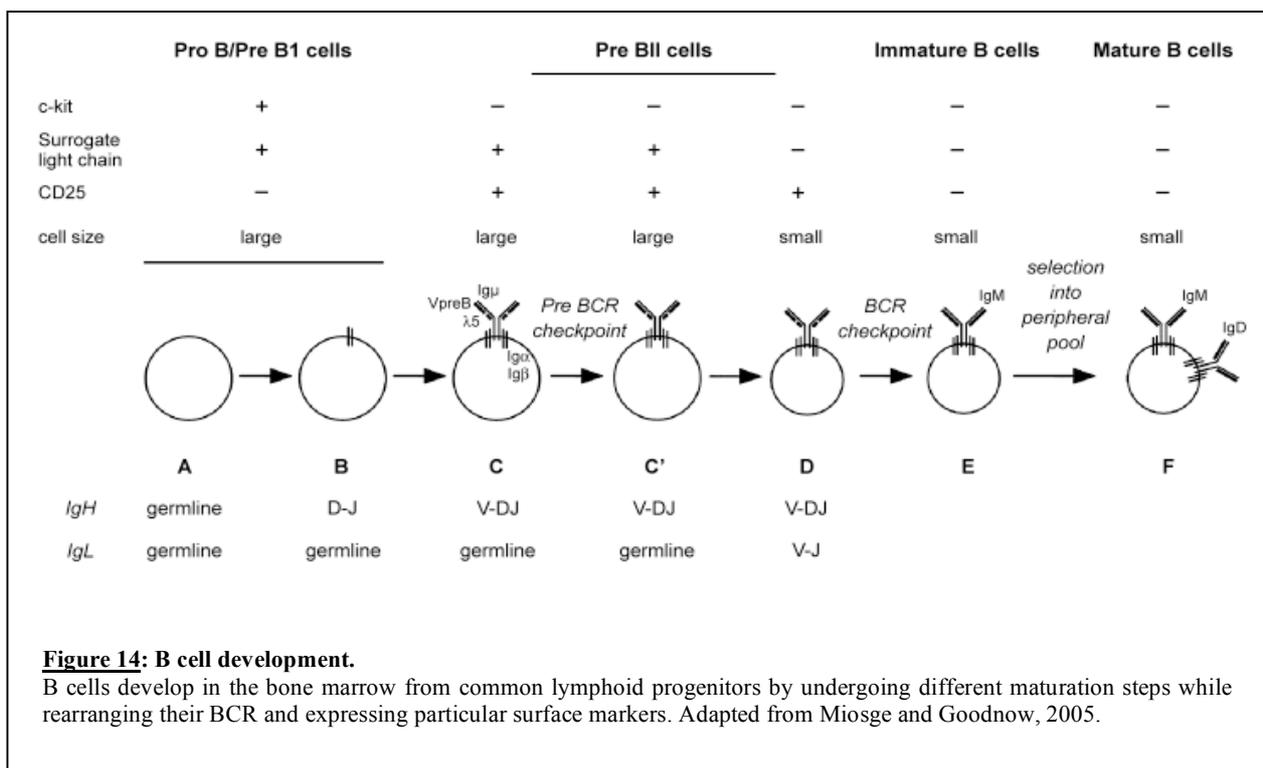
The production of DAG induces the activation of protein kinases C (Brose et al., 2004), while InsP $_3$  will bind to the InsP $_3$  receptors that are present in the endoplasmic reticulum membrane and will

induce the opening of the  $\text{InsP}_3\text{R}$  channels and the release of  $\text{Ca}^{2+}$  into the cytoplasm (Jun and Goodnow, 2003; Lewis, 2001). The depletion of  $\text{Ca}^{2+}$  from the intracellular stores triggers the flux of  $\text{Ca}^{2+}$  through the plasma membrane into the cytosol via the store-operated calcium channels (Cahalan et al., 2007; Lewis, 2007; Parekh, 2006; Parekh and Penner, 1997). This signaling cascade will ultimately induce the expression of specific genes (Gallo et al., 2006).

## 1.5.2 Development and functions of B cells

B cells derive from common lymphoid progenitors and their development takes place in the bone marrow while further maturation occurs in the periphery.

The earliest B cell precursor is the pro-B cell that expresses the pan B cell marker B220, the receptor for interleukin 7 and the receptor for stem cell factor (c-kit). These pro-B cells then undergo D-J rearrangement and become pre-B-I cells. They then rearrange the V region and the resulting V-DJ exon allows them to present on their surface a pre-BCR and to become pre-B-II cells. The pre-BCR is made of the rearranged heavy chain and a surrogate light chain composed of  $\lambda_5$  and VpreB; the  $\text{Ig}\alpha/\text{Ig}\beta$  heterodimer is also associated to the pre-BCR. The successful assembly of the pre-BCR at the surface is necessary for proliferation of pre-B-II cells into smaller cells that will undergo V-J rearrangement of the light chain (figure 14).



When cells express a conventional IgM on their surface, the so-called immature B cells, they can leave the bone marrow if this BCR is functional and if they are not autoreactive. Otherwise, these cells are either deleted, become anergic or initiate receptor editing to produce a different light chain (Clark et al., 2005; Miosge and Goodnow, 2005; Rolink and Melchers, 1996; Rolink et al., 2001; Rolink et al., 1999; Zhang et al., 2004).

Immature B cells reach the spleen where they can undergo further maturation. The immature splenic B cells can be divided in 2 categories: the transitional 1 (T1) and the transitional 2 (T2) B cells (Loder et al., 1999). The transitional B cells are characterized by the expression of CD93 on their surface. The T1 B cells highly express IgM but low levels of IgD and they are negative for CD21 and CD23, while the T2 B cells still express high levels of IgM and are positive for IgD, CD21 and CD23. Some T2 B cells are expressing intermediate levels of IgM and are referred as T3 B cells (Loder et al., 1999; Rolink et al., 2004).

The mature splenic B cells are CD93 negative and can be subdivided into follicular (FO) and marginal zone (MZ) according to their localization, surface markers expression and function.

Follicular B cells express both CD23 and CD21 and present high amounts of IgD and low levels of IgM on their surface. They represent most of the recirculating mature B cells and reside in the follicles of spleen, lymph nodes or Peyer's patches. They can participate in reactions against T cell independent antigens; however, they mainly play a role in T cell dependent antibody responses and germinal center formation (Casola, 2007; Rajewsky, 1996; Rolink et al., 2004). It was proposed that strong BCR signals would favor development of mature follicular B cells compared to marginal zone B cells (Cariappa et al., 2001; Casola et al., 2004; Pillai et al., 2004).

Marginal zone B cells are CD23 negative and express high levels of CD21, low levels of IgD and high levels of IgM. They represent 5 to 10 % of the splenic B cells and are localized in the spleen next to the periarterial lymphatic sheath (PALS) and the follicles, between the red and the white pulp. Because of this location, they can efficiently sense blood-borne antigens and can therefore initiate a T cell independent response. Marginal zone B cells are also named "innate-like" lymphocytes since they can produce antibodies without T cell help and can shorten the temporal gap between innate and acquired immune responses (Bendelac et al., 2001; Lopes-Carvalho and Kearney, 2004; Martin and Kearney, 2000; Pillai et al., 2005). Marginal zone B cells also contribute to the humoral response to T cell dependent antigens (Song and Cerny, 2003). Mutant

mice in which BCR signals are increased are deficient in marginal zone B cells suggesting that weak BCR signals favor development of marginal zone B cells (Cariappa et al., 2001; Pillai et al., 2004; Wang et al., 1998).

Another type of B cells that are CD5 and CD11b positive, IgM high and IgD low are named B-1 B cells. These are mainly found in the peritoneal and pleural cavities. They are not developing from bone marrow progenitors like the B-2 B cells (FO and MZ B cells) do but originate from fetal liver progenitors. They are producing antibodies against foreign antigens at the mucosal sites without T cell help and are considered to be the main source of natural antibodies (Berland and Wortis, 2002; Casola, 2007; Hardy, 2006; Lam and Rajewsky, 1999; Martin and Kearney, 2000; Wortis and Berland, 2001).

## **1.6 The coronin proteins**

The members of the coronin family are proteins containing WD40 repeats. The first coronin isoform was described in *Dictyostelium discoideum* (de Hostos et al., 1991) and coronin proteins were afterwards discovered in yeast and mammals (Utrecht and Bear, 2006). The coronin proteins share similar sequence, structure and localization; relatively little is known about their function.

### **1.6.1 Coronin in *Dictyostelium* and yeast**

The first coronin (coronin A) was identified in *Dictyostelium discoideum* as a 55 kDa protein co-precipitating with actin-myosin complexes (de Hostos et al., 1991). It concentrated at the cell cortex of the amoeba and was therefore called coronin because of its localization in the crown-shaped structures. *Dictyostelium discoideum* mutants lacking coronin showed defects in cytokinesis and motility (de Hostos et al., 1993). Phagocytic ability was also abolished in the absence of coronin (Maniak et al., 1995). Using expression of GFP-tagged coronin, it was shown that coronin accumulates at the phagocytic cup (Maniak et al., 1995), at the leading edge towards chemoattractant (Gerisch et al., 1995), at the forming macropinosome (Hacker et al., 1997) and into the cleavage furrow during cytokinesis (Fukui et al., 1997; Fukui et al., 1999). Since deletion of coronin altered several actin-dependent processes and since coronin was visualized in areas where

actin cytoskeleton remodeling frequently occurs, it was proposed that coronin A functions in the modulation of F-actin cytoskeleton in *Dictyostelium discoideum*.

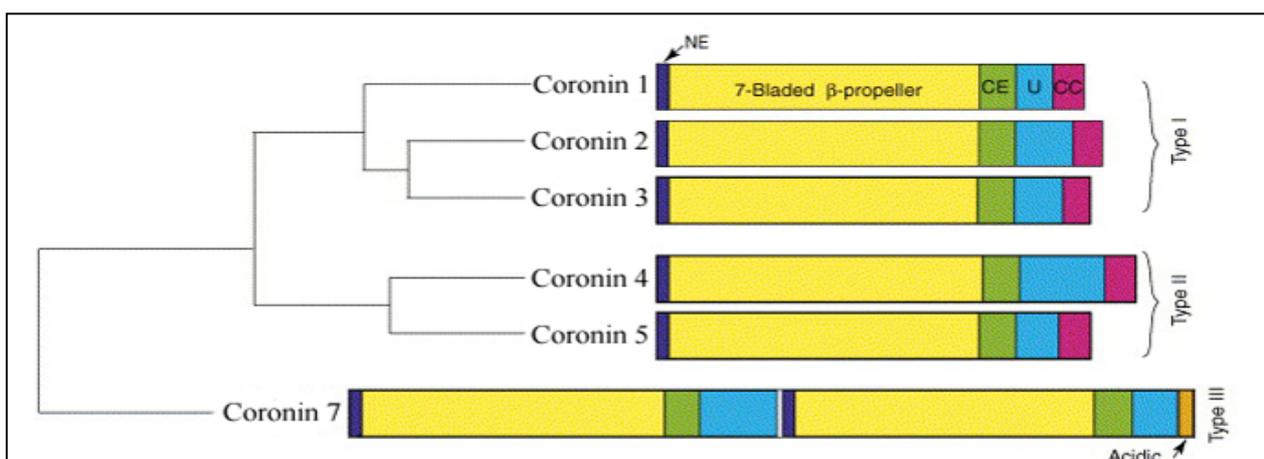
*Dictyostelium discoideum* expresses 2 other coronin like proteins, coronin 7 or DCrn7 and villidin, whose functions are not yet clearly defined (Shina and Noegel, 2008).

The yeast *Saccharomyces cerevisiae* possesses only one coronin gene, called *crn1*. Unlike *Dictyostelium discoideum*, deletion of yeast coronin does not lead to any defects in actin-related processes (Heil-Chapdelaine et al., 1998). However, yeast coronin was localized to cortical actin patches and was shown to bind *in vitro* to actin, microtubules and Arp2/3 complex (Goode et al., 1999; Humphries et al., 2002).

## 1.6.2 Coronins in mammals

### 1.6.2.1 The coronin family

Seven coronin proteins have been identified in mammals and these all have in common the WD40 repeats (Rybakin and Clemen, 2005; Utrecht and Bear, 2006). Except coronin 7, they share similar structural domains: a short N-terminal extension, the 5 WD40 repeats forming a 7-bladed  $\beta$ -propeller, a short C-terminal extension, a unique and variable region, and a C-terminal coiled-coil domain (Appleton et al., 2006; Gatfield et al., 2005; Utrecht and Bear, 2006). Coronin 7 is a larger isoform containing 2 WD40 domains and no coiled-coil region but an acidic C-terminal domain (Utrecht and Bear, 2006).



**Figure 15: The mammalian coronin family.**

Mammalian coronin proteins can be classified in 3 categories. The Type I and II coronins present a N-terminal extension (NE), a WD40 domain organized in a 7-bladed  $\beta$ -propeller, a C-terminal extension (CE), a unique region (U) and a coiled-coil domain (CC). Coronin 7 consists of a double WD40 domain and an acidic C-terminal domain instead of the coiled-coil domain. Adapted from Utrecht and Bear, 2006.

The coronins can be divided in 3 groups: the type I coronins representing coronin 1, 2, 3 and 6; the type II coronins consisting of coronins 4 and 5 and having a different C-terminal domain; and the type III coronin made of coronin 7 with its double WD40 domain (figure 15).

### **1.6.2.2 Expression and functions of coronins in mammalian cells**

Coronin 1 is specifically expressed in leukocytes and has been the most extensively studied coronin. Its functions will be discussed in the next chapter.

Coronin 2, originally called coronin<sub>se</sub>, coronin 1B or p66, is ubiquitously expressed in mammalian cells with higher levels in gastrointestinal mucosa, spleen, lung, liver and kidney (Okumura et al., 1998; Parente et al., 1999). Coronin 2 was shown to localize at the leading edge of migrating fibroblasts where it interacts with the Arp2/3 complex in a PKC-regulated manner (Cai et al., 2005). Coronin 2 also regulates cofilin activity and its depletion alters F-actin dynamics (Cai et al., 2007b). It also binds directly to F-actin and it has been demonstrated that this binding site is necessary for coronin 2 functions (Cai et al., 2007a).

Coronin 2 plays also an important role in the nervous system since it has been linked to neurite outgrowth after spinal cord injury (Di Giovanni et al., 2005; Di Giovanni et al., 2006). Coronin 2 was as well found in a screen of phagosome-associated proteins (Morrissette et al., 1999).

Coronin 3, or coronin 1C, is expressed in every mammalian cell but most prominently in brain, lung, intestine and kidney (Iizaka et al., 2000; Okumura et al., 1998; Spoerl et al., 2002). It was identified in the cytosol and at membrane ruffles and its C-terminal domain is responsible for oligomer formation and F-actin interaction (Spoerl et al., 2002). Coronin 3 has an essential function during neuron morphogenesis (Hasse et al., 2005) and was proposed to be associated with malignant progression of diffuse gliomas (Thal et al., 2008).

Coronin 4, also named coronin 2A, clipinB or IR10, expression is restricted to colon, prostate, testis, ovary, uterus, brain, lung and epidermis (Nakamura et al., 1999; Okumura et al., 1998; Zaphiropoulos and Toftgard, 1996) but its role has not yet been identified except its copurification with the nuclear receptor corepressor complex (Yoon et al., 2003).

Coronin 5, also referred as coronin 2B, clipinC or FC96, is mainly expressed in the brain and at a lesser extent in the heart and ovary (Nakamura et al., 1999; Okumura et al., 1998). It was proposed to link the actin cytoskeleton to the membrane however no further studies defined the precise function of coronin 5 (Nakamura et al., 1999).

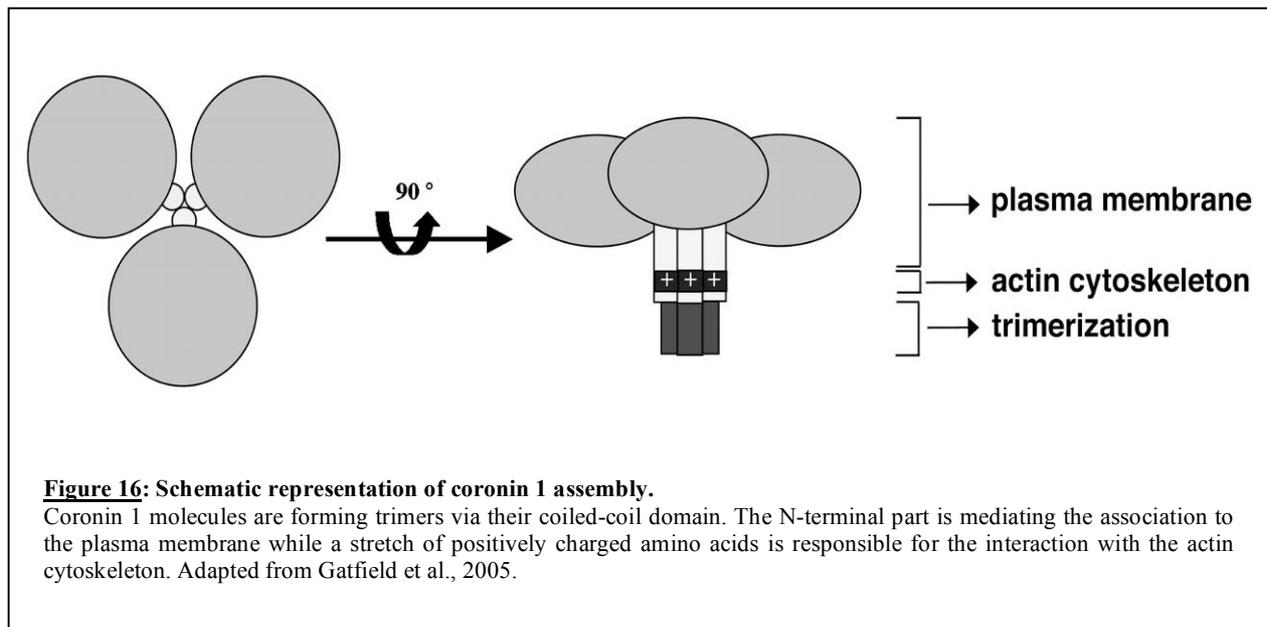
Coronin 6 is the least known coronin and has been detected in the brain with 3 possible splicing variants (Rybakin and Clemen, 2005).

Coronin 7, or *crn7*, is ubiquitously expressed at low levels but strongly enriched in the thymus, the spleen, the kidneys and the brain (Muralikrishna et al., 1998; Rybakin, 2008; Rybakin et al., 2004). The coronin 7 is the only type III coronin and presents a characteristic structure; it is a long coronin with a double WD40 motif. Coronin 7 is highly homologous to POD-1 proteins from *Drosophila* and *C. elegans* and was found to reside at the outer side of Golgi membranes (Rybakin, 2008; Rybakin et al., 2004). Coronin 7 does not interact with actin but rather with Src kinase and AP-1 (Rybakin et al., 2006; Rybakin et al., 2004). Coronin 7 is actually phosphorylated by Src kinase and this is required for the recruitment of coronin 7 from the cytosol to the Golgi membranes (Rybakin, 2008; Rybakin et al., 2004). Using RNAi, it has been shown that the depletion of coronin 7 resulted in arrest of protein export from the Golgi and in an altered Golgi morphology (Rybakin et al., 2006).

### **1.6.2.3 Role of coronin 1 in leukocytes**

Coronin 1, also called coronin 1A, TACO, p57, clabp or clipinA, is the best characterized mammalian coronin and was found to be exclusively expressed in cells of the hematopoietic system (Ferrari et al., 1999; Okumura et al., 1998; Suzuki et al., 1995). The coronin 1 WD40 domain was predicted to form a 7-bladed  $\beta$ -propeller (Gatfield et al., 2005) which was confirmed by determination of the crystal structure of coronin 1 (Appleton et al., 2006). Coronin 1 molecules are differentially phosphorylated *in vivo* and form trimers via the C-terminal coiled-coil domain (Gatfield et al., 2005; Kammerer et al., 2005). This trimerization is necessary for the association of coronin 1 with the plasma membrane and the actin cytoskeleton (see figure 16).

Coronin 1 is the closest homolog to *Dictyostelium* coronin and is recruited to nascent phagosome upon internalization by macrophages or neutrophils (Ferrari et al., 1999; Itoh et al., 2002; Schuller et al., 2001). The recruitment of coronin 1 to the phagosome was proposed to be mediated by its phosphorylation by protein kinase C (Itoh et al., 2002; Reeves et al., 1999). Coronin 1 was also shown to be exclusively retained by phagosomes containing pathogenic mycobacteria (Ferrari et al., 1999).



Transduction of dominant-negative fragments of coronin 1 into macrophage-like cell lines or human neutrophils led to the partial alteration of phagocytosis of opsonized red blood cells and a diminished chemotaxis (Yan et al., 2005; Yan et al., 2007). However, based on the study of Kupffer cells lacking naturally coronin 1 expression, macrophages expressing coronin 1 specific siRNA and macrophages from coronin 1 deficient mice it was concluded that coronin 1 does not play a role in F-actin dependent processes but rather in mycobacterial trafficking (Ferrari et al., 1999; Jayachandran et al., 2008; Jayachandran et al., 2007). It turned out that the coronin 1-mediated phagosomal arrest is caused by the activation of the phosphatase calcineurin, which is abolished in the absence of coronin 1 (Jayachandran et al., 2007).

Coronin 1 was also identified in neutrophils as a p40phox, a subunit of the NADPH complex, interacting partner (Grogan et al., 1997).

Coronin 1 expression is tightly regulated during T cell development and it accumulates at the immunological synapse (Das et al., 2002; Mueller et al., 2008; Nal et al., 2004). Studies using coronin 1 *-/-* mice revealed a dramatic depletion of peripheral T cells in the absence of coronin 1 while the development of T cells in the thymus was not affected (Foger et al., 2006; Mueller et al., 2008). Coronin 1 appeared to be necessary for the survival of T cells via inducing the mobilization of calcium from the intracellular stores after TCR triggering (Mueller et al., 2008). Coronin 1 was also recently proposed to be required for the development of autoimmune disease in animal models (Haraldsson et al., 2008).

## **1.7 Aim of the thesis**

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, is one of the most successful pathogens known today. One third of the global population is infected by *M. tuberculosis* however only a minority of them develops the disease. Healthy individuals can actually control the infection due to an efficient immune response.

Macrophages are phagocytic cells internalizing bacteria inside phagosome that are then maturing into lysosomes, vacuoles containing digestive enzymes, where the bacteria are degraded. Mycobacteria are also engulfed by macrophages but they can reside and survive intracellularly by preventing the fusion of their phagosomes with lysosomes. Pathogenic mycobacteria employ different strategies to subvert immune surveillance. The eukaryotic-like serine/threonine protein kinase G was found to be necessary for mycobacteria to block the phagosome maturation and was therefore defined as an essential virulence factor (Walburger et al., 2004). The goal of the first study was to investigate the influence of the mycobacterial trafficking on antigen presentation. To which organelles are PknG deficient mycobacteria directed inside professional Antigen Presenting Cells (APCs)? Are peptides from wild type or PknG deficient mycobacteria presented on Major Histocompatibility Complex (MHC) molecules with the same efficiency upon infection? Does the success of pathogenic mycobacteria in avoiding the immune system rely on an altered mycobacterial peptide presentation?

Mycobacteria take also advantage of host proteins to manipulate their trafficking inside phagocytic cells. A leukocyte specific protein, termed TACO or coronin 1, was described to be recruited upon mycobacteria internalization and to be actively retained only around phagosomes containing pathogenic mycobacteria (Ferrari et al., 1999). To better understand the role of coronin 1 in macrophages upon infection and in other leukocyte-specific functions, a mouse lacking coronin 1 expression was generated (Jan Massner thesis). The goal of the second part of this thesis was to investigate the activation of signaling pathways and the fate of mycobacteria inside coronin 1 deficient macrophages.

Coronin 1 is a member of the coronin protein family, of which up to 7 members are expressed in mammalian cells. Coronin 1 the closest homologue to *Dictyostelium* coronin that was described to influence several actin-dependent processes (de Hostos et al., 1993; Maniak et al., 1995). Coronin 1

was proposed to be required for chemotaxis and phagocytosis in human neutrophils (Yan et al., 2007) and to associate with cytosolic phospholipase C proteins (Grogan et al., 1997). The goal of the third study presented in this thesis was to analyze the role of coronin 1 in murine neutrophil functions.

Coronin 1  $-/-$  mice present a dramatic depletion of peripheral T lymphocytes due to a deficient cytosolic calcium mobilization upon T cell receptor activation (Mueller et al., 2008). B cells were not strongly affected in mice lacking coronin 1. However, given the analogy between T- and B cell receptor signaling, B cell function in the absence of coronin 1 was analyzed in the fourth part of this thesis.

To summarize, the studies presented in this thesis provide a better understanding on the interaction between mycobacteria and cells of the host immune system. This might help in the development of more efficient vaccines against tuberculosis. These studies should also allow us to better define the versatile roles of coronin 1 in leukocytes.

## 2 MATERIALS AND METHODS

### 2.1 Chemicals and reagents

Acetic acid	Merck
Acrylamide	BioRad
Agarose	Eurogentec
Alkaline phosphatase yellow liquid substrate (pNPP)	Sigma
Aluminum hydroxide gel	Sigma
Ammonium chloride (NH <sub>4</sub> Cl)	Fluka
Ammonium persulfate (APS)	BioRad
BAPTA-AM	Molecular Probes
BCA	Pierce
β-mercaptoethanol	Sigma
Bisacrylamide	Serva
Boric acid	Merck
Bovine serum albumin (BSA)	Equitec-Bio
Bradford	BioRad
Bromophenol blue	Merck
Calcein AM	Molecular Probes
Calcimycin	
CpG DNA	Microsynth
Cyclosporin A	Novartis
Dextran	Amersham
Dihydrorhodamine 1,2,3	Molecular Probes
Disodium hydrogen phosphate (Na <sub>2</sub> HPO <sub>4</sub> )	Merck
Dimethylsulfoxide (DMSO)	Fluka
Dithiothreitol (DTT)	Sigma
dNTPs	Roche
ECL	Amersham
ECL+	Pierce
Ethidium bromide	Sigma
Ethylenediamine tetraacetate sodium salt (EDTA)	Fluka

Fibronectin	Sigma
Ficoll	Amersham
Fluo-3-AM	Molecular Probes
FluoroGuard antifade reagent	BioRad
Glycerol	Fluka
Glycine	Fluka
Horseradish peroxidase particles	Sigma
Hydrochloric acid	Merck
Hyflo Super powder	Fluka
Interleukin 4 (IL4)	RD Systems
Interleukin 8 (IL8)	RD Systems
Isobutanol	Merck
Isopropanol	Merck
Lipopolysaccharide (LPS)	Sigma
Lipoteichoic acid	Sigma
Low-Tox M rabbit complement	Cedarlane
Methanol	Merck
Middlebrook 7H9 Broth	Difco
Microscint 40	Packard
Milk powder	Hochdoef
Monopotassium phosphate (KH <sub>2</sub> PO <sub>4</sub> )	Merck
Mycobacteria 7H11 Agar	Difco
N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)	Sigma
Nycodenz	Axis Shield
OCT Compound	Tissue-Tek
O-dianisidine	Sigma
PAM3Cys	EMC Microcollections
Paraformaldehyde	Fluka
Peptidoglycan	Fluka
Percoll	Amersham
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Phorbol-12-myristate-13-acetate (PMA)	Sigma
P-nitrophenyl-N-acetyl-β-D-glucosaminidine (PNP)	Sigma
Polylysine	Sigma
Ponceau S solution	Sigma

Potassium bicarbonate (KHCO <sub>3</sub> )	Sigma
Potassium chloride (KCl)	Merck
Potassium permanganate	Merck
Protease inhibitor	Roche
Proteinase K	Roche
Saponin	Sigma
Sodium acetate (NaAc)	Fluka
Sodium azide (NaN <sub>3</sub> )	Sigma
Sodium chloride (NaCl)	Merck
Sodium dodecylsulfate (SDS)	BioRad
Sodium hydroxide (NaOH)	Merck
Sucrose	Fluka
Taq DNA Polymerase	Sigma
TB Auramine-Rhodamine T solution	BD
N,N,N',N'-tetramethylethylenediamine (TEMED)	Serva
Thioglycolate	Sigma
TNP-BSA	Biosearch Technologies
TNP-Ficoll	Biosearch Technologies
TNP-OVA	Biosearch Technologies
Triethanolamine	Fluka
Tritiated thymidine	Amersham
Triton X-100	Roche
Trizma base	Sigma
Trypan blue	Sigma
Trypsin	Calbiochem
Trypsin inhibitor	Calbiochem
Tumor Necrosis Factor alpha (TNF $\alpha$ )	RD Systems
Tween-20	Fluka
Tween-80	Fluka
Xylene cyanol	Sigma
Zymosan A	Sigma

## 2.2 Buffers and solutions

Ammonium Chloride Potassium (ACK)	0.15 M NH <sub>4</sub> Cl
Lysis buffer (pH 7.2)	10 mM KHCO <sub>3</sub> 0.1 mM EDTA filter sterile (0.22 μm)
Density Gradient Electrophoresis (DGE) buffer (pH 7.4)	10 mM triethanolamine 10 mM acetic acid 1 mM EDTA 0.25 M sucrose
DNA loading buffer (6x)	0.25 % bromophenol blue 0.25 % xylene cyanol 1 mM EDTA pH 8 30 % glycerol
ELISA binding buffer	PBS containing 4 % BSA, 0.1 % Tween-20 and 10 mM NaN <sub>3</sub>
ELISA washing buffer	PBS containing 0.05 % Tween-20
FACS buffer	PBS containing 2 % FBS
Hypotonic buffer (pH 7.4)	0.83 % NH <sub>4</sub> Cl 10 mM Hepes-NaOH pH 7.0
PBS (pH 7.2)	137 mM NaCl 2.7 mM KCl 8 mM Na <sub>2</sub> HPO <sub>4</sub> 1.5 mM KH <sub>2</sub> PO <sub>4</sub>
PBST (pH 7.2)	PBS containing 0.2 % Tween-20

Paraformaldehyde (3 %)	Dissolve while stirring and heating at 60°C 3 g of PFA in 90 ml distilled water containing 30 $\mu$ l 1 M NaOH. Let the solution cool down and add 10 ml of 10x PBS. Check the pH (7.2)
PNP solution	4 mM P-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminidine dissolved at 50°C in 0.2 M sodium acetate (pH 5.4)
SDS-PAGE running buffer	25 mM Trizma base 0.19 M glycine 0.1 % SDS
SDS-PAGE transfer buffer	48 mM Trizma base 39 mM glycine 0.0375 % SDS 20 % methanol
SDS-SB (5x)	300 mM Tris-HCl (pH 6.8) 500 mM DTT 10 % SDS 20 % glycerol 0.1 % bromophenol blue
Stripping buffer	62.5 mM Tris-HCl (pH 6.8) 2 % SDS 100 mM $\beta$ -mercaptoethanol
Tail buffer	100 mM Tris-HCl (pH 8.5) 200 mM NaCl 5 mM EDTA 0.2 % SDS
TB Decolorizer	0.5 % hydrochloric acid 70 % isopropanol

TB Potassium Permanganate	0.5 % potassium permanganate
TBE (0.5x, pH 8.3)	0.54 % Trizma base 0.275 % boric acid 0.0372 % Na <sub>2</sub> EDTA
TX-100 buffer	50 mM Tris-HCl (pH 7.5) 137 mM NaCl 2 mM EDTA 10 % glycerol 1 % Triton X-100

### **2.3 Media and supplements**

7H9 medium	0.52 % Middlebrook 7H9 Broth 0.28 % glycerol 0.056 % Tween-80
7H11 plates	2.1 % Mycobacteria 7H11 Agar 0.5 % glycerol 10 % OADC
β-mercaptoethanol (50 mM, 1000x)	Gibco
DMEM	Sigma
Glutamax DMEM	Gibco
FBS	PAA Laboratories
FBS ultra-low IgG	Gibco
HBSS	Gibco
Calcium-free HBSS	Gibco
Hepes (1 M, 100x)	Gibco
IMDM	Sigma
Kanamycin (25 mg/ml, 1000x)	Sigma
L-glutamine (200 mM, 100x)	Gibco
OADC	home-made
PBS	Sigma
Penicillin/Streptomycin (100x)	Sigma

RPMI 1640	Sigma
Sodium pyruvate (100 mM, 100x)	Sigma
Trypsin-EDTA in HBSS	Gibco

## 2.4 Oligonucleotides

Name	Sequence (5' – 3')	Source of sequence	Application
WTCor1	CTG TTG TAG GGG CTG ATG GT	murine coronin 1 locus: bp 229 - 210	PCR genotyping of coronin 1 -/- mice
WTCor2	CAC TGG CCT CAC AGA TCA GA	murine coronin 1 locus: bp 143 - 162	PCR genotyping of coronin 1 -/- mice
KOCor1	CTT CAT GTG GTC GGG GTA G	pEGP-N2: bp 904 - 922	PCR genotyping of coronin 1 -/- mice
IFNGWT1	ACA GAT GCA ACG GTT TCC AC	murine IFN $\gamma$ R locus: bp 14163 - 14182	PCR genotyping of IFN $\gamma$ R -/- mice
IFNGWT2	CTT CTC CGA GGA AAG GGA CT	murine IFN $\gamma$ R locus: bp 14554 - 14535	PCR genotyping of IFN $\gamma$ R -/- mice
IFNGKO1	CCA CAC GCG TCA CCT TAA TA	pMC1neopA: bp 701 - 682	PCR genotyping of IFN $\gamma$ R -/- mice

## 2.5 Antibodies and dyes

Name	Isotype	Antigen	Source	Application (dilution)
1D4B	Rat IgG <sub>2a</sub>	LAMP-1	Developmental Studies Hybridoma Bank, University of Iowa	IF (1:40)
4G10	Mouse monoclonal	P-Tyrosine	Upstate	WB (1:1000)
antiserum 1002	Rabbit polyclonal	GST-coronin 1	(Gatfield et al., 2005)	WB (1:10,000) IF (1:500)
anti-actin (MAB1501)	Mouse monoclonal IgG <sub>1κ</sub>	F- and G-actin	Chemicon International	WB (1:1000) IF (1:50)
anti-B220-PE	Rat monoclonal IgG <sub>2a</sub>	B220	BD Pharmingen	FACS (1:200)
anti-BCG	Rabbit polyclonal	<i>M. bovis</i> BCG	DakoCytomation	IF (1:20,000)
anti-CD3-APC	Hamster monoclonal IgG <sub>1</sub>	CD3	BD Pharmingen	FACS (1:200)
anti-CD4	Rat monoclonal IgM	CD4	Supernatant from RL172 hybridoma	T cell depletion
anti-CD4-PECy7	Rat monoclonal IgG <sub>2a</sub>	CD4	BD Pharmingen	FACS (1:200)
anti-CD8	Rat monoclonal IgM	CD8	Supernatant from 31M hybridoma	T cell depletion
anti-CD8-APC	Rat monoclonal IgG <sub>2a</sub>	CD8	BD Pharmingen	FACS (1:200)
anti-CD11b-PECy7	Rat monoclonal IgG <sub>2b</sub>	CD11b	BD Pharmingen	FACS (1:200)
anti-CD11b-APC	Rat monoclonal IgG <sub>2b</sub>	CD11b	BD Pharmingen	FACS (1:200)
anti-CD19-PE	Rat monoclonal IgG <sub>2a</sub>	CD19	BD Pharmingen	FACS (1:200)
Name	Isotype	Antigen	Source	Application

				<b>(dilution)</b>
anti-CD40	Rat monoclonal IgG <sub>2a</sub>	CD40	Serotec	Stimulation
anti-IgA	Goat polyclonal	IgA	Southern Biotech	ELISA
anti-IgA-AP	Goat polyclonal	IgA	Southern Biotech	ELISA
anti-IgD	Rat polyclonal	IgD	BD,obtained from Daniela Finke, DKBW, Basel	IF (1:4000)
anti-IgG	Goat polyclonal	IgG	Southern Biotech	ELISA
anti-IgG-AP	Goat polyclonal	IgG	Southern Biotech	ELISA
anti-IgM	F(ab') <sub>2</sub> fragment	IgM, $\mu$ chain	Jackson ImmunoResearch	Stimulation
anti-IgM	Goat polyclonal	IgM	Southern Biotech	ELISA
anti-IgM-AP	Goat polyclonal	IgM	Southern Biotech	ELISA
anti-I $\kappa$ B $\alpha$	Rabbit polyclonal	I $\kappa$ B $\alpha$	Cell Signaling Technology	WB (1:1000)
anti-P-I $\kappa$ B $\alpha$	Rabbit polyclonal	P-I $\kappa$ B $\alpha$	Cell Signaling Technology	WB (1:1000)
anti-Ly-6G-biotin	rat monoclonal IgG <sub>2b</sub>	Ly-6G	BD Pharmingen	FACS (1:200)
anti-NIMP-R14	Rat monoclonal IgG <sub>2b</sub>	murine neutrophils	Santa Cruz	IF (1:50)
anti-p38	Rabbit polyclonal	p38 MAPK	Cell Signaling Technology	WB (1:1000)
anti-P-p38	Rabbit polyclonal	P-p38 MAPK	Cell Signaling Technology	WB (1:1000)
anti-p44/42	Rabbit polyclonal	ERK 1/2	Cell Signaling Technology	WB (1:1000)
anti-P-p44/42	Rabbit polyclonal	P-ERK 1/2	Cell Signaling Technology	WB (1:1000)
anti-PLC $\gamma$ 2	Rabbit polyclonal	PLC $\gamma$ 2	Cell Signaling Technology	WB (1:500)
<b>Name</b>	<b>Isotype</b>	<b>Antigen</b>	<b>Source</b>	<b>Application</b>

				<b>(dilution)</b>
anti-P-PLC $\gamma$ 2	Rabbit polyclonal	P-PLC $\gamma$ 2	Cell Signaling Technology	WB (1:500)
anti-SAPK/JNK	Rabbit polyclonal	SAPK/JNK	Cell Signaling Technology	WB (1:1000)
anti-P-SAPK/JNK	Rabbit polyclonal	P-SAPK/JNK	Cell Signaling Technology	WB (1:1000)
anti-tubulin (clone E7)	Mouse monoclonal IgG <sub>1</sub>	tubulin	Developmental Studies Hybridoma Bank, University of Iowa	IF (1:4000)
anti-mouse Alexa 488	Goat polyclonal	Mouse IgG (H+L)	Molecular Probes	IF (1:200)
anti-rabbit Alexa 488	Goat polyclonal	Rabbit IgG (H+L)	Molecular Probes	IF (1:200)
anti-rabbit Alexa 633	Goat polyclonal	Rabbit IgG (H+L)	Molecular Probes	IF (1:200)
anti-rat Alexa 568	Goat polyclonal	Rat IgG (H+L)	Molecular Probes	IF (1:200)
anti-rat Cy3	Goat polyclonal	Rat IgG	Obtained from Daniela Finke, DKBW, Basel	IF (1:200)
anti-mouse-HRP	Goat polyclonal	Mouse IgG (H+L)	Southern Biotech	WB (1:20,000)
anti-rabbit-HRP	Goat polyclonal	Rabbit IgG (H+L)	Southern Biotech	WB (1:20,000)
7-AAD	-	-	BD Pharmingen	FACS (1:20)
Annexin V-APC	-	-	BD Pharmingen	FACS (1:20)
Biotinylated Peanut Agglutinin	-	-	BD,obtained from Daniela Finke, DKBW, Basel	IF (1:2000)
<b>Name</b>	<b>Isotype</b>	<b>Antigen</b>	<b>Source</b>	<b>Application (dilution)</b>
Streptavidin	-	biotin	BD,obtained from	IF (1:1000)

Alexa 488			Daniela Finke, DKBW, Basel	
Streptavidin-PE	-	biotin	BD Pharmingen	FACS (1:400)
Phalloidin 488	-	F-actin	Molecular Probes	IF (1:40) FACS (1:50)

## 2.6 *Mycobacteria and mammalian cells*

Name	Source	Culture medium	Application
<i>M. bovis</i> BCG WT	(Walburger et al., 2004)	7H9 + 10 % OADC	Montreal strain expressing GFP used for infection
<i>M. bovis</i> BCG $\Delta$ <i>pkng</i>	(Walburger et al., 2004)	7H9 + 10 % OADC	Montreal strain lacking <i>pkng</i> and expressing GFP used for infection

Name	Source	Culture medium	Cell type
Bone marrow derived macrophages	BALB/c or C57BL/6 mice	DMEM 10 % heat-inactivated FBS 30 % L929 supernatant 2 mM L-glutamine 50 $\mu$ M $\beta$ -mercaptoethanol	murine macrophages obtained after 5-day differentiation of bone marrow cells
Bone marrow derived dendritic cells	BALB/c mice	RPMI 1640 10 % heat-inactivated FBS 2 ng/ml M-CSF 2 mM L-glutamine 50 $\mu$ M $\beta$ -mercaptoethanol	murine dendritic cells obtained after 7-day differentiation of bone marrow cells
L929	(Wiltshcke et al., 1989) obtained from M. Kopf, Zürich, Switzerland	DMEM 10 % heat-inactivated FBS 2 mM L-glutamine 50 $\mu$ M $\beta$ -mercaptoethanol	fibroblasts producing GM-CSF
RL172	(Ceredig and Rolink, 2002) obtained from Ton Rolink, DKBW, Basel	Glutamax DMEM 10 % FBS ultra-low IgG 50 $\mu$ M $\beta$ -mercaptoethanol 1 mM sodium pyruvate penicillin/streptomycin	hybridoma producing anti-CD4
31M	(Ceredig and Rolink, 2002) obtained from Ton Rolink, DKBW, Basel	Glutamax DMEM 10 % FBS ultra-low IgG 50 $\mu$ M $\beta$ -mercaptoethanol 1 mM sodium pyruvate penicillin/streptomycin	hybridoma producing anti-CD8

## **2.7 Cell culture methods**

### **2.7.1 Determination of cell numbers**

To determine the cell numbers, 10  $\mu$ l of a cell suspension was mixed with 90  $\mu$ l of Trypan blue solution (0.4%) in a 96-well plate and a droplet was transferred onto the extremity of a Neubauer chamber mounted with a coverslip. The viable cells, i.e. cells that are not stained with the blue dye, present in the 16 small squares were counted 4 times (upper left, upper right, lower right and lower left quadrant) and the number of cells per ml was calculated by dividing the counted value by 4 and multiplying by  $10^5$ .

### **2.7.2 Freezing and thawing of cells**

For long-term storage of cell lines, cells in culture were harvested and pelleted for 10 min at 200  $\times$  g. They were then resuspended in freezing medium (FBS containing 10% DMSO) and aliquoted into cryotubes (1 ml corresponding to 5-10 million cells) that were then put into Cryo Freezing container (Nalgene) overnight at  $-80^{\circ}\text{C}$ . The tubes could afterwards be transferred into a liquid nitrogen tank.

For thawing of cells, the tubes were removed from the liquid nitrogen tank and rapidly warmed up to  $37^{\circ}\text{C}$  in a water bath. The cells were transferred into a 15 ml Falcon tube containing 10 ml of prewarmed medium and centrifuged for 5 min at 200  $\times$  g. The cell pellet was resuspended in 1 ml medium and added to the appropriate cell culture flask containing prewarmed medium. The cells were splitted within the next 2 days.

### **2.7.3 Preparation of L929 medium**

A dish containing L929 cells in culture was washed 3 times with sterile PBS and 5 ml of Trypsin-EDTA in HBSS was added to the cells. The dish was incubated for 5 min at  $37^{\circ}\text{C}$  and the cells were then pipetted into a 15 ml tube containing 10 ml of L929 medium (DMEM with 10% FBS, 2 mM L-glutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol). The cells were centrifuged for 5 min at 200  $\times$  g, resuspended in 10 ml L929 medium and counted. Ten million L929 cells were plated into a 15 cm diameter cell culture dish with 100 ml of medium. After one week incubation at  $37^{\circ}\text{C}$ , the

supernatant was harvested and 100 ml of fresh medium was added to the cells. This procedure was repeated up to 4 times and the supernatants were sterile filtered (0.22  $\mu\text{m}$ ) and stored at 4°C.

L929 supernatant, which contains GM-CSF, was then used to prepare the bone marrow macrophages medium.

#### **2.7.4 Preparation of anti-CD4 and anti-CD8 cell supernatant**

Anti-CD4 (RL172) and anti-CD8 (31M) hybridomas were plated into 45 cm<sup>2</sup> T flasks containing 20 ml of hybridoma medium (Glutamax DMEM supplemented with 10 % FBS ultra-low IgG, 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, 1 mM sodium pyruvate and penicillin/streptomycin). The cells were then transferred to a 150 cm<sup>2</sup> T flask already containing 80 ml of medium and after 3-4 days of culture to a roller bottle with a final volume of 500 ml. After around 2 weeks of incubation, the medium turned yellow-orange and more than 90% of the cells were dead as analyzed by Trypan blue exclusion. The supernatant was passed through a 0.22  $\mu\text{m}$  filter filled with Hyflo Super powder (a celite based filter aid) and 40 ml aliquots were stored at -20°C. Each batch was titrated to be used for T cell depletion of the splenocytes; reproducibly 5 ml of each supernatant was enough to deplete T cells from 100 million splenocytes.

#### **2.7.5 Mycoplasma detection**

Mycoplasma are common cell culture contaminants and since they compete with the cells for the nutrients one of the contamination sign is the reduction of cell proliferation. To test the presence of mycoplasma, cell cultures were routinely assayed with the Mycoalert kit (Cambrex), which is a test that exploits the activity of certain mycoplasma enzymes. Briefly, the viable mycoplasma are lysed and the enzymes react with Mycoalert Substrate catalyzing the conversion of ADP to ATP. The level of ATP is measured with a luciferase assay. Mycoalert Reagent and Mycoalert Substrate were reconstituted by addition of Mycoalert Assay Buffer and equilibrated at room temperature for 15 min. Two ml supernatant of cells, which were cultured without antibiotics for at least 2 days, was centrifuged 5 min at 300 x g and 100  $\mu\text{l}$  of the cleared supernatant was transferred into a luminescence 96-well plate (white-walled plate, Greiner). One hundred  $\mu\text{l}$  of Mycoalert Reagent was added and the luminescence was read (reading A) in a luminometer (Biotek) after 5 min incubation. One hundred  $\mu\text{l}$  of Mycoalert Substrate was added and the luminescence was measured

again (reading B) after 10 min. Cells which are infected with Mycoplasma produce ratio (reading B / reading A) greater than 1.

## 2.8 Animal experiments

### 2.8.1 Genotyping of coronin 1 <sup>-/-</sup> mice

The coronin 1 <sup>-/-</sup> mice used in the present study were backcrossed 5 to 8 times to C57/BL6 mice and originated from either homozygous breedings (for the backcross 8 mice) or heterozygous breedings (for the backcross 5 mice). In that case, the mice had to be genotyped before use. To do so, a small piece of the tail of the 3-5 week old mice was cut and the mice were simultaneously earmarked for further identification. The tail pieces were digested in 500 µl of tail buffer containing 100 µg/ml Proteinase K overnight at 55°C in an Eppendorf Thermomixer, 600 rpm. The tubes were then spun down at full speed (16,000 x g) in a table top centrifuge for 5 min and 10 µl of the supernatant was diluted 10 x in distilled water to obtain the DNA template. The PCR was set up by adding 1 µl of DNA template to 19 µl of MasterMix into 8-tubes PCR strips.

		MM (20)
DNA template	1 µl	
10x PCR buffer	2 µl	40 µl
distilled H <sub>2</sub> O	15.2 µl	304 µl
10 mM dNTPs	0.4 µl	8 µl
Primer 1 (WTCor1, 10 µM)	0.4 µl	8 µl
Primer 2 (WTCor2, 10 µM)	0.4 µl	8 µl
Primer 3 (KOCor1, 8 µM)	0.4 µl	8 µl
Taq DNA Polymerase (5 U/µl)	0.2 µl	4 µl

The tubes were briefly vortexed and transferred into a Biometra Thermocycler to run the following program:

1x	96°C	5 min
34x	95°C	30 sec
	56°C	30 sec
	72°C	45 sec

1x	72°C	10 min
1x	4°C	pause

At the end of the PCR, 4  $\mu$ l of 6x DNA loading buffer was added to each tube that was subsequently vortexed. 10  $\mu$ l of each sample was loaded on a 1.8% agarose gel containing ethidium bromide (made in 0.5x TBE) and the PCR products were separated by electrophoresis for 1 hour at 80V. The DNA from wild type animals gave a single at 392 bp, the heterozygous 2 bands at 392 bp and 478 bp, and the coronin 1  $-/-$  a single band at 478 bp.

### **2.8.2 Preparation of bone marrow derived macrophages**

BALB/c mice, C57BL/6 mice or coronin 1  $-/-$  mice were euthanized and the femur was removed and cleaned from muscles. The bones were sprayed with ethanol, opened on one side and put inside a cut yellow tip (the open part of the bone facing down). The tip was transferred into a 5 ml tube containing 500  $\mu$ l of bone marrow macrophage medium (DMEM with 10 % heat-inactivated FBS, 30 % L929 supernatant, 2 mM L-glutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol) and spun down at 800  $\times$  g for 10 min at room temperature. The tip was then removed and the bone marrow, which has been pushed out of the bone during the centrifugation, was resuspended and added into a 15 cm diameter Teflon dish (Rowatec) with 30 ml medium. The bone marrow derived macrophages were differentiated and usually used after 5 days but could be kept until day 7.

### **2.8.3 Preparation of bone marrow derived dendritic cells**

Bone marrow from BALB/c mice was obtained the same way as for the bone marrow derived macrophages. It was then resuspended and splitted into Optilux dishes (5 dishes for one bone) containing 10 ml of bone marrow dendritic cell medium (RPMI 1640 with 10 % heat-inactivated FBS, 2 ng/ml M-CSF, 2 mM L-glutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol). The dishes were then incubated for 7 days at 37°C to allow the precursor cells to differentiate into bone marrow derived dendritic cells.

#### **2.8.4 Purification of splenic B cells by T cell depletion**

Spleens from wild type and coronin 1 <sup>-/-</sup> mice were dissected and single cell suspension was prepared by placing the spleen in a cell culture dish and mashing it between two nylon nets using the plunger of a 2 ml syringe. The splenocytes were then centrifuged at 300 *x g* and resuspended into 1 ml of ACK lysis buffer to lyse the erythrocytes for 1 min at room temperature. The cells were then washed 3 times with RPMI-FBS (5%) and resuspended into anti-CD4/anti-CD8 medium (for 100 million splenocytes, 5 ml of both anti-CD4 and anti-CD8 supernatants). After 30 minutes incubation on ice to allow binding of the antibodies on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the excess of antibody was removed and the splenocytes washed once with RPMI-FBS (5%) and once with RPMI. They were then resuspended into RPMI medium containing Low-Tox M rabbit complement (for 100 million cells, 10 ml RPMI and 1 ml complement) and incubated for 45 min at 37°C to allow lysis of the T cells. The cells were then washed in RPMI-FBS (5%) and the quality of the T cell depletion was analyzed by staining the total splenocytes and the purified cells for CD19, CD4 and CD8 and analyzing them by flow cytometry.

#### **2.8.5 Isolation of peripheral neutrophils**

For isolation of peripheral neutrophils, blood was collected into EDTA containing tubes (50 µl of 2% EDTA in saline solution) by eye bleeding of 2-4 month old wild type and coronin 1 <sup>-/-</sup> mice. Briefly, mice were anesthetized in a chamber containing dry ice and around 500-700 µl of blood was collected by disruption of the orbital sinus. The death of the mice was then ensured by cervical dislocation. The blood containing tubes were inverted several times to avoid coagulation. The blood samples from the wild type and coronin 1 <sup>-/-</sup> mice were pooled in 2 different 15 ml polypropylene tubes and the leukocytes were extracted by dextran (2%) sedimentation of the erythrocytes for 1 hour at room temperature. The leukocyte-rich plasma was then layered onto a Nycodenz gradient according to the manufacturer instructions (0.25 vol. of 20% Nycodenz + 0.5 vol. of Nycodenz 1.077A + 1 vol. of leukocyte-rich plasma) and centrifuged at 600 *x g* for 20 min at room temperature. The neutrophils were harvested from the lower interface and the residual erythrocytes were lysed by hypotonic shock (5 min at room temperature in lysis buffer: 0.83% NH<sub>4</sub>Cl, 10 mM Hepes-NaOH, pH 7.0).

### **2.8.6 Isolation of bone marrow neutrophils**

For preparation of bone marrow neutrophils, mice were sacrificed in CO<sub>2</sub> chamber and both femur and tibia were removed. The bones were chopped on one side and put in a cut yellow tip. This yellow tip was transferred into a 5 ml tube containing 200 µl of HBSS and centrifuged at 800 *x g* for 10 min at room temperature. The bone marrow cells were resuspended, pooled and centrifuged for 5 min at 300 *x g*. They were then resuspended into 10 ml of hypotonic buffer to lyse red blood cells for 1 min by inverting the tube several times. The leukocyte preparation was then washed 3 times in HBSS, layered on a Percoll gradient (75%-65%-52%, equal volume) and centrifuged for 30 min at 1500 *x g* with low brake. The upper interface mainly contains lymphocytes as checked by flow cytometry and the lower interface, where the neutrophils are concentrated, was collected. The neutrophils were then washed once in HBSS and the quality of the preparation was routinely controlled by flow cytometry and immunofluorescence.

### **2.8.7 Blood collection and serum preparation**

To prepare serum, mice were placed briefly under a red warming lamp and the murine blood was obtained into a 1.5 ml Eppendorf tube by cutting the tail vein with a scalpel. The blood was allowed to coagulate overnight at 4°C and then centrifuged 10 min at 20,000g. The serum was then collected and analyzed by ELISA or stored at -80°C.

### **2.8.8 Thymus independent immunization of mice**

To analyze the immune response of mice upon encounter with T independent antigens, mice were intraperitoneally injected with TNP-Ficoll. TNP-Ficoll was dissolved in sterile saline solution at 0.1 mg/ml and 500 µl (corresponding to 50 µg) was injected using a 27G needle. Saline solution was used for the negative control mice. Blood samples were taken at days 5, 9, 12, 26 and 33 after immunization and the antibody titers in the serum were evaluated. The mice were euthanized at the end of the experiment in a CO<sub>2</sub> chamber.

### **2.8.9 Thymus dependent immunization of mice**

To study the immune response of mice upon encounter with T dependent antigens, mice were injected in the footpad with TNP-OVA. The immunogen was dissolved in saline solution at 2.5 mg/ml and then mixed with aluminum hydroxide gel at room temperature during half an hour to obtain a final concentration of 1 mg/ml. 30  $\mu$ l corresponding to 30  $\mu$ g of TNP-OVA was injecting per footpad and only alum adjuvant was used for the negative control mice. After 14-week immunization, mice were boosted by injection of 30  $\mu$ g of TNP-OVA in alum adjuvant per footpad. The immune response was followed by taking blood samples on a weekly basis, preparing serum and determining the antibody contents by ELISA. A group of mice was sacrificed after one-week immunization and the other ones 2 weeks after the reboost.

### **2.8.10 Leukocyte recruitment into the peritoneum**

Wild type and coronin 1 deficient mice were intraperitoneally injected (27G needle) with 500  $\mu$ l of sterile PBS or thioglycolate (3.85%). After 4 hours, the mice were sacrificed and the leukocytes recruited into the peritoneal cavity were harvested with 5 ml of RPMI-FBS (10%)-EDTA (0.5mM). The cell numbers were determined using a Neubauer counting chamber; they were then stained for CD11b and Ly6G and analyzed by FACS.

### **2.8.11 Analysis of peripheral blood composition**

To determine the number of neutrophils present in the blood of wild type and coronin 1  $-/-$  mice, tail blood was drawn into EDTA containing tubes (Microvette 500, Sarstedt), the volume was measured and the blood was diluted to 500  $\mu$ l using saline solution. The sample was then analyzed on a fully automated ADVIA hematology system (Bayer) and the number of neutrophils per  $\mu$ l of blood was calculated taking into account the dilution factor.

## **2.9 Flow cytometry**

### **2.9.1 Staining of cells for FACS analysis**

For flow cytometric analysis, cell suspensions were adjusted to 10 millions per ml and 100  $\mu$ l transferred into a round-bottom 96-well plate to obtain 1 million cells per staining. The plate was centrifuged for 3 min at 300  $\times$  g, 4°C, and the medium was discarded by inverting the plate. The cells were washed in FACS buffer (PBS containing 2 % FBS) and then resuspended in 100  $\mu$ l of FACS buffer containing the FACS antibody, usually at a 1:200 dilution. The plate was incubated on ice in the dark and after 30 min 100  $\mu$ l of FACS buffer was added into each well. The plate was then centrifuged and the cells washed twice in FACS buffer. When biotinylated antibodies were used, a second incubation step was included where conjugated streptavidin (1:400) was added to the cells for 20 min. The cells were then washed, resuspended into 200  $\mu$ l of FACS buffer and transferred into FACS tubes (Matrix). The samples were then analyzed on a FACSCalibur (BD) and with the software FlowJo (4.3.2).

### **2.9.2 Determination of phagocytosis efficiency of neutrophils**

Neutrophils were incubated in suspension with opsonized yellow-green fluorescent polystyrene 1  $\mu$ m beads for 45 min at 4°C or 37°C and the tubes were periodically inverted. The cells were then transferred onto ice to stop phagocytosis, washed with ice-cold PBS-FBS (2%) 5 times in large volume (15 ml), resuspended in 500  $\mu$ l PBS-FBS (2%) and analyzed by flow cytometry. The number of beads taken up by the wild type and coronin 1  $-/-$  neutrophils was determined by measuring the fluorescence intensity (channel 2) of the cells that underwent phagocytosis.

### **2.9.3 Analysis of NADPH oxidase activity**

Wild type and coronin 1 deficient neutrophils were purified from the bone marrow and resuspended in RPMI-FBS (10%) at a density of 1 million cells / ml. One million neutrophils were then treated either with DMSO (10  $\mu$ l) or with PMA (100 nM and 1000 nM) for 30 min at 37°C. Dihydrorhodamine 1,2,3 (2  $\mu$ M) was then added to the cells for 15 min at 37°C. The cells were then washed 3 times with PBS-FBS (2%), resuspended in 500  $\mu$ l and analyzed on a FACSCalibur

instrument. To determine the NADPH activity, the fluorescence of the cells was measured in channel 2.

## **2.9.4 Calcium measurements**

Single cell suspensions from wild type and coronin 1 *-/-* spleen were prepared and the erythrocytes were lysed using ACK buffer. The cells were then resuspended into RPMI without phenol red supplemented with 3% FBS and 10 mM Hepes and loaded with Fluo-3-AM (4  $\mu$ M) by incubating them for 45 min at 37°C in the dark. The cells were washed twice and incubated 30 min on ice in medium containing anti-CD3-APC (1:200) and anti-CD11b-APC (1:200). The cells were washed 3 times to remove the unbound antibodies and 1 ml aliquots corresponding to 3 million cells were prepared. Before measurement, the cells were equilibrated for 10 min at 37°C, a 30-second baseline was acquired and a stimulus was added. The levels of intracellular calcium were recorded for 5 min on the FL-2 channel of a FACSCalibur instrument (BD) after gating on the APC-negative cells.

Alternatively, to distinguish between calcium release from the intracellular stores and calcium entry through the plasma membrane, T cell depleted splenocytes were prepared as previously described and loaded with Fluo-3-AM. The cells were then washed in calcium-free HBSS and resuspended to obtain 1 million cells per ml. After 10 min prewarming of 1 million cells, a one-minute baseline was recorded, the stimulus was added in the absence of calcium for 9 min and calcium was then added to a final concentration of 1 mM. The calcium fluxes were recorded on the FL-2 channel on a FACSCalibur instrument (DB).

## **2.10 Microscopy**

### **2.10.1 Staining of leukocytes for immunofluorescence analysis**

The cells adhered on teflon-coated microscopy glass slides were fixed by incubating them in 3% paraformaldehyde in PBS for 10 min at 37°C. The slides were further washed at room temperature for 5 min in PBS, 5 min in PBS containing 5 mM Glycine to quench the remaining PFA and finally 5 min in PBS. The cells were permeabilised by incubating them 15 min in PBS containing 0.1% saponin (PBS-SAP) and then blocked 15 min in PBS containing 0.1% saponin and 2% BSA (PBS-SAP-BSA). Slides were put inside a wet chamber and 25  $\mu$ l of the first antibody diluted in PBS-SAP-BSA was added into each well. After a 30 min incubation, the slides were washed in jars

containing PBS-SAP-BSA (5 min), PBS-SAP (2 x 5 min) and PBS-SAP-BSA (15 min). The slides were transferred back inside the wet chamber and the secondary antibody (40 µl per well) diluted in PBS-SAP-BSA was added for 20 min. The slides were subsequently washed in PBS-SAP-BSA (5 min), PBS-SAP (2 x 5 min), PBS (3 x 5 min), the surrounding of the wells were dried using a cotton stick, a droplet of antifade reagent was added on the top of each well, a coverslip was applied onto the slide and sealed with nail polish. The slides were then stored at 4°C or immediately analyzed using a confocal laser scanning microscope (LSM510 Meta, Zeiss).

Alternatively, the slides were fixed with methanol for 4 min at -20°C. They were then washed with PBS (3 x 5 min) and blocked for 15 min at room temperature in PBS-FBS (5%). First antibody diluted in blocking solution was added for 30 min onto slides in wet chamber. The slides were washed in PBS (2 x 5 min), in blocking solution (15 min) and the slides were incubated with the secondary antibody for 20 min. They were then washed with PBS (3 x 5 min), mounted with antifade reagent and analyzed using a confocal microscope.

### **2.10.2 Mycobacterial infection and confocal laser scanning microscopy**

Dendritic cells, at day 7 of culture, were incubated with GFP-expressing wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$  ( $OD_{600\text{ nm}} = 0.1$ ) in the cell culture dishes for 3 hours at 37°C. For subsequent staining, adherent and non-adherent dendritic cells were harvested, resuspended in PBS, and transferred onto Polylysine-coated glass slides. Following incubation for 20 min on ice to allow the cells to adhere to the slides, cells were fixed in methanol (4 min at -20°C) and stained for LAMP-1 using a mouse anti-rat IgG (H + L)-Alexa 568 as a secondary antibody.

Infection of 5-day old macrophages with GFP-expressing wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$  ( $OD_{600\text{ nm}} = 0.1$ ) was performed directly onto the glass slide after letting the macrophages adhere for 1 hour. After a 3-hour incubation, cells were washed, fixed with methanol, and stained for LAMP-1. Slides were analyzed by confocal microscopy LSM510 Meta (Zeiss).

*Mycobacterium bovis* BCG were grown in 7H9 medium supplemented with OADC (10%), washed in PBS, resuspended in RPMI-FBS (10%) and added at an  $OD_{600\text{ nm}}$  of 0.05 to neutrophils that adhered on Polylysine-coated slides. After one hour infection at 37°C, the cells were washed 3 times to remove the non-phagocytosed mycobacteria and incubated for an extra hour at 37°C. The cells were then fixed in PFA (3%), permeabilised by saponin (0.1%), stained for BCG and neutrophil marker and analyzed by confocal microscopy for the presence of phagocytosed mycobacteria inside the neutrophils.

### **2.10.3 Analysis of the neutrophil and B cell cytoskeleton by immunofluorescence**

Neutrophils isolated from wild type and coronin 1 <sup>-/-</sup> bone marrow were mixed and seeded onto Polylysine-coated 10-well microscopy slides (Polysciences) for 10 min at room temperature. The non-adherent cells were then removed and the slides were transferred into jars containing PFA (3%) for 10 min fixation at 37°C. The cells were then washed with PBS, permeabilized with saponin and blocked with BSA. The slides were incubated for 30 min with anti-actin, anti-coronin 1 and anti-NIMP-R14 antibodies, followed by 20 min with anti-mouse Alexa 488, anti-rabbit Alexa 647 and anti-rat Alexa 568 secondary antibodies respectively. The slides were then mounted with antifade reagent and analyzed by confocal microscopy (LSM510 Meta, Zeiss).

B lymphocytes isolated from spleen were adhered onto Polylysine-coated 10-well slides by incubating them for 20 min on ice. The cells were then fixed in PFA (3%) and stained for coronin 1 and either for actin or tubulin.

### **2.10.4 Acid fast staining of mycobacteria**

To determine the numbers of mycobacteria present in the organelle electrophoresis fractions, the bacteria were cytospun and acid fast stained.

Four hundreds  $\mu$ l of each fraction was added into 1 ml of cold PBS, mixed well and centrifuged at 20,000  $\times$  g for 15 min, 4°C. The pellets were then taken in 50  $\mu$ l PBS, added into cytospin apparatus and centrifuged for 15 min at 1600  $\times$  g. The slides were dried overnight, fixed for 20 min in 3% paraformaldehyde at room temperature and washed in PBS, PBS-Glycine (5 mM) and PBS (5 min each). The slides were then placed in a staining jar containing TB Auramine-Rhodamine T solution for 25 min. After gentle washes under tap water, the slides were incubated for 2 min in TB Decolorizer solution, rinsed and counterstained with TB Permanganate Potassium for 5 min. The slides were finally extensively washed with water and allowed to air-dry. They were then mounted with antifade reagent and coverslips and analyzed on either a confocal laser scanning microscope LSM510 Meta (Zeiss) or an Axioplan 2 fluorescence microscope (Zeiss). Using a grid ocular and a 40x objective, the number of stained mycobacteria in 10 areas was counted to obtain the relative amount of mycobacteria present in each fraction.

### **2.10.5 Analysis of neutrophil spreading**

Neutrophils were isolated either from the blood or from the bone marrow and resuspended in RPMI medium. The cells from wild type and coronin 1 <sup>-/-</sup> origin were then mixed 1:1 and added onto 10-well microscopy slides for 1 min at room temperature (25  $\mu$ l, 20000 cells per well). 25  $\mu$ l of 2x stimuli were then supplemented to these cells for another 5 min to allow cell spreading. The cells were then fixed, stained for F-actin, coronin 1 and for a neutrophil marker and analyzed by confocal microscopy. A line was drawn at the surrounding of the cell and the area of each cell was then calculated using the LSM510 Meta software.

### **2.10.6 Opsonization of beads and phagocytosis by neutrophils**

Yellow-green fluorescent polystyrene 1  $\mu$ m beads and monodisperse polystyrene 2.8  $\mu$ m microspheres were washed in PBS, resuspended in 1 ml PBS containing 50  $\mu$ g/ml rabbit IgG and incubated at 37°C for 2 hours in a thermoshaker. Beads were then washed in PBS, resuspended in RPMI-FBS (10%) and added to the neutrophils which were allowed to adhere on Polylysine-coated slides for 15 min at 37°C and washed to remove non-adhered cells. After 15 min incubation on ice, the cells were washed 4 times with ice-cold medium to remove the free beads and either kept on ice or prewarmed medium was added and the slides were shifted to 37°C for 45 min. Cells were then fixed in PFA (3%), permeabilised with saponin (0.1%) and stained with phalloidin, anti-NIMP-R14 and anti-coronin 1. The phagocytosis of beads was then analyzed by confocal microscopy.

### **2.10.7 Immunohistology**

Popliteal Lymph Nodes were embedded in OCT Compound (Tissue-Tek), frozen and 5  $\mu$ m sections were prepared using a cryostat. Sections were then fixed for 10 min with acetone, rehydrated with PBS and blocked in PBS-BSA (1%). Rat anti-IgD and biotinylated Peanut Agglutinin (PNA) were added for one hour and secondary antibodies, anti-rat Ig Cy3 and Streptavidin Alexa 488, were afterwards used during 30 min. Sections were then washed and mounted in FluoroGuard antifade reagent (BioRad). Slides were analyzed using the confocal laser scanning microscope LSM510 Meta (Zeiss) with the corresponding software.

### **2.10.8 Coating of the slides**

For microscopy analysis of non-adherent cells, coated slides had to be used. Slides or 96-well plates were either coated with Polylysine (mainly for dendritic cells, neutrophils and B lymphocytes) or with fibronectin (only for neutrophils).

Ten-well microscopy slides were first cleaned with 70% ethanol and 50  $\mu$ l of Polylysine solution (obtained by diluted the commercial solution 1:10 with distilled water) was added per well for 5 min at room temperature. The solution was then removed by aspiration and the slides were dried for 1 hour at 60°C or overnight at room temperature. The slides were washed once with medium prior to use.

Slides or 96-well plates were alternatively coated by adding 50  $\mu$ l of 20  $\mu$ g/ml fibronectin in PBS per well. After 1 hour at room temperature, the fibronectin solution was removed and the slides or plates were washed once with PBS and once with the working medium just before using them.

## **2.11 Biochemical techniques**

### **2.11.1 Preparation of cell lysates**

To prepare cell lysates, leukocytes were washed twice in ice-cold PBS and then resuspended in TX-100 buffer containing PMSF (1 mM), protease inhibitor and phosphatase inhibitor when necessary. After 15 min incubation on ice with occasional inversion of the tubes, the cells debris were pelleted at 20,000  $\times$  g for 15 min at 4°C and 5x SDS-SB was added to the supernatant.

### **2.11.2 Determination of protein concentration**

The protein concentrations were determined either by using the Bradford assay (Bradford, 1976) for example after organelle electrophoresis or by using the BCA method (Smith et al., 1985) when the samples were containing detergents such as Triton X-100.

Different volumes of the samples (1 to 10  $\mu$ l) and of the standard solution (1, 2, 4, 6, 8 and 10  $\mu$ l of 1 mg/ml BioRad Protein Assay Standard I) were added into 96-well plate, flat bottom, containing 200  $\mu$ l of either BCA solution (prepared by mixing 1 vol. of BCA Protein Assay Reagent B with 50 vol. of BCA Protein Assay Reagent A) or Bradford solution (prepared by diluting the BioRad

Protein Assay 5 times with water). The samples were well mixed using a multichannel pipette. After 5 min incubation at room temperature, the results of the Bradford assay was measured at 595nm using a microplate reader (Bucher). In the case of BCA assay, the plate was incubated for 30 min at 37°C and then read at 562nm. The protein concentration was then calculated by using the standard curve and taking into account the dilution factors of the samples.

### 2.11.3 Discontinuous SDS polyacrylamide gel electrophoresis.

The glass plates of the Protean II minigel system (BioRad) were assembled after cleaning with 70% ethanol. The running gel was prepared, poured into the glass plates assembly and overlaid with water-saturated isobutanol. After polymerization, the isobutanol was fully removed, the stacking gel was poured and the comb was inserted on the top. After complete polymerization, the comb was removed, the slots were washed and the gel inserted into the electrophoresis chamber that was further filled with SDS-PAGE running buffer. Samples containing 1x SDS-SB were boiled for 7 min, quickly centrifuged at full speed and loaded into the gels using a Hamilton syringe. The proteins were then separated by applying a current with constant voltage (70V during the separation in the stacking gel and 100-150V afterwards).

	<b>Running gel 12.5% (Vol. in ml)</b>	<b>Running gel 10% (Vol. in ml)</b>	<b>Running gel 7.5% (Vol. in ml)</b>	<b>Stacking gel 4% (Vol. in ml)</b>
30% acrylamide/bisacrylamide	4.2	3.3	2.5	0.65
2 M Tris/HCl, pH 8.8	2	2	2	-
0.5 M Tris/HCl, pH 6.8	-	-	-	1.25
20% SDS	0.05	0.05	0.05	0.025
dH <sub>2</sub> O	3.7	4.6	5.4	3.05
10% APS	0.05	0.05	0.05	0.025
TEMED	0.01	0.01	0.01	0.005

## **2.11.4 Western blot analysis**

### **2.11.4.1 Semi-dry protein transfer onto nitrocellulose membranes**

After SDS-PAGE separation, the proteins were transferred onto Hybond C super nitrocellulose membrane (Amersham). To do so, the running gel, the membrane and Whatman filter papers were soaked in transfer buffer and a transfer sandwich was realized onto the anode of the semi-dry transfer cell apparatus (BioRad) in the following order from the bottom: thick paper, thin paper, membrane, gel, thin paper and thick paper. The air bubbles present in the sandwich were removed before adding the cathode on the top. The transfer was performed during 36 min at 20V and 0.17A per gel. Afterwards the membrane was stained with Ponceau S solution to verify the quality of the protein transferred, the extra solution was washed out with water, the bands of the molecular weight standard were marked and the membrane was scanned.

### **2.11.4.2 Immunodetection**

To further detect the proteins with specific antibodies, the nitrocellulose membrane was incubated with PBST-milk (5%) for 2 hours at room temperature or overnight in the cold room to block further unspecific binding of the antibodies. The membranes were agitated on a rocking table and the blocking buffer was exchanged at least twice. The membranes were then incubated with first antibodies diluted in blocking buffer for 1 hour at room temperature. The unbound antibodies were further washed out (6 x 5 min with PBST-milk) and HRP-conjugated secondary antibodies were added (1:20,000) for 30 min onto the membranes. The membrane was then washed with PBST-milk (3 x 5 min), PBST (3 x 5 min), PBS (2 x 5 min) and subsequently incubated with ECL solution during 1 min. The excess of detection reagent was then drained out, the membrane was placed inside a plastic bag, transferred into an autoradiography cassette and exposed to ECL films (Amersham) for different time points. If the detection was not sensitive enough, the membrane could be further incubated with ECL+ solution (Pierce) and reexposed to films.

When phosphorylated proteins had to be detected, milk was replaced by BSA and the incubation with the first antibody was performed overnight in the cold room.

### **2.11.4.3 Stripping of membranes for reprobing**

For reprobing of membrane with other antibodies, the membrane was first washed twice with PBST to remove the remaining ECL solution and then incubated in stripping buffer for 30 min at 50°C with slight agitation on a rocking table. The membrane was then washed with PBST (3 x 10 min) and blocked with PBST-milk (5%) before repeating the immunodetection as previously described.

### **2.11.5 Separation of organelles by density gradient electrophoresis**

The dishes containing the infected leukocytes were transferred onto ice and the cells were harvested using a rubber scraper. The dishes were washed once and the cells twice with DGE buffer and pelleted at  $450 \times g$  for 7 min at 4°C. The cells were then resuspended in 1 ml DGE buffer and homogenized using a cell cracker (8.020 with a 8.002 ball, EMBL) on ice and in the cold room. Twenty-five strokes were usually necessary to obtain a correct homogenization (around 90% of the cells) as controlled by microscopy. The homogenized cells were centrifuged at  $240 \times g$  for 15 min at 4°C and the amount of protein present in the postnuclear supernatant (PNS) was determined by a Bradford assay. The PNS was then trypsinized for 5 min at 37°C in a water bath with frequent inversions of the tube (25 µg trypsin per mg of PNS protein) and the reaction was stopped by the addition of ice-cold soybean trypsin inhibitor in excess (625 µg per mg of PNS protein). The organelles were then sedimented for 1 hour at  $100,000 \times g$  (4°C, TLA 102.2 rotor), the supernatant was carefully removed and the pellet was taken in 6% Ficoll in DGE: first with 100 µl using a yellow tip and 400 µl of 6% Ficoll were then added and the organelles well resuspended with a yellow tip and finally with a 22G 1<sup>1/4</sup> needle.

During the ultracentrifugation step, the Ficoll gradient was prepared in the column. First of all, a cellophane membrane (Pharmacia) was tightly applied at the bottom of the column and the lower part of the apparatus, containing the cathode, was firmly screwed. The outer chamber was then filled with DGE buffer up to the membrane. The column (i.e. the inner chamber) was then filled to the top with 10% Ficoll in DGE buffer using an Auto Densi Flow pump while DGE buffer was added to the outer chamber at the same speed. A 10% to 7% Ficoll gradient (2ml + 2ml) was then realized in the top of the column using a gradient mixer. The organelles, in 6% Ficoll, were added on the top of this gradient and a 5% to 0% Ficoll gradient (10ml + 10ml) was made afterwards. DGE buffer was finally added at the top of the column, the anode was inserted and the electrophoresis was achieved by applying a 10.4 mA current for 80 min. Some air bubbles

accumulated at the anode and were regularly removed. The anode was detached afterwards and 60 fractions (12 droplets, around 500  $\mu$ l) were collected from the top.

The fractions were assayed for protein concentration to detect the phagosomal fractions. In brief, 40  $\mu$ l of each fraction was added to 200  $\mu$ l of Bradford solution in a 96-well plate using a multichannel pipette. After mixing, the absorbance at 595 nm was measured on a microplate reader. The lysosomal fractions were defined by their  $\beta$ -hexosaminidase activity. 30  $\mu$ l of each fraction was added to 120  $\mu$ l of PNP solution (a substrate for the lysosomal hydrolase) in a 96-well plate and incubated for 1 hour at 37°C. The enzymatic reaction was then stopped by adding 60  $\mu$ l of 2M Glycine-NaOH (pH 10) and the absorbance at 405 nm was measured.

To determine the presence of mycobacteria, the different fractions were cytopun and acid fast stained as previously described.

For endosome labeling of the dendritic cells, the dishes were first washed with PBS and then with internalization medium (DMEM containing 10 mM Hepes-NaOH and 10 mM Glucose). HRP (0.5 mg/ml) were then added to the cells in internalization medium for 30 min at 37°C. The dishes were afterwards put on ice and washed 6 times with PBS containing FBS (5%) to remove the non-internalized particles. After 3 hour incubation with internalization medium at 37°C, the cells were homogenized and the organelles separated by density gradient electrophoresis. The repartition of the HRP was determined by mixing 50  $\mu$ l of the fractions with 200  $\mu$ l of HRP reagent (0.1 mg/ml o-dianisidine, 0.003 % H<sub>2</sub>O<sub>2</sub>, 50 mM NaPi pH 5.0 and 0.3 % TritonX-100) and the absorbance at 450 nm was measured.

### **2.11.6 Macrophage stimulation and lysate preparation**

Bone marrow derived macrophages were used at day 5-6 and washed once in bone marrow macrophage medium (DMEM with 10 % heat-inactivated FBS, 30 % L929 supernatant, 2 mM L-glutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol). They were then plated (one million in 6 ml) into a 6 cm diameter dish and allowed to adhere for 4 hours. Non-adherent cells were washed out and 1.5 ml of medium containing the stimuli was added for a define time. The dishes were then transferred onto ice and washed twice with ice-cold PBS. Three hundreds  $\mu$ l of 1x SDS-SB was added per plate and the cell lysates were harvested using a rubber scraper and transferred into an Eppendorf tube. The sample was then vortexed and boiled for 5 min, centrifuged 15 min at 20,000  $\times$  g and the supernatant was transferred into a new tube. The samples were immediately separated by SDS-PAGE or frozen and stored at -80°C.

### **2.11.7 Adhesion assay**

Special optics 96-well plate (black, flat bottom, Costar) were coated either with fibronectin or with Polylysine and then washed once with HBSS. Wild type and coronin 1 deficient neutrophils were labeled with Calcein AM (5  $\mu$ M) for 30 min at 37°C, washed in HBSS and then added to the coated plates (100  $\mu$ l, 10000 cells per well) for 10 min at 37°C. Fluorescence (excitation: 490 nm, emission: 515 nm) was then read on a Synergy2 microplate reader (BioTek) to measure the fluorescence of the total cells. The plate was then centrifuged upside down (1 min, 100  $\times$  g), new HBSS medium was added and the fluorescence was read again. The % of adherence was determined as the ratio of the second measurement (fluorescence of adherent cells) to the first one (total fluorescence).

### **2.11.8 Transwell migration assay**

Wild type and coronin 1  $-/-$  neutrophils were purified from the bone marrow and resuspended in RPMI at a density of 6 million cells / ml. 600  $\mu$ l of medium containing either fMLP, IL8 or PMA was added into 24-well plate. Transwell permeable membranes (8 $\mu$ m pore, Corning) were placed on the top of the stimuli containing medium and 100  $\mu$ l of cell suspension was added onto the membrane. After 2 hour incubation of the plate at 37°C, the Transwell membrane insert was removed and the number of cells present in the lower chamber was counted using a Neubauer chamber.

### **2.11.9 Enzyme-Linked ImmunoSorbent Assay**

Microtest 96-well ELISA plates (flat bottom, BD) were coated overnight at 4°C with either TNP-BSA (50  $\mu$ l per well, 10  $\mu$ g/ml in PBS) for detection of TNP-specific antibodies or with anti-IgA, anti-IgM or anti-IgG (50  $\mu$ l per well, 10  $\mu$ g/ml in PBS) for detection of specific isotypes. The following day, the plates were washed 5 times with ELISA washing buffer (PBS containing 0.05% Tween-20) and 100  $\mu$ l of ELISA binding buffer (PBS containing 4% BSA, 0.1% Tween-20 and 10 mM NaN<sub>3</sub>) was added to each well except in the first row where 150  $\mu$ l of serum diluted in ELISA binding buffer (1:50 or 1:100 depending on the experiment) was used. A serial dilution (1:3) of the serum was performed by transferring 50  $\mu$ l of diluted serum from the first row to the second using a multichannel pipette. After having well mixed (5 strokes), 50  $\mu$ l of the second row was transferred

to the third one and so on till the penultimate row. The last row only contained buffer and was used as a negative control. After overnight incubation of the plate at 4°C, the wells were washed 5 times with ELISA washing buffer and 50 µl of revealing antibodies was added for 2 hours at 4°C. The revealing antibodies were either anti-IgA, anti-IgM or anti-IgG coupled to alkaline phosphatase and were diluted 1:1000 in ELISA binding buffer. The plate was then washed 5 times with ELISA washing buffer and 50 µl of alkaline phosphatase yellow (pNPP) liquid substrate was added per well. After 5 min for anti-IgG and 10 min for anti-IgM, the reaction was stopped by addition of 15 µl of 3 M NaOH and the absorbance at 405 nm was measured. The OD<sub>405nm</sub> was plotted against the dilution factor and the EC<sub>50</sub> values were determined to obtain a relative antibody titer of the serum.

#### **2.11.10 B cell proliferation**

T cell depleted splenocytes were prepared from wild type and coronin 1 <sup>-/-</sup> mice and resuspended in proliferation medium (IMDM supplemented with 5 % FBS, 50 µM β-mercaptoethanol, 2 mM glutamine and penicillin/streptomycin). The quality of the purification was analyzed by flow cytometry and the cell preparation from each mouse was diluted to 1 million B cells per ml. The stimulation medium was prepared at a 2 x concentration and 100 µl was added per well into flat bottom 96-well plate. After 30 min incubation of the plate at 37°C, 100,000 B cells (100 µl) were added per well and the plate was incubated at 37°C for 72 hours. Tritiated thymidine (50 µl per well corresponding to 0.5 µCi) was added and the plate was incubated for an extra 12 hours at 37°C. The cells were then harvested into glassfiber plates (Perkin Elmer) and the incorporation of the isotope was measured in a liquid scintillation counter (Packard TopCount). The results are expressed as the average cpm from 3 wells from 4 independent B cells preparation.

## 3 RESULTS

### 3.1 Phagosome maturation and mycobacterial peptide presentation

*Mycobacterium tuberculosis* is one of the most successful pathogens known today, killing millions of individuals worldwide each year. Importantly, while about one-third of the global population is infected with *M. tuberculosis*, only a relatively small percentage of infected individuals develop disease. The reason for this lies within the robust host immunity that is generated within healthy individuals, resulting in the establishment of a latent infection that is kept under control by both innate and adaptive immunity. Host control of mycobacterial infections relies on the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells against epitopes generated in professional antigen presenting cells (APC) (Kaufmann, 2006).

In APC, proteolysis of antigens leads to the generation of peptides able to bind to recycled or newly synthesized MHC class II molecules. Newly synthesized MHC class II molecules associated with invariant chain (Ii) are transported to endocytic vesicles, the so-called MHC-II compartment or MIIC, where the loading of the antigenic peptides occurs (Amigorena et al., 1994; Bryant et al., 2002; Peters et al., 1991; Tulp et al., 1994). However, phagosomes containing particulate antigens possess MHC class II molecules and other components involved in MHC class II presentation machinery. Thus, phagosomes, like MIIC, are equipped with the machinery to generate appropriate peptide-MHC-II complexes (Ramachandra et al., 1999). In addition, cross-presentation of peptides, derived from particulate antigens, can occur via a putative phagosome-to-cytosol mechanism involving the conventional MHC class I pathway (Kovacsovics-Bankowski and Rock, 1995). Alternatively, it was suggested to occur by fusion and fission of phagosomes with endoplasmic reticulum-derived vesicles containing newly synthesized MHC class I molecules (Gagnon et al., 2002; Guermonprez et al., 2003; Roy et al., 2006).

Presentation of mycobacterial antigens to the immune system involves the internalization, intracellular transport, and proteolytic processing of mycobacteria in macrophages and dendritic cells (Kaufmann, 2006). However, the hallmark of pathogenic mycobacteria is that following phagocytosis, they resist lysosomal delivery, instead residing within mycobacterial phagosomes that do not fuse with lysosomes. Retention within the nonlysosomal mycobacterial phagosome allows the mycobacteria to replicate (Armstrong and Hart, 1971; Clemens and Horwitz, 1995). Mycobacterial phagosomes acquire markers of early endosomes but display limited acquisition of the late endosomal/lysosomal markers rab 7 and lysosome-associated membrane proteins (LAMP)

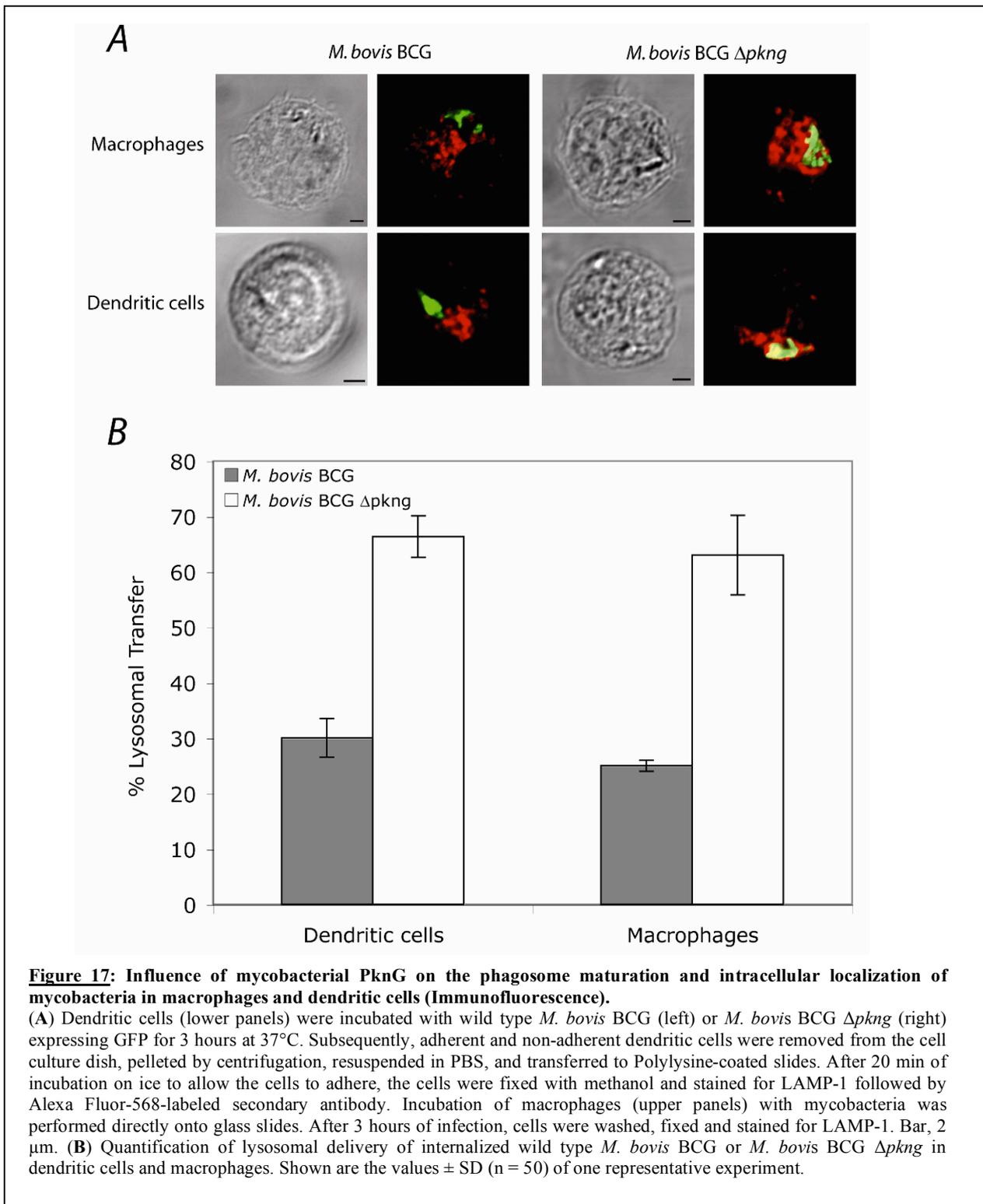
(Amigorena et al., 1994; Clemens and Horwitz, 1995, 1996; Clemens et al., 2000; Xu et al., 1994) and exclude vacuolar ATP-dependent proton pumps ( $vH^+$ -ATPases), explaining their limited acidification (Sturgill-Koszycki et al., 1994). Moreover, these phagosomes retain coronin 1 (also known as p57 or TACO, for tryptophan aspartate-containing coat protein) (Ferrari et al., 1999). In parallel to these events, processing and presentation of mycobacterial antigens by MHC class II is hampered in the case of infection with live mycobacteria compared to their heat-killed counterparts (Ramachandra et al., 2001). Because the latter are unable to inhibit phagosome maturation (Barker et al., 1997)), it is important to determine whether phagosome maturation is required for efficient antigen presentation.

The eukaryotic-like serine/threonine protein kinase G (PknG) is an important mycobacterial virulence factor, because it is responsible for blocking the fusion of the mycobacterial phagosome with lysosomes, allowing the bacteria to survive intracellularly (Av-Gay and Everett, 2000; Walburger et al., 2004). The fact that wild type *M. bovis* BCG and *M. bovis* BCG  $\Delta pkng$  are sorted to different organelles might influence the antigen presentation process. In this chapter, the following questions are addressed: are *M. bovis* BCG and *M. bovis* BCG  $\Delta pkng$  trafficking in the same manner in macrophages and dendritic cells? Are mycobacterial peptides from these two strains presented the same way *in vivo* and *in vitro*? Does the fact that pathogenic mycobacteria escape from lysosomal delivery influence antigen presentation and therefore explains the successful strategy of mycobacteria in escaping the immune system?

### **3.1.1 Influence of protein kinase G on intracellular trafficking of mycobacteria in professional Antigen Presenting Cells**

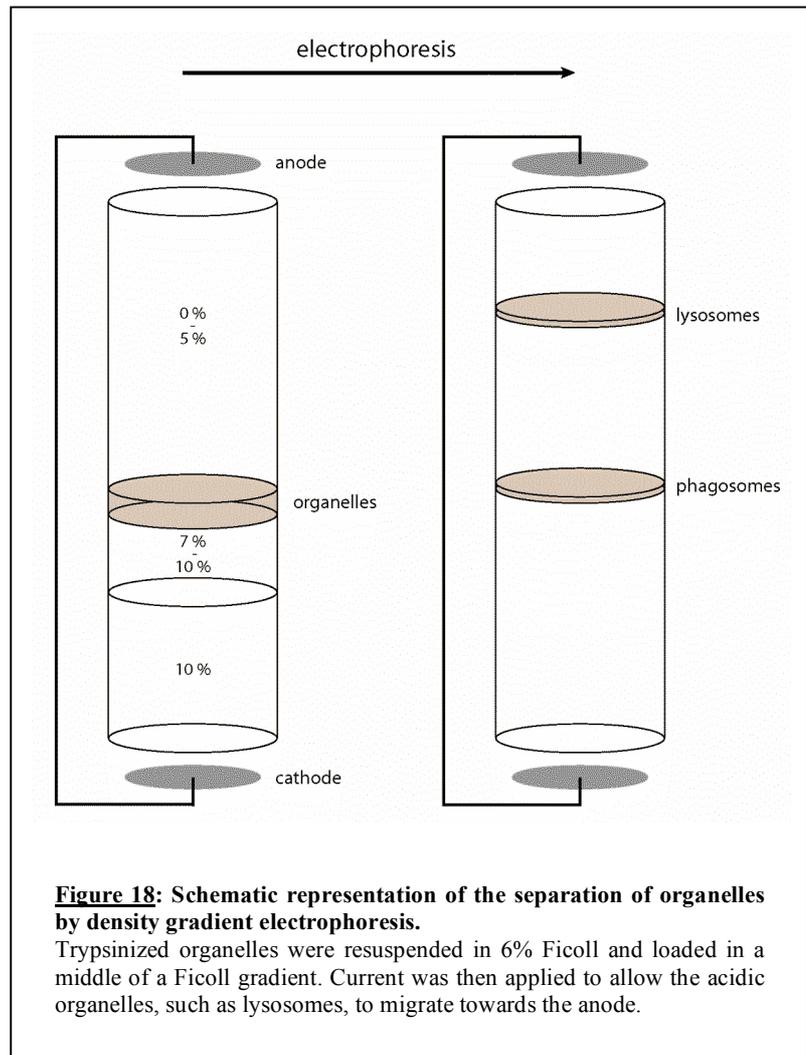
Pathogenic mycobacteria survive within macrophages by expressing the eukaryotic-like serine/threonine protein kinase G (PknG). PknG is secreted within the cytosol of macrophages and blocks the fusion of mycobacterial phagosomes with lysosomes, preventing intracellular degradation of the mycobacteria (Walburger et al., 2004). To analyze whether PknG also blocks phagosome-lysosome fusion in dendritic cells, bone marrow derived macrophages and bone marrow derived dendritic cells were incubated with either wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$  expressing GFP for 3 hours. Following fixation and permeabilization, the cells were stained for the lysosomal marker protein LAMP-1 and analyzed by confocal laser scanning microscopy. As shown in figure 17, wild type *M. bovis* BCG remained largely in nonlysosomal phagosomes both in macrophages and in dendritic cells. In contrast, in the absence of PknG, the

majority of the bacilli were delivered to lysosomes, as shown by the colocalization with LAMP-1 (figure 17).



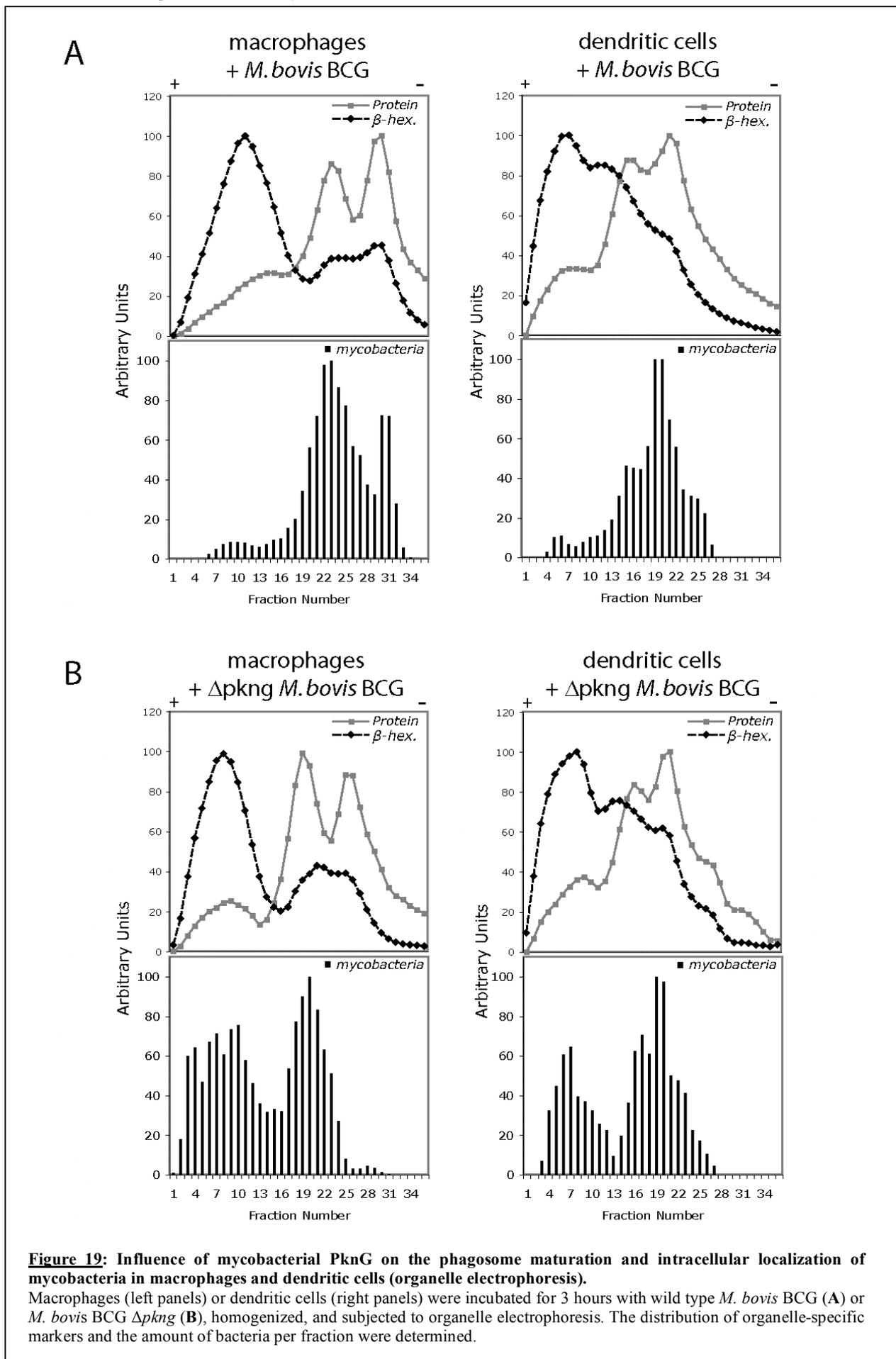
To independently analyze the intracellular transport of wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pkng* in macrophages versus dendritic cells, subcellular fractionation by organelle electrophoresis

was used (Ferrari et al., 1999; Hasan et al., 1997; Tulp et al., 1994; Tulp et al., 1993). To that end, macrophages and dendritic cells were infected with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pknG$  for 3 hours, followed by homogenization and organelle separation by electrophoresis. Briefly, the infected APCs were harvested in DGE buffer and then homogenized using a cell cracker. The postnuclear supernatant was then trypsinized to disrupt the F-actin cytoskeleton and to obtain single organelles. The organelles were sedimented by ultracentrifugation and resuspended in 6% Ficoll-70. They were then loaded in a middle of a Ficoll gradient (figure 18) and a current was applied. During organelle electrophoresis, negatively-charged organelles such as the acidic lysosomes migrate toward the anode, while nonacidified organelles, including mycobacterial phagosomes, remain deflected toward the cathode (Tulp et al., 1993). After electrophoresis, the different fractions were collected and analyzed for organelle-specific markers:  $\beta$ -hexosaminidase for lysosomes and Bradford assay for phagosomes. Part of the fractions was analyzed by cytospin followed by acid fast staining to detect the presence of mycobacteria.



As seen in figure 19, in both macrophages as well as dendritic cells, as expected, lysosomes were shifted toward the anode following electrophoresis as analyzed using the lysosomal marker  $\beta$ -hexosaminidase (Cella et al., 1997; Engering et al., 1997). Importantly, while in macrophages as well as in dendritic cells, wild type *M. bovis* BCG were recovered in nonshifted fractions, mycobacteria lacking PknG were largely transferred to fractions positive for the lysosomal marker

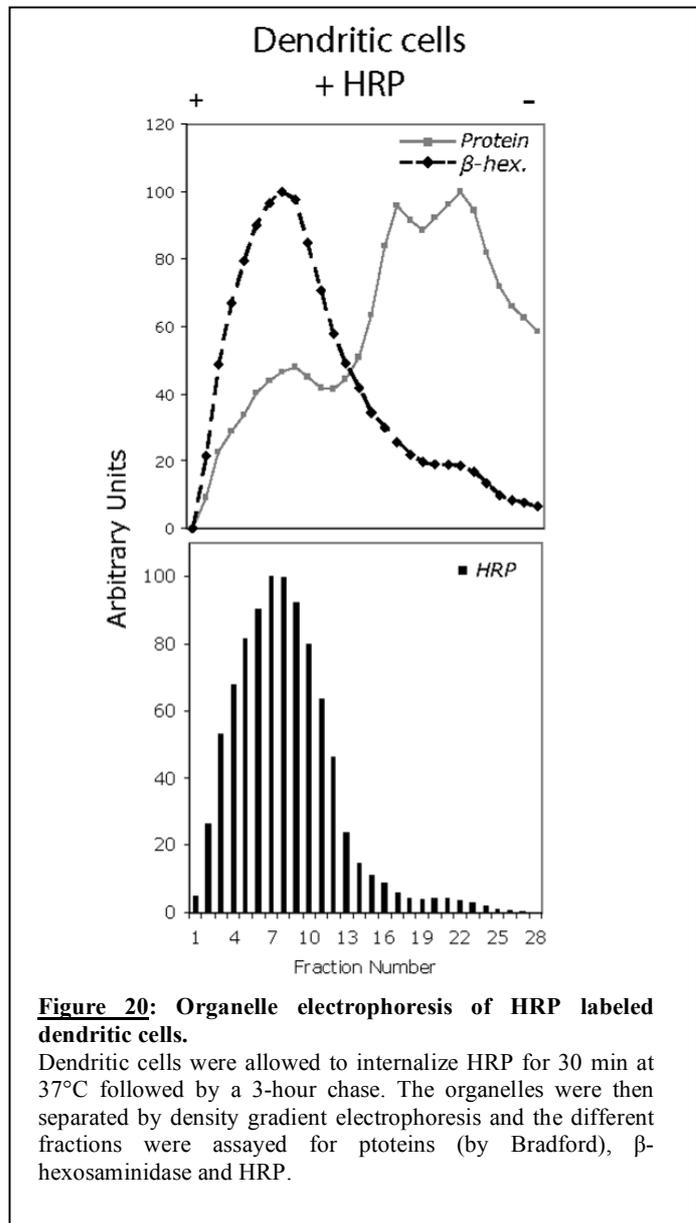
$\beta$ -hexosaminidase (Barker et al., 1997; Ferrari et al., 1999; Nguyen et al., 2005; Ramachandra et al., 2001; Walburger et al., 2004).



**Figure 19: Influence of mycobacterial PknG on the phagosome maturation and intracellular localization of mycobacteria in macrophages and dendritic cells (organelle electrophoresis).**

Macrophages (left panels) or dendritic cells (right panels) were incubated for 3 hours with wild type *M. bovis* BCG (A) or *M. bovis* BCG  $\Delta$ pkng (B), homogenized, and subjected to organelle electrophoresis. The distribution of organelle-specific markers and the amount of bacteria per fraction were determined.

To analyze the efficacy of the organelle electrophoresis technique with bone marrow derived dendritic cells, 7-day old dendritic cells were washed with internalization medium (DMEM containing 10 mM HEPES-NaOH and 10 mM Glucose) and were then incubated with Horseradish Peroxidase (HRP) for half an hour at 37°C. The non-internalized HRP was removed by extensive washing and the cells were further incubated in internalization medium for 3 hours at 37°C to allow the HRP particles to be directed to the lysosomes. The cells were harvested, homogenized and the organelles separated by organelle electrophoresis. The fractions were assayed with Bradford and  $\beta$ -hexosaminidase activity and the presence of HRP was analyzed. As shown in figure 20, lysosomes and phagosomes were separated into distinct fractions as analyzed with  $\beta$ -hexosaminidase and Bradford assays respectively. Moreover, the fractions having a  $\beta$ -hexosaminidase activity corresponded to the one containing HRP. Thus the dendritic cell subcellular fractions that were positive for  $\beta$ -hexosaminidase represent lysosomal organelles.

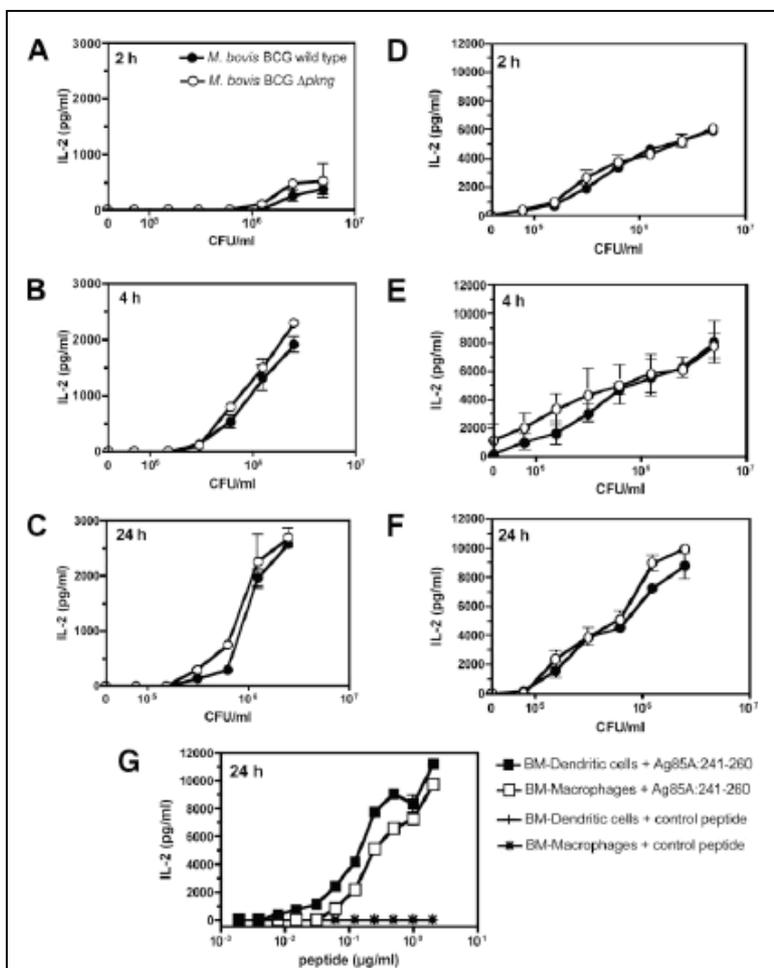


Together, these data suggest that in both macrophages and dendritic cells, PknG is essential to avoid lysosomal delivery.

### 3.1.2 Presentation of mycobacterial antigens upon infection with wild type *M. bovis* BCG or *M. bovis* BCG $\Delta$ pkng

The finding that mycobacteria lacking PknG are readily delivered to lysosomes prompted us to compare the antigen processing and presentation of wild type and PknG-deficient mycobacteria after uptake by macrophages or dendritic cells. In collaboration with the laboratory of Claude Leclerc at the Pasteur Institute, Paris, France, wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ pkng were used to infect APC derived from C57BL/6 (H-2<sup>b</sup>) mice. To analyze antigen processing and

presentation, the well-defined Ag85A was chosen as a reporter mycobacterial antigen (Wiker and Harboe, 1992) and the DE10 T cell hybridoma, specific to Ag85A:241–260 peptide and restricted by I-A<sup>b</sup> (Majlessi et al., 2006), was used to detect the presentation by the MHC class II molecules of the corresponding immunodominant epitope. At different time points after infection with various concentrations of wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ pkng, APC from C57BL/6 (H-2<sup>b</sup>) were fixed to arrest antigen processing and presentation and were cocultured with the DE10 T cell hybridoma to assess the amount of surface MHC class II-peptide complexes. In this model, the efficiency of the presentation of Ag85A by MHC class II molecules of macrophages

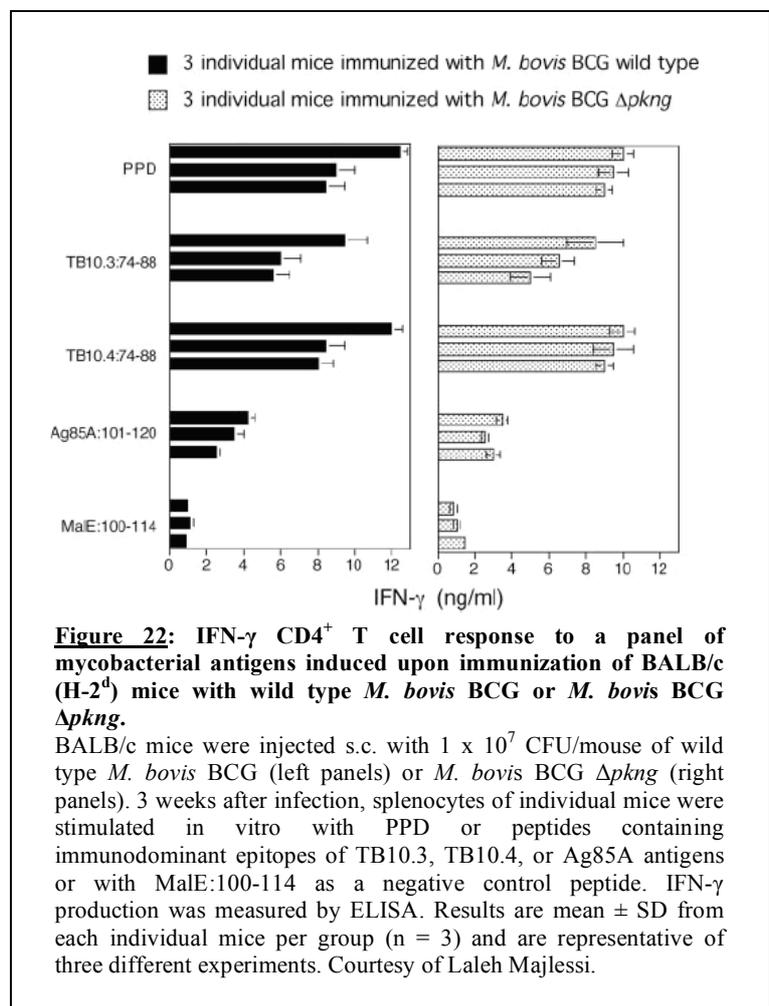


**Figure 21:** In vitro presentation of mycobacterial Ag85A by C57BL/6 (H-2<sup>b</sup>) macrophages or dendritic cells infected with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ pkng.

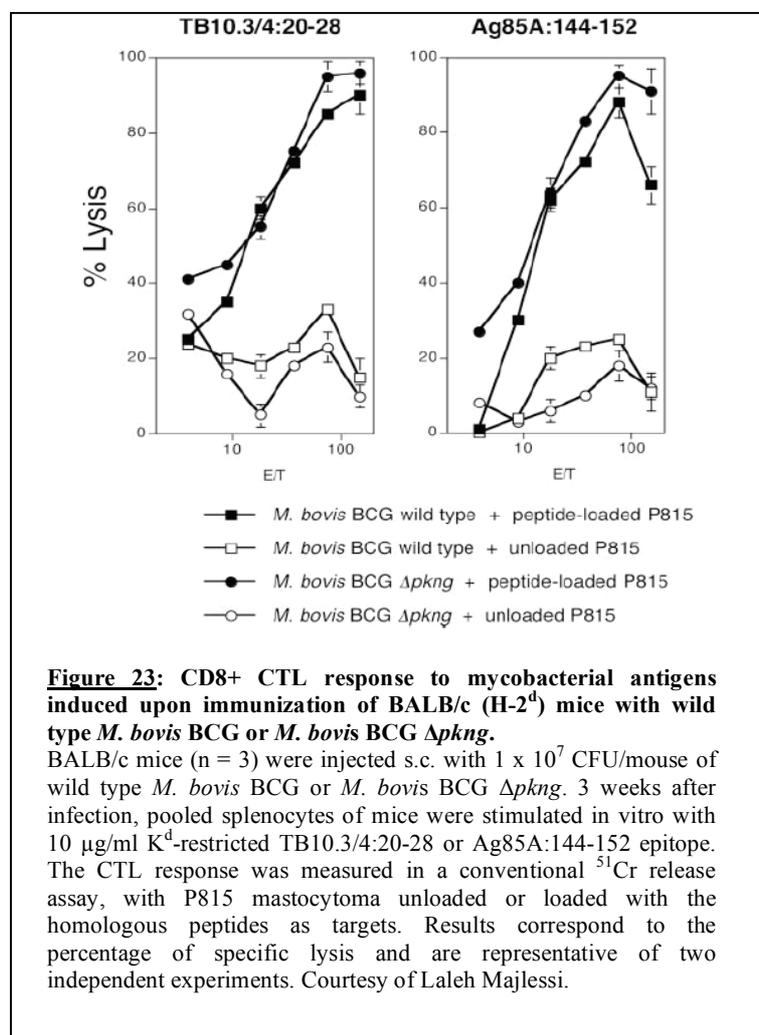
Macrophages (A-C) or dendritic cells (D-F) from C57BL/6 (H-2<sup>b</sup>) mice were infected with various amounts of wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ pkng for 2, 4, or 24 hours. (G) Macrophages or dendritic cells were incubated with various concentrations of Ag85A:241-260 or a negative control peptide for 24 hours. Cells were then washed, fixed, and cocultured overnight with the I-A<sup>b</sup>-restricted, Ag85A:241-260-specific, DE10 T cell hybridoma. The IL-2 production detected by ELISA was used as the readout of hybridoma activation. Results are mean  $\pm$  SD and are representative of two independent experiments. Courtesy of Laleh Majlessi

infected either with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$  strain was comparable after 2 hours of infection and increased similarly after 4 or 24 hours of infection (figure 21, A–C). The presentation of Ag85A by MHC class II molecules on dendritic cells was also comparable upon infection by wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$  after 2 hours and increased in a similar manner after 4 or 24 hours of infection (figure 21, D–F). Important to note and as expected, the efficiency of antigen presentation by dendritic cells was considerably higher as compared with macrophages (see different scales of IL-2 production used for macrophages (figure 21, A–C) and for dendritic cells (figure 21, D–F)). However, macrophages and dendritic cells showed the same efficiency in the presentation of the synthetic Ag85A:241–260 peptide (figure 21G), suggesting that the difference in antigen presentation between dendritic cells and macrophages was not due to any other culture parameters than the intrinsic capacity of these two cell types in antigen processing and presentation. Importantly, comparable data were obtained by using the CG11 T cell hybridoma specific to another Ag85A epitope, i.e., Ag85A:101–120 and restricted by I-E<sup>d</sup>, following the presentation of Ag85A by macrophages or dendritic cells generated from BALB/c (H-2<sup>d</sup>) mice (data not shown). These data demonstrate that the *in vitro* presentation of Ag85A through the MHC class II-dependent processing and presentation pathway of either macrophages or dendritic cells is not influenced by the marked differential intracellular localization of mycobacteria due to the PknG function.

We further sought to determine *in vivo* the possible consequence of different intracellular trafficking of mycobacteria on the presentation of a panel of various mycobacterial antigens in both MHC class II and MHC class I pathways. To that end, we investigated the efficiency of induction of CD4<sup>+</sup> or CD8<sup>+</sup> T cell responses against several mycobacterial antigens upon immunization of mice with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta pkng$ . BALB/c (H-2<sup>d</sup>) mice were chosen for these experiments due to the availability of not only MHC class



II-, but also well-defined MHC class I-restricted mycobacterial T cell epitopes in the H-2<sup>d</sup> haplotype (Denis et al., 1998; Majlessi et al., 2003). CD4<sup>+</sup> T cell responses were studied against PPD and a panel of well-defined BCG antigens, i.e., Ag85A and immunogens from the early secreted antigenic target-6-kDa (ESAT-6) family (Brodin et al., 2004), namely, TB10.3 and TB10.4. CD4<sup>+</sup> T cell responses were evaluated at 3 weeks post immunization with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pkng*. Splenocytes were *in vitro* stimulated with PPD, Ag85A:101–120 (Huygen et al., 1994), TB10.3:77–84, TB10.4:77–84 (Hervas-Stubbs et al., 2006), or the unrelated Male:100–114 peptide (Lo-Man et al., 2000). All of these peptides have been previously shown to contain only MHC class II-restricted T cell epitopes. Comparable intensities and sensitivities of proliferative (data not shown) as well as IFN- $\gamma$  (figure 22) responses were observed in individual BALB/c mice immunized with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pkng*. We then compared the CD8<sup>+</sup> CTL responses induced in BALB/c mice immunized with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pkng*. Three weeks after immunization, splenocytes of immunized mice were investigated for their CTL activity against K<sup>d</sup>-restricted Ag85A:144–152 (Denis et al., 1998) or TB10.3/4:20–28 peptides, the latter being shared by TB10.3 and TB10.4 immunogens (Majlessi et al., 2003). Comparable lytic activities against Ag85A:144–152 or TB10.3/4:20–28 epitopes were detected with splenocytes of mice immunized with either wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pkng* (figure 23).



Taken together, our results show that the differential trafficking of mycobacteria within professional antigen presenting cells has no consequence for the efficiency of induction of MHC class II-restricted CD4<sup>+</sup> Th1 or MHC class I-restricted CD8<sup>+</sup> CTL responses in mice.



### **3.2 Activation of signaling pathways and mycobacteria trafficking in coronin 1 deficient macrophages**

The innate immune system developed to fight against intruders by recognizing and clearing them before they cause disease inside the host. However, some pathogens evolved to escape this immune surveillance. Pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, are one of the most successful. While the microbes are engulfed inside phagocytic cells and normally delivered to lysosomes for degradation, pathogenic mycobacteria can block phagosome maturation and therefore reside and survive inside host cells (Armstrong and Hart, 1971; Russell, 2001).

Mycobacteria have developed different strategies to survive intracellularly. They express several virulence factors such as PknG (Walburger et al., 2004) or SapM (Saleh and Belisle, 2000; Vergne et al., 2005) that are necessary for them to resist to lysosomal delivery. Pathogenic mycobacteria can also take profit of host cell components to modulate phagocytosis or intracellular trafficking. For example, cholesterol was shown to play an important role during mycobacterial phagosome formation (Gatfield and Pieters, 2000; Peyron et al., 2000). It was also proposed that the activation of Toll-like receptor (TLR) signaling pathways by bacteria might regulate early steps in phagocytosis as well as phagosome maturation (Blander and Medzhitov, 2004). Notably, coronin 1, a leukocyte-specific protein also known as TACO (tryptophan aspartate-containing coat protein) or p57, was found to be strongly recruited around mycobacteria-containing phagosomes (Deghmane et al., 2007; Ferrari et al., 1999; Itoh et al., 2002; Schuller et al., 2001). Coronin 1 is only actively retained by pathogenic mycobacteria, suggesting that this coronin family member could be directly involved in phagosome maturation and therefore in mycobacterial survival inside the host cells (Ferrari et al., 1999).

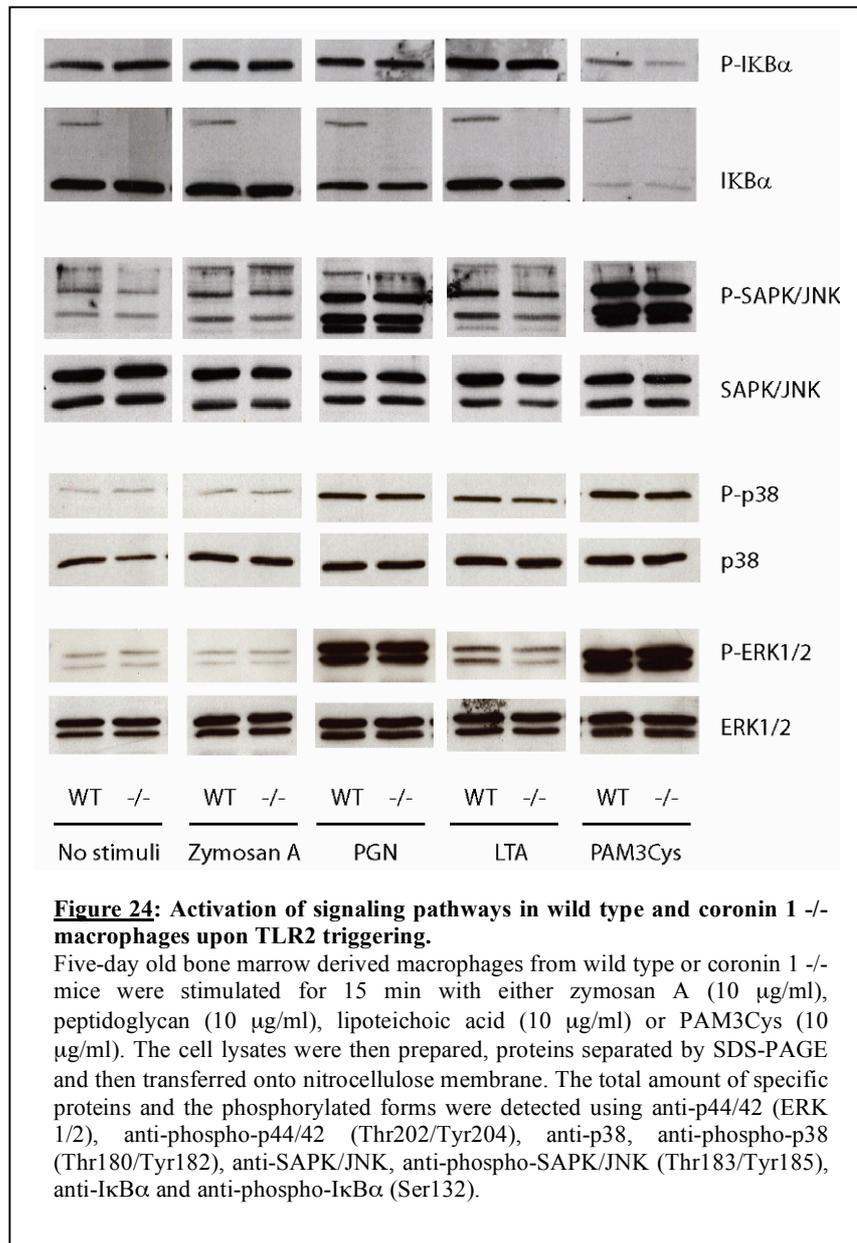
The role of mammalian coronin 1 is not well understood and therefore a mouse lacking the expression of coronin 1 was engineered by homologous recombination (Jayachandran et al., 2007; Mueller et al., 2008). The absence of coronin 1 did not result in any obvious phenotype and the actin-dependent processes were not impaired in coronin 1 *-/-* macrophages, in accordance with results obtained in cell culture with siRNA (Jayachandran et al., 2008; Jayachandran et al., 2007).

We then asked if the TLR signaling pathways were altered in the absence of coronin 1. Are pathogenic mycobacteria still able to interfere with phagosome maturation without their phagosome-coating protein? What is the mechanism behind coronin 1 dependent blockage of lysosomal delivery?

### 3.2.1 Activation of signaling pathways upon recognition of pathogen-associated molecular patterns in the absence of coronin 1

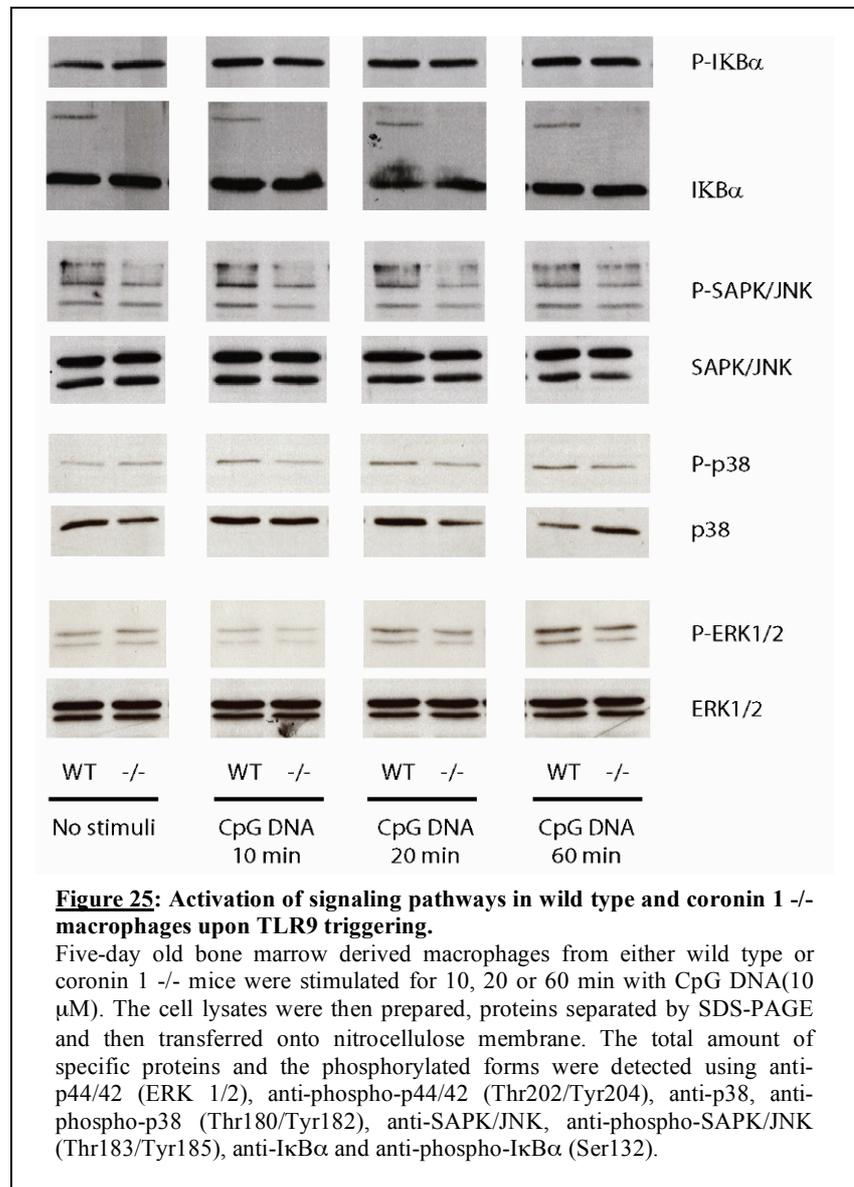
Coronin 1 and the Toll-like receptors (TLRs) were both proposed to play a role in phagosome maturation. We therefore investigated the response of macrophages lacking coronin 1 upon encounter of pathogen-associated molecular patterns (PAMPs) that are recognized by TLRs (Kawai and Akira, 2007). Bone marrow derived macrophages were either prepared from coronin 1 *-/-* mice or from wild type littermates and were used after 5 days of differentiation. Cells were seeded into 6 cm dishes and activated with different stimuli after allowing them to adhere. The cell lysates were then prepared, the proteins separated by SDS-PAGE and the activation of different signaling pathways analyzed by immunoblotting using specific phospho-antibodies. ERK 1/2, SAPK/JNK, p38MAPK and NF- $\kappa$ B, the

main signaling pathways activated after TLR stimulation (figure 5), were especially investigated. The amount of total protein was used as a loading control while the phosphorylation of proteins represented the activation of the signaling pathways. As seen in figure 24, both wild type and coronin 1 *-/-* macrophages responded similarly following stimulation of their TLR2. Addition of zymosan A mainly induces phosphorylation of SAPK/JNK resulting in activation of AP-1 (activator protein-1), a transcription factor (figure 5). Peptidoglycan (PGN), lipoteichoic acid (LTA) and the lipopeptide PAM3Cys



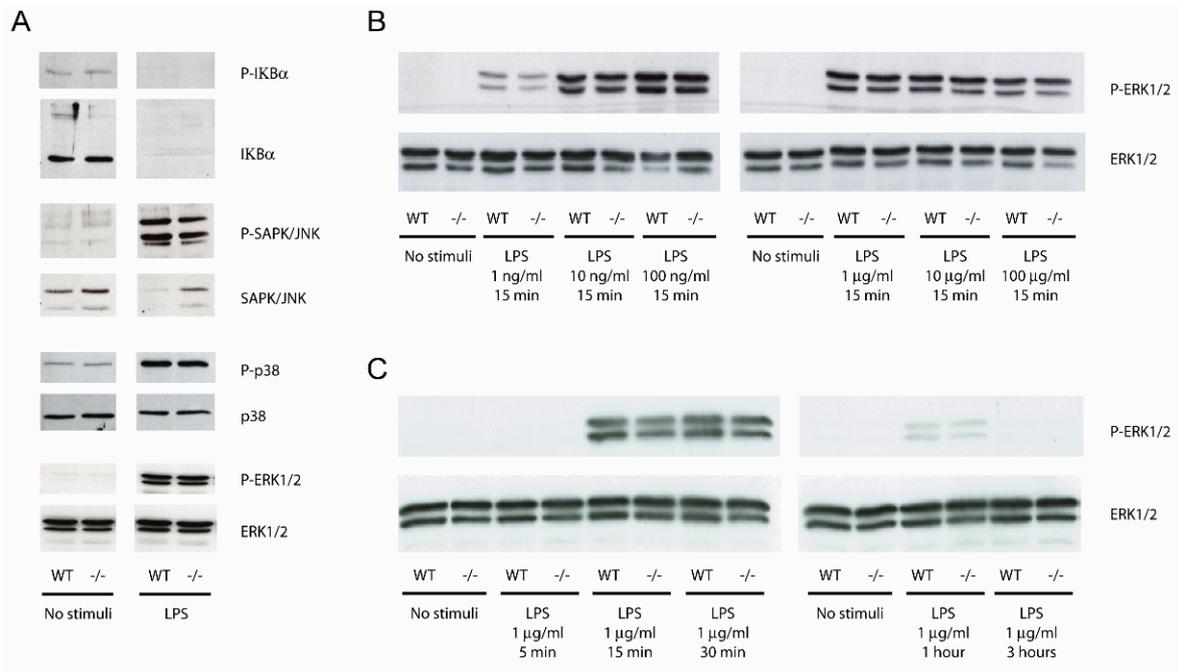
activate all the studied pathways of both wild type and coronin 1  $-/-$  macrophages to the same extent, what will upregulate transcription of inflammatory genes (West et al., 2006). These data suggest that the activation of TLR2 via fungic zymosan, peptidoglycan and bacterial lipoproteins from Gram-positive bacteria was not altered in the absence of coronin 1.

Wild type and coronin 1  $-/-$  macrophages were also stimulated with CpG DNA (bacterial DNA containing unmethylated CpG motifs) to trigger TLR9 in the endosomal compartment. No differences in activation of the main signaling pathways were observed at the several time points between wild type and coronin 1  $-/-$  macrophages (figure 25). We concluded that coronin 1  $-/-$  macrophages were normally activated when recognizing bacterial DNA through their TLR9.



We also investigated the response of coronin 1  $-/-$  macrophages via TLR4 upon encounter of Gram-negative bacteria. As shown in figure 26A, wild type and coronin 1  $-/-$  macrophages were equally stimulated by addition of lipopolysaccharide (LPS). ERK 1/2, SAPK/JNK and p38MAPK were similarly phosphorylated in the presence or absence of coronin 1 in the macrophages as judged by immunoblotting. We further tried different amounts of LPS and the activation of ERK 1/2 pathway was not altered in the absence of coronin 1 (figure 26B). The kinetics of activation was also similar (figure 26C); ERK 1/2 were phosphorylated shortly after stimulation and the activation was

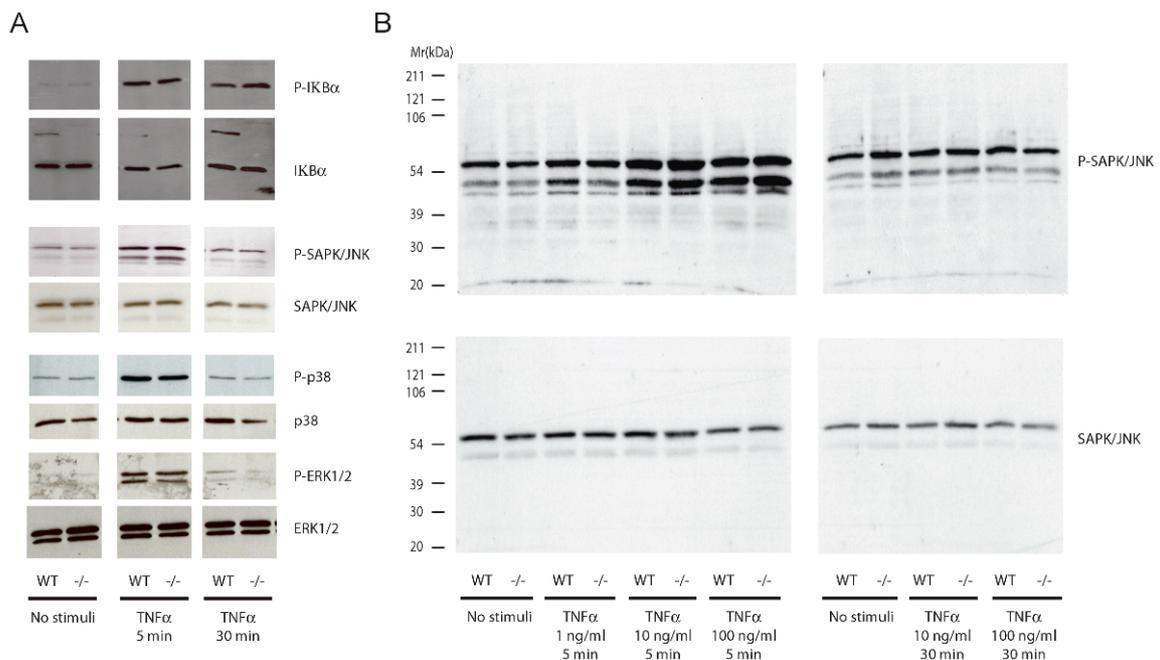
downregulated after an hour. These results would suggest that the coronin 1  $-/-$  macrophages are not deficient in signaling upon TLR4 triggering.



**Figure 26: Activation of signaling pathways in wild type and coronin 1  $-/-$  macrophages upon TLR4 triggering.**

(A) Five-day old bone marrow derived macrophages from either wild type or coronin 1  $-/-$  mice were stimulated for 15 min with LPS (10  $\mu$ g/ml). The cell lysates were then prepared, proteins separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The total amount of specific proteins and the phosphorylated forms were detected using anti-p44/42 (ERK 1/2), anti-phospho-p44/42 (Thr202/Tyr204), anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-I $\kappa$ B $\alpha$  and anti-phospho-I $\kappa$ B $\alpha$  (Ser132). (B-C) Wild type and coronin 1  $-/-$  macrophages were stimulated with different amounts of LPS for the indicated time. The total amount of p44/42 and P-p44/42 were determined.

We also analyzed the response of macrophages lacking coronin 1 upon stimulation with Tumor Necrosis Factor alpha (TNF $\alpha$ ), a pleiotropic inflammatory cytokine. As seen in figure 27, no significant difference in signaling pathways activation was detected between wild type and coronin 1  $-/-$  macrophages, as judged by immunoblotting with specific phospho-antibodies. ERK 1/2, SAPK/JNK, p38MAPK and NF- $\kappa$ B were strongly activated (figure 27A). Moreover, we observed comparable activation of SAPK/JNK in the absence or presence of coronin 1 upon stimulation with different amount of TNF $\alpha$  and similar kinetics (figure 27B). We concluded that the responses of wild type and coronin 1  $-/-$  macrophages upon triggering of the TNF $\alpha$ R on their surface were indistinguishable.



**Figure 27: Activation of signaling pathways in wild type and coronin 1 <sup>-/-</sup> macrophages upon TLR4 triggering.** (A) Five-day old bone marrow derived macrophages from either wild type or coronin 1 <sup>-/-</sup> mice were stimulated for 5 or 30 min with TNF $\alpha$  (10 ng/ml). The cell lysates were then prepared, proteins separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The total amount of specific proteins and the phosphorylated forms were detected using anti-p44/42 (ERK 1/2), anti-phospho-p44/42 (Thr202/Tyr204), anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-I $\kappa$ B $\alpha$  and anti-phospho-I $\kappa$ B $\alpha$  (Ser132). (B) Wild type and coronin 1 <sup>-/-</sup> macrophages were stimulated with different amounts of TNF $\alpha$  for the indicated time. The total amount of SAPK/JNK and P- SAPK/JNK were determined.

Taken together, these data suggest that the capacity of macrophages to respond upon TLR or TNF $\alpha$ R triggering is not affected by the absence of coronin 1. We next considered the activation of coronin 1 <sup>-/-</sup> macrophages upon infection with mycobacteria.

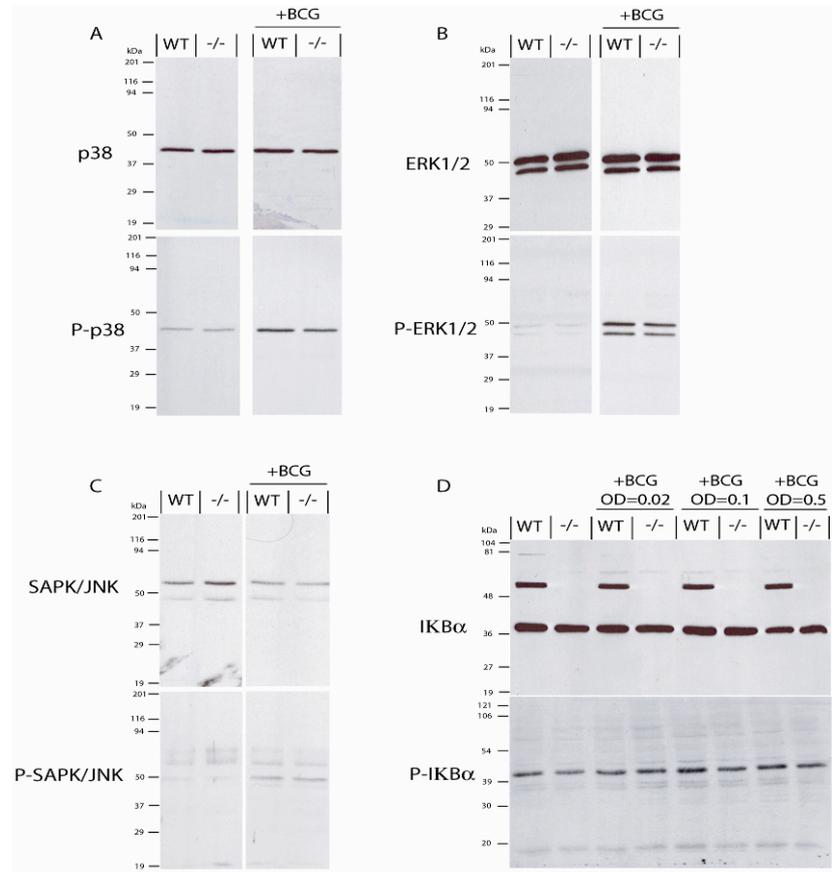
### 3.2.2 Activation of signaling pathways upon encounter with mycobacteria in the absence of coronin 1

Coronin 1 <sup>-/-</sup> macrophages show normal phagocytic activity of mycobacteria (Jayachandran et al., 2007). To analyze whether coronin 1 <sup>-/-</sup> macrophages can respond accurately towards mycobacterial stimulation, wild type as well as coronin 1 deficient macrophages were infected for 1 hour with *M. bovis* BCG. The cells were then washed, lysates were prepared, and proteins were separated on a SDS-PAGE gel and transferred onto nitrocellulose membrane. The membranes were then probed with different specific antibodies to study the signaling pathways activated upon mycobacterial infection. As shown in figure 28, p38MAPK, ERK1/2 and SAPK/JNK pathways were activated upon stimulation with mycobacteria. However, no significant difference was

observed between bone marrow macrophages from wild type or coronin 1  $-/-$  origin. These results therefore suggest that the absence of coronin 1 does not affect the main signaling pathways upon mycobacterial infection.

**Figure 28: Activation of signaling pathways in wild type and coronin 1  $-/-$  macrophages upon encounter with *M. bovis* BCG.**

Five-day old bone marrow derived macrophages from either wild type or coronin 1  $-/-$  mice were incubated for 1 hour with *M. bovis* BCG at an OD of 0.5 (also 0.02 and 0.1 for D). The cell lysates were then prepared, proteins separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The total amount of specific proteins and the phosphorylated forms were detected using anti-p44/42 (ERK 1/2), anti-phospho-p44/42 (Thr202/Tyr204), anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-I $\kappa$ B $\alpha$  and anti-phospho-I $\kappa$ B $\alpha$  (Ser132).

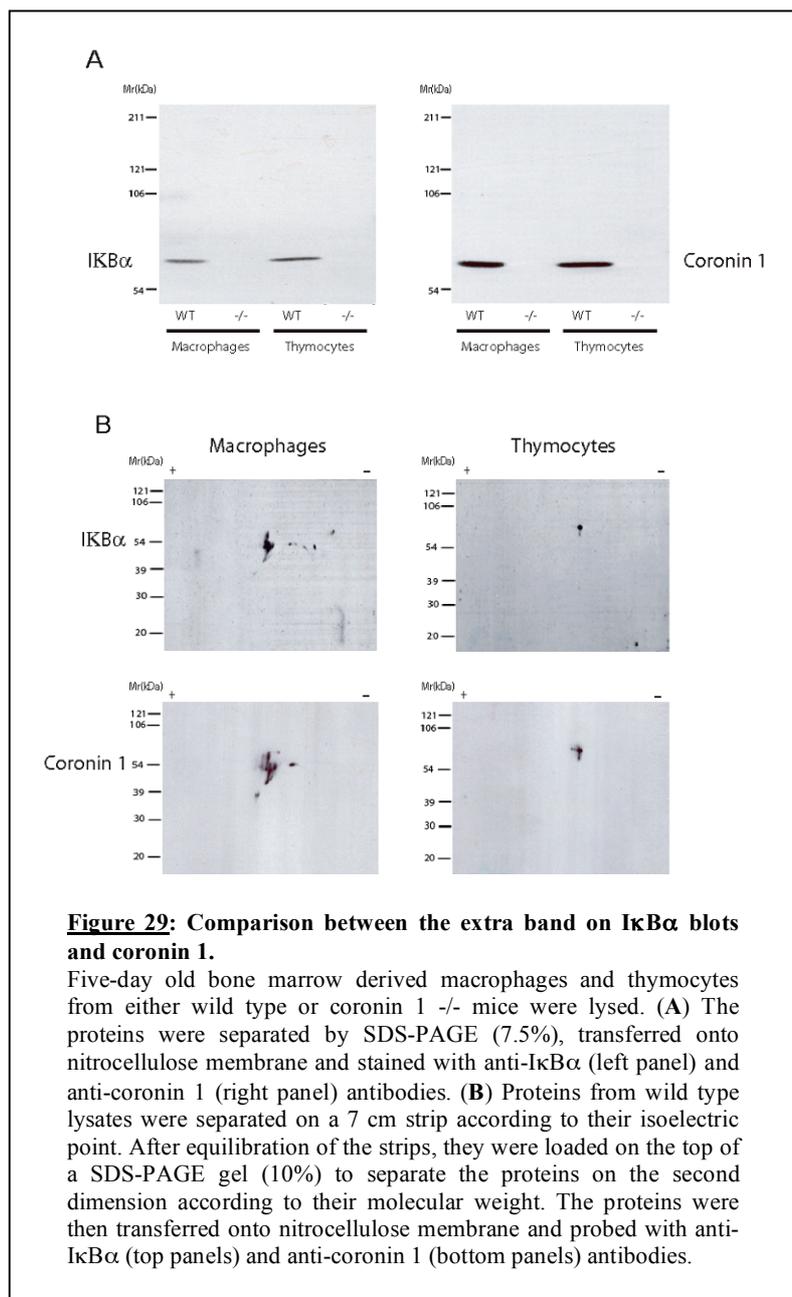


We also noticed that in every blot probed for I $\kappa$ B $\alpha$ , an extra band at around 50-60 kDa was specifically detected in the lysates from wild type macrophages but not from coronin 1  $-/-$  macrophages. This band could represent I $\kappa$ B $\alpha$  dimers or other complexes that would not be formed in the absence of coronin 1. Alternatively, this band may correspond to coronin 1 and could reflect a cross-reaction of the anti-I $\kappa$ B $\alpha$  antibody with coronin 1.

To solve this question, lysates from wild type and coronin 1  $-/-$  macrophages and thymocytes were prepared and the proteins were separated by SDS-PAGE (7.5% big gel) and transferred onto nitrocellulose membrane. Immunoblotting using anti-I $\kappa$ B $\alpha$  antibody revealed the extra band at around 55-60 kDa exclusively in the lanes corresponding to macrophages and thymocytes from wild type mice (figure 29A, left panel). The membrane was then stripped to detach the bound antibodies and was reprobed with anti-coronin 1. As shown in figure 29A, right panel, the coronin

1 specific band was observed at around 55-60 kDa and when the film was superposed to the one probed with anti-I $\kappa$ B $\alpha$  the extra band and the coronin 1 specific band were perfectly matching.

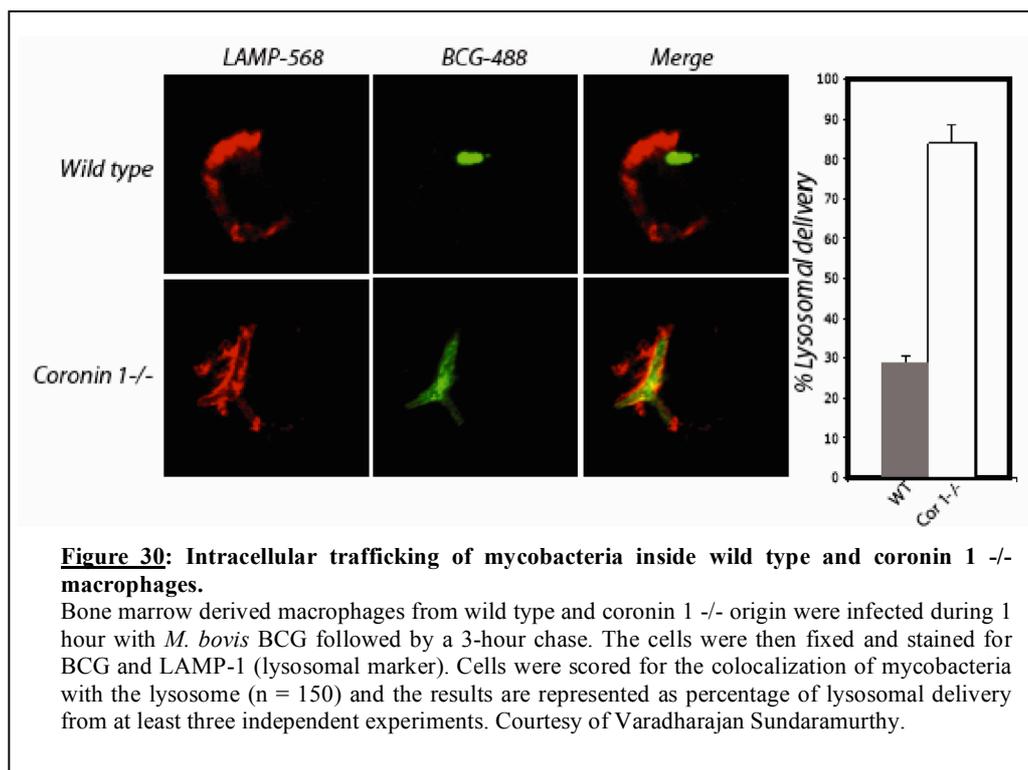
To confirm a cross-reactivity of the anti-I $\kappa$ B $\alpha$  antibody with coronin 1, macrophages and thymocytes proteins were separated by two-dimensional gel electrophoresis. Native proteins were first resolved by isoelectric focusing and then by SDS-PAGE after equilibration of the strips in SDS. As shown in figure 29B, the patterns obtained with anti-I $\kappa$ B $\alpha$  and anti-coronin 1 antibodies were similar confirming that the extra band observed in wild type lysates which has the same isoelectric point and molecular weight as coronin 1 is due to a cross-reaction of the anti-I $\kappa$ B $\alpha$  antibody with coronin 1.



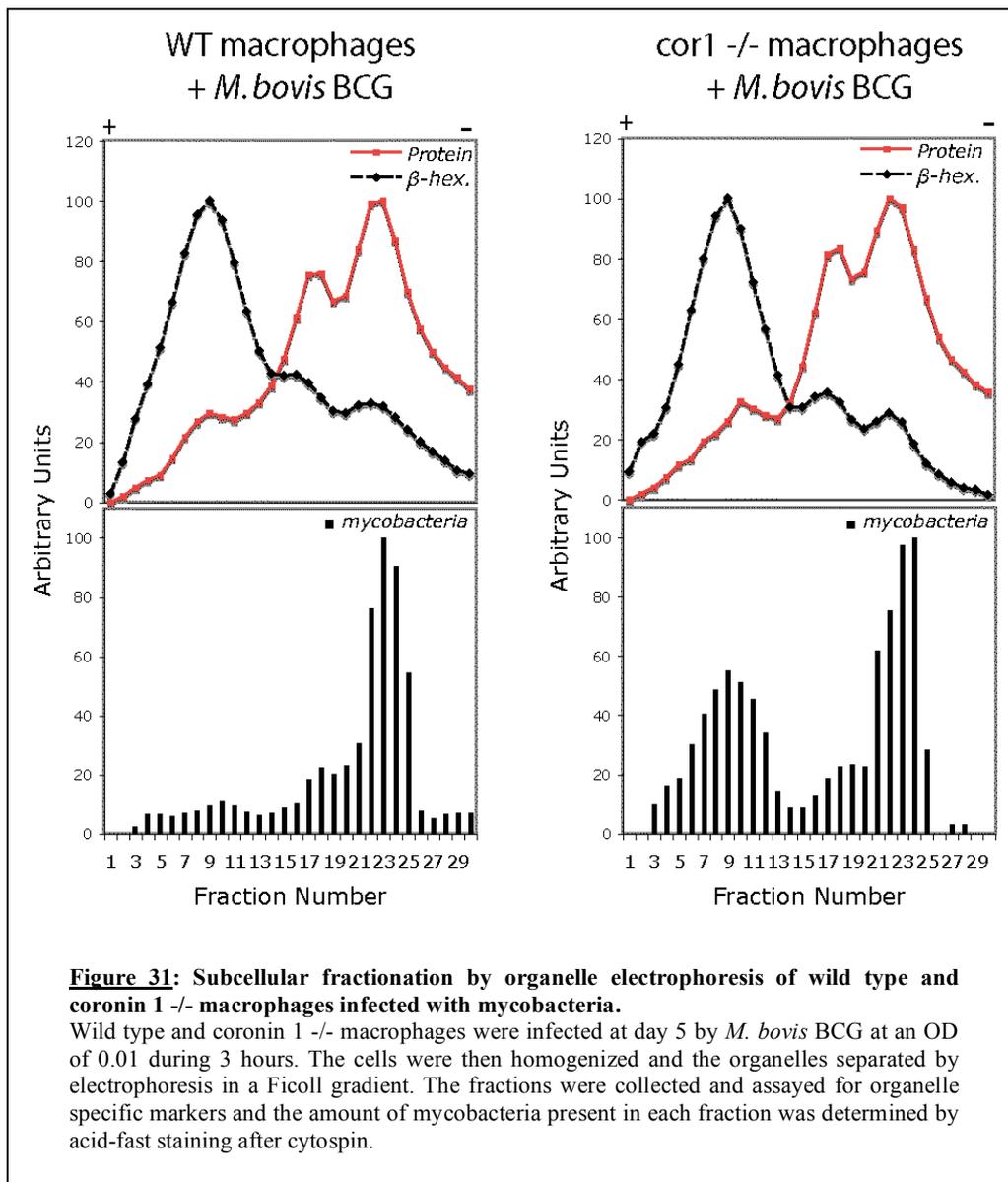
### 3.2.3 Mycobacterial trafficking in coronin 1 $-/-$ macrophages

Coronin 1  $-/-$  macrophages did not present any intrinsic deficiency in term of mycobacterial phagocytosis and activation of signaling pathways upon stimulation with pathogen-associated molecular patterns or mycobacteria. We further studied the fate of mycobacteria when entering macrophages lacking coronin 1. To do so, wild type and coronin 1  $-/-$  macrophages were incubated during 1 hour with *M. bovis* BCG followed by a chase for 3 hours after having removed the non-internalized mycobacteria. The intracellular localization of the bacteria was then analyzed by

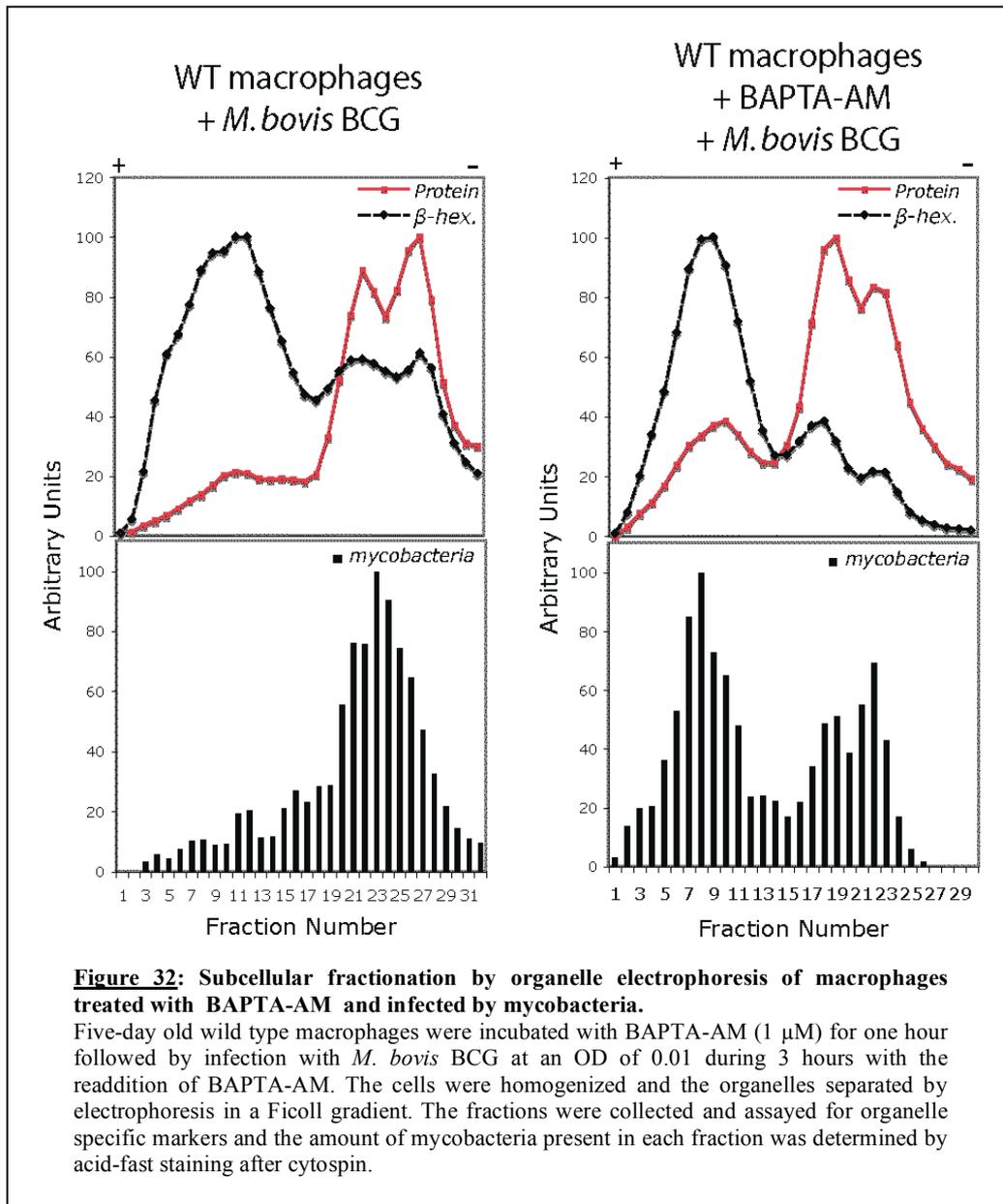
immunofluorescence with the staining of the lysosomes with LAMP-1 specific antibody. As expected, the mycobacteria were not delivered to lysosomes inside wild type macrophages since they were mainly found in phagosomes (LAMP-1-negative vacuoles) (figure 30, upper panels). However, when coronin 1 deficient macrophages were infected with *M. bovis* BCG they did not localize to phagosomes and were readily transferred in lysosomes as judged by colocalization of mycobacteria with the lysosomal marker (figure 30, lower panels). This suggests that coronin 1 is essential for blocking the maturation of mycobacterial phagosome.



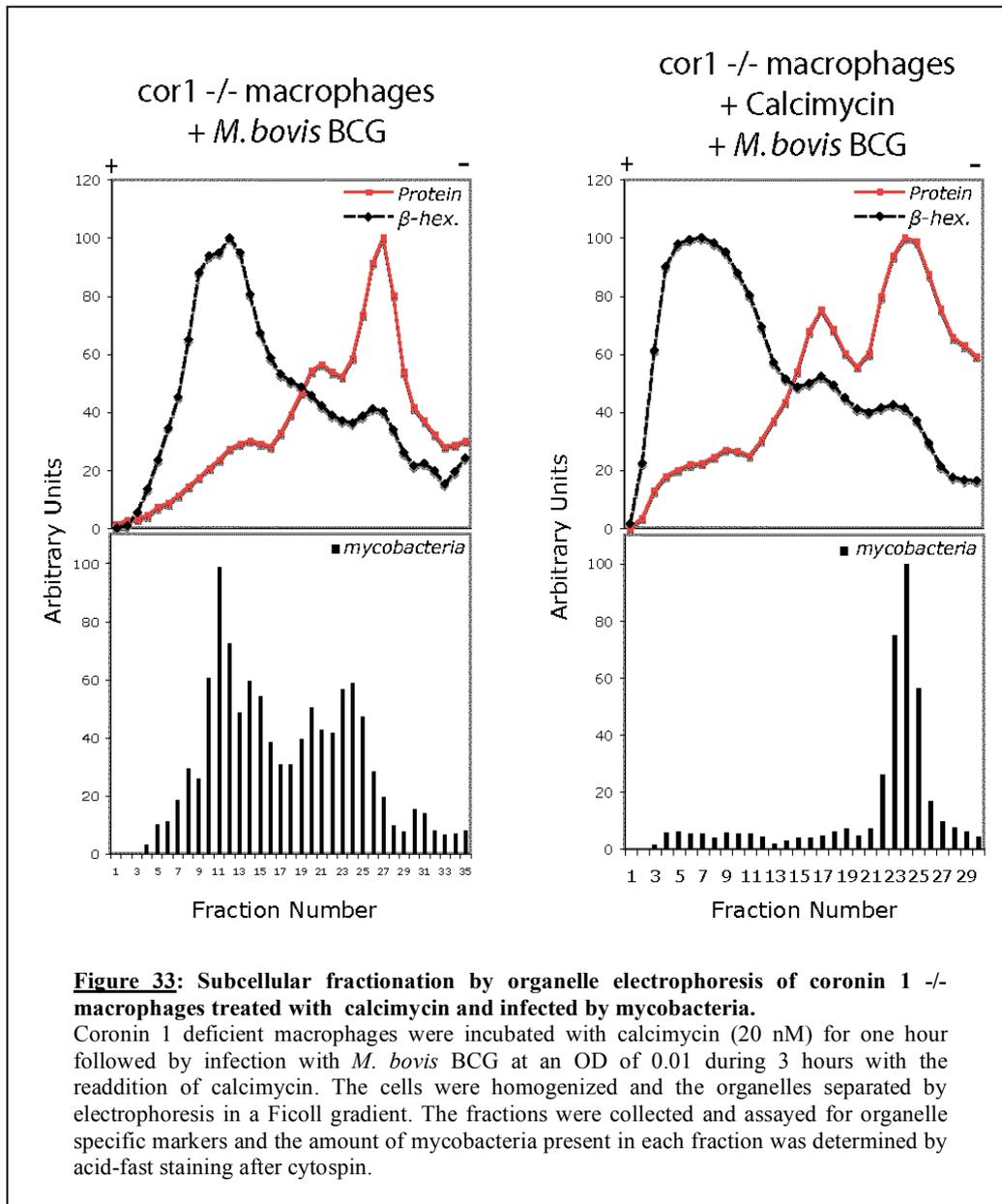
To independently confirm these observations, wild type and coronin 1  $-/-$  macrophages were infected with *M. bovis* BCG for 3 hours, the cells were homogenized and the organelles separated by density gradient electrophoresis. As expected in the case of wild type macrophages (figure 31, left panels), most of the mycobacteria were found in the phagosomal fractions (red line) whereas almost none of the bacteria were delivered to the lysosomes (black line) (Ferrari et al., 1999). However, when coronin 1 deficient macrophages were infected with *M. bovis* BCG and subjected to organelle electrophoresis a large proportion of the internalized mycobacteria were found in the shifted fractions (black line) corresponding to the lysosomes (figure 31, right panels). Taken together, these data define a role for coronin 1 in the regulation of phagosome maturation upon mycobacterial infection.



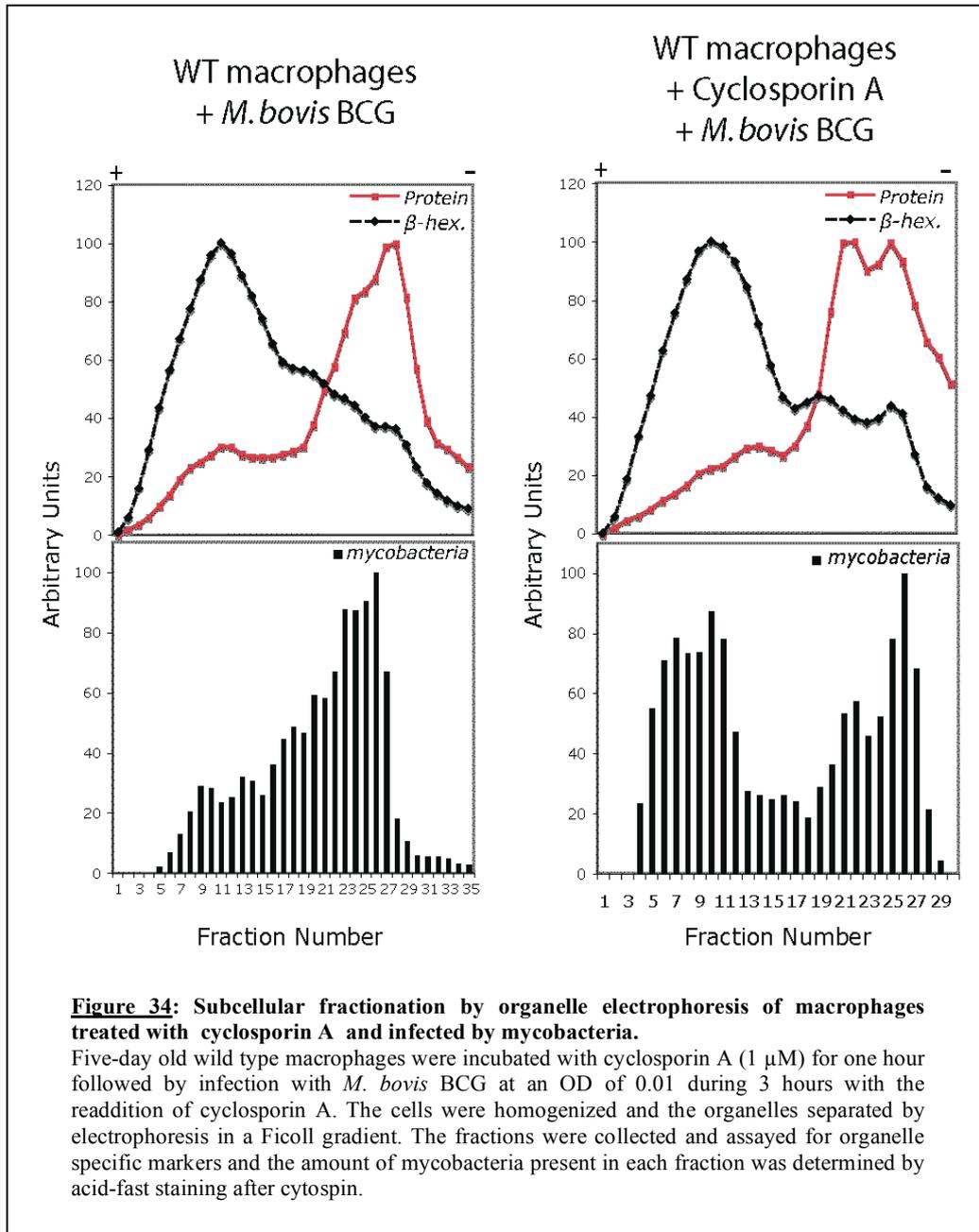
Since in T cells (Mueller et al., 2008) coronin 1 was shown to play a role in regulating calcium fluxes, we hypothesized that the coronin 1 dependent phagosome maturation arrest might be linked to the regulation of intracellular calcium. To verify this assumption, wild type bone marrow derived macrophages were treated with BAPTA-AM, a cell permeable calcium chelator, before and during mycobacterial infection. The cells were then harvested and the organelles separated by density gradient electrophoresis. While in non-treated wild type macrophages, the mycobacteria were mostly retained in the non-shifted fractions (figure 32, left panels), the mycobacteria were largely colocalizing with lysosomal fractions upon intracellular calcium chelation with BAPTA-AM (figure 32, right panels). These results show that the intracellular calcium levels play a dramatic role in mycobacterial phagosome maturation.



The above-described results suggested that the mycobacteria might be rescued from lysosomal delivery inside coronin 1  $-/-$  macrophages by increasing cytosolic calcium levels. To test this idea, coronin 1  $-/-$  macrophages were incubated prior and during infection by mycobacteria with a low dose of calcimycin, a calcium ionophore. Mycobacterial trafficking was then analyzed by organelle electrophoresis. As shown in figure 33, right panels, upon treatment with calcimycin of coronin 1  $-/-$  macrophages mycobacteria resisted phagosome maturation since they were predominantly found in the phagosomal fractions. These results suggest that the lysosomal delivery of mycobacteria inside coronin 1 deficient macrophages is due to a loss in cytosolic calcium increase in the absence of coronin 1 upon phagocytosis.



The immediate result of an increase in intracellular calcium levels is the activation of calcineurin, a protein phosphatase responsible for the activation of various transcription factors. To analyze the result of calcineurin inhibition, wild type macrophages were treated with cyclosporin A, a calcineurin inhibitor, and then infected with mycobacteria. As shown in figure 34, right panels, upon inhibition of calcineurin, mycobacteria were directly transferred to lysosomes.



Together these data suggest that coronin 1 is dispensable for the activation of the main signaling pathways upon bacterial encounter but is predominantly a host factor responsible for mycobacterial pathogenesis by the activation of calcineurin.

### **3.3 Coronin 1 and polymorphonuclear leukocytes functions**

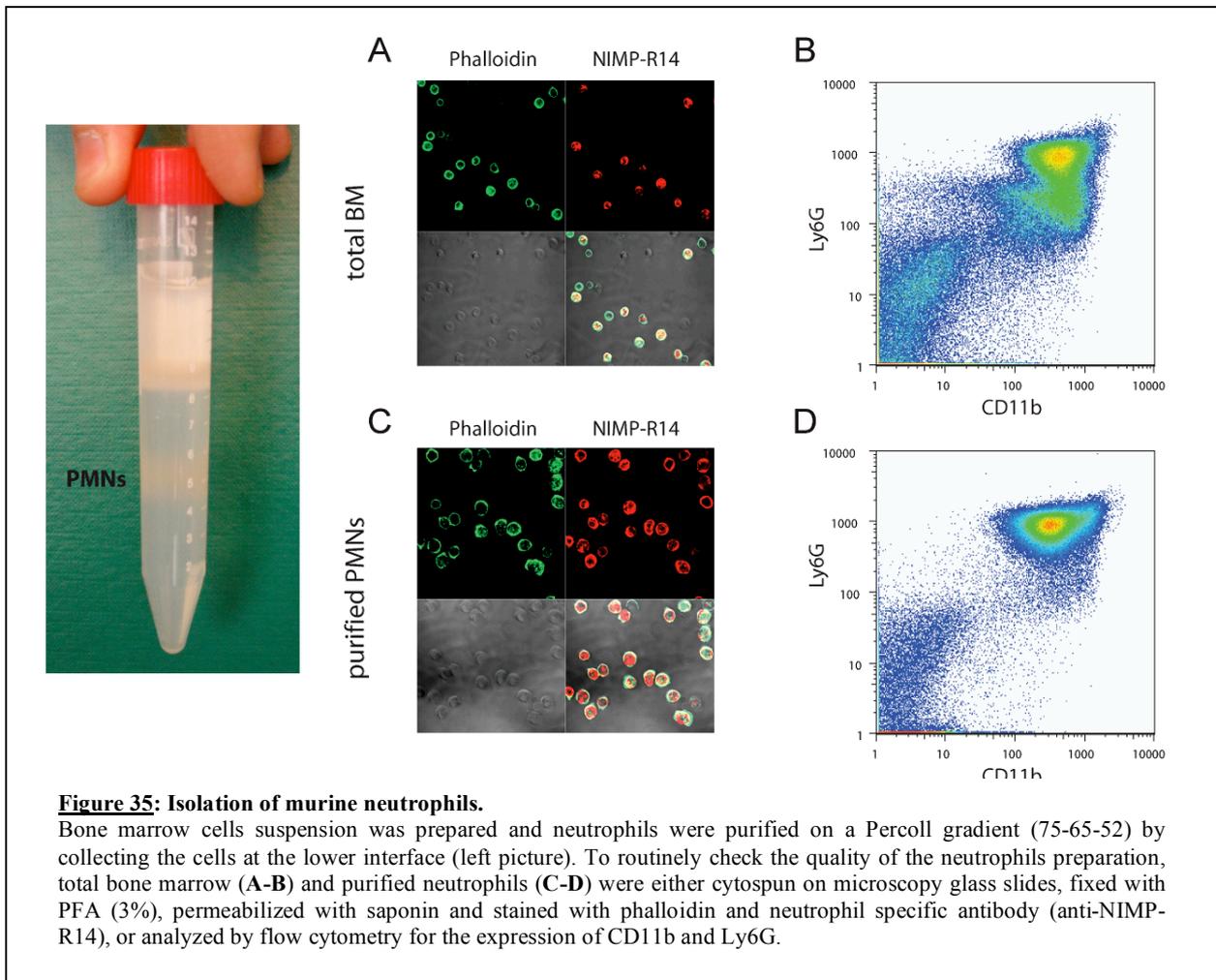
Coronins constitute a family of seven proteins that are conserved throughout the eukaryotic kingdom. While lower eukaryotes contain one or two coronin family members, in mammals up to seven coronins have been described (Rybakin and Clemen, 2005). The first member of the protein family was identified in *Dictyostelium discoideum*, where coronin was identified as an actin/myosin interacting protein (de Hostos et al., 1991). Since deletion of coronin from *Dictyostelium* affected several actin-dependent processes such as phagocytosis (Maniak et al., 1995), cell motility and cytokinesis (de Hostos et al., 1993; Fukui et al., 1999), it was concluded that in *Dictyostelium* coronin functions in the modulation of F-actin. In yeast, deletion of the single coronin gene *crn1* results in a mutant strain that does not show any phenotype (Goode et al., 1999; Heil-Chapdelaine et al., 1998), suggesting that the *crn1* gene product is dispensable for actin-mediated processes in yeast.

Of the seven mammalian coronin isoforms, coronin 1 (also known as p57 or TACO, for tryptophan aspartate-containing coat protein), that is exclusively expressed in leukocytes, is the closest related to *Dictyostelium* coronin, showing about 35% identity (Ferrari et al., 1999). Based on this homology as well as the colocalization of coronin 1 with actin at the cell cortex of leukocytes, coronin 1 was proposed to perform a role in the modulation of actin-dependent processes in leukocytes (Foger et al., 2006; Yan et al., 2005; Yan et al., 2007). However, analysis of several leukocyte populations from coronin 1 deficient mice, including T cells, B cells and macrophages, failed to show any defects associated in F-actin dependent processes. Instead, coronin 1 regulates cytosolic  $Ca^{2+}$  mobilization upon a variety of leukocyte stimuli, suggesting that coronin 1 is involved in  $Ca^{2+}$ -dependent signaling (Jayachandran et al., 2007; Mueller et al., 2008).

Neutrophils are granulocytes with significant migration capacity and are the first cells arriving at the site of inflammation thereby playing a crucial role in response to microbial infections. It was previously proposed that coronin 1 would be required for chemotaxis and phagocytosis in human neutrophils based on studies using cells transduced with dominant-negative fragments of coronin 1 (Yan et al., 2007) and that coronin 1 interacts with cytosolic phox proteins (Grogan et al., 1997). We examined the exact role of coronin 1 in murine neutrophils using a mouse lacking coronin 1 expression. The questions addressed in this part of the study were: are the morphology and F-actin cytoskeleton of neutrophils modified in the absence of coronin 1? Are the actin-dependent processes such as adhesion, spreading or phagocytosis altered in coronin 1 deficient neutrophils? Is the NADPH oxidase activity of neutrophils maintained in the absence of coronin 1? What is the effect of coronin 1 deletion on neutrophils chemotaxis in vitro and in vivo?

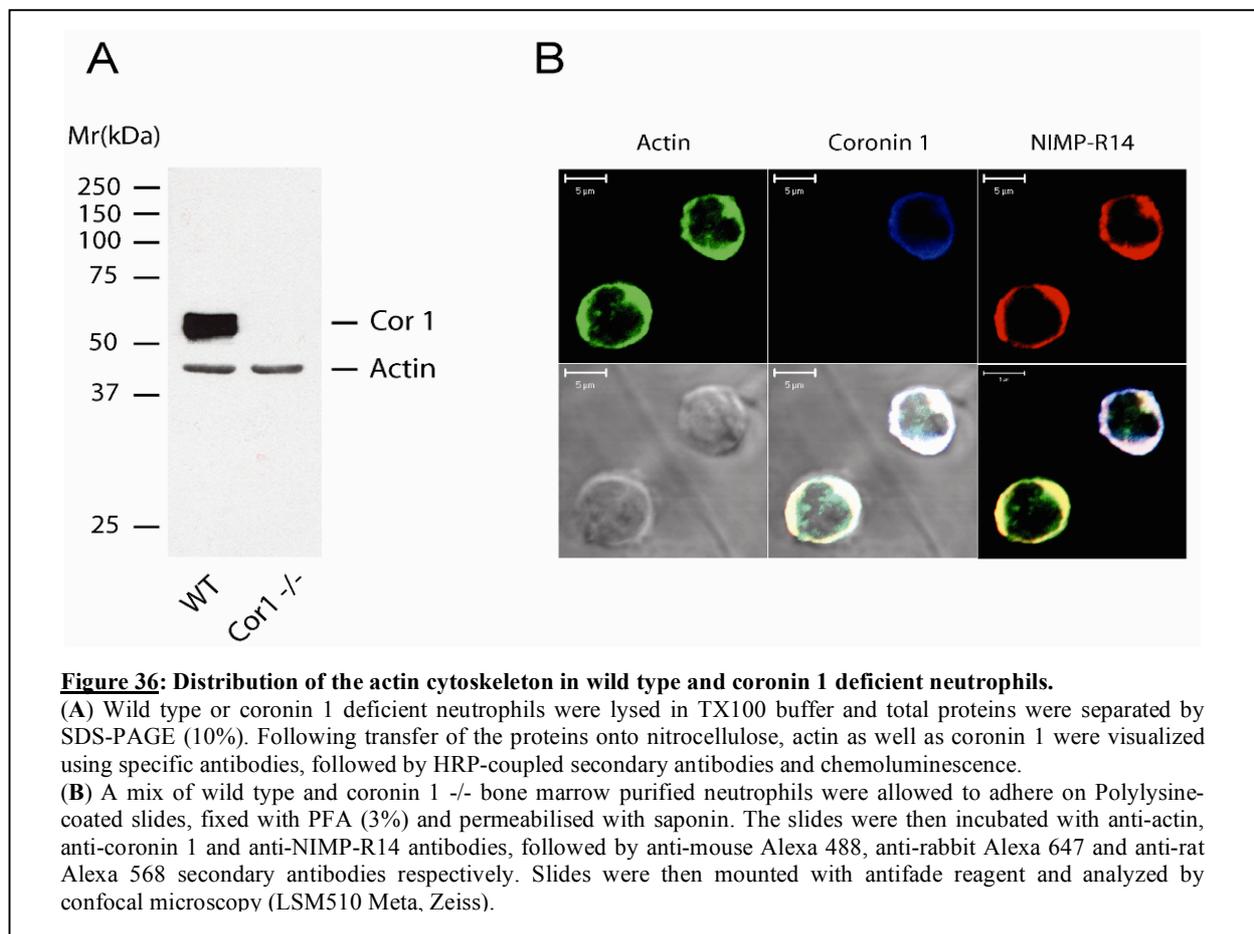
### 3.3.1 Neutrophil morphology and F-actin distribution in the presence and absence of coronin 1

To analyze a role for coronin 1 in murine neutrophils, cells were isolated from the bone marrow and purified by Percoll gradient. Briefly the bone marrow cells were taken from both femur and tibia from wild type and coronin 1  $-/-$  mice and the red blood cells were lysed in hypotonic buffer. The cells were resuspended in HBSS, layered onto a Percoll gradient (75-65-52) and centrifuged for 30 min at  $1500 \times g$ . The neutrophils were collected at the lower interface (figure 35, left picture) and washed several times with HBSS to remove the Percoll. The neutrophil preparation was at least 90% pure as routinely checked by immunofluorescence after cytopspin onto microscopy glass slides (figure 35, A and C) and by flow cytometry (figure 35, B and D). Neutrophils isolated from the bone marrow were used for the experiments described below unless otherwise mentioned. Alternatively, neutrophils were isolated from the blood after Dextran sedimentation of the erythrocytes and purification on a Nycodenz gradient.



Since neutrophils express besides coronin 1 also coronin 2, 3, 4 and 7 (Yan et al., 2007), it was analyzed whether coronin 1 antibodies would cross-react with any of these coronin isoforms. To that end, neutrophils as isolated above from wild type or coronin 1 deficient mice were lysed, and proteins separated by SDS-PAGE followed by immunoblotting using anti-coronin 1 and anti-actin antibodies. As shown in figure 36A, no signal was obtained in case coronin 1  $-/-$  neutrophil lysates were probed using anti-coronin 1 antibody, indicating the specificity of the antibody used. As a control, the immunoblot was also stained using anti-actin antibodies, revealing a similar signal in both wild type as well as coronin 1 deficient neutrophils and suggesting that actin levels are not modified in the absence of coronin 1 (figure 36A).

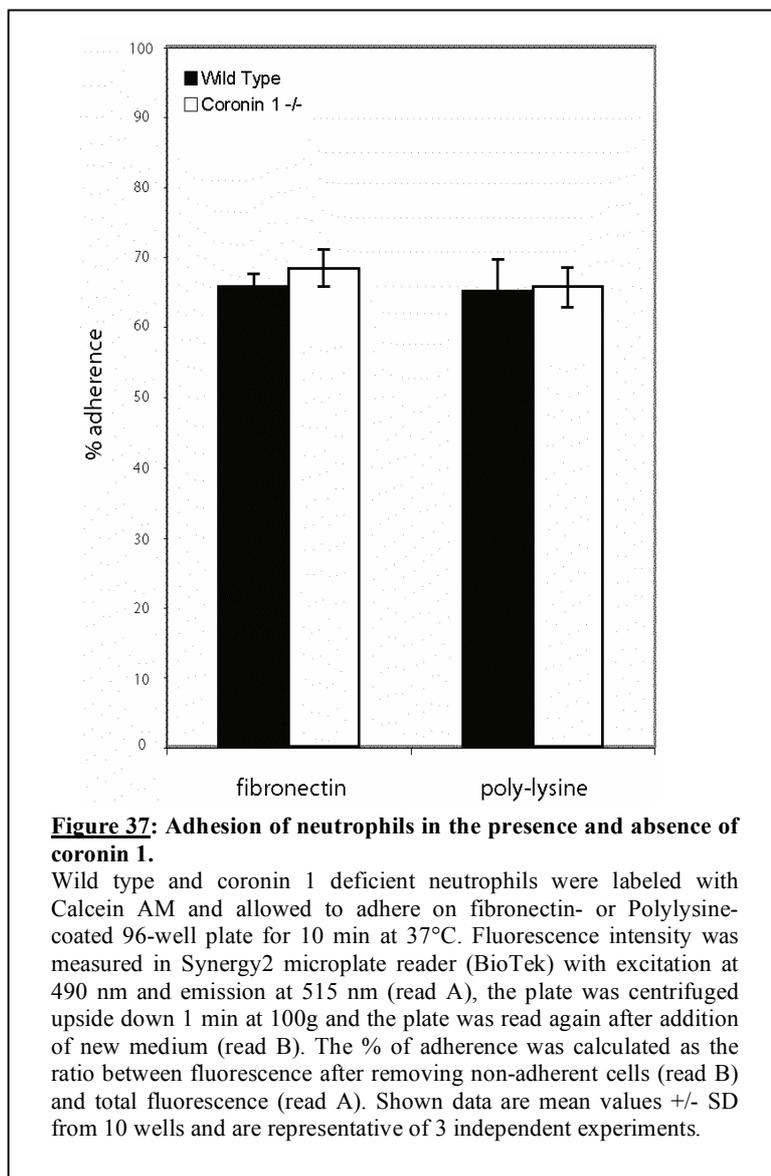
Coronin 1 is the closest homologue to *Dictyostelium* coronin that was identified as an actin-myosin interacting protein (de Hostos et al., 1991). Since in *Dictyostelium*, coronin has been implicated in F-actin mediated processes, it has been generally assumed that coronin 1 performs an F-actin regulatory function in leukocytes as well. To analyze the morphology and the F-actin cytoskeleton of neutrophils in the presence and absence of coronin 1, neutrophils were allowed to adhere to coverslips, fixed, and stained for actin, NIMP-R14, a neutrophil marker, and coronin 1. As shown in figure 36B, both the overall morphology as well as the distribution of actin was similar in wild type as well as coronin 1 deficient neutrophils.



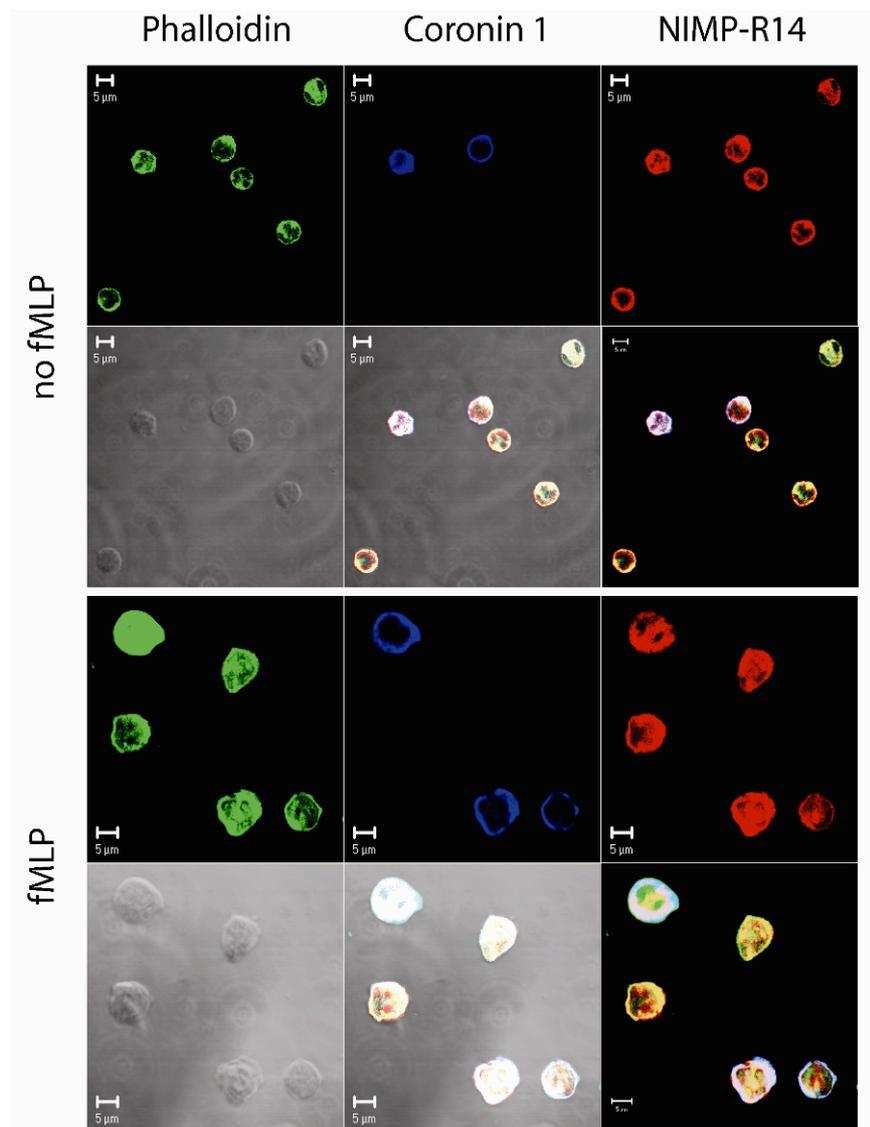
### 3.3.2 Adhesion and spreading of neutrophils in the absence and presence of coronin 1

Neutrophils are highly polymorphic cells, and since the F-actin cytoskeleton is heavily involved in cytoskeleton rearrangements the effect of coronin 1 deletion on neutrophil adhesion and spreading was analyzed.

To analyze adhesion, neutrophils from wild type and coronin 1 *-/-* origin were labeled with Calcein AM, a cell-permeant dye, and allowed to adhere on either fibronectin- or Polylysine-coated 96-well plate. The plate was then centrifuged upside down to remove non-adherent neutrophils and fluorescence intensity was measured before and after centrifugation. The % of adherence was expressed as the ratio of fluorescence intensity after and before removing non-adherent cells. No quantitative or qualitative differences in adherence were apparent between neutrophils from wild type or coronin 1 deficient mice on fibronectin- or Polylysine-coated surface (figure 37).



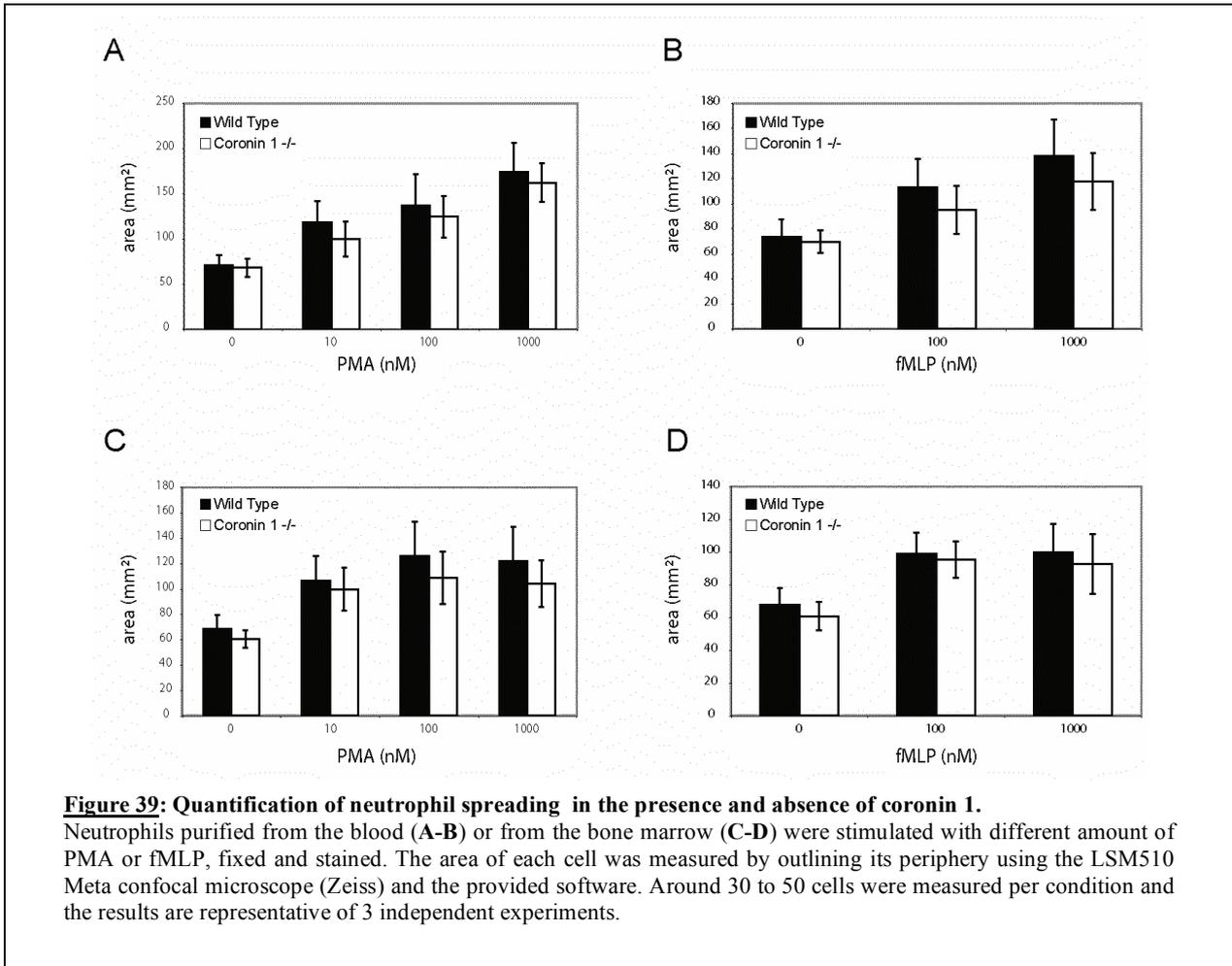
To analyze neutrophil spreading, either Phorbol-12-myristate-13-acetate (PMA) or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) was added to a mixture of wild type and coronin 1 *-/-* cells seeded on Polylysine-coated microscopy slides. After 5 min incubation at 37°C, the cells were fixed, permeabilised and stained to detect F-actin, coronin 1 and NIMP-R14. As shown in figure 38, both wild type and coronin 1 deficient neutrophils could efficiently spread upon stimulation (lower panel).



**Figure 38: Spreading of neutrophils in the presence and absence of coronin 1.**

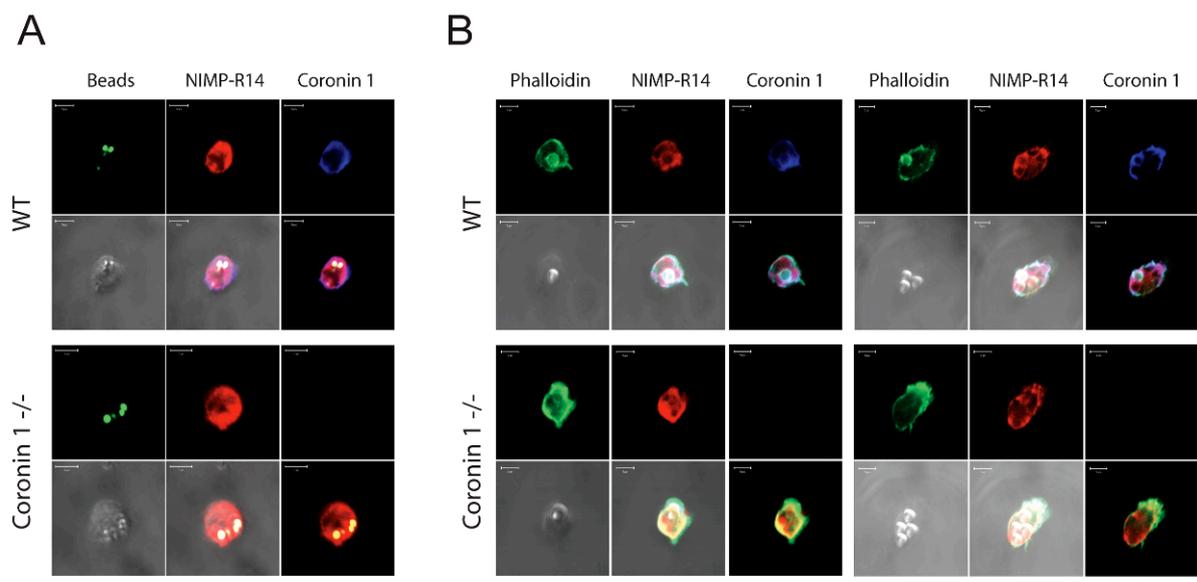
A 1:1 mixture of wild type and coronin 1 <sup>-/-</sup> neutrophils were added onto Polylysine-coated microscopy slides for 1 min at room temperature. The stimulus (1  $\mu$ M of fMLP) was then added to these cells for 5 min to allow cell spreading and the cells were fixed in PFA (3%) and stained for phalloidin, coronin 1 and the neutrophil marker NIMP-R14

The spreading was further quantified by measuring the area of neutrophils upon stimulation by drawing a line at the surrounding of the cells using the LSM510 Meta software (Zeiss). As can be seen in figure 39, the addition of PMA (A and C) as well as fMLP (B and D) resulted in a concentration-dependent increase in the surface area of neutrophils isolated from blood (A-B) or bone marrow (C-D) that was independent on the presence or absence of coronin 1. Together these results indicate that coronin 1 is dispensable for neutrophil adhesion and spreading.



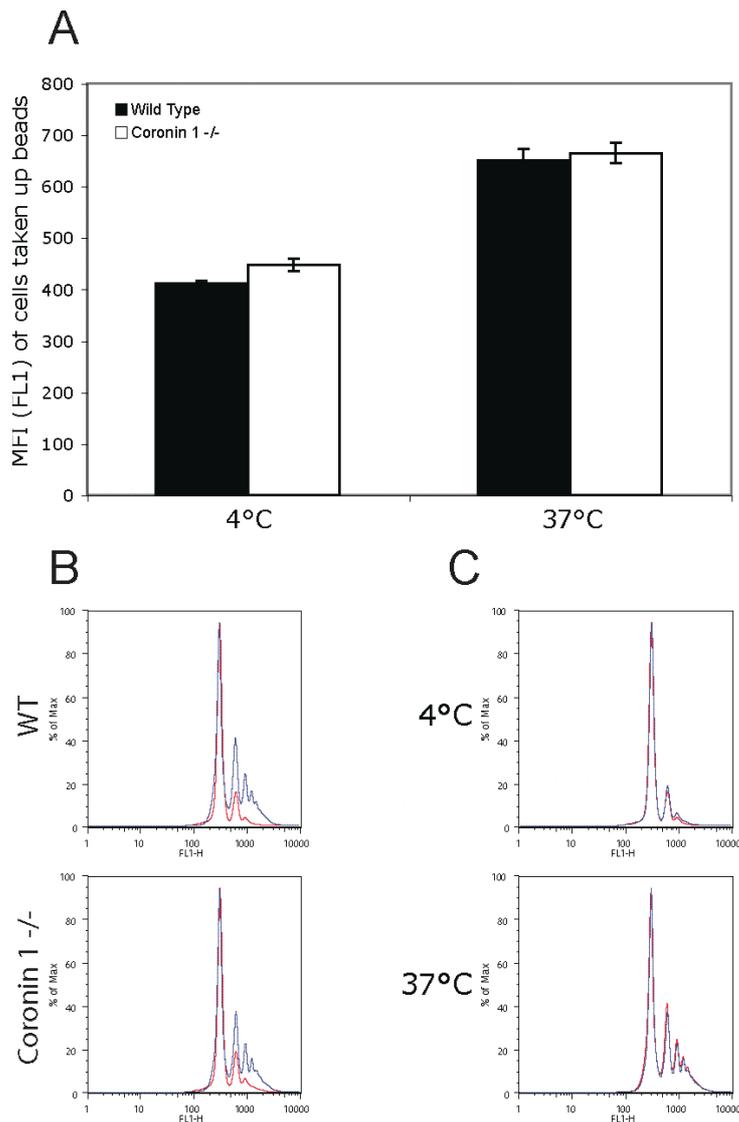
### 3.3.3 Phagocytosis of neutrophils lacking coronin 1

Coronin 1 transiently accumulates at the nascent phagosome and dissociates from the phagosomal membrane prior to phagosome-lysosome fusion (Ferrari et al., 1999; Yan et al., 2005). To analyze a role for coronin 1 in phagocytosis by neutrophils, opsonized beads were added to purified neutrophils on ice and the cells were then transferred to 37°C to allow them to internalize the beads bound on their surface. As shown by immunofluorescence, both wild type and coronin 1 -/- neutrophils phagocytosed several 1 μm beads per cell (figure 40A). They could also internalize a single or several larger particles (2.8 μm) despite the absence of coronin 1 (figure 40B).



**Figure 40: Phagocytosis of opsonised beads by wild type and coronin 1 <sup>-/-</sup> neutrophils.**  
 Wild type and coronin 1 deficient neutrophils were allowed to adhere onto Polylysine-coated slides for 15 min at 37°C; the slides were then washed once and transferred to ice. Yellow-green fluorescent 1 µm beads (A) and 2.8 µm beads (B) were opsonised with IgG and added on ice to the neutrophils for 15 min. The cells were then washed 4 times with cold medium to remove the unbound beads and either cold medium was added and the slides kept on ice (cold control) or prewarmed medium was used and the slides shifted to 37°C for 45 min. Cells were then fixed in PFA (3%) and stained with phalloidin (only B), anti-NIMP-R14 and anti-coronin 1, and further analyzed by confocal microscopy.

The number of 1 µm beads internalized per cell was further quantified by incubating fluorescent beads with neutrophils in suspension. After washing to remove the non phagocytosed beads, the cells were analyzed by flow cytometry and the fluorescence intensity of those cells having internalized beads was measured. As can be seen in figure 41A, the Mean Fluorescence Intensity (MFI) of wild type (filled bars) and coronin 1 deficient neutrophils (open bars) was similarly increased when the cells were allowed to phagocytose fluorescent beads at 37°C suggesting that the phagocytic capacity of the neutrophils was not altered upon deletion of coronin 1. As represented in figure 41B, both wild type (top panel) and coronin 1 <sup>-/-</sup> neutrophils (bottom panel) phagocytosed several beads when shifted to 37°C (blue line) compared to the cells incubated at 4°C (red line). As shown in figure 41C top panel, when the fluorescence of the wild type neutrophils (red line) was compared to the one of the neutrophils lacking coronin 1 (blue graph) no difference was detected indicating that the binding of the beads at 4°C was not affected in the absence of coronin 1. The fluorescent profiles of wild type (red line) and coronin 1 <sup>-/-</sup> neutrophils (blue line) when incubated with beads at 37°C (figure 41C, bottom panel) were also similar, confirming that the number of beads internalized per cell was not influenced by the presence or the absence of coronin 1.



**Figure 41: Quantification of opsonised bead phagocytosis by wild type and coronin 1  $-/-$  neutrophils by flow cytometry.**

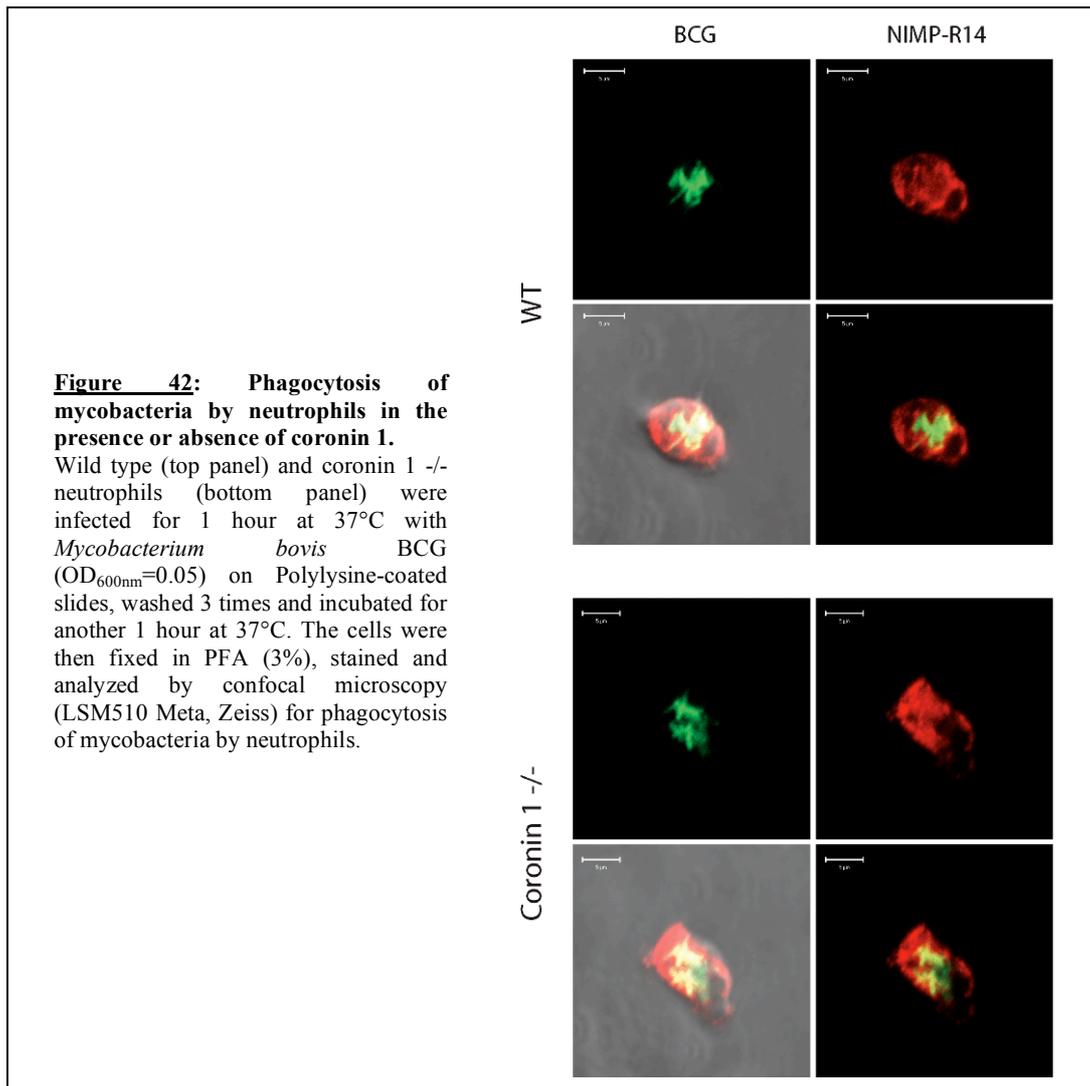
(A) Wild type and coronin 1  $-/-$  neutrophils were incubated with opsonised FITC 1  $\mu$ m beads for 45 min at 4°C or 37°C. Cells were then extensively washed in PBS-FBS (2%) to remove the non-internalized beads and the samples were analyzed using a FACSCalibur (BD). The Mean Fluorescence Intensity (MFI) of the cells that phagocytosed beads was calculated to evaluate the number of beads internalized per neutrophils. The data are mean values  $\pm$  SD from triplicate samples and are representative of 4 independent experiments.

(B) Representation of the beads phagocytosis in wild type (top panel) and coronin 1  $-/-$  (bottom panel) neutrophils at either 4°C (red graph) or 37°C (blue graph).

(C) Representation of the beads phagocytosis at 4°C (top panel) and 37°C (bottom panel) by either wild type (red graph) or coronin 1  $-/-$  (blue graph) neutrophils.

Coronin 1 has been previously demonstrated to be recruited and retained around phagosomes containing pathogenic mycobacteria (Ferrari et al., 1999) and to be essential for mycobacterial survival inside macrophages (Jayachandran et al., 2007). To study the ability of coronin 1  $-/-$  neutrophils to phagocytose mycobacteria, bone marrow purified neutrophils were infected with *M. bovis* BCG, fixed, stained and analyzed by confocal laser scanning microscopy. As shown in figure 42, both in the presence and absence of coronin 1, neutrophils were capable of internalizing mycobacteria.

Taken together, these data show that coronin 1 is dispensable for phagocytosis in neutrophils.

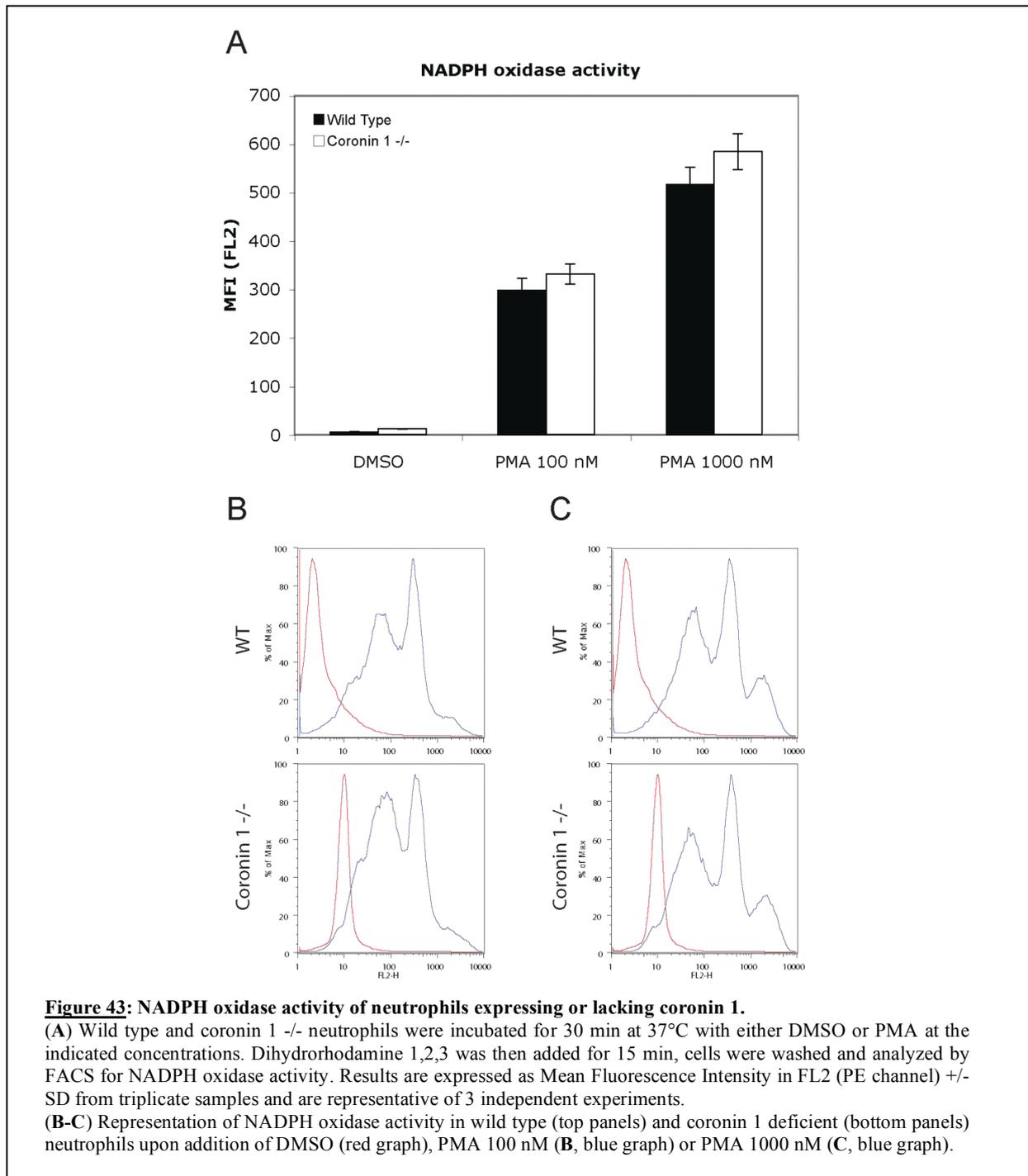


### 3.3.4 Oxidase activity of coronin 1 <sup>-/-</sup> neutrophils

Coronin 1 has been shown to be transiently associated with the NADPH oxidase during phagocytosis and was suggested to participate in the stabilization of the NADPH complex (Allen et al., 1999; Grogan et al., 1997). To directly investigate a contribution of coronin 1 to NADPH oxidase activity, neutrophils prepared from wild type and coronin 1 <sup>-/-</sup> bone marrow were stimulated with phorbol-12-myristate-13-acetate (PMA). Dihydrorhodamine 1,2,3 (DHR 1,2,3) was further supplemented and the cells were analyzed by flow cytometry after washing out the extracellular dye. In the presence of reactive oxygen species, DHR 1,2,3 is converted into rhodamine 1,2,3, a brightly fluorescent dye. Upon addition of PMA, the Mean Fluorescence

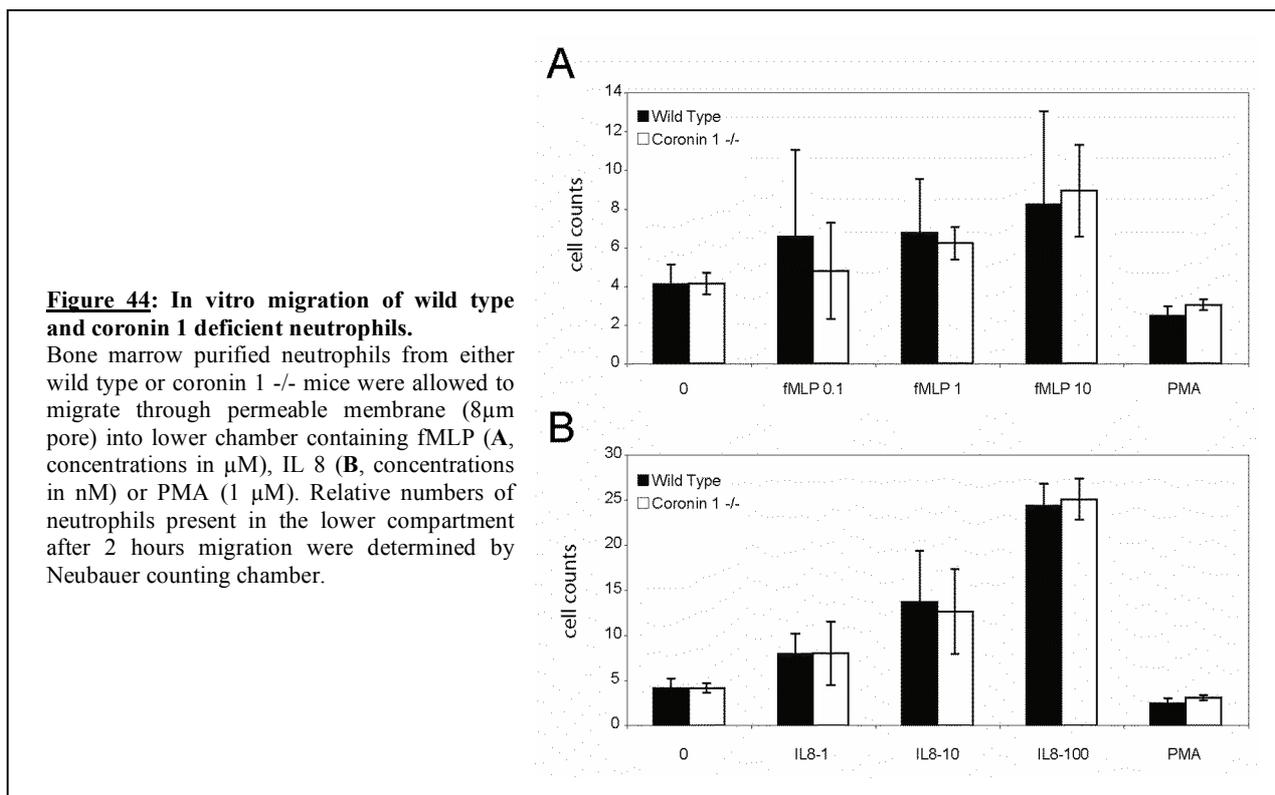
Intensity of DHR 1,2,3 loaded cells increased dramatically but no difference was observed in the absence or the presence of coronin 1 (figure 43A). The fluorescence profiles of wild type (top panels) and coronin 1  $-/-$  neutrophils (bottom panels) were similar upon addition of PMA at concentrations of 100 nM (figure 43B, blue lines) or 1000 nM (figure 43C, blue lines).

These results suggest that coronin 1 is dispensable for PMA-mediated NADPH oxidase activity.



### 3.3.5 Chemotaxis of neutrophils in the presence and absence of coronin 1

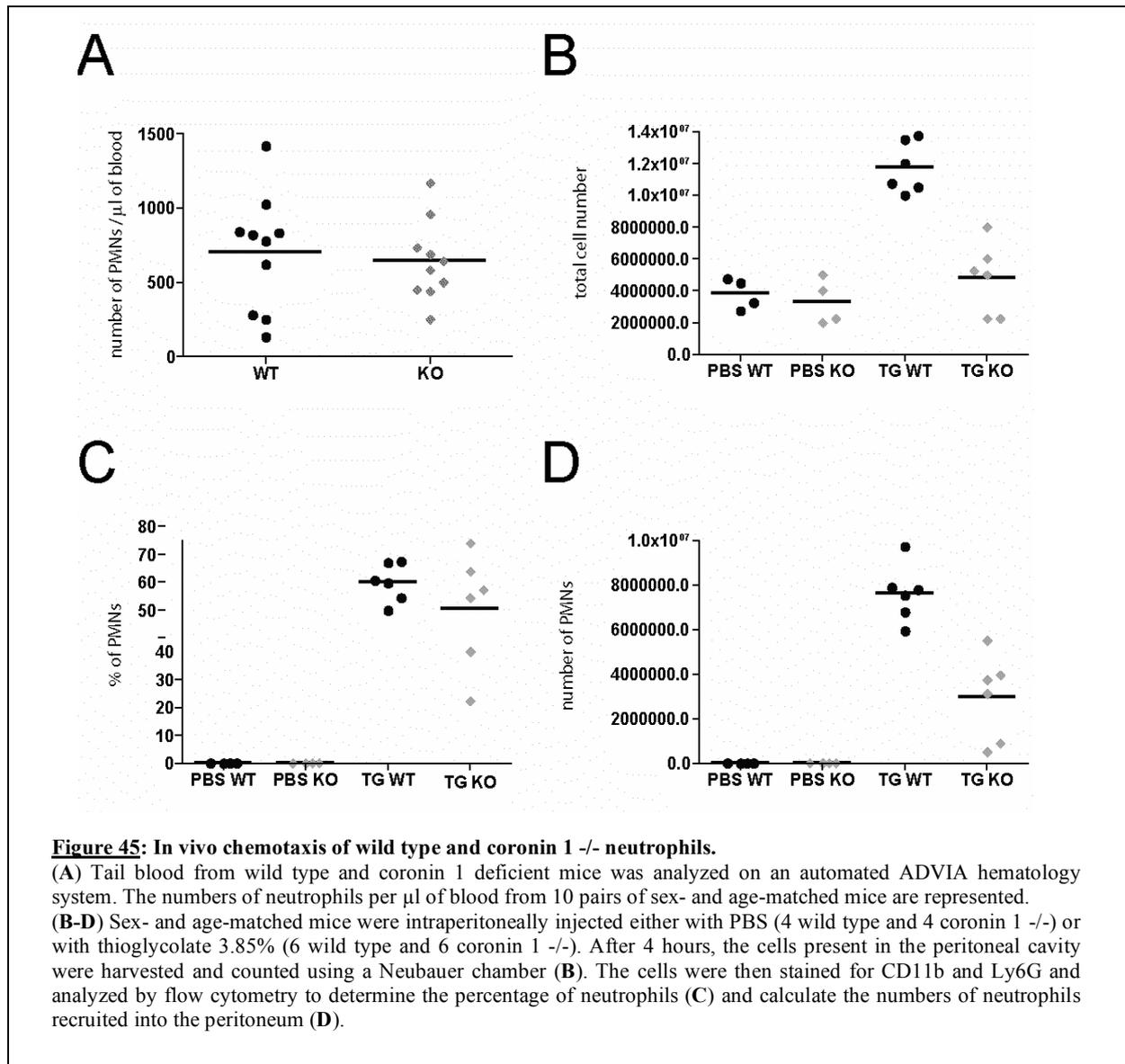
We next analyzed a possible involvement of coronin 1 in chemotaxis. To that end, wild type or coronin 1 deficient neutrophils were incubated on top of a permeable transwell membrane and allowed to migrate through an 8  $\mu\text{m}$  pore into the lower compartment that contained the strong neutrophil chemoattractants fMLP or IL-8. After 2 hours incubation at 37°C, while both chemoattractants stimulated the migration of neutrophils to the lower chamber, no differences were observed in the chemotactic activity of wild type versus coronin 1 deficient neutrophils (figure 44).



Prior to the analysis of *in vivo* chemotaxis, the number of circulating neutrophils was determined by analyzing the blood composition of wild type and coronin 1  $-/-$  mice using an ADVIA hematology system. As shown in figure 45A, the number of neutrophils in the blood was not dramatically influenced by the absence of coronin 1 while, as expected (Mueller et al., 2008), the number of lymphocytes was severely reduced (data not shown).

Interestingly, when migration was analyzed *in vivo* 4 hours after thioglycolate injection, it appeared that leukocyte recruitment to the peritoneal cavity was less efficient in coronin 1 deficient mice as compared to wild type mice (figure 45B). The leukocytes recruited in the peritoneum of wild type and coronin 1  $-/-$  mice were mainly neutrophils as analyzed by flow cytometry (figure 45C)

implicating that specific recruitment of neutrophils after short thioglycolate injection is diminished in coronin 1  $-/-$  mice (figure 45D).



Together these results suggest that whereas in coronin 1 deficient mice neutrophils are less efficiently recruited to the peritoneum, coronin 1 is dispensable for chemotactic activity as triggered by fMLP and IL-8.

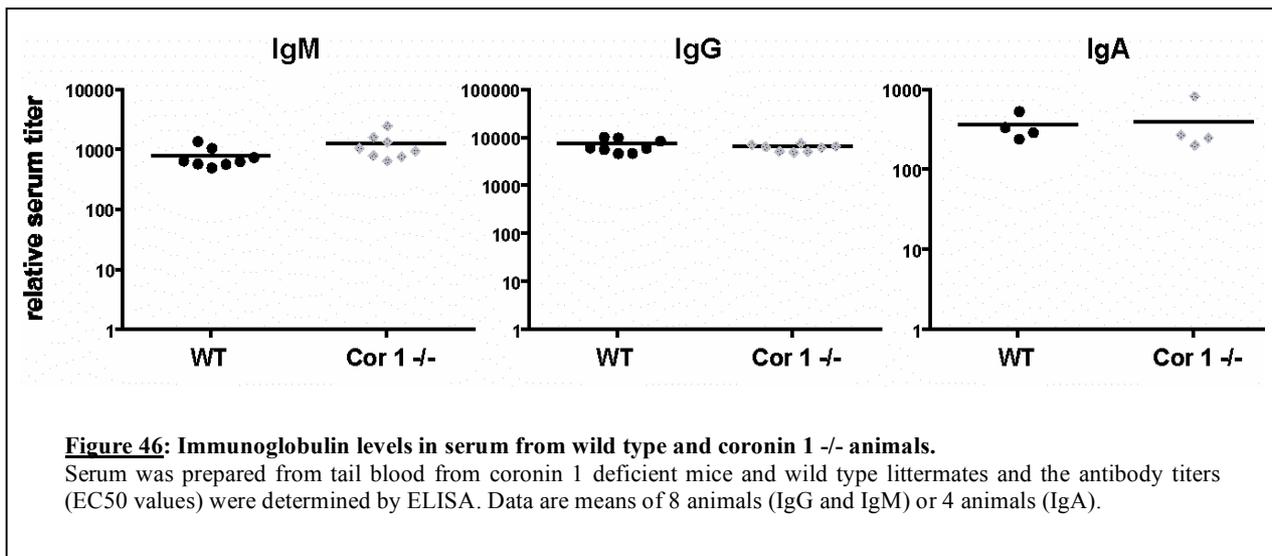
### **3.4 Role of Coronin 1 in B lymphocytes functions**

The immune response defends the host organism against pathogens or dysfunctions by coordinating innate and acquired immunity. B lymphocytes are responsible for the humoral adaptive immune response after binding specific foreign antigens via their receptors leading to proliferation and differentiation into antibody-producing cells. The B-cell antigen receptor (BCR) consists of an antigen binding subunit, a membrane-bound immunoglobulin, and a subunit transducing the signal, non-covalently linked Ig $\alpha$ /Ig $\beta$  heterodimers (Geisberger et al., 2003). Recognition of antigens induces BCR aggregation and transduction of the signals via phosphorylation of the Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) present on the cytoplasmic tail of the Ig $\alpha$  and Ig $\beta$  subunits by Lyn kinase. This initial signaling step results in the recruitment and activation of other signaling elements such as Syk, Btk, BLNK and PLC $\gamma$ -2 (Dal Porto et al., 2004; Fruman et al., 2000; Kurosaki et al., 2000). Finally, the phosphorylation of PLC $\gamma$ -2 triggers the conversion of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into two lipid second messengers: inositol 1,4,5-triphosphate (InsP<sub>3</sub>) and diacylglycerol (DAG). DAG activates protein kinase C  $\beta$  (PKC $\beta$ ) and InsP<sub>3</sub> induces calcium release from the internal stores by interacting with the IP<sub>3</sub>R present on endoplasmic reticulum. This increase in intracellular calcium level promotes calcium entrance through the plasma membrane via calcium channels (Gallo et al., 2006; Parekh, 2006, 2007).

The mechanisms and proteins that allow the transduction of B cell receptor cross-linking into an increase of cytosolic calcium remain poorly defined. Recently, coronin 1 (also known as p57 or TACO, for tryptophan aspartate-containing coat protein) a protein specifically expressed in leukocytes, was found to be essential for the induction of cytosolic calcium fluxes following T cell receptor activation (Mueller et al., 2008). Given the similarities of T- and B cell receptor signaling, a role for coronin 1 in B cell receptor-mediated cytosolic calcium mobilization was analyzed. In this work, we show that *in vitro*, coronin 1 is necessary for the generation of calcium fluxes as well as B cell proliferation upon BCR triggering. In the presence of costimulation however, coronin 1 dependent calcium mobilization was dispensable for B cell proliferation, consistent with the generation of normal immune responses in coronin 1 deficient mice.

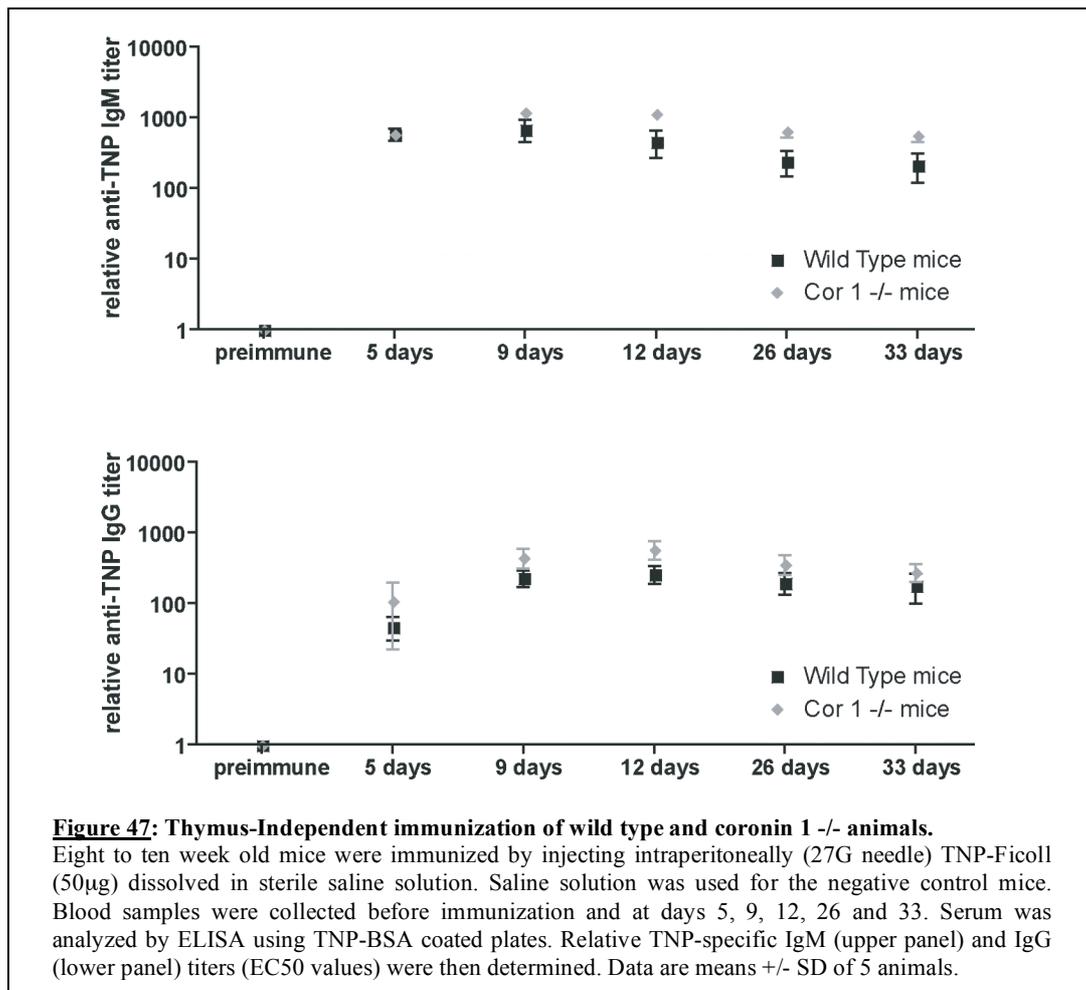
### 3.4.1 Immune responses in mice lacking coronin 1

To study the functionality of the immune system in the absence of coronin 1, the amount of immunoglobulin in blood serum was determined by an Enzyme-Linked Immunosorbent Assay (ELISA). Antibody titers of coronin 1  $-/-$  mice were found to be similar for the different isotypes (IgM, IgG and IgA) when compared to wild type littermates (figure 46).

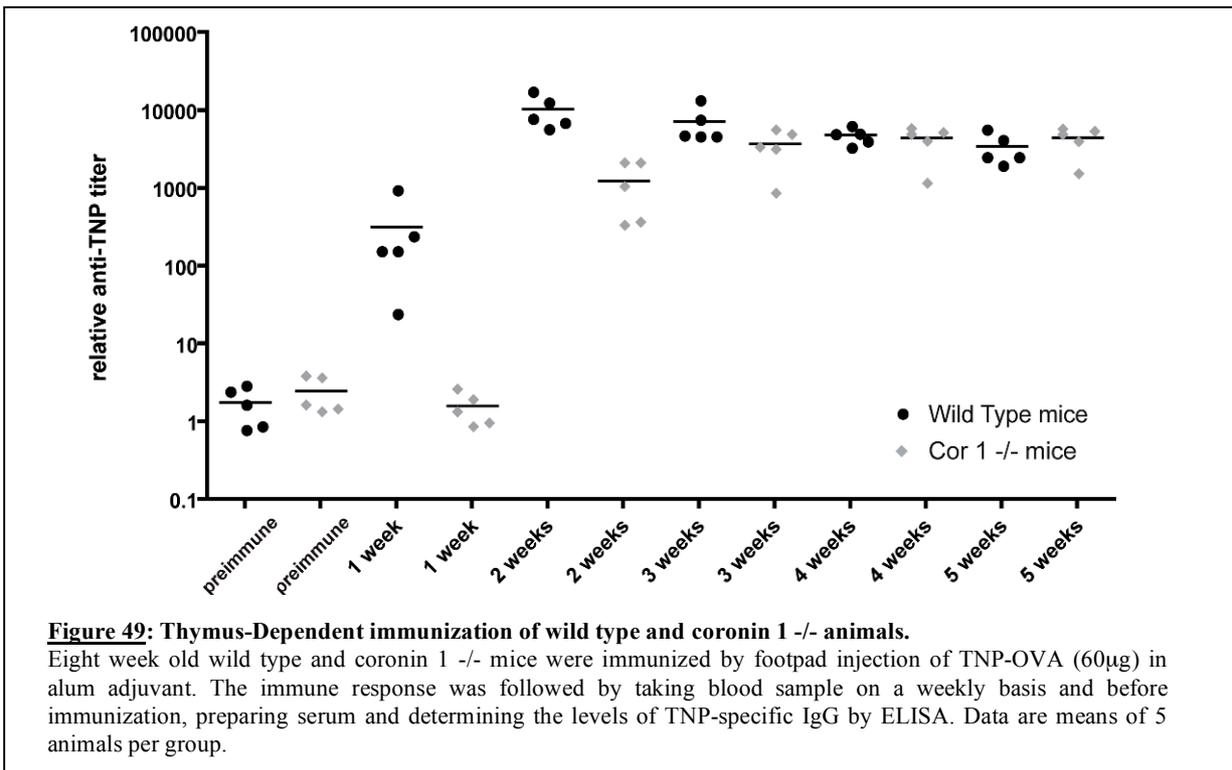
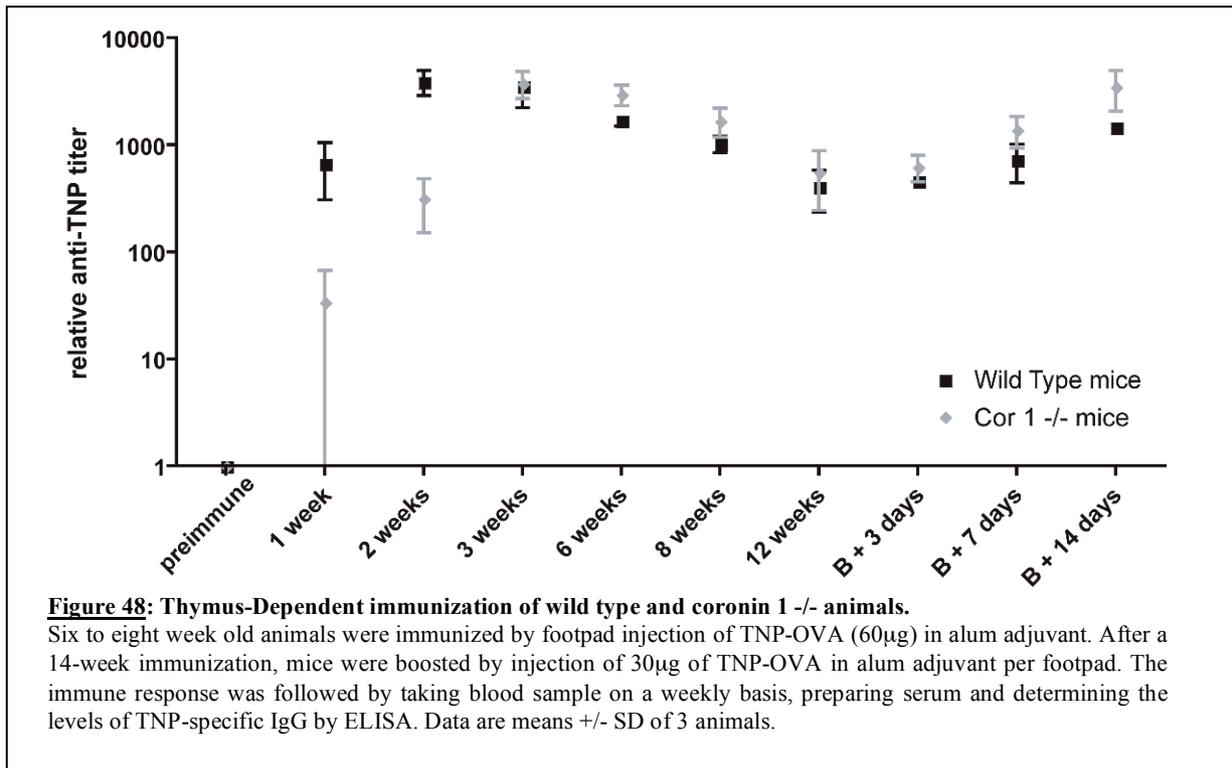


To analyze the capacity to mount an immune response in the presence and absence of coronin 1, wild type or coronin 1-deficient mice were immunized with the thymus-independent (TI) antigen TNP-Ficoll (Mond et al., 1995a; Mond et al., 1995b) and the humoral response was followed over a period of 33 days by ELISA. As shown in figure 47 (upper panel), both wild type as well as coronin 1 deficient mice were able to produce IgM specific for TNP to the same degree and with similar kinetics following immunization. After 5 days of immunization, TNP-specific IgM titers were elevated and reached a maximum after 9 days. Similarly, when the amounts of TNP-specific IgG were analyzed, both wild type as well as coronin 1 deficient animals produced anti-TNP IgG quantitatively and kinetically to the same degree (figure 47, lower panel). The peak in intensity of IgG was obtained after 12 days of immunization, which is in accordance with a normal delay of IgG production compared to IgM (MacLennan et al., 1997).

These data demonstrate that coronin 1  $-/-$  animals can generate specific immunoglobulins similar to wild type littermates in terms of isotype, quantity and kinetics against TI antigens.

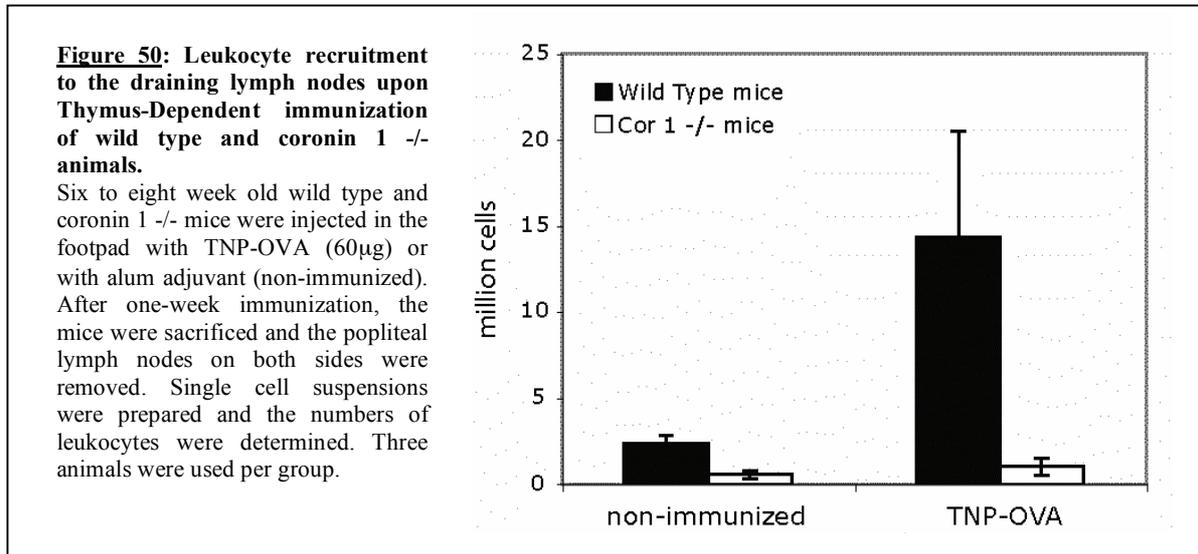


To analyze the immune response upon thymus-dependent (TD) antigen encounter, mice were immunized with TNP-OVA (Parker, 1993). Six to eight week old wild type and coronin 1  $-/-$  were injected in the footpad with TNP-OVA in alum adjuvant. The humoral response was followed by taking blood samples on a weekly basis and analyzing the antibody titer in the serum by ELISA. As shown in figure 48, while initially the quantity of TNP-specific antibodies was lower in coronin 1 deficient animals, after a three-week period both wild type as well as coronin 1 deficient mice generated equal amounts of TNP-specific IgG. The immunization was repeated with more animals (5 mice per group) and the same phenomenon was observed. As illustrated in figure 49, wild type mice could produce TNP-specific IgG after one-week immunization while coronin 1  $-/-$  mice were not responsive at this time point. However, coronin 1  $-/-$  mice were able to mount a delayed immune response against TNP-OVA and reach antibody titers similar to wild type animals after 3 weeks.



Since the efficiency of an immune response depends on the presence of primed T cells in lymph nodes, we analyzed whether the delay in immune responses could be due to the reduced number of leukocytes in the periphery (Mueller et al., 2008). To that end, the draining lymph nodes of immunized wild type or coronin 1 deficient mice were analyzed after one week for cellularity. While in immunized wild type animals, immunization resulted in a 5-10 fold increase in leukocyte

counts in the lymph nodes analyzed, no significant cell recruitment was observed in the popliteal lymph nodes of immunized coronin 1 deficient animals (figure 50), suggesting that the delayed immune response in the absence of coronin 1 was probably due to partial depletion of peripheral T cells.



To analyze memory immune responses, immunized mice were boosted 14 weeks after initial injection followed by the analysis of TNP-specific IgG. As can be seen in figure 48, no significant differences were observed in the memory responses of either wild type or coronin 1 deficient animals in term of specific immunoglobulin production. As an alternative analysis of the memory immune response the formation of germinal centers was studied. The germinal centers develop in secondary lymphoid organs after exposure to thymus-dependent antigens. Within germinal centers, B lymphocytes proliferate, undergo affinity maturation and isotype class switching. Two weeks after the boosting, mice were sacrificed and the leukocytes present in the draining lymph nodes were counted and analyzed by flow cytometry. The number of B lymphocytes forming germinal centers ( $CD19^+$ ,  $PNA^+$  and  $IgD^-$ ) was 3-fold increased in both wild type and coronin 1  $-/-$  mice (figure 51A). Furthermore, analysis of the morphology of the germinal centers in the draining lymph nodes at day 14 after boosting revealed a similar histology for either wild type or coronin 1 deficient animals (figure 51B).

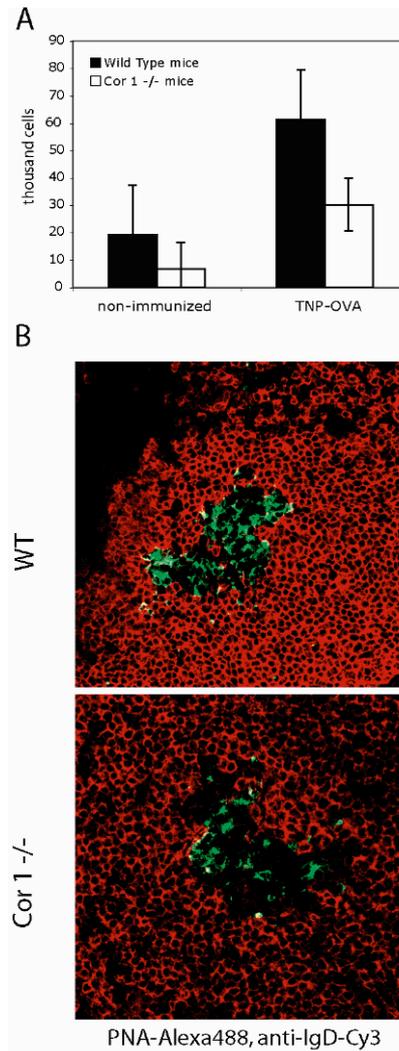
These data suggest that *in vivo*, coronin 1 deficient B cells are functional and coronin 1 deficient mice are able to mount an immune response against both thymus independent as well as thymus dependent antigens to a similar degree.

**Figure 51: Germinal center formation in wild type and coronin 1  $-/-$  lymph nodes upon Thymus-Dependent immunization.**

Six to eight week old animals were immunized by footpad injection of TNP-OVA (60 $\mu$ g) in alum adjuvant. After a 14-week immunization, mice were boosted by injection of 30 $\mu$ g of TNP-OVA in alum adjuvant per footpad. Two weeks after boosting, the mice were sacrificed and the popliteal lymph nodes were removed.

(A) The popliteal lymph nodes were homogenized, the cells were counted and analyzed by flow cytometry to determine the number of cells forming germinal center (CD19 $^+$ , PNA $^+$  and IgD $^-$ ). Data are means  $\pm$  SD of 3 animals per group.

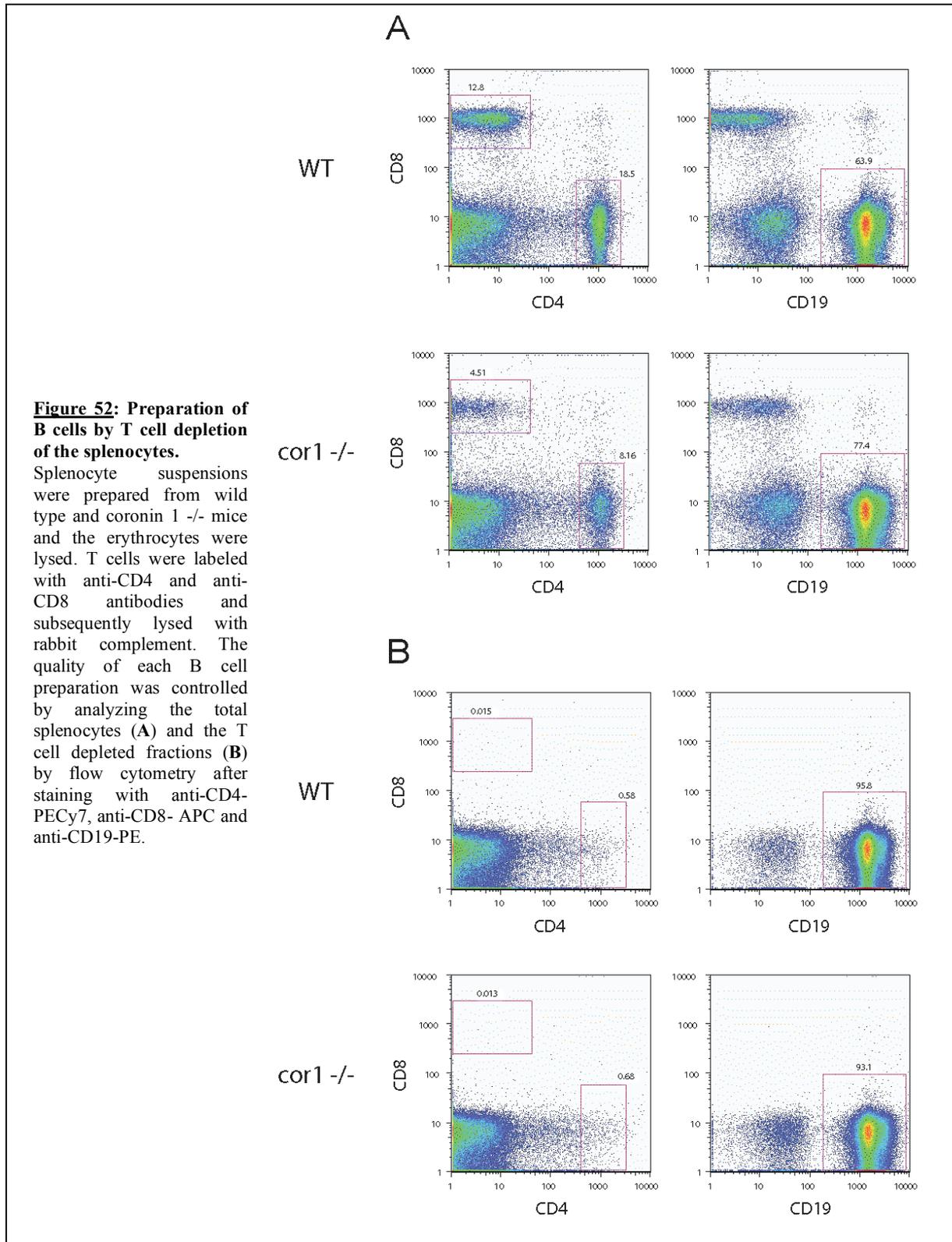
(B) Alternatively, lymph nodes were embedded in OCT Compound, frozen and 5  $\mu$ m sections were prepared using a cryostat. The sections were then fixed, rehydrated and stained with anti-IgD and peanut agglutinin (PNA). The slides were analyzed on a confocal microscope using a 20x magnification.

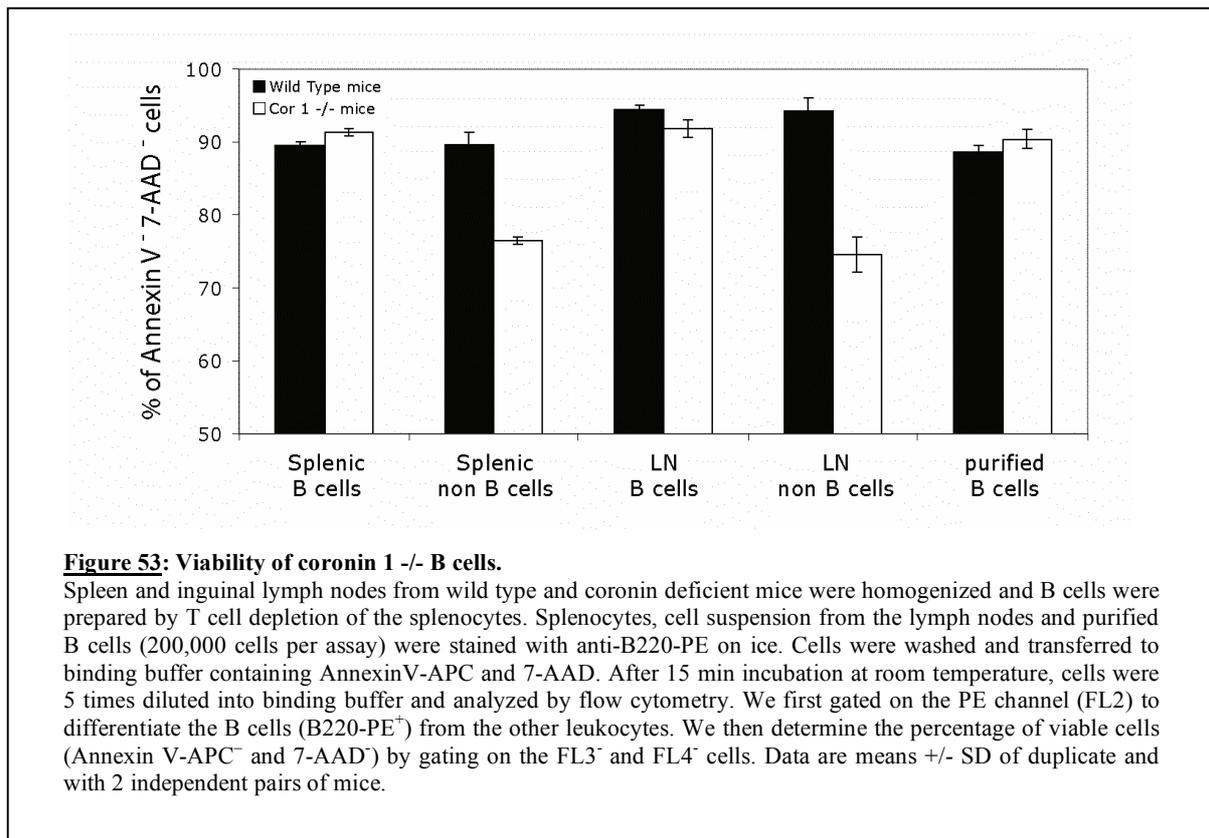


### 3.4.2 B cell proliferation in the presence and absence of coronin 1

The above results suggest that mice lacking coronin 1 were able to respond to antigenic challenge. In addition, the development of the B cells in the bone marrow did not present any defect and the different subsets appeared also normally in the periphery (Mueller et al., 2008). To address more specifically the role of coronin 1 in B lymphocytes functions, we prepared B cells from wild type and coronin 1  $-/-$  mice. Erythrocytes depleted splenocytes were incubated with anti-CD4 and anti-CD8 antibodies and the T cells were removed by complement lysis. The quality of the preparation was routinely controlled by flow cytometry and the B cells were found to be around 95% pure (figure 52). Furthermore, the viability of the purified B cells was analyzed using Annexin V staining, which is a sensitive assay for apoptosis. As can be seen in figure 53, both wild type and coronin 1  $-/-$  B lymphocytes were around 90% viable and comparable to the values obtained for B

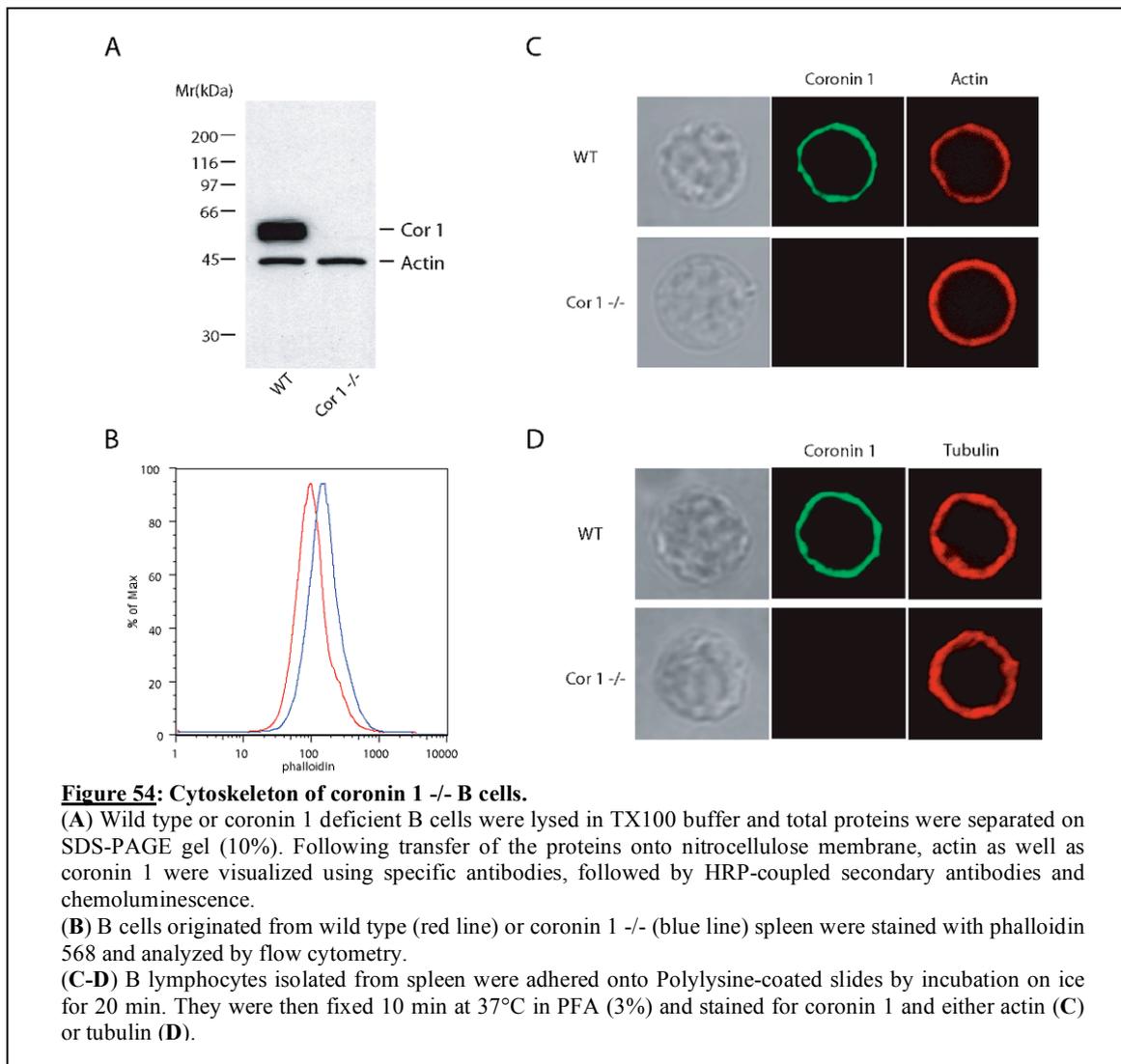
cells before purification. Interestingly, the non B cell population (i.e. the B220<sup>-</sup> cells, mainly T lymphocytes) showed an increase in apoptosis of the coronin 1 <sup>-/-</sup> cells from the spleen and the lymph nodes confirming the importance of coronin 1 in T cell homeostasis (Foger et al., 2006; Mueller et al., 2008).





Since *Dictyostelium* coronin was defined as an actin-myosin interacting protein (de Hostos et al., 1991), we studied if the absence of coronin 1, the closest homologue to *Dictyostelium* coronin, would modify the B cell cytoskeleton. First, wild type and coronin 1<sup>-/-</sup> B lymphocytes were lysed, the proteins separated by SDS-PAGE and followed by immunoblotting using anti-coronin 1 and anti-actin antibodies. As shown in figure 54A, the levels of actin were similar in wild type and coronin 1<sup>-/-</sup> B cell lysates as judged by Western blotting. B cells were alternatively stained with phalloidin and analyzed by flow cytometry. The phalloidin staining of coronin 1<sup>-/-</sup> B cells appeared to be elevated compared to wild type cells (figure 54B). However the same phenomenon was observed in other cell types lacking coronin 1. This appeared to be due to an artifact of phalloidin staining in the absence of coronin 1 (Mueller et al, manuscript in preparation).

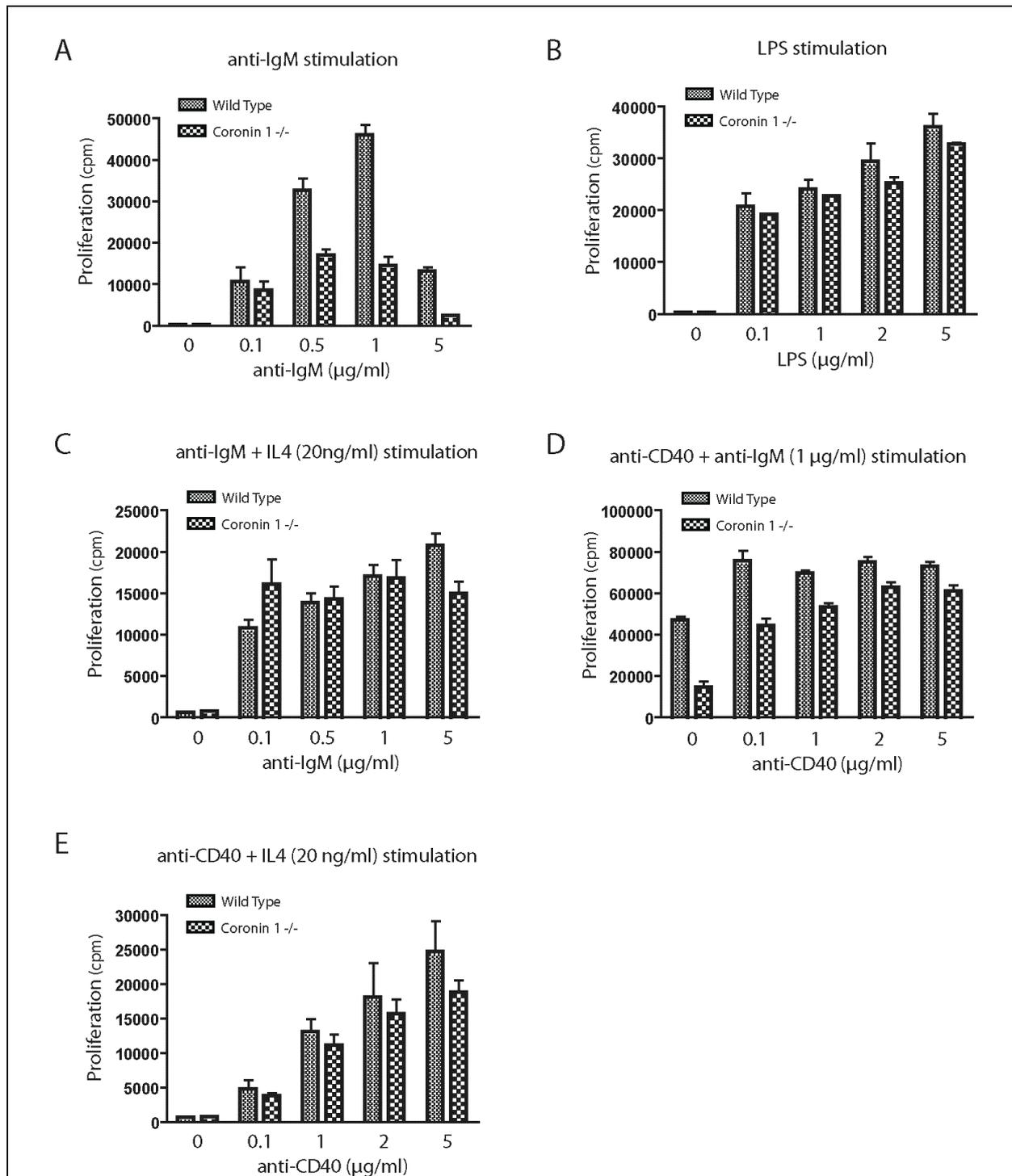
To analyze the cytoskeletal arrangement of B cells in the presence and absence of coronin 1, lymphocytes were seeded onto Polylysine-coated microscopy slides, fixed and stained for coronin 1 and either actin or tubulin. The F-actin cytoskeleton (figure 54C) as well as the tubulin organization (figure 54D) were not modified by deletion of coronin 1 as judged by confocal microscopy analysis.



To analyze proliferation in the presence and absence of coronin 1, B cells were isolated from the spleen of either wild type or coronin 1 deficient animals, and the B cell receptors were cross-linked with anti-IgM at the concentrations indicated in figure 55A. After 72 hours, proliferation was analyzed by incubating the cells with <sup>3</sup>H-thymidine for 12 hours, followed by liquid scintillation counting. Proliferation of coronin 1 deficient B cells was severely depressed upon stimulation of the B cell receptor using anti-IgM when compared to wild type B cells (figure 55A). The defective proliferation was not due to a general defect in B cell cycling since the polyclonal activator lipopolysaccharide (LPS) induced a similar B cell proliferation on both wild type and coronin 1 deficient B cells (figure 55B). However, providing a co-stimulatory signal with anti-IgM either through the addition of interleukin 4 (IL-4) to mimic the *in vivo* cytokine milieu (figure 55C) or anti-CD40 to reproduce T cell help (figure 55D) resulted in proliferation rates similar to wild type B cells (Banchereau et al., 1994; Coffman et al., 1988; Parker, 1993; Paul and Ohara, 1987; Rabin

et al., 1985). The use of anti-CD40 and IL-4, a model for TD activation in vitro, gave rise to the same stimulation of coronin 1 <sup>-/-</sup> and wild type B cells (figure 55E).

We conclude that while proliferation induced by B cell receptor triggering alone is strongly dependent on coronin 1, in the presence of a second signal coronin 1 becomes dispensable for B cell proliferation.

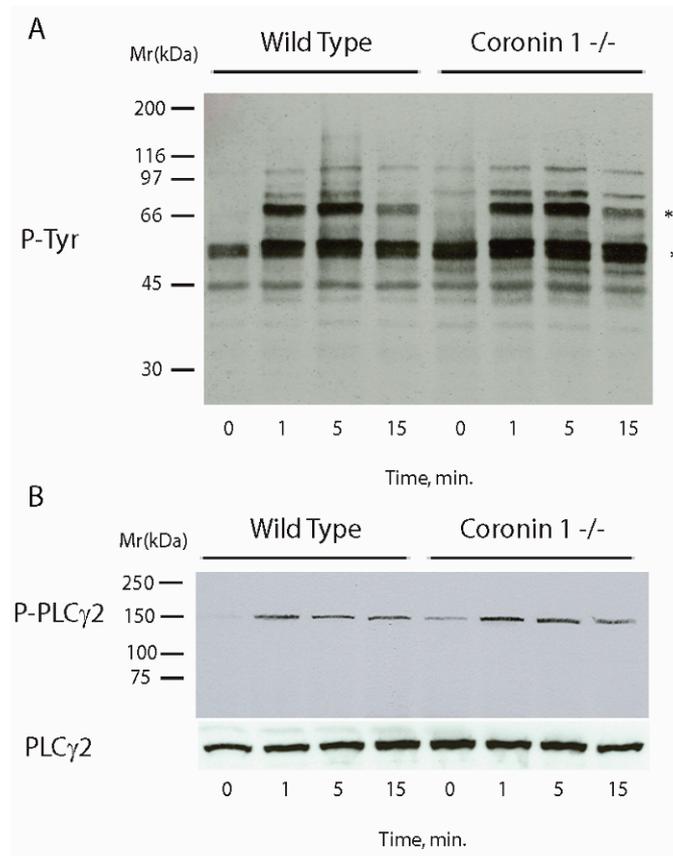


**Figure 55: Proliferation of B cells in the absence or presence of coronin 1.**

Single cell suspension of the spleen was depleted for T cells by complement lysis, after removal of red blood cells, to obtain B lymphocytes with an average purity of 95%. B cells were cultured for 3 days in the presence of anti-IgM (A), lipopolysaccharide (B), various amounts of anti-IgM with 20 ng/ml of IL4 (C), various amounts of anti-CD40 with 1 µg/ml of anti-IgM (D) or with 20 ng/ml of IL4 (E). [<sup>3</sup>H]-thymidine was then added for 12 hours and its incorporation was determined. Shown data are mean values +/- SD from triplicate samples from 4 different animals and are representative of at least 3 independent experiments.

### 3.4.3 B cell signaling in the presence and absence of coronin 1

Coronin 1 is essential for the mobilization of  $\text{Ca}^{2+}$  in T lymphocytes following stimulation of T cell receptors (Mueller et al., 2008). Given the parallels between T cell and B cell receptor signaling (Feske, 2007; Gallo et al., 2006), we investigated whether coronin 1 is required for B cell receptor mediated signaling which might explain the defect in proliferation after BCR crosslinking. To that end, B cells were prepared from wild type or coronin 1 deficient mice and stimulated using anti-IgM for the times indicated in figure 56A. Cells were lysed and total proteins separated by SDS-PAGE followed by immunoblotting for phosphotyrosine. As shown in figure 56A, B cell receptor stimulation resulted in similar induction of phosphotyrosine, including phosphorylation of Lyn (55kDa, shown as \*) and Syk (70 kDa, \*\*) confirming that early signaling events are not affected in B cells lacking coronin 1. Similarly, phosphorylation of phospholipase  $\text{C}\gamma 2$  that is required for the generation of  $\text{InsP}_3$  was independent of coronin 1, although in the absence of coronin 1 dephosphorylation appeared to be slightly faster (see figure 56B).

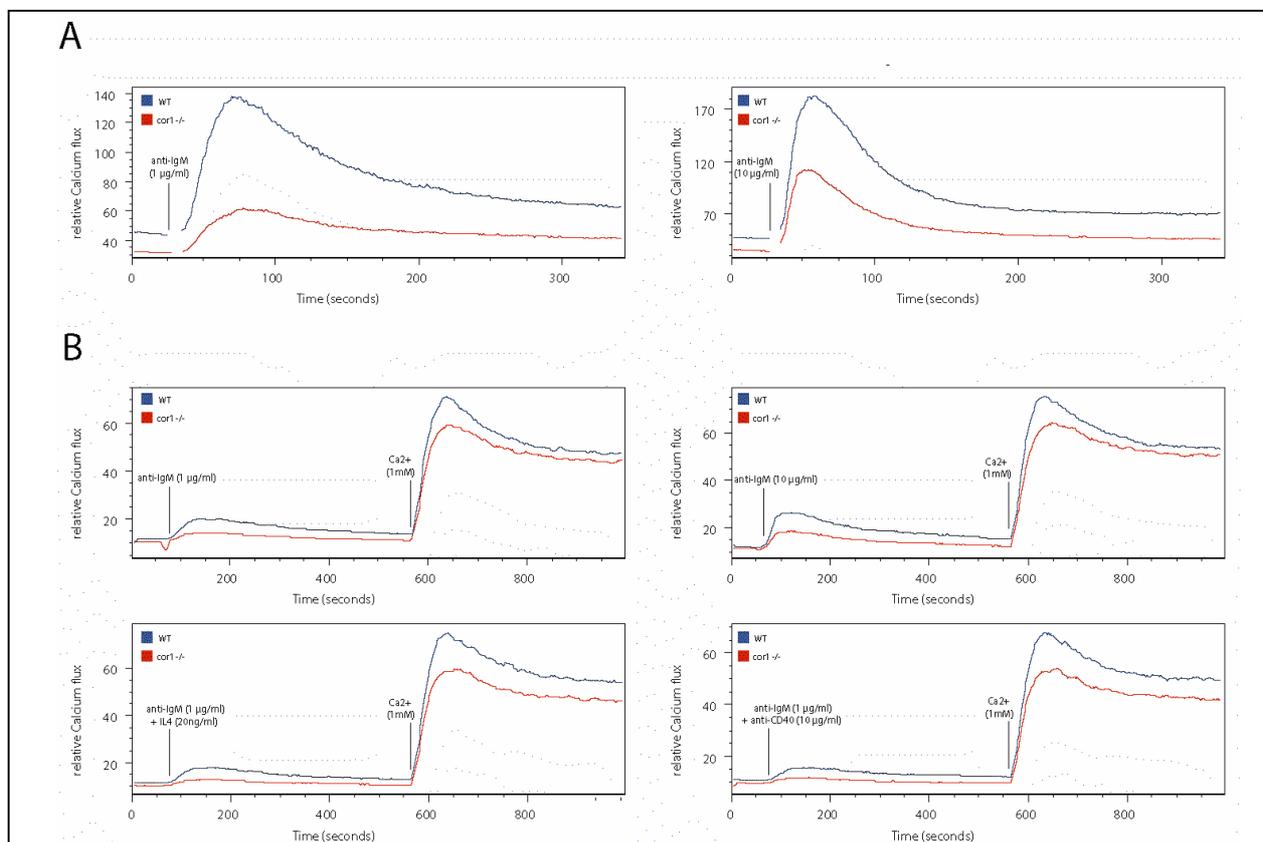


**Figure 56: B cell signaling pathway in wild type and coronin 1 deficient lymphocytes.**

Wild type and coronin 1  $-/-$  B cells were stimulated with  $1 \mu\text{g/ml}$  of anti-IgM for the indicated time, cell lysates were prepared in TX-100 buffer, proteins were separated on a SDS-PAGE gel (10%) and then transferred onto nitrocellulose membrane. Phospho-Tyrosine proteins were detected by using 4G10 antibodies; phosphorylation of Lyn (55kDa) and Syk (70kDa) are respectively marked by \* and \*\* (A).  $\text{PLC}\gamma 2$  and P- $\text{PLC}\gamma 2$  were detected by using specific antibodies for each isoform (B).

These results therefore suggest that the signaling cascade until PLC $\gamma$ 2 upon BCR triggering is not dependent on coronin 1.

The binding of antigens on the surface mIg conducts to the crosslinking of the BCR and then the transduction of the signal to the cytoplasm via phosphorylation of tyrosines within the ITAMs starting the signaling cascade and finally leading to calcium release from the internal stores followed by calcium entry (see figures 12 and 13). To investigate calcium mobilization upon B cell stimulation, total splenocytes were negatively labeled to select B cells, loaded with the fluorescent calcium indicator Fluo-3-AM and calcium release was recorded after BCR crosslinking. As in T cells, B cell receptor stimulation of coronin 1 deficient B cells resulted in a severely depressed Ca<sup>2+</sup> mobilization (figure 57A, red line), in contrast to normal Ca<sup>2+</sup> mobilization observed after stimulation of wild type B cells (figure 57A, blue line).



**Figure 57: BCR-mediated calcium mobilization in the absence or presence of coronin 1.**

(A) Single cell suspensions from wild type and coronin 1<sup>-/-</sup> spleen were prepared and the erythrocytes were lysed using ACK buffer. Cells were loaded with Fluo-3-AM for 45 min at 37°C and then incubated on ice during 30 min with anti-CD3-APC and anti-CD11b-APC. They were washed and taken up into RPMI without phenol red supplemented with 3% FBS and 10 mM Hepes and equilibrated for 10 min at 37°C before measurement. After acquisition of the baseline for 30 seconds, either 1 µg/ml (left panel) or 10 µg/ml (right panel) of anti-IgM was added to the cells and the calcium fluxes were recorded for an extra 5 min on the FL-2 channel of a FACSCalibur instrument after gating on the APC-negative cells.

(B) T cell depleted splenocytes were loaded with Fluo-3-AM and then taken up in calcium-free HBSS. The cells were then prewarmed for 10 min at 37°C and a one-minute baseline was recorded before adding the stimuli: anti-IgM 1 µg/ml (top left panel), anti-IgM 10 µg/ml (top right panel), anti-IgM 1 µg/ml with 20 ng/ml of IL4 (bottom left panel) or anti-IgM 1 µg/ml with 10 µg/ml anti-CD40 (bottom right panel). Extracellular calcium was added after 9 min at a final concentration of 1 mM. The calcium fluxes were recorded on the FL-2 channel on a FACSCalibur instrument. Wild type B cells are represented in blue and coronin 1<sup>-/-</sup> B cells in red.

To study the contribution of internal calcium store depletion and calcium entry through the plasma membrane, purified splenic B cells loaded with Fluo-3-AM were stimulated in the absence of extracellular calcium and the calcium release into the cytosol was measured. BCR crosslinking of the wild type B cells provoked the release of calcium from the internal store (figure 57B, upper panels, blue line) whereas this was deficient in the coronin 1  $-/-$  B cells (figure 57B, upper panels, red line). Calcium was then added to the external milieu and calcium entry across the plasma membrane was monitored. It turned out that calcium entry after BCR crosslinking of the coronin 1  $-/-$  B cells was not as efficient as in the case of wild type B cells (figure 57B, upper panels). These data suggested that both calcium release from the internal store and extracellular calcium entry were affected in coronin 1  $-/-$  B cells.

Addition of helper components (IL-4 or anti-CD40) restored B cell proliferation after BCR stimulation (figure 55C and D) but this did not rescue the calcium signaling defect (figure 57B, lower panels) suggesting that IL-4 and anti-CD40 enable the coronin 1  $-/-$  B cells to proliferate by calcium-independent alternative pathways

We conclude that in B cells, coronin 1 is essential for the generation of  $Ca^{2+}$  mobilization, similar to its role in T cells.

## 4 DISCUSSION

### **4.1 Inhibition of phagosome maturation by mycobacteria does not interfere with presentation of mycobacterial antigens by MHC molecules**

Inhibition of phagosome-lysosome fusion has been long proposed to represent a mechanism by which pathogenic mycobacteria bias the efficiency of antigen presentation by host MHC molecules to evade immune surveillance (Hmama et al., 1998; Noss et al., 2001; Pancholi et al., 1993). In addition to the fact that a low phagosomal pH can be directly toxic to microorganisms, the terminal degradation of proteins into peptides, i.e., unfolding of antigens by thiol reductases and acidic endopeptidases such as the cathepsins, occurs at low pH. Moreover, the removal of the Ii chaperone from MHC class II heterodimers and interaction of the latter with H-2M for replacement of class II-associated Ii-derived peptide by antigenic peptides into the groove of mature MHC class II molecules, all take place in acidified endosomes (Bryant et al., 2002; Ferrari et al., 1997; Pieters, 1997a, b). In the present study, however, we demonstrate that the presentation of mycobacterial antigens is identical whether or not mycobacteria are transferred to lysosomes or remain sequestered in nonlysosomal phagosomes.

*In vivo* processing and presentation of mycobacterial antigens occur by both macrophages as well as dendritic cells (Kaufmann, 2006). However, the relative contribution of each of these types of professional APC in the generation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses *in vivo* are not clear. We therefore investigated both of these APC types in this study. We first established that mycobacterial PknG was able to block lysosomal fusion in dendritic cells as occurs in macrophages (Walburger et al., 2004).

Subsequently, we compared the efficiency of mycobacterial antigen presentation by host macrophages or dendritic cells infected with wild type or PknG-deficient mycobacteria. As evaluated *in vitro* by use of anti-Ag85A T cell hybridomas (Majlessi et al., 2006), macrophages or dendritic cells infected with wild type *M. bovis* BCG or *M. bovis* BCG  $\Delta$ *pknG* presented this mycobacterial reporter antigens with similar efficiency, independent of the multiplicity of infection and following short (2 or 4 hours) or long (24 hours) time periods subsequent to infection. Moreover, following infection, the presentation of Ag85A was similar in the context of two distinct restricting elements (I-A<sup>b</sup> and I-E<sup>d</sup>) and in two different mouse genetic backgrounds (C57BL/6 (H-2<sup>b</sup>) and BALB/c (H-2<sup>d</sup>)) whether wild type or PknG-deficient mycobacteria were used. Also, *in*

*in vivo* immunization with either wild type or PknG-deficient BCG strains induced similar proliferative and IFN- $\gamma$  CD4<sup>+</sup> T cell responses to the PPD antigen mixture as well as to diverse well-defined mycobacterial immunogens. Exogenous protein antigens may display different intracellular trafficking as well as different sensitivity to proteolysis and can give rise to epitopes with various affinities for the restricting elements (Bryant et al., 2002). To understand the capacity of distinct mycobacterial antigens to trigger CD4<sup>+</sup> T cell responses upon immunization with wild type or PknG-deficient mycobacteria, we selected a subset of mycobacterial antigens, i.e., the 32-kDa Ag85A expressed in complex with Ag85B and C (Wiker and Harboe, 1992), and 10-kDa small proteins from the ESAT-6 family of proteins (TB10.3 and TB10.4), which most probably are secreted by an ESAT-6–1-like secretion machinery (Okkels and Andersen, 2004; Pym et al., 2003; Tekaiia et al., 1999). Regardless of the nature of the antigen, these were presented in a similar fashion whether the mycobacteria were retained in phagosomes (wild type *M. bovis* BCG) or transported to lysosomes (*M. bovis* BCG  $\Delta$ pknG). It should also be noted that the precise organelles of the endocytic pathway where the processing from phagocytosed antigens and peptide loading to MHC-II molecules occurs is still unclear (Trombetta and Mellman, 2005). However, the results reported here suggest that the lack of delivery of mycobacteria to lysosomes does not limit the ability of the APC to stimulate T cell responses. Indeed, wild type mycobacteria that resist lysosomal delivery can give rise to identical MHC class I- and class II-restricted T cell responses as compared with PknG-deficient bacilli that are immediately shuttled to lysosomes.

Although we cannot exclude that also *in vivo*, a small proportion of mycobacteria are routed to lysosomes, our data show that the capacity of mycobacteria to resist lysosomal delivery does not prevent the generation of an immune response. In fact, when all of the infecting mycobacteria are directly transferred to lysosomes, as in the case of PknG-deficient mycobacteria, an immune response identical to the one generated by wild type mycobacteria was found. This is surprising in light of previous work showing that the route of antigen uptake and the final subcellular localization defines the outcome of an immune response (Davidson and Watts, 1989; Delamarre et al., 2005; Engering et al., 1997; Watts and Davidson, 1988). For example, for both macrophages and dendritic cells, it has been established that lysosomal transfer is important for the processing and presentation of antigens (Delamarre et al., 2005; Engering et al., 1997). The results presented here show that in the case of an infection with mycobacteria, the degree of lysosomal transfer of mycobacteria does not influence the extent to which responses are generated against mycobacterial antigens. These results are not only important for the understanding of the immune escape mechanisms of pathogenic mycobacteria but are also relevant for strategies aimed at improving existing vaccines.

Our results also show that immunization of mice with wild type or PknG-deficient *M. bovis* BCG induces comparable CD8<sup>+</sup> T cell responses to Ag85A and TB10.3/TB10.4 mycobacterial immunogens. Therefore, the distinct trafficking of mycobacteria within the host APC and the marked phagosome maturation due to the loss of function of PknG do not affect the presentation of mycobacterial antigens within the MHC class I presentation pathway. Little is known about the mechanisms of presentation of mycobacterial antigens by MHC class I molecules. It has been shown that phagosomes, via interaction with endoplasmic reticulum-derived vesicles, can acquire the MHC class I processing and presentation machinery (Guermónprez et al., 2003; Houde et al., 2003). Maturation of phagosomes into phagolysosomes, as occurs upon infection with PknG-deficient mycobacteria, would therefore impair epitope editing and loading onto MHC class I molecules, the latter being unstable and binding peptides inefficiently in acidic conditions (Ojcius et al., 1993). However, the comparable CD8<sup>+</sup> T cell induction following immunization with wild type or PknG-deficient mycobacteria suggests that MHC class I loading does not occur in phagolysosomes.

Cross-presentation of mycobacterial antigens may also occur via the cytosolic pathway by proteasome- and TAP-dependent mechanisms (Lewinsohn et al., 2006), most probably following phagosome-to-cytosol translocation of mycobacterial antigens. According to this scheme, degradation of antigens depends on the proteasome and not on the endosome/lysosome. Thus, maturation of phagosomes, for instance upon infection with PknG-deficient mycobacteria, would have no consequence on mycobacterial antigen presentation by MHC class I molecules. Recently, the presentation of mycobacterial antigens by MHC class I and II molecules has been shown to involve uptake by bystander dendritic cells of apoptotic vesicles generated from primary infected macrophages (Schaible et al., 2003; Winau et al., 2006). Mycobacterial antigens then enter the MHC class I or II pathway of dendritic cells. Therefore, in this model, the cells that present mycobacterial antigens in vivo are not those initially infected with mycobacteria. Maturation of phagosomes, as induced by PknG-deficient BCG in primarily infected macrophages, would not have an appreciable consequence on the antigen presentation by dendritic cells which capture subsequently the apoptotic vesicles that are generated.

In conclusion, the present investigation demonstrates that the inhibition of phagosome-lysosome fusion by mycobacteria and the sequestration of the bacilli in APC (Pancholi et al., 1993) does not affect mycobacterial antigen presentation by MHC class I or II molecules and that these phenomena cannot explain how this successful intracellular pathogen can escape from immune surveillance.

## **4.2 Resistance of pathogenic mycobacteria to lysosomal delivery is mediated by Coronin 1 dependent activation of calcineurin**

Coronin 1 is a member of the coronin proteins family and is specifically expressed in leukocytes. To analyze the role of coronin 1 in cells of the immune system and its importance in mycobacteria pathogenesis, a mouse lacking coronin 1 was generated (Jayachandran et al., 2007; Mueller et al., 2008). The coronin 1  $-/-$  mice were viable and fertile and did not present any obvious phenotype. Coronin 1 is recruited during mycobacterial phagocytosis (Ferrari et al., 1999; Schuller et al., 2001) which was suggested to be related to modulation of the F-actin cytoskeleton (Fratti et al., 2000). Indeed, coronin 1 has been classified as an actin-binding protein due to this homology to the *Dictyostelium* coronin (de Hostos et al., 1991) that was found to associate with actin/myosin complexes and was proposed to be essential in F-actin modulation. However, macrophages lacking coronin 1 did not present any defect in actin-dependent processes such as spreading, migration, motility, membrane ruffling, macropinocytosis and phagocytosis (Jayachandran et al., 2007). In addition, when coronin 1 is knocked down in macrophage-like cell lines using siRNA (Jayachandran et al., 2008) no F-actin phenotype is observed. We next showed that coronin 1  $-/-$  macrophages respond normally when encountering mycobacteria or when triggering Toll-Like Receptors. Finally, a broad range of stimuli was analyzed to study several signaling pathways without detecting any significant differences in the presence or absence of coronin 1.

Coronin 1 was also proposed to play a role in phagosome maturation since it was actively retained by pathogenic mycobacteria around their phagosomes upon internalization leading to their survival inside host cells. In contrast, non-pathogenic mycobacteria could not keep this coating and were directly transferred to lysosomes for degradation (Ferrari et al., 1999). Pathogenic mycobacteria could actually not block phagosome maturation in coronin 1  $-/-$  macrophages.

Coronin 1 is essential for T cell survival due to its role in calcium mobilization after TCR triggering (Mueller et al., 2008). We hypothesized that coronin 1 dependent phagosome maturation might be related to the regulation of intracellular calcium. In fact, wild type macrophages responded with an increase in cytosolic calcium levels upon infection with mycobacteria while no cytosolic calcium release was observed in the absence of coronin 1 (Jayachandran et al., 2007). We next showed that mycobacteria did not resist to lysosomal delivery when intracellular calcium of wild type macrophages was chelated suggesting that the cytosolic calcium levels were central in mycobacterial trafficking. The fate of mycobacteria in coronin 1  $-/-$  macrophages could actually be rescued when a calcium ionophore was used. It was finally demonstrated that the inhibition of

calcineurin, a protein phosphatase activated upon increase of intracellular calcium concentration, allowed us to mimic the coronin 1  $-/-$  macrophages. In conclusion, coronin 1 is responsible for the activation of calcineurin upon mycobacteria internalization and therefore for the inhibition of lysosomal delivery.

This is again an example of the ability of pathogenic mycobacteria to successfully utilize host components in order to survive in a hostile environment (Houben et al., 2006; Nguyen and Pieters, 2005). This would also mean that coronin 1 has been maintained during evolution even if this leukocyte-specific protein makes the patients more vulnerable to mycobacterial infection. This suggests that it was either beneficial for the host not to entirely clear mycobacteria to regularly stimulate the immune system (Falkow, 2006; Pieters and Gatfield, 2002) or that coronin 1 might have additional roles in other leukocytes, such as B and T cells, that made it valuable for the overall immune system.

It was also proposed that the activation of the Toll-like receptor (TLR) signaling pathways by bacteria might regulate phagosome maturation (Blander and Medzhitov, 2004). However, we showed that the absence of coronin 1 led to a dramatic change in mycobacterial trafficking while activation of signaling pathways upon TLR triggering was not affected. This would suggest that the pathways regulating mycobacterial trafficking dependent on coronin 1 or on TLR stimulation are autonomous (Krutzik and Modlin, 2004; Pai et al., 2004).

### ***4.3 Chemotaxis, phagocytosis and oxidase activity in neutrophils are independent of Coronin 1***

The role for coronin 1 in leukocytes remains a matter of debate. Whereas the analysis of a variety of cell types from coronin 1 deficient mice suggest a specific role for coronin 1 in the modulation of intracellular signaling by regulating cytosolic calcium mobilization (Jayachandran et al., 2007; Mueller et al., 2008), leukocytes expressing dominant-negative fragments of coronin 1 show a variety of phenotypes when F-actin dependent functions are analyzed (Yan et al., 2005; Yan et al., 2007). Here we analyzed neutrophils from coronin 1 deficient mice, and found no evidence for any contribution of coronin 1 to F-actin dependent functions. In these coronin 1 deficient neutrophils, adherence, spreading, motility, phagocytosis, chemotaxis and the generation of an oxidative burst were identical to neutrophils obtained from wild type littermates. The coronin 1  $-/-$  neutrophils were not depleted from the periphery in contrast to lymphocytes. We conclude that in neutrophils, as in lymphocytes and macrophages, coronin 1 is dispensable for F-actin mediated processes.

The results presented here are in sharp contrast to those from a recent study based on the expression of a dominant negative fragment of coronin 1, showing pleiotropic defects in several F-actin dependent processes (Gatfield et al., 2005; Yan et al., 2007). However, in our hands, expression of coronin 1 fragments results in accumulation of misfolded protein inside the transfected cell (Gatfield et al., 2005). Since no data exists on the distribution or expression pattern of the dominant negative coronin 1 fragment in neutrophils, it cannot be excluded that the observed phenotypes result from aberrant expression of coronin 1 domains.

Furthermore, the data presented here are in full agreement with the lack of an F-actin-dependent phenotype in macrophages in which coronin 1 expression is ablated by siRNA. Also in those cells, knocking down coronin 1 to undetectable levels results in macrophages that are fully functional in terms of motility, membrane ruffling, macropinocytosis and phagocytosis (Jayachandran et al., 2008). The only phenotype observed in these cell lines expressing siRNA specific for coronin 1 is the differential trafficking of pathogenic mycobacteria; in coronin 1 knock down cell lines these bacteria are transferred to lysosomes instead of residing within phagosomes, as expected (Ferrari et al., 1999; Jayachandran et al., 2007).

Interestingly, while lymphocytes are severely depleted from the periphery in the absence of coronin 1 (Mueller et al., 2008), circulating neutrophils are not affected. However the recruitment of neutrophils to the peritoneum was strongly reduced in coronin 1 *-/-* mice whereas the purified neutrophils did not present any chemotactic deficiencies *in vitro*. This could be explained by the absence of certain lymphocytes population that might influence the cytokine environment. T cells play an important role in regulating the amount of neutrophils recruited to the site of infection by controlling the production and release of neutrophils from the bone marrow (Nathan, 2006; Stark et al., 2005). As illustrated in figure 11, macrophages and mature dendritic cells get activated by neutrophils at the site of inflammation and therefore release IL-23 that will trigger T cells to produce IL-17 (Aggarwal et al., 2003). IL-17 induces the production of granulocyte colony-stimulating factors (G-CSF) by stromal cells in the bone marrow that promote the proliferation of neutrophils precursors and the release of neutrophils to the circulation and subsequently to the inflammatory site (Nathan, 2006; Semerad et al., 2002).

#### **4.4 Coronin 1 is essential for IgM mediated Ca<sup>2+</sup> mobilization in B cells but dispensable for *in vivo* B cell functions**

Coronin 1 is a member of the WD repeat protein family exclusively expressed in leukocytes (Ferrari et al., 1999). While initially, coronin 1 was believed to be necessary for the regulation of F-actin dynamics (de Hostos, 1999) recent work has shown that in macrophages and T cells, coronin 1 is fully dispensable for F-actin mediated processes, instead regulating calcium-dependent signaling (Jayachandran et al., 2007; Mueller et al., 2008). Here, we show that in B cells, coronin 1 regulates the intracellular mobilization of Ca<sup>2+</sup> upon B cell receptor triggering. In the absence of coronin 1, early signaling processes such as tyrosine phosphorylation and phosphorylation of PLC $\gamma$ 2 were unaffected. However, in the absence of coronin 1, B cell receptor triggering failed to result in intracellular Ca<sup>2+</sup> mobilization, independent of the presence of the co-stimulatory signals provided by IL4 and anti-CD40. Furthermore, proliferation of B cells lacking coronin 1 was altered upon B cell receptor triggering but this could be rescued by the addition of co-stimulatory signals. Interestingly, despite defective Ca<sup>2+</sup> mobilization, the *in vivo* generation of immune responses against both thymus independent as well as dependent antigens was normal. We conclude that coronin 1 is dispensable for B cell functions *in vivo* but, as in macrophages and T cells, is necessary for the proper mobilization of intracellular calcium.

The phenotype of coronin 1 deficient B cells is fully consistent with recent work showing a defect in the intracellular mobilization of Ca<sup>2+</sup> upon triggering T cell receptors (Mueller et al., 2008). Also in the case of coronin 1 deficient T cells, triggering of the T cell receptor does not affect tyrosine phosphorylation or the phosphorylation of phospholipase C $\gamma$ 1. However, in the absence of coronin 1, calcium is not mobilized from intracellular stores, resulting in defective T cell receptor-mediated proliferation, IL2 production and cell cycling which is translated *in vivo* in a strong depletion of peripheral T cells. In coronin 1 deficient mice B cells can develop normally in the bone marrow however their numbers are reduced in the circulation (Mueller et al., 2008), but the results shown here strongly suggest that this depletion may be a result from a paucity of T cells through a process of homeostatic depletion.

The results presented here support a role for coronin 1 in signaling rather than the modulation of the F-actin cytoskeleton. As in T cells (Mueller et al., 2008), macrophages (Jayachandran et al., 2008; Jayachandran et al., 2007) and neutrophils (Combaluzier et al., manuscript in preparation), coronin 1 does not modulate the actin cytoskeleton in B cells. The purported role for coronin 1 in

modulating F-actin dependent functions is likely to be a result of analyses based on the overexpression of coronin 1 domains within leukocyte cell types, possibly resulting in the expression of misfolded proteins, which may compromise cellular functions such as spreading, membrane ruffling and phagocytosis (Jayachandran et al., 2008; Yan et al., 2005; Yan et al., 2007). Alternatively, expression of coronin 1 domains that are sufficiently homologous to domains also present in other coronin family members may result in the inhibition of the function of coronin isoforms that are involved in the regulation of cytoskeletal dynamics.

While the results presented here provide evidence for a role for coronin 1 in B cell receptor mediated signaling and  $Ca^{2+}$  mobilization, they also suggest that  $Ca^{2+}$  mobilization in B cells is less critical for signal transduction, proliferation and B cell function than it is for T cells. A few recent works also emphasized the fact that alternative pathway for BCR signaling could be triggered by use of IL4 or CD40 ligand independent of PI3K and phospholipase  $C\gamma$  activation (Guo et al., 2007; Guo and Rothstein, 2005; Mizuno and Rothstein, 2005)

## 5 SUMMARY

The immune system developed to prevent infections with pathogenic intruders without inducing uncontrolled reactions that could be harmful to the host. As a result, the activation of the immune system is a well-controlled process including recognition of the invading bacteria and cooperation between different cell types. However, some pathogens can still avoid this immune surveillance by using multiple and sophisticated mechanisms. A particular notorious example is *Mycobacterium tuberculosis*. About one third of the global population has been exposed to this pathogen and while a healthy person can deal with this infection it can lead to severe symptoms in immunocompromised patients.

Pathogenic mycobacteria are recognized and internalized inside macrophages where they can block the maturation of their phagosomes into lysosomes thereby residing and surviving inside host cells. Firstly, we showed that mycobacteria lacking the virulence factor PknG, an eukaryotic-like serine/threonine protein kinase, could not resist to lysosomal delivery inside professional antigen presenting cells. It was then demonstrated that this efficient transfer of the mycobacterium to the lysosomes did not modify the presentation of mycobacterial peptides on MHC molecules when compared to wild type mycobacteria residing inside phagosomes. This suggests that the trafficking of mycobacteria to different organelles does not influence the quality of the antigen presentation.

Coronin 1 is a leukocyte-specific protein that is recruited and retained around phagosomes containing pathogenic mycobacteria. In the second part of this thesis, we demonstrate that mycobacteria cannot resist lysosomal delivery when infecting macrophages lacking coronin 1 expression. However, this dramatic change in trafficking is not due to an altered signaling upon TLRs triggering but to the calcineurin activity that is affected in the absence of coronin 1.

Thirdly we showed that the absence of coronin 1 did not affect the main functions of neutrophils. The morphology of the neutrophils lacking coronin 1 was not affected as well as several actin-dependent processes such as adhesion, spreading, phagocytosis and chemotaxis. The oxidative burst was also not disturbed in the absence of coronin 1.

Fourthly, this thesis describes the capacity of the immune system to respond against antigens in mice lacking coronin 1. The absence of coronin 1 did not compromise the ability of the animals to mount an immune response even if coronin 1 was essential for calcium release in B lymphocytes upon BCR triggering. These results argue for a general role of coronin 1 in mediating cytosolic  $Ca^{2+}$  mobilization rather than regulating actin-dependent processes.

Taken together, the results presented in this thesis provide indications for a better understanding of mycobacteria-host interactions and coronin 1 functions in leukocytes.

## 6 LITERATURE

- Abadie, V., Badell, E., Douillard, P., Ensergueix, D., Leenen, P.J., Tanguy, M., Fiette, L., Saeland, S., Gicquel, B., and Winter, N. (2005). Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood* 106, 1843-1850.
- Ackerman, A.L., and Cresswell, P. (2004). Cellular mechanisms governing cross-presentation of exogenous antigens. *Nature immunology* 5, 678-684.
- Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *The Journal of biological chemistry* 278, 1910-1914.
- Agramonte-Hevia, J., Gonzalez-Arenas, A., Barrera, D., and Velasco-Velazquez, M. (2002). Gram-negative bacteria and phagocytic cell interaction mediated by complement receptor 3. *FEMS immunology and medical microbiology* 34, 255-266.
- Allen, L.A., DeLeo, F.R., Gallois, A., Toyoshima, S., Suzuki, K., and Nauseef, W.M. (1999). Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. *Blood* 93, 3521-3530.
- Amigorena, S., Drake, J.R., Webster, P., and Mellman, I. (1994). Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature* 369, 113-120.
- Andoniou, C.E., Andrews, D.M., and Degli-Esposti, M.A. (2006). Natural killer cells in viral infection: more than just killers. *Immunological reviews* 214, 239-250.
- Apostolopoulos, V., and McKenzie, I.F. (2001). Role of the mannose receptor in the immune response. *Current molecular medicine* 1, 469-474.
- Appelberg, R., Castro, A.G., Gomes, S., Pedrosa, J., and Silva, M.T. (1995). Susceptibility of beige mice to *Mycobacterium avium*: role of neutrophils. *Infection and immunity* 63, 3381-3387.
- Appleton, B.A., Wu, P., and Wiesmann, C. (2006). The crystal structure of murine coronin-1: a regulator of actin cytoskeletal dynamics in lymphocytes. *Structure* 14, 87-96.

Armstrong, J.A., and Hart, P.D. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *The Journal of experimental medicine* *134*, 713-740.

Armstrong, J.A., and Hart, P.D. (1975). Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *The Journal of experimental medicine* *142*, 1-16.

Av-Gay, Y., and Everett, M. (2000). The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends in microbiology* *8*, 238-244.

Baba, Y., Hashimoto, S., Matsushita, M., Watanabe, D., Kishimoto, T., Kurosaki, T., and Tsukada, S. (2001). BLNK mediates Syk-dependent Btk activation. *Proceedings of the National Academy of Sciences of the United States of America* *98*, 2582-2586.

Babior, B.M. (2004). NADPH oxidase. *Current opinion in immunology* *16*, 42-47.

Babior, B.M., Kipnes, R.S., and Curnutte, J.T. (1973). Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *The Journal of clinical investigation* *52*, 741-744.

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J.P., van Kooten, C., Liu, Y.J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annual review of immunology* *12*, 881-922.

Barker, L.P., George, K.M., Falkow, S., and Small, P.L. (1997). Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. *Infection and immunity* *65*, 1497-1504.

Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. *Cell* *92*, 367-380.

Benard, V., Bokoch, G.M., and Diebold, B.A. (1999). Potential drug targets: small GTPases that regulate leukocyte function. *Trends in pharmacological sciences* *20*, 365-370.

Bendelac, A., Bonneville, M., and Kearney, J.F. (2001). Autoreactivity by design: innate B and T lymphocytes. *Nature reviews* *1*, 177-186.

- Bennouna, S., Bliss, S.K., Curiel, T.J., and Denkers, E.Y. (2003). Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol* *171*, 6052-6058.
- Berland, R., and Wortis, H.H. (2002). Origins and functions of B-1 cells with notes on the role of CD5. *Annual review of immunology* *20*, 253-300.
- Beutler, B. (2004). Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* *430*, 257-263.
- Beutler, B., Jiang, Z., Georgel, P., Crozat, K., Croker, B., Rutschmann, S., Du, X., and Hoebe, K. (2006). Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annual review of immunology* *24*, 353-389.
- Bevan, M.J. (1976). Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J Immunol* *117*, 2233-2238.
- Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* *329*, 506-512.
- Blander, J.M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. *Science (New York, NY)* *304*, 1014-1018.
- Bonneau, M., Epardaud, M., Payot, F., Niborski, V., Thoulouze, M.I., Bernex, F., Charley, B., Riffault, S., Guilloteau, L.A., and Schwartz-Cornil, I. (2006). Migratory monocytes and granulocytes are major lymphatic carriers of Salmonella from tissue to draining lymph node. *Journal of leukocyte biology* *79*, 268-276.
- Borregaard, N., and Cowland, J.B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* *89*, 3503-3521.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* *72*, 248-254.
- Brennan, P.J., and Nikaido, H. (1995). The envelope of mycobacteria. *Annual review of biochemistry* *64*, 29-63.

- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science (New York, NY)* *303*, 1532-1535.
- Brodin, P., Rosenkrands, I., Andersen, P., Cole, S.T., and Brosch, R. (2004). ESAT-6 proteins: protective antigens and virulence factors? *Trends in microbiology* *12*, 500-508.
- Brose, N., Betz, A., and Wegmeyer, H. (2004). Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Current opinion in neurobiology* *14*, 328-340.
- Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* *364*, 33-39.
- Bryant, P.W., Lennon-Dumenil, A.M., Fiebiger, E., Lagaudriere-Gesbert, C., and Ploegh, H.L. (2002). Proteolysis and antigen presentation by MHC class II molecules. *Advances in immunology* *80*, 71-114.
- Cahalan, M.D., Zhang, S.L., Yeromin, A.V., Ohlsen, K., Roos, J., and Stauderman, K.A. (2007). Molecular basis of the CRAC channel. *Cell calcium* *42*, 133-144.
- Cai, L., Holoweckyj, N., Schaller, M.D., and Bear, J.E. (2005). Phosphorylation of coronin 1B by protein kinase C regulates interaction with Arp2/3 and cell motility. *The Journal of biological chemistry* *280*, 31913-31923.
- Cai, L., Makhov, A.M., and Bear, J.E. (2007a). F-actin binding is essential for coronin 1B function in vivo. *Journal of cell science* *120*, 1779-1790.
- Cai, L., Marshall, T.W., Uetrecht, A.C., Schafer, D.A., and Bear, J.E. (2007b). Coronin 1B coordinates Arp2/3 complex and cofilin activities at the leading edge. *Cell* *128*, 915-929.
- Cariappa, A., Tang, M., Parng, C., Nebelitskiy, E., Carroll, M., Georgopoulos, K., and Pillai, S. (2001). The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. *Immunity* *14*, 603-615.
- Casola, S. (2007). Control of peripheral B-cell development. *Current opinion in immunology* *19*, 143-149.

- Casola, S., Otipoby, K.L., Alimzhanov, M., Humme, S., Uyttersprot, N., Kutok, J.L., Carroll, M.C., and Rajewsky, K. (2004). B cell receptor signal strength determines B cell fate. *Nature immunology* 5, 317-327.
- Castandet, J., Prost, J.F., Peyron, P., Astarie-Dequeker, C., Anes, E., Cozzone, A.J., Griffiths, G., and Maridonneau-Parini, I. (2005). Tyrosine phosphatase MptpA of *Mycobacterium tuberculosis* inhibits phagocytosis and increases actin polymerization in macrophages. *Research in microbiology* 156, 1005-1013.
- Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388, 782-787.
- Ceredig, R., and Rolink, T. (2002). A positive look at double-negative thymocytes. *Nature reviews* 2, 888-897.
- Chertov, O., Ueda, H., Xu, L.L., Tani, K., Murphy, W.J., Wang, J.M., Howard, O.M., Sayers, T.J., and Oppenheim, J.J. (1997). Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *The Journal of experimental medicine* 186, 739-747.
- Chertov, O., Yang, D., Howard, O.M., and Oppenheim, J.J. (2000). Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. *Immunological reviews* 177, 68-78.
- Cicchetti, G., Allen, P.G., and Glogauer, M. (2002). Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. *Crit Rev Oral Biol Med* 13, 220-228.
- Clark, M.R., Cooper, A.B., Wang, L.D., and Aifantis, I. (2005). The pre-B cell receptor in B cell development: recent advances, persistent questions and conserved mechanisms. *Current topics in microbiology and immunology* 290, 87-103.
- Clemens, D.L., and Horwitz, M.A. (1995). Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *The Journal of experimental medicine* 181, 257-270.
- Clemens, D.L., and Horwitz, M.A. (1996). The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *The Journal of experimental medicine* 184, 1349-1355.

- Clemens, D.L., Lee, B.Y., and Horwitz, M.A. (2000). Mycobacterium tuberculosis and Legionella pneumophila phagosomes exhibit arrested maturation despite acquisition of Rab7. *Infection and immunity* 68, 5154-5166.
- Coffman, R.L., Seymour, B.W., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Shrader, B., Cherwinski, H.M., Savelkoul, H.F., Finkelman, F.D., Bond, M.W., *et al.* (1988). The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunological reviews* 102, 5-28.
- Cole, S.T., Eiglmeier, K., Parkhill, J., James, K.D., Thomson, N.R., Wheeler, P.R., Honore, N., Garnier, T., Churcher, C., Harris, D., *et al.* (2001). Massive gene decay in the leprosy bacillus. *Nature* 409, 1007-1011.
- Cosma, C.L., Sherman, D.R., and Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. *Annual review of microbiology* 57, 641-676.
- Dal Porto, J.M., Gauld, S.B., Merrell, K.T., Mills, D., Pugh-Bernard, A.E., and Cambier, J. (2004). B cell antigen receptor signaling 101. *Molecular immunology* 41, 599-613.
- Das, V., Nal, B., Roumier, A., Meas-Yedid, V., Zimmer, C., Olivo-Marin, J.C., Roux, P., Ferrier, P., Dautry-Varsat, A., and Alcover, A. (2002). Membrane-cytoskeleton interactions during the formation of the immunological synapse and subsequent T-cell activation. *Immunological reviews* 189, 123-135.
- Davidson, H.W., and Watts, C. (1989). Epitope-directed processing of specific antigen by B lymphocytes. *The Journal of cell biology* 109, 85-92.
- Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity* 17, 693-702.
- de Hostos, E.L. (1999). The coronin family of actin-associated proteins. *Trends in cell biology* 9, 345-350.
- de Hostos, E.L., Bradtke, B., Lottspeich, F., Guggenheim, R., and Gerisch, G. (1991). Coronin, an actin binding protein of Dictyostelium discoideum localized to cell surface projections, has sequence similarities to G protein beta subunits. *The EMBO journal* 10, 4097-4104.

- de Hostos, E.L., Rehfuess, C., Bradtke, B., Waddell, D.R., Albrecht, R., Murphy, J., and Gerisch, G. (1993). Dictyostelium mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility. *The Journal of cell biology* 120, 163-173.
- DeFranco, A.L., Locksley, R.M., and Robertson, M. (2007). *Immunity: The Immune Response in Infectious and Inflammatory Disease*. New Science Press Ltd.
- Deghmane, A.E., Soualhine, H., Bach, H., Sendide, K., Itoh, S., Tam, A., Noubir, S., Talal, A., Lo, R., Toyoshima, S., *et al.* (2007). Lipoamide dehydrogenase mediates retention of coronin-1 on BCG vacuoles, leading to arrest in phagosome maturation. *Journal of cell science* 120, 2796-2806.
- Delamarre, L., Pack, M., Chang, H., Mellman, I., and Trombetta, E.S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science (New York, NY)* 307, 1630-1634.
- Denis, O., Tanghe, A., Palfliet, K., Jurion, F., van den Berg, T.P., Vanonckelen, A., Ooms, J., Saman, E., Ulmer, J.B., Content, J., *et al.* (1998). Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4<sup>+</sup> and CD8<sup>+</sup> T-cell epitopic repertoire broader than that stimulated by Mycobacterium tuberculosis H37Rv infection. *Infection and immunity* 66, 1527-1533.
- Di Giovanni, S., De Biase, A., Yakovlev, A., Finn, T., Beers, J., Hoffman, E.P., and Faden, A.I. (2005). In vivo and in vitro characterization of novel neuronal plasticity factors identified following spinal cord injury. *The Journal of biological chemistry* 280, 2084-2091.
- Di Giovanni, S., Knights, C.D., Rao, M., Yakovlev, A., Beers, J., Catania, J., Avantaggiati, M.L., and Faden, A.I. (2006). The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. *The EMBO journal* 25, 4084-4096.
- Di Noia, J.M., and Neuberger, M.S. (2007). Molecular mechanisms of antibody somatic hypermutation. *Annual review of biochemistry* 76, 1-22.
- Eberle, M., Traynor-Kaplan, A.E., Sklar, L.A., and Norgauer, J. (1990). Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? *The Journal of biological chemistry* 265, 16725-16728.
- Engering, A.J., Cella, M., Fluitsma, D., Brockhaus, M., Hoefsmit, E.C., Lanzavecchia, A., and Pieters, J. (1997). The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *European journal of immunology* 27, 2417-2425.

- Ernst, J.D. (1998). Macrophage receptors for Mycobacterium tuberculosis. *Infection and immunity* 66, 1277-1281.
- Ethuin, F., Gerard, B., Benna, J.E., Boutten, A., Gougereot-Pocidallo, M.A., Jacob, L., and Chollet-Martin, S. (2004). Human neutrophils produce interferon gamma upon stimulation by interleukin-12. *Laboratory investigation; a journal of technical methods and pathology* 84, 1363-1371.
- Falcone, F.H., Zillikens, D., and Gibbs, B.F. (2006). The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. *Experimental dermatology* 15, 855-864.
- Falkow, S. (2006). Is persistent bacterial infection good for your health? *Cell* 124, 699-702.
- Faurschou, M., and Borregaard, N. (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes and infection / Institut Pasteur* 5, 1317-1327.
- Ferrari, G., Knight, A.M., Watts, C., and Pieters, J. (1997). Distinct intracellular compartments involved in invariant chain degradation and antigenic peptide loading of major histocompatibility complex (MHC) class II molecules. *The Journal of cell biology* 139, 1433-1446.
- Ferrari, G., Langen, H., Naito, M., and Pieters, J. (1999). A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* 97, 435-447.
- Feske, S. (2007). Calcium signalling in lymphocyte activation and disease. *Nature reviews* 7, 690-702.
- Flo, T.H., Smith, K.D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S., and Aderem, A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 432, 917-921.
- Foger, N., Rangell, L., Danilenko, D.M., and Chan, A.C. (2006). Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science (New York, NY)* 313, 839-842.
- Fratti, R.A., Vergne, I., Chua, J., Skidmore, J., and Deretic, V. (2000). Regulators of membrane trafficking and Mycobacterium tuberculosis phagosome maturation block. *Electrophoresis* 21, 3378-3385.
- Fruman, D.A., Satterthwaite, A.B., and Witte, O.N. (2000). Xid-like phenotypes: a B cell signalosome takes shape. *Immunity* 13, 1-3.

- Fu, C., Turck, C.W., Kurosaki, T., and Chan, A.C. (1998). BLNK: a central linker protein in B cell activation. *Immunity* 9, 93-103.
- Fu, L.M. (2003). The potential of human neutrophil peptides in tuberculosis therapy. *Int J Tuberc Lung Dis* 7, 1027-1032.
- Fukui, Y., de Hostos, E.L., and Inoue, S. (1997). Dynamics of GFP-coronin and eupodia in live *Dictyostelium* observed with real-time confocal optics. *The Biological bulletin* 193, 224-225.
- Fukui, Y., Engler, S., Inoue, S., and de Hostos, E.L. (1999). Architectural dynamics and gene replacement of coronin suggest its role in cytokinesis. *Cell motility and the cytoskeleton* 42, 204-217.
- Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., Paiement, J., Bergeron, J.J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119-131.
- Gallo, E.M., Cante-Barrett, K., and Crabtree, G.R. (2006). Lymphocyte calcium signaling from membrane to nucleus. *Nature immunology* 7, 25-32.
- Gatfield, J., Albrecht, I., Zanolari, B., Steinmetz, M.O., and Pieters, J. (2005). Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled coil-mediated trimeric coronin 1 molecules. *Molecular biology of the cell* 16, 2786-2798.
- Gatfield, J., and Pieters, J. (2000). Essential role for cholesterol in entry of mycobacteria into macrophages. *Science (New York, NY)* 288, 1647-1650.
- Gatti, E., and Pierre, P. (2003). Understanding the cell biology of antigen presentation: the dendritic cell contribution. *Current opinion in cell biology* 15, 468-473.
- Geisberger, R., Cramer, R., and Achatz, G. (2003). Models of signal transduction through the B-cell antigen receptor. *Immunology* 110, 401-410.
- George, K.M., Chatterjee, D., Gunawardana, G., Welty, D., Hayman, J., Lee, R., and Small, P.L. (1999). Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science (New York, NY)* 283, 854-857.

- Gerisch, G., Albrecht, R., Heizer, C., Hodgkinson, S., and Maniak, M. (1995). Chemoattractant-controlled accumulation of coronin at the leading edge of Dictyostelium cells monitored using a green fluorescent protein-coronin fusion protein. *Curr Biol* 5, 1280-1285.
- Glogauer, M., Hartwig, J., and Stossel, T. (2000). Two pathways through Cdc42 couple the N-formyl receptor to actin nucleation in permeabilized human neutrophils. *The Journal of cell biology* 150, 785-796.
- Goldsby, R.A., Kindt, T.J., Osborne, B.A., and Kuby, J. (2003). Immunology, fifth edition. WH Freeman and Company.
- Goode, B.L., Wong, J.J., Butty, A.C., Peter, M., McCormack, A.L., Yates, J.R., Drubin, D.G., and Barnes, G. (1999). Coronin promotes the rapid assembly and cross-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast. *The Journal of cell biology* 144, 83-98.
- Gough, P.J., and Gordon, S. (2000). The role of scavenger receptors in the innate immune system. *Microbes and infection / Institut Pasteur* 2, 305-311.
- Grogan, A., Reeves, E., Keep, N., Wientjes, F., Totty, N.F., Burlingame, A.L., Hsuan, J.J., and Segal, A.W. (1997). Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *Journal of cell science* 110 ( Pt 24), 3071-3081.
- Guermonez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397-402.
- Gunn, M.D. (2003). Chemokine mediated control of dendritic cell migration and function. *Seminars in immunology* 15, 271-276.
- Guo, B., Blair, D., Chiles, T.C., Lowell, C.A., and Rothstein, T.L. (2007). Cutting Edge: B cell receptor (BCR) cross-talk: the IL-4-induced alternate pathway for BCR signaling operates in parallel with the classical pathway, is sensitive to Rottlerin, and depends on Lyn. *J Immunol* 178, 4726-4730.
- Guo, B., and Rothstein, T.L. (2005). B cell receptor (BCR) cross-talk: IL-4 creates an alternate pathway for BCR-induced ERK activation that is phosphatidylinositol 3-kinase independent. *J Immunol* 174, 5375-5381.

- Hacker, U., Albrecht, R., and Maniak, M. (1997). Fluid-phase uptake by macropinocytosis in *Dictyostelium*. *Journal of cell science* *110 (Pt 2)*, 105-112.
- Haraldsson, M.K., Louis-Dit-Sully, C.A., Lawson, B.R., Sternik, G., Santiago-Raber, M.L., Gascoigne, N.R., Theofilopoulos, A.N., and Kono, D.H. (2008). The lupus-related *Lmb3* locus contains a disease-suppressing *Coronin-1A* gene mutation. *Immunity* *28*, 40-51.
- Hardy, R.R. (2006). B-1 B cell development. *J Immunol* *177*, 2749-2754.
- Hasan, Z., Schlax, C., Kuhn, L., Lefkovits, I., Young, D., Thole, J., and Pieters, J. (1997). Isolation and characterization of the mycobacterial phagosome: segregation from the endosomal/lysosomal pathway. *Molecular microbiology* *24*, 545-553.
- Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T., and Tsukada, S. (1999). Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK--functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood* *94*, 2357-2364.
- Hasse, A., Rosentreter, A., Spoerl, Z., Stumpf, M., Noegel, A.A., and Clemen, C.S. (2005). *Coronin 3* and its role in murine brain morphogenesis. *The European journal of neuroscience* *21*, 1155-1168.
- Heil-Chapdelaine, R.A., Tran, N.K., and Cooper, J.A. (1998). The role of *Saccharomyces cerevisiae coronin* in the actin and microtubule cytoskeletons. *Curr Biol* *8*, 1281-1284.
- Hervas-Stubbs, S., Majlessi, L., Simsova, M., Morova, J., Rojas, M.J., Nouze, C., Brodin, P., Sebo, P., and Leclerc, C. (2006). High frequency of CD4<sup>+</sup> T cells specific for the TB10.4 protein correlates with protection against *Mycobacterium tuberculosis* infection. *Infection and immunity* *74*, 3396-3407.
- Hmama, Z., Gabathuler, R., Jefferies, W.A., de Jong, G., and Reiner, N.E. (1998). Attenuation of HLA-DR expression by mononuclear phagocytes infected with *Mycobacterium tuberculosis* is related to intracellular sequestration of immature class II heterodimers. *J Immunol* *161*, 4882-4893.
- Houben, E.N., Nguyen, L., and Pieters, J. (2006). Interaction of pathogenic mycobacteria with the host immune system. *Current opinion in microbiology* *9*, 76-85.

- Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M.F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402-406.
- Humphries, C.L., Balcer, H.I., D'Agostino, J.L., Winsor, B., Drubin, D.G., Barnes, G., Andrews, B.J., and Goode, B.L. (2002). Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. *The Journal of cell biology* 159, 993-1004.
- Huygen, K., Lozes, E., Gilles, B., Drowart, A., Palfliet, K., Jurion, F., Roland, I., Art, M., Dufaux, M., Nyabenda, J., *et al.* (1994). Mapping of TH1 helper T-cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live *Mycobacterium bovis* BCG. *Infection and immunity* 62, 363-370.
- Iizaka, M., Han, H.J., Akashi, H., Furukawa, Y., Nakajima, Y., Sugano, S., Ogawa, M., and Nakamura, Y. (2000). Isolation and chromosomal assignment of a novel human gene, CORO1C, homologous to coronin-like actin-binding proteins. *Cytogenetics and cell genetics* 88, 221-224.
- Inaba, K., Turley, S., Iyoda, T., Yamaide, F., Shimoyama, S., Reis e Sousa, C., Germain, R.N., Mellman, I., and Steinman, R.M. (2000). The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *The Journal of experimental medicine* 191, 927-936.
- Inderlied, C.B., Kemper, C.A., and Bermudez, L.E. (1993). The *Mycobacterium avium* complex. *Clinical microbiology reviews* 6, 266-310.
- Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A.C., and Kurosaki, T. (1999). BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity* 10, 117-125.
- Itoh, S., Suzuki, K., Nishihata, J., Iwasa, M., Oku, T., Nakajin, S., Nauseef, W.M., and Toyoshima, S. (2002). The role of protein kinase C in the transient association of p57, a coronin family actin-binding protein, with phagosomes. *Biological & pharmaceutical bulletin* 25, 837-844.
- Iwasaki, H., and Akashi, K. (2007). Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 26, 726-740.
- Jayachandran, R., Gatfield, J., Massner, J., Albrecht, I., Zanolari, B., and Pieters, J. (2008). RNA Interference in J774 Macrophages Reveals a Role for Coronin 1 in Mycobacterial Trafficking but Not in Actin-dependent Processes. *Molecular biology of the cell* 19, 1241-1251.

- Jayachandran, R., Sundaramurthy, V., Combaluzier, B., Mueller, P., Korf, H., Huygen, K., Miyazaki, T., Albrecht, I., Massner, J., and Pieters, J. (2007). Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell* *130*, 37-50.
- Jiang, H., Kuang, Y., Wu, Y., Smrcka, A., Simon, M.I., and Wu, D. (1996). Pertussis toxin-sensitive activation of phospholipase C by the C5a and fMet-Leu-Phe receptors. *The Journal of biological chemistry* *271*, 13430-13434.
- Johnson, S.A., Pleiman, C.M., Pao, L., Schneringer, J., Hippen, K., and Cambier, J.C. (1995). Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J Immunol* *155*, 4596-4603.
- Jun, J.E., and Goodnow, C.C. (2003). Scaffolding of antigen receptors for immunogenic versus tolerogenic signaling. *Nature immunology* *4*, 1057-1064.
- Kammerer, R.A., Kostrewa, D., Progiás, P., Honnappa, S., Avila, D., Lustig, A., Winkler, F.K., Pieters, J., and Steinmetz, M.O. (2005). A conserved trimerization motif controls the topology of short coiled coils. *Proceedings of the National Academy of Sciences of the United States of America* *102*, 13891-13896.
- Karakousis, P.C., Bishai, W.R., and Dorman, S.E. (2004). Mycobacterium tuberculosis cell envelope lipids and the host immune response. *Cellular microbiology* *6*, 105-116.
- Katsura, Y. (2002). Redefinition of lymphoid progenitors. *Nature reviews* *2*, 127-132.
- Kaufmann, S.H. (2006). Tuberculosis: back on the immunologists' agenda. *Immunity* *24*, 351-357.
- Kawai, T., and Akira, S. (2007). TLR signaling. *Seminars in immunology* *19*, 24-32.
- Kim, S.I., and Bresnick, E.H. (2007). Transcriptional control of erythropoiesis: emerging mechanisms and principles. *Oncogene* *26*, 6777-6794.
- Klebanoff, S.J. (2005). Myeloperoxidase: friend and foe. *Journal of leukocyte biology* *77*, 598-625.
- Kloetzel, P.M., and Ossendorp, F. (2004). Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Current opinion in immunology* *16*, 76-81.
- Kovacsovics-Bankowski, M., and Rock, K.L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science (New York, NY)* *267*, 243-246.

- Krutzik, S.R., and Modlin, R.L. (2004). The role of Toll-like receptors in combating mycobacteria. *Seminars in immunology* 16, 35-41.
- Kulathu, Y., Hobeika, E., Turchinovich, G., and Reth, M. (2008). The kinase Syk as an adaptor controlling sustained calcium signalling and B-cell development. *The EMBO journal*.
- Kurosaki, T., Maeda, A., Ishiai, M., Hashimoto, A., Inabe, K., and Takata, M. (2000). Regulation of the phospholipase C-gamma2 pathway in B cells. *Immunological reviews* 176, 19-29.
- Lam, K.P., and Rajewsky, K. (1999). B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. *The Journal of experimental medicine* 190, 471-477.
- Lambeth, J.D. (2004). NOX enzymes and the biology of reactive oxygen. *Nature reviews* 4, 181-189.
- Lanzavecchia, A. (1987). Antigen uptake and accumulation in antigen-specific B cells. *Immunological reviews* 99, 39-51.
- Lanzavecchia, A. (1990). Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annual review of immunology* 8, 773-793.
- Lewinsohn, D.M., Grotzke, J.E., Heinzl, A.S., Zhu, L., Owendale, P.J., Johnson, M., and Alderson, M.R. (2006). Secreted proteins from *Mycobacterium tuberculosis* gain access to the cytosolic MHC class-I antigen-processing pathway. *J Immunol* 177, 437-442.
- Lewis, R.S. (2001). Calcium signaling mechanisms in T lymphocytes. *Annual review of immunology* 19, 497-521.
- Lewis, R.S. (2007). The molecular choreography of a store-operated calcium channel. *Nature* 446, 284-287.
- Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V., and Wu, D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science (New York, NY)* 287, 1046-1049.
- Lo-Man, R., Langeveld, J.P., Deriaud, E., Jehanno, M., Rojas, M., Clement, J.M., Meloen, R.H., Hofnung, M., and Leclerc, C. (2000). Extending the CD4(+) T-cell epitope specificity of the Th1

immune response to an antigen using a *Salmonella enterica* serovar typhimurium delivery vehicle. *Infection and immunity* 68, 3079-3089.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *The Journal of experimental medicine* 190, 75-89.

Lopes-Carvalho, T., and Kearney, J.F. (2004). Development and selection of marginal zone B cells. *Immunological reviews* 197, 192-205.

MacLennan, I.C., Gulbranson-Judge, A., Toellner, K.M., Casamayor-Palleja, M., Chan, E., Sze, D.M., Luther, S.A., and Orbea, H.A. (1997). The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunological reviews* 156, 53-66.

Majlessi, L., Rojas, M.J., Brodin, P., and Leclerc, C. (2003). CD8<sup>+</sup>-T-cell responses of *Mycobacterium*-infected mice to a newly identified major histocompatibility complex class I-restricted epitope shared by proteins of the ESAT-6 family. *Infection and immunity* 71, 7173-7177.

Majlessi, L., Simsova, M., Jarvis, Z., Brodin, P., Rojas, M.J., Bauche, C., Nouze, C., Ladant, D., Cole, S.T., Sebo, P., *et al.* (2006). An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. *Infection and immunity* 74, 2128-2137.

Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J., and Gerisch, G. (1995). Coronin involved in phagocytosis: dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag. *Cell* 83, 915-924.

Martin, F., and Kearney, J.F. (2000). B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunological reviews* 175, 70-79.

May, M.E., and Spagnuolo, P.J. (1987). Evidence for activation of a respiratory burst in the interaction of human neutrophils with *Mycobacterium tuberculosis*. *Infection and immunity* 55, 2304-2307.

Mayer-Scholl, A., Averhoff, P., and Zychlinsky, A. (2004). How do neutrophils and pathogens interact? *Current opinion in microbiology* 7, 62-66.

McCluskey, J., Rossjohn, J., and Purcell, A.W. (2004). TAP genes and immunity. *Current opinion in immunology* 16, 651-659.

- Melchers, F., and Rolink, A. (1998). B-lymphocyte development and biology. WE Paul, Editor, *Fundamental Immunology*, Lippincott-Raven, Philadelphia, 183-224.
- Mellman, I. (2005). Antigen processing and presentation by dendritic cells: cell biological mechanisms. *Advances in experimental medicine and biology* 560, 63-67.
- Mellman, I., and Steinman, R.M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106, 255-258.
- Mempel, T.R., Henrickson, S.E., and Von Andrian, U.H. (2004). T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427, 154-159.
- Miller, J.F., and Osoba, D. (1967). Current concepts of the immunological function of the thymus. *Physiological reviews* 47, 437-520.
- Miosge, L.A., and Goodnow, C.C. (2005). Genes, pathways and checkpoints in lymphocyte development and homeostasis. *Immunology and cell biology* 83, 318-335.
- Mizuno, T., and Rothstein, T.L. (2005). B cell receptor (BCR) cross-talk: CD40 engagement creates an alternate pathway for BCR signaling that activates I kappa B kinase/I kappa B alpha/NF-kappa B without the need for PI3K and phospholipase C gamma. *J Immunol* 174, 6062-6070.
- Mond, J.J., Lees, A., and Snapper, C.M. (1995a). T cell-independent antigens type 2. *Annual review of immunology* 13, 655-692.
- Mond, J.J., Vos, Q., Lees, A., and Snapper, C.M. (1995b). T cell independent antigens. *Current opinion in immunology* 7, 349-354.
- Morrisette, N.S., Gold, E.S., Guo, J., Hamerman, J.A., Ozinsky, A., Bedian, V., and Aderem, A.A. (1999). Isolation and characterization of monoclonal antibodies directed against novel components of macrophage phagosomes. *Journal of cell science* 112 ( Pt 24), 4705-4713.
- Mueller, P., Massner, J., Jayachandran, R., Combaluzier, B., Albrecht, I., Gatfield, J., Blum, C., Ceredig, R., Rodewald, H.R., Rolink, A.G., *et al.* (2008). Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. *Nature immunology* 9, 424-431.
- Mukhopadhyay, S., and Gordon, S. (2004). The role of scavenger receptors in pathogen recognition and innate immunity. *Immunobiology* 209, 39-49.

- Muralikrishna, T., Begum, Z., Swamy, C.V., and Khar, A. (1998). Molecular cloning and characterization of a tumor rejection antigen from rat histiocytoma, AK-5. *DNA and cell biology* *17*, 603-612.
- N'Diaye, E.N., Darzacq, X., Astarie-Dequeker, C., Daffe, M., Calafat, J., and Maridonneau-Parini, I. (1998). Fusion of azurophil granules with phagosomes and activation of the tyrosine kinase Hck are specifically inhibited during phagocytosis of mycobacteria by human neutrophils. *J Immunol* *161*, 4983-4991.
- Nakamura, T., Takeuchi, K., Muraoka, S., Takezoe, H., Takahashi, N., and Mori, N. (1999). A neurally enriched coronin-like protein, ClipinC, is a novel candidate for an actin cytoskeleton-cortical membrane-linking protein. *The Journal of biological chemistry* *274*, 13322-13327.
- Nal, B., Carroll, P., Mohr, E., Verthuy, C., Da Silva, M.I., Gayet, O., Guo, X.J., He, H.T., Alcover, A., and Ferrier, P. (2004). Coronin-1 expression in T lymphocytes: insights into protein function during T cell development and activation. *International immunology* *16*, 231-240.
- Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nature reviews* *6*, 173-182.
- Nauseef, W.M. (2007). How human neutrophils kill and degrade microbes: an integrated view. *Immunological reviews* *219*, 88-102.
- Nguyen, L., and Pieters, J. (2005). The Trojan horse: survival tactics of pathogenic mycobacteria in macrophages. *Trends in cell biology* *15*, 269-276.
- Nguyen, L., Walburger, A., Houben, E., Koul, A., Muller, S., Morbitzer, M., Klebl, B., Ferrari, G., and Pieters, J. (2005). Role of protein kinase G in growth and glutamine metabolism of *Mycobacterium bovis* BCG. *Journal of bacteriology* *187*, 5852-5856.
- Noss, E.H., Pai, R.K., Sellati, T.J., Radolf, J.D., Belisle, J., Golenbock, D.T., Boom, W.H., and Harding, C.V. (2001). Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J Immunol* *167*, 910-918.
- Okkels, L.M., and Andersen, P. (2004). Protein-protein interactions of proteins from the ESAT-6 family of *Mycobacterium tuberculosis*. *Journal of bacteriology* *186*, 2487-2491.

- Okumura, M., Kung, C., Wong, S., Rodgers, M., and Thomas, M.L. (1998). Definition of family of coronin-related proteins conserved between humans and mice: close genetic linkage between coronin-2 and CD45-associated protein. *DNA and cell biology* 17, 779-787.
- Ooi, C.E., Weiss, J., Doerfler, M.E., and Elsbach, P. (1991). Endotoxin-neutralizing properties of the 25 kD N-terminal fragment and a newly isolated 30 kD C-terminal fragment of the 55-60 kD bactericidal/permeability-increasing protein of human neutrophils. *The Journal of experimental medicine* 174, 649-655.
- Orabona, C., Grohmann, U., Belladonna, M.L., Fallarino, F., Vacca, C., Bianchi, R., Bozza, S., Volpi, C., Salomon, B.L., Fioretti, M.C., *et al.* (2004). CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nature immunology* 5, 1134-1142.
- Orkin, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 1, 57-64.
- Orkin, S.H., and Zon, L.I. (2008). SnapShot: hematopoiesis. *Cell* 132, 712.
- Pai, R.K., Pennini, M.E., Tobian, A.A., Canaday, D.H., Boom, W.H., and Harding, C.V. (2004). Prolonged toll-like receptor signaling by *Mycobacterium tuberculosis* and its 19-kilodalton lipoprotein inhibits gamma interferon-induced regulation of selected genes in macrophages. *Infection and immunity* 72, 6603-6614.
- Pancholi, P., Mirza, A., Bhardwaj, N., and Steinman, R.M. (1993). Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. *Science (New York, NY)* 260, 984-986.
- Parekh, A.B. (2006). On the activation mechanism of store-operated calcium channels. *Pflugers Arch* 453, 303-311.
- Parekh, A.B. (2007). Functional consequences of activating store-operated CRAC channels. *Cell calcium* 42, 111-121.
- Parekh, A.B., and Penner, R. (1997). Store depletion and calcium influx. *Physiological reviews* 77, 901-930.
- Parente, J.A., Jr., Chen, X., Zhou, C., Petropoulos, A.C., and Chew, C.S. (1999). Isolation, cloning, and characterization of a new mammalian coronin family member, coroninse, which is regulated

- within the protein kinase C signaling pathway. *The Journal of biological chemistry* 274, 3017-3025.
- Park, C.S., and Choi, Y.S. (2005). How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology* 114, 2-10.
- Parker, D.C. (1993). T cell-dependent B cell activation. *Annual review of immunology* 11, 331-360.
- Paul, W.E., and Ohara, J. (1987). B-cell stimulatory factor-1/interleukin 4. *Annual review of immunology* 5, 429-459.
- Peters, P.J., Neefjes, J.J., Oorschot, V., Ploegh, H.L., and Geuze, H.J. (1991). Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349, 669-676.
- Peyron, P., Bordier, C., N'Diaye, E.N., and Maridonneau-Parini, I. (2000). Nonopsonic phagocytosis of *Mycobacterium kansasii* by human neutrophils depends on cholesterol and is mediated by CR3 associated with glycosylphosphatidylinositol-anchored proteins. *J Immunol* 165, 5186-5191.
- Philips, J.A., Rubin, E.J., and Perrimon, N. (2005). *Drosophila* RNAi screen reveals CD36 family member required for mycobacterial infection. *Science (New York, NY)* 309, 1251-1253.
- Pieters, J. (1997a). MHC class II compartments: specialized organelles of the endocytic pathway in antigen presenting cells. *Biological chemistry* 378, 751-758.
- Pieters, J. (1997b). MHC class II restricted antigen presentation. *Current opinion in immunology* 9, 89-96.
- Pieters, J., and Gatfield, J. (2002). Hijacking the host: survival of pathogenic mycobacteria inside macrophages. *Trends in microbiology* 10, 142-146.
- Pieters, J., Horstmann, H., Bakke, O., Griffiths, G., and Lipp, J. (1991). Intracellular transport and localization of major histocompatibility complex class II molecules and associated invariant chain. *The Journal of cell biology* 115, 1213-1223.
- Pillai, S., Cariappa, A., and Moran, S.T. (2004). Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunological reviews* 197, 206-218.

- Pillai, S., Cariappa, A., and Moran, S.T. (2005). Marginal zone B cells. *Annual review of immunology* 23, 161-196.
- Pym, A.S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K.E., Marchal, G., Leclerc, C., and Cole, S.T. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nature medicine* 9, 533-539.
- Rabin, E.M., Ohara, J., and Paul, W.E. (1985). B-cell stimulatory factor 1 activates resting B cells. *Proceedings of the National Academy of Sciences of the United States of America* 82, 2935-2939.
- Radel, S.J., Genco, R.J., and De Nardin, E. (1994). Structural and functional characterization of the human formyl peptide receptor ligand-binding region. *Infection and immunity* 62, 1726-1732.
- Rajewsky, K. (1996). Clonal selection and learning in the antibody system. *Nature* 381, 751-758.
- Ramachandra, L., Chu, R.S., Askew, D., Noss, E.H., Canaday, D.H., Potter, N.S., Johnsen, A., Krieg, A.M., Nedrud, J.G., Boom, W.H., *et al.* (1999). Phagocytic antigen processing and effects of microbial products on antigen processing and T-cell responses. *Immunological reviews* 168, 217-239.
- Ramachandra, L., Noss, E., Boom, W.H., and Harding, C.V. (2001). Processing of Mycobacterium tuberculosis antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. *The Journal of experimental medicine* 194, 1421-1432.
- Reeves, E.P., Dekker, L.V., Forbes, L.V., Wientjes, F.B., Grogan, A., Pappin, D.J., and Segal, A.W. (1999). Direct interaction between p47phox and protein kinase C: evidence for targeting of protein kinase C by p47phox in neutrophils. *The Biochemical journal* 344 Pt 3, 859-866.
- Reth, M., and Wienands, J. (1997). Initiation and processing of signals from the B cell antigen receptor. *Annual review of immunology* 15, 453-479.
- Rickert, P., Weiner, O.D., Wang, F., Bourne, H.R., and Servant, G. (2000). Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. *Trends in cell biology* 10, 466-473.
- Rincon, M., and Flavell, R.A. (1997). T-cell subsets: transcriptional control in the Th1/Th2 decision. *Curr Biol* 7, R729-732.

- Robbie-Ryan, M., and Brown, M. (2002). The role of mast cells in allergy and autoimmunity. *Current opinion in immunology* 14, 728-733.
- Rolink, A., and Melchers, F. (1996). B-cell development in the mouse. *Immunology letters* 54, 157-161.
- Rolink, A.G., Andersson, J., and Melchers, F. (2004). Molecular mechanisms guiding late stages of B-cell development. *Immunological reviews* 197, 41-50.
- Rolink, A.G., Schaniel, C., Andersson, J., and Melchers, F. (2001). Selection events operating at various stages in B cell development. *Current opinion in immunology* 13, 202-207.
- Rolink, A.G., ten Boekel, E., Yamagami, T., Ceredig, R., Andersson, J., and Melchers, F. (1999). B cell development in the mouse from early progenitors to mature B cells. *Immunology letters* 68, 89-93.
- Rooyackers, A.W., and Stokes, R.W. (2005). Absence of complement receptor 3 results in reduced binding and ingestion of *Mycobacterium tuberculosis* but has no significant effect on the induction of reactive oxygen and nitrogen intermediates or on the survival of the bacteria in resident and interferon-gamma activated macrophages. *Microbial pathogenesis* 39, 57-67.
- Rothenberg, M.E., and Hogan, S.P. (2006). The eosinophil. *Annual review of immunology* 24, 147-174.
- Roy, C.R., Salcedo, S.P., and Gorvel, J.P. (2006). Pathogen-endoplasmic-reticulum interactions: in through the out door. *Nature reviews* 6, 136-147.
- Rus, H., Cudrici, C., and Niculescu, F. (2005). The role of the complement system in innate immunity. *Immunologic research* 33, 103-112.
- Russell, D.G. (2001). *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol* 2, 569-577.
- Rybakin, V. (2008). Role of mammalian coronin 7 in the biosynthetic pathway. *The Coronin Family of Proteins*, Landes Bioscience and Springer Science.
- Rybakin, V., and Clemen, C.S. (2005). Coronin proteins as multifunctional regulators of the cytoskeleton and membrane trafficking. *Bioessays* 27, 625-632.

- Rybakin, V., Gounko, N.V., Spate, K., Honing, S., Majoul, I.V., Duden, R., and Noegel, A.A. (2006). Crn7 interacts with AP-1 and is required for the maintenance of Golgi morphology and protein export from the Golgi. *The Journal of biological chemistry* *281*, 31070-31078.
- Rybakin, V., Stumpf, M., Schulze, A., Majoul, I.V., Noegel, A.A., and Hasse, A. (2004). Coronin 7, the mammalian POD-1 homologue, localizes to the Golgi apparatus. *FEBS letters* *573*, 161-167.
- Sada, K., Takano, T., Yanagi, S., and Yamamura, H. (2001). Structure and function of Syk protein-tyrosine kinase. *Journal of biochemistry* *130*, 177-186.
- Saito, K., Scharenberg, A.M., and Kinet, J.P. (2001). Interaction between the Btk PH domain and phosphatidylinositol-3,4,5-trisphosphate directly regulates Btk. *The Journal of biological chemistry* *276*, 16201-16206.
- Sakaguchi, S., Wing, K., and Miyara, M. (2007). Regulatory T cells - a brief history and perspective. *European journal of immunology* *37 Suppl 1*, S116-123.
- Saleh, M.T., and Belisle, J.T. (2000). Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. *Journal of bacteriology* *182*, 6850-6853.
- Sarmay, G., Angyal, A., Kertesz, A., Maus, M., and Medgyesi, D. (2006). The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. *Immunology letters* *104*, 76-82.
- Scapini, P., Bazzoni, F., and Cassatella, M.A. (2008). Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunology letters* *116*, 1-6.
- Schaible, U.E., Winau, F., Sieling, P.A., Fischer, K., Collins, H.L., Hagens, K., Modlin, R.L., Brinkmann, V., and Kaufmann, S.H. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nature medicine* *9*, 1039-1046.
- Schamel, W.W., and Reth, M. (2000). Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity* *13*, 5-14.
- Scherr, N., Honnappa, S., Kunz, G., Mueller, P., Jayachandran, R., Winkler, F., Pieters, J., and Steinmetz, M.O. (2007). Structural basis for the specific inhibition of protein kinase G, a virulence

factor of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* *104*, 12151-12156.

Schlesinger, L.S. (1993). Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J Immunol* *150*, 2920-2930.

Schlesinger, L.S. (1996). Entry of *Mycobacterium tuberculosis* into mononuclear phagocytes. *Current topics in microbiology and immunology* *215*, 71-96.

Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R., and Horwitz, M.A. (1990). Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J Immunol* *144*, 2771-2780.

Schuller, S., Neefjes, J., Ottenhoff, T., Thole, J., and Young, D. (2001). Coronin is involved in uptake of *Mycobacterium bovis* BCG in human macrophages but not in phagosome maintenance. *Cellular microbiology* *3*, 785-793.

Segal, A.W. (2005). How neutrophils kill microbes. *Annual review of immunology* *23*, 197-223.

Selsted, M.E., and Ouellette, A.J. (2005). Mammalian defensins in the antimicrobial immune response. *Nature immunology* *6*, 551-557.

Semerad, C.L., Liu, F., Gregory, A.D., Stumpf, K., and Link, D.C. (2002). G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity* *17*, 413-423.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science (New York, NY)* *287*, 1037-1040.

Shina, M.C., and Noegel, A.A. (2008). Invertebrate Coronins. *The Coronin Family of Proteins*, Landes Bioscience and Springer Science.

Smith, E., Zarbock, A., Stark, M.A., Burcin, T.L., Bruce, A.C., Foley, P., and Ley, K. (2007). IL-23 is required for neutrophil homeostasis in normal and neutrophilic mice. *J Immunol* *179*, 8274-8279.

- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analytical biochemistry* 150, 76-85.
- Song, H., and Cerny, J. (2003). Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *The Journal of experimental medicine* 198, 1923-1935.
- Spicuglia, S., Franchini, D.M., and Ferrier, P. (2006). Regulation of V(D)J recombination. *Current opinion in immunology* 18, 158-163.
- Spoerl, Z., Stumpf, M., Noegel, A.A., and Hasse, A. (2002). Oligomerization, F-actin interaction, and membrane association of the ubiquitous mammalian coronin 3 are mediated by its carboxyl terminus. *The Journal of biological chemistry* 277, 48858-48867.
- Stark, M.A., Huo, Y., Burcin, T.L., Morris, M.A., Olson, T.S., and Ley, K. (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22, 285-294.
- Stinear, T.P., Jenkin, G.A., Johnson, P.D., and Davies, J.K. (2000). Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. *Journal of bacteriology* 182, 6322-6330.
- Striz, I., and Trebichavsky, I. (2004). Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiological research / Academia Scientiarum Bohemoslovaca* 53, 245-253.
- Strober, W., Murray, P.J., Kitani, A., and Watanabe, T. (2006). Signalling pathways and molecular interactions of NOD1 and NOD2. *Nature reviews* 6, 9-20.
- Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994). Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science (New York, NY)* 263, 678-681.
- Sun, R., Iribarren, P., Zhang, N., Zhou, Y., Gong, W., Cho, E.H., Lockett, S., Chertov, O., Bednar, F., Rogers, T.J., *et al.* (2004). Identification of neutrophil granule protein cathepsin G as a novel chemotactic agonist for the G protein-coupled formyl peptide receptor. *J Immunol* 173, 428-436.

- Sundaramurthy, V., and Pieters, J. (2007). Interactions of pathogenic mycobacteria with host macrophages. *Microbes and infection / Institut Pasteur* 9, 1671-1679.
- Suzuki, K., Nishihata, J., Arai, Y., Honma, N., Yamamoto, K., Irimura, T., and Toyoshima, S. (1995). Molecular cloning of a novel actin-binding protein, p57, with a WD repeat and a leucine zipper motif. *FEBS letters* 364, 283-288.
- Taguchi, T., Kiyokawa, N., Takenouch, H., Matsui, J., Tang, W.R., Nakajima, H., Suzuki, K., Shiozawa, Y., Saito, M., Katagiri, Y.U., *et al.* (2004). Deficiency of BLNK hampers PLC-gamma2 phosphorylation and Ca<sup>2+</sup> influx induced by the pre-B-cell receptor in human pre-B cells. *Immunology* 112, 575-582.
- Tan, B.H., Meinken, C., Bastian, M., Bruns, H., Legaspi, A., Ochoa, M.T., Krutzik, S.R., Bloom, B.R., Ganz, T., Modlin, R.L., *et al.* (2006). Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* 177, 1864-1871.
- Tekaia, F., Gordon, S.V., Garnier, T., Brosch, R., Barrell, B.G., and Cole, S.T. (1999). Analysis of the proteome of *Mycobacterium tuberculosis* in silico. *Tuber Lung Dis* 79, 329-342.
- Thal, D., Xavier, C.P., Rosentreter, A., Linder, S., Friedrichs, B., Waha, A., Pietsch, T., Stumpf, M., Noegel, A., and Clemen, C. (2008). Expression of coronin-3 (coronin-1C) in diffuse gliomas is related to malignancy. *The Journal of pathology* 214, 415-424.
- Tonegawa, S., Steinberg, C., Dube, S., and Bernardini, A. (1974). Evidence for somatic generation of antibody diversity. *Proceedings of the National Academy of Sciences of the United States of America* 71, 4027-4031.
- Tonjum, T., Welty, D.B., Jantzen, E., and Small, P.L. (1998). Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. *Journal of clinical microbiology* 36, 918-925.
- Traynor-Kaplan, A.E., Harris, A.L., Thompson, B.L., Taylor, P., and Sklar, L.A. (1988). An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature* 334, 353-356.
- Trombetta, E.S., and Mellman, I. (2005). Cell biology of antigen processing in vitro and in vivo. *Annual review of immunology* 23, 975-1028.

- Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H.L., and Pieters, J. (1994). Isolation and characterization of the intracellular MHC class II compartment. *Nature* *369*, 120-126.
- Tulp, A., Verwoerd, D., and Pieters, J. (1993). Application of an improved density gradient electrophoresis apparatus to the separation of proteins, cells and subcellular organelles. *Electrophoresis* *14*, 1295-1301.
- Turley, S.J., Inaba, K., Garrett, W.S., Ebersold, M., Unternaehrer, J., Steinman, R.M., and Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. *Science (New York, NY)* *288*, 522-527.
- Utrecht, A.C., and Bear, J.E. (2006). Coronins: the return of the crown. *Trends in cell biology* *16*, 421-426.
- van Berkel, T.J., Out, R., Hoekstra, M., Kuiper, J., Biessen, E., and van Eck, M. (2005). Scavenger receptors: friend or foe in atherosclerosis? *Current opinion in lipidology* *16*, 525-535.
- van Gisbergen, K.P., Sanchez-Hernandez, M., Geijtenbeek, T.B., and van Kooyk, Y. (2005). Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *The Journal of experimental medicine* *201*, 1281-1292.
- Velasco-Velazquez, M.A., Barrera, D., Gonzalez-Arenas, A., Rosales, C., and Agramonte-Hevia, J. (2003). Macrophage--Mycobacterium tuberculosis interactions: role of complement receptor 3. *Microbial pathogenesis* *35*, 125-131.
- Vergne, I., Chua, J., Lee, H.H., Lucas, M., Belisle, J., and Deretic, V. (2005). Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America* *102*, 4033-4038.
- von Andrian, U.H., and Mempel, T.R. (2003). Homing and cellular traffic in lymph nodes. *Nature reviews* *3*, 867-878.
- Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., Klebl, B., Thompson, C., Bacher, G., and Pieters, J. (2004). Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science (New York, NY)* *304*, 1800-1804.
- Wang, D., Feng, J., Wen, R., Marine, J.C., Sangster, M.Y., Parganas, E., Hoffmeyer, A., Jackson, C.W., Cleveland, J.L., Murray, P.J., *et al.* (2000). Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity* *13*, 25-35.

- Wang, J.H., Avitahl, N., Cariappa, A., Friedrich, C., Ikeda, T., Renold, A., Andrikopoulos, K., Liang, L., Pillai, S., Morgan, B.A., *et al.* (1998). Aiolos regulates B cell activation and maturation to effector state. *Immunity* 9, 543-553.
- Watanabe, D., Hashimoto, S., Ishiai, M., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T., and Tsukada, S. (2001). Four tyrosine residues in phospholipase C-gamma 2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling. *The Journal of biological chemistry* 276, 38595-38601.
- Watts, C., and Davidson, H.W. (1988). Endocytosis and recycling of specific antigen by human B cell lines. *The EMBO journal* 7, 1937-1945.
- Weiss, J., Elsbach, P., Olsson, I., and Odeberg, H. (1978). Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *The Journal of biological chemistry* 253, 2664-2672.
- West, A.P., Koblansky, A.A., and Ghosh, S. (2006). Recognition and signaling by toll-like receptors. *Annual review of cell and developmental biology* 22, 409-437.
- Wiker, H.G., and Harboe, M. (1992). The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiological reviews* 56, 648-661.
- Wiltshcke, C., Nemet, H., Holzinger, C., Gessl, A., Pernerstorfer, T., Forster, O., and Boltz-Nitulescu, G. (1989). Murine recombinant GM-CSF-driven rat bone marrow cell differentiation and factors suppressing cell proliferation. *Immunobiology* 179, 145-158.
- Winau, F., Weber, S., Sad, S., de Diego, J., Hoops, S.L., Breiden, B., Sandhoff, K., Brinkmann, V., Kaufmann, S.H., and Schaible, U.E. (2006). Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* 24, 105-117.
- Wittamer, V., Bondue, B., Guillabert, A., Vassart, G., Parmentier, M., and Communi, D. (2005). Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* 175, 487-493.
- Wong, P., and Pamer, E.G. (2003). CD8 T cell responses to infectious pathogens. *Annual review of immunology* 21, 29-70.
- Wortis, H.H., and Berland, R. (2001). Cutting edge commentary: origins of B-1 cells. *J Immunol* 166, 2163-2166.

- Wright, G.W., Ooi, C.E., Weiss, J., and Elsbach, P. (1990). Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A2 that participate in the destruction of *Escherichia coli* in a rabbit inflammatory exudate. *The Journal of biological chemistry* *265*, 6675-6681.
- Wu, D., Huang, C.K., and Jiang, H. (2000). Roles of phospholipid signaling in chemoattractant-induced responses. *Journal of cell science* *113 (Pt 17)*, 2935-2940.
- Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D.G. (1994). Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. *J Immunol* *153*, 2568-2578.
- Xu, Y., Harder, K.W., Huntington, N.D., Hibbs, M.L., and Tarlinton, D.M. (2005). Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity* *22*, 9-18.
- Yan, M., Collins, R.F., Grinstein, S., and Trimble, W.S. (2005). Coronin-1 function is required for phagosome formation. *Molecular biology of the cell* *16*, 3077-3087.
- Yan, M., Di Ciano-Oliveira, C., Grinstein, S., and Trimble, W.S. (2007). Coronin function is required for chemotaxis and phagocytosis in human neutrophils. *J Immunol* *178*, 5769-5778.
- Yoon, H.G., Chan, D.W., Huang, Z.Q., Li, J., Fondell, J.D., Qin, J., and Wong, J. (2003). Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. *The EMBO journal* *22*, 1336-1346.
- Zachariae, C.O. (1993). Chemotactic cytokines and inflammation. Biological properties of the lymphocyte and monocyte chemotactic factors ELCF, MCAF and IL-8. *Acta dermatovenereologica* *181*, 1-37.
- Zaphiropoulos, P.G., and Toftgard, R. (1996). cDNA cloning of a novel WD repeat protein mapping to the 9q22.3 chromosomal region. *DNA and cell biology* *15*, 1049-1056.
- Zhang, M., Srivastava, G., and Lu, L. (2004). The pre-B cell receptor and its function during B cell development. *Cellular & molecular immunology* *1*, 89-94.

## 7 ABBREVIATIONS

ACK	Ammonium chloride potassium
APC	Antigen presenting cell
APS	Ammonium persulfate
BAFF	B cell activating factor
BCA	Bicinchoninic acid
BCG	Bacille Calmette-Guerin
BCR	B cell receptor
BPI	Bactericidal permeability increasing protein
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLIP	Class II linked invariant chain peptide
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CpG DNA	DNA containing unmethylated CpG motifs
CR3	Complement receptor type 3
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DGE	Density gradient electrophoresis
DHR 1,2,3	Dihydrorhodamine 1,2,3
dH <sub>2</sub> O	Distilled water
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetate sodium salt
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
FO	Follicular
G-CSF	Granulocyte colony-stimulating factor

GFP	Green fluorescent protein
GIPLs	Glycoinositol-phospholipids
GMP	Granulocyte/macrophage progenitor
GPCR	G protein-coupled receptor
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
IF	Immunofluorescence
IFN- $\gamma$	Interferon gamma
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate or InsP3
IRAK	Interleukin-1 receptor-associated kinase
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilodalton
LAMP-1	Lysosomal-associated membrane protein 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MEP	Megakaryocyte/erythroid progenitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
min	Minute (s)
MM	Master mix
MMPs	Matrix metalloproteinases
MptpA	Mycobacterial phosphotyrosine phosphatase A
MR	Mannose receptor
MZ	Marginal zone
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
OADC	Oleic acid, albumin, dextrose and catalase supplement
OD	Optical density

OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PALS	Periarterial lymphatic sheath
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBS-SAP	PBS containing 0.1% saponin
PBS-SAP-BSA	PBS containing 0.1% saponin and 2% BSA
PBST	Phosphate buffered saline with Tween-20
PFA	Paraformaldehyde
PGN	Peptidoglycan
PIP2	Phosphatidylinositol 4,5-biphosphate or PtdIns(4,5)P <sub>2</sub>
PknG	Serine/threonine protein kinase G
PLC $\gamma$ 2	Phospholipase C gamma 2
PMA	Phorbol-12-myristate-13-acetate
PMNs	Polymorphonuclear leukocytes
PMSF	Phenylmethylsulfonylfluoride
PNA	Peanut agglutinin
PNS	Postnuclear supernatant
PNP	P-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminidine
pNPP	P-nitrophenyl phosphate
PRR	Pathogen-recognition receptor
RSV	Respiratory syncytial virus
SapM	Secreted acid phosphatase of <i>M. tuberculosis</i>
SDS	Sodium dodecylsulfate
SDS-SB	SDS sample buffer
SR	Scavenger receptor
TACO	Tryptophan aspartate-containing coat protein
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TD	Thymus-dependent
TEMED	N,N,N',N'-tetramethylenethyldiamine
TI	Thymus-independent
TIR	TLR/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein

TLR	Toll-like receptor
TNF- $\alpha$	Tumor necrosis factor alpha
TNP	2,4,6-Trinitrophenyl
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN- $\beta$
UPEC	Uropathogenic <i>Escherichia coli</i>
WB	Western blot
WD	Tryptophan-aspartate
WT	Wild type

## 8 ACKNOWLEDGEMENTS

First I would like to thank my supervisor Jean Pieters. He gave me the opportunity to work in his laboratory and to perform the studies presented in this thesis. He allowed me to get familiar with multiple and diverse research topics and to develop fruitful collaborations with other laboratories.

My thanks also go to Daniela Finke and Martin Spiess for being part of my thesis committee and for their advices during my PhD studies.

I am especially grateful to Daniela Finke for her constant support, her dedicated interest and her valuable guidance in studying B lymphocytes and immune response of the coronin 1  $-/-$  mice. I also want to thank Sandrine Schmutz and Stephane Chappaz from her lab and other members of the DKBW for their help, the discussions and the good time we had together.

I thank Laleh Majlessi and Claude Leclerc from the Pasteur Institute in Paris with whom we had an effective collaboration about presentation of mycobacterial peptides by MHC molecules.

I am also thankful to Hans-Reimer Rodewald who welcomed me in his lab to do some exciting experiments with the help of Thorsten Feyerabend.

Furthermore I want to express my gratitude to:

Current and former lab mates for the time we spent together.

Jan Massner, for generating the coronin 1  $-/-$  mice and introducing me to animal work. He is also a good “friend of science”.

Imke Albrecht, for helping with dendritic cells studies.

Varadharajan Sundaramurthy, for his assistance when injecting the mice.

Edith Houben, for her scientific approach and the enjoyable atmosphere she brought to the lab.

Nicole Scherr, for her patience in proofreading my thesis and listening to my stories. We started our PhD studies at the same time and shared some good moments.

Susan Treves, for her advice in calcium measurements with neutrophils.

The hematology laboratory at the Kantonsspital Basel, for the study of coronin 1  $-/-$  mice blood composition.

All my friends (especially Angele, Ana and Guillaume), for the great moments spent in Basel or Zürich.

Finally I want to thank my family members for their continuous support. Without them this thesis would not have been possible.