

***The Role of Complement Properdin in Murine Infection with  
Listeria monocytogenes***

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Properdin or complement factor P is a conserved serum glycoprotein of the immune defence. It plays a role in strengthening the activation of complement, a system of proteins essential in the first line defence against infection.

Properdin is the only positive regulator and plays a major role in regulating the alternative pathway of the complement system, an effector system of the innate immune response, by binding and stabilising two specific converting enzyme complexes, which are normally labile (C3bBb and C3bBbC3b).

Mouse models have shown that complement, in particular complement receptor 3 (CR3) and complement 5 (C5), contributes to survival of infection with *Listeria monocytogenes*.

The purpose of the project was to characterise the contribution of properdin in the response to *L. monocytogenes* (EGD-e), a Gram-positive, intracellular pathogen, which can cause severe infectious disease in human and animals, by using *in vitro* and *in vivo* methods.

*In vitro* assays for the first time point to the significant role of properdin in infection with *L. monocytogenes*: using dendritic cells and macrophages derived from the bone marrows of properdin-deficient (KO) and wild type mice (WT), cells from WT mice showed greater intracellular load of viable *L. monocytogenes* at an early time point. Cells from KO mice produced less IFN- $\gamma$  and nitric oxide compared to cells from WT mice and showed less surface expression of CD40. In addition properdin is found to react as a hypoxia sensitive gene; its expression in WT macrophages was also significantly decreased after infection compared to uninfected cells.

*In vivo* experiments demonstrated for the first time that properdin is necessary in the survival of acute murine listeriosis: properdin-deficient mice were more susceptible to intravenous infection with *L. monocytogenes* compared to wild type mice, and had a greater systemic IFN-gamma and splenic IL-17A response and greater disease severity with impaired M1 type activation.

In conclusion, these findings show that properdin is essential in survival of murine listeriosis and in sustaining a cellular response to the intracellular pathogen *L. monocytogenes*.

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*To the spirit of my husband Hassoun (God mercy be upon him)*

*To my lovely kids, two little stars, my two sweethearts,*

*Mohammed and Retaj*

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## List of Abbreviations

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Ab	Antibody
BHI	Bovine serum albumin
bp	Base pairs
BSA	Brain heart infusion
C3	Complement 3
C5aR	C5a receptor
CFU	Colony Forming Unit
CR3	Complement receptor 3
DC	Dendritic cells
DNase I	Deoxyribonuclease I
dNTP	Deoxy-nucleotide tri-phosphate
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting (Flow cytometry)
FcR	Fc receptor
Fc $\gamma$ R	Fc-gamma receptor
FCS	Fetal calf serum
FITC	fluorescein isothiocyanate
PE	phycoerythrin
IFN- $\gamma$	Interferon-gamma
IL-17	Interleukin-17
KDa	Kilodaltons
KO	knock out
LPS	Lipopolysaccharide
LM	<i>Listeria monocytogenes</i>
Mac	Macrophage
MBL	Mannan-binding lectin
MFI	Mean fluorescence intensity
T <sub>M</sub>	Melting temperature
mRNA	messenger ribonucleic acid
$\mu$ l	microlitre

## List of Abbreviations

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µm	micrometer
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
mIL-4	mouse Interleukin 4
MOI	Multiplicity of infection
NE	Norepinephrine /Noradrenaline
OD	Optical density
PAMP	pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PBS	phosphate-buffered saline
rmGM-CSF	recombinant mouse Granulocyte Monocyte Colony-Stimulating Factor
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
SDS	Sodium dodecyl sulphate
TLR	Toll-like receptor
TEM	Transmission Electron Microscopy
Tris	Tris (hydroxymethyl) aminomethane
TBS	Tris buffered saline
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
V	volts
v/v	volume/volume
w/v	weight/volume
WT	Wild type

## *Chapter One- Introduction*

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### 1. Introduction

#### 1.1 The complement system

Bacteria, viruses, fungi and parasites activate the immune system which is a system of biological processes that involves many interdependent components in order to protect the body against damage by microbial infections and to elicit immunological memory.

There are two categories of immune system: innate and adaptive immunity. The innate immune response acts as the first line of host defense against invading microbial pathogens (Hoffmann *et al.*, 1999). It has its role in the early recognition and triggering of an appropriate proinflammatory response. The adaptive immune response is antigen-specific and acts in a target specific manner which involves humoral response by using antibodies to recognise the specific pathogen, and cell mediated response to recognise the infected cells.

The complement system (C) is part of the innate immunity but also contributes to acquired immunity (Menges *et al.*, 2005). This dual importance is described for other recognition systems as well, such as toll-like receptors (TLRs) which not only bind to pathogen-associated molecular patterns (PAMPs) but also control adaptive immune responses for instance the differentiation of T helper cells (Medzhitov, 2001).

Complement was identified more than 100 years ago and is a cascade of carefully regulated enzymatic reactions. Complement proteins are found in blood and body fluids. Specific complement receptors that mediate cellular effects have been characterised including complement receptor 2 (CR2) on B cells (Dempsey *et al.*, 1996) and complement C5a receptors (C5aR) on immune cells such as neutrophils, macrophages, and dendritic cells (Gutzmer *et al.*, 2006; Connelly *et al.*, 2007), and also C3R on monocytes and macrophages (Ross & Vetvicka, 1993). Recent major advances in our

understanding of complement have resulted from the use of genetically engineered mouse lines. These for example have demonstrated the role of complement in cellular integrity and tissue homeostasis (Zipfel & Skerka, 2009). Its discovered roles are becoming more and more varied, but its agreed function is in host defence by recognising altered surfaces and pathogens and removing pathogens by direct killing, opsonisation, activation of phagocytosis, and initiation of local inflammatory reactions. Due to its immunoregulatory functions, the complement system is assigned a pathogenic role during ischemic infections, and autoimmune diseases. The common denominator for these diseases is inflammation, which is initiated in response to infection, cell surface changes, or injury. The function of inflammation is to protect the host against microbes, to repair injuries, and to contribute to the removal of apoptotic cells or immune complexes. However, this procedure can initiate or contribute to several pathologies including the development of cancer, which may be because the activation of complement during an inflammatory reaction contributes to inflammation driven tissue injury. Furthermore, a deficiency in complement components, even those necessary for efficient complement activation, may result in tissue injury, as observed in autoimmune reactions because immune complexes are not solubilised and removed but lead to Fc $\gamma$ R-mediated macrophage activation. In addition, alterations in the expression of complement regulatory proteins, which lead to the excessive complement activation, can also contribute to tissue injury because they no longer protect host cells from self-attack (Markiewski & Lambris, 2007).

Complement activation promotes inflammation by generating anaphylatoxins (C3a, C4a and C5a) and facilitates phagocytosis and lysis by membrane attack complex formation (Zipfel & Skerka, 2009; Walport, 2001).

Complement is activated via three pathways, the classical pathway, the lectin pathway and alternative pathway (Figure 1.1). Each pathway responds to a different set of activators and each pathway leads to the formation of C3 convertases that cleave C3 to C3a and C3b. The C3 convertases are central to the activity of the cascade. The effects of C3 split products are as follows: C3a mediates degranulation of mast cells, bronchial constriction, and vasodilatation. The role of C3b is in opsonisation. C3dg binds to B cells (CR2) lowering the activation threshold to produce antibodies. C3a, C4a, and C5a are all chemoattractants. Recently, the C3a peptide, at relevant concentrations, was shown to exert direct bactericidal activity against *Streptococcus pyogenes* (Nordahl *et al.*, 2004).

### 1.1.1 The classical pathway

The classical pathway (CP) is activated by antigen-antibody complexes and other molecular patterns such as histones and DNA. This was the first complement pathway to be discovered and is initiated for example by the recognition of IgG and IgM bound to antigen, complement fixing isotypes of IgG, and several other proteins, via the C1 complex (formed by C1q, and dimeric C1r and C1s respectively). The C1 complex is subsequently activated and cleaves complement C4 into C4a and C4b. The C4b cleaves bound complement component C2a, to form enzymatically active C4bC2a complex which cleaves complement C3 and is the C3 convertase of classical pathway. C4bC2a cleaves C3 into C3a and C3b. This C3 convertase promotes generation of further C3b molecules which bind to the cell surface and leads to formation of the C5 convertase C4b2aC3b that cleaves C5 into C5a and C5b. C5b initiates the non-enzymatic formation of the membrane attack complex (MAC).

### 1.1.2 The lectin pathway

The lectin pathway (LP) of complement activation is activated by recognition and binding of pathogen-associated molecular patterns with lectin proteins, such as mannose binding lectin (MBL) and ficolins. Each of these is found complexed with three serine proteases MASPs (mannose-binding protein-associated serine protease) called MASP-1, MASP-2, and MASP-3. In addition, the MASP-2 gene encodes a non-enzymatic protein called MAp19. MBL-MASP-2 and ficolin-MASP-2 complexes bind to the pathogen surface which activates MASP-2. Activated MASP-2 cleaves complement C4 and C2. C4 complement cleaves into C4a and C4b, C4b bind to the cell surface. C2 complement cleaves into C2a and C2b. As with the classical pathway, the formation of the C3 and C5 convertases is via C4b and C2a binding, with the final stages of complement again concluding with the establishment of the MAC (Thiel *et al.*, 1997). Another component protein was recently discovered called MAp1 (also known as MAp44) (Degn *et al.*, 2009) was recently discovered and shown to inhibit the activation of the lectin complement pathway (Pavlov *et al.*, 2012). MASP-1 is involved in activation of both the lectin and the alternative pathways, because mice deficient in MASP-1 and MASP-3 show a lack of alternative pathway activation (Iwaki *et al.*, 2011).

### 1.1.3 The alternative pathway

The alternative pathway of complement (AP) forms an amplification loop of complement activation (Figure 1.1). It is initiated by spontaneous hydrolysis of C3 with the Factor B to form C3(H<sub>2</sub>O)B complex caused to constant low-level auto-activation in the fluid phase (so-called tickover). Factor B itself can in turn be cleaved by activated

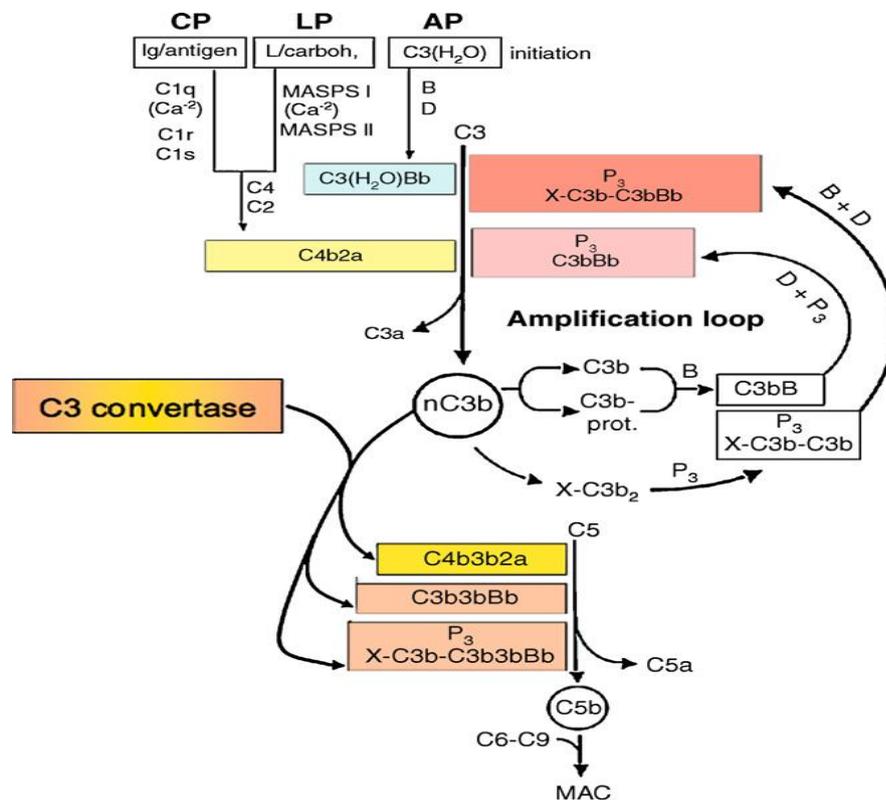
Factor D into Ba and Bb. Its activity is enhanced by the binding of properdin to the C3bBb complex, thereby stabilising the C3 convertase.

During complement activation, C3b binds to microbial or altered self target cells and can then initiate assembly of the C3 convertase. In addition, C3b can bind directly to C3 convertase to form the C5 convertase, which in turn cleaves to C5a and C5b. The C5a fragment acts as a chemo-attractant for leukocytes. C5a acts by their receptor C5aR and in addition to this receptor there is another receptor for C5a is C5L2 receptor. C5L2 receptor (previously known as GPR77) also acts as a receptor for C5a and is activated after binding to the C5a. C5aR is a classical G protein-coupled receptor, but C5L2 is structurally homologous and does not link to G protein coupling (Li *et al.*, 2012).

The convertases of complement are assembled on target surfaces, which activate their proteolytic potential, marking the target for phagocytosis or lysis. Complement dependent recognition of apoptotic cells can take place via the classical, lectin, and alternative pathways (Mevorach *et al.*, 1998; Ogden *et al.*, 2001).

The alternative pathway has been shown to activate without any antibody participation. However, in a recent study using *Candida albicans*, a yeast-like fungal organism, an opportunistic pathogen proved to be an exception to this rule. A role of antibody-dependent to activate the alternative pathway has been identified involving monoclonal human recombinant anti-mannan Fab fragment (M1) and its full-length IgG1 antibody M1g1. It was also established that M1g1 activates the complement system in mice and increases their resistance of mice to systemic candidiasis (Zhang *et al.*, 2006). As expected, Fc-free anti-mannan Fab fragment M1 is not capable to activating the classical pathway. M1 enhances the formation of the alternative pathway C3 convertase (C3bBb) to activate the alternative pathway on the cell surface (Boxx *et al.*, 2009).

The alternative pathway functions as an amplifier of the classical and the lectin pathways. It can be activated by the two other complement pathways because it is initiated by a hydrolysed form of C3 and C3b molecules formed after activation of both classical and lectin pathways. It will bind to the surface of a pathogen with the presence of both factors B and D, generating alternative C3 convertases (Brouwer *et al.*, 2006). Properdin contributes to the activation of the alternative pathway by stabilizing C3 and C5 convertases, and the complement activation amplifying ongoing by any of the three pathways.



**Figure 1.1:** Diagrammatic presentation of the activation cascade of complement with emphasis on the amplification loop. Initiations of the three pathways sequential enzymatic and non-enzymatic reactions are shown, the amplification loop start from nascent C3b (nC3b). (Properdin= $P_3$  (trimeric), IgG- or F(ab')<sub>2</sub>-containing immune complexes =X). (The diagram taken from Lutz *et al.*, 2007 with permission).

### 1.1.4 Properdin

Properdin or complement factor P is a soluble, plasma glycoprotein found in blood, is secreted by leukocytes and activated endothelial cells, and has a designated role in the alternative complement pathway of the innate immune system. Properdin was first discovered as an important component of an antibody-independent complement activation pathway in 1954 by Dr Louis Pillemer and collaborators (PILLEMER *et al.*, 1954), and then in the 1970s it was shown to be a stabilising component of the alternative pathway C3 convertase. The name properdin is derived from the Latin word *perdere*, meaning to destroy (Kemper *et al.*, 2008). Properdin is necessary also in the amplification of the classical and mannose-binding lectin pathways (Kimura *et al.*, 2010).

Contrary to its hypothesised role as a pattern-recognition molecule in its own right, a recent study showed that C3b was essential for properdin to bind to zymosan, a component of yeast cells, or *Escherichia coli* (Harboe *et al.*, 2012).

#### 1.1.4.1 Structure of properdin

Properdin exists as a basic glycoprotein in plasma at a concentration of about 15-25µg/ml, formed rod-like by head-to-tail associations of identical monomers. It is released from neutrophil granules after stimulation and consists of a mixture of oligomers of a 53-kDa monomer, which are mostly as dimer, trimer, or tetramer in a constant ratio of 1:2:1. For this reason, properdin is shown indicated as P3 in Figure 1.1. Each properdin monomer is composed of an N-terminal domain and seven thrombospondin repeats (TSRs) of type one domain, each about 60 amino acids long, representing independently folded modules. The TSRs are numbered in order from the

N-terminus from TSR-0 until TSR-6. TSR-4 and TSR-5 are involved in stabilisation of the C3bBb complex (Higgins *et al.*, 1995) and were chosen as the target in the generation of the properdin-deficient mouse line (Stover *et al.*, 2008) used in this thesis. Myeloid cells were shown to be the main source of properdin in the blood which was released from neutrophils after activation (Kimura *et al.*, 2010).

### **1.1.4.2 Properdin's properties and functions**

#### **1.1.4.2.1 Amplification role**

Properdin is the only positive regulator of the alternative pathway of the complement system and acts by binding and stabilising the inherently labile C3 convertases C3bBb and C3bBbC3b. It is able to bind to a surface ligand of C3b alone or to iC3b, C3bB or C3bBb complex by mean of one of its subunits. Because of its oligomeric structure, properdin which is bound to C3bB and C3bBb enables more C3b to bind. The C3bB is a short-lived complex ( $t_{1/2}$  about 90 seconds), which is cleaved by Factor D in the presence of  $Mg^{2+}$  at a single site in the Factor B subunit, resulting in the release of the Factor B amino-terminal fragment (Ba) and the activation of the serine protease domain. Properdin substantially extends their half-lives and protects the alternative pathway convertases (C3bBb and C3bBb5b) from rapid inactivation (Fearon & Austen, 1975). Properdin attains its the full stabilising effect on C3bBb when it binds to more than one C3b or more ligands at a time on the surface (Hourcade, 2006). It also stabilises the C5 convertases by binding to C3b in C3bnBb and C3b2b4b (Figure 1.1). This interaction inhibits the deactivation of the C3 convertase by Factors I and H. By stabilising the C3 convertase, properdin amplifies deposition of more C3b on the activating surfaces of

pathogens and generates more of anaphylatoxins C3a and C5a, thus the alternative pathway is called an amplification loop of complement activation.

The alternative pathway amplifies effector functions of the complement system (Lutz *et al.*, 2007). It has been shown that blocking the alternative pathway via neutralising factor D using human serum (diluted 1:2), was shown to inhibit more than 80% of C5a and terminal complement complex (TCC) formation. This has been demonstrated using anti-factor D mAb 166–32 and block the classical pathway by anti-C2 MAb 175–62. Factor D is the rate-limiting serine protease in an amplification loop of the alternative pathway. C5a and the TCC formation were induced by IgM in solid phase and IgG solid- and human aggregated IgG in fluid-phase (Harboe *et al.*, 2004).

### **1.1.4.2.2 The role of complement in clearance of apoptotic cells**

The complement system plays a role in the recognition and removal of apoptotic cells (Fadok *et al.*, 1998). Apoptosis, or programmed cell death, plays an essential role in morphogenesis, regulation of cellular immunity, and cellular homeostasis, and in the removal of cells which are virus-infected cells and injured, dying cells. Furthermore, apoptosis plays an important role in shaping and controlling the immune response. C1q is able to bind to apoptotic cells either directly, or by interaction with IgG or IgM (Korb & Ahearn, 1997), MBL binds to late-apoptotic and necrotic cells (Nauta *et al.*, 2003). The study by Kemper *et al.* 2008 shows that properdin binds to glycosaminoglycans on early apoptotic T cells. This is thought to enhance complement activation and phagocytosis via macrophages and dendritic cells using CR3, an integrin composed of CD11b and CD18.

Moreover, a recent study by (Xu *et al.*, 2008) demonstrates that properdin is able to bind to late apoptotic and necrotic cells prior to C3 deposition on the cell surface but not to bind to early apoptotic cells. This suggests that early apoptotic cells are cleared by anti-inflammatory macrophages.

In addition, properdin binds to late apoptotic and necrotic cells by ligands for example DNA, which is one of the main autoantigens exposed on apoptotic cell surfaces. During apoptosis, properdin can bind to small fragments of DNA, although it does not bind to the complete nucleosomal units of DNA. This occurs in the absence of C3 or C3b. Binding of properdin to dying cells does not compete with C1q and MBL because both of these C1q and MBL interact with structures of DNA in a different way from those that properdin recognises (Xu *et al.*, 2008).

Properdin binds to the eukaryotic cell surface via glycosaminoglycans (GAGs) and to the altered surfaces of apoptotic cells but the physiological relevance is uncertain (Kemper *et al.*, 2010).

It has been reported in numerous of studies that septic shock is associated with cellular immune defects, including T-cell apoptosis (Ayala *et al.*, 1996 ; Hotchkiss *et al.*, 2000). Septic shock is the cause of the expansion of a localised infection into the blood stream resulting in a systemic inflammatory reactions. In some cases this may affect the cardiovascular and heamostatic systems causing multi-organ dysfunction, injury, and death (Angus *et al.*, 2001). Properdin-deficient mice are more susceptible to worse outcome from polymicrobial septic peritonitis (Stover *et al.*, 2008).

Properdin that is released from activated, degranulated neutrophils may respond differently when compared to neutrophils in blood that release properdin in serum.

Properdin in serum does not bind to apoptotic T cells compared to properdin expelled by degranulating neutrophils (Kemper *et al.*, 2008).

It has been shown that the role of properdin direct binding to certain microbial surfaces possibly as an innate pattern-recognition molecule to initiate the AP (Hourcade, 2006; Kemper & Hourcade, (2008).

This binding initially promotes C3b-deposition then formation of the C3bBbP-complex. It thus provides a platform for new convertase assembly and function of the alternative pathway C3 convertase. Convertase assembly begins with properdin binding to a target surface. This directs complement activation of the alternative pathway because properdin, via its multiple protein subunits is able to potentiate both, the binding to ligands clustered on a microbial surface and the binding of C3b molecules. Properdin is bound by one ligand-binding site and provides additional sites for C3b binding via the remaining polypeptide chains (between 1 and 3) and C3bBb assembly.

Some studies have suggested that properdin does not bind directly to deposited C3, but to glucan particles and zymosan as shown by flow cytometry using an anti-properdin monoclonal antibody (Agarwal *et al.*, 2011)

Until now it is shown that alternative pathway could be activated by wherever properdin is bound. However, a recent study showed that properdin binds to *Neisseria*, *Escherichia coli*, lipopolysaccharide (LPS) mutants (component endotoxin of Gram-negative bacteria, and zymosan) (Spitzer *et al.*, 2007).

Properdin binds differently to different types of LPS and lipooligosaccharide (LOS) modifications. While it was observed to bind strongly to *Salmonella typhosa* LPS, its binding to *S. minnesota* and *E. coli* LPS was weak (Kimura *et al.*, 2008). Properdin's ability to recognise and bind to certain bacterial surfaces enhances activation of the

alternative pathway, whereas other bacterial surfaces that do not bind properdin activate the alternative pathway more slowly (Spitzer *et al.*, 2007). This may indeed be the basis of the observation that a deficiency of properdin coincides with fulminant *N. meningitidis* sepsis. Complement activation with an intact alternative pathway is necessary for complement activation in response to *Neisseria* (Kimura *et al.*, 2008). However, research shows that not all microbial activators that activate the alternative pathway need properdin, because zymosan was able to activate the alternative pathway of a mouse in the absence of properdin (Kimura *et al.*, 2008).

Host cells are able to protect themselves against complement attack by using multiple complement inhibitors thus properdin is required as a positive regulator to balance and overcome these inhibitory mechanisms (Kimura *et al.*, 2010). It also has been shown that systemic properdin derived from myeloid lineage cells proved to be responsible for arthritis pathogenesis. Moreover, properdin plays a role in the activation of the alternative complement pathway by fungal glycans (Agarwal *et al.*, 2011).

### **1.1.4.3 Properdin deficiency**

Human deficiency of properdin was first reported in a Swedish family in 1982 and is associated with a high susceptibility to meningococcal meningitis with high mortality (Sjoholm *et al.*, 1982). This emphasises the important role of the alternative pathway in controlling the growth of meningococci (Kemper *et al.*, 2010). Properdin deficiency is the most common genetic defect of the alternative pathway components, and is inherited in an X-linked manner, with all index cases for properdin deficiency being male. There are three types of properdin deficiency phenotypes type I, type II and type III.

Type I complete deficiency, is the most common type with no properdin in serum (less than 0.01 mg/l) in spite of a normal mRNA level, Type II is an incomplete deficiency with less than 10% of the normal level of properdin in serum. Type III is characterised by a normal serum concentration but a dysfunction in properdin protein and to date has been detected in one family only. All three types are associated with an increased susceptibility to meningococcal disease.

Properdin deficiency results in an increased risk of infection with *N. meningitidis*. Individuals with properdin deficiency have several infections in the early years of life. However, not all of individuals with properdin deficiency get meningococcal infections, and it has been suggested that may be because there is a high concentration of subclasses IgG2 as contributory susceptibility factor. Moreover, a lack of the subclasses IgG2 has previously been associated with reduced immune responses to T-independent antigens like the meningococcal antigen (Fijen *et al.*, 1993).

This disease often complicated by sepsis, frequently caused by uncommon serogroups W-135 and Y and it occurs especially in young age. The alternative pathway has been shown to play a very important role in anti-capsular antibody-dependent immunity to *N. meningitidis in vitro* and this may explain the association of properdin deficiency with meningococcal disease (Sjoholm *et al.*, 2006) together with the fact that recurrent infection are rare. In addition, properdin deficiency is connected with recurrent otitis media and pneumonia (Schejbel *et al.*, 2009).

### **1.1.4.4 Properdin-deficient mouse line**

Properdin deficient mice have been generated by gene specific targeting and are on pure C57BL/6 background. This line (intellectual property of University of Leicester) was

used to assess the role of properdin in complement activation after infection with *Listeria monocytogenes* *in vitro* and *in vivo*. Evidence for the absence of functional properdin in the properdin-deficient mice was provided by testing sera of targeted mice in comparison with littermate controls using rabbit red blood cell lysis. This is a test classically used to quantify alternative pathway activity.

The generation of properdin-deficient mice provided additional knowledge in order to understand the properdin function. Human and mouse properdin sequences show a high degree of identity (about 76%) (Goundis & Reid, 1988; Maves *et al.*, 1995).

Another properdin-deficient mouse line was created, as mented in Kimura *et al* 2008, using mixed background 129/C57BL/6 to study the role of properdin in complement activation.

Properdin-deficient mice are impaired in their survival after sublethal of cecal ligation and puncture (CLP). A properdin-deficient mouse line in a model of CLP for acute polymicrobial septic peritonitis found that over an observation period of 14 days, therefore, properdin-deficient mice were significantly impaired in their survival compared with wild-type littermates (Stover *et al.*, 2008). Moreover, properdin-deficient mice showed significantly higher mortality in nonseptic shock models of LPS injection (Ivanovska *et al.*, 2008). It has been shown that C5-deficient mice show increased survival in zymosan-induced shock (Miller *et al.*, 1996). In properdin-deficient mice also were more resistant to zymosan-induced shock than wild type mice (Ivanovska *et al.*, 2008).

### **1.1.5 Complement and Fc $\gamma$ receptors**

Immunoglobulin Fc receptors (FcR) are a key player in the regulation of innate and adaptive immune responses since they provide a link between antibody-antigen

complexes and cellular effector machinery leading to phagocytosis, endocytosis of IgG-opsonised particles and release of inflammatory mediators.

There are a distinct receptors for the Fc portion of the IgG molecule, so-called Fc $\gamma$ Rs, which are expressed by immune cells and can transmit activating or inhibitory signals to the cells. Structurally, four different classes of Fc $\gamma$ Rs have been recognised in mice (varying from human), known as Fc $\gamma$ RI (CD64), Fc $\gamma$ RIIb (CD32), Fc $\gamma$ RIII (CD16) and Fc $\gamma$ RIV (CD16-2). Their function is determined by the presence of so-called ITAM or ITIM motifs, as follows: Fc $\gamma$ RI, Fc $\gamma$ RIII and Fc $\gamma$ RIV share a common  $\gamma$ -chain containing an intracellular ITAM (immunoreceptor tyrosine-based activation motif) sequence necessary to mediate activation, especially of phagocytosis but also release of inflammatory mediators, unlike Fc $\gamma$ RII which contains a cytoplasmic ITIM sequence (immunoreceptor tyrosine-based inhibition motif). Both Fc $\gamma$ RII and Fc $\gamma$ RIII are low-affinity receptors for IgG and are expressed on haemopoietic cells. Mature B cells only express Fc $\gamma$ RII (CD32), which functions as an inhibitor of B-cell activation.

Complement receptor and C3b are effective in immune adherence and Fc $\gamma$ R is effective in mediating internalisation. In mice deficient of different early components of the complement pathways like C3, the antibody activity remained largely unchanged. On the contrary, the late components of the complement pathway for example C5a were shown to act as a pro-inflammatory cytokine to up-regulate activating Fc $\gamma$ Rs. Moreover, the generation of C5a was dependent on activating Fc $\gamma$ Rs and independent of known pathways of complement activation (Gerard & Gerard, 1994).

Dendritic cell and macrophage functions are co-determined by the balance of expression between activating and inhibiting Fc $\gamma$  receptors. Three of these are of particular interest in this work, namely Fc $\gamma$ RIIb, Fc $\gamma$ RIII, Fc $\gamma$ RIV. As this thesis will demonstrate,

properdin-deficient mice have a lower mRNA expression of Fc $\gamma$ RIIb in their uninfected spleen compared to wild type mice. The Fc $\gamma$ RIIb appears to have the role of controlling cytokine release, antibody production, and phagocytosis by balancing the inflammatory response to infection. Higher phagocytic ability is seen in cells with low level of Fc $\gamma$ RII expression, leading to higher pro-inflammatory cytokines with susceptibility to septic shock. However, higher levels of Fc $\gamma$ RIIb coincide with less phagocytosis leading to lower pro-inflammatory cytokines and resistance to septic shock (Clatworthy & Smith, 2004).

Murine models of Fc $\gamma$ R deficiency have been used in different studies to investigate the important role of Fc $\gamma$ RIIB in combating intracellular pathogens. The immune response of Fc $\gamma$ RIIb-deficient mice infected with *Mycobacterium tuberculosis* (MTB) in comparison with infected wild type showed reduced bacterial burdens and decreased pathology (Maglione *et al.*, 2008). This is important because *M. tuberculosis* causes primarily cell-mediated, not antibody-mediated responses.

Complement and Fc $\gamma$ R reportedly interact in shaping the cellular immune response (Atkinson, 2006). CR3 initiates a phagocytic response when iC3b opsonised particles are bound, while the ratio of expression of Fc $\gamma$ RIIb and Fc $\gamma$ RIII determines uptake of immunoglobulin opsonised antigens. In the fluid phase, complement assures removal of immunoglobulin-antigen complexes.

*In vitro*, using blocking antibodies, it was shown that these two phagocytic receptors interact functionally in a FcR driven manner (Huang *et al.*, 2011). The activation of ITIM or ITAM motifs directs activity of the CR3 integrin pathway.

Complement and immunoglobulins are important opsonins in the blood. The cross talk between complement and immunoglobulins has been reported in Kumar *et al.* (2006)

which C3b and IgG and their receptors cooperating in promoting phagocytosis. It was demonstrated in a study using a mouse model of the acute pulmonary IC hypersensitivity reaction that initial C5a production and C5aR activation in IC inflammation in the lung showed an increase in the number and enhanced function of FcγRIII and inverse regulation which decreased the inhibitory receptor FcγRII. C5aR is expressed by neutrophils, macrophage and monocytes (Gerard & Gerard, 1994). Lack of properdin causes changes in the function of neutrophils. It has been reported that neutrophils from blood properdin-deficient collagen antibody-induced arthritis (CAIA) mice showed a significantly decreased expression of FcγRs but higher C5aR expression compared to wild type (CAIA) mice (Dimitrova *et al.*, 2012).

### 1.1.6 *Listeria monocytogenes*

*Listeria monocytogenes* (LM) is a Gram-positive bacterium which can cause a serious disease called Listeriosis. It is rapidly growing, ubiquitous in the environment, a facultative intracellular pathogen and can principally infect any immune and non-immune cell type in tissue and in the blood stream. It is a non-spore forming, motile, and rod-shaped bacterium measuring approximately 0.4-0.5 μm in diameter and 1-2 μm in length. *L. monocytogenes* may form chains of multiple cells of similar size (Farber & Peterkin, 1991; Romick *et al.*, 1996; Rowan *et al.*, 2000).

*Listeria monocytogenes* was first discovered as the microorganism causing a septicemic disease in rabbits and guinea pigs in 1926 by E. G. D Murray, Webb and Swan and was named *Bacterium monocytogenes* (Murray *et al.*, 1926). In 1929 the first human case was reported (Nyfelt, 1929). In 1940 it was renamed *L. monocytogenes* by Harvey (Harvey, 1940).

There are different environmental sources of growth of *L. monocytogenes* but the major source of infection in humans is contaminated ready-made food. *L. monocytogenes* is one of the most important causes of death from food-borne pathogens especially in those with altered immune surveillance (pregnant women, newborns, the elderly and immunocompromised individuals) (Ramaswamy *et al.*, 2007). After gastrointestinal transfer *L. monocytogenes* is taken up, resides intracellularly, and replicates in vacuoles then escapes into the cytoplasm, spreading between cells to cause the most severe pathologies, such as meningitis, meningoencephalitis and septicemia. *L. monocytogenes* has the ability to multiply in the cytoplasm of varieties of human and animal cell types which reside primarily in the intracytoplasmic compartment of host cells, including the primary APCs, such as macrophages and dendritic cells. However, not all the mechanism of survival and persistence of *L. monocytogenes* in the environment are known. The bacteria grow and reproduce at temperatures between 1°C and 45°C under aerobic and facultative anaerobic condition with the optimum growth temperature being 30°C-37°C with pH 4.1–9.6. There are 13 serotypes (serovars) of *L. monocytogenes* based on the somatic (O) and flagella (H) antigen which are potentially capable of infecting humans (Rowan & Anderson, 1998; Jemmi & Stephan, 2006; Nadon *et al.*, 2001). A fatality rate of up to 30% has been reported with listeriosis, and because *L. monocytogenes* is widespread in the environment, it has physiological resistance, and has the ability to adapt to external stresses and different temperatures (Lungu *et al.*, 2009).

Adhesion of bacteria to the cell surface is one of several distinct steps by which the host cells are infected followed by internalisation through active phagocytosis of the host cells.

There are two *L. monocytogenes* surface proteins, internalins A (InlA) and internalins B (InlB), which are used by *L. monocytogenes* to enter into non-phagocytic cells (Braun *et al.*, 1998; Cossart & Toledo-Arana, 2008) such as epithelial cells. Listeriolysin O (LLO) is required to escape from the phagosome, and phospholipases and actin assembly-inducing protein (ActA) are required for cell-cell mobility and invasion. All of these proteins are coordinated by positive regulatory factor A (PrfA) which is a transcriptional activator protein of *L. monocytogenes*. Functionally this protein works as an important regulatory factor which is required for different expressions of virulence gene product of bacteria in order to survive and grow within infected host cells. Initial levels of PrfA direct bacterial escape from host cell vacuoles whilst the higher levels promote spread of intracellular bacteria to adjacent host cells. Therefore, the PrfA product may play an important role in controlling multiple virulence properties of *L. monocytogenes*.

Phagocytic cells play an important role in the defense against *L. monocytogenes* infection (Pamer, 2004). Infection with *L. monocytogenes* leads to activation of phosphoinositide (PI) 3-kinase and enhances the uptake of *L. monocytogenes* but is unclear. PI 3-kinase controls different processes in host cells including survival, cell growth and motility. During Fc $\gamma$  receptor-mediated phagocytosis in macrophages PI 3-kinase is required for pseudopod extension. It has been demonstrated that PI 3-kinase plays a role in Fc $\gamma$ R-mediated phagocytosis (Greenberg, 2001). *L. monocytogenes* enter into host cells in a non-phagocytic way, the major pathway being by interaction of the bacterial surface protein InlB with the host receptor, and one of the signalling proteins for internalisation of *L. monocytogenes* is type IA phosphoinositide (PI) 3-kinase.

*Listeria monocytogenes* can enter host cells after infection by phagocytosis and initially reside inside a vacuole, which has become trapped within a single-membrane vacuole which secretes its main virulence factor LLO. LLO is the key for *L. monocytogenes* virulence and shortly after infection LLO acts on the *L. monocytogenes* vacuole within 5 minutes (Beauregard *et al.*, 1997), and after 30 to 45 minutes of internalisation, *L. monocytogenes* escape from these vacuoles into the macrophage cytosol (Myers *et al.*, 2003). LLO is a protein toxin which is required for the intracellular life cycle of *L. monocytogenes* to escape from the vacuole in most cells, in addition to the formation of vacuoles, for cell-to-cell spread, and replication of bacteria in the cytoplasm of host cells. LLO is a protein toxin which is required for *L. monocytogenes* to escape from vacuoles, for cell-to-cell spread, and replication of bacteria in the cytoplasm of host cells. LLO determines the extent of the host cellular response. Before the vacuole membrane lyses, the pH acidifies in the vacuole to an average pH of 5.9, the optimum pH for activation of listeriolysin O. Activation of LLO lyses the vacuolar membrane of the host cells allowing the bacteria to escape into cytosol where it replicates.

*Listeria monocytogenes* can be recognised by a number of pattern recognition receptors (PRRs) on cell surfaces and in the cytosol. Two main types of PRRs have been described, the membrane-bound toll-like receptors (TLRs) and soluble, cytosolic nuclear oligodimerization domain (NOD)-like receptors (NLRs). TLRs are transmembrane signalling proteins that detect pathogen associated molecular patterns (PAMPs) in the extracellular or vacuolar spaces and promote transcriptional responses. TLRs are expressed on many different types of cells including macrophages and dendritic cells and can be activated by lipoteichoic acid (LTA) (a surface-associated adhesion amphiphile) from Gram-positive bacteria.

The NOD-like receptors are a group of intracellular receptors that respond to PAMPs in the cell cytosol and identify bacterial DNA and toxins. *L. monocytogenes* is detected on the surface of the cells or in phagosomes by TLR2, then in the cytosol of the cell by nuclear oligodimerisation domain NOD-like receptors (NOD1, NOD2) and NALP3 following LLO degradation of the phagosome. Moreover, caspase-1 activation is necessary for the clearance of *L. monocytogenes* in murine infection by processing and release of biologically active and pro-inflammatory cytokine (Tsuji *et al.*, 2004). Activated caspase-1 was observed inside the cytosol in macrophage. TLR2-deficient mice are shown to be more susceptible to systemic infection by *L. monocytogenes* compared to wild type mice (Torres *et al.*, 2004). Recognition of the bacteria by PRRs on antigen-presenting cells leads to an inflammatory response, which is followed by phagocytosis (Areschoug & Gordon, 2008). Moreover, recent studies showed that after intraperitoneal (i.p.) and intravenous (i.v.) infection with *L. monocytogenes* in the early period of infection, macrophages from TLR2-deficient mice show greater numbers of phagocytosed *L. monocytogenes* cells compared to macrophage from wild type mice (Shen *et al.*, 2010).

*In vivo* in response to *L. monocytogenes* infection TLR2-deficient mice showed a partial deficit in their capacity to produce IFN- $\gamma$  and TNF- $\alpha$  (Seki *et al.*, 2002). IFN- $\gamma$  production was reduced in TLR2-deficient mice during early infection of *L. monocytogenes* ( $1-3 \times 10^5$  CFU/mouse) (Torres *et al.*, 2004).

*L. monocytogenes* is able to survive and replicate in a large variety of cells. It can resist early intravascular killing and spread intracellularly to evade the immune system. It is able to avoid detection by PRRs by modifying peptidoglycan (PG) in its cell wall (Bishop *et al.*, 2007). As another strategy, *L. monocytogenes* secretes LLO in the host

cells in the vacuole during infection. Moreover, *L. monocytogenes* may evade autophagy in macrophages. Autophagy is an essential part of innate immunity against intracellular pathogens and stimulation of autophagy by peptidoglycan-recognition protein (PGRP-LE) inhibited intracellular growth of the pathogen and improved resistance to infection. *L. monocytogenes* escapes from the autophagic vacuoles and replicates in the cytosol. Another strategy for avoiding the immune response is to induce apoptosis, infection with *L. monocytogenes* and release of LLO in early innate immunity cause lymphocyte apoptosis that leads to bacterial growth. Naturally, apoptosis of immune cells occurs to limit inflammation. *L. monocytogenes* is thought to use the strategy of macrophage phagocytosis to cross the blood brain barrier and infect the meninges (Southwick & Purich, 1996).

In the bloodstream, *L. monocytogenes* may associate with CD41<sup>+</sup> platelets, and translocate to splenic CD8 $\alpha$ <sup>+</sup> DCs (Verschoor *et al.*, 2011). Splenocyte populations were analysed by flow cytometry including CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> CD11c<sup>+</sup> DCs, and using an intracellular CD41 marker for platelets (Verschoor *et al.*, 2011).

Transfer of cells from mice that had treatment with a neutralising monoclonal antibody against virulence factor LLO, but not serum, provided defence against *L. monocytogenes* infection. Infection with *L. monocytogenes* does not generate high antibody titers, but it has been reported that a monoclonal antibody against LLO provides a defence intracellularly to neutralise the virulence factor of *L. monocytogenes*, and block bacterial escape from the phagosome (Edelson & Unanue, 2001).

### 1.1.6.1 Evidence of the role of complement in listeriosis

In normal mouse serum, *L. monocytogenes* activates the alternative pathway of complement and becomes coated on the surface with C3, which leads to uptake by phagocytes such as macrophages, but to activate the macrophage, the presence of complement receptor CR3 is required. This was shown by blocking the CR3-mediated phagocytosis of normal mouse serum that opsonise *L. monocytogenes* and anti-CR3 monoclonal antibody to inhibit the phagocytosis of *L. monocytogenes* by mouse inflammatory macrophages (Drevets & Campbell, 1991; Drevets *et al.*, 1993). It was reported that human complement alternative pathway is activated by *L. monocytogenes* (Croize *et al.*, 1993).

The key complement activation products in listeriosis are C3b for opsonophagocytosis of *L. monocytogenes* by CR3, and C5a for attraction of leukocytes to tissue resident *L. monocytogenes*. These activation products are the result of the activation of the alternative pathway stabilised by properdin. Early studies using complement C5-deficient mice showed that the viable count of *L. monocytogenes* as determined from their spleens was greater from 24 to 27 hours than was the case for C5 sufficient mice. This difference was abolished when C5-deficient mice were subjected to bone marrow transfer from C5 sufficient mice (Petit, 1980). Another group showed there was less macrophage accumulation in the peritoneum of *L. monocytogenes* infected C5-deficient mice as compared to C5 sufficient mice and there was impairment of chemotaxis, bactericidal activity and IL-1 production by C5-deficient macrophages (Gervais *et al.*, 1989).

Innate immune responses have an important role in early control of *L. monocytogenes* infection. After infection of mice with *L. monocytogenes* using the intravenous route,

these are removed from the bloodstream in the liver and spleen (Conlan, 1996; Gregory *et al.*, 1992). In the spleen bacteria are uptake by macrophage in the marginal zone between the T cell-loaded white pulp and the B cell-loaded red pulp and *L. monocytogenes* quickly increased in the spleen but this increase took 48 hours in the case of the liver. However, after this initial phase, listerial numbers in the liver increased significantly. In the liver granuloma are formed in response to the presence of *L. monocytogenes* containing leukocytic cells which are attracted from the blood.

In mice, *L. monocytogenes* quickly associates with platelets in the bloodstream in a manner dependent on Glycoprotein Ib (GPIb) and complement C3. Mice deficient of C3 were more susceptible to *L. monocytogenes* infection (Verschoor *et al.*, 2011). C3-deficient and wild type mice (C57bl/6) were infected with *L. monocytogenes* via the intravenous route ( $1 \times 10^4$  CFU). After one day of infection there were significantly less bacteria in the spleen of C3-deficient mice compared to their wild type counterparts. However, after three days of infection the bacteria were increased in wild type but not in C3-deficient mice, with the same dose after seven days of infection bacteria were undetectable in both genotypes (Verschoor *et al.*, 2011).

A recent study showed that after intravenous injection with *L. monocytogenes* into C3-deficient mice, macrophage-depleted mice and wild type mice, all mice showed clearance of *L. monocytogenes* in 10-30 minutes. Circulation was significantly accelerated in C3-deficient mice compared to wild type and macrophage-depleted mice. This gives the complement a role in early bacterial clearance by phagocytes (Verschoor *et al.*, 2011). Complement exerts an influence in clearance of bacteria from the bloodstream and their localisation to the spleen while C3 complement allows splenic colonisation with *L. monocytogenes* during the early systemic infection.

C3-deficient and C5a receptor-deficient mice were used to investigate the requirement for C3 and C5aR in T cell responses to *L. monocytogenes*. C3-deficient mice had reduced antigen-specific CD8 and CD4 T cells. Therefore, during a primary *L. monocytogenes* infection the clearance of bacteria by CD8 T cells and activation of CD8 and CD4 T cells does not require C5aR but requires C3 (Nakayama *et al.*, 2009).

### **1.1.6.2 Inflammatory response to *L. monocytogenes***

Secretion of cytokines, including interferon-gamma (IFN- $\gamma$ ), interleukins (IL) and tumour-necrosis factor (TNF), is necessary in the primary defence against infection with *L. monocytogenes*, because the cytokines coordinate cell function within the immune response. This is underlined by the fact that mice deficient in these cytokines are susceptible to *L. monocytogenes* infection (Dai *et al.*, 1997).

Interferon- $\gamma$  has an important role in both innate and acquired immunity. It is released from cells after infection, and is particularly important in the immunological defence against different pathogens, particularly intracellular bacteria *L. monocytogenes*, in addition to viruses and fungi. At first, the only important source of IFN- $\gamma$  was thought to be the activated natural killer (NK) cells but it was demonstrated that macrophages and dendritic cells could also be stimulated to produce IFN- $\gamma$  *in vitro* under different conditions (Thale & Kiderlen, 2005).

Function of IFN- $\gamma$  is by direct effect on pathogen growth or by indirect mechanisms activating host cells to produce effective immune responses against intracellular bacteria (Decker *et al.*, 2002; Taylor *et al.*, 2004).

IFN- $\gamma$  production occurs early within the first 24 hours post infection with *L. monocytogenes*. However, the production of IFN- $\gamma$  fails to clear or limit growth of *L.*

*monocytogenes* which continue to grow. This may be because the early IFN- $\gamma$  production is not enough to activate the macrophage bactericidal activity (Humann *et al.*, 2007; Kang *et al.*, 2008; Rayamajhi *et al.*, 2010). IFN- $\gamma$  production is more important during *L. monocytogenes* infection for defensive immunity than against re-infection according to study by Zenewicz & Shen (2007). Immunisation of IFN- $\gamma$ -deficient mice with *L. monocytogenes* and subsequent challenge with virulent *L. monocytogenes* lead to these mice being protected against the challenge. The number of *L. monocytogenes* does not increase after macrophage exposure to IFN- $\gamma$  for 20 hours although macrophage plays a role in activation and killing of *L. monocytogenes* (Higginbotham *et al.*, 1992).

IL-4 and IL-10 inhibit antilisterial resistance (Dai *et al.*, 1997; Haak-Frendscho *et al.*, 1992). Th1 cells are needed in the immune response towards *L. monocytogenes* to produce cytokines which are able to activate macrophages and cytotoxic T cells. It has been reported that disrupted IFN- $\gamma$  or their receptor genes in mice with high levels of IL-4 production are more susceptible to Mycobacterial infections (Cooper *et al.*, 1993). However, mice resistant to *mycobacterium bovis* produce high levels of IFN- $\gamma$  with low IL-4 production (Erb *et al.*, 1998). It has been reported that the level of complement activity affects IL-17 production by T cells and macrophages (Graier *et al.*, 2012).

IL-17A is necessary for host defense against extracellular and intracellular pathogens. IL-17A is released from the cells of the innate immune system such as neutrophils, natural killer T (NKT) cells and  $\gamma\delta$ T cells (gamma delta T cells) and highly specialised epithelial cells during acute inflammatory response despite the fact that IL-17A is known to be released from Th-17. IL-17 increases in the host in response to infection with *L. monocytogenes* (Hamada *et al.*, 2008) and helps in the polarisation of M1

macrophages (Zhang *et al.*, 2013). It was reported in some studies that IL-17A is unnecessary for protection against infection with intracellular bacteria such as *Mycobacterium tuberculosis* (Khader *et al.*, 2005) but other studies show that IL-17A is required for host resistance to the intracellular pathogens such as *Francisella tularensis* (Lin *et al.*, 2009). Furthermore, the role of IL-17A has been demonstrated in defense against *L. monocytogenes* infection by regulating the adaptive cytotoxic-T-lymphocyte (CTL) responses which show less CTL response in the absence of IL-17A compared to wild type mice (Xu *et al.*, 2010). IL-17 also plays an important role in activating and recruitment of neutrophils in the innate immune system (Kolls & Linden, 2004).

Moreover, IL-17A was shown to be required for Th1 immunity in *F. tularensis* infection (Lin *et al.*, 2009) but showed no difference during listeriosis. IL-17A is required for innate response and the most important source of IL-17A production *in vivo* are  $\gamma\delta$ T cells in response to *L. monocytogenes* infection. Complement C5a can regulate IL-17 by affecting the crosstalk between dendritic cells and  $\gamma\delta$ T cells in a murine model of cecal ligation and puncture CLP-induced sepsis (Xu *et al.*, 2010).

The other source of IL-17A has been demonstrated to be macrophage which has been shown *in vivo* from a section of spleen from wild type endotoxemic mice with IL-17A seen F4/80 macrophage by confocal microscopy. On the other hand, bone marrow derived macrophages were shown to be negative for IL-17 release after LPS stimulation (Bosmann *et al.*, 2012).

The role of IL-17A during secondary infection with intracellular bacteria was studied and it was reported that IL-17A is involved in the memory T cell response to secondary infection with *M. tuberculosis* (Khader *et al.*, 2007). However, mice infected with secondary *L. monocytogenes* show no difference between wild type and IL-17A-

deficient mice in terms of the amount of memory CD8<sup>+</sup> T cells and both cells secreted the same level of IFN- $\gamma$  and TNF- $\alpha$  (Xu *et al.*, 2010).

### **1.1.6.3 The cellular immune response to *L. monocytogenes***

Immune cells are derived from a hematopoietic stem cells in the bone marrow, and are differentiate into myeloid cells (e.g. macrophages and dendritic cells) and lymphoid cells (B cells, T cells and natural killer (NK) cells) which form the cellular components of the innate and adaptive immunity. Host defences against *L. monocytogenes* require coordinated interactions between a number of innate and adaptive components to clear an infection (Zenewicz & Shen, 2007). *L. monocytogenes* induces the Th1 cell response in the infected host, which produces cytokines such as IFN- $\gamma$  which are able to activate macrophages and cytotoxic T cells and are involved in the cellular immune response by controlling or as host defense against intracellular pathogens. Based on cytokine production, effector T helper cells can be differentiated into two types of cytokine profiles. Th1 (proinflammatory) which can produce interleukin-2 (IL-2), gamma interferon (IFN- $\gamma$ ) and nitric oxide, and Th2 (anti-inflammatory) which can produce IL-4, IL-5, IL-10 and IL-13.

Dendritic cells (DC) are the most active professional antigen presenting cells (APC) and are found in most tissues. They activate resting T cells and thereby link innate and adaptive immune system. They were described for the first time by Steinman & Cohn, 1973. Mouse mature dendritic cells express a large number of complement receptors, namely CD11c and the co-stimulator molecules CD80, CD86 and CD40, and have moderate to high surface levels of MHC II. However, the levels of all of these can be increased after activation (Baruah *et al.*, 2009).

The importance of dendritic cells in an immune response has been shown in many studies (Grouard *et al.*, 1997). They play a fundamental role in the initiating and regulating of complement in infectious and inflammatory disease models and have an important role during infection with *L. monocytogenes*, inducing of effective T cell immunity and colonization by *L. monocytogenes*. They are also required for the immune response against *L. monocytogenes* for infection *in vivo* and *in vitro*.

*In vitro* dendritic cells can be differentiated mostly from blood and bone marrow by a mixture of cytokines (GM-CSF and IL-4). These culture conditions are possible to generate a more heterogeneous immature population of cells. It has been shown that dendritic cells derived from murine bone marrow in presence of GM-CSF similar to dendritic cells studied *in vivo* and *in vitro* in phenotype and function (Inaba *et al.*, 1992; Lutz *et al.*, 1999; Pierre *et al.*, 1997; Winzler *et al.*, 1997). Cultured mouse bone marrow-derived dendritic cells express a wide range of complement components, regulators and receptors after stimulation with and without LPS (Peng *et al.*, 2008).

Moreover, dendritic cells derived from bone marrow generated by using GM-CSF and IL4 have been used in various studies. Xu *et al.* (2007) investigated the relationship between GM/IL4-DCs and inflammatory/Tip-DCs (Called Tip-DCs because produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and nitric oxide). In their study they provided evidence that dendritic cells generated by GM-CSF and IL-4 are the equivalents of the induced inflammatory or Tip-dendritic cells *in vivo*. As a result *in vitro* GM/IL4-DCs represent inflammatory dendritic cells that are normally found *in vivo*. A recent study showed that in the absence of C3 in serum during differentiation of dendritic cells from human monocytes and in the presence of GM-CSF and IL-4, response to LPS was impaired by reducing MHC-II, co-stimulatory molecules and cytokine production.

Dendritic cells play an important role in linking the innate and adaptive systems. They are important in priming the T cell response to *L. monocytogenes* infection, because of its primarily intracellular localisation of *L. monocytogenes*. In innate response T cells are required for clearance of the pathogen, and CD4 and CD8 T cells mediate the adaptive immune response, and are essential for long-term immunity after initial *L. monocytogenes* infection.

The costimulatory molecules CD80, CD86, CD40 and MHCII were up-regulated after infected dendritic cells derived from mice bone marrow with *L. monocytogenes* (Brzoza *et al.*, 2004). These costimulatory molecules were induced only when the *L. monocytogenes* invaded the cytoplasm. CD40 signaling in APCs lead to changes in various phenotypes, and served as a trigger for the expression of costimulatory molecules, such as CD80/CD86 for efficient T-cell activation and the induction or increased expression of chemokines and cytokines (Koch *et al.*, 1996; Cella *et al.*, 1996). It has been shown that deficient of C3 in bone marrow-derived dendritic cells expressed lower levels of MHC class II and CD80, CD86 and CD40 (Nakayama *et al.*, 2009).

During *L. monocytogenes* infection mouse macrophages play a central role in the early innate immune responses which act as primary host cells and as the main defenses against listeriosis. Macrophages are phagocytes of the innate immune system as described for the first time by Metschnikoff in 1884. They present the first line of defence against different microorganisms and play a role in both innate and adaptive immunity. *In vivo* infection with *L. monocytogenes* is found preferentially within the cytosol of macrophages and hepatocytes in the spleen and liver (Gregory *et al.*, 1997). In the liver, macrophage (especially Kupffer cells) plays a key role during infection.

The phenotype and function of the macrophage are influenced by its surrounding tissue type and microenvironment (Crowther *et al.*, 2001). Activation of macrophage has been shown to play a role in quickly killing *L. monocytogenes* and clearing the infection (Shaughnessy & Swanson, 2007; Myers *et al.*, 2003).

Research demonstrates that murine macrophage derived from bone marrow die by necrosis after several hours of cytoplasmic replication of *L. monocytogenes* and apoptosis does not occur during *L. monocytogenes* infection (Barsig & Kaufmann, 1997). *L. monocytogenes* induces cell death in the murine macrophage through caspase-1 (Cervantes *et al.*, 2008). Caspase-1 activation is dependent on the cytosolic presence of bacteria. This was concluded from studies using LLO mutant *L. monocytogenes* which had a reduced doubling time, escaped to a significantly lower extent from the phagosomes and did not cause caspase-1 activation (Westcott *et al.*, 2007).

Dendritic cells and macrophages are comparable in the intracellular life cycle of *L. monocytogenes*. However, dendritic cells present a less desirable environment for *L. monocytogenes* growth in compare to macrophages and the maturation status of the dendritic cells influences the result of infection. It has been shown that dendritic cells provide fewer reservoirs for replication of *L. monocytogenes* in terms of increasing bacteria number and doubling time in comparison to bone marrow macrophage which affects the outcome of infection. Also larger percentages of *L. monocytogenes* were restricted to vacuoles in bone marrow dendritic cells as compared to bone marrow macrophage. In macrophage more *L. monocytogenes* were present in the cytosol compared to dendritic cells. Indeed, this may be due to LLO having a greater effector function on macrophage vacuoles than on dendritic cell vacuoles (Westcott *et al.*, 2007; Stier *et al.*, 2005).

*L. monocytogenes* induces an M1 program which prevents phagosomal escape and activating intracellular killing of bacteria *in vitro* and *in vivo* (Shaughnessy & Swanson, 2007).

### **1.1.7 Effect of hypoxia on dendritic cells and macrophages**

Hypoxia or low oxygen tension is a common characteristic of many inflammatory diseases, and this has been described at almost every site of inflammation and found in pathological tissues. The change of the function of dendritic cells to hypoxia has been studied: hypoxia strongly favours the innate immune function of dendritic cells by reducing their maturation. This means a lower expression of molecules involved in antigen presentation (Rius *et al.*, 2008).

Generally in healthy tissues, the oxygen tension is between 2.5-9%, however inadequate perfusion of diseased tissue can cause transient or chronic hypoxia, in which oxygen tension falls below 1% oxygen. The hypoxia-inducible transcription factor (HIF-1 $\alpha$ ) has a role in increasing a full inflammatory response in myeloid cells in low oxygen conditions and due to HIF-1 $\alpha$  activation, macrophages phagocytose and kill bacteria better under hypoxic conditions than they do under normoxic conditions (Zinkernagel *et al.*, 2007).

The effect of the hypoxia was reported to impair immune responses to bacterial pathogens for example *Klebsiella pneumoniae*, *Salmonella typhimurium*, *E. coli* and *Chlamydia trachomatis*.

Immune cells are quickly recruited to pathological tissues and are therefore severely affected by lack of oxygen. Different cell behaviours have been observed in response to hypoxia for example in T cells hypoxia may block many effector functions like cytokine

production (Sitkovsky & Lukashev, 2005). The transcription factor HIF1 $\alpha$  is expressed in almost all mammalian cells and is a key regulator in responses to hypoxia, being essential to the functioning and survival of immune cells. Studies of infected mice have reported that macrophage from HIF1 $\alpha$ -deficient mice showed less ability to kill bacteria (Cramer *et al.*, 2003; Peyssonnaud *et al.*, 2005). Furthermore, the activity of HIF1 $\alpha$  showed an increase in phagocytosis and in NO production in phagocytes (Peyssonnaud *et al.*, 2005; Nizet & Johnson, 2009). Moreover, stabilisation of HIF1 $\alpha$  interferes the *Chlamydia pneumoniae* in the early phase of infection with host cell HIF1 $\alpha$  (Yaraei *et al.*, 2005).

When mouse macrophages exposed to Gram-positive or Gram-negative bacteria quickly up-regulate their expression of HIF-1 $\alpha$  (Murdoch *et al.*, 2005). Moreover, studies *in vitro* reported that removal of HIF-1 $\alpha$  could decrease myeloid cell bactericidal activity (Peyssonnaud *et al.*, 2005).

Macrophage up-regulates expression of TNF- $\alpha$  in hypoxia. However, another study showed that the determination of protein release by using ELISA, production of TNF- $\alpha$  was decreased from macrophage in supernatants from RAW cells exposed to hypoxia in comparison to normoxia for 24 hours (Yun *et al.*, 1997).

### **1.1.8 Effects of norepinephrine on immune cells and *L. monocytogenes* infection**

Bacteria living in environments such as a human or animal host are subject to stressful conditions and need to adapt to these to survive. Work by Dr Freestone *et al.* (2008) has reproducibly demonstrated that stress hormones influence the outcome of infections by modulating the immune response and increasing the growth and virulence of bacteria.

Norepinephrine, one of the stress hormones, is released from the medulla of the adrenal glands into the blood, from noradrenergic neurons during synaptic transmission. Norepinephrine has the ability to increase the *E. coli* growth both *in vivo* and *in vitro* and induces the expression of virulence determinants in enteric pathogens, thereby leading to higher virulence, which means higher infectivity and invasion with greater survival of antibiotic attack. Invasion by *L. monocytogenes* of intestinal epithelia has been shown to respond to stress hormone (Freestone *et al.*, 1999).

Conversely, stress hormone affects immune cells and influences the ability of the host to fight infection. Macrophages have receptors for the stress-related neurohormones adrenaline and noradrenaline (Reiche *et al.*, 2004). The impact of stress hormone levels on the outcome of infections in general is not well researched but is certainly taken into account in modern husbandry practices involving mouse models of infection. Infection with *L. monocytogenes* in general is short lived in healthy individuals who present flu-like symptoms. The infective dose of *L. monocytogenes* is not known and is probably dependent on the virulence potential and the susceptibility of the individual.

### **1.1.8.1 Stress hormones and immune function**

It has been demonstrated that the effect of stress hormones on immune response is mainly inhibitory, affecting the trafficking of neutrophils, macrophages and antigen-presenting cells. Stress hormone inhibits the production of pro-inflammatory cytokines and chemokines by these cells and impairs the effector functions of immune cells (Padgett & Glaser, 2003).

There is interaction between the immune system and central nervous system (CNS) and both of them have shown to be important in maintaining homeostasis and in regulating

immune responses in disease conditions. This interaction is involved in two different pathways, the hypothalamic-pituitary-adrenal axis (HPA) activation and the sympathetic nervous system (SNS) activation (Steinman, 2004). Both activations result in systemic increases in stress hormones like noradrenaline (norepinephrine), which directly affect the immune cell activity (Webster *et al.*, 2002; Straub, 2004). Norepinephrine binds to adrenergic receptors and is expressed by different cell types such as monocytes, macrophages, lymphocytes, granulocytes and natural killer cells.

Bacteria-infected host cells have been reported to activate the sympathetic nervous system for the possibility of host survival by inducing a change in immune cell activity. Activation by the immune cells leads them to secrete cytokines which influence central nervous system activity which responds by regulating the levels of immune cell activity.

### 1.1.9 The aims of this study

The aim of this study was to characterise *in vitro* and *in vivo* the role of properdin in murine infection with *L. monocytogenes*. Properdin-deficient and wild type mice were used in parallel. Theoretically, one might expect a similar outcome in properdin-deficient mice to C5-deficient mice (which are naturally deficient, not targeted) because the deficiency of properdin accompanies impaired C5a production and limited C3b deposition (Ivanovska *et al.*, 2008). However, as the C5-deficient mice were from a genetic background that was different from the wild type controls they were compared against, a better designed control study was needed. Traditionally, the role of properdin has been confined to host defence against *N. meningitidis*, because it was the properdin-deficient individuals who showed high susceptibility to *N. meningitidis* infection with fatal outcome. Moreover, *in vitro* infection of primary bone marrow-derived macrophages and dendritic cells allow dissection of cellular immune responses.

#### **The objectives of the project, comprising *in vitro* and *in vivo* analyses:**

1. To investigate the bactericidal effect of dendritic cells and macrophages derived from the bone marrow of wild type and properdin-deficient mice against *L. monocytogenes* in normoxia and hypoxia and also in the presence of the stress hormone norepinephrine using cell culture and viable bacterial count.
2. To study the role of properdin in intracellular localisation of *L. monocytogenes* in dendritic cells and macrophages by electron microscopy.
3. To analyse gene expression by using reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qPCR) using cDNA prepared

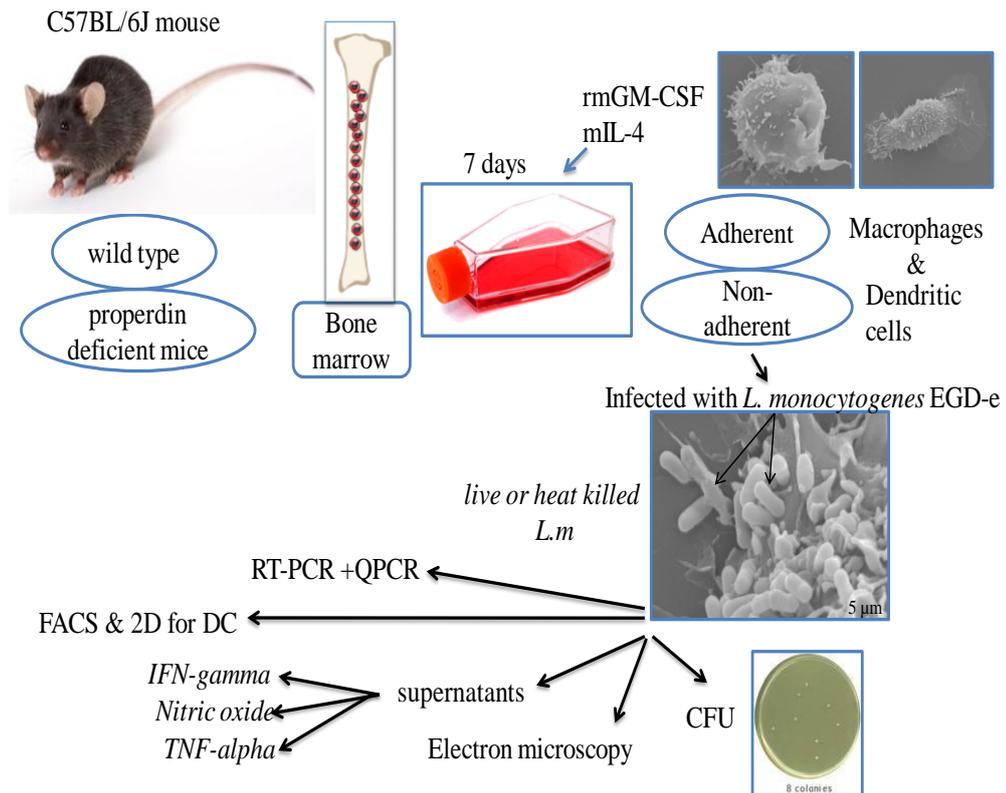
from mouse dendritic cells and macrophages after infection with *L. monocytogenes*.

4. To investigate the cellular reaction of dendritic cells and macrophages from wild type and properdin deficient mice after infection with *L. monocytogenes* using cell culture, together with cytokine and nitric oxide measurements.
5. To study the cellular reaction of dendritic cells derived from bone marrow by 2-D gel electrophoresis and flow cytometry.
6. To investigate the extent of *L. monocytogenes*-mediated complement activation in serum of wild type and properdin-deficient mice using Western blot.
7. To analyse the expression of cellular reaction using mouse splenic B cells by flow cytometry
8. To investigate the role of properdin in the severity of *L. monocytogenes* infection by scoring of animals.
9. To investigate the role of properdin in attracting leukocytes to infected tissue, in organising these cells, in clearance of apoptotic cells, and in tissue specific responses (spleen, liver) using histology study.
10. To study the role of properdin in controlling *L. monocytogenes* numbers by viable counts.
11. To analysis the mRNA expression of IFN- $\gamma$ , IL-17A, CD11b, TLR2, C5aR, Fc $\gamma$ RIIb, Fc $\gamma$ RIII, and Fc $\gamma$ RIV by qPCR using cDNA prepared from the spleen of infected mice

## *Chapter Two Material and Methods*

2. Material and Methods

2.1 *In vitro* infection of murine bone marrow derived dendritic cells and macrophages with *L. monocytogenes*

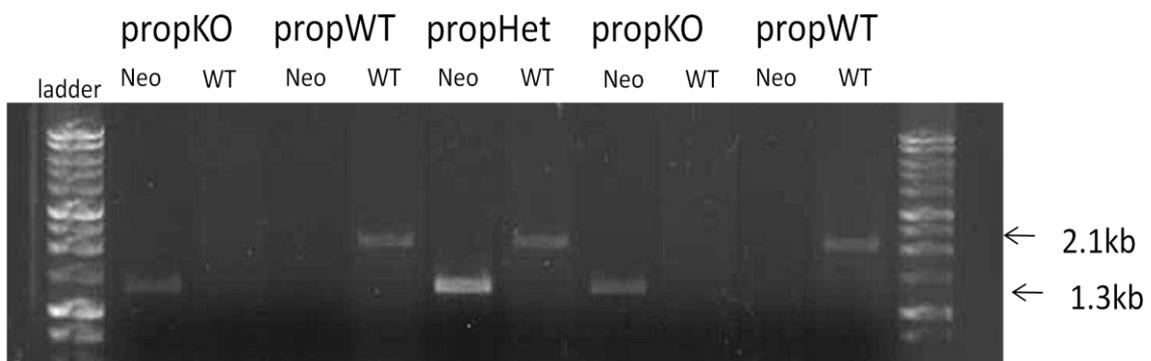


**Figure 2.1:** Schematic overview of the methods contained in this chapter. Mouse picture was taken from [http://www.isgtw.org/sites/default/files/img\\_2011/Black\\_Mouse.JPG](http://www.isgtw.org/sites/default/files/img_2011/Black_Mouse.JPG). The mouse cells and *L. monocytogenes* image from this study. Initially non-adherent, dendritic cells are loosely adherent after 7 days' differentiation. L.m= *L. monocytogenes*, EGD-e= (E. G. D. Murray). RT-PCR= reverse transcriptase polymerase chain reaction, QPCR=quantitative real-time polymerase chain reaction.

### 2.1.1 Infection of dendritic cells and macrophages derived from mouse bone marrows with *L. monocytogenes* strain EGD-e

#### 2.1.1.1 Mice

Properdin-deficient mice (KO) in parallel with littermate wild type (WT) mice were used in this study. The colony was maintained by Dr. Cordula Stover (University of Leicester). The mouse line was genetically engineered to be deficient only of properdin by specifically targeting the mouse properdin gene (described in Stover *et al.*, 2008). The mice are fertile and not susceptible to infection in the barrier unit of the transgenic facility at the University of Leicester. The mice are on C57BL/6J background, a genetic background typically chosen in studies interrogating cellular responses because of their M1/Th1 bias. Figure 2.2 shows typical agarose gel electrophoresis of PCR products obtained from wild type, homo- or hemizygous properdin-deficient and heterozygous mice by target specific amplification. Then heterozygous mice were backcrossed to C57BL/6 mice to obtain male wild type and properdin-deficient littermates. These were used in the experiments wherever possible.



**Figure 2.2:** Exemplary electrophoresis of amplicons obtained from wild type (propWT), homo- or hemizygous properdin-deficient (propKO) and heterozygous mice (propHet). Genomic DNA is prepared from ear snips by proteinase K digestion and amplified using primers specific for wild type (WT) and targeted (Neomycin resistance gene, Neo) sequences, respectively, as described in Stover *et al.*, 2008.

### 2.1.1.2 Bone marrow preparation

The preparation of bone marrow cells culture is based on methods described in (Westcott *et al.*, 2007) with modifications as follows:

Genotyped mice were culled using a schedule 1 method (cervical dislocation) by experienced animal technicians.

Bone marrows were flushed from the tibias and femurs, which had been removed and purified from the surrounding muscle. Intact bones were put in 100 % IMS (Industrial methylated spirit) for a few minutes before washing in phosphate-buffered saline (PBS). Both ends of bones were cut and flushed with PBS using a 1 ml syringe with a 0.45 mm diameter needle. The bone marrow suspension was vigorously pipetted gently to disintegrate any clusters, then left to settle down to the bottom of the collection tube. The supernatant was transferred to a new tube and centrifuged at 433g for 5 minutes. Red blood cells were lysed by adding 1 ml hypotonic 0.87% Tris-NH<sub>4</sub>CL (pH 7) and incubated at 37°C in 5% CO<sub>2</sub> for 5-10 minutes. To neutralise the lysis, the cells were suspended in 10 ml RPMI 1640 media (Gibco) and centrifuged again at 433g for 5 minutes to obtain the bone marrow cells and re-suspended with 10 ml culture media RPMI-1640 supplemented with 10% (v/v) fetal calf serum (FCS). For sterility all bone marrow preparations were performed in a tissue culture hood.

