Multi-nucleated glial cells and the implication for neural health

Multi-nucleated glial cells and the implication for neural health

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Dedicated to my parents who gave me life, love and endless support.

Abstract

The term glioma encompasses all tumours that originate from glial cells and accounts for almost 80% of primary malignant brain tumours. Gliomas typically present as a heterogeneous mass of cells that include the presence of multi-nucleated cells. Infectious agents are a significant risk factor in gliomagenesis and brain cancer, particularly those agents that are known to cause multi-nucleation of cells. To date, the infectious agents that have been implicated in gliomagenesis include viruses, protozoans, and possibly *Brucella* species. However, there is currently no evidence of bacteria directly causing multi-nucleation of glia resulting in gliomagenesis. The bacteria *Burkholderia pseudomallei* and *Neisseria meningitidis*, the causative agents of melioidosis and meningitis respectively can penetrate the central nervous system (CNS) via infection of the olfactory and trigeminal nerves. Importantly, the penetration of the CNS by *B. pseudomallei* and *N. meningitidis* can occur at very low bacterial levels such that CNS infection can be asymptomatic, and the bacteria can persist within the body for many years or even decades. The effect of long-term presence of subclinical levels of the bacteria within the CNS is unknown and *B. pseudomallei* and *N. meningitidis* infections have not yet been implicated in gliomagenesis. The *in vitro* work in our laboratory has determined that *B. pseudomallei* rapidly initiates the formation of multinucleated glial cells, including olfactory ensheathing cells (OECs), Schwann cells and astrocytes. These results suggest that the bacteria may initiate a cascade of events leading to multi-nucleation of glial cells; as multi-nucleated cells are a pathology that is a characteristic of glioma it raises the question of whether the glia cells exhibit other characteristics associated with inflammation and/or glioma. The goal of this thesis is to characterise the cellular and molecular events resulting from bacterial initiation of multi-nucleated glial cells.

The project consists of three aims. The first aim is the study of multinucleated cell (MNC) formation in Schwann cells after infection with *B. pseudomallei* and *N. meningitidis* serogroup B *in vitro*. The second aim is to determine the changes in the expression of molecules associated with gliomagenesis in multinucleated glial cells. The third aim is to study the energy production in Schwann cells following the infection with *B. pseudomallei*.

Trigeminal Schwann cells were infected with *B. pseudomallei* and *N. meningitidis* serogroup B in various ratios and formats and examined for formation of MNCs. The immunofluorescence microscopy results showed that Schwann cells formed MNCs following the infection with both bacteria. The results also demonstrated that infection with both bacteria is associated with the expression of proteins and markers associated with inflammation and glioma. Immunoblotting, proteomics and qRT-PCR results showed that the expression of inflammatory and gliomagenesis

markers were upregulated significantly after the infection with *B. pseudomallei* and *N. meningitidis* serogroup B. These results suggest that the bacterial infection of glia leads to responses consistent with inflammation and/or initiation of gliomagenesis. We also demonstrated that *B. pseudomallei* infection can mis-regulate the expression of Warburg effect genes in favour of aerobic glycolysis energy production which is a characteristic feature of carcinogenesis.

In conclusion, in this study we showed that infection of glia with facultative intracellular bacteria such as *B. pseudomallei* and *N. meningitidis* results in molecular and cellular changes consistent with inflammatory responses and potentially associated with glioma.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Seyed Ali Delbaz

Table of Contents

Index of Tables

Index of Figures

List of Abbreviations

B.pseudomallei	Burkholderia pseudomallei
BBB	Blood brain barrier
BimA	Burkholderia intracellular motility A
CifBP	Cell-cycle inhibitory factor
CNS	Central nervous system
Cox-2	Cyclooxygenase 2
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
EMT	Epithelial mesenchymal transition
FinC	FibronectinC
GLUT-1	Glucose transporter1
HIF-1	Hypoxia inducible factor
IARC	International Agency for Research on Cancer
IL-6R	Interleukin 6 receptor
LDH-A	Lactate dehydrogenase A
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MIF	Macrophage migration inhibitory factor
MMP-2	Matrix-metalloproteinase 2
MNC	Multinucleated cell
N.meningitidis	*Neisseria meningitidis*
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OEC	Olfactory ensheathing cell
PAI-2	Plasminogen Activator Inhibitor, Type II
PAMPs	Pathogen-associated molecular patterns
PDH-A	pyruvate dehydrogenase A
PGAM1	Phosphoglycerate mutase 1
PNS	peripheral nervous system
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
SWATH-Proteomics	Sequential window acquisition of all
T6SS	Type VI secretion system
TLR-4	Toll-like receptor
VINC	Vinculin
VPs35	Vacuolar protein sorting-associated protein

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Publications arising from this thesis

1) Ali Delbaz, Johana Tello Velasquez, James St John, Michael Batzloff, Jenny Ekberg. Infection with *Burkholderia pseudomallei* induces cancer-like cellular and molecular changes in trigeminal Schwann cells. To be submitted to Glia – expected submission date 10 November 2017.

2) Ali Delbaz, Freda Jen, Benjamin Schulz, Michael Jennings, James St John, Jenny Ekberg. *Nisseria meningitidis* induces cancer-like cellular and molecular changes in trigeminal Schwann cells. To be submitted to Molecular and Cellular Neuroscience – expected submission date 8 December 2017.

Chapter 1: Introduction

1 Introduction

Glioma is a broad category of brain and spinal cord tumours that originate from glial cells. Glioma is the most common primary intracranial tumour, representing around 81 % of malignant brain tumours (Ostrom, Bauchet et al. 2014). Gliomas usually consist of a heterogeneous cell mass which contains glial cells with multiple nuclei (Fujita, Mizuno et al. 2004). Multinucleation is thought to be due to cell cycle arrest secondary to dysregulation of the tumour suppressing p53 protein, but the underlying causes are not known (Temme, Geiger et al. 2010). Incidence rates of glioma vary significantly by age, gender, histologic type, race and country (Ostrom, Bauchet et al. 2014). Although several potential risk factors for gliomagenesis have been identified, including environmental, genetics, and immunological, various infections also constitute an under-appreciated but significant risk (Vittecoq, Elguero et al. 2012, Alibek, Kakpenova et al. 2013). Based on the International Agency for Research on Cancer (IARC) estimation, around 16 % of total cancers in 2008 were due to infections, however, the potential link between infections and glioma is largely unexplored. Histopathological studies have revealed that certain viral infections may be associated with glioma and brain cancer, including human cytomegaloviruses and papilloma viruses, but almost no information on the association between brain cancer/gliomagenesis and bacterial infection exists (Alibek, Kakpenova et al. 2013), with the exception of one report of a link between *Brucella* spp., which can cause neurobrucellosis, and medulloblastoma (Zhang, Izadjoo et al. 2011).

Our group showed, for the first time, that intranasal inoculation of mice with the gram negative bacterium *Burkholderia pseudomallei* led to rapid penetration of the trigeminal nerve which projects into the brainstem. After 24 h, the bacteria had penetrated the brainstem, and another 24 h later were found in the spinal cord; the trigeminal nerve was the direct route of entry into the central nervous system (CNS) (Fig. 1.1). Importantly, the bacteria were present at low levels and did not elicit an immune response. This work is the first direct evidence of bacteria directly invading the brain via the trigeminal nerve undetected by the immune system (St John, Walkden et al. 2016)

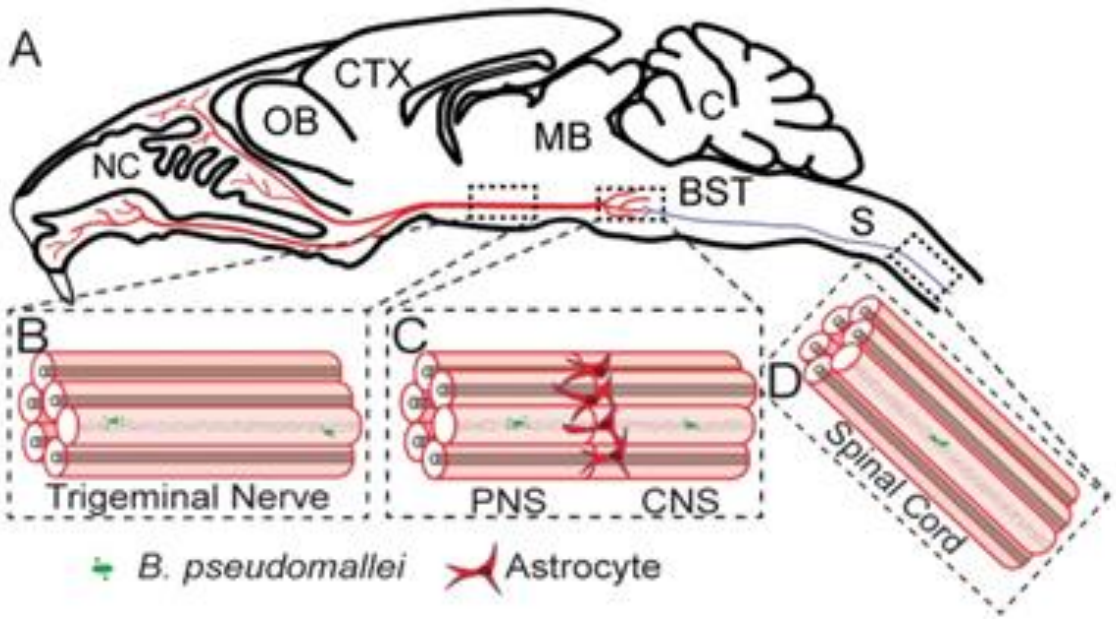


Figure 1-1. ***B. pseudomallei*** **rapidly penetrates the brain via the trigeminal nerve.**

Adapted from (St. John, Walkden et al. 2016). A) Overall structure of brain and trigeminal nerve in mouse. B) A magnified trigeminal nerve, axon degradation (grey) in small proportion of nerves. C) A layer of astrocytes assists the transition of bacteria between trigeminal nerve and brainstem. D) *B. pseudomallei* found within the spinal cord. NC: Nasal cavity, OB: Olfactory bulb, CTX: Cortex , MB: medulla brain, BST: Brain stria terminals, S: Spinal cord

As it is now clear that the bacteria can invade the peripheral nerves and CNS without activating an immune response, it raises the question of what the long-term effect of subclinical pathogenic bacterial infections have on the fate of nervous system cells, such as glial cells. Many glial cell types are phagocytic (Inoue, Terashima et al. 1980, Bjerknes, Bjerkvig et al. 1987, Wagner and Raymond 1991, Bechmann and Nitsch 1997, Schuetz and Thanos 2004, Panni, Ferguson et al. 2013, Schafer and Stevens 2013, Nazareth, Lineburg et al. 2015, Nazareth, Tello Velasquez et al. 2015) and can harbor microorganisms intracellularly, but the long-term effects on the host glia have not been characterised (Massa and ter Meulen 1987, Bowman, Rasley et al. 2003, Rasley, Tranguch et al. 2006, Vincent, Choi-Lundberg et al. 2007, Liu, Chauhan et al. 2010, Lamirand, Ramauge et al. 2011, Li, Chen et al. 2016). Our preliminary research, part of which is presented in this thesis, has shown that *B. pseudomallei* can initiate the formation of multi-nucleated glial cells using *in vitro* assays. Multi-nucleated cells (MNCs) are a characteristic component of glioma cells but what initiates the formation of MNC and how they contribute to the progression of glioma are unknown (Fujita, Mizuno et al. 2004). To the best of our knowledge, there are no reports of bacteria causing the formation of multi-nucleated glial cells. This therefore places our research at

the forefront of the potential discovery of bacteria-induced cellular changes that may contribute to the formation of gliomas.

In this project we also investigated whether the gram negative bacterium *Neisseria meningitidis* affected glial cells in manners that may be associated with gliomagenesis. *N. meningitidis* as a commensal bacterium is a natural inhabitant of the human nasopharynx mucosa, part of which is covered by olfactory epithelium (Brooks, Woods et al. 2006). The olfactory epithelium contains the cell bodies of olfactory neurons, axons from which come together to form the olfactory nerve that terminates in the olfactory bulb in the brain. Previous studies have revealed that *N. meningitidis* invades the meninges and CNS in the absence of bloodstream dissemination, most likely via the olfactory and trigeminal nerve, and that the bacteria invaded the olfactory epithelium via binding to CD46 (Johansson, Rytkonen et al. 2003, Sjölinder and Jonsson 2010) (Fig. 1.2). As the nasal cavity is also innervated by branches of the trigeminal nerve, and we previously showed the trigeminal nerve to be more sensitive than the olfactory nerve to bacterial infection in one mouse model (St John, Walkden et al. 2016), we tested whether trigeminal Schwann cells were also susceptible to *N. meningitidis* infection. We showed that this was the case, and subsequently hypothesised that infection of trigeminal Schwann cells with *N. meningitidis* can induce cellular changes in these cells. In the research of the current thesis, we showed that not only did *N. meningitidis* infection cause the formation of multinucleated cells, but also induced atypical nuclei and the upregulation of cancer related molecules

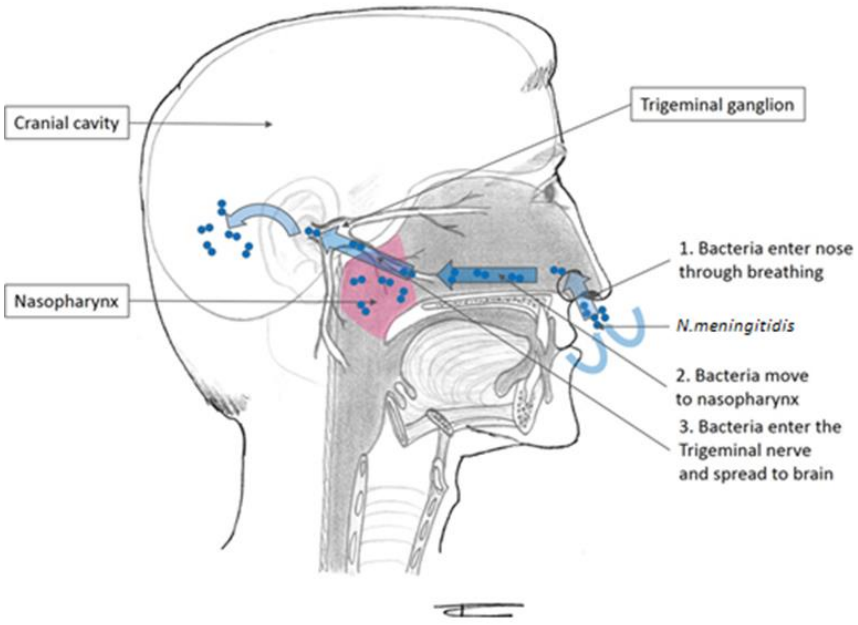


Figure 1-2. *N. meningitidis* is able to invade the CNS through the trigeminal nerve in the absence of bloodstream dissemination.

The current study was focussed on multinucleation of trigeminal Schwann cells. Schwannoma tumours do form in the trigeminal nerve, but only constitute ~0.2 % of all intracranial tumours and are usually benign (Zhang, Yang et al. 2009, Fukaya, Yoshida et al. 2010, Coniglio, Miller et al. 2013), however, understanding the processes that initiate cancer-related changes in these glial cells can then be translated to other glial types, such as astrocytes which are the primary cells involved in glioma formation (Dai and Holland 2003). Further, we have shown that *B. pseudomallei* can traverse the astrocytic glia limitans layer between the trigeminal nerve and the brainstem (St John, Walkden et al. 2016) and *N. meningitidis* is well known to infect the CNS which is populated by astrocytes (Coureuil, Join-Lambert et al. 2012). Thus, astrocytes come in direct contact with these bacteria. We have also shown that trigeminal Schwann cells readily interact with astrocytes via lamellipodia (Nazareth et al., unpublished data), and hence it is possible that bacteria-infected Schwann cells exhibit altered interactions with astrocytes that can promote astrocytic cellular changes (Fig. 1.3).

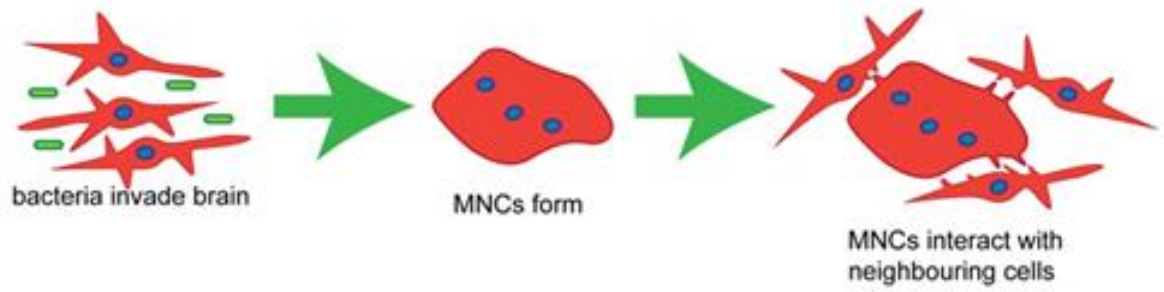


Figure 1-3. Phagocytosis of bacteria creates multi-nucleated glial cells *(in particular multinucleated astrocytes), which then interact with filopodia and lamellipodia with neighbouring cells, resulting in a cascade of events leading to the formation of a glioma*

Chapter 2: Literature Review

2 Literature Review

2.1 Glial cells

Since 1955 when Albert Einstein died, many studies have been performed on his brain to understand what made him so intelligent. It was widely expected that the histological studies would show that his brain size or number of neurons were greater than in the average person, but surprisingly, this was not the case (Diamond, Scheibel et al. 1985). What, then, made his brain so exceptional? In the 1980s, scientists discovered that his brain had considerably more glial cells than the average brain, especially in the association cortex (Diamond, et al. 1985), an area of the brain involved in imagination, personality and complex thinking. This finding altered the way scientists viewed glia; glial cells had long been thought to only serve as supporters of neurons with specific duties such as supplying nutrients and cleaning up dead nerve cells and debris. Recent studies have now revealed that glial cells are important players in more complex processes such as the formation of synapses and their function. For instance, astrocytes release thrombospondin, which increases synapse formation in neurons (Cheng, Lau et al. 2016). Moreover, rodent models have shown that when there is a lack of glial cells, neurons and their synapses fail to function properly (Diamond, Scheibel et al. 1985).

In the central nervous system (CNS), the main glial cell types are astrocytes, oligodendrocytes and microglia whereas in the peripheral nervous system (PNS), Schwann cells and olfactory ensheathing cells (OEC) constitute the main glial populations (Su and He 2010). These different glial cells are important neural cells with varied functions including regulating neuronal migration in early development, neuroimmunity and neural regeneration (Su and He 2010, Toy and Namgung 2013).

Like other cancers, brain cancers are the consequence of abnormal growths of cells. In the case of glial-origin cancers, glioma is the most common primary brain tumour (Ohgaki 2009). Despite extensive efforts and significant progress in our knowledge regarding the molecular mechanism and signalling pathways involved in gliomagenesis, high-grade glioma remains a fatal disease with dismal prognosis (Jiang and Uhrbom 2012). Over a hundred years ago, the recognition of the histopathological similarities between glioma cells and normal glial cells of the brain has led to the agreement that gliomas originate from glial cell types (Jiang and Uhrbom 2012). The location of the glioma tumour depends on the type of cells from which it originates. Within the CNS, astrocytoma originates from astrocytes and its location can be in the various parts of the brain and nervous system, including the cerebellum, the cerebrum, and the central areas of the brain, the brainstem, and the spinal cord (Jiang and Uhrbom 2012).

Oligodendroglioma is the other type of glioma of the CNS and is derived from oligodendrocytes. These tumours can be found anywhere within the cerebral hemisphere of the brain, although the frontal and temporal lobes are the most common locations (Persson, Petritsch et al. 2010). In the peripheral nervous system, Schwannomas are derived from Schwann cells, which usually have single place of origin; however, 10% originate from multiple locations. Schwannomas often arise in the head and neck (about 40%) and rarely located in the retroperitoneum (about 6%) (Samarakoon, Weerasekera et al. 2012). Interestingly, OECs rarely or never form tumours (Yamaguchi, Fujii et al. 2010). In summary, the complexity of gliomas is derived from the diverse characteristic of each glial cell type; the following section explains the morphological and physiological properties and function of glial cells.

2.2 Astrocytes

Astrocytes display a remarkable heterogeneity in their morphology and function. Normal mature astrocytes are classified into two groups based on shape (Su and He 2010): (1) star shaped astroglia, which include protoplasmic astrocytes in the grey matter and fibrous astrocytes in the white matter (Kimelberg and Nedergaard 2010), and (2) elongated astroglia which include Muller cells spanning the retina and Bergmann glia in the molecular layer of the cerebellum (Kimelberg and Nedergaard 2010). Astrocytes have several key functions within the central nervous system which are listed in Table2.1.

Table 2-1. *The functions of astrocytes*

Function	Explanation
Controlling extracellular K^+ homeostasis	Astrocytes can control K^+ concentration in the brain, and have higher capacity for K^+ uptake than neurons (Walz and Hertz 1984). By a mechanism termed ``spatial buffering'' they remove excess extracellular K^+. Thus, astrocytes either take up the K^+ from the site of higher concentration and release at the lower concentration site, or they can remove extracellular K^+ by increasing Na+/K+-ATPase pump activity and increasing intracellular K^+ and water (Walz and Hertz 1984).
Removing excess glutamate	Glutamate as a major excitatory neurotransmitter can be a powerful neurotoxin when released in excess for a long time and may trigger neuronal cell death. Following the uptake of glutamate by glutamate transporter1 (GLT-1), astrocyte-specific glutamine synthase converts glutamate to glutamine. Glutamine can be taken up and consumed by neighbouring neurons (Walz and Hertz 1984).
Control of cerebral blood flow	Astrocytes are the central elements of the neurovascular units that integrate neural circuitry with local blood flow. Astrocytes surround all blood vessels in the brain including

	the precapillary arterioles (Gordon, Choi et al. 2008). Astrocytes can transduce neuronal activity affecting arteriole diameter and blood flow. For instance, mGluR-related increases in intracellular Ca^2 can activate and release vasoactive agent phospholipase II to generate arachidonic acid and prostaglandin II by COX-I to widen vascular smooth muscle (Gordon, Choi et al. 2008).
Anti-oxidant activity	Because of the large quantities of myelin and high rates of oxidative metabolic activities, the CNS is subjected to the damaging effects of reactive oxygen species (ROS). Astrocytes possess an antioxidant GSSG-GSH system (Aschner 2000).
Transporting water	Astrocytes express high levels of water channels namely aquaporin 4 (AQP4). The polarized expression of AQP4 in vascular end feet suggests that astrocytes constitute the major route for water transport into and out of the brain (Badaut, Brunet et al. 2007)
Controlling synaptogenesis and synaptic maintenance	Astrocytes control the connectivity of neuronal circuits by regulating the formation, maturation, maintenance and stability of synapses. Synaptic formation is dependent on astrocyte-derived cholesterol, which is needed for membrane synthesis. Synaptogenesis also depends on several signalling pathways and soluble factors, released from astrocytes. For example, TNFα regulates the insertion of glutamate receptors into the postsynaptic membranes or activity-dependent neurotrophic factor (ADNF) increase the density of NMDA receptors in the membrane of neighbouring postsynaptic neurons (Badaut, Brunet et al. 2007)
Ameliorating brain injury	Following ischemic insult to the brain, some synaptic rearrangement occurs by expression of TSP1/2 in astrocytes. It has been shown that in animals which lack TSP1/2 expression in their astrocytes, synaptic rearrangement is reduced following stroke (Badaut, Brunet et al. 2007), (Liauw, Hoang et al. 2008)

2.2.1 Oligodendrocytes

Oligodendrocytes are the major myelinating cells and insulator of the axons in the CNS, but there are also satellite oligodendrocytes that are perineuronal and serve to regulate the microenvironment around the neurons rather than myelination of the axons (Ludwin 1997, Baumann and Pham-Dinh 2001). The origin of oligodendrocytes in the spinal cord and brain are different. In the spinal cord, the majority of oligodendrocytes derive from oligodendrocyte precursor cells/progenitors (OPCs) in the ventral ventricular zone, a transient embryonic layer that lines the ventricles and contains neural stem cells. In addition, 10-15% of spinal cord oligodendrocytes arise from OPC in the dorsal spinal cord (Cai, Qi et al. 2005). In the brain, the

origin of oligodendrocytes is heterogeneous and is characterized by expression of different transcription factors. The initial population consists of cortical OPCs, which express Dlx2 and Nkx2.1 transcription factors; the second population is derived from the more dorsal parts of the cortex express neither Dlx2 nor Nkx2.1. Finally, the third population is found in the ganglionic eminences and the cortical subventricular zone, and express PDGFRα, Oig1 and NG2, the typical OPC markers (Cai, Qi et al. 2005).

2.2.2 Microglia

Microglia are the resident macrophage-like cells within the CNS, and constitute 5-12% of the total glial population (Ling 2014). Upon infection, traumatic or ischemic insults within the CNS, microglia either phagocytose the pathogens and dying cells, or release effector molecules to recruit other immune cells from the blood, or both (Saijo and Glass 2011). Furthermore, microglia also secrete growth factor and anti-inflammatory molecules to facilitate the regeneration of damaged CNS tissue (Saijo and Glass 2011).

It is well accepted that in early embryonic development microglia are derived from myeloid lineage cells that originate in the bone marrow (Morris, Graham et al. 1991). Microglia are divided into three morphological types: (1) ameboid, (2) ramified and (3) reactive. During brain development, most of the microglia are amoeboid but in the postnatal stage of development, ameboid microglia transform into ramified resting microglia. Upon induction of pathological conditions or brain injury, ramified microglia undergo a series of morphological and molecular changes to transform into activated microglia (Ling and Leblond 1973).

However, while this transformation is a hallmark of brain pathology, it is not clear whether this activation has beneficial or detrimental functions in neuropathological conditions by secretion of several potentially cytotoxic molecules such as proinflammatory cytokines and reactive oxygen intermediates (Dheen, Kaur et al. 2007). Apart from the pathogens, there are several inflammatory stimuli, which can activate microglial cells. These stimuli include bacteria-derived lipopolysaccharide (LPS), β-amyloid, interferon-γ (IFNγ), thrombin, and macrophage colony stimulating factor and prion proteins (Dheen, Kaur et al. 2007). The microglial response to the stimuli is mediated by kinase and phosphatase cascades such as p38 and p44/42 families of mitogen activated protein kinase (MAPK) pathways. It has been shown that stimuli such as LPS and IFNγ can activate MAPK-activated protein kinase 2 (MK2) to induce pro-inflammatory mediators in microglia (Dheen, Kaur et al. 2007).

2.2.3 Schwann cells

Schwann cells are found in the peripheral nervous system (PNS) and have similar functions to oligodendrocytes within the CNS. Schwann cells are involved in myelin production, maintenance, and repair of peripheral nerves (Table 2.2). Unlike oligodendrocytes, which only reside within the CNS, Schwann cells can traverse the PNS-CNS border to invade the CNS in order to make new myelin sheaths around demyelinated axons (Graca, Bondan et al. 2001). Based on their morphology, biochemical markers and the neuronal types of their association, Schwann cells can divided into four groups: (1) myelinating cells, (2) nonmyelinating cells, (3) perisynaptic Schwann cells and (4) satellite cells of peripheral ganglia (Graca, Bondan et al. 2001).

Schwann cells originate from neural crest in early embryonic development (Ledouarin 1986). The crest cells develop into precursor cells, which ultimately transform into the immature Schwann cells, which go through the myelination stage and then become non-myelinating Schwann cells (Jessen, Brennan et al. 1994). Schwann cells can either myelinate or ensheathe (enwrap, but not myelinate) axons based on their diameters. During development, Schwann cells in contact with small-diameter axons (less than 1 μm in diameter), will differentiate into mature non-myelinating Schwann cells, and enwrap several unmyelinated axons into a small fascicle termed a Remak bundle (Kaplan, Odaci et al. 2009). Ultrastructural analysis has revealed that Schwann cells engage in 1:1 ratio with axons. Most of these axons are sensory, in particular nociceptive (pain-sensing) neurons, along with postganglionic sympathetic axons. In contrast, Schwann cells that come into contact with large axons (>1 μm), including all motor neurons and some sensory neurons, differentiate into myelinating Schwann cells. The myelinating process is mediated by lamellipodial membrane protrusions from the Schwann cell, which after establishing contact enwrap the axon in a jelly-roll like manner. As the thickness of the myelin envelope increases, so too will the conduction speed of the nerve fibre, due to the insulating properties of the fatty membranous myelin layer (Voyvodic 1989, Corfas, Velardez et al. 2004)

Several molecules including myelin-associated glycoprotein (MAG), the neurotrophin receptor p75 (p75NTR), insulin-like growth factor 1 (IGF1), ß1 integrins, transforming growth factor β (TGF-β) and neuregulin 1 (NRG-1) play critical roles in axon-Schwann cells interaction (Table2.2) (Yin, Crawford et al. 1998, Cosgaya, Chan et al. 2002, Feltri, Graus Porta et al. 2002). Among them, only NRG1 and its receptor are key factors in axon-Schwann cell interactions (Meyer, Yamaai et al. 1997).

Table 2-2 Regulatory molecules and their roles in Schwann cell functions

Regulatory molecules	Functions
Myelin associated glycoprotein (MAG)	This glycoprotein is a member of the I-type lectin subgroup of the Ig gene superfamily, which is highly expressed by myelinating cells enriched in the axonal membrane of the myelin internode (Arquint, Roder et al. 1987, Trapp, Andrews et al. 1989, Yin, Crawford et al. 1998). It has been demonstrated that MAG-deficient mice develop a chronic atrophy of myelinated PNS axons, which leads to axonal degeneration (Yin, Crawford et al. 1998).
Neurotrophin receptor p75 (p75NTR)	Neutrophins, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5) are key PNS myelination mediators which bind to the p75 receptors on Schwann cells and exert their action by activating divergent intracellular pathways (Cosgaya, Chan et al. 2002).
Insulin-Like Growth Factor-1 (IGF-1)	The IGF-1 receptor on Schwann cells is a tyrosine kinase receptor which can bind to the neurotrophic factor IGF-1 and exert its action by controlling Schwann cell viability during postnatal development. It has been postulated that this survival regulation may switch from an axon-dependent mechanism to autocrine and/or paracrine signalling during development (Syroid, Zorick et al. 1999).
β1 integrin	The myelin-forming Schwann cells synthesize laminin receptors, including α6β4, α6β1 and α2β1 integrins, which play various roles in peripheral nerve development and myelination (Previtali, Feltri et al. 2001). β1 integrins are vital for Schwann cell-axon interactions. It has been shown that in null β1 integrin mice, Schwann cells survive normally but are unable to segregate axons and attain the proper 1:1 relationship with an axon, which is a prerequisite for radial axonal sorting and myelination (Previtali, Feltri et al. 2001).
TGF-β (transforming growth factor beta)	TGF-β is expressed by Schwann cells and regulates Schwann cell differentiation and neurite myelination. TGF-β blocks Schwann cell myelination on dorsal root ganglion (DRG) neurites and inhibits the expression of myelin-related molecules such as GC, P0, MAG, and MBP, however, the molecular mechanisms behind this activity are not clear. It has been speculated that blockage of myelination is due to the interference of TGF-β with intracellular signalling pathways resulting downregulation of myelin-related molecules genes (Guenard, Gwynn et al. 1995).

NRG1 (Neuregulin1)	NRG1-erbB2/3 interactions regulate many aspects of Schwann cells biology, including differentiation from neural crest cells, survival, motility, proliferation, expression of Na^+ channels and myelination (Shah, Marchionni et al. 1994, Mahanthappa, Anton et al. 1996). The NRG1-erbB2/3 interaction can activate MAPK to induce cell proliferation and PI3K/Akt to inhibit apoptosis (Bhatheja and Field 2006).

2.2.4 Olfactory ensheathing cells (OECs)

OECs are the glia of the peripheral olfactory system, and are present from the olfactory mucosa all the way up to the nerve fibre layer of the olfactory bulb (OB). OECs support the growth and regeneration of olfactory axons, and are thought to be crucial for the continuous regeneration of the olfactory nerve that occurs throughout life (Raisman 2001, Zhu, Cao et al. 2010). Similar to Schwann cells, OECs are also derived from the neural crest (Barraud, Seferiadis et al. 2010) but OECs do not myelinate olfactory axons. Instead, OECs ensheathe bundles of numerous unmyelinated axons and intermingle with them (Ekberg and St John 2015). Here, OECs form a stable channel which forms a strong mechanical supportive conduit for the axons (Doucette 1984, Nazareth, Lineburg et al. 2015). OECs are highly plastic in their morphology, which makes them able to rapidly react to injury and infection. They are the main phagocytic cells within the olfactory system, as macrophages are largely excluded from the olfactory nerve. Thus, OECs are the principal cell type that eliminates axon debris (Doucette 1984, Nazareth, Lineburg et al. 2015).The ability of OECs to facilitate the regeneration of olfactory axons in adult mammals makes them promising candidates for repairing nervous system damage including spinal cord injuries (reviewed by(Ekberg and St John 2015).

OECs and Schwann cells share several similar characteristics including antigenic and morphological similarity. They both express p75NTR, S100ß *in vivo* and GFAP *in vitro* (Yan, Bunge et al. 2001, Barnett and Riddell 2004) and show similar responses to growth factors like GGF, forskolin and FGF2 (Yan, Bunge et al. 2001). The gap junctional communication of OECs closely resembles that of Schwann cells as they both display a similar connexin expression profile (Barnett and Riddell 2004). However, there are two distinguishing properties between OECs and Schwann cells including ErbB receptor expression profile and *in vitro* interactions with astrocytes (Thompson, Roberts et al. 2000)

The ErbB receptor tyrosine kinase family are a group of receptor tyrosine kinases (RTKs) involved in key cellular functions such as cell proliferation, migration, differentiation, apoptosis,

and cell motility. This family consists of four cell surface receptors including ErbB1/ EGFR/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. The ligands are varied and can be either specific to the receptor (i.e. EGF, TGF-α, AR, which bind to the ErbB1/ EGFR/HER1) or versatile binding ability (i.e. binding of neuregulins 1–4 to ErbB3 and ErbB4 or HB-EGF, epiregulin, and β-cellulin binds to ErbB1/ EGFR/HER1 and ErbB4/HER4)(Arteaga and Engelman 2014). By using molecular techniques such as qRT-PCR and also immunocytochemistry in rat, OECs show distinctly higher expression of mRNA and protein for ErbB2 and ErbB4, while Schwann cells express more ErbB3 (Barnett and Riddell 2004).

In vitro assays demonstrated that in co-culture, OECs and Schwann cells display strikingly different behaviour in contact with astrocytes, with OECs easily intermingling with astrocytes while Schwann cells do not. This is consistent with their *in vivo* interactions as OECs mix with astrocytes within the nerve fibre layer of the olfactory bulb. Some of the features of OECs and Schwann cells in co-culture with astrocytes are listed below (Lindholm, Cullheim et al. 2002).

1) OECs and astrocytes interact with each other, but OECs and SCs separate into two populations (Lakatos, Franklin et al. 2000);
2) OECs spend more time in contact with astrocytes than Schwann cells (Lakatos, Franklin et al. 2000);
3) Astrocytes undergo hypertrophy in contact with Schwann cells but not OECs (Li, Xu et al. 2012);
4) Astrocytes express more chondroitin sulphate proteoglycans (CSPG) when co-cultured with SCs than OECs; CSPGs inhibit axonal extension (Lakatos, Franklin et al. 2000).

In summary, while Schwann cells and OECs share many similarities, it is clear that there are many important morphological and functional differences between OECs and Schwann cells.

2.3 Glioma

The term of glioma encompasses all tumours that originate from glial cells. Of tumours that occur in the intracranial CNS region, glioma account for 30% to 40% of all tumours (Schwartzbaum, Fisher et al. 2006). Within the CNS, astroglioma involve astrocytes, whilst oligodendroglioma involves oligodendrocytes. In the PNS Schwannoma involve Schwann cells, and in very rare cases OECs give rise to olfactory ensheathing cell tumours.

The incident rate of glioma differs between countries ranging from high incidence in highly developed countries (e.g. Australia, Canada, United States, Denmark, and New Zealand) to low incidence in India or Philippines. Such differences may be the result of the diagnostic practices

and reporting differences in different countries (Wrensch, Minn et al. 2002). The mortality rate of patients with the most aggressive CNS glioblastomas is very high, with only 2% of patients over 65 and 35% of patients under 45 years surviving for two years or more (Central Brain Tumour Registry of the United States [http://www.CBTRUS.org]). The demographic aspects of glioma vary between age, sex and race. For instance, Caucasian populations have the highest incidence rate, while south-eastern Asian populations have the lowest rate. The male/female ratio is 1.4 /1.5 in pre-menopausal ages (Darefsky and Dubrow 2009).

Gliomas are heterogeneous tumours, which are categorised into four grades (I, II, III, IV) (The 2007 WHO Classification of Tumours of the Central Nervous System) (Ostrom, Gittleman et al. 2015). Grade I tumours are least malignant tumours and are usually associated with long-term survival. Pilocytic astrocytoma, craniopharnygioma and ganglioglioma are the examples of this grade. Grade II are slow-growing with an abnormal appearance under microscope and capacity to spread into near normal tissues. Although grade III do not show big morphological differences compared to grade II, they actively produce abnormal cells and tend to give rise to grade IV tumours. Grade IV tumours are the most malignant gliomas, which appear highly unusual under the microscope and easily grow into nearby normal brain tissue. Angiogenesis is the most important characterisation of these tumours. Glioblastoma multiforme is the main category IV glioma (Urbańska, Sokołowska et al. 2014).

Like progenitor cells, glioma cells can migrate throughout the central nervous system. The migration of glioma cells throughout the CNS, which is related to their ability to degrade the extracellular matrix, integrin signalling, activation of growth factor such as PGDF, EGF and activation of neurotransmitter receptors (AMPA and GABA), may reflect the similarity between glioma cells and neuronal stem cells/progenitors (Westphal and Lamszus 2011). The evolution in molecular approaches led to the identification of glioma based on molecular signature or gene expression patterns. These approaches contributed to elucidating the molecular oncogenic pathways involved in glioma, for example EGF receptor (EGFR)/platelet derived growth factor (PDGF) pathways, as well as cellular cycle control elements such as p53, the retinoblastoma gene, phosphatase and tensin homologue (PTEN) signalling pathway. Although all of these approaches are important to understand the original genetic events that lead to glioma, it is crucial to elucidate in which order these events occur (Westphal and Lamszus 2011).

The brain neural stem cell reservoirs, in particular the subventricular zone and the subgranular zone of the dentate gyrus, are essential for adult neurogenesis. The mouse model has shown that glial progenitor cells are highly distributed in the subventricular zone which may also constitute an origin of glioma cells (Guglielmetti, Praet et al. 2014). By understanding the regulatory

mechanisms involved in maintaining the progenitor cells, we can determine which steps are responsible for turning progenitor cells into cancerous cells (Westphal and Lamszus 2011). It has been shown that the maintenance of progenitor cells requires the suppression of proliferation and differentiation. To activate proliferation, inhibitory pathways must be inhibited, such as the Notch signalling pathway which is responsible for the generation astrocytes and neurons from ependymal cells (Carlen, Meletis et al. 2009, Imayoshi, Sakamoto et al. 2010, Westphal and Lamszus 2011). According to the two different research approaches, there is a tight correlation between inactivation of Notch pathway and progression of ependymoma, glioblastoma and oligodendroglioma (Puget, Grill et al. 2009, Xu, Qiu et al. 2010).

2.3.1 Schwannomas

Schwannomas are nerve sheath tumours, which are derived from Schwann cells and arise particularly from peripheral, cranial or visceral nerves. These tumours are usually large and infiltrating and histologically have perineural and intraneural spread pattern (Machairiotis, Zarogoulidis et al. 2013). Schwannomas typically express the S-100β protein, glial fibrillary acid protein (GFAP), padoplanin, calretinin, and SOX-10 (Machairiotis, Zarogoulidis et al. 2013).

2.3.2 Schwannoma variants

Cellular Schwannoma: Although this type of Schwannoma is uncommon, it is an important variant of Schwannoma. This type of Schwannoma possesses high level of cellularity and fascicular growth pattern with highly mitotic activity and damaging behaviour including bone destruction. This variant shows foamy histiocytic aggregates and diffuses S-100 protein. Cellular Schwannomas lack malignant potential and never metastasize (Rodriguez, Folpe et al. 2012).

Plexiform Schwannoma: This type of Schwannoma usually occurs in superficial locations and is characterised by intraneural-nodular pattern of growth. This type of Schwannoma is less restricted than other Schwannoma but more problematic in that this type of Schwannoma usually arises in deep anatomic locations and soft tissues (Samarakoon, Weerasekera et al. 2012, Machairiotis, Zarogoulidis et al. 2013).

Trigeminal Schwanomma: This type of Schwannoma is the slow-growing tumour containing Schwann cells which is the second most common intracranial schwannoma (McCormick, Bello et al. 1988). Trigeminal Schwannoma accounts for about 2% of all intracranial schwannomas and tend to occur predominantly in the late decades of life. This tumour presents with facial pain along with paraesthesia in the distribution of trigeminal nerve division on the location of the tumour (Borges and Casselman 2010, Bathla and Hegde 2013, Agarwal 2015) .

2.3.3 Glioma risk factors

The aetiology of gliomas is complex with a multitude of risk factors that ultimately result in malignant transformation of glial cells, many that are uncharacterised. The most common known risk factors are listed below.

Environmental genetic risk factors and genetic syndromes

Exposure to therapeutic or high-dose radiation is the main environmental risk factor for gliomagenesis (Ohgaki and Kleihues 2005). However, genetics can play a significant role in determining the degree of risk associated radiation exposure. For instance, children with germline polymorphisms leading to decrease in thiopurine methyltransferase activity are significantly more susceptible to develop brain cancer than those without this polymorphism (Relling, Rubnitz et al. 1999). A small proportion of glioma cases are accounted for genetic syndromes, including neurofibromatosis 1 and 2, tuberous sclerosis, retinoblastoma, Li–Fraumeni syndrome, Turcot's syndrome and multiple hamartoma (Schwartzbaum, Fisher et al. 2006).

Immunologic risk factors and germline polymorphisms

Epidemiological studies have revealed that there is significant inverse association between glioma and allergies, high level of serum IgE, IgG antibodies to varicella-zoster virus (VZV) and chicken pox. By comparing allergy-related polymorphism and glioma, researchers found that interleukin 4 receptor alpha (IL-4RA) SNPs T478C TC, CC and A551G AG, AA were significantly positively associated with glioma, while IL-13 SNP C1112T CT, TT was inversely associated with glioma. In another study, glioma has been shown to have a positive association with human leukocyte antigen (HLA) genotype B*13, the HLA haplotype B*07-Cw*07 and negatively with the genotype Cw*01 (Schwartzbaum, Fisher et al. 2006).

Cell cycle regulation polymorphisms

Similar to other neoplasms, dysregulation of cell cycle is a hallmark feature of most gliomas. It has been shown that SNP in the Mouse double minute 2 homolog (MDM2) promoter as an important negative regulator of the p53 leads to increasing expression of MDM2 and simultaneous reduction of P53 expression and subsequently tumour development especially in patients with Li–Fraumeni syndrome (Bond, Hu et al. 2004).

2.3.4 Infectious agents and cancer

The relationship between carcinogenesis and infectious agents is not fully understood, however the International Agency for Research on Cancer (IARC) estimated that 20% of deaths from cancers are due to infectious agents. The carcinogenesis mechanism of infectious agents is thought to involve the dysregulation of DNA repair mechanisms, cell cycle modulation, DNA mutations, chronic inflammation and immune system impairment (Alibek, Kakpenova et al. 2013). To date, no infectious agents have been accepted as a direct oncogenic cause of brain cancer or glioma, but a number of viruses, parasites and bacteria are accepted as being associated with the malignancy.

When Peyton Rous first extracted cell-free filtrate from chicken sarcoma, he was credited as the first person to show that cancer may have an infectious origin (Rous 1983). Almost a century after his finding, our knowledge of association between infectious agents and malignancies has been improved but is still not clear, and incidents may be under-reported (Alibek, Kakpenova et al. 2013). As there is no direct link between infectious agents and brain cancer or glioma, it is useful to examine which infectious agents cause cancer in other cell types. Latest estimations, suggests around 16% of all malignancies can be attributed to infections (de Martel, Ferlay et al. 2012). By using epidemiological tools and serological methods, 1.2 million malignancies per year have been attributed to infectious agents. The mechanism that infectious agents uses to initiate carcinogenesis are different and usually divided into the three mechanisms: persistence in the host cells, insertion of oncogenes into the host genome (HPV, HIV), and immunosuppression (HIV) (Ohshima and Bartsch 1994).

Chronic inflammation caused by bacteria, viral and parasitic infections increase cancer risk by eliciting inflammatory cytokines, which alter DNA integrity and suppress apoptosis. This mechanism is usually initiated by reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) formation at the site of the inflammation. ROS and RNOS produced by neutrophils and macrophages induce DNA damage by DNA base oxidation, deamination or base alkylation via lipid peroxidation (LPO) (Coussens and Werb 2002). ROS-sensitive signalling pathways such as MAPK/Erk cascade, PI3K/Akt-regulated signalling cascades and IKK/NF-kB signalling pathway are highly elevated in many cancers, which regulate cell growth and proliferation, differentiation and cell survival (Storz 2005).

Infection within cells induces chronic inflammation by recruiting leukocytes and other phagocytic cells. One of the critical genes which are affected by chronic inflammation is p53. For instance, *H. pylori*, an established carcinogen for the development of gastric cancer, triggers an inflammatory

response by inducing macrophage migration inhibitory factor (MIF) from macrophages and T-cells and suppressing p53 transcriptional activity (Zaika, Wei et al. 2015). Due to the clear relationship between infectious agents and carcinoma risk, the International Agency for Research on Cancer (IARC) have classified the infectious agents listed in table 2.3 as carcinogenic in humans.

Table 2-3 *Established carcinogen viruses*

Pathogen	Structure	Carcinogenesis
Hepatitis B virus (HBV)	HBV is an enveloped circular DNA virus with a 3.2-kb partially double-stranded DNA genome (Liang 2009). The genome contains 4 open frame reading (ORF) including (1) viral surface (HBs Antigen), (2) core (HBc Antigen and HBe Antigen), (3) polymerase encoded by ORF P. (4) ORF X encodes (HBx Antigen) which has a versatility in its function including signal transduction, DNA repair and protein degradation (Liang 2009).	Chronic HBV infection is one of the main risk factors of hepatocellular carcinoma. HBs Antigen carriers are approximately 14 times more susceptible to develop hepatocellular carcinoma than the rest of the population (Donato, Boffetta et al. 1998). The synergy between HBV, HCV, hepatitis C virus (HCV) and aflatoxin a fungal toxic metabolite is the other hepatocellular carcinoma risk factor (Kaklamani, Trichopoulos et al. 1991),(Moudgil, Redhu et al. 2013). The HBV-DNA is capable to integrating into the human genome resulting in insertional mutagenesis and alteration of the host DNA structure by deletion, translocations, and DNA amplification (Bruix and Llovet 2003). The protein X remains intact in the integration event and plays a critical role in carcinogenesis (Balsano, Avantaggiati et al. 1991).This protein enhance the activity of cellular genes via interactions with transcription factors such as *c-myc* and also interacts with p53 which interrupts its function (Uchida, Takahashi et al. 1996).
Hepatitis C virus (HCV)	HCV is a single- stranded RNA virus belongs to the *Flaviviridae* family. This virus contains structural and non-structural proteins. Structural proteins include core and envelope glycoproteins E1 and E2, and non-structural proteins including p7 viroporin, the NS2 protease, the NS3-4A complex harboring	Although there is not any evidence about HCV genome integration into the host genome or about HCV sequences similarity to known oncogenes or tumour suppressor genes, direct carcinogenesis of HCV is possible. Based on the observations of interaction activation of between core and proto-oncogene proteins such as, ras gene and interruption of apoptosis by core protein, it is possible that HCV

	protease and RNA helicase activities, NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (Freedman, Logan et al. 2016)	might may induce hepatocarcinoma (Ray, Lagging et al. 1995, Ray, Lagging et al. 1996, Kim, Ryu et al. 2004)
Epstein–Barr virus (EBV)	The Epstein–Barr virus (EBV) belongs to the *herpes viridae* family and is well known in for causing mononucleosis. The genome is composed of linear double-stranded DNA. Over 100 genes are encoded by the genome including the Epstein–Barr nuclear antigen 1 (EBNA1) needed for EBV episome replication; EBNA2, which activates EBV latency and immortalizes genes as well as lymphocyte membrane-associated oncoprotein (LMP1) which interacts with cell signalling molecules, and may even interfere with p53 mediated apoptosis (Zhang, Gutsch et al. 1994)	Based on the WHO International Agency for Research on Cancer, EBV has been classified as a group 1 carcinogen with conclusive evidence with respect to non-Hodgkin's lymphoma, and Hodgkin's disease (IARC, Lyon, France: World Health Organization; 1997).
Human papilloma virus (HPV)	Human papilloma virus (HPV) is a non-enveloped DNA virus with a closed, circular double-stranded genome which contain genes (E1-E8), involved in viral DNA replication, transcriptional regulation, and cellular transformation and (L1, L2) responsible for translation of major (L1) and a minor (L2) capsid proteins (Bernard 2002).	HPV is a sexually transmitted agent, which has been associated with cancer of the vulva, penis, anus, head and neck, but most persuasively with cervical cancer (zur Hausen 2000). The carcinogenic potential of HPV is directly associated with the ability of random integration of viral genes into the host genome. E6 and E7 have been shown to immortalize primary human genital keratinocytes in vitro (Pao, Yao et al. 1996). E6 can form a complex with p53 and increase the degradation of p53 (Scheffner, Takahashi et al. 1992)
Human herpes virus 8 (HHV8)	Human herpes virus 8 (HHV8), or Kaposi's sarcoma-associated herpes virus (KSHV), is a sexually transmited gamma herpes virus belonging to the *herpes viridae* family (Rohner, Wyss et al. 2016).	HHV8 is associated with numbers of malignancies including Kaposi's sarcoma, non-Hodgkin's B-cell lymphoma (Zhang, Gutsch et al. 1994). The risk of Kaposi sarcoma among homosexual male HIV- positive patients is higher than other HIV-positive patients (Rohner, Wyss et al. 2016). HHV8 contains v-cyclin and v-bcl-2 which is similar to cellular proto-oncogenes, inhibits retinoblastoma

		and apoptosis, respectively (Ensoli and Sturzl 1998).
Human thymus-derived-cell leukaemia/lymphoma virus-1 (HTLV-1)	HTLV-1, a single stranded RNA virus, is the only human retrovirus belonging to the Oncovirus family known to be an aetiological agent of cancer.	In human, this virus causes Adult T-cell leukaemia/lymphoma (ATLL) (Yoshida, Miyoshi et al. 1982) and neurological disorder tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM)(Izumo, Umehara et al. 2000)
Human Immunodeficiency Virus (HIV)	HIV is composed of two strands of RNA, 15 types of proteins, a few proteins from the last infected cell and lipid bilayer membrane.	According to the IARC, HIV-1 has been deemed carcinogenic to humans (IARC, Lyon, France 1996), with Kaposi sarcoma and non-Hodgkin's lymphoma being the most prevalent cancers in people infected with HIV, especially in the late stage of AIDS. The role of HIV in developing and progression of Kaposi sarcoma is due to its ability of sustaining and increasing survival of transformed cells. In AIDS- related Kaposi sarcoma, the HIV-1- Tat protein has been directly implicated in pathogenesis. Tat protein can act as an angiogenic factor *in vivo*. This protein activates the vascular endothelial cell growth factor (VEGF) receptor on Kaposi sarcoma cells (Albini, Fontanini et al. 1994, Albini, Soldi et al. 1996), Moreover, Tat protein may transform endothelial cells to a Kaposi cell- like phenotype by cooperating with cytokines to release VEGF and basic fibroblastic growth factor (bFGF) (Ensoli, Gendelman et al. 1994).

Helicobacter pylori (*H. pylori*) as the only established carcinogen bacteria

H. pylori is a gram-negative bacterium, which was first isolated from gastric biopsies in 1982 (Warren and Marshall 1983). This bacterium is strongly associated with peptic ulcer, gastritis, gastric adenocarcinoma and lymphoma (Cave 1997). Epidemiological studies have shown that gastric cancer in *H. pylori*-infected patients is significantly more common than in *H. pylori*-negative individuals (Cave 1997). *H. pylori* contains several virulence factors including cag PAI which is strongly associated with peptic ulceration (Cover, Dooley et al. 1990). The CagA protein is a 120 to 140-kDa protein that is translocated into the host cells by type IV Cag secretion system following bacterial attachment. The CagA protein induces cell morphological changes and

increased cellular migration by tyrosine phosphorylation at its glutamate-proline-isoleucine-tyrosine-alanine motif (Asahi, Azuma et al. 2000). In human gastric adenocarcinoma, CagA translocation into cells causes a so called "hummingbird phenotype" which is associated with cell elongation and cell scattering (Moese, Selbach et al. 2004). The interaction between CagA and tyrosin phosphatase SHP-2 increase the duration of Erk activation in a Ras and PI3K independent manner, which result in cell elongation (Higashi, Lee et al. 2007).

Peptidoglycan and VacA toxin are other virulence factors of *H. pylori*. Peptidoglycan can interact with Nucleotide-binding oligomerization domain (Nod1) in host cell, leading to activation of NF-κB-dependent proinflammatory responses, such as secretion of IL-8 or β-defensin-2. These pro-inflammatory responses can promote carcinogenesis by increasing vasculogenesis (Conejo-Garcia, Benencia et al. 2004). In addition, *H. pylori* peptidoglycan can activate PI3K-AKT signalling, leading to decreased apoptosis and increased cell migration (Viala, Chaput et al. 2004, Boughan, Argent et al. 2006). The VacA toxin assists the longevity of *H. pylori* infection by suppressing the T-cell response (Boncristiano, Paccani et al. 2003). In addition, VacA can converse the effect of CagA by inactivating the epidermal growth factor receptor (EGFR) and HER2/Neu leading to suppression of MAPK activation and hummingbird phenotype (Tegtmeyer, Zabler et al. 2009).

2.3.5 Infectious Agents and Glioma

Infectious agents constitute under-appreciated but significant risk factors for glioma and brain cancer including glioblastoma multiforme and medulloblastoma (Alibek, Kakpenova et al. 2013). Glioblastoma multiforme which develops from diffuse astrocytoma is highly anaplastic among neuroepithelial tumours. This tumour is usually found in the cerebral hemispheres with high incidence rate in age of 45–70 years. Medulloblastoma, which originates from primitive undeveloped cells is, the other frequent brain tumour usually found in the cerebellum, which can spread to other CNS regions (the WHO classification of tumours of the central nervous system in 2007) (Louis, Ohgaki et al. 2007).

Human cytomegalovirus which is prevalent in the human population is detected at a high frequency in both glioblastoma multiforme (>90-95%) and medulloblastoma. By inducing COX-2 expression, STAT3 phosphorylation, prostaglandin E2 (PGE2) formation, expression of VEGF and production of IL-6 (the role of these molecules in carcinogenesis are explained in table2.4), HCMV may promote the formation of glioblastoma multiforme and medulloblastoma. By using valganciclovir and celecoxib, which selectively prevent HCMV replication and PGE2 production respectively, medulloblastoma tumour cell growth is reduced *in vitro* and *in vivo* (Baryawno,

Rahbar et al. 2011). The viral chemokine receptor protein US28, which is constitutively expressed in HCMV, induces COX-2 expression andSTAT3 phosphorylation, leading to an increase in production of VEGF and IL-6 and finally tumour formation *in vivo* (Maussang, Langemeijer et al. 2009)

Table 2-4 *Carcinogenic molecules associated with HCMV infection*

Molecule	Role in carcinogenesis
COX-2	Cox-2 can modulate cell proliferation and apoptosis especially in the early of tumorigenesis (Sobolewski, Cerella et al. 2010). This molecule is discussed in detail later in this chapter.
STAT3	Signal transducer and activator of transcription 3 (STAT3) is an transcription factor that plays a critical role in mediating cytokine-induced changes in gene expression (Johnston and Grandis 2011). STAT3 is constitutively phosphorylated in several cancers including glioma. this phosphorylation is vital in controlling invasion, cell survival, self-renewal, angiogenesis, and tumour-cell immune evasion in most of the tumours(Devarajan and Huang 2009).
prostaglandin E2 (PGE2)	Prostaglandin E2 (PGE2) is an inflammatory and carcinogenic bioactive lipid that elicits a wide range of biological effects such as cell proliferation, apoptosis, angiogenesis, inflammation and immune surveillance (Nakanishi and Rosenberg 2013).
Vascular endothelial growth factor (VEGF)	VEGF is a key regulatory factor in angiogenesis which increase the endothelial cell survival, proliferation and migration (Chung and Ferrara 2011). Paraclinical studies have shown that VEGF-driven angiogenesis is an early, crucial event in carcinogenesis and implicated VEGF-regulated angiogenesis is an important component of most of the cancer growth (Mu, Abe et al. 1996).
IL-6	IL-6, one of the major cytokines found in the tumour microenvironment, is overexpressed in almost all types of the cancer (Kumari, Dwarakanath et al. 2016). High concentration level of IL-6 reflects the association between inflammation and tumorigenesis by regulating multiple signalling pathways and hallmarks of the carcinogenesis including apoptosis, survival, proliferation, angiogenesis, invasiveness, metastasis and metabolism.

Toxoplasma gondii

Recent research has showed that the protozoan parasite *Toxoplasma gondii* can increase the risk of brain cancer by provoking inflammation and inhibiting apoptosis by impairing activation of the initiator caspase 8 and inhibit the activation of the two pro-apoptotic Bax and Bak proteins (Hippe, Weber et al. 2009). These two proteins pierce the mitochondrial outer membrane to mediate cell death by apoptosis. However, the molecular mechanism of these two proteins to puncture the mitochondrial outer membrane is far from being understood (Vutova, Wirth et al. 2007, Hippe, Weber et al. 2009, Thomas, Lafferty et al. 2012). Moreover, it has been shown that *T. gondii* upregulates anti-apoptotic proteins such as Bcl2, Bfl1, Bcl-Xl, Bcl-w, Mcl-1, Bad and Bax in host cells which are important in gliomagenesis (Molestina, Payne et al. 2003, Carmen, Hardi et al. 2006).

MicroRNAs (miRNAs) have been implicated in the aetiology of number of cancers by their essential regulatory effects on cell differentiation and proliferation. Although the effect of miRNAs in brain cancer is poorly understood, miR-17~92 has been shown to be highly expressed in glioblastoma.

miR-17~29 which is highly conserved among vertebrates plays an important role in vertebrate development homeostasis. It has been shown that mutation or deregulation of miR-17~29 contributes to the tumorigenesis. For instance, truncated miR-17~29 cooperates with c-Myc in a mouse model of lymphoma, thus providing convincing evidence that miR-17~92 is a bona fide oncogene (He, Thomson et al. 2005). It has been shown the inhibition of miR-17~92 leads to reduction of cell viability, cell proliferation and increased apoptotic rate (Zhang, Hu et al. 2016). *T. gondii* infection modulates and increases by 2–3 fold the levels of mature miR-17~92 in primary human foreskin fibroblasts (Zeiner, Norman et al. 2010). Phosphatase and tensin homolog (PTEN) is a widely expressed phosphatase protein in human cells, which dephosphorylate PIP3 and leads to inhibition of the AKT signalling pathway. PTEN is one of the miR-17~92 target, which is downregulated by miR-17~92 during the *T. gondii* infection. This downregulation could activate AKT pathways, which may result in the development of brain cancer or decrease negative modulators of cancer cell migration, E-Cadherin and tissue inhibitor of metalloproteinases 2 (TIMP2) proteins (Laliberte and Carruthers 2008, Grunder, D'Ambrosio et al. 2011).

2.3.6 Bacteria and brain cancer

So far, there is limited information on association of bacteria and brain cancer but in some cases, mycoplasma infections have been found in brain cancer tissues including glioma. Mycoplasmas

are the smallest replicative organisms which cause a wide variety of diseases. Continuous infection of murine embryonic C3H cells with *Mycoplasma penetrans* or *Mycoplasma fermentans* can lead to malignant transformation and upregulation in some oncogenic genes (Tsai, Wear et al. 1995). The ability of tumour cells to invade through the extracellular matrix is a critical event in tumour invasion and metastasis. The P37 lipoprotein, as the major immunogen of *Mycoplasma hyorhinis*, promotes cell motility, migration, and invasion *in vitro* by inducing the expression of genes implicated in inflammation and cancer progression (Gong, Meng et al. 2008). The *in vitro* experiments revealed that P37 rapidly increase the expression of the factors IL-6, Vcam, Dcn and LIF which are all implicated in inflammation and cancer progression. P37 utilize TLR4-NF-κB signalling to induce epithelial-mesenchymal transition (EMT) to initiate metastasis(Gomersall, Phan et al. 2015). Moreover, p37 up-regulates the activity but not the expression of matrix metalloproteinase-2 MMP-2.

MMPs have fundamental roles in cellular biology such as tissue remodelling, bone development, wound healing, tumour angiogenesis, metastasis and modulation the activity of several growth factors, such as TNF-α, insulin-like growth factor-1, EGF, and fibroblast growth factors (Manes, Mira et al. 1997, Afzal, Lalani et al. 1998, Haro, Crawford et al. 2000). Further, the stimulatory effect of mycoplasma infection on tumour formation is associated with the upregulation the MMPs activity by p37 (Gong, Meng et al. 2008).

Brucella species, which are gram-negative coccobacilli, produce lesions in multiple organs including the brain (Sohn, Probert et al. 2003). Drinking or eating unpasteurized milk products, meat, or contact with infected animals are the main routes of brucellosis. Positive DNA findings for certain Brucella species (spp.) in medulloblastomas suggest a direct association between Brucella spp. and CNS tumour formation (Zhang, Izadjoo et al. 2011).

Burkholderia pseudomallei, is the causative agent for melioidosis (Whitemore's disease). This gram-negative organism is usually found in soil and standing water in tropical areas such as south Asia and northern Australia (McRobb, Kaestli et al. 2014). The bacteria penetrate the body via percutaneous inoculation and inhalation of contaminated water droplets or soil and have recently been shown to infect the olfactory and trigeminal nerves within the nasal cavity following intranasal inoculation (St John, Ekberg et al. 2014, St. John, Walkden et al. 2016). *B. pseudomallei* uses olfactory and trigeminal nerve bundles to cross the cribriform plate by actin-mediated motility facilitated by an unusual actin-recruiting bacterial protein called BimA to enter the central nervous system in the cranial cavity within 24 hours (St John, Ekberg et al. 2014). While there is no known link between *B. pseudomallei* and glioma, the rapid low-level penetration of bacteria into the nerves and brain raise the possibility that long-term low-level bacterial

infections may adversely affect cells of the nervous system and induce molecular and cellular changes as occurs with inflammation and/or the development of glioma.

B. pseudomallei is a unique facultative intracellular bacterium which can survive inside the phagocytic and non-phagocytic cells by inducing giant multinucleated cells (Kespichayawattana, Rattanachetkul et al. 2000)(this unique characteristic will be discussed in detail later). Giant glial multinucleated cell formation is one of the major nuclear and cytological alterations which are usually seen in most of the glioma cells (Fujita, Mizuno et al. 2004).

Neisseria meningitidis

Since 1877, when Anton Weichselbaum identified gram-negative meningococcal bacteria from the cerebrospinal fluid (CSF) of a patient with meningitis, meningococcal infection has been a serious health problem (Rouphael and Stephens 2012). Approximately 10% of people carry meningococcal bacteria in throat and nose without any symptoms but occasionally these bacteria can cause serious disease (Caugant, Hoiby et al. 1994, Johri, Gorthi et al. 2005). Our knowledge of meningococcal pathogenesis and the availability of therapeutic and prophylactic antibiotics and immunizations against important serogroups have been expanded during the last decades, meningococci remains a leading cause worldwide of bacterial meningitis and meningoencephalitis (Rouphael and Stephens 2012). More than 50% of survivors of *N. meningitidis* infection suffer from neuronal loss and neuropsychological disabilities (van de Beek, Schmand et al. 2002). However, the exact mechanism of neuronal damage following *N. meningitidis* infection is not fully clear; bacteria toxins and some cytotoxic products of the immune system may have a role in meningitis neural damage (Braun, Blackwell et al. 2002). *N. meningitidis* frequently colonizes the human nasopharynx asymptomatically, and only a small proportion of bacterial infections proceed to a sustained bacteraemia. In the bloodstream, the bacteria can cause either a deadly septic shock and purpura fulminant and/or cross the blood brain barrier (BBB) to invade the meninges (Coureuil, Join-Lambert et al. 2012). Human genetic polymorphisms are likely to be important determinants of meningococcal infection outcome (Brouwer, Read et al. 2010). *N. meningitidis* is a natural inhabitant of the human nasopharynx mucosa, caudally containing the olfactory epithelium including the dendrites of olfactory neurons, and also innervated by the trigeminal nerve. *N. meningitidis* has been suggested to be capable of invading the brain via the olfactory nerve (Sjölinder and Jonsson 2010). Prior to this thesis, no known direct links between gliomagenesis and *N. meningitidis* were known.

Among limited neuroinvasive bacteria, *N. meningitidis* is one of the few bacteria which can traverse the olfactory nerve to reach the brain and CNS (Sjölinder and Jonsson 2010). The olfactory nerve is the shortest cranial nerve with the olfactory neurons located within the olfactory mucosa. From olfactory mucosa the nerve fascicles cross the cribriform plate to reach the forebrain (Rea 2014). The olfactory nerve fascicles consist of the olfactory nerve axons which are ensheathed by the olfactory ensheathing cells (Doucette 1984). As *N. meningitidis* is among the few known neuroinvasive bacteria which can penetrate the brain via olfactory nerves, we suggest that *N. meningitidis* is a strong candidate to infect the neuroglial cells and alter their morphology and functions towards the acquisition of a malignant phenotype.

2.3.7 Inflammatory responses and gliomagenesis

Since 1863 when Rudolf Virchow noticed leukocytes in neoplastic tissues and suggested that "lymphoreticular infiltrate'' reflects the origin of cancer at sites of chronic inflammation, our understanding of inflammation and inflammatory response in malignancies has been improved. Microbial infections are the most important trigger of chronic inflammation, which can lead to cancer (Parsonnet 1995). Inflammatory disease may increase the risk of developing cancers such as bladder and cervical cancer, and non-steroidal anti-inflammatory drugs decrease the risk of developing certain cancers and mortality (Balkwill and Mantovani 2001).

The role of inflammation in glioma is less clear than other types of cancers and needs more research to elucidate the links between glioma and inflammation. Due to the blood-brain barrier (BBB) that excludes circulating immune cells, microglia constitutes the main cells with immune functions in the CNS, while astrocytes and oligodendrocytes contribute phagocytic roles. In the healthy CNS, microglia are activated by interaction between their receptors such as Toll-like receptors (TLRs) and c-type lectin receptors with pathogen-associated molecular patterns (PAMPs) such as LPS (Galvao and Zong 2013). Activated microglia express MHCII to activate T cells and secrete pro-inflammatory cytokines such as IL-6, interleukin 1 β (IL1β) and tumour necrosis factor α (TNFα). IL1β can allow the CD8+ cytotoxic T-cells and CD4+helper T-cells (Th1, Th2, Th3) to enter the CNS by modulating the BBB (Shaftel, Carlson et al. 2007). Th1 activate pro-inflammatory response by secreting IFNγ, IL12 and nitric oxide by activating microglia and macrophages. They can also activate CD8+ cytotoxic T-cells, which are critical component of the cellular immune response. Th2 has contrary effect on Th1 by secreting IL4, IL10, and IL13, which leads to the activation of B-cells. Th3 which secret TGFβ supress the immune response (Sakaguchi 2005).

Astrocytes also have a significant role in modulating immune responses in the CNS (Farina, Aloisi et al. 2007) and like microglia, astrocytes express TLRs, which can be activated by PAMPs and secrete pro-inflammatory cytokines such as IL-6 to recruit the immune cells to the region (Farina, Aloisi et al. 2007) In the peripheral nervous system, Schwann cells and OECs also have important immune cell functions with OECs being the main phagocytic cell of the olfactory system(Nazareth, Lineburg et al. 2015, St John, Walkden et al. 2016), and Schwann cells also being capable of phagocytosing pathogens. Schwann cells also recruit macrophages to aid the removal of cell debris after injury or infection (Wagner and Myers 1996, Sawada, Sano et al. 2007). Thus, the glia in both the CNS and PNS are responsive to infections and injury, and initiate a cascade of signalling events that influence neighbouring cells. Indeed, it has been shown that inflammatory response may influence neural stem cells and oligodendrocytes precursors which can lead to gliomagenesis (Fitch and Silver 2008). Inflammatory responses play crucial role at different stages of tumour development especially initiation. Inflammation can increase the mutation rates by production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) by neutrophils and macrophages which are capable to induce mutagenesis in DNA (Hussain, Hofseth et al. 2003). Moreover, inflammatory responses can trigger the DNA damage and mutagenesis by producing dextran sodium sulphate (DSS) (Meira, Bugni et al. 2008).

Another mechanism which enhance the tumour initiation by inflammation is the production of growth factors and cytokines which can stimulate the transformation of stem cells to tumour progenitors (Grivennikov, Greten et al. 2010). Some of the inflammatory molecules which are associated with gliomagenesis of neural stem cells are listed in table 2.5

***Table 2-5**. Inflammatory molecules in neural stem cells that have been associated with gliomagenesis*

Inflammatory molecule	Cell of origin	Function
MCP1/CCL2	microglia and astrocytes	neural stem cell migration to inflamed regions
TNFα	macrophages/monocytes	neural stem cell and oligodendrocyte proliferation
Polyamines	activated microglia	neural stem cell proliferation
SDF1	microglia and astrocytes	neural stem cell migration to inflamed regions

Neuro invasive and meningitis bacterial pathogens such as *N. meninigitidis* can cause an inflammatory response by increasing the expression of cytokines and chemokines along with the expression of IκBζ (Borkowski, Li et al. 2014). IκBζ is a transcriptional regulator, involved in NFκB signalling pathway, activation and expression, resulting in production of inflammatory cytokines such as IL6, IL12 p40, GM-CSF, G-CSF (Trinh, Zhu et al. 2008). On the other hand activation of IκBζ can activate the degradation of inhibitory κB (IκB) of NF-κB transcription factor which leads to

the nuclear localization of NF-κB and the expression of regulatory carcinogenic genes (Borkowski, Li et al. 2014),(Trinh, Zhu et al. 2008).

2.4 Multinucleated giant cells

Multinucleated giant cells (MGCs) result from fusion of monocytes or macrophages during inflammation, but the mechanism of formation is not yet elucidated. Langerhans first described these cells in tuberculoid granulomas (Helming and Gordon 2007). In healthy individuals, MGC formation is a part of the natural hybridization of cells to modulate macrophage function. However, multinucleated giant cell formation can also be a result of chronic inflammation due to the persistent pathogens or presence of apoptotic debris following some infections (Quinn and Schepetkin 2009). Although the exact mechanism of multinucleated giant cell formation is not fully understood, it is clear that a number of inflammatory cytokines such as interferon γ, several interleukins (IL-1, IL-3, IL-4 and IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth factors, calcium, integrins and other unknown components of a cellular fusion machinery, participate in formation of multinucleated giant cells (Fais, Burgio et al. 1994),(McInnes and Rennick 1988)(Enelow, Sullivan et al. 1992, Orentas, Reinlib et al. 1992). Out of the cytokines, interferon γ (IFN-γ) and IL-4 have a particularly crucial role in MGC formation. IFN-γ enhances the formation of multinucleated giant cells in a concentration- and time-dependent fashion by increasing the expression of adhesion molecules such as ICAM-1, leading to clustering and adhesion of cells that eventually fuse (Fais, Burgio et al. 1994). IL-4 directly promotes the formation of multinucleated giant cells and engulfment of foreign bodies in STAT6 dependant manner by inducing the expression of proteins such as E-cadherin and dendritic cell-specific transmembrane (Moreno, Mikhailenko et al. 2007). Despite poor knowledge of molecular mechanisms of multinucleated cells formation, some bacterial infection is suggested to induce multinucleation in limited number of cell types (Gasser and Most 1999, Dean and Kenny 2013). *B. pseudomallei* has been shown to induce cell fusion, leading to multinucleate cell formation in RAW264.7 cells, a murine macrophage-like cell line (Kespichayawattana, Rattanachetkul et al. 2000). The role of *B. pseudomallei* infection in multinucleation will be discussed in detail later in this chapter.

2.4.1 Multinucleated giant cells and glioma

The origin of multinucleated giant cells in glioma is not yet understood. It has been shown that these cells remain in the early mitotic phase, undergoing neither fusion nor degeneration (Maeda, Kamata et al. 2005), however, it has not been completely ruled out that cell-cell fusion can also occur in glioma MNCG formation. One possibility of this phenomenon might be the dysfunction of Aurora-B, which affects cytoplasmic cleavage without effect on nuclear division (Goepfert and

Brinkley 2000, Fujita, Mizuno et al. 2004). Aurora-related mitotic kinases regulate cell division and its checkpoints. These kinases are conserved in eukaryotic cells and have been shown to play a key role in cell division (Nigg 2001). Mammals have at least three Aurora-related mitotic kinases subfamilies including; Aurora-A, -B, and –C. However, Aurora A only has a role in centrosome separation and spindle bipolarity, Aurora B plays crucial roles in chromosome segregation/condensation, and cytokinesis. During mitosis, Aurora-B acts by phosphorylating the histone H3 at Ser28 and vimentin at Ser72 in nuclear division and cytokinetic process respectively (Nigg 2001, Carmena and Earnshaw 2003). Aurora-B has been shown to be present but inactivated at the early mitotic phase in glioma multinucleated giant cells, suggesting the aberration of cytoplasmic cleavage is due to the Aurora-B dysfunction (Anderson 1991). The exact mechanism of Aurora-B inactivation is not yet fully understood but one possibility might be auto phosphorylation of Aurora-B which leads to aberration of cytokinesis (Yasui, Urano et al. 2004).

It has been demonstrated that the formation of multinucleated cells requires not only dysfunction of Aurora-B but also downregulation of the tumour p53 protein (p53) (Tsuno, Natsume et al. 2007), a key tumour suppressor. p53 has a key regulatory effect on cell cycle by arresting the cell cycle at G1 in response to DNA damage or preventing polyploidization following mitotic spindle damage which leads to maintenance of euploidy (Kastan, Zhan et al. 1992, Cox and Lane 1995). It has also been demonstrated that polypoidization and aneuploidization are prevented by p53-dependent G1 and G2 checkpoints. Altogether, p53 prevents the formation of multinucleated cells by maintaining euploidy (Harvey, Sands et al. 1993). p53 has an indispensable role in maintaining cell metabolism and cell integrity (Siegl and Rudel 2015). Most intracellular pathogens have evolved multiple strategies to bypass p53 signalling to ensure host cell survival and optimal bacterial replication during infection. To enhance their replication, many pathogens manipulate p53 by enhancing its degradation or suppressing its transcriptional activity (Siegl and Rudel 2015). For instance, HPV and EBV, increase the ubiquitination of p53 leading to degradation by proteasome system (Siegl and Rudel 2015) *Helicobacter pylori*, *Chlamydia trachomatis*, *Shigella flexneri,* respiratory syncytial virus (RSV) and the malarial parasite *Plasmodium yoelii* induce p53 ubiquitination by the activation of phosphoinositide 3-kinase (PI3K)–AKT signalling and human double minute 2 (HDM2) (Groskreutz, Monick et al. 2007).

In the setting of glioma, the loss of p53 function may dysregulate Aurora B to induce the development of multinucleated giant cells by aberrantly arresting the cell cycle and permitting multiple replication of genome without completion of cytokinesis. Intracellular pathogens may therefore synergistically work with the dysregulated Aurora B to induce the multinucleated giant

cell formation via p53 inhibition. When investigating whether neuroinvasive pathogens cause glioma, it is therefore crucial to determine whether they cause the formation of MNGCs.

2.5 Expression of gliomagenesis markers in glial cells

Similar to oncogenesis where multiple cancer oncogene alterations occur, gliomagenesis could potentially represent with modification of oncogenic markers such as TLRs, IL-6 receptor, Cox-2 and MMPs (Weissenberger, Loeffler et al. 2004, Elkington, O'Kane et al. 2005, Kirkby, Zaiss et al. 2013). These markers are explained in more detail below.

2.5.1 TLRs in Microglia and Glioma Cells

The expression of Toll-like receptors (TLRs) on glioma cells and microglia have been under tight investigation to elucidate their contribution in tumour development. The Toll gene, which was first described in Drosophila, has a role in dorsoventral embryonic development and innate immune functions (Anderson 1991). TLRs are evolutionally conserved protein family with 10 functional members (TLR1-TLR10) which recognise pathogen-associated molecular patterns (PAMPs) and initiate an immune response (Deng, Zhu et al. 2014).

TLR2, TLR4 and TLR9 are the only TLRs expressed on glioma cells. Activation of TLR2 with its agonist, peptidoglycan, may activate NF-κB signalling pathway, which ultimately leads to increased cell growth (Echigo, Sugimoto et al. 2012). Activation of TLR4 following interaction with its agonist, LPS, may activate signalling pathways which are involved in regulating cell survival, migration and immune evasion, and resistance to TNF-α treatment. Expression of TLR4 on glioma cells may also influence the expression of specific cytokines that protect cells from apoptosis such as IL-6, IL-8, and MCP-1 (Brat, Bellail et al. 2005, Tewari, Sharma et al. 2009). Interaction of TLR9 and its agonist CpG dinucleotide enhances cell invasion and promotes the growth of glioma stem-like cells by activation of STAT signalling pathway (Brat, Bellail et al. 2005)

2.5.2 The IL-6 pathway in gliomagenesis

The IL-6 pathway plays an important role in tumour and glioma cell growth. The canonical signal transduction via the IL-6 pathway triggers by binding IL-6 cytokine to the IL-6 receptor on the glial cells. Upon receptor activation, Jak kinase family members and ultimately the activation of transcription activators STAT family, particularly STAT3, propagate intracellular signalling cascades. It has been demonstrated that in glioblastoma multiforme, the IL6 pathway is stimulated, resulting in STAT3 activation (Rahaman, Harbor et al. 2002).

Cox-2 and gliomagenesis

It is recently accepted that the alteration in expression of cyclooxygenase-2 (COX-2) has a key role in influencing the development of gliomagenesis in mice (Fujita, Kohanbash et al. 2011). The exact mechanism of Cox-2 in gliomagenesis is not yet fully understood but it has been suggested that Cox-2 can alter the expression of Id1. Id1 belongs to the helix-loop-helix (HLH) family of transcriptional repressors that act as dominant-negative inhibitors of basic-HLH factors (Fujita, Kohanbash et al. 2011). A recent study has shown that Cox-2 is overexpressed in some gliomas, in particular in glioblastoma multiforme (Xu, Wang et al. 2014).

2.5.3 MMP-2 and gliomagenesis

Matrix metalloproteinases (MMPs) have been implicated to play a critical role in gliomagenesis and invasiveness. MMPs exert key functions in Epithelial-Mesenchymal Transition (EMT) in cancer and tumour progression. This biological process allows epithelial cells to undergo multiple biochemical changes to enable them to express mesenchymal phenotypes including enhanced extracellular matrix production, increased migratory capacity, invasiveness and enhanced resistance to the apoptosis (Kalluri and Neilson 2003, Kalluri and Weinberg 2009). A recent study showed that there is a positive correlation between glioma and MMP-2 expression and that MMP-2 expression is significantly correlated with the degree of malignancy of glioma (Wang, Wang et al. 2003).

2.5.4 *Burkholderia pseudomallei* and glioma

As discussed earlier, *Burkholderia pseudomallei* is the causative agent of neurological melioidosis and can infect the olfactory and trigeminal nerves within the nasal cavity following intranasal inoculation. As also covered earlier, the glia of olfactory nerve are OECs and the glia of the trigeminal nerve are Schwann cells (St John, Ekberg et al. 2014). In the case of olfactory nerve infection, the infection leads to the formation of open channels lined by OECs resulting in a direct open conduit from the epithelium into the olfactory bulb in the forebrain through which the bacteria can penetrate the brain (St John, Ekberg et al. 2014). Similarly, in the trigeminal nerve infection by *B. pseudomallei* leads to rapid bacterial entry into the nerve and penetration of the brain after the bacteria traverse the astrocytic glia limitans layer at the junction of the trigeminal nerve and the brainstem (St John, Walkden et al. 2016).

The link between *B. pseudomallei* infection and cancer is to date unknown and unexplored. *B. pseudomallei* is known to cause the formation of MNGCs both via fusion mediated by the protein BimA (Benanti, Nguyen et al. 2015) and cell cycle arrest via the protein CifBP (cell-cycle inhibitory

factor) (Galyov, Brett et al. 2010), suggesting that it is possible *B. pseudomallei* causes cellular changes relating to carcinogenesis. Even if a direct link between *B. pseudomallei* and cancer cannot be established, the fact that it induces MNGC formation therefore renders this pathogen very useful for laboratory studies of MNGC formation.

2.6 Major virulence factors of *Burkholderia pseudomallei*

Like other pathogenic bacteria, *B. pseudomallei* must overcome the host defence system including cellular and humoral immune responses. By using its arsenal of the virulence factors, *B. pseudomallei* evades host immune responses and persists *in vivo*. Known virulence factors for this pathogen are listed in Table 2.6.

Table 2-6 *Known virulence factors of Burkholderia pseudomallei*

Virulence factor	Description
Capsule	Capsule production is strongly related to virulence of many invasive bacteria. *B. pseudomallei* produces an extracellular polysaccharide capsule -3(-2-O-acetyl-6-deoxy-Beta-D-manno-heptopyranose-)1- which is an essential virulence factor. *B. pseudomallei* capsule contributes to the persistence of the organism in the serum by evasion of the complement cascade and reducing C3b-mediated opsonization and phagocytosis (Isshiki, Matsuura et al. 2001),(Reckseidler-Zenteno, DeVinney et al. 2005).
Quorum sensing	Like other gram negative bacteria, *B. pseudomallei* regulates gene expression in cell density mechanism incorporating N-acyl-homoserine lactone (AHL) signal molecules including N-octanoyl-homoserine lactone, N-decanoyl-homoserine lactone, N-(3-hydroxyoctanoyl)-L-homoserine lactone, N-(3-hydroxydecanoyl)-L-homoserine lactone and N-(3-oxotetradecanoyl)-L-homoserine lactone which promotes bacterial pathogenicity. These products are encoded by *luxI* homologs while *LuxR* encodes transcriptional regulators which mediate gene repression or expression following association with their cognate AHL(s) (Schuster, Lostroh et al. 2003, Ulrich, Deshazer et al. 2004)
LPS	Lipopolysaccharide (LPS) is one of the most important virulence factors and antigenic components of *B. pseudomallei.* LPS consists of unbranched heteropolymers with repeating D-glucose and l-talose units with the structure -3)-β-D-glucopyranose-(1, 2, 3)-6-deoxy-α-l-talopyranose-(1. The only difference between *B. pseudomallei* LPS and that of other gram-negative pathogens is that it contains *O*-acetyl or *O*-methyl substitutions at the 2′ position of the talose residue. The LPS can increase the surveillance of *B. pseudomallei* in serum by preventing the alternative pathway of complement system and resistance to macrophage killing (Arjcharoen, Wikraiphat et al. 2007),(Wikraiphat, Charoensap et al. 2009).
Type III secretion system	T3SSs are "molecular syringes" that inject the effector protein molecules into the host to invert host cell processes to the benefit of bacteria (Burtnick, Brett et al. 2008). *B. pseudomallei* possesses three T3SS gene clusters (*T3SS-1, T3SS-2* and *T3SS3*), but only T3SS3 has been implicated in animal pathogenesis. T3SS3 is necessary for bacterial escape from phagosomes and endosomes into the cytosol and also caspase 1-induced pyroptosis (Stevens, Haque et al. 2004),(Sun, Lu et al. 2005).

Type VI secretion system (T6SS)	In *B. pseudomallei*, T6SSs accounts for approximately 2% of whole coding region of the organism genome. The studies showed T6SSs assists organism's survival in the macrophages, replication and spreading to other cells by actin motility and **multi nucleated giant cells formation**. The cell-to-cell spreading helps the organism to be safe from antimicrobial substances in the extracellular milieu (Schwarz, West et al. 2010).
Flagella	Like other bacteria, the flagellum of *B. pseudomallei* is considered a virulence factor. The 43 kDa flagellin protein on *B. pseudomallei* surface is required for motility and invasion (DeShazer, Brett et al. 1998),(Brett, Mah et al. 1994).The major subunit of flagellum or FliC is encoded by *fliC*. FliC plays an important role in innate and adaptive immune response (Cullender, Chassaing et al. 2013). In animal models, it has been shown that antibodies against *B. pseudomallei* flagellin remarkably reduced the motility of the organism and provide the passive protection against the organism infection (Brett, Mah et al. 1994). *fliC* mutants shows attenuated virulence during intranasal infection of mice which might be due to the loss of motility or adhesion or both (Chua, Chan et al. 2003). Moreover, flagella assist the organism to be taken up by the cells in peritoneal cavity, facilitate the intracellular replication, and spread which is attenuated in *filC* mutants(Chua, Chan et al. 2003).
Type II secretion system (T2SS)	In *B. pseudomallei* T2SS is required for secretion of several exoproduct enzymes such as lipase, phospholipase C and protease (DeShazer, Brett et al. 1999).
Type IV pili (TFP)	Similar to other gram-negative bacteria type IV pili (TFP) is an important virulence factor responsible for survival, replication and attachment of bacteria to the host cells. Based on the presence of conserved motifs TFP is divided into two subclasses, IVa and IVb (Strom and Lory 1993). It has been shown the mutation in TFP *pliA*, which encodes a type IVA pilin subunit, decreased the attachment of organism to cultured respiratory cell lines, which means PilA is necessary for attachment of bacteria to the host cells (Strom and Lory 1993).
Type III O-PS and Type IV O-PS	In addition to the capsule and LPS O-antigen, *B. pseudomallei* encodes type III O-PS and type IV O-PS. Mice studies revealed that the intraperitoneal injection of type III O-PS or type IV O-PS mutants increased the mean times to death in comparison with wild-type *B. pseudomallei* (rom 3 days to 7- 11 days) (Sarkar-Tyson, Thwaite et al. 2007).
Phospholipase C (Plc-3)	As for many other bacteria, phospholipase C plays an important role in the pathogenesis of *B. pseudomallei.* This enzyme cleaves phospholipid phosphatidylcholine (PC) to produce phosphorylcholine and diacylglycerol (DAG). Analysis of *plc* mutant behaviour in eukaryotic system model revealed that both Plc1 and Plc2 contribute to the plaque formation by lysis of the cells in culture media However, only Plc2 is required for cell cytotoxicity (Korbsrisate, Tomaras et al. 2007).

2.6.1 Host-pathogen interaction at cellular level

B. pseudomallei is a facultative intracellular pathogen, which replicates inside phagocytic cells and epithelial cells. The following section focuses on host-pathogen interaction at cellular level.

Adhesion to host Cells

Adhesion is considered a vital initial step in pathogenesis of many pathogens including *B. pseudomallei*. A number of *in vivo* and *in vitro* assays showed the significant role of PilA in the adherence of *B. pseudomallei* to the host cells. The mutated organism in PilA showed reduced

adhesion of organism in compare to the wild type, which suggests that type IV pili plays significant role in *B. pseudomallei* adhesion and virulence (Essex-Lopresti, Boddey et al. 2005, Stone, DeShazer et al. 2014)

Type III Secretion System

An important virulence factor in *Burkholderia* spp. is the type III secretion system (T3SS). *B. pseudomallei* possesses a body of T3SSs to secrete and deliver subset of effector proteins into the host cell cytosol. *B. pseudomallei* contains three T3SSs, out of which only *Burkholderia* secretion apparatus (Bsa) T3SS is required for bacterial virulence (Brett, Mah et al. 1994). Bsa T3SS forms a secretion body by spanning both bacterial membranes, constituting a so-called needle complex. Upon receiving appropriate *in vivo* signals, two types of proteins are secreted via the Bsa T3SS: translocators and effectors. Following the contact of bacteria with host cells, three translocator proteins (BipB, Bipc,and BipD) localize at the tip of the needle complex and form a pore in the host cell membrane, resulting in delivery of effector proteins into the host cells (Gong, Lai et al. 2015). The effector proteins then interfere with host cell signal transduction pathways in a manner that benefits the pathogen. It has been postulated that *B. pseudomallei* possess several effector proteins such as BopA, CifBp/CHBP but BopE is the most important organism effector protein secreted by Bsa T3SS (Galyov, Brett et al. 2010) (Stevens, Haque et al. 2004).

T3SS Translocators

The Bip translocator proteins of Bsa T3SS (BipB, BipC, and BipD) facilitate effector proteins delivery across the host cell membranes into the host cell cytosol. Studies on bip mutants showed that the organism virulence is attenuated and also the appropriate delivery of effector proteins is prevented (Stevens, Haque et al. 2004)

T3SS Effectors

Bsa T3SS and secreted effector protein BopE induce the uptake of *B. pseudomallei* into non-phagocytic cells. BopE exerts its function by acting as a guanine nucleotide exchange factor for host cell GTPases to initiate the host cell membrane ruffling and actin rearrangement (Stevens, Stevens et al. 2005). T3SS effector proteins, especially BopA, are also important to subvert the autophagy mechanism (an important host defence mechanism against intracellular pathogens that delivers cytoplasmic constitutes to the lysosome) and survival of the organism within the host cells. The *bopA* mutant studies showed that in the lack of *bopA* the survival of the organism reduced dramatically and co-localization of autophagy markers such as LC3 increased (Gong, Cullinane et al. 2011)

CifBP is the other effector *B. pseudomallei* protein, which belongs to the cyclomudolins family. Cyclomodulins can interfere with the eukaryotic cell cycle to promote host cell proliferation or inhibit it by blocking cell cycle progression. it has been suggested that CifBP is secreted by Bsa T3SS and contribute to form or maintain the multinucleated giant cells (MNGC; discussed below) during *B. pseudomallei* infection (Galyov, Brett et al. 2010).

Type VI secretion system (T6SS)

T6SS is a recently reported secretion system in gram-negative bacteria that exports two main proteins including hemolysin-coregulated protein (Hcp) and valine-glycine repeat protein G (VgrG). It has been demonstrated that T6SS mutants exhibit growth, actin-based motility, MNGC formation (discussed below) and intracellular replication defects in RAW 264.7 murine macrophages (Burtnick, Brett et al. 2008). However, the exact mechanism of T6SS effectors is not fully understood, *B. mallei* model suggest that T6SS is active in phagocytic vacuoles and effector proteins such as VgrG translocated into the cytosol in a process that requires bacterial endocytosis (Burtnick, Brett et al. 2008).

Actin-Based Motility

Following the lysis of entry vacuole, *B. pseudomallei* enter the cytosol and induce their propulsion by polymerizing actin at one bacterial pole. It has been shown that actin polymerization by *B. pseudomallei* is different from those evolved by Listeria, shigella and Rickettsia (Ma, McAuley et al. 2009). It has been demonstrated that *B. pseudomallei* actin based motility is mediated by protein BimA which is located at the one pole of bacteria (Stevens, Haque et al. 2004).

Multinucleated Giant Cell Formation

One of the unique features of the *B. pseudomallei*, which has not been observed in other intracellular pathogens possessing T3SS, is formation of MNGCs. Although the exact molecular mechanism of MNGC formation is not yet fully elucidated, this phenomenon may be due to the BimA-mediated actin-based motility (Suparak, Kespichayawattana et al. 2005, Galyov, Brett et al. 2010). Moreover, genetic analysis has demonstrated that T3SS effector protein BipB induces MNGC formation (Suparak, Kespichayawattana et al. 2005). In addition, vacuolar escape is the other critical factor in development and formation of MNGC, as *B. pseudomallei* Bsa T3SS mutants demonstrated delayed vacuolar escape and ultimately delayed MNGC formation in RAW 264.7 cells (Galyov, Brett et al. 2010). MNGC formation may be a key factor of supporting the growth of the organism inside the host cell, cell-to-cell spread, persistence of organism in the host cell and protection of the organism from humoral and innate immune responses (Galyov, Brett et al. 2010).

However, the function of T6SS is more diverse than T3SS, its role in bacterial virulence, actin polymerization, and formation of multinucleated giant cells (MNGCs) *in vitro* is critical (Burtnick, Brett et al. 2008, Chen, Wong et al. 2011) Previous studies on RAW 264.7 macrophage cells following the infection with *B. pseudomallei* showed that the multinucleation is a unique phenotype of T6SS (Bonemann, Pietrosiuk et al. 2009). On the other hand, the role of T3SS in multinucleation may be due to its regulatory effect on T6SS expression, because the expression of T6SS is dependent on bsaN a central regulatory gene located inside the T3SS cluster (Chen, Wong et al. 2011)The fact that *B. pseudomallei* via several mechanisms can induce MNGC formation suggests that this bacterium may in fact cause cellular changes relating to cancer. As key bacterial proteins are known to cause MNGC formation via either fusion or cell cycle arrest, it suggests that selective mutation to delete specific bacterial proteins can allow us to study how fusion or cell cycle inhibition in isolation affects cells in the laboratory, rendering *B. pseudomallei* a useful tool for studying downstream effects of multinucleation.

2.7 *N. meningitidis* and olfactory epithelial infection

As discussed earlier, *N. meningitidis* is the causative agent of septic shock and meningitis. *N. meningitidis* can infect the nasal cavity, olfactory nerve and potentially trigeminal nerves and enter into the CNS. Upon nasopharynx bacterial infection, N-cadherin remodelling and tissue lesion especially at the olfactory epithelium assist the bacterial entry into the CNS (Sjölinder and Jonsson 2010). *N. meningitidis* binds to CD46 and carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) expressed on the basolateral surface of the nasopharyngeal epithelium (Johansson, Rytkonen et al. 2003). Binding and colonization of *N. meningitidis* on nasopharynx epithelium leads to conspicuous damage and tissue atrophy by attenuation of N-cadherin expression especially at the olfactory epithelial region and facilitate bacterial invasion to CNS through the olfactory nerve.

2.7.1 Major virulence factor of *N. meningitidis*

Traditionally, *N. meningitidis* has been classified into the different serogroups (A,B,C,Y,W135 and X) based on the antigenic variation of the capsular polysaccharide, serotypes based on the PorB outer membrane protein (OMP), serosubtypes based on the PorA OMP and immunotype based on the lipopolysaccharide(LPS) (Frasch, Zollinger et al. 1985).The major virulence factors of *N. meningitidis* are listed in Table 2.7.

Table 2-7 *The main virulence factors of N. meningitidis*

Virulence factor	Description
Capsule	Capsular polysaccharide is the main virulence factor determinant for pathogenic *N. meningitidis*. So far, 13 serogroups have been described for *N. meningitidis* based on the differences of their capsule. Out of the 13 serogroups only 6(A,B,C,Y,W135,X) cause invasive meningococcal disease (Hill, Griffiths et al. 2010). The invasive meningococcal polysaccharide capsule except serogroup A is composed of sialic acid derivatives. Serogroup A capsular polysaccharide is composed of N-acetyl-mannosamine-1-phosphate(Blacklow and Warren 1962). Serogroup B has a distinct capsular polysaccharide contains α(2–8)-linked sialic acid homopolymer which is identical in structure to the human fetal neural cell-adhesion molecule (NCAM). Due to this similarity immune response generated against serogroup B capsule is poor (Zimmer and Stephens 2006).
Cell Envelope	Like all gram negative bacteria, cell envelope in *N.meninigitidis* consists of an outer membrane (OM), a peptidoglycan layer, and a cytoplasmic or inner membrane. The outside of the outer membrane consists of LPS and proteins and inside composed of phospholipids especially phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) (Nikaido 1999). The peptidoglycan of *N. meningitidis* consists of two layers with varied cross linking (almost 40%) and O-acetylation which is resistant to the lysozyme and other muramidase. The peptidoglycan is usually recognized by innate immune system (Clarke and Dupont 1992, Quintela, Caparros et al. 1995)
Lipopolysaccharide (LPS)	Lipopolysaccharide in *N. meningitidis* is different from other gram negative bacteria. In *N. meningitidis*, LPS lacks a repeating O-side chain of LPS found in entire gram negative bacteria and called lipooligosaccharide (LOS) (Kahler and Stephens 1998). LOS in *N. meningitidis* plays an important role in adherence and inactivation of innate immune responses (Plant, Sundqvist et al. 2006). Meningococcal LOS consists of 3 parts: lipid A, Core and short oligosaccharides which is very highly variable. Binding of LPS to the series of receptors and transfer molecules such as LPS-binding protein (LBP), CD14, and myeloid differentiation protein 2 (MD2), Toll-like receptor 4 (TLR4) can induce the secretion of various cytokines such as IL-6 and TNF-α which result in endothelial damage and capillary leakage (Braun, Blackwell et al. 2002). Also LOS can trigger the release of the reactive oxygen species (ROS) and nitric oxide (NO)(Rouphael and Stephens 2012).
Pili	The attachment on human upper respiratory mucosal surfaces by capsulated *N. meningitidis* is mediated by pili (Pinner, Spellman et al. 1991). Pileated meningococci attachment to the human nasopharyngeal cells is more achievable than non-pileated meningococci attachment (Proft and Baker 2009). Pili in *N. meningitidis* consist of type IV pilin families and undergo both antigenic, and phase variation (Kahler, Martin et al. 2001).
Opacity proteins	Opacity (Opa) proteins mediate a variety of interactions between the bacterium *N. meningitidis* and the host which is crucial in both asymptomatic colonization of the nasopharynx and the sporadic occurrence of meningococcal disease (Callaghan, Jolley et al. 2006). Opa proteins assists the cellular attachment and invasiveness by interacting with multiple members of the CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) family (Virji, Watt et al. 1996).
PorA and PorB	*N. meningitidis* expresses two distinct porins, PorA and PorB which are involved in host cell interactions and as targets for bactericidal antibodies (Tzeng and Stephens 2000). While PorA is a major component of outer membrane vesicle-based vaccines and a target for bactericidal antibodies, PorB is the major outer membrane porin that inserts in membranes, induces Ca2+ influx, the TLR2 activation and apoptosis (Tzeng and Stephens 2000)

2.7.2 Host- pathogen interaction at cellular level

The *N. meningitidis* as an obligatory commensal organism usually residing within the nasopharynx. Adherence plays an important role in the colonization of the nasopharyngeal mucosal surface. This crucial step depends on a number of bacterial surface structures such as Type IV pili and opacity proteins (Chen and Seifert 2011).

Pili

The isolates from both asymptomatic carriers and patients are heavily piliated and adhere more efficiently to epithelial cells than non-piliated isolates (Higashi, Lee et al. 2007). Similar to other Type IV pilus family, meningococcal pili undergo rapid extension, retraction that consequently leads to the twitching motility (Merz, So et al. 2000). The major feature of the pathogenic *N. meningitidis* pili is that they are highly glycosylated. This glycosylation is not a requirement for pilus biogenesis but it has been shown that non-glycosylated pilins are more aggregative and tend to form larger bundles (Parge, Forest et al. 1995). Large bundle formation might increase the adhesiveness by promoting the bacteria-bacteria interactions and strengthen the interaction of other adhesins presented on bundles with cellular receptors (Marceau, Beretti et al. 1995) (Marceau and Nassif 1999). CD46 has been recently recognized as a pilus receptor for pathogenic *N. meningitidis* attachment but the associated molecules for this attachment have not yet been identified. The consequence of this initial attachment might be sending the signals to the host cells (Nassif 1999).

Opa/Opc proteins

The outer membrane Opa-associated (class V) proteins mediate interaction between only unencapsulated meningococci and eukaryotic cells (Nassif 1999) (de Vries, Cole et al. 1998).These proteins facilitate interaction and attachment of unencapsulated *N. meningitidis* with CD66 family expressed on epithelial, leukocytes and endothelial cells (Virji, Alexandrescu et al. 1992). Cell surface proteoglycans are the main and major receptors of Opc. However the exact mechanism of this interaction is not yet fully understood; Opc might assist the attachment via vitronectin binding in a trimolecular complex (Opc, vibronectin and integrin αvβ3) on the epithelial surfaces (Virji, Makepeace et al. 1994). The other receptors of Opa proteins on epithelial and endothelial cells are CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) which belongs to the immunoglobulin superfamily (Virji, Watt et al. 1996). Several studies have shown that LOS sialyation negatively regulates Opa and Opc interactions with the host cells (van Putten 1993, de Vries, Cole et al. 1998).

Porins

Porins (PorA and PorB) are the other outer membrane components which promotes bacterial entry, nucleating actin and reorganization during infection in the host cells (Nassif 1999).

IgA protease

The other crucial factor in bacteria-host interaction is IgA 1 protease which impact the mucosal colonization and possess significant role in the intracellular survival by cleaving the LAMP1 and preventing phagolysosomal fusion (Ayala, Lin et al. 1998), Colonization and penetration of the respiratory mucosa

Capsule

Neisseria meningitidis polysaccharide capsule is the main component of the organism to enhance the survival of the organism outside the host (Diaz Romero and Outschoorn 1994). In most of the non-epidemic situations, most of the asymptomatic individuals carry both capsulated and acapsulated strains, whereas, in epidemic situations which are usually seen in sub-Saharan meningitis belt, carriage of capsulate phenotypes is more common (van Deuren, Brandtzaeg et al. 2000, Leimkugel, Hodgson et al. 2007).

Pili and adhesins

It is reasonable that firm and fast adhesion of *N. meningitidis* to mucosal epithelial cells would be the essential step to avoid the pathogen being flushed away by the flow of mucus. However, the main adhesive property of capsulated *N. meningitidis* belongs to the pili which initiate attachment to the epithelial cells (Virji, Alexandrescu et al. 1992, Proft and Baker 2009). The capsule is the essential component for the meningococcal survival in the environment, but it may have adverse effects on the bacterial colonization ability by hindering the surface-expressed adhesins. Thus, phase variation, which is usually seen in capsule genes expression, may be beneficial following pili attachment (Hill, Griffiths et al. 2010). Pili is, however, considered as the primary adhesin which can initiate attachment in capsulated strains, outer membrane proteins such as PorA, PorB, Opa and Opc might be counted as essential for adhesion (Hill, Griffiths et al. 2010).

As mentioned before, CEACAM is one of the main Opa receptors on epithelial and endothelial cells. It has been demonstrated that CEACAM expression levels may be influenced by inflammatory cytokines and be subjected to up-regulation following the inflammation (Dansky-Ullmann, Salgaller et al. 1995, Fahlgren, Baranov et al. 2003). Thus, at high level of inflammatory response, increasing the Opa–CEACAM interaction may assist the bacteria to invade the host cells (Griffiths, Bradley et al. 2007, Rowe, Griffiths et al. 2007). Thus, inflammation induced by a prior viral

infection may assist the meningococci penetration into the epithelium and blood without capsule assistance (Muenzner, Rohde et al. 2005).

The molecular phase and antigenic variations on surface molecules allow the organism to evade from immune system during the infection which leads to generate the variants with altered abilities to colonize and augmented mucosal penetration ability(Virji, Alexandrescu et al. 1992).

2.8 Energy production in cancerous cells, Glycolysis versus oxidative phosphorylation (OXPHOS)

Unlike normal cells, most of the cancerous cells rely on the glycolysis to generate ATP for their energy (Zheng 2012). This phenomenon for energy production was describe for the first time by Otto Warburg in the 1920s, which is known as the Warburg effect (Koppenol, Bounds et al. 2011). It has long been believed that the aerobic glycolysis was due to the impairment in the mitochondria function in cancerous cells. However, recent investigations have shown that the mitochondrial OXPHOS is intact in most cancerous cells (Zheng 2012).

Increasing the glycolysis in cancerous cells provide selective advantage for growing and proliferation through rapid ATP production and maintaining constant levels of glycolytic intermediates as macromolecular precursors. In Warburg effect, cancer cells can metabolise the glucose 10-100 times faster than complete metabolism of glucose in mitochondria (Liberti and Locasale 2016). Moreover, increased glucose consumption in glycolysis provides more carbon sources to synthesize cellular blocks such as nucleotides, amino acids and lipids for supporting the biosynthesis of the highly proliferative cancerous cells (Lunt and Vander Heiden 2011).

2.8.1 Glucose Metabolism in glioma cells

In recent years, our understanding of the metabolism regulation in tumour cells has dramatically improved. Enhanced glycolysis, resulting in increased glutaminolysis and lipogenesis are the prominent features of the glioma cells (Wolf, Agnihotri et al. 2010, Maher, Marin-Valencia et al. 2012). In normal conditions and normal tissues, most of the ATP is generated in mitochondria via OXPHOS of glucose; conversely, less than 10% of ATP is generated from glycolysis during conversion to lactate (Ainscow and Brand 1999). Increased glucose utilization is one of the main characteristic and early recognized biochemical markers of the gliomagenesis (Hatanaka 1974). Glucose transporter 1 and 3 (GLUT-1 and 3) are the main glucose transporters to uptake and transport glucose into the glioma cells (Griguer, Oliva et al. 2005). Unlike normal brain cells, glioma cells are exposed to varied oxygen gradients that directly influence their metabolism. Glioma cells are usually exposed to metabolic stresses such as hypoxia. In hypoxia conditions,

glial tumours are adapted to the stressed conditions by activating the Hypoxia inducible transcription factors (Hif-1α and Hif-2α), resulting in a shift towards glycolysis and angiogenesis (Ziello, Jovin et al. 2007). Hypoxia conditions can coordinate the adaptation of glioma cells to metabolic stresses by induction of several glycolytic enzymes, glucose transporters, and lactate transporters (Wang, Jiang et al. 1995, Brat and Mapstone 2003). Hif1α transcription factor in hypoxia promotes key contributor of Warburg effect such as Hexokinase-2 (Wolf, Agnihotri et al. 2010). HIF-1α has four major effects on glioma cells; first, this enzyme increases the GLUT-1 expression during the hypoxia, GLUT1 is activated as a response to HIF-1α in hypoxic conditions especially in glioblastoma multiforme (Rooj, Bronisz et al. 2016). In addition, HIF-1α contributes to epithelial-to-mesenchymal transition observed in most of the glioma cells (Xu, Jiang et al. 2015). As mentioned before, this transition is an essential step in hyper-plasticity that most glioma cells exhibit. Hif1-α also induce the transcription of vascular endothelial growth factor (VEGF) which is crucial in gliomagenesis (Carroll and Ashcroft 2006). In addition to the angiogenesis, VEGF receptors can upregulate the GLUT-1 synthesis and trafficking to its location on the cellular membrane (Sone, Deo et al. 2000). It shows that HIF-1α has ability to increase the glucose uptake via upregulation of GLUT-1 expression (Suh and Han 2013). Finally, HIF-1α upregulates the pyruvate dehydrogenase kinase α (PDK1) expression, which normally increases the ATP production via glycolysis (Kim, Tchernyshyov et al. 2006).

In comparison with normal brain cells, glioma cells express higher level of hexokinase-2, which is the first enzyme in glycolysis (Wolf, Agnihotri et al. 2010). On the other hand, Hif-1α can activate lactate dehydrogenase and shuffling lactate into the extracellular space (Semenza 2003)

Hypoxia and Warburg effect ultimately increase lactate production, which is known as an important factor in tumour progression and maintenance (Romero-Garcia, Moreno-Altamirano et al. 2016). Lactate production provokes inflammatory responses that attract macrophages to the site of the glioma cells(Doherty and Cleveland 2013). In inflammation sites, macrophages can secrete cytokines and growth factors, which lead to the tumour cell growth, invasion and metastasis (Shime, Yabu et al. 2008), and lactate can induce signalling molecules to trigger cell migration, tube formation, and tumour angiogenesis (Vegran, Boidot et al. 2011, Sonveaux, Copetti et al. 2012).

2.9 Aims

Our group has previously shown that pathogenic bacteria can rapidly penetrate the brain via the olfactory and trigeminal nerves. Importantly, the bacteria can enter at such low levels that host immune responses are not always activated. We have also shown that the glial cells of the

peripheral nerves, OECs and Schwann cells can phagocytose and destroy the bacteria, but in some conditions the bacteria can persist and replicate intracellularly within the glia. Glioma are cancers of the nervous system in which the glial cells are the primary affected cell type. While some viruses have been identified as causative agents of some glioma, there are few reports of bacteria as causative agents.

Preliminary work in our group has found that *B. pseudomallei* infection of peripheral glia can lead to the formation of multi-nucleated cells. As multi-nucleated cells are a characteristic of glioma, it raises the question of whether bacterial infections of glia can initiate a cascade of events consistent with the formation of glioma.

2.10 Hypothesis

We suggest that certain neuroinvasive bacteria can infect glial cells and induce inflammatory and/or cancer-related molecular and cellular alterations in these cells. The overall hypothesis addressed in this thesis was that low-level *B. pseudomallei* and *N. meningitidis* invasion of glial cells (trigeminal Schwann cells) lead to the formation of MNCs via the mis-regulation of gliomagenesis related molecules, and that the formation of glial MNCs may be the initiating factor in the formation of gliomas (Fig. 1.3).

Significance

Of all cancers, 16 % are thought to have a microbial cause (de Martel, Ferlay et al. 2012). What remain unknown are the cellular mechanisms by which microorganisms cause cancer, in particular within the brain. Brain cancer constitutes a large socioeconomic burden in Australia and overseas, is the most common cancer causing death in children, and the most lethal cancer in adults under 40 years old in Australia; a person in Australia is diagnosed with brain cancer every five hours (Australian Institute of Health and Welfare 2017). Gliomas constitute 81 % of primary malignant brain tumours (Goodenberger et al., 2012), and therefore, determining causative factors in the initiation of gliomas will lead to new strategies to treat cancers of glial origin. While bacteria have been suggested to be associated with gastric cancer (*Helicobacter pylori*) (Marshall et al., 1984), and may be a causative agent of some lung cancers (*Chlamydia pneumoniae*) (Chaturvedi et al., 2010) no evidence of bacterial initiation of brain cancers has been reported, except for one study suggesting that *Brucella* species may be implicated in medulloblastoma (B. Zhang et al., 2011a). Understanding how neuroinvasive bacteria modulate glial cells also provides an increased understanding of the pathophysiology of glia in CNS infections, and may have implications in other CNS disease. For example, bacterial infections are now a recognised factor

in the development of Alzheimer's disease (Shima et al., 2010), as well as a strong contributing factor to demyelinating disorders (Libbey et al., 2014).

The overall hypothesis was addressed using the following specific Aims/Hypotheses:

AIM 1: To determine whether trigeminal Schwann cells form from mice multinucleated cells (MNCs) following infection with *B. pseudomallei* and *N. meningitidis* .

Hypothesis 1. Trigeminal Schwann cells will become multinucleated after infection with these bacteria.

Justification: *B. pseudomallei* induces multinucleation of other cell types (Kespichayawattana et al., 2000a) and preliminary data from our laboratory has previously shown that glial cells can become multinucleated following *B. pseudomallei* infection (Nazareth, Delbaz et al., unpublished data), as well as after infection with *Chlamydia muridarum*, another neuroinvasive pathogen (Nazareth, Walkden et al., unpublished data). We therefore hypothesised that a common factor for bacteria capable of invading the nervous system, including *N. meningitidis*, is the ability to induce multinucleation of glial cells.

AIM 2: To determine the changes in expression of molecules associated with gliomagenesis and cancer markers in trigeminal Schwann cells following the infection with *B. pseudomallei* and N. meningitidis

Hypothesis 2. *B. pseudomallei* and *N. meningitidis* will alter the expression of gliomoagenesis-related molecules, potentially accompanied by other cellular changes such as atypical nuclei.

Justification: *B. pseudomallei* can alter the expression of inflammatory mediators in glial cells, some of which are also implicated in cancer (Vincent, Choi-Lundberg et al. 2007, Leung, Chapman et al. 2008, Harris, West et al. 2009, Herbert, Harris et al. 2012). Epithelial-Mesenchymal Transition (EMT) as a main biological process involved in initiation of carcinogenesis was studied following the infection with *B. pseudomallei* and *N. meningitidis* serogroup B. Our results suggest that following the infection with *B. pseudomallei* and *N. meningitidis*, EMT occurs by overexpression of both inflammatory mediators EMTand EMT markers in trigeminal Schwann cells.

AIM 3: To assess the regulation of glycolysis related enzymes in trigeminal Schwann cells infected with *B. pseudomallei*.

Hypothesis 3. *B. pseudomallei* will induce Warburg effect (the phenomenon in which cancer cells produce additional energy through high rate of oxidative glycolysis, this phenomenon has been fully described in the introduction in) in trigeminal Schwann cells.

Justification: *B. pseudomallei* can shift the energy production in trigeminal Schwann cells mainly from oxidative phosphorylation (OXPHOS) to glycolysis in aerobic condition known as Warburg effect. As a result, there is lactic acid production which decreases the cellular pH and ultimately triggers the bim-A gene (responsible for multinucleation) expression in *B. pseudomallei*.

Chapter 3: Material and Methods

3 Methodology

3.1 *Burkholderia pseudomallei* strains and growth conditions

Wild type (wt) MSHR520 and *ΔbimA* mutant *B. pseudomallei* MSHR520 *ΔbimA* (generously donated by Professor Ifor Beacham, Institute of Glycomics, Griffith University) were grown in liquid Luria broth (LB) media with shaking at 37 °C. Following overnight incubation, bacterial dilutions were prepared in Dulbecco's phosphate buffered saline (DPBS) to yield a multiplicity of infection (MOI) of 75:1 (75 bacteria per cell) based on the number of seeded glial cells.

As determined by the University Biosafety Committee, due to the potential risk factor of handling *B. pseudomallei*, all the experiments needed to be performed within PC3 standard laboratories.

3.2 *Neisseria meningitidis* strain and growth conditions

N. meningitidis serogroup B (C311#3) were grown in Brain Heart Infusion (BHI) agar at 37 °C in the presence of 5 % CO_2 for 16 -18 h. Following overnight incubation, one bacterial colony was used to inoculate a 3-mL culture to an optical density at 600 nm (OD600) of 0.05. Following the inoculation the dilutions were prepared in DPBS to yield MOI 10:1 based on the number of seeded glial cells.

3.3 Animal and Cell culture

For the primary culture of glia, we used S100ß-DsRed transgenic mice, as the S100ß promoter drives expression of DsRed resulting in glial cells being easily visualised by DsRed expression (Windus, Claxton et al. 2007). All procedures were carried out with the approval of the Griffith University Animal Ethics Committee under the guidelines of the Australian Commonwealth Office of the Gene Technology Regulator (NLRD/009/15_Var4).

Postnatal day 7 pups were decapitated. The olfactory mucosa overlying the nasal septum was dissected out for preparations of olfactory ensheathing cells (OEC) and the trigeminal nerve lying on the basal surface of the cranial cavity was dissected out for trigeminal Schwann cells. Explants of the different glia preparations were separately plated in wells that had been coated with Matrigel basement membrane matrix (BD Bioscience, 1:10) and were maintained in glia medium containing Dulbecco's Modified Eagle Medium containing 10 % foetal bovine serum, G5 supplement (Gibco), gentamycin (Gibco, 50 mg/mL) and l-glutamine (200 μM) at 37 °C with 5 % CO_2 for 5 days. Cells were replated into plastic 24-well plates and grown to 80 % confluency (Windus, Claxton et al. 2007, Amaya, Ekberg et al. 2015).

3.4 Cell debris preparation

Axonal cell debris was generated by dissecting out the nerve fibre layer of the olfactory bulb of an OMP-ZsGreen mouse and using TrypLE express (Life Technologies) and collagenase (0.1 mg/ml, Life Technologies) for 30 min to partial digesting it. The reaction was stopped by addition of fetal bovine serum (Bovogen), followed by centrifuging for 5 min and resuspending in DMEM and triturated using a syringe with a 27 gauge needle.

3.5 Formation of multinucleated cells in trigeminal Schwann cell and olfactory ensheathing cell (OECs) cultures after infection with *B. pseudomallei*

Immunofluorescence microscopy on trigeminal Schwann cells and OECs was performed in order to study the multinucleated cell formation after infection with *B. pseudomallei*. The primary trigeminal Schwann cells and OECs were seeded and cultured at the density of 5000 cells in 8-well chambers, incubated and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10 % Fetal Bovine Serum (GIBCO) and 1x G5 (ThermoFisher scientific) for 12 h in 5 % CO_2 at 37 °C in a humidified incubator. After 12 h, cell debris were added and then cells were infected with *B. pseudomallei* (MOI 75:1) for 24 h. on the other hand to study the effect of cell debris only and bacteria only on the cells, cell debris and bacteria were added separately to the cells. Following the incubation time, cells were rinsed in 1x HBSS and were fixed for 20 min in 4 % paraformaldehyde (PFA) in Dulbecco-Phosphate Buffered Saline (DPBS). After fixation, the cells were rinsed in DPBS 3 times for 5 min. Subsequently, cells were washed and incubated in blocking buffer for 1 h before the application of primary antibody for *B. pseudomallei*. Rabbit anti-*B. pseudomallei* antibody raised against the sarkosyl-insoluble fraction of outer membrane vesicle of *B. pseudomallei* were diluted in blocking buffer, added to the wells and the cells were incubated overnight at 4 °C. Following the incubation, the cells were washed 3 times for 3 min each in DPBS and then were incubated for 1 h in secondary antibody (Donkey anti-rabbit Alexa Fluor 488) at room temperature. After 1-h incubation, cells were washed 3 times for 3 min each in DPBS and then nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI; nuclear stain) for 5 min at room temperature. Followed by 2 times washes in DPBS for 3 min each, the cells visualised using confocal microscopy (Olympus FluoView FV1000 microscope).

3.6 Determining the percentage of trigeminal Schwann cells containing more than one nuclei following the infection with *B.pseudomallei* at different time points

To study how the percentage of multinucleated Schwann cells changed over the different incubation time (6h, 12h, 18h, 24h) with *B.pseudomallei,* immunofluorescence microscopy was performed. The cells were infected with *B.pseudomallei* MOI 75:1 for 6h, 12h, 18h and 24 h. following each time point the cells were fixed with 4% PFA and incubated with primary antibody for *B.pseudomallei,* secondary antibody and DAPI as described in section 3.4

3.7 Investigation of the role of BimA in Schwann cell multinucleation

To study the role of the *B. pseudomallei* protein BimA protein (or actin-polymerizing protein known as the main inducer of multinucleation in host cells) in Schwann cell multinucleation, immunofluorescence microscopy was performed after infection of trigeminal Schwann cells with a *B. pseudomallei* BimA deletion mutant (*B. pseudomallei ΔbimA*) previously constructed in our laboratory (Horton, Grant et al. 2013). The primary trigeminal Schwann cells were grown as discussed in 3.3 and infected with GFP-tagged *B. pseudomallei ΔbimA* (MOI 75:1) for 24 h. Following the infection, cells were fixed with 4 % PFA and then nuclei were stained using DAPI for 5 min at room temperature, and later imaged using confocal microscopy (Olympus Fluoview FV1000 microscope).

3.8 Multinucleation of trigeminal Schwann cells and olfactory ensheathing cells after infection with *N. meningitidis* serogroup B

The formation of multinucleated Schwann cells and OECs after infection with *N. meningitidis* serogroup B was studied using immunofluorescence microscopy. The primary trigeminal Schwann cells and OECs were seeded and cultured at the density of 5000 cells in 8-well chambers, incubated and grown in Dulbeco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10 % Fetal Bovine Serum (GIBCO) and 1x G5 for 12 h in 5 % CO_2 at 37 °C in a humidified incubator. After 12 h, cells were infