Host response and bacterial virulence during acute and persistent Burkholderia cepacia complex infection using zebrafish embryos

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THÈSE

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Réponse de l'hôte et virulence bactérienne durant une infection aiguë ou persistante causée par le complexe *Burkholderia cepacia* chez l'embryon de poisson-zèbre (*Danio rerio*)

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ACRONYMS

APC: antigen presenting cell

B cell: lymphocyte B

Bcc: *Burkholderia cepacia* complex

BcCV: *Burkholderia cepacia*-containing vacuole

BPI: bactericidal/permeability-increasing protein

CARD: caspase-recruitment-and-activation domain

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CFU: colony forming unit

CGD: Chronic Granulomatous disease

CHT: caudal hematopoietic tissue

CLR: C-type lectin receptor

Cm: chloramphenicol

DAMP: danger-associated molecular pattern

EMP: erythromyeloid progenitors

EPS: Exopolysaccharides

Hpf: hours post fertilization

Hpi: hours post infection

HSC: hematopoietic stem cells

ICM: intermediate cell mass

IL: interleukin

Iv: intravenous

K56-2: *Burkholderia cenocepacia* K56-2

LAP: LC3-associated phagocytosis

LB: Luria Bertani

LC3: light chain 3

LPS: lipopolysaccharide

MAC: membrane attack complex

MAP1: microtubule-associated protein 1

MBL: mannan-binding lectin

MHCII: major histocompatibility complex II

MLST: Multilocus sequence typing

MMP-9: matrix metalloproteinase 9

Mo: morpholino

MPO: myeloperoxidase

Mpx: myeloid-specific peroxidase

MyD88: myeloid differentiation factor 88

NET: Neutrophil extracellular trap

NK: Natural killer cell

NLR: NOD-like receptor

NO: nitric oxide

NOD: Nucleotide-binding oligomerization domain

PAMP: pathogen-associated molecular pattern

PBI: posterior blood island

PBS: phosphate-buffered saline

PMN: plolymorphonuclear neutrophil

PR: phenol red

PRR: pattern recognition receptor

RBI: rostal blood island

RLR: RIG-I-like receptor

ROS: reactive oxygen species

ST: sequence type

Th cell: lymphocyte T helper

TIR: Toll/IL1 receptor

TIRAP: MyD88 adaptor-like (Mal)

TLR: Toll-like receptor

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor-inducing interferon-β

VDA: ventral wall of the dorsal aorta

Zmp: zinc-dependent metalloprotease

ABSTRACT

Bacteria belonging to the *Burkholderia cepacia* complex (Bcc) can cause severe infection in individuals with Cystic fibrosis (CF). Infection can vary from asymptomatic infection to progressive worsening of lung function, and sudden acute fatal necrotizing pneumonia and septicaemia ("cepacia syndrome"). The high intrinsic resistance of these bacteria to clinically used antibiotics complicates treatment and the Bcc are associated with poor prognosis. The role of host phagocytes, and the immune pathways involved in the pro-inflammatory character of the infection and sudden transitions to acute infection are not well known.

Earlier, we developed a new infection model to study virulence of the Bcc using zebrafish embryos. Whereas *B. cenocepacia* K56-2 was able to replicate in macrophages and cause a rapidly fatal response, less pathogenic strains such as *B. stabilis* LMG14294 induced persistent infection. In the present study, we further exploited the exciting possibilities for in vivo imaging of infection in real time in the transparent embryos. The embryos have a developing innate immune system with remarkable similarity to that of humans, and genetic approaches and global host transcriptome analysis allow studying the role of phagocytes and the innate immune response during Bcc infection.

We show that macrophages play a critical role in multiplication of K56-2 and induction of a MyD88-dependent fatal inflammatory response, characterised by strong induction of *cxcl8* and *il1b* gene expression. Surprisingly, in the absence of macrophages the bacteria were unable to replicate in the first 24 h, which resulted in a significant pro-survival advantage to the host compared to wild type embryos. This is in sharp contrast to the situation found for infections with other important pathogens, including *Mycobacterium marinum* or *Staphylococcus aureus*, where macrophages are required for host defence. Thus *B. cenocepacia* requires macrophages for its replication and the induction of a rapidly fatal inflammatory response in this model. The Toll-like receptor (TLR) pathway is a major arm of the cell-mediated innate immune response with both Myd88-dependent and –independent signaling cascades, resulting in the production of pro-inflammatory cytokines. Here we show

that in contrast to *S*. Typhimurium, and *M. marinum* infections, the absence of Myd88 provided a pro-survival effect to the embryos after infection with K56-2. The bacteria replicated better in *myd88*$^{-/-}$ mutant than wild type embryos, suggesting that it is not bacterial burden per se, but the inflammatory response that kills the embryos. Interestingly, *cxcl8* and *il1b* expression were not significantly induced during the first 7 hours in the *myd88*$^{-/-}$ mutant while a strong induction was seen in control embryos, suggesting that a Myd88-dependent inflammatory response during early macrophage stages significantly contributes to fatal infection.

To gain more insight into global changes in host gene expression we performed an RNAseq screen. As expected, acute infection was characterised by strong modulation of host gene expression that increased over time, in contrast to persistent infection which showed modulation of only a small set of genes. The innate immune response, specifically TLR and apoptosis signaling pathways were strongly activated during acute infection, in line with the strong inflammatory character of *B. cenocepacia*. During persistent infection by *B. stabilis* the major differentially expressed gene set concerned genes encoding complement proteins. The obtained data set will help us to further unravel specific host-responsive gene expression that may aid in finding targets to develop new therapeutics against this pathogen.

The critical role for macrophages in Bcc infection in zebrafish is in line with recent clinical observations. This suggests that the intracellular stages of *B. cenocepacia* and the ensuing inflammatory response are unexplored targets for the development of new therapies to combat this infection.

CHAPTER 1

INTRODUCTION

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This thesis describes the analysis of the interaction of bacteria belonging to the *Burkholderia cepacia* complex (Bcc), with a focus on *Burkholderia cenocepacia* and *Burkholderia stabilis*, with the host innate immune system using zebrafish as a model. In this introduction, I will first discuss the innate arm of the human immune system, followed by a section describing our current knowledge of the innate immune response in zebrafish and the zebrafish infection model. A third section will describe in more detail the biology of the Bcc and our current knowledge on the capacity of the Bcc to survive and replicate in host cells, followed by the outline of this thesis.

Part I – The human innate immune response

1.1. General information

The immune system exists since the emergence of multicellular life. It is a complex system built up of different proteins, cells and signalling cascades that protect the organism against microbial attack and danger signals. In jawed vertebrates it is divided into an innate and an adaptive immune arm (Pancer and Cooper, 2006).

The innate response is present in all animals and plants, and the complexity of the immune system increases with the evolution of the species. The presence of proteins and cells that structure the innate immune system allows a rapid response to signals, and mobilisation to the site of infection or danger (Mayer, 2010). It can roughly be divided in four parts that will also be discussed in more detail in section 1.2.

1) The physical and chemical surface barrier with for example the skin, the gastrointestinal epithelial lining, the respiratory tract also the epithelial and the mucus. 2) The complement system that is activated through a triggered enzyme-cascade with an important role for the so-called “membrane attack complex” (MAC) resulting in damage of bacterial membranes and death of the pathogen. 3) The cellular barrier that makes up the most important part of the innate immune response, and consists of an arsenal of phagocytic cells including macrophages, neutrophils and dendritic cells, but also mast cells, eosinophils, basophils and Natural Killer (NK) cells. 4) Inflammation which is a combination of cellular response and chemical attack, and involves the production of eicosanoids and cytokines (Mayer, 2010).

Adaptive immunity is a specific response to microbes that exhibits immunological memory better than innate memory, and takes more time to efficiently kill pathogens. Adaptive immunity contains both humoral and cell-mediated responses involving lymphocytes. There are three types of B lymphocytes, naive B lymphocytes, plasma B lymphocytes that produce antibodies as part of the humoral response, and memory B cells. The T lymphocyte, with a major role in cell-mediated immunity, functions as a bridge between the innate and the adaptive immune system like antibodies, and exists as CD4+ T cells (or helper-T cell, Th), CD8+ T cells (or cytotoxic T cell) and γδ T cells. Th cells are recruited and activated by antigen presenting cells (APC), such as macrophages or dendritic cells, through the major histocompatibility complex II (MHC II). After activation, Th cells produce cytokines and activate B and T cells (Figure 1). For the purpose of my thesis work, I will further focus on the innate immune response.

1.2. The innate immune response

This part describes in more detail the crucial role of neutrophils, macrophages, complement and inflammation as part of the innate immune response during host protection. It is followed by a description of how some bacteria are able to avoid being degraded or killed by the host defence system and thrive, either inside cells or systemically. Bacteria of the Bcc are pathogens of immunocompromised persons and notorious for infection of individuals with cystic fibrosis (CF) and chronic granulomatous disease (CGD). The last part of this section describes the defective immune response in CF and CGD.

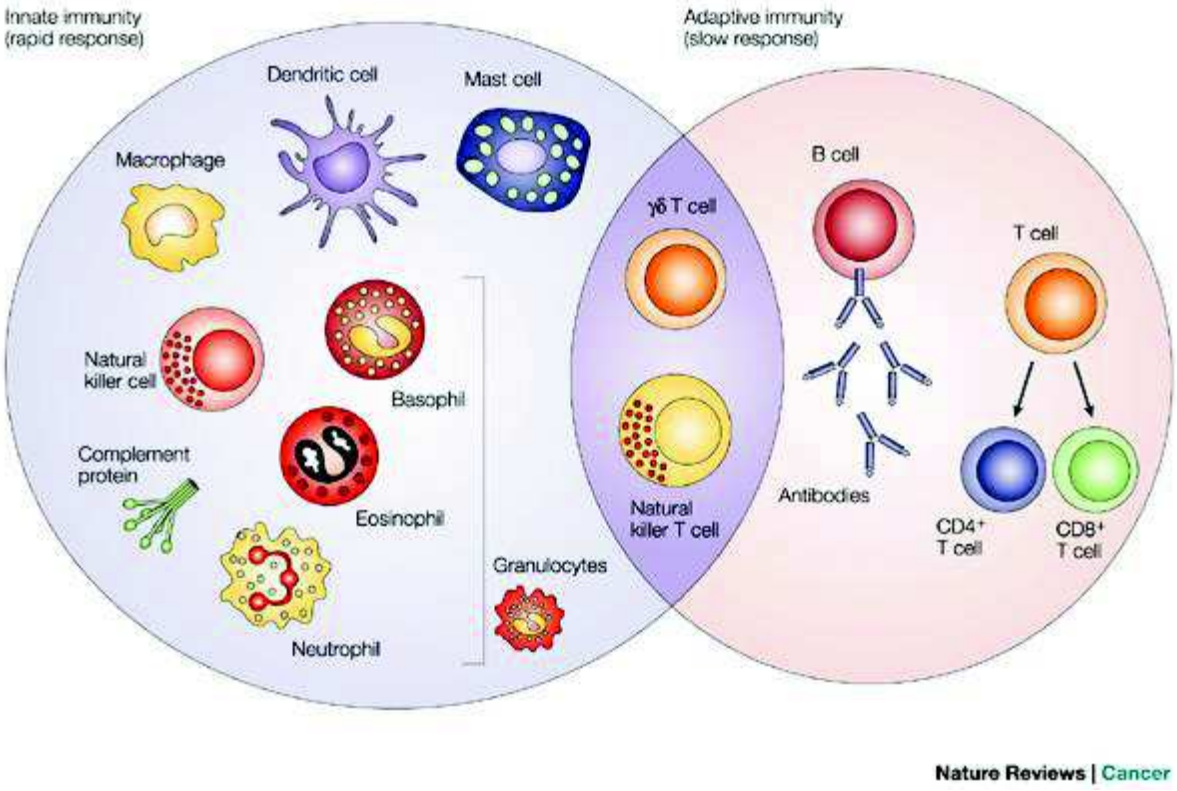

Figure 1. The innate and adaptive immune response.

The immune system consists of the innate immunity and the adaptive immunity; both systems possess specific cells except Natural killer (NK), T cells and γδ T cells that are the interface of innate and adaptive immunity. The innate immune response is the first line of defence against infection and is composed of macrophages, dendritic cells, mast cells, NK cells, granulocytes (neutrophils, eosinophils and basophils) and complement protein. It is composed of lymphoid cells, such as B cells and T cells (Reproduced from Dranoff G. 2004)(Dranoff, 2004).

1.2.1. The role of neutrophils

Neutrophils or polymorphonuclear leukocytes (PMN) are the most abundant immune cells. They phagocytose and kill microorganisms via oxidative and non-oxidative mechanisms, either directly in the phagocytic compartment or extracellularly (see Figure 2) (Nauseef and Borregaard, 2014).

PMNs possess granules containing antimicrobial compounds. Granules can fuse with the phagocytic compartment or directly with the plasma membrane resulting in extracellular release of their contens. There are three types of granules that contain molecules with different antimicrobial compounds (Nauseef and Borregaard, 2014). The azurophilic granules (or primary granules) contain myeloperoxidase (MPO), bactericidal/permeability-increasing protein (BPI), defensins, and the serine proteases neutrophil elastase and cathepsin G. The specific granules (or secondary granules) contain alkaline phosphatase, lysozyme, NADPH oxidase, collagenase, lactoferrin and cathelicidin. The two first granule types are released at site of infection. Tertiary granules contain cathepsin and gelatinase B (or metalloproteinase 9, MMP-9), and is released during neutrophil transmigration.

The major way for oxidative killing is the production of reactive oxygen species (ROS) through activation of NADPH oxidase. Electron transfer via NADPH oxidase produces superoxide that is converted by superoxide dismutase (SOD) to hydrogen peroxide and other ROS. The NADPH oxidase complex is formed by five subunits and Rac-GTP. The catalytic core of the complex is composed of two transmembrane subunits, $gp91^{phox}$ (NOX2 or CYBB) and $p22^{phox}$ (CYBA) that are localised in vesicles and the plasma membrane. The three other regulatory subunits $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ form a complex in the cytosol and upon

activation translocate with Rac-GTP to the membrane to form an active NADPH oxidase complex (Figure 2) (Gardiner 2013).

Eleven years ago, a new neutrophil activity was discovered, named Neutrophil Extracellular Trap (NET) formation (Brinkmann et al., 2004; Nauseef and Borregaard, 2014). “NETosis” is the release by neutrophils of granule proteins and chromatin to form an extracellular matrix with antimicrobial proteins such as neutrophil elastase, cathepsin G and histones (Thomas et al., 2014). NETs entrap extracellular bacteria and can kill them. “NETosis” is a new death pathway that is not yet clearly understood, but ROS has been shown to play a role in this (Fuchs et al., 2007)(Figure 2).

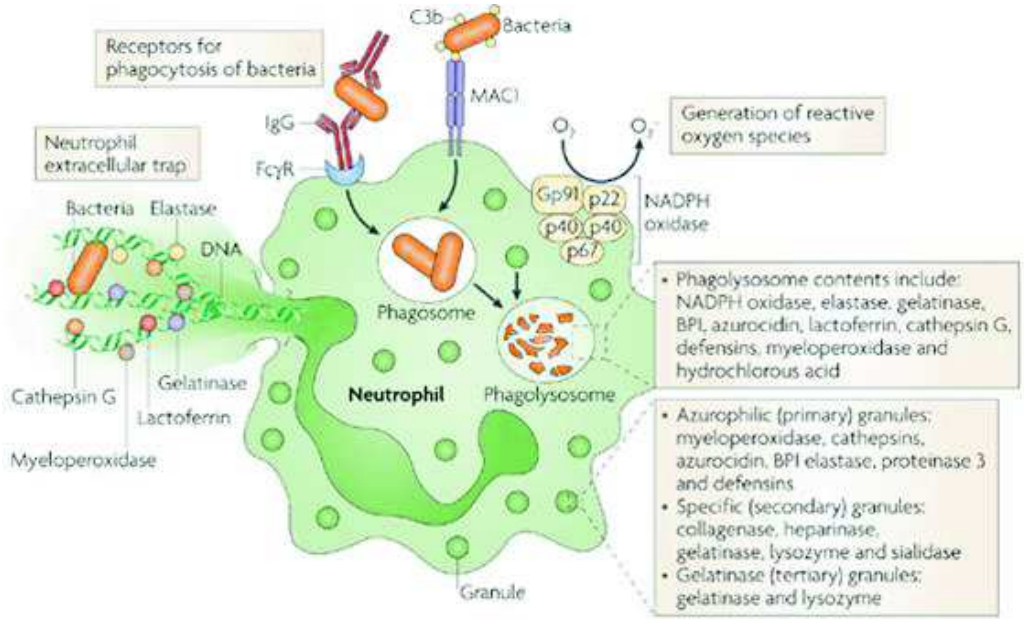

Figure 2. Schematic overview of neutrophil antibacterial systems.

Neutrophils possess different efficient antibacterial systems. Different receptors that will induce bacterial phagocytosis are present in the membrane. Phagosomes subsequently fuse with lysosomes and neutrophil granules, which contain a wide range of proteolytic and antibacterial enzymes and peptides, thereby forming phagolysosomes. NADPH oxidase, which is present in the membrane of the phagolysosome, generates reactive oxygen species that destroy the bacteria. In addition, neutrophils can extrude neutrophil extracellular traps (NETs), which are fibrous extracellular meshes that consist of chromatin coated with proteases, including myeloperoxidase, elastase and cathepsin G. These trap and kill bacteria and degrade bacterial virulence factors. (Reproduced from Hickey and Kubes, 2009)

1.2.2. The role of macrophages

Macrophages form an interface between the innate and the adaptive immune system. Macrophages can be activated and polarized in two different types: classical activated macrophage M1 or alternative activated macrophage M2. Macrophage polarization could be induce in the context of infection and mainly by the cytokines produced by other cells. M1 macrophages kill microorganisms, infected cells and tumour cells, and they produce pro-inflammatory cytokines that induce the inflammatory response. In contrast, M2 macrophages control the inflammatory response and down-regulate M1 cells and the adaptive immune response. M2 cells are also involved in tissue repair and angiogenesis but they promote tumour development and could become Tumour-Associated Macrophages (TAMs) (Figure 3) (Van den Bossche et al., 2014).

Cells have different mechanism to internalize particles, liquid, cellular debris or microorganisms. Endocytosis is the actin-dependent process that permits to take up small molecules (Sandvig et al., 2011). Macropinocytosis or “cell drinking” allows cells to engulf surrounding liquid (Kerr and Teasdale, 2009). Phagocytosis or “cell eating” is the actin-dependent process that internalizes microorganisms, dead cells or debris by different mechanisms, including receptor-mediated uptake (see below). Phagocytosis is specific to certain cells (macrophages, neutrophils, dendritic cells and B cells). Two non-usual processes have also been described, cell “cannibalism” where cells actively “eat” healthy live cells (found in tumours) and entosis where one cell “burrows” into a neighbouring cell (Overholtzer et al 2008).

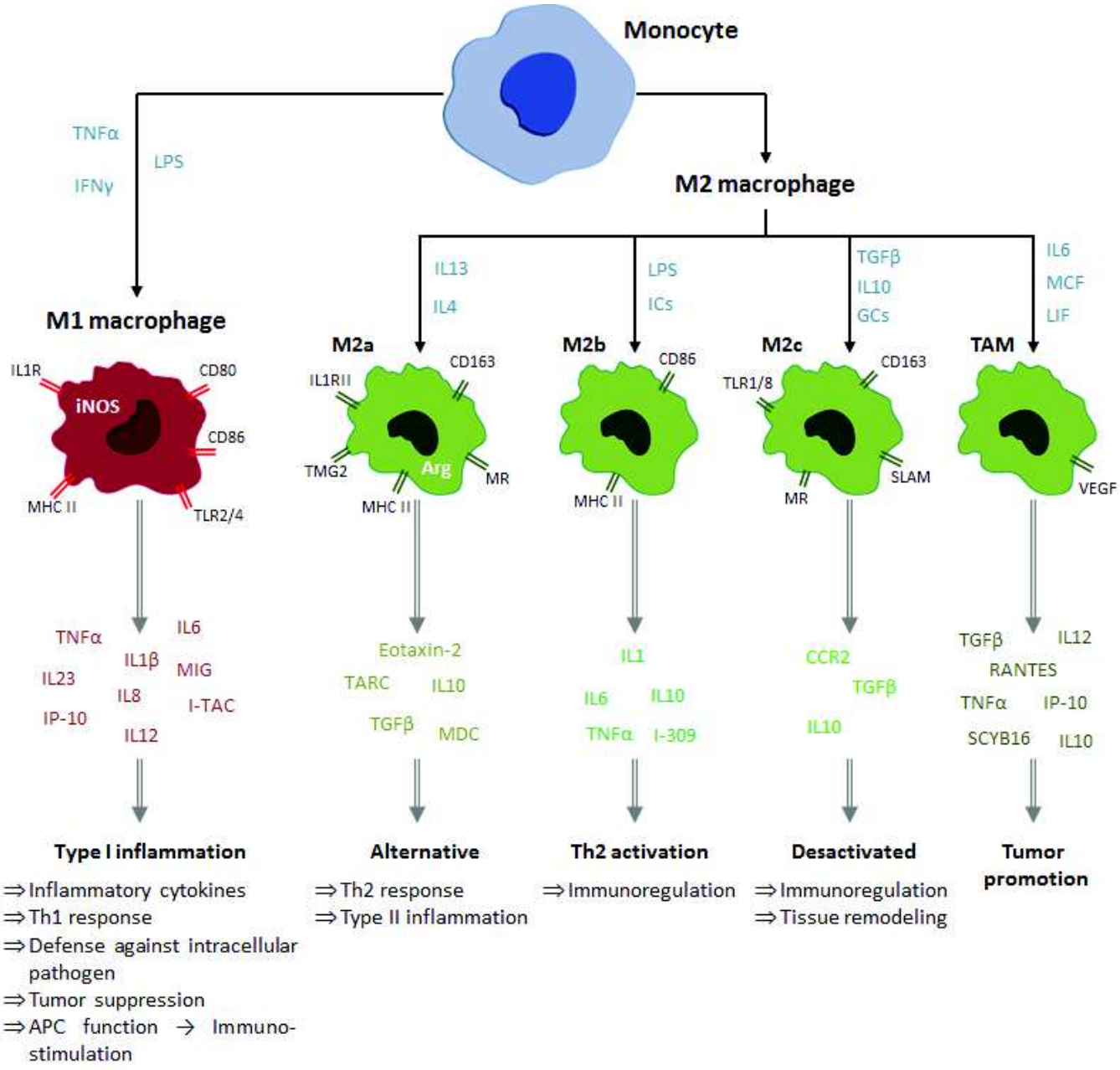

Figure 3. Macrophage polarization and activation.

After tissue injury or microbial attack, cells around the site will produce different molecules that recruit monocytes and induce their polarization in M1 or M2 macrophages. The polarization is context-dependent. M1 are type I inflammation macrophages, they induce proinflammatory cytokine production and activate Th1 cells by antigen-presentation. They are the first defence against intracellular pathogens and also mediate tumour suppression. M2 macrophages are subdivided in four different groups. M2a induce type II inflammation and activate Th2 cells. M2b and M2c are involved in inflammation resolution by production of anti-inflammatory cytokines (IL-10), and help tissue remodelling and the angiogenesis. M2d or Tumour associated macrophage (TAM), a recently identified subgroup, promote tumour development.

Macrophages are professional phagocytes and play an important role in the control of pathogen invasion. They possess three groups of receptors on their membrane that can be involved in phagocytosis (See Table 1). The first group includes complement and antibodies receptors, and induces opsonic phagocytosis. The second group of receptors include the macrophage receptor MARCO (Macrophage Receptor With Collagenous Structure) and the mannose receptor. The last group involves the Pattern-Recognition Receptor (PRR) that recognise Microbe-Associated Molecular Patterns (MAMPs) through phagocytosis (see 1.2.3). Adherence of microorganisms to the cell membrane through specific receptor engagement induces pseudopodia formation that extends around the microorganism resulting in the uptake of the microorganism by a "zipper" like mechanism in a vacuole called the phagosome (Alberts et al., 2002). One of the major host defence mechanisms against ingested microbes, the phagolysosomal degradation pathway, is a sequential series of membrane fusion events with vesicles of the endocytic processing pathway, which result in the maturation of the phagosome into a phagolysosome. Briefly, the ordered membrane fusion events that take place with early endosome, late endosomes and lysosomes require the activation of small GTPases (including Rab5-early endosomes and Rab7-late endosomes) and specific SNARE proteins, inserted in the membranes of the phagosome and the target vesicles. Maturation of the phagolysosome is characterized by progressive acidification, involving recruitment of a proton pump, the vacuolar-type ATPase (V-ATPase) to the phagosome membrane, and together with the high concentration of proteases this results in the degradation of the ingested microorganism. The digested content is then eliminated by exocytosis.

Autophagy is one of the major proteolysis systems that helps maintain cellular homeostasis by engulfment of cytosolic material and ensures its intracellular recycling. In addition to the "classical" phagolysosomal degradation pathway, autophagy has also been shown to be involved in the degradation process of microorganisms. After uptake, double membrane vesicles called autophagosomes are formed by the action of a specific set of autophagy-related proteins, including p62, a ubiquitin-binding protein that marks ubiquitinated material (including microorganisms) for recognition by the autophagy component Atg8/microtubule-associated protein 1 (MAP1)-light chain 3 (LC3). A cytosolic form of LC3 (LC3-I) is converted into LC3-II by conjugation with phosphatidylethanolamine (PE), which is subsequently targeted to autophagosomal membranes, followed by maturation into an autophagolysosome, and resulting in degradation of the engulfed material (Vural and Kehrl, 2014). The recent discovery that the LC3 protein can also be recruited to non-autophagy related single membrane phagosomes, termed LC3-associated phagocytosis (LAP), exemplifies the multitude of pathways that cells employ to maintain cellular homeostasis, and as a consequence the many possibilities intracellular microorganisms may use to avoid degradation by these immune defence systems (for review see Lai and Devenish, 2012).

Table 1. Receptors involved in phagocytosis and their ligands

Adapted from (Jaumouillé and Grinstein, 2011; Underhill and Goodridge, 2012)

Type of phagocytosis	Receptors	Ligands
Opsonic phagocytosis	Fc receptor family : FcγRI (CD64), FcγRIIa (CD32a), FcαRI (CD89), FcεRI	Antibody-opsonized targets (IgG1-4, IgA1 and IgA2, IgE)
	Complement receptors : CR1 (CD45), CR3 (CD11b/CD18) and CR4 (CD11c/CD18)	Complement opsonized targets (Mannan-binding lectin, C1q, C B, C3b, iC3b)
	α5β1 integrin	Fibronectin, Vitronectin
Non-opsonic phagocytosis	Dectin-1	B-glucan
	Mannose receptor (CD206)	Mannan
	Macrophage receptor MARCO	Bacteria
	Scavenger receptor A (CD204)	LPS, Lipoteichoic acid
	CD14	LPS-binding protein
	αVβ5 integrin	Apoptotic cells
Nonspecific phagocytosis	Toll-like receptors	Pathogen associated molecular patterns

In addition to receptors involved in phagocytosis, macrophages possess pattern recognition receptors (PRRs) that will recognize different microbial ligands termed microbe-associated molecular patterns (MAMPs), as well as danger-associated molecular patterns (DAMPs). There are three types of PRRs with different localizations. Membrane-bound PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and kinase receptors. Cytoplasmic PRRs comprise Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and RIG-I-like receptors (RLRs). A third and last group contains secreted PRRs such as the

mannan-binding lectin (MBL) and also the complement factors that bind to a wide range of bacteria, viruses, fungi and protozoa (Kawai and Akira, 2010).

NLR is a family of 22 cytoplasmic proteins involved in recognition of stress signals from endogenous or exogenous origins. NOD proteins recognize bacterial peptidoglycan in the eukaryotic cell. NOD1 and NOD2 are the most study NOD proteins; after activation they induce pro-inflammatory cytokine production through the NF-κB signalling pathway and can activate autophagy mechanisms (Zhong et al., 2013).

1.2.3. The Toll-Like receptor pathway and the inflammatory response

Toll-like receptors (TLR) play an important role in innate immunity and induction of the inflammatory response. Toll was first discovered in *Drosophila* and this PRR was shown to play an important role in antifungal defence (Lemaitre et al., 1996). One year later, homologues of this PRR were found in mammals, forming the TLR family (Medzhitov et al., 1997). TLRs are composed of an extracellular portion containing leucine-rich repeats that recognize MAMPs, a transmembrane domain, and the intracellular Toll/interleukine-1(IL-1) receptor (TIR) domain which mediates signal transduction into the cell (Kawai and Akira, 2010). Different TLRs recognize different microbial components or MAMPs and induce a signalling cascade, resulting in the production of pro-inflammatory cytokines. Some TLRs are present in the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6) (Figure 4) and others in endosomes (TLR3, TLR7, TLR8, TLR9) (Figure 5) (Kawai and Akira, 2010).

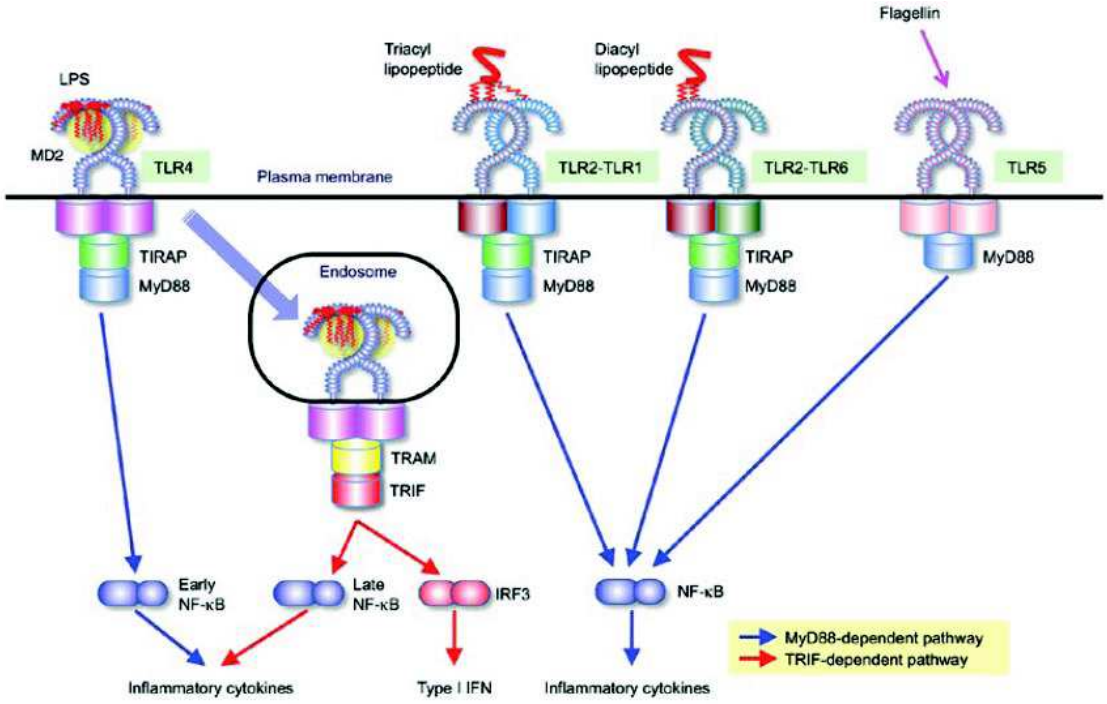

Figure 4. MAMP recognition by cell surface TLRs.

TLR1, TLR2, TLR4, TLR5 and TLR6 are cells surface receptors. The interaction with their specific MAMPs will initiate a signal transduction cascade that will activate of NF-κB or IRF3 and induce the innate immune response. TLR5 recognizes flagellin and activates NF-κB through MyD88. TLR4, TRL2/TLR1 and TLR2/TLR6 recruit TIRAP and MyD88 that also induce NF-κB activation. The TLR4--LPS complex can be internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase NF-κB for the induction of type I interferon (TRIF-dependent pathway). (Reproduced from Kawai and Akira, 2010)

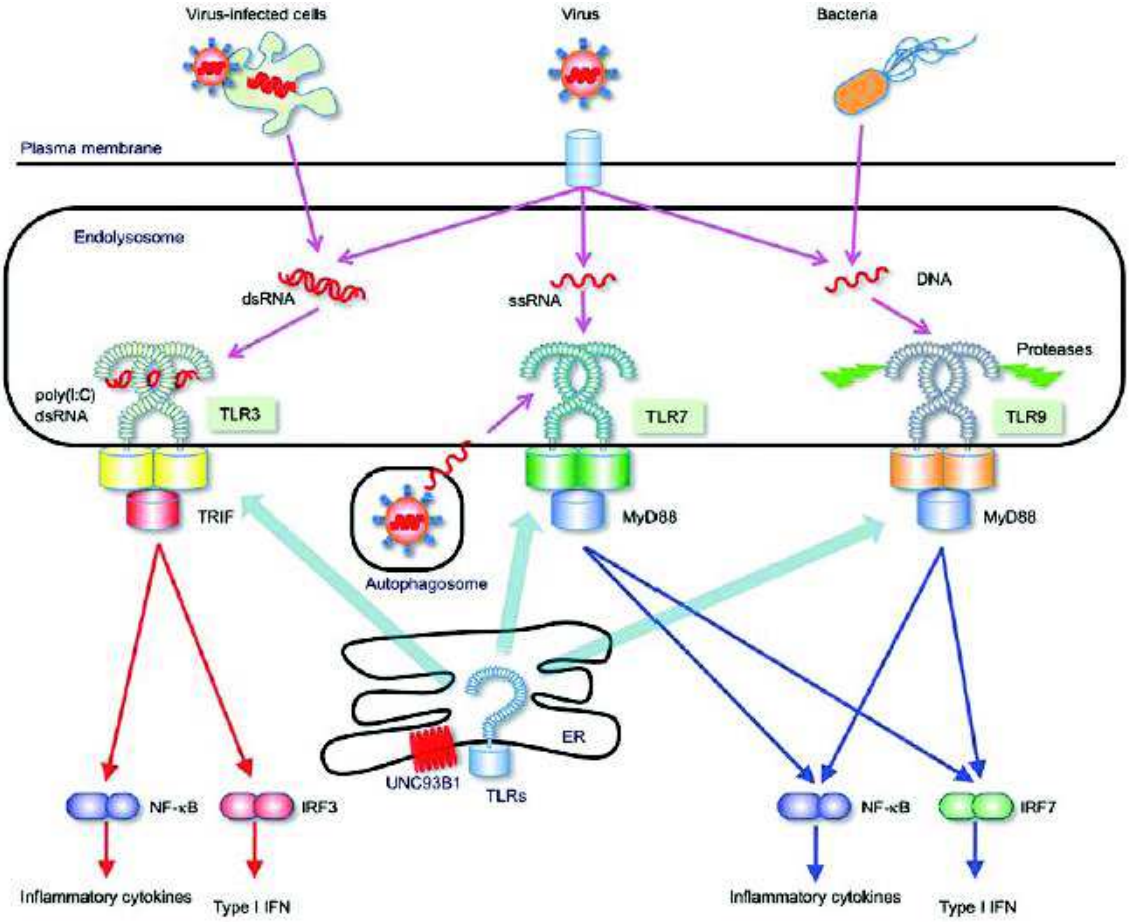

Figure 5. PAMP recognition by intracellular TLRs.

TLR3 recognizes dsRNA derived from viruses or virus-infected cells. TLR3 activates the TRIF-dependent pathway to induce type I interferon and inflammatory cytokines. TLR7 recognizes ssRNA derived from ssRNA viruses in endolysosomes and activates NF-κB and IRF7 via MyD88 to induce inflammatory cytokines and type I interferon, respectively. In addition, autophagy is involved in delivering ssRNA to TLR7-expressing vesicles. TLR9 recognizes DNA derived from both DNA viruses and bacteria. TLR9 recruits MyD88 to activate NF-κB and IRF7. (Reproduced from Kawai and Akira, 2010)

After interaction with MAMPs, TLRs induce a signal transduction cascade using different TIR adapter proteins that are recruited to distinct TLRs to mount an innate immune response. The TLR adaptor proteins are myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), MyD88 adaptor-like (Mal) or TIRAP, and SARM, the only TIR domain-containing adaptor that can negatively regulate TLR signalling (Zhang et al 2011). TLR4 is the only TLR that can use MYD88, TRIF, TRAM and TIRAP adaptors and induce both MyD88 and TRIF-dependent pathways (Figure 6).

TLR signalling leads to the synthesis and production of inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-12 and TNFα. To be active and secreted, pro-IL-1β has to be cleaved by caspase-1. Caspase-1 is activated though the action of the inflammasome, which is build-up of a sensor molecule (a NLR protein), an adaptor molecule ASC and caspase-1 (Latz et al., 2013). ASC possess an amino-terminal caspase-recruitment-and-activation domain (CARD). This CARD domain recruits procaspase-1 monomers and will form an active heterotetrameric caspase-1. Caspase-1 cleaves the proinflammatory precursor prointerleukin-1β to produce active form of IL-1β (Franchi et al., 2009; Gross, 2012). The composition of the inflammasome depends on the activating signal, for instance *Salmonella* flagellin induces the macrophage NLRC4 inflammasome that results in a potent Caspase-1 dependent IL-1β response and pyroptosis (Broz et al., 2010).

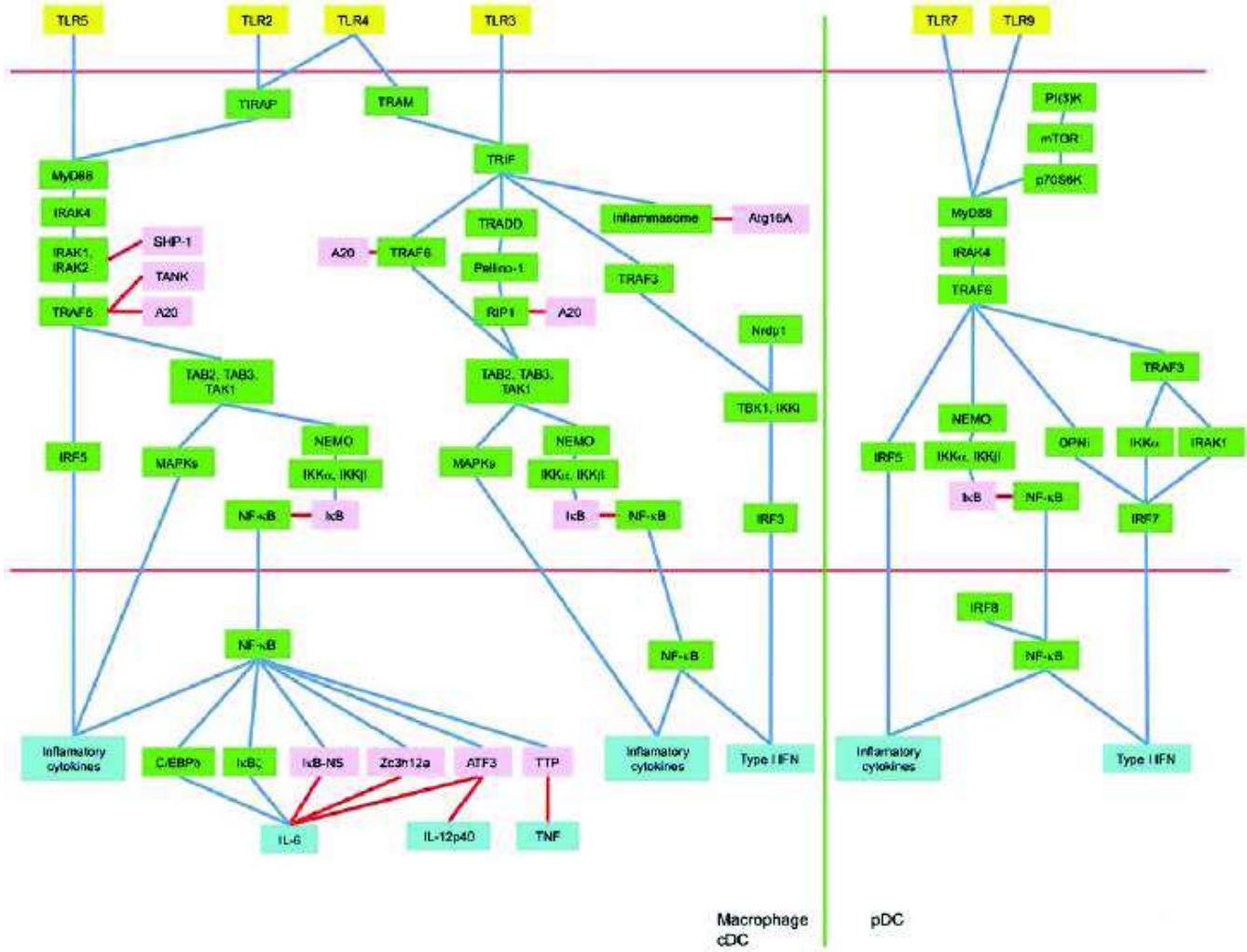

Figure 6. Overview of TLR signalling pathways.

TLR-mediated responses are controlled mainly by the MyD88-dependent pathway, which is used by all TLRs except TLR3, and the TRIF-dependent pathway, which is used by TLR3 and TLR4. TRAM and TIRAP are sorting adaptors used by TLR4 and TLR2-TLR4, respectively. The TRIF-dependent pathway leads to inflammasome activation during TLR4 signalling. Yellow, TLRs; green, stimulators; pink, negative regulators; blue, target genes. (Reproduced from Kawai T and Akira S *Nature Immunology* 2010) (Kawai and Akira, 2010)

Inflammation mediated by the activation of the complement cascade, the production of pro-inflammatory cytokines and recruitment and activation of macrophages and neutrophils, is the major activity of the innate immune response to microbial invasion and damaged cells. Resolution of inflammation is crucial to restore tissue integrity and function. Failure to resolve inflammation processes results in dramatic tissues damage, organ failure and even death of the host. Under normal conditions, neutrophils that go in apoptosis express specific surface markers as “eat me” or “find me” signals, which leads to their controlled elimination by pro-inflammatory M1 macrophages in a process called efferocytosis. During inflammation, ROS production by neutrophils reduces their life time, and triggers pre-mature apoptosis, as part of the resolution process (Geering et al 2011). After phagocytosis of apoptotic neutrophils, these macrophages switch to the M2 type, characterized by an anti-inflammatory programme, including the secretion of IL10 and TGF-β, and promote return to tissue homeostasis (Ortega-Gómez et al., 2013). Any problems to resolve inflammation can result in disease and damage.

1.2.4. The complement system

The complement system in humans consists of over 30 serum proteins produced by hepatocytes and plays a crucial role in immunity (Rus et al., 2005). The complement system has four major junctions: opsonisation (C3b fragment, binds extracellular pathogens), chemotaxis (C5a, attracts phagocytic cells), accretion (C3a and C5a, induce mast cell degranulation) and cell lysis with the formation of the MAC (C5b, C6, C7, C8, and polymeric C9). There are three different pathways for activation of the complement system:

the classical complement pathway, the alternative complement pathway and the lectin pathway (See Figure 7). The classical pathway is activated by the interaction of the C1q fragment with IgM or IgG complexed with antigens. The alternative pathway is constantly activated at low level and does not need antibodies for activation, and it takes an important place in the innate immune response. The lectin pathway is homologous to the classical pathway but it is activated by the binding of mannose to mannose-binding lectin (MBL) instead of C1q (Rus et al., 2005).

1.2.5. Avoidance of host immune responses by bacterial pathogens

The immune system has evolved with a selective pressure from microorganisms that colonize the host. In return, certain viruses, bacteria, fungi and parasites that have co-evolved with their host have also developed mechanisms to avoid and counteract the host immune response. Opportunistic and environmental micro-organisms may be pre-adapted to survive the different host environments, or adapt during the relatively short infection periods in a specific host. We can classify these mechanisms in five major groups; structural modification that helps escape recognition by TLRs, the immunosuppression process, escape from phagocyte killing, evasion of autophagy, and evasion of neutrophil killing by NETs and ROS.

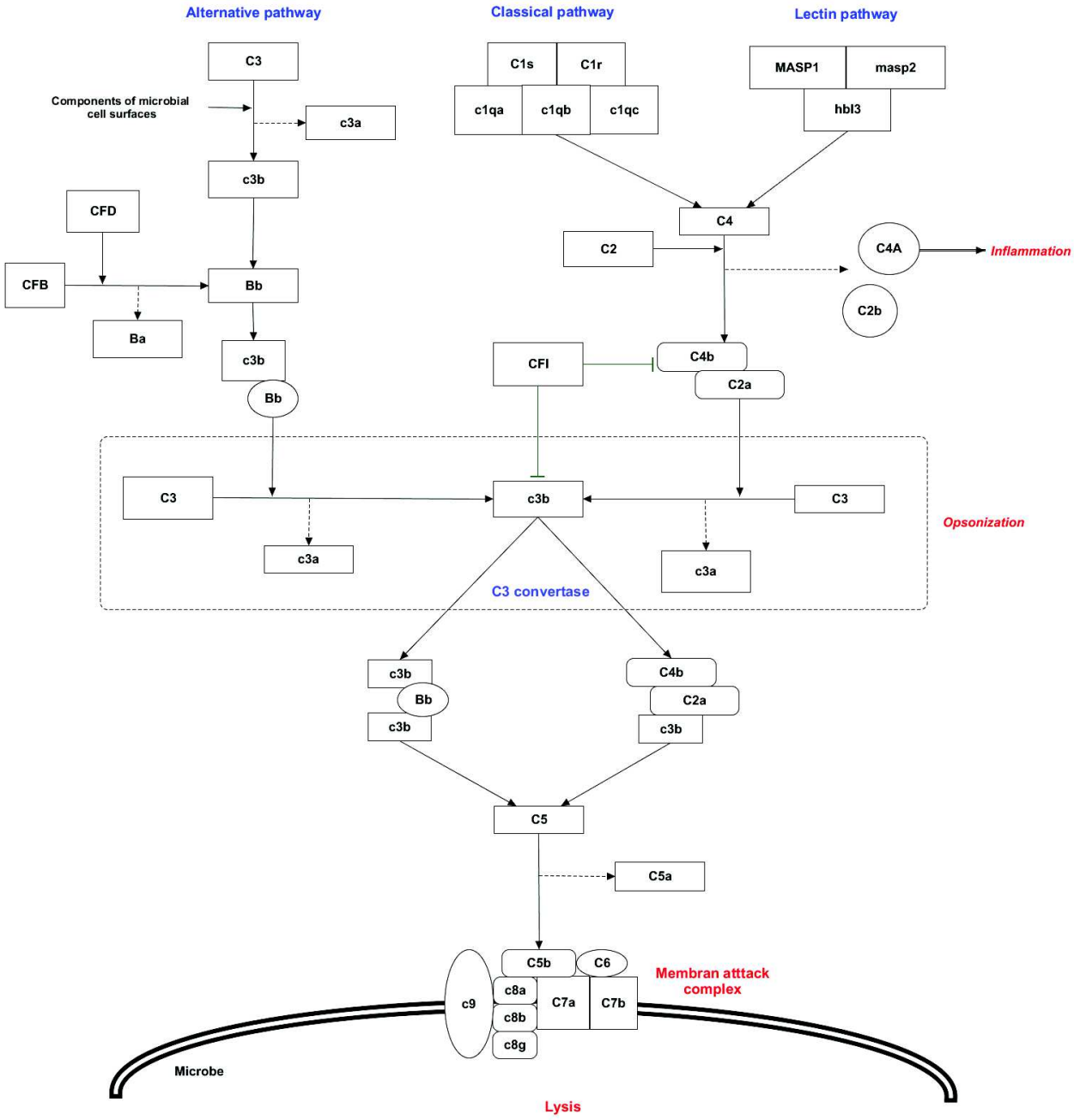

Figure 7. The complement system

The complement system can be divided in three different pathways: the classical pathway activated by interaction with IgM or IgG; the alternative pathway that is constantly activated and takes an important part in the innate immune response; the lectin pathway, homologous to the classical pathway but activated by interaction with mannose-binding lectin.

1.2.5.1. How not to be recognized by TLRs

The recognition of MAMPs by TLRs is crucial for induction of the innate immune response and pro-inflammatory cytokine production. TLR4 is the best characterised TLR and recognises lipopolysaccharide (LPS). LPS is a large molecule composed of three parts; the O antigen, the core oligosaccharide and the lipid A. LPS is an essential component of the Gram-negative bacterial outer membrane. The lipid A is the most toxic part of the LPS; upon bacterial lysis excess LPS can cause endotoxic shock. Modification of the lipid A could change the regulation of the inflammatory process. For example, the lipid A of *Shigella flexneri* promotes inflammation and facilitates cell invasion (D'Hauteville et al., 2002). In contrast, the lipid A of *Salmonella* and *Yersinia pestis* reduces immunostimulation and increases avoidance of inflammation (Kawasaki et al., 2004; Rebeil et al., 2004). Interestingly, *Porphyromonas gingivalis* can synthesize multiple forms of lipid A and avoid host recognition (Darveau et al., 2002).

1.2.5.2. Immunosuppressive actions

Another mechanism is to immunosuppress the innate immune response. *Y. pestis* and *Mycobacterium tuberculosis* for example can actively down-regulate IFNγ production with the help of secreted bacterial effector proteins. *Y. pestis* injects type III secretion system (T3SS) effectors directly into the host cell that block macrophage phagocytosis and alter the inflammatory response (Portnoy, 2005). This immunomodulation results in acute infection. In contrast, *M. tuberculosis* induces a persistent infection with granuloma formation with a balance between high inflammation and infection control. Other pathogens, including

Brucella spp, introduce effector proteins into the host cell, where they directly interfere with TLR-mediated signalling cascades to suppress innate immune responses (for review see Johannessen et al 2013).

1.2.5.3. Avoidance of phagocytic killing

Phagocytosis is one of the most important processes to maintain host homeostasis and defence. Intracellular pathogens use different strategies to escape from phagocytic killing, including modification of phagosome maturation (Figure 8), escape from the phagosome and the phagocytic cell (Figure 9), and persistence in the phagolysosome at low pH.

To modify the phagosome maturation process the most common mechanism is to avoid fusion between the phagosome and endocytic vesicles and lysosomes. This mechanism is used by different bacteria, fungi and parasites, which have each been shown to block phagosome maturation at different stages (see Smith and May, 2013). Intracellular pathogens often use bacterial effector proteins, secreted into the host cell through T3SS or T4SS to modulate host cell biology and promote bacterial survival (Angot et al., 2007). For instance, *Legionella pneumophila* uses an arsenal of T4SS effector proteins to build an intracellular replication niche. One of these, VipD, has recently been shown to be involved in depleting endosomes from PI(3)P in a Rab5-dependent manner. This results in an altered protein composition of endosomes, which blocks further fusion with *Legionella*-containing vacuoles (Gaspar and Machner, 2014).

With the help of T4SS effectors, *Brucella melitensis* modifies the phagosome and forms an ER-like replicative vacuole by interacting with ER exit sites (ERES)(Celli, 2015). Escape from

the phagosome into the cytosol is another strategy adopted by for instance *Burkholderia pseudomallei* and *Francisella tularensis. S. flexneri, Listeria monocytogenes* and *Rickettsia ricketsii* developed a very smart mechanism to escape from the phagosome and invade other cells by recruiting host actin to form a tail that permits bacterial motility. *Cryptococcus neoformans,* a fungus that can cause lethal infection in immunocompromised patients, can escape from the phagocytic cell by vomocytosis or phagosome extrusion/expulsion. Vomocytosis is a new non-lytic escape mechanism induced by fungi and a similar process has been described for *Candida albicans* (Smith and May, 2013). And finally, some bacteria like *Streptococcus agalactiae* or *B. melitensis* , are capable of resisting to acidic conditions, allowing them to persist and multiply inside acidified phagosomes.

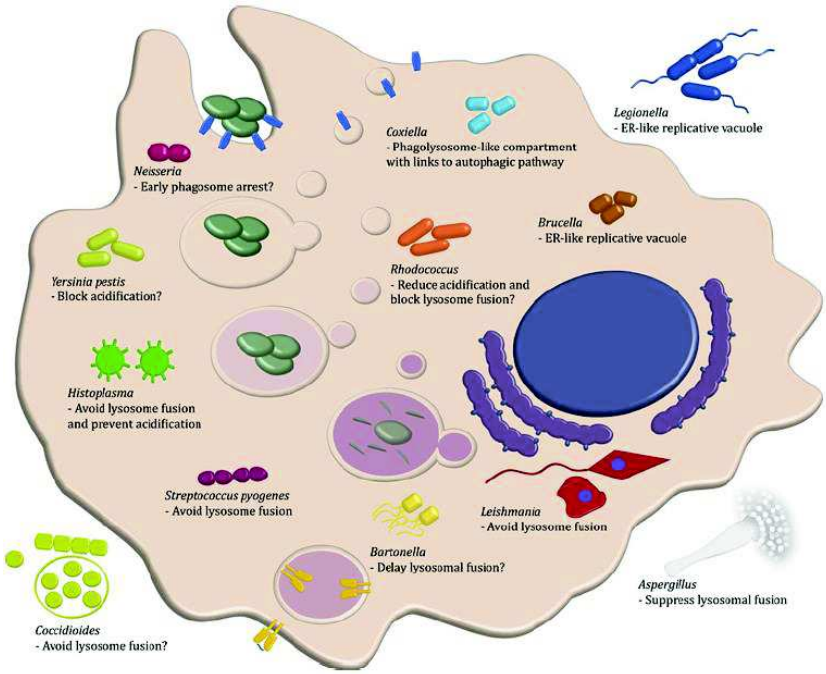

Figure 8. Strategies used by different pathogens to modify phagosome maturation.
Many pathogens are able to manipulate the phagosome. (Reproduced from Smith LM and May RC *Biochemical Society Transaction* 2013) (Smith and May, 2013)

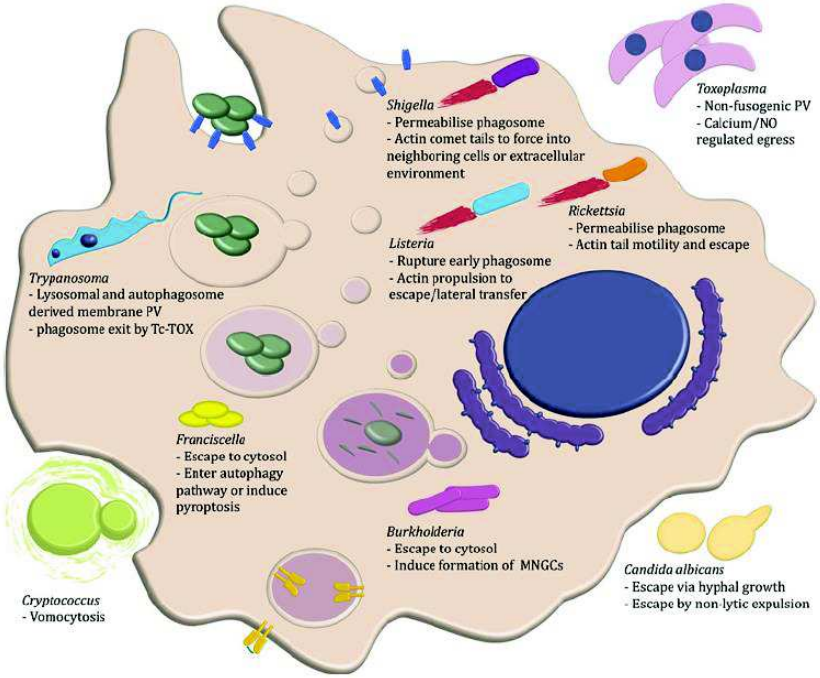

Figure 9. Strategies used by pathogens to escape the phagosome and phagocyte.
There are a surprising number of pathogens that are able to escape either the phagosome or the phagocytic cell entirely. (Reproduced from Smith LM and May RC *Biochemical Society Transaction* 2013) (Smith and May, 2013)

1.2.5.4. Escape from the autophagy pathway

Escape from the autophagy pathway, either by evasion or inhibition, is another important mechanism to avoid the immune response. *S. flexneri* and *B. pseudomallei* inject T3SS protein effectors IcsB (Ogawa et al., 2005) and BopA (Gong et al., 2011), respectively that helps them escape from the autophagosome. *L. monocytogenes* recruits the host cell proteins Arp2/3 complex and Ena/VASP in order to evade the autophagy recognition with the help of the ActA bacterial protein (Yoshikawa et al., 2009). Absence of ActA resulted in ubiquitination, and recognition by the host proteins p62 and LC3, as part of the autophagy process.

1.2.5.5. Specific mechanisms against neutrophil killing

Many intracellular and/or opportunistic bacteria are able to escape from macrophage recognition and killing, and create a replicative niche in these cells. Neutrophils also play an important role in control of microorganisms, however, few pathogens, including *Y. enterocolitica* and *Ehrlichia* have evolved ways to survive or even replicate in these cells (Allen, 2003). In addition to their ability to phagocytose, neutrophils produce NETs, ROS and granules to kill pathogens. A strain of Group A *Streptococcus* produces DNAse that destroys the NET matrix, allowing the bacteria to survive this host defence mechanism (Buchanan et al., 2006). *S. pneumoniae* and *Staphylococcus aureus* also produce endonucleases that allow them to evade ensnarement by NETs (Beiter et al., 2006; Berends et al., 2010). *P. aeruginosa* also produces scavengers of ROS that aloid to avoid neutrophil killing (Genestet et al., 2014).

1.2.6. The innate immune response of CF and CGD patients

CF is the most common genetic disease in the Caucasian population and it is caused by a mutation in a gene encoding a membrane-embedded chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (Lubamba et al., 2012). The most common mutation is a small deletion (3 nucleotides) in the CFTR gene, CFTR ΔF508, which is present in 70% of the patients. Although the disease affects many organs, the lungs are the major affected organ, congested by mucus, and the pancreas has an abnormal function. Loss of function of this chloride channel causes a reduction in innate immune responses and a mucus accumulation in the airways. An excessive neutrophil-mediated inflammation develops in lung patients with several exacerbation phases. The immune system is unable to

efficiently resolve inflammation that results in important tissue damage. Patients are highly susceptible to chronic pulmonary bacterial infections that increase morbidity and mortality of these patients. A major pathogen of CF is *Pseudomonas aeruginosa.* A non-functional CFTR also has an important impact on neutrophil activity. The CFTR mediates chloride transport into neutrophil phagosomes and it has been shown that CF neutrophils were defective in killing of *Pseudomonas aeruginosa* (Painter et al., 2006). Moreover, CF neutrophils have an excessive and prolonged superoxide production when they are stimulated and are unresponsive to anti-inflammatory signals through IL-10 that have as consequence an excessive inflammatory response and significant tissue damage (Allen, 2003; Jundi et al., 2015; Pedersen et al., 1990). It has recently been shown that the CFTR defect in patients with a ΔF508 and/or G551D mutation caused decreased cytosolic magnesium levels that resulted in decreased levels of GTP-bound Rab27a (Pohl et al., 2014). Defects in Rab27 activation affected degranulation of type 1 and 3 granules, reducing the killing effect of neutrophils. The defect in degranulation was corrected by using the ion channel potentiator invocator (Pohl et al., 2014).

Chronic Granulomatous disease (CGD) is a genetic disease caused by a non-functional phagocyte NADPH oxidase complex. Mutation in one of the five genes encoding the subunits of NADPH oxidase complex result in non-active NADPH oxidase and deficient ROS production, and makes patients more susceptible to infection by ineffective pathogen clearance. Bacteria belonging to the *Burkholderia cepacia* complex are specifically virulent for CGD patients.

The most common mutations are in the transmembrane subunit $gp91^{phox}$ or in $p47^{phox}$. The ion flux of for instance Ca^{++} or Cl^{-} inside cells is also important for NADPH oxidase activity (Dewitt et al., 2003) and for fusion of granules with the phagosome (Jaconi et al., 1990). It was shown that NETosis depends of the presence of neutrophil elastase, myeloperoxidase (Metzler et al., 2011; Papayannopoulos et al., 2010) and active NADPH oxydase (Hakkim et al., 2011). Thus, in CGD patients, NETosis has a minor role in host defence.

Part II – The zebrafish embryo model

2.1. General description

During the last decade, a new non-mammalian vertebrate animal model, the zebrafish (*Danio rerio*), has emerged as a powerful complementing model to study host-pathogen interactions and the host immune response during microbial infection (Meijer et al., 2013; Sullivan and Kim, 2008; Torraca et al., 2014). It has been shown to be a good model for pathogens of both human and fish including *Mycobacterium marinum* (Davis et al., 2002), *Salmonella enterica* Typhimurium (Sar et al., 2003), *Listeria monocytogenes* (Levraud et al., 2009), *Staphylococcus aureus*, *Aeromonas salmonicida* (Lin et al., 2007) and *Burkholderia cenocepacia* (Vergunst et al., 2010) for bacterial infection; Chikungunya Virus (Palha et al., 2013) and viral haemorrhagic septicemia virus (Encinas et al., 2010) for viral infection; *Candida albicans* (Chen et al., 2013) and *Aspergillus fumigatus* (Knox et al., 2014) for fungal infection; *Toxoplasma gondi* (Sanders et al., 2015) for parasitic infection.

Studies have shown that the zebrafish immune system is highly similar to that of humans: the innate immune response consisting of the inflammatory response, pro-inflammatory cytokine production, Toll-Like receptor (TLR) signalling (Meijer et al., 2004), the complement system (Zhang and Cui, 2014) and phagocytic cells (neutrophils and macrophages), are present in fish from early stage (28-30 hpf)(Herbomel et al., 1999; Lieschke, 2001). The adaptive immune system is functional 4-6 weeks after fertilization (Figure 10). The temporal separation of adaptive and innate responses allows the study of infections in the young embryos in the presence of an innate immune system with no adaptive immunity. Many genetic tools have been developed, such as gene knockdown technology using antisense morpholinos (MO), overexpression using mRNA injection, transgenesis with the Tol2 transposon system, and generation of knockout mutants using CRISPR/CAS or TALEN technologies (Heintze 2013 – Hwang 2013). A key asset is the transparent nature of the embryos which allows real time visualisation of host pathogen interactions using transgenic reporter fish and fluorescent bacteria in a manner that is unprecedented in other models (Tobin et al., 2012). Although zebrafish specific antibodies are only now becoming available in large numbers, other useful cellular and molecular approaches have been developed (qRT-PCR, whole mount in situ hybridization, immune fluorescence (Cui et al., 2011; Trede et al., 2004). The small size of the embryos makes it a perfect model for high throughput studies, including following disease progression, host transcriptomic analysis by microarrays or RNAseq, and drugs screens (Carvalho et al., 2011; Hegedus et al., 2009; Spaink et al., 2013; Stockhammer et al., 2009; Tan and Zon, 2011; Veneman et al., 2013; Yang et al., 2013).

2.2. Vertebrate model to study bacterial, viral, fungal and parasitic infections

The zebrafish embryo has been shown to be an excellent model for biomedical research, and the study of human infectious disease. Pioneering work by the Ramakrishnan group has shown that granulomas, long thought to be the result of a host defence mechanism against mycobacterial infection, may in fact facilitate bacterial spread (Davis and Ramakrishnan, 2000). It is now clearly recognized that the zebrafish embryo is a very interesting model for tuberculosis and mycobacterial infection (Cronan and Tobin, 2014; Ramakrishnan, 2013). The zebrafish model has improved our understanding of early granuloma formation and the crucial role macrophages play in this (Torraca et al., 2014). More recently it was shown, for example, that *Mycobacterium* preferentially recruits permissive, but not microbicidal macrophages by using cell-surface-associated phithocerol dimycoceroserate (PDIM) lipids to cover underlying PAMPs (Cambier et al 2014).

Until now, zebrafish model has been developed to study infection caused by four human viruses: *Herpes simplex* virus-1 (Burgos et al., 2008), Hepatitis C virus (Ding et al., 2011, 2015), Chikungunya virus (Palha et al., 2013) and recently Influenza A virus (Gabor et al., 2014). This model permits studying the virology and the host response to infection. Viral gene expression and viral burden could be quantified while analysis of host mortality, histological modification and immune response (phagocyte behaviour and the IFN signalling pathway) showed the evolution of the viral infection in the *in vivo* context (Goody et al., 2014). In addition to viruses, the fungi *Candida albicans* (the most used and studied) (Torraca et al., 2014), *Penicillium marneffei, Aspergillus fumigatus* and *Cryptococcus neoformans* (Gratacap and Wheeler, 2014) also infect zebrafish, and these new models have

shown how the fungi interact with host phagocytes. Very recently, a zebrafish model was developed to study infection caused by *Toxoplasma gondii,* one of the most prevalent protozoan parasites (Sanders et al., 2015). Zebrafish embryos can also be used for high throughput drugs screening against pathogens (Bowman and Zon, 2010; Ding et al., 2011; Tan and Zon, 2011).

Earlier, we have shown that differences in virulence caused by different *Burkholderia cenocepacia* complex strains (Bcc) strains could be observed in zebrafish embryos. Several epidemic CF isolates, including *B. cenocepacia* K56-2, J2315 and BC1 caused acute infection that became rapidly fatal, whereas other isolates including *B. stabilis* LMG14294 and *B. vietniamensis* FC441 caused persistent infections. We also showed that *B. cenocepacia* could survive and multiply in macrophages prior to systemic fatal infection (Vergunst et al., 2010). These results suggest that zebrafish embryos are a promising model to study the intracellular life of Bcc bacteria in an *in vivo* model, as well as the host innate immune response during *Burkholderia* infection especially the role played by macrophages and neutrophils.

2.3. The zebrafish innate immune response

2.3.1. Zebrafish haematopoiesis and macrophage activity

The zebrafish immune system is highly similar to the human immune system (Meeker and Trede, 2008; Renshaw and Trede, 2012; van der Vaart et al., 2012). Haematopoiesis, also

with high similarity to the human process, can be divided in two different phases for the production of immune cells, and at distinct anatomical localisations during fish development (Jagannathan-Bogdan and Zon, 2013; Stachura and Traver, 2011)(Figure 10). The primitive haematopoiesis takes place in the rostal blood island (RBI) and the intermediate cell mass (ICM). Primitive macrophages and neutrophils start to be produced in the RBI at 18-22 hours post fertilization (hpf). They are able to phagocytose and kill bacteria from around 28 hpf (Herbomel et al., 1999). Mast cells are mainly produced in the RBI and *cpa5*, a mast cell marker, has been detected as early as 24 hpf. Few *cpa5* positive cells are found in the ICM. The ICM also produces erythrocytes. The PBI produces erythromyeloid progenitors (EMPs) that will differentiate in erythrocytes and myeloid cells (macrophages, mast cells and neutrophils) (Bertrand et al., 2007). Neutrophils have been shown to contain granules from 48 hpf (Le Guyader et al., 2008; Lieschke, 2001). The definitive hematopoietic stem cells (HSCs) start to be produced in the ventral wall of dorsal aorta (VDA) and are able to differentiate into myeloid cells. The definitive haematopoiesis is the last phase of hematopoietic development. HSC migrate from the caudal hematopoietic tissue (CHT) and colonize the kidney at 3 and half dpf. HSC can finally generate all cell types: erythrocytes, myeloid cells and lymphocytes (Jin et al., 2009). During this step, the thymus is developing and the first early lymphocytes appear (4 dpf). Eight dpf, dendritic cells are produced and can help other immune cells (Wittamer et al., 2011). Finally, 4 to 6 weeks post fertilization the adaptive immune system is fully developed and fully functioning (Lam et al., 2004). At 22 hpf, the first primitive macrophages appear, these primitive cells are still able to proliferate (Herbomel et al., 1999). Macrophage precursors are released in the blood circulation, colonize all tissues and will differentiate in tissue macrophages. Macrophages participate to

the host development by degrading apoptotic bodies, extracellular matrix with mmp proteins. Zebrafish macrophages have the same functions as human macrophages, they are able to recognize and phagocytose pathogens through TLRs and can produce pro-inflammatory cytokines in order to attract and activate other immune cells (Kanwal et al., 2014). After phagocytosis, they produce reactive oxygen and nitrogen species, antimicrobial compounds and proteases.

2.3.2. Zebrafish embryo neutrophil function

Neutrophils appear at ~33 hpf during the intermediate haematopoiesis. Zebrafish neutrophils are very similar to mammalian neutrophils and have similar morphological, biochemical and functional features. They have a polymorphic nucleus with two lobes compared to human neutrophils that have three lobes. They have primary, secondary and tertiary granules, myeloid-specific peroxidase (mpx) equivalent to MPO in mammals (Lieschke, 2001), and a functional NADPH oxidase to produce ROS (Brothers et al., 2011). Zebrafish neutrophils are able to phagocytose and kill pathogens, as in humans, from 30 hpf. Importantly, Colucci-Guyon and colleagues showed that neutrophils need a surface for efficient phagocytosis, and do not efficiently phagocytose liquid borne microbes. NET formation has also been detected in neutrophils, isolated from fish in *in vitro* assays (Colucci-Guyon et al., 2011; Palić et al., 2007).

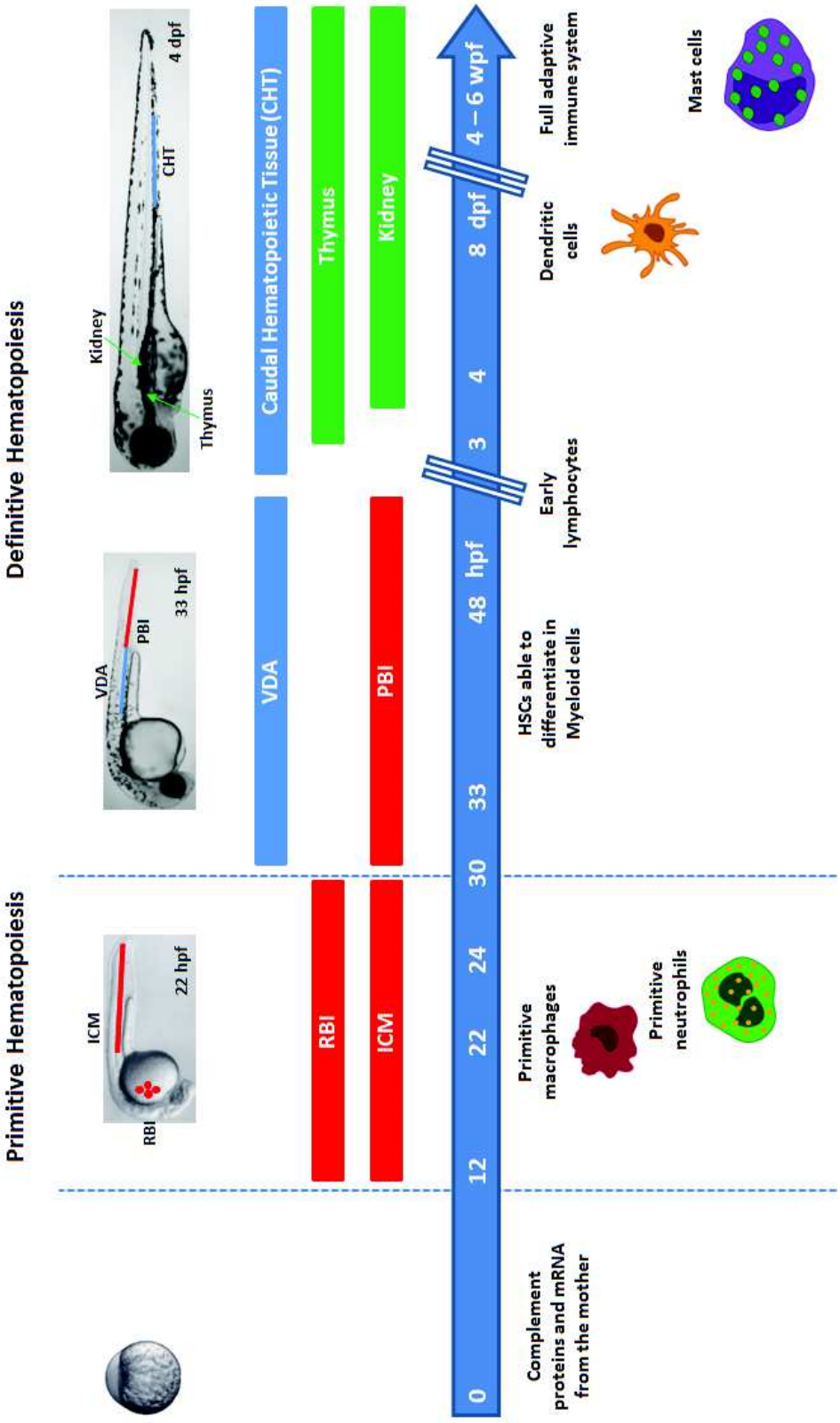

Figure 10. Zebrafish haematopoiesis and immune system development.

RBI: Rostral blood island; ICM: Intermediate cell mass; VDA: Ventral wall of dorsal aorta; PBI: Posterior blood island.

2.3.3. The complement system of zebrafish

The complement system of zebrafish is structurally and functionally similar to the mammalian complement system. All orthologues of the mammalian complement proteins, including receptors and regulatory proteins, have been found in zebrafish (Boshra et al., 2006) (Zhang and Cui, 2014). The three different activation pathways (classical, alternative and lectin pathway) have been shown to be functional in zebrafish. Due to maternal transfer of complement proteins and mRNA, the alternative pathway is active early in the embryo development and induces an immune-protection against extracellular bacteria (Wang et al., 2009). Zhang S and Cui P (2014) provide an extensive comparison between the human and zebrafish complement system (Zhang and Cui, 2014).

2.3.4. The TLR pathway and the inflammatory response of zebrafish embryo

TLR signalling is one of the major arms of the innate immune response. Orthologues of most of the human TLRs have been found in zebrafish except TLR6-10-11-12 and 13, but as with other fish species, zebrafish possess several fish-specific TLRs (Figure 11), with a total of 6 non-mammalian TLRs (Tlr14, Tlr18, Tlr19,Tlr20 in four copies, Tlr21 and Tlr22) (Matsuo et al., 2008; Meijer et al., 2004; Pietretti et al., 2014; Yeh et al., 2013). Also some of the mammalian TLR counterparts, including *Tlr4*, *Tlr5* and *Tlr8*, have been duplicated in fish (Jault et al., 2004; Meijer et al., 2004; Sullivan et al., 2009). TLR4 recognition is specific in zebrafish, and it seems that TLR4 is not involved in LPS recognition in a similar way as in humans and fish are highly tolerant to LPS (Novoa et al., 2009). However, contradictory

results have been reported about the implication of Myd88 in LPS response in fish: LPS has been shown to activate a MyD88-independent pathway, and, in contrast, to negatively regulate the MyD88-dependent pathway (Sepulcre et al., 2009). Also, *md2*, *cd14* and *lipid A binding protein* genes have not been detected in zebrafish and could explain the tolerance of zebrafish to bacterial LPS. However, van der Vaart et al showed that the expression of both *il1b* and *mmp9* after injection with LPS was significantly lower in m*yd88* mutant embryos compared with wild types showing that that recognition of LPS in zebrafish embryos requires MyD88 (van der Vaart et al., 2013). The TLR5 receptor recognizes the flagellin protein as in humans (Kanwal et al., 2014), and its dependency on Myd88 was shown using *myd88* mutant fish (van der Vaart et al 2013).

Recently, three studies have shown that zebrafish embryos are a good model to study autophagy activated by TLR signalling, another defence mechanism against intracellular bacteria (Hosseini et al., 2014; Mostowy et al., 2013; van der Vaart et al., 2014).

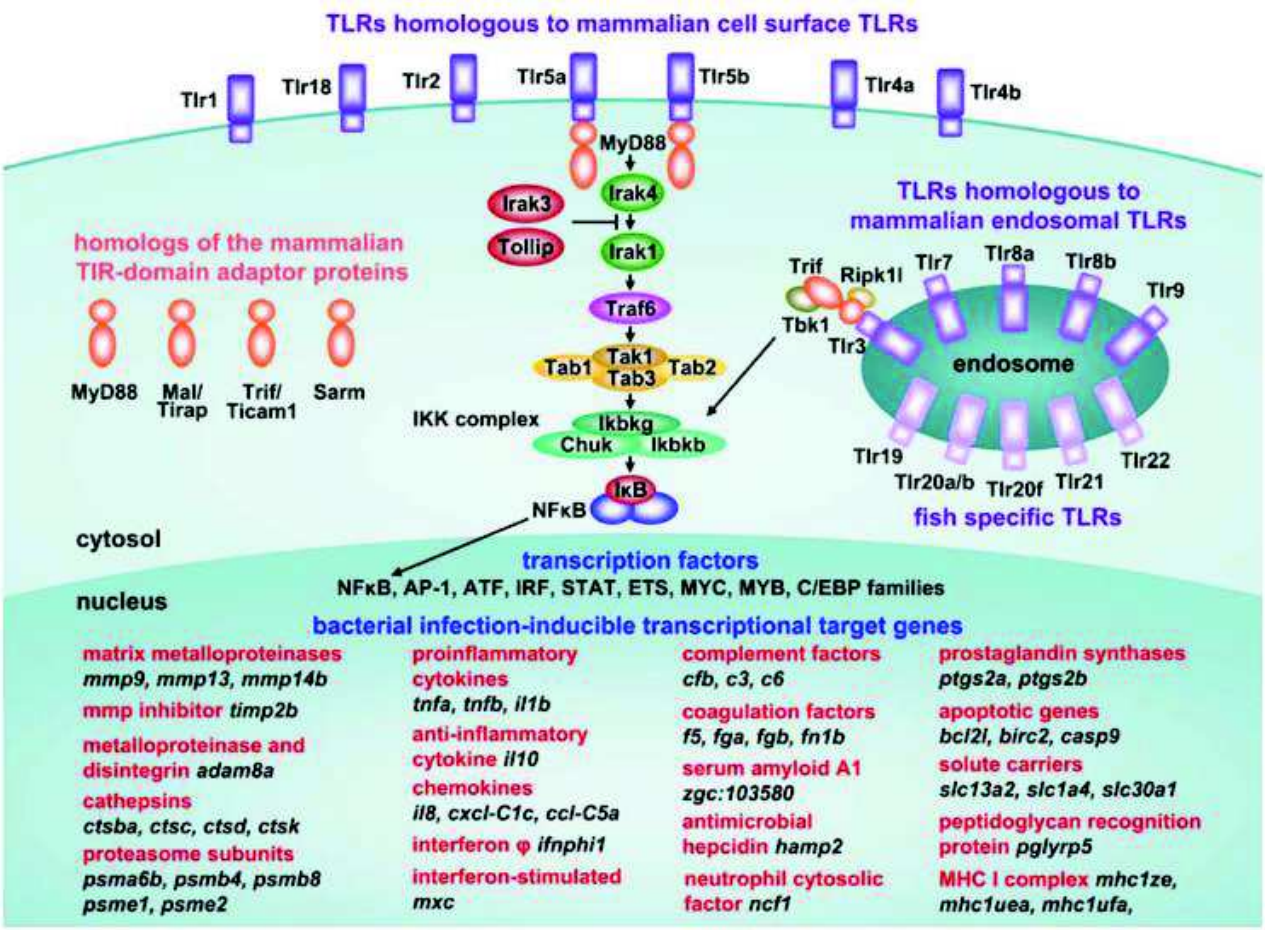

Figure 11. Components of the TLR pathway and genes commonly induced during the innate immune response of zebrafish to bacterial infection.

Annotation of the zebrafish TLRs is based on Meijer *et al* (Meijer et al., 2004). Cell surface or endosomal localizations of the zebrafish TLRs are hypothetical, based on localization of their mammalian homologs. The fish specific TLRs are tentatively placed on the endosome, since members of this group have been shown to recognize DNA or RNA ligands similar to the mammalian endosomal TLRs. (Reproduced from Meijer et al 2004).

Part III – The *Burkholderia cepacia* complex and interaction with the immune system

3.1. Description of the Bcc

The *Burkholderia cepacia* complex is a group of opportunistic pathogens, now containing 18 species: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia*, *B. ubonensis*, *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica*, *B. contaminans*, *B. lata* and *B. uronensis* (Papaleo et al., 2010; Vanlaere et al., 2009) (Figure 12). In 1948, Walter Burkholder described a plant pathogen and the causative agent of sour skin in onions, and proposed to name it *Pseudomonas cepacia* (Burkholder W, 1950), which was renamed in 1992 into *B. cepacia* (Coenye et al., 2001). Bcc species can live in diverse ecological niches and can be pathogenic for plants (Jacobs et al., 2008) but they can also be beneficial for plant growth or live in symbiosis with sugar cane for example (Govindarajan et al., 2006; Ramette et al., 2005). *B. cepacia* is also known for its use in bio-remediation of polluted soils (Chavan and Mukherji, 2008). Like other environmental bacteria, Bcc are highly resistant to antibiotics and stress conditions. Recently, Agnoli *et al* showed that the third chromosome, which they suggested to be a large virulence plasmid, pC3, rather than a chromosome, is present in almost all Bcc strains and is important for virulence. It carries genes that encode factors that increase bacterial resistance to different types of stress, including oxidative stress, osmotic stress, high-temperature and chlorhexidine-induced stress (Agnoli et al., 2011; Agnoli et al., 2014).

To classify Bcc species, different methods were developed. Sequence polymorphisms in the *recA* gene permit to differentiate most of the species (Mahenthiralingam et al., 2000). Multilocus sequence typing (MLST) was based on the polymorphism of seven housekeeping genes (*atpD, gltB, gyrB, recA, lepA, phaC* and *trpB*) and permits a better identification and determination of an allelic profile which can be used to assign a clonal sequence type (ST) (Baldwin et al., 2005) (Figure 12). Based on *recA* sequencing, *B. cenocepacia* (previously Genomovar III) can be divided into four groups, IIIA, IIIB, IIIC and IIID. More recently, another technique has been used to differentiate between Bcc species, which is based on sequence analysis of the *hisA* gene (Papaleo et al., 2010).

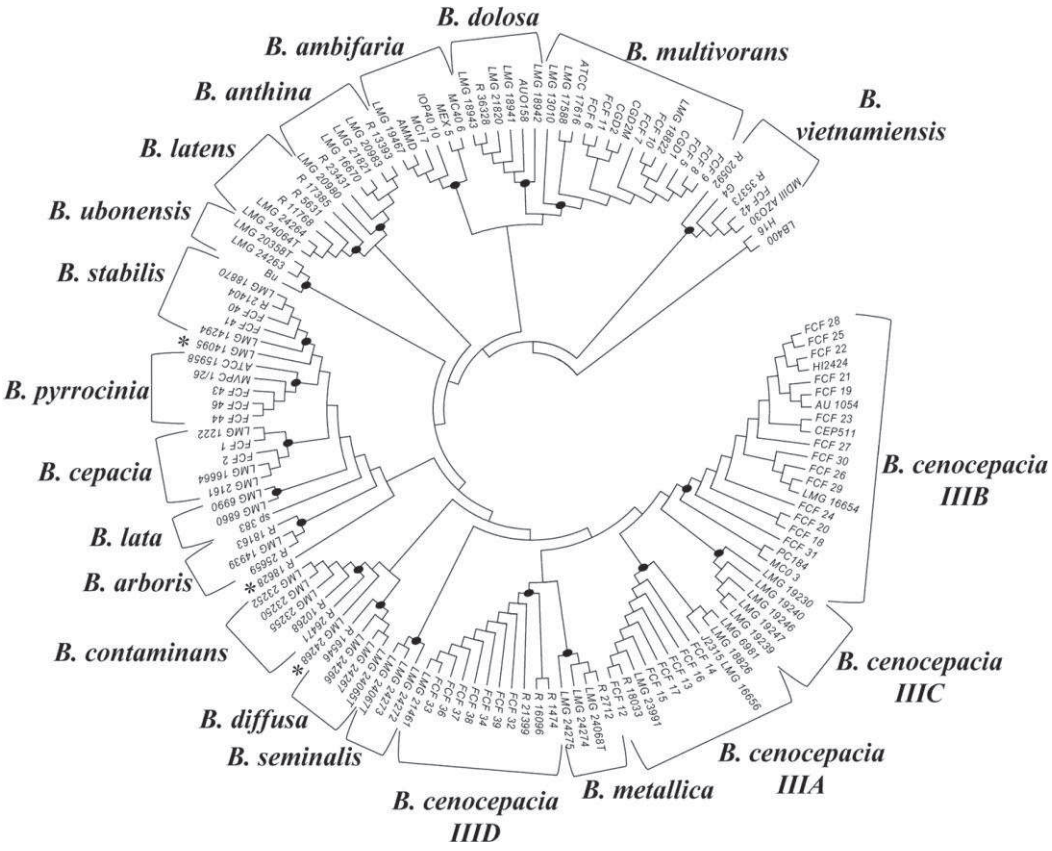

Figure 12. Phylogenetic tree constructed using the nucleotide sequence of the 442 bp hisA region from 132 Bcc strains. The orthologous sequences from *B. xenovorans* LB400 and *R. eutropha* H16 were used as outgroups. (Reproduced from Papaleo et al., 2010)

3.2. *B. cenocepacia,* an opportunistic pathogen

In immunocompromised persons, mainly CF and CGD patients, Bcc can cause chronic infections, with periods of exacerbation. Infection with bacteria belonging to the Bcc is worrisome, since they can cause sudden and unpredictable acute infection with often fatal necrotizing pneumonia, sometimes associated with septicaemia, known as the "cepacia syndrome". Most Bcc members were isolated from CF patients and from the environment except *B. latens* and *B. metallica* that were detected only in CF patients (Vial et al., 2011). Several highly transmissible strains have caused important epidemic outbreaks in Canadian and European CF populations (Drevinek and Mahenthiralingam, 2010). Most *B. cenocepacia* isolates from CF patients belong to former genomovars IIIA and IIIB, and include the major epidemic lineages. One of these *B. cenocepacia* lineages is known as Electrophoretic Type-12 (ET-12), and caused epidemics amongst patients in the UK and in Canada. Two clonal and highly virulent ET-12 isolates, J2315 and K56-2, are often used to study Bcc infection and bacterial virulence. *B. cenocepacia* and *B. multivorans* are the most prevalent species isolated from patients in Europe and North America (Drevinek and Mahenthiralingam, 2010). Bcc bacteria are highly resistant to antimicrobial peptides produced by immune cells such as neutrophils and have intrinsic multiple drug resistance, which causes major problems for treatment of patients (Kooi and Sokol, 2009; Loutet and Valvano, 2010). Currently it is not known what causes the transition of the bacteria from chronic to acute, sometimes fatal infection. Different model systems are used to better understand the bacterial virulence factors, immune signalling pathways and intracellular survival strategies in order to find new drug targets and antimicrobial treatments.

3.3. Models used to study *Burkholderia cepacia* complex infection

3.3.1. *In vitro* models

To study *Burkholderia cepacia* complex infection and virulence, several *in vitro* and *in vivo* models have been developed. It has now been firmly established using *in vitro* cell culture models that Bcc are intracellular pathogens (Burns et al., 1996; Saini et al ., 1999; Lamothe et al., 2007; Martin and Mohr, 2000; Sajjan et al., 2008; Schmerk and Valvano, 2012). Importantly, a role of intracellular stages in persistence and invasiveness of the bacteria during infection has been suggested by clinical studies (Sajjan et al., 2001; Schwab et al., 2014). Cell models have been used for the study of Bcc infection and identification of virulence factors. Airway epithelial cells from CF or healthy patients are often used for *in vitro* studies (Burns et al., 1996; Schwab et al., 2002). Some Bcc species have been shown to induce cell apoptosis. A haemolysin produced by *B. cepacia* can induce apoptosis in non-infected human neutrophils and in the mouse-derived macrophage cell line J774 (Hutchison et al., 1998). *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. vietnamiensis* increased cell apoptosis in infected human bronchial epithelial cell line BEAS-2b (Moura et al., 2008). *B. cenocepacia* can also infect and survive in phagocytic cells, such as dendritic cells (MacDonald and Speert, 2008), macrophages (Saini et al., 1999) and CGD neutrophils (Bylund et al., 2005, 2006). Interactions between *B. cenocepacia* and macrophages or neutrophils will be described in more detail in the next paragraph. To better investigate Bcc virulence in CF conditions, CFTR mutant lines have been developed, including CFTR ΔF508 macrophages (Lamothe and Valvano, 2008) and epithelial cells, IB3, C38 and S9 cells (Fink et al., 2003).

3.3.2. Plant and invertebrate models

Four plant models have been developed to study virulence of the Bcc; alfalfa seedlings (Bernier et al., 2003), onion tissue (Yohalem and Lorbeer, 1994), duckweed (*Lemna minor*) (Thomson and Dennis, 2013) and *Arabidopsis thaliana* (Madala et al., 2012). Also three invertebrate animal models, *Caenorhabditis elegans* (Köthe et al., 2003), *Galleria mellonella* (Seed and Dennis, 2008) and *Drosophila melanogaster* (Castonguay-Vanier et al., 2010) have been used for the study of Bcc pathogenicity.

3.3.3. Vertebrate models

To study chronic lung infection, rat and mice agar bead models have been used (Cieri et al., 2002; Sokol et al., 1999). A piece of agar with bacteria is inserted into the animal lung and causes chronic but nonlethal lung infection (Tomich et al., 2003).

All these infection models have largely contributed to the identification of bacterial genes involved in virulence including *cepR* and *cepI* from the *cepIR* quorum sensing system, type II, III, IV, and VI secretion systems, zinc metalloproteases (*zmpA* and *zmpB*), MgtC, transcriptional regulators, flagella, proteins involved in exopolysaccharide biosynthesis, and lipopolysaccharide (see Fazli et al., 2014; Ganesan and Sajjan, 2011; Loutet and Valvano, 2010; Subramoni and Sokol, 2012; Sokol et al., 2003; for overview). O'Grady and Sokol addressed bacterial gene expression during infection in a rat chronic lung infection model (O'Grady et al., 2011). They analysed differential gene expression of bacteria in the rat lung compared to that of bacteria in densely grown cultures, and surprisingly found that the

majority of genes encoding known virulence factors were not differentially expressed. Genes encoding N-acylhomoserine lactone synthase CepI, the orphan LuxR homolog CepR2, zinc metalloproteases ZmpA and ZmpB, the LysR-type transcriptional regulator ShvR, and the nematocidal protein AidA, as well as genes associated with flagellar motility, Flp type pilus formation, and type VI secretion were expressed at lower levels *in vivo*, compared to culture grown bacteria. Interestingly, T4SS genes, and genes predicted to be involved in osmotic stress adaptation or intracellular survival, metal ion, and nutrient transport, as well as those encoding outer membrane proteins were significantly induced *in vivo.*

The rat agar bead model has also been used to identify bacterial mutants *in vivo.* Using signature tagged mutagenesis several genes important for virulence, including regulatory genes, genes encoding efflux proteins and ABC transporters heme-binding proteins, MgtC and LPS biosynthesis were identified (Hunt et al 2004*)*.

As with cell lines, a mouse model of CGD has been developed. The X-linked CGD model, $gp91^{phox-/-}$, with a mutation in the gp91 subunit of NADPH oxidase (Pollock JD 1995 - Sousa S 2007) and autosomal recessive model of CGD, $p47^{phox-/-}$, with a mutation in the gp47 subunit (Jackson SH 1995, Vethanayagam RR 2011 and Pizzolla A 2012) have been used to study bacterial virulence in a context of CGD. These two models show a phenotype similar to human CGD with increased susceptibility to the development of infection and especially neutrophil lung inflammation. $p47^{phox-/-}$ or $gp91^{phox-/-}$ mice are highly susceptible to Bcc infection. In the absence of a functional NADPH oxidase, these mice produce less ROS and die faster than wild type mice, suggesting an important role for ROS-mediated killing by

neutrophils in host defence (Pizzolla et al., 2012; Vethanayagam et al., 2011). Infected mice developed neutrophil-dominated lung abscesses and died in 3 days (Sousa et al., 2007).

A new vertebrate model was developed a few years ago, the zebrafish model. Only few virulence assays have been done with adult zebrafish, and these experiments showed that adult zebrafish can develop lethal infections with *B. cenocepacia* J2315 and confirmed the crucial role of CepI/CepR quorum sensing for virulence (Deng et al., 2009). We developed the zebrafish embryo model to study Bcc infections (Vergunst et al., 2010). The optical transparency of zebrafish embryos allows real time non-invasive imaging of infection *in vivo* using fluorescent bacteria and cell-specific transgenic reporter fish (Mesureur and Vergunst, 2014; Vergunst et al., 2010). Using this model it was shown that *B. cenocepacia* replicates in zebrafish macrophages, followed by induction of a strong lethal inflammatory response, in agreement with results obtained in other models that suggested an important role for macrophages in virulence. Other Bcc strains caused persistent, non-lethal infections in zebrafish embryos, allowing the comparison of these different infection phenotypes at both the host and bacterial level in more detail. In addition, a role for the CepR quorum sensing system in virulence was shown.

3.4. Role of macrophages and neutrophils during *Burkholderia cenocepacia* infection

3.4.1. The role of macrophages

Bcc bacteria can interact with phagocytic cells and adopt an intracellular lifestyle. It has been shown that *B. cenocepacia* can enter, survive and replicate in healthy (non-CF, and CF) macrophages *in vitro* in cell cultures (Saini et al., 1999), such as in the RAW 264.7 murine macrophage-like cell line (Maloney and Valvano, 2006; Shimomura et al., 2001) and PU5-1.8 (Saini et al., 1999), in human macrophage cell lines such as U937(Martin and Mohr, 2000) or THP-1 (Gavrilin et al., 2012) and also *in vivo* in macrophages of zebrafish embryos (Vergunst 2010). After phagocytosis, live bacteria reside in a specific compartment: the *Burkholderia cepacia*-containing vacuole (BcCV). In macrophages, this compartment is very spacious and the pH has been determined to be around 6.4 (Lamothe et al., 2004). This compartment fuses with early endosomes visualised by recruitment of early endosome markers, EEA1 (early endosome autoantigen 1) protein and Rab5 (Huynh et al., 2010). CD63, a marker for late endosomes is also recruited to BcCVs but the acquisition of LAMP-1 is delayed compared to *E. coli* and heat-killed bacteria, and therefore maturation of the phagosome is delayed or blocked (Huynh et al., 2010; Lamothe et al., 2007). Recruitment of late endosomes is marked by the transition between the small GTPase Rab5 to Rab7. The majority of BcCVs acquired Rab7. However, it was shown that *B. cenocepacia* impaired activation of Rab7 on the vacuole, blocking further fusion with lysosomes and maturation into a phagolysosome.

Several bacterial factors have been shown to be crucial for BcCV formation and bacterial intracellular survival. The sigma factor RpoN regulates *B. cenocepacia* K56-2 motility and this motility is essential for biofilm formation (Saldias et al 2008). RpoN is also required for intracellular trafficking and bacterial survival in mouse macrophages. As with RpoN, RpoE is also required for intracellular trafficking and essential for the maturation delay of the BcCV and thus survival of K56-2 in macrophages (Flannagan et al 2008). Via introduction of yet unknown effector proteins of the bacterial type VI secretion system (T6SS), *B. cenocepacia* disrupts the actin cytoskeleton by inhibition of two Rho GTPases, Rac1 and CDC42 (Rosales-Reyeset al. 2012; Flannagan et al. 2012). In addition, Rosales-Reyes *et al* showed that the T6SS may help the secretion of T2SS effector proteins into the host cell (Rosales-Reyeset al. 2012). The global regulator AtsR, which has been shown to be important for bacterial virulence and survival, represses *Hcp* and induces more biofilm formation (Aubert et al., 2008). MgtC, a regulator of the F1/F0 ATPase (Lee et al., 2013), is also essential for the bacteria to survive in macrophages. Interestingly, *B. cenocepacia* K56-2 *ΔmtgC* is more resistant to stress environments including oxidative (ROS), nitrosative (RNS, reactive nitrogen species), cationic peptides, or low pH, and it was suggested that there is an inverse correlation between virulence and stress resistance (Maloney and Valvano, 2006).

During phagocytosis, one way to eliminate pathogens is the production of ROS via NADPH oxidase and the acidification of the phagosome. BcCVs can delay the assembly or the recruitment of the NADPH oxidase complex on the BcCV membrane and reduce ROS production in infected macrophages (Keith et al., 2009). Furthermore SodC, a periplasmic superoxide dismutase produced by *B. cenocepacia*, can protect the bacteria from ROS generated in the phagosome and contributes to intracellular survival in macrophages (Keith

et al., 2009). *B. cenocepacia* has an effect on the amount of superoxide produced by macrophages and can reduce this production.

3.4.2. *B. cenocepacia* and autophagy

Using cell cultures it has been shown that *B. cenocepacia* replicates more efficiently in *cftr* ΔF508 macrophages and induces a higher IL-1β production than in WT macrophages (Abdulrahman et al., 2011a). It was shown that *cftr* ΔF508 macrophages have reduced autophagic activity (Abdulrahman et al., 2011b). When autophagic activity was induced with rapamycin or by the depletion of ubiquitin binding adaptor protein SQSTM1/p62 in *cftr* ΔF508 macrophages, the autophagy molecule beclin1 (BECN1) was released. As a result, *B. cenocepacia* was transferred to the autophagy pathway and the inflammatory response and the infection decreased (Abdulrahman et al., 2011b, 2013). Recently, however, AL-Khodor *et al* (Al-Khodor et al., 2014) found that *B. cenocepacia* J2315 engages autophagy components during its intracellular life cycle: J2315 is able to disrupt the endocytic process and escape into the cytosol of the THP-1 line of human differentiated macrophages. This triggers selective autophagy, with the recruitment of p62, and LC3, but the bacteria are able to block autophagosome completion, and replicate in the cytoplasm with the recruitment of ER markers (Al-Khodor et al., 2014). Despite the avoidance of autophagosomal degradation, pre-induction of autophagy decreased bacterial burden, providing a putative therapeutic approach.

Summarizing, *B. cenocepacia* is able to subvert macrophage immune responses; the bacteria can interfere with the endocytic and autophagy pathways, delay or even completely block

their degradation, and multiply inside the cell. The CF defect also plays a crucial role in the inability of macrophages to control and kill bacteria, as the CFTR mutation enhances the bacterial infection.

3.4.3. Neutrophil activity against *B. cenocepacia* in CF and CGD context

Bcc can persist in CGD neutrophils due to the absence of a functional NADPH oxidase, and the fact that they are not killed by non-oxidative killing mechanisms (Speert et al 1994). Moreover, in cell culture, infected neutrophils from CGD patients become necrotic and show a reduced pro-inflammatory response explaining why CGD patients are highly susceptible to Bcc (Bylund et al., 2005). Although CF neutrophils do not have a defect in ROS production, redox imbalance in the CF lung might contribute to the reduced killing of the Bcc. Smith *et al* found that airway epithelial cells from CF patients do not express nitric oxide (NO) synthase and cannot produce NO in the lungs (Smith et al., 1999). Neutrophils from CF patients spontaneously released more elastase than neutrophils from healthy individuals, and induced more inflammation and tissues injury (Brockbank et al., 2005).

Moreover, *B. cenocepacia* produces factors that could contribute to persistence and survival in the CF lung and avoid neutrophil killing, including exopolysaccharides (EPS) or haemolysin. EPS can inhibit neutrophil chemotaxis and ROS production (Bylund et al., 2006), and has been shown to interfere with cell-surface interactions and prevent bacterial clearance in mice (Conway et al., 2004; Sousa et al., 2007). Haemolysin induces spontaneous degranulation of human neutrophils and neutrophil death (Hutchison et al., 1998). The CepIR quorum sensing system is a major virulence factor of *B. cenocepacia*, and CepR-

regulated expression of the zinc-dependent metalloproteases A (*ZmpA*) and ZmpB produced by *B. cenocepacia* K56-2 can result in the cleavage of the neutrophil proteases elastase and cathepsin G. This could increase bacterial resistance and maintenance of chronic respiratory infection (Kooi and Sokol, 2009; Sokol et al., 2003).

3.5. Role of inflammation and the Toll-Like Receptor signalling pathway during *Burkholderia cenocepacia* infection

Bcc bacteria induce potent pro-inflammatory responses. It has been shown that *B. cenocepacia* induces NF-κB activation and pro-inflammatory cytokine production predominantly via MyD88-dependent signalling (Ventura et al., 2009), but in contrast *B. multivorans* can activate immune signalling by MyD88-independent pathways, possibly via activation of the adaptors TRIF and TRAM (Bamford et al., 2007). Ventura *et al* showed that during *B. cenocepacia* infection immunocompromised *myD88*$^{-/-}$ mice survived longer than wild type mice, with reduced production of TNFα, IL-6, MIP-2 and G-CSF. Also corticosteroid treatment could decrease inflammation and death of the mice (Ventura et al., 2009). These paradox changing results showed that the Bcc-infected host has a survival advantage in the absence of Myd88, and that inflammatory responses mediated through MyD88 were harmful for the host. During infection of human lung epithelial cells by *B. cenocepacia* TNFα, IL-8, IL-6 and IL-1β mRNA was up-regulated (Gillette et al., 2013). In cell culture, *B. cenocepacia* have been shown to induce the production of TNFα, IL-1β and IL-8 by CFTR-

positive and CFTR-negative bronchial epithelial cell lines, although the production is more increased in CFTR-negative cells compared to CFTR-positive cells.

Two major TLR/ligand interactions have been shown to be involved in induction of the strong inflammatory responses in Bcc infection: TLR4/LPS and TLR5/flagellin (Hanuszkiewicz et al., 2014).

LPS from *B. cenocepacia* and *B. multivorans* induced IL-8 and IL-1β production through TLR4/CD14 signalling and TLR4/MyD88-dependent pathways. The LPS/TLR4 interaction induced IκB-α degradation and NFκB activation (Bamford et al., 2007; McKeon et al., 2010; Reddi et al., 2003; De Soyza et al., 2004), resulting in non-secreted pro-IL-1β expression. Flagella are essential for bacterial motility and invasion of epithelial cells by *Burkholderia*. *B. cenocepacia* Δ*fliG* or Δ*fliI* mutants are less virulent than wild type bacteria in mice (Tomich et al., 2002). *B. cenocepacia* flagellin interact with TLR5 and induce IL-8 production via NF-κB activation (de C Ventura et al., 2008; Urban et al., 2004). For *Pseudomonas aeruginosa*, it has been shown that the p38 MAPK signalling pathway plays a major role in gene regulation in response bacterial infection via TLR5 (Zhang et al., 2007). Moreover the inhibition of TLR5 abolishes the inflammatory response during *B. cenocepacia* and *P. aeruginosa* infection (Blohmke et al., 2008). Recently, Hanuszkiewicz et al (2014) showed that glycosylation of flagellin reduced its interaction with TLR5 and reduced pro-inflammatory immune responses. Although induction of the innate immune response through TLR signalling may contribute to enhanced inflammation seen during infection by *B. cenocepacia*, the precise mechanisms that lead to the excessive pro-inflammatory responses seen in humans and in experimental animal models remain to be elucidated.

OUTLINE OF THE THESIS:

The use of infection models has improved our understanding of virulence of bacteria belonging to the Bcc, but major questions about the ability of this pathogen to cause persistence, to cause sudden transition leading to fatal inflammatory infection, and the role of host phagocytes during infection *in vivo*, are still largely unanswered. One major outcome is that Bcc are not exclusively extracellular pathogens, and the intracellular strategy of the Bcc has been suggested in many publications to be important for virulence in CF patients.

To help address these questions in an *in vivo* model, we have made use of the excellent imaging possibilities of the zebrafish embryo, with an innate immune system highly similar to that of humans, and the ease of performing large scale global transcriptomic analyses to get an insight into the changes in host gene expression during persistent and acute infection. This thesis describes an infection protocol for the study of Bcc virulence (Chapter 2), analysis of the role of host phagocytes and the early innate immune response and the involvement of MyD88 in inflammation induced by *B. cenocepacia* (Chapter 3), and a global host transcriptomics study using RNAseq to analyse differential host gene expression during persistent and acute Bcc infection (Chapter 4), followed by a General Discussion (Chapter 5).

This work shows:

- A critical role of macrophages for *Burkholderia cenocepacia* K56-2 infection;
- The role of a MyD88-dependent inflammatory response to induce fatal infection;
- A minimal role for neutrophils *in vivo;*
- Global host gene expression changing during acute and persistent Bcc infection.

CHAPTER 2

ZEBRAFISH EMBRYOS AS A MODEL TO STUDY BACTERIAL VIRULENCE

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Abstract

In recent years the zebrafish has gained enormous attention in infection biology, and many protocols have been developed to study interaction of both human and fish pathogens, including viruses, fungi and bacteria, with the host. Especially the extraordinary possibilities for live imaging of disease processes in the transparent embryos using fluorescent bacteria and cell-specific reporter fish combined with gene knockdown, transcriptome and genetic studies have dramatically advanced our understanding of disease mechanisms. The zebrafish embryo is amenable to study virulence of both extracellular and facultative intracellular pathogens introduced through the technique of microinjection. Several protocols have been published that address the different sites of injection, antisense strategies, imaging and production of transgenic fish in detail. Here we describe a protocol to study the virulence profiles of clinical isolates, ranging from acute fatal to persistent, of bacteria belonging to the Burkholderia cepacia complex. This standard operating protocol combines simple survival assays, analysis of bacterial kinetics, analysis of the early innate immune response with qRT-PCR, and the use of transgenic reporter fish to study interactions with host phagocytes, and is also applicable to other pathogens.

Key words: zebrafish, *Burkholderia cepacia complex*, *Burkholderia cenocepacia*, bacterial virulence, intracellular bacteria, infection profiles

1. Introduction

Opportunistic microbial infections are a major cause of respiratory failure in cystic fibrosis (CF). Bacteria of the *Burkholderia cepacia* complex (Bcc), specifically *B. cenocepacia*, are particularly harmful for CF patients, and infection leads to increased morbidity and mortality (1). Although infection with Bcc can be asymptomatic, it can unpredictably result in chronic progressive worsening of lung function and sometimes acute fatal necrotizing pneumonia and sepsis, termed cepacia syndrome. Several highly transmissible strains, including *B. cenocepacia* J2315 (2) and K56-2 (3) have caused a lot of havoc amongst patients, and since these bacteria have a high intrinsic resistance to antibiotics there is no effective treatment. The development of cell culture, non-vertebrate (*Galleria mellonella*, nematodes, *Drosophila*), vertebrate (zebrafish), and mammalian infection models (4–12) has contributed to a better understanding of the behaviour of this pathogen, and bacterial virulence factors including lipopolysaccharide (LPS), flagella, secretion systems, and catalases (13) have been identified; however, the precise mechanisms underlying the disease and causing these sudden exacerbations and the induction of an excessive pro-inflammatory response are still not clearly understood.

Here we describe in great detail the protocol that we developed to study virulence of the Bcc using zebrafish embryos, although the method is generally applicable to other bacterial pathogens. The zebrafish has now been firmly established as an infection model in the study of human infectious disease (14), including for a number of facultative intracellular pathogens, such as *Mycobacterium, Salmonella, Listeria* and *Burkholderia* (11,15–17). Especially the unprecedented possibilities to follow the infection of fluorescently labelled bacteria in real time in the transparent embryos allows the analysis of the role of host phagocytes during infection of cell-specific reporter fish, for instance those expressing GFP in neutrophils (14,18) or mCherry in macrophages (19). In addition, the embryos have an innate immune system that is very similar to that of humans (20–23) and this

allows studying the role of the innate immune response during infection in great detail, whereas an adaptive system develops in 2-3 weeks. The research community's efforts to develop this animal as a valuable additional and important tool for disease studies and drug screens (24) have produced many useful techniques (e.g., antisense RNA gene knockdown using morpholinos (MO) (25,26) and transgenesis (27,28) and materials (e.g., transgenic zebrafish lines) that will help the researcher to address relevant biological questions from both the host and the bacterial side using bacterial mutants. Recent global transcriptome studies have also greatly extended our understanding of the host response to infection of adult zebrafish or embryos in response to pathogens using microarrays or RNAseq (29–32), and dual RNAseq combined with proteomics will soon allow the simultaneous identification of host and bacterial factors essential during specific disease stages in the whole animal, or specific cells.

Earlier we have shown that different clinical Bcc isolates, introduced by microinjection directly in the blood circulation of 30 hours post-fertilization (hpf) embryos, are rapidly phagocytosed and can cause infection in zebrafish embryos that ranges from symptomless persistent, with bacteria (e.g., *B. stabilis* LMG1429) surviving in macrophages but unable to disseminate, to acute pro-inflammatory infection (e.g., *B. cenocepacia* K56-2), with bacteria surviving and multiplying in macrophages, followed by a highly pro-inflammatory infection that becomes rapidly fatal (11). This model therefore offers great possibilities for detailed analysis of the early innate immune response, interaction with host phagocytes, and the importance of intracellular stages in disease development during both persistent and acute infection. In this chapter, we describe a protocol that allows analysing virulence potential of environmental and clinical isolates, ranging from simple CFU counts to determine bacterial load in the embryos and survival assays to real time imaging and qRT-PCR analysis of important host immune response genes. Several zebrafish infection protocols have recently been published, including very elegant JoVe videos (33) and other interesting publications that describe techniques including transfection and morpholino injections in fine detail (34,35). Our aim is to

present a protocol for newcomers in the field, and provide useful suggestions that might help to quickly adopt the protocol. The method will also be applicable to other bacterial pathogens, although some details may not be valid for other bacterial pathogens (for instance growth conditions, counting strategies, interesting time points).

2. Materials

2.1. Obtaining eggs and preparation for infection

1. Zebrafish facilities (*see* **Note 1** and **Note 2**).
2. Adult wild-type AB, Golden (*see* **Note 3** and **Note 4**) and Tg(*mpx::GFP*) transgenic fish (18).
3. Spawning tanks (*see* **Note 5**).
4. Incubator at 29°C.
5. Embryo water (E3 medium) (36): 5 mM Nacl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (from sterilized stock solutions) in sterilized water, supplemented with 1µl of 10% methylene blue (MB) per litter (*See* **Note 6**).
6. Stereomicroscope.
7. Petri dishes 90x14 mm.
8. Very fine forceps, Tweezers #5 Dumont, Dumoxel/Biology Grade.
9. Tissue culture-quality Petri dishes 60/15mm (*See* **Note 7**).
10. Plastic Pasteur pipettes (7 mL) (*See* **Note 8**).

2.2. Bacterial culture and preparation of inoculum

1. *Burkholderia cenocepacia* K56-2 and *B. stabilis* LMG1429, expressing a fluorescent reporter. Here we use strains harbouring plasmid pIN29, encoding DsRed (11). (*See* **Note 9** and **Note 10).**

2. Luria Bertani (LB) broth: 10 g Bacto-tryptone, 5g yeast extract, 10 g NaCl. Add to 800mL H2O. Adjust pH to 7.5 with NaOH. For agar plates add 1.5% Bacto agar. Adjust volume to 1L with dH2O and sterilize by autoclaving (121 °C, 20 minutes).
3. Chloramphenicol (Cm), 100 mg/mL stock solution in 100% ethanol (store at -20°C): For *Burkholderia*, we use Luria Broth (LB) medium with 100 mg/l of chloramphenicol (*See* **Note 11**).
4. Phosphate-buffered saline (PBS) 1x.
5. Phenol red (PR) solution, 0.5%.
6. Spectrophotometer.
7. Inoculation loops.
8. 18x180mm glass tubes with loose-fitting metal lids for bacterial growth.
9. 37 °C shaking incubator.
10. 37 °C incubator.

2.3. Micro injection

1. Microinjector, e.g. Femto Jet (Eppendorf).
2. Mechanical xyz micromanipulator arm, e.g., M-152 (Narishige).
3. Stereo microscope.
4. Micro loader pipette tips.
5. Borosilicate glass capillaries, e.g. with filament O.D.:1 mm, I.D.: 0.78 mm, 10 cm length (*See* **Note 12**).
6. Agarose plates (1- 1.5 % agarose in E3 medium) for injection containing slots of 1 by 1 mm (*See* **Note 13**).
7. Tricaine (MS222) 20x, 400 mg in 100 mL of sterile water, adjust pH 7.0 with Tris-HCl 1M pH9 (around 2.1 mL). Aliquot in 15 mL tubes and freeze at -20°C. After use, keep at 4°C.
8. Pasteur pipettes and latex bulbs. 24 and 48 well tissue culture plates.

2.4. Analysis of bacterial multiplication

1. LB 1.5% agar plates (*See* **section 2.2 step 2**) with appropriate antibiotics (*See* **Note 14**).

2. LB 1.5% agar plates, square dishes 125/15mm (~50 mL per plate) with appropriate antibiotics (*See* **Note 15)**.

3. Bacterial safety cabinet.

4. Eppendorf tubes.

6. Pipette man and tips.

7. 10x Trypsin/EDTA, diluted 10x in sterile H_2O.

8. Triton X-100 2% in H_2O.

2.5. Tools for survival assays and intravital imaging

1. Inverted fluorescence and fluorescence multizoom microscopes with camera and supplied imaging software (*See* **Note 16**).
2. 24- and 48-well tissue culture plate (*see* **Note 17**).
3. 35mm Glass-bottom dishes.
4. Microscope depression slides.

2.6. Tools for qRT-PCR and RNA-Seq analysis

1. RNase-free work zone.

2. RNase-eliminating solution such as RNase Away.

3. RNase, DNA free 2ml.

4. 1.5 and 0.5ml Eppendorf tubes.

5. RNase, DNA-free water.

6. TRIzol® (Invitrogen).

7. Chloroform.

8. Isopropanol 100%.

9. Ethanol 70%.

10. DNase I, RNase free.

11. RNeasy MinElute Cleanup kit (Bio Rad).

12. Photospectrometer to quantify RNA.

13. IScript cDNA Synthesis kit (Bio Rad).

14. qPCR Primers with Tm of 60°C (see Note 48).

15. Lightcycler 480 with software: Light Cycler 480 (Roche).

16. 96 well white plates with transparent sealing foils for Lightcycler 480 (Roche).

17. SYBR Green mix adapted for your machine: For the LC480: Light Cycler 480 SYBR Green I Master (Roche).

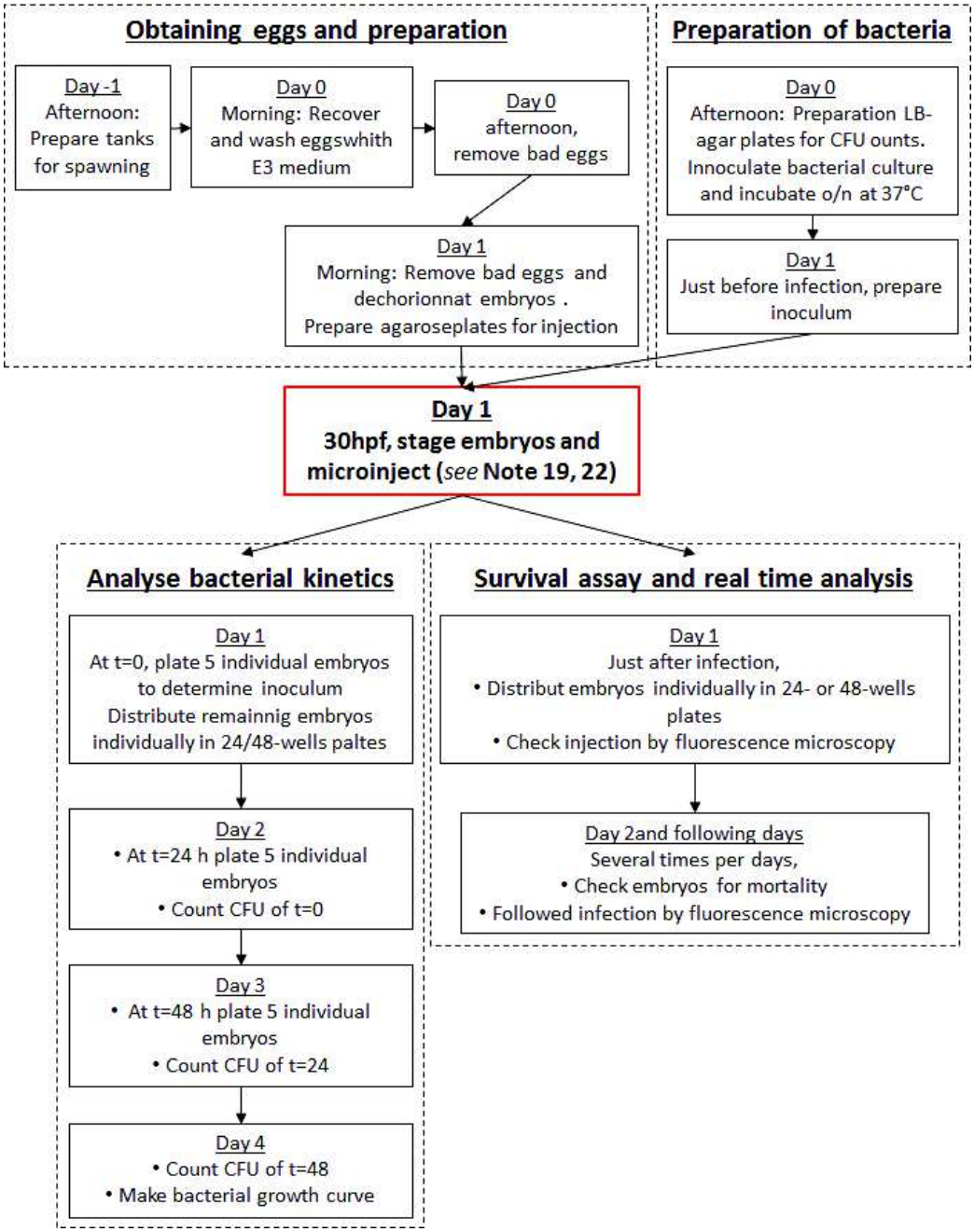

Figure 13. Typical work plan for an infection experiment.

OD: optical density at 600 nm; hpf: hours post-fertilization; hpi: hours post-injection/infection; CFU: colony-forming unit

3. Methods

Figure 1 shows a typical work plan of an infection experiment.

3.1. Obtaining eggs and preparation of embryos for infection

1. To obtain eggs, prepare several tanks containing one or two adult couples of the desired line (WT or Tg(*mpx:GFP*)) (*see* **Note 5**).
2. Carefully rinse eggs with tap water using a fish net with very fine maze, and transfer eggs in a standard Petri dish with E3 medium. Remove remaining waste (*see* **Note 18**).
3. Put the plates in the incubator at 29°C. During the day remove bad eggs (abnormal embryo development or infected eggs).
4. The next day, 2 h before micro-injection (*see* **Note 19**), remove the protective membrane of the embryos under a dissecting microscope by delicately opening the chorion with very fine forceps (*see* **Note 20**).
5. After dechorionation, place the embryos in a tissue culture plate 60/15mm with fresh E3 medium (*see* **Note 6** and **Note 7**), and maintain at 29 °C until the time of injection.

3.2. Growth of **B. cenocepacia** *for infection and preparation of inoculum*

1. One day before the infection experiment, grow *B. cenocepacia* in LB medium from frozen glycerol stock (*see* **Note 11**). To maintain plasmid pIN29, add 100 mg/L Cm (LB-Cm100). Pipette 5 mL LB-Cm100 medium into a sterile glass tube and inoculate with a loop full of bacteria. Incubate in an orbital shaker (200 rpm) for 16 - 18 h at 37°C.
2. Just before infection, measure the OD_{600} and prepare a dilution to obtain 50 bacteria per nL (*see* **Notes 21, 22** and **23)** as follows**:** Transfer the required volume of bacterial culture to obtain 1 mL of an OD 1 to an Eppendorf tube, and centrifuge at 3500 x g for 2 min. Remove supernatant and add 1 mL PBS to the bacterial pellet. Resuspend by gently pipetting up and down using a pipette

man. Add 50 µL of the suspension OD=1 to 850 µL PBS. To be able to visualize the injection, add 5 µL phenol red 10x to 45 µL of the bacterial dilution.

3.3. Micro injection (see Fig. 2 for set up)

1. Prepare agarose plates for injection and Tricaine 2x in E3.
2. Switch on the microinjector (*see* **Note 24**).
3. Pipette 5 mL of E3 containing 2x Tricaine onto the agarose plate and position 60 30hpf embryos in the slots (*see* **Note 25** and **26**).
4. Position the micromanipulator next to the stereomicroscope (**Fig. 2a**).
5. Load 3 to 4 µL of the bacterial injection suspension (from **section 3.2 step 2**) into a pulled capillary pipette using a micro loader tip, and place on the micromanipulator.
6. With the micromanipulator, place the tip of the needle in the centre of the image. Adjust time and/or pressure to obtain an injection volume of around 1 nL (*see* **Note 27** and **28**).
7. Place the agarose plate with embryos on the microscope, and place the needle above the caudal vein or the blood island region. Pierce the skin with the capillary needle (**Fig. 2b**), by descending the z-axis of the micromanipulator. When the capillary needle is at the correct location and in the blood circulation, inject the bacteria with a single pulse. The phenol red allows visualization of correct injection directly in the blood circulation (*see* **Note 29**) (**Fig. 2c and d**). Remove any embryos that are not properly injected.
8. Place five embryos in 10-20 mL sterile E3 medium to rinse embryos (*see* **Notes 30** and **31)** to determine inoculum, T=0.
9. Transfer the embryos individually to a sterile 1.5 mL tube with a Pasteur pipette, and remove any E3 with a micropipette. Immediately add 100 µL trypsin, disrupt the embryo by pipetting up and down (30-40 times) with a micropipette.

10. Incubate for 20 min at room temperature and pipette up and down (30-40 times) to completely disrupt the tissue.
11. Plate the total lysate from each embryo on LB-Cm100 plate and count colonies after an overnight incubation at 37°C (*see* **Note 32** and **33**).
12. Proceed with the remaining injected embryos to 3.4 (determination of survival rates), 3.5 (determination of bacterial multiplication), 3.6 (real-time analysis), and/or 3.7 (analysis of host gene expression). Using a fluorescence microscope (with 10x objective) discard any embryos that do not have any fluorescent bacteria.

3.4. Embryo survival studies

1. Just prior to injection prepare 24- or 48-well tissue culture plates with 1 mL E3 medium per well.
2. Transfer a minimum of 20 infected embryos individually into wells and put in the incubator at 29 °C.
3. Observe the embryos at least once a day and every 2-3 hours during the critical phase of infection under a stereomicroscope and record mortality. An embryo is considered dead when the blood circulation has stopped and the heart no longer beats (*see* **Note 34**).
4. Represent mortality/survival rates using Kaplan-Meier graphic representations and analyse data with a log-rank or other appropriate statistical tests (**Fig. 3c**).

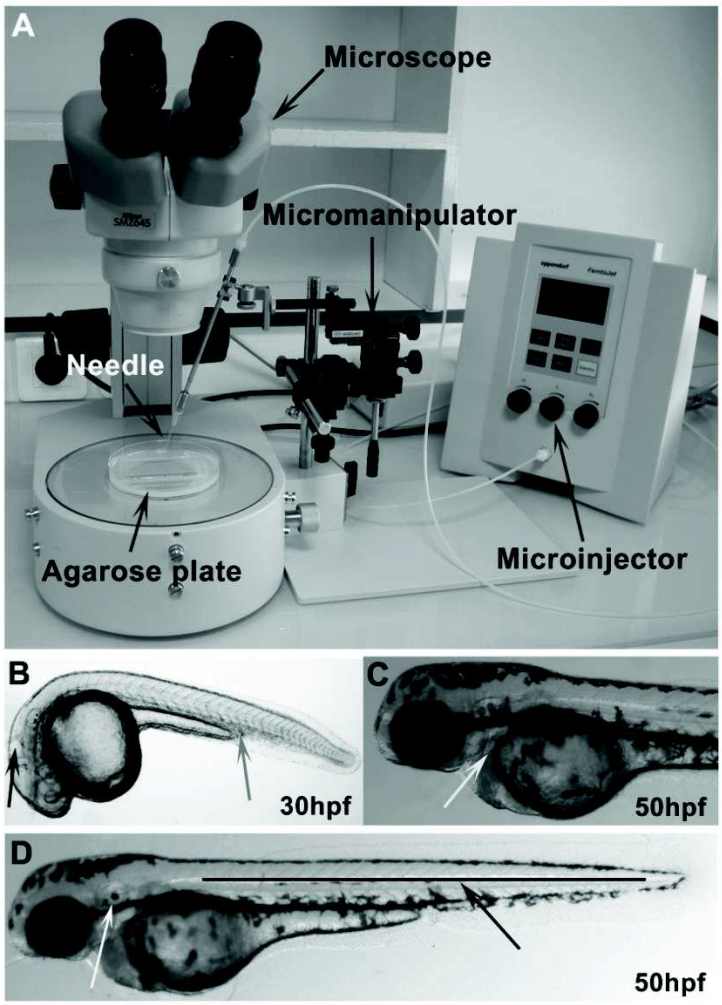

Figure 14. Injection setup and injection sites.

(**a**) Typical injection setup. (**b-d**) Injection sites for systemic (**b** *grey arrow*, **c**) or local (**b** *black arrow*, **d**) bacterial infection in the zebrafish embryo (35). (**b**) At 30 hpf, two preferred sites are used for microinjection; the blood island (*grey arrow*) or the hindbrain (*black arrow*). (**c**) Duct of Cuvier injection site at 50 hpf. (**d**) In 50 hpf embryos, the otic vesicle (*white arrow*), subcutaneously (*black arrow*, (44)), and the notochord (*black line*).

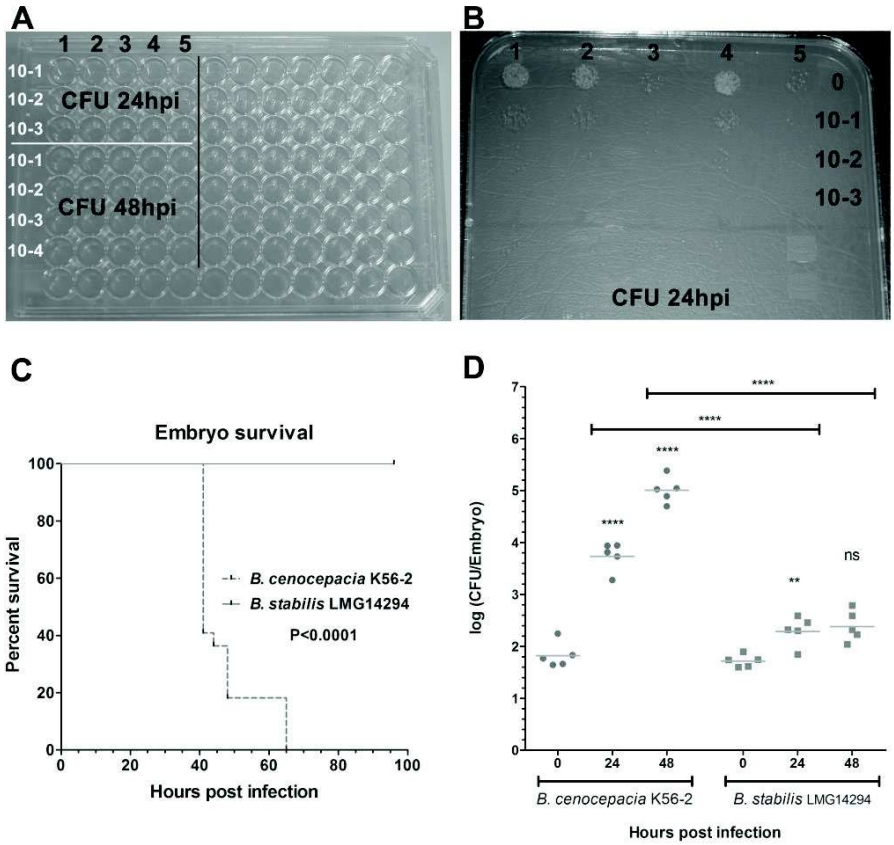

Figure 15. Determination of bacterial multiplication and embryo survival.

(a) A 96-well plate can be used to prepare serial dilutions for CFU counts at 24 and 48 hpi. (b) Plating method to determine bacterial CFU in tenfold serial dilutions. (c) Embryo survival following infection with *B. cenocepacia* K56-2 and *B. stabilis* LMG 14,294 (n=20 for each strain) with Kaplan-Meier representation. Significance is determined with a log-rank (Mantel–Cox) test. (d) Bacterial multiplication of *B. cenocepacia* K56-2 and *B. stabilis* LMG 14,294 during infection. Five embryos per time point per experiment, with grouped column scatter representation. Each dot represents CFU per embryo; geometric means are indicated by bars. Comparison between 0 and 24 hpi, 24 and 48 hpi for the same strain and the difference between two different strains at 24 and 48 hpi with unpaired student T-test *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

3.5. Determination of bacterial multiplication as readout for virulence

1. Prepare LB-agar-Cm100 plates.
2. After microinjection (section 3.3, **step 7**), transfer 5 infected embryos per time point per strain to be analysed individually into wells of a 24- or 48-well culture plate, and place in the incubator at 29 °C.
3. Wash 5 embryos at the desired time point by transferring the embryos to 10-20 mL E3 (*see* **Note 35)**.
4. Transfer the washed embryos individually to sterile 1.5 mL tubes with a Pasteur pipette, and withdraw the E3 with a micropipette. In contrast to T=0, add 45 µL trypsin, disrupt the embryo by pipetting up and down with a yellow tip (30-40 times), add 50 µL 2% Triton X-100, mix by flicking the tube, and incubate 30 min at room temperature. Repeat disruption by pipetting up and down 30-40 times (*see* **Note 36**)**.**
5. Plate the total lysate on LB Cm100 agar plates (10 cm), or plate serial dilutions as follows (**Steps 6-10**; *see* **Note 37**).
6. Prepare a sterile 96-well plate with 90 µL of PBS in each well.
7. For each embryo lysate (**Fig. 3a**) add 10 µL to a well in row A containing 90 µL of PBS, pipette 20 times up and down to mix evenly.
8. Transfer 10 µL from well A to well B, and pipet 20 times.
9. Repeat **step 8** until the desired number of dilutions is obtained.
10. Change pipette tip and spot 10 µL of each dilution and 10 µL of embryo lysate (non-diluted) on LB-Cm 100cm^2 square plates as in **Fig. 3b**. Let the drops dry into the plate without spreading (normally takes 30 minutes), and incubate the plate overnight at 37°C (*see* **Note 38).**
11. Count colonies of each dilution (*see* **Note 39)** and determine total CFU per embryo.
12. Use an appropriate software program for analysis of bacterial multiplication rates and plot data using a semi logarithmic scale (*see* **Note 40**) (**Fig. 3d**).

3.6. Real-time observation during infection

1. For global observations, embryos can be visualized directly in the 24-wells plate with a 10x objective. If the embryos move, add Tricaine (1x final concentration in E3).
2. For observation at higher magnification embryos can be placed in a microscope depression slide or in a glass-bottom dish in a small drop of 1x Tricaine in E3 (make sure the liquid does not evaporate during imaging).
3. Use 40x, 63x, or 100x oil objectives and bring embryo in focus using bright field.
4. Take representative images with bright field, Nomarski, and/or the different filter sets to visualize GFP and DsRed or mCherry (*see* **Note 16**, **Fig.4**).
5. After observation and imaging, pipette the embryo back into E3 medium in the 24-well plate and return to the 29 °C incubator.

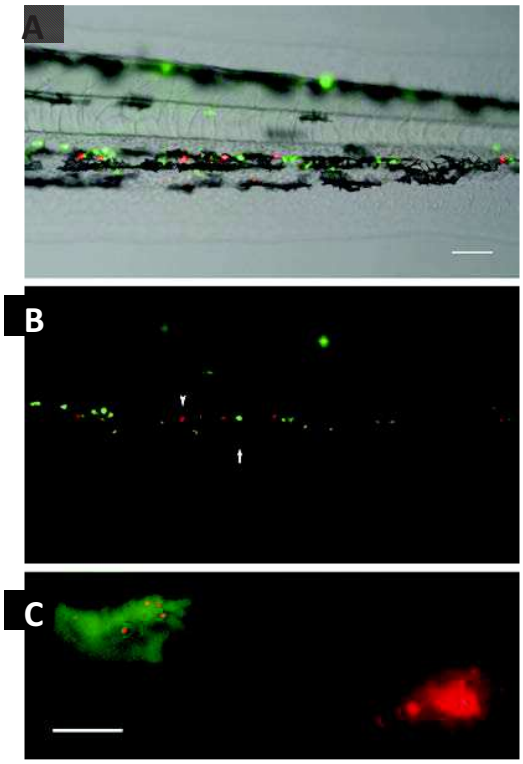

Figure 16. Real-time imaging using fluorescence microscopy. (a, b) Images 2 hpi of the tail region of a Tg(*mpx-GFP*) embryo, with neutrophils expressing GFP, injected at 60 hpf with K56-2, expressing DsRed. (**a**) Representative bright-field and fluorescence (*green* and *red channel)* overlay image. *Arrowhead*: K56-2 bacteria (*red*) inside a phagocytic cell that is not a neutrophil. *Arrow*: Individual bacterium. *Scale bar* 50 μm. (**b**) Image as in A taken with *red* and *green channel* only. (**c**) Fluorescence overlay image (*red* and *green filters*) 18 hpi representing an individual GFP-expressing neutrophil that contains several red fluorescently labelled K56-2 bacteria. On the right, a GFPminus cell containing many bacteria. *Scale bar* 10 μm. (11)

3.7. Extraction of RNA for qRT-PCR and RNA-Seq analysis

1. Follow the steps for preparation of the embryos, preparation of inoculum, and microinjection until Subheading 3.3**, step 7** (*see* **Note 41**).
2. After injection, for each time point required for analysis at each of the conditions (for instance: strain K56-2, T=3hpi (=target sample A), PBS, T= 3hpi (=control sample), transfer a

pool of 10 to 25 embryos to 500µl of TRIzol. Vortex well until all tissue has dissolved. Transfer the tubes immediately to -80 °C.

3. Extract and purify the RNA from each pool using the RNeasy MinElute Cleanup kit according to the manufacturer's instructions or as previously described (35). The final volume of purified RNA is 13 µl (*see* **Note 42**).

4. Analyse the quantity and the quality of RNA using 1 µl of the sample (*see* **Note 43**).

3.8. qRT-PCR analysis *(see* ***Note 44)***

1. Reverse transcribe each sample (500ng total RNA is enough) with a cDNA synthesis kit following the manufacturer's instructions (*see* **Note 45**). The final volume is 25 µL.
2. Dilute each cDNA sample 10 times to obtain the final volume required to set up the qRT-PCR reaction (*see* **Note 46**).
3. Prepare the SYBR Green mix for each condition (target and control samples at each of the different time points). For each condition, both the target gene and the reference genes should be analysed (*see* **Note 47).** Volume per well: Pipette 5µl master mix (2x), 1µl primer mix (5µM Fw and Rev primers, *see* **Note 48**), 0.5µl PCR water. Prepare the SYBR Green mix in an Eppendorf tube for the number of wells that you need plus two volumes to be sure to have enough.
4. Put the 7.5µl of SYBR Green mix in each well and then add 2.5µl of cDNA (*see* **Note 49**).
5. Use the following qPCR program (**Table 1**).
6. Analyse the qRT-PCR results (*see* **Note 50**).

Table 1. qPCR program used

Pre-incubation

Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:10:00	4,4	

Amplification

Cycles	45	Analysis Mode	Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:15	4,4	
60	Single	00:00:40	2,2	

Melting Curve

Cycles	1	Analysis Mode	Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	4,4	
65	None	00:01:040	2,2	
97	Continuous		0,11	5

Cooling

Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
40	None	00:00:10	1,5	

4. Notes

1. Although more extensive zebrafish facilities are needed when keeping multiple transgenic fish lines (we acquired a Zebtec stand-alone system from Tecniplast), if only wild-type fish are needed for egg production a few aquaria (30L) with adults are sufficient.
2. Authorization to keep and handle animals should be acquired according to your local and national regulations. In addition, permits should be obtained if you wish to produce transgenic animals or perform infection experiments (GMO regulations, ethical committees). Infection experiments using embryos and larvae that have not yet reached the free feeding stage are not considered as animal experiments (directive 2010/63/EU.

3. Pigment formation in the young larvae can interfere with imaging (fluorescence, light microscopy, histology). We suggest using Golden adults (37), which have a mutation in the gene *slc24a5* that prevents pigment formation. Another possibility is to use Casper fish. We avoid using phenylthiourea (PTU) to prevent melanisation as it has an effect on fish biology (38) and immune signalling.
4. Zebrafish can be obtained from several stock centres, including the ZIRC (http://zebrafish.org/zirc/home/guide.php). We normally compare virulence of at least one bacterial strain in the different genetic backgrounds to exclude differences in virulence.
5. Male and females are put together in the evening in spawning tanks, which contain an inner tank with holes. The next morning, usually half an hour (sometimes longer) after the light in the room is turned on automatically (fish are maintained with a regime of 14-h light, 10-h dark); the females will lay eggs which fall through the holes. This avoids the parents from eating the eggs. If no spawning tanks are available, glass containers filled with glass beads or marbles can be placed in the aquarium the night before. We noticed the fish prefer dark underground, and it may help also to include (plastic) plants underneath the inner container. Although we have had good spawning with females that had been separated from males for longer time periods, as a general rule do not keep females separate for longer than 1 or 2 weeks as they may become egg bound. A female can lay between 50 to 200 eggs. We usually set up several couples to be sure to have enough eggs. If it is difficult to obtain eggs it might help to separate males and females in the same tank a few days before.
6. Methylene blue (MB) is an antiseptic used in aquaria against fungal growth, but with low antimicrobial activity. It is therefore necessary to wash the eggs well before putting them in the E3 medium. We use a very fine fish net to rinse the eggs under running tap water, and then further separate the eggs from faecal materials under a dissecting microscope, before placing them in E3 medium. When you plan to work with fixed embryos (e.g., for in situ

hybridization, immune staining), or image fluorescent phagocytes in live animals for further analysis with software tools (e.g., Image J) (39)**,** avoid using MB since it can accumulate in the yolk and cause auto fluorescence. For qRT-PCR analysis of host gene expression, it is better to use E3 without MB.

7. Once dechorionated, the embryo tail can stick to the plastic of Petri dishes damaging the tail. This may increase variation in infection outcome, and can induce wound-induced immune responses. We suggest using cell culture-quality dishes, such as Greiner Cellstar 60-15 mm, after the embryos have been dechorionated. To prevent this, an ambient room temperature of around 23-25 °C is optimal for handling embryos.
8. We use 7 mL plastic Pasteur pipettes to transfer eggs. At later stages, after dechorionating the embryos, glass Pasteur pipettes should be used instead to prevent sticking of the embryo to the plastic pipette. Take great care taking up the embryos with the pipette and try to avoid wounding the embryos. Pipetting the embryos “head-first” works well.
9. Following the infection in real time requires the use of fluorescently labelled bacteria. For this we have constructed a series of plasmids with the genes encoding a selection of fluorescent proteins (GFP, DsRed or mCherry, CFP, YFP, mTurquoise, E2-Crimson) expressed from a strong constitutive *tac* promoter sequence. In the examples described here we use very bright, red fluorescent K56-2 that were obtained by transforming bacteria with the pIN29 (DsRed) vector by electroporation (11). For colocalization studies, the actual fluorescent strain used will depend on the fluorescent proteins expressed in host phagocytes, or other immune-labelled host proteins. Avoid the use of CFP or mTurquoise for imaging of live embryos, since the high-energy light with the blue filter set affects the embryos.
10. Bacteria can be obtained from culture collections such as the BCCM/LMG bacterial culture collection.

11. Prepare overnight bacterial cultures directly from glycerol stocks kept at -80 °C, or from fresh agar plates started from -80 stocks. Do not keep Bcc strains on LB agar plates by repeatedly re-streaking the bacteria, as mutations resulting in attenuated virulence are likely to accumulate. Do not use bacteria that have been kept on agar plates or in liquid medium for longer times than needed, and do not store Bcc at 4 °C. For some Bcc strains (like *B. stabilis* LMG14294, or *B. cenocepacia* J415), liquid cultures directly from -80 glycerol stocks often do not grow, and we suggest in that case to first streak out bacteria from frozen glycerol stocks onto LB plates containing appropriate antibiotics 2 days before an infection experiment and incubate at 37°C for 1 day. In the evening prior to infection inoculate a loop full of bacteria in liquid LB medium and incubate on a rotary shaker with good aeration at 37°C overnight. Avoid using airtight plastic tubes.
12. To prepare pulled microcapillary pipettes (borosilicate glass capillaries, e.g., with filament O.D.:1 mm, I.D.: 0.78 mm, 10 cm length) we use a micropipette puller (Sutter Instruments Inc., Flaming/Brown p-97) with settings to get a relatively short needle tip. We use air pressure 550, heat 990, pull 40, velocity 80, time 200. This should be tried experimentally though for personal preference. In addition, the settings depend on the type of heating filament. The tip of the needle is closed after pulling, and should be opened by gently touching the fine point with a fine forceps under a stereomicroscope.
13. To prepare agarose plates with slots of 1 by 1 mm (6 cm in length) that hold the embryos in place for microinjection we use a house-made aluminium mold (kindly provided by Nicolas Cubedo, CNR, Université de Montpellier 2). We have recently seen similar glass molds available commercially (MidSci).
14. We do not bleach the eggs for each experiment. To enumerate CFU, it is important to use LB agar plates containing an antibiotic to allow growth of Bcc only, and avoid growth of bacteria from the natural microflora that might obscure the counts. Usually the antibiotic resistance

marker present on the reporter plasmid is used for this purpose. Due to the high intrinsic resistance to antibiotics of Bcc, you can also use polymyxin B or gentamicin when the bacteria do not contain a reporter plasmid. Of course it should first be analysed whether the strains or mutants used are resistant to these antibiotics. We have never encountered changes in virulence due to the presence of the reporter plasmid. We have found that the pBBR series of plasmids can be stably maintained in the Bcc during infection of zebrafish embryos in the absence of antibiotic pressure for over 5 days. We suggest to test this in the beginning by for instance comparing CFUs in individual embryos plated on LB with polymyxin B and LB with antibiotics used for selection of the plasmid, and then analysing the colonies for expression of the reporter plasmid under a fluorescence microscope. All Bcc colonies should express the fluorescent reporter, indicating the plasmid has been maintained in the absence of selection.

15. We use square plates (140 mm) to apply 10 µl drops of 10x dilution series per individual embryo (*See* **Fig. 3**). This method is highly reproducible and avoids having to plate multiple dilutions per individual embryo on single Petri dishes. It is very important that the square plates are level when the agar solidifies and when applying the bacterial drops, to avoid the drops running into each other once applied to the plate. It is equally important to properly dry the plates. We dry the plates without lid in a microbiological safety cabinet for exactly 30 minutes, not longer. Bcc do not grow well on plates that are too dry. For Bcc, use freshly prepared agar plates (do not use plates older than 1-2 days), and store the plates at room temperature to avoid condensation when stored at 4 °C.
16. An inverted fluorescence microscope with possibilities for large magnification is required for detailed analysis of bacteria-cell interactions. Our microscope is equipped with 5, 10, 40, 63, and 100 x objectives. The possibility to use Nomarski optics will greatly enhance the quality of the imaging of host cells and tissue. The microscope should also be equipped with the

different filter sets needed to visualize the different fluorescent markers, such as GFP and DsRed. For our Leica microscope, we use filter sets L5 (band pass (BP) 480/40; beam splitter (BS) 505; emission BP527/30) and N2.1 (515-560; BS 580; emission long pass (LP) 590), respectively. For imaging we used a Coolsnap fx (Roper Scientifique) and MetaVue software, and images are further processed using Adobe Photoshop. In addition, a stereomicroscope equipped for fluorescence (the Nikon AZ100, or alike) which allows rapid analysis of embryos and imaging of complete embryos (1x, 2x), but also allows more detailed imaging, is very useful. A minor disadvantage of this microscope is that it is difficult to focus the embryos due to light distortion (at the borders of the well, and the plastic) and the embryos should be transferred to glass bottom dishes or be observed in larger Petri dishes. Placement of embryos on agarose plates in E3 medium with Tricaine 1x is also a good option for imaging (see (33)). In this case, to mobilize the embryos on the plate, use a loading tip (that can be shortened if needed).

17. Six-, 12-, 24- or 48-well tissue culture plates can be used to culture embryos after injection. If possible, avoid using 96-well plates because the wells are too small for embryos meaning that they will not develop correctly if kept for more than 2 days in the wells.
18. Do not keep more than 100 eggs per Petri dish. Use multiple plates or larger 150mm Petri dishes to reduce the risk of contamination and allow normal embryo development. At this stage remove non-fertilized and empty eggs under a dissecting microscope as they may be a source of infection. Prevent transgenic eggs from being washed away through the sink and dispose of them properly.
19. The time point after fertilization that the embryos are best injected depends on the site of injection, the pathogen used, and the questions to be addressed. We usually microinject embryos in the blood circulation (blood island, or caudal vein, *See* **Fig. 2**) between 28 and 32 hours post-fertilization (hpf). At this stage immature macrophages and neutrophils are

capable of phagocytosing and killing non-pathogenic bacteria, and expression of genes involved in innate immune response, resembling those in human infections, can be detected by qRT-PCR. For the Bcc we can easily distinguish between strains that induce a strong inflammatory response, and kill embryos within 48 hours (e.g., *B. cenocepacia* K56-2), and less virulent strains that do not kill embryos within the duration of the experiment (5-6 days) but remain persistent in macrophages (e.g., *B. stabilis*). *Mycobacterium marinum* infections will require several days for the development of granuloma-like structures (15). Microinjection in the inner ear or hindbrain (to study recruitment of phagocytes) and in the notochord is typically performed in older (>50 hpf) embryos. When studying the role of certain virulence factors by micro injecting bacterial mutants, it may be advisable to compare virulence with the WT strain by injecting at different time points after fertilization. A type 3 secretion mutant of *Pseudomonas* was for example attenuated when injected 50 hpf, but not at 30 hpf (40).

20. Two fine tweezers can be used, each "gripping" the chorion, placing them close next to each other. By gently moving the tweezers apart the chorion will open and liberate the embryo. Avoid touching the yolk, as it will "disintegrate" upon touching. Alternatively, one pair of tweezers can be used; gently touch the chorion with the closed points of the tweezers, then immediately but gently open the tweezers to disrupt the membrane. We do not use proteolytic enzymes such as pronase to remove the chorion, as it can damage the embryos if left too long, and may have an effect on immune responses.
21. The calculations are based on a total number of $1x10^9$ bacteria per mL for an OD =1. A 20-fold dilution will result in 50 bacteria per nL. We prepare 50 µL total volume of inoculum (this can be reduced to 10 µL). The number of bacteria in a culture at OD_{600} 1 should be determined experimentally for each bacterial strain by plating different dilutions. The number of colony-forming units (CFU) can differ between strains under different growth

conditions. For *B. cenocepacia* K56-2 the culture density can sometimes reach an OD_{600} of 8 with almost all bacteria in the culture viable (contributing to CFU), whereas with other strains bacteria may start to die at this high density resulting in a discrepancy in the relationship between the OD and the number of CFU. For injections in other sites, such as the otic vesicle, higher bacterial inoculum will be required, since pressure and time of injection have to be reduced (See N**ote 27**).

22. For *B. cenocepacia*, as is the case for *Mycobacterium* (15) and *Salmonella* (16), a low infectious dose (<10 CFU) is sufficient to cause a lethal infection. We generally inject around 50 CFU to determine virulence of new isolates or mutants. For other pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (41,42), much higher inocula (>1,200 and 1,500 CFU, respectively) are needed to induce virulent infections. When initiating experiments with new bacterial species, we suggest finding the optimal inoculum, as well as different bacterial growth stages, as differences in virulence outcome may be apparent after infection of log-phase compared to stationary-phase-grown bacteria
23. Although we do not have this problem with Bcc, some bacterial species tend to form aggregates in culture. This could result in rapid clogging of the injection needle. In this case, pass the bacteria through a gauge needle several times to break up the aggregates.
24. Switch on the microinjector with the injection cable unplugged several minutes before injection to allow it to reach the correct pressure.
25. One could transfer the embryos prior to injection to a Petri dish containing E3 with 1% Tricaine, and subsequently position the embryos on the agarose plate for microinjection. However, we transfer embryos directly from E3 onto the agarose plate containing 5 mL of 2x Tricaine in E3. When doing this try to avoid adding a too large volume of E3 with the embryos with a Pasteur pipette. The embryos will “sink” to the bottom of the tip by holding it briefly in a vertical position, allowing positioning the embryos very rapidly in a small volume. We

have found that that incubation of embryos in Tricaine (without injection) reduces expression of some innate immune response genes (IL-8 and IL-1β) during the first hour after incubation compared to non-treated control embryos. Therefore it is essential when performing qRT-PCR to always treat PBS control embryos in an identical manner, including the time of incubation in Tricaine. As soon as embryos start to twitch when touching them with the injection needle, you should replace the Tricaine solution, as it is not working efficiently. A 20x solution can be stored at 4 °C for a maximum of 2 weeks. Do not reuse or store 2x dilutions, as the activity will go down.

26. Stage the embryos at 30 hpf: They should have a consistent blood circulation and a straight tail (43). To analyse virulence potential, we typically inject 60 embryos per bacterial strain. Five embryos are sacrificed at different time points to determine CFU (T= 0 (*see* **Note 30**), 24 and 48hpi (72, and later if desired), at least 24 embryos are used for mortality assays (*see* **section 3.4**), and around 10 to 20 embryos are kept for real-time observation of infection. Each experiment should be repeated at least 3 times. If host gene expression is to be analysed by qRT-PCR the number of infected embryos should be adjusted as required (*see* **section 3.7**).
27. There are several options to determine the correct injection volume. Due to the fact that pulled capillaries are used there will be significant variation in the bore of each needle. With the help of a scale bar on a microscope slide or in the ocular, adjust the pressure and or pulse time to obtain the desired diameter of a drop (34). Alternatively, a test injection can be done into a drop of glycerol on a glass slide, and fluorescent bacteria can be counted using a fluorescence microscope. But through experience, the trained eye will be able to rapidly determine the injection volume after performing a test injection in liquid E3 in a Petri dish. Typically, we use setting at 400 hPa and an injection time of 0.6 seconds. As noted below (*see* **Note 28**) different settings will be required when injecting in different anatomical sites (such

as the otic vesicle), normally reducing both time and pressure, thus injecting a smaller volume to avoid rupture of the vesicle. The bacterial density in the inoculum should be adjusted to give the correct infectious dose.

28. In order to reduce problems with clogging of needles and to ensure injection of reproducible numbers of bacteria, apply a low output pressure (<10 hPa) for a continuous flow.

29. The injection site can influence the observed virulence outcome, and this should be taken into account when analysing virulence potential by analysing embryo survival only. Neutrophils for instance were shown to need a surface to efficiently sweep up bacteria, including *Bacillus* (44), whereas in the blood circulation macrophages were the major phagocytosing cells. Bacteria that are normally killed by neutrophils may therefore be avirulent when injected subcutaneously in zebrafish embryos, but survive when injected in the blood and phagocytosed primarily by macrophages. This injection site-dependent phagocyte behaviour permits the study of the interaction of pathogens with different host cells, and identification of different host cell-specific mechanisms in virulence. Intravenous injection permits to study macrophage behaviour and subcutaneous injection in the embryo tail permits to study neutrophil behaviour. There are other sites for injection, each offering different experimental possibilities (**Fig. 2b-d**, *see* **Note 19**) (35). Injection into the Duct of Cuvier is an attractive alternative to injection into the blood island to introduce bacteria into the blood circulation. *M. marinum* can be injected into the yolk sac, and an automated injection system was developed for yolk injection (24). The tail muscle is a good site to study the migration of innate immune cells toward a local bacterial infection. The otic vesicle is a closed cavity that is use to study innate immune cell migration. The hindbrain ventricle is a closed cavity which contains very few macrophages (0 to 2 at 30 hpf). After injection into the hindbrain ventricle it is possible to follow the migration of macrophages and neutrophils to the infection site. Recently, the notochord has been described as another compartment for

infection. Macrophages are unable to enter the notochord because they cannot cross the collagen sheath (45).

30. To determine the precise inoculum (T=0), five embryos are “plated” individually immediately after micro injection for each strain. We found this more accurate than microinjection directly in a drop of LB or PBS on an LB agar plate. Collect embryos at different times during an injection series (for instance when injecting 50 embryos with one needle, take an embryo every other 10 embryos directly for determination of the inoculum). This will allow showing whether the inoculum is constant throughout the injection series. Do not wait too long before disrupting the embryo, as non-virulent bacteria may be phagocytosed and killed rapidly, and this would result in an underestimation of the inoculum size.
31. Prior to disrupting each embryo in trypsin/Triton, transfer the embryo to a Petri dish with 10 mL fresh E3. Taking embryos directly from the injection plate and transferring to an Eppendorf tube carries the risk of carrying over bacteria present on the injection plate.
32. For some Bcc strains longer incubations are needed. Bacteria can display different morphotypes. Sometimes ghostlike or egg-like colonies can be observed at different frequencies after passing through the animals. These are Bcc, and should be included in the CFU counts (and could be verified after longer growth with a fluorescence microscope for presence of the reporter plasmid). Such morphotype variations may give interesting information about the bacterial strains and interaction with host cells.
33. If bacterial numbers greater than 250 are expected (inoculum, or during infection by increasing bacterial numbers) bacteria are plated in serial dilutions, as described in section 3.5.
34. Infection experiments using zebrafish embryos are not considered animal experimentation until they reach the free feeding stage. Experiments should therefore be terminated before

this stage, unless the experiment requires longer infection times and authorization has been obtained (*see* **Note 2**).

35. For determination of bacterial multiplication rates, CFU in five individually treated embryos per time point per strain should be analysed. Pooling of the embryos will not allow statistical analysis of variation in virulence. We generally determine CFUs for Bcc at 24 and 48 hpi. For each additional time point, for example to analyse the onset of bacterial replication in more detail (*B. cenocepacia* K56 is taken up by macrophages and survives and starts to replicate intracellularly around 6 to hpi (11) five extra embryos are required. Large differences in bacterial counts can be expected between different Bcc strains (*See* **Fig. 3B**). For Bcc, it is not useful to try to calculate intracellular bacteria by using gentamicin or another antibiotic treatment, as Bcc are resistant to most antibiotics.
36. Bcc are generally resistant to treatment with trypsin and Triton-X-100 at the indicated concentration. We advise however to check whether bacterial survival is affected by this treatment by incubating bacterial dilutions from an overnight culture in E3 with and without trypsin/Triton for 10, 20, and 30 minutes, prior to plating on LB agar plates.
37. Depending on the virulence of the strains, the bacteria can multiply to high numbers during infection, and it may be necessary to dilute the samples for counting CFU. Whether a strain is multiplying and to which extent can be visualized by fluorescence microscopy. For K56-2 at 24hpi 10 µl drops of 10^{-1}, 10^{-2} and 10^{-3} dilutions and at 48hpi 10 µl drops of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of infected embryos are typically plated (*see* **Fig. 3**). For *B. stabilis*-infected embryos 10^{-1} dilutions and the undiluted remaining 90 µl are plated.
38. It is important to know the growth rate of the bacterial strain used. When incubated too long, the colonies will start overgrowing each other very rapidly. The small colonies should be counted under a stereomicroscope when visible and not yet touching each other. For

some bacterial species it may be required to incubate the plates at 30 °C overnight (such as *Salmonella*).

39. It is important to count each (countable) dilution for each embryo; this allows seeing whether dilutions were performed correctly. Spots with more than 150 colonies should not be considered due to the risk of overlapping colonies and underestimating CFUs. Use the dilution with a value of between 20 and 150 CFU to calculate the total CFU per embryo. Do not forget to prepare the dilution to multiply by 10 because only 10 µL from a 100 µL embryo lysate was used.
40. For statistical analysis we use GraphPad Prism software, which we find very useful to represent both bacterial multiplication data and Kaplan-Meier survival assays, including statistical analysis.
41. To study the global host immune response at the transcriptional level during infection, qRT-PCR (quantitative reverse transcription PCR) and RNA-seq experiments can be performed. Extreme care should be taken in handling and manipulating the embryos because any wounding during embryo development can induce innate immune responses, and will add to experimental variation. Inject each embryo only once and very carefully. We found that the anaesthetic has an immunosuppressive effect on embryos and we can see this effect on cytokine gene expression at least up to 1 hour after injection. Therefore, do not leave embryos too long in the anaesthetic solution, and treat all controls (PBS injection) in exactly the same manner. Try to inject the different pools without too much time difference, since also the development of the embryos during this stage is very fast. We use a pool of at least 10 embryos (up to 25 works well) per condition for each time point. For qRT-PCR or RNA-seq analysis, a PBS-injected control group is essential. For Bcc, we generally analyse embryos 3-4, 7- 8 and 24 hpi.

42. It is important to work in RNAse- and DNA-free environment. It is possible to treat the bench and objects with RNAse inactivator such as RNase Away. After extraction, RNA can be stored for a long time at -80°C.
43. For ten embryos we obtain around 200-300ng/µl of RNA. Especially for RNA-seq experiments in which highly pure and non-degraded RNA is required it is essential to analyse the quality of the RNA, for instance with a Bioanalyzer (Agilent).
44. Before the experiment, determine the number of genes you wish to analyze. Always include a housekeeping gene. It is imperative that the expression level (CP value) of the housekeeping gene does not vary from one condition to the other (infected, PBS, etc.). For zebrafish qRT-PCR, several housekeeping genes such as *PPIAL* or *EF1a* genes have been used. We have good results with *PPIAL*. Results with *EF1a* were less reproducible during Bcc infection.
45. It is important to use exactly the same quantity of RNA in all samples; otherwise the different levels of gene expression cannot be compared. cDNA can be conserved for several months at -20°C.
46. A volume of 2.5µl cDNA is used per reaction, each condition should be set up in triplicate. Be careful, diluted cDNA is less stable and it is advised to store only one night at 4°C.
47. We use a Light Cycler 480 from Roche for qPCR analysis, with the SYBR Green kit from Roche. Using another light cycler, other kits may be optimal.
48. The Tm of the primers must be around 60 °C and the PCR product should be between 100 and 150 nucleotides (nt) in order to reach maximum efficiency during the PCR reaction. The size of the primers should be between 20 and 25 nt. Manual technique to calculate Tm for oligonucleotides : [(A+T)x2 + (G+C)x4] x [1+(N-20)/20] = Tm in °C, N=number of nucleotides. This formula work for an oligonucleotide with more than 20 nt. Programs can be used to calculate the Tm.

49. Avoid bubbles; if there are bubbles in the wells perform a short centrifugation step: 2 min at 700g.

50. Before starting the analysis, check whether the housekeeping gene gives reproducible results under the different conditions and that all replicates have a reproducible CP (small variations of ~0.5 cycle are allowed). Then, perform a melting curve analysis; there must be only one peak per gene for all reactions. If there are several peaks this indicates that there are several PCR products; either the sample is not pure or the primers are not specific. Next, analyze the data with the second derivative or ΔΔCt method (46). As a (simplified) example: PBS, T= 3hpi (=control sample), strain K56-2, T=3hpi (=target sample). Reference gene = PPIAL, target gene = IL-8.

    For each sample (control and target), calculate 1^{st} ΔCT : c = b – a, where

    a = average of replicates of reference gene of sample A (e.g., PBS 3hpi/PPIAL)

    b = average of replicates of target gene of sample A (e.g., PBS 3 hpi/IL-8)

    The c-value of the control sample (in our case PBS, 3hpi), become the 2^{nd} ΔCT = C.

    The normalized value for target gene expression is then $2^{-\Delta\Delta CT}$ where ΔΔCT = c – C

    In the example (**Table 2**) below the IL-8 expression is enhanced 3.58 times during K56-2 infection at 3 hpi.

Table 2. Example of qRT-PCR comparing target and reference gene expression under one experimental condition (K56-2 infection at 3hpi)

	CP n°1	CP n°2	CP n°3	AVG Reference gene
PBS 3hpi	21,31	21,36	21,37	21,35
K56-2 3hpi	21,19	21,23	21,20	21,21

	CP n°1	CP n°2	CP n°3	AVG target gene
PBS 3hpi	24,61	24,67	24,65	24,64333333
K56-2 3hpi	22,69	22,6	22,7	22,66333333

	1st Δct	2nd Δct	ΔΔct	$2^{-\Delta\Delta CT}$	Normalized Values
PBS 3hpi	3,30	3,30	0,00	1	1
K56-2 3hpi	1,46	3,30	-1,84	3,580100284	3,580100284

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CHAPTER 3

Macrophages are a critical site for replication of *Burkholderia cenocepacia* and ensuing MyD88-dependent inflammation

The work presented in this chapter describes the largest part of my thesis work, in which I have performed experiments to study the role of phagocytes during infection with different species belonging to the Bcc, and the inflammatory response, specifically the role of Cxcl8 and Myd88. For this chapter, I performed all experiments except those presented in Figure 2, Figure 3 (B-D), Figure S3 and Figure S4 (A to C).

Submitted for publication

CHAPTER 3

MACROPHAGES ARE A CRITICAL SITE FOR REPLICATION OF *BURKHOLDERIA CENOCEPACIA* AND ENSUING MYD88-DEPENDENT INFLAMMATION

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Running title (50 characters): role phagocytes during *B. cenocepacia* infection

SUMMARY

Bacteria of the *Burkholderia cepacia* complex (Bcc) can cause devastating pulmonary infections in cystic fibrosis (CF) patients, yet the precise mechanisms underlying inflammation, recurrent exacerbations and transition from chronic stages to acute infection and septicemia, are not known. Here we show for the first time *in vivo* that macrophages are a critical site for multiplication of *B. cenocepacia* and development of a rapidly fatal MyD88-dependent pro-inflammatory response in zebrafish embryos. Inflammation is enhanced by Cxcl8-dependent recruitment of neutrophils that are unable to clear the bacteria, and coincides with systemic phagocyte death. In agreement with recent clinical observations showing Bcc bacteria in alveolar macrophages of CF patients, we suggest that paradigm-changing approaches are needed for development of new antimicrobials to efficiently disarm this group of bacterial intracellular pathogens.

Highlights

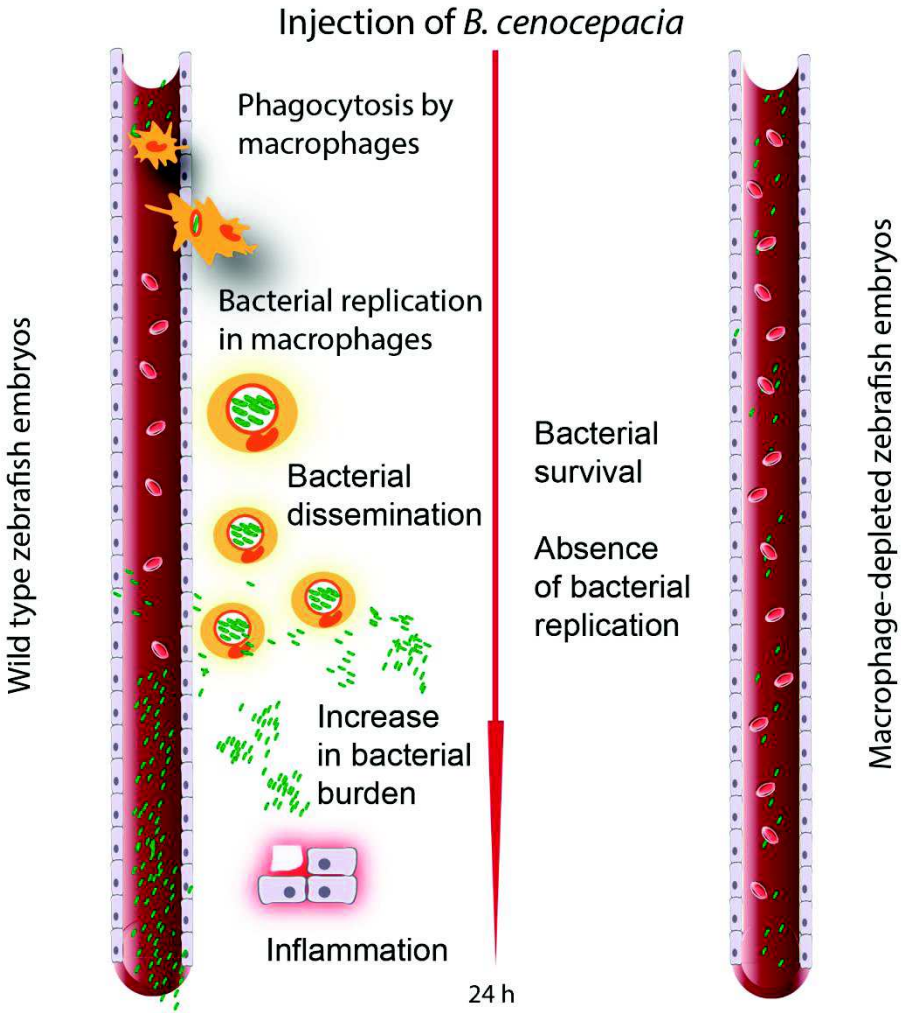
-*B. cenocepacia* exploits macrophages for replication and induction of fatal inflammation

-Cxcl8-dependent neutrophil-recruitment enhances Bcc-elicited inflammation

-Surface-associated Bcc resist ROS-dependent eradication by neutrophils

-Absence of Myd88 increases resistance of zebrafish to *B. cenocepacia* challenge

GRAPHICAL ABSTRACT



INTRODUCTION

Pulmonary disease is the most important cause of morbidity and mortality in cystic fibrosis (CF) patients. Vicious cycles of pulmonary obstruction, chronic infection, and ineffective airway inflammation result in progressive deterioration of lung function (Hartl et al., 2012). Infections with bacteria belonging to the *Burkholderia cepacia* complex (Bcc) are particularly worrying. They aggravate clinical outcome with recurrent exacerbations and often result in fatal necrotizing pneumonia and septicemia known as Cepacia Syndrome, which is further complicated by their intrinsic multiple resistance to most clinically used antibiotics (Drevinek & Mahenthiralingam 2010).

CF lung disease is characterized by massive neutrophil infiltration and excessive production of pro-inflammatory cytokines, including IL-1β, TNFα and the major neutrophil chemo attractant CXCL8 (IL8). Neutrophils are fundamental in fighting infections as part of the innate immune response. However, dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) in neutrophils and ineffective resolution of inflammation are considered as major causes of many of the pathological consequences seen in CF through repeated cycles of ineffective bacterial clearance and neutrophil death (Pohl et al., 2014). Although human neutrophils have been shown to be able to reduce *B. cenocepacia* numbers in a ROS-dependent manner *in vitro* (Speert et al., 1994), neutrophil activation has been suggested to play an important role in the observed clinical deterioration of the CF lung in *B. cenocepacia*-colonized patients (Hughes et al., 1997).

Initially thought to have an exclusively extracellular lifestyle, *B. cenocepacia* can also build an intracellular niche and evade host immune killing, as has been shown extensively using

macrophage and epithelial cell culture models (reviewed by Saldías & Valvano 2009; Ganesan & Sajjan 2011). In macrophages, *B. cenocepacia* has been shown to interfere with the phagolysosomal maturation process, membrane trafficking and, more recently, the autophagy machinery (Abdulrahman et al., 2011; Al-Khodor et al., 2014). Several bacterial virulence factors and regulators, including bacterial secretion systems, MgtC and RpoE play a role in the bacterium's intracellular survival strategy (Maloney & Valvano 2006; Flannagan & Valvano 2008; Flannagan et al., 2012). *B. cenocepacia* has also been detected in macrophages in experimental rodent models (Chiu et al., 2001; Sajjan et al., 2001a), and we have shown that *B. cenocepacia* can replicate in macrophages in experimentally infected zebrafish embryos (Vergunst et al., 2010). Importantly, different Bcc species have been detected in alveolar macrophages of lung tissue samples from infected CF patients (Sajjan et al., 2001b; Schwab et al., 2014). While macrophages have been suggested to be involved in the early onset of inflammation in CF infants (Hubeau et al., 2001) and CF macrophages stimulated with LPS or infected with *B. cenocepacia* display a significantly increased cytokine production (Bruscia et al., 2009; Kopp et al., 2012; Gavrilin et al., 2012), the direct contribution of macrophages to the exaggerated inflammation in CF, specifically that of Bcc infected macrophages *in vivo*, is poorly understood.

The rise of the zebrafish (*Danio rerio*) as a suitable vertebrate model for infectious disease studies is for a large part due to the high similarity of its immune system to that of humans (van der Vaart et al., 2012), Macrophages and neutrophils are involved in protection against experimentally introduced microbes from an early developmental stage, and the excellent possibilities of intravital imaging in the transparent zebrafish embryos have shown the power of this model in better understanding microbe/host phagocyte interactions (Cambier

et al., 2014; Mostowy et al., 2013). Here, we demonstrate that macrophages are critical for bacterial multiplication of intravenously (iv) injected *B. cenocepacia* in zebrafish embryos. Moreover, we show that macrophage-driven fatal inflammation requires the Myd88-dependent arm of the innate immune system. And that neutrophils, even when dominating phagocytosis of surface-associated bacteria, are unable to eradicate the bacteria. We will discuss the implications of our findings that macrophages are crucial for disease development in the context of development of therapeutic strategies to control infections with the Bcc.

RESULTS

Macrophages are critical for *B. cenocepacia* multiplication and fatal infection

To better understand the explicit role of macrophages during infection with Bcc strains we used two strategies to deplete macrophages from zebrafish embryos; knockdown of Pu.1[22], a transcription factor involved in early myeloid progenitor formation, and chemical ablation. Intravenous injection with *B. cenocepacia* K56-2, J2315 or *B. cepacia* CEP509, epidemic strains that cause rapidly fatal systemic infection in zebrafish embryos[12], surprisingly resulted in a significant pro-survival effect when macrophages were depleted from embryos by pu.1 knockdown (Fig. 1a, Supplementary Fig. 1a and 1c). This correlated with significantly reduced bacterial burden at 24 and 48 hours post infection (hpi) (Fig. 1b, Supplementary Fig. 1b, 1d) in a Pu.1-specific manner (Supplementary Fig. 1e). Intravital imaging of iv injected Tg(*mpeg1:mCherry-F*) embryos, expressing membrane-localized mCherry in macrophages, confirmed that in control embryos bacteria were rapidly seen associated with macrophages and that the embryos had become heavily infected by 24 hpi, (Fig. 1c), in contrast to pu.1 knockdown embryos which contained far fewer bacteria (Fig. 1d). Of note, macrophage-associated mCherry expression was lost in infected control embryos at 24 hpi (Fig. 1c and described further below). The applied concentration of MOs[22] resulted in depletion of macrophages (Fig. 1d compared to Fig. 1c, 30 mpi), but not neutrophils (Supplementary Fig. 1f), although the effect was temporary as mCherry positive macrophages re-appeared during infection (Fig. 1d). These data suggest that macrophages are required for efficient bacterial multiplication and fatal infection..

Next, we adopted a chemically-inducible targeted cell ablation strategy based on the metronidazole/nitroreductase (Mtz/NTR) system[23]. Treatment of zebrafish embryos which expressed a *mCherry-NTR* fusion protein (umn^{+}) in macrophages (Supplementary Fig. 2a) with the prodrug Mtz resulted in efficient specific ablation of macrophages (Supplementary Fig. 2b and 2c). Such Mtz-treated embryos lived significantly longer than control embryos after iv injection of *B. cenocepacia* K56-2, with 40% still alive at 5 dpi (Fig. 1e). Strikingly, no significant multiplication of *B. cenocepacia* was seen in the macrophage-depleted embryos up to 24 hpi (Fig. 1f), showing the iv-injected bacteria do not replicate extracellularly. Infected DMSO-control or Mtz-treated non-fluorescent umn^{-} embryos showed no significant difference in bacterial burden or embryo survival compared to non-treated infected control embryos (Fig. 1e, 1f). Together, these data consolidate the pu.1 knockdown results, and demonstrate that macrophages are critical for multiplication of iv injected *B. cenocepacia* K56-2, J2315 and *B. cepacia* CEP509 and the development of a rapidly fatal infection.

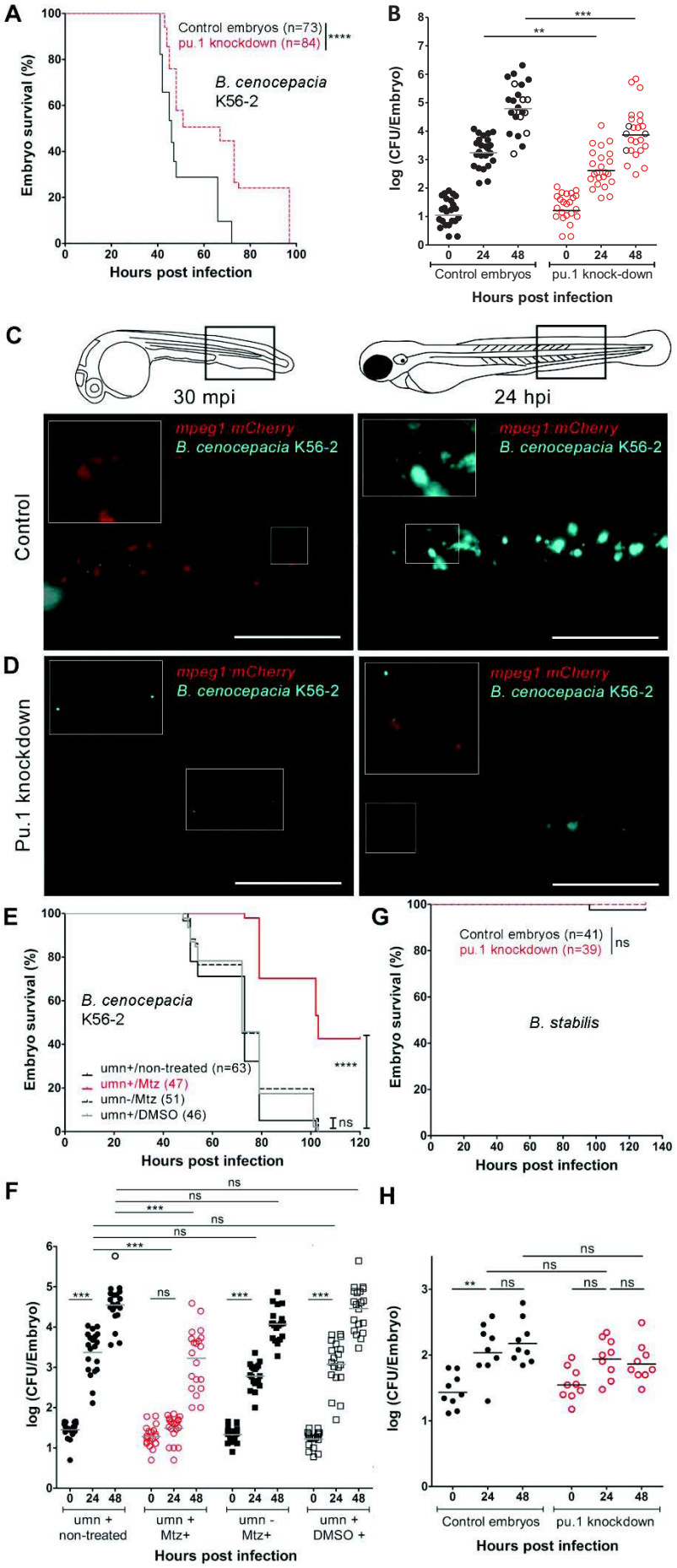


Figure 1. Macrophages are critical for virulence of *B. cenocepacia*

(A and B) Embryo survival (A) and bacterial burden over time (B) of control (black) and *pu.1* knockdown embryos (red) injected iv with *B. cenocepacia* K56-2 (~15 CFU and ~20 CFU respectively). Five independent experiments.

(C and D) Representative bright field and fluorescence overlay (left) and fluorescence (right) images of an *mpeg1:mCherry* control (C) and *pu.1 mpeg1:mCherry* knockdown embryo (D) at 30 min and 24 h after injection with ~40 CFU *B. cenocepacia* K56-2 (blue). (C) *mpeg1:mCherry*-positive macrophages (red) colocalise with K56-2 at 30 mpi (inset), and are no longer detected at 24 hpi. (D) Macrophages are absent in knockdown embryos at 30 mpi and re-appear at 24 hpi. Scale bar, 100 µm.

(E and F) Embryo survival (E) and bacterial burden (n=10 per time point) over time (F) of umn^{+} embryos, untreated or treated with 5mM Mtz or 0.2% DMSO, and umn^{-} embryos treated with 5mM Mtz iv injected with *B. cenocepacia* K56-2 (~40 CFU). Two independent experiments.

(G and H) Embryo survival (G) and bacterial burden over time (H) of control (black) and pu.1 knockdown embryos (red) iv injected with *B. stabilis* LMG14294 (~20 CFU). Two independent experiments.

(B, F, H) Geometric means with each data point representing an individual embryo. Dead embryos marked as black open circles. (A, B, E, F G and H) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; **** $p \leq 0.0001$. ns: not significant. See materials and methods for statistical tests. (Also see Figure S1 and S2).

Macrophages inefficiently clear less virulent Bcc strains

We were interested to determine the effect of macrophage depletion on the fate of *B. stabilis* LMG14294, *B. cenocepacia* LMG16654 (J415), and *B. vietnamiensis* FC441, which we have shown earlier to cause persistent infection in macrophages (Vergunst et al., 2010). The absence of macrophages had no significant effect on host survival for any of the three strains (Figure 1G, S1G and S1K, respectively). For *B. stabilis*, pu.1 knockdown resulted in slightly but not significantly lower bacterial numbers at 48 hpi (Figure 1H), showing that macrophages are not contributing to elimination of the bacteria. *B. cenocepacia* J415, however, was cleared in the presence, but not absence of macrophages at 48 hpi in 50% of the embryos (Figure S1H). CFU counts were also slightly, but not significantly higher at 48 hpi for *B. vietnamiensis* after pu.1 knockdown compared to control embryos (Figure S1I). These data show that macrophages may have some, although insignificant protective role against less virulent strains. Macrophages do not contribute to efficient bacterial clearance, which might result in increased intracellular persistence.

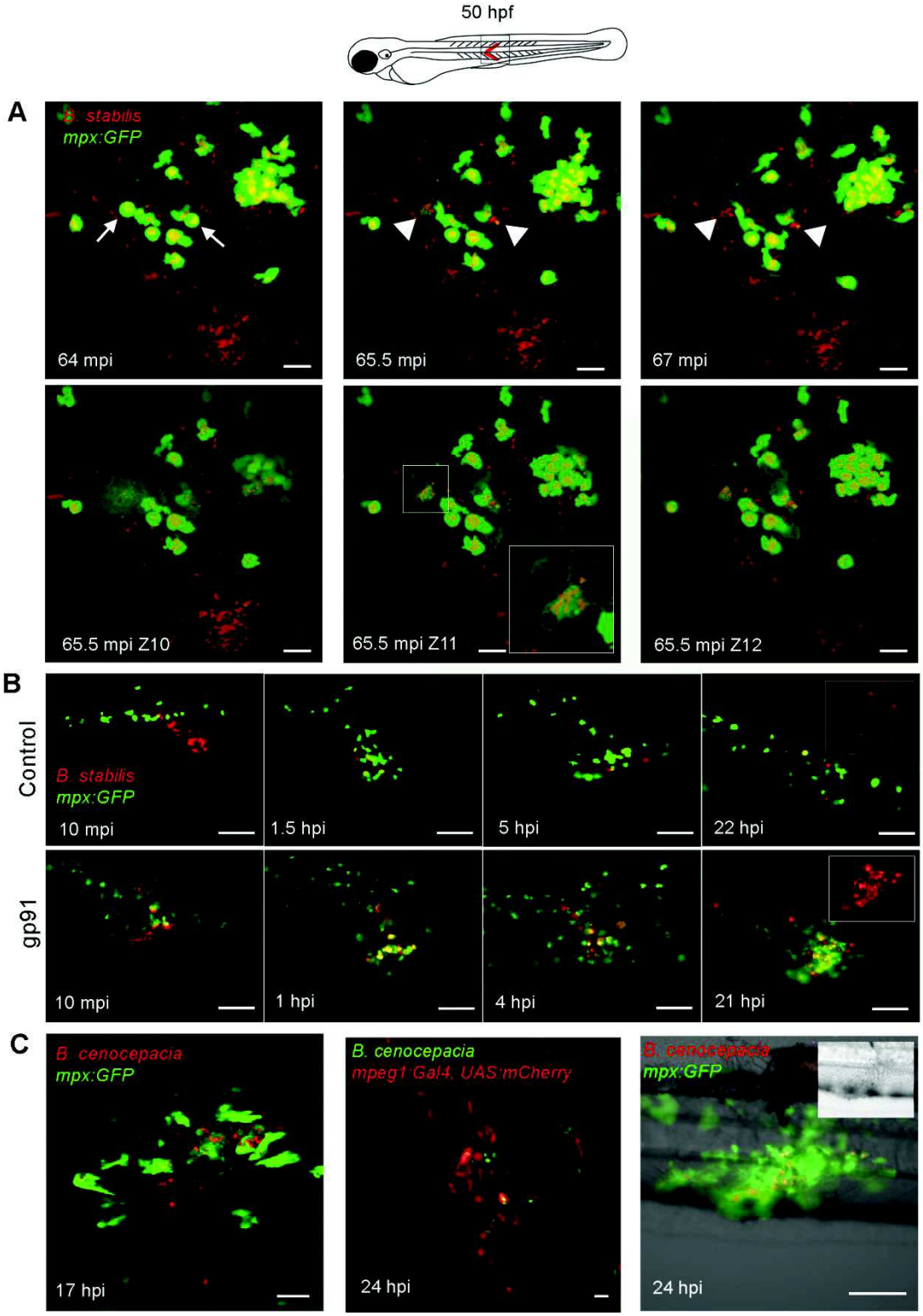


Figure 2. Neutrophils phagocytose surface-associated Bcc but are unable to eradicate the bacteria

(A) Confocal stack after subcutaneous infection *B. stabilis* (STD intensity projection, 2µm x19 steps; T=35-37 in movie S2) in *mpx:GFP* embryos. Arrows, rounded neutrophils (green), with vacuoles full of bacteria 64 mpi), eject their cell contents in the surroundings (arrow heads 65.5 mpi, diffuse GFP signal), leaving bacterial clusters and cell debris (arrow heads 66 min). Lower panels, individual slices at 105.5 mpi. Scale bar, 50 µm. (B) Images of a representative *mpx:GFP* control MO and *gp91* knockdown embryo in time after subcutaneous injection with *B. stabilis.* Inset shows bacterial load at ~20 hpi. Scale bar, 100 µm. (C) *mpx:GFP* and *mpeg1:mCherry* (umn^{+}) embryos infected subcutaneously with *B. cenocepacia* K56-2. Left, confocal stack of *mpx:GFP* embryo (2 µm x 19 steps, T=9 in Movie S1) at 17 hpi. Middle, confocal stack at 24 hpi showing bacteria associated with macrophages in *mpeg1:mCherry* embryo. Scale bar 50 µm. Right, representative image at 24 hpi showing tissue inflammation in a *mpx:GFP* embryo, scale bar 100 µm. See also Figure S3.

Neutrophils rapidly phagocytose surface-associated Bcc but are unable to eradicate the bacteria *in vivo*

Intravenous injection of several strains belonging to the Bcc in embryos is marked by macrophage-dominated phagocytosis (Vergunst et al., 2010). To better understand the fate of the bacteria in neutrophils upon phagocytosis *in vivo* we performed experiments based on the elegant work by Colucci-Guyon and colleagues who showed that neutrophils very efficiently sweep up surface-associated bacteria in contrast to liquid-borne bacteria (Colucci-Guyon et al., 2011). Intravital imaging of Tg(*mpx:GFP*) embryos subcutaneously injected with high doses of fluorescent *B. cenocepacia* K56-2 or *B. stabilis* LMG14294 showed that neutrophils were rapidly attracted to live and heat-killed (HK) Bcc, but not to PBS, and infiltrated the infected area (Videos S1, S2), indicating that neutrophils were responding specifically to bacterial signals. To our surprise, we observed spontaneous cell lysis of bacteria-containing neutrophils after about 2-3 hpi; once the cells had rounded up, cell contents were ejected into the extracellular space covering a large area, reminiscent of neutrophil extracellular trap (NET) (Figure 2A, Video S2). We observed the same phenomenon with neutrophils that had taken up surface-associated *B. cenocepacia* K56-2 (not shown). Recruited $GFP^{positive}$ neutrophils, and GFP^{minus} macrophages, phagocytosed the bacteria at the infection site within 3-5 hours. A functional NADPH oxidase complex, required for production of reactive oxygen species (ROS), was important to reduce bacterial burden and neutrophil numbers at the site of infection in *B. stabilis*-infected embryos (Fig. 2b); knockdown of the $gp91^{phox}$ (or *cybb*) NADPH oxidase subunit[25] resulted in increased bacterial numbers and persistent neutrophil infiltration at the infection site. Neutrophils

were still massively recruited at later time points in 71.3% (±14.43) of the gp91 knockdown embryos (3 independent experiments, n= 8, 10, and 22 respectively), compared to 13.2% (±6.85% SEM) of control embryos (n=8, 12, and 26, respectively). In control embryos bacteria persisted in small clusters, possibly in GFP^{minus} macrophages and in clusters released by neutrophils, or as individual bacteria (Supplementary Fig. 3). Together the data suggest that during neutrophil-dominated subcutaneous injections ROS are important for controlling *B. stabilis* burden and resolution of inflammation, although neutrophils are unable to completely eradicate these bacteria. In contrast to *B. stabilis*, even in the presence of a functional NADPH oxidase complex, persistent neutrophil infiltration, tissue inflammation and increased bacterial burden were observed in 100 % of the *B. cenocepacia* K56-2-infected embryos. *B. cenocepacia* K56-2 was associated with macrophages (Fig. 2d).

Acute infection is correlated with systemic cell death of non-infected phagocytes

Although neutrophils do not phagocytose iv injected Bcc efficiently (Vergunst et al, 2010), real time imaging showed that from the onset of bacterial dissemination from infected macrophages, patrolling neutrophils started to approach such cells, often completely engulfing them without apparent success, and took up extracellular bacteria seen close to infected cells (Figure S4A). Although it is currently not clear how K56-2 exits from infected macrophages, neutrophils were seen to degranulate close to infected cells which might contribute to release of intracellular bacteria (Figure 3A). Subsequent neutrophil infiltration was followed by tissue damage (Figure 3B), and the infection progressed with massive

reduction in total numbers of GFP^{positive} cells leading to complete neutropenia by the time of death (Figure 3C). In contrast to the massive neutrophil infiltration seen during K56-2 infection, neutrophils were only occasionally seen near *B. stabilis*-infected macrophages (not shown), and an increase in neutrophil numbers was observed in about 50% of the embryos by 48 hours (Figure 3C, S4B, S4C). Neither treatment of embryos with the ROS scavenger N-acetyl cysteine (NAC) nor *gp91*^{phox} knockdown significantly affected survival and bacterial burden in embryos iv injected with *B. cenocepacia* K56-2, J2315 or with *B. stabilis* LMG14294 (Figure 3D and not shown). This suggests that ROS-mediated responses do not play a significant role in host defence during infections with macrophage-dominated phagocytosis.

Using Tg(*mpeg1:mCherry*) embryos, we found that non-infected mCherry^{positive} macrophages also disappeared during infection with K56-2 (Figure 1C, 3E, S4D). qRT-PCR analysis showed that both the neutrophil-specific *mpx* and macrophage-specific *mpeg1* genes were rapidly down regulated in embryos injected with K56-2, but not *B. stabilis*-injected embryos (Figure 3F). This was not simply due to reduced expression levels of the transgenes, since immune labeling with the leukocyte specific marker L-plastin or the dye neutral red (NR) also revealed drastically reduced phagocyte numbers in infected embryos (not shown), while infected macrophages stained positive for L-plastin (Figure S4E). In addition, staining with the cell impermeable dye SYTOX Green showed that the circulation of K56-2 infected embryos was loaded with extracellular DNA (Figure 3G), in contrast to non-infected control embryos that only showed few SYTOX-positive cells at the site of DMSO injection. Of note, infected macrophages were not positive for SYTOX, showing membrane integrity. Thus, excessive inflammation correlates with systemic phagocyte death, yet infected macrophages stay alive.

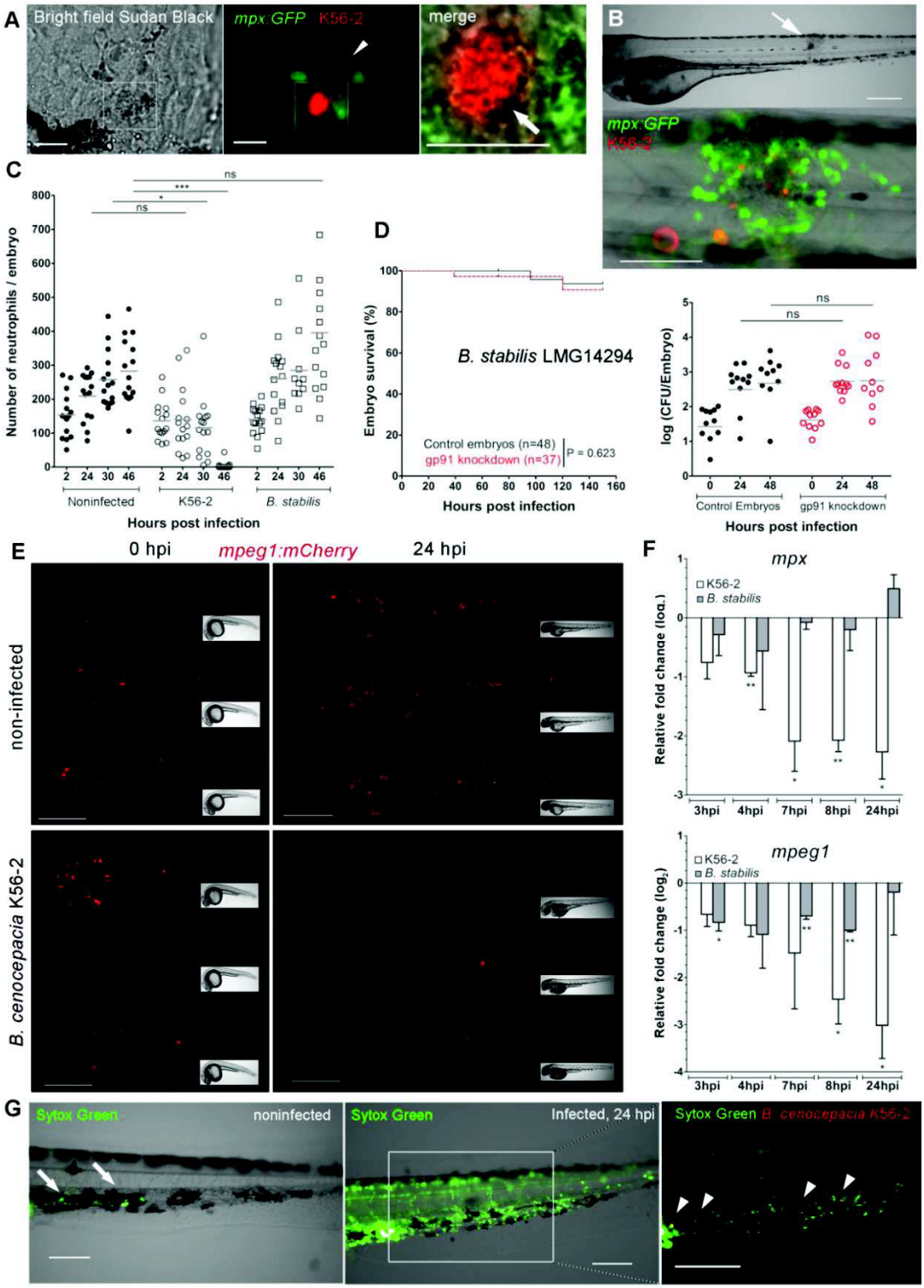


Figure 3. Acute, but not persistent infection results in systemic phagocyte death.

(A) Sudan black staining of an *mpx:GFP* embryo at 24 hpi, injected with ~45 CFU *B. cenocepacia* K56-2. Recruited neutrophils (green), release their granules (black deposit, white arrow) close to an infected cell loaded with bacteria (red). Note individual bacteria above infected cell (arrow head). Bright field, fluorescence (red and green channel) and merged images. Scale bar, 50 µm.

(B) Representative image of an m*px:GFP* embryo, 28 hpi, infected with *B. cenocepacia* K56-2 (red), showing infiltrated neutrophils (green) to an infection site (arrow) with multiple infected cells. Scale bar top 200 µm, bottom 50 µm.

(C) Mean neutrophil numbers, determined by pixel counting in *mpx:GFP* embryos injected at 50 hpf with ~58 CFU *B. cenocepacia* K56-2 or ~30 CFU *B. stabilis* LMG14294. See also Figure S4B and C.

(D) Embryo survival (left) and bacterial burden over time (right, geometric mean) of control and gp91 knockdown embryos iv injected with *B. stabilis* (~ 49 CFU). Three independent experiments.

CXCL8 enhances neutrophil recruitment, neutropenia and inflammation during acute infection

To better understand the immune signalling pathways playing a role during persistent and inflammatory infection caused by different Bcc species *in vivo*, we first quantified global *cxcl8*, *il1b* and *tnfa* gene expression levels. Whereas iv-injected heat-killed (HK) *B. cenocepacia* K56-2 induced a rapid 3 to 4 fold relative increase in pro-inflammatory cytokine expression that returned to control levels within 4 hours, live bacteria induced a strong increase in pro-inflammatory gene expression which peaked at 3 to 5 hpi, and remained high until at least 24 hpi (Figure 4A,B and S5A). The level of induction was bacterial dose-dependent (Figure S5B and S5C). In contrast, injection with *B. stabilis* resulted in only slightly enhanced expression levels of pro-inflammatory cytokines (Figure 4C, D and S5D), in agreement with the persistent infection phenotype.

Knockdown of *cxcl8* in Tg(*mpx:GFP*) embryos showed a significantly delayed neutrophil recruitment to infection sites and delayed neutropenia (Figure 4E, S5E), and *cxcl8* knockdown embryos died significantly later than control embryos (Figure 4F). This shows that cxcl8-dependent recruitment of neutrophils is contributing to fatal systemic infection. Interestingly, significantly fewer bacteria were present at 48 hpi after *cxcl8* knockdown compared to control embryos (Figure 4G).

(E) Representative images of *mpeg1:mCherry* embryos showing reduced numbers of macrophages (red) in *B. cenocepacia* K56-2-infected (~45 CFU) compared to non-infected control embryos. Scale bar, 0.5 mm. See also Figure S4D.

(F) Mean relative *mpx* and *mpeg1* gene expression (qRT-PCR) with 95% confidence interval (error bars) in embryos injected with ~50 CFU *B. cenocepacia* K56-2 (white) or *B. stabilis* LMG14294 (grey) normalised to a PBS-injected control group at each time point. Three independent experiments.

(G) Representative images of non-infected and *B. cenocepacia* K56-2 (~50 CFU, turquoise indicated in red for better visualization) infected embryos at 24hpi with the cell-impermeable dye Sytox Green. Arrow, dead cells due to DMSO injection. Arrow heads, bacteria. Scale bar, 100 µm.

(C,D). Each data point represents an individual embryo.

(C,D,F,G) * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; ns : non-signifiant. See materials and methods for statistical tests. See also Figure S4.

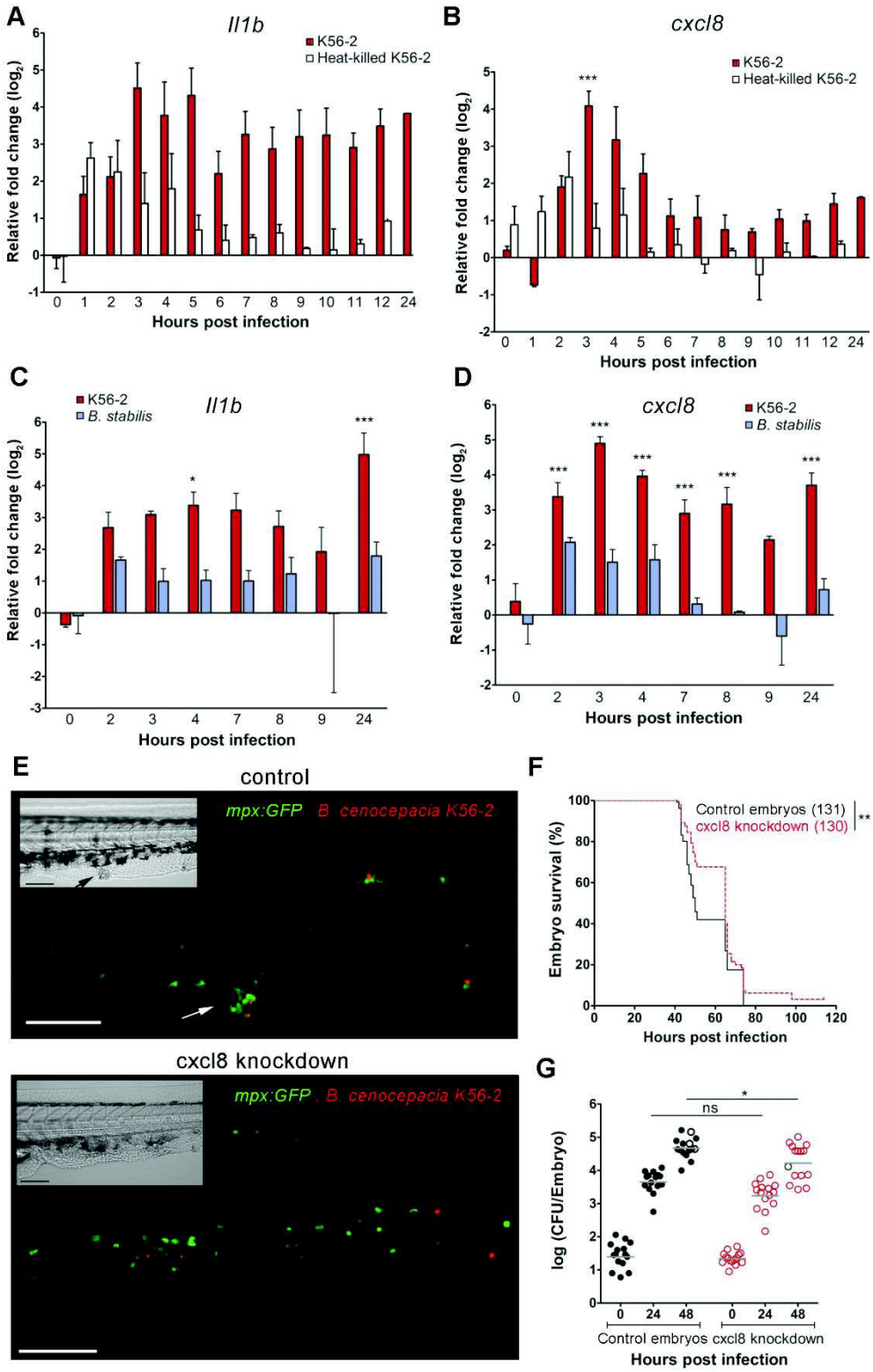


Figure 4. C*xcl8* knockdown delays neutrophil recruitment, neutropenia and inflammation during acute infection.

(A-D) Mean relative *il1b* (A,C) and *cxcl8* (B,D) gene expression levels (qRT-PCR) in embryos injected with ~50 CFU live or heat-killed (HK) *B. cenocepacia* K56-2 (A,B), or ~200 CFU *B. cenocepacia* K56-2 or *B. stabilis* LMG14294 (C,D), normalized to a PBS-injected control group at each time point. Error bars represent a 95% confidence interval of the mean of three biological replicates. For *tnfa* see Figure S5. (E-G) *mpx:GFP* control and cxcl8 knockdown embryos were injected with ~50 CFU *B. cenocepacia* K56-2 (red). (E) Representative images at 22 hpi show recruited neutrophils (green) in control embryos (top), but not in *cxcl8* knockdown embryos (bottom). Scale bar, 100 µm. (F,G) Embryo survival (F) and bacterial burden over time (G) of control (black) and *cxcl8* knockdown (red) embryos iv injected with ~40 CFU *B cenocepacia* K56-2. Three independent experiments. Geometric means are shown with each data point representing an individual embryo. Dead embryos are indicated with black open circles. (A-D, F, G).* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns: non-significant. See materials and methods for statistical tests. See also Figure S5.

Myd88 contributes to pro-inflammatory fatal infection caused by *B. cenocepacia*

Myd88 is a key adaptor protein in the inflammatory response and the production of pro-inflammatory cytokines, and we analyzed its role in acute infection caused by iv injected *B. cenocepacia* K56-2. Surprisingly, *myd88* knockdown embryos lived significantly longer than control embryos (Figure 5A). Paradoxically, the absence of Myd88 resulted in significantly higher CFU at 24 and 48 hpi compared to control embryos (Figure 5B), suggesting a Myd88-dependent control of bacterial multiplication. We have shown earlier that following rapid phagocytosis by macrophages, K56-2 replication starts after 6 to 8 hours (Vergunst et al., 2010). During the first 7 hours following iv infection no replication was observed in the presence or absence of Myd88, however by 18 hpi K56-2 had replicated faster in myd88 knockdown embryos (Figure S6A). Thus, despite increased bacterial replication rates, myd88 morphants lived significantly longer than control embryos, suggesting that Myd88-dependent inflammation, rather than bacterial numbers contributes to enhanced mortality in control embryos.

Recently Van der Vaart et al (2013) characterized a *myd88*$^{-/-}$ mutant zebrafish line and showed that the mutant was more susceptible to *Edwardsiella tarda*, *S.* Typhimurium and *M. marinum* infection. In agreement with our *myd88* knockdown experiments, but in sharp contrast to *S.* Typhimurium and *E. tarda* infections, *myd88*$^{-/-}$ embryos infected with *B. cenocepacia* K56-2 lived longer than *myd88*$^{+/+}$ embryos (Figure 5C), and *B. cenocepacia* K56-2 replicated better in *myd88*$^{-/-}$ than in wild type embryos (Figure 5D). This survival advantage was correlated with a delay in neutropenia and tissue damage (Figure 5E). Interestingly,

cxcl8 and *il1b* gene expression was not significantly induced during the first 8 hours in the *myd88* mutant compared to PBS-injected control embryos. However, from 8 hpi, when bacteria started to replicate intracellularly, a Myd88-independent increase in expression of both cytokines was observed (Figure 5F, 5G, S6B, S6C). Thus, our data show that the absence of Myd88 provides a survival advantage to zebrafish embryos, and suggest that an early Myd88-dependent pro-inflammatory response by macrophages significantly contributes to fatal infection caused by *B. cenocepacia* K56-2 in zebrafish embryos.

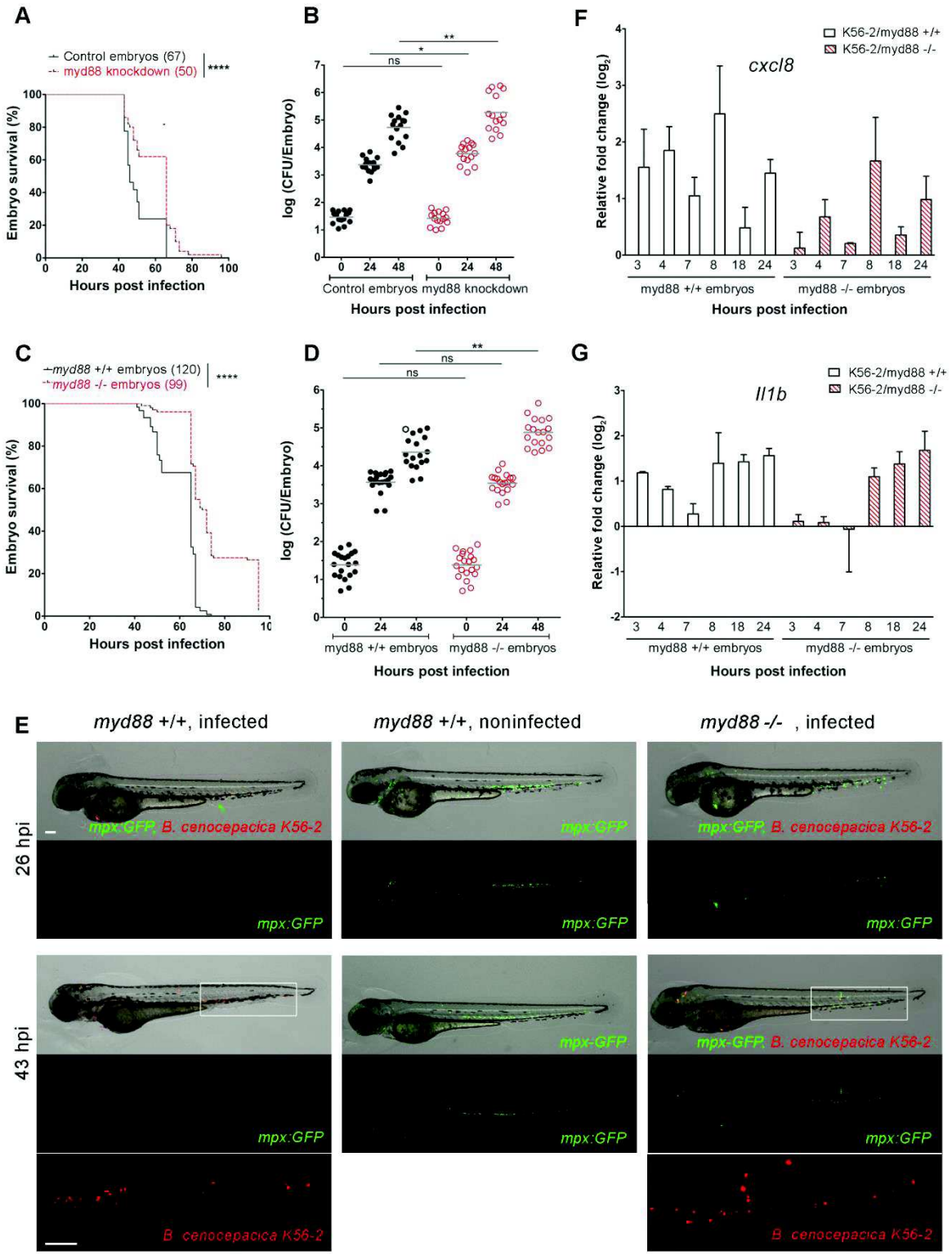


Figure 5. Myd88 is important for induction of a pro-inflammatory fatal infection caused by *B. cenocepacia*.

(A and B) Embryo survival (A) and bacterial burden over time (B) of control and *myd88* knockdown embryos iv injected with ~ 45 CFU *B. cenocepacia*. Three independent experiments.(C-E) Wild type (*myd88* +/+) control and *myd88* mutant (*myd88* -/-) embryos were iv injected with ~35 CFU *B. cenocepacia* K56-2. (C, D) Embryo survival (C) and bacterial burden over time (D, n=6). Results of 3 independent experiments. (E) Representative images of noninfected and infected *myd88* +/+ and *myd88* -/- embryos at 26 hpi and 43 hpi. The bottom panel shows a close up of the bacterial burden (red) in infected embryos at 43 hpi (white box). Few GFP^+ neutrophils (green) were seen at 26 hpi in infected *myd88* +/+ embryos in contrast to *myd88* -/- mutant embryos, and at 43 hpi neutrophils in the wildtype were practically absent. At 43 hpi, tissue damage in *myd88* embryos was reduced compared to wild type embryos, and fewer GFP^+ neutrophils were present compared to non-infected embryos. Scale bar 200 µm

(F, G) Mean relative *cxcl8* (F) and *il1b* (G) gene expression levels (qRT-PCR) in wildtype (*myd88* +/+) and *myd88* mutant (*myd88* -/-) embryos injected with ~35 CFU *B. cenocepacia* K56-2, normalised to the corresponding PBS-injected control group (wildtype/PBS and *myd88* -/- /PBS, respectively) at each time point. Error bars represent a 95% confidence interval of the mean of three biological repeats. See supplemental figure S6 B and C for corresponding graphs comparing PBS mutant relative to PBS wildtype control. (B, D) Geometric means are shown with each data point representing an individual embryo. Dead embryos are indicated with black open circles. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: non-significant. See materials and methods for statistical tests. See also Figure S6.

DISCUSSION

Macrophages are sentinels of the innate immune system that defend the host against intruding microbes. Intracellular pathogens have evolved mechanisms to counteract host defences and evolved ways to survive in phagocytic cells. Here we provide important *in vivo* evidence that macrophages are critical for multiplication of *B. cenocepacia* and subsequent induction of pro-inflammatory, acute infection (See Figure 6). Macrophages have been suggested to be important for Bcc virulence and invasiveness since it was shown in the late 90's that Bcc can survive in macrophages (Saini et al., 1999). Importantly, two different clinical studies have shown a high association of Bcc with cells in lung tissue samples taken from transplant and deceased patients (Sajjan et al., 2001b; Schwab et al., 2014). Schwab et al (2014) identified Bcc predominantly in phagocytes, mainly macrophages, and mucus, and were unable to detect Bcc in biofilm-like structures that are generally believed to be the predominant life form of Bcc in the infected lungs. However, the contribution of macrophages to virulence *in vivo* is still unexplored.

Despite the fact that many intracellular bacterial species exploit macrophages for their virulence, these phagocytes still have been shown to be important to protect the host against fatal systemic infection; in the absence of macrophages higher bacterial loads and increased mortality of zebrafish embryos were observed with bacterial pathogens, including *M. marinum*, *S. aureus* and *S.* Typhimurium (Clay et al., 2007; Prajsnar et al., 2008). In sharp contrast, using two different methods to ablate macrophages, we show that macrophage-depleted embryos have a survival advantage when injected with otherwise highly virulent Bcc strains. In addition, our data show that macrophages are not efficient in eradicating

persistent bacteria, but may in fact contribute to their persistence. In addition, we find that macrophages cannot eradicate persistent bacteria, but may in fact contribute to their persistence. Strikingly, in contrast to for instance *Salmonella enterica* serovar Typhimurium and *P. aeurginosa* that replicate efficiently in the absence of macrophages, the presence or absence of specific host- and/or bacterial factors seemed to prevent extracellular replication of all analyzed Bcc strains in the circulation of macrophage-depleted embryos following iv infection. We are currently analyzing in which other cell types, in the absence of phagocytosis by macrophages, Bcc enter and replicate.

In embryos that were chemically depleted of macrophages, bacteria survived but did not replicate for at least 24 hours, in sharp contrast to control embryos. Despite the absence of macrophages in such embryos, however, the bacteria started to multiply after 24 hpi, in association with cells. We are currently analyzing whether in the absence of phagocytosis by macrophages, Bcc enters and replicates in other cell types.

To fight infection, the host employs a multitude of innate immune responses, including rapid killing by neutrophils (phagocytosis-mediated degradation, NET) and macrophages, and rapid local induction of cytokines resulting in a pro-inflammatory response. Here we show that neither macrophages, nor neutrophils are able to eradicate bacteria belonging to the Bcc *in vivo*, but instead contribute to high inflammatory responses induced by virulent strains.

Neutrophils have been shown to readily phagocytose Bcc and reduce bacterial numbers *in vitro* (Speert et al., 1994; Porter & Goldberg 2011). In agreement with the finding that neutrophils need a surface for efficient phagocytosis (Colucci-Guyon et al., 2011), we show that neutrophils were massively recruited to and readily phagocytosed surface-associated *B.*

cenocepacia K56-2 and *B. stabilis* delivered by subcutaneous injection in zebrafish embryos. Neutrophils which are unable to produce ROS through defects in the NADPH oxidase complex, as is the case in chronic granulomatous disease (CGD) patients, showed reduced killing of ingested Bcc, and were unable to protect mice against *Bcc* challenge (Speert et al., 1994; Dinauer et al., 2001; Sousa et al., 2007). In agreement with this, in our study visual reduction in bacterial numbers and resolution of inflammation in most *B. stabilis*-infected embryos was ROS-dependent. Inflammation failed to resolve in *gp91* knockdown embryos, reminiscent of neutrophil dominated abscesses found in the lungs of rats lacking ROS (Sousa et al., 2007), and might be caused by Bcc-induced necrosis of neutrophils lacking a functional NADPH oxidase (Bylund et al., 2005). Intriguingly, starting one hpi, neutrophils that had taken up *B. stabilis* projected their cell contents in the environment in a process resembling NETosis (Brinkmann & Zychlinsky 2012). NETosis depends on the production of ROS by NADPH oxidase (Fuchs et al., 2007), and suggests that ROS-dependent release of NETs might be a host-protective mechanism to control *B. stabilis* infection. NET induction by Bcc has not yet been described to our knowledge, and it will be interesting to analyse whether the release of neutrophil DNA entraps the bacteria that become unavailable for further phagocytosis, and how the bacteria are able to resist NETs. In contrast to *B. stabilis*, subcutaneously injected *B. cenocepacia* induced a pro-inflammatory response, even in the presence of functional NADPH oxidase, and bacterial burden increased within 20 hpi. Internalization of *B. cenocepacia* by macrophages even during neutrophil-dominated phagocytosis likely contributes to the observed high inflammatory response with this pathogen.

Detection of bacterial ligands through Toll-like receptors (TLR) is essential for initiation of downstream immune signaling, principally through the key adaptor protein Myd88, resulting in expression of immune genes. Flagellin has been suggested as a key ligand in TLR5-mediated induction of the immune response to infection with Bcc (Ventura et al., 2008; Urban et al., 2004). However, recently it has also been described that glycosylation of *B. cenocepacia* flagellin reduces immune responses (Hanuszkiewicz et al., 2014), and it remains essential to identify other bacterial ligands and host factors that are involved in the induction of this high, deregulated immune response, and inability to resolve the inflammation. We found that intracellular *B. cenocepacia* caused a rapid (within 2 hours) and strong induction of important pro-inflammatory cytokine genes in zebrafish embryos, in contrast to the much lower increase for *B. stabilis*, which correlated with the outcome of infection (acute, neutropenia versus persistent, neutrophilic inflammation). This high pro-inflammatory state during *B. cenocepacia* infection was followed by massive Cxcl8-dependent neutrophil recruitment, neutrophil degranulation at the site of infection, and tissue damage, culminating in a Myd88-dependent fatal inflammation with systemic death of phagocytes. In contrast to other pathogens, including *E. tarda* and *S.* Typhimurium that have recently been shown to be more virulent in a zebrafish (van der Vaart et al., 2013), we found that *B. cenocepacia* infected *myd88* mutants have a pro-survival advantage. These findings are in perfect agreement with the new paradigm for the role of Myd88 in Bcc infection described by Ventura et al (2009), who showed that immunodeficient mice lacking Myd88 were protected against fatal challenge with *B. cenocepaci*a. Paradoxically, bacterial replication, as in mice (Ventura et al., 2009), was enhanced in the *myd88* mutant suggesting a Myd88-dependent inhibition of bacterial replication in wild type embryos. Thus, a Myd88-

dependent contribution to inflammation, not bacterial numbers, leads to increased mortality.

B. cenocepacia-increased expression of *cxcl8* and *il1b* during the first 8 hours, just prior to onset of bacterial intracellular multiplication, was dependent on the presence of Myd88, but increase in cytokine expression at later stages was Myd88-independent. This suggests a critical, early Myd88-dependent effect that occurs when bacteria are contained by macrophages and that contributes to fatal inflammation. In apparent contrast, knockdown of *cxcl8* resulted in reduced bacterial multiplication, suggesting that bacterial burden may depend on the concentration of Cxcl8 (Kaza et al., 2011).

Zebrafish have been instrumental in better understanding the interaction of several important human pathogens with host macrophages, and several therapeutic strategies against intracellular pathogens have been driven by zebrafish research (Cronan & Tobin 2014). To date the molecular mechanisms that are at the basis of exacerbation, lung function decline, and often fatal septicemia in CF patients caused by Bcc are not known. Our data show that macrophages are critical for bacterial multiplication, persistence, but also development of acute fatal inflammation *in vivo*, in agreement with recent clinical observations, and those (healthy) neutrophils do not contribute to bacterial clearance *in vivo*. To improve our understanding of the strong pro-inflammatory character of the infection seen in CF patients it is important to further unravel the molecular basis of the Myd88-dependent signaling cascade. Based on our results that macrophages are critical for virulence in an *in vivo* model, and results from others, we suggest that the intracellular stages of *B. cenocepacia* and the ensuing inflammatory response are essential targets to explore for the development of new therapies to combat this infection.

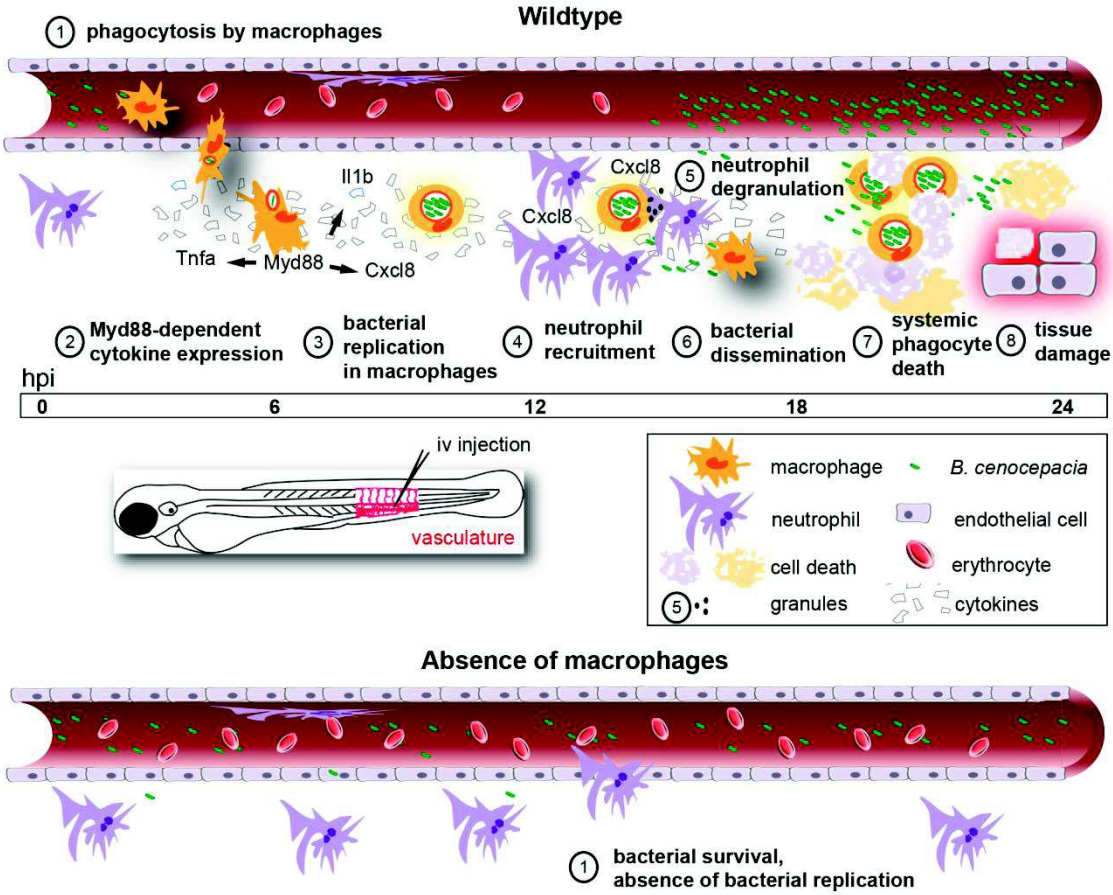


Figure 6. Schematic showing the role of macrophages during development of acute infection by *B. cenocepacia* K56-2 in zebrafish embryos.

Macrophages are the major phagocytosing phagocytes of iv injected bacteria (1), for which they provide a critical replication niche (3). In their absence, K56-2 does not replicate for 24 h (bottom panel, 1). Myd88-dependent cytokine expression during the first 6 hours (2) is important for the development of a pro-inflammatory response, characterized by massive neutrophil infiltration (4) neutrophil degranulation (5), bacterial dissemination from infected cells (6), phagocyte death (7), and tissue damage (8).

EXPERIMENTAL PROCEDURES

Zebrafish husbandry

Zebrafish (*Danio rerio*) were kept and handled in compliance with national regulations for animal welfare and approved by the departmental and ethical committee (ID 30-189-4 and CEEA-LR-12186). See Table S1 for fish lines.

Bacterial strains and plasmids

Bcc strains containing DsRed or turquoise expression plasmids (see Table S2) were grown in 5 ml of LB medium in the presence of 100 µg.ml^{-1} chloramphenicol at 37°C overnight. The *mTurquoise2-C1* gene was amplified from pmTurquoise-C1[42] using primers TurquoiseNdeI-Fw (5'–GGAATTCCATATGGTGAGCAAGGGCGAGGAGC-3'; *NdeI* underlined) and TurquoiseXbaI-Rv (5'– GCTCTAGACTACTTGTACAGCTCGTCCATGCCGAG-3'; *XbaI* underlined), and cloned as an *NdeI*/*XbaI* fragment in pIN29, replacing the DSRed gene[12], resulting in pIN289. The plasmid was transferred to *B. cenocepacia* by electroporation as described[12], and fluorescence verified.

Injection conditions

One nL of MOs (Genetools, Eugene, OR) dissolved in H_2O with 0.05% Phenol Red (see Table S3) was micro injected in the yolk at the 1-2 cell stage and eggs were incubated at 28°C in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33 mM MgSO4, 0.00005% methylene blue). Methylene Blue was omitted when embryos were used for pixel count analysis to avoid strong auto fluorescence of the yolk. Embryos were manually dechorionated 2 hours

before injection with Bcc bacteria. Bacterial solutions were injected iv into zebrafish embryos (30 hpf, unless mentioned otherwise) as described (Vergunst et al., 2010; Mesureur & Vergunst 2014). Embryos in each group were randomized after injection and attributed to survival assays (generally 20 embryos per experiment, exact numbers are indicated in survival graphs), or to CFU counts (n=5 per experiment per indicated time point, unless mentioned otherwise). Inoculum (T=0) and bacterial burden were determined by plating individually lysed embryos for CFU counts as described (Mesureur & Vergunst 2014). Subcutaneous injection was performed using 50 hpf embryos as described (Colucci-Guyon et al. 2011) with bacterial suspensions at an OD of 10. See Supplemental Procedures.

Macrophage ablation

Fluorescent (umn^{+}) and non-fluorescent **(**umn^{-}) embryos (see Table S1, and Figure S2) were dechorionated and either left untreated (infection control), or incubated in 0.2%DMSO (DMSO effect) or 5mM Mtz (ablation and non-specific Mtz control, respectively) at 33 hpf for 15 h prior to iv injection of bacteria. Embryos were incubated at 28°C in the dark. Ablation was verified by fluorescence microscopy, and CFU counts and mortality determined as described above. See Supplemental Procedures.

Analysis of gene expression

At the indicated time points, 10 to 25 embryos were processed for RNA isolation, cDNA synthesis, and qRT-PCR analysis. The peptidylprolyl isomerase A-like (*ppial*) gene was used as a reference housekeeping gene. Results were analyzed using the ΔΔCt method and represented as column bar graphs normalized to a PBS-injected control group at each time

point, unless mentioned otherwise. Three biological control experiments were performed, each with two technical replicates, unless stated otherwise. See Supplemental procedures for details, and Table S4 for primers.

Cell death assay and Sudan black staining

Embryos were injected with 50-100 CFU red fluorescent *B. cenocepacia* K56-2, and 20 hr later with 1 nL of the live cell impermeable nucleic acid stain SYTOX Green (1mM in DMSO, Invitrogen). As a control embryos were injected with 1 nL of DMSO. Embryos were analysed between 30 min and 6 hours after injection of the dye by fluorescence microscopy. Sudan black staining was performed as described (Le Guyader et al., 2008).

Antibody labeling

Zebrafish embryos were fixed in 4% Paraformaldehyde (PFA) in PBS with 0.4% Tween20 during 2 hours at room temperature or overnight at 4°C, washed, and incubated in blocking buffer, primary antibody buffer (anti-L-plastin, 1:500) and secondary antibody buffer (anti-rabbit coupled with Alexa 350, 1:250 (Life Technologies). See Supplemental Experimental Procedures.

Microscopy and Fluorescent Pixel Quantification

Embryos were transferred to E3 withMS222 (inverted microscope) or embedded in 0.5% E3-agarose (confocal) in glass-bottom dishes (MatTek Corp., Ashland, MA). A Leica DM IRB inverted microscope (bright-field, differential interference contrast (DIC), and fluorescence

imaging) coupled to a Coolsnap fx camera (Roper Scientific) was used (Vergunst et al., 2010). A Nikon AZ100 equipped for bright-field and fluorescence imaging, coupled with Coolsnap HQ2 (Roper Scientific) using MetaVue software was used to record full size embryos. Confocal microscopy was performed with an Olympus FV10i and images and videos were processed with Fluoview and Image J. Images were processed further using Adobe Photoshop, and time-lapse videos made with image J (see specific details in movie legends). To quantify phagocytic cells, the fluorescent pixel quantification method was used as described (Ellett & Lieschke 2012). Graphs depict macrophage and neutrophil numbers.

Statistical analysis

Statistical analysis was performed using Prism 5.01 and 6 (GraphPad). Survival assays are represented in Kaplan-Meier graphs and analyzed with a Log rank test. For CFU, macrophage and neutrophil counts, normal Gaussian distribution was verified with the D'Agostino and Pearson omnibus normality test. Significance between multiple groups was determined using one-way Anova, with Tukey as Multiple Comparison Test (Figure 1B, 1H, 5B, S1B, S2C and S4D). For experiments in which the data were not normally distributed a non-parametric Kruskal-Wallis test was used with Dunn's Multiple Comparison Test (Figures 1F, 3C, 3D 4G, 5D, S1D, S1H, S1J, S1L, S4B and S5A). qRT-PCR data were $\log_2$-transformed, and significance of the data was analysed using one-way Anova, with Tukey's Multiple Comparison Test. Significance is indicated with: ns, non-significant, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

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Supplemental Information

Supplemental Tables

Table S1. Related to experimental procedures: Zebrafish lines used in this study

Name	Description	Reference
AB	Wild type	-
AB "Golden"	Pigment mutation (*slc24a5*)	(Lamason et al., 2005)
Tg(*mpx:eGFP*^{i114})	Neutrophil marker - GFP	(Renshaw et al., 2006)
Tg(*mpeg1:mCherryF*) ^{ump2Tg}	Macrophage marker with membrane localised mCherry-F	(Nguyen-Chi et al., 2014)
Tg(fms:Gal4-VP16-$^{il86/}$*/UAS-E1b:nfsB-mCherry*^{i149})	Line expressing a fusion protein of nitroreductase and mCherry under control of the Gal4 regulated UAS promoter in macrophages	(Gray et al., 2011)
Tg(*mpeg1:Gal4-VP16/ UAS-E1b:kaede*^{s1999t})	Line expressing the photo convertible protein Kaede under control of the Gal4-regulated UAS promoter in macrophages	(Ellett et al., 2011)
myd88^{hu3568}	Mutant line with truncated MyD88	(van der Vaart et al., 2013)
Tg(mpeg1:*Gal4-VP16-*$^{il86/}$*; UAS-E1b:nfsB-mCherry*^{i149})	Offspring from a cross between Tg(*fms:Gal4; UAS-E1b:nfsB-mCherry*^{i149}) and Tg(*mpeg1:Gal4-VP16 /UAS-E1b:kaede*^{s1999t}) fish, named *umn* in this study for simplification.	This study

Table S2. Related to experimental procedures: Description of bacterial strains and plasmids

Bacterial species or plasmid	Isolate	Description[a]	Reference
Species			
B. cenocepacia	K56-2 (LMG18863)	ET12, Toronto, Canada, CF	(Darling et al. 1998)
	J2315 (LMG16656)	ET12 index strain, Edinburgh, UK, CF	(Govan et al. 1993)
	J415 (LMG16654)	Not associated with patient-to-patient spread, CF	(Glass & Govan 1986)
B. stabilis	LMG14294	Belgian CF patient, stable condition; detected in one other patient	(Revets et al. 1996)
B. vietnamiensis	FC441 (LMG18836)	9-year-old boy with X-linked recessive CGD who survived septicaemia	(Mahenthiralingam et al. 2000)
B. cepacia	CEP509 (LMG18821)	CF patient, Sydney, Australia; recovered from three other patients	(Mahenthiralingam et al. 2000)
Plasmids			
pIN29		ori_{pBBR} Δ*mob*, Cm^r, DsRed	(Vergunst et al. 2010)
pIN289		ori_{pBBR} Δ*mob*, Cm^r, pm-Turquoise2	This study

[a] CF: cystic fibrosis, CGD: chronic granulomatous disease, Cmr: chloramphenicol resistance, Δmob: mobilization deficient

Table S3. Related to experimental procedures. Morpholinos used in this study

Gene	Accession #	Name	Sequence	Target[a]	[ ] (mM)[b]	Reference
pu.1 (spi1)	ENSDARG00000000767	tMO_pu.1	5'-CCTCCATTCTGTACGGATGCAGCAT-3'	atg/ 5'UTR	0,1	(Clay et al. 2007)
		sMO_E4I5_pu.1	5'-GGTCTTTCTCCTTACCATGCTCTCC-3'	E4-I4	0,38	
cxcl8	ENSDARG00000100007	tMO_IL8	5'-TTTGCTGGTCATTTTGCCTAAGTGA-3'	atg/ 5'UTR	0,45	(Sarris et al. 2012)
		sMO_E2I2_IL8	5'-ACTCACATGATCTCTAAATCTTTAC-3'	E2-I2	0,45	This study
cybb (gp91)	ENSDARG00000056615	sMO2_cybb	5'-CATAATCCCGATAGCTTACGATAAC-3'	E1-I1	0,8	(Yang et al. 2012)
myd88	ENSDARG00000010169	tMO1-myd88	5'-TAGCAAAACCTCTGTTATCCAGCGA-3'	atg/ 5'UTR	0,5	(Sar et al. 2006)
		sMO2-myd88	5'-GTTAAACACTGACCCTGTGGATCAT-3'	E2-I2	0,5	(Vaart et al. 2013)
		standard control morpholino	5'-CCTCTTACCTCAGTTACAATTTATA-3'		0,8	

[a] For *pu.1*, *il8* and m*yd88* knockdown, the injection solution was a mix between tMO and sMO. For pu.1, the indicated combination of sMO/tMO allows the specific ablation of macrophages, without affecting neutrophils (Su et al. 2007).

[b] [ ] = concentration; all MOs were injected at 1nL.

Table S4. Related to experimental procedures. Primers used for qRT-PCR experiments.

Gene	Accession #	Forward qPCR primer	Reverse qPCR primer
ppial	ENSDARG00000042247	5'-ACACTGAAACACGGAGGCAAAG-3'	5'-CATCCACAACCTTCCCGAACAC-3'
mpeg1	ENSDARG00000055290	5'-GTCTTATATCTCCAACAGTCAG-3'	5'-GATGCCTGGGTAGAATAAAGC-3'
mpx	ENSDARG00000019521	5'-ACCATTGCGAACGTCTTTGC-3'	5'-ACTGGGAAACTGAGGATGGTTC-3'
cxcl8	ENSDARG00000100007	5'-TGTGTTATTGTTTTCCTGGCATTTC-3'	5'-GCGACAGCGTGGATCTACAG-3'
il1b	ENSDARG00000005419	5'-GAACAGAATGAAGCACATCAAACC-3'	5'-ACGGCACTGAATCCACCAC-3'
tnfa	ENSDARG00000009511	5'-AGACCTTAGACTGGAGAGATGAC-3'	5'-CAAAGACACCTTGGCTGTAGAC-3'

Supplemental figure inventory

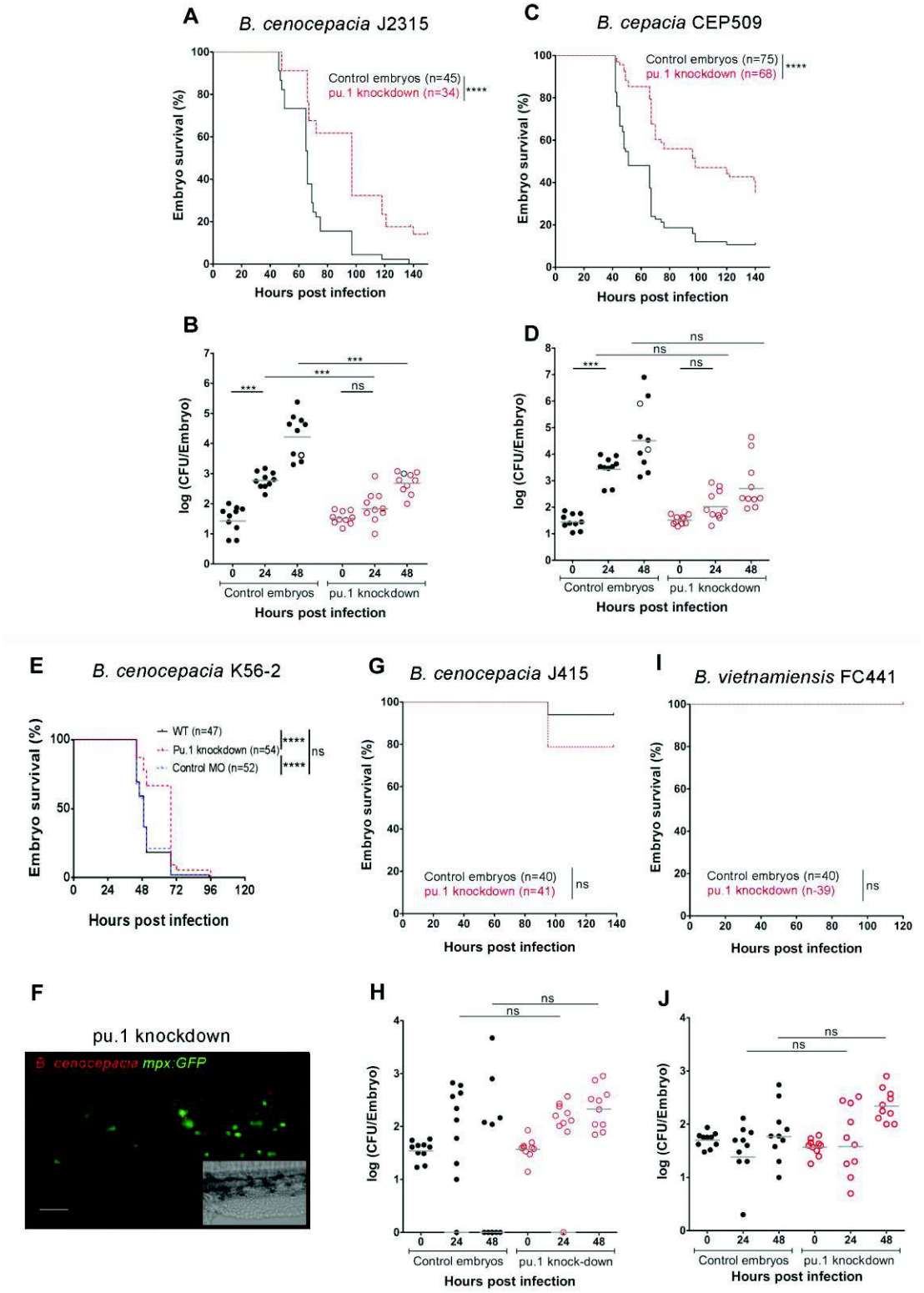

Figure S1 (related to Figure 1). Macrophages are critical for virulence of *B. cenocepacia*.

(A and B) Control embryos (black) and pu.1 knockdown embryos (red) were injected with *B. cenocepacia* J2315 (average 35 CFU). Results from 2 independent experiments are shown. In each group, the embryos were randomized and attributed to survival assays (A, n=45 (control) and n=34 (pu.1 knockdown)) or to CFU counts (B, n=5 per experiment per time point).
(C and D) Control embryos (black) and pu.1 knockdown embryos (red) were injected with *B. cepacia* CEP509 (average 40 CFU). Results from 2 independent experiments are shown. In each group, the embryos were randomized and attributed to survival assays (C, n=75 (control), n=68 (pu.1 knockdown)) or to CFU counts (D, n=5 per experiment per time point).

(E) Embryo survival of control embryos (n=24), pu.1 knockdown (n=20) and nonspecific control MO (n=22), injected with *B. cenocepacia* K56-2 (average 45 CFU). One representative experiment is shown.

(F) Representative fluorescence image at 24 hpi showing neutrophils (green) in an *mpx:GFP pu.1* knockdown embryo injected with *B. cenocepacia* J2315 (red) (~50 CFU). Inset shows corresponding bright field image. Scale bar, 100 µm.
(G, H) Control and *pu.1* knockdown embryos were injected with *B. cenocepacia* J415 (average 60 CFU). Results from 2 independent experiments are shown. The embryos were randomized and attributed for survival assays (G, n=40 (control) and n=41 (*pu.1* knockdown)) or for CFU counts (H, n=5 per experiment per time point). Note that 50% of the control embryos have cleared the infection at 48 hpi.
(I, J) Control and *pu.1* knockdown embryos were injected with *B. vietnamiensis* FC441 (average 60 CFU). Results from 2 independent experiments are shown. The embryos were randomized and attributed to survival assays (I, n=40 (control) and n=39 (*pu.1* knockdown)) or for CFU counts (J, n=5 per experiment per time point).
(B, D, H H, and J) Geometric mean with each data point representing an individual embryo. Dead embryos are indicated with a black open circle.
(A-E, and G-I) * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: non-significant. See materials and methods for statistical tests used.

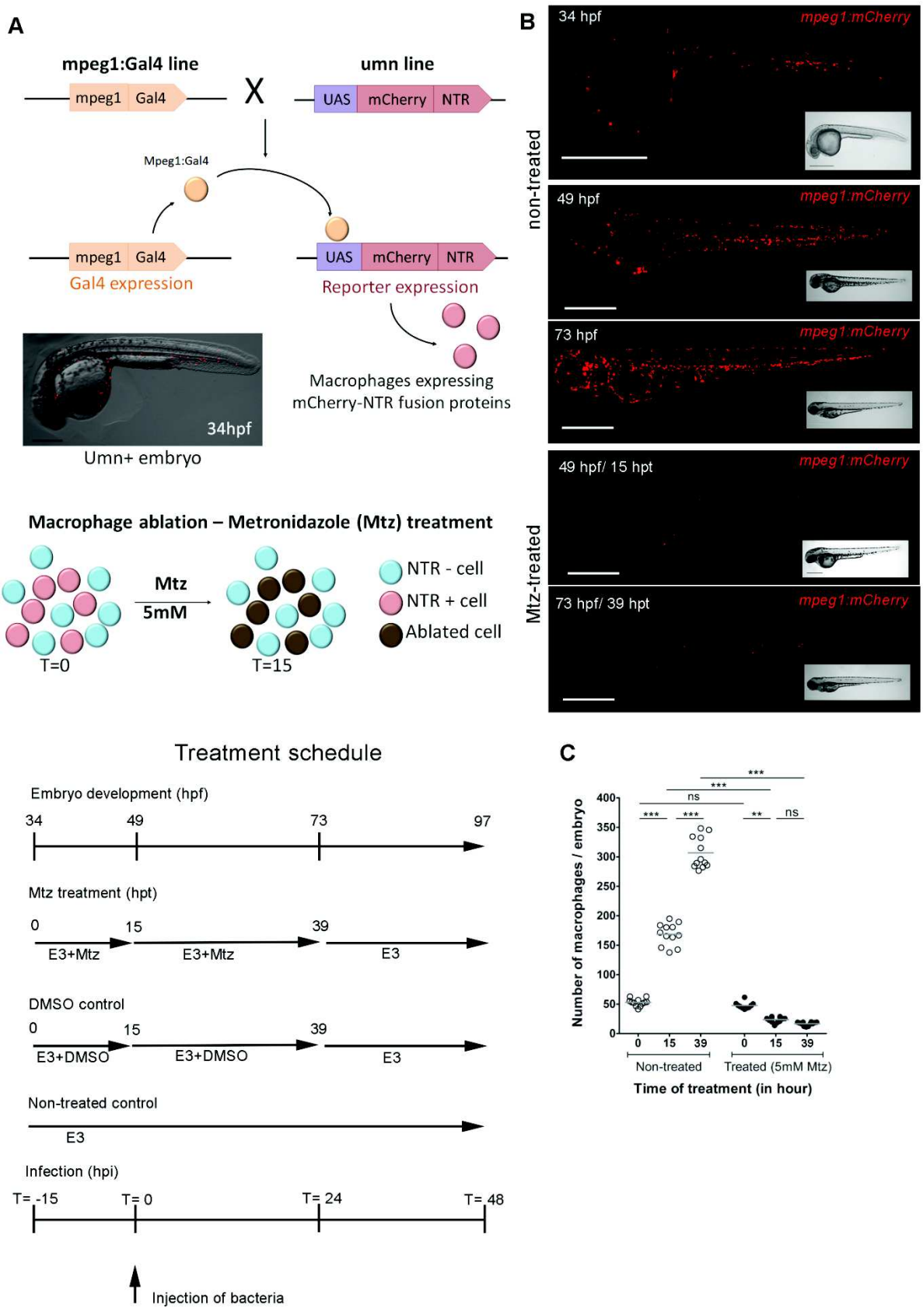

Figure S2 (related to Figure 1). Chemical ablation of macrophages using the NTR/Mtz system.

(A) Schematic representation and treatment schedule of the chemical ablation strategy based on the nitroreductase (NTR)/ metronidazole (Mtz) system. Offspring was raised from a cross between *mpeg1::Gal4* driver and *UAS::mCherry::NTR* responder fish, which specifically expressed the mCherry-NTR fusion protein in macrophages, further named umn^{+}. For infection experiments, such fluorescence positive embryos were crossed with wild type, or heterozygote siblings were intercrossed. The resulting embryos were screened and divided in umn^{+} and umn^{-} groups for each experiment. The incubation of embryos in E3 medium containing 5 mM Mtz according to the indicated time schedule resulted in the specific apoptosis of NTR^{+} macrophages by the conversion of the prodrug Mtz by NTR (B and C). Embryos were pre-treated at 34 hours post fertilization (hpf) (T= -15), for 15 hours prior to iv injection with bacteria (T=0). After injection of bacterial suspension, the embryos were incubated for an additional 24 hours with Mtz, and then transferred to fresh E3 water. CFU (Figure 1F) and macrophage counts (S2C) were performed at the indicated time points.

(B) Representative fluorescence images of non-treated control and Mtz-treated embryos, showing the efficacy of the Mtz treatment. Residual red fluorescence in treated embryos represented apoptotic cells. Scale bar, 0.5 mm.

(C) Quantification of macrophage numbers in umn^{+} embryos (untreated or treated at 34 hpf with 5mM Mtz) at 0, 15 and 39 hours after treatment as indicated in (A). The efficacy of macrophage ablation by Mtz treatment was evaluated by pixel counting. The average macrophage numbers of two independent experiments (n=6) are shown. ns, non-significant, ** $p \leq 0.01$, *** $p \leq 0.001$. See material and methods for statistical analysis used.

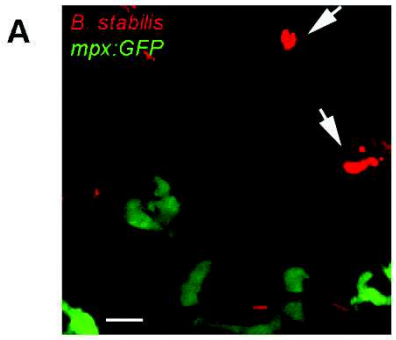

Figure S3 (related to Figure 2). Neutrophils phagocytose surface-associated Bcc but are unable to eradicate the bacteria

Confocal stack (24 slices) of an *mpx::GFP* embryo at 25 hpi with *B. stabilis* (red). Scale bar, 50 µm. Arrows points are bacterial clusters, possibly in GFP^{minus} macrophages.

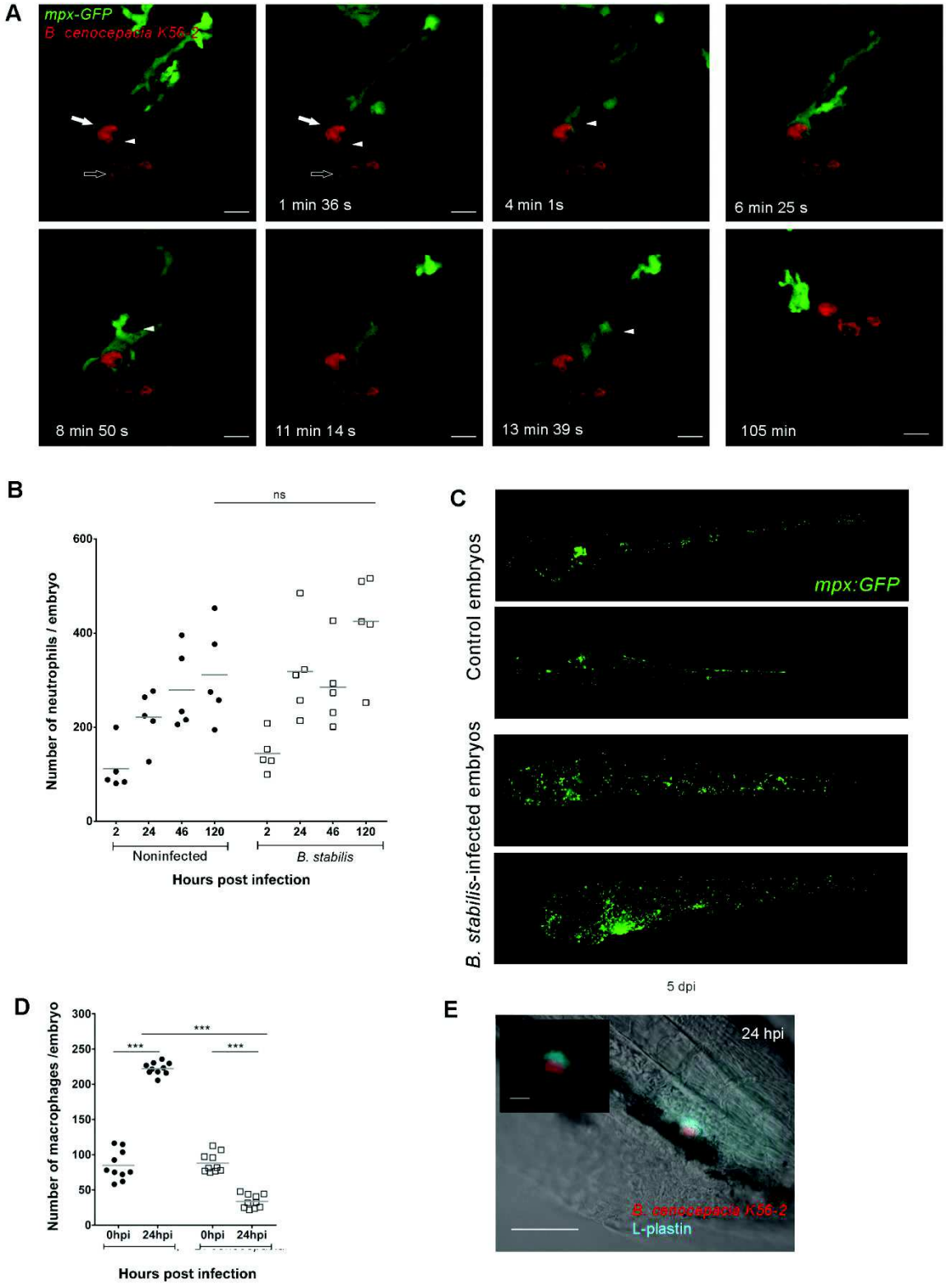

Figure S4 (related to Figure 3) Behaviour and fate of neutrophils and macrophages during acute and persistent infection

(A). Confocal stack images of a time series, first image ~20 hpi of *mpx::GFP* embryos *with B. cenocepacia K56-2.* Patrolling neutrophil inspects heavily infected macrophage (white arrow). Individual bacteria (arrow head),

possibly released from infected cell nearby (open arrow), are moved around by the neutrophil, but it is not clear if they are taken up. The last image shows the same area 90 minutes later with the infected macrophage still intact.

(B, C) *Mpx::GFP* embryos (50 hpf) were injected with *B. stabilis* LMG14294. (B) The number of neutrophils in infected and non-infected control embryos was determined by pixel counting at different time points after injection. Each data point represents one embryo. The graph represents one of the three experiments represented in Figure 3C, but includes an additional 5 dpi time point, which was not determined in the other 2 experiments. Although not significant (Kruskal Wallis), most embryos injected with *B. stabilis* contained more neutrophils than control embryos (see also (C)). (C) Representative images of control and *B. stabilis* infected *mpx::GFP* embryos with increased neutrophil (green) numbers. Neutrophils were more dispersed in *B. stabilis*-infected compared to non-infected control embryos, where most neutrophils were resting in the caudal hematopoietic tissue.

(D) *Mpeg1::mCherry* embryos were injected with *B. cenocepacia* K56-2 (~45 CFU) and the number of macrophages was evaluated by pixel counting at 0 and 24 hpi (30 and 54 hpf, respectively). The results are related to the corresponding experiment shown in Figure 3E. Each data point represents one embryo. *** $p \leq 0.001$. Two independent experiments (n=5). See materials and methods for statistical tests used.

(E) Image showing *B. cenocepacia* K56-2 (red) in an L-plastin labelled macrophage (blue) at 24 hpi. Scale bar, 50 µm. Inset, magnification presented in red and blue channels, scale bar 10 µm.

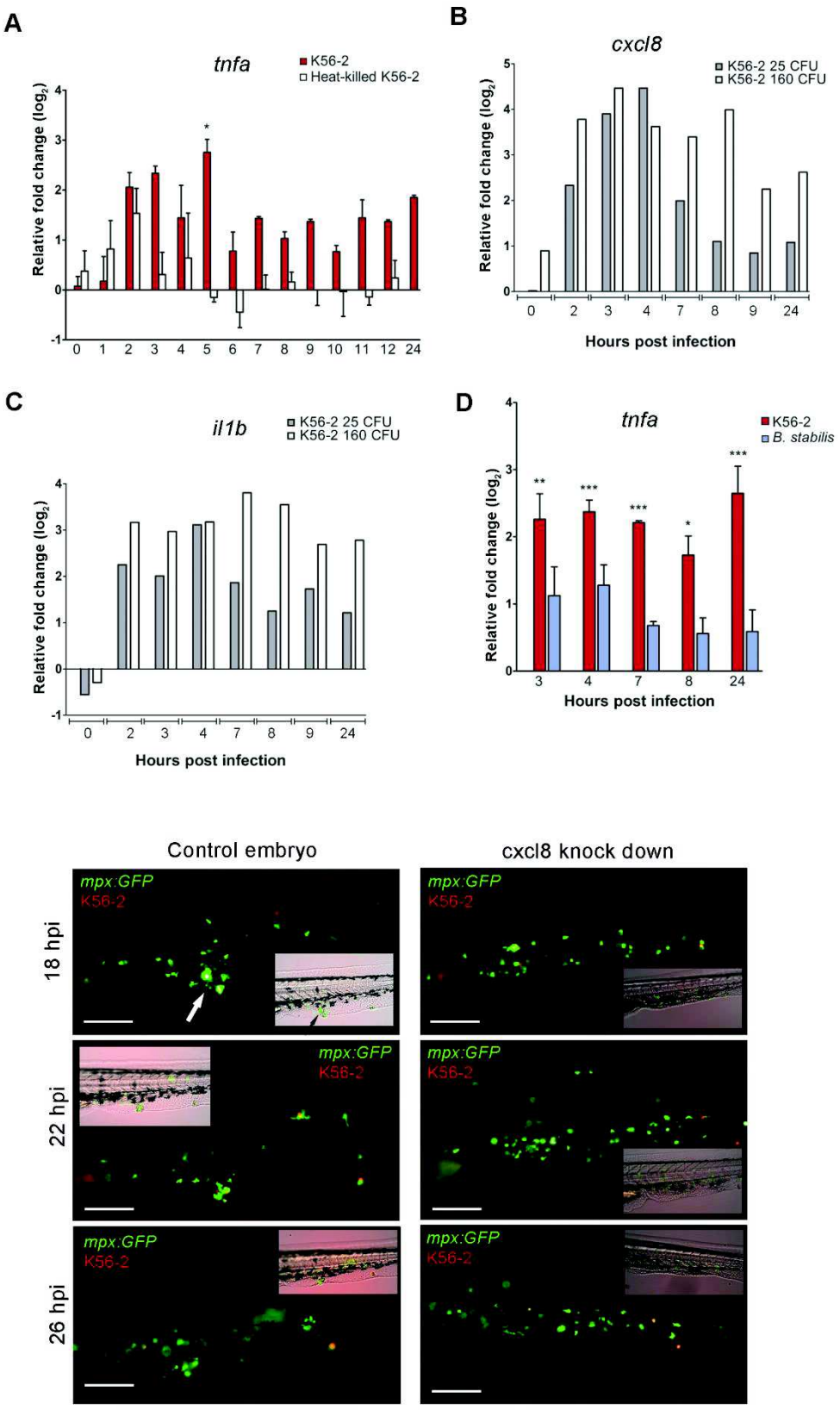

Figure S5 (related to Figure 4). The up-regulation of pro-inflammatory cytokine gene expression during Bcc infection is bacterial dose-dependent.

(A-D) qRT-PCR analysis of *cxcl8*, *il1b*, and *tnfa* expression at the indicated time points.

(A) Analysis of expression of *tnfa* expression in embryos injected with life or heat-killed (HK) *B. cenocepacia* K56-2 (~50 CFU). Error bars represent a 95% confidence interval of the mean of three independent infection experiments.
(B, C) Analysis of expression of *cxcl8* and *IL1b* expression using embryos injected with on average 25 CFU and 160 CFU respectively, of *B. cenocepacia* K56-2. One representative experiment is shown.
(D) Analysis of expression of *tnfa* expression in embryos injected with *B. cenocepacia* K56-2 (200 CFU) or *B. stabilis* LMG14294 (200 CFU). Error bars represent a 95% confidence interval of the mean of three independent infection experiments.
(A-D) Relative gene expression was analysed by the 2 -ΔΔCt method using *PPIAL* as a reference housekeeping gene for normalization of the data. Gene expression values of the different time points were calculated relative to a PBS-injected control group. Significant difference in expression is indicated with respect to the PBS control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. See materials and methods for statistical tests used.
(E) Correlated to Figure 4E. *Mpx::GFP* control and cxcl8 knockdown embryos were injected with ~50 CFU *B. cenocepacia* K56-2 (red). Representative fluorescence images at 18, 22 (identical to Figure 4E) and 26 hpi, show recruited neutrophils (green) in control embryos (top) from 18 hpi (white arrow), but not in cxcl8 knockdown embryos (bottom). Scale bar, 100 µm.

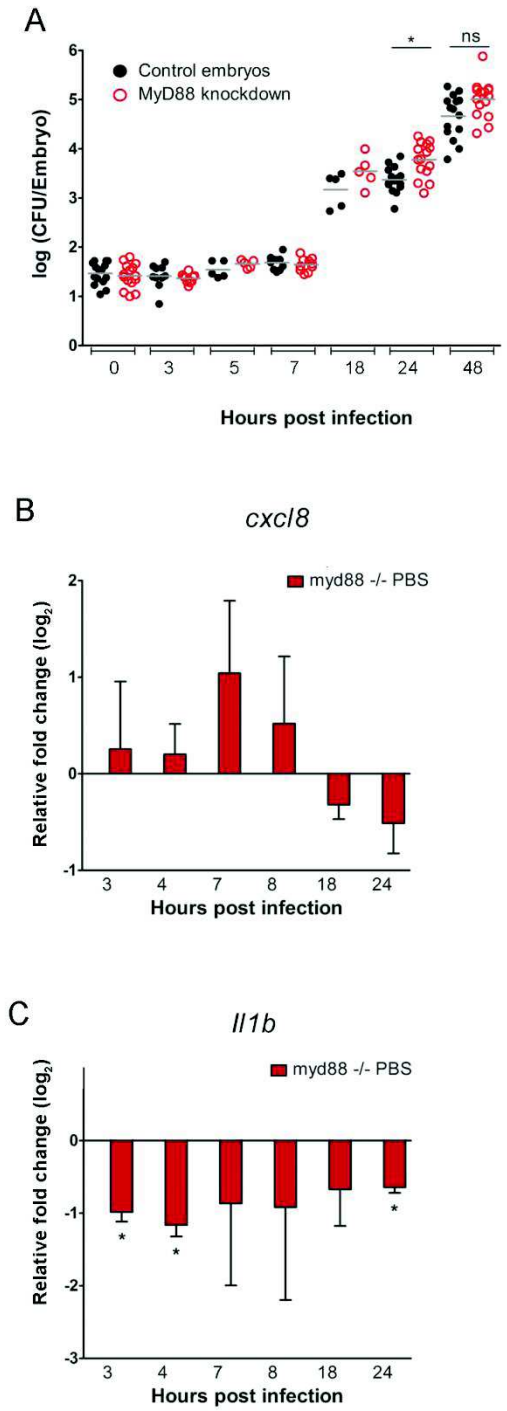

Figure S6 (related to Figure 5). Replication kinetics of *B. cenocepacia* K56-2 in the presence or absence of MyD88

(A) Control embryos (black) and *myd88* knockdown embryos (red) were injected with *B. cenocepacia* K56-2 (average 60 CFU), and CFU were determined at the indicated time points (n= 5 at 5 and 18 hpi, n=10 at 3 and 7 hpi, and n=15 at 0, 24 and 48 hpi). Each data point represents an individual embryo. Results of a single experiment are shown. * $p \leq 0.05$; ns : non-significant. See materials and methods for statistical analysis used.

(B,C) qRT-PCR control of *cxcl8* (B) and *il1b* (C) expression in myd88 mutant (*myd88* -/-) embryos injected with PBS relative to wildtype (*myd88* +/+) expression levels at each of the indicated time points. The data correspond to the experiments shown in Figure 5F and G. Error bars represent a 95% confidence interval of the mean of three biological control experiments. See materials and methods for statistical tests used.

Supplemental Experimental Procedures

Injection conditions

Just prior to injection of bacteria the dechorionated embryos were anesthetized in 0.04% buffered 3-aminobenzoic acid ethyl ester (Tricaine) in E3. Embryos (28 to 32 hpf) were micro injected in the caudal vein or subcutaneously (50 hpf) with 1 to 2 nL (generally 50-100 CFU), and 0.5 nL respectively, of bacterial inoculum. In knockdown experiments, MO-injected embryos were compared to wild type control embryos (Control embryos). No differences were found between embryos injected with aspecific control morpholinos compared to non-treated embryos (Figure SF, and data not shown).

To prepare the inoculum, bacteria expressing fluorescent proteins (see Table 1) were grown overnight in LB medium. Bacteria were collected by centrifugation at 3,000 g for 3 min and resuspended in PBS (Gibco). Bacterial inoculum (generally 50 CFU/nL) was prepared in PBS with 0.05% phenol red solution for visualization of the injection (Sigma). After injection, embryos were rinsed in E3 medium, anesthetized in Tricaine, and individually transferred to 1.5 ml Eppendorf tubes containing 45µl of 1x Trypsin-EDTA. Embryos were disrupted by pipetting (20-30 times) and 50 µL 2% Triton X-100 was added, mixed by flicking the tube, and incubate for 30 min at room temperature. To complete disruption the mixture was pipetted up and down 20-30 times. Depending on the number of bacteria, total lysate was plated on LB-agar plates with 100µg.ml^{-1} chloramphenicol or serial dilutions were made and 10µl of each dilution was deposited on a square LB-agar plate. Plates were incubated overnight at 37°C. The next day the number of colonies was determined. Graphs represent the individual

number of CFU per embryo at the different time points. At least 5 embryos per time point per experiment, and 3 or more independent experiments, unless otherwise mentioned.

Macrophage ablation

Offspring from a cross between Tg(*fms::Gal4; UAS-E1b::nfsB-mCherry*^{i149}) and Tg(*mpeg1::Gal4-VP16/UAS-E1b::kaede*^{s1999t}) fish (see Table 1) was selected for strong expression of mCherry in macrophages (Umn^{+}), and raised to adulthood. Umn^{+} fish were crossed to AB, or heterozygote siblings were incrossed. For efficient ablation of macrophages, *umn*$^{+}$ embryos were preselected at 30 hpf for strong mCherry expression, and embryos with only few mCherry-positive macrophages were discarded. Non-fluorescent embryos from the same crosses were used as umn-negative (unm^{-}) control. Metronidazole (Mtz; Sigma), freshly prepared in DMSO, was used at 5 mM in E3. The schedule for Mtz treatment and injection of bacterial suspensions, including controls, is shown in Figure S2. Treatment of 15 h prior to infection, followed by an addition 24 h in Mtz, resulted in specific ablation of macrophages (see results).

Analysis of gene expression by qRT-PCR

For each condition embryos (10 to 25) were transferred to 500µl of TRIzol, homogenized and stored at -80°C, as described (Mesureur & Vergunst 2014). RNA for each pool was extracted as described (Cui et al. 2011) and purified using the RNeasy MinElute Cleanup kit (Bio-Rad). Reverse transcription of each sample (500ng total RNA) was performed with the IScript

cDNA synthesis kit (Bio-Rad) according to the manufacture. Quantitative RT-PCR was performed using the LightCycler® 480 SYBR Green I Master mix (Roche), on a LightCycler 480. Each reaction was performed in a 10µl volume comprised of 2.5µl 10-fold diluted cDNA, 5µl of master mix and 10pMol of each of the primer (Table S4). Cycling parameters were: 95°C during 10 min to activate the polymerase, followed by 45 cycles of 95°C for 15 s and 60°C for 40 s. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers and non-specific product were amplified. Stability of the house keeping gene *ppial* was verified for each experiment.

Antibody staining

After fixation in PFA, embryos were washed 4 times during 30 min with PBST (0.1% Tween 20 in PBS). Blocking was performed during 2 hr at room temperature in 5% goat serum in PBST and embryos were incubated with 5% goat serum/PBST containing the primary antibody anti-L-plastin (1:500) with gentle shaking at 4°C overnight (Feng et al., 2010). The antibody solution was removed and stored at 4 ˚C in the solution containing 0.001% sodium azide (re-use max 3 times). Embryos were washed (3 x 30 min) with PBST at RT. Embryos were incubated with 2% goat serum/PBT containing secondary antibody anti-rabbit coupled with Alexa 350 (1:250) (Life Technologies). Embryos were rinsed twice with PBST and prepared for microscopy analysis.

Construction of reporter plasmid pIN288

The *mTurquoise2-C1* gene was amplified from pmTurquoise-C1 (Goedhart et al., 2012) using primers TurquoiseNdeI-Fw (5'– GGAATTCCATATGGTGAGCAAGGGCGAGGAGC; *NdeI*

underlined) and TurquoiseXbaI-Rv (5’ – GCTCTAGACTACTTGTACAGCTCGTCCATGCCGAG; *XbaI* underlined), and cloned as an *NdeI*/*XbaI* fragment in pIN29, replacing the DsRed gene (Vergunst et al., 2010), resulting in pIN289. The plasmid was transferred to *B. cenocepacia* by electroporation as described (Vergunst et al 2010), and fluorescence verified.

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CHAPTER 4

GLOBAL HOST TRANSCRIPTOME ANALYSIS REVEALS DIFFERENCES IN INNATE IMMUNE RESPONSE BETWEEN ACUTE AND PERSISTENT INFECTION CAUSED BY DIFFERENT *BURKHOLDERIA CEPACIA* COMPLEX STRAINS IN ZEBRAFISH EMBRYOS

In this chapter I describe the experiments, including bioinformatic analysis and representations I performed to better understand the host transcriptome to infection with Bcc strains causing persistent or acute infection. The RNA sequencing was outsourced to ZF Screens (Leiden, The Netherlands). To confirm these data qRT-PCR experiments were done with the help of Cyril Perrier, who I supervised during his BSc training.

CHAPTER 4

GLOBAL HOST TRANSCRIPTOME ANALYSIS REVEALS DIFFERENCES IN INNATE IMMUNE RESPONSE BETWEEN ACUTE AND PERSISTENT INFECTION CAUSED BY DIFFERENT *BURKHOLDERIA CEPACIA* COMPLEX STRAINS IN ZEBRAFISH EMBRYOS

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Abstract

The innate immune response is the first line of defence against pathogen attack. The ensuing interaction between host and pathogen is marked by sometimes rapid changes in gene expression profiles of both the invader and the host. Although many studies describe the transcriptome of bacteria belonging to the *Burkholderia cepacia* complex (Bcc) under different conditions, nothing is known to date about global gene expression changes in the host during infection caused by these opportunistic pathogens, mainly of cystic fibrosis (CF) patients. The unpredictable outcome of infection, varying from chronic with periods of exacerbation, to acute, necrotic lung infection causes enhanced morbidity and mortality in CF patients. Without existing efficient treatment, infection is correlated with poor prognosis. The molecular basis for these infection types and transition from chronic to acute infection are not understood. We used zebrafish embryos, which have been successfully used for high throughput global host transcriptome analysis, to identify host-specific changes in gene expression during acute and persistent infection caused by two different Bcc strains. Acute infection caused by *B. cenocepacia* K56-2 caused strong modulation of the host transcriptome, in contrast to persistent infection, induced by *B. stabilis*, which had only minor impact on host gene expression. As expected, during acute infection the inflammatory response through Toll-like receptor (TLR) signalling and NOD-like signalling pathways was strongly upregulated. An increase in apoptosis-related gene expression at 24 hours post infection correlated with previously described cell death and tissue damage in infected embryos. In contrast, persistent infection was characterized by minor changes in innate immune signalling, but the complement system was significantly modulated. Here, we describe in more detail the different gene sets affected during infection, and discuss how

these results may contribute to a better understanding of the interaction between Bcc bacteria and the host, with the aim to find new targets for development of anti-infectious therapies to fight this infection.

Introduction

Since some decades, bacteria belonging to the *Burkholderia cepacia* complex (Bcc) have caused devastating lung infections in cystic fibrosis (CF) patients. This group of opportunistic pathogens, now consisting of 18 bacterial species, can cause severe and fatal lung infection sometimes associated with septicaemia called "*cepacia* syndrome" (Glass and Govan, 1986; Mahenthiralingam et al., 2008). *B. cenocepacia* and more recently *B. multivorans* are the most prevalent species in CF patients, and several highly transmissible *B. cenocepacia* strains have caused epidemic outbreaks in the USA and in Europe (Mahenthiralingam et al., 2008; Reik et al., 2005; Speert et al., 2002). The Electrophoretic Type-12 (ET-12) lineage (Drevinek et al., 2005), for example caused major problems for CF patients in the UK and Canada in the late 1980's (Johnson et al., 1994) and the *B. cenocepacia* sequence type (ST) 32 clone CZ1 caused an epidemic outbreak in a CF centre in Prague infecting a high percentage of patients (Dedeckova et al., 2013; Kalferstova et al., 2015). Bcc bacteria are highly resistant to most of the clinically used antibiotics which further complicates treatment (Drevinek and Mahenthiralingam, 2010; Podnecky et al., 2015). Bcc infection is correlated with poor survival prognosis and as a consequence patients infected with *B. cenocepacia* are excluded from lung transplant options in many CF centres (Alexander et al., 2008; Chaparro et al., 2001; Jones et al., 2004; de Perrot et al., 2004; De Soyza et al., 2010). Therefore, a better understanding of the molecular basis of this host-pathogen interaction, including the host response to infection is important for the identification of new therapeutic targets.

To study the pathogenesis of Bcc, different *in vitro* and *in vivo* models have been developed, including cell cultures (macrophages, epithelial cells for example)(Martin and Mohr, 2000), *Caenorhabditis elegans* (Köthe et al., 2003), *Galleria mellonella* (Seed and Dennis, 2008),

mammalian models (rats and mice) (Cieri et al., 2002; Sokol et al., 1999) and more recently, zebrafish embryos (Vergunst et al., 2010). The zebrafish embryo is now recognized as a good animal model to study host-pathogen interactions and the immune response during viral, bacterial and fungal infection (Chen et al., 2013; Ramakrishnan, 2013; Sullivan and Kim, 2008; Tobin et al., 2012; Torraca et al., 2014).

Earlier, we have developed the zebrafish embryo model to study Bcc infection both from the host and bacterial perspective in the context of an innate immune response (Mesureur and Vergunst, 2014; Vergunst et al., 2010). We have shown that the outcome of infection in zebrafish embryos is as variable as that seen in CF: It can vary from acute and rapidly fatal (for instance *B. cenocepacia* strains belonging to the epidemic ET12 lineage, including J2315 and K56-2), to persistent infection (including *B. stabilis* LMG14294 and *B. cenocepacia* J415) with bacteria persisting inside macrophages and no visual signs of inflammation. Our very recent data show that macrophages play a critical role as a niche for the multiplication of *B. cenocepacia* K56-2 during early infection stages and the subsequent initiation of fatal inflammation in zebrafish embryos (**Chapter 3**). The *B. cenocepacia/*macrophage interaction is responsible for a strong Myd88-dependent and Myd88-independent induction of proinflammatory cytokine expression. In contrast, persistent infection seen with *B. stabilis* show a reduced increase in pro-inflammatory cytokine gene expression compared to K56-2 infection. Here, we aimed to get more insight into the differences in the global host response during acute and persistent infection in zebrafish embryos.

Zebrafish have gained remarkable interest as an infection model for human infectious disease, especially to study the innate immune response and interaction of pathogens with host phagocytes in the transparent embryos. A major reason is the high similarity of the

zebrafish immune system to that of humans, with Toll-like receptor signalling pathways (Meijer et al., 2004), complement system (Zhang and Cui, 2014), and presence of all counterparts of human immune cells (Herbomel et al., 1999; Lieschke, 2001). The delay between development of the innate and adaptive immunity, allows studying the innate immune response in the absence of an adaptive immune system in the embryos and early larval stages. The small size of the embryos makes it an interesting model for high throughput sequencing and global host transcriptome studies. Different transcriptomic and microarray studies have shown the potential of the zebrafish embryo model to study in detail host infection-responsive gene expression patterns during bacterial infections (Ordas et al., 2013; Veneman et al., 2013, 2015). Analysis of the host transcriptome to infection with *Salmonella* Typhimurium for example identified an important role for MicroRNA-146, that was shown to be up-regulated during infection in a *myd88/traf6* dependent-manner, and to play a role in lipid regulation during infection (Ordas et al., 2013). Comparative transcriptome analysis between *Staphylococcus epidermidis* and *Mycobacterium marinum* identified common functions that were modulated during both infections, but also detected infection-specific gene expression (Veneman et al., 2013). These approaches permitted to better understand the host response during infection and identified several genes that were important for infection.

Here, we studied the global host transcriptome during acute and persistent Bcc infection caused by *B. cenocepacia* K56-2 and *B. stabilis* LMG14294, respectively. We describe in more detail the differential effect on the infection-specific gene expression, with a major focus on TLR signalling and apoptosis-related signalling for acute infection and the involvement of complement during persistent infection.

Results

Experimental design and validation of RNA-Seq data

We have shown that *B. cenocepacia* K56-2 (hereafter referred to as *B. cenocepacia*) causes a rapid and strong pro-inflammatory response that kills zebrafish embryos in 2 days. In contrast, *B. stabilis* LMG14294 (hereafter *B. stabilis*) shows a persistent phenotype with little inflammation and bacteria persisting in macrophages (Vergunst et al., 2010, Mesureur et al, submitted). To determine changes in the global host transcriptome profile during acute and persistent infection we performed RNA sequencing (RNA-seq) on total mRNA from embryos isolated at different time points during infection. Earlier, we found that the pathophysiology of these two infection types correlated with changes in global gene expression levels of the pro-inflammatory genes *cxcl8, il1b* and *TNFa,* quantified by qRT-PCR (Mesureur et al, submitted); we observed a rapid and strong induction of all three pro-inflammatory cytokine genes with a peak at 3 to 4 hours after intravenous (iv) delivery of *B. cenocepacia*, that was still upregulated compared to non-infected control embryos at 24 hpi. In contrast, *B. stabilis* induced a lower increase in expression during this time interval. Importantly, all iv injected bacteria were rapidly phagocytosed, mainly by macrophages, and started to replicate intracellularly between 6 and 8 hpi. We therefore prepared a library of RNA isolated from infected embryos during the peak of pro-inflammatory cytokine expression (3 and 4 hpi, pooled), the onset of bacterial intracellular replication (7 and 8 hpi, pooled), and at 24 hpi. At 24 hpi, embryos were heavily infected with *B. cenocepacia* (2-log increase in bacterial burden, systemic spread, reduced blood flow, tissue damage, and systemic neutrophil and macrophage death), whereas no anatomical signs of infection were observed in *B. stabilis*

infected embryos at this time point, with persistent numbers of intracellular bacteria (Mesureur et al, submitted). Two biological replicates were performed using PBS-injected embryos as a control group.

Illumina sequencing was performed and raw sequence reads were mapped to the zebrafish reference genome Zv9. The Pearson correlation coefficient (R^2) ranged from 0.967 (*B. cenocepacia*, 7-8 hpi) to 0.99 (PBS, 7-8hpi) (Figure 1A), showing strong correlation between the two biological replicates. Taking all conditions into account, 65 to 70% of the genes were found to be expressed (Figure 1A). As expected a much larger gene set was differentially expressed after infection with *B. cenocepacia* compared to *B. stabilis*. Therefore, we compared the EdgeR package, which tends to detect outliers that have a wide distribution with extreme values (up or down regulated), and DESeq, that generally detects more genes with small significant changes in expression compared to control conditions, to obtain differential expression data sets (Figure 1A).

For each treatment, between 55-60 % and 60-70 % of differentially expressed genes of *B. stabilis* and *B. cenocepacia,* respectively, were detected by both methods. In general, more *B. cenocepacia* genes were identified with the EdgeR method, whereas DESeq detected more genes with significantly altered expression levels for *B. stabilis* (Figure 1B and 1C). For the further analysis we combined the genes obtained with both methods.

A

Library	Correlation duplicats R^2	Uniquely mapping reads	Normalization Factors (EdgeR)	Size Factors (DEseq)	Genes expressed
PBS_3_4_hpi	0.978253	17 157 483	1.0139846	0.9806091	21 171 (65.24%)
PBS_3_4_hpi_(2)		13 327 977	1.0077632	0.7546081	
PBS_7_8_hpi	0.990157	37 111 196	0.9673212	0.9864623	22 077 (68.04%)
PBS_7_8_hpi_(2)		10 520 337	1.0220264	0.7586201	
PBS_24_hpi	0.974129	9 249 752	0.9697223	1.8370903	23 082 (71.13%)
PBS_24_hpi_(2)		50 486 967	0.9664113	0.5451752	
K56_3_4_hpi	0.979833	9 545 233	1.0193429	0.5457628	21 832 (67.28%)
K56_3_4_hpi_(2)		46 267 760	0.9600411	2.5087624	
K56_7_8_hpi	0.967392	8 691 427	1.0329687	0.5826750	22 230 (68.51%)
K56_7_8_hpi_(2)		44 524 866	0.9792491	2.3773341	
K56_24_hpi	0.976494	13 539 973	1.0391886	0.4552915	22 671 (69.87%)
K56_24_hpi_(2)		13 775 004	1.0268245	2.2220952	
stab_3_4_hpi	0.987148	10 208 265	1.0225453	0.5826750	21 743 (67.01%)
stab_3_4_hpi_(2)		43 262 371	0.9436927	2.3273341	
stab_7_8_hpi	0.974217	51 688 808	0.9961989	2.6730158	22 299 (68.72%)
stab_7_8_hpi_(2)		6 968 794	1.0044041	0.3635116	
stab_24_hpi	0.985515	42 581 312	0.9827436	2.1566587	22 925 (70.65%)
stab_24_hpi_(2)		7 231 345	1.0144801	0.3780868	

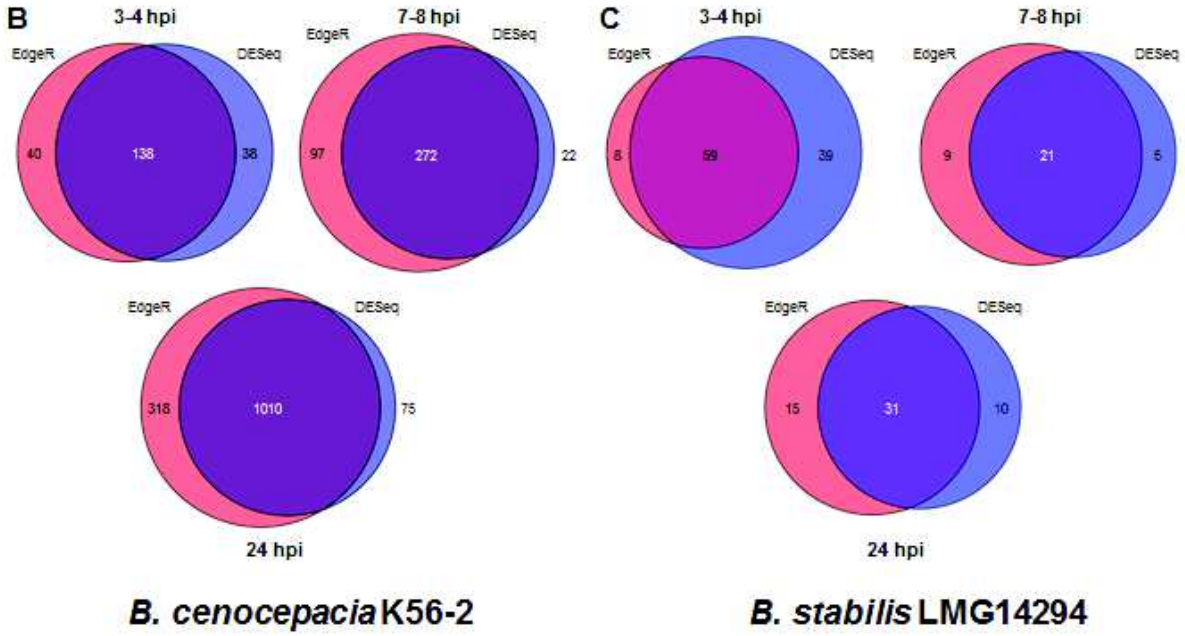

Figure 1. Overview of the RNAseq analysis

A. Table summarizing general RNA-Seq statistics using DESeq and EdgeR packages.

B, C. Venn diagram representation of the total number of differentially expressed genes determined with EdgeR (pink) and/or DESeq (blue) packages at 3/4, 7/8 and 24 after infection with *B. cenocepacia* K56-2 (B), or *B. stabilis* LMG14294 (C).

Modulation of the host transcriptome profile largely differs between acute and persistent infection

The obtained set of host-responsive genes showed that a high number of genes were modulated during acute infection caused by *B. cenocepacia*, in contrast to only a small set of genes that were modulated during persistent infection with *B. stabilis*. In addition, the number of genes that were differentially expressed during acute, but not persistent infection increased over the time course of infection (Figure 2A). Infection with *B. cenocepacia* resulted in the differential expression of 1372 host genes at 24 hpi (985 up-regulated and 418 down-regulated), compared to only 56 genes at 24 hpi after infection with *B. stabilis*. In both cases, more genes were up-regulated than down-regulated during bacterial infection. Although most genes that were differentially expressed during *B. stabilis* infection also showed changes in gene expression levels during *B. cenocepacia* infection (Figure 2B, 2C), several genes were specifically upregulated during *B. stabilis* infection (3/4 hpi, 11 genes (20%); 7/8 hpi, 3 genes (11%); and 24 hpi, 11 genes (22%)). Interestingly, of the few host genes that were down-regulated during persistent infection, most seemed specific for this infection type (3/4 hpi, 14 genes (42%); 7/8 hpi 2 genes (29%); and 24 hpi, 2 genes (40 %)).

Most genes that were specifically up-regulated during *B. stabilis* infection were related to innate immunity and bacterial recognition (Figure 2B). For example, the intelectin 1 (*itln1*) gene encodes a receptor for bacterial arabinogalactan and lactoferrin (Suzuki et al., 2001; Tsuji et al., 2001), and *cyp1a,* encoding a member of the cytochrome P450 family*,* shows TLR2-dependence (Do et al., 2012; Ma and Lu, 2007). Early during infection, *cybb* that encodes $gp91^{phox}$, a subunit of the NADPH oxidase complex involved in production of

reactive oxygen species (ROS), was up-regulated. This could explain our previous findings (Mesureur et al, submitted) that *B. stabilis* was more sensitive to NADPH oxidase activity than *B. cenocepacia,* and suggests that *B. cenocepacia* might prevent upregulation of this host response gene. *Fgl2* is an immune regulator of both innate and adaptive immune response (Marazzi et al., 1998), and *tnmd* encodes tenomodulin that inhibits angiogenesis and induces a high level of systemic immune mediators, including complement components. Complement is one of the major upregulated groups during persistent infection as described below in more detail. In contrast several down regulated genes have been described to be involved in cancer, such as *serbp1, her4* or *mafbb*.

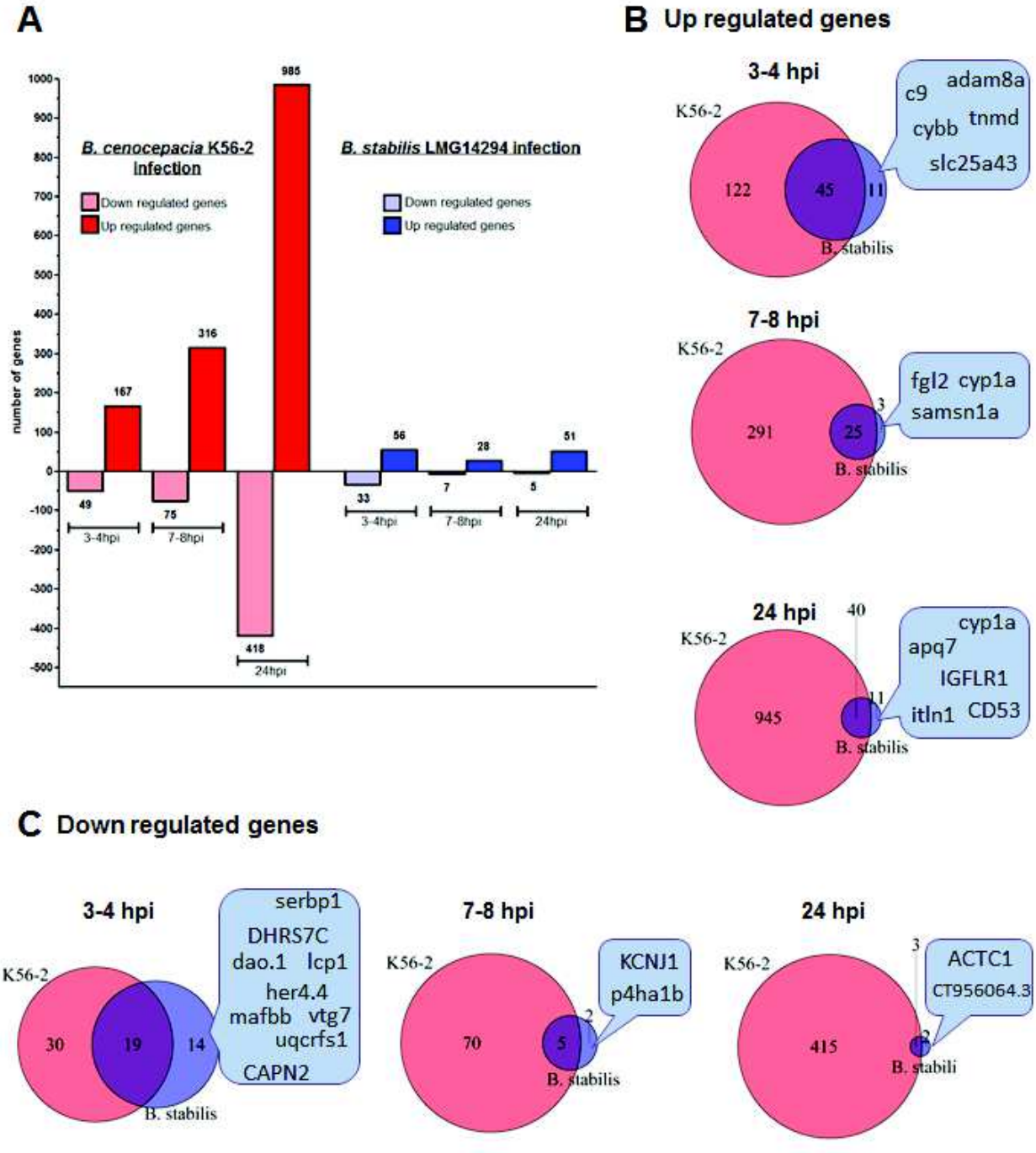

Figure 2. Acute and persistent Bcc infections induce two different transcriptome profiles.

A. Overview of the total number of up- and down-regulated genes (combining DESeq and EdgeR results) in *B. cenocepacia* or *B. stabilis*-infected embryos at 3/4, 7/8 and 24 hpi.

B,C. Venn diagrams showing the overlap between upregulated gene sets (B) and down regulated gene sets (C) during infection with *B. cenocepacia* K56-2 (pink) and *B. stabilis* LMG14294 (blue) at 3/4, 7/8 and 24 hpi. Known gene names of those differentially regulated only after *B. stabilis* infection are indicated.

Gene Ontology (GO) analysis was performed to determine GO term enrichment and host signalling pathways involved during bacterial infection. GO terms were divided in three different groups, biological process, molecular function and cellular component. Genes that were up-regulated during *B. cenocepacia* infection were part of 36 GO terms at 3/4 hpi, 57 at 7/8 hpi and 109 at 24 hpi. For genes that were down-regulated, 14 GO terms were found at 3/4 hpi, 12 at 7/8 hpi and 69 at 24 hpi. Here, the most significantly enriched GO terms are presented (Table 1). The GO term "Immune system process" (GO:0002376) was significantly enriched during acute and persistent infection, but not in the same proportion. During acute infection, the number of genes in "Immune system process" increased in time with a maximum of 33 genes at 24 hpi, whereas just 3 genes were found during *B. stabilis* infection at 24 hpi. "Response to bacterium" (GO:0009617) and "defence response" (GO:0006952) were found during acute and persistent infection. The number of genes involved in these responses increased during acute infection but remained stable during persistent infection. The enrichment of these three GO terms showed that *B. cenocepacia* infection induced a strong host immune response in agreement with in vivo observations. Interestingly, GO terms "cell death" (GO:0008219) and "regulation of apoptosis" (GO:0042981) were not enriched at the early time points of infection, but 23 genes showed changes in gene expression at 24 h of *B. cenocepacia* infection. Analysis of genes that were down-regulated during acute infection showed enrichment in "Oxidation reduction" (GO:0055114) and "cytoskeleton" (GO:0005856) GO terms (Table 1).

Table 1. GO term enrichment during *B. cenocepacia* K56-2 and *B. stabilis* infection.

A. Up-regulated genes

GO	Term	K56-2 3-4 hpi		K56-2 7-8 hpi		K56-2 24 hpi		*B. stabilis* 3-4 hpi		*B. stabilis* 7-8 hpi		*B. stabilis* 24 hpi	
		# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value
GO:0002376	**Immune system process**	**11**	**<0.0001**	**23**	**<0.0001**	**33**	**<0.0001**	**0**		**0**		**3**	**0.0427**
GO:0006955	Immune response	11	<0.0001	18	<0.0001	29	<0.0001	0		0		3	0.0427
GO:0019882	Antigen processing and presentation	0		6	0.0005	10	<0.0001	0		0		0	
GO:0050896	**Response to stimulus**	**37**	**<0.0001**	**60**	**<0.0001**	**160**	**<0.0001**	**8**	**<0.0001**	**2**	**0.0381**	**4**	**<0.0001**
GO:0009617	Response to bacterium	10	<0.0001	12	<0.0001	14	<0.0001	6	<0.0001	2	0.0376	4	<0.0001
GO:0006952	Defence response	9	<0.0001	10	<0.0001	13	<0.0001	5	<0.0001	2	0.0555	3	0.0063
GO:0008219	**Cell death**	**0**		**0**		**12**	**0.0003**	**0**		**0**		**0**	
GO:0012501	Programmed cell death	0		0		11	0.0009	0		0		0	
GO:0042981	Regulation of apoptosis	0		0		23	<0.0001	0		0		0	

B. Down-regulated genes

GO	Term	K56-2 3-4 hpi		K56-2 7-8 hpi		K56-2 24 hpi		*B. stabilis* 3-4 hpi		*B. stabilis* 7-8 hpi		*B. stabilis* 24 hpi	
		# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value	# genes	p-value
GO:0055114	Oxidation reduction	4	0.0253	6	0.0071	22	<0.0001	5	0.0011	0		0	
GO:0005856	Cytoskeleton	4	0.0111	6	0.0011	11	0.0467	0		0		0	

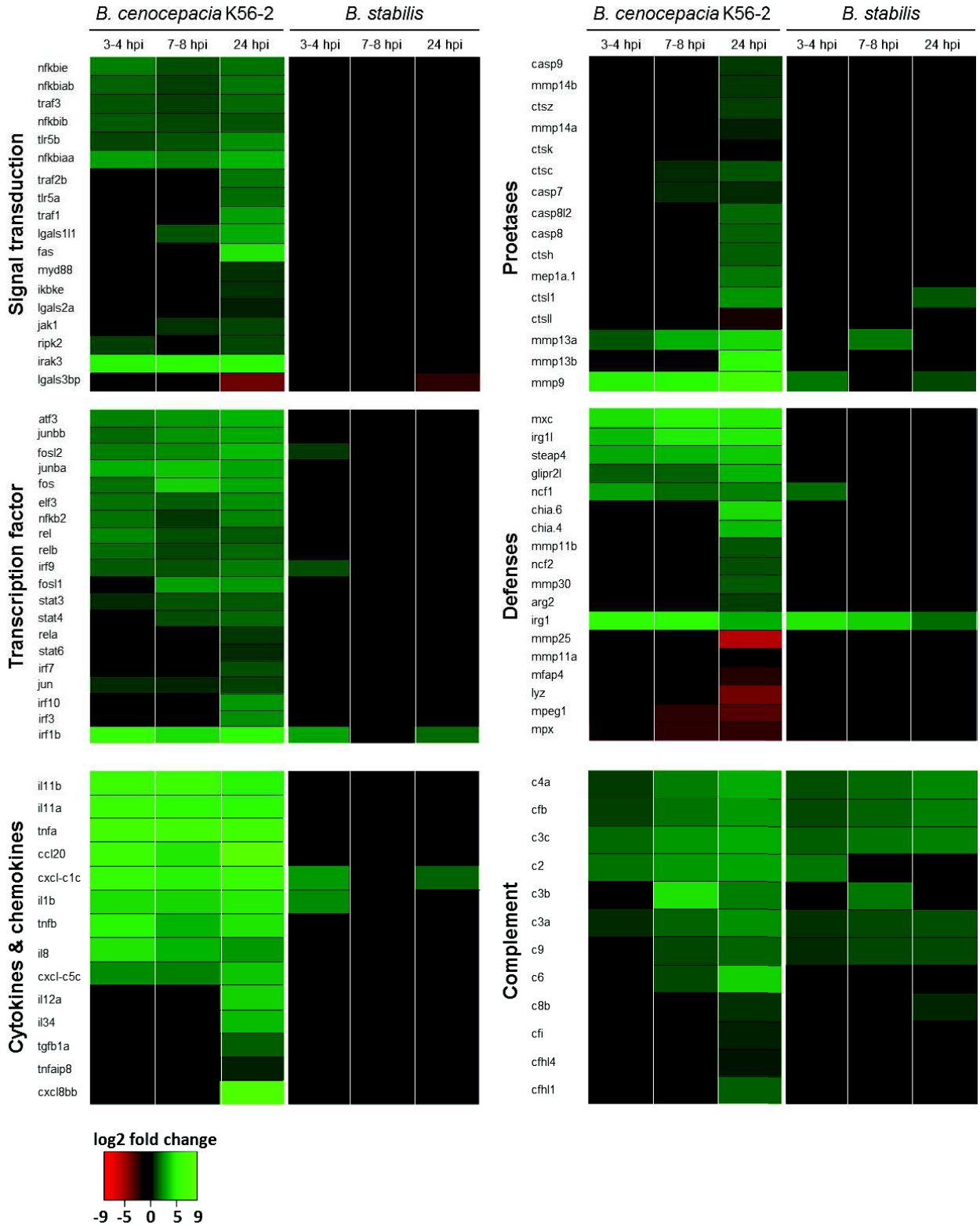

Figure 3. Expression profiles of the innate immune response

Heatmaps showing relative changes in transcription level of genes in six different groups (signal transduction, transcription factor, cytokines and chemokines, proteases, defences and complement system) during *B. cenocepacia* K56-2 or *B. stabilis* LMG14294 infection. Levels of expression are represented as $\log_2$-fold change compared to the PBS-injected condition at each time point.

We were specifically interested in differences in host immune response during *B. cenocepacia* and *B. stabilis* infection. The evolution of differential gene expression during both infections is presented in heat maps (Figure 3) and confirms the strongly induced changes in global host gene expression during infection with *B. cenocepacia* compared to *B. stabilis.* Signal transduction, transcription factors, proteases, cytokines and chemokines were mainly up-regulated during *B. cenocepacia* infection. Interestingly, although also upregulated during acute infection, of these 6 groups mainly genes with a role in the complement system were up-regulated during persistent infection (see further below).

Several genes were specifically down regulated. The genes myeloperoxidase (*mpx*), macrophage expressed 1 (*mpeg1*), lysozyme (*lyz*) and microfibrillar-associated protein 4 (*mfap4*) were all rapidly down regulated during *B. cenocepacia* infection. These genes are specifically expressed in macrophages and neutrophils (Renshaw et al., 2006; Zakrzewska et al., 2010), and this result is in agreement with our previous qRT-PCR results that *mpx* and *mpeg1* gene expression are rapidly down regulated during *B. cenocepacia* infection, and that at 24 hpi most neutrophils and macrophages have disappeared from the infected embryos (Mesureur et al, submitted).

Interestingly, whereas *lgals1* (galectin 1) expression was up-regulated during *B. cenocepacia* infection at 7/8 hpi, *lgals3* (galectin 3) was down-regulated during both acute and persistent infection at 24 hpi. The down regulation of *lgals3* was confirmed by qRT-PCR (Figure 4E). Galectins are lectins involved in functions including mediation of cell–cell interactions, cell–matrix adhesion and transmembrane signalling. Galectin 3 (Gal-3) is important for the inflammatory response during bacterial, viral and parasitic infections and has been shown to be up regulated during infection except for *E. tarda* (Curciarello et al., 2014; Garcin et al.,

2015; Sato et al., 2014; van der Vaart et al., 2013). In zebrafish, Gal-3 promotes inflammatory responses and the ROS response in neutrophils, and, in contrast to galectin 1, has a pro-apototic activity on host cells, and helps macrophages to control intracellular infection by inhibiting microbial activity (Vasta et al., 2004). Several ligands were found to interact with Gal-3, as for example matrix metalloproteinase 9 (MMP-9) and Mac-2 binding protein (Mac-2bp). Mac-2bp can interact with extracellular Gal-3 and induces regulation of cell adhesion (Dumic et al., 2006). Down regulation of *lgals3* suggests that both *B. cenocepacia* and *B. stabilis* actively repress the expression of this gene to evade host immune killing, but further experimental evidence is needed.

The matrix metalloproteinase family is a large group of zinc-dependent endopeptidases that are able to degrade extracellular matrix. During *B. cenocepacia* infection, 7 MMPs were up-regulated and only 2 down-regulated. *Mmp9* is one of the stronger up regulated genes and is also up regulated during *B. stabilis* infection. This up regulation was confirmed by qRT-PCR (Figure 4A). It has been suggested that MMP-9 plays an important role in development of infection. During *Mycobacterium tuberculosis* and *Mycobacterium marinum* infections for example, MMP9 is required for recruitment of macrophages and granuloma formation (Taylor et al., 2006; Volkman et al., 2010), and it has been suggested that MMP-9 prevents return to tissue homeostasis during *B. cenocepacia* infection (Wright et al., 2011). MMP25 is one of the more strongly down regulated genes, and is involved in the breakdown of extracellular matrix proteins, such as type-IV collagen, gelatin, fibronectin and fibrin in normal physiological processes (English et al., 2001). The nephrosin (*npsn* gene) is a zinc metalloendopeptidase which is responsible for immune and hematopoietic functions in fish.

In contrast to *mmp9*, *npsn* gene expression was down-regulated during *Burkholderia* infection (Figure 4C).

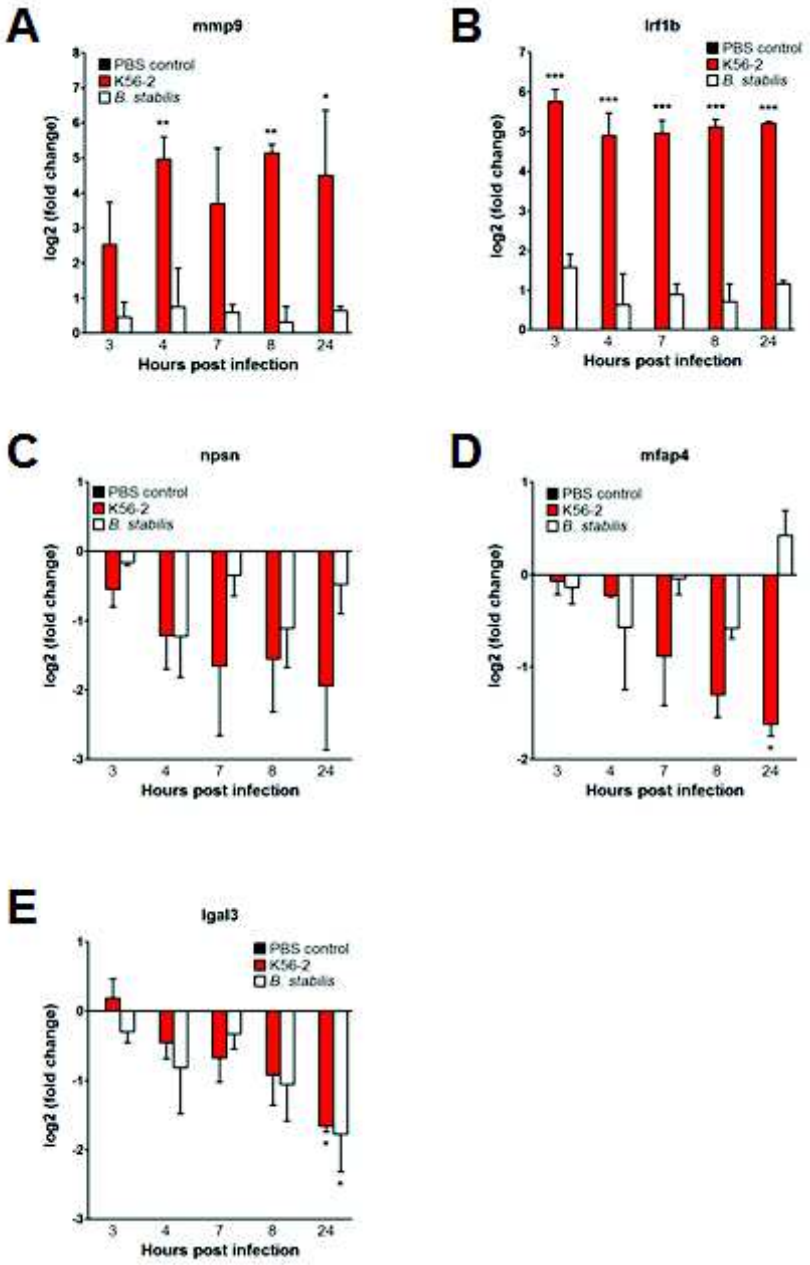

Figure 4. Validation of RNA-Seq expression.

qRT-PCR results of gene expression of 5 selected genes confirm the RNA-Seq expression profile. Data are presented in $\log_2$ fold change normalised to a PBS control group at each time point. Expression levels obtained by qRT-PCR of *mmp9* (A), *irf1b* (B), *npsn* (C), *mfap4* (D) and *lgal3* (E) genes during *B. cenocepacia* or *B. stabilis* infection. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Burkholderia cenocepacia K56-2 highly modulates host innate immune response and TNF-mediated cell death

We looked in more detail at the different innate immune signalling pathways that were activated during *B. cenocepacia* infection at 24 hpi (Figure 5A). The most important is the Toll-Like Receptor (TLR) signalling pathway that induces pro-inflammatory cytokine production and has a major role in the innate immune response. At 24 hpi, genes involved in the MyD88-dependent and independent pathways were up-regulated, which was confirmed with qRT-PCR for the *myd88* gene (Figure 5B). *Irak3*, encoding an inhibitor of the MyD88 pathway, was highly up-regulated from the beginning of the infection, as confirmed by qRT-PCR (Figure 5C). Downstream of MyD88, *nfκb* (Figure 5D) gene expression was activated and enhanced expression of this gene could amplify the pro-inflammatory response though activation of pro-inflammatory cytokine expression. Cxcl-c1c was also up-regulated very early in infection (Figure 5E). Three other important signalling pathways were upregulated; NOD-like receptor, MAP-kinase and Jak-STAT signalling. The activation of these in total four pathways involved in the immune response, specifically the inflammatory response, underscores the highly pro-inflammatory character of *B. cenocepacia*.

The strong inflammation caused by *B. cenocepacia* resulted in severe tissue damage and phagocytic cell death (Mesureur et al, submitted). Our RNA-Seq analysis showed that the apoptosis pathway was up-regulated downstream of p53 signalling and TNFa activation (Figure 5A). Caspase 8 (*casp8* gene) was up-regulated and could induce apoptosome activation.

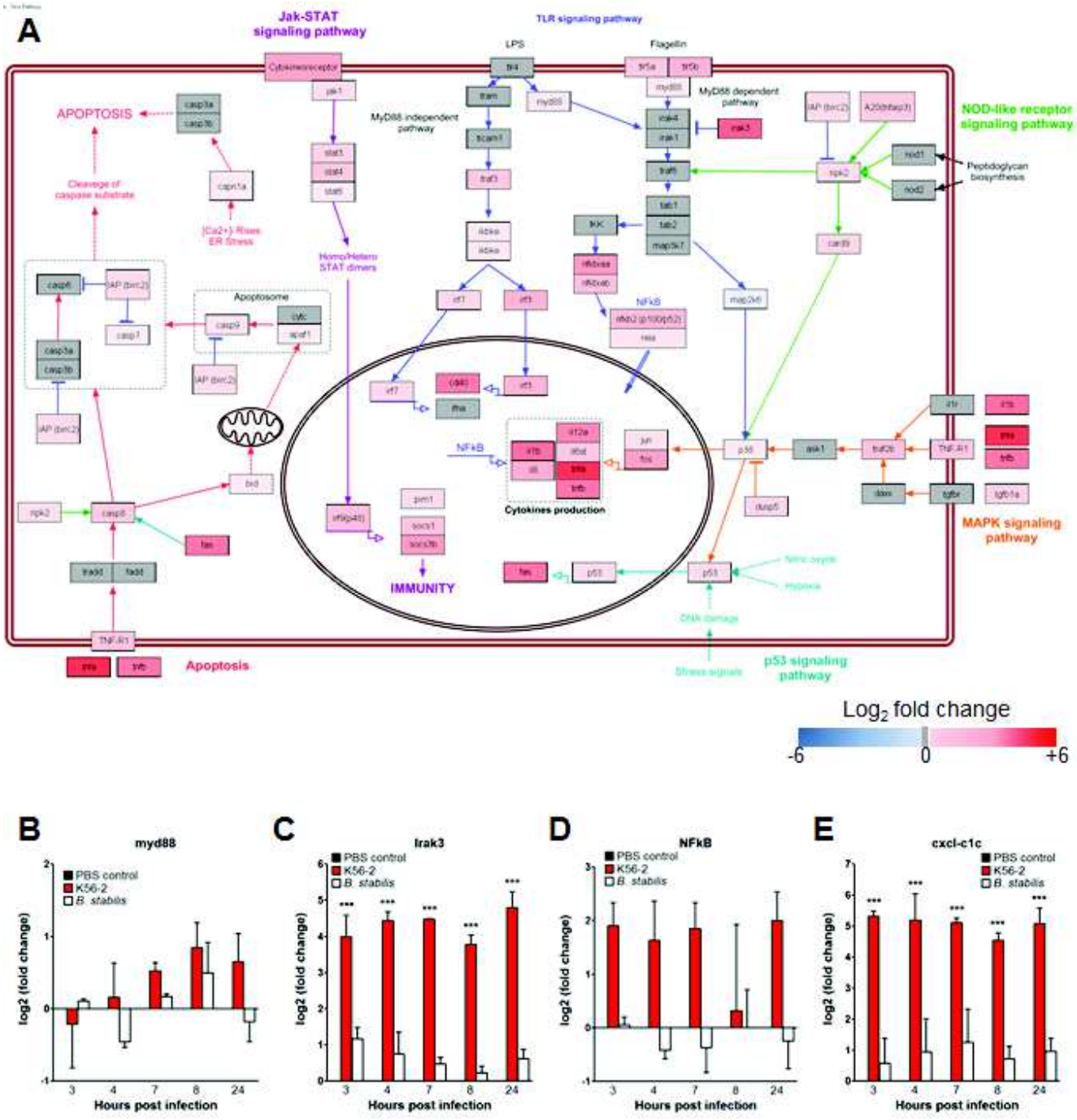

Figure 5. The inflammatory response is up regulated during *B. cenocepacia* K26-2 infection

A. Drawing representing different innate immune pathways and the apoptosis pathway activated in cells during *B. cenocepacia* infection at 24 hpi, based on data obtained in this study. Data are presented in $\log_2$ fold change relative to a PBS control group at each time point.

B-E. Expression levels obtained by qRT-PCR of *myd88* (B), *irak3* (C), *nfkb* (D) and *cxcl-c1c* (E) genes during *B. cenocepacia* or *B. stabilis* infection. *** $p \leq 0.001$.

***Burkholderia stabilis* LMG14294 induces up-regulation of complement pathway genes.**

As shown in the beginning of this study *B. stabilis* infection caused a limited modulation of the host transcriptome. The complement system constitutes an important part of the innate immune response through the alternative and the lectin pathway. *B. stabilis* infection induced the alternative pathway with the up-regulation of *c3a*, *c3b* and *c3c* genes (Figure 6A). Results for *c3c* were confirmed by qRT-PCR (Figure 6B). The complement factor B (*cfb* gene) was also up-regulated (Figure 6C). CfB associated with c3b formed the c3 convertase. C3 convertase provokes the formation of the membrane attack complex (c5b, c6, c7a-b, c8a-b-g and c9 subunit). C4a, an element of the lectin pathway, was up-regulated (Figure 6D) and is known to induce inflammation responses.

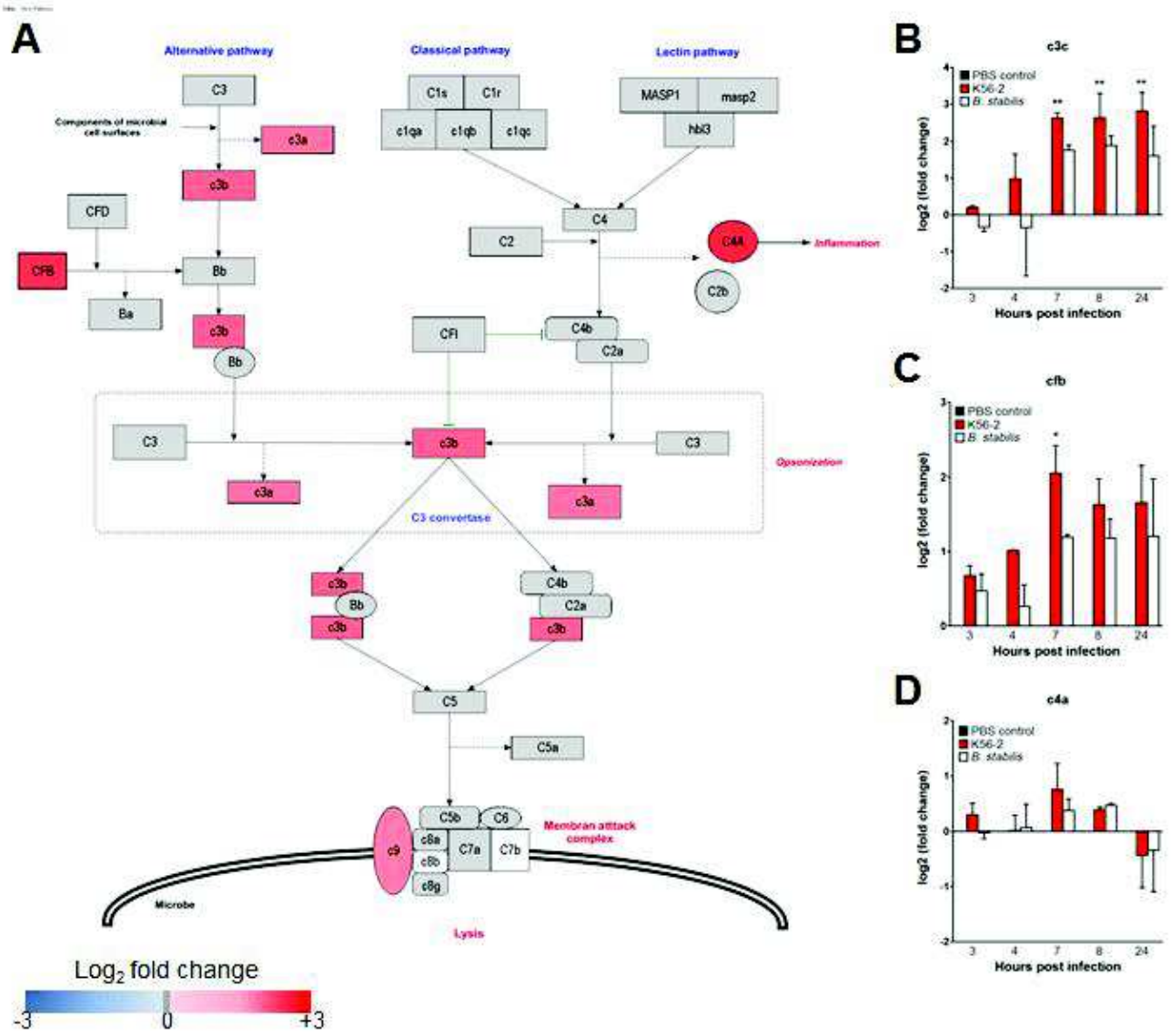

Figure 6. The complement system is up regulated during *B. stabilis* infection

A. Drawing representing complement system activation during *B. stabilis* infection at 24 hpi. Data are presented in $\log_2$ fold change relative to a PBS control group.

Expression levels obtained by qRT-PCR of *c3c* fragment (B), *cfb* gene (C) and *c3a* fragment (D) during *B. cenocepacia* or *B. stabilis* infection. * $p \leq 0.05$ and ** $p \leq 0.01$.

Discussion

Transcriptome studies to determine changes in gene expression during Bcc infection have thus far focused on the bacterial genes involved in virulence, using conditions mimicking cystic fibrosis sputum, a chronic lung infection model in rats, or a cell culture model (Kalferstova et al., 2015; O'Grady and Sokol, 2011; Tolman and Valvano, 2012; Yoder-Himes et al., 2010). Mariappan et al were the first to study host transcriptome variation during infection of A549 epithelial cells with *B. cepacia* (Mariappan et al., 2013). Here, we describe the first global host transcriptome response study in an animal model to Bcc infection.

In our study we identified two different host transcriptome profiles that are in perfect accordance with the infection phenotypes induced by *B. cenocepacia* K56-2 and *B. stabilis* LMG14294. We have shown earlier that *B. cenocepacia* K56-2 causes a rapidly fatal infection with hallmarks of a strong MyD88-dependent pro-inflammatory response that kills zebrafish embryos in 2 days (Vergunst et al., 2010, Mesureur et al, submitted). After iv injection of bacteria, they are rapidly phagocytosed by macrophages, and during the first 7 hours the bacteria adapt to the intramacrophage environment, after which they start to replicate intracellularly.

Our transcriptomic study reveals a change in expression of genes involved in the innate immune response, especially the inflammatory response which is in accordance with the strong pro-inflammatory character of the infection. Recently, we described that an increase in pro-inflammatory cytokine expression during the first 6 hours of infection was critically dependent on the presence of MyD88 (Mesureur et al, submitted). Our RNAseq and qRT-PCR data indicate that *myd88* gene expression itself is not induced during these early stages but its induction at later stages suggests the inflammatory response is further amplified during

acute infection. Major cytokine genes were upregulated, including *cxcl8* and *tnfa,* in agreement with our earlier finding in zebrafish (Mesureur et al, submitted), and those in other models (O'Grady and Sokol, 2011; Reddi et al., 2003). Also the genes encoding Il11*,* *C*cl20 (or macrophage inflammatory protein-3α) and *C*xcl-c1c were strongly upregulated at 3/4 hpi. CXCL-C1C and IL11play an important role in for example activation of Jak and acute phase proteins, the recruitment of neutrophils and induction of degranulation, and the chemo attraction of immature dendritic cells (CCL20, (Schutyser et al., 2003) Indeed, key genes in all major signalling pathways, TLR, NOD-like, MAPK, Jak/Stat, Fos/Jun, were already upregulated during the first 3 to 4 hours of infection. This is in accordance with results obtained in a micro array study in A549 cells (Mariappan et al 2013). Despite reports that describe that zebrafish show tolerance to bacterial LPS and have an altered TLR4/LPS response (Sepulcre et al., 2009; Novoa et al., 2009), we found a strong inflammatory response of *B. cenocepacia* K56-2 in zebrafish embryos, as found in murine models and seen in patients. Perhaps zebrafish signal bacterial LPS differently, but still in a Myd88-dependent manner (van der Vaart et al., 2013). It will be interesting to determine whether *B. cenocepacia* induces a TLR4-Myd88-dependent signalling cascade also in zebrafish, or whether the inflammatory response is induced by a different set of bacterial ligands, including flagellin. It has been shown in fish that the TLR5 receptor recognizes the flagellin protein as in humans (Kanwal et al., 2014), and its dependency on Myd88 was shown using *myd88* mutant fish (van der Vaart et al 2013). Interestingly, TLR5, a receptor of bacterial flagellin, is strongly upregulated early during infection, and this is in line with data showing that *B. cenocepacia* flagella contribute to virulence in mice and that flagellin interacts with

TLR5 and induce IL-8 production via NF-κB activation (de C Ventura et al., 2008; Urban et al., 2004).

Although at early time points no difference in expression profile for apoptosis-related processes was obvious, they were activated later during infection. This profile correlates with the development of the acute infection: at 24 hpi inflammation sites with severe tissue damage can be observed in zebrafish embryos (Vergunst et al., 2010, Mesureur et al, submitted), and macrophage- and neutrophil- specific genes were down regulated from an early time point, in line with a dramatic effect on the observed massive phagocyte death during acute infection. In A549 cells, *B. cepacia* infection induced down-regulation of apoptosis-related gene expression at 3 hpi (Mariappan et al 2013). Although we do not find evidence for specific down regulation, small difference of differential expression, in for instance macrophage, may have been missed in our global host response analysis. In fact, our infection studies show that infected macrophages are kept alive by *B. cenocepacia*, suggesting indeed the induction of anti-apoptotic programmes at early time points.
Although we currently do not know the reason for this massively observed phagocyte death. The strong upregulation of TNFa specific signalling suggests that increased TNFa levels might be involved, maybe by inducing necroptosis (Fuchs and Steller, 2015).

In contrast to *B cenocepacia*, *B. stabilis* LMG14294 shows a persistent phenotype with bacteria residing in macrophages (Vergunst et al., 2010, Mesureur et al, submitted), an increase in neutrophil numbers, and little signs of inflammation during the first 5 days of infection. Indeed, only a limited number of genes were differentially regulated. Interestingly,

genes involved in mainly complement system, and bacterial recognition were upregulated. The induction of the complement, also during acute infection, is in agreement with the microarray study by Mariappan et al (Mariappan et al., 2013). This suggests that *B. stabilis* does not induce excessive inflammation possibly by avoiding host immune detection. It will be interesting to determine if the induction of complement has a specific role in keeping the bacterial infection under control, or whether *B. stabilis* is lacking factors that would result in enhanced acute virulence. Interesting is the observation that *B. stabilis*, which is persistent in macrophages, can cause acute pro-inflammatory infection in a small percentage of the embryos from 5 dpi (Vergunst et al., 2010). It has been shown that serum resistance of Bcc isolates is not linked to the capacity of Bcc strains to induce bacteraemia in CF patients (Zlosnik et al., 2012). We propose the zebrafish embryo/*B. stabilis* interaction may serve as a model to study the involvement of complement in more detail.

Although the mounting of an effective innate immune response is crucial for host defence, an excessive activation may be harmful to the host. In a review of 32 transcriptomics studies involving 77 different host-pathogen interactions, Jenner and Young highlighted groups of general infection-response genes that were always up regulated during infection. One of these gene sets contains genes that code for pro-inflammatory cytokines and chemokines, including TNF, IL1β, IL8, GCSF, CCL3, CCL4, CXCL1 and CXCL3 (Jenner and Young, 2005). They hypothesize that this group of genes represents the common program by which inflammation is induced during infection. In our study, *il8, il1b* and *tnfa* genes were also highly up regulated, as was the case during *S.* Typhimurium infection in zebrafish embryos (Stockhammer et al., 2009) suggesting the crucial role of these genes to induce the

inflammatory response also in zebrafish. To be activated the inflammatory response depends on transcriptional regulators and signal transduction pathways, a second group of genes. NF-κB and its family (BCL3, AP1, JUN, JUNB and FOSL2) were up regulated by different pathogens (Jenner and Young, 2005; van der Vaart et al., 2013) as is also the case for *B. cenocepacia* K56-2 infection. In this group *myd88, tradd, traf1* and *traf6* genes were also included. Further, it is interesting that the *lgals3* gene, encoding the lectin Gal3, and which has been shown to be up regulated in different infections, from bacterial to parasitic, was down regulated in our study, as also found during infection by *Edwardsiella tarda* (Curciarello et al., 2014; Garcin et al., 2015; Sato et al., 2014; van der Vaart et al., 2013). Specfic down regulation of Gal3 early during infection might be a way of *B. cenocepacia* to specifically avoid the innate immune functions of this protein, including the described role in ROS response, its pro-and control of intracellular infection in macrophages by inhibiting microbial activity (Vasta et al 2004).

Summarizing, we showed that acute and persistent infection induced by *B. cenocepacia* K56-2 and *B. stabilis* LMG14294, respectively, correlated with the identified differential gene expression data sets we obtained. In this study we focused in more detail on the innate immune response, and we identified several signalling pathways that are important for the observed inflammation in zebrafish embryos infected with *B. cenocepacia*. Our study identified interesting host genes to further explore as potential drug targets, such as *lgals.* Our RNA-seq data also highlighted different metabolic pathways that were modified during Bcc infection and it will be important to study in more detail these different pathways in order to better understand the general host modification induced by the infection.

Materials and methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) were kept and handled in compliance with national regulations for animal welfare and approved by the departmental and ethical committee (ID 30-189-4 and CEEA-LR-12186).

Bacterial strains

Burkholderia cenocepacia K56-2 and *Burkholderia stabilis* LMG14294 containing the DsRed reporter plasmid pIN29 (Vergunst et al., 2010) were grown overnight in 5 ml of LB in the presence of 100 $\mu g.ml^{-1}$ chloramphenicol at 37°C in a rotary shaker.

Infection experiments

Embryos (27 hours post fertilization, hpf) were dechorionated manually precisely 1 hour before injection and kept at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$). Methylene blue was omitted from the medium. Just prior to injection, embryos were anesthetized in E3 medium containing 0.02% of MS222 (Tricaine, Sigma). Around 1 nL (200 CFU) of bacterial suspension in PBS, or 1X PBS as a control, was injected intravenously as described (Mesureur and Vergunst, 2014). All groups were treated similarly (time of incubation in Tricaine, temperature, time of injection) and experiments were performed by two people to keep the variation due to experimental and developmental cues to a minimum. Special care was taken to avoid wounding the embryos by pipetting. The

exact bacterial inoculum was confirmed by individually plating 5 embryos as described (Mesureur and Vergunst, 2014). PBS-injected embryos were used as a control condition.

RNA extraction and purification

At 3, 4, 7, 8 and 24 hpi 20 embryos of each condition (PBS-injected, *B. cenocepacia* injected and *B. stabilis* injected embryos) were transferred to 500 µl of TRIzol, homogenized by rigorous vortexing and stored at -80°C. From each pool, RNA was extracted as described by Cui *et al* (Cui et al., 2011) and purified using the RNeasy MinElute Cleanup kit (BioRad). The amount and the purity of RNA were measured with a NanoPhotometer (IMPLEN).

RNA-Seq experiment

For RNA sequencing, RNA samples from 3 and 4 hpi, and 7 and 8 hpi were pooled. RNA integrity was analysed by Lab-on-a-chip analysis (Agilent). The average RIN value of the RNA samples was 9.7 with a minimum of 9.5. A total of 3 µg of RNA was used to make RNA-Seq libraries with the Illumina TruSeq RNA Sampl Preparation Kit v2 (Illumina Inc.). Small changes have been made to the manufacturer's instruction. Only 1 µl of adaptor was used and the library fragments were isolated with a double Ampure XP purification with a 0.7x beads to library ratio. Samples were sequenced at paired-end 2 x 50 nt run (PE50) and using an Illumina Hiseq2000 to a minimum of 10 million paired reads per sample. Sequenced reads were filtered and mapped to ENSEMBL transcript (Zv9_63) using the ref_assemle_short module in the CLCbio Assemby Cell v4.0.6. For more details see Veneman *et al* (Veneman et al., 2013). RNA sequencing was performed by ZFScreens (Leiden, The Netherlands). The data will be deposited in GEO before submission of this chapter.

Gene expression analysis

Based on recent comparative studies of normalization methods for RNA-Seq analysis (Dillies et al., 2013; Maza et al., 2014), we performed normalization of the data with EdgeR and DESeq packages using R programming software. Prior to statistical analysis, we removed genes with less than 10 total reads over both replicates. For EdgeR, we normalised the data using the TMM (Trimmed Mean of M-values), UpQ (Upper Quartile) and RLE (Relative Log Expression) methods. We found no differences between these three methods, and here we used the data obtained with the UpQ method. For DESeq the normalization method was implemented in the DESeq Bioconductor package, and based on the hypothesis that most of genes are not differently expressed (DE) (Dillies et al., 2013). The reason we used both methods is that EdgeR permits to diminish the impact of extreme values, and DESeq is better able to minimize the false positive DE genes. Genes that we found upregulated or down regulated more than two fold (p-value < 0.5) with either method were combined and used for GO analysis. The global views of gene expression patterns are visualized by Venn diagrams (VennDiagram package) and graphical column representations.

Gene ontology and functional annotation

Gene Ontology (GO) analysis was performed using the DAVID Bioinformatics Resources web site. For analysis, we separated up- and down-regulated genes. Heatmap representations were made using gplots packages. Biological pathway representations in figure 6 and 7 were made using PathVisio 3.2.0 software.

qRT-PCR analysis

Reverse transcription of each sample (500 ng in total) was performed with the IScript cDNA synthesis kit (BioRad). qRT-PCR was performed on a LightCycler 480 (Roche), using the LightCycler 480 SYBR Green I Master Mix (Roche) and with white LightCycler 480 96 multiwell plates (Roche). Primers used for qRT-PCR are described in Table 2. The peptidylprolyl isomerase A-like (*ppial*) gene was used as reference housekeeping gene. Results were analysed using the ΔΔCt method and normalized to the PBS-injected condition at each time point. The ΔΔCt values were $\log_2$ transformed and represented as histograms. Before performing the actual RNA-Seq experiment, each condition was verified by qRT-PCR (not shown). After RNA-seq analysis, three biological experiments were performed to verify the RNA-seq data of selected genes, two were also used for RNA-seq analysis and one new external biological experiment was performed. Data were analysed using one-way ANOVA with Tukey as Multiple Comparison Test.

Table 2. qRT-PCR primers

Gene	Accession #	Forward primer	Reverse primer
ppial	ENSDARG00000042247	5'-ACACTGAAACACGGAGGCAAAG-3'	5'-CATCCACAACCTTCCCGAACAC-3'
mmp9	ENSDARG00000042816	5'-GCACTACAGAAGGACGAAATGAC-3'	5'-CCACCATCACAGCCGTATCTC-3'
irf1b	ENSDARG00000032768	5'- CTCAGCCTGACCCCAAAACCTG-3'	5'- CCGCAGCCCTTGTTGATGCTT-3'
npsn	ENSDARG00000010423	5'-CCACACAGCTCCACGGGAAGTAG-3'	5'-GATAGTTCCTCTCTCCAGTATGAGGCACA-3'
mfap4	ENSDARG00000090783	5'-AATCGTGCTGTTCTTGGC-3'	5'-CATCCTCCGTTCTCTTCATC-3'
lgal3	ENSDARG00000077850	5'-ACAACATGGCAGTGATTCCA-3'	5'- GTCAAATCGCTGAGCATTCA-3'
myd88	ENSDARG00000010169	5'-GATAGTGATGCCTGTGATTTTCAGA-3'	5'- ACGGCCTCTTCATGGATTTGTA-3'
irak3	ENSDARG00000053131	5'-TGAGGTCTACTGTGGACGATGG-3'	5'-ATGTTAGGATGCTGGTTGAGTTGG-3'
nfkb	ENSDARG00000038687	5'-CACAGTCTCAGTCGAACTCACAAATA-3'	5'-GCTGTACTGTAATAAAGCACGAGCAT-3'
cxcl-c1c	ENSDARG00000075045	5'- TCAAGAGTGGGAATTCTGGG-3'	5'- AGTGATCCGGGTGTTTTCAG-3'
c3c	ENSDARG00000052207	5'- ACGGACAAGCCCATCTACAC-3'	5'- TGCCTTGTGGATTCGTGATA-3'
cfb	ENSDARG00000055278	5'-GTCCTCTCATTGCAGGTGCTCCAT-3'	5'-GCAGATAACTTCCATCCGAATAGCCA-3'
c4a	ENSDARG00000038424	5'-CCCTCCTTCCACCTCTACTCCTCCT-3'	5'-CATTGGCTGGCTTGGTAAGGACTAGA-3'

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

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Bacteria belonging to the *Burkholderia cepacia* complex form a versatile group of bacteria that have been found in many different ecological niches, ranging from soil, water, and the rhizosphere to industrial environments. Importantly, they are opportunistic pathogens, mainly of cystic fibrosis patients. These bacteria are notorious for individuals with CF, due the existence of highly transmissible strains, their inherent resistance to antibiotics, sudden unpredictable transitions from chronic to acute, sometimes rapidly fatal pneumonia, together leading to reduced life expectancy. *B. cenocepacia* has been responsible for different epidemic outbreaks in the CF community (Drevinek and Mahenthiralingam, 2010).

Bcc have a large genome (8 Mb) composed of three circular replicons and can adapt to many different conditions. The third chromosome has recently been described as a large virulence plasmid, pC3 that is important for stress tolerance *in vitro* and virulence (Agnoli et al., 2012). The Bcc are highly resistant to stress conditions, and express many factors that have been shown to be important for virulence in different experimental model systems. These factors include secretion system, quorum sensing, biofilm formation, and protease production (Loutet and Valvano, 2010). Importantly, research in different laboratories has shown that Bcc are able to survive and replicate intracellularly, showing they have acquired the ability to adapt to the harsh host environment, and evade host defence mechanisms.

To better understand the molecular basis of this often fatal host-pathogen interaction, and to identify new drug targets and antimicrobials, different *in vivo* models have been

developed: plants (including Alfalfa), nematodes (*Caenorhabditis elegans*), the larvae of the wax moth (*Gallaria mellonella*), Drosophila (Castonguay-Vanier et al., 2010), mammals (mice and rats) (Uehlinger et al., 2009) and more recently zebrafish (Vergunst et al., 2010; Mesureur and Vergunst, 2014 - **Chapter 2**). Uehlinger *et al* showed the importance to use multiple *in vivo* models to study bacterial virulence, because unlike *Pseudomonas aeruginosa*, *B. cenocepacia* has host-specific virulence mechanisms (Uehlinger et al., 2009). For example, the *aidA* mutant H111-A was greatly attenuated in *C. elegans* but not in other models which suggested a host-specific role for this gene in nematodes. The choice of infection model depends on the aim of the study and the advantages and inconveniences for each of them.

The zebrafish embryo model has been shown to be an exciting model to study the innate immune response and the intracellular bacterial stages in an *in vivo* context (Torraca et al., 2014). Zebrafish have counterparts of all the immune cells present in humans, and especially the optical transparency of the young embryos with a developing innate immune system permits non-invasive visualization that is more complicated in mammalian models.

We developed zebrafish embryos as a model to study Bcc infection, and found that different strains could induce persistent or acute infection (**Chapter 2,** Mesureur and Vergunst, 2014). This is in agreement with what has been described in other models, including mice (Bragonzi, 2010; Chung and Speert, 2007; Conway et al., 2004; Pirone et al., 2008). *B. stabilis* for instance persists in macrophages (Vergunst et al., 2010), however, it can suddenly cause more severe infection by 5 dpi in about 10% of the embryo, and it would be interesting to find out which factors determine these sudden transitions in the infected embryos, and to find out whether these transitions resemble transition to acute infection seen in CF patients.

In this study, we used the advantages of this model to better understand the role of host phagocytes, especially macrophages during *B. cenocepacia* infection (**Chapter 3**). In a second part zebrafish embryos were used to study the global host transcriptome by RNAseq during acute and persistent Bcc infection (**Chapter 4**).

Role of host phagocytes during Bcc infection

Macrophages are the first line of defence against pathogen invasion. Bcc have been shown to adopt an intracellular life style (Saldías and Valvano, 2009). Schwab et al (2014) identified *Bcc* bacteria inside macrophages in a clinical context and did not find bacteria in biofilm-like structures (Schwab et al., 2014), the paradigm of Bcc infection. Our results demonstrate that macrophages play a critical role in bacterial virulence and induction of fatal inflammatory infection in zebrafish embryos (**Chapter 3**). After intravenous injection and phagocytosis by macrophages, *B. cenocepacia* creates an intracellular niche that is crucial for bacterial replication, and the induction of the subsequent pro-inflammatory response. Generally, macrophages form an important protection against extracellular and also intracellular bacteria; without macrophages zebrafish embryos are more susceptible to infection with pathogens, including *Mycobacterium* and *Staphylococcus* (Clay et al., 2007; Prajsnar et al., 2008). In sharp contrast, after macrophage ablation using two different methods, we showed that macrophage-depleted embryos have a survival advantage when injected with highly virulent Bcc strains, compared to wild type control embryos. Intriguingly, all strains analysed survived extracellularly but were unable to replicate freely in the blood circulation during the first 24 hours of infection. These data unambiguously demonstrate the

importance of macrophages as a site for bacterial multiplication and development of a rapidly fatal inflammatory response caused by Bcc infection *in vivo*. Interestingly, a transcriptome study in a chronic lung infection model using rats indicated that genes involved in intracellular stages were up regulated suggesting an important intracellular life stage for *B. cenocepacia* (O'Grady et al, 2011).

In zebrafish embryos, bacteria, including *Salmonella,* are more virulent and can replicate much better in the absence of macrophages. The inability of the Bcc to replicate freely in the circulation after iv infection is remarkable. In control embryos, once the infection has progressed and bacteria have started to spread, they re-enter the blood circulation where they can then replicate. This suggests that maybe bacteria need an intracellular stage to change their transcriptome and be able to multiply in the blood circulation, or, inhibiting factors are present at early but not later stages of infection, or, the high inflammatory state allows the bacteria to replicate freely.

Neutrophils also play a crucial role in host defence, and it has been shown that healthy neutrophils can phagocytose Bcc and reduce bacterial numbers, depending on the bacterial strain *in vitro* (Porter and Goldberg, 2011; Speert et al., 1994). In this study, we show that neutrophils are also able to rapidly phagocytose surface-associated *B. cenocepacia* K56-2 and *B. stabilis* after subcutaneous injection. Thus, in zebrafish embryos, dependent on the site of injection we can study macrophage behaviour (after intravenous injection) or the neutrophil behaviour (after subcutaneous injection) in more detail. Although bacterial numbers visibly reduced at the infection site, more so for *B. stabilis* than for *B. cenocepacia*, neutrophils were unable to efficiently kill the bacteria. Intriguingly, within one hour after phagocytosis of subcutaneously injected *B. stabilis,* neutrophils projected their cell contents

in the environment in a process resembling NETosis (Brinkmann and Zychlinsky, 2012). Based on microscopic observations, it seems that Bcc can resist those NETs, which have been described as a new host immune response against microbes. This would be the first report describing NETosis *in vivo* during Bcc infection. In CF patients, lungs are filled with extracellular DNA originating from NET formation and could obstruct the airflow (Marcos et al., 2015). *Pseudomonas aeruginosa* infection induces macrophage migration-inhibitory factor (MIF)-dependent NET formation and exacerbation of inflammation (Yoo et al., 2014a, 2014b). NET formation increases the mucus viscosity that amplifies lung failure (Marcos et al., 2015; Zawrotniak and Rapala-Kozik, 2013). Further studies are needed to further confirm NETosis, and it would be interesting to study the mechanism of activation of NETosis during Bcc infection and how bacteria can resist to this.

We also showed that neutrophils were able to resolve inflammation during *B. stabilis*, but not *B. cenocepacia* infection, in a NADPH oxidase-dependent manner. However, the bacteria persisted, also in macrophages, showing again an important role for macrophages, also in neutrophil dominated phagocytosis. *B. cenocepacia* induced an inflammatory response, and the host was unable to resolve the inflammation, even in the absence of a functional NADPH oxidase. This again shows the highly virulent character of these bacteria and their ability to escape macrophage and neutrophil immune functions.

Burkholderia cenocepacia induces strong inflammation and neutrophil infiltration in the lungs of CF patients that contribute to chronic infection. We found that *B. cenocepacia* K56-2 induces a strong up-regulation of pro-inflammatory cytokine genes, *cxcl8* (or IL-8)*, il1b* and

tnfa, in zebrafish embryos, in contrast to *B. stabilis.* Acute infection caused rapid up-regulation (2 hpi) that remained high even at 24hpi. The up-regulation of *cxcl8* (*IL-8*) expression is correlated with neutrophil recruitment and degranulation around infected cells once the bacteria have replicated intracellularly. In the absence of *cxcl8*, neutrophil recruitment was delayed, and zebrafish embryos lived longer, suggesting that neutrophils have an important role in the pro-inflammatory character, also in zebrafish. Degranulation seen at the site of heavily infected macrophages might help bacteria to escape, although at this time we do not know the precise mechanism for bacterial escape from infected cells.

Induction of *cxcl8* could also have a direct effect on Bcc since Kaza *et al* showed that the concentration of IL-8 can influence bacterial multiplication (Kaza et al., 2011). Low concentrations (<10 ng/ml) of IL-8 enhanced extracellular growth of *Burkholderia* and high concentrations (10 ng/ml) of IL-8 reduced bacterial growth. In contrast, high extracellular concentrations of IL-8 enhanced intracellular growth and bacterial survival in CFTR-positive and CFTR-negative bronchial epithelial cells, and low concentrations reduced intracellular growth (Kaza et al., 2011). In our study we observed reduced bacterial replication in *cxcl8* knock down experiments. This reduction was observed during the early stages of the infection (6 to 8 hpi) when bacteria are intracellular (data not shown), suggesting a delay in the onset of bacterial intracellular replication. The results that blocking Cxcl8 results in delayed intracellular bacterial growth are in line with the results described by Kaza *et al*. It would be interesting to block or overexpress *cxcl8* in macrophage-ablated embryos to see the effect of Cxcl8 on extracellular growth *in vivo.*

The TLR signalling pathway is one of the major pathways to induce the innate immune response, specifically pro-inflammatory cytokine production, essentially through the MyD88

adaptor protein. Van der Vaart *et al* showed that *myd88* mutant zebrafish embryos were more susceptible to infection caused by *E. tarda* and *S.* Typhimurium (van der Vaart et al., 2013). In contrast to these pathogens, we found that *B. cenocepacia*-infected *myd88* mutants have a pro-survival advantage. Our result is in perfect agreement with results obtained by Ventura et al (2009), who showed that immunodeficient mice lacking Myd88 were protected against *B. cenocepacia* challenge (Ventura et al., 2009). Unexpectedly, bacterial replication was enhanced in both studies in the *myd88* mutant, indicating a Myd88-dependent inhibition of bacterial replication in wild type embryos. This also suggests that a Myd88-dependent signalling cascade and not bacterial numbers per se, contributes significantly to fatal inflammation. I found that glucocorticoid treatment of infected embryos slightly enhanced embryo survival and reduced tissue damage and inflammation (not shown). This suggests that excessive inflammation is key to the progression of Bcc infection and the cause of embryo death. In addition we found a Myd88-dependent induction of pro-inflammatory cytokine gene expression during the early intracellular stages (up to 6 hpi), whereas later stages of intracellular replication correlated with a Myd88-independent pro-inflammatory response. The early stage inside macrophages is Myd88-dependent and it affects the infection outcome. It is an important step for Bcc to induce fatal infection. Therefore, a better understanding of all bacterial factors that might play a role in the induction of this pro-inflammatory response could permit to develop new drugs against these factors and help the host to control the inflammation.

Host gene expression during acute and persistent Bcc infection

Until now, only one study looked at the host transcriptome during Bcc infection. Mariappan *et al* found that genes related to apoptosis, inflammation, cell cycle or other metabolic pathways were modulated by *B. cepacia* infecting human epithelial cell line A59 (Mariappan et al., 2013). We showed that Bcc infection has an impact on phagocyte activity and induced up-regulation of pro-inflammatory cytokine genes. In order to have a better idea of the impact of Bcc infection on global host gene expression, we performed a transcriptomic analysis using RNAseq during acute and persistent infection. Statistical analysis allowed distinction of two different host transcriptome profiles. Acute infection induced by *B. cenocepacia* K56-2 caused high modulation of the host transcriptome in contrast to persistent infection induced by *B. stabilis* LMG14294. This is in correlation with the different infection profiles observed in zebrafish embryos. During acute infection, the innate immune response was highly up-regulated and increased over time, with more genes modulated at 24 hpi than 3-4 hpi. The TLR pathway, as expected, was rapidly up-regulated. The NOD-like signalling pathway and MAPK signalling pathway were also modulated during infection. These data are in agreement with *B. cenocepacia* K56-2 being an inflammatory pathogen and inducing a massive inflammation that becomes fatal for the host. Furthermore, at 24 hpi the apoptosis pathway was up-regulated. Apoptosis could be activated through TNFa receptor signalling; indeed we found that *tnfa* gene expression is up-regulated during infection. Upregulation of the apoptosis pathway is in line with our microscopical observations that showed a dramatic reduction in phagocyte number, confirmed by staining with the cell impermeable dye SYTOX Green that showed that the circulation of *B. cenocepacia* K56-2 infected embryos was loaded with extracellular DNA. At this point we do not know whether this massive cell death is caused directly by bacterial factors, or due to

the strong uncontrolled inflammation and excessive TNFa production. Macrophage death prevents proper resolution of inflammation by efferocytosis of apoptotic neutrophils, and cell contents, including elastases and gelatinase B (or MMP-9) are released inside in the host. Wright *et al* showed that the activation of MMP-9 in human lung epithelial cells is responsible for a delay in wound repair and increased the inflammatory phenomenon (Wright et al., 2011). *Mmp9* is one of the major up regulated genes found in our study, and *B. cenocepacia* K56-2 infection is correlates with massive tissues damage. Thus, also in zebrafish, Mmp9 may play an important role in fatal inflammation found during acute infection.

In contrast to *B cenocepacia*, *B. stabilis* induced just a small host transcriptome modulation that was stable during the infection course. This was in agreement with the observed persistent phenotype with *B. stabilis* surviving and sometimes replicating inside macrophages, but without major pro-inflammatory signs. Interestingly, the complement system is the only innate immune system component modulated during persistent infection. The alternative pathway is quickly up-regulated. It would be interesting to study in more detail why *B. stabilis* induces the expression of just few host genes, and to focus on genes specifically expressed during persistent infection.

Concluding remarks and perspectives

The zebrafish is a powerful model to study host-pathogen interaction and innate immune response. In this work, we showed the critical role played by macrophages to induce fatal infection by Bcc; based on our observations, and results obtained in cell culture and clinical studies, we propose that paradigm changing approaches are needed for development of new antimicrobials to disarm these intracellular pathogens. Our finding that MyD88-dependent inflammation matches the results found in mice further validate the zebrafish model to study Bcc infection. Also the global host transcriptome analysis permits to analyse the host response during infection in greater detail. Here, we focused on the immune response but this RNAseq experiment generated a lot of information about the host metabolism and general host activities that form a basis for future studies. To develop new therapies against *B. cenocepacia*, we propose that the intracellular stages and the ensuing inflammatory response are essential targets to explore. The zebrafish model will be a good platform to screen for drugs that will target the bacterial intracellular stages.

CHAPTER 6

RÉSUMÉ EN FRANÇAIS

RESUME EN FRANÇAIS

Le modèle animal du poisson zèbre (*Danio rerio*)

Au cours de la dernière décennie, un nouveau modèle animal vertébré non-mammifère, le poisson zèbre (*Danio rerio*), a émergé comme un modèle complémentaire puissant pour étudier les interactions hôte-pathogène et la réponse immunitaire de l'hôte lors d'infections microbiennes (Meijer et al, 2013; Sullivan et Kim, 2008; Torraca et al, 2014). Il a été montré comme étant un bon modèle pour l'étude des agents pathogènes infectant à la fois l'humain et le poisson, y compris *Mycobacterium marinum* (Davis et al., 2002), *Salmonella enterica* Typhimurium (Sar et al., 2003), *Listeria monocytogenes* (Levraud et al., 2009), *Staphylococcus aureus*, *Aeromonas salmonicida* et *Burkholderia cenocepacia* (Vergunst et al., 2010) pour les infections bactériennes (Lin et al, 2007); le virus du Chikungunya et le virus de la septicémie hémorragique virale (Encinas et al, 2010) pour les infections virales (Palha et al, 2013); *Candida albicans* et *Aspergillus fumigatus* (Knox et al., 2014) pour les infections fongiques (Chen et al., 2013); *Toxoplasma gondi* (Sanders et al., 2015) pour les infections parasitaires.

Des études ont montré que le système immunitaire du poisson zèbre est très similaire à celui des humains: la réponse immunitaire innée, comprenant la réponse inflammatoire, production de cytokine pro-inflammatoire, les Toll-Like Receptors (TLRs) et leur voie de signalisation (Meijer et al., 2004), le système du complément (Zhang et Cui, 2014) et les cellules phagocytaires (neutrophiles et les macrophages), sont présents dans les poissons dès le stade précoce (28-30 heures post fécondation) (Herbomel et al., 1999; Lieschke,

2001). Le système immunitaire adaptatif est fonctionnel 4-6 semaines après la fécondation (Lam et al., 2004). La séparation temporelle des réponses adaptatives et innées permet l'étude des infections dans les jeunes embryons avec juste la présence d'un système immunitaire inné. De nombreux outils génétiques ont été développés, tels que le knock-down de gènes utilisant les morpholinos (MO) et la stratégie d'ARN antisens, la surexpression de gènes utilisant l'injection d'ARN messager, la transgénèse avec le système de transposon Tol2, et la génération de mutants knock-out en utilisant les technologies CRISPR/CAS ou Talen (Heintze 2013 - Hwang 2013). Un atout majeur de ce modèle est le caractère transparent des embryons qui permet la visualisation en temps réel des interactions hôte-pathogène grâce à des poissons transgéniques rapporteurs et des bactéries fluorescentes. C'est un avantage majeur par rapport aux autres modèles animaux (Tobin et al., 2012). Bien que les anticorps spécifiques de poisson zèbre commencent à être disponibles en grand nombre, d'autres approches cellulaires et moléculaires utiles ont été développées (qRT-PCR, hybridation in situ, la fluorescence immunitaire) (Cui et al., 2011; Trede et al., 2004). La petite taille des embryons en fait un modèle parfait pour les études et le screening à haut débit, y compris le suivi de la progression d'une maladie, l'analyse du transcriptome par microarrays ou RNAseq, et le test et le criblage de nouveaux médicaments (Carvalho et al., 2011; Hegedus et al., 2009; Spaink et al., 2013; Stockhammer et al., 2009; Tan et Zon, 2011; Veneman et al., 2013; Yang et al., 2013).

Le complexe *Burkholderia cepacia* et son interaction avec le système immunitaire

Le complexe *Burkholderia cepacia* (Bcc) est un groupe d'agents pathogènes bactériens opportunistes, contenant maintenant 18 espèces : *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia*, *B. ubonensis*, *B. latens*, *B. diffusa, B. arboris*, *B. seminalis*, *B. metallica*, *B. contaminans*, *B. lata* et *B. uronensis* (Papaleo et al., 2010; Vanlaere et al., 2009). En 1950, Walter Burkholder décrit un agent pathogène de la plante, agent causal de la peau aigre dans les oignons, et a proposé de le nommer *Pseudomonas cepacia* (Burkholder W, 1950). Cet agent a été rebaptisé en 1992 en *B. cepacia* (Coenye et al, 2001). Les espèces du Bcc peuvent vivre dans diverses niches écologiques et peuvent être pathogènes pour les plantes (Jacobs et al., 2008), mais ~~ils~~ elles peuvent aussi être bénéfiques pour la croissance des plantes ou vivre en symbiose avec la canne à sucre par exemple (Govindarajan et al., 2006; Ramette et al., 2005). *B. cepacia* est également connue pour son utilisation dans les bio-remédiations des sols pollués (Chavan et Mukherji, 2008). Comme d'autres bactéries environnementales, les Bcc sont hautement résistantes aux antibiotiques et aux conditions de stress. Récemment, Agnoli *et al.* ont montré que le troisième chromosome pC3 est présent dans presque toutes les souches du Bcc et est important pour la virulence. Ils ont aussi suggéré qu'il est un grand plasmide de virulence plutôt qu'un chromosome, notamment du fait des différences au niveau de l'origine de réplication et de son système de maintien et de réplication. Il porte des gènes qui codent pour des facteurs qui augmentent la résistance bactérienne aux différents types de stress, y compris le stress oxydatif, le stress osmotique, la haute température et le stress induit par la chlorhexidine (Agnoli et al, 2013).

Pour classer les espèces Bcc, différentes méthodes ont été développées. Le polymorphisme de séquence dans la séquence du gène recA a permis de différencier la plupart des espèces (Mahenthiralingam et al., 2000). Le Multilocus Sequence Typing (MLST) a été basé sur le polymorphisme de sept gènes de ménage (atpD, LGBT, gyrB, recA, Lepa, l'ASPC et trpB) et permet une meilleure identification et la détermination d'un profil allélique qui peut être utilisé pour attribuer une séquence clonale type (ST) (Baldwin et al., 2005). basée sur le séquençage recA, *B. cenocepacia* (précédemment genomovar III) peut être divisée en quatre groupes, IIIA, IIIB, IIIC et IIID. Plus récemment, une autre technique a été utilisée pour différencier les espèces du Bcc, elle est basée sur l'analyse de la séquence du gène hisA (Papaleo et al., 2010).

***Burkholderia cenocepacia*, un pathogène opportuniste**

Chez les personnes immunodéprimées, principalement des patients atteints de mucoviscidose et de maladie granulomateuse chronique, les bactéries du Bcc peuvent causer des infections chroniques, avec des périodes d'exacerbation. L'infection par des bactéries appartenant au Bcc est inquiétante, car elles peuvent provoquer une infection aiguë soudaine et imprévisible entraînant d'une pneumonie nécrosante souvent fatale, parfois associée à une septicémie, connue sous le nom de «syndrome cepacia». La plupart des membres du Bcc ont été isolés à partir de patients atteints de mucoviscidose et de l'environnement, sauf *B. latens* et *B. metallica* qui ont été détectées seulement chez des patients (Vial et al., 2011). Plusieurs souches hautement transmissibles ont provoqué des épidémies importantes dans les populations canadiennes et européennes de personnes atteintes de mucoviscidose (Drevinek et Mahenthiralingam, 2010). La plupart des isolats de

B. cenocepacia provenant de patients appartiennent aux genomovars IIIA et IIIB, et comprennent les grandes lignées épidémiques. Une des lignées de *B. cenocepacia*, identifiée comme étant de type électrophorétique 12 (ET-12), ~~et~~ a causé des ravages parmi les patients au Royaume-Uni et au Canada. Deux isolats clonaux et hautement virulents du ET-12, J2315 et K56-2 sont souvent utilisés pour étudier l'infection par Bcc et la virulence bactérienne. *B. cenocepacia* et *B. multivorans* sont les espèces les plus répandues et isolées chez les patients en Europe et en Amérique du Nord (Drevinek et Mahenthiralingam, 2010). Les bactéries du Bcc sont très résistantes aux peptides antimicrobiens produits par les cellules immunitaires telles que les neutrophiles et ont une résistance intrinsèque multiple aux médicaments, ce qui provoque des problèmes majeurs pour le traitement des patients (Kooi et Sokol, 2009; Loutet et Valvano, 2010). Actuellement, les éléments qui provoquent le passage d'une maladie chronique à une infection aiguë, parfois mortelles, sont inconnus.

Des modèles différents sont utilisés pour mieux comprendre les facteurs de virulence bactériens, voies de signalisation immunitaire, et les stratégies de survie intracellulaires afin de trouver de nouvelles cibles médicamenteuses et des traitements antimicrobiens.

Le poisson zèbre comme modèle d'infection pour étudier le Bcc

Nous avons développé l'embryon de poisson zèbre comme modèle pour étudier l'infection causée par le Bcc, et on a constaté que différentes souches pouvaient provoquer une infection persistante ou aiguë (Vergunst et al., 2010 ; Mesureur et Vergunst, 2014/**Chapter 2**). Ceci est en accord avec ce qui a été décrit dans d'autres modèles, y compris les souris (Bragonzi, 2010; Chung et Speert, 2007; Conway et al., 2004; Pirone et al., 2008). *B. stabilis* par exemple persiste dans les macrophages (Vergunst et al., 2010), et peut soudainement

causer une infection plus sévère 5 jours post infection dans environ 10% des embryons. Il serait intéressant de savoir quels facteurs déterminent cette soudaine transition dans les embryons infectés, et de savoir si cette transition ressemble à celle observée chez les patients atteints de mucoviscidose.

Dans cette étude, nous avons utilisé les avantages de ce modèle pour mieux comprendre le rôle des phagocytes de l'hôte, en particulier les macrophages lors de l'infection causée par *B. cenocepacia*. Dans une deuxième partie, les embryons de poisson zèbre ont été utilisés pour étudier le transcriptome de l'hôte par RNAseq au cours d'une infection aiguë et persistante causée par *B. cenocepacia* et *B. stabilis*.

Rôle des phagocytes lors de l'infection par les bactéries appartenant au Bcc

Les macrophages sont la première ligne de défense contre l'invasion de pathogènes. Il a été montré que les bactéries du Bcc pouvaient avoir un style de vie intracellulaire (Saldías et Valvano, 2009). Schwab et al. (2014) ont identifié des bactéries du Bcc dans les macrophages dans un contexte clinique et n'ont pas trouvé de bactéries dans le biofilm (Schwab et al., 2014), le paradigme de l'infection par le Bcc. Nos résultats démontrent que les macrophages jouent un rôle crucial dans la virulence bactérienne et l'induction d'une infection inflammatoire fatale dans les embryons de poisson zèbre. Après injection par voie intraveineuse et la phagocytose par les macrophages, *B. cenocepacia* crée une niche intracellulaire qui est déterminante pour la réplication bactérienne, et l'induction de la réponse pro-inflammatoire. Ceci suggère que les macrophages peuvent jouer un rôle plus important durant l'infection que ce qui est généralement admis. En général, les macrophages constituent une protection importante contre les bactéries intracellulaires et

extracellulaires ; sans macrophages, les embryons de poisson zèbre sont plus sensibles aux infections par des agents pathogènes, y compris *Mycobacterium* et *Staphylococcus* (Clay et al., 2007; Prajsnar et al., 2008). À l'opposé, après l'ablation des macrophages en utilisant deux méthodes différentes, nous avons montré que les embryons dépourvus de macrophages ont un avantage de survie par rapport aux embryons contrôles sauvages lorsqu'ils sont infectés avec des souches très virulentes du Bcc (**Chapter 3**). Curieusement, toutes les souches analysées ont survécu mais étaient incapables de se répliquer librement dans la circulation sanguine au cours des 24 premières heures de l'infection. Ces données démontrent clairement l'importance de macrophages comme un site pour la multiplication bactérienne et le développement d'une réponse inflammatoire rapide et mortelle causée par une infection par le Bcc dans des conditions *in vivo*. Fait intéressant, une étude du transcriptome chez les rats a indiqué que les gènes impliqués dans le stade intracellulaire ont été surexprimés, suggérant que l'étape intracellulaire de la vie de *B. cenocepacia* (O'Grady et al., 2011) est importante .

Les bactéries *Salmonella* sont plus virulentes et peuvent mieux se répliquer en l'absence de macrophages. L'incapacité du Bcc à se multiplier librement dans la circulation après injection intraveineuse est remarquable. Dans les embryons contrôles, une fois que l'infection a progressé et que les bactéries ont commencé à se répandre, elles retournent dans la circulation sanguine où elles peuvent ensuite se répliquer. Cela laisse penser que peut-être les bactéries ont besoin d'une étape intracellulaire pour changer leur transcriptome et être capables de se multiplier dans la circulation sanguine ; ou bien des facteurs inhibeurs sont présents au début de l'infection, mais plus dans les stades tardifs de l'infection, ou l'état inflammatoire élevé permet aux bactéries de se reproduire librement.

Les neutrophiles jouent également un rôle crucial dans la défense de l'hôte, et il a été montré que les neutrophiles en bonne santé peuvent phagocyter les bactéries du Bcc et réduire le nombre de bactéries *in vitro*, en fonction de la souche bactérienne (Porter et Goldberg, 2011; Speert et al., 1994). Dans cette étude (**Chapitre 3**), nous montrons que les neutrophiles sont également capables de phagocyter rapidement *B. cenocepacia* K56-2 et *B. stabilis* quand ils sont associés à une surface après l'injection sous-cutanée. Ainsi, en fonction du site d'injection, nous pouvons étudier plus en détail le comportement des macrophages (après injection intraveineuse) ou le comportement des neutrophiles (après l'injection sous-cutanée). Bien que le nombre de bactéries semble visiblement réduit au niveau du site d'injection, plus pour *B. stabilis* que pour *B. cenocepacia*, les neutrophiles ont été incapables de tuer efficacement les bactéries. Curieusement, une heure après la phagocytose de *B. stabilis*, les neutrophiles projettent leur contenu cellulaire dans l'environnement dans un processus ressemblant à la NETosis (Brinkmann et Zychlinsky, 2012). Ce serait le premier rapport décrivant la NETosis *in vivo* lors de l'infection par le Bcc. Chez les patients atteints de mucoviscidose, les poumons sont pleins de l'ADN extracellulaire provenant de la formation de NET et peut obstruer le flux d'air (Marcos et al., 2015). L'infection à *Pseudomonas aeruginosa* induit la migration des facteurs inhibiteurs de macrophages (MIF) dépendant de la formation NET et de l'exacerbation de l'inflammation (Yoo et al., 2014A, 2014b). La formation de NET augmente la viscosité du mucus qui amplifie une insuffisance pulmonaire (Marcos et al., 2015; Zawrotniak et Rapala-Kozik, 2013). D'autres études sont nécessaires pour confirmer la NETosis, et il serait intéressant de savoir si la NETosis pourrait avoir un rôle dans le contrôle de l'infection par le Bcc et comment les

bactéries du Bcc sont capables de rersister à cette réponse immune innée récemment découverte.

Nous avons également montré que les neutrophiles sont en mesure de résoudre l'inflammation au cours de l'infection par *B. stabilis*, mais pas celle causée par *B. cenocepacia*, grâce au complexe NADPH oxydase. *B. cenocepacia* induit une réponse inflammatoire, et l'hôte était incapable de résoudre l'inflammation, même en l'absence d'une NADPH oxydase fonctionnelle. Cela montre encore une fois le caractère hautement virulent de ces bactéries et leur capacité à échapper aux fonctions immunitaires des macrophages et des neutrophiles.

Burkholderia cenocepacia induit une forte inflammation et l'infiltration de neutrophiles dans les poumons des patients atteints de mucoviscidose, ce qui contribue à l'infection chronique. Nous avons constaté que B. *cenocepacia* K56-2 induit une forte sur-régulation des gènes codant pour les cytokines pro-inflammatoires, Cxcl8 (ou IL-8), Tnfa, et Il1b dans les embryons de poisson zèbre, contrairement à *B. stabilis*. L'infection aiguë cause une sur-régulation rapide (2 hpi) qui est restée élevée même à 24hpi. La sur-régulation de l'expression de Cxcl8 est en corrélation avec le recrutement et la dégranulation des neutrophiles vers les cellules infectées une fois que les bactéries se sont répliquées intracellulairement. En l'absence de CxclL8, le recrutement de neutrophiles a été retardé, et les embryons de poisson zèbre vivent plus longtemps, ce qui suggère que les neutrophiles ont un rôle également important chez le poisson zèbre dans le caractère pro-inflammatoire. La dégranulation autour des macrophages fortement infectés pourrait aider les bactéries à

s'échapper, même si en ce moment le mécanisme précis d'évasion des bactéries à partir de cellules infectées n'est pas connu.

L'induction de Cxcl8 chez les poissons pourrait également avoir un effet direct sur les bactéries depuis que Kaza et al. ont montré que la concentration de l'IL-8 peut influencer la multiplication bactérienne (Kaza et al., 2011). De faibles concentrations à l'intérieur de la cellule (<10 ng / ml) d'IL-8 améliorent la croissance extracellulaire de *Burkholderia* et de fortes concentrations (10 ng / ml) d'IL-8 réduisent la croissance bactérienne. En revanche, les concentrations extracellulaires élevées d'IL-8 améliorent la croissance intracellulaire et la survie des bactéries dans les cellules épithéliales bronchiques CFTR-positives et négatives, et de faibles concentrations réduisent la croissance intracellulaire (Kaza et al, 2011). Dans notre étude, nous avons observé une petite réduction de la réplication bactérienne dans les expériences où Cxcl8 est bloquée. Cette diminution a été observée au cours de la phase précoce de l'infection (6 à 8 hpi) lorsque les bactéries sont intracellulaires, ce qui suggère un retard dans le début de la réplication bactérienne intracellulaire (résultats préliminaires non montrés). Ces résultats de l'influence du blocage de CXCL8 sur la croissance bactérienne intracellulaire sont cohérents avec les résultats décrits par Kaza et al. Il serait intéressant de bloquer ou sur-exprimer Cxcl8 dans des embryons dépourvus de macrophages pour voir l'effet *in vivo* de Cxcl8 sur la croissance extracellulaire.

La voie de signalisation des TLR est l'une des principales voies pour induire la réponse immunitaire innée, en particulier la production de cytokines pro-inflammatoires, essentiellement grâce à la protéine adaptateur MyD88. Van der Vaart *et al.* ont montré que les embryons de poisson zèbre mutant pour MyD88 étaient plus sensibles à l'infection causée par *E. tarda* et *S.* Typhimurium (Van der Vaart et al., 2013). Contrairement à ces

agents pathogènes, nous avons constaté que les mutants MyD88 infectés par *B. cenocepacia* survivent plus longtemps que les contrôles sauvages. Notre résultat est en parfait accord avec les résultats obtenus par Ventura et al. (2009), qui ont montré que les souris immunodéficientes pour Myd88 étaient protégés contre *B. cenocepacia* (Ventura et al., 2009). De façon inattendue, la réplication bactérienne a été améliorée dans les deux études avec les mutants MyD88, ce qui indique une inhibition de la réplication bactérienne dépendante de MyD88 dans les embryons de type sauvage. Ces données en outre valident l'embryon de poisson zèbre comme modèle pour étudier le rôle de la réponse immunitaire innée lors d'infections causées par le Bcc. Nous avons aussi montré une induction MyD88-dépendante de l'expression des cytokines pro-inflammatoires au cours des étapes intracellulaires précoces de l'infection (jusqu'à 6 hpi), tandis que les derniers stades de la réplication intracellulaire sont corrélés avec une réponse pro-inflammatoire MyD88-indépendant. Par conséquent, une meilleure compréhension de tous les facteurs bactériens qui pourraient jouer un rôle dans l'induction de cette réponse pro-inflammatoire pourrait permettre de développer de nouveaux médicaments contre ces facteurs et aider l'hôte à contrôler l'inflammation.

Expression des gènes de l'hôte lors de l'infection aiguë et persistante causée par *B. cenocepacia* et *B. stabilis*

Jusqu'à présent, une seule étude a porté sur le transcriptome de l'hôte lors de l'infection par le Bcc. Mariappan *et al* ont montré que les gènes liés à l'apoptose, l'inflammation, le cycle cellulaire et des voies métaboliques ont été modulés par *B. cepacia* infectant la lignée cellulaire épithéliale humaine A59 (Mariappan et al., 2013). Nous avons montré que

l'infection par le Bcc a un impact sur l'activité phagocytaire et induit une régulation des gènes de cytokines pro-inflammatoires. Afin d'avoir une meilleure idée de l'impact de l'infection par le Bcc sur l'expression génique globale de l'hôte, nous avons effectué une analyse transcriptomique utilisant le RNAseq au cours d'une infection aiguë et persistante (**Chapitre 4**). L'analyse statistique a permis la distinction de deux profils différents de transcriptome. L'infection aiguë induite par *B. cenocepacia* K56-2 cause une forte modulation du transcriptome de l'hôte contrairement à une infection persistante induite par *B. stabilis* LMG14294. Ceci est en corrélation avec les différents profils d'infection observés dans les embryons de poisson zèbre. Lors d'une infection aiguë, la réponse immunitaire innée est hautement sur-régulée et augmente au fil du temps, avec plus de gènes modulés à 24 hpi qu'à 3-4 hpi. La voie TLR, comme prévu, a été rapidement sur-régulée. La voie de signalisation NOD-like et la voie de signalisation MAPK ont également été modulés lors de l'infection. Ces données sont en accord avec le fait que *B. cenocepacia* K56-2 est un agent pathogène inflammatoire qui induit une inflammation massive fatale pour l'hôte. En outre, à 24 hpi, la voie de l'apoptose a été surexprimée. L'apoptose pourrait être activée par la signalisation via le récepteur du TNFa. En effet, nous avons constaté que le TNFa est surexprimé au cours de l'infection. La régulation positive de la voie de l'apoptose est en accord avec nos observations microscopiques qui ont montré une réduction spectaculaire du nombre de phagocytes, confirmée par un marquage de viabilité au SYTOX green qui a montré que la circulation des embryons infectés par *B. cenocepacia* K56-2 était chargée de l'ADN extracellulaire. À ce stade, nous ne savons pas si cette mort cellulaire massive est causée directement par des facteurs bactériens, et ou à cause de la forte inflammation incontrôlée et une production excessive de TNFa. La mort des macrophages empêche une

bonne résolution de l'inflammation par l'efferocytose de neutrophiles apoptotiques, et le contenu des cellules, y compris les élastases et gélatinase B (ou MMP-9) sont libérés à l'intérieur dans l'hôte. Wright *et al.* a montré que l'activation de MMP-9 dans des cellules épithéliales de poumon humain est responsable d'un retard dans la cicatrisation des plaies et augmente le phénomène inflammatoire (Wright et al., 2011). Mmp9 est l'un des principaux gènes surexprimés trouvés dans notre étude, et l'infection par *B. cenocepacia* K56-2 est corrélée avec des dommages de tissu massifs. Ainsi, également chez le poisson zèbre, Mmp9 peut jouer un rôle important dans l'inflammation mortelle lors d'une infection aiguë.

Contrairement à *B. cenocepacia*, *B. stabilis* induit juste une petite modulation du transcriptome de l'hôte qui est donc plus stable au cours de l'infection. C'est également en accord avec le phénotype persistant observé avec *B. stabilis*. Fait intéressant, le système du complément est le seul composant du système immunitaire inné surexprimé pendant l'infection persistante. La voie alternative est rapidement sur-régulée et trois composants du complément (BFC, C3a et C3b) qui forment le complexe d'attaque membranaire sont également surexprimés. Il pourrait être intéressant d'étudier plus en détail pourquoi *B. stabilis* induit seulement quelques gènes et se concentrer sur les gènes exprimés spécifiquement lors de l'infection persistante.

Remarques et perspectives

Le poisson zèbre est un modèle puissant pour étudier l'interaction hôte-pathogène et la réponse immunitaire innée. Dans ce travail, nous avons montré le rôle essentiel joué par les macrophages pour induire une infection mortelle par les bactéries appartenant au Bcc; sur la

base de nos observations, et les résultats obtenus en culture cellulaire et les études cliniques, nous proposons qu'une évolution des approches de ce paradigme est nécessaire pour le développement de nouveaux antimicrobiens afin de lutter contre ces pathogènes intracellulaires. Notre recherche sur l'inflammation MyD88-dépendante correspond aux résultats trouvés dans les souris, ce qui valide davantage le modèle de poisson zèbre pour étudier l'infection par le Bcc. L'analyse globale du transcriptome de l'hôte permet également d'analyser la réponse de l'hôte lors de l'infection plus en détail. Ici, nous nous sommes concentrés sur la réponse immunitaire, mais cette expérience de RNAseq a généré beaucoup d'informations sur le métabolisme de l'hôte et les activités générales de l'hôte, ce qui forme une bonne base pour de futures études. Pour développer de nouvelles thérapies contre *B. cenocepacia*, nous proposons que les stades intracellulaires et la réponse inflammatoire qui s'ensuit soient des cibles essentielles à explorer. Le modèle de poisson zèbre sera une bonne plate-forme pour le criblage de médicaments qui cibleront le stade intracellulaire des bactéries.

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Résumé en français

Les bactéries appartenant au complexe *Burkholderia cepacia* (Bcc) provoquent des infections sévères chez les personnes atteintes de mucoviscidose. L'infection peut varier d'une forme asymptomatique à une forme plus aiguë pouvant entraîner une pneumonie nécrosante et une septicémie, connue sous le nom de syndrome *cepacia*. Afin d'étudier les infections causées par le Bcc, nous avons développé un nouveau modèle *in vivo*, l'embryon de poisson zèbre. Nous avons montré que *B. cenocepacia* K56-2 pouvait se répliquer dans les macrophages et causer une infection aiguë mortelle pour les embryons. En revanche, *B. stabilis* LMG14294 induit une infection persistante chez les embryons. Dans cette étude, nous avons montré que les macrophages jouaient un rôle-clé dans la multiplication de K56-2 et dans l'induction d'une réponse inflammatoire MyD88-dépendante, caractérisée par la surexpression des gènes codant pour Cxcl8 (ou IL-8) et l'IL-1b. En l'absence de macrophages, les bactéries sont incapables de se multiplier durant les premières 24h de l'infection, ce qui donne un avantage pour la survie des embryons. L'absence de MyD88 induit aussi l'augmentation de la survie des embryons infectés par K56-2. Mais de manière paradoxale, les bactéries se multiplient mieux chez les embryons myd88$^{-/-}$ mutants que chez les embryons sauvages. Ceci suggère que ce n'est pas le nombre de bactéries qui est important pour l'infection, mais que c'est la réponse inflammatoire excessive causée par cette infection qui entraîne la mort des embryons. Afin d'avoir une vision globale des changements d'expression des gènes de l'hôte durant l'infection, nous avons effectué une expérience de RNAseq. Comme attendu, l'infection aiguë se caractérise par une importante modulation du transcriptome de l'hôte qui augmente avec le temps. A l'opposé, l'infection persistante n'induit que très peu de changements. La réponse immunitaire innée, et en particulier la voie des TLR, ainsi que l'apoptose sont très fortement activées durant une infection aiguë. Pour sa part, *B. stabilis* module essentiellement les gènes codant pour le système du complément.

Le rôle critique des macrophages lors d'une infection par Bcc chez les poissons zèbre est en accord avec les récentes observations cliniques. Ceci suggère que le stade intracellulaire de *B. cenocepacia* et la réponse inflammatoire qui s'ensuit peuvent être des cibles pour le développement de nouvelles thérapies permettant de lutter contre cette infection.

Mots clés : *Burkholderia cenocepacia*, Poisson zèbre, mucoviscidose, interaction hôte-pathogène

Abstract

Bacteria belonging to the *Burkholderia cepacia* complex (Bcc) can cause chronic infection with periods of acute exacerbation and sometimes fatal necrotizing pneumonia ("cepacia syndrome") in individuals with cystic fibrosis (CF), and are associated with poor prognosis. Here, we exploited the exciting possibilities for *in vivo* non-invasive imaging of Bcc infection in transparent zebrafish embryos, with an innate immune system with remarkable similarity to that of humans, and numerous genetic and genomic tools to study the role of host phagocytes and the innate immune response in the pro-inflammatory character of the infection.

We show that macrophages play a critical role in intracellular multiplication of *B. cenocepacia* K56-2 and induction of a MyD88-dependent fatal inflammatory response, characterised by high levels of *cxcl8* and *il1b* expression. Surprisingly, in sharp contrast to the situation found for infections with other pathogens including *Mycobacterium marinum* and *Staphylococcus aureus,* in the absence of macrophages, K56-2 survived but was unable to replicate in the first 24 h, which resulted in a significant pro-survival advantage to the host compared to wild type embryos that died within 2 to 3 days. The Toll-like receptor (TLR) pathway is a major arm of the cell-mediated innate immune response with MyD88 as a key adaptor protein involved in the production of pro-inflammatory cytokines. We found that the absence of MyD88 also provided a pro-survival effect to the embryos after infection with K56-2. Paradoxically, the bacteria replicated better in *myd88*$^{-/-}$ mutant than wild type embryos, suggesting that it is not bacterial burden per se, but the inflammatory response that kills the embryos. Interestingly, *cxcl8* and *il1b* expression were not significantly induced during the first 7 hours in the *myd88*$^{-/-}$ mutant while a strong induction was seen in control embryos, suggesting that a Myd88-dependent inflammatory response during early macrophage stages significantly contributes to fatal infection.

Next, we performed RNAseq to analyse global changes in host gene expression during acute and persistent infection induced by K56-2 and *B. stabilis* LMG14294 respectively. Whereas acute infection was characterised by strong modulation of host gene expression increasing over time, persistent infection showed modulation of only a small set of genes. TLR and apoptosis signaling pathways were amongst the strongly activated groups during acute infection, in line with the strong inflammatory character of *K56-2*. During persistent infection, the major differentially expressed gene set concerned genes encoding complement proteins.

The critical role for macrophages in Bcc infection in zebrafish is in agreement with recent clinical observations. We suggest that the intracellular stages of *B. cenocepacia* and the ensuing inflammatory response are essential targets to explore for the development of new therapies to combat this infection.

Key words: *Burkholderia cenocepacia*, zebrafish embryo, cystic fibrosis, host-pathogen interaction