IL-4 was used in combination with GM-CSF (Menges *et al.*, 2005). It has been demonstrated that dendritic cells derived from bone marrow generated by GM-CSF and IL-4 are more efficient antigen presenting cells *in vitro* and *in vivo* than dendritic cells derived from bone marrow generated with GM-CSF alone (Masurier *et al.*, 1999; Lu *et al.*, 1995; Dillon *et al.*, 1997). Dendritic cells and macrophage populations derived from bone marrow, which is a widely used population, because differentiation *in vitro*

permits the generation of relatively homogeneous cells. The macrophage is an important cell subset in mediating clearance of bacteria. These cells were used to assess the role of properdin in immune response to *L. monocytogenes* infections.

### **2.1.1.3 Differentiation of dendritic cells and macrophages from mouse bone**

#### **marrows**

Dendritic cells and macrophage can be generated *in vitro* by culture in growth medium, dendritic cells can be derived from bone marrow or splenocytes or from blood-derived monocytes in the presence of GM-CSF (Inaba *et al.*, 1992; Sallusto & Lanzavecchia, 1994; Liu *et al.*, 2002) and IL-4 (Inaba *et al.*, 1992; Sallusto & Lanzavecchia, 1994; Liu *et al.*, 2002). Bone marrow derived dendritic cells were generated as described (Inaba *et al.*, 1992; Lutz *et al.*, 1999). Also bone marrow derived macrophages were generated as showed in (Zhang *et al.*, 2008) with some modifications.

After bone marrow preparation, progenitor cells were counted using the Neubauer chamber slide to adjust at  $2 \times 10^6$  cells/ml in 15 or 20 ml culture medium (RPMI 1640 medium with penicillin (100U/ml), streptomycin (100 $\mu$ g/ml), L-glutamine (2mM) and 10% FCS and seeded in tissue culture 75 cm<sup>2</sup> flask, then cytokines, 10 ng/ml rmGM-CSF (recombinant mouse Granulocyte Monocyte Colony-Stimulating Factor) and 10ng/ml mL-4 (mouse Interleukin 4) (PEPROTECH) were added, as described in (Xu *et al.*, 2007) but different in IL-4 concentration. Over a period of 6 days at 37°C in 5% CO<sub>2</sub>, rmGM-CSF and mL-4 were used to differentiate dendritic cells and macrophages. On day 3 cells were centrifuged and the pellet was re-suspended with new media and cytokines. The cells appeared morphologically as on day zero but some cells had become adherent (about 20%). There was no apparent difference between cell types from wild type and properdin-deficient mice in the population of cells.

On day 6 for the infection experiments, adherent and non-adherent cells were separated. According to this method, the morphological differences between adherent and non-adherent cells is used to assign the two separate populations into two types, namely dendritic cells and macrophages.

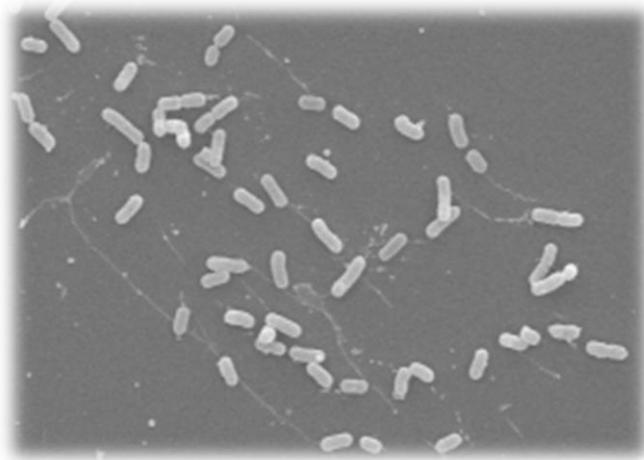
An improved, recent method relies on antibody selection of the specific cells from this population. This, however, was not done as part of this project, so the populations termed dendritic cells or macrophages, which will be those that are merely enriched in cells with these phenotypes. Of note, the cells are viable in the presence of IL-4 and Gm-CSF and have a uniform appearance in light microscopy, SEM and TEM even though the dendritic cells populations is positive for CD11c, a dendritic cells maturation marker to only 20 %. This, however, does increase after infection (page 162 section 3.1.16).

To obtain dendritic cells, the culture supernatants were centrifuged. For macrophages, the adherent cells were harvested using a cell scraper. Dendritic cells and macrophages were counted and adjusted to an approximately 800000 cells/well in all experiments that use viable count numbers of the cells were the same, then added in 12-well plates or for some experiments cells were seeded in 60 mm<sup>2</sup> tissue dishes containing 12 mm<sup>2</sup> sterile glass cover slips, fed again with the medium and cytokines and incubated overnight at 37 °C to let the dendritic cells to adhere.

### **2.1.2 *Listeria monocytogenes* strain EGD-e (serotype 1/2a), growth and preparation**

Rod-shaped *L. monocytogenes* strain EGD-e were used in this study and was obtained from the American Type Culture Collection (ATCC catalogue number BAA-679) in lyophilised form, revived in brain heart infusion (BHI) broth, and handled in CAT 2 certified laboratory areas.

As shown in Figure 2.3 *L. monocytogenes* present as a short coccobacillus, occasionally as short chains or single. The edges of its colonies grown on BHI-agar plates at 37°C typically have a smooth outline. This appearance matches that described by (Murdoch *et al.*, 2005).



**Figure 2.3:** Scanning electron micrograph of EGD-e *L. monocytogenes*, were used in this study, from original magnification 30  $\mu\text{m}$  (image was taken from this study).

Starting from one fresh colony, *L. monocytogenes* were grown to log phase as follows; after overnight incubation (220 rpm, 37°C), 1 ml of a 10 ml pre-culture in BHI was transferred to 100 ml BHI (220 rpm, 37°C). *L. monocytogenes* in log phase are most metabolically active compared to *L. monocytogenes* in stationary phase. The optical density (OD) was measured at 30-minute intervals reproducibly. After 3-4 hours, the bacteria reached (OD) mid-logarithmic phase of growth. Bacteria were pelleted by centrifugation (771g for 20 minutes) and stored in BHI with 10% (v/v) glycerol at -80°C in 0.5 ml aliquots to be used for infection studies. Bacterial numbers were established by a viable cell count method using BHI-agar plates and expressed as CFU (colony forming unit)/ml.

### **2.1.2.1 The efficiency of gentamicin**

The bacteriocidal efficiency of 10µg/ml gentamicin in the time frame used in the subsequent experiments was quantified by a viable count of *L. monocytogenes*. *L. monocytogenes* ( $1 \times 10^6$  /ml medium) were incubated with and without gentamicin at time point 30 minutes, 1 hour and 2 hours at 37°C, then CFU dilutions were plated on BHI-agar after 24 h at 37°C and counted.

### **2.1.2.2 Enumeration of mouse cells and viable bacteria**

Number of cells per well were the same between wild type and properdin-deficient mice in all experiments. Stock of *L. monocytogenes* and each dilution of the stock as well as the viable bacteria after each time point of infection were counted using colony forming units (CFU). 10 µl in triplicate from each well (1 ml dH<sub>2</sub>O) were counted. The count was performed after incubation for 24 hours at 37°C. Only dilutions that resulted in counts of between 25-300 colonies were enumerated. The number of colony forming units per ml is calculated as follows:

Number of colonies counted in the sector x (1000/volume plated 30 µl) x sector dilution.

Mouse cell count was performed using Neubauer chamber slide and the cell concentration (cells per ml) calculated as follows:

Number of cells /number of bacteria x MOI x 1000= (/ml)

### **2.1.3 Infection of mouse cells**

On day 7 after differentiation of dendritic cells and macrophages from mouse bone marrows, cells were infected with *L. monocytogenes* (log phase of known OD and CFU). For this experimental infection of mouse cells, an aliquot of bacteria was thawed

and washed once by adding RPMI medium to remove freezing medium, pelleted and re-suspended in culture medium (RPMI 1640 without antibiotics) at a number to achieve the indicated MOI (multiplicity of infection). Cells were infected with *L. monocytogenes* at MOI 0.2 following a previous study investigating the differential susceptibility of bone marrow-derived dendritic cells and macrophages to produce infection with *L. monocytogenes* by Westcott *et al.*, 2007 with some adjustments. The MOI was confirmed by plating dilutions of inoculated medium on BHI agar and enumerating CFU/ml after 24 h at 37°C.

The cells were infected for an hour and after 1-hour incubation at 37°C, the infected cells were gently washed with PBS. Fresh medium containing 10µg/ml gentamicin was added to kill extracellular bacteria.

For the experiment with cover slips, the cover slips were removed in 30 minutes, 1 hour, and 2 h and the adherent cells were lysed by transferring the cover slips to wells containing 1ml or 2ml sterile water and vortexing vigorously. For the experiment without cover slips the adherent cells were lysed by adding 1ml or 2 ml of sterile water to each well in the plate and viable counts were determined on BHI-agar.

Supernatants were kept in -80 °C from experiment that culture the cells and infected in the flask were kept separately and analysed individually and supernatants were pooled from experiments that were used wells.

The experiments were repeated with and without cover slips. The number of mouse cells that were used was the same for all experiments and 1 ml medium was added to each well.

### **2.1.3.1 Infection of mouse cells in normoxia and hypoxia**

The aim of this experimental setup was to determine the intracellular bacteria under normoxic and hypoxic conditions. It is used in hypoxia-related changes because in the mouse model *in vivo*, hypoxia is a relevant feature on *L. monocytogenes* infection of organs.

The experiment was repeated as above (Section 2.1.3) but after 1 hour of incubating cells with *L. monocytogenes* at 37°C, the medium with gentamicin after another hour was removed and fresh medium without antibiotic was added to each well. Then the cells were incubated for 24 hours at 37°C and viable counts were determined, as above, after incubation in normoxia or hypoxia. Hypoxia was induced by exposing the cells to 0.2% O<sub>2</sub> with 5% CO<sub>2</sub>, 94.8% N<sub>2</sub> at 37°C, using modular incubator chambers a hypoxia incubator (Galaxy<sup>®</sup> CO<sub>2</sub> incubator, RS Biotech Ltd). Mini O<sub>2</sub> oxygen analyser (Analox Sensor Technology) was used by placing it inside the hypoxic incubator to insure the level of oxygen was 0.2%.

The normoxia group was cultured in parallel under standard incubator conditions at 37°C. Further incubations done in 75 cm<sup>2</sup> tissue culture flasks for another experiment such as RT-PCR and qPCR (where more cells were needed), the supernatants from these experiments were used to measure the cytokines TNF- $\alpha$  and IFN- $\gamma$ .

### **2.1.3.2 Infection of mouse cells with *L. monocytogenes* in the presence of norepinephrine (NE)**

Stress hormones affect the immune response mainly by inhibiting the functions of neutrophils, macrophages, antigen-presenting cells (Padgett & Glaser, 2003). Norepinephrine *in vitro* increases the growth of *E. coli* in a serum-based medium (serum-SAPI medium), moreover, numbers of Gram-positive bacteria including *L.*

*monocytogenes* have been reported to respond to norepinephrine (Freestone *et al.*, 1999). *L. monocytogenes* react towards exposure of stress hormones present during sepsis (Freestone *et al.*, 2008) therefore, dendritic cells and macrophages derived from mouse bone marrow were infected using *L. monocytogenes* pre-incubated with 100  $\mu$ M (stress relevant dose, personal communication Dr. P. Freestone) at MOI= 0.2 and the infection carried out as reported in section 2.1.3. After gentle mixing, the bacteria were added to each well and infected for 1 hour, and then viable counts were taken at time points 30 min, 1 hour, and 2 hours after treatment with gentamicin.

### **2.1.3.3 Determination of cellular viability after infection with *L. monocytogenes***

Viability of primary mouse macrophages and dendritic cells after 1-hour infection with *L. monocytogenes* and 1 hour after addition of gentamicin was determined by using trypan blue stain as previously described (Parra *et al.*, 2008), also after 24 hours infection in normoxia and hypoxia. Floating and attached cells were analysed microscopically for viability by trypan blue exclusion. The floating cells (representing detached, infected cells) were collected and centrifuged at 3000g then re-suspended in 20  $\mu$ l of 1 x PBS. The adherent cells (for macrophages and dendritic cells) were rinsed carefully with 1 x PBS and cells detached with 0.2% trypsin for 30 minutes at 37°C then centrifuged at 10000g and the resulting pellet was re-suspended in 200  $\mu$ l of 1 x PBS. Then 10  $\mu$ l of each cell suspension were mixed with the same volume of trypan blue solution 0.4% (Sigma). After 1-2 min at room temperature, (white) intact and compromised (blue) cells were counted by using the Neubauer chamber slide.

#### **2.1.3.4 Analysis of infected and uninfected dendritic cells and macrophages by electron microscopy**

Dendritic cells and macrophage derived from bone marrow were prepared for electron microscopy, on 6 days of differentiation the cells, the uninfected cells were adjusted to the same number between wild type and properdin-deficient and incubated overnight into 12 wells plate containing 12 mm sterile glass cover slips.

For infected cells, dendritic cells and macrophages from both genotypes were infected with mid-logarithmically grown *L. monocytogenes*. Before infection, aliquots of bacterial suspensions were thawed and washed once with RPMI 1640. The bacteria were diluted in RPMI medium and added, at the desired multiplicity of infection (MOI) 2.5 bacteria/cells, to 60 mm tissue dishes containing 12 mm sterile glass cover slips (Westcott *et al.*, 2007). After 4 and 24 hours incubation, the infected cells were washed with 1 ml/well PBS and were fixed with 600 µl/well 2.5% Glutaraldehyde/0.05 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.2 for 15 minutes at room temperature then samples were kept at 4°C before processing for electron microscopy.

Moreover, dendritic cells and macrophage were infected with *L. monocytogenes* with MOI= 0. 2 without and in present with stress hormone for 4 hours, uninfected cells control were used at the same time. The cells were prepared for transmission electron microscopy.

Electron microscopy was used to study the role of properdin in intracellular localisation of *L. monocytogenes* in dendritic cells and macrophages. After setting up the experiments (cell purification and infection), Stefan Hyman and Natalie Allcock of the Electron Microscopy Laboratory at University of Leicester School of Biological Sciences, processed the samples and blindly captured images of processed samples.

Representative images for uninfected and infected cells were chosen from a total number of about 400 images for scanning electron microscopy and about 900 images for transmission electron microscopy.

Transmission electron microscopy (TEM) of infected and control cells. After removing the fixative by washing (0.05 M HEPES) 3 times, 10 minutes, cover slips were immersed in 1 % osmium tetroxide in 0.05 M HEPES for 90 minutes. The plate was washed 3 times for 10 minutes in distilled de-ionised water and passed through ascending alcohol concentration (70% (v/v) Ethanol 15 minutes , 70% (v/v) Ethanol overnight at 4°C, 90% (v/v) Ethanol 20 minutes, 100% Absolute Ethanol for 30 minutes, 100% Absolute Grade Ethanol for 15 minutes two times). To embed, cover slips were first incubated in propylene oxide, two times for 15 minutes, then passed through propylene oxide agar mixtures (3 Propylene oxide: 1 Agar Low Viscosity Resin-30 minutes, 1 Propylene oxide: 1 Agar Low Viscosity Resin-30 minutes, 1 Propylene oxide: 3 Agar Low Viscosity Resin-30 minutes) and left in 100% Agar Low Viscosity Resin overnight. After a change in fresh Agar Low Viscosity Resin for 5 hours, the samples were polymerised at 60°C for 24 hours.

Thin sections, of approximately 80 nm thickness, were cut from each sample using a Reichert Ultracut S ultramicrotome, collected on copper mesh grids, counter stained with 2 % Uranyl Acetate and Reynolds' lead citrate and observed using a JEOL 1220 transmission electron microscope using an accelerating voltage of 80 kV.

Digital images were recorded using a SIS Megaview III Digital Camera with analysis Software.

Using scanning electron microscopy (SEM) of infected and control cells, the cells, on cover slips, supplied in 2.5 % Glutaraldehyde/0.05 M HEPES pH 7.2 were washed in 0.05 M HEPES buffer 3 times for 10 minutes. Cover slips were taken through ascending

series of ethanol concentrations (70% Ethanol for 90 minutes, 90% Ethanol for 20 minutes, 100% Ethanol for 20 minutes, 100% Analytical grade ethanol for 20 minutes) to dehydrate, then 2:1 ethanol/HMDS (hexamethyldisilazane) for 20 minutes, 1:2 ethanol/HMDS for 20 minutes, 100% HMDS for 20 minutes two times, HMDS was removed from cells in the wells and allowed to air dry in fume hood before mounting cultures on specimen stubs for light sputter coating with gold/palladium.

### **2.1.3.5 Quantitative measurement of mouse IFN- $\gamma$ secreted from dendritic cells and macrophages in response to infection with *L. monocytogenes***

The cytokine IFN- $\gamma$  is released from dendritic cells and macrophages in response to infection with *L. monocytogenes*. Murine IFN- $\gamma$  ELISA (Enzyme-Linked Immunosorbent Assay) development Kit was used to quantitatively measure mouse IFN- $\gamma$ .

Mouse cells were isolated as described and infected with *L. monocytogenes*, the cell culture supernatants were kept in -80°C at 2 and 24 hours after adding gentamicin, and ELISA protocol was used according to the manufacturer's instructions using ELISA micro-plates (Nunc Maxisorp).

After 1 hour incubation at 37°C of mouse cells in 12 wells plate with *L. monocytogenes* the media was removed and the cells were gently washed with PBS, and fresh medium containing gentamicin was added to kill the extracellular bacteria. After another 1 hour incubation with gentamicin, the medium removed and fresh medium without antibiotic were added to each well then incubated for 24 hours at 37°C. After infection the supernatants was kept and used to measure IFN- $\gamma$ .

To prepare the ELISA plate, capture antibody was diluted with PBS to a concentration of 1 µg/ml and immediately 100 µl were added to each well. The plates were sealed and incubated overnight at room temperature. Subsequently, the plate wells were aspirated to remove liquid and washed 4 times using 300 µl of wash buffer (0.05% (v/v) Tween-20 in PBS) per well.

After the last wash the plates were inverted to remove the residual buffer and were blotted on paper towels. Then 300 µl of the block buffer (1% (w/v) Bovine Serum Albumin (BSA) in PBS) were added to each well and incubated for at least 1 hour at room temperature after that the plate was aspirated and washed 4 times.

Murine IFN- $\gamma$  standard was diluted starting from 1.5 ng/ml in diluent (0.05% (v/v) Tween-20, 0.1% BSA in PBS) and the samples were used without dilution (neat). Then immediately, 100 µl of standard or sample were added to each well in triplicate and incubated at room temperature for at least 2 hours. The plate was aspirated and washed 4 times by using wash buffer. Detection antibody was diluted in diluent to a concentration of 0.25 µg/ml, and then 100 µl were added per well and incubated at room temperature for 2 hours. After that, the plate was aspirated and washed 4 times using a wash buffer. 5.5 µl of Avidin-HRP conjugate was diluted 1:2000 in diluent for total volume of 11 ml and 100 µl were added per well and incubated 30 minutes at room temperature. The plate was aspirated and washed 4 times and 100 µl of substrate solution ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (Sigma) were added to each well and incubated at room temperature for colour development. Colour development was monitored using an ELISA plate reader (680 Microplate Absorbance Reader) at 405 nm with wavelength correction set at 650 nm.

**2.1.3.6 Quantitative measurement of mouse TNF- $\alpha$  secreted from dendritic cells and macrophages in response to infection with live and heat-killed *L. monocytogenes* in normoxic and hypoxic conditions**

TNF- $\alpha$  cytokine released from dendritic cells and macrophages from wild type and properdin-deficient mice were measured in response to infection with live and heat killed *L. monocytogenes* for 24 hours in normoxia and hypoxia. Mouse cells (dendritic cells and macrophages) were isolated and infected with *L. monocytogenes* as described in section 2.1.2 and 2.13, the cell culture supernatants were kept at - 80°C. Murine TNF- $\alpha$  ELISA (PeproTech Cat# 900-M54) development Kit was used to measure mouse TNF- $\alpha$  levels (Tumor Necrosis Factor) and ELISA protocol was used according to the manufacturer's instructions using ELISA microplates (Nunc Maxisorp) as described in murine IFN- $\gamma$  ELISA Development Kit. Murine TNF- $\alpha$  standard was diluted from 2 ng/ml to zero in diluent.

To inactivate *L. monocytogenes*, *L. monocytogenes* were heat killed at 60 °C for 1 hour. Heat killed *L. monocytogenes* were used in this study because a large number of cells was required especially of dendritic cells, to analyse by FACS or PCR. Live *L. monocytogenes* secrete e.g. listeriolysin O (LLO), which makes cells very sensitive to lysis even at a low concentration of *L. monocytogenes*. LLO binds to cholesterol in membrane rafts and interferes with signal transduction and makes holes (Gekara *et al.*, 2005). The heat killing of *L. monocytogenes* was verified by plating on BHI (CFU) and there was no growth.

**2.1.3.7 Nitric Oxide (NO) determination**

Nitric oxide production from dendritic cells and macrophages in response to infection with *L. monocytogenes* was measured indirectly by assaying the concentration of nitrite

using Griess Reagent kit (Promega, Wisconsin, USA) in the cell culture medium after 24 hours infection of macrophages and dendritic cells with *L. monocytogenes*. Using 96-well flat-bottom enzymatic assay plate reader, 50µl of each samples were added to the wells in triplicate. Then 50µl of the sulfanilamide solution were added to all samples and wells containing the dilution series for the Nitrite Standard reference curve. The plate was incubated 5–10 minutes at room temperature with protection from light. Then 50µl of the NED solution (N-1-naphthylethylenediamine dihydrochloride) was added to all wells, and incubated for 5–10 minutes at room temperature with protection from light. The absorbance was measured within 30 minutes in a plate reader with a filter setting between 520 nm and 550 nm.

### **2.1.3.8 Measurement of TNF- $\alpha$ bioactivity**

Bone marrow-derived dendritic cells and macrophages were infected with *L. monocytogenes* for 5 and 24 hours. The cell culture supernatants after infection were kept on -80°C, and used to measure TNF- $\alpha$  activity. This was determined by a cytotoxicity assay using TNF- $\alpha$  sensitised L929 cells, following a modified version of the technique (Delahooke *et al.*, 1995). L929 cells are mouse fibroblast cell line and become sensitised to TNF- $\alpha$  when exposed to actinomycin-D. L929 cells are maintained in DMEM medium supplemented with 5% (v/v) FCS and penicillin, streptomycin and glutamine containing actinomycin-D (1µg/ml) after disaggregation using the trypsin/EDTA. L929 cells were seeded at a density of  $4 \times 10^5$  cells/ml per well in 100µl/well of a 96 well tissue culture flat bottom microtiter plate and left in the incubator for 3 hours to adhere.

The standard curve was prepared by addition of recombinant murine TNF- $\alpha$  (PeproTech) in 1:2 sequential dilutions. 10µl of each sample supernatant from infected

cells were added to the wells of the L929 plate in triplicate, and positive (supernatant from LPS stimulated macrophages) and negative (medium) controls were included in the plate. The plate was placed in 37°C, 5% CO<sub>2</sub> incubator overnight.

The following day, the plates were inverted over a sink to remove media from the cells. Then 50 µl of crystal violet solution were added to each well and cells were left to stain for 3-4 minutes (TNF- $\alpha$  causes the L929 sensitised cells to die and this is measured with the stain). The plate was washed thoroughly under the tap to make sure all traces of free dye were removed. The plate was dried briefly on tissues to remove excess water then 100 µl of 20% (v/v) acetic acid (in dH<sub>2</sub>O) were added to each well to solubilise the stained cells, then the absorbance was measured at 540 nm. The standard curve was determined by plotting absorbance of recombinant TNF- $\alpha$  series against concentrations.

### **2.1.3.9 Detection of listerial haemolytic activity**

The aim of this experiment was to measure any different level of hemolytic activity released by *L. monocytogenes* infection in both genotypes. Haemolytic assays were performed to detect the biological activity of *L. monocytogenes* in supernatants from dendritic cells and macrophages from wild type and properdin-deficient mice. Cells were infected with *L. monocytogenes* MOI= 0.2 in 10 ml medium with number of cells  $5 \times 10^6$ /ml.

#### **2.1.3.9.1 Haemolysis Assays**

Haemolytic assay was performed to assess the biological activity of *L. monocytogenes* using horse erythrocytes. 10 ml of horse blood was centrifuged at 1500 x g for 15 minutes at 4°C, and then diluted in PBS (pH 7.4) to obtain a 4% (v/v) suspension. In 96-wells microtitre plate (round bottom) serial dilution of two fold dilutions (1:2) of the

culture supernatant of infected cells with *L. monocytogenes* for 24 hours in PBS were mixed with 50 µl of 4% RBC and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator for 45 minutes. The ELISA plate was centrifuged for 10 minutes at 300g, then supernatant was removed and transferred to the new 96 wells plate and after that the absorbance of released haemoglobin was read at 541 nm in ELISA plate reader (BIORAD).

### **2.1.3.11 The role of exogenous IFN- $\gamma$ to reduce intracellular counts of dendritic cells and macrophages**

The aim of this experiment was to see whether properdin has a role in the IFN- $\gamma$  mediated response to *L. monocytogenes*.

Bone marrow derived dendritic cells and macrophages from wild type and properdin-deficient mice were infected with *L. monocytogenes* (MOI 0.2). Numbers of the cells per well were the same. After 1 hour, the medium was removed and new medium with gentamicin was added, and incubated for another 1 hour; then this was removed and new medium was added with exogenous IFN- $\gamma$  (1ng/ml) (Mouse IFN- $\gamma$  recombinant protein, eBioscience Cat# 14-8311) in 10% (w/v) BSA-PBS. For the control, DTT (1mM) with 10% (w/v) BSA-PBS was added. Plates were incubated for 30 minutes and 24 hours at 37°C and viable counts of *L. monocytogenes* were determined.

### **2.1.3.12 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Bone marrow derived dendritic cells and macrophage from wild type and properdin-deficient mice were used to analyse the changes of mRNA expression using different genes related to infection with *L. monocytogenes* under different oxygen conditions. Uninfected dendritic cells and macrophages were used as controls. Dendritic cells were

infected for 24 hours with heat killed *L. monocytogenes*. Macrophages were infected with live *L. monocytogenes* at 5 and 24 hours. The numbers of cells counted for each experiment and the MOI was 0.2. 75 cm<sup>2</sup> flasks were used.

After infection, the cells were washed with PBS. After harvesting using cell scraper, cells were centrifuged at 433g for 5 minutes. The cells were washed with PBS before preparation of RNA of dendritic cells and macrophage followed by extraction quantification.

Total RNA was extracted using Trizol Reagent (Ambion) following the manufacturer's instruction. 1 ml Trizol was added to each sample to lyse the cells then incubated for 5 minutes at room temperature. 200 µl of chloroform were added and the tubes were shaken for 5 seconds and incubated for 3 minutes then centrifuged at 12,000g for 15 minutes. The aqueous phase containing nucleic acids was collected in new tubes. To the transferred, aqueous phase was added 500 µl isopropanol to precipitate the RNA for 10 minutes. After centrifugation for 10 minutes at 12,000g the supernatant was removed and RNA was washed with 1 ml of 75 % (v/v) ethanol followed by centrifugation for 5 minutes at 7,500g. After drying, the RNA pellets were resuspended in DEPC-treated water. At this point, possible contamination with genomic DNA can be removed by DnaseI digest (37°C, 30 minutes) and nucleic acids are re-purified using Trizol reagent. RNA concentration in samples was estimated by diluting the samples into Diethylpyrocarbonate 0.2% (v/v) treated water and the concentration of RNA samples were measured using a Nano Drop spectrophotometer ND-1000 (Thermo Scientific) according to the manufacturer's protocol.

From total RNA, first strand cDNA was synthesised by using superscript first strand synthesis system (RETROScript, Ambion and BioScript Bioline kit) following

manufacturer's instruction. 1 µg of total RNA with random decamer primers (0.2 µg) were denatured for 5 minutes at 70°C (2 µl of random decamer primers and DEPC water were added up to final volume 12 µl), after that were chilled on ice. The following master-mix containing 4 µl of 5 x reaction buffer, 1 µl of 10 mM dNTP mix, 0.5 µl of an RNase inhibitor (20 unit/µl) and 0.5 µl of BioScript enzyme (200u/µl) were added to a final volume 20 µl for one reaction. After that the mixture was incubated at 42 °C for 1 hour then the reaction were stopped by heating at 70 °C for 10 minutes. The cDNA for the samples and negative control (water without samples) were synthesised at the same time.

Moreover, alternative experiment and the program were used, cDNA was synthesised using (Invitrogen) following manufacturer's instructions, 1 µg of total RNA was used for template RNA, as before, then 2 µl of oligo dT primers (50-250 ng), and 1 µl of dNTP mix (stock 10mM) were added in final volume of 12 µl. After heating the mix to 65°C for 5 minutes and chilling on ice, the following master-mix was added: 4 µl of 5 x buffer first-strand, 2 µl of 0.1M DTT, 1 µl of RNase OUT to final volume 20 µl, then the mixture were incubated at 25 °C for 2 minutes. After that 1 µl of SuperScript Reverse Transcriptase was added and incubated for 10 minutes at 25 °C and then incubated at 42 °C for 50 more minutes. After that the mixture was incubated at 70 °C for 15 minutes to inactivate the enzyme. To degrade the RNA template RNAase H were added to newly synthesised cDNA and incubated for 37 °C for 20 minutes. At the end the cDNA was kept on -20°C until further use.

Then cDNA mixture was used for amplification of target genes. Gene expression of C3, CD11b, TLR2 and β-actin (100µM stock) were analysed by RT-PCR in dendritic cells from wild type or properdin-deficient mice after 24 hours' infection with heat killed *L.*

*monocytogenes*. The same genes, including IFN- $\beta$  were expressed in macrophages from wild type or properdin-deficient mice after 5 hours' infection with live *L. monocytogenes*.

$\beta$ -actin was chosen as a housekeeping gene because its expression that does not change in hypoxia (Mancino *et al.*, 2008) as some experiments were expressed the genes in hypoxia compared to normoxia. VEGF gene was used as a positive control of hypoxia (Shweiki *et al.*, 1992). In addition, expression of C3, CD11b, Toll-like receptors (TLR2) and IFN-  $\beta$  genes were interrogated after infection with *L. monocytogenes*.

To optimise amplification, pilot reactions are set up to include a non-template control and a positive control (cell line expressing the gene of interest). In the case of low amplification, cycle numbers can be increased, annealing temperature can be decreased, and amount of template changed. Amplification of a housekeeping gene is run in parallel.

To assess amplification efficiency, cDNA was serially diluted and analysed for gene amplification using agarose gel electrophoresis.

**Table 2.1:** Primer sequences, annealing temperatures and the expected sizes of each amplicon generated in this study.

Gene Name	Sequence of primers	T <sub>A</sub>	Product size (bp)	Reference sequence accession number NCBI
β-actin	5' GTGGGCCGCTCTAGGCACCAA-3' 5' CTCTTTGATGTCACGCACGATTTC-3'	55°C	540 bp	X03765.1
TLR2	5' GGCCAGGTTCCAGTTTTTCAC-3' 5' GGAACAACGAAGCATCTGGG-3'	55°C	548 bp	NM_011905
CD11b	5' GATGGTGTTCGAGCTCTCTGCG-3' 5' TTGTCTCAACTGTGATGGAGCA-3'	55°C	397bp	NM_008401
C3	5' GAATACGTGCTGCCAGTTT-3' 5' TGAGTGACCACCAGCACTTT-3'	55°C	500 bp	NM_009778
IFN-β	5' ATGAGTGGTGGTTGCAGGC-3' 5' TGACCTTTCAAATGCAGTAGATTCA-3'	55°C	82 bp	NM_010510
VEGF	R&D systems	55°C	235 bp	NM_009505

The reaction was performed by mixing the following reagents: 1 x of 10 x reaction buffer IV, 1.5 mM of 25 mM MgCl<sub>2</sub>, 0.2 mM of 1.5 mM dNTPs mix, Thermoprime enzyme 5U/μl, 0.5 μM of each primer (forward and reverse) (100 μM stock), 2 μl of template cDNA and water to 25 μl final volume of each PCR reaction.

After mixing each reaction was placed into a thermocycler (Gene Amp PCR System 9700, PE Applied Biosystems) and followed the program shown as an example in the table.

**Table 2.2:** Program of the amplification in thermocycler.

Number of steps	Temperature	Time	Number of cycles
1	95°C	3 minutes	1
2	95°C	3 minutes	30
	55°C	1 minutes	
	72°C	1 minutes	
3	72°C	10 minutes	1
	4°C	Hold	

PCR products were analysed using agarose gel electrophoresis. Using 1% (w/v) agarose gel and this was prepared by adding a solution of a suitable percentage of agarose into 1 x TBE Buffer (Tris Borate EDTA) and heated it up in a microwave to dissolve the agarose. After the solution cooled down, then ethidium bromide was added to give a final concentration of 0.25 µg/ml. The solution was poured immediately into a casting apparatus with comb and was left to set for about 15 minutes. The samples were loaded into the agarose gel wells into running tank with 1 x TBE buffer and electrophoresis was performed at a constant voltage of 90 V for 45 minutes. Then the DNA on the gel was visualised using (UVP) UV Transilluminator and pictures for the gel were taken by the DC120 digital camera. 1.8 % (w/v) agarose was made to analyse amplification of IFN-β. In addition, BenchTop 1kb DNA ladder (promega) and 50bp DNA ladder Biolabs (1 µl gel loading dye with 1 µl of 1mg/ml 50 bp DNA ladder and 4 µl dH<sub>2</sub>O) were loaded into the gel at the same time with the samples and negative control.

**2.1.3.13 Quantitative analysis of mRNA expression by Real-Time RT-PCR (qPCR)**

Bone marrow derived dendritic cells and macrophages from wild type and properdin-deficient mice were prepared and infection with *L. monocytogene* as described in section 2.1.1.2 and 2.1.1.3. Q-PCR was used to measure the mRNA levels of a number of genes in response to *L. monocytogenes* due to its high sensitivity.

Total RNA was isolated and extracted with Trizol and cDNA was synthesised as described in section 2.1.3.12. After cDNA was synthesised from extracted RNA from each sample was used as templates in qPCR using SYBR Green PCR kit (BioScript Bioline) to see any difference in genotypes after infection with *L. monocytogenes* in mRNA expression. The primers were the same as used in reverse transcriptase RT-PCR (C3, TLR2 and  $\beta$ -actin) with same annealing temperature as shown in table 2.1. mRNA expression levels of C3 and TLR2 were determined by qPCR in macrophages from both genotypes after infection for 5 and 24 hours with live *L. monocytogenes* but in dendritic cells from both genotypes after infected 24 hours with heat killed *L. monocytogenes*.

In addition, expression of properdin was studied to see whether there is any change of the properdin expression after infection compared to uninfected and the primer sequences used were as shown in table 2.3. Moreover, expression of mFc $\gamma$ RIIb and mFc $\gamma$ RIV were studied in macrophage from wild type and properdin-deficient mice after 24 hours infection with *L. monocytogenes*.

The qPCR reactions were performed in 20  $\mu$ l total volume using 2  $\mu$ l of cDNA (previously diluted 1:4 in dH<sub>2</sub>O), 10  $\mu$ l SYBRGreen (containing MgCl<sub>2</sub>) and 2 $\mu$ l of mix of forward, reverse primers for genes of interest (100 $\mu$ M stock) (5  $\mu$ l forward with 5  $\mu$ l reverse in 40 dH<sub>2</sub>O for total volume 50  $\mu$ l) and up to 20  $\mu$ l by adding 6 $\mu$ l nH<sub>2</sub>O.

## Chapter Two Material and Methods

**Table 2.3:** Primer sequences, annealing temperatures and the expected size of properdin, mFcγRIIb and mFcγRIV generated in this study.

Gene Name	Sequence of primers	T <sub>A</sub>	Product size (bp)	Reference sequence accession number NCBI
Properdin	5'-TTCACCCAGTATGAGGAGTCC-3` 5'-GCTGACCATTGTGGAGACCT-3`	62°C	149bp	NM_008823.3
mFcγRIIb	5'-CTGAGGCTGAGAATACGATC-3` 5'-GTGGATCGATAGCAGAAGAG-3`	60°C	307 bp	NM_001077189.1
mFcγRIV	5'-GTGACCCTCAGATGCCAAGGC-3` 5'-TGGATGGAGACCCTGGATCGC-3`	68°C	461 bp	NM_144559.2

Negative control (non-template control) using water instead of the cDNA template was used. Gene expression levels are presented relative quantification based on comparison to  $\beta$ -actin mRNA expression. Strip tubes and caps 0.1 ml (strips of 4 tubes and caps) were used.

The samples of 20  $\mu$ l total volume qPCR reaction were run on the Corbett: Rotor-Gene™ 6000 machines and software. The temperature cycling conditions was 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 20 seconds, optimized annealing temperature for 30 seconds, 72°C for 20 seconds and 74°C for 20 seconds, the melting curve program consisted of temperatures between 55°C to 99°C.

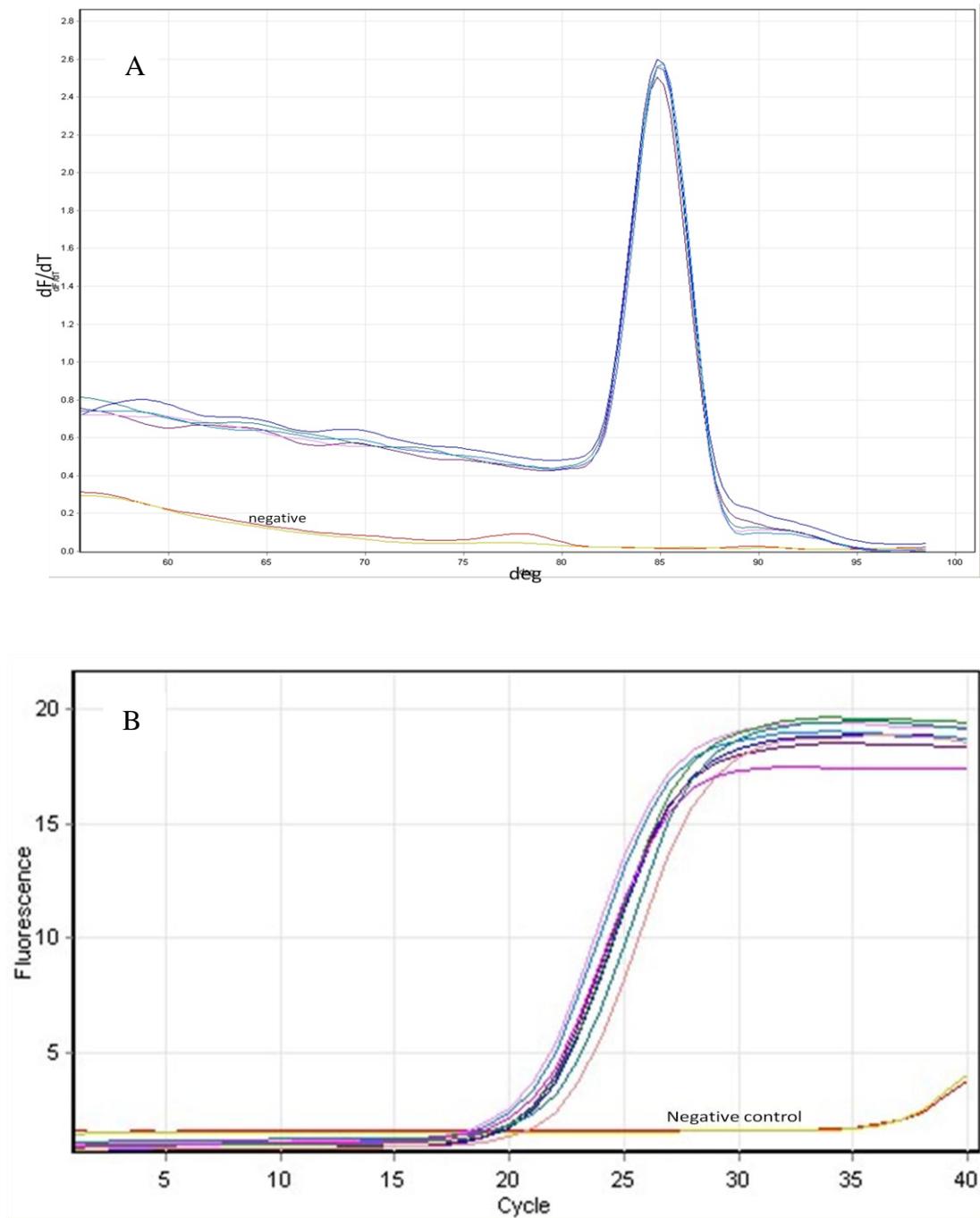
The fluorescence signal was measured at the end of each elongation step. The melting curve analysis allows determining that only one product was amplified and to verify the absence of contaminants or primer dimer products when only one distinct peak for each sample for each gene is seen.

The light cycler software plot the single peak ( $T_m$ ) at around 80-90 °C as example in Figure 2.4 (A) show optimization of good amplification.

Reaching of melting temperature ( $T_m$ ) will separate the strands of DNA and lose the binding to the dye SYBR Green I which lead to a rapidly reduced fluorescent signal (Ginzinger, 2002).

All experiments were run in duplicate and non-template controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. Figure 2.4 (B) shows the amplification curve of each sample after 40 cycles. The threshold cycle (CT) of each target product was determined and set manually in relation to the amplification plot of  $\beta$ -actin. The detection threshold is set to the log linear range of the amplification curve. In addition, the expected size and the absence of nonspecific products were confirmed by running the real-time PCR products on agarose gels.

The CT value is number of PCR cycles and the point at which the product's detected fluorescence crosses the threshold. 40 cycles are run and CT value under the threshold during all the 40 cycles means a negative result, or the expression is so low or undetectable.



**Figure 2.4:** Example of the melting curve and amplification curves from the qPCR analysis. (A) example of the melting curve analysis of the macrophage expression of Fc $\gamma$ RIV gene of bone marrow derived macrophae infected *in vitro* with *L. monocytogenes*. Temperature was plotted against dF/dT [rate of change of fluorescence (F) in the reaction with time (T)]. (B) The graph shows the amplification curves from the qPCR analysis; each assay target is represented by a different colour.

**2.1.3.14 Analysis of complement C3 activation in mouse serum incubated with *L. monocytogenes***

The ability of *L. monocytogenes* to activate the alternative pathway of complement and the role of properdin was studied in this *in vitro* experiment. Western blot analysis was used to evaluate the generation of C3 fragments in buffer conditions favouring alternative pathway activity. Live and heat-killed *L. monocytogenes* were used to activate mouse serum.

*L. monocytogenes* were washed once with PBS, to remove residual BHI-medium, and then equilibrated with the buffer needed for the alternative pathway activation assay (GVB-Mg<sup>2+</sup>-EGTA) (10 mM) (Sigma). Live and heat-killed *L. monocytogenes* were analysed in parallel for their ability to activate complement C3. The final concentration of mouse serum was 5% (1:20 dilution, final volume 80 µl). After 30 minutes incubation at 37°C, the samples were centrifuged for 2 minutes at 16.000g, then the supernatants were separated using a 12% resolving gel SDS-PAGE (denaturing condition) and analysed by western blotting. Before loading, the samples were diluted in β-mercaptoethanol SDS loading dye and were boiled at 95 °C for 5 minutes, then put directly in ice. They were separated on 1 x SDS-PAGE at 90 volts for about 10-15 minutes then the voltage was increased to 120 V until the dye reached the end. The separated proteins on the gel were electrophoretically transferred to a nitrocellulose membrane (0.2 µm trans-blot transfer medium; Bio-Rad Laboratories) using transfer buffer (1 x SDS running buffer with 10 % methanol). Prestained protein marker (Broad range cat: P7708V New England Biolabs) was used.

After blotting at 30 V overnight, the membrane was blocked with 5% (w/v) semi-skimmed milk and PBS with slowly shaking for 30 minutes, then blocking solution was changed and biotin labelled rat anti-mouse complement component C3 antibody

(Cedarlane) (1:100) was added and incubated for 2 hours with slow shaking. Then the membrane was washed with PBS-Tween 20 0.1% (v/v) (three times, 10 minutes each wash). After that, the biotin label was conjugated with streptABCComplex/HRP (ABC kit, DakoCytomation) (Code# K 0377) according to the manufacturer's instructions. After that the membrane was washed as above, and exposed to ECL reagent (Pierce kit) (Pierce ECL western Blotting Substrate Cat# 32209). In plastic foil the membrane was exposed to ECL by mixing equal volume of detection reagent 1 (Peroxide solution) and detection reagent 2 (Luminol enhancer solution) to yield a final volume sufficient to cover the membrane for a minute. After that the membrane was exposure to X-ray film (autoradiography) by using the developer solution and fix solution, for 10 seconds each solution then washed with water.

### **2.1.3.15 Bactericidal effect of mouse serum on live *L. monocytogenes***

To study the bactericidal effect of mouse serum on *L. monocytogenes*, pooled serum from uninfected wild type and properdin-deficient mice were incubated with *L. monocytogenes* for 30 minutes and 24 hours.

Viable count after complement C3 activation in mouse serum incubated with *L. monocytogenes* were determined in BHI-agar after 30 minutes incubation with *L. monocytogenes* in GVB-Mg<sup>2+</sup>-EGTA buffer/ 5% mouse serum. However, GVB-Mg<sup>2+</sup>-EGTA buffer contains Mg<sup>2+</sup> and *L. monocytogenes* used it to grow more, therefore serum were added to PBS. 50µl of serum from each genotype was added to 50µl PBS with *L. monocytogenes* (CFU log 8.21 for 30 minutes number of bacteria start with and total volume start with 100µl) in each tube, after 30 minutes 20µl were taken and added to 80µl of total volume 1 ml, then serial dilutions were made by 10 fold CFU/ml. The

remaining 80µl (log 7.119 for 24 hours number of bacteria start with and total volume start with 80µl) were incubated for 24 hours.

**2.1.3.16 Bactericidal effect of whole human blood on live *L. monocytogenes***

To study the contribution of whole human blood to control of *L. monocytogenes*, whole human blood was incubated with different doses of *L. monocytogenes*  $2.3 \times 10^3$ /ml and  $1.7 \times 10^6$ /ml and  $6.3 \times 10^6$ /ml for 30 minutes, 1 hour and 24 hours at 37 °C, with slow shaking or without shaking. Viable counts were determined on BHI-agar CFU/ml with or without lysing cells with water.

**2.1.3.17 Antigenic maturation of dendritic cells from wild type and**

**properdin-deficient mice after infection with heat-killed *L. monocytogenes***

The maturity of dendritic cells can be characterised by surface expression of key molecules, which are involved in antigen presentation. Flow cytometry was used to measure the expression of surface markers of uninfected and infected dendritic cells. Cells from bone marrow were isolated and dendritic cells were differentiated for 7 days *in vitro*. After 1.5 hours infection with heat-killed *L. monocytogenes* (MOI=0.2), dendritic cells were harvested and dispensed at  $5 \times 10^5$  cells per pellet (per tube), then the cells were washed once with ice cold PBS and were centrifuge at 277g for 10 minutes. The supernatant was removed and the cell pellets were re-suspended in the drop of buffer remaining in the tube. Specific antibodies were added to the cell samples with a concentration of up to 1µg/µl and incubated for 30 minutes at 4°C in the dark. The cells were typed for CD11c and surface markers (FITC (fluorescein isothiocyanate) labelled anti-mouse CD40, CD80, CD86 and MHCII) (BD Biosciences Pharmingen, Oxford, UK). CD11c represents the main surface marker for the dendritic cells lineage.

An isotype control of mouse IgG, FITC and PE (phycoerythrin) labelled, were used. The cells were washed twice with wash buffer (PBS with 2% BSA) and centrifuged at 277g for 10 min. The supernatant was removed and pellets were re-suspended and flicked in the remaining drops of wash buffer and then fixed in 2% paraformaldehyde and incubated at 4°C in the dark before acquiring using Becton Dickinson FACS Scan and CellQuest pro software.

In all cases, the dendritic cells were evaluated using the following 2-color combinations: IgG FITC with PE anti-mouse CD11c and IgG PE with MHC-II-FITC. Data were collected for 10000 cells/sample.

### **2.1.3.18 Expression of Fc $\gamma$ RIII/II (CD16/CD32)**

Flow cytometry was used to analyse the expression levels of Fc $\gamma$ RIIB on B-cells from mouse spleen after infected with *L. monocytogenes*.

The aim of this experiment was to measure surface expression of CD16/CD32 in mouse splenic macrophages and neutrophils but these were difficult to detect even with the help of an expert (Dr L. Machado, Department of Genetics). Therefore, B-cells were used because Fc $\gamma$ RIIB is the only Fc $\gamma$ R which is expressed by B cells (Smith & Clatworthy, 2010).

To prepare splenocytes from spleen, whole spleens from wild type and properdin-deficient mice were harvested from mice and were placed in PBS in a petri dish. By using sterile forceps, the spleens were placed on a sterile cell strainer mesh (BD falcon 70 um Nylon) and RPMI 1640 media with 10% FCS were used to wet the cell strainer first, then the spleens were pushed through the cell strainer with the plunger of a 10 ml syringe into the 50 ml tubes and were rinsed with media every time to get the cells through the suspension. Next the cells were centrifuged at 277g for 5 minutes. After that

supernatant were discarded and red blood cells were lysed by adding 1 ml lysing buffer (hypotonic 0.87% Tris-NH<sub>4</sub>CL pH 7) for 5 minutes at room temperature and within the 5 minutes, tubes were shaken gently. Then, to neutralise the cells lysis, 10 ml of media was added and centrifuged again at 277g for 5 minutes two times. After that the cells were counted using a hemocytometer or cell counter.

Next, splenocytes from wild type and properdin deficient mice were infected with heat killed *L. monocytogenes* with MOI of 20 and 80 overnight in 12 wells plate. After infection the numbers of cells were adjusted at a concentration of  $5 \times 10^5$ / tube for each genotype, and then cells were stained.

Uninfected and infected splenocytes were subsequently stained with PE-labelled B cell-specific rat anti-mouse CD45R/B220 and FITC rat anti-mouse CD16/CD32 (Fc $\gamma$ III/II receptor) (2.4G2) (BD Pharmingen) (BD Biosciences). An isotype control of FITC rat IgG 2a was used with B-cells. All antibodies that used were listed in the table below. After adding the antibodies incubated for 30 minutes at 4°C. After incubation, the cells were washed in PBS with 2% (w/v) BSA. Then, the cells were fixed in PBS containing 2% (w/v) paraformaldehyde. Fluorescence intensity was measured using a FACS flow cytometry and CellQuest software.

Isotype-matched controls were included to determine background fluorescence in each experiment, after cells were stained with antibodies against CD32, were analysed on a FACSCalibur with CellQuest Software (BD Biosciences) and the Fc $\gamma$ RIIb expression was analysed on gated B cells.

Uninfected cells were stained with different titration of the antibody to determine the optimal titration of the antibodies therefore specific antibodies were added to the cell samples with a concentration of up to 1 $\mu$ g/ $\mu$ l, 0.5  $\mu$ g/ $\mu$ l, 0.25  $\mu$ g/ $\mu$ l and 0.05  $\mu$ g/ $\mu$ l. So the concentration of 0.05  $\mu$ g/ $\mu$ l are shown.

Null tubes without antibodies were used and for compensation, FITC rat anti-mouse CD16/CD32 and PE rat anti-mouse CD45R/B220 were used. For FITC compensation, FITC rat anti-mouse CD16/CD32 were used and for PE compensation, PE rat anti-mouse CD45R/B220 were used.

The following FITC- and PE-conjugated antibodies were used:

**Table 2.4:** FITC- and PE-conjugated antibodies.

NO		Antibody	Stock concentration
1	B cell	PE rat anti-mouse CD45R/B220	0.2 mg/ml
2	CD32	FITC rat anti-mouse CD16/CD32	0.5 mg/ml
3	neutrophil	PE rat anti-mouse Ly-6G	0.2 mg/ml
4	Macrophage	CD11b PE	0.2 mg/ml
5	Isotype	FITC rat IgG 2a	0.5 mg/ml

### 2.1.3.19 Proteomic analysis of dendritic cells from wild type and properdin-deficient mice after infection with *L. monocytogenes*

The idea behind this experiment was to identify the protein changes in dendritic cells that occur during infection with *L. monocytogenes* using two-dimensional gel electrophoresis.

Dendritic cells were purified and cultured, then infected with heat-killed *L. monocytogenes* for 1.5 hours. Then the medium was removed after centrifuged and rinsed with PBS to wash away any excess *L. monocytogenes* or medium, the cells were scraped and washed with PBS and centrifuged again to pellet at 433g for 5 minutes, then the cells pellet were re-suspend with lysis buffer, 40 mM Tris with DnaseI and

Rnase A then were sheared with a needle. The proteins were frozen in  $-80^{\circ}\text{C}$  until used, and uninfected cells were used as controls.

Protein concentrations were adjusted to 50 mg/ml and samples frozen (The concentration was used that had been optimised in the adjoining laboratory whose equipment was used). The protein concentration of each sample was determined by recording the absorbance at 280nm using a NanoDrop ND-1000 spectrophotometer according to the manufacturer's protocol. For each 7cm strip ReadyStrip IPG Strip (BIO-RAD, UK) 100 $\mu\text{g}$  protein was used. Then the samples were solubilised and desalted following the instruction manual of PIERCE for desalting column: 2D sample prep for insoluble proteins (from Pierce perbio Bioscience cat. No: 89866). The total volume to load onto column is 50 $\mu\text{l}$ . The samples were then applied onto isoelectric focusing immobiline DryStrips of 7cm, the IPG-strips were rehydrated with rehydration buffer (0.850g urea, 0.304g thiourea, 0.08g chaps, 10 $\mu\text{l}$  ampholyts, 24 $\mu\text{l}$  destreak and 0.25 % of bromophenol blue solution and up to 2ml with nanopure  $\text{H}_2\text{O}$ ) for each strip were loaded a maximum 125 $\mu\text{l}$  (50 $\mu\text{l}$  of sample and 75 $\mu\text{l}$  of rehydration buffer).

After rehydration of IPG-strips for 17 hours at  $20^{\circ}\text{C}$ , proteins were focused for 20,000 Vh at room temperature, after that were run on SDS gel electrophoresis (vertical direction) Cast 12% SDS electrophoresis gel, with only resolving gel 30 mA per gel. The strip was applied on top of the gel and overlain with the blue overlay agarose.

Prior to the second dimension, the strips were equilibrated in buffer containing (0.3g DTT, 5.40g urea, 3.75 ml 1.5M Tris-HCl pH 8.8, 3 ml 10% (w/v) SDS, 3 ml glycerol up to 15ml with np $\text{H}_2\text{O}$ ) for 10 minutes on a shaker at room temperature by adding 2 ml on each strip, and then in same buffer but containing 0.375g Iodoacetamide instead of DTT for another 10 minutes.

The proteins were then separated according to their molecular mass using standard SDS-PAGE. An ultrasensitive Coomassie-based reagent (RAPIDstain™ Reagent Cat# 553215) was used. The gel was washed three times in ultra-pure water 5-10 minutes per wash and the water discarded from the gel, then RAPIDStain were added to cover the gel, and incubated with shaking gently in stain for about 1 hour. After that the gel was washed three times with nanopure water. The gels were quantified by densitometry (G5-710 instrument with Quantity One software; BioRad).

### **2.1.3.20 Expression of LAMP-1 by western blot**

Bone marrow derived dendritic cells and macrophage from wild type and properdin-deficient mice were cultured in tissue culture 75 cm<sup>2</sup> flasks and infected with heat-killed *L. monocytogenes* MOI 0.2 for 1.5 hours, after infection, cells were washed with PBS then lysed with lysis buffer (40 mM Tris with DnaseI and Rnase A), and were sheared with a needle. The proteins were frozen in – 80 °C until used, the uninfected cells were also used. The aim of this experiment was to investigate the difference of lysosome in both genotypes by expression of lysosomal-associated membrane protein 1 (LAMP-1) because there seemed to be differences in numbers of lysosome observed in EM.

20µg sample protein concentration was used for total volume 12µl and 3µl of 4 x loading dye (B-Me SDC). Samples were boiled at 95 °C for 5 minutes, and were loaded on the gel then transferred to the membrane as described (western blot protocol as described in section 2.1.3.14). After blocking the membrane with 5% semi-skimmed milk and PBS with slow shaking for 30 minutes, the primary antibody (LAMP-1) lamp1 (C54H11) cell signaling Rabbit mAb (mouse antibody) dilute 1/1000 were added in 10 ml PBS and left with slow shaking overnight in 4 °C.

## Chapter Two Material and Methods

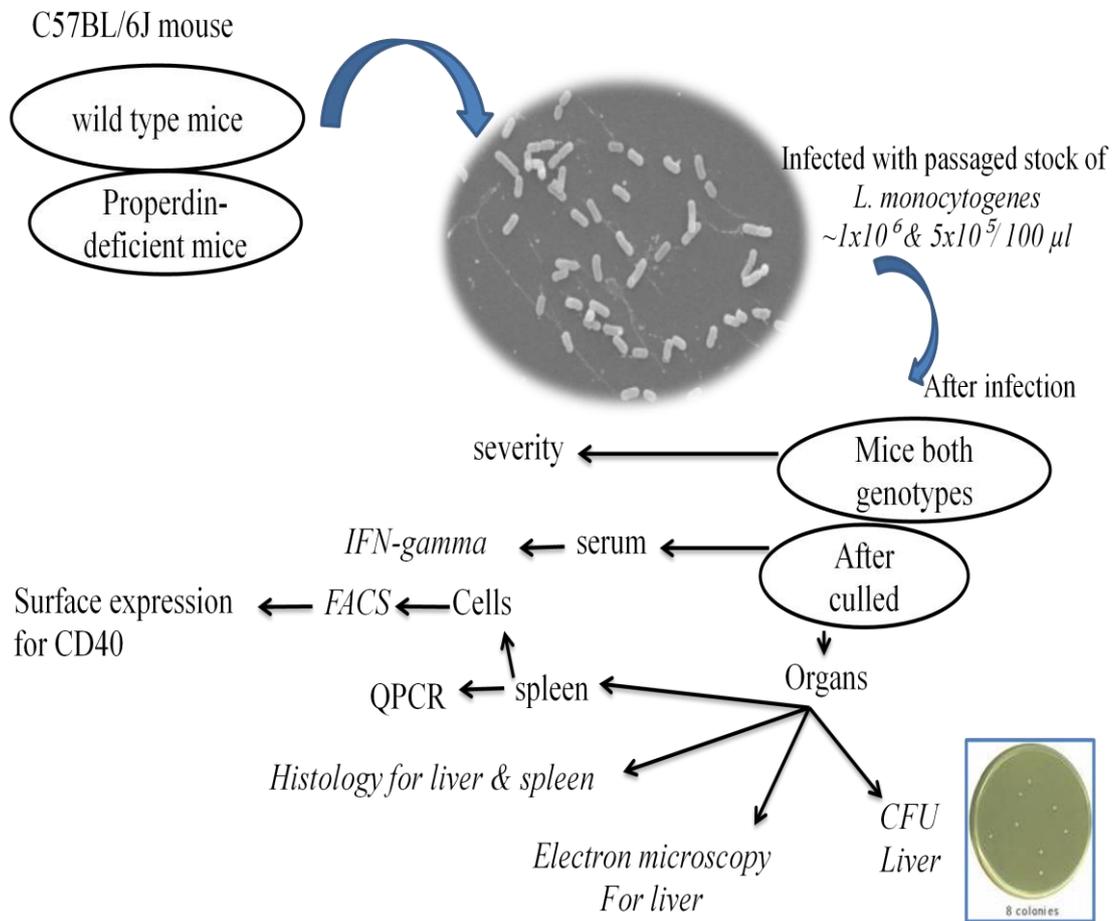
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Next day, the membrane was incubated with secondary antibody Goat anti-Rabbit HRP (diluted 1:5000) while shaking at room temperature for 1 hour. As described, the membranes were washed, was exposed to ECL reagent then exposed to X-ray film.

*Material and methods in vivo model  
of murine Listeriosis*

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2.2 *In vivo* model of murine Listeriosis



**Figure 2.5:** Overview of *in vivo* experiments. Wild type and properdin-deficient mice were infected with passaged *L. monocytogenes*. Mice were culled when they were lethargic or at an earlier end time point. Liver, spleen and blood were collected and different experiments with different methods were done to answer the question if there is any difference between wild type and properdin-deficient mice. Image of *L. monocytogenes* was taken from this study.

### 2.2.1 Generation of passaged bacterial stock for infection

The purpose of this experiment was to generate a stock of virulent *L. monocytogenes*.

The inoculum for infection was prepared from an overnight culture of single colonies of *L. monocytogenes* in 10 ml BHI broth volume, 37°C, with shaking 220 rpm then the pellet was re-suspended in 10 ml PBS. Two wild type mice were injected intraperitoneally (i.p.) with  $1 \times 10^9$  CFU/ml in 100  $\mu$ l PBS from stock that used in *in vitro* study.

The mice were culled when lethargic (about 3 hours), blood was collected by cardiac puncture and 50 $\mu$ l was used to inoculate 10 ml of BHI broth then incubated overnight with shaking at 37°C. The bacteria were grown to mid-log phase at 37°C with shaking in BHI-broth to generate a frozen stock from these passaged bacteria, and this was made by centrifugation at 3000xg for 15 minutes and re-suspended in BHI containing 10% (v/v) glycerol, before freezing at -80°C in 1 ml aliquots.

### 2.2.2 Murine model of listeriosis-intravenous infections of mice with *L.*

#### *monocytogenes*

Frozen vials were thawed and the bacteria were washed once with sterile PBS, and then diluted. A comparable number of wild type and properdin-deficient mice were infected intravenously (i.v.) with the passaged stock adjusted to  $5 \times 10^5$  CFU per 100 $\mu$ l of sterile PBS. In other experiments, the mice were injected with  $1 \times 10^6$  and  $9 \times 10^5$  CFU of sterile PBS. The numbers of bacteria inoculated were determined by colony counts performed from the stock used for infection.

Moreover, 3 wild type and 3 properdin-deficient mice were infected with  $1 \times 10^6$  *L. monocytogenes* and culled after 28-29 hours (matched endpoints). This time was chosen

because earlier experiments showed in this time numbers of mice from properdin-deficient group were lethargic after infection more than wild type mice.

Intravenous injection was used because it results in rapid systemic infection, and bacteria isolated from the liver and spleen result from parenchymal infection. Infection via the gastric route is less reliable to cause systemic disease at this dose.

After the infection, mice were culled then liver and spleen were removed and also bloods were collected by cardiac puncture. Then bloods were centrifuged at 700g for 5 minutes and the supernatant were transferred to new 1.5 ml tube and this was repeated one more time. Then sera were kept in -80 °C.

### **2.2.3 Survival of *L. monocytogenes* infection**

Mice after infection with *L. monocytogenes* were observed for signs of disease, such as hunched, starey or lethargic. Survival of mice was monitored over 5 days post infection or for 14 days, depending on the experiment, and expressed as a percentage of survival for individual infections using between  $5 \times 10^5$  and  $1 \times 10^6$  CFU of *L. monocytogenes*.

When mice reached a lethargic state or became moribund they were culled immediately and the time of death was recorded. In some experiments, the mice survived till the endpoint. In general infection, any mice alive at 96 hours were considered to have survived the infection. The first experiment was a pilot experiment and both experiments using a dose of  $1 \times 10^6$  and  $5 \times 10^5$  of *L. monocytogenes* were performed twice. In addition, one experiment with  $1 \times 10^6$  of *L. monocytogenes* mice was culled at the same time after 28-29 hours.

Age-matched groups of mice were used to study the role of properdin in sex differences, seven female and male mice from properdin-deficient and eight female and male mice from wild type were infected with  $5 \times 10^5$  of *L. monocytogenes*, and mice were

observed for about 7 to 14 days after infection. This experiment was done based on study by Pasche *et al.*, 2005 that showed different susceptibility between male and female mice after i.v. infection.

### **2.2.4 Determination of number of viable counts of *L. monocytogenes* in the organs**

Most bacteria like *L. monocytogenes* that enter the bloodstream by i.v. injection are taken up by the liver and the reason to use this organ is macrophage-phagocyte system (MPS). Liver and/or heart in some experiment were removed, weighed and homogenized in 10 ml PBS (for extracellular bacterial count) or in some experiment in distilled de-ionised water (dH<sub>2</sub>O) (for intracellular bacterial count) using Ultra-turax T8 (IKA-WERKE) for a few seconds. The numbers of *L. monocytogenes*/g in the organs of infected animals were established by plating serial 10-fold dilutions of organ homogenates on BHI-agar plate and incubating at 37°C for 24 hours. Counts were performed on the homogenates and the number of CFU determined per gram of tissue.

### **2.2.5 Quantitative measurement of mouse IFN- $\gamma$ from mice after infection with *L. monocytogenes***

To determine the level of serum IFN- $\gamma$  of infected mice, bloods were collected by cardiac puncture at the indicated days after infection. Sera from mice wild type and proportion-deficient mice were pooled and stored at -80°C. Cytokine assays were performed by ELISA and murine IFN- $\gamma$  ELISA Development Kit was used as described before (section 2.1.3.5).

### 2.2.6 Determination of anti-listeria IgM and IgG antibodies

Whole blood was collected from wild type and properdin deficient-mice by cardiac puncture after infection with  $1 \times 10^6$  and  $5 \times 10^5$  CFU of *L. monocytogenes* and serum were isolated as described to determine the presence of specific anti-listeria immunoglobulins IgG and IgM.

The ELISA method was adapted from (Russell *et al.*, 2000; Wernette *et al.*, 2003). Microtiter 96-well plates (Nunclon Maxisorb, Wilford UK) were coated overnight at 4°C with 100µl of *L. monocytogenes*  $1 \times 10^8$ / ml in 0.1M carbonate buffer, pH 8.5, to adhere to wells. The next day, the plate was left for 30 minutes at room temperature, and was washed four times with washing buffer (1 x PBS, 0.05% Tween 20). The plate was blocked with a 200µl blocking solution to each well (1 x PBS, 1% BSA) for 1 ½ hour at 37°C. Then, the plate was washed four times and serum samples were added to the plate with serial dilution (100 µl or 50 µl of serum were diluted started with 1:1.5). The plate was incubated for 1 hour at 37°C, and then it was washed four times. To detect serum IgM specific against *L. monocytogenes*, 100 µl of goat anti-mouse IgM antibodies HRP labelled (Bethyl Laboratories) diluted 1:10,000 with conjugate diluent (1 x PBS, 1% BSA, 0.05% Tween 20), and goat anti-mouse IgG antibodies HRP conjugated were added and incubated for 1 hour at 37°C. Again after incubation, the plate was washed for four times. 100 µl of 3,3',5,5'-tetramethylbenzidine chromogen substrate (TMB, Bio-Rad Hemel Hempstead UK) was added. The reaction was stopped after appearance of an optimal blue colour by adding 100 µl stop solution (0.8M H<sub>2</sub>SO<sub>4</sub>), which produced a yellow colour. The optical density was read at 450 nm using an ELISA plate reader (model 680, Bio-Rad).

### **2.2.7 Measurement of complement components C3**

Serum levels of C3 were determined by sandwich ELISA according to the manufacturer's manual using a mouse C3 ELISA kit (E-90C3) (ICL, Inc). Serum samples from individual mice from wild type and properdin-deficient mice were diluted 1/50.000. The standard curve of mouse C3 with stock concentration of 2.74 µg/ml was diluted in diluent at concentration started of 200 ng/ml to zero. Then 100 µl of each sample and standard were added in duplicate into each well. The plate was incubated at room temperature for 20 minutes then the plate was washed 3 times with washing solution. After that 100 µl of TMB substrate solution were added to each well and incubated in the dark at the room temperature for 10 minutes and the reaction was stopped by adding 100 µl of stop solution to each well. Next, the absorbance was determined at 450nm by using microplate reader (680 Bio-Rad). Values were plotted against the standard curve and calculated concentrations multiplied with the dilution factor. The antibody concentration is expressed in mg/ml.

### **2.2.8 Histology of the liver and spleen of mice infected with *L. monocytogenes***

Livers and spleen of the infected mice were analysed histologically for localisation of *L. monocytogenes* and granuloma formation.

Mice from wild type and properdin-deficient were killed after 69 hours infected with *L. monocytogenes*  $5 \times 10^5$  of sterile PBS and after dissection their liver lobes and spleen were removed. Livers immediately were fixed in 10% buffered formal saline solution and were kept in fridge until further treatment. The tissues were then dehydrated, after cut into small pieces, using Citadel 2000 tissue processor (Shandon). The samples were first immersed into IMS 70%, 90%, and 100%, then into a chloroform solution and then into polywax (TCS bioscience Ltd). This was done by Mrs J Edwards. (Department of

Toxicology, MRC Unit). Then paraffin blocks were cut into 5  $\mu\text{m}$  thick tissue sections using microtome (Leitz Wetzlar), then were mounted on microscope slides. After that were dewaxed with xylene and rehydrated with (100%, 90%, 70% IMS), then the slides were stained using hematoxylin and eosin, and also stained with Twort's Gram stain (Twort 1924) for detecting bacteria in the section.

### **2.2.9 Antigenic maturation of splenic dendritic cells *in vivo* after infection with *L. monocytogenes***

Whole spleens from wild type and properdin-deficient mice were harvested after infected with  $1 \times 10^6$  *L. monocytogenes* and splenocytes were prepared as described before *in vitro* part in section 2.1.3.18. Splenocytes cells were re-stimulated overnight with heat-killed *L. monocytogenes* at MOI=200. Splenocytes were counted and aliquots of  $1 \times 10^6/\text{ml}$ , cells were made for each staining combination for flow cytometry as well as compensation and were subsequently stained. The cells were typed for CD11c and surface markers CD40 for the FACS as described *in vitro* part in section 2.1.3.17. Dendritic cells were evaluated using the following 2-color combinations: IgG FITC with CD11c PE and IgG PE with CD40 FITC and Fc-block (AbD serotec) was added (diluted 1:200) to minimise nonspecific binding.

### **2.2.10 Analysis of mRNA expression by Quantitative Real-Time RT-PCR**

Samples of RNA extracted from the spleen of experimental mice were used in qRT-PCR analyses. cDNA was synthesized from 1  $\mu\text{g}$  of RNA as described before (section 2.1.3.13). Total RNAs were obtained from uninfected spleens and also after infected with approximately  $5 \times 10^5$  and  $1 \times 10^6$  of *L. monocytogenes*. Spleens were homogenised by glass dounce homogenisation and use of TRIzol® Reagent (Invitrogen

Cat NO: 15596-018) according to the TRIzol protocol for isolation of RNA from tissues. Quantitative real-time PCR was performed using the detection system for quantification with SYBR Green and melting curve analysis as described in section 2.1.3.13. Expression of IFN- $\gamma$ , IL-17A, CD11b, TLR2, C5aR, Fc $\gamma$ RIIb, Fc $\gamma$ RIV, Fc $\gamma$ RIII was determined from spleens of infected wild type and properdin-deficient mice. Fold changes in expression of genes of interest can then be compared with fold changes in the control gene. The expression of  $\beta$ -actin was chosen as normaliser. The thermal cycling conditions were as described *in vitro* part as well. Mice primer sequences for genes and the reference gene used with optimised annealing temperature were shown in the table below. Sequence of  $\beta$ -actin, CD11b and TLR2 were used as in material and methods *in vitro* chapter (Table 2.1), but for CD11b T<sub>A</sub> was changed to 62°C for qPCR. Sequence of Fc $\gamma$ RIIb and Fc $\gamma$ RIV were shown in Table 2.3. Sequences of primers used for amplification of IFN- $\gamma$ , IL-17A, C5aR and Fc $\gamma$ RIII are shown in the Table below.

**Table 2.5:** Primers sequences with T<sub>A</sub> and the sizes used in this study.

Gene name	Sequence of primers	T <sub>A</sub>	Product size (bp)	Reference sequence accession number NCBI
mIFN- $\gamma$	5'-TACTGCCACGGCACAGTCATTGAA-3' 5'-GCAGCGACTCCTTTTCCGCTTCCT-3'	57°C	405 bp	NM_008337.3
mIL-17A	5'-GGCTGACCCCTAAGAAACC-3' 5'-CTGAAAATCAATAGCACGAAC-3'	59°C	79 bp	NM_010552.3
mFc $\gamma$ RIII	5'-ATCACTGTCCAAGATCCAGC-3' 5'-GCCTTGAAGTGGTGATCCTA-3'	60°C	346 bp	NM_010188.4
mC5aR	5'-TATAGTCCTGCCCTCGCTCAT-3' 5'-CTACCACTTTGAGCGTCTTCTTGG-3'	60°C	410 bp	NM_001173550.1

All QPCR analyses were run in duplicate and including a negative control (non-RNA just water). Comparative  $C_t$  method was used in this study and this involves comparing the  $C_t$  values of the samples of interest with calibrator negative control (Schmittgen & Livak, 2008). The  $C_t$  values of the samples of interest are normalized to an appropriate endogenous housekeeping gene  $\beta$ -actin.

### **2.2.11 Localisation of *L. monocytogenes* by transmission electron microscopy of liver**

Liver samples were placed in fixative buffer 2% (v/v) formaldehyde / 4% (v/v) glutaraldehyde / 0.1M Sodium Cacodylate buffer / 2mM Calcium chloride pH 7.4 overnight at 4°C, and processed by Stefan Hyman and Natalie Allcock, EM Facility University of Leicester according to an established methods, as follows:

Samples were washed in 0.1M Sodium Cacodylate buffer / 2mM Calcium chloride pH 7.4 and stored at 4°C. After that with same washing buffer samples were washed 3 times for 20 minutes, and then washed 2 times for 20 minutes in distilled de-ionised water. Samples were secondary fixed with 1% Osmium tetroxide / 1.5% Potassium ferricyanide in double distilled water for 3 hours. Then samples were washed 3 times for 20 minutes in distilled de-ionised water. Tertiary fix in 2% aqueous uranyl acetate for 1 hour at 4°C, then washed 2 times for 10 minutes in distilled de-ionised water, followed with serial alcohols, 30% Ethanol 15 minutes, 50% Ethanol 15 minutes, 70% Ethanol 15 minutes, 70% Ethanol stored overnight at 4°C, 90% Ethanol 30 minutes, 100% Analytical Grade ethanol for 30 minutes for 3 times. Propylene oxide, 2 times for 10 minutes and 2 Propylene oxide: 1 Modified Spurr's Low Viscosity Resin (Hard Formula) for 90 minutes. 1 Propylene oxide: 1 Modified Spurr's Low Viscosity Resin for 90 minutes also 1 Propylene oxide: 2 Modified Spurr's Low Viscosity Resin for another

90 minutes, followed by 100% Modified Spurr's Low Viscosity Resin for 30 minutes and again for overnight after that Fresh Modified Spurr's Low Viscosity Resin were added for 3 hours 2 times. Finally the samples were embedded and polymerise at 60°C for 16 hours. Samples sectioned were embedded using a Reichert Ultracut S ultramicrotome. Then samples sections of approximately 90 nm thickness were cut from each sample and were collected onto copper mesh grids after that were counterstained with 2 minutes in Reynold's Lead citrate. Samples were viewed on the JEOL 1400 TEM with an accelerating voltage of 80kV. Overall, the images were captured using Mageview III digital camera with TEM software.

### **2.2.12 Cytospin of liver homogenates**

To study the differential or counts of neutrophils and macrophages, livers from individual mice were homogenised in 10 ml PBS then slides were prepared by centrifugation using a Shandon cytospin 2 cytocentrifuges. 50 µl or 30 µl were spun in cytospin centrifuge at 150 rpm for lower speed for 3 minutes, then the slides were left to dry overnight. Next day the slides were stained with stain Quick-Diff kit and evaluated microscopically.

### **2.2.13 Immunohistochemistry**

Paraffin sections of livers of 5 µm from both genotypes after infection with *L. monocytogenes* were analysed by immunohistochemistry.

The sections were dewaxed two times in xylene for 5 minutes and rehydrated in serial alcohols 2 times for 5 minutes in 100 % MIS, once for 5 minutes in 90 % and 70% . Then the section was blocked for 10 minutes to inhibit the activity in endogenous peroxidase (2.4 ml of 30 % hydrogen peroxide in 400 mls PBS). The antigen retrieval

were per-heat in microwave for 10 minutes using the pressure cooker then the slides were placed into heated retrieval solution and were microwaved for 12 minutes. After that the slides were cooled for 20 minutes by lay them on the base of the humidity chamber after prepared by damping paper towels. After that slides were placed in tap water. Then 100 µl of blocking serum (0.5% BSA in diluted serum) were added to each sample and incubated for at least an hour at room temperature in the humidity chamber. After that the blocking solution were drained and dried around the tissue sample and 100 µl of primary antibody solution (Rat MAb to F4/80 antibody, Abcam Cat. No. ab6640 (1mg/ml)) were added onto each sample and were incubated overnight at 4 °C. The next day the second antibody (goat anti rat IgG: HRP (mouse absorbed) (Serotec) was added after dilution (1:200) in serum. Then samples were drained off from serum and washed in three changes of 1 x PBS 5 minutes with slow shaking. Next after the slides were dried (back and around the sample) 100 µl of the second antibody were added over the tissue, including the controls and incubated for 25 minutes at room temperature.

After the incubation the sections were washed in three changes of 1xPBS for 10 minutes each then dried. Next, a DAB solution from Vector lab DAB substrate kit for peroxide were added to the sections and incubated for 2-10 minutes. The DAB solution was prepared as follows depending on number of samples:

1 ml of distilled water was measured, and then each of the following were added and mixed well, one drops of the buffer stock solution, 2 drops of DAB stock solution, 1 drop of the Hydrogen peroxide solution, 1 drop of Nickel solution. After that the section was washed in water and then counter stained with haematoxylin for no more than 10 seconds then washed in running water for 2-5 minutes. Next, the sections were dehydrated in serial dilution of alcohols (70%, 90%, 100%) and were clear in two

change of xylene and then 3 minutes each in 70%, 90%, 100 % also 5 minutes in 100% xylene. After that mounted with DPX mounting medium and slides dried for 3 hours, then were evaluated under microscope.

### **2.2.14 Data Analysis**

Paired t-test was used to compare the means of two groups. One-way ANOVA was used to compare the mean of four groups, dendritic cells and macrophages from wild type and properdin-deficient mice, respectively. The Tukey's multiple comparison tests was chosen to compare all pairs of columns. Values of  $p < 0.05$  were deemed significant. Statistical significance was evaluated with the Graph-Pad prism 5 and 6 software packages (Graphpad, San Diego California, USA). Geometric means were applied to data collected on a logarithmic scale e.g. flow cytometry. For the statistic survival curve the Log-rank (Mantel-Cox) test was used.

## *Chapter Three Results*

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*In vitro studies*

*Role of properdin in the response of murine bone marrow cells to *L. monocytogenes* infection*

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### 3 Results

#### 3.1 The role of complement properdin in infection of murine bone marrow derived macrophages and dendritic cells with *Listeria monocytogenes*

Dendritic cells and macrophages were differentiated from bone marrows prepared from wild type and properdin-deficient mice to study their infectivity with *L. monocytogenes*. Direct comparison between cells from wild type and properdin-deficient mice allowed evaluation of a role for properdin in bactericidal activity, cytokine and nitric oxide release, as well as sub-cellular location of *L. monocytogenes*.

The macrophage provides a first line of innate immune protection response against infecting pathogens. It plays a role in early immune responses against intracellular *L. monocytogenes*. *In vitro* infection of macrophage with *L. monocytogenes* showed that *L. monocytogenes* initiates a sequence of responses at transcriptional and post-transcriptional levels that are essential for host survival (Schnitger *et al.*, 2011).

Dendritic cells respond to infection with *L. monocytogenes* by up-regulating surface expression of molecules necessary for antigen presentation and T cell interaction (CD40, CD80, CD86 and MHCII) (Muraille *et al.*, 2005) and by increasing production of cytokines such as IFN- $\gamma$ . In the murine intravenous model dendritic cells are required for the initiation of an adaptive immune response (Jung *et al.*, 2002). Dendritic cells and macrophages derived from bone marrow have been studied in relation to *L. monocytogenes* infection (Westcott *et al.*, 2007). Remarkably, dendritic cells had the capacity to trap large numbers of *L. monocytogenes* and limit the growth of bacteria. By contrast, in bone marrow-derived macrophages bacteria can grow in cytosol more than in vacuoles. Because the outcome of *L. monocytogenes* infection of dendritic cells is different from that of macrophage (Westcott *et al.*, 2010), both cell types were used to study the response and behavior of intracellular *L. monocytogenes*.

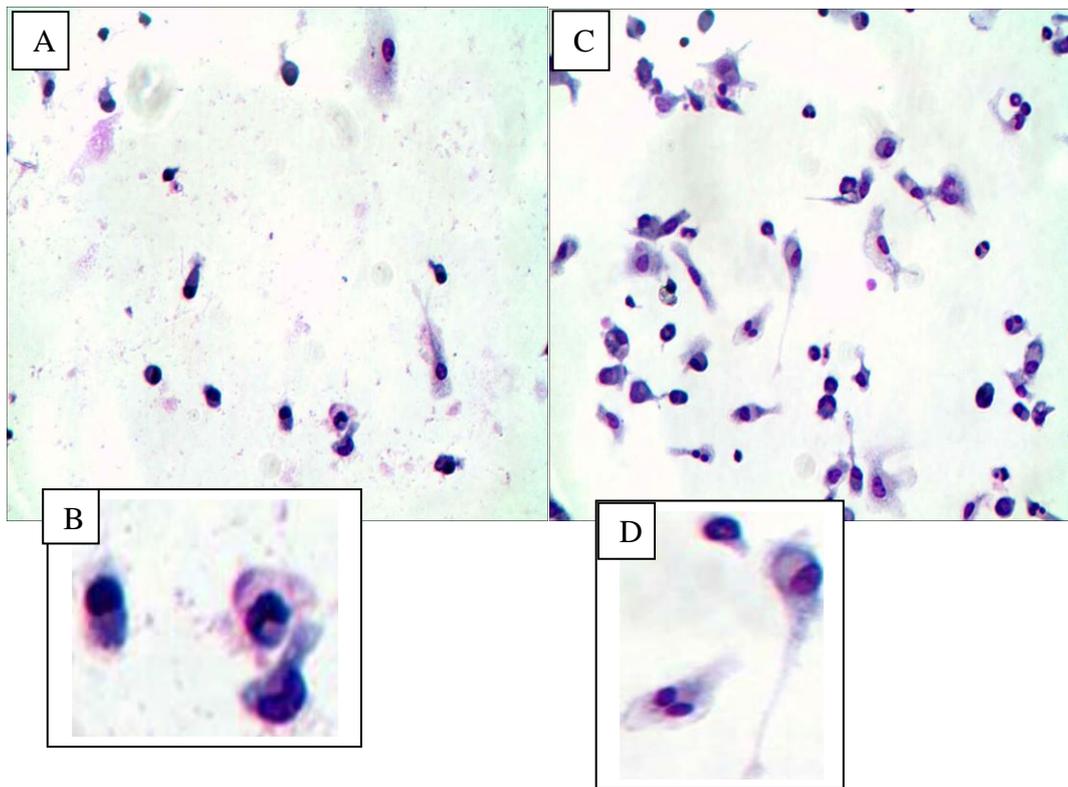
### 3.1.1 Generation of bone marrow-derived dendritic cells and macrophages

The protocol used for generation of these two myeloid cell types required continuous stimulation with the cytokines IL-4 and GM-CSF (Xu *et al.*, 2007, Westcott *et al.*, 2007), which reportedly drive differentiation of bone marrow precursor cells to a population of predominately dendritic cells and macrophages, as determined by flow cytometry (Westcott *et al.*, 2007). These researchers showed that the two cell types can be told apart by their adherence characteristics, and this is the method that was used for this study: while macrophages adhere to tissue culture plastic, dendritic cells do not or only loosely and can therefore be separated from the macrophages by withdrawing the culture supernatant. Typically, the dendritic cells are immature but acquire cell surface expression of markers on stimulation.

Approximately 40% adherent cells are obtained from a standard preparation of bone marrows as described *in vitro* material and methods chapter. The yield of dendritic cells was quantified during flow cytometric analysis of the non-adherent cell population (section 3.1.16). About 20% of the population were positive for the dendritic cells marker CD11c. This is ten fold more than the yield achieved by purifying dendritic cells from mouse blood as is related to the immature phenotype of the isolated cells (Adachi *et al.*, 2002).

Primary macrophages and dendritic cells from wild type were stained with hematoxylin and eosin stain (Figure 3.1.1). Macrophages appeared mononuclear with oval or kidney-shaped nucleus in cytoplasm with less processes compared to dendritic cells. Dendritic cells were a more heterogenous population appearing with different shapes. Dendritic cells are relatively large cells with an oval nucleus and a large cytoplasm, had an irregular shape and a number of dendritic protrusions, mostly occurred as single cells or formed small clusters.

About the same numbers of dendritic cells were derived from bone marrows from wild type and properdin-deficient mice. There was no obvious difference in cell morphology between the genotypes.

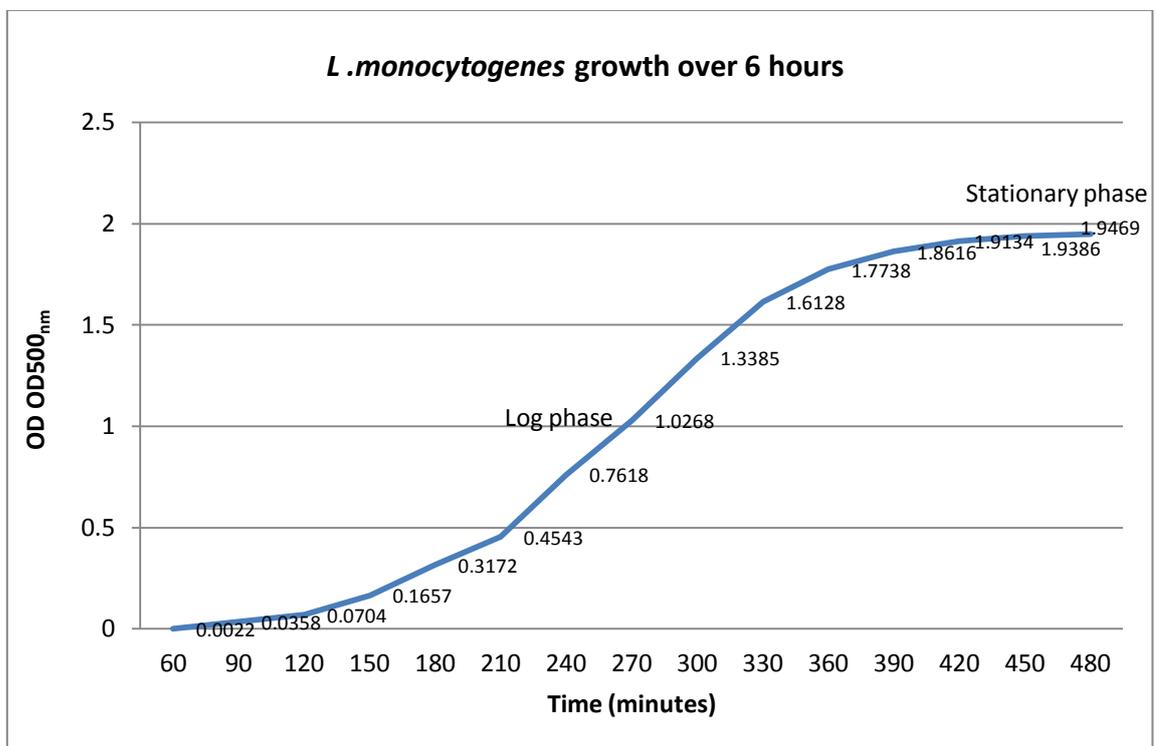


**Figure 3.1.1:** Representative pictures of the sections show characteristic uninfected macrophage and dendritic cells. Macrophages (A) and dendritic cells (C) derived bone marrow from wild type mice were differentiated and cultured in GM-CSF and IL-4 for 7 days stained with hematoxylin and eosin stain. Original magnification 40 X, B are magnified of section in A, D are magnified of section in C.

### 3.1.2 Preparation of an infective stock of *L. monocytogenes*

Growth of *L. monocytogenes* was monitored over 6 hours in order to determine the logarithmic and stationary phases of growth. The optical density was determined every 30 minutes, and the readings were used to plot a growth curve. Typical results of 2

independent experiments are shown in Figure 3.1.2. The curve depicts a typical logarithmic growth behavior between OD<sub>500nm</sub> of 0.4 and 0.9. *L. monocytogenes* were spun down and frozen in aliquots. By viable counting, the MOI of these aliquots was determined to be approximately 1 x 10<sup>9</sup> CFU/ml. This procedure was repeated as required with comparable results. As before the stock were frozen and used for *in vitro* as well as the primary infection.



**Figure 3.1.2:** Logarithmic and stationary phase of *L. monocytogenes* growth, performed from 100 ml culture inoculated with 1ml overnight cultures (220 rpm, 37°C) started from one colony. The logarithmic phase was reached after about 3 hours.

### 3.1.3 *In vitro* infection with *L. monocytogenes*

The aim of this part of the study was to determine host pathogen interactions in term of bacterial growth, cell viability, and their modulation by hypoxia and stress hormone.

The analysis is based on establishing the viable count of *L. monocytogenes* after lysis of host cells. Viable counts therefore represent *L. monocytogenes* which are located intracellularly and are able to replicate. There could be some bacteria attached to the membrane. Cells were infected with *L. monocytogenes* for 1 hour at 37°C and after washing with PBS, cells were treated with gentamicin for 30 minutes to eliminate all extracellular bacteria as described in Inaba *et al.* 1992 and Westcott *et al.*, 2007 with some modifications delineated in “Materials and Methods”.

The MOI of an approximately 0.2 bacteria/cell was used in this study for the viable count and MOI of an approximately 2.5 bacteria/cell was used for electron microscopy based on Westcott *et al.*, 2007. However, for without and in addition of 100 µM norepinephrine the MOI of an approximately 0.2 bacteria/cell were used.

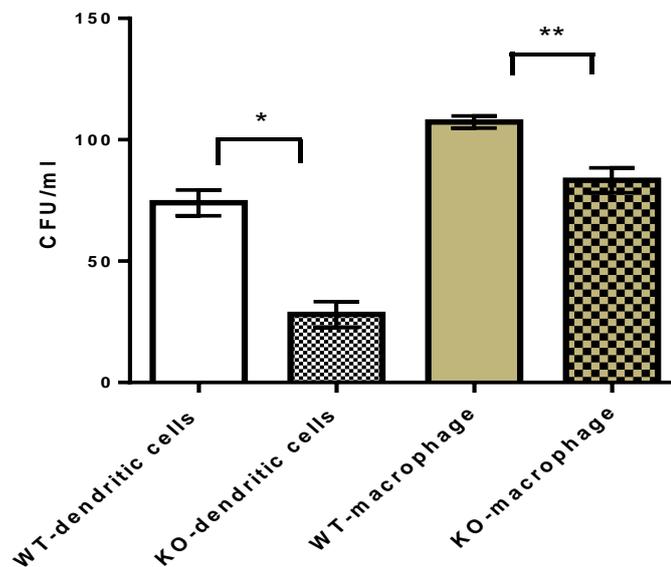
The gentamicin was efficient as described in material and methods *in vitro*. *L. monocytogenes* were incubated in RPMI medium with gentamicin for time point 30 minutes, 1 hour and 2 hours. There was no growth of *L. monocytogenes* for all time points subsequent to gentamicin treatment.

**Table 3.1.1:** Viable count of *L. monocytogenes* obtained with and without gentamicin treatment and showed that gentamicin treatment is working at time point 30 minutes 1 hour and 2 hours.

NO	Time of infection	CFU/ml Listeria Without Gentmicin	log CFU/ml	CFU/ml Listeria with Gentamicin	Log
1	Start time	1.01 x 10 <sup>6</sup> /ml	6.004321	1.6 x 10 <sup>4</sup> /ml	4.20412
2	30 min	1.9 x 10 <sup>6</sup> /ml	6.278754	Zero	Zero
3	1hr	2.1 x 10 <sup>6</sup> /ml	6.322219	Zero	Zero
4	2hrs	3.7 x 10 <sup>6</sup> /ml	6.568202	Zero	Zero

Typical results of viable counts of *L. monocytogenes* (expressed as CFU/ml) obtained from infected dendritic cells and macrophages are shown in Figure 3.1.3.

As can be seen, there were significantly reduced *L. monocytogenes* numbers in dendritic cells from properdin-deficient mice compared to dendritic cells from wild type mice ( $p= 0.0154$ ). Likewise, there were significantly more *L. monocytogenes* in macrophages from wild type mice compared to macrophages from properdin-deficient mice ( $p= 0.0040$ ). In all experiments, the numbers of dendritic cells and macrophage used for both genotypes were the same.



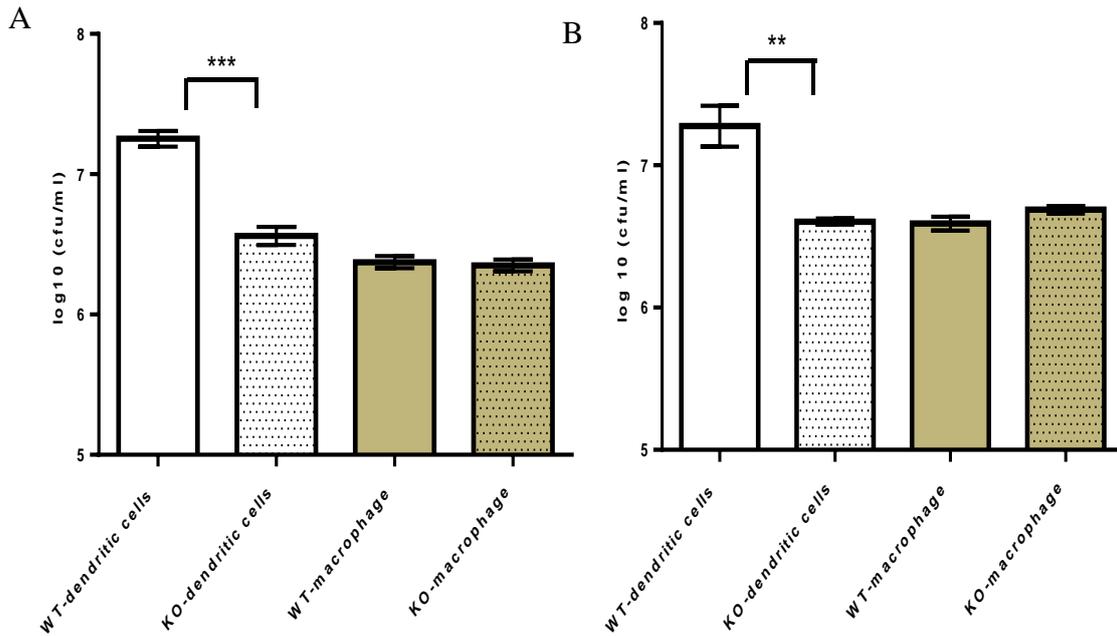
**Figure 3.1.3:** Analysis of bone marrow derived dendritic cells and macrophages for their intracellular viable load of *L. monocytogenes*. Dendritic cells and macrophages from wild type and properdin-deficient mice (WT= wild type and KO= properdin-deficient mice) were analysed for 1 hour infection and after 30 minutes of adding gentamicin. This is a representative graph of 4 independent experiments. The data are presented as means with standard deviation (SD).

However, the antibacterial effect of dendritic cells and macrophages isolated from wild type and properdin-deficient mice were studied after infection with *L. monocytogenes*

for 1 and 2 hours, respectively, was inconclusive, and therefore a longer incubation of 24 hours was studied. Because a longer incubation time was studied, parallel experiments were set up under normoxic and hypoxic condition in which dendritic cells and macrophages from properdin-deficient mice and wild type were infected with *L. monocytogenes*, incubated for 24 hours and analysed for their bactericidal/bacteriostatic activities as shown in Figure 3.1.4.

In both conditions (Figure 3.1.4 A and B), there were significantly elevated numbers of *L. monocytogenes* in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice ( $p < 0.05$ ), but there were no differences in bacterial loads between macrophages from wild type and macrophages from properdin-deficient mice. This is different from the previous Figure 3.1.3, after 30 minutes of infection there were a higher number of *L. monocytogenes* in macrophage from wild type compared to properdin-deficient. *L. monocytogenes* were diluted in 10-fold as shown on the y-axes in Figure 3.1.4 (A and B) while in Figure 3.1.3 is showing the number of bacteria.

The total number of *L. monocytogenes* retrieved from the cells in hypoxia and normoxia was very similar. The low oxygen tension in hypoxia shows no effect on cell survival or the clearance of *L. monocytogenes* compared to normoxia conditions, even though the presence of hypoxia was confirmed by amplifying readily VEGF (vascular endothelial growth factor) by RT-PCR (see Figures 3.2.29 and 3.1.31). This was unexpected because in hypoxia dendritic cells and macrophage increase their functions and interactions and improved defence against *Leishmania amazonensis* (Degrossoli *et al.*, 2007).



**Figure 3.1.4:** Mouse cells infected with *L. monocytogenes* strains after 24 hours incubation in normoxic (A) and hypoxic (B) conditions. This is a representative graph of 3 independent experiments. The data are presented as means with standard deviation (SD).

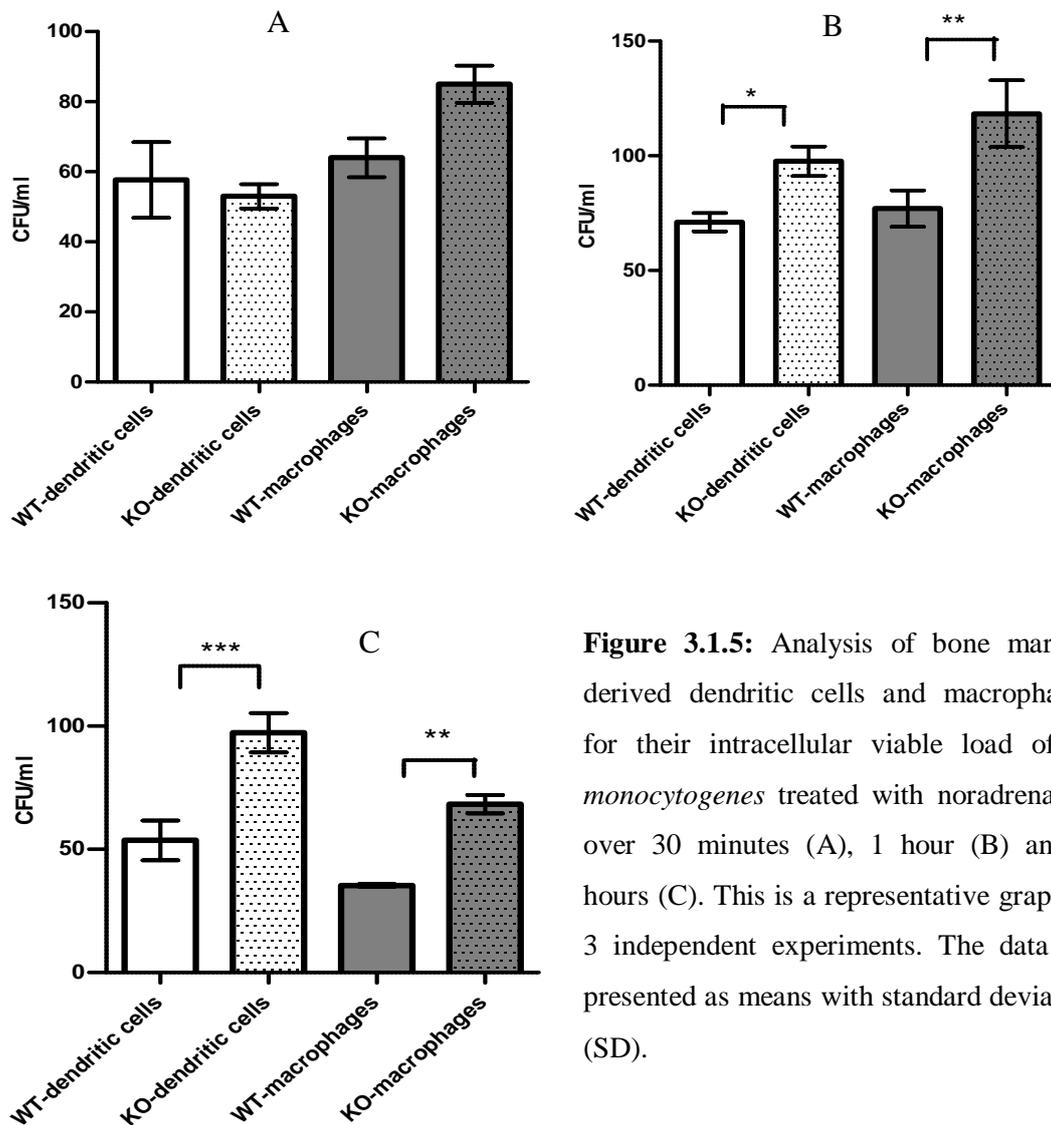
The viable counts of *L. monocytogenes* from splenocytes from wild type and properdin-deficient mice after infected *in vitro* with MOI = 0.02 for 15.5 hours has been studied in collaborating with another project in Dr. Stover's group. splenocytes were prepared in parallel from infected wild type and properdin-deficient mice (one mouse each) and cultured overnight. The splenocytes from wild type mice released more bacteria to the supernatant (CFU (lg10) = 2.114) compared to splenocytes from properdin-deficient mice (CFU (lg10) = 1.452).

Stress hormones reportedly influence susceptibility to infection by acting not only on host cells but also on infectious organisms directly (Freestone *et al.*, 2008). The aim of this investigation was to study the role of properdin in the presence of norepinephrine, to see whether it affects *L. monocytogenes* growth or in dendritic cells and macrophages of both genotypes. *L. monocytogenes* in the presence of 100  $\mu$ M norepinephrine

(noradrenaline) in RPMI medium were used to infect mouse cells as described and viable counts determined for 1 hour infection and 3 time points (30 minutes, 1 hour and 2 hours) after addition of gentamicin for 30 minutes and washing.

The numbers of viable *L. monocytogenes* (CFU) after 30 minutes were determined and representative results are shown in Figure 3.1.5. Dendritic cells from wild type and properdin-deficient mice showed comparable intracellular bacterial loads. This is in contrast with findings in Figure 3.1.3 (Absence of stress hormone). On the other hand, there were higher *L. monocytogenes* numbers in macrophages from properdin-deficient mice compared to macrophages from wild type mice but significantly there was no difference. This also contrasts with Figure 3.1.3 (Absence of stress hormone).

Next, viable counts of *L. monocytogenes* after 1-hour incubation with gentamicin were studied because there were no differences in the presence of stress hormone after 30 minutes compared to absence of stress hormone in both genotypes. After 1-hour incubation and the addition of gentamicin for another hour, there were significantly more viable counts obtained from dendritic cells from properdin-deficient mice than dendritic cells from wild type mice ( $p < 0.05$ ). There were significantly more viable counts in macrophages from properdin-deficient mice than wild type macrophages ( $p < 0.05$ ) as shown in the Figure 3.1.5 panel B. A similar observation is made after 2 hours. Viable counts of *L. monocytogenes* were studied after 2 hours' incubation with gentamicin. There were significantly more intracellular *L. monocytogenes* in dendritic cells from properdin-deficient mice ( $p = 0.0001$ ) compared to dendritic cells from wild type mice. There were also more intracellular *L. monocytogenes* in macrophages from properdin-deficient mice ( $p = 0.0037$ ) compared to macrophages from wild type mice, as shown in Figure 3.1.5 panel C.



**Figure 3.1.5:** Analysis of bone marrow derived dendritic cells and macrophages for their intracellular viable load of *L. monocytogenes* treated with noradrenaline over 30 minutes (A), 1 hour (B) and 2 hours (C). This is a representative graph of 3 independent experiments. The data are presented as means with standard deviation (SD).

Taken together, addition of norepinephrine to *L. monocytogenes* and infecting murine Dendritic cells and macrophages for 1 hour, led to a comparable amount of viable of *L. monocytogenes* intracellularly in bone marrow derived dendritic cells from wild type and properdin-deficient mice. This is likely to be due to greater virulence of *L. monocytogenes* due to norepinephrine (compare Figure 3.1.5 with Figure 3.1.3) because *L. monocytogenes* numbers are not affected by norepinephrine alone after 1 and 2 hours

(tested separately) as shown in the table 3.1.2 ( $p>0.05$ ), and a change in susceptibility specific for properdin-deficient cells are unlikely. However, after 24 hours, *L. monocytogenes* numbers increased one fold with stress hormone compared to *L. monocytogenes* numbers grown without stress hormone ( $p<0.05$ ). Next, measured after another 1 hour of incubation, norepinephrine increases further *L. monocytogenes* viability in dendritic cells from properdin-deficient mice. Interestingly, the same was observed in macrophages from properdin-deficient mice in comparison with macrophages from wild type mice. This effect is further increased at 2 hours after infection as shown in 3.1.5. Therefore, this observation is likely to point to a direct effect of norepinephrine on *L. monocytogenes*.

Reportedly, *L. monocytogenes* responds moderately to norepinephrine: A study showed that norepinephrine added to the culture broth (Tryptic soy broth with 0.6% yeast extract) can stimulate slightly the growth of *L. monocytogenes* during 10 hours without added iron source (Coulanges *et al.*, 1997).

**Table 3.1.2:** Viable count of *L. monocytogenes* with and without norepinephrine, representative of 2 independent experiments.

NO	Time	Without norepinephrine	log	With norepinephrine	log
1	Start time	$2.6 \times 10^5$ /ml	5.414973	$3 \times 10^5$ /ml	5.477121
2	1 hr	$4.5 \times 10^5$ /ml	5.653213	$3.5 \times 10^5$ /ml	5.544068
3	2hrs	$5 \times 10^5$ /ml	5.69897	$4 \times 10^5$ /ml	5.60206
4	24hrs	$7.3 \times 10^6$ /ml	6.863323	$2.8 \times 10^7$ /ml	7.447158

### 3.1.4 Determination of cellular viability after infection with *L. monocytogenes*

Viabilities of mouse bone marrow-derived macrophages and dendritic cells after infection were determined using the trypan blue exclusion method. Cells were counted in a Neubauer chamber. The viability of uninfected cells is about 98%. Viability was calculated using the following equation: % viable = [number of live cells/(number of live cell + numbers of dead cells)] x 100%.

The trypsinised cells were the only shown in this experiment because these are the ones that are analysed in the CFU part of the experiment after lysing the cells with water. The cell counts in the table 3.1.3 and table 3.1.4 are from those cells that have stayed attached to the bottom of the well till the end of the experiment.

The numbers of cells from macrophages and dendritic cells of properdin-deficient and wild type mice that started with were the same.

The viability of mouse macrophages retrieved by trypsin detachment after infection with *L. monocytogenes* for 1 hour and 24 hours (in normoxia and hypoxia) is shown in the table 3.1.3. The majority of cells appeared negative for trypan blue.

The total numbers counted of (live and dead) cells after 1 hour for WT-macrophages =  $38.8 \times 10^4/\text{ml}$  and KO-macrophages =  $39.6 \times 10^4/\text{ml}$  and showed the same viability between them as shown in the table 3.1.1 therefore, there was no impairment in acute, infection-mediated adherence of macrophages from properdin-deficient mice. The total numbers counted for the cells adherent to the wells (live and dead) for normoxia after 24 hours of infection are as shown in the table 3.1.3 macrophages from wild type mice ( $24 \times 10^4/\text{ml}$ ) compared to macrophages from properdin-deficient mice ( $54 \times 10^4/\text{ml}$ ). In hypoxia the count of WT-macrophages ( $34 \times 10^4/\text{ml}$ ) was similar to that of KO-macrophages ( $24 \times 10^4/\text{ml}$ ). There was no difference between macrophages from

properdin-deficient and wild type in percentage viability after 1 hour and 24 hours in normoxia and hypoxia.

The *L. monocytogenes* numbers obtained from these experiments were comparable (CFU in normoxia after 24 hours log 6.3 from wild type and log 6.3 from properdin-deficient mice and CFU in hypoxia after 24 hours log 6.5 from wild type and log 6.6 from properdin-deficient mice).

**Table 3.1.3:** The viability of the small proportion of mouse macrophages (Mac) from wild type and properdin-deficient mouse retrieved after infection with *L. monocytogenes* incubated for 1 hour, 24 hours in normoxic and 24 hours in hypoxic condition. These percentages are worked out from the mean of 4 squares per experiment.

No	Mouse cells	1 hour		24 hours normoxia		24 hours hypoxia	
		%	Total	%	Total	%	Total
		viability	Number	viability	Number	viability	Number
1	WT-Mac	88%	n=39	75%	n=24	68%	n=34
2	KO-Mac	85%	n=40	70%	n=54	75%	n=24

The same analysis was performed for infected mouse dendritic cells (Table 3.1.4). Varying numbers were retrieved for dendritic cells, generally low, with the exception of dendritic cells from wild type after 24 hours infection under normoxia. In this experiment, there were more *L. monocytogenes* in dendritic cells from wild type than from properdin-deficient mouse, the average log in normoxia (log 7.2 wild type mice and log 6.5 properdin-deficient mice). Greatest impairment in viability was observed in this group.

The total numbers (live and dead) counted of cells after 1 hour for wild type dendritic cells and dendritic cells from properdin-deficient mice show about the same and the percentage viability no difference between them. Moreover, in the table 3.1.4 the total numbers counted for the cells adhere to the wells (live and dead) for normoxia are: dendritic cells from wild type mice  $232 \times 10^4/\text{ml}$  dendritic cells from properdin-deficient mice  $60 \times 10^4/\text{ml}$ . The total numbers counted for the cells adhere to the wells (blue and white) for hypoxia are: dendritic cells from wild type mice  $53 \times 10^4/\text{ml}$  dendritic cells from properdin-deficient mice  $59 \times 10^4/\text{ml}$ .

**Table 3.1.4:** The viability of mouse dendritic cells (DC) after infection with *L. monocytogenes* incubated for 1 hours, 24 hours in normoxia and 24 hours in hypoxia condition. These percentages are worked out from the mean of 4 squares per experiment.

No	Mouse cells	1 hour		24 hours normoxia		24 hours hypoxia	
		%	Total	%	Total	%	Total
		viability	Number	viability	Number	viability	Number
1	WT-DC	92%	n=21	38%	n=232	75%	n=53
2	KO-DC	79%	n=22	50%	n=60	61%	n=59

The experiment was repeated for dendritic cells from wild type and properdin-deficient mice, after infection with *L. monocytogenes* for 24 hours as shown in the table below:

**Table 3.1.5:** Shows the viability of mouse dendritic cells after infection with *L. monocytogenes* incubated for 24 hours in normoxia these percentages are worked out from the mean of 4 squares per experiment.

No	Mouse cells	24 hours normoxia	
		% viability	Total Number
1	WT-DC	34%	n=628
2	KO- DC	30%	n=764

After 1 hour to 24 hours in normoxia the viability decreases over time for dendritic cells and macrophage from wild type and properdin-deficient mice. This infection-induced impairment of cell viability goes along with more detachment of dead cells into the supernatants for macrophages and dendritic cells from properdin-deficient than for wild type macrophages and dendritic cells. At 1 hour, there is no difference in viability of cells shed into the supernatants of infected wild type and properdin-deficient cultures.

Those cells which detached during the infection (dendritic cells and macrophage) showed no difference in viability. These percentages are worked out from the mean of 4 squares per experiment. However, there is greater viability percentage in macrophage after 24 hours normoxia compared to dendritic cells.

Viabilities of both, dendritic cells from properdin-deficient and wild type mice in hypoxia are increased compared to normoxia.

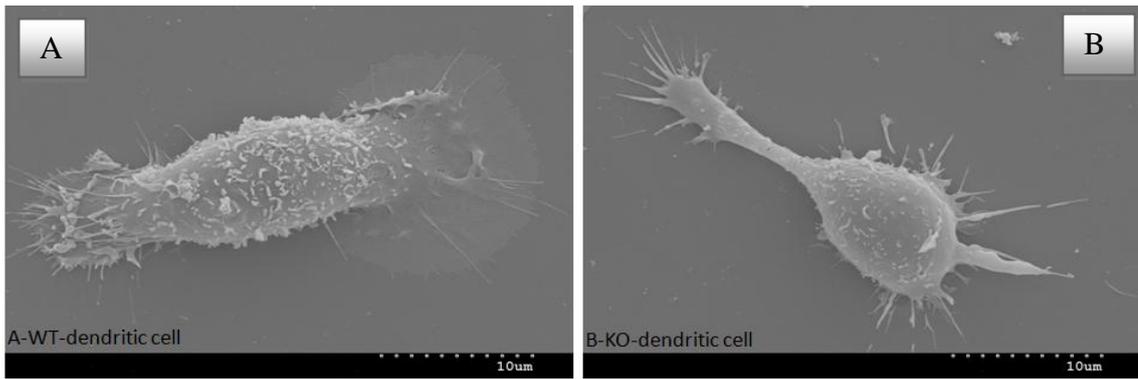
Numbers of *L. monocytogenes* in normoxia and hypoxia are unchanged, but the immune cells have adapted to the condition. Hypoxia reduces maturation of dendritic cells (Rius *et al.*, 2008) and they become more like macrophages under hypoxia (functionally) and apparently from the point of view of viability.

### **3.1.5 Analysis of intracellular localisation of *L. monocytogenes* in dendritic cells using scanning and transmission electron microscopy**

Dendritic cell populations derived from bone marrows of wild type and properdin-deficient mice were isolated as described in “Materials and Methods,” and infected for 4 hours and 24 hours with mid-logarithmically grown *L. monocytogenes* at an MOI of approximately 2.5 bacteria/cell.

Representative images for uninfected and infected cells were chosen from a total number of about 400 images for scanning electron microscopy and about 900 images for transmission electron microscopy, which were generated by Stefan Hyman and Natalie Allcock at the Electron Microscopy Unit. All cells, infected and uninfected, were fixed by 2.5 % Glutaraldehyde/0.05 M HEPES pH 7.2 for at least 15 min and used for electron microscopy as described in “Materials and Methods.”

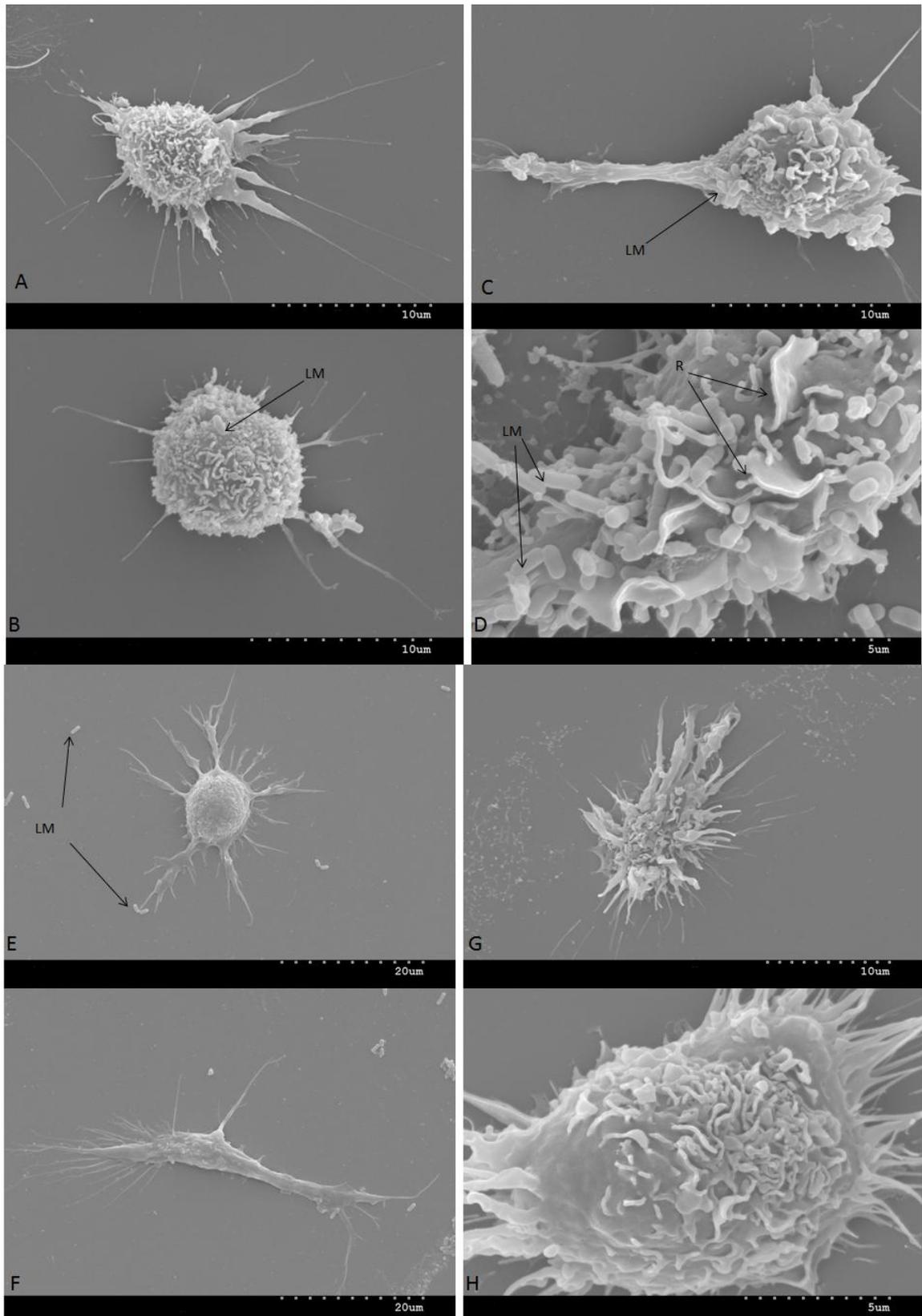
Two images characteristic of the observed morphology are presented. Uninfected dendritic cells present with cell bodies showing typical villi and ruffles and protrusions (Figure 3.1.6). There was no obvious difference between cells from wild type or properdin-deficient mice. The image of bone marrow derived dendritic cells using GM-CSF and IL-4 and their general appearance is comparable to study by Xing *et al.*, 2011.



**Figure 3.1.6:** Scanning electron micrographs show control (uninfected) dendritic cells from wild type (A) and properdin-deficient mice (B), dendritic cells were generated in the presence of GM-CSF and IL-4 and were cultured for 7 days. Typical villi and ruffles and dendrites were showing in the cells. Representative images are shown.

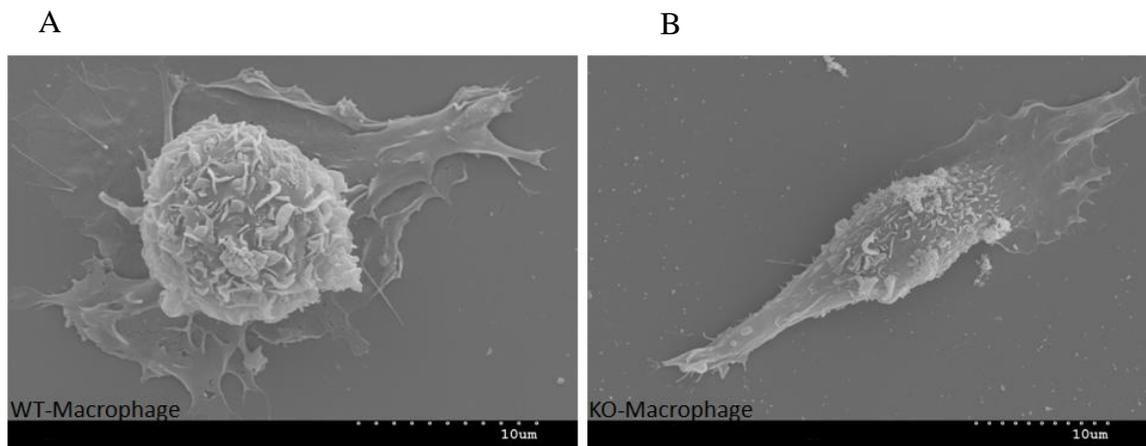
Dendritic cells derived from bone marrows prepared from wild type and properdin-deficient mice after infection with *L. monocytogenes* show features comparable to those presented in a previous study (Kolb-Maurer & Brocker, 2003) which investigated adherence of *L. monocytogenes* to human dendritic cells and will be explained hereafter. Figure 3.1.7 shows dendritic cells from wild type and properdin-deficient mice infected for 4 hours with *L. monocytogenes*. *L. monocytogenes* adhere singly or in clusters to the ruffles of dendritic cells from wild type mice. The ruffles appear plumper compared to the uninfected control cells (Figure 3.1.6).

There were less *L. monocytogenes* around the dendritic cells from properdin-deficient mice compared to dendritic cells from wild type, mainly single *L. monocytogenes* and not in groups. Again the ruffles appear plumper compared to the control and some cells had fewer ruffles as shown in E and F. The number of *L. monocytogenes* (CFU) that was prepared for infection was the same for dendritic cells from wild type and properdin-deficient mice.



**Figure 3.1.7:** Typical scanning electron micrographs showing activate population of dendritic cells from wild type (A, B, C, D) and properdin-deficient mice (E, F, G, H) after 4 hours infection with *L. monocytogenes*. (Shown in D: the R=ruffle, LM= *L. monocytogenes*).

Uninfected macrophages appear as cell bodies with typical ruffles and protrusions (dendrites). Two characteristic images are presented in Figure 3.1.8, there was no obvious difference between cells from wild type or properdin-deficient mice. Their general appearance is comparable to that available online for this type of cells at the time this study was conducted.



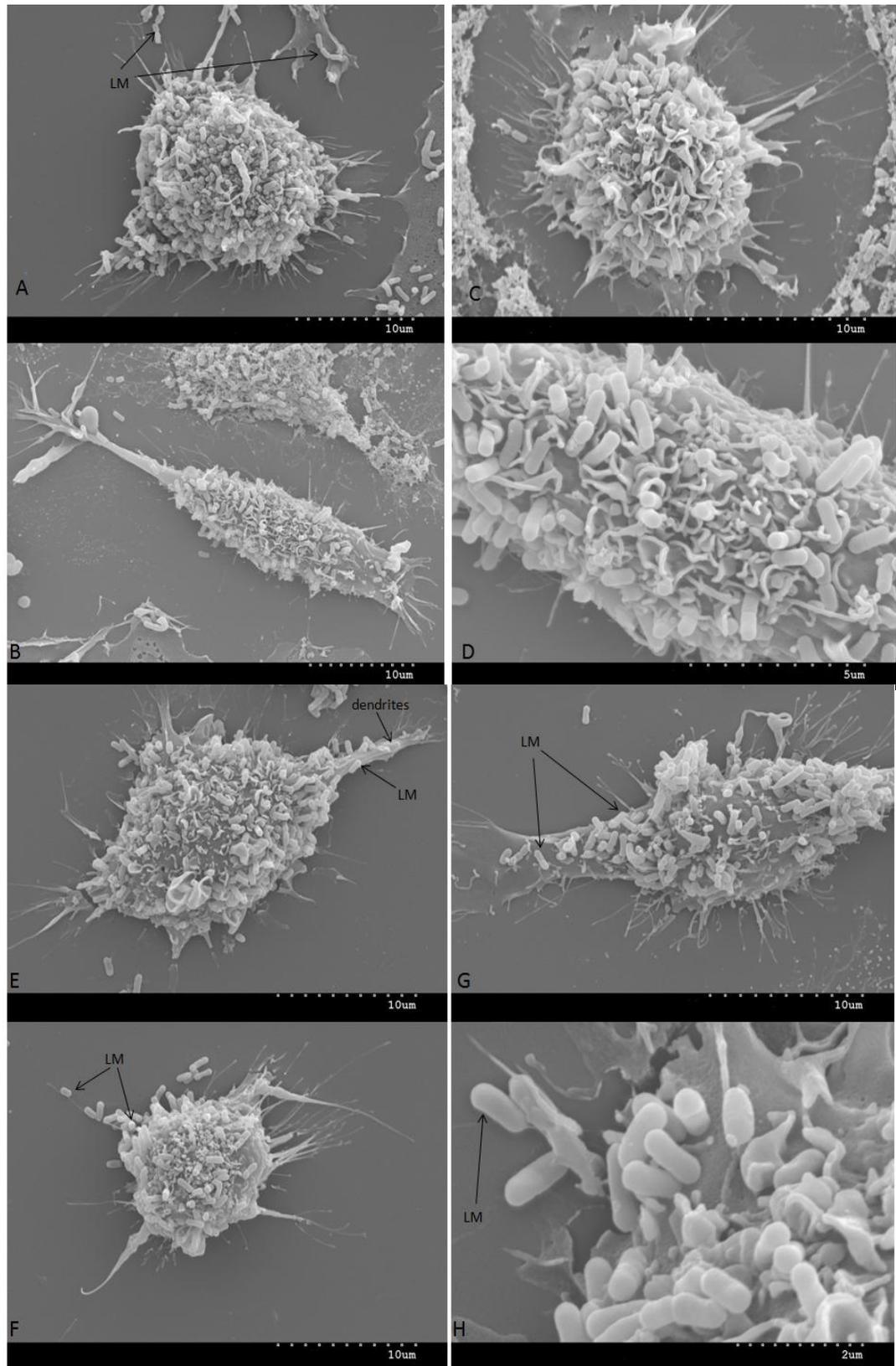
**Figure 3.1.8:** Scanning electron micrographs of control macrophages from wild type (A) and properdin-deficient mice (B) after cultured in GM-CSF and IL-4 for 7 days.

Macrophages from wild type and properdin-deficient mice infected with *L. monocytogenes* show features comparable to those presented in a previous study (Pierce *et al.*, 1996) which investigated the binding and uptake of *L. monocytogenes* by murine peritoneal macrophages in the absence of opsonins. The similarities are the morphology of macrophage as shown in scanning electron micrograph, and the numerous of *L. monocytogenes* bound directly to the cytoplasmic membrane. Moreover, observations of monolayers showed numerous of *L. monocytogenes* adherent to filopodia extending from macrophage.

Figure 3.1.9 shows wild type macrophages infected with *L. monocytogenes* for 4 hours. There were more *L. monocytogenes* around the macrophages compared to the dendritic cells presented above (Figure 3.1.7). The number of bacteria (as determined by viable counting) that was prepared for infection was the same for both genotypes. Moreover, the cells were densely ruffled and covered with *L. monocytogenes*, also some *L. monocytogenes* were in direct contact with each other as shown in Figure 3.1.9 panel A. *L. monocytogenes* located around the wild type macrophages similarly to macrophages from properdin-deficient mice. The cells were densely ruffled and covered with *L. monocytogenes* and some *L. monocytogenes* follow dendrites as shown in Figure 3.1.9 panels A and B.

A small number of globular bodies, most likely representing apoptosing cells were observed in both infected cell populations, macrophages and dendritic cells infected with *L. monocytogenes*. *L. monocytogenes* appeared to be intact and appeared uniformly as rods as expected (Figure 3.1.9 D).

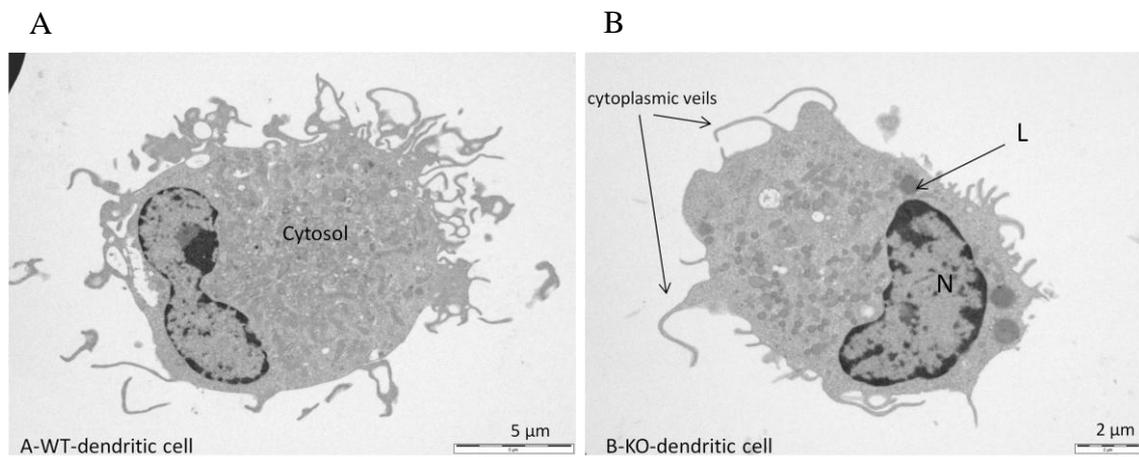
Dendritic cells and macrophage from wild type and properdin-deficient mice show the expected appearance of the type of cells. Intact cells show membrane attached *L. monocytogenes* and signs of cellular reactions (ruffling). The selected images that are shown are representative.



**Figure 3.1.9:** Scanning electron micrographs showing macrophages after infection for 4 hours with *L. monocytogenes*, from wild type (A, B, C, D) and properdin-deficient mice (E, F, G, H). Numerous *L. monocytogenes* are bound in both genotypes and villus-like structures can be observed. LM= *L. monocytogenes*.

Transmission and scanning electron micrographs were set up in parallel within the same experiment.

Dendritic cells were differentiated from bone marrow prepared from wild type and properdin-deficient mice as described in “Materials and Methods” and processed for transmission electron micrographs. Figure 3.1.10 shows uninfected bone marrow derived dendritic cells, representative cross-sections are chosen, and show cell bodies with dendrites and lysosomes as described in Inaba *et al.*, 1992. The processes are called cytoplasmic veils.



**Figure 3.1.10:** Typical transmission electron micrographs of control (uninfected) mouse dendritic cells derived from bone marrow from wild type (A) and properdin-deficient mice (B) at day 7 of culture shown many cytoplasmic veils, there are few lysosoms. N= nucleus, L= lysosomes.

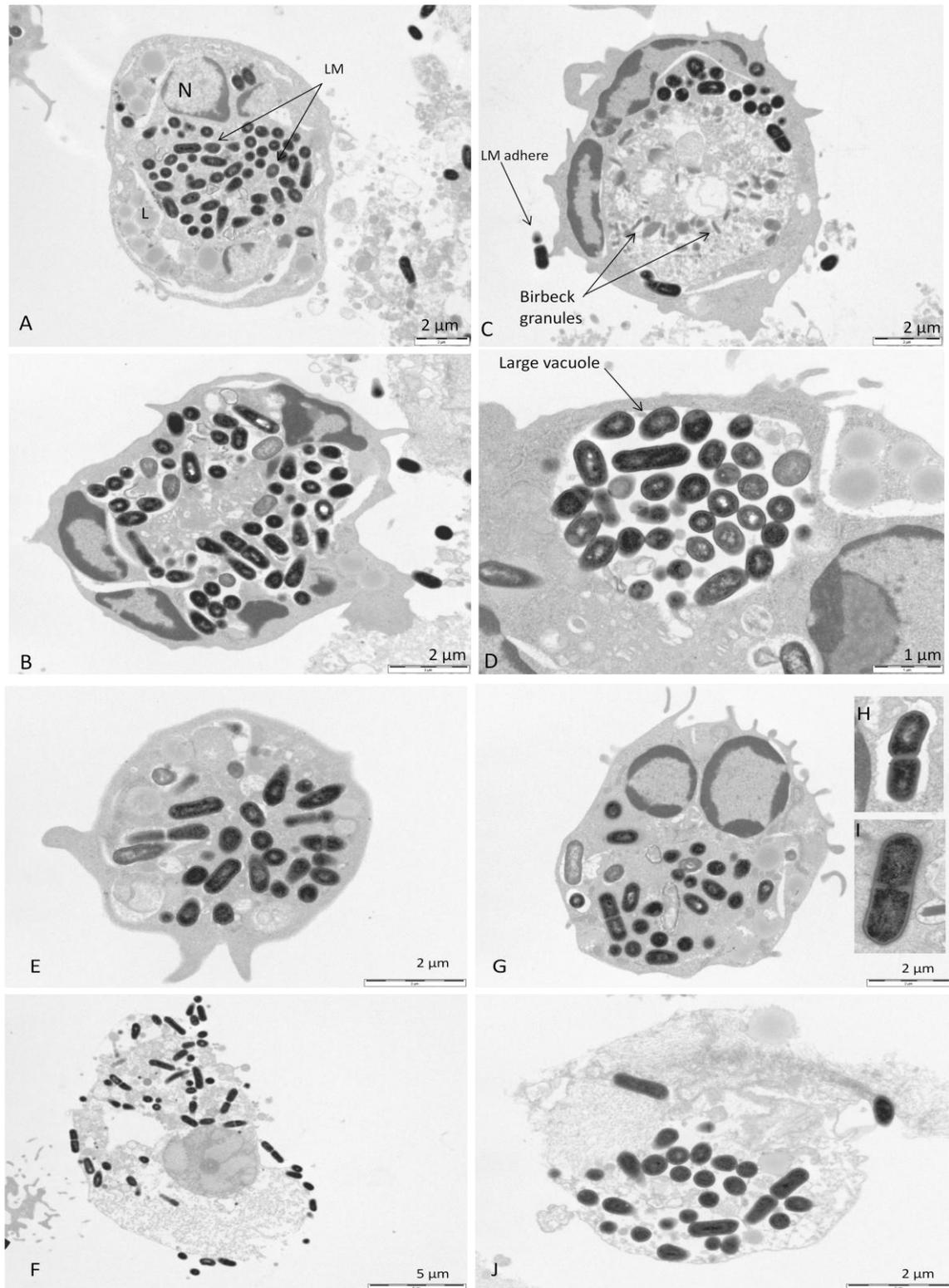
Figure 3.1.11 shows dendritic cells from wild type and Figure 3.1.12 shows dendritic cells from properdin-deficient mice, both after infection 4 hours with *L. monocytogenes*. There were more *L. monocytogenes* in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice. The different electron densities with which *L. monocytogenes* present in the phagosomes (Figure 3.1.11 panels A, B and D) may be indicative of different stages of degradation. There were dividing (segmented) bacteria and fusion of phagosomes producing large vacuoles as shown in panel D. There

were mixtures of cytosolic (A) and vacuolic locations (D) of *L. monocytogenes*, as expected from the literature (Paschen *et al.*, 2000; Guzman *et al.*, 1995). Birbeck granules, characteristic of dendritic cells, were identified in both genotypes and speak in supports of the methods of cell purification. (An example is shown in panel C). There are some *L. monocytogenes* in the cytosol, which have initiated actin polymerisation as shown in panel D and H. *L. monocytogenes* in dendritic cells from wild type were observed in membrane-bound compartments as in (1) panel G and free in the cytosol (2) panel G (Figure 3.1.11).

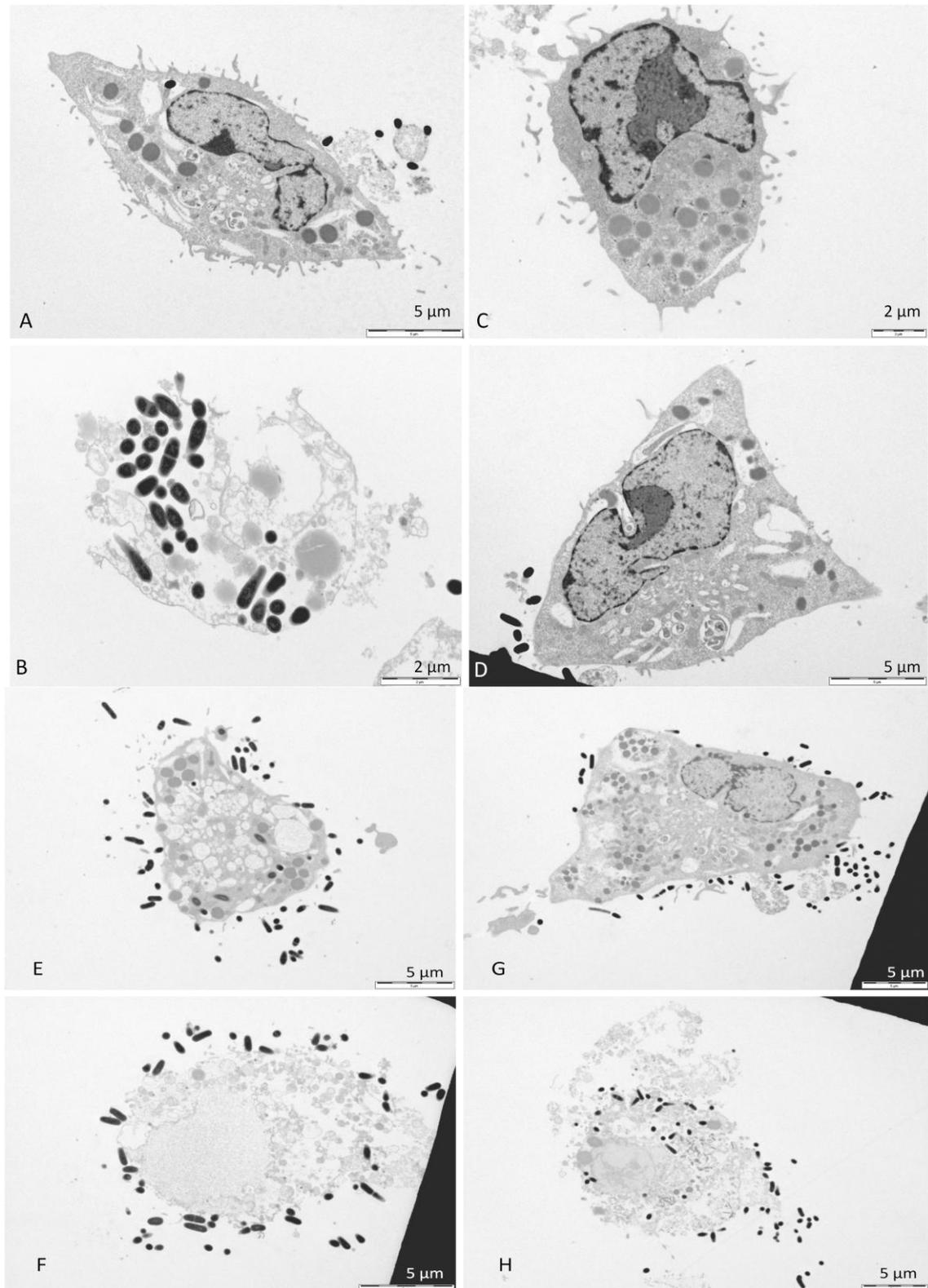
Overall less *L. monocytogenes* were observed in dendritic cells from properdin-deficient mice, with some cells showing dissolved, ultrastructural loss of the nucleus and the cell membrane (Panels B, F, and H) in the presence of *L. monocytogenes*. In addition, there appear to be more lysosomes, possibly due to the paucity of *L. monocytogenes* compared to dendritic cells from wild type. The predominant location of *L. monocytogenes* in dendritic cells from properdin-deficient mice showed less *L. monocytogenes* inside and less around the host cells, some show no *L. monocytogenes* around as shown in Figure 3.1.12, panel G. There were less vacuoles compared to cells from wild type.

Across the analysed cells, there are viable intracellular loads of *L. monocytogenes* but generally there are more of *L. monocytogenes* in dendritic cells from wild type (Figure 3.2.11) than in dendritic cells from properdin-deficient mice (Figure 3.1.12), which is consistent with result of viable bacteria obtained from infection of dendritic cells as shown in Figure 3.1.3.

Generally, the cytoplasmic processes appear less in infected cells compared to uninfected cells, this matches corresponding scanning electron micrographs.

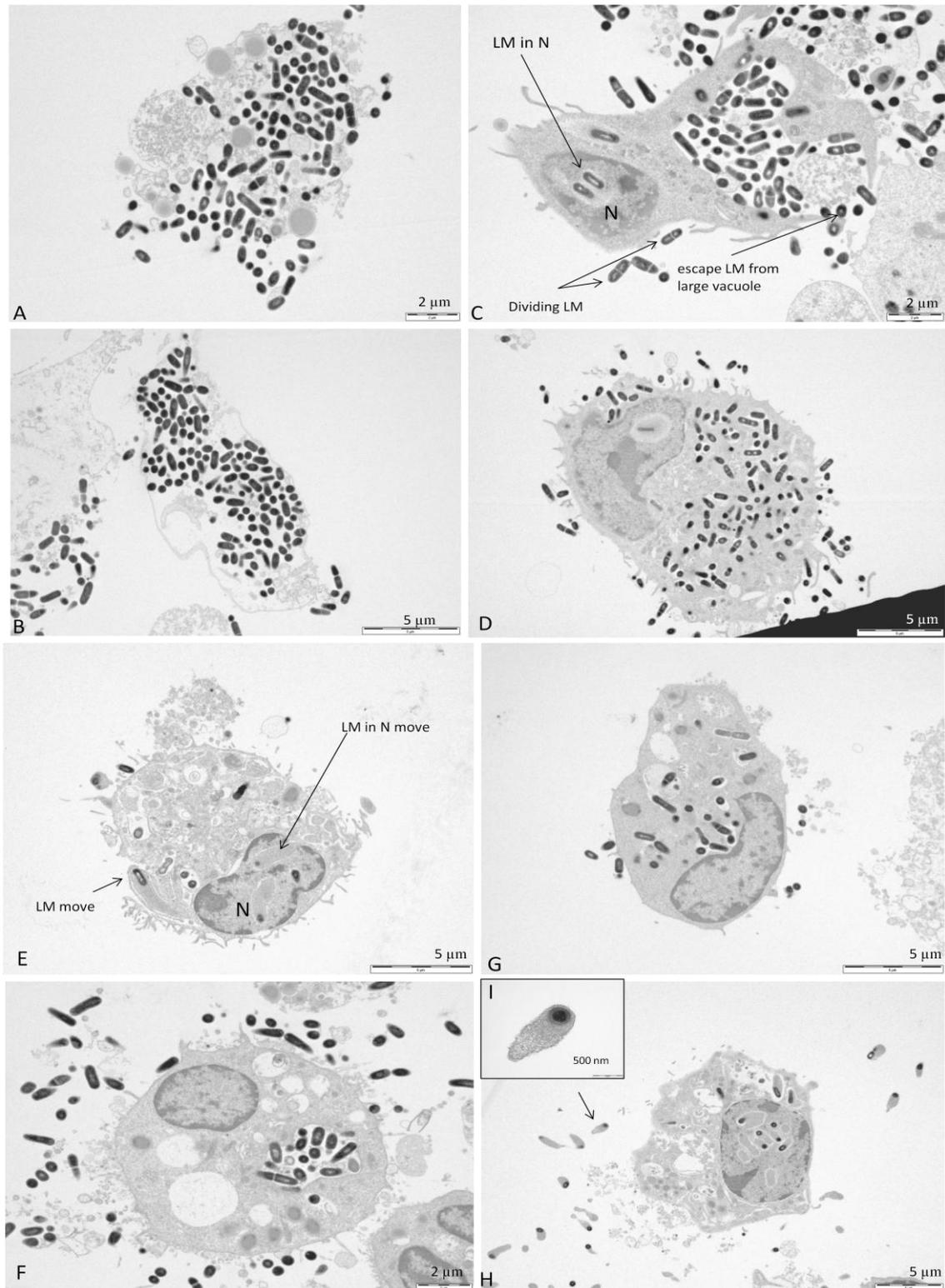


**Figure 3.1.11:** Typical transmission electron micrographs analysis of dendritic cells from wild type mice after infected with *L. monocytogenes* for 4 hours' incubation. There are different location of *L. monocytogenes* in the dendritic cell, (I) a dividing *L. monocytogenes* in cytosol and (H) dividing *L. monocytogenes* in vacuolar. F and J destroyed dendritic cells in J show *L. monocytogenes* with actin.



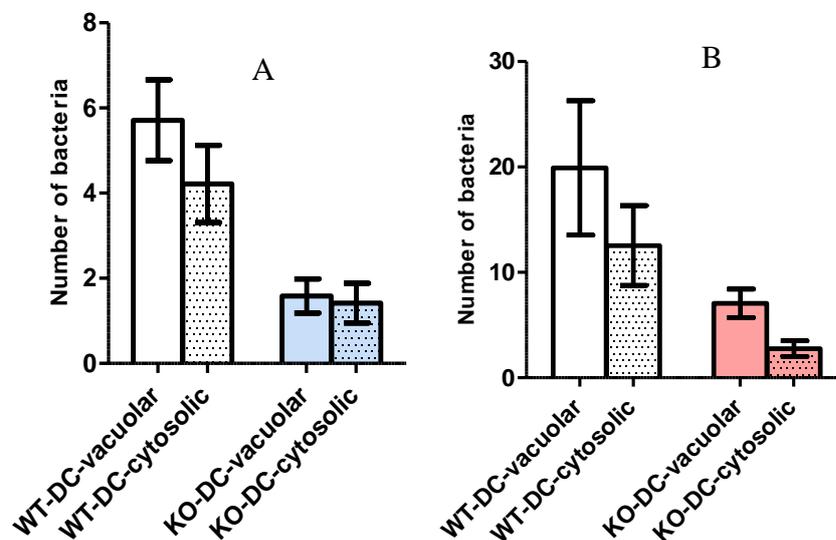
**Figure 3.1.12:** Representative transmission electron micrographs analysis of dendritic cells from properdin-deficient mice after infected with *L. monocytogenes* after 4 hours' incubation.

Next, Figure 3.1.13 shows mouse cells infected with *L. monocytogenes* for 24 hours and analysed for their ultrastructure by transmission electron microscopy. Dendritic cells from properdin-deficient mice (Panels E, F, G and H) show less *L. monocytogenes* than those from wild type mice (Panels A, B, C and D). Moreover, *L. monocytogenes* were present in the nucleus as shown in panels E and H. In panel H the insert (I) shows that the material enveloping *L. monocytogenes* has the same electron density as seen surrounding *L. monocytogenes* present in nucleus. Division of *L. monocytogenes* is seen in panels F and G. Panels E and F show cytosolic and vacuolar presence of *L. monocytogenes*. In panel C, *L. monocytogenes* escapes from large vacuoles.



**Figure 3.1.13:** Representative transmission electron micrograph analysis of dendritic cells after 24 hours' infection with *L. monocytogenes*, from wild type (A, B, C and D) and properdin-deficient mice (E, F, G and H). The fewer bacteria shown in dendritic cells from properdin-deficient mice compared to dendritic cells from wild type. Dendritic cells from properdin-deficient mice are intact compared to dendritic cells from wild type panels (A and B). Some *L. monocytogenes* are dividing as in panel (C and F) and others are moving panel (E). *L. monocytogenes* found in the nucleus with material surround it, panels (E, H and I).

Sub-cellular location of *L. monocytogenes* present in the vacuolar and cytosolic compartments of dendritic cells was scored after 4 and 24 hours of infection. Figure 3.1.14 in panel (A) varying number of dendritic cells remained intact for the genotypes at the two time points: there were 73 intact cells from wild type and 12 from properdin-deficient mice after 4 hour infection contrasting with 11 for wild type and 16 for properdin-deficient after 24 hours' infection. The numbers of *L. monocytogenes* in vacuolar and cytosolic compartments were higher in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice, and in wild type there were somewhat more *L. monocytogenes* in the vacuolar compartment than in the cytosolic compartment after 4 and 24 hours. In properdin-deficient the numbers of bacteria were equally distributed between the two compartments at 4 hours but after 24 hours were higher in the vacuolar than the cytosolic compartment.



**Figure 3.1.14:** Enumeration of *L. monocytogenes* in dendritic cells from wild type and properdin-deficient mice, vacuolar and cytosolic localisation after 4 hours (A) and 24 hours of infection (B). DC = dendritic cells. The data are presented as means with SEM.

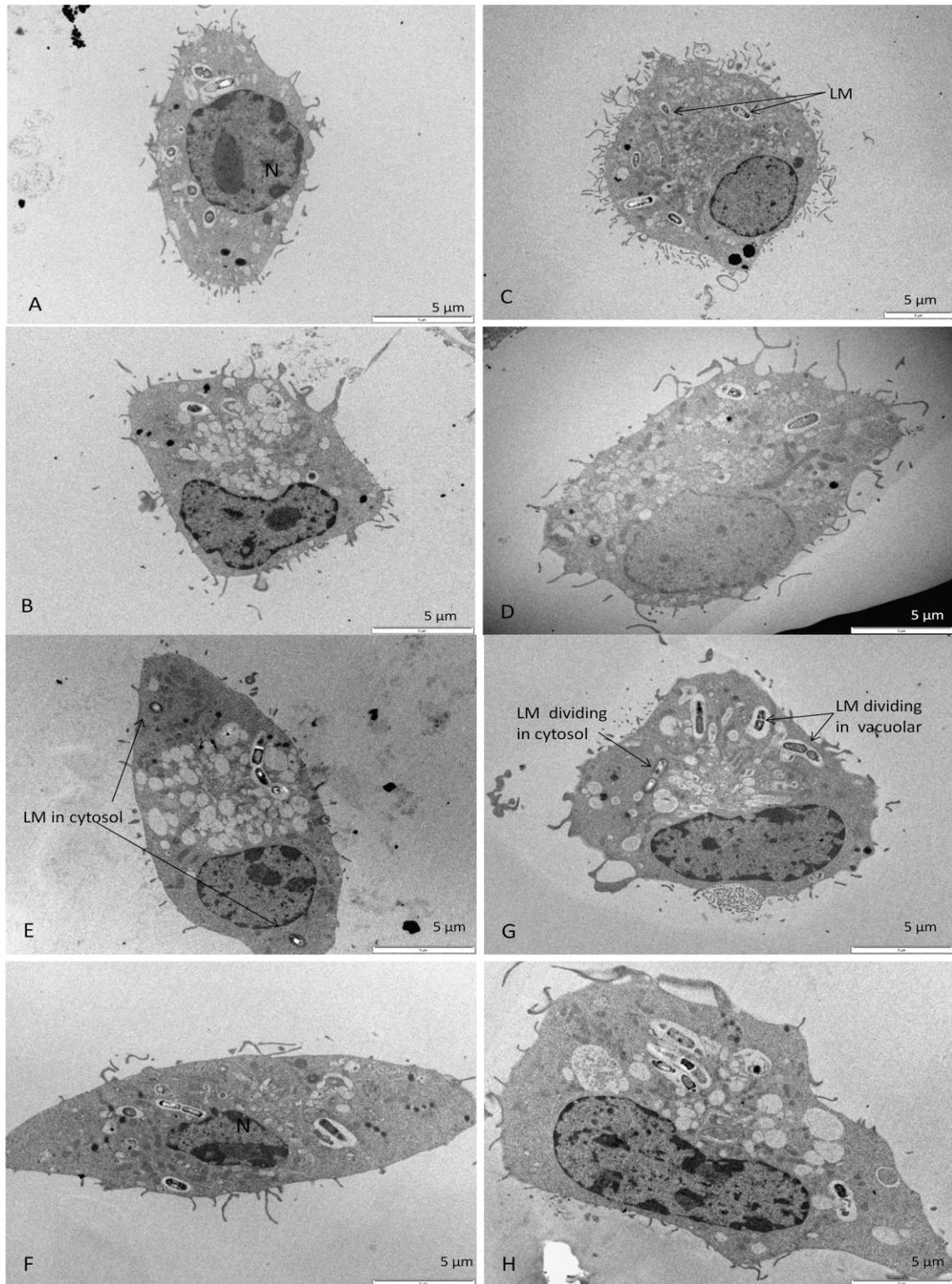
It has been shown that norepinephrine may regulate the function of immune cells that protect the body against pathogens (Kohm & Sanders, 2001).

To analyse the intracellular localisation of *L. monocytogenes* in dendritic cells using electron microscopy without and in the presence of norepinephrine, dendritic cell derived from bone marrows of wild type and properdin-deficient mice were infected for 4 hours with *L. monocytogenes* at MOI of an approximately 0.2 bacteria/cell without and in addition of 100  $\mu$ M norepinephrine. Infected cells with *L. monocytogenes* without and in the presence with norepinephrine were processed in parallel. Approximately 169 images were evaluated in this experiment with norepinephrine and without presence of norepinephrine approximately 149 images.

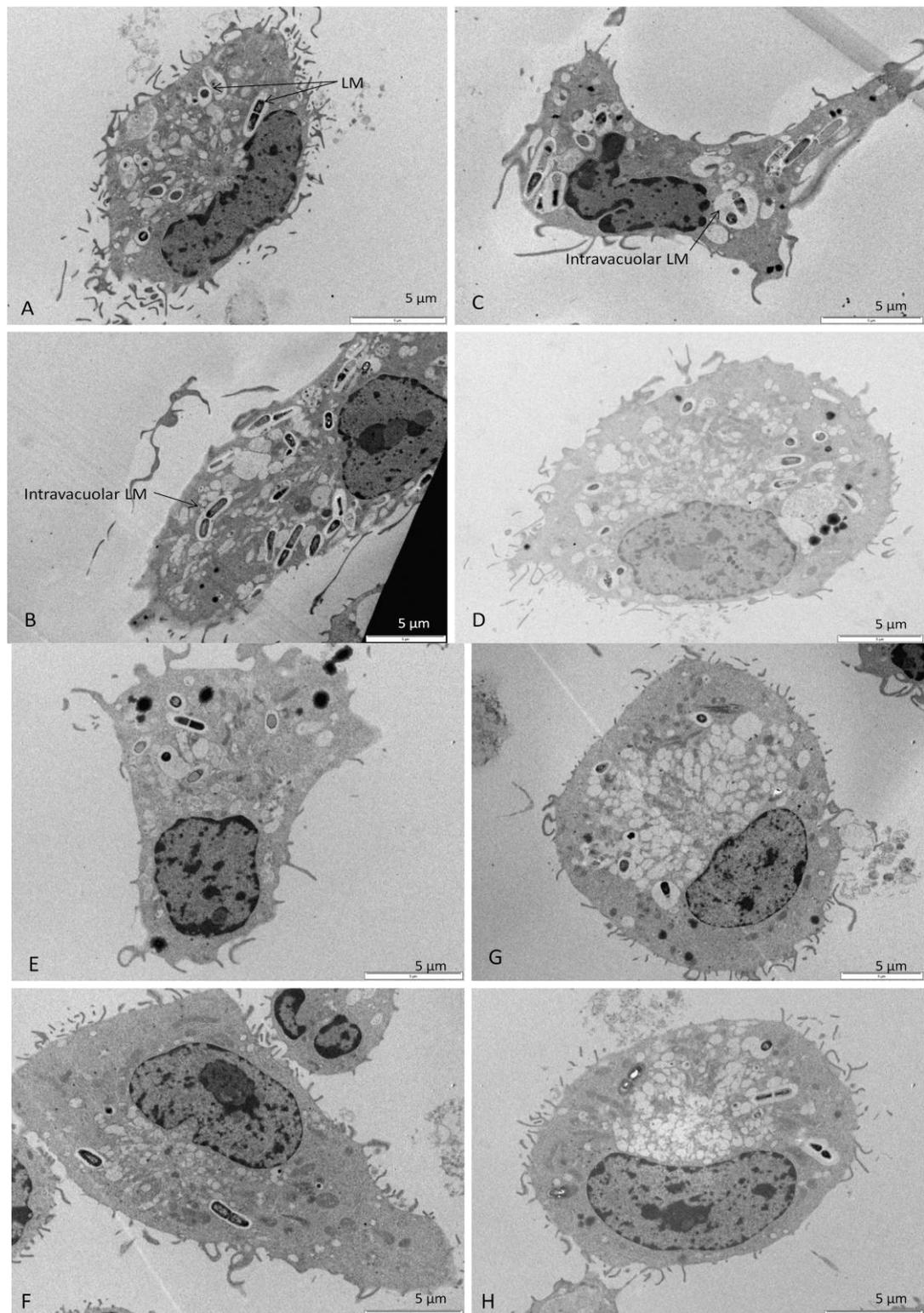
Dendritic cells infected with *L. monocytogenes* without norepinephrine as shown in the Figure 3.1.15 appeared intact compared to dendritic cells infected higher MOI (2.5) as shown in Figure 3.1.11 panel F and J and Figure 3.2.12 panel F and H. There were less *L. monocytogenes* in dendritic cells from wild type panels (A, B, C and D) compared to dendritic cells from properdin-deficient mice as shown in panels (E, F, G and H) so they were either more able to kill *L. monocytogenes* or they phagocytosis less. Most of *L. monocytogenes* were in vacuoles in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice with some bacteria in cytosol as shown in panel E (Figure 3.1.15). There were more bacterial division in some vacuoles in dendritic cells from properdin-deficient mice as shown in most panels (E, F, G and H) in compared to wild type (A, B, C and D) in the Figure 3.1.15.

Next, Figure 3.1.16, dendritic cells infected with *L. monocytogenes* MOI 0.2 in the presence of norepinephrine show more dividing *L. monocytogenes* compared to infected dendritic cells without norepinephrine and more *L. monocytogenes* in wild type in vacuoles (Panels A, B, C and D) compared to dendritic cells from properdin-deficient

mice (panels E, F, G and H). In both genotypes *L. monocytogenes* appears in cytosol and in vacuoles as shown in all panels and there is evidence of bacterial division in some vacuoles (Figure 3.1.16 panels A, B, E and H). (See appendix, Figures V and VI). For the quantitative analysis of *L. monocytogenes* were scored for their location in vacuolar and cytosolic compartments of dendritic cells after 4 hours as in Figure 3.1.17. In wild type, there are more *L. monocytogenes* in vacuoles compared to properdin-deficient.

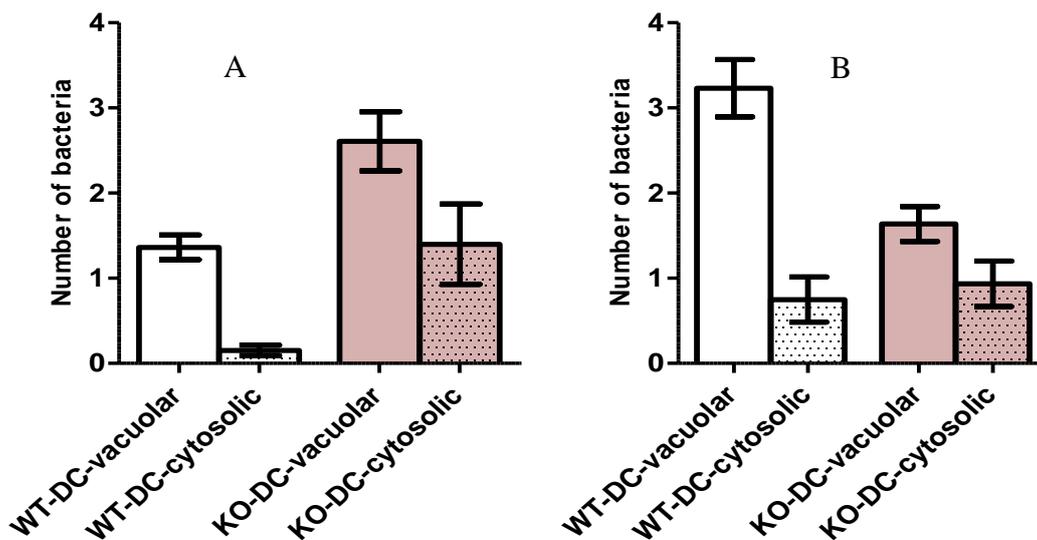


**Figure 3.1.15:** Representative transmission electron micrographs of dendritic cells after 4 hours infected with *L. monocytogenes* (MOI=0.2) without presence of norepinephrine. Dendritic cells from wild type mice (A, B, C and D) and from properdin-deficient mice (E, F, G and H). Most *L. monocytogenes* in dendritic cells from wild type shows in single form and in phagocytotic vacuoles but in cells from properdin-deficient *L. monocytogenes* are dividing in vacuole more than cells from wild type as showing in panels (E, F, G and H) and there some *L. monocytogenes* in cytosol (panel E). All cells are intact.



**Figure 3.1.16:** Representative transmission electron micrographs of dendritic cells after 4 hours infected with *L. monocytogenes* (MOI= 0.2) in presence with norepinephrine. Dendritic cells from wild type mice (A, B, C and D) and properdin-deficient mice (E, F, G and H). More *L. monocytogenes* in phagocytotic vacuoles in wild type and more dividing *L. monocytogenes* compared to cells from properdin-deficient. With a lower MOI all cells appeared intact.

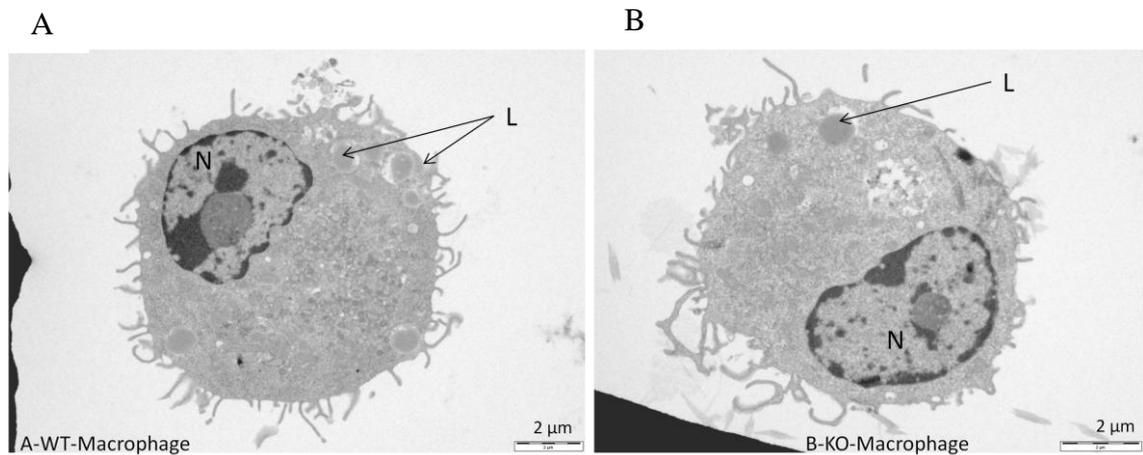
Figure 3.1.17 (A) shows greater escape in dendritic cells from properdin-deficient as proportion of those in vacuoles. Figure 3.1.17 (B) may point increase phagocytic activity of dendritic cells from wild type as a reaction to norepinephrine. The dendritic cells from properdin-deficient mice do not react in the same way, relatively more *L. monocytogenes* escape.



**Figure 3.1.17:** Enumeration of *L. monocytogenes* in dendritic cells from wild type and properdin-deficient mice (A) without norepinephrine and (B) with 100 µM norepinephrine, vacuolar and cytosolic localisation after 4 hours. The cells were set up in parallel. The data are presented as means with SEM.

### 3.1.6 Analysis of intracellular localisation of *L. monocytogenes* in macrophage using scanning and transmission electron microscopy

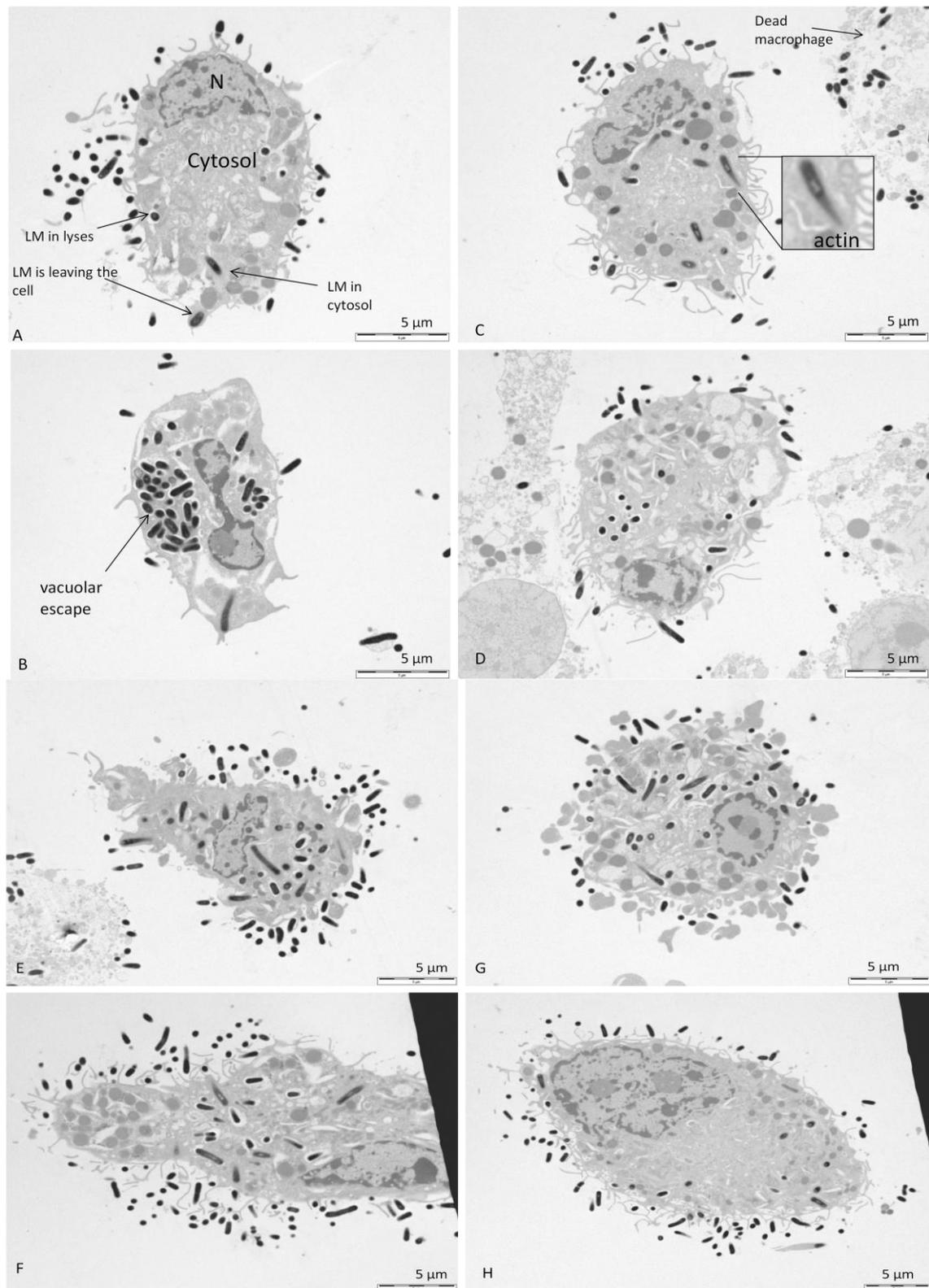
Macrophages were differentiated from bone marrow prepared from wild type and properdin-deficient mice and processed for transmission electron micrographs. Figure 3.1.18 shows uninfected macrophages, representative cross section are chosen, and show mononuclear cells with membrane processes and lysosomes, comparable to those murine macrophage from C57BL/6 described in (Zwaferink *et al.*, 2008).



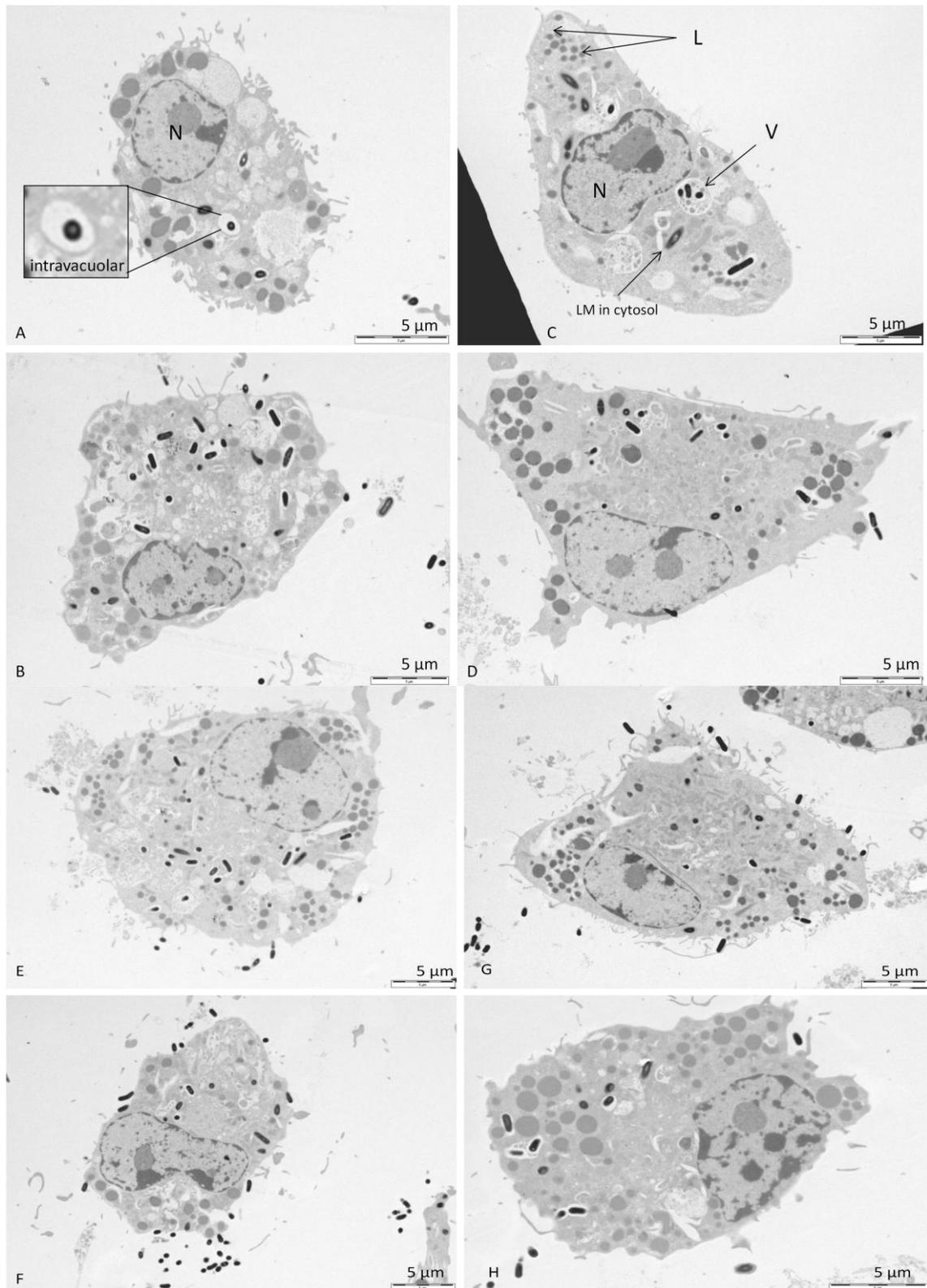
**Figure 3.1.18:** Shows transmission electron micrographs of control macrophages from wild type and properdin-deficient mice. N= cell nucleus, L= lysosomes.

Figure 3.1.19 shows macrophages from wild type and Figure 3.1.20 from properdin-deficient mice infected with *L. monocytogenes* for 4 hours in representative pictures of a blinded random choice of cells.

Electron micrographs show intact cells and bacteria which appear to have been fixed at different stages of infection: adsorption (Figure 3.1.19 A), vacuolar location (Figure 3.1.19 A and B) (Figure 3.1.20 C, and D), cytosolic location (Figure 3.1.19 A, B) (Figure 3.1.20 D), budding (Figure 3.1.19 A). There were more *L. monocytogenes* in macrophages from wild type compared with macrophages from properdin-deficient mice.



**Figure 3.1.19:** Typical transmission electron micrographs analysis of macrophages from wild type mice (all panels) after 4 hours infected with *L. monocytogenes*. (N= Nucleus, V= vacuole) MOI = approximately 2.5 bacteria/cell. *L. monocytogenes* appeared active there a lots of them moving (as in all panels) and almost in a single form. There is evidence of severely compromised cells (loss of cytoplasmic integrity) as in Panels C, D, E.



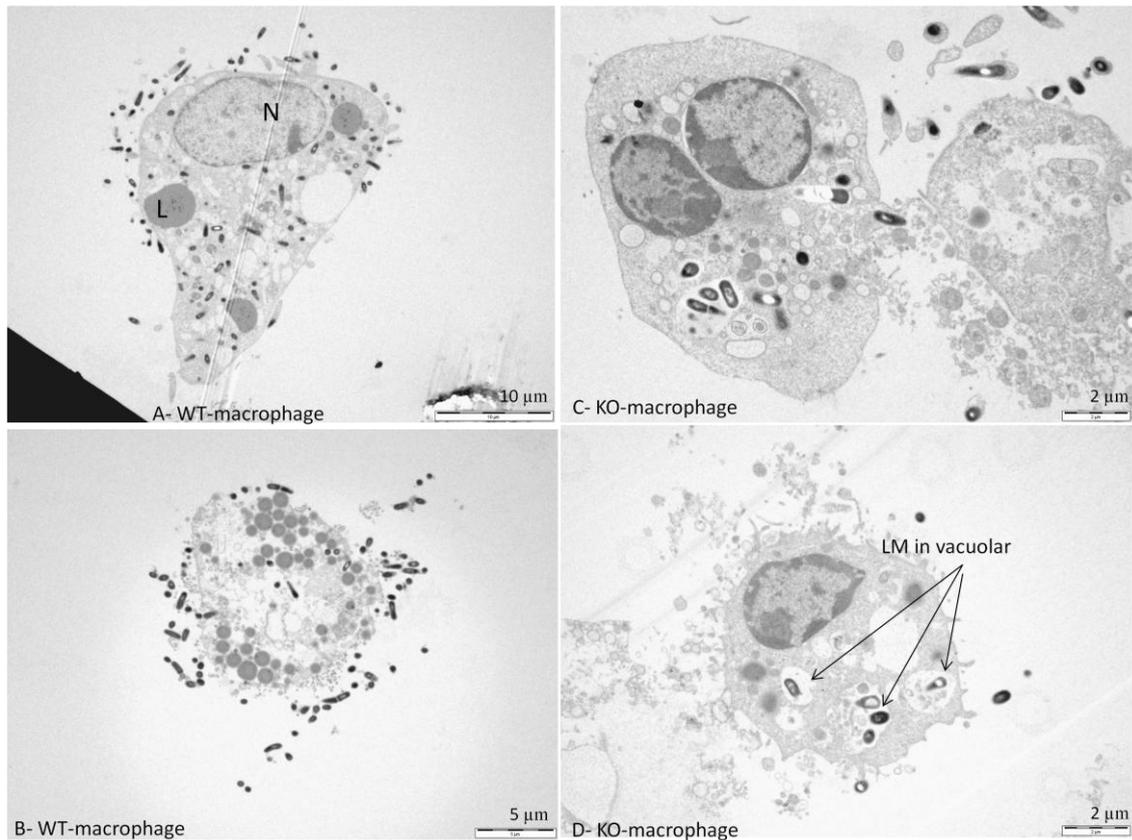
**Figure 3.1.20:** Typical transmission electron micrographs analysis of macrophages from properdin-deficient mice (all panels) after 4 hours infected with *L. monocytogenes*. MOI= 2.5 bacteria/cells. (N=Nucleus, V, vacuole) less *L. monocytogenes* surround or inside the cells compared to wild type (Figure 3.1.19).

Figure 3.1.21 show electron micrographs after 24 hours' incubation show a mixture of intact (A, C left cell and D) and compromised (B and C right cell) cells as judged by the loss of cell membrane.

Comparable characteristics have been described in Zwaferink *et al.*, 2008 which showed *L. monocytogenes* distributed throughout the cytoplasm in infected macrophages, with some of the cells (Figure 3.1 21 panel B) having disintegrated of plasma membrane in many places, with spilling -out of cytoplasmic contents. Moreover, in study by Pierce *et al.*, 1996 show localisation of *L. monocytogenes* in macrophage, *L. monocytogenes* were seen in the cytoplasm of peritoneal macrophages after 10 minutes of the infection, also numbers of bacteria were seen within phagosomes.

The electron micrographs documented various steps of the intracellular life cycle of *L. monocytogenes* that are known from the literature (Join-Lambert *et al.*, 2005; Kolb-Maurer *et al.*, 2000). In macrophage from wild type and properdin-deficient mice (Figure 3.1.9 and Figure 3.1.20) after infection for 4 hours with *L. monocytogenes* showed the intravacuolar bacteria, vacuolar escape and actin polymerisation (Guzman *et al.*, 1995). Intracellular growth or dividing bacteria were observed in dendritic cells more than in macrophage after 4 and 24 hours.

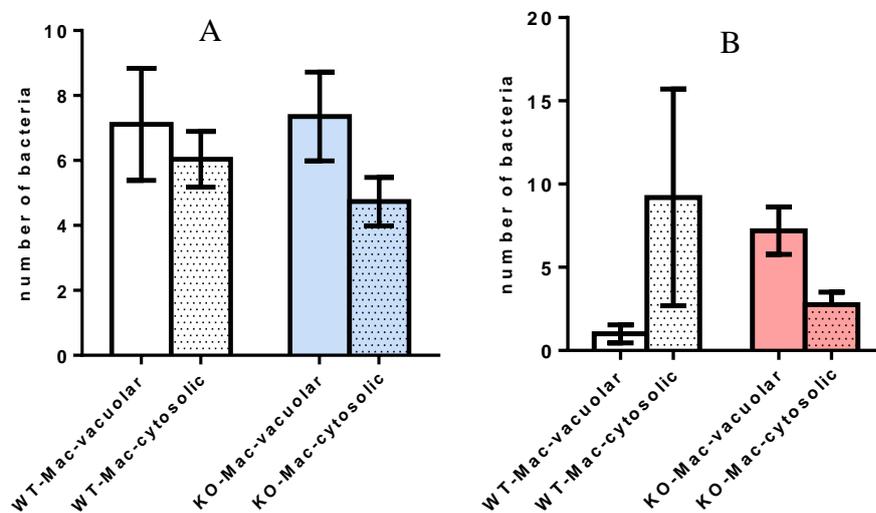
Importantly, cells infected with *L. monocytogenes* were washed as indicated in the protocol so the attachment of extracellular bacteria is wash-resistant, consistent with earlier reports (Pierce *et al.*, 1996).



**Figure 3.1.21:** Representative transmission electron micrographs analysis of macrophages after 24 hours infected with *L. monocytogenes*. Macrophages from wild type (A and B) and from properdin-deficient mice (C and D) (MOI=2.5). Some bacteria are surrounded with material (C). (B) Destroyed macrophage. Some *L. monocytogenes* in macrophages from wild type are extracellular, in vacuoles (D) or cytosolic (A). *L. monocytogenes* in macrophages from properdin-deficient mice are mainly located in vacuoles. N= nucleus, L= lysosomes.

Figure 3.1.22 shows enumeration of *L. monocytogenes* in macrophages, determined in vacuolar and cytosolic compartments after 4 and 24 hours' infection. Intact cells were analysed. The number of *L. monocytogenes* in vacuolar was comparable in macrophages from wild type compared to macrophages from properdin-deficient mice after 4 hours infection, and higher numbers of bacteria in cytosolic in macrophage from wild type than in macrophage from properdin-deficient mice but statistically there were no difference ( $p>0.05$ ). In wild type there were higher numbers of *L. monocytogenes* in vacuolar and less in cytosolic after 4 but after 24 hours the number of *L. monocytogenes*

were higher in cytosolic in macrophage from wild type and less in vacuolar. This may indicate escape from vacuoles. By contrast, in properdin-deficient cells, the numbers of bacteria remained higher in vacuoles after 24 hours compared to the cytosolic ( $p>0.05$ ). In macrophages, there were more *L. monocytogenes* in cytosol than in vacuoles. This is in contrast to dendritic cells, which showed more *L. monocytogenes* in vacuoles than cytosol at 4 hours of infection. This observation has been shown in a study by Westcott *et al.*, 2007. Compare macrophage and dendritic cells derived from bone marrow after infected with wild type *L. monocytogenes* at MOI approximately 2.5 at 4 hours. The more bacteria were observed in vacuole in dendritic cells compared to macrophage which mean that dendritic cells were restrictive for *L. monocytogenes* escape to the cytosol compared to macrophage the majority of *L. monocytogenes* were observed in cytosol.



**Figure 3.1.22:** Numbers of *L. monocytogenes* in macrophage from wild type and properdin-deficient mice, vacuolar and cytosolic localisation after 4 hours (A) and after 24 hours (B) ( $p>0.05$ ). Mac = macrophage. (26 for wild type and 34 for properdin-deficient cells mice after 4 hours' infection, and 5 for wild-type and 16 for properdin-deficient cells after 24 hours' infection).

*Listeria monocytogenes* in both dendritic cells and macrophage has not seen a double membrane vacuole but just only single ones in TEM (Vazquez-Boland *et al.*, 2001).

By using CFU and electron microscopy higher intracellular load of *L. monocytogenes* in dendritic cells from wild type compared to properdin-deficient mice which show properdin deficient mice have significantly less intracellular *L. monocytogenes*. By CFU but not electron microscopy the load can be increased in the presence of norepinephrine compared to wild type that shows unchanged intracellular *L. monocytogenes* in the presence of stress hormone. The escape of *L. monocytogenes* from lysosomes seems unimpaired in dendritic cells from properdin-deficient mice.

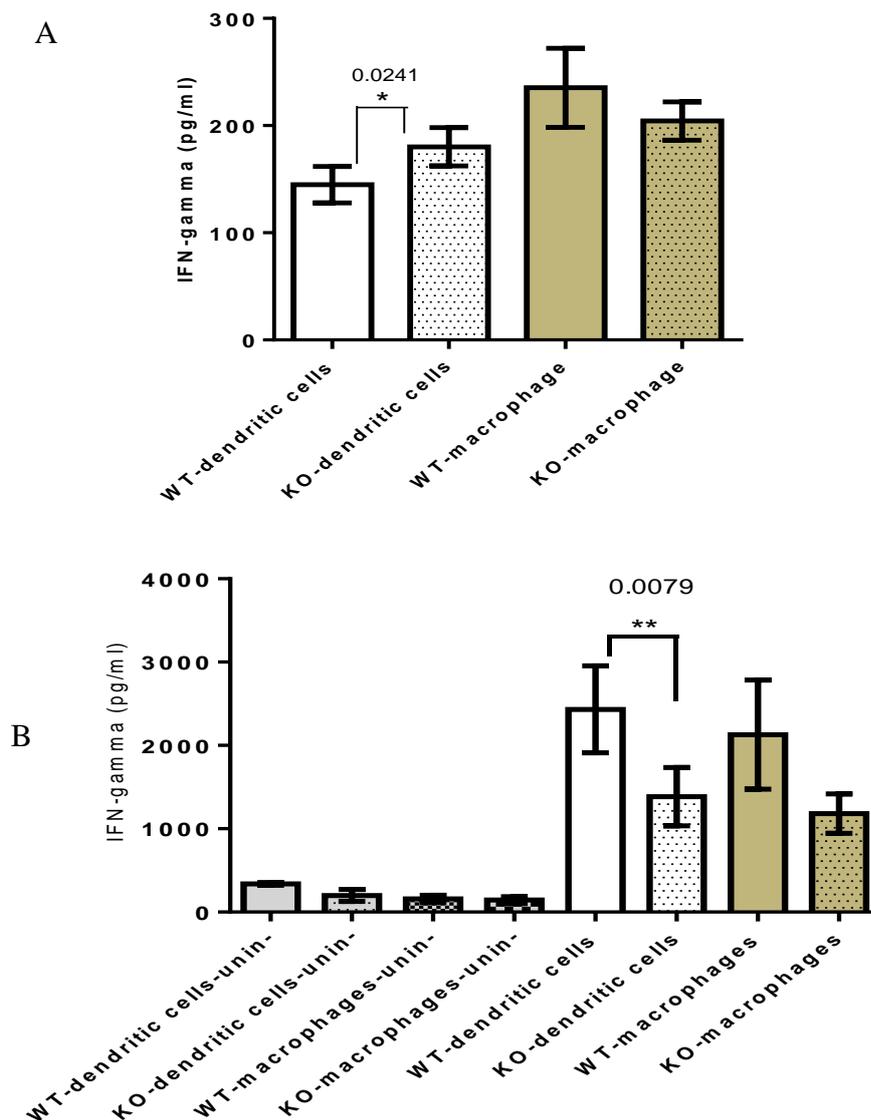
### **3.1.7 IFN- $\gamma$ response of dendritic cells and macrophages towards infection with *L. monocytogenes***

Production of IFN- $\gamma$  is normal anti-listerial host cell response produce by activated T cells and natural killer (NK) also *in vitro* under certain conditions. Macrophages and dendritic cells can also be stimulated to produce IFN- $\gamma$  (Thale & Kiderlen, 2005).

To study the role of properdin in IFN- $\gamma$  cytokine response from dendritic cells and macrophages in response to infection with *L. monocytogenes*, supernatants of infected cells from wild type and properdin-deficient mice were used for comparative analysis (Figure 3.1.23). After 2 hours infection (Figure 3.1.23 A), there was no significant difference IFN- $\gamma$  level between groups. After 24 hours infection (Figure 3.1.23 B), however, there was significantly increased IFN- $\gamma$  level in supernatants from infected dendritic cells from wild type compared to dendritic cells from properdin-deficient mice ( $p=0.0079$ ), on the other hand there was higher level of IFN- $\gamma$  in macrophages from wild type mice compared to macrophage from properdin-deficient mice but no significant difference ( $p>0.05$ ). The detection range for IFN- $\gamma$  using this ELISA format was 0.016-2ng/ml, statistically there was a significant difference. Other studies report a

much greater increase in cytokine production by macrophages infected with *L. monocytogenes* (Kupfahl *et al.*, 2006), so to determine the biological significance of the cytokine levels measured in this study, more experiments are needed.

There is a limitation of the *in vitro* test and only the mouse model would answer the question how relevant differences for IFN- $\gamma$  are in properdin-deficient and wild type mice. Mice when relating there to their survival.



**Figure 3.1.23:** IFN- $\gamma$  production of mouse cells in response to infection with *L. monocytogenes* after 2 hours (A) and after 24 hours (B). Each bar represents triplicate determinations. This experiment was set up in duplicate. The data are presented as means with standard deviation (SD). Unin= uninfected cells.

### 3.1.8 Responsiveness of dendritic cells and macrophages to exogenous IFN $\gamma$

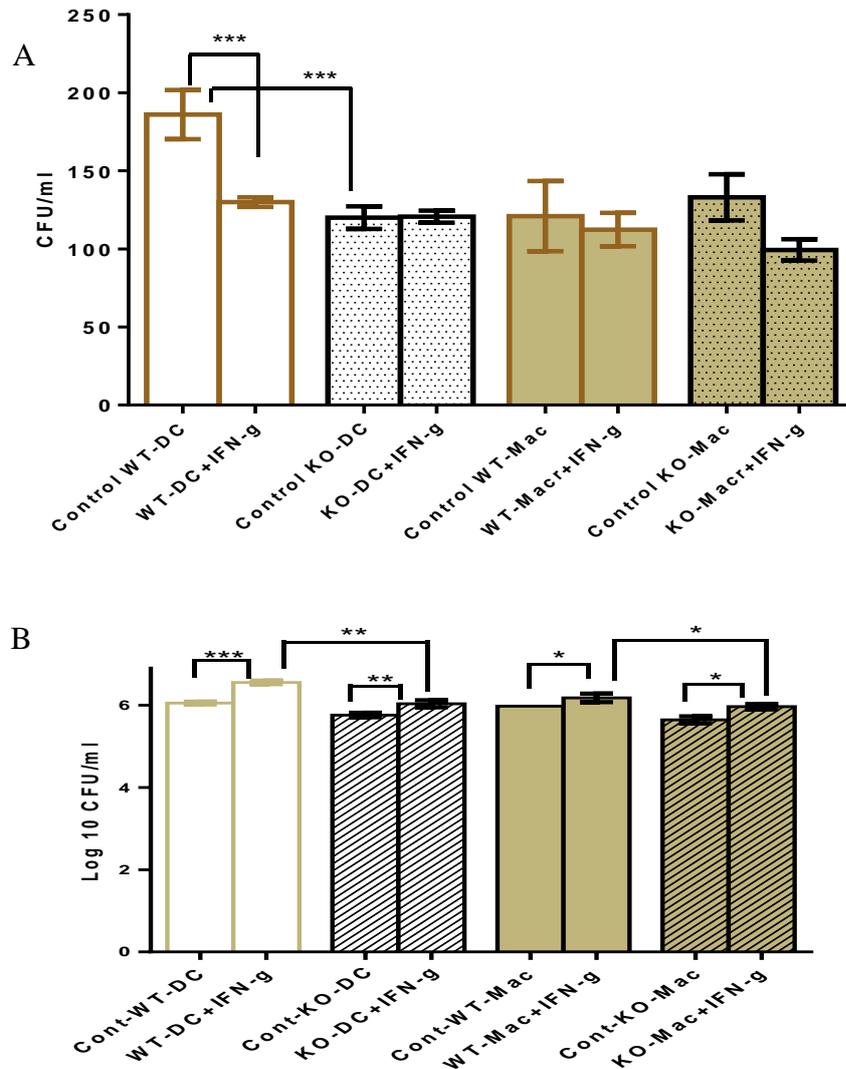
Exogenous IFN- $\gamma$  has been shown to limit *L. monocytogenes* numbers (Portnoy *et al.*, 1989). The analysis is based on establishing the viable count of *L. monocytogenes* after lysis by immune cells. The aim of this experiment was to see whether there was a difference in dendritic cells and macrophages from the two genotypes in responding in this way.

Viable counts were determined after treatment with gentamicin and addition of mouse IFN- $\gamma$  for 30 minutes and 24 hours.

There is an acute response to exogenous IFN- $\gamma$  in wild type dendritic cells viable counts of *L. monocytogenes*. After 30 minutes (A), as expected, in Figure 3.1.24 without the addition of IFN- $\gamma$  there were significantly more *L. monocytogenes* in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice ( $p < 0.05$ ). After cells were treated with IFN- $\gamma$ , there were significantly more *L. monocytogenes* in dendritic cells from wild type mice compared to dendritic cells from wild type mice treated with IFN- $\gamma$  ( $p < 0.05$ ). However, there was no difference in bacterial load in both wild type and properdin-deficient mice in the presence of IFN- $\gamma$ , and there were no significant difference in numbers of *L. monocytogenes* in dendritic cells from properdin-deficient mice compared to dendritic cells from properdin-deficient mice treated with IFN- $\gamma$ . By contrast, there were comparable amounts of viable *L. monocytogenes* intracellularly in bone marrow derived macrophages from wild type and properdin-deficient mice without or in presence of IFN- $\gamma$ . Dendritic cells from properdin-deficient mice do not show this sensitivity at 30 minutes.

After 24 hours addition of IFN- $\gamma$ , there were significantly higher number of *L. monocytogenes* (note y-axes A compare to B) in dendritic cells and macrophages in the presence of IFN- $\gamma$  compared to control without the addition of IFN- $\gamma$  (Figure 3.1.24 B).

There were significantly higher numbers of *L. monocytogenes* in dendritic cells from wild type compared to dendritic cells from properdin-deficient mice ( $p < 0.05$ ). In addition, the same was observed in macrophages from properdin-deficient mice in comparison with macrophages from wild type mice.



**Figure 3.1.24:** Analysis of bone marrow derived dendritic cells and macrophages from wild type (WT) and properdin-deficient mice (KO) for their intracellular viable load of *L. monocytogenes* obtained after 1 hour infection treated by adding IFN- $\gamma$  over (10 ng/ml) and treat with gentamicin for 30 minutes in (A) and for 1 hour gentamicin and infected for 24 hours with new media (B). One-way ANOVA test was used (Tukey compare all pairs of columns) ( $p < 0.05$ ). This experiment was set up in duplicate. The data are presented as means with standard deviation (SD).

After 30 minutes, the numbers of bacteria were able to count and expressed as CFU/ml however, the *L. monocytogenes* were grown more after 24 hours and the number of bacteria were expressed as log<sub>10</sub> CFU/ ml as shown on the axes (B) compared to (A). Moreover, after 24 hours, IFN- $\gamma$  was also released (previous experiment, section 3.1.7) but still there were differences with and without the addition of IFN- $\gamma$ .

The conclusion from this experiment is that initially, IFN- $\gamma$  restricts *L. monocytogenes* growth in dendritic cells from wild type and later, the intracellular load at 24 hours in wild type is higher than in properdin-deficient mice in the presence of IFN- $\gamma$  and compared to control.

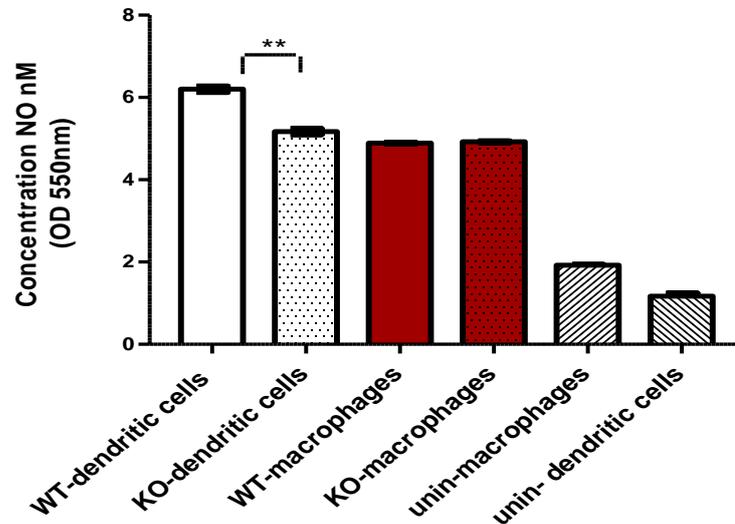
So, exogenous IFN- $\gamma$  as expected to be present *in vivo*, will be a modifier of the infection, just like stress-related hormones, which would be present in sepsis (Everest, 2007).

### **3.1.9 Quantitative measurement of nitric oxide (NO) from dendritic cells and macrophages in response to infection with *L. monocytogenes***

Nitric oxide is produced during murine listeriosis (Boockvar *et al.*, 1994), and important for the control of infection *in vivo* and *in vitro* (Torres *et al.*, 2004) and produced by activated macrophages. To study the role of properdin in nitric oxide from dendritic cells and macrophages in response to infection with *L. monocytogenes*, supernatants of infected cells from both genotypes were used for analysis in parallel.

Figure 3.1.25 shows the photometric measurement of nitric oxide in supernatants of dendritic cells and macrophages from wild type and properdin-deficient mice in response to 24 hours' infection with *L. monocytogenes*. There were significantly increased NO levels in supernatants from infected dendritic cells from wild type compared to those from properdin-deficient mice, on the other hand there was no

significant difference in NO level from infected macrophages from wild type and properdin-deficient mice.



**Figure 3.1.25:** Nitric oxide release from mouse cells in response to infection with *L. monocytogenes* after 24 hours. Each bar represents triplicate determinations. This experiment was set up in duplicate. The data are presented as means with standard deviation (SD).

### 3.1.10 TNF- $\alpha$ response of dendritic cells and macrophages towards infection with

#### *L. monocytogenes*

Tumor necrosis factor (TNF) plays an important role in the host response to the intracellular pathogen *L. monocytogenes*. TNF- $\alpha$  is a key pro-inflammatory cytokine which orchestrates inflammatory cells and reactions. TNF- $\alpha$  is produced by the host defence against bacterial infections and involved in antilisterial protection. It is produced by macrophages, dendritic cells, NK cells, and other non immune cells such as keratinocytes and neurons.

Supernatants from infected dendritic cells and macrophage derived from bone marrow from wild type and properdin-deficient mice with live and heat-killed *L. monocytogenes* (MOI=0.2) for 24 hours in normoxia and hypoxia, were used to measure TNF- $\alpha$  levels by ELISA. Uninfected supernatants were used to compare with infected supernatants.

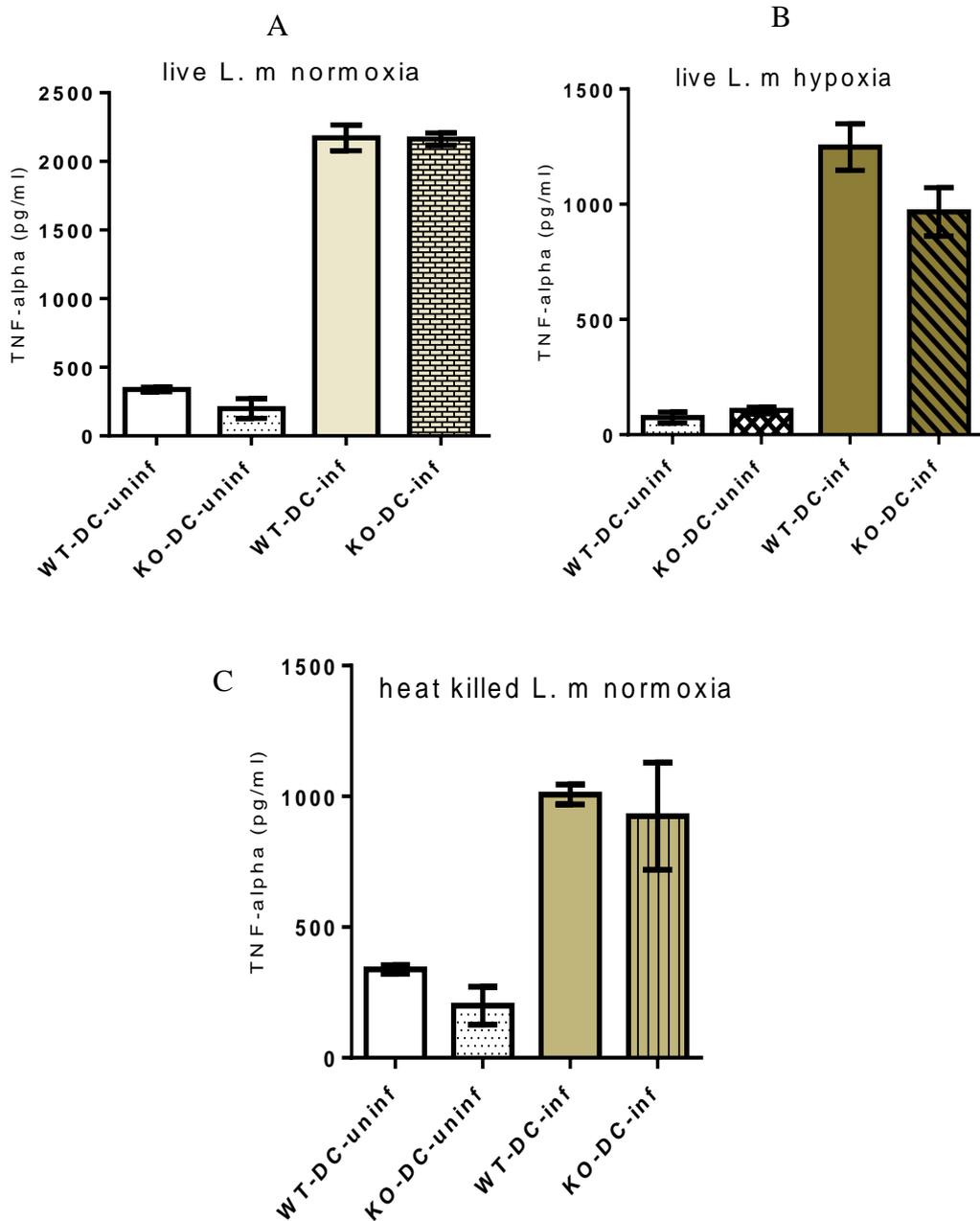
Compared to uninfected cells, dendritic cells from both genotypes produced more TNF- $\alpha$  after 24 hours infection in normoxic and hypoxic conditions (Figure 3.1.26). Moreover, as expected, the TNF- $\alpha$  level was higher in dendritic cells infected with live *L. monocytogenes* compared to cells infected with heat-killed *L. monocytogenes* (Figure 3.1.26 A and C), the increase TNF- $\alpha$  on the y-axes. Because as in literature shows heat-kill *L. monocytogenes* does not up-regulate TNF- $\alpha$  (Popov *et al.*, 2006).

The TNF- $\alpha$  level was less in both genotypes infected with live *L. monocytogenes* in hypoxia compared to dendritic cells infected with live *L. monocytogenes* in normoxia (Figure 3.1.26 A and B). There was no significant difference in TNF- $\alpha$  level from wild type dendritic cells compared to properdin-deficient mice in normoxic and hypoxic condition.

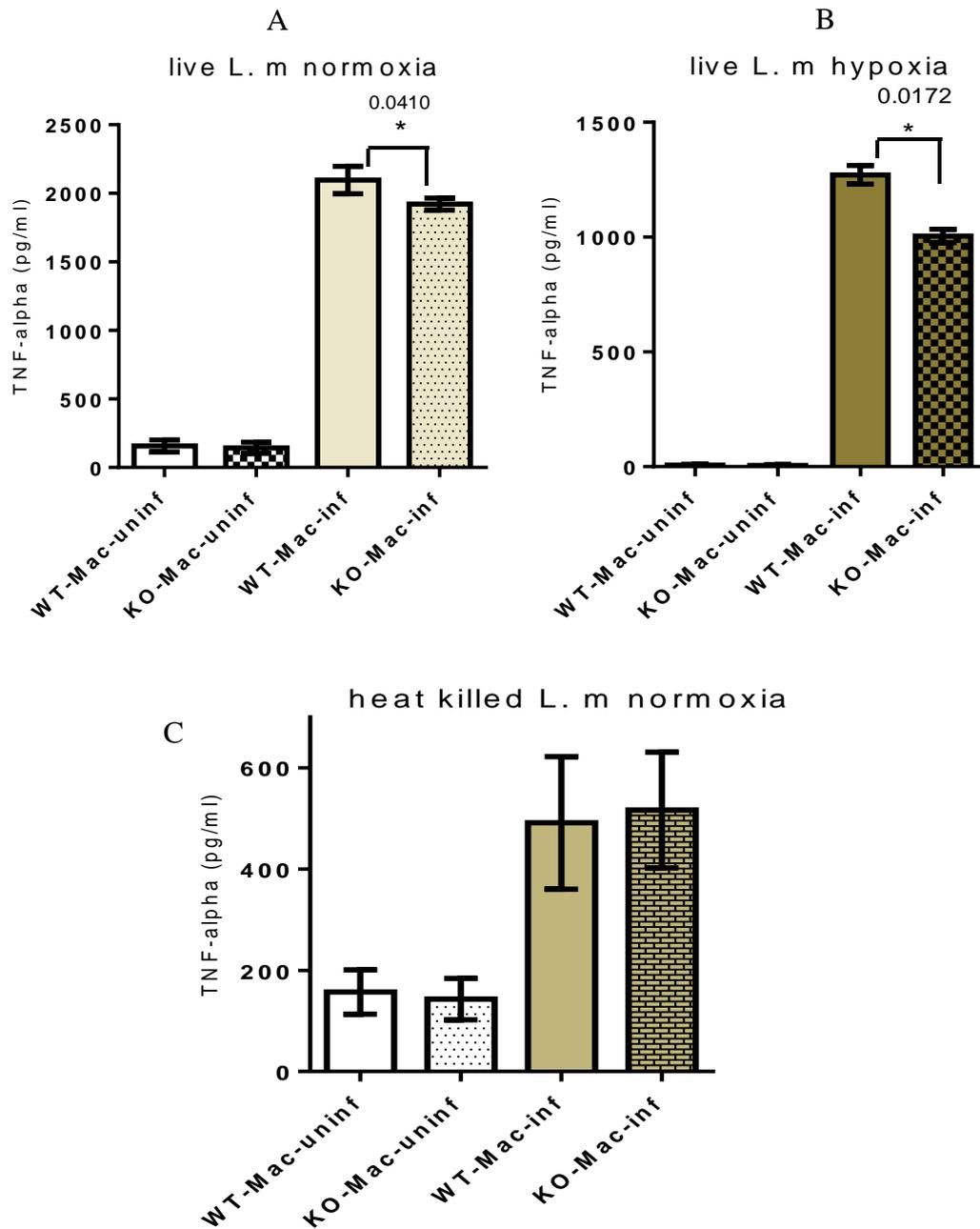
When studying the TNF- $\alpha$  response of bone marrow derived macrophage (Figure 3.1.27) a similar pattern emerges: the TNF- $\alpha$  level from macrophages was increased in supernatants in both genotypes after infection with *L. monocytogenes* compared to uninfected cells in both normoxia and hypoxia. Also, TNF- $\alpha$  level was higher in macrophages infected with live *L. monocytogenes* compared to infected cells with heat-killed *L. monocytogenes*, and was less in both genotypes infected with live bacteria in hypoxia compared to cells infected with live *L. monocytogenes* in normoxia. The decrease production of TNF- $\alpha$  in hypoxia has been shown in a study by (Yun *et al.*, 1997) using supernatants from murine macrophage-like RAW 264.7 (RAW) cells that exposed to hypoxia (2% O<sub>2</sub>) compared to normoxia (for 24 hours), and the protein release was determined by using a TNF- $\alpha$  ELISA kit.

Importantly, infected macrophages from wild type produced significantly more TNF- $\alpha$  compared to macrophage from properdin-deficient mice in both normoxia ( $p= 0.0410$ ) and hypoxia ( $p=0.0172$ ) when infected with live *L. monocytogenes*.

The viability of dendritic cell and macrophage in hypoxia after 24 hours were comparable for both genotypes (see section 3.1.4).



**Figure 3.1.26:** TNF- $\alpha$  level in supernatants from mouse dendritic cells in response to infection with live *L. monocytogenes* in normoxia (A) and in hypoxia (B) and with heat-killed *L. monocytogenes* in normoxia (C) for 24 hours (in hypoxia cells were infected with just live *L. monocytogenes* only). Each bar represents triplicate determinations. This experiment was set up in duplicate. Error bars were represents the mean with standard deviation (SD). The detection range for TNF- $\alpha$  using this ELISA format was 16-2000pg/m. Uninfected samples in (A) were represented same in (C). inf= infected, uninf= uninfected, L. m= *L. monocytogenes*.



**Figure 3.1.27:** TNF- $\alpha$  level in supernatants from mouse macrophage cells in response to infection with live *L. monocytogenes* in normoxia (A) and hypoxia (B) and with heat-killed *L. monocytogenes* in normoxia (C) for 24 hours. Each bar represents triplicate determinations. This experiment was set up in duplicate. The data are presented as means with standard deviation (SD). The detection range for TNF- $\alpha$  using this ELISA format was 16-2000pg/ml. Uninfected measurement was represented in (A) same as in (C). Mac= macrophage, inf= infected, uninf= uninfected, L. m= *L. monocytogenes*.

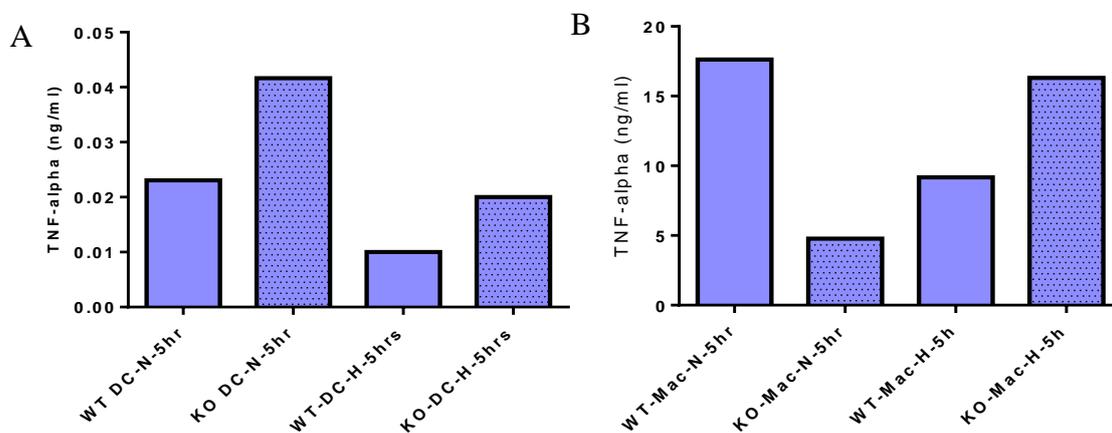
### **3.1.11 Determination of bioactive tumor necrosis factor-alpha (TNF- $\alpha$ ) levels using L929 assay**

This assay estimates the amount of TNF- $\alpha$  in a sample that is able to cause apoptosis in sensitised indicator cells unlike an ELISA which measures protein levels, therefore detect early bioactive TNF- $\alpha$ . Values are calculated from triplicate determination in relation to the effect of recombinant TNF- $\alpha$  on the density of indicator cells.

The extent of TNF- $\alpha$  release from dendritic cells and macrophages from wild type and properdin-deficient mice in response to infection was studied using live *L. monocytogenes* under normoxic and hypoxic conditions. The TNF- $\alpha$  were less in hypoxia compared to normoxia (Figure 3.2.18 A) and production is higher by macrophages than dendritic cells which is a general observation that is independent of properdin, and this has been reported previously, that hypoxia reduced the secretion and biological activity of TNF- $\alpha$  (Lahat *et al.*, 2008). TNF- $\alpha$  levels was higher in dendritic cells from properdin-deficient mice infected with *L. monocytogenes* compared to wild type in both hypoxia and normoxia. TNF- $\alpha$  levels from dendritic cells after infected with *L. monocytogenes* for 24 hours was less in both genotypes  $< 0.0002$  ng/ml. Moreover, the TNF- $\alpha$  level was somewhat increased in supernatant after infected 24 hours with *L. monocytogenes* in the presence with stress hormone, 0.0006 ng/ml in dendritic cells from wild type compared to 0.00015 ng/ml in dendritic cells from properdin-deficient mice (data not shown) but was less compared to without stress hormone. It has been reported that norepinephrine inhibits the production of cytokines including IFN- $\gamma$  and TNF- $\alpha$  (van der Poll *et al.*, 1994) using spleen cells from mice that were injected intravenously with an immunising inoculum of *L. monocytogenes* and incubated *in vitro* in the presence of norepinephrine. Norepinephrine showed no effect on cytokine production on unstimulated dendritic cells derived from murine bone

marrow, but it suggests that norepinephrine had an effect on activated dendritic cells at a physiologically relevant dose by beta- and alpha<sub>2</sub>-adrenergic receptors (Maestroni, 2002). Importantly, infected macrophage from wild type produced significantly more TNF- $\alpha$  in wild type compared to macrophage from properdin-deficient mice in normoxia (Figure 3.1.28 B). On the other hand, in hypoxia the TNF- $\alpha$  level was higher in infected macrophage from properdin-deficient mice compared to wild type. The wild type and properdin-deficient mice behave differently on a cell level. TNF- $\alpha$  secreted from macrophage was higher in both genotypes compared to dendritic cells in both genotypes after infection.

In summary, the TNF- $\alpha$  activity after 5 hours show a decrease of TNF- $\alpha$  in hypoxia in dendritic cells and macrophage from wild type compared to normoxia. The TNF- $\alpha$  activity in dendritic cells was higher in properdin-deficient in both conditions and in both 5 and 24 hours even with stress hormone norepinephrine.



**Figure 3.1.28:** TNF- $\alpha$  bioactivity concentration release of dendritic cell (A) and macrophage (B). Cells were cultured in RPMI medium and infected with *L. monocytogenes* for 5 hours in hypoxia and normoxia. Culture supernatants were assayed for TNF- $\alpha$  production using a bioassay (TNF- $\alpha$  sensitive L929 cells). Data representative of two independent experiments performed in triplicate (N=normoxia H=hypoxia). As described for this bioassay in section 2.1.3.8, the mean of triplicate reading is related to the activity of the TNF- $\alpha$  standard and therefore, no standard deviation (SD) appear here.

Protein levels of TNF- $\alpha$  measured by ELISA shows no difference in supernatants from dendritic cells in both genotypes after infection 24 hours with live and heat-killed *L. monocytogenes* in normoxia and hypoxia. TNF- $\alpha$  bioactivity using the L929 cell line shows that TNF- $\alpha$  activity in supernatants after 5 hours infection with *L. monocytogenes* were higher in dendritic cells (in normoxia and hypoxia) and macrophage (in hypoxia) from properdin-deficient mice compared to dendritic cells from wild type. In macrophage TNF- $\alpha$  bioactivity was higher in wild type than in properdin-deficient mice in normoxia and this was the same observation of protein levels of TNF- $\alpha$  using ELISA in normoxia and hypoxia. TNF- $\alpha$  bioactivity were higher in macrophage than dendritic cells. Summary of mediators released from dendritic cells and macrophages after infected with *L. monocytogenes* for 24 hours' are shown in the table below.

**Table 3.1.6:** Summary of mediators released from dendritic cells and macrophages after infection with *L. monocytogenes* for 24 hours'.

Mediator	Dendritic cells -24 hours	Macrophages-24 hours
IFN- $\gamma$ (by ELISA)	Significantly more IFN- $\gamma$ in WT compared to KO.	Increased IFN- $\gamma$ in WT compared to KO.
NO (by enzymatic assay)	More nitric oxide release in WT compared to KO.	Increased, but no significant difference between WT and KO.
TNF- $\alpha$ (by ELISA)	There were no differences between WT and KO in both conditions.	More TNF- $\alpha$ in WT compared to KO in normoxia and hypoxia

So in total, there are more antilisterial reaction (IFN- $\gamma$  and NO) and pro-inflammatory reaction (TNF- $\alpha$ ) in cells from wild type mice.

### **3.1.12 Basal expression and infection-induced changes of properdin, CD11b, C3 and TLR2 mRNA expression in bone marrow derived dendritic cells**

Dendritic cells derived from bone marrow of mice from wild type and properdin-deficient mice were infected with *L. monocytogenes* and were used to investigate gene specific immune response. C3 expression was analysed because it is an acute phase reactant (Wenger *et al.*, 1995). CD11b and TLR2 are involved in recognition and uptake phase of *L. monocytogenes* (Torres *et al.*, 2004), and properdin the gene of interest.

The mRNAs expressions were analysed by reverse transcriptase polymerase chain reaction (RT-PCR), and quantitative RT-PCR. Total RNA was isolated from control and infected cells at different time-points. Expression of  $\beta$ -actin was used as a reference (housekeeping gene) and expression of this gene stays constant upon infection and this was shown by qPCR. VEGF was used as a control for hypoxia. Gene expression of C3, CD11b, TLR2 and  $\beta$ -actin were analysed by RT-PCR on dendritic cells from wild type or properdin-deficient mice after 24 hours infected with heat-killed *L. monocytogenes*. This experiment used heat killed *L. monocytogenes* because of the particular sensitivity of dendritic cells to be damaged by live *L. monocytogenes*. *Listeria monocytogenes* are phagocytosed by dendritic cells and normally are quickly destroyed within phagosome (Popov *et al.*, 2006).

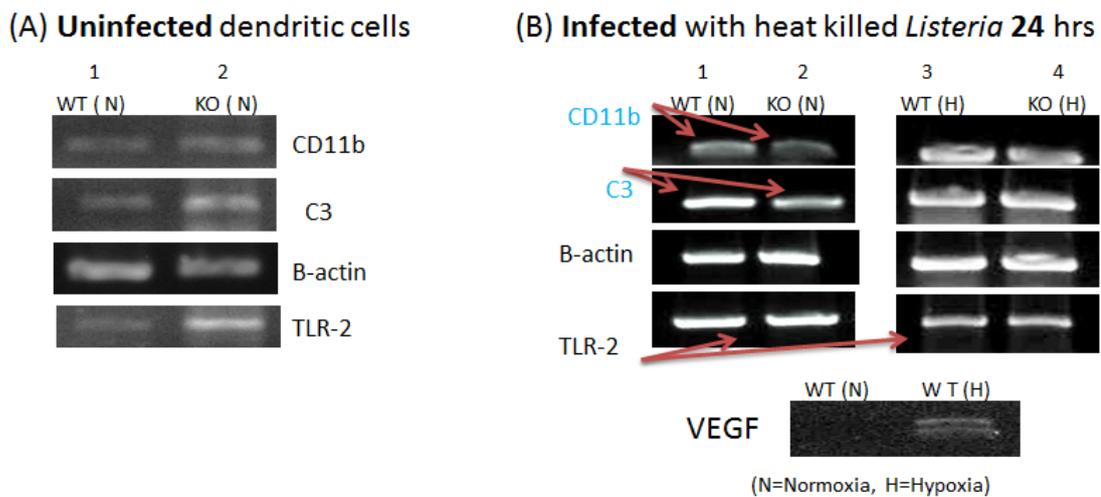
RT-PCR was used as a screen to determine the expression (level) and possibility of regulation.

The mRNA expression of C3, CD11b and TLR2 in uninfected and infected dendritic cells were detected as shown in Figure 3.1.29 and related to expression of  $\beta$ -actin.

As can be seen in Figure 3.1.29 (A), the mRNA expression of C3, CD11b and TLR2 in uninfected dendritic cells from wild type and properdin-deficient mice in normoxia were compared to  $\beta$ -actin and, all genes were expressed less compared to infected

dendritic cells. Expression of C3 and TLR2 appeared higher in dendritic cells from properdin-deficient compared to wild type but there was no significant difference in expression of CD11b in both genotypes ( $p>0.05$ ).

Next, in the Figure 3.1.29 (B), after infection the expression of all genes were up-regulated compared to uninfected, the expression levels of both genes C3 and CD11b in dendritic cells from wild type appeared higher than in dendritic cells from properdin-deficient mice, given that  $\beta$ -actin intensity was comparable. All genes were abundantly expressed in hypoxia. CD11b is needed to activate killing of *L. monocytogenes* by recruitment, mediated phagocytosis, and signaling *L. monocytogenes* activity. Therefore, quantitative qRT-PCR was needed to determine the role of properdin in an expression and up-regulation of C3 and TLR2.



**Figure 3.1.29:** RT-PCR products obtained using dendritic cells, uninfected (A) and infected (B) with *L.monocytogenes*. Sizes were as expected for  $\beta$ -actin gene, 540 bp, C3 gene, 500 bp, TLR2 gene, 548 bp, CD11b gene, 397 bp and VEGF gene, 235 bp, and relevant section of gel images used for composite figure. Dendritic cells were infected with heat-killed *L. monocytogenes* for 24 hours. In (A and B) 1=WT- in normoxia, 2= WT- in hypoxia, 3= KO- in normoxia, 4= KO- in hypoxia. VEGF was used as a control for hypoxia in WT. The data are representative of two independent experiments.

Expression of properdin by RT-PCR was undetectable therefore, properdin expression was analysed by qPCR to investigate whether properdin change or un-regulated in wild type after infection. The mRNA expression of properdin in uninfected and infected dendritic cells after 24 hours with heat-killed *L. monocytogenes* was shown in the Figure 3.1.30 (A). As expected, expression of properdin was significantly higher in dendritic cells from wild type in uninfected and infected with *L. monocytogenes*. However, the expression of properdin was down-regulated after infection with *L. monocytogenes* in wild type compared to uninfected cells. This expression was unexpected and there was no significant difference in dendritic cells from wild type after infection with *L. monocytogenes* compared to uninfected.

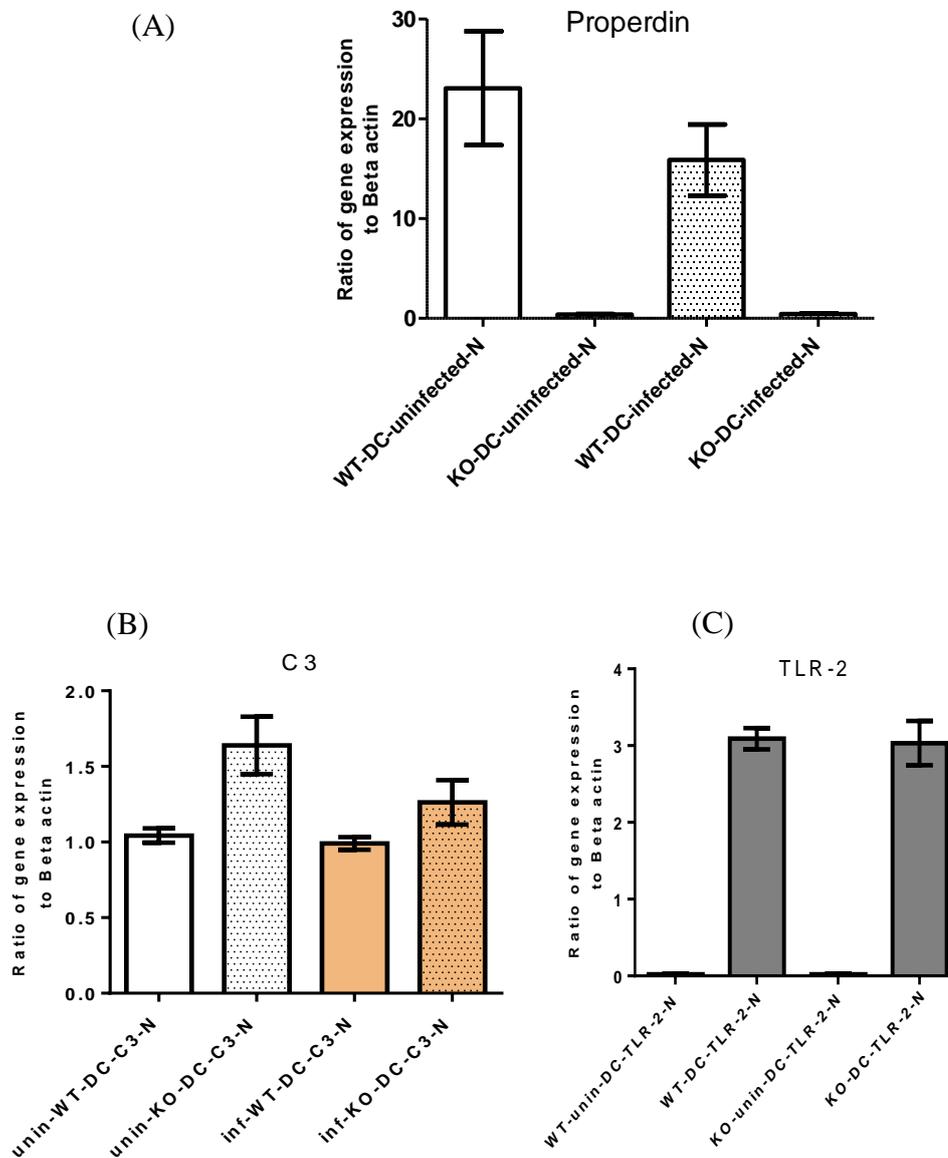
Dendritic cells from properdin-deficient mice uninfected and infected were used as properdin-deficient control.

The qPCR expression, as can be seen in Figure 3.1.30 expression of C3 (B) and TLR2 (C). Expression of C3 in (B) in uninfected dendritic cells show unexpected higher expression in both genotypes and appeared higher in dendritic cells from properdin-deficient mice compared to dendritic cells from wild type mice. However, after the infection expression of C3 was the same unchanged in uninfected in dendritic cells from wild type mice, in dendritic cells from properdin-deficient mice appear less expression after infection.

The same pattern was observed for expression of C3 by mRNA qPCR in RT-PCR in uninfected dendritic cells also the same pattern after infection in normoxia.

Expression of TLR2 (C) was up-regulated after 24 hours infection with *L. monocytogenes* in normoxia compared to uninfected and the expression was higher than other genes but was shown no difference between both genotypes. Overall in dendritic cells there was no significant difference in all genes expression between both genotypes.

Analysis of the real-time PCR results *in vitro* and *in vivo*, each gene level was measured by dividing the normalised expression value obtained for the  $\beta$ -actin gene by the normalised expression value obtained for the each gene.



**Figure 3.1.30:** Quantitative values of mRNA expression levels of properdin (A), C3 (B) and TLR2 (C) were determined in bone marrow derived dendritic cells uninfected and infected with heat-kill *L. monocytogenes* after 24 hours. Gene expression levels are presented relative to  $\beta$ -actin mRNA expression. This experiment was set up in duplicate from triplicate measurement. The data are presented as means with standard deviation (SD).  $\Delta\Delta C_t$  methods were used. (N=normoxia).

### **3.1.13 Basal expression and infection-induced changes of properdin, CD11b, C3, TLR2, IFN- $\beta$ mRNA expression in bone marrow derived macrophages**

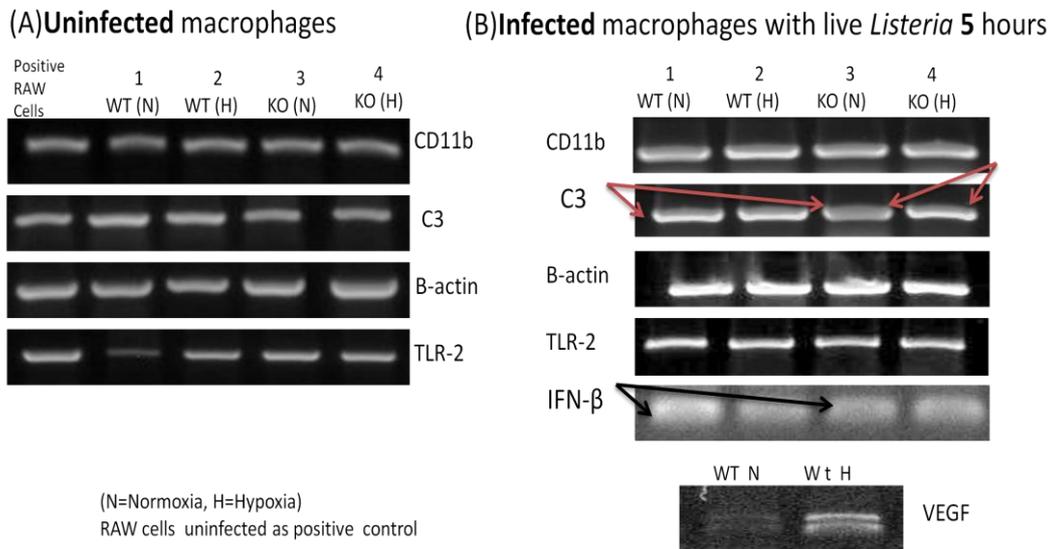
Expression of C3, CD11b, IFN- $\beta$  and TLR2 mRNA was analysed in bone marrow derived macrophages (infected after 5 hours with live *L. monocytogenes* and control from both genotypes) by RT-PCR initially. A mouse macrophage cell line RAW 264.7, was used as control in normoxia. It has been shown that higher levels of IFN- $\beta$  can be induced in infected macrophage cells by *L. monocytogenes* once inside the cytoplasm of the cells (Cossart, 2002; Havell, 1986).

Uninfected macrophages from wild type and properdin-deficient mice showed basal expression for C3, CD11b and TLR2 under normoxia and hypoxia (Figure 3.1.31 A).

Expression of TLR2 in uninfected macrophage (1= WT (N)) were repeated and showed no difference in expression compared to macrophage from properdin-deficient mice not as showing in (Figure 3.1.31 A).

After infection with live *L. monocytogenes*, however, expression of C3, CD11b, TLR2 and IFN- $\beta$  appeared higher (Figure 3.1.31 B). Expression of C3 mRNA in macrophages from wild type appeared higher compared to macrophages from properdin-deficient mice in normoxia (arrow in Figure 3.1.31 B) and showed that qPCR analysis was required. Expression of CD11b appeared higher in macrophage from wild type in hypoxia compared to macrophage from wild type in normoxia but no difference was apparent compared to properdin-deficient mice. CD11b mRNA is stably expressed in hypoxia and normoxia once it is induced by infection. TLR2 expression after infection appeared to be the same in all genotypes. IFN- $\beta$  expression was induced after infection (it was undetectable in uninfected cells) especially in wild type cells (Figure 3.1.31 B).

Detection of VEGF in hypoxia treated macrophages from wild type, as control for both genotypes, is a clear indication that the cells had indeed been exposed to hypoxic condition as it is a hypoxia inducible gene (Xiong *et al.*, 1998).



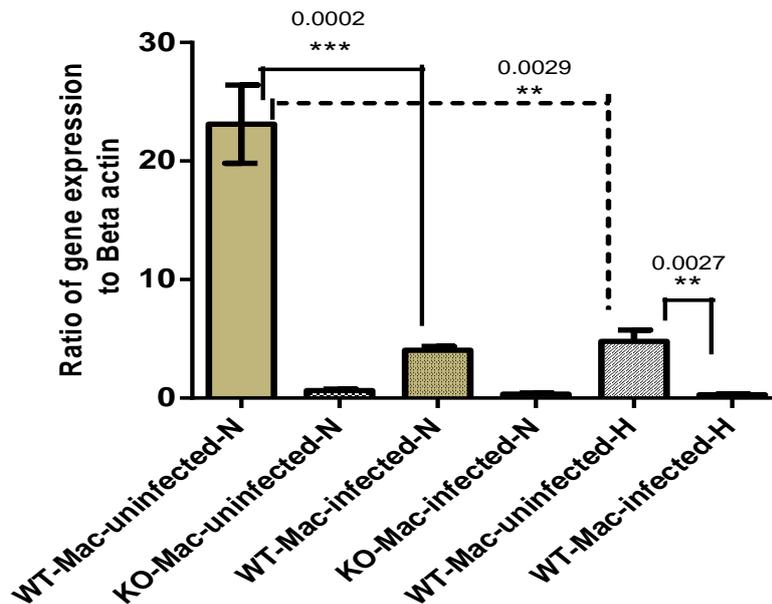
**Figure 3.1.31:** RT-PCR products obtained using macrophages, uninfected and infected with live *L. monocytogenes* (5 hours). RAW cells were used as positive control for basal expression. Sizes were as expected for  $\beta$ -actin gene, 540 bp, C3 gene, 500 bp, TLR2 gene, 548 bp, IFN- $\beta$  gene, 82 bp, CD11b gene, 397 bp and VEGF gene, 235 bp, and relevant section of gel images used for composite figure. In (A and B) 1=WT- in normoxia (N), 2= WT- in hypoxia (H), 3= KO- in normoxia, 4= KO- in hypoxia. VEGF was used as control for hypoxia in WT.

QPCR was performed for quantitative analysis, to study the potential difference of mRNA expressions in macrophages from both genotypes. A distinct difference may be lost in performing the amplification at a defined cycle number as in normal, not quantitative RT-PCR.

As in dendritic cells, the mRNA expression of properdin in uninfected and infected macrophage after 24 hours as shown in the Figure 3.1.32 was a significantly higher expression in wild type. However, after infection the properdin expression was down-

regulate in macrophage from wild type compared to uninfected, this was unexpected and there was significant difference between uninfected and infected in normoxic ( $p=0.0002$ ) and hypoxic ( $p= 0.0027$ ). The expression of properdin in hypoxia was less compared to normoxia this difference show that properdin as a hypoxia sensitive gene. The decrease of properdin expression was observed in RWA cells after stimulation with LPS (with concentration of 100 ng/ml for 4 hours) compared to un-stimulated as shown in another project conducted in Dr, Stover' group. Further studies are needed to assess the abundance of properdin protein expression, but at the time of this study mouse specific antibodies were not available.

Uninfected and infected macrophage from properdin-deficient shows that macrophage from mice genotyped as KO, control, was indeed properdin-deficient.



**Figure 3.1.32:** Quantitative value of mRNA expression level of properdin was determined in macrophages uninfected and infected with live *L. monocytogenes* after 24 hours. Gene expression levels are presented relative to  $\beta$ -actin mRNA expression. This experiment was set up in duplicate from triplicate measurement. The data are presented as means with standard deviation (SD) and  $\Delta\Delta$ CT value CT method was used. (N=normoxia, H=hypoxia).

mRNA expression levels of C3 and TLR2 were determined by qPCR in macrophages from both genotypes after infection for 5 and 24 hours with live *L. monocytogenes* (Figure 3.1.33). Expression of C3 in uninfected (panel A) was higher in both genotypes in normoxia and hypoxia compared to infected that show slightly higher, and uninfected appeared higher in wild type compared to properdin-deficient mice, but in hypoxia the expression of C3 in uninfected cells appeared to be higher in cells from properdin-deficient compared to wild type. Moreover, expression of C3 in macrophages from properdin-deficient in hypoxia was increased compared to macrophages from properdin-deficient in normoxia in both times after 5 hours ( $p=0.0325$ ) (panel B) and 24 hours ( $p=0.0260$ ) (panel C). Expression of C3 in macrophage from wild type mice was the same expression in uninfected and infected compared normoxia to hypoxia. Also the same pattern was observed in uninfected in both genotypes and infected cells.

Even it appears a higher expression between wild type and properdin-deficient but there were no difference in expression of C3 in both genotypes and in both conditions.

Expression of TLR2 were higher as expected after infection for 5 and 24 hours with live *L. monocytogenes* compared to uninfected cells in both genotypes. Expression of TLR2 in the Figure 3.1.33 (panel D after 5 hours and panel E after 24 hours) shows TLR2 expression in uninfected macrophage was detectable in hypoxia and normoxia. No difference was shown after infected 5 hours with *L. monocytogenes* in both genotypes and in both conditions. On the other hand, after 24 hours infection shows significantly lower expression of TLR2 in macrophages from properdin-deficient mice compared to macrophage from wild type mice in normoxia ( $p<0.05$ ) as the wild type response by definition in the normal response. But there were no difference between macrophages from wild type and properdin-deficient mice after infection with in hypoxia (Figure

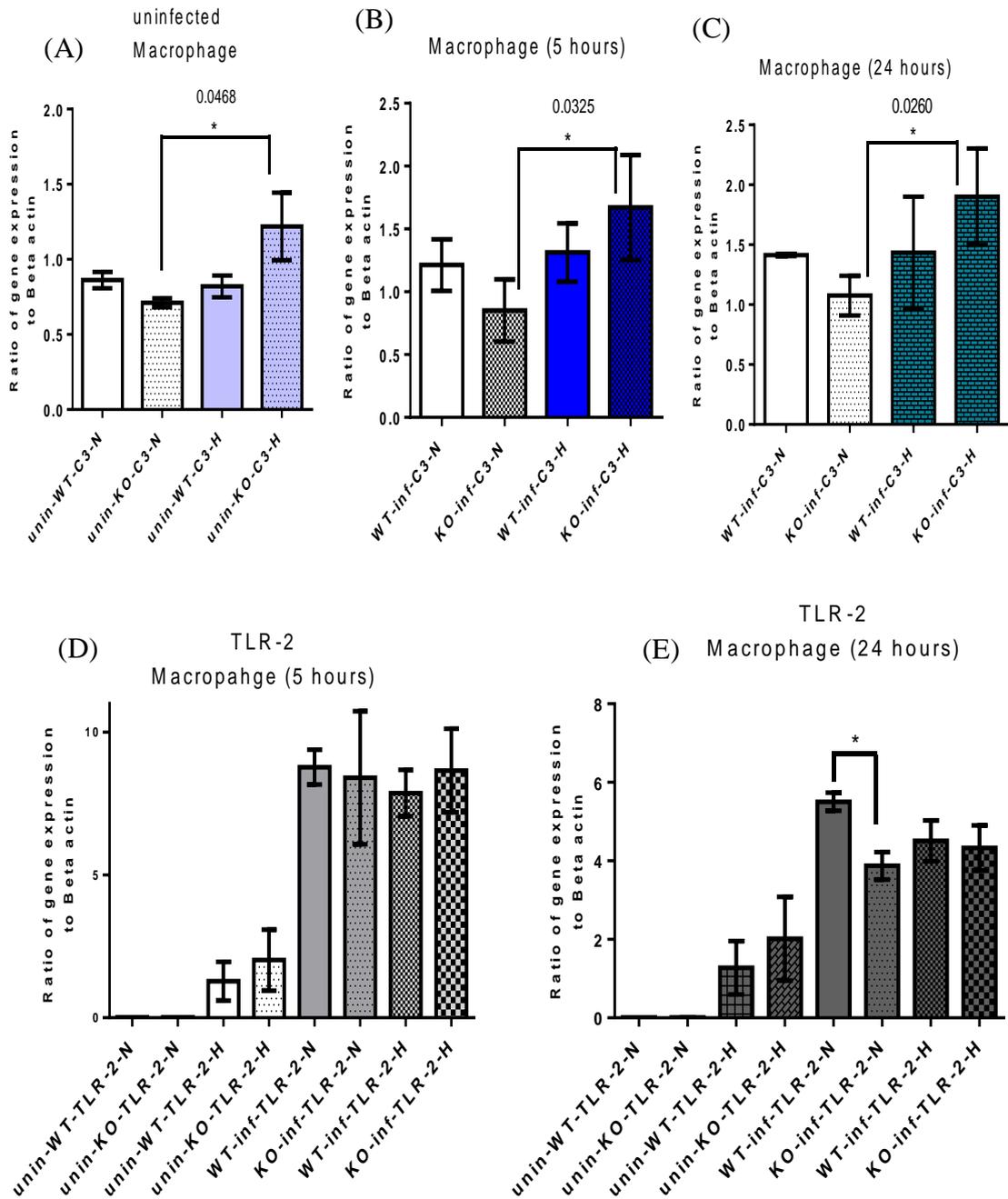
3.1.33 D and E). During the time of infection the expression of TLR2 was decreased after 24 hours compared to 5 hours (axes in panels D and E).

In macrophage from properdin-deficient mice, where expression as hypoxia-sensitive feature is missing, C3 appears as a hypoxia inducible gene. No explanation, further researches is needed. Transcription of TLR2 is increased after infection because TLR2 has a role in the control of *L. monocytogenes* infection (Torres *et al.*, 2004).

TLR2 is hypoxia inducible as shown in uninfected macrophage expression of TLR2 is higher in hypoxia compared to normoxia (Selejan *et al.*, 2012).

It has been shown in a study by (Kuhlicke *et al.*, 2007) that dendritic cells derived from mouse bone-marrow after exposed *in vitro* to normoxia and hypoxia 2% oxygen for 24 hours and express of TLR2 using real-time RT-PCR analysis showed higher levels of mRNA TLR2 expression in hypoxia compared to normoxia.

A summary of the main findings of expression analyses for bone marrow derived macrophages and dendritic cells of wild type and properdin-deficient mice for different conditions and stimuli are tabulated in table 3.1. 7.



**Figure 3.1.33:** Quantitative values of mRNA expression levels of C3 and TLR2 were determined in bone marrow derived macrophages. (A) expression of C3 in uninfected macrophage, (B) C3 expression in macrophage after 5 hours and (C) after 24 hours infection. (D) expression of TLR2 after 5 hours, and (E) after 24 hours infection. Gene expression levels are presented relative to  $\beta$ -actin mRNA expression. This experiment was set up in duplicate from triplicate measurement. The data are presented as means with standard deviation (SD) and  $\Delta\Delta CT$  was used. (N=normoxia, H=hypoxia).

**Table 3.1.7:** Summary of main findings of RT-PCR and qPCR analyses in dendritic cells and macrophages for different genes related to infection with *L. monocytogenes*.

Dendritic cells	Macrophages
<ul style="list-style-type: none"> <li>• By qPCR expression of properdin in WT was less after infected compared to uninfected dendritic cells but there was no significant difference.</li> <li>• By RT-PCR, expressions of C3 and CD11b after 24 hours infection were higher in WT compared to KO.</li> <li>• Expression of C3 and CD11b after 24 hours infection in hypoxia were higher compared to normoxia</li> <li>• By qPCR, no difference in expression of TLR2 after 24 hours of infection between WT and KO.</li> <li>• By RT-PCR, expression of TLR2 was less in hypoxia compared to normoxia in both genotypes.</li> <li>• Expression of C3 in uninfected DC was higher in KO compared WT.</li> </ul>	<ul style="list-style-type: none"> <li>• Expression of properdin in WT macrophages was significantly decreased after infection in normoxia and hypoxia compared to uninfected cells.</li> <li>• Expression of C3 after 5 hours of infection were higher in WT compared to KO but no difference in expression of CD11b.</li> <li>• There were no differences in the expression of C3 or CD11b in hypoxia compared to normoxia after 5 hours.</li> <li>• Higher expression of TLR2 after 24 hours of infection in normoxia in WT compared to KO.</li> <li>• By qPCR, in hypoxia, higher expression of C3 in KO after 5 and 24 hours compared to KO in normoxia.</li> <li>• In hypoxia, decreased expression of properdin in WT compared to normoxia.</li> </ul>

Bone marrow derived dendritic cells and macrophages have the role *in vitro* infection with *L. monocytogenes*.

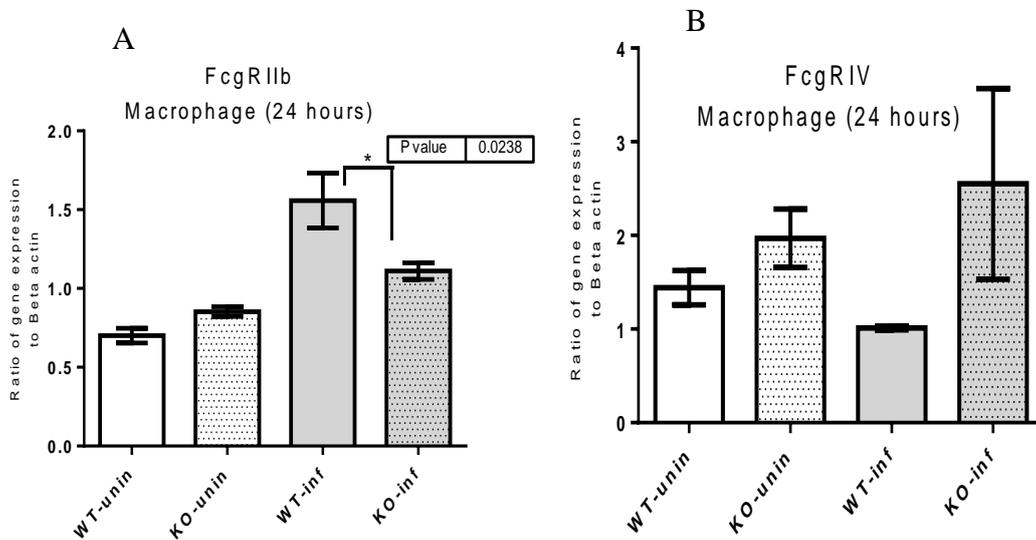
### **3.1.14 Expression of FcγRIIb and FcγRIV mRNA by bone marrow derived macrophages**

Bone marrow-derived macrophages from wild type and properdin-deficient mice were infected with *L. monocytogenes* for 24 hours and total RNA was isolated as described. qPCR was used to investigate the role of activating FcγR (FcγRIIb and FcγRIV) in the bone marrow derived macrophage after infection with *L. monocytogenes*, this analysis was not done for dendritic cells.

These receptors were used in this study because my supervisor, as part of other projects, discovered lower FcγRIIb expression in properdin-deficient mice compared to wild type mice by normal qualitative RT-PCR. This is most likely an expression of altered macrophage activity independent of a direct IgG mediated effect. For this reason, this study investigated the expression of FcγRIIb and FcγRIV as markers, in relation to infection with *L. monocytogenes*.

As can be seen in the Figure 3.1.34, expressions of both FcγRIIb and FcγRIV in uninfected cells are somewhat higher in properdin-deficient compared to wild type. After infection, expression of FcγRIIb was increased but was less expression in properdin-deficient compared to wild type. On the other hand, expression of FcγRIV was decreased in wild type compared to properdin-deficient. Although CFU/ml from infected macrophage at 24 hours (Figure 3.1.4) shows there were no differences in bacterial loads in both genotypes, lack of properdin leads to decreased FcγRIIb expression and inverted relationship FcγRIIb/FcγRIV in wild type compared to properdin-deficient, meaning that the genotypes do differ in their macrophage phenotype.

There is more expression of Fc $\gamma$ RIV than Fc $\gamma$ RIIb in macrophage from both genotypes in uninfected and infected after 24 hours (see axes panels A and B).



**Figure 3.1.34:** Quantitative values of mRNA expression of Fc $\gamma$ RIIb (A) and Fc $\gamma$ RIV (B) on bone marrow derived macrophage from wild type and properdin-deficient mice in uninfected and infected 24 hours with *L. monocytogenes*. Gene expression levels are presented relative to  $\beta$ -actin mRNA expression. This experiment was set up in duplicate from triplicate measurement. The data are presented as means with standard deviation (SD) and  $\Delta\Delta$ Ct methods were used.

### 3.1.15 Expression of Fc $\gamma$ R III/II receptor (CD16/CD32) in mouse splenic B-cells

B cells were used as a model system for the control of immune activation. Fc $\gamma$ RIIB is the only Fc $\gamma$ R that expressed by B cells. Moreover, B-cells do not express Fc $\gamma$ RIII, so antibody shows only Fc $\gamma$ RII. Fc $\gamma$ RIIb is a protein that found on the surface of particular cells as B lymphocytes.

To study further the role of properdin in activation of immune cells through Fc $\gamma$ Rs contributes to the disease pathology. This *in vitro* experiment focused on expression of Fc $\gamma$ RIIb inhibitory in B lymphocytes and to investigate any differences between wild type and properdin-deficient mice cells after infection with *L. monocytogenes* reflected at the protein level, therefore FACS was used to analysed cell surface expression of

Fc $\gamma$ RII in B-cells and this different to the expression of transcription or regulatory factors can be determined by differences of standard mRNA methods, including qRT-PCR.

Splenocytes from wild type and properdin-deficient mice were prepared as described in material and methods. Splenocytes were infected overnight with heat-killed *L. monocytogenes* MOI of 20 and 80 the higher MOI was used as relevant to a previous study in the lab, then were stained for B-cells with PE- rat anti-mouse CD45R/B220 (B cell marker) and FITC rat anti-mouse CD16/CD32 (Fc $\gamma$ III/II receptor) and were analysed by FACS. CD16/CD32 is an antibody that recognises both Fc $\gamma$ RIII and Fc $\gamma$ RII and was used to detect Fc $\gamma$ RIIb on the B cells because this receptor is the only Fc $\gamma$  receptor which is expressed by B cells (Smith & Clatworthy, 2010). Uninfected splenocytes were used as control. The geometric mean was used and analysis by calculated the geometric mean levels comparing them to their relevant individual isotype controls. Normally Fc block is used to stop non-specific binding or decrease background but in this case in this experiment, this was the receptor that was investigated.

As can be seen in Figure 3.1.35 panels A and D in uninfected the Fc $\gamma$ RIIB receptor protein expression was measurably less in the splenic B cells from properdin-deficient mice compared to wild type, this finding was not the same results by QPCR expression of Fc $\gamma$ RIIb which appeared less in macrophage from wild type compared to macrophage from properdin-deficient mice. The histogram shows a shift of fluorescence signal between wild type and properdin-deficient in uninfected cells meaning that the genotype may influence basal B cells phenotype. The expression of Fc $\gamma$ RIIB was increased after infection in both genotypes but to the same extents, splenocytes from properdin-

deficient uninfected expresses less than splenocytes from wild type mice and this was the same after infection.

The histograms are presented by an overlay of wild type and properdin-deficient with their isotypes for uninfected and infected splenocytes. And in panel D show uninfected and infected present the mean fluorescence intensity value (MFI).

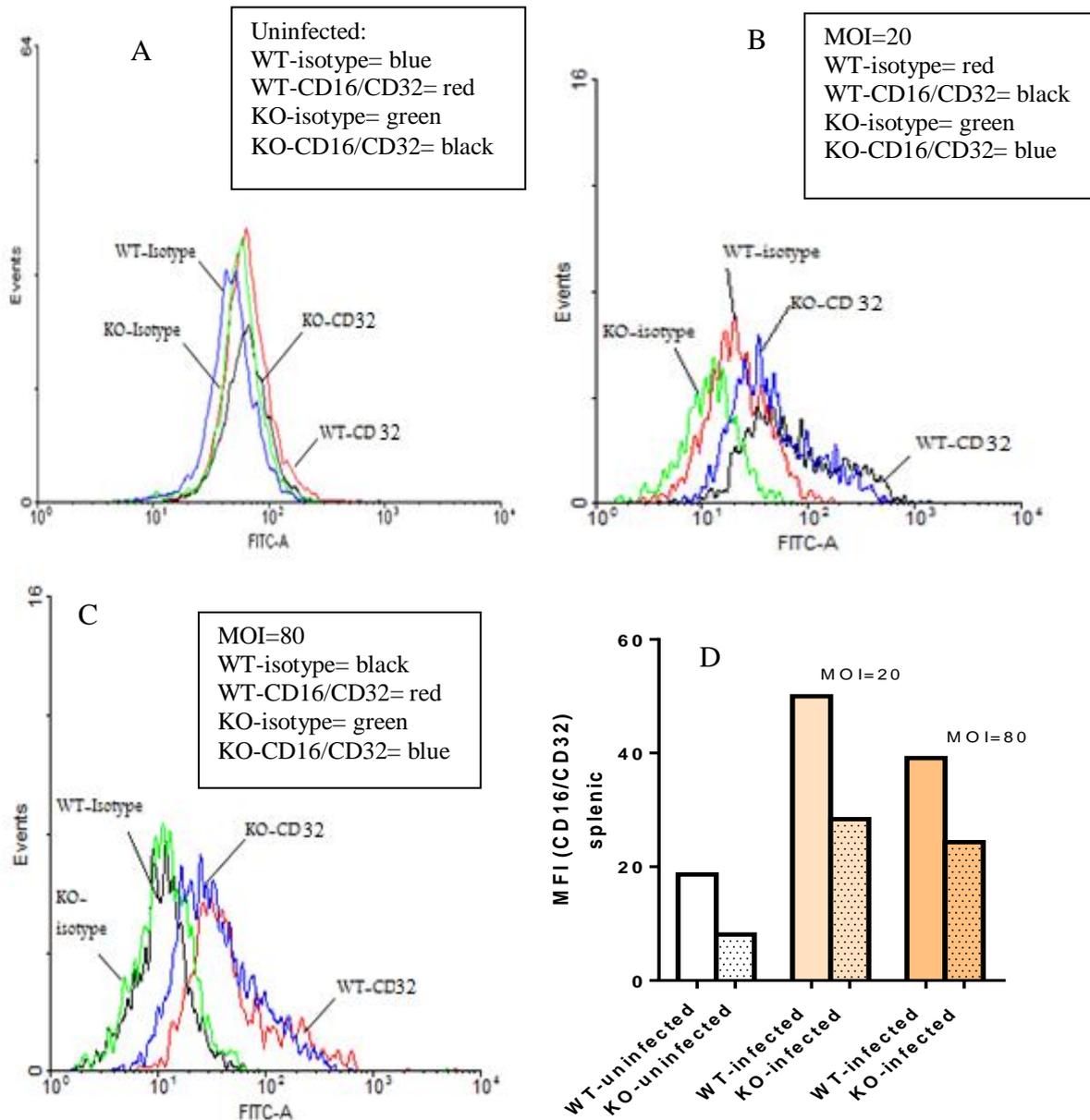
After infection with *L. monocytogenes* (MOI=20 and 80) (Figure 3.1.35 B, C and D) the expression of Fc $\gamma$ RIIB receptor increased compared to uninfected in both genotypes and were as uninfected measurably less expression in the splenic B cells from properdin-deficient mice compared to wild type.

The population of B cells was identified by SSC and FSC based on which region they appear or based on bright expression of B220 marker, the aim was also to expression of CD16/CD32 but for monocytes and neutrophils were a bit ambiguous perhaps maybe because the cell numbers compared to lymphocytes were just too low.

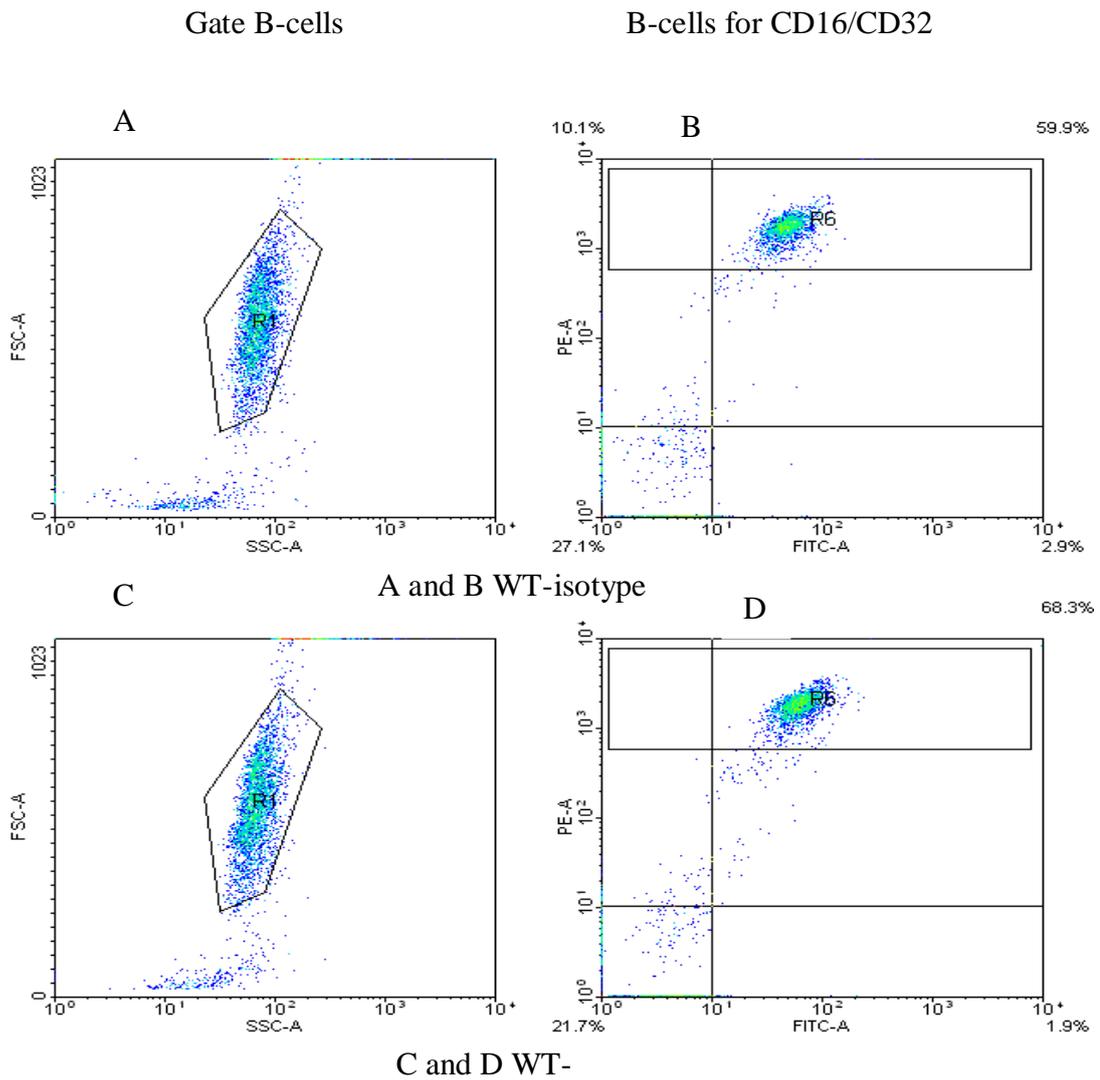
Once the populations are identified and gate on the populations of B cells in the dot plot (R2) shown in Figures 3.1.36-3.1.39 in panels A, and in panels B the FITC/PE plot that gated on B lymphocytes so show positive B-cells for CD16/CD32 are demarcated within FACS plots by a box in the upper right for uninfected as show in (R6) and in infected the gates R2 show positive for B220 and CD16/CD32. This was done for wild type and properdin-deficient for isotypes as background control and for positive expression B-cells for CD16/CD32.

In the uninfected cells the isotypes appear to be sticking to the cells and makes it hard to interpret but there were decreases of Fc $\gamma$ RIIB on the MFI on B cells. Therefore, the concentration of the isotype was titration from 1  $\mu$ g, 0.5  $\mu$ g, 0.25  $\mu$  and 0.05  $\mu$ g then the best concentration was used 0.05  $\mu$ g.

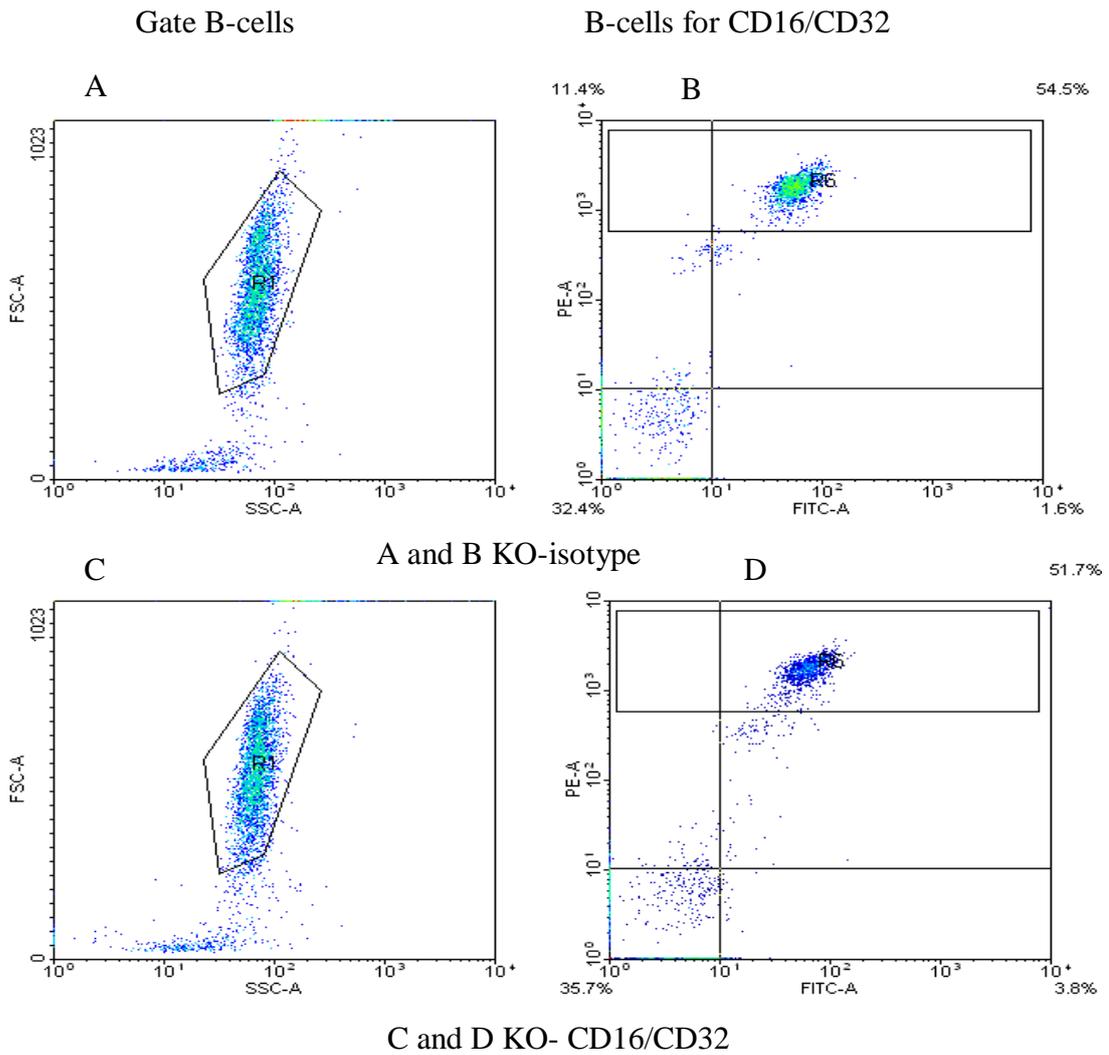
In infected splenocytes cells, there were different population of B cells compared to uninfected. The B cells were gated by the size for B-cells PE B220 in SSC and FSC in panels A and C then the population PE B220 and FITC for CD16/CD32 were present in the density plot as in panels B and D. After that the dot plots were present as histogram, without the antibody isotype control show in panels A and B (Figures 38 and 39). Positive expression in both genotypes for both B220 and FcγRIIb were shown in panel panels D and F in (Figures 38 and 39). Then MFI were present in panel D Figure 3.1.35.



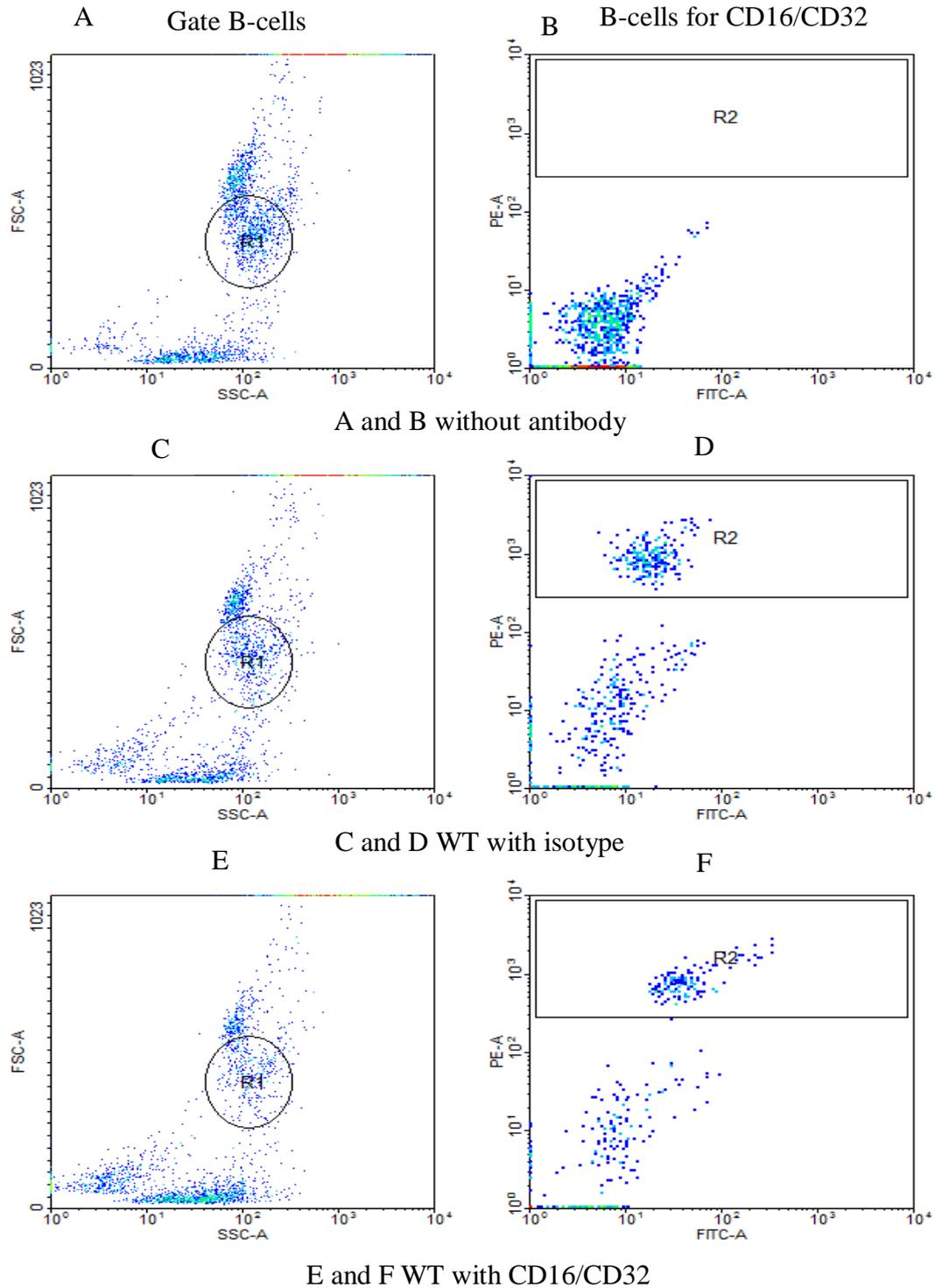
**Figure 3.1.35:** Flow cytometric analysis of CD16/CD32 surface expression in splenic B220 positive cell population. Overlay flow cytometry histogram of the MFI value of uninfected cells (A) and cells infected with heat-killed *L. monocytogenes* at MOI 20 (B) and 80 (C) were analysed ( $5 \times 10^5$  cells/tube). MFI of these experiments are compiled in D. The population were gated by the size for B-cells PE B220 and FITC for CD16/CD32 in density plot (The histograms show the data for 10,000 gated events from each sample) after the FITC/PE plot is gated then present in the histograms. Results are representative of two experiments. Results are expressed mean using MFI (mean fluorescence intensity) value. (C) Both genotypes were shift of fluorescence from their isotypes WT-CD32 and KO-CD32 were shifted from isotypes WT-isotype and KO-isotype.



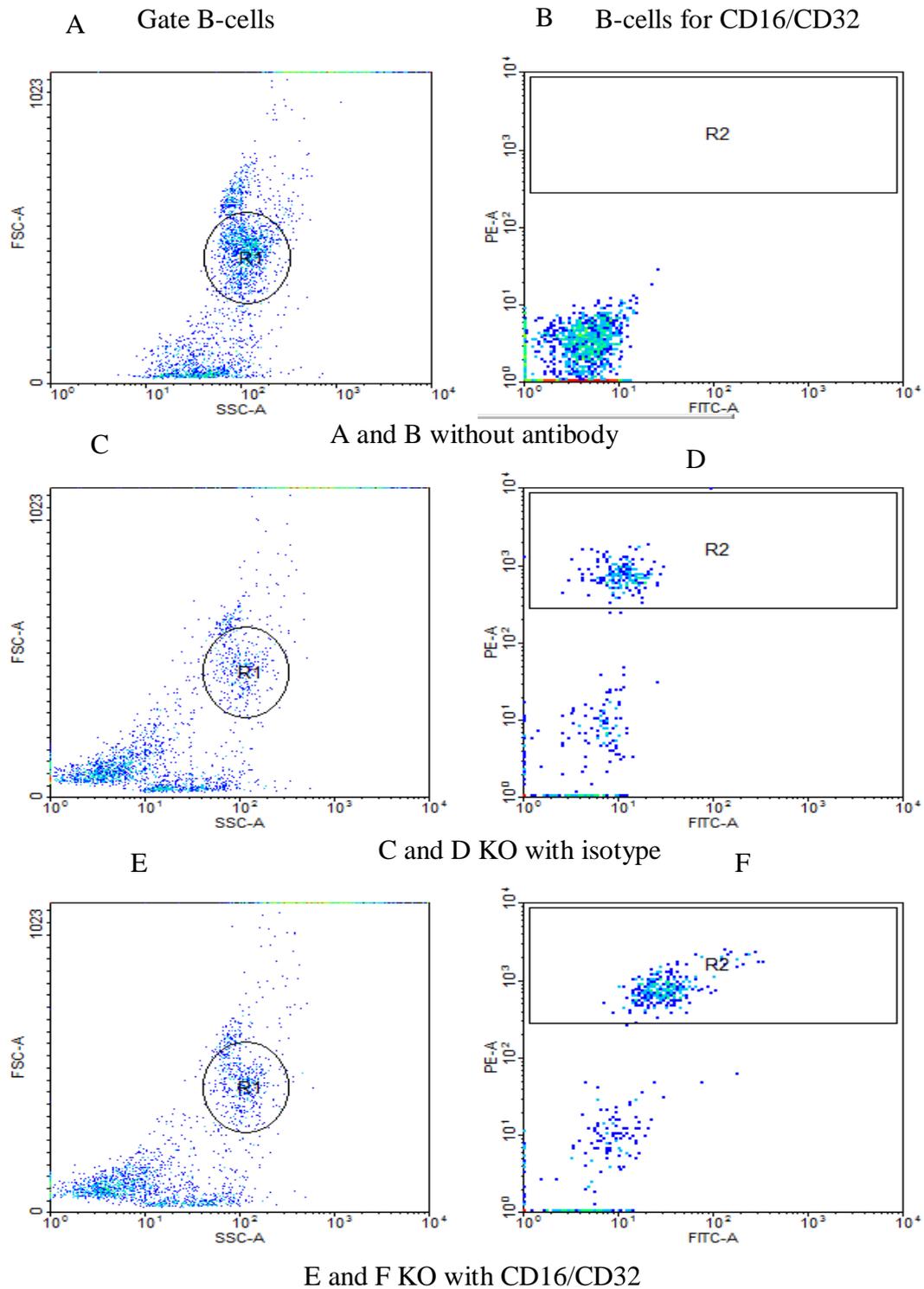
**Figure 3.1.36:** Flow cytometric analysis of B-cells from uninfected spleen from wild type mice. Representative dot plot shows expression of Fc $\gamma$ RIIb (CD16/CD32) in uninfected splenocytes from wild type mice. The B-cells were identified by forward and side scatter and were gated (R1) panels A and C.



**Figure 3.1.37:** Flow cytometric analysis of B-cells from uninfected spleen of properdin-deficient mice. Representative dot plot shows expression of Fc $\gamma$ RIIb (CD16/CD32) in uninfected splenocytes from properdin-deficient mice. The B-cells were identified by forward and side scatter and were gated (R1) panels A and C.



**Figure 3.1.38:** B-cells from spleen of wild type mice after infection with heat-killed *L. monocytogenes* MOI=20 for overnight was measured by flow cytometric. Representative dot plot shows expression of Fc $\gamma$ RIIb (CD16/CD32) in infected splenocytes from wild type mice. The B-cells were identified by forward and side scatter and were gated (R1) pane A, C and E.



**Figure 3.1.39:** B-cells from the spleen of properdin-deficient mice after infection with heat-killed *L. monocytogenes* MOI=20 for overnight was measured by flow cytometric. B-cells from the spleen of properdin-deficient mice after infection with heat-killed *L. monocytogenes* MOI=20 for overnight was measured by flow cytometric. Representative dot plot shows expression of Fc $\gamma$ RIIb (CD16/CD32) in infected splenocytes from properdin-deficient mice. The B-cells were identified by forward and side scatter and were gated (R1) pane A, C and E.

### **3.1.16 The role of properdin in maturation of dendritic cells after infection with *L. monocytogenes***

CD40, CD80, CD86 and MHCII are surface markers that characterise antigen presenting cells. After stimulation of dendritic cells, these up-regulate the molecules necessary for their role within the adaptive immune response. Antibodies specific for these molecules can be used to characterise the extent of maturation of dendritic cells. To study the surface expression of dendritic cells from wild type mice compared to properdin-deficient mice after infection with *L. monocytogenes*, an approach of double staining with the dendritic cell marker CD11c was taken. The expression was measured by flow cytometry.

The experiments in uninfected were done separately from infected because live *L. monocytogenes* were used and numbers of dendritic cells after infected were few after processing for the staining. Therefore, heat-killed of *L. monocytogenes* were used.

The analysis was done on cells that were double positive for CD40 and CD11c, CD80 and CD11c, CD86 and CD11c, MHC class II and CD11c with the double positive quadrant determined by the isotype control profile. The level of expression of CD40, CD80, CD86 and MHC class II was calculated using the geometric mean levels and comparing them to their relevant individual isotype controls (Figure 3.1.42).

In the Figure 3.1.40 expression of CD40, CD80, CD86 and MHCII in uninfected dendritic cells were the same between wild type and properdin-deficient.

The percentage of dendritic cells positive for CD11c in uninfected both genotypes are about 20%.

Figure 3.1.41 shows as expected the levels of surface markers CD40 was higher in dendritic cells after infection compared to uninfected dendritic cells (Muraille *et al.*, 2005).

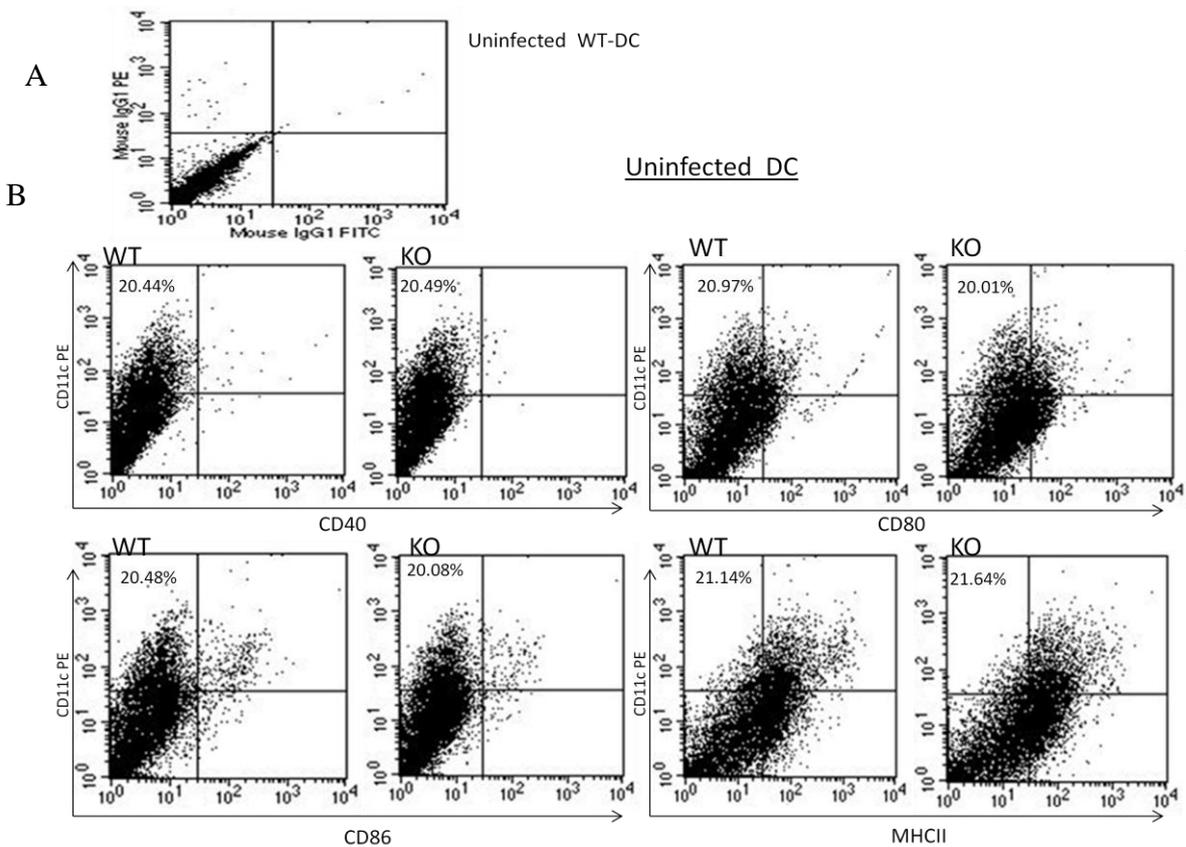
The percentages of positive CD11c in both genotypes were comparable in uninfected dendritic cells but after infection the percentages of CD11c were higher in dendritic cells from properdin-deficient compared to dendritic cells from wild type, although the percentages of CD11c after infection is less in wild type compared to properdin-deficient but CD40 is higher.

The percentage of dendritic cells positive for CD11c after infection the percentage of dendritic cells positive for CD11c in wild type is about 36.01% and in properdin-deficient mice are about 48.49 % for each of the experiments. This speaks for a marked maturation of dendritic cells.

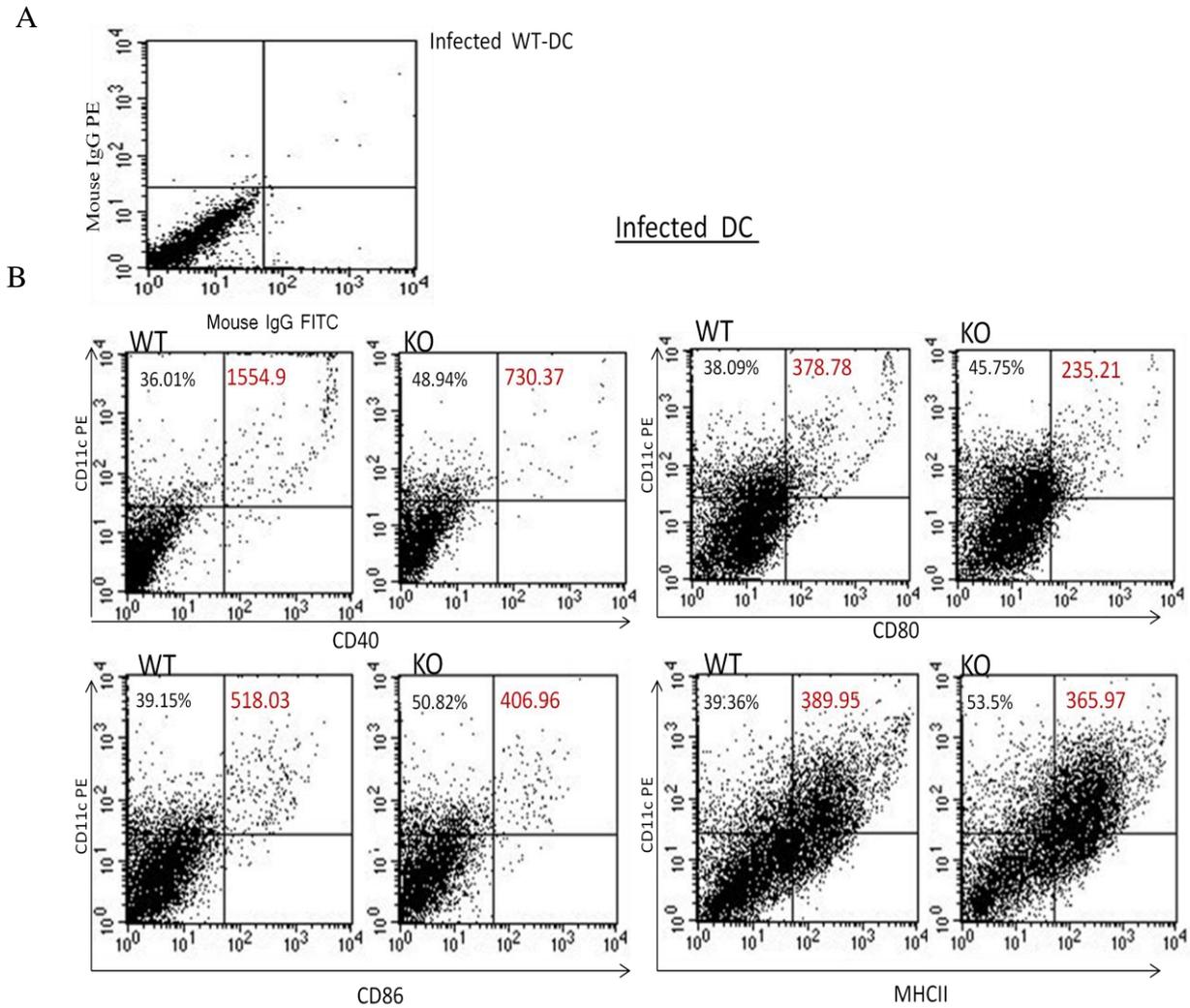
Expression of CD86 and CD80 with CD11c in wild type dendritic cells after infection with heat-killed *L. monocytogenes* (Figure 3.1.41 panels A and B) appeared slightly higher compared to dendritic cells from properdin-deficient but statically there was no difference. Expression of MHCII + CD11c (panel C) shows no difference between both genotypes. The dendritic cells express high levels of CD40 with CD11c in wild type compared to properdin-deficient mice after infection with heat-killed *L. monocytogenes*. Infection with *L. monocytogenes* of mouse dendritic cells derived from bone marrow for 24 hours has been studied previously using MOI between 3 to 10 (Westcott *et al.*, 2010). This study showed that infected immature dendritic cells (using a low MOI of *L. monocytogenes*) increased their expression of CD86 after 24 hours. In another study by Guzman *et al.*, 1995, dendritic cells infected with *L. monocytogenes* after 24 hours. Using murine spleen dendritic cells line infected with *L. monocytogenes* in order to study the uptake and intracellular survival of bacteria within the cells and to investigate the interaction of *L. monocytogenes* it was shown that dendritic cells are tolerant hosts for *L. monocytogenes* and *L. monocytogenes* attack murine spleen dendritic cells intracellularly was shown at 24 hours.

Figure 3.1.42, also as expected, the levels of all surface markers (CD80, CD86 and MHCII) were higher after infection (Muraille *et al.*, 2005) compared to uninfected dendritic cells. This means that the dendritic cells have undergone so-called maturation.

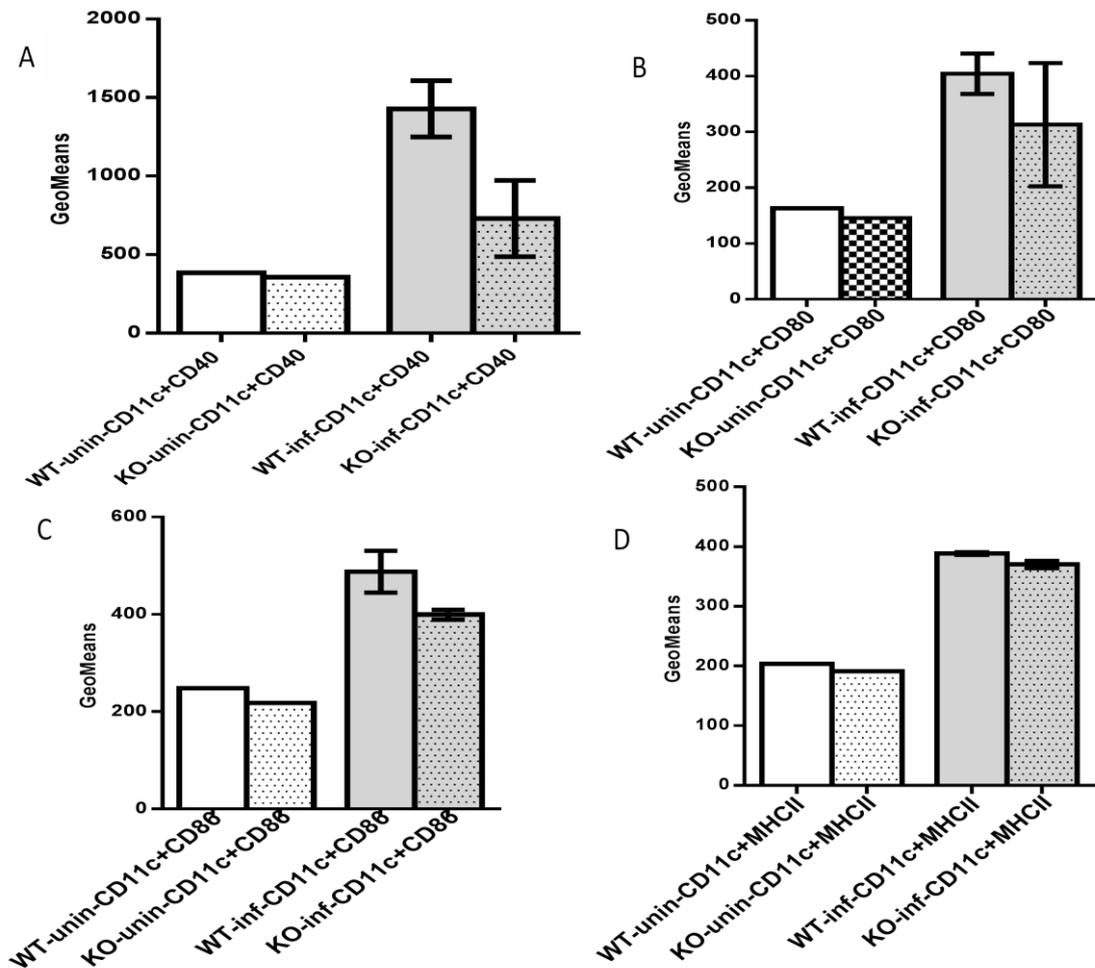
Independently, the same result in CD86 expression was observed analysing splenocytes obtained from infected mice (Appendix, Figure II). The expression of CD86 was higher in dendritic from wild type compared to dendritic cells from properdin-deficient, dendritic cells obtained from spleen infected with heat-killed *L. monocytogenes*.



**Figure 3.1.40:** One of two dot plots showing expression of CD40 and CD11c, CD80 and CD11c, CD86 and CD11c, MHCII and CD11c on uninfected dendritic cells. This was measured by flow cytometry on dendritic cells isolated from bone marrow after 1.5 hours infection with heat-killed *L. monocytogenes*, dendritic cells  $5 \times 10^5$  cells per pellet were analysed. (A) WT-DC uninfected isotype background control. The autofluorescence was used to set the gate. (B) expression the CD40, CD80, CD86 and MHCII, percentage of total events over two quadrants is indicated e.g. (UL + UR= 20.44%). UR=up right, UL= up left.



**Figure 3.1.41:** One of two dot plots showing expression of CD40 and CD11c, CD80 and CD11c, CD86 and CD11c, MHCII and CD11c on infected dendritic cells. Representative FFplot shows expression of CD40 and CD11c, CD80 and CD11c, CD86 and CD11c, MHCII and CD11c on infected dendritic cells. Dendritic cells isolated from bone marrow after infection with heat-killed *L. monocytogenes* for 1.5 hours was measured by flow cytometry, DC  $5 \times 10^5$  cells per pellet were analysed. Data for infected are representative of two separate experiments in duplicate. The autofluorescence was used to set the gate. (B) expression the CD40, CD80, CD86 and MHCII, the numbers of X Geo Mean of surface markers expression + Y Geo Mean of CD11c is indicated e.g. (UR X Geo Mean of CD40+ UR Y Geo Mean of CD11c = 1554.9). UR=up right.



**Figure 3.1.42:** Flow cytometry analysis show surface staining of dendritic cells from wild type and properdin-deficient mice after staining with the CD40 and CD11c, CD80 and CD11c, CD86 and CD11c, MHCII and CD11c after infection with heat-killed *L. monocytogenes* compared to uninfected cells. The extent of surface staining with specific antibodies is expressed using Geometrical Means.

Unfortunately, sorting or magnetic selection could not be part of this project. This would have allowed for a pure population.

In another project in the lab (See Appendix Figure IV A) splenocytes from wild type and properdin-deficient mice were stimulated for 3 days with heat-killed *L. monocytogenes* at MOI=200. T cells were stained with CD4+ and CD 8+ and were used to analysis CD69 expression. CD69 is a marker of early T cell activation.

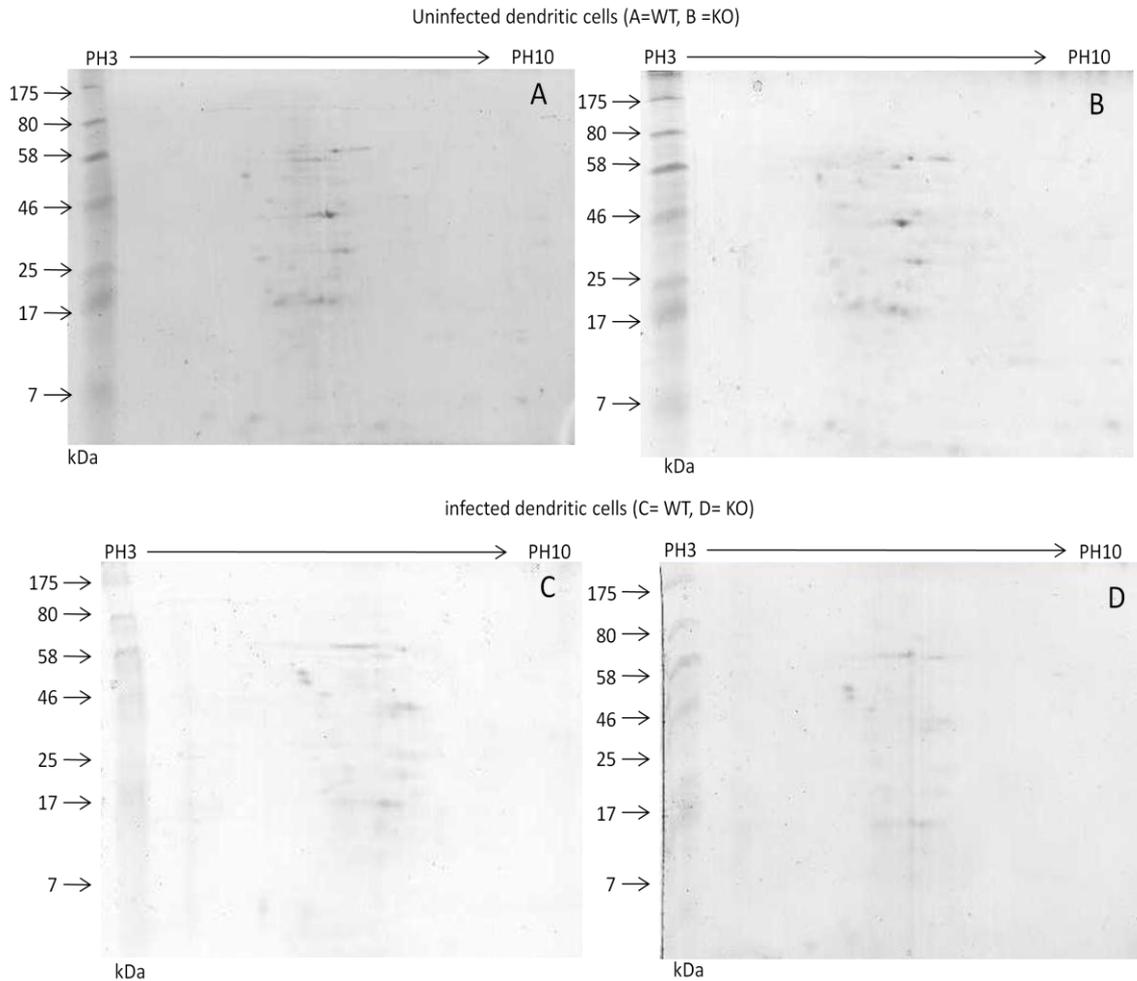
Increased CD69 CD4<sup>+</sup> and CD69 CD8<sup>+</sup> double positive for splenocytes of properdin-deficient and wild type after stimulation with heat-killed *L. monocytogenes*. Expression of CD69 CD4<sup>+</sup> and CD69 CD8<sup>+</sup> after stimulation with heat-killed *L. monocytogenes* was increased in splenocytes from wild type mice compared to splenocytes from properdin-deficient mice. Moreover, the expression of IFN- $\gamma$  were measured and also were increased in CD69 CD4<sup>+</sup> and CD69 CD8<sup>+</sup> after stimulation splenocytes from wild type compared to splenocytes from properdin deficient mice (See appendix Figure I).

### **3.1.17 Proteomic analysis of dendritic cells from wild type and properdin-deficient mice after infection with *L. monocytogenes***

At the time of this part of the project, the accepted understanding was that properdin acts solely as a released serum protein without direct cellular impact. Previous experiments have shown that dendritic cells from wild type have a higher number of *L. monocytogenes* compared to dendritic cells from properdin-deficient mice. Because these infection experiments were carried out in the presence of intact serum (FCS), a cellular difference was surmised. Therefore, a proteomic approach using 2 D-gel electrophoresis was taken. Protein extracts were prepared by lysing uninfected and infected dendritic cells. The mixture of Tris-soluble proteins was separated by high-resolution isoelectric focusing and SDS- PAGE. *L. monocytogenes* proteins are included and it maybe the lysis method it misses any membrane protein or there were a less concentration of the proteins. Gels were stained with colloidal Coomassie blue in order to be able to proceed with sequencing if needed. The expectation was to see any difference between wild type and properdin-deficient in proteomic analysis in dendritic cells after infection.

## Chapter Three Results

As can be seen in Figure 3.1.43 there was resolution of protein according to their IP and weight, but overall the abundance was very low. There was no obvious difference in protein spots for uninfected and infected wild type or properdin-deficient mice.



**Figure 3.1.43:** Blots of 2-dimensional gels uninfected (A-B) and infected (C-D) for dendritic cells from wild type run in parallel with properdin-deficient mice. Dendritic cells from bone marrow mice from wild type and properdin-deficient mice infected with heat-killed *L. monocytogenes* for 1.5 hours and cells were lysied were with lysis buffer, 40 mM Tris with DnaseI and Rnase A. (A-B-C-D) Coomassie-stained two-dimensional gel showing separated proteins. Vertical and horizontal axes indicate the molecular weight in kilodaltons and isoelectric points (pH3–10) of separated proteins, respectively. Data are representative of two separate experiments.

Unfortunately, this method did not appear sensitive enough to detect a significant difference in protein abundance between the cells (uninfected and infected) of the two genotypes and was discontinued in favor of analyses of candidate properdin-dependent modifiers of infection (Fc $\gamma$ R).

### **3.1.18 The role of properdin in alternative pathway-mediated activation by *L.***

#### ***monocytogenes***

*L. monocytogenes* activates the alternative pathway of mouse complement (Drevets & Campbell, 1991). Incubation of macrophages with *L. monocytogenes* in the presence of serum led to deposition of C3 on the bacterial cell wall (as shown by immunofluorescent-antibody staining) and internalisation of the bacteria by macrophages, largely by CR3, as shown by using specific blocking antibodies.

The effect of properdin in complement C3 activation after incubation of mouse serum from wild type and properdin-deficient with *L. monocytogenes* was studied in this experiment.

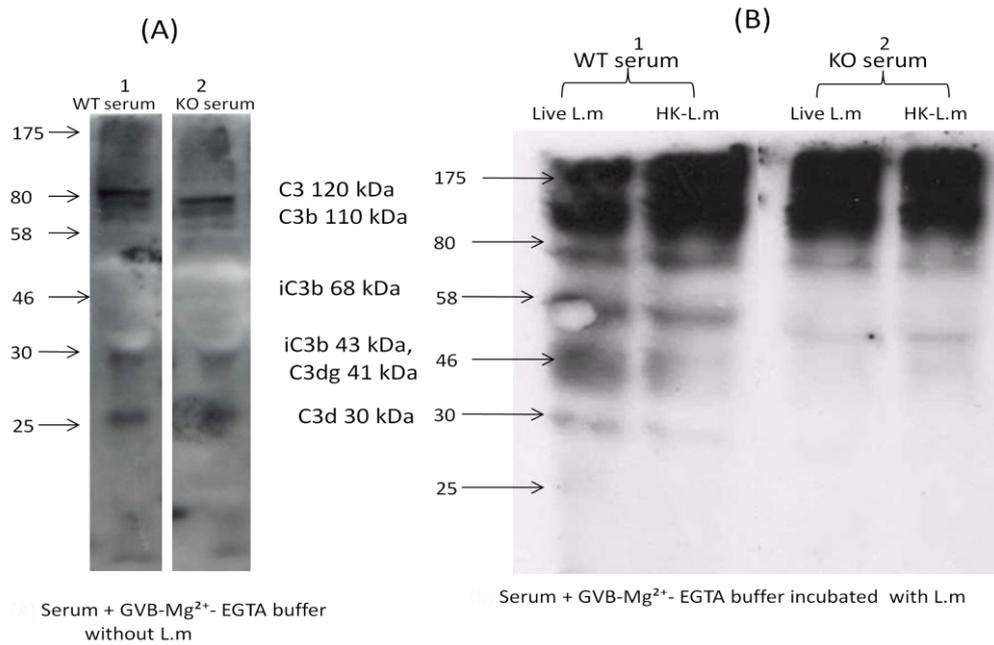
The aim of this experiment was to study the activation of complement in mouse serum from wild type and properdin-deficient mice by *L. monocytogenes*. By analyse C3 fragments after serum activation which can be separated by size and are reactive with rat anti mouse C3 antibody. Using serum from wild type and properdin-deficient mice and buffer conditions which favour alternative pathway activation, the role of properdin in this enzymatic C3 conversion activation can be concluded.

Properdin stabilises the C3 convertase of the alternative pathway by binding to C3b of C3bBb via two of its thrombospondin repeat modules, thereby counteracting Factor H mediated dissociation. In the absence of properdin, C3 convertase activity has a shortened half-life, resulting in less C3 activation products.

This experiment seemed relevant in the context of the planned infection model, when *L. monocytogenes* comes in contact with mouse blood through i.v. administration because it gives an indication of the initial extent of C3 activation products generated by the alternative pathway in wild type and properdin-deficient mice.

Alternative pathway mediated activation of complement by live and heat-killed *L. monocytogenes* was investigated by western blot. Some bacteria have the ability to cleave C3 directly through membrane bound proteases without activation of the complement cascade. Therefore, the effect of live and heat-killed *L. monocytogenes* in the generation of C3 cleavage products was tested in parallel. Pooled sera from wild type and properdin-deficient mice were incubated with live and heat-killed *L. monocytogenes* for 30 minutes incubation at 37°C, the samples were centrifuged for 2 minutes at 16.000g, then the supernatants were used and separated. C3 fragments were detected with rat anti-mouse complement component C3 antibody. Heat-inactivated and native serum samples were analysed. This antibody reacts not only with uncleaved C3 but also with the activation (and degradation) products.

There is always some spontaneous C3 degradation through activity of tryptic enzymes present in serum. This can be seen in Figure 3.1.44 panel A (serum without *L. monocytogenes*). Activation of serum C3 by *L. monocytogenes* showed additional of C3 reactive fragments after incubation with *L. monocytogenes* at 58 kDa (Figure 3.1.44 B). C3 reactive fragments were comparable in wild type and properly serum regardless whether live or heat-killed *Listeriai* were used. There were less anti-C3 reactive bands in serum from properdin-deficient mice after activation with live and heat-killed *L. monocytogenes* (between 58 kDa to 25kDa) so less iC3b, C3dg, C3d compared to activated serum from wild type mice as expected.



**Figure 3.1.44:** Western blot analysis of C3 reactive products in serum with or without incubation with *L. monocytogenes*. (A) Serum without *L. monocytogenes* 1= wild type 2= properdin-deficient mice. (B) Serum from wild type=1 and from properdin-deficient mice=2 incubated with live *L. monocytogenes* and heat-kill *L. monocytogenes* (HK-L.m). The membrane was probed with biotin anti mouse C3 mAb and developed with streptavidin-HRP (Dakocytomation). Films were exposed for 10 seconds.

Having found an impaired alternative pathway-mediated C3 activation in serum from properdin-deficient mice compared to serum from wild type mice in the presence of *L. monocytogenes*, the bactericidal effect of mouse serum against *L. monocytogenes* was assessed next.

Sera from wild type and properdin deficient mice were incubated with *L. monocytogenes* for 30 minutes and 24 hours. As can be seen in table 3.1.9, there was no differences in viable count from serum between wild type and properdin-deficient and compared to control.

Taken together, while there are differences in complement activation at the C3 level, this does not translate to a measurable membrane attack complex (MAC)-dependent

lytic effect. This may not have been unexpected, as *L. monocytogenes* are gram positive bacteria and effective MAC insertion has only been described for gram negative bacteria.

**Table 3.1.8:** Viable count of *L. monocytogenes* after incubation with wild type and properdin deficient serum for 30 minutes and 24 hours. CFU results were the average sera from 3 mice each genotypes. After 30 minutes 50µl of serum from each genotype was added to 50 µl PBS with *L. monocytogenes* but after 24 hours remained 80 µl after taking 20 µl from total 100 µl to do serial CFU for 30 minutes.

NO	Sample types	30 min log		NO	Sample types	24 hrs log
1	Control	8.212454		1	Control	7.11991541
2	WT	8.226592		2	WT	7.46869453
3	KO	8.009644		3	KO	7.46487007

### 3.1.19 Functional detection of Listeriolysin O by hemolysis assay

A hemolysis assay was performed to quantify LLO activity in supernatants from dendritic cells and macrophages derived from bone marrow from wild type and properdin-deficient mice after infection with *L. monocytogenes* MOI= 0.2 in an approximately 10 ml RPMI 1640 medium with number of cells  $5 \times 10^4$  /ml.

No hemolysis could be detected in supernatants of these infected dendritic cells and macrophages. By contrast, when splenocytes were infected as part of another project in Dr. Stover' group (Appendix, Figure III), their supernatants reproducibly showed haemolytic activity at MOI 0.02 and for 13 and 18 hours with MOI 0.1 and 0.02, to a somewhat larger extent in wild type than in properdin-deficient mice. This coincided with immoreactive bands for LLO on Western blot analysis.

The fact that no haemolytic activity was found in supernatants from bone marrow derived cells most likely means that their infection induced lysis was less than that of splenocytes.

In addition, sera of infected mice were analysed in parallel and were found to be negative for haemolytic activity. It is possible that LLO is quickly neutralised in the living organism and therefore is not available in circulation.

Hemolytic activity was detected in a project conducted in the corporate in Dr. Freestone' group using - without host cells - only *L. monocytogenes* grown in serum-based minimal medium (serum-SAPI) designed to mimic the *in vivo* conditions a bacterium would experience in its host. There was haemolytic activity but no difference between conditions in the presence or absence of norepinephrine.

*In vitro* analysis of bone marrow derived dendritic cells from properdin-deficient showed less bacterial load at an early time point and at 24 hours compared to wild type. Less IFN- $\gamma$  and nitric oxide were released after 24 hours from infected dendritic cells from properdin-deficient mice compared to dendritic cells from wild type mice. There was less surface expression of CD40 in dendritic cells from properdin-deficient cells compared to wild type. The surface marker CD40 was shown to be induced in dendritic cells also in macrophage during infection with *L. monocytogenes* (Popov *et al.*, 2008). Properdin has the role in the *in vitro* of dendritic cells and macrophage in response to *L. monocytogenes*. Next, *in vivo* model was to test the importance of these differences in the survival of disease (model of *L. monocytogenes* sepsis). The disease model analysed was *L. monocytogenes* sepsis because properdin is a serum protein and the alternative pathway is activated on the surface of *L. monocytogenes*. (Drevets *et al.*, 1991) see page 68 section 2.1.3.14 (C3 activation). In addition, murine listeriosis is the classical model with which to analyse the cellular component of an infection response. A very little was known about the role of complement in determining the cellular (macrophage and dendritic cells-mediated) immune response to infection with *L. monocytogenes* at the start of this project. Previous studies on the role of complement in response to murine *L. monocytogenes* infection did not investigate the role of complement in survival, rather analysed activation in serum and tissue infiltration. This is the first study on survival and humoral response in mice genetically engineered to be deficient of complement properdin and their controls.

*In vivo studies*

*The role of complement properdin in murine  
infection with *Listeria monocytogenes**

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### 3.2 Justification and outline of *in vivo* experimentation

Previous mouse models of complement C5-deficient mice showed a greater viable count of *L. monocytogenes* determined from their spleens compared to that from spleens of C5 sufficient mice (Petit, 1980). Moreover, *ex vivo*, anti-CR3 blocking antibodies significantly reduced the ability of mouse macrophages to kill *L. monocytogenes* EGD (Drevets *et al.*, 1993). *In vitro* experiments using dendritic cells and macrophages derived from bone marrow from properdin-deficient and wild type mice were conducted in order to characterise the importance of intact complement amplification and properdin production or a timeframe in which this plays a role. *In vitro* analysis of bone marrow-derived macrophage from wild type and properdin-deficient mice revealed differences in bacterial load at an early time point only.

*In vivo*, a murine model of systemic *L. monocytogenes* infection was used to study the immune response to these intracellular bacteria in the absence of properdin. Mice from wild type and properdin-deficient mice were infected intravenous (i.v.) with a passaged stock of *L. monocytogenes*. Different doses of  $5 \times 10^5$  and  $1 \times 10^6$  *L. monocytogenes* were used to study the role of properdin in survival of mice, in bactericidal activity, cytokine release response to bacteria and localisation of bacteria in liver and spleen by electron microscopy and histology, and these different doses will be presented separately. A dose of  $10^6$  CFU led to better responses in mice than a dose of  $10^5$  CFU. These different doses were used in this study because at a low dose of  $1 \times 10^3$  of *L. monocytogenes*, the mice will clear the bacteria with no signs of disease (Conlan, 1996). This has been observed in the properdin-deficient line as well, that a certain minimum dose of infectious inoculum of *S.pneumoniae* is needed to produce disease (PhD thesis Dupont A, 2008). In addition, the expression of different genes related to infection were analysed such as IFN- $\gamma$  and IL-17A as reactants to *L. monocytogenes* infection,

receptors CD11b, TLR2, C5aR related to phagocytes and receptors of Fc $\gamma$  that may be related to phagocytosis. Home office training was undertaken in order to carry out these studies (personal license number PIL 40/9662). The project license was entitled “Studies of Infectious Diseases” (PPL 80/2111, holder: Prof. P.W. Andrew).

For the survival study, to determine the ability of mice to survive an i.v. infection with *L. monocytogenes*, mice were assessed frequently, about 3-4 times daily. In total, the experiments were done 6 times and showed that wild type mice survived the infection with *L. monocytogenes* to a greater extent compared to properdin-deficient mice. Consistent with this, properdin-deficient mice showed greater disease severity within 28-29 hours.

### **3.2.1 Setting up listeriosis experiment for mice of the properdin-deficient colony**

A stock of passaged *L. monocytogenes* EGD-e was used in this study, following standard operating procedures established by Prof. Peter Andrew’s group and advised by Dr. Sarah Glenn. Therefore, passage through wild type mice selects a population of *L. monocytogenes* that has reacted to the host response, and is therefore, more virulent than an unpassaged population. Wild type and properdin-deficient mice were infected with a dilution of a washed stock of passaged *L. monocytogenes*. Mice were culled when lethargic or at the point of the experiment where indicated, which was earlier than when mice became moribund.

Mice were infected intravenously through a lateral vein with *L. monocytogenes* on day 0 and observed over a five days’ period, another experiment was conducted for 14 days. Viable counts of extracellular and intracellular *L. monocytogenes* were determined from liver homogenates and compared between the two groups. Livers from mice infected with *L. monocytogenes* were removed, homogenised in PBS and in some experiments

with distilled water, and aliquots then bacteria were plated on BHI-agar to determine CFU per gram of tissue.

*Listeria monocytogenes* are typically cleared from C57BL/6 mice more quickly from the spleen than the liver and that clearance does not happen until five days after infection (Wang *et al.*, 2011). Therefore, the CFU of *L. monocytogenes* were determined from the liver. However, as can be seen from another project conducted in Dr. Stover' group, the CFU from homogenised spleen were not different from those from liver and did not appear different between the two wild type mice and the single properdin-deficient mouse analysed. The result is shown in the table below.

**Table 3.2.1:** Systemic infection of mice with  $5 \times 10^5$  *L. monocytogenes* for 69 hours. From other project conducted in Dr. Stover' group.

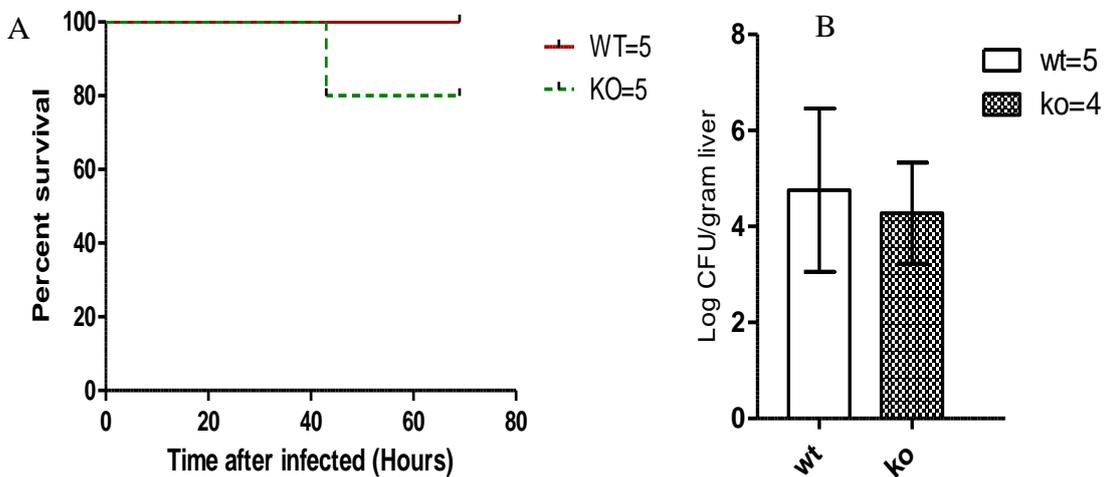
Mouse strain	CFU spleen extracellular (lg10)	CFU spleen intracellular (lg10)	CFU extracellular /CFU intracellular	CFU liver (lg10)	CFU liver/ CFU spleen intracellular
<b>WT</b>	2.029	1.606	1.263	6.52	5.162311955
<b>WT</b>	1.501	1.204	1.247	5.43	4.35445068
<b>KO</b>	1.420	1.079	1.316	5.96	4.52887537

### 3.2.2 Infection of properdin-deficient and wild type mice with $5 \times 10^5$ *L. monocytogenes*

#### 3.2.2.1 Pilot experiment

Groups contained 5 mice of each genotype were used in this experiment. Mice were infected and the experiment was ended at 69 hours. After mice were culled blood, liver, spleen were collected.

The survival curve, as can be seen in Figure 3.2.1 panel (A) shows no mice died (n=5) from wild type mice and 1 of 5 died from properdin-deficient mice at 43 hours. The difference between wild type and properdin-deficient mice was not statistically significant. Figure 3.2.1 panel (B) shows there was no difference in the numbers of viable count of *L. monocytogenes* that were obtained from homogenised livers of wild type and properdin deficient mice.



**Figure 3.2.1:** Survival curve of wild type (red —) and properdin-deficient mice (green ----) group of each genotypes panel (A) after infection (i.v) with  $5 \times 10^5$  *L. monocytogenes*. (B) Viable count obtained from the livers homogenised in PBS. A group of 5 WT and 4 KO these mice were killed at end time point except one were died from properdin-deficient. (B) The data are presented as means with standard deviation (SD).

Disease severities of experimental mice for individual mice over 69 hours of infection are shown in the table II in the appendix.

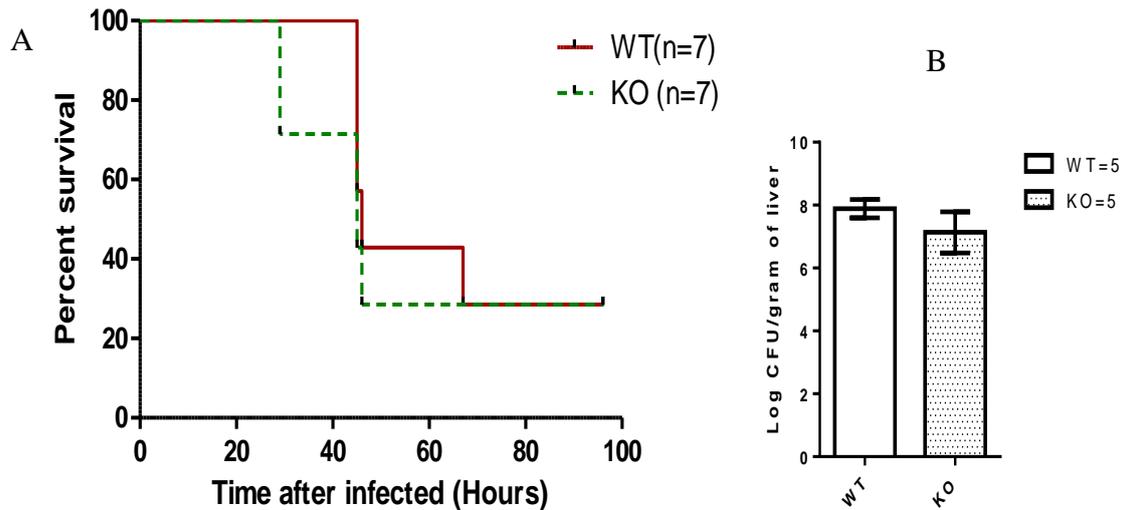
### **3.2.2.2 Systemic infection of mice with $5 \times 10^5$ *L. monocytogenes* for more than 100 hours**

The aim of this experiment was to see any difference between wild type and properdin-deficient mice for a longer time point, when a significant inflammatory reaction could be expected.

Wild type and properdin-deficient mice were infected with  $5 \times 10^5$  *L. monocytogenes*, and were monitored for survival and liver and serum were analysed. Groups of 7 of properdin-deficient mice and 7 to 9 of wild type were used in this experiment and these were infected with  $5 \times 10^5$  *L. monocytogenes*; this was repeated twice using different mice from the colony.

As can be seen in Figure 3.2.2 panel (A) the survival was reduced to 70% in the properdin-deficient mice (2 out of 7 dead) at 29 hours and 30% (out 5 of 7 dead) at 45 hours, while 40% of the wild type mice survived (4 of 7) at 45 hours and 30% at 67 hours (5 of 7). Properdin-deficient mice were lethargic and culled early after 29 hours and at the end time point, the experimental end at 100 hours, and properdin-deficient mice appeared normal. The survival curve shows there was no significant difference between wild type and properdin-deficient mice, but initially there were 2 out of 7 dead compared to 5 out of 7 dead at 28-29 hours, but at endpoint properdin-deficient mice were same compared to wild type mice.

The viable count obtained from homogenate livers show numbers of *L. monocytogenes* organisms were appeared slightly higher in wild type compared to properdin-deficient. However, statistically there was no difference between both genotypes.



**Figure 3.2.2:** Survival curve of wild type (red —) and properdin-deficient mice (green - - - -) panel (A) after infection (I.V) with  $5 \times 10^5$  *L. monocytogenes* (Subacute model). Panel (B) viable count of *L. monocytogenes* from livers homogenised in PBS, the data are presented as means with standard deviation (SD).

Disease severities of experimental mice for individual mice up to 100 hours of infection the severities are shown in the table III. Wild type and properdin-deficient mice were culled when they were lethargic, and mice that survived up to 100 hours appeared normal.

In C57BL/6J wild type mice start to die by day 4 after infection with  $1 \times 10^5$  CFU of *L. monocytogenes* (Pasche *et al.*, 2005).

Female C57BL/6J mice as reported in a study by Pasche *et al.*, 2005 showed increased susceptibility to *L. monocytogenes* compared to male mice that were infected intravenously with  $1.3 \times 10^3$  to  $2.0 \times 10^4$  CFU of *L. monocytogenes*. Female mice have the ability to reduce and control replicating bacteria. In order to assist this difference in this study, other experiment was set up with sex matched mice. Then the experiment was put together on the other groups. In this experiment the severity and survival of mice were compared between male and female as also shown in section 3.2.2.3.

The progression of disease started after 19 hours which is more manifest in properdin-deficient mice than in wild type. The time of highest severity was between 29 to 100 hours, and this resulted in death and culling mice in both genotypes, but more properdin-deficient mice were culled than wild type. Survival is possible, to comparable extent between wild type and properdin-deficient mice, and the surviving mice were either hunched or normal in both genotypes.

### **3.2.2.3 Infection of mice with $5 \times 10^5$ to analyse the role of properdin in males and females**

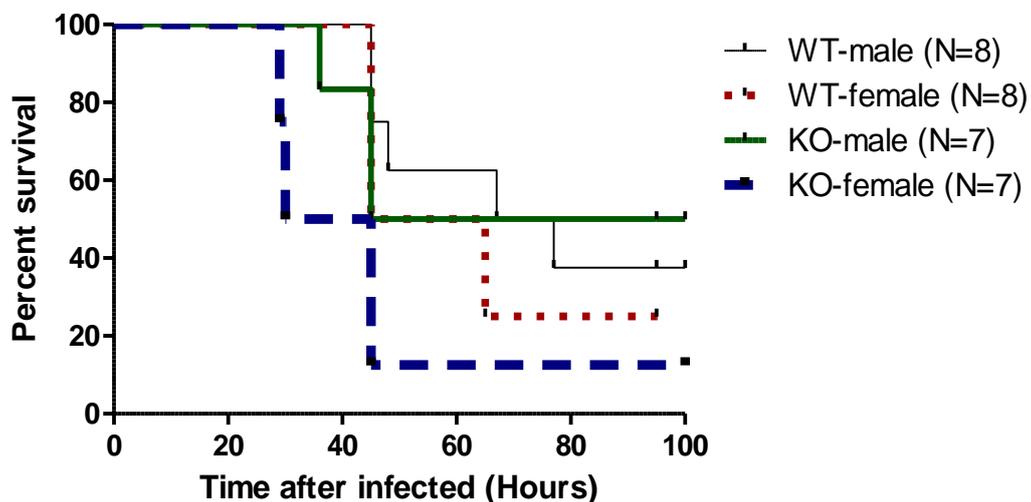
To study the role of properdin in sex-dependent susceptibility, age-matched groups of males and females mice from 16 wild type and 14 properdin-deficient mice were infected with  $5 \times 10^5$  *L. monocytogenes* up to 100 hours from 2 independent experiments, and mice were culled when they were lethargic for survival experiments, mice were observed for 10 to 14 days after infection.

As can be seen in Figure 3.2.3 female from wild type mice are more susceptible than males from wild type mice. This is the same observation was shown in a study by Pasche *et al.*, 2005. Female mice from wild type and properdin-deficient mice shows higher susceptibility to infection with *L. monocytogenes* compared to male. However, female from properdin-deficient showed higher susceptibility to *L. monocytogenes* compared to others. 7 of 14 female mice (50%) and 3 of 14 male mice (70%) from properdin-deficient were dead or culled. 6 of 16 female mice (60%) and 5 of 16 male mice (70%) from wild type were dead or culled. Female mice from properdin-deficient mice succumbed to the infection sooner at 28 hours than female mice from wild type at 45 hours. Female from properdin-deficient mice, 4 of 14 were culled or dead at 29 hours, 3 of 14 culled at 45 hours and 1 of 14 were survival at 100 hours. Female from wild type mice, 4 of 16 were culled at 45 hours, 2 of 16 culled at 65 hours and 2 of 16 at

100 hours. Male from properdin-deficient mice, 1 of 14 culled at 36-43 hours, 2 of 14 at 45 hours and 3 of 14 were culled at 100 hours. Male from wild type mice, 3 of 16 were culled at 45-48 hours, 2 of 16 at 67 hours and 3 of 16 were culled at 100 hours (Table 3.2.3 and table 3.2.4).

The relative impairment in the outcome of properdin-deficient mice is maintained against the different levels of susceptibility of males and females. Males are usually bigger than females; females were more susceptible than males which compared to the survival data of mice infected with same bacterial doses per gram of body weight (Pasche *et al.*, 2005). The severities of mice were shown in table 3.2.4.

As expected (see Figure 3.2.2), mice infected with  $5 \times 10^5$  *L. monocytogenes* showed (10 of 14 properdin-deficient mice and 11 of 16 wild type mice dead or culled). The survival mice were 4 of 14 properdin-deficient mice and 5 of 16 wild type mice. But again statistically there was no difference between mice from wild type and properdin-deficient (See table IV in appendix).



**Figure 3.2.3:** Comparison of survival curve between male and female from of wild type and properdin-deficient mice after infection (i.v.) with  $5 \times 10^5$  *L. monocytogenes*. The figure data were from two independent experiments. Seven mice per group of properdin-deficient and eight mice per group of wild type were monitored for about 100 hours.

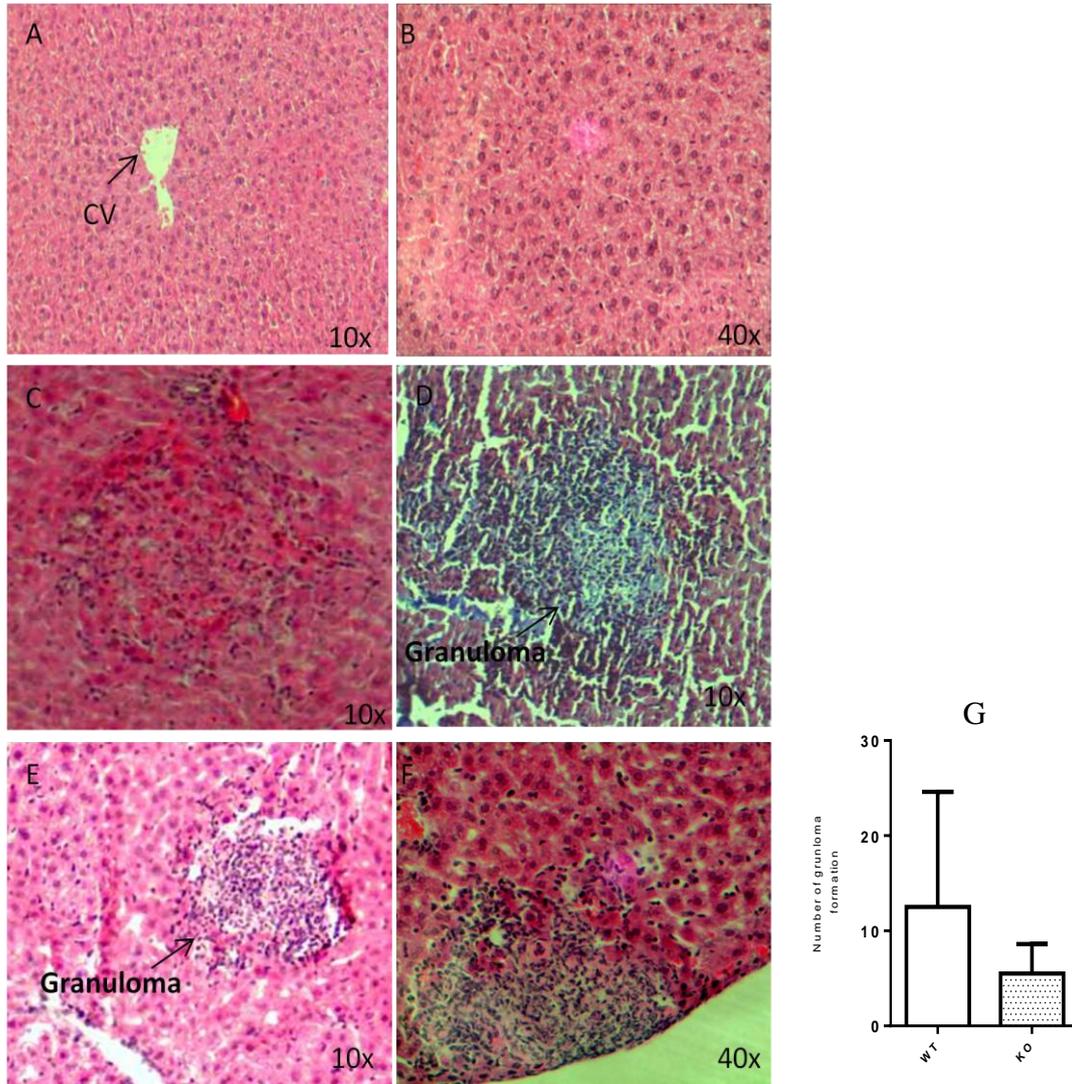
#### 3.2.2.4 Histological evidence of listeriosis-induced immune response

To study the role of properdin in infectious granuloma formation, liver and spleen was removed from mice infected with  $5 \times 10^5$  *L. monocytogenes*, sectioned and stained with haematoxylin and eosin. Twort's stain was used to stain gram-positive bacteria in formalin-fixed section for light microscopy in order to study the localisation of bacteria and granuloma in both genotypes.

The liver is an important site of the immunological response to *L. monocytogenes* infection in mice. It harbors part of the monocyte-phagocyte system like spleen and bone marrow (Van Furth *et al.*, 1972) and *L. monocytogenes* are taken up in the liver where they multiply in parenchymal cells. In both wild type and properdin-deficient mice injected intravenously with  $5 \times 10^5$  *L. monocytogenes* granulomas are identified (Figure 3.2.4 arrow D and E). Representative images were chosen from a total of 5 mice each genotype. In both genotypes, few Twort positive bacteria were seen in granulomas. Overall, in the random sections, the diameters of granulomas in wild type mice appeared larger than those in properdin-deficient mice.

The normal spleen is divided into red pulp, and white pulp. *L. monocytogenes* localise to T cell zones of the white pulp (Conlan, 1996). In this study location of *L. monocytogenes* in spleen of some mice in both genotypes shows most of them in white pulp (images not shown). Bacteria that enter the spleen by blood are removed in white pulp first by mononuclear phagocytes before accessing the red pulp. Intravenous infections of *L. monocytogenes* that are taken up by the spleen are removed from the blood predominantly by mononuclear phagocytes in the marginal zone of the white pulp (Conlan, 1996).

In the absence of properdin, granulomas are formed but overall to a lesser extent than in wild type (5-10 KO and 14-29 WT) (Figure 3.2.4 in panel G) after infection with a dose of  $5 \times 10^5$  of *L. monocytogenes*.

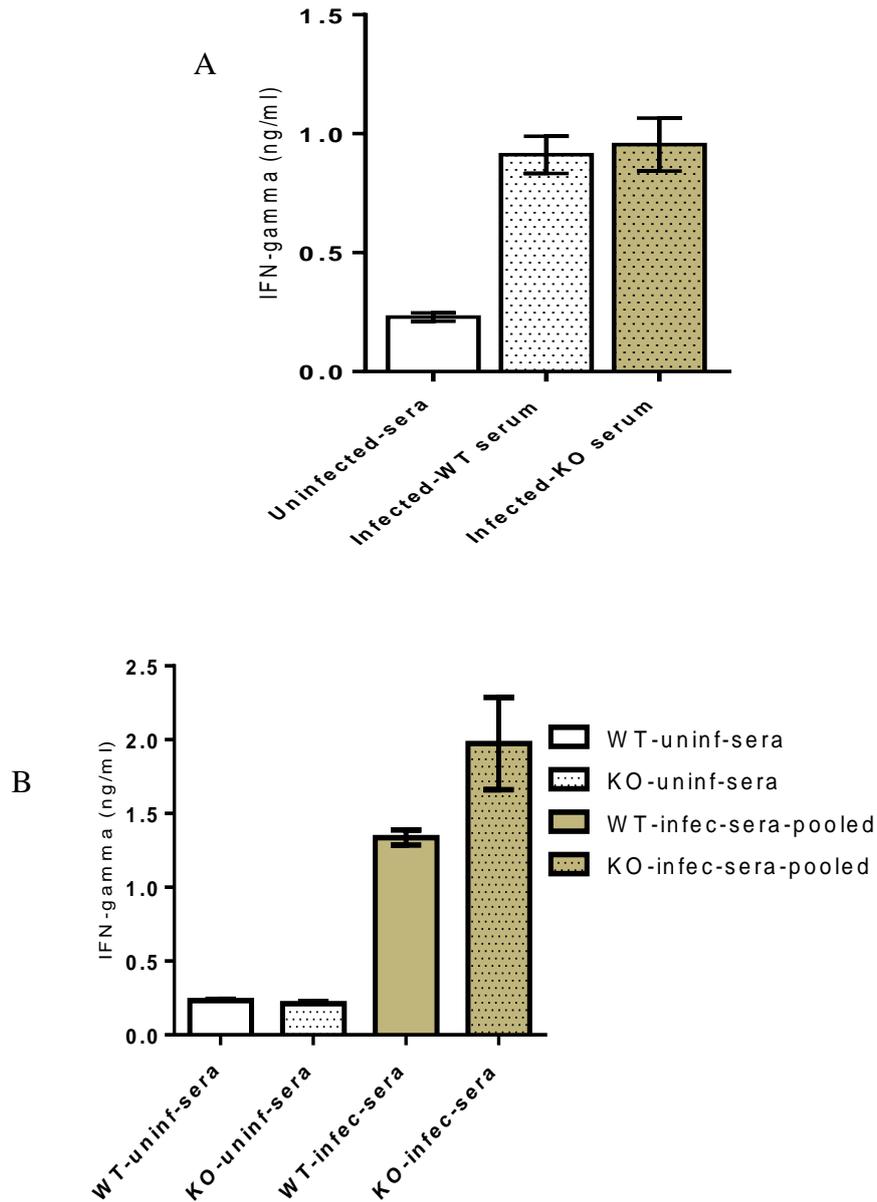


**Figure 3.2.4:** Histological examination of the liver of the wild type and properdin-deficient mice on day 4 after i.v. infection with  $5 \times 10^5$  *L. monocytogenes* (pilot experiment). Representative images of the liver sections from the WT (Panel C and D) and properdin-deficient mice (Panel E and F) is demonstrated, panels A, B, C and E were used haematoxylin and eosin staining. Panels D and F were used Twort's stain of the liver sections from the WT (Panel D) and properdin-deficient mice (Panel F). (A and B uninfected liver). Magnification of 10X, in panels B and F was 40X, CV= central veinule. In panel G numbers of granuloma were compound (4 mice each genotypes) the data are presented as means with standard deviation (SD).

**3.2.2.5 Quantitative measurement of IFN- $\gamma$  in mouse serum in response to infection with  $5 \times 10^5$  *L. monocytogenes***

To study the role of properdin in IFN- $\gamma$  cytokine response in response to infection with *L. monocytogenes*, as IFN- $\gamma$  is crucial for the early control of bacterial infections, blood from infected wild type and properdin-deficient mice was collected by cardiac puncture. Serum was separated and used for analysis by ELISA.

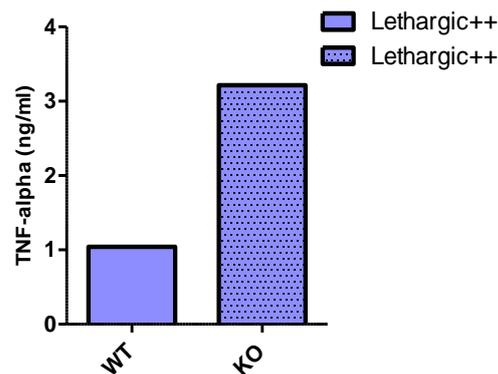
In the experiment that gave no impairment of properdin-deficient mice compared to wild type mice in their survival after infection with  $5 \times 10^5$  *L. monocytogenes* (Pilot experiment), there was an increase of IFN- $\gamma$  over normal in both genotypes. There was no difference in IFN- $\gamma$  levels between wild type and properdin-deficient mouse serum as shown in Figure 3.2.5 panel A, the severity of those mice were not lethargic (See Table 3.2.2) and the serum were from individual mice not pooled serum. In panel B, the levels of IFN- $\gamma$  from pooled sera were analysed and are a summary of individual experiments with groups of 7 each genotype. Pooled sera were used, from wild type mice (two mice were culled after 45 hours) and properdin-deficient mice (one mouse after 28 hours and another after 45 hours). Level of IFN- $\gamma$  appeared higher in sera from lethargic properdin-deficient mice compared to sera from lethargic wild type mice.



**Figure 3.2.5:** Serum IFN- $\gamma$  level from wild type and properdin-deficient mice after infection with  $5 \times 10^5$  *L. monocytogenes* were determined by ELISA. In (A) the data presented from individual experiments from groups of five each genotype not pooled sera (pilot experiment). In (B) the data presented from 2 times pooled sera and are a summary of individual experiments with groups of 7 each genotype. Pooled sera from wild type mice were culled after 45 hours and properdin-deficient mice, one mouse after 28 hours and another after 45 hours all were culled when they were lethargic. Data present two independent experiments performed in triplicate. The data are presented as means with standard deviation (SD).

### 3.2.2.6 Quantitative measurement of TNF- $\alpha$ activity in mouse serum in response to *L. monocytogenes* infection

TNF- $\alpha$  activity was measured using a functional bioassay in serum from wild type and properdin-deficient mice after infection with  $5 \times 10^5$  *L. monocytogenes*. These mice were lethargic and culled after 49 hours in both wild type and properdin-deficient mice. As can be seen in Figure 3.2.6 the level of bioactive TNF- $\alpha$  was higher in serum from properdin-deficient mouse which was ++lethargic compared to wild type mouse that have the same severity ++lethargic. The same trend of this difference was observed when mice were +lethargic (data not shown).



**Figure 3.2.6:** TNF- $\alpha$  activity in serum from wild type and properdin-deficient mice after infection with  $5 \times 10^5$  *L. monocytogenes* after 45 hours, sera from individual mouse. As described for this bioassay in section 2.1.3.8, the mean of triplicate reading is related to the activity of the TNF- $\alpha$  standard and therefore, no standard deviation (SD) appear here.

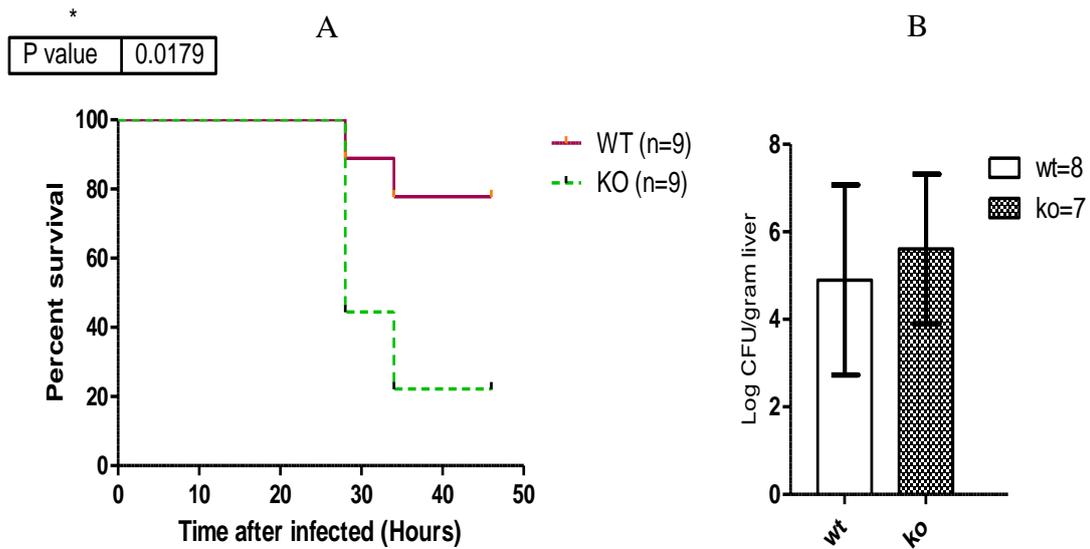
### 3.2.3 Infection of properdin-deficient and wild type mice with $1 \times 10^6$ *L. monocytogenes*

A group of 4 mice each and another experiment with 5 mice each genotype were infected with  $1 \times 10^6$  and  $9 \times 10^5$  *L. monocytogenes*.

When mice were infected with  $9 \times 10^5$  and  $1 \times 10^6$  *L. monocytogenes*, there was a significant difference in survival between wild type and properdin-deficient mice as shown in Figure 3.2.7 (A). Using a larger group size of 9 each, survival was reduced to (20%) in the properdin-deficient mice (7 of 9 were culled), while (80%) of the wild type mice survived (2 of 9). Most properdin-deficient mice were lethargic and culled early after 29 hours and later were ended the experiment after 46 hours. Importantly, the time point of observed significant severity is the same as previously observed (Figure 3.2.2). The viable count of *L. monocytogenes* per organs from all wild type and properdin-deficient mice showed no significant difference between wild type and properdin-deficient mice (Figure 3.2.7 B) although properdin-deficient mice have a somewhat higher number of bacteria compared to wild type mice.

Bacteria were counted from the liver after homogenisation in distilled water to establish extracellular and intracellular bacterial count (data not shown). Although there was a slightly higher number of *L. monocytogenes* in homogenised liver from properdin-deficient mice compared to homogenised liver wild type mice (Figure 3.2.7 B), statistically there was no difference in viable count between homogenised livers from wild type compared to properdin-deficient mice.

For properdin-deficient mice the median survival was 28 hours with SD 7.61, for wild type the median survival was 46 hours with SD 6.78.



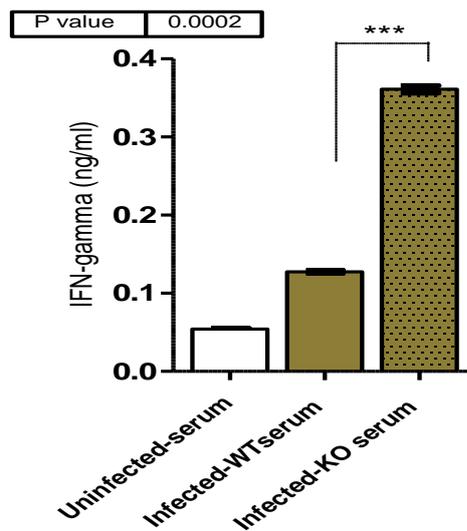
**Figure 3.2.7:** Survival curve (A) of wild type (red —) and properdin-deficient mice (green ----) after infection (I.V) with  $1 \times 10^6$  *L. monocytogenes*. (B) Viable count of *L. monocytogenes* obtained from the livers homogenised in PBS were determined at indicated time points by plating tissue homogenates in 10-fold serial dilutions on BHI-agar plates. The data are presented as means with standard deviation (SD). In (A) the log rank test ( $P= 0.0179$ ). Two independent experiments (Group of 5 each and another group of 4 each).

The results indicate that properdin-deficient mice were severely impaired in the *L. monocytogenes* sepsis model even though the viable count of *L. monocytogenes* in (liver only) both genotypes were comparable. Therefore, it is hypothesised that the difference in mortality may be due to a properdin-dependent immune response to the infection, and not a result of different bacterial viability in wild type and properdin-deficient mice.

The overall severity of the mice model of wild type and properdin-deficient show 3 of 18 were dead or culled, 6 of 18 were starey and +lethargic, 9 of 18 were hunched. Wild type shows 1 of 9 was culled, 1 of 9 was lethargic, 4 of 9 were +hunched, and 3 of 9 were ++hunched. Properdin-deficient mice 2 of 9 were dead or culled, 5 of 9 were +lethargic and 2 of 9 were +hunched (see appendix table V).

### 3.2.3.1 Quantitative measurement of IFN- $\gamma$ in mouse serum in response to infection with $1 \times 10^6$ *L. monocytogenes*

IFN- $\gamma$  was measured in mouse serum by ELISA at the endpoint of the infection study using  $1 \times 10^6$  CFU of *L. monocytogenes*, (lethargic mice culled after 29 hours) (Figure 3.2.8). As expected, the level of IFN- $\gamma$  was higher in serum from infected mice compared to serum from uninfected mice. In lethargic mice, sera from infected mice showed a higher level of IFN- $\gamma$  from properdin-deficient mice and had significantly elevated levels than sera from lethargic wild type mice. There was significant difference of IFN- $\gamma$  level between wild type serum compared to properdin-deficient mice ( $p=0.0002$ ).



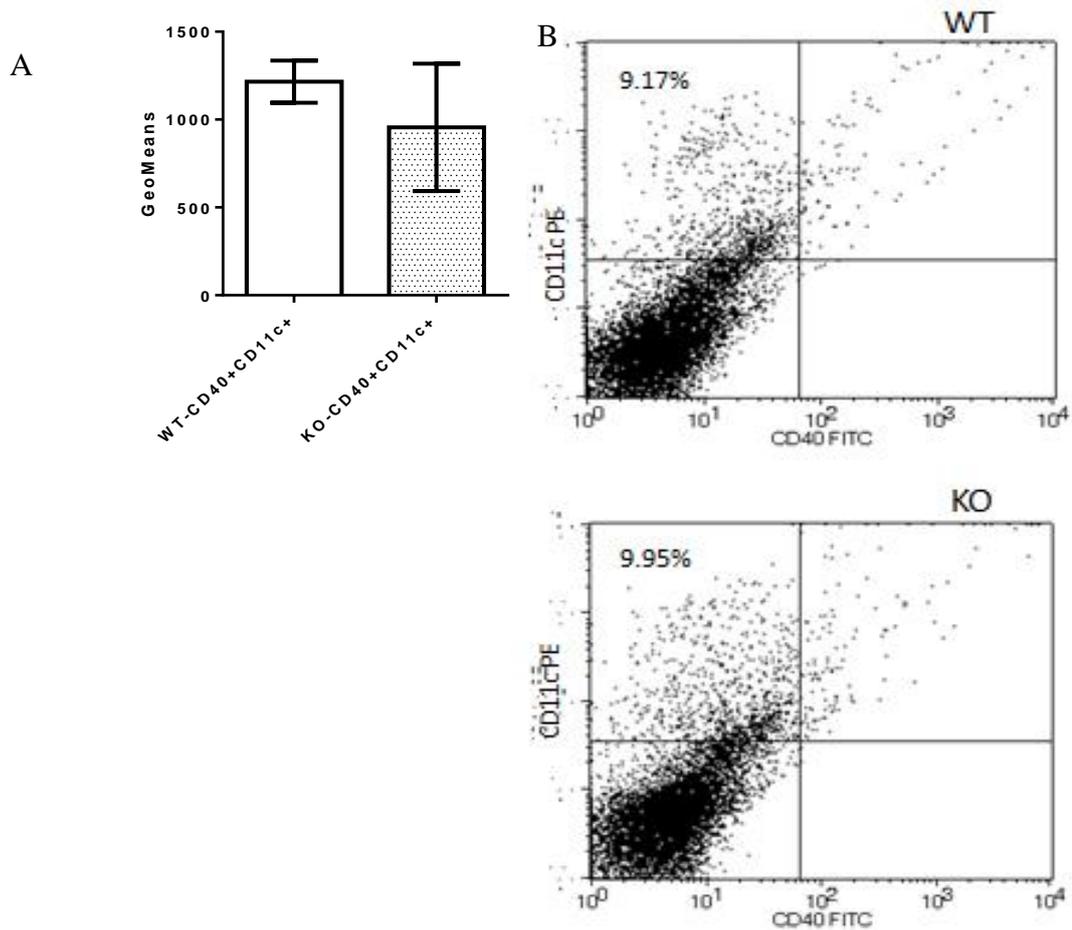
**Figure 3.2.8:** Sera IFN- $\gamma$  level from lethargic wild type and lethargic properdin-deficient mice after infection with  $1 \times 10^6$  *L. monocytogenes* were determined by ELISA. The data presented from 2 mice pooled sera each genotype and are a summary of individual experiments with groups of five each genotype. Data representative of two independent experiments performed in triplicate. The data are presented as means with standard deviation (SD).

The higher serum level of IFN- $\gamma$  in properdin-deficient mice are coincident with their greater severity compared to infected wild type mice at doses of  $1 \times 10^6$  and  $9 \times 10^5$  CFU of *L. monocytogenes*.

### **3.2.3.2 Surface expression of CD40 of splenic dendritic cells**

CD40 was more abundantly expressed in bone marrow-derived dendritic cells from wild type after *in vitro* infection with heat-killed *L. monocytogenes*. Therefore, the expression of CD40 by CD11c<sup>+</sup> dendritic cells was assessed after *in vivo* infection with  $1 \times 10^6$  of *L. monocytogenes* from spleens of both genotypes using flow cytometry.

CD40 was higher in dendritic cells from wild type splenocytes compared to dendritic cells from properdin-deficient splenocytes (Figure 3.2.9 A). However, there was no significant difference in expression of CD40 in CD11c<sup>+</sup> cells of both genotypes. Content of CD11c<sup>+</sup> cells in splenocyte preparation was comparable between wild type and properdin-deficient as indicated in the upper left quadrants of Figure 3.2.9 (B).



**Figure 3.2.9:** Flow cytometric analysis of dendritic cells from spleen after infection with  $1 \times 10^6$  *L. monocytogenes* stained with the mouse CD11c and CD40 antibody. (B) Representative dot plot shows expression of (CD40 and CD11c) from dendritic cells induced in the spleen of the wild type (WT) and properdin-deficient mice (A) (The data are presented as means with standard deviation (SD)). Representative data from triplicate determination performed on matched mice infected in parallel are shown. The extent of surface staining with specific antibodies CD40 and CD11c is expressed using Geometrical Means. Fc block was used.

### 3.2.3.3 Characterisation of the inflammatory mRNA response in *L. monocytogenes* infected mice

The expressions of inflammatory splenic IFN- $\gamma$  and IL-17A as reactants to *L. monocytogenes* infection were determined from spleens of mice infected with  $5 \times 10^5$  *L. monocytogenes*. Wild type (after 49 hours) and properdin-deficient mice (after 29 hours and one mouse after 49 hours) were analysed using (qPCR) real-time quantitative PCR.

Pathological tissue infiltration in the liver was macroscopically evident for properdin-deficient mice and wild type culled on day 4 after i.v. infection with  $5 \times 10^5$  *L. monocytogenes*.

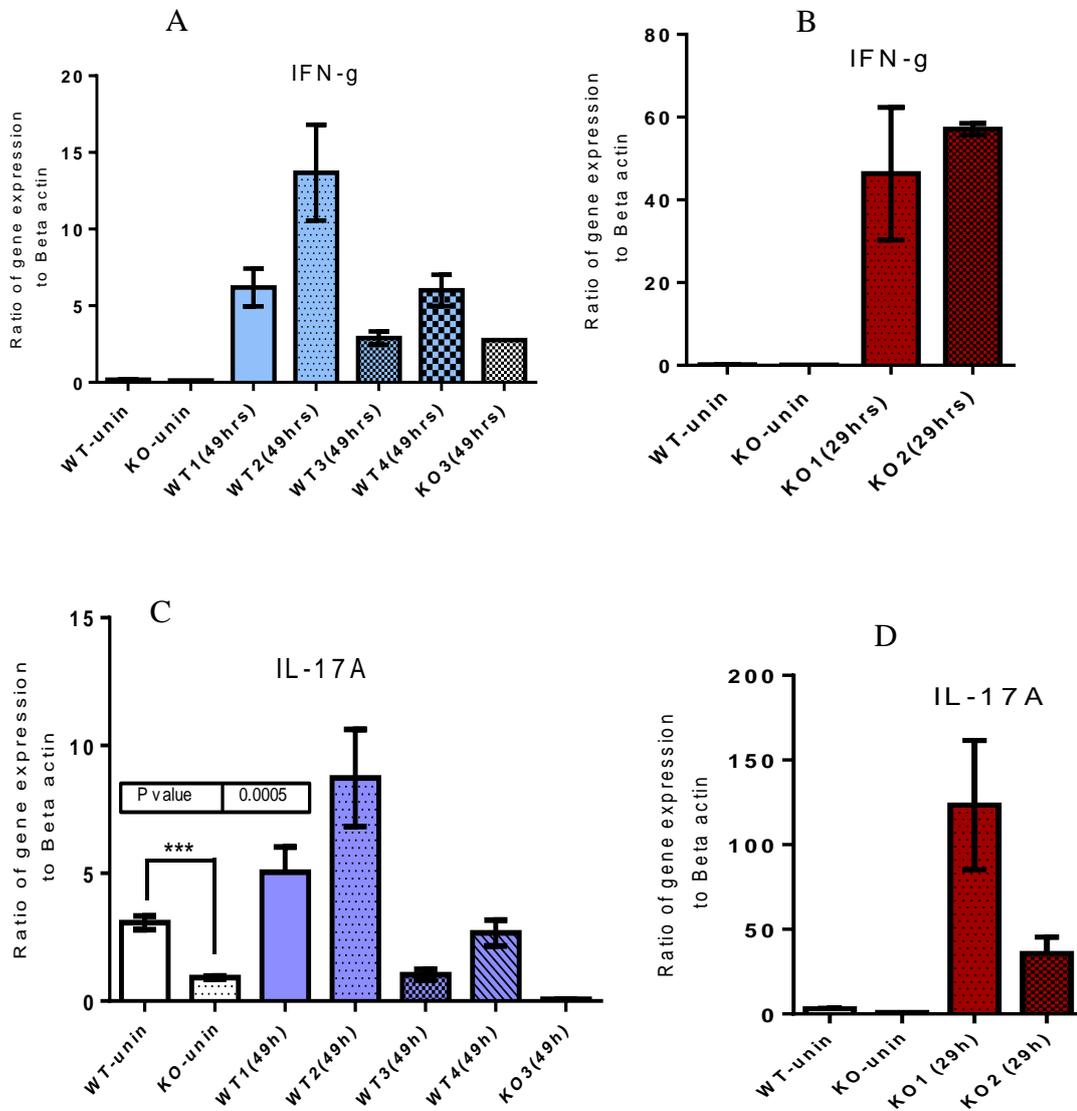
In uninfected mice, expression of IFN- $\gamma$  was undetectable but after infection expression was up-regulated in both genotypes.

The expression was detectable in mice after infection lasting at least 29 hours. IFN- $\gamma$  production by the spleen from the wild type mice and one mouse from properdin-deficient was lower after 49 hours (Figure 3.2.10 A) than the spleen from the properdin-deficient mice at the endpoint of 29 hours (panel B). In wild type lethargic mouse (WT2 panel A) IFN- $\gamma$  expression was higher compared to hunched wild type mice. The IFN- $\gamma$  mRNA expression in the spleen of properdin-deficient mice was up-regulated 5 fold at 29 hours after infection (panel B) compared with wild type mice at 49 hours (panel A). Overall, mRNA expression of IFN- $\gamma$  by qPCR in spleen from properdin-deficient mice was the same observation of the level of protein IFN- $\gamma$  measured by ELISA which higher in serum from properdin-deficient mice after 29 hours.

Expression of IL-17A (Figure 3.2.10 C) in spleen from uninfected mice was detectable and there was a higher expression level and significant difference in spleen from wild type compared to spleen from properdin-deficient mice ( $P = 0.0005$ ).

Similarly, the IL-17A mRNA expression in the spleen of properdin-deficient mice were significantly higher about 4 or 5 fold after 29 hours infection (Figure 3.2.10 panel D). However, the levels of IFN- $\gamma$  and IL-17A mRNA in the spleen of properdin-deficient mouse at endpoint 49 hours were comparable with those in wild type mice at endpoint 49 hours during *L. monocytogenes* infection.

Properdin-deficient mice after 29 hours also showed higher levels of IL-17A production (panel D) than those of the wild type mice and one properdin-deficient mouse after 46 hours (panel C).



**Figure 3.2.10:** mRNA expression of IFN- $\gamma$  (A and B) and IL-17A (C and D) from spleen from properdin-deficient mice after 29 hours (B and D), and (A and C) from wild type and mouse from properdin-deficient after 49 hours i.v infection with  $5 \times 10^5$  of *L. monocytogenes*.  $\Delta\Delta\text{CT}$  value were used, the mRNA expression corrected for  $\beta$ -actin and compared with uninfected spleen. The QPCR results are from analysis was set up in duplicate from triplicate expression. The data are presented as means with standard deviation (SD). (Numbers of mice after 49 hours are WT1, 2, 3, 4 and KO3, after 29 hours KO1 and KO2).

Disease severities of experimental mice for the indicated end time points are tabulated hereafter.

**Table 3.2.2:** Severity of mice that were used to measure the cytokine IL-17 and IFN- $\gamma$  response to *L. monocytogenes*.

Score	Condition/disease severity	Score	Condition/disease severity
<b>wild type mice</b>		<b>properdin-deficient mice</b>	
<b>WT1</b>	++hunched at 49 hours	<b>KO1</b>	Lethargic at 29 hours
<b>WT2</b>	Lethargic at 49 hours	<b>KO2</b>	Lethargic at 29 hours
<b>WT3</b>	++hunched and starey coat at 49 hours	<b>KO3</b>	++hunched at 49 hours
<b>WT4</b>	++hunched and starey coat at 49 hours		

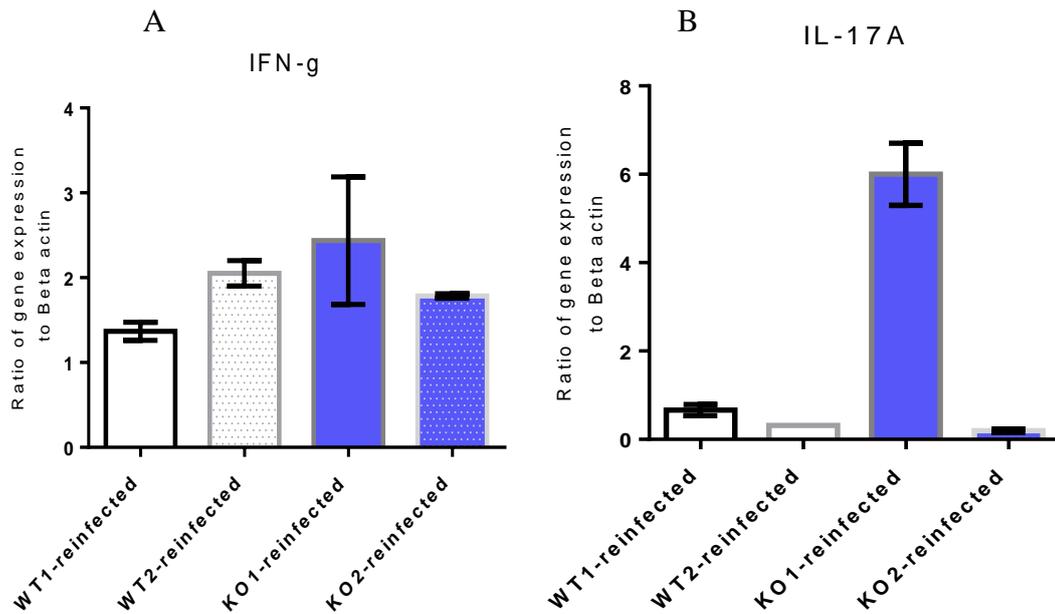
The detection of mRNA expression of IFN- $\gamma$  and IL-17A in spleen follows the overall severity of the mice.

The role of IL-17A in *L. monocytogenes* infection has been studied. A study by Xu *et al.*, 2010 using C57BL/6J mice they demonstrated that IL-17A is required for innate and adaptive response against bacterial infection, they showed that IL-17A-deficient mice (liver and spleen) have more *L. monocytogenes* compared to wild type mice after day 3 of infection with i.v. with  $1 \times 10^6$  *L. monocytogenes* that secret OVA protein ( $\Delta actA$  LM-OVA). Moreover, a study by Hamada *et al.*, 2008 used wild type C57BL/6 mice infected by i.p. inoculation of  $5 \times 10^4$  CFU *L. monocytogenes* strain EGD they showed that mRNA expression of IL-17A in the liver of infected mice increased from day 1 and maintained high levels of expression to day 5 but decreased over the days, which

indicate that in the infected liver, *L. monocytogenes* infection induces IL-17A expression at very early stages. In addition, *L. monocytogenes* counts were more than 100 times higher in the liver of IL-17A-deficient mice compared to wild type mice on day 5 of the infection.

### **3.2.3.3.1 Secondary immune responses to *L. monocytogenes***

To study secondary immune responses, the surviving 2 mice of each genotype were re-infected i.v with the same dose of *L. monocytogenes* ( $5 \times 10^5$ ) and were culled after 5 days. All mice survived after challenge and their severity were normal. The viable count obtained from homogenate liver show no growth of *L. monocytogenes* in wild type mice and one of properdin-deficient mouse (KO2), another mouse from properdin-deficient (KO1) show number of bacteria  $\log = 4.276671$ . The spleens, however, appeared enlarged compared to spleens obtained from mice infected only once (Section 3.2.3.2). The expressions of IFN- $\gamma$  and IL-17A were determined from the spleen of re-infected mice from wild type and properdin-deficient mice individually using qPCR as shown in the Figure 3.2.11, the expression of IFN- $\gamma$  was similar expression in both genotypes but IL-17 were higher in re-infected mice from properdin-deficient group compared to wild type and higher in KO1 which has the growth of bacteria number in liver.



**Figure 3.2.11:** mRNA expression of IFN- $\gamma$  (A) and IL-17A (B) from spleen from wild type and properdin deficient mice after i.v infection with  $5 \times 10^5$  *L. monocytogenes* and re-infection with the same dose.  $\Delta\Delta\text{CT}$  value was used, the mRNA expression corrected for  $\beta$ -actin and compared with uninfected spleen. The QPCR results are from analysis set up in duplicate from two independent expressions. The data are presented as means with standard deviation (SD).

mRNA expression of IFN- $\gamma$  and IL-17A from mice spleen after re-infected with *L. monocytogenes* showed less in both genotypes compared to previous analysis (see y-axes), the levels of expression in re-infected mice were as normal mice or in hunched mice after infection.

### 3.2.4 Analysis of entire groups of mice infected with $1 \times 10^6$ *L. monocytogenes* at an early time point (28-29 hours)

3 wild type and 3 properdin-deficient mice were infected with  $1 \times 10^6$  of *L. monocytogenes* and culled after 28-29 hours because from last experiments numbers of mice from properdin-deficient were lethargic after 28-29 hours more than wild type.

Because the time to measure the cytokines were an important and at stated in *in vivo* experiment, it was unknown the severity of properdin-deficient mice, therefore, mice were culled when they were lethargic and the organs were used. Thus, when the time was shown for properdin-deficient mice were lethargic at 29 hours, another experiment was designed to end the experiment at same time point and mice were culled at 28-29 hours after infection. For this experiment, the number of viable counts in liver and levels of serum IFN- $\gamma$  matched the individual severity. Similarly, mRNA expression of IFN- $\gamma$  and TLR2 were more increased in those mice (wild type mice or properdin-deficient mice), which showed greater disease severity, but generally higher in lethargic wild type mice compared to properdin-deficient mice.

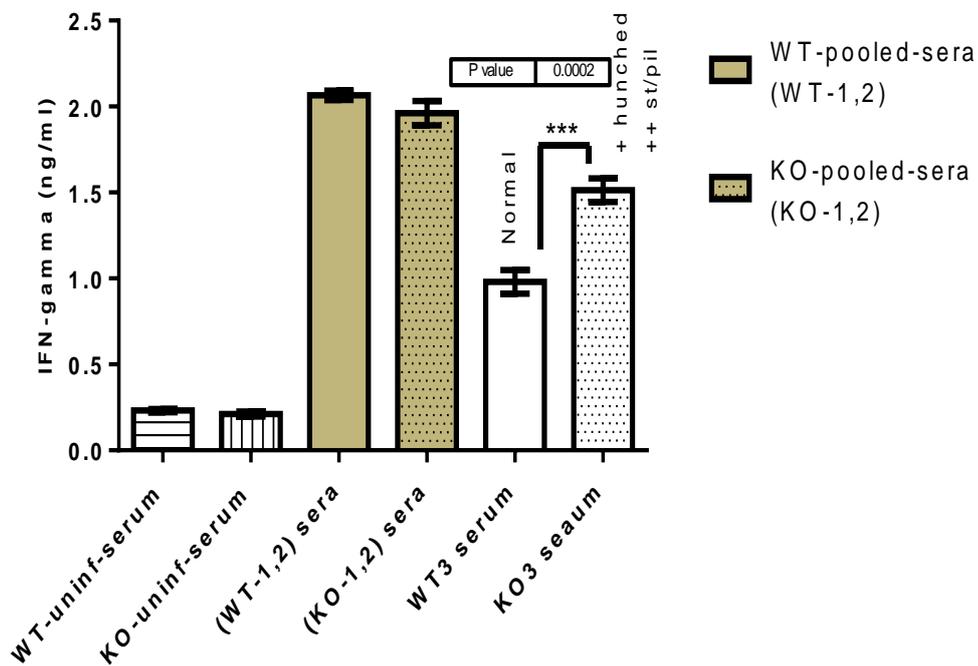
The viable count of *L. monocytogenes* was slightly higher in properdin-deficient mice compared to wild type mice.

### **3.2.4.1 Quantitative measurement of IFN- $\gamma$ in response to infection with $1 \times 10^6$ *L. monocytogenes* in mouse serum**

To study the role of properdin in IFN- $\gamma$  cytokine response from individuals and pooled mouse sera in response to infection with *L. monocytogenes*, sera from 3 mice each genotypes wild type and properdin-deficient after infection were collected by cardiac puncture and were used for analysis.

Figure 3.2.12 shows the measurement of IFN- $\gamma$  from individual sera from wild type and properdin-deficient mice in response to i.v. infection with  $1 \times 10^6$  of *L. monocytogenes*. The level of IFN- $\gamma$  was higher in infected sera compared to uninfected sera. The mice were culled after 28-29 hours when they were lethargic after infection, serum from infected mice showed a higher level of IFN- $\gamma$  from properdin-deficient mice (severity +Hunched and starey/piloerect) and had significantly elevated levels then that serum from wild type mice (severity normal), there was significant difference of IFN- $\gamma$  level

between wild type serum compared to properdin-deficient mice ( $p=0.0002$ ). On the other hand, IFN- $\gamma$  from pooled sera 2 wild type (one was +lethargic and ++starey/piloerect another was +hunched and +starey/piloerect) and 2 properdin-deficient (one was +lethargic and ++starey/piloerect and other was ++ hunched and +starey/piloerect) shows no difference between both genotypes.



**Figure 3.2.12:** Serum IFN- $\gamma$  level from wild type and properdin-deficient mice after infection with  $1 \times 10^6$  of CFU *L. monocytogenes* determined by ELISA. The data presented from individual experiments with groups of 3 each genotype. Mice were culled at the same time point. The data are presented as means with standard deviation (SD).

The severities of 3 mice each genotype is shown in the table VI in the appendix.

Compared the IFN- $\gamma$  level from individual mice with severity normal in wild type and hunched in properdin-deficient, was higher in properdin-deficient mouse. This confirms the previous finding, higher level of IFN- $\gamma$  in lethargic properdin-deficient mice sera compared to lethargic wild type mice (Figure 3.2.8).

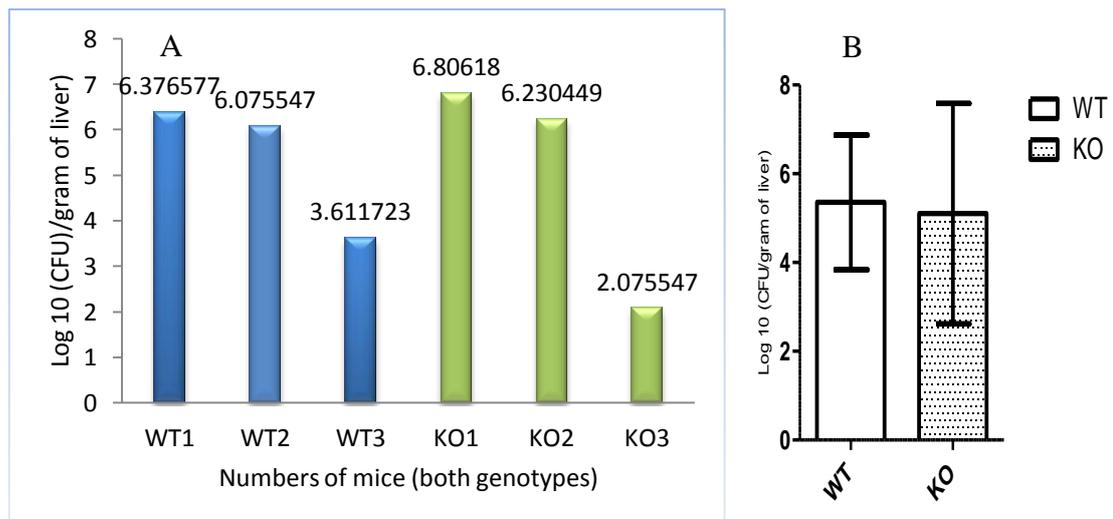
IFN- $\gamma$  level in pooled serum with different severity although the mice were culled at matched endpoint but show no difference between wild type and properdin deficient mice (one technical limitation is that not the same volume of serum was taken from each mouse).

### 3.2.4.2 Determination of number of viable counts of *L. monocytogenes* in the liver

To investigate any differences between wild type and properdin-deficient mice in bacteria numbers, liver were removed from culled mice aseptically and after weighing were homogenised in 10 ml PBS and serially diluted on BHI-agar.

3 mice each genotype were infected with  $1 \times 10^6$  *L. monocytogenes* and culled at the same time after 28-29 hours. This matched endpoint approached over the previous experiments, which showed that mice from properdin-deficient dead early after 28-29 hours compared to wild type mice.

As can be seen in the Figure 3.2.13 (A) the CFU from the homogenized liver of individual mice was matched the severity but in (B) with groups of wild type and properdin-deficient mice there were no difference between both genotypes.

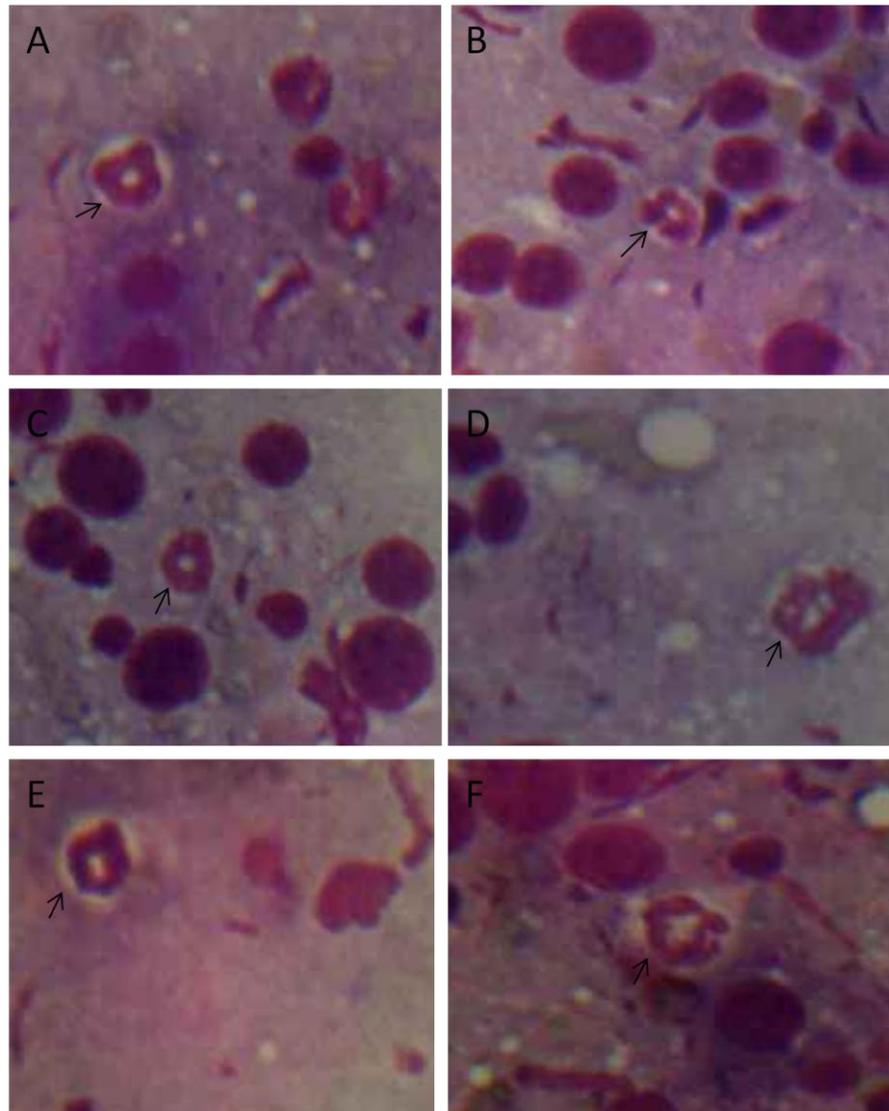


**Figure 3.2.13:** Viable count obtained from homogenised mice liver in PBS (A) individual mice (B) all mice, after infection (i.v) with  $1 \times 10^6$  of CFU *L. monocytogenes*. The data are presented as means with standard deviation (SD).

### 3.2.4.3 Cytological analysis of liver homogenates

This experiment was done in parallel to determining the viable count from homogenates liver. The aim of this experiment was to see any evidence of inflammatory cells in homogenates liver, because cells composition of the granuloma was hard to make out and immunohistochemistry was not set up in the lab to characterise them by antibody reactivity.

Cytospins were prepared from homogenised liver in PBS from wild type and properdin-deficient mice after infection with  $1 \times 10^6$  *L. monocytogenes* (Figure 3.2.14). These mice were culled at 29 hours. Typical for bacterial infections, there are neutrophils, characterised by a polymorphic nucleus, invading the tissues (indicated by arrows). Images were taken from infected mouse from wild type with normal appearance and properdin-deficient mouse with hunched appearance. The numbers of granulocytes were 3 granulocytes in both of them 6 in total. This was compared to images that taken from lethargic mice from both genotype, which showed the numbers of granulocytes were more in normal and hunched appearance mice compared to lethargic mice from both genotype that have numbers of granulocytes 1 in KO and 2 in WT. Also granulocytes appeared more in wild type mouse with lethargic appearance compared to properdin-deficient mouse with lethargic appearance. This was suggested the capacity to mount an inflammatory response in wild type.



**Figure 3.2.14:** Cytopsin preparations of cell suspension from homogenized liver from wild type and properdin-deficient mice after infected with  $1 \times 10^6$  *L. monocytogenes*. Arrows in all panels show granulocytes. Panels A, B, C, and D from infected wild type mice. A and B from mouse with normal severity, C and D from lethargic mouse. Panels E and F from infected properdin-deficient mice, E from mouse with +hunched and F was from lethargic mouse.

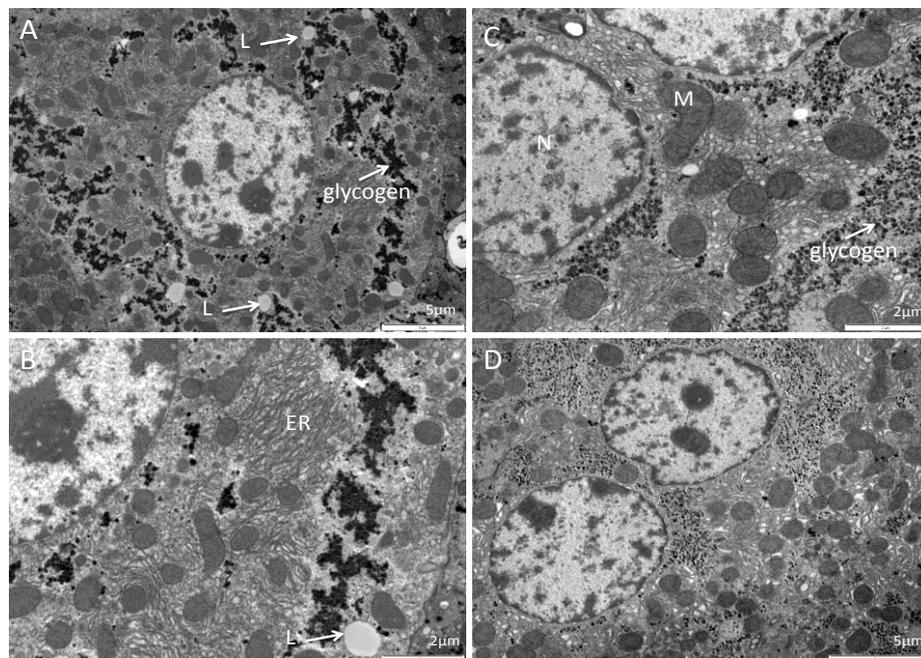
### 3.2.4.4 Transmission electron microscopy (TEM) of liver for localisation of *L. monocytogenes* and presence of granuloma

#### *monocytogenes* and presence of granuloma

The liver sections were prepared after the livers were fixed then processed for transmission electron micrographs as described in material and methods.

Livers from wild type and properdin-deficient mice infected with  $1 \times 10^6$  of *L. monocytogenes*. Representative images for uninfected and infected liver were chosen from a total number of about 264 images. Generally, from the images the liver from both mice appear damaged in infected cells compared to uninfected liver.

The controls are shown in the Figure 3.2.15: in both genotypes hepatocytes appear with one or more nucleus in panel D, many mitochondria, and rough endoplasmic reticulum, and some lipid globules (panels A and B). In addition, there is abundance of glycogen, comparable to those described in relevant textbooks (Ross *et al.*, 1995).



**Figure 3.2.15:** Transmission electron micrographs of control mouse liver from wild type (A and B) and properdin-deficient mice (C and D), representative image shows mitochondrion and endoplasmic reticulum-rich, normal hepatic ultrastructure and indicates normal abundance of glycogen. N= hepatocytes nucleus, M= mitochondria, ER= endoplasmic reticulum L=lipid globules.

When investigating liver sections prepared from infected mice, there were more *L. monocytogenes* in liver from properdin-deficient mouse (Figure 3.2.16) compared to wild type mouse (Figure 3.2.17). Both mice, when culled, were lethargic. In liver from properdin-deficient mouse, *L. monocytogenes* were seen surrounding and inside the granuloma (Figure 3.2.16 A, B, C, D) and within leukocytes (panels A and H (6)) and in hepatocytes. Some *L. monocytogenes* were confined within large phagolysosomes (A and B), and many bacteria were located free in the cytoplasm of hepatocytes (panel H), with some present in vacuoles or in the cytosol.

Leukocytes were present in granuloma. Dividing bacteria in single vacuoles or large vacuoles were observed in tissue specimens from properdin-deficient mouse even in cytoplasm of the leukocytes or hepatocytes as shown in (Figure 3.2.16

Figure 3.2.16 B). *L. monocytogenes* with actin tails, indicative of movement, were observed in leukocytes panel (C and H (1)) and in hepatocytes as in panel (G).

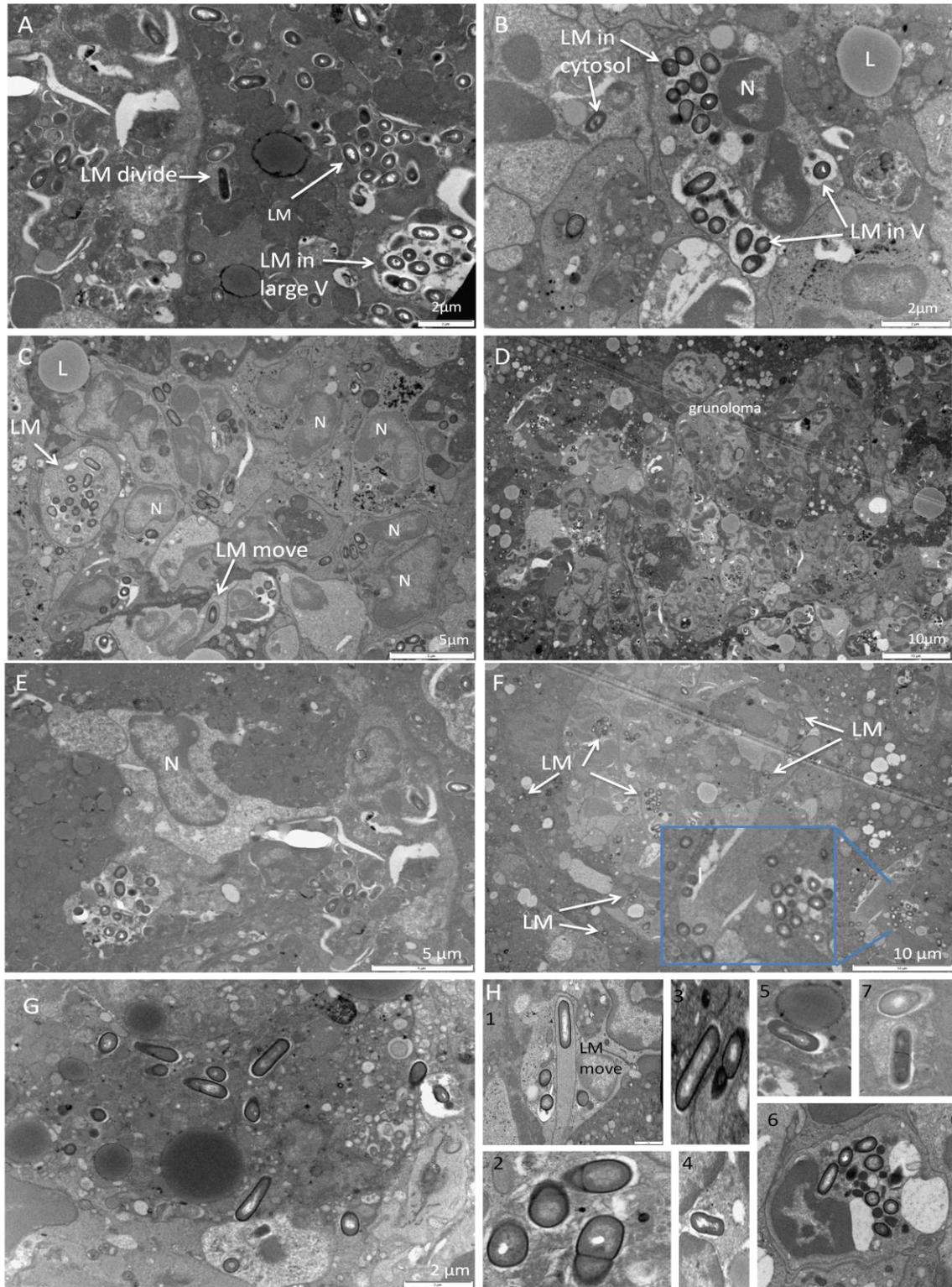
The numbers of granuloma were counted based from plastic section. The numbers of granuloma were higher in liver from properdin-deficient mouse KO1 (lethargic), having 5 granuloma per section and KO2 (++hunched) having 4 granuloma compared to WT1 mouse (lethargic) with 2 granuloma per section and WT2 mouse (+hunched) with one granuloma. There were no granulomas in wild type mouse (normal) and properdin-deficient mouse (+hunched).

Across the analysed livers, (Figure 3.2.17) *L. monocytogenes* in liver from wild type mouse were less compared to properdin-deficient and are observed sparsely in the granulomas. In Figure 3.2.17 (2 in panel H) the *L. monocytogenes* appeared to undergo degradation inside a vacuole in the leukocytes cells or were in the process of being killed in granuloma (1 and 2 in panel C) as seen in (Gaillard *et al.*, 1996), but some *L. monocytogenes* appeared intact and were seen with actin tails (Figure 23.2.16 C, H (1)).

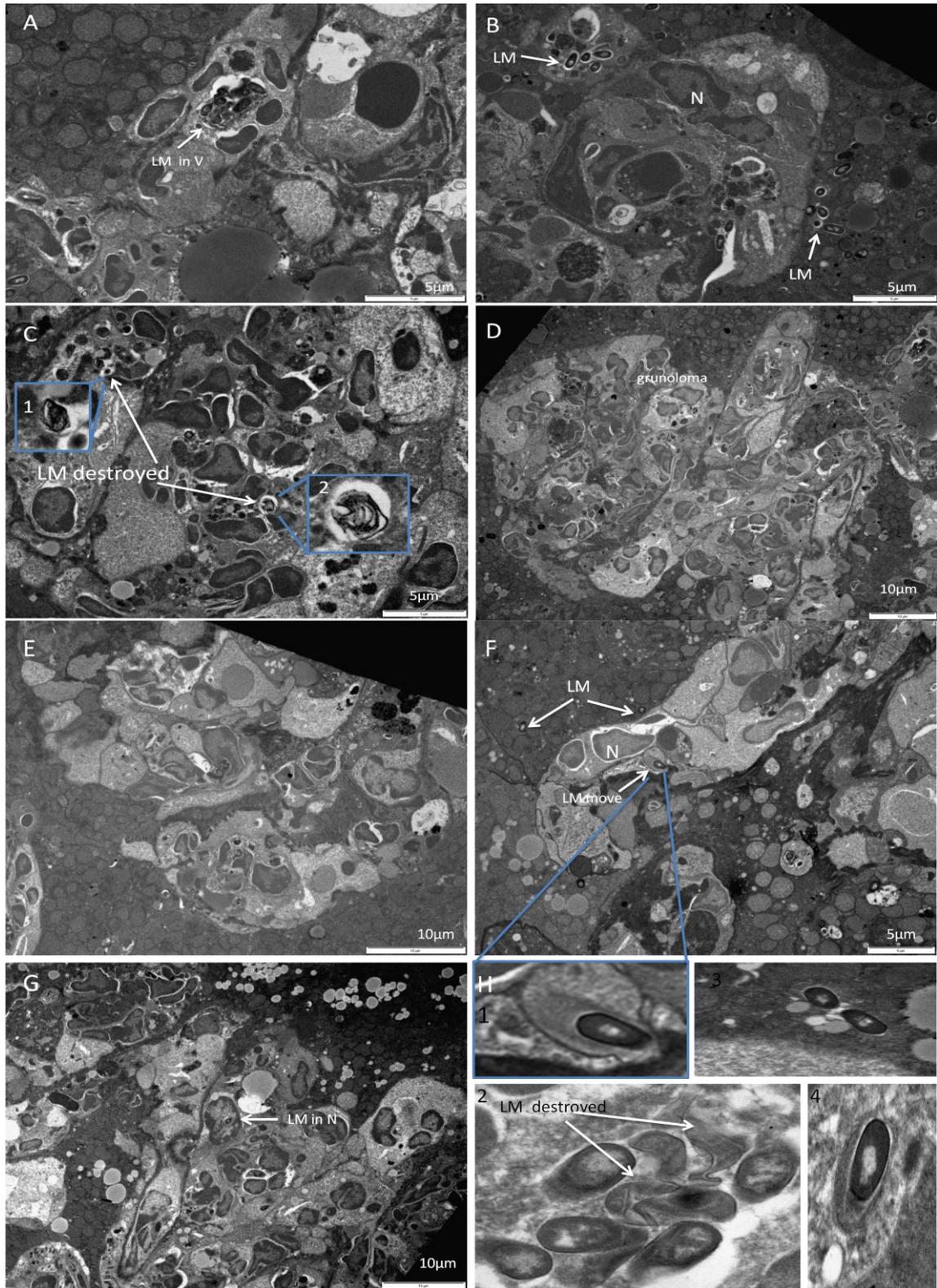
In properdin-deficient and wild type mouse with lower severity (hunched) there were less *L. monocytogenes* and they were located around the granuloma with the occasional destroyed *L. monocytogenes* seen. In wild type mouse with no severity scoring and properdin-deficient mouse with the hunched severity score there were no bacteria alive and just dead or destroyed *L. monocytogenes* were observed panel B (5).

After infection extensive abnormalities were observed in the section: there was an increase in lipid globules in both genotypes compared to uninfected liver.

The replication of bacteria (concluded from the presence of septa, indicative of division) and the movement (concluded from electron dense material surrounding *L. monocytogenes*, typical of polymerised actin) also appeared in both genotypes but more *L. monocytogenes* matched these criteria in properdin-deficient mice compared to wild type and were located inside leukocytes cells. Some *L. monocytogenes* are remaining in a single form (Figure 23.2.16 panel G, H (7)), and some are divided in the cytoplasm of the hepatocyte and leukocytes, panel H (2), and were apparent even in the nucleus (Figure 3.2.17 G).

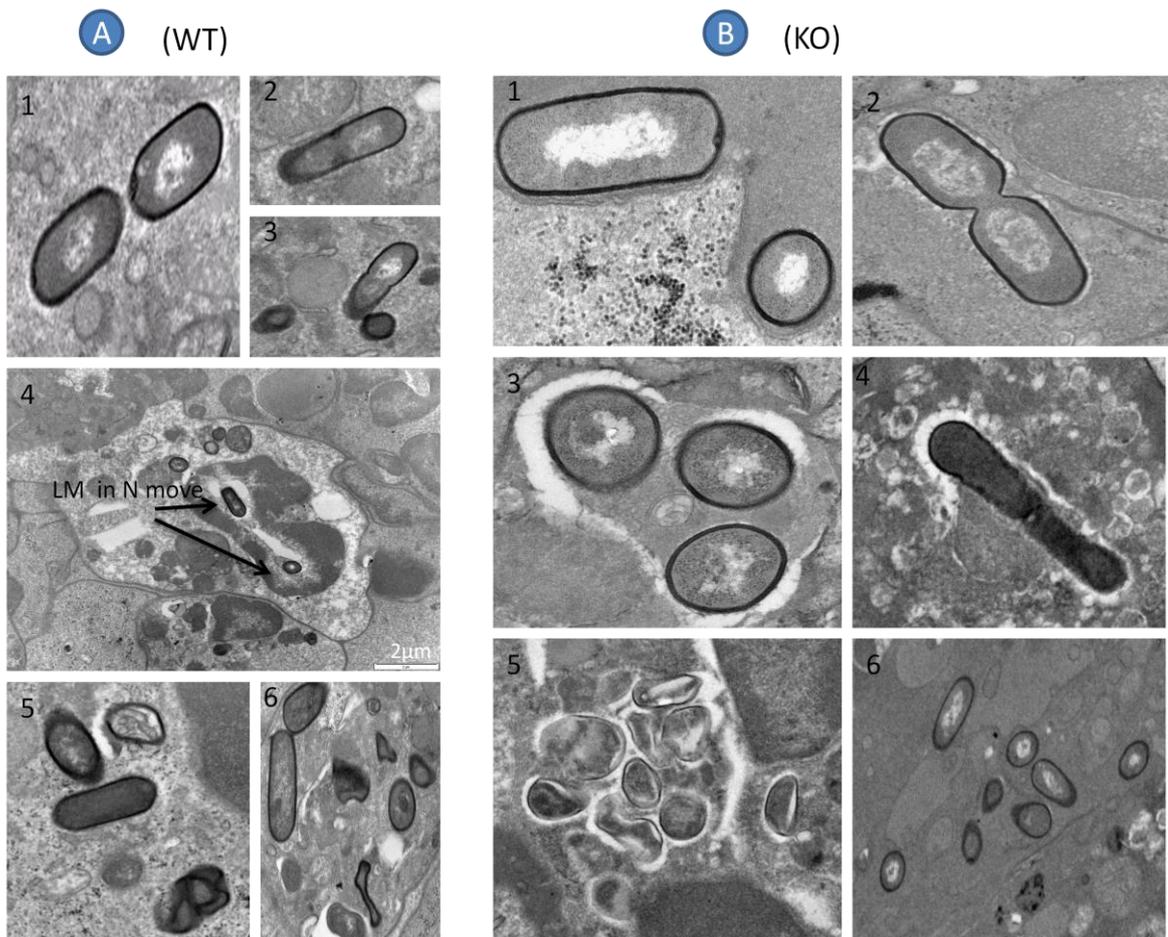


**Figure 3.2.16:** Representative transmission electron micrographs analysis of liver tissues shown localization of *L. monocytogenes* inside the liver. Liver of properdin-deficient mice infected with  $1 \times 10^6$  *L. monocytogenes*, and were removed after 29 hours.



**Figure 3.2.17:** Representative transmission electron micrographs analysis of liver from wild type mouse with lethargic severity after infected with  $1 \times 10^6$  *L. monocytogenes* after 29 hours. 1, 2, 4 in panel J taken from images with magnification of  $5 \mu\text{m}$  and  $2 \mu\text{m}$  size bars are indicated.

As can be seen in the Figure 3.2.18 there were a different form *L. monocytogenes* in the liver mice with hunched severity in both genotypes. The *L. monocytogenes* were seen free within the cytoplasm surrounded by a particularly electron dense layer (dark black colour surrounds the bacteria) as in wild type and properdin-deficient (Figure 3.2.18 1, 2, and 3) to multiply in cytosol after escaped from the cytoplasmic vacuole. In addition some *L. monocytogenes* were seen destroyed in both genotype in panel B (5) and panel A (5 and 6) also *L. monocytogenes* were seen in nucleus in wild type panel A (4) and lots of bacteria were free in cytosol of hepatocytes cells.

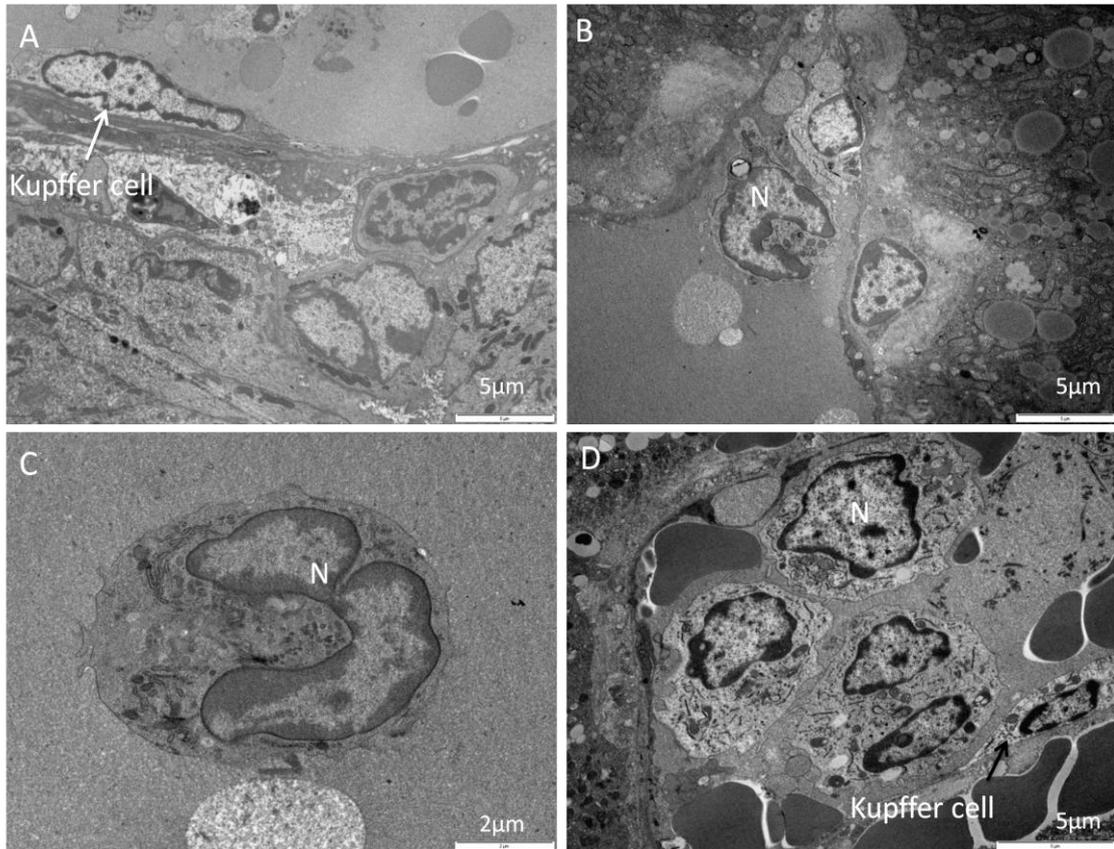


**Figure 3.2.18:** Selective image for *L. monocytogenes* in different form from livers wild type and properdin-deficient mice after infection mice 29 hours. The mice appeared hunch.

The fixation was identical between all liver samples. Overall, there were less *L. monocytogenes* in liver from lethargic wild type mouse compared to the liver from lethargic properdin-deficient mouse, and this result matched with previous results for CFU, which showed higher numbers of *L. monocytogenes* in lethargic properdin-deficient mouse compared to lethargic wild type mouse.

### **3.2.4.4.1 Evidence of infiltrating leukocytes**

After infection, leukocytes were seen around blood vessels in the liver. These pictures (Figure 3.2.19) were taken from mice after infection with *L. monocytogenes* after 28-29 hours and for these mice their severities were, in properdin-deficient mouse was ++hunched and +starey and wild type was +hunched and + starey. Panel A from properdin-deficient mouse and B, C and D panels were from wild type. It appeared from the images that less white cells were seen in lethargic mice maybe the reason that almost white cells appeared in graunloma and it is hard to organise the type of cells, but in mice with less severity like +hunched show clear image of cells derived from the blood stream (Panel D) and there appeared to be more cells compared to properdin-deficient mice.



**Figure 3.2.19:** Representative transmission electron micrographs of leukocytes from infected mice with *L. monocytogenes* cells around blood vessels in the liver.

### 3.2.4.5 Analysis of mRNA expression of IFN- $\gamma$ and IL-17A in spleen of infected mice

Expression of IFN- $\gamma$  and IL-17A was determined from spleens of infected wild type and properdin-deficient mice with  $1 \times 10^6$  (after 28-29 hours) using qPCR.

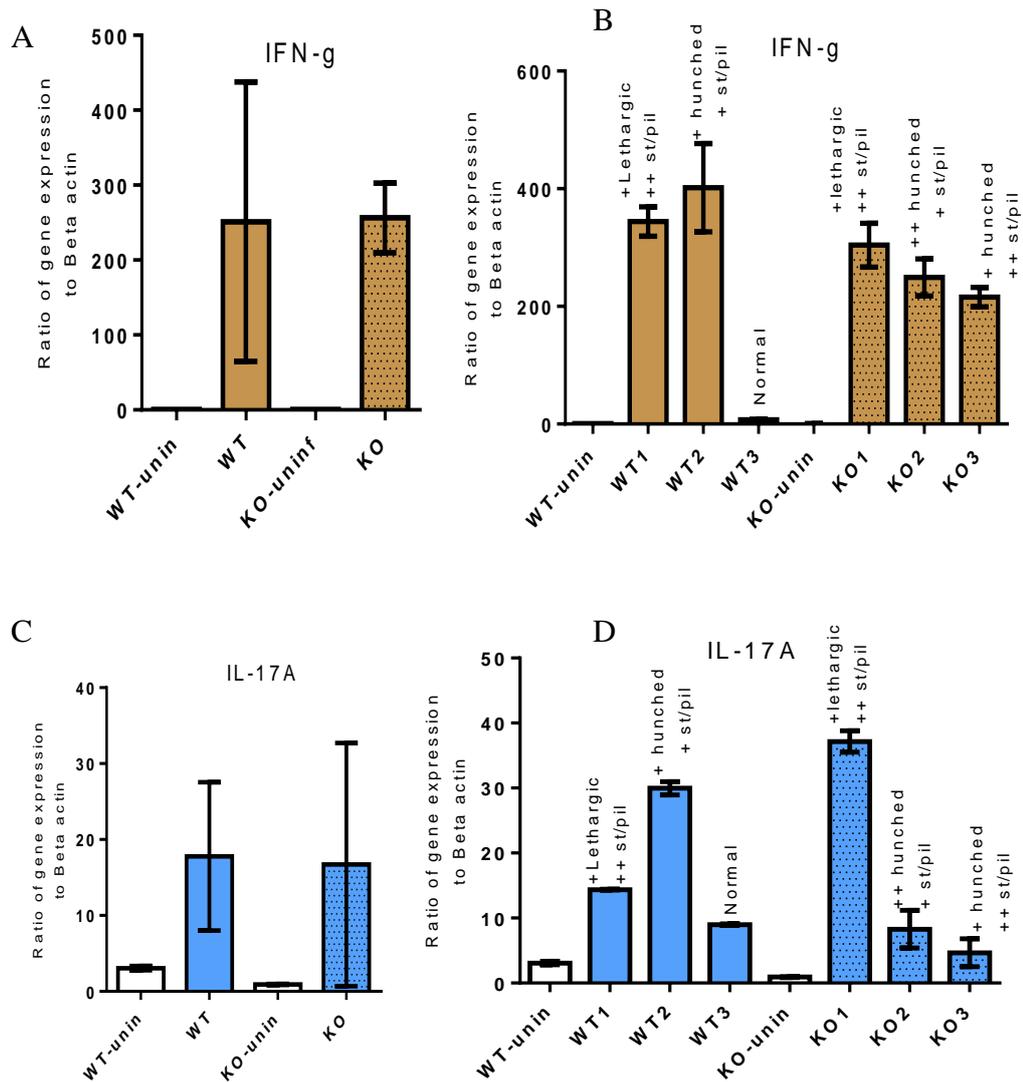
As can be seen in Figure 3.2.20 expression of all genes in uninfected spleen was up-regulated after infection and it follows the viable counts per liver which were higher in all lethargic or starey mice in both wild type and properdin-deficient mice and less in normal or hunched mice.

No mRNA expression of IFN- $\gamma$  production was detected in uninfected spleen from wild type and properdin-deficient mice. Infect mice with *L. monocytogenes* resulted in a remarkable increase expression in IFN- $\gamma$  production. There were no difference in expression of IFN- $\gamma$  in both genotypes (Panel A) but in individual mice, lower expression levels of IFN- $\gamma$  were detected in wild type mouse which appeared normal (Panel B).

In panel C there was no difference in expression of IL-17A in both genotypes but in individual mice expression of mRNA IL-17A in properdin-deficient lethargic mouse was produced higher levels compared to wild type lethargic mouse and shows less expression in other properdin-deficient mice with less severity. On the other hand, there was a higher expression of IL-17A in wild type mouse with +hunched and +starey compared to hunched wild type mice. This was the same observation, the relative reduction of IL-17A mRNA expression in properdin-deficient mice were also found when analysing mice infected with a dose  $5 \times 10^5$  *L. monocytogenes* and survival at 49 hours (Figure 3.2.10).

Expression of IL-17A in uninfected spleen was higher in wild type compared to properdin-deficient (Figure 3.2.19 C). The difference in control of wild type and properdin-deficient mice in IL-17A expression was conform before as shown in the Figure 3.2.10 in section 3.2.3.3.

In collaboration in the lab, T cell populations were analysed by FACS of re-stimulated *ex vivo* splenocyte populations of wild type and properdin-deficient mice infected with heat-killed *L. monocytogenes*. CD4<sup>+</sup> cells were gated and analysed for expression of intracellular IL-17A and showed increased the expression in wild type compared to properdin-deficient (Appendix, Figure IV B).



**Figure 3.2.20:** mRNA expression of IFN- $\gamma$  and IL-17A by qPCR (B and D, shows expression of individual mice) from mice spleen from wild type and properdin-deficient mice after (29 hours) after i.v infection with  $1 \times 10^6$  CFU of *L. monocytogenes*. Panels A and C were combined the expression wild type mice together and properdin-deficient mice together.  $\Delta\Delta CT$  value were used, the mRNA expression corrected for  $\beta$ -actin and compared with uninfected spleen. The qPCR results represent one analysis set up in duplicate and expressed separately. The data are presented as means with standard deviation (SD). St/pil= Starey/Piloerect.

### 3.2.4.6 Expression of TLR2, CD11b and C5aR receptors

Spleen from wild type and properdin-deficient mice after infected with *L. monocytogenes* for 28-29 hours were used to express mRNA level of different receptor

related to infection with *L. monocytogenes*, and compared to the uninfected spleen of normal mice. To see any difference between wild type and properdin-deficient mice after infected with *L. monocytogenes*.

Figure 3.2.21 panel (A) expression level of mRNA TLR2 was increased upon infection compared to spleens from uninfected mice (panel A). In individual mice the expression of TLR2 appeared to relate to the severity of sepsis: those that were hunched or lethargic showed more TLR2 expression compared to those that appeared normal in both genotypes.

(Panel C and D) mRNA CD11b expression increased the expression in properdin-deficient and wild type after infection and appeared higher in properdin-deficient compared to wild type (panel C) however, in individual mice expression of mRNA CD11b, which also follows the viable count and the severity, shows less expression of CD11b in properdin-deficient mouse was lethargic compared to wild type lethargic mouse but there is variability (panel D) and were less expression in mouse with normal severity in wild type compared to other severity. In properdin-deficient mice the expression of CD11b was higher in hunched mouse compared to others.

The high-affinity C5a receptor (C5aR) is a G protein-coupled receptor for the anaphylatoxin C5a that mediates pro-inflammatory reactions. C5a is an important mediator of the inflammatory response and acts by binding to C5aR.

CR3 is an integrin, composed of two parts, CD11b and CD18 and is expressed on the surface of phagocytes (Mobberley-Schuman & Weiss, 2005).

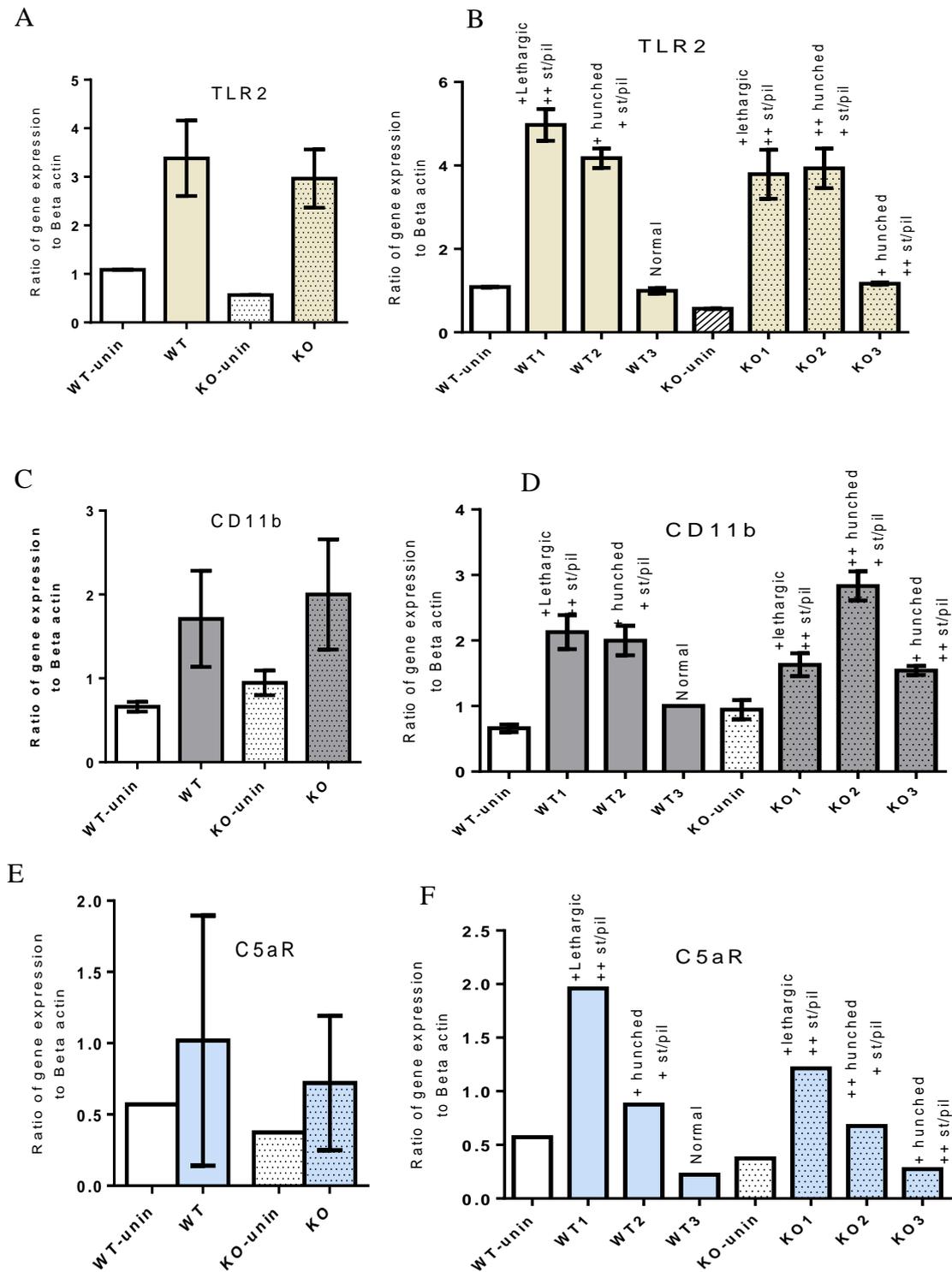
mRNA expression of C5aR was studied and was increased after infected compared to uninfected mice (Figure 3.2.21). In individual mice expression of C5aR from infected mice in wild type mouse with mild disease and properdin-deficient mouse with

Uninfected severity were the same mRNA expression in spleen of uninfected mice in both genotypes. However, the expression of C5aR was higher in both lethargic mice from wild type and properdin deficient, but was higher expression in wild type compared to properdin-deficient. There was less expression of C5aR in other mice with other severity in both genotypes.

There was no obvious relation of C5aR expression with severity of disease. Another receptor, which has not been measured in this study, C5L2, may be important in understanding this.

There were no significant difference in expressions of all genes compared wild type and properdin-deficient after infection.

There is variation within the experimental groups but overall, wild type and properdin-deficient increased their expression levels of the relevant receptors significantly in response to the infection.



**Figure 3.2.21:** mRNA expression of, TLR2 , CD11b and C5aR from spleen from wild type and properdin-deficient mice after 29 hours i.v infection with  $1 \times 10^6$  *L. monocytogenes* B, D and F shows expression of individual mice, A, C and E were compared the expression wild type mice together and properdin-deficient mice together.  $\Delta\Delta CT$  value were used, the mRNA expression corrected for  $\beta$ -actin. QPCR results are from analysis set up in duplicate and a separate expression. The data are presented as means with standard deviation (SD). St/pil= starey/piloerect (piloerection hairs).

### 3.2.4.7 mRNA expression of Fcγ receptors using qPCR

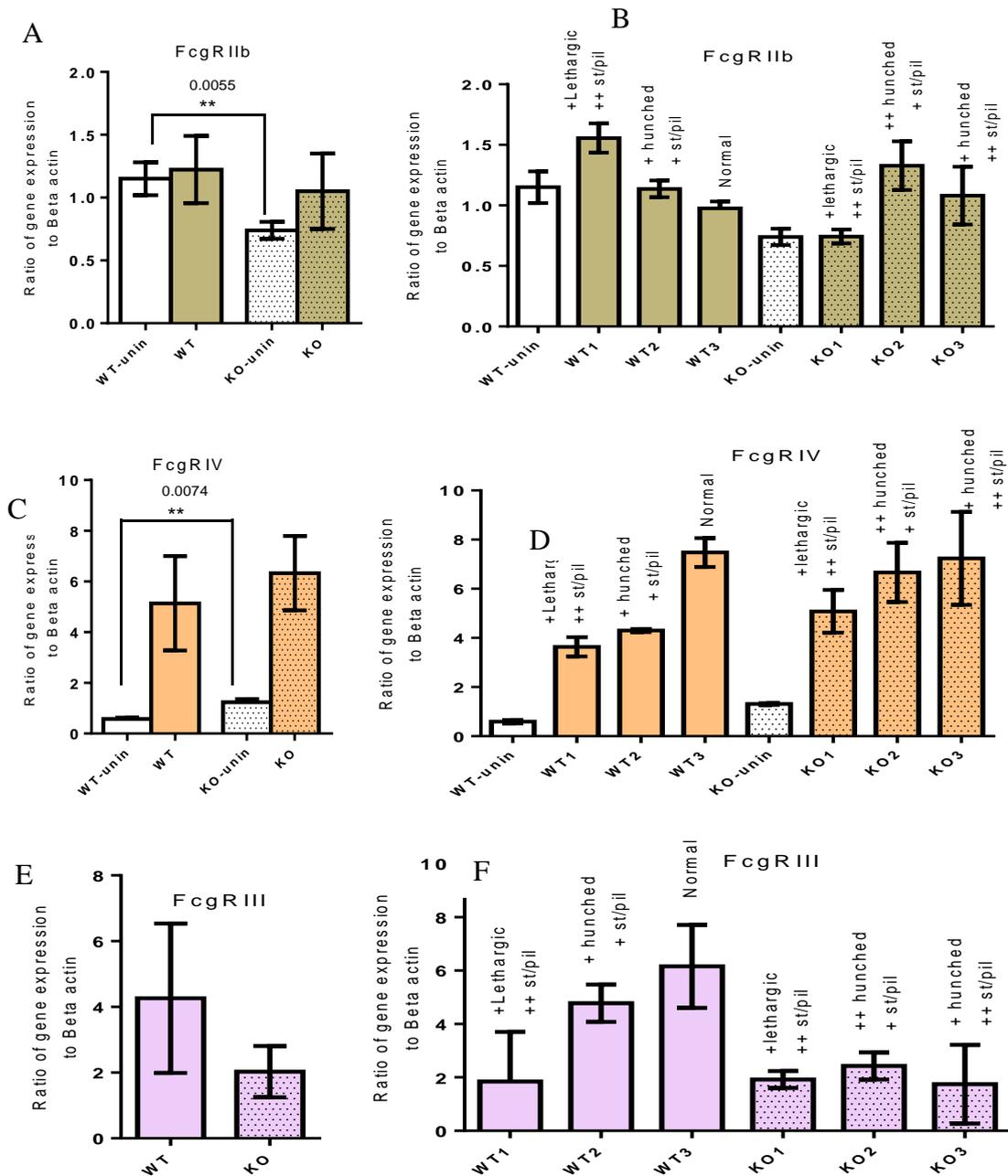
The background for this experiment was another project, which used splenic macrophages and discovered lower FcγRIIb expression in properdin-deficient mice compared to wild type mice. Expression of Fcγ receptors was used to see whether any differences between wild type and properdin-deficient in expression of FcγR after 29 hours infection i.v with  $1 \times 10^6$  CFU of *L. monocytogenes*.

Whole spleen from wild type and properdin-deficient mice from 3 mice each genotype was used to analyse the relative mRNA expression levels of FcγRIIb, FcγRIII, and FcγRIV by qPCR.

As can be seen in the Figure 3.2.22 (A) uninfected spleen FcγRIIb were expressed in both genotype but were significantly different in wild type compared to properdin-deficient ( $P= 0.0055$ ). Expression of FcγRIIb in spleen from uninfected wild type mice show no difference compared to infected (A). But in individuals wild type mouse after infection with lethargic severity shows higher compared to other severity (hunched, normal mice or uninfected). Expression of FcγRIIb in spleen from properdin-deficient mice were higher after infected compared to uninfected (A) however, in individuals, mouse with lethargic severity from properdin-deficient shows no different compared to uninfected but were higher in two other mice with less severity (panel B). In uninfected spleen from properdin-deficient mice (A) leads to decreased FcγRIIb expression which inverted relationship of FcγRIIb/FcγRIV in wild type comparing to properdin-deficient. This was different as observed previously *in vitro* part FcγRIIb and FcγRIV in macrophage. This showed the expression of FcγRIIb was the same between both genotypes but expression of FcγRIV was higher in macrophage from properdin-deficient mice compared to macrophage from wild type.

In panels (C and D) the Fc $\gamma$ RIV expression was higher in infected spleen compared to uninfected spleen. In uninfected spleen the expression of Fc $\gamma$ RIV was higher and significantly different in spleen from properdin-deficient mice compared to spleen from wild type mice ( $P= 0.0074$ ). After infection the expression was increased and was higher in spleen from properdin-deficient mice compared to spleen from wild type mice (C), in individual mice the expression was higher in mice with less severity compared to lethargic mice in both genotypes (D). Fc $\gamma$ RIII and Fc $\gamma$ RIV receptors are activation receptor which required for different effector functions including phagocytosis and the release of inflammatory mediators. Expression of Fc $\gamma$ RIII in panels E and F in uninfected wild type and properdin-deficient were unable to detect it, this because maybe it was less Fc $\gamma$ RIII products. In infected spleen, the expression of Fc $\gamma$ RIII was higher in all wild type mice compared to properdin-deficient mice, and higher in mice with the severity starey/piloerect (piloerection hairs) and normal, and less in lethargic mouse. Expression of Fc $\gamma$ RIV (D) also was the same extend of Fc $\gamma$ RIII. The expressions of Fc $\gamma$ RIII and Fc $\gamma$ RIV were higher compared to Fc $\gamma$ RIIb.

After combining 3 individual mice in each genotype in expression of Fc $\gamma$ RIIb (A) and Fc $\gamma$ RIV (C) in the Figure 3.2.22, this showed higher levels of expression of Fc $\gamma$ RIV compared to expression of Fc $\gamma$ RIIb. Expression of Fc $\gamma$ RIV seemed higher in spleen from properdin-deficient mice compared to spleen wild type mice. This is congruent with the observation made during the *in vitro* studies of this thesis which showed that macrophage from properdin-deficient have higher expression of Fc $\gamma$ RIV compared to macrophage from wild type mice, and have less expression of Fc $\gamma$ RIIb compared to macrophage from wild type mice (Section 3.1.14). Moreover, expression of Fc $\gamma$ RIII after combined show higher in spleen from wild type compared to spleen from properdin-deficient mice.

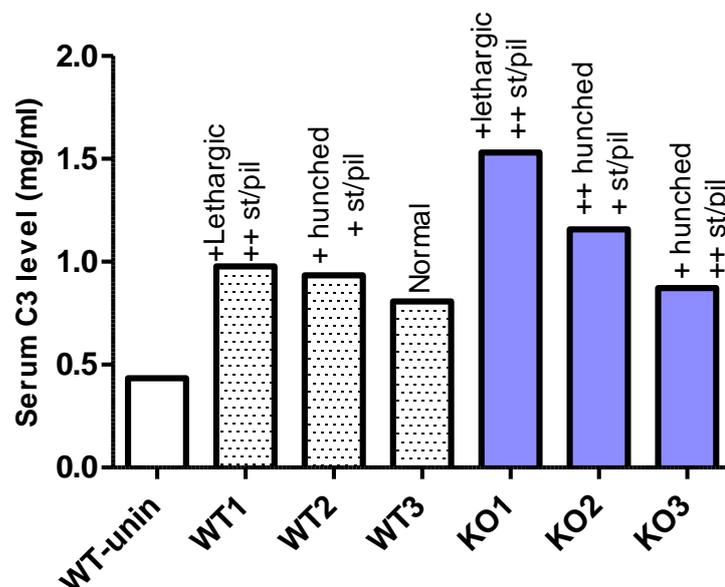


**Figure 3.2.22:** mRNA expression of FcγRIIb (A and B), FcγRIV (C and D) and FcγRIII (E and F) from mice spleen from wild type and properdin-deficient from uninfected and infected after 29 hours i.v with  $1 \times 10^6$  CFU of *L. monocytogenes*. (Panels A, C and E) combined the expression wild type mice together and properdin-deficient mice together. (Panels B, D and F) expression of FcγRIIb, FcγRIV and FcγRIII from individuals mice wild type and properdin-deficient.  $\Delta\Delta CT$  value were used, the mRNA expression corrected for  $\beta$ -actin and compared. The QPCR results are from analysis set up in duplicate and separate expression. The data are presented as means with standard deviation (SD). St/pil= starey/piloerect (piloerection hairs).

### 3.2.4.8 Measurement of complement C3 from wild type and properdin-deficient mice

Serum levels of complement C3 were measured by ELISA in individual mouse sera from wild type and properdin-deficient mice after i.v. infection with  $1 \times 10^6$  *L. monocytogenes*. Sera from 3 mice each genotype were used.

As can be seen in the Figure 3.2.23, as expected, the level of C3 was higher in sera from infected mice compared to uninfected mice (Pepys *et al.*, 1980; Giclas *et al.*, 1985). These mice were culled after 28-29 hours when mice started to be lethargic after infection, serum from infected mice show a higher level of C3 from properdin-deficient mice see table 3.2.6 for severity of mice (KO1 mouse was +lethargic and KO2 mouse was ++hunched) compared to wild type (WT1 +lethargic and WT2 +hunched). There were no difference in the levels of C3 in all wild type although there were a difference in the severity and were the same level of properdin-deficient mouse (KO3) that has the +hunched severity.



**Figure 3.2.23:** Concentration of C3 (mg/ml) level in sera from wild type and properdin-deficient mice after 28-29 hours i.v infection with  $1 \times 10^6$  *L. monocytogenes* measured by ELISA. Each bar represents triplicate determinations. St/pil= starey/piloerect (piloerection hairs).

### 3.2.4.9 Anti-listeria IgG and IgM

Anti-listeria IgG and IgM were detected in mice serum after infection of mice with *L. monocytogenes*. The IgM are natural antibodies play an important role in the first innate immune defence against infection of bacteria. The levels of immunoglobulins IgM and IgG present in serum of uninfected and infected wild type and properdin-deficient mice was measured by ELISA. Complement fixation assay and ELISA using intact bacteria as the antigen.

As seen in Figure 3.2.24 the anti-listeria IgM levels were determined from mice sera (n=2) for uninfected. For infected mice sera, the sera were taken from individual mice by cardiac puncture. WT1 and KO1 sera from experiment that mice were killed at the same time after 28-29 hours with  $1 \times 10^6$  CFU of *L. monocytogenes*, the severity of WT1 mouse was normal and KO1 mouse was ++hunched and +starey. The level of anti-listeria IgM in these mice appeared higher in KO compared to WT. WT2 and KO2 were from experiment that infected with  $5 \times 10^5$  CFU of *L. monocytogenes*, WT2 mouse was culled after 45 hours and was lethargic, KO2 mouse was culled after 95 hours and was ++hunched + st/pil, the level of anti-listeria IgM appeared higher in WT compared to KO.

Levels of anti-listeria IgG as in the Figure 3.2.25 were determined from mice sera after infected with *L. monocytogenes*.

In panel A, mice sera from the experiment were mice infected with  $5 \times 10^5$  CFU of *L. monocytogenes*. WT1 pooled sera from mouse severity was lethargic culled after 48 hours and mouse was lethargic after 77 hours. KO1 mouse severity was lethargic after 45 hours and hunched mouse culled after 95 hours. WT2 hunched culled after 69 hours, KO2 lethargic after 45 hours.

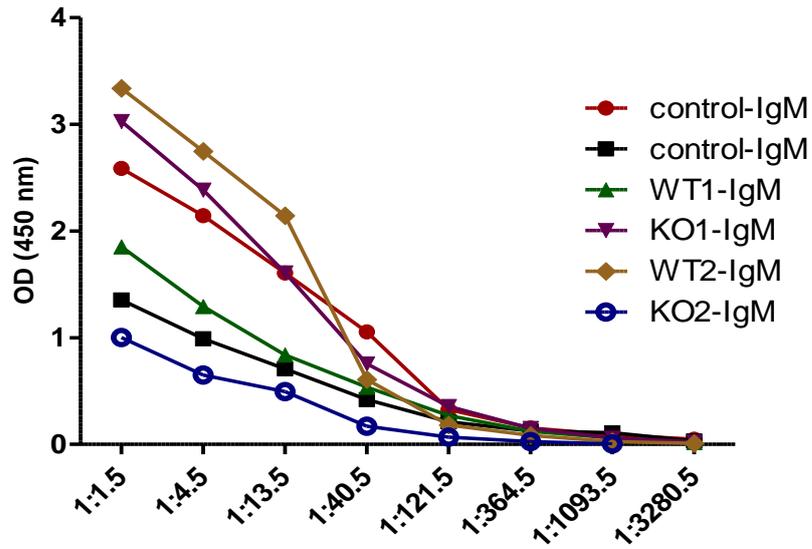
In panel B, WT1 and WT2 mice were lethargic and culled after 45 hours from experiment were mice infected with  $5 \times 10^5$  CFU of *L. monocytogenes*. WT3 was hunched culled after 69 hours, KO1 mouse was lethargic after 45 hours, KO2 mouse was lethargic 28hr, KO3 lethargic 45 hours from same experiment with  $5 \times 10^5$ . KO4 was hunched severity from experiment that mice killed same time after 28-29 hours with  $1 \times 10^6$  *L. monocytogenes*. In B started with the dilution 1:40.5.

From panel A KO2 appeared higher level of anti-listeria IgG compared to the rest the shows no difference between infected and uninfected in both genotypes.

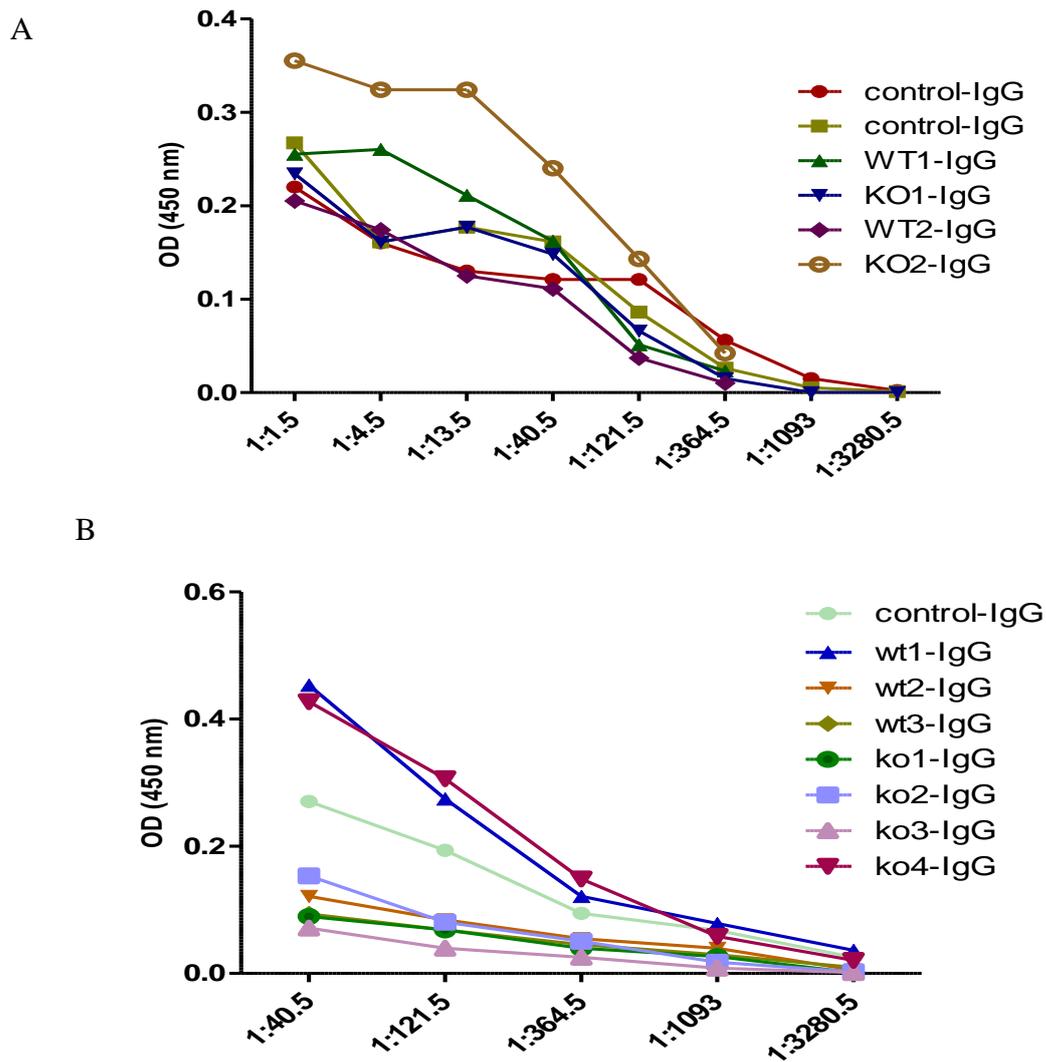
From panel B WT1 and KO4 appeared with higher levels of IgG compared to the others.

Overall there were similar titres of anti-listeria IgM and IgG in sera from wild type compared to properdin-deficient mice. No significant difference in the total of IgG and IgM levels were observed between wild type and properdin-deficient mice. ODs were similar obtained from both genotypes, with a similar ODs decreased following serial dilutions. These data suggest that level of IgM and IgG were same in both genotypes.

It is debatable whether the infection has induced specific antibodies, as the titres are not very different from the uninfected controls. This could indicate that the response in this model is predominately macrophage ( $T_H1$ ) driven.



**Figure 3.2.24:** levels of anti-listeria IgM were detected in sera from wild type and properdin-deficient mice after infection with *L. monocytogenes*. WT1 and KO1 sera from experiment when mice were killed at 28-39 hours after infected with  $1 \times 10^6$  CFU of *L. monocytogenes*. Serum from uninfected mice was used as control.



**Figure 3.2.25:** levels of anti-*Listeria* IgG in sera from wild type and properdin-deficient mice after infection with *L. monocytogenes*. Panel (A) sera pooled WT1 lethargic mice after 48hr and 77 hrs, KO1 lethargic mouse after 45hr and +hunched mouse after 95 hrs. Serum not pooled WT2 ++ hunched mouse 69 hrs, KO2 lethargic mouse 45 hrs. All were from experiment infected with  $5 \times 10^5$  *L. monocytogenes*. In panel (B) WT1 and WT2 mice lethargic after 45 hrs, WT3 hunched mouse after 69 hrs, KO1 mouse lethargic after 45 hrs, KO2 mouse lethargic 28 hrs, KO3 mouse lethargic 45 hrs, KO4 hunched mouse after 28 hrs. All were infected with  $5 \times 10^5$  *L. monocytogenes* except KO4 infected with  $1 \times 10^6$  *L. monocytogenes*. Panel B started with the dilution 1:40.5. Serum from uninfected mice was used as control.

## *Chapter Four Discussion*

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### 4. Discussion

Using mice genetically engineered to be deficient of selected complement genes, studies have revealed that intact complement activation is an integral part of the innate immune defence against bacterial infections such as *Streptococcus pneumoniae* (Brown *et al.*, 2002) and *Pseudomonas aeruginosa* infection as single pathogens (Mueller-Ortiz *et al.*, 2004) or mixtures such as polymicrobial peritonitis (Stover *et al.*, 2008; Celik *et al.*, 2001). Deficiencies of C3 lead to an increased risk of bacterial infections especially with pneumococcus, *N. meningitidis* or haemophilus, all encapsulated microorganisms (Figuroa & Densen, 1991).

Properdin, a normal serum protein, is a major contributor to the activity level of complement activation. Based on previous *in vivo* studies, intact complement limits *L. monocytogenes* infectious burden in tissue and ensures granuloma formation (Petit, 1980; Gervais *et al.*, 1989).

The aim of this study was to characterise *in vitro* and *in vivo* the role of properdin in murine infection with *L. monocytogenes*. Properdin-deficient mice were compared to wild type mice in order to deduce the specific role played by properdin during *L. monocytogenes* infection, and to determine whether properdin deficiency leads to a cellular phenotype that is different from the wild type. *L. monocytogenes* infection is the classical model used to address this (Czuprynski & Haak-Frendscho, 1997). Experimental infection of mice with *L. monocytogenes* has been used as a model to study cell-mediated immune responses and inflammation (Garifulin & Boyartchuk, 2005).

Phagocytes from C57BL/6J mice (the genetic background of the mice in this study) can kill *L. monocytogenes* more effectively than phagocytes from *L. monocytogenes*-susceptible strains of mice, such as A/J mice. This is because the inflammatory

responses upon infection are directed into the Th1 pathway in C57BL/6 mice (Su *et al.*, 2001).

There is past evidence for a role of complement in response to murine *L.monocytogenes* infection: the alternative pathway of complement is activated on the surface of *L. monocytogenes* (Drevets & Campbell, 1991; Croize *et al.*, 1993) and this was confirmed in the present study. C3b mediated opsonophagocytosis of *L. monocytogenes*, and CR3-mediated phagocytosis of *L. monocytogenes* is required for macrophages to kill this bacterium (Rosen *et al.*, 1989; Conlan & North, 1992). C5a mediated attraction of leukocytes to tissue resident *L. monocytogenes* is needed in granuloma formation (Petit, 1980; Gervais *et al.*, 1989).

This study has produced evidence of the important role played by properdin in control of *L. monocytogenes*. *In vitro*, in response to infection, the absence of properdin led to a significant decrease in the presence of intracellular *L. monocytogenes* in dendritic cells from properdin-deficient mice compared to dendritic cells from wild type mice, and early after 30 minutes in macrophage. Also in dendritic cells with absence of properdin there was less IFN- $\gamma$ , and nitric oxide released, and decreased surface expression of CD40. Moreover, macrophages from properdin-deficient mice showed less TNF- $\alpha$  production in normoxia and hypoxia.

The consistent *in vitro* finding demonstrating an impaired antilisterial immune response in macrophages and dendritic cells justified the study of the specific role played by properdin during *L. monocytogenes* infection of mice *in vivo*. *In vivo* studies demonstrated that properdin-deficient mice infected with *L. monocytogenes* showed increased mortality after 29 hours of infection when compared to wild type controls.

This work shows for the first time that complement properdin determines significantly the Th1 response in the survival of listeriosis.

#### **4.1 Role of properdin in control of *L. monocytogenes* by primary dendritic cells and macrophages**

Dendritic cells and macrophages are important in the early infection of *L. monocytogenes* (Rayamajhi *et al.*, 2010; Westcott *et al.*, 2010; Kapadia *et al.*, 2011). In the current study, bone marrow derived dendritic cells and macrophages from properdin-deficient mice in parallel with wild type mice were used to understand the role of properdin in responding to *L. monocytogenes*. Following optimisation of protocols, both dendritic cells and macrophages were successfully differentiated from bone marrows prepared from wild type and properdin-deficient mice. In the presence of hematopoietic growth factors GM-CSF (Inaba *et al.*, 1992) and IL-4 (Sallusto & Lanzavecchia, 1994), two main populations were distinguishable after seven days, adherent cells which corresponded phenotypically to macrophages and non-adherent cells which corresponded phenotypically to dendritic cells, in line with the previous literature (Inaba *et al.*, 1992).

Westcott *et al.* 2007 investigated the intracellular growth of *L. monocytogenes* using murine bone marrow-derived dendritic cells and macrophages. Their study reported that dendritic cells derived from bone marrow have the ability to restrict large numbers of *L. monocytogenes* in vacuoles, and restrict them from escaping to the cytosol, in order to limit their intracellular growth to a membrane-bound compartment. The same observation was noted in this study in wild type dendritic cells compared to macrophage, and electron microscopy (TEM) confirmed that there was more *L. monocytogenes* in vacuoles of dendritic cells compared to macrophages. In addition, this study discovered a role of properdin in determining the cellular phenotype of dendritic cells during infection with *L. monocytogenes*.

#### 4.1.1 Role of properdin in intracellular viability of *L. monocytogenes*

Dendritic cells prepared from wild type and properdin-deficient mice were infected with *L. monocytogenes* and numbers of intracellular *L. monocytogenes* were measured by the colony-forming unit assay. Previous laboratory-based work (PhD thesis Salehen N., 2011) showed that bone marrow derived dendritic cells from properdin-deficient mice were less able to control the numbers of *Mycobacterium tuberculosis* (Mtb) at MOI of 5 to 10 after 24 hours' incubation compared to dendritic cells from wild type bone marrow. It appeared therefore that properdin deficiency led to a cellular phenotype, which was unexpected at the time as properdin is a soluble, plasma protein, with no identified receptor. Another laboratory-based study conducted showed that macrophages from properdin-deficient mice released less nitric oxide compared to those of wild type mice (Ivanovska *et al.*, 2008). Nitric oxide is a significant mediator of innate immune response.

Westcott in 2010 showed that the outcome of *L. monocytogenes* infection of dendritic cells is different from that of macrophages, as dendritic cells have the capacity to limit the extent of growth of *L. monocytogenes* compared to macrophages, both early and late in infection. Kapadia *et al.* (2011) have studied the contribution of antigen presenting cells, CD8 $\alpha$ <sup>+</sup> dendritic cells and plasmacytoid dendritic cells, in inducing immune responses against *L. monocytogenes*. They found that the cells susceptible to infection are CD8 $\alpha$ <sup>+</sup> dendritic cells compared to plasmacytoid dendritic cells that are not infected. In both, dendritic cells and macrophages, *L. monocytogenes* were seen to be present in the cytosol, dividing in cytosol and in vacuoles, and spreading from cell to cell. Dendritic cells, however, appeared to provide a less desirable environment for growth of *L. monocytogenes* in comparison to macrophage. After 30 minutes there were more *L. monocytogenes* in macrophage from wild type than in dendritic cells from wild type.

Higher intracellular loads of *L. monocytogenes* were seen in macrophages and dendritic cells from wild type compared to properdin-deficient mice. This was detected one-hour after infection and as early as 30 minutes after killing of extracellular bacteria with gentamicin to quantify the intracellular bacterial load efficiently. This observation could be due to altered uptake or altered intracellular killing. Importantly, the numbers of mouse cells and of *L. monocytogenes* were kept constant for all incubations. The viability of mouse bone marrow-derived macrophages and dendritic cells after infection were somewhat decreased but the same for the genotypes.

Stress increases susceptibility to infectious diseases in both human and animals (Peterson *et al.*, 1991). In the current study the role of properdin was investigated for acute *in vitro* infection with *L. monocytogenes* in the presence of norepinephrine. Both norepinephrine and the sympathetic nervous system have been linked with the "fight or flight" response (Kohm & Sanders, 2001). Stress hormones influence cells via adrenergic receptors. These have been described also for macrophages and dendritic cells. Catecholamines bind to cells and initiate IP<sub>3</sub> mediated signalling involving diacylglycerol and leading to calcium release, and activation of further pathways. *In vitro* studies have shown that noradrenaline can influence the growth of bacteria and expression of virulence factors (Freestone *et al.*, 1999; Lyte *et al.*, 1997). Mice that cannot produce noradrenaline have impaired host resistance against *L. monocytogenes* (Alaniz *et al.*, 1999).

The viable intracellular load of *L. monocytogenes* was higher in dendritic cells and macrophage from wild type mice after 30 minutes' infection with *L. monocytogenes* (see Figure 3.1.45 in results *in vitro*). However, the presence of noradrenaline in dendritic cells after 30 minutes of infection with *L. monocytogenes* led to a comparable amount of viable *L. monocytogenes* intracellularly when comparing bone marrow

derived dendritic cells from wild type and properdin deficient mice. Next, when measured after a further hour of incubation, norepinephrine further increases the viability of *L. monocytogenes* viability in dendritic cells from properdin-deficient mice and this has once again further increased at two hours after infection (see Figure 3.1.46). Interestingly, the same is observed in macrophages from properdin-deficient mice which therefore show an impairment in containing *L. monocytogenes* numbers as the dendritic cells from properdin deficient mice. This may point to a direct effect of noradrenaline on *L. monocytogenes*. Dendritic cells and macrophage from wild type mice have approximately the same numbers of *L. monocytogenes* (by CFU) with or without norepinephrine treatment; no major change was observed. However, the number of *L. monocytogenes* were increased in dendritic cells and macrophage from properdin-deficient mice. There was greater virulence of *L. monocytogenes* due to noradrenaline especially in the context of properdin deficiency. Unfortunately, this characterisation could not be pursued further.

Thus, norepinephrine may lead to greater virulence (invasiveness) of *L. monocytogenes* which becomes apparent in the absence of properdin that is in a state of immune deficiency. It is also possible that noradrenaline mediated signalling intersects with complement mediated activation, but this was not investigated in this study.

It has been shown that after incubating *L. monocytogenes in vitro* with norepinephrine the bacterial growth did not change (Rice *et al.*, 2001). In the current study *L. monocytogenes* numbers were not affected by norepinephrine alone after one and two hours.

At 24 hours after infection of dendritic cells and macrophages, it appears that properdin-deficient mice provide a cellular phenotype for dendritic cells but not macrophages because the presence or absence of properdin did not seem to impact on the clearance of

*L. monocytogenes*, as similar bacterial loads were found in macrophage in both genotypes conditions of normoxia and hypoxia, measured as intracellular prokaryotic and viability of eukaryotic cells.

The fact that no difference in intracellular viable counts after 24 hours was observed in both wild type and properdin-deficient mice under normoxia and hypoxia was unexpected, because hypoxia is thought to influence the clearance of pathogens. Macrophages exposed to hypoxia reduced susceptibility to infection with *Leishmania amazonensis*, an intracellular parasite, that causes cutaneous and cutaneous metastatic lesions, using a different source of macrophages (human cell line U937, murine cell line J774, and murine peritoneal macrophages) (Degrossoli *et al.*, 2011; Colhone *et al.*, 2004). Moreover, hypoxia induced dendritic cells to reduce survival of *L. amazonensis* (Bosseto *et al.*, 2010). It seems that the ability to kill bacteria depends on a range of considerations, for example cell type, differentiation and maturation status of the cell species in addition to the type of bacteria.

Furthermore, after cells were treated with IFN- $\gamma$ , dendritic cells and macrophage from wild type and properdin-deficient mice had more intracellular *L. monocytogenes* compared to unstimulated cells. However, there were significantly more *L. monocytogenes* in dendritic cells and macrophages from wild type mice compared to those dendritic cells and macrophages from wild type mice as observed in CFU without treatment with IFN- $\gamma$ . It was concluded that properdin had a role in responding to *L. monocytogenes* in the presence of exogenous IFN- $\gamma$ . Treatment with IFN- $\gamma$  stimulates the cells to phagocytose more *L. monocytogenes*. Similarly, the growth of *Chlamydia trachomatis* was reduced after activating the peritoneal mouse macrophages *in vitro* with recombinant murine IFN- $\gamma$  (Zhong & de la Maza, 1988).

#### 4.1.2 The role of properdin in association and sub-cellular location of *L.*

##### *monocytogenes* using primary murine dendritic cells and macrophage

Both transmission and scanning (TEM and SEM respectively) electron microscopy were used in this study. While TEM allows evaluation of sections, SEM visualises the surface of the cells. The overall aim was to obtain characteristics of both macrophages and dendritic cells and capture in a snapshot the location of *L. monocytogenes* within the cell types. This was also applied to infections in the presence of norepinephrine (NE). Evaluation of the TEM micrographs supported the conclusions drawn from determining the CFU from permeabilised cells. After counting the number of *L. monocytogenes* in dendritic cells and macrophages from both genotypes, properdin-deficient mice had significantly less intracellular *L. monocytogenes* in both vacuolar and cytosolic localisation after 4 hours than the corresponding cell type from wild type mice. However, after 24 hours there were less *L. monocytogenes* in macrophages compared to dendritic cells.

The numbers of *L. monocytogenes* in macrophages from wild type after 24 hours were higher in cytosol than in vacuolar compartment. This observation could be due to escape from the lysosomes. In dendritic cells from wild type mice, *L. monocytogenes* were present more in vacuoles which may be because dendritic cells are prevent LLO from functioning effectively, leading to the observation by CFU and electron microscopy that there are more *L. monocytogenes* in dendritic cells from wild type. Greater bacterial retention in vacuoles means less lysosomal escape and less transcellular infection.

Unfortunately in this project it was unable to analyse what these differences in location signify for gene expression of *L. monocytogenes*. This is due to the fact that any changes which may relate to altered bacterial virulence are likely to be lost due to

adaptation to nutrient source and conditions in the culture which are necessary to obtain a suitable number of bacteria for RNA analysis.

TEM analyses show more *L. monocytogenes* in wild type phagosomes and more dividing *L. monocytogenes* in dendritic cells together with more cytosolic escape and less *L. monocytogenes* extracellularly in macrophages and dendritic cells from properdin-deficient mice. It has been reported that macrophages are able to recognise *L. monocytogenes* and can distinguish between *L. monocytogenes* within a vacuole or in the cytosol, the so-called surveillance pathway (O'Riordan *et al.*, 2002). In cytosol *L. monocytogenes* induces IFN- $\beta$  expression but not in vacuolar *L. monocytogenes* (O'Riordan *et al.*, 2002). Macrophage from properdin-deficient mice show less IFN- $\beta$  expression compared to macrophage from wild type mice.

In addition, dendritic cells from both genotypes were infected in the presence of norepinephrine with fewer numbers of *L. monocytogenes* (MOI 0.2). Dendritic cells from wild type mice show higher numbers of bacteria in both vacuolar and cytosol compared to dendritic cells from wild type without norepinephrine, but the numbers were higher in vacuolar compared to cytosolic compartment. In dendritic cells from properdin-deficient mice in the presence of norepinephrine they show less numbers of *L. monocytogenes* compared to those without norepinephrine. However, the numbers of bacteria in the presence of norepinephrine were less in dendritic cells from properdin-deficient mice compared to dendritic cells from the wild type. Without norepinephrine, numbers of bacteria were higher in dendritic cells from properdin-deficient mice compared to wild type. The amount of bacteria still in vacuoles may be due in part to a failure of large numbers of bacteria to escape to the cytosol.

In summary, norepinephrine treatment moderately influenced the virulence of *L. monocytogenes* in terms of its escape and division of *L. monocytogenes* in both dendritic cells from wild type and properdin-deficient mice.

### **4.1.3 The role of properdin in the antilisterial inflammatory response of primary dendritic cells and macrophages**

There was no difference in the viable counts of *L. monocytogenes* in macrophages after 24 hours, consistent with there being no difference in the levels of IFN- $\gamma$  and nitric oxide in macrophage between both genotypes. IFN- $\gamma$  was significantly decreased in properdin-deficient compared to wild type dendritic cells but not significantly in macrophages. The role of properdin in dendritic cells and macrophage mediated killing of *L. monocytogenes* was investigated, by measuring the concentration of nitric oxide in the culture supernatants of macrophages and dendritic cells derived from bone marrow infected with *L. monocytogenes*. The concentration of nitric oxide was lower in dendritic cells from properdin deficient mice.

The nitric oxide production by peritoneal macrophages at 24 hours after the injection of LPS or zymosan was decreased in properdin-deficient mice in both models (Ivanovska *et al.*, 2008).

The nitric oxide increased significantly approximately 3 to 4 fold above the level of uninfected control. ELISA was used to measure the release of IFN- $\gamma$  after 24 hours from dendritic cells and macrophages from wild type and properdin-deficient mice supernatants in response to live *L. monocytogenes*. Moreover, the extent of TNF- $\alpha$  release was studied using live and heat-killed *L. monocytogenes* under normoxic and hypoxic conditions.

Macrophages and dendritic cells isolated from properdin-deficient mice behave like M2 polarised cells, while cells isolated from wild type mice maintain the M1 phenotype, which is characteristic of C57Bl/6 background.

*L. monocytogenes* induce a predominant Th1 response. It has reported that microorganisms that induce Th1 responses can direct macrophages and change in the proportion of M1 to M2, possibly trying to better evade the immune response. M1 cells promote Th1 responses and M2 cells support Th2-associated effector functions (Lolmede *et al.*, 2009).

The fact that IFN- $\gamma$  is important for host resistance to *L. monocytogenes* and other intracellular pathogens is well known. It drives the differentiation of resting macrophage into an activated macrophage (M1) that controls the growth of intracellular pathogens (Rayamajhi *et al.*, 2010). However, properdin has no role in the 24 hours increase of TNF- $\alpha$  after infection with live and heat-killed *L. monocytogenes* of dendritic cells when exposed to normoxia or hypoxia. By contrast, the lack of properdin seems to compromise TNF- $\alpha$  secretion at this time point in macrophages. Macrophages secrete TNF- $\alpha$ , which stimulates other cells such as natural killer cells to produce IFN- $\gamma$  and to activate macrophages in turn to increase their bactericidal activity and kill bacteria through the production of nitric oxide (Beckerman *et al.*, 1993; Edelson & Unanue, 2000). Contrasting the role of properdin in macrophage derived TNF- $\alpha$  production, Bohlson *et al.* (2001) showed that complement C3-deficient macrophages have a similar level cytokine of TNF- $\alpha$  in response to *M. avium* infection compared to wild type control.

The level of TNF- $\alpha$  was decreased in hypoxia compared to normoxia in both dendritic cells and macrophages of both genotypes, and was decreased with heat-killed *L. monocytogenes* in both genotypes from both types of cells. Heat-killed *L.*

*monocytogenes* induce low levels of TNF- $\alpha$  production from unprimed macrophages (Mitsuyama *et al.*, 1990). The reduction of TNF- $\alpha$  secretion in hypoxia has been observed in macrophage (Yun *et al.*, 1997; Lahat *et al.*, 2008). Culture supernatants from mouse peritoneal macrophage cell lines (RAW 264.7) exposed to hypoxia (O<sub>2</sub> 2%) for 24 hours showed reduction of TNF- $\alpha$  compared to normoxia (Yun *et al.*, 1997). Similarly, RAW cells in hypoxia (O<sub>2</sub> < 0.3%) reduced the secretion of LPS-induced TNF- $\alpha$  compared to normoxia (Lahat *et al.*, 2008)

As evidenced in previous studies, live *L. monocytogenes* induce protective immunity but not heat-killed *L. monocytogenes* (Lauvau *et al.*, 2001; von Koenig *et al.*, 1982). TNF- $\alpha$  production was less (by 2-3 fold) after infecting dendritic cells and macrophages with heat-killed *L. monocytogenes* when compared to infection with live bacteria. It has been observed in a study by Feng *et al.*, (2005) that after infecting dendritic cells with live *L. monocytogenes* TNF- $\alpha$  production was higher than after infecting with heat-killed bacteria.

Supernatants from bone marrow derived dendritic cells and macrophage from wild type and properdin-deficient mice were analysed for TNF- $\alpha$  bioactivity using L929 after 5 hours' infection with *L. monocytogenes* in two independent experiments, TNF- $\alpha$  bioactivity was higher in dendritic cells from properdin-deficient mice in normoxia and hypoxia. However, in macrophages the TNF- $\alpha$  bioactivity was higher in properdin-deficient in hypoxia compared to macrophages from wild type mice, TNF- $\alpha$  bioactivity was higher in macrophages from wild type mice compared to those from properdin-deficient mice in normoxia. These results do not readily mirror the viable counts obtained from *in vitro* infection. It is important to note that since this bioassay is not specific for TNF- $\alpha$ , it may detect any cytotoxic activity present in the tested

supernatants, such as LLO. However, the haemolytic assay to demonstrate this activity was negative. Another possibility is that the production of cytokines is determined not only by the infectious dose but by the cellular bacterial burden as well as the extent of vacuolar or cytoplasmic sublocalisation.

#### **4.1.4 The role of properdin in maturation of dendritic cells after infection with *L. monocytogenes***

Dendritic cells direct T cell responses by up-regulating the surface molecules necessary for antigen presentation and efficient T cell interaction. A previous study (Salehen & Stover, 2008) showed that dendritic cells from properdin-deficient mice up-regulated CD40 after LPS stimulation to a lesser extent than dendritic cells from wild type mice, while expression of MHCII and of accessory molecules CD80 and CD86 was comparable in mature dendritic cells of both genotypes.

Bone marrow derived dendritic cells after infection with live *L. monocytogenes* show slightly more CD40 and CD80 on the surface compared to those cells infected with heat-killed *L. monocytogenes* (Brzoza *et al.*, 2004).

Flow cytometry was used to study the expression of CD40, CD80, CD86 and MHCII in dendritic cells from both genotypes after infection. CD11c is used as a marker for murine dendritic cells. Dendritic cells showed up-regulation of cell surface markers CD40, CD80, CD86 and MHCII after infection with *L. monocytogenes*. There was an increased surface expression of CD40 in dendritic cells from wild type compared to dendritic cells from their properdin-deficient equivalents. Because CD40 is needed for binding between antigen presenting cells (dendritic cell) and T cells, a reduction of

CD40 could imply less efficient antigen presentation. *In vivo*, this may translate to prolonged disease or greater severity.

#### **4.1.5 The role of properdin in mRNA expression of candidate genes in dendritic cells and macrophages infected with *L. monocytogenes***

The expression of properdin, C3, CD11b and TLR2 mRNA in dendritic cells and macrophages from wild type and properdin-deficient mice infected with *L. monocytogenes* were investigated.

Expression of properdin mRNA by qPCR was decreased after infection with *L. monocytogenes* in both dendritic cells and macrophage from wild type mice. Decreasing the expression of mRNA properdin after infection with *L. monocytogenes* compared to uninfected was an unexpected finding. This was also observed in RAW cells after stimulation with LPS compared to un-stimulated as shown in another project conducted in the lab. Macrophage after infection expressed less properdin compared with uninfected macrophage and compared to uninfected and infected dendritic cells with *L. monocytogenes*, but expression levels of properdin in uninfected was comparable between dendritic cells and macrophage (see Figure 3.1.30 (A) and Figure 3.1.32) (the ratio of properdin expression to  $\beta$ -actin). In hypoxia, macrophages expressed even less properdin mRNA compared to normoxia. By contrast, the expression level of mRNA C3 by qPCR in macrophage from wild type mice remains unchanged after infection in normoxia and hypoxia. Although it appears there is less expression of C3 in macrophage from properdin-deficient mice in normoxia after being infected with *L. monocytogenes* compared to macrophage from wild type mice (as shown also by RT-PCR). However, in hypoxia the level of C3 was significantly increased in macrophage from properdin-deficient mice compared to the results for normoxia which suggest

properdin plays this role. The stimulation of C3 production was shown in hypoxia (Wenger *et al.*, 1995).

Studies on the protein level would be needed to increase our understanding. For now, it is worth noting that in an acute phase, properdin and C3 mRNA do not increase.

TLR2 is important in the immune recognition of *L. Monocytogenes* and in the initiation of a protective immune response. TLR2-deficient mice are more susceptible to systemic infection by *L. monocytogenes* than their wild type counterparts (Torres *et al.*, 2004). TLRs are expressed on many cells types including innate immune cells, B cells in the adaptive immune system and also on T cells (Komai-Koma *et al.*, 2004; Gelman *et al.*, 2004). Expression of TLR2 by qPCR was significantly higher in normoxia after 24 hours compared to hypoxia. A higher level of mRNA TLR2 was expressed in uninfected macrophage in hypoxia than normoxia but it is unclear why this is higher in macrophage from properdin deficient mice than from wild type ones. Another study shows that TLR2 is needed in survival for the *in vivo* model (Liu *et al.*, 2002; Kuhlicke *et al.*, 2007).

Expression of the TLR2 receptor has been reported to be important for the optimal induction of IFN- $\gamma$  during *L. monocytogenes* infection. In the absence of TLR2, IFN- $\gamma$  levels were significantly lower after 24 hours post infection (Torres *et al.*, 2004).

mRNA expression of IFN- $\beta$  was higher in macrophage from wild type mice after infection for 5 hours with *L. monocytogenes* compared to macrophage from properdin-deficient mice. Electron microscopy (TEM) determined that numbers of *L. monocytogenes* were higher in cytosolic in macrophage from wild type mice and less in vacuolar after 4 and 24 hours infection compared to macrophage from properdin-deficient mice. In macrophages the innate host surveillance mechanism can distinguish between the presence of *L. monocytogenes* in the cytosol and in a vacuole. This lead to

specifically up-regulate expression of IFN- $\beta$ , which is induced by the presence of *L. monocytogenes* in the cytosol because p38 MAP kinase plays an important role in responding to cytosolic bacterial products and is required for induction of gene expression (O'Riordan *et al.*, 2002).

Expression of C3 and CD11b mRNA by RT-PCR appeared higher in dendritic cells from wild type mice in normoxia when compared with dendritic cells from properdin-deficient mice. However, it appeared to be the same in hypoxia after 24 hours of infection, although in hypoxia, mRNA expression of C3 and CD11b appeared higher than in normoxia. This same observation was noted in macrophage when using qPCR in expression of C3 which was higher in wild type mice compared to properdin-deficient mice. An unexpected finding in uninfected cells was that the expression of C3 was higher in dendritic cells from properdin-deficient mice compared to wild type mice but after infection the expression was similarly decreased.

In macrophage the expression of properdin in hypoxia was less than in normoxia, and expression of C3 was produced a different and was higher in hypoxia compared to normoxia. Both of these findings show for the first time that properdin is a hypoxia sensitive gene.

In general Gram-positive bacteria are resistant to lysis by complement because the thick peptidoglycan layer in their cell wall that prevents insertion of the membrane-attack complex (Marques *et al.*, 1992). However, complement deposition on gram-positive bacteria leads to deposition of C3 fragments that act as opsonins. Furthermore, C5a is an effective chemotaxin that attracts leukocytes for phagocytosis. Less C3 deposition causes inflammation by microbes, although some intracellular microbes like *M. tuberculosis*, can use C3 fragments deposited on their surface to enter the cells.

This study was unable to identify which cell population which *L. monocytogenes* infects so quickly or whether *L. monocytogenes* preferentially infects leukocytes as this could not be determined using light microscopy. Thus it was carried out using human blood incubated with *L. monocytogenes* in order to contribute serum only to the control of *L. monocytogenes* as mouse serum did not kill or contribute of whole blood to this control. In addition, *L. monocytogenes* was labelled with FITC to answer these questions but unfortunately there was a technical problem with this experiment as there was no methodology to answer these questions and the bacteria increased both with and without shaking.

In summary, the investigation using dendritic cells and macrophage derived from properdin-deficient mice and wild type mice has shown that properdin plays a role in responding to *L. monocytogenes* infection. However, this role was observed in dendritic cells more than macrophage. This may be due to a difference between of macrophage and dendritic cells which have been reported at the location of the *L. monocytogenes*, showing that dendritic cells restrict larger percentages of *L. monocytogenes* in vacuoles compared to the macrophages, and there is more *L. monocytogenes* in cytosol in macrophage in comparison to dendritic cells, also dendritic cells as antigen presenting cells and macrophage showed to be more in phagocytosis. The data showed that there are significantly more intracellular *L. monocytogenes* in dendritic cells from wild type mice compared to properdin-deficient mice, and more IFN- $\gamma$  and nitric oxide release, creating an increase in the surface expression of CD40, and TNF- $\alpha$  activity was also increased in properdin-deficient mice compared to wild type mice in both normoxia and hypoxia.

This is the first time that a cellular phenotype of properdin deficiency has been shown. In the absence of properdin the intracellular *L. monocytogenes* load is less than in dendritic cells and macrophages, but this is accompanied by an impaired immune response and a decreased chance of survival of the whole organism.

In macrophage, *L. monocytogenes* were located more in cytosol than in vacuoles, whilst dendritic cells are more restrictive for *L. monocytogenes* in vacuoles than in cytosol. This finding reflects the results of the study by Stier *et al.* (2005), who suggest that the reason for differences between dendritic cells and macrophage in their location of *L. monocytogenes* is that LLO function, in macrophage vacuoles was more effective than in dendritic cells vacuoles (Westcott *et al.*, 2007).

The difference between mature and immature dendritic cells have been examined and it has been shown that phenotypically mature dendritic cells restricted bacteria to vacuoles and prevented growth while phenotypically immature supported bacterial escape to the cytosol and subsequent growth.

The characterisation of adaptation of *L. monocytogenes* has been examined in this study, characterise changes of gene expression after infection *L. monocytogenes* was studied to see whether *L. monocytogenes* it changes its gene expression before and after infection by extraction of mRNA of *L. monocytogenes* but this experiment failed and instead it was decided to look for transcriptional changes in *L. monocytogenes* towards the host's response because *L. monocytogenes* needs to grow in large culture overnight which will have an effect in gene expression.

## **4.2 Role of properdin in survival and immune response in *vivo* after infection with *L. monocytogenes***

The disease model analysed in *vivo* was *L. monocytogenes* sepsis. *L. monocytogenes* activate the alternative pathway of complement in mouse serum and are opsonophagocytosed (Drevets & Campbell, 1991; Drevets *et al.*, 1993).

This is the first study on survival and humoral response in properdin-deficient mice compared to their controls, because previous studies on the role of complement in response to murine *L. monocytogenes* infection did not investigate the role of complement in survival, but instead analysed activation in serum and tissue infiltration.

A principal aim of this work was to investigate whether there are differences between wild type and properdin-deficient mice after infection with *L. monocytogenes*. Therefore, wild type and properdin-deficient mice were infected with  $5 \times 10^5$  and  $1 \times 10^6$  of previously passaged *L. monocytogenes*.

### **4.2.1 Impairment of properdin-deficient mice in surviving listeriosis**

The present study showed that properdin-deficient mice are significantly impaired in surviving listeriosis. In previous in *vivo* work properdin-deficient mice have been shown to be significantly impaired in their survival in a model of polymicrobial septic peritonitis induced by sublethal caecal ligation and puncture (CLP), and in non-septic shock models (LPS) compared to their wild type controls (Stover *et al.*, 2008; Ivanovska *et al.*, 2008).

In this study, after infection with  $1 \times 10^6$  or  $5 \times 10^5$  of *L. monocytogenes*, properdin-deficient mice developed to a lethargic state and had to be culled early at approximately 29 hours, which was significantly earlier than wild type mice which developed this

severity at 49 hours. Occasionally wild type mice had to be culled early for welfare reasons at 29 hours but less than properdin-deficient mice (2 wild type and 6 properdin-deficient mice).

At a dose of  $5 \times 10^5$  of *L. monocytogenes*, properdin-deficient mice showed signs of severe illness during early infection and developed to a lethargic state and were culled early in comparison to their wild type counterparts but the remainder recovered and finally survived. However, this dose did not cause many deaths of mice from properdin-deficient and showed more survival mice over the time (100 hours) for both genotypes.

At a dose of  $1 \times 10^6$  of *L. monocytogenes*, properdin-deficient mice were culled more and they were impaired in comparison to wild type mice. For survival of mice after infection with relatively  $1 \times 10^6$  infectious doses, properdin-deficient mice were more lethargic than wild type mice mostly on day two, infected mice were monitored for 4 days. Therefore, an important finding in this study was that properdin-deficient mice succumbed early at 28-29 hours, this was clearly observed after infecting the mice with a dose of  $1 \times 10^6$  *L. monocytogenes*. Properdin-deficient mice were more susceptible to *L. monocytogenes*, but their mortality was enhanced only with the dose of  $1 \times 10^6$  viable passaged *L. monocytogenes*.

The qualitative difference in response to doses of  $5 \times 10^5$  and  $1 \times 10^6$  is documented in the literature when studying resistance and susceptibility of mice, this was determined by using a single dose of bacteria that was capable of distinguishing between susceptible and resistance of different strains of mice C57BL/6 and BALB/c on the basis of death or survival (Cheers *et al.*, 1979). A group of five C57BL/6 mice were infected intravenously with graded doses of *L. monocytogenes*  $1.5 \times 10^4$ ,  $7.5 \times 10^4$ ,  $3.8 \times 10^5$  and  $1.9 \times 10^6$  and their deaths were recorded daily over a period of two weeks.

Mice were resistant to doses of  $1.5 \times 10^4$  and  $7.5 \times 10^4$ . After one week a slight effect was seen with  $3.8 \times 10^5$  but  $1.9 \times 10^6$  led to the death of mice on day two. In this study the same observation was shown in wild type mice with a dose of  $1 \times 10^6$  of *L. monocytogenes*.

Another, interesting finding was that female properdin-deficient mice showed greater sensitivity when infected with  $5 \times 10^5$  *L. monocytogenes* in comparison with all groups of mice (male and female wild type mice and male properdin-deficient mice). Female mice, whether from wild type or properdin-deficient showed higher susceptibility to infection with *L. monocytogenes* compared to males. This study confirms the previous finding that female mice from wild type show higher susceptibility to infection with *L. monocytogenes* compared to their male counterparts (Pasche *et al.*, 2005). This is aggravated in the absence of properdin. Given that properdin deficiency leads to a greater polarisation towards M2 phenotype, the worse outcome for female properdin-deficient mice could have been expected.

Mice were immunised with a dose of  $5 \times 10^5$  of viable *L. monocytogenes* because heat-killed *L. monocytogenes* are less able to induce a detectable and protective immune response (Mitsuyama *et al.*, 1990; Miller *et al.*, 2003). But properdin-deficient and wild type mice did not differ in their outcome from this experiment. Both groups survived and showed no signs of disease severity.

### *Role of properdin in inflammatory responses to L. monocytogenes*

Mice deficient in IFN- $\gamma$  and TNF- $\alpha$  are highly susceptible to *L. monocytogenes* infection (Harty & Bevan, 1995; Huang *et al.*, 1993). But some protective effects of IFN- $\gamma$  and TNF- $\alpha$  might be complement dependent against *L. monocytogenes* infection (Drevets *et al.*, 1996; Nakane *et al.*, 1993). A study by Nakane *et al.* (1993) showed the

increased expression of CR3 after stimulation of macrophages with IFN- $\gamma$  and TNF- $\alpha$  enhanced the phagocytic activity to kill *L. monocytogenes*. Production of IFN- $\gamma$  is essential in inducing protective immunity in response to *L. monocytogenes* infection (Yang *et al.*, 1997; Rolph & Kaufmann, 2001).

The IFN- $\gamma$  was measured *in vivo* because the production of IFN- $\gamma$  is increased *in vivo* by antigen-presenting cells in early host defence in response to infection with intercellular pathogens (Suzue *et al.*, 2003). In this study the serum level of IFN- $\gamma$  and activity of TNF- $\alpha$  were higher in properdin-deficient mice in response to *L. monocytogenes* which showed that these mice were more impaired (matching with their appearance of severity), even though the overall viable counts were comparable to those of the wild type. Moreover, the systemic IFN- $\gamma$  response to *L. monocytogenes* was greater after measuring by ELISA from mice sera compared to uninfected mice. It has been shown that IFN- $\gamma$  may have different effects of on the proliferation of *L. monocytogenes* due to the differences in the number of injected bacteria (Langermans *et al.*, 1992). The higher levels of IFN- $\gamma$  may be an attempt by the properdin-deficient mice to counteract M2 type activity in listeriosis (Benoit *et al.*, 2008).

In addition, the serum levels of TNF- $\alpha$  activity using L929 cells in sera was higher in the lethargic mouse from properdin-deficient compared to lethargic mouse than its wild-type counterpart. *In vivo* study demonstrated that TNF- $\alpha$  is important for clearing infections with *L. monocytogenes* and acts with IFN- $\gamma$  to increase macrophage killing of *L. monocytogenes* (Edelson & Unanue, 2000). However it has been reported that TNF- $\alpha$  is quickly cleared from the serum as observed during infection with  $2 \times 10^4$  *L. monocytogenes*. A lower level of TNF- $\alpha$  were detected in serum from BALB/c and IFN- $\alpha/\beta$ R-deficient mice (Sheehan *et al.*, 1989). Consistent with this, low levels of TNF- $\alpha$  were detected in the serum from both genotypes. Moreover, adipose tissues from both

genotypes are able to produce cytokine IFN- $\gamma$  when analysed in *ex vivo* from mice infected with *L. monocytogenes*.

In order to investigate the effect of inflammatory responses in the survival of wild type and properdin-deficient mice, the mRNA expression levels of inflammatory mediators were analysed and compared in both groups after *L. monocytogenes* infection.

The results of the real time PCR analysis of mRNA expression of cytokines in spleen tissues showed that IFN- $\gamma$  and IL-17A expression level was higher in properdin-deficient mice that were culled when they were lethargic at 29 hours after mice were infected with  $5 \times 10^5$  of *L. monocytogenes*. However, levels of expression at 49 hours were higher in wild type mice which displayed more severe lethargic in comparison to wild type mice and properdin-deficient mouse with less severity. So the expression abundance and levels reflect the degree of severity more than they relate to the gene deficiency.

At matched time points after mice has been infected mice with  $1 \times 10^6$  *L. monocytogenes* at 29 hours, levels of IFN- $\gamma$  mRNA expression in mice with lethargic severity were increased in the spleen of the wild type mouse compared to the properdin-deficient mouse, while the levels of IL-17A mRNA increased in the properdin-deficient mouse compared to the wild type mouse with identical severity culled at 29 hours. This was observed after measuring the expression of IL-17A in mice infected with dose  $5 \times 10^5$  of *L. monocytogenes* and surviving at 49 hours.

Interestingly, the expression levels of mRNA IL-17A in the uninfected spleen from the wild type and properdin-deficient mice show significantly impaired basal expression of IL-17A mRNA in properdin-deficient mice and this was higher in wild type (see *in vivo* results Chapter Three Figure 3.2.11 C). This difference on the protein level was observed in a study by Dimitrova *et al.* (2012), which noted determinate of cytokine IL-

IL-17 production by ELISA from supernatants uninfected splenocytes after this had been cultured for 48 hours (WT= 216±22, KO=174±24). ( $p<0.05$ ) KO compared to WT (Dimitrova *et al.*, 2012).

At the matched time point (3 mice each comparing mice with the same severity) the properdin-deficient mouse showed significantly higher levels of mRNA expression for IL-17A at the same time this mouse had a higher bacterial load in the liver. In contrast, the wild type mouse showed a significantly lower mRNA expression level for the inflammatory mediators IL-17A while a slightly lower bacterial load was found in the liver. This was in line with the finding of a significantly higher serum level of IFN- $\gamma$  and activity of TNF- $\alpha$  in the properdin-deficient mice compared to the wild type.

IL-17A is produced *in vivo* in response to *L. monocytogenes* infection, and it is required for both innate and optimal adaptive response against bacterial infection (Xu *et al.*, 2010). Both, IL-17A mRNA in the spleen and IL-17A in the serum increased significantly from day one. IL-17A is involved in the protective immune responses against *L. monocytogenes* infection and the expression of IL-17A from the spleen in the wild type mice compared to the properdin-deficient mice analysed. The production of IL-17A plays an essential role in protection during the early stage of infection of *L. monocytogenes* in the liver as showed the aggravation of the protective response in IL-17A-deficient mice (Hamada *et al.*, 2008).

Therefore, the TNF- $\alpha$  results in addition to those for IFN- $\gamma$  and IL-17A support the argument that the properdin-deficient mice were more impaired, even though their overall viable counts were comparable to those of the wild type mice.

### *Role of properdin in inflammatory response of the liver*

The largest target organ of *L. monocytogenes* in both humans and mice is the liver. *L. monocytogenes* are cleared quickly from the bloodstream after intravenous injection and the majority of the bacteria are found in the liver and spleen within 10 to 20 minutes (Gregory *et al.*, 1996), since these represent the main targets of this pathogen. This is consistent with the concept of activation of the macrophage phagocyte system in these organs.

In the absence of properdin viable counts from organs (after they had been infected with  $5 \times 10^5$  *L. monocytogenes*) are the same as in wild type mice. Histology study shows that wild type mice have well-formed granulomas in their liver, and in the absence of properdin, granuloma formation appeared less in comparison with the higher numbers of granuloma in wild type mice (2 of 4 mice). Although the viable counts obtained from the liver after infection with *L. monocytogenes* shows no difference, the survival study shows that mice from properdin deficient mice were more susceptible to *L. monocytogenes* compared to wild type mice. Infected C57BL/6 mice with *L. monocytogenes* showed well-defined granulomas in the liver after 3 days postinfection (Boyartchuk *et al.*, 2001). Moreover, with dose  $5 \times 10^5$  showed less C5aR in properdin-deficient mice leading to less granuloma formation.

Transmission electron micrographs obtained from infected livers of both genotypes showed fewer kupffer cells in lethargic mice and more in hunched mice in both genotypes, cells were seen around blood vessels in liver. Kupffer cells play a role in the immediate clearance of *L. monocytogenes*, but neutrophils are required to kill the trapped *L. monocytogenes* (Gregory *et al.*, 1996; Gregory *et al.*, 2002). There was evidence of inflammatory cells in homogenates liver which showed more granuocytes

in wild type mice and in hunched or uncompromised mice compared to lethargic mice in both genotypes. In early defences against *L. monocytogenes* neutrophils accumulate in large numbers in both livers and spleens (Conlan, 1999; Cousens & Wing, 2000) and increased numbers of neutrophils may enhance killing of *L. monocytogenes*. There are numbers of cell types clumped in granulomas in the liver from both genotypes which makes it hard to organise them. These cells are involved in control of *L. monocytogenes*. Properdin-deficient and wild type mice were culled at a matched endpoint based on the previous experiment that showed that properdin-deficient mice culled more at 29 hours. Therefore, mice were culled after having been infected intravenously with *L. monocytogenes*  $1 \times 10^6$  CFU of *L. monocytogenes*. The lethargic properdin-deficient mouse was characterised by a higher IFN- $\gamma$  level in serum and a higher serum level of complement C3 compared to the lethargic wild-type mouse. In the absence of properdin the viable counts from homogenised liver were higher in the properdin-deficient mice than in wild type mice but there was no significant difference between both genotypes. The high cytokine levels in the properdin-deficient mice on day two might be partially explained by the higher bacterial load in the liver.

C3 plays a role in promoting T cell responses to an intracellular bacterial infection. This was, shown in mice lacking C3, which had reduced expansion of antigen specific CD4 and CD8 T cells in response to *L. monocytogenes* (Nakayama *et al.*, 2009). Using TEM of liver after  $1 \times 10^6$  infection mice showed more *L. monocytogenes* in the liver from the properdin-deficient mouse in comparison to the wild type mouse. It appeared that these mice were trying to clear the *L. monocytogenes* from the tissue by informing the granuloma, although there was no significant difference in bacterial load in homogenised liver. Importantly, the same pattern was observed for expression of

mRNA (see qPCR data presented in chapter 3 *in vivo* results) in both genotypes dependent on the severity scores.

### *Role of properdin in expression of selected receptors cells in response to L. monocytogenes*

In the absence of properdin, expression of TLR2, CD11b and C5aR were less in the lethargic mouse compared to the wild type mouse at the matched time point. The expression of CD11b was higher in the properdin deficient mice (combined sample) than the wild type mice, but it was not higher in the lethargic mouse.

Mice deficient in CR3 (Gregory *et al.*, 2002) or immunoglobulin superfamily complement receptor (Helmy *et al.*, 2006) are more susceptible to *L. monocytogenes* infection but not to *Mycobacterium tuberculosis* (Hu *et al.*, 2000; Melo *et al.*, 2000). However, a study by Jagannath *et al.* (2000) showed that C5-deficient mice are more susceptible to *M. tuberculosis* infection compared to wild type control mice. CD11b, is part of CR3 (CD11b/CD18) an important receptor for phagocytosis and killing of *L. monocytogenes* (Drevets & Campbell, 1991; Drevets *et al.*, 1993; Drevets *et al.*, 1992). In this study there was less expression of CD11b in properdin-deficient mice as opposed to wild type mice.

Deficient mice in TLR2 are more susceptible to systemic infection by *L. monocytogenes* (Torres *et al.*, 2004). Moreover, a study by Nakayama *et al.* (2009) showed that the interactions of C5a and C5aR are unnecessary for primary activation and expansion of CD8 and CD4 T-cells in C5aR-deficient mice after systemic infection with *L. monocytogenes*. Blocking of C5a receptors in a mouse model of cecal ligation and puncture (CLP) using neutralising antibodies significantly improved the survival of mice in sepsis (Czermak *et al.*, 1999).

In the lethargic properdin-deficient mouse with a less abundant expression of C5aR there was a higher IL-17A expression than in the lethargic wild type mouse. This finding is in accordance with the result of a study by Weaver *et al.* (2010), their findings demonstrated that increased production of Th17 promotes cytokines in the absence of C5aR signalling.

After TLR2-deficient C57BL/6 mice were infected with  $5 \times 10^5$  *L. monocytogenes* strain EGD intraperitoneally showed normal innate immune response (Edelson & Unanue, 2001). Moreover, there was no difference in the CFU in the liver of TLR2 deficient mice and wild type mice after day three. However, TLR2 plays a role in the protective immune response to *L. monocytogenes* (Torres *et al.*, 2004).

### *The role of properdin in re-infection with L. monocytogenes*

Mice re-infected or immunised with *L. monocytogenes* were given a second injection of *L. monocytogenes*, which would be more effective against *L. monocytogenes* challenge. In order to study secondary immune responses, mice from both genotypes were infected with  $5 \times 10^5$  of *L. monocytogenes*. Then the two surviving mice of each genotypes were re-infected intravenous (i.v) with the same dose of *L. monocytogenes*  $5 \times 10^5$  and were culled after 5 days at the end time point. Mice from both genotypes survived after being immunised. Interestingly, this study also showed that re-infected mice were able to clear the bacteria as shown no growth of bacteria was visible in homogenise liver in wild type (two individual mice) and properdin-deficient mouse (one individual mouse).

In addition, expression of IL-17 in the spleen was higher in the properdin-deficient mice compared to wild type mice. However, expression of IFN- $\gamma$  was the same in both genotypes since IFN- $\gamma$  has a less essential role in protective immunity against re-infection (Zenewicz & Shen, 2007). Therefore, the speculation about the T cell response

based on IFN- $\gamma$  and IL-17 in these animals was to reduce the bacterial infection. The role of IL-17A in intracellular bacterial infection is controversial because it appears to be unnecessary for protection against intracellular primary infection with *Mycobacterium tuberculosis* (Khader *et al.*, 2005) but is involved in the secondary challenge with *M. tuberculosis* (Khader *et al.*, 2007) and in host defense against primary *Francisella tularensis* (Lin *et al.*, 2009).

### *Role of properdin in expression of Fc $\gamma$ receptors*

The previously observed differences in Fc $\gamma$ RIIb and Fc $\gamma$ RIV expressions of uninfected properdin-deficient mice and wild type mice spleens were confirmed in this study, involving a higher expression of Fc $\gamma$ RIIb in wild type compared to properdin-deficient mice and inverted expression of Fc $\gamma$ RIV. The expression levels change after infection with *L. monocytogenes*, expression of the inhibitory Fc $\gamma$ RIIb was higher in both wild type and properdin-deficient lethargic mice, while expression of the activating Fc $\gamma$ RIII and Fc $\gamma$ RIV was lower.

This depends on the degree of severity in both genotypes, as the lethargic mouse has less Fc $\gamma$ RIV compared to other levels of severity such as hunched.

Expression of Fc $\gamma$ RIII was less in properdin-deficient mice compared to wild type mice. It has been showed that the uptake of *L. monocytogenes* is Fc $\gamma$ RIII receptor mediated because the Fc $\gamma$ RIII monoclonal antibody reduces phagocytosis of *L. monocytogenes* by monocyte-derived dendritic cells (MoDC) in humans (Kolb-Maurer *et al.*, 2001).

Analysis of production of Fc $\gamma$ RIIb mRNA by real-time RT-PCR and Fc $\gamma$ RII protein by flow cytometry using the anti-Fc $\gamma$ RIII/II mAb conjugated to FITC showed that the expression of Fc $\gamma$ RIIb in uninfected spleen *in vivo* and *in vitro* is less in properdin-deficient mice than in wild type mice. Therefore, further work will be needed to

determine whether the observed differences in the levels of the Fc $\gamma$ RIIb receptor between properdin-deficient and wild type mice are connected with increases or decreases in expression in specific populations of the immune cells. However, another study reported that when stimulated Fc $\gamma$ RIIb receptor lead to down-regulation of much Fc $\gamma$ R signaling which does not mediate phagocytosis (Huang *et al.*, 2011).

Furthermore, expression of CD16/CD32 (Fc $\gamma$ RIII/Fc $\gamma$ RIIb) was investigated *in vitro* in mouse splenic macrophage and neutrophil but since it was not possible to detect the population in macrophages and neutrophils, B-cells were used because Fc $\gamma$ RIIB the inhibitory low-affinity FcR for IgG is the only Fc $\gamma$ R, that is expressed by B cells (Smith & Clatworthy, 2010; Nimmerjahn & Ravetch, 2008).

Moreover, it was reported in the study by Clatworthy & Smith, (2004) that macrophages from mice Fc $\gamma$ RIIb-deficient demonstrated increased phagocytosis of pneumococci *in vitro* and *in vivo* after infected Fc $\gamma$ RIIb-deficient mice with pneumococci showed increased bacterial clearance and survival. Following on from this finding, the present study tried to understand why dendritic cells and macrophages from wild type mice have a higher viable count of *L. monocytogenes in vitro* but *in vivo* properdin-deficient mice died more. Expression of mRNA Fc $\gamma$ RIIB from uninfected mice was higher in wild type mice. However, after infected lethargic mice from properdin-deficient showed decreased Fc $\gamma$ RIIB compared to wild type mice and compared to other mice with less severe symptoms.

Furthermore, the expression of CD40 in splenic dendritic cells from wild type and properdin-deficient mice after infection *in vivo* with  $1 \times 10^6$  *L. monocytogenes* was higher in the wild type but there were no significant difference. Although *in vitro* study observed higher expression of CD40 in dendritic cells derived from bone marrow from

wild type mice compared to those from properdin-deficient mice, this may be due to differences between dendritic cells from bone marrow and those of the spleen. However, the survival study showed that properdin-deficient mice were more susceptible to intravenous infection with *L. monocytogenes* compared to wild type mice. This difference in survival rates was reflected in higher uptake of bacterial load *in vitro* in wild type mice with higher cytokine measurement.

### *Role of properdin in anti-listeria IgG and IgM*

Natural antibodies are required for the optimal clearance of bacteria by complement. IgM binds to bacterial antigens and is protective in models of septic shock. There was no difference between properdin-deficient and wild type mice in their total serum IgM levels. This finding was reflected in this present study which showed that both properdin-deficient and wild type mice had the same in total IgG and IgM levels after infection with *L. monocytogenes*, at time points between 29-100 hours, compared to uninfected mice as measured by ELISA. It has been shown in (Ochsenbein *et al.*, 1999) that wild type C57BL/6 mice have detectable levels of *L. monocytogenes*-specific natural IgM but at low titres in their serum. However, no detectable *L. monocytogenes*-specific IgG was found.

### **Conclusion**

From the data generated, a working model has been developed (see Figure 4.1). Properdin-deficient mice are shown in the diagram in the crosstalk of factors determining the severity of disease development over time. The higher cytokine levels in properdin-deficient mice at 28-29 hours after *L. monocytogenes* infection was consistent with the increase in bacterial burden in liver showed by TEM and the

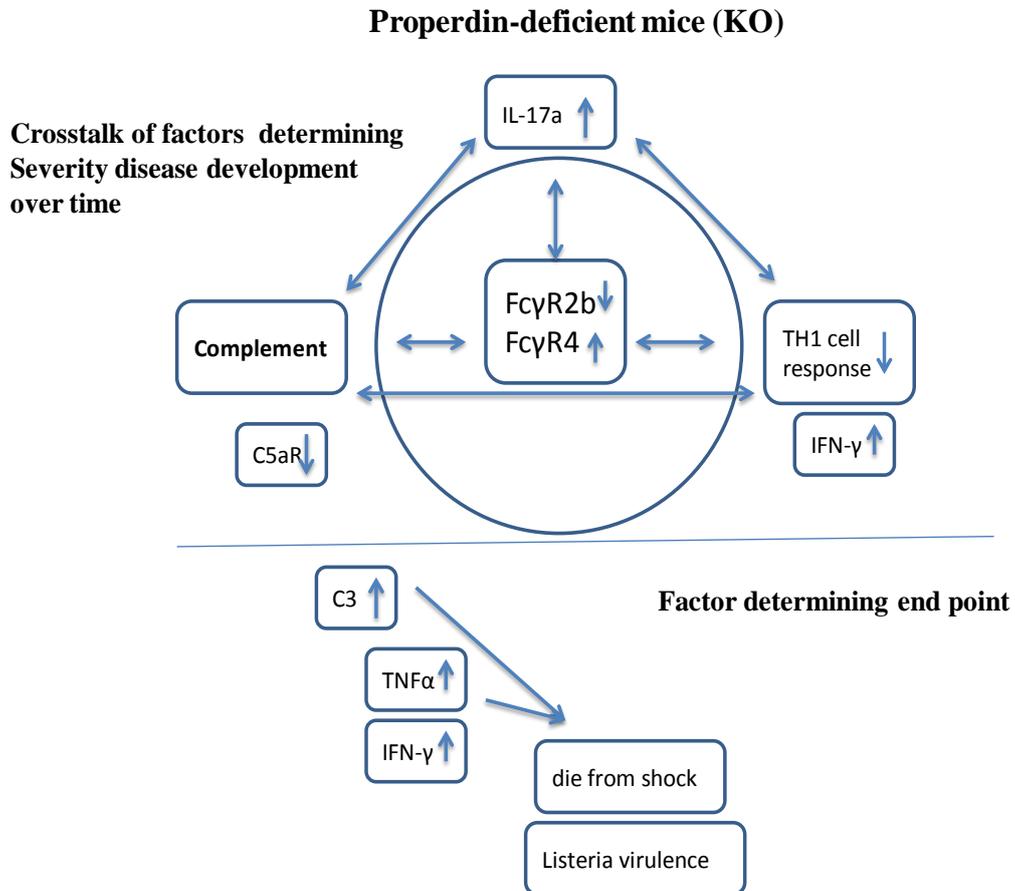
increased expression of IL-17A level in the spleen and the increased the concentration of C3 level in sera.

Most deaths of properdin-deficient mice from infection with *L. monocytogenes* seem to be due not to the infection itself but to higher proinflammatory cytokine production like IFN- $\gamma$ , TNF- $\alpha$  and expression of IL-17A which result in uncontrolled cytokine production and septic shock. Alternatively *L. monocytogenes* may have developed greater virulence in a M2 showed response in the properdin-deficient mice.

The death of mice after intravenous injection is possibly due to impaired immune response as observations confirm study showed complement C5-deficient mice are more susceptible to *L. monocytogenes* infection with a higher viable count in their spleen (Petit, 1980) this is probably due to the activation of macrophage and intact chemotaxis (Gervais *et al.*, 1989), as well as the particular great virulence of *L. monocytogenes*, which was demonstrated that after intravenous injection. Virulence factors such as LLO are needed by *L. monocytogenes* to escape from host cells to other cells and to multiply intracellularly and to avoid being destroyed by host defenses. But the dose of *L. monocytogenes* is still an important factor as demonstrated by the observation of the differing degree of severity of symptoms in mice after infection using varying dose of *L. monocytogenes*. One reason that the wild type mice could have the advantage in survival is because they have a greater Th1 (M1) response compared to properdin-deficient mice.

It is possible that the mice die of severe tissue damage due to overproduction of IFN- $\gamma$ . There was a higher IFN- $\gamma$  level was in properdin-deficient mice and this fails to limit *L. monocytogenes* growth because these mice died earlier than their wild type mice counterparts. This may the reason as indicated in the study by Rayamajhi *et al.* (2010)

which observed down-regulation of IFN- $\gamma$  receptor (IFNGR) APC populations during the early stages of systemic infection with *L. monocytogenes*. They used cell suspensions from the spleen of infected C57BL/6 mice i.v. with  $0.5\text{--}2 \times 10^4$  CFU of wild type *L. monocytogenes*, this were stained for IFNGR1 expression on the indicated cell populations by flow cytometry. They showed that APC populations of (splenic myeloid (CD11b<sup>+</sup>) and B lymphocyte (B220<sup>+</sup>CD19<sup>+</sup>) had significantly reduced IFNGR1 staining after 24 to at least 48 hours post infection. Dendritic cells also showed the reduction IFNGR1 staining for 79 hours after infection. In properdin-deficient mice infection may lead to lower phagocytic function, and decreased T helper cell type 1 (Th1) cytokine release.



**Figure 4.1:** Diagrammatic representation of properdin-deficient mice in the crosstalk of factors determining the severity of disease development over time.

This is the first study to show that intact complement is essential for the survival of murine listeriosis and for sustaining a cellular response to the intracellular pathogen *L. monocytogenes*. *In vitro*, bone marrow-derived dendritic cells in the absence of properdin have less intracellular listeria compared to dendritic cells from wild type mice, and showed impaired maturation to function as antigen presenting cells, leading possibly to a lower IFN- $\gamma$ <sup>+</sup> CD<sup>+</sup> T cell response. *In vivo*, properdin-deficient mice have

greater disease severity and an altered T cell phenotype consistent in impaired M1 polarisation.

In summary, both *in vitro* and *in vivo* results clearly demonstrated that the properdin plays a role in protecting against *L. monocytogenes* infection. Overall, these findings showed that properdin-deficient mice were significant increase in mortality after *L. monocytogenes* when compared to their wild type counterparts. The findings of this thesis add to the knowledge that properdin is essential to help the complement system as part of a defence mechanism against infection, in particular infection with primarily intracellular pathogens. The aim of this study was to investigate the role of properdin and the data suggest that properdin is necessary in order to survive acute murine listeriosis.

### 4.3 Future Work

A number of potentially interesting avenues of research arose in the process of this work, but could not be undertaken due to time constraints.

Adipose tissue contributes to the inflammatory response during systemic infection and in listeriosis in particular (Desruisseaux *et al.*, 2007). Complement can be activated and target the adipose tissue (Ram *et al.*, 2010), and properdin is expressed in adipose tissue of mice (Patrick *et al.*, 2009). The role of properdin in adipose tissues from both genotypes was analysed in response to infection with *L. monocytogenes* and there was an attempt to investigate MCP-1 (Monocyte chemoattractant protein-1) production by adipose tissue (MCP-1 is an M2 marker), unfortunately, it was not possible for technical reasons to do so (see appendix, Figure VII and table I).

Further investigation is needed on a molecular level to explain the behaviour of properdin and C3 as hypoxia-sensitive genes, as discovered using primary macrophages exposed to hypoxia for 24 hours (also 5 hours for C3). Analysis of promoter activities by reporter assays would be one way to do this.

One limitation of the analyses was that no properdin antibody was available to evaluate the abundance of the translation product compared with the message detected by PCR. mRNA may be decreased when properdin protein production and export increases. This would have important implications for the understanding of the regulation of the activation of complement.

The expression of Fc $\gamma$ RIIb in uninfected spleen *in vivo* and *in vitro* was less in properdin-deficient mice than in their wild type equivalents. This was after analysis of production of Fc $\gamma$ RIIb mRNA by real-time RT-PCR and Fc $\gamma$ RII protein by flow cytometry using the anti-Fc $\gamma$ RIII/II mAb conjugated to FITC. Therefore, further work is needed to determine whether the observed differences in the levels of the Fc $\gamma$ RIIb

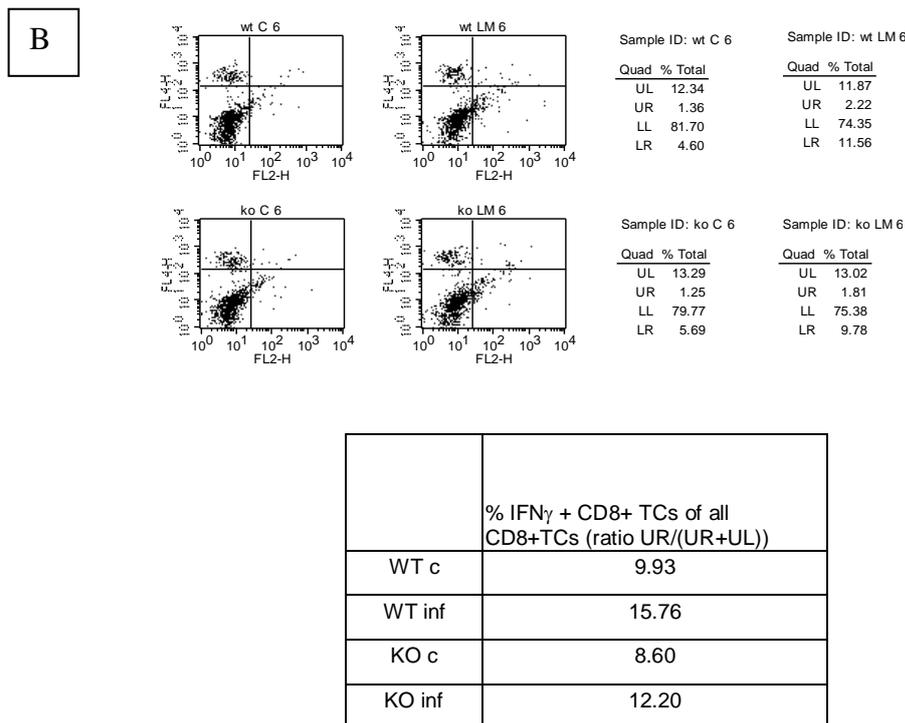
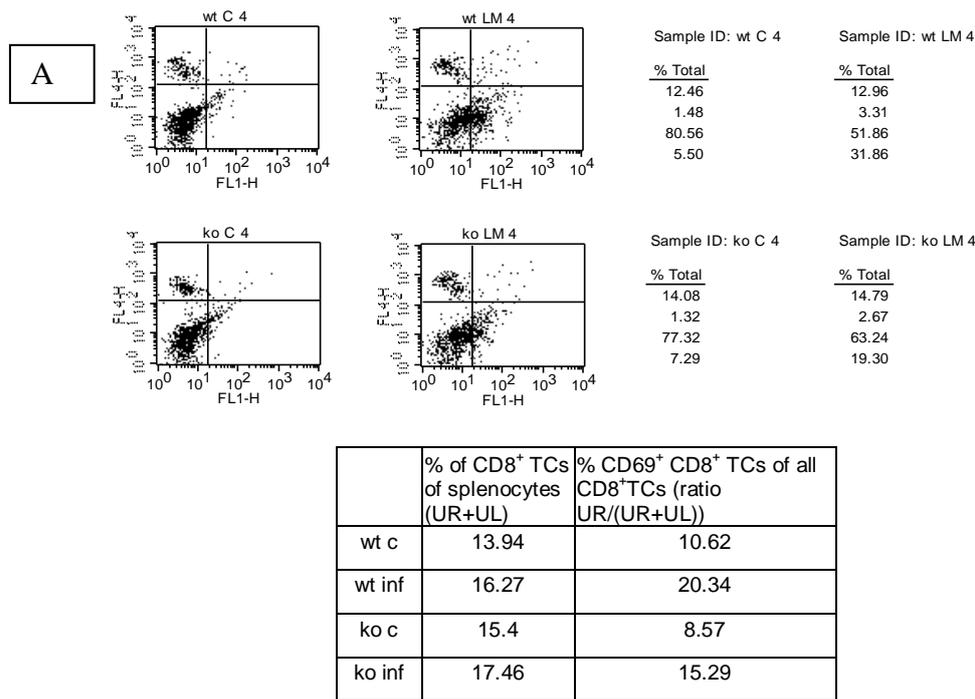
receptor between properdin-deficient and wild type mice are connected with increases or decreases expression in specific populations of the immune cells or whether this altered expression is merely a byproduct of a different scope of cell activities.

In addition, for further study it would be interesting to firm up the characterisation of the antilisterial T cell response, which could only figure as a small project performed during a student placement. The implications for other diseases are important if an influence of the innate immune component properdin is reproducibly shown on the quality of the adaptive T cell response.

Finally, the observation that properdin-deficient mice die more than wild type mice, even though the actual numbers of viable bacteria retrieved from their livers do not differ, is worth pursuing. As discussed (page 261), the virulence of bacteria may differ notably and this could be addressed *in vitro* using serum and cells from properdin-deficient and wild type mice and measuring the gene expression profiles of the adapting bacteria.

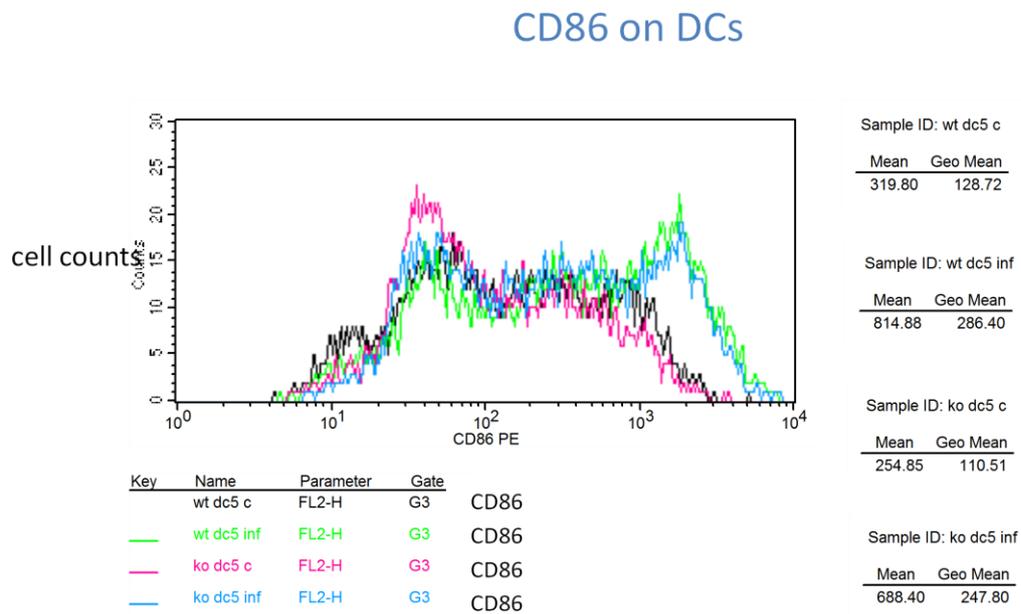
*Chapter Five Appendix*

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**Figure I:** Analysis of T cell populations of wild type and properdin-deficient mice within splenocyte populations stimulated with heat-killed *L. monocytogenes*. A. Compared to uninfected controls, splenocytes from wild type and properdin-deficient mice show an increase of CD8<sup>+</sup> T cells after stimulation with heat-killed *L. monocytogenes*, whereas CD69<sup>+</sup>CD8<sup>+</sup> T cells are more increased in

wild type. B. There is a relatively higher increase in IFN $\gamma$  producing CD8<sup>+</sup> T cells in wild type than in properdin-deficient mice after infection.

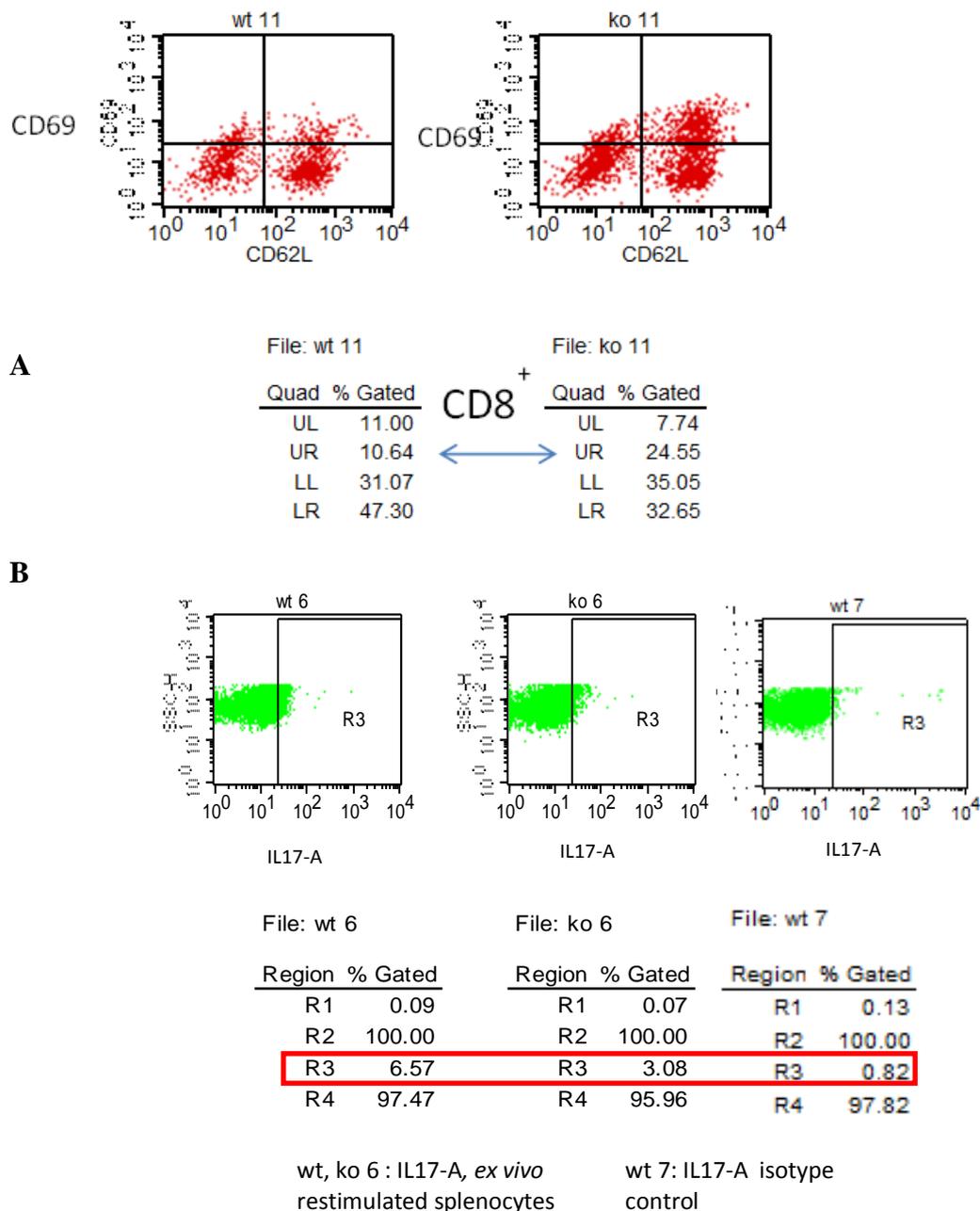


**Figure II:** Flow cytometric analysis of CD11c<sup>+</sup> cells from spleens infected *in vitro* with heat-killed *Listeria*: CD86 expression was higher in WT compared to KO Dendritic cells.

Time	13 h			18 h		
MOI	0.1	0.02	0	0.1	0.02	0
genotype	WT KO					

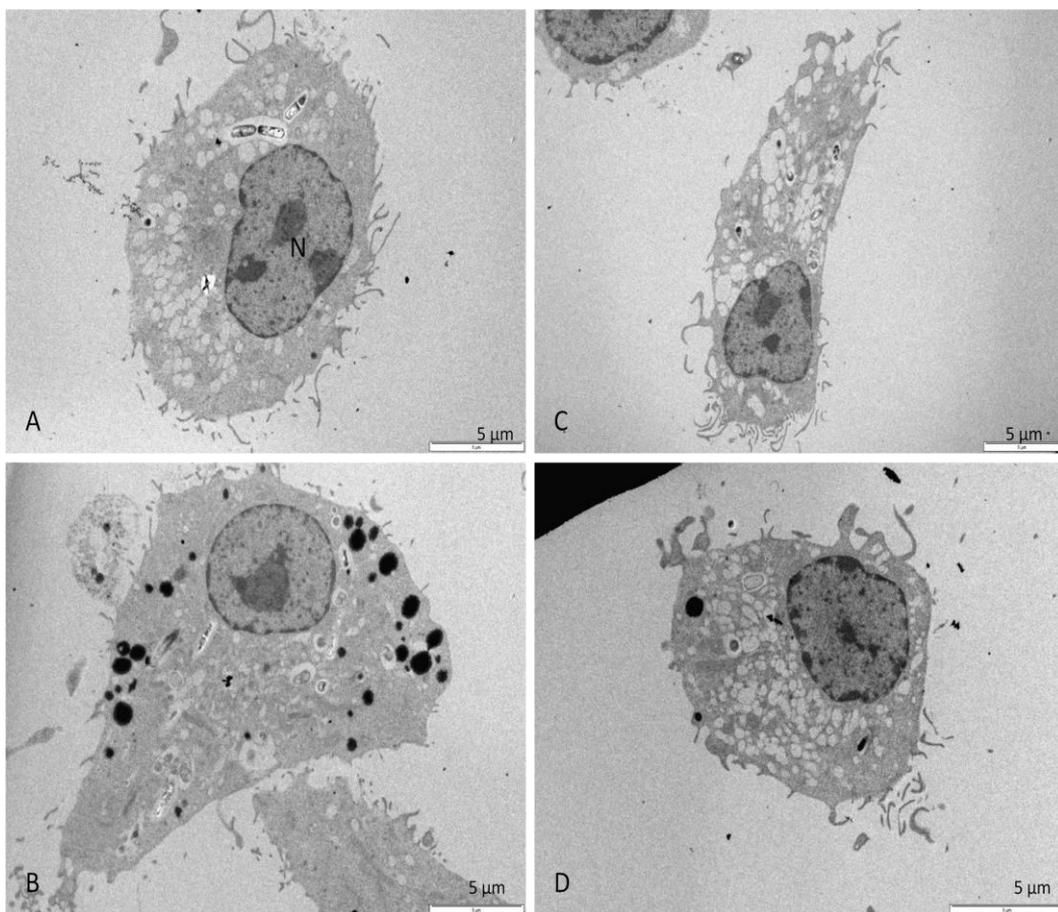


**Figure III:** Western blot analysis of supernatants from splenocytes infected with *L. monocytogenes* at different MOI (indicated) with anti-LLO antibody. A singular band at the expected size is observed to comparable extents between genotypes.

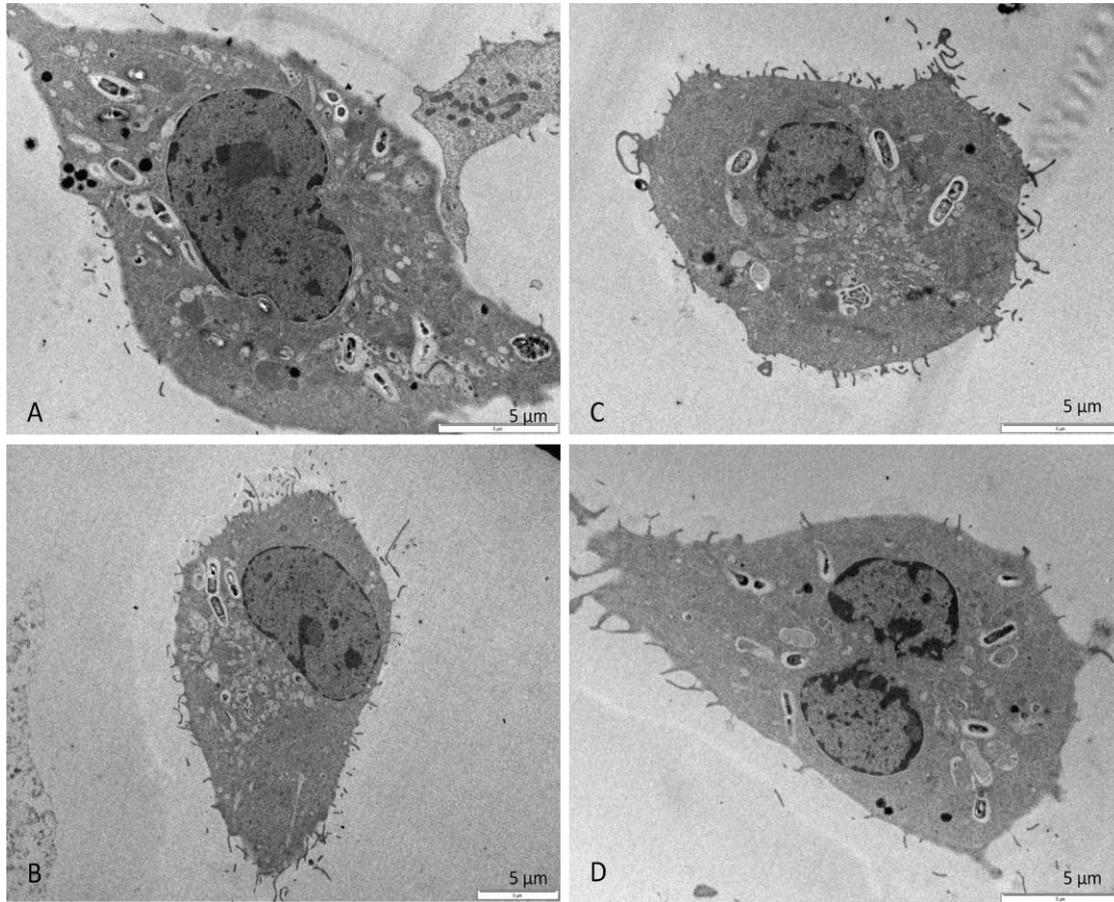


**Figure IV:** Analysis of T cell populations within re-stimulated splenocyte populations of wild type and properdin-deficient mice infected with *L. monocytogenes*. A *Ex vivo* re-stimulation of splenocytes with heat-killed *L. monocytogenes*. CD8<sup>+</sup> cells were gated and analysed for expression of CD69 (an early activation marker) and CD62L (a marker of naïve T cells). A representative analysis of two is shown. B. *Ex vivo* re-stimulation of splenocytes with heat-killed *L. monocytogenes*. CD4<sup>+</sup> cells were gated and analysed for expression of intracellular IL-17A.

Figures I- IV were generated by a placement student in the lab (summer 2012): Nina Lapke, Dipl.biol., Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany. I had a part in providing samples from *in vivo* and *in vitro* experiments, and in discussion of results.



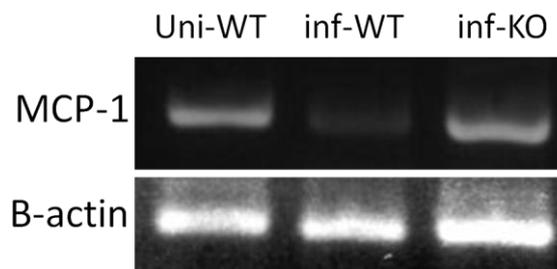
**Figure V:** Representative transmission electron micrographs of dendritic cells from wild type mice after 4 hours infected with *L. monocytogenes* (MOI=0.2).



**Figure VI:** Representatives transmission electron micrographs analysis of dendritic cells from properdin-deficient mice after 4 hours infected with *L. monocytogenes* (MOI=0.2).

**Table I:** Primer sequences, annealing temperatures and the expected sizes of MCP-1 generated in this study.

Gene	Sequence of primers	T <sub>A</sub>	Product size (bp)
MCP-1	5` CACTCACCTGCTGCTACTCATTCAC-3` 5` GGATTCACAGAGAGGGAAAAATGG-3`	57°C	488 bp



**Figure VII:** RT-PCR products obtained using adipose tissue from infected mice with  $5 \times 10^5$  *L.monocytogenes*. Sizes were as expected for  $\beta$ -actin gene and MCP-1.

**Table II:** Severity of mice from wild type and properdin-deficient mice after infection with  $5 \times 10^5$  *L. monocytogenes*, 5 mice each genotype (pilot experiment). End= mice were culled.

Condition	Start time		18h		25h		43h		67h		69h	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Normal	All	All	5	4	5	3	5	2	4	2	4end	2end
Hunched				1		2		2	1	3	1end	2end
Dead								1				
Total	5/5											

**Table III:** Severity of mice after i.v. infected with  $5 \times 10^5$  *L. monocytogenes*, groups of 7 each genotype. F= female and M= male

Condition	Start time		19hrs		29hrs		45-46hrs		67hrs		96hrs		100hrs	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Normal	All	All	6	3	1			1		1		1	2M	1F+1M
													End	End
+Hunched			1	4	4	3	3	1	1	1	2	1		
++ Hunched					2	2			1					
+Lethargic						2F	3F	2F	1M					
						End	End	End	End					
++ Lethargic							1M	1M						
							End	End						
Total	7/7													

**Table IV:** Severity of mice after i.v. infection with  $5 \times 10^5$  *L. monocytogenes*, 9 wild type and 7 properdin-deficient mice. The table data were from two independent experiments. F=female and M=male

Condition	Start time		29-30hrs		36-43hrs		45h		48hrs		65h		95h	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Normal	All	All	2M+F										2F end	
+Hunched			4M+F	3M+F										
++ Hunched			3M+F	2M+F									1M end	2M end
+Lethargic														
++ Lethargic							1M+1F End	1M+1F End	1M end		2F+(1M77hr) end			
Dead				2F		1M								
Total	9WT/7KO													

**Table V:** Severity of wild type and properdin-deficient mice after injection of  $9 \times 10^5$  and  $1 \times 10^6$  *L. monocytogenes*. Data represent of two independent experiments.

Condition	Start time		20-22h		24h		29h		39h		46h	
	WT	KO	WT	KO	WT	KO	WT	KO	W T	KO	WT	KO
Type of mice												
Normal	All	All	3									
+Hunched			6	9	9	2	8	2		1	4end	2end
++ Hunched						7	1				3end	
+Lethargic							1end	5 end				
Dead									1	2		
Total	9/9											

**Table VI:** Severity of wild type and properdin-deficient mice after i.v. infection with  $1 \times 10^6$  *L. monocytogenes*, culled at matched endpoints (28-29 hours).

Condition	Start time (0)		~29hrs	
	WT	KO	WT	KO
Type of mice				
Normal	All	All	1(WT3)	
+Hunched			1(WT2)	1(KO3)
++ Hunched				1(KO2)
+ Starey			1(WT2)	1(KO2)
++ Starey			1(WT1)	2(KO1, KO3)
+Lethargic			1(WT1)	1(KO1)
Total	3/3			

## *Chapter Six References*

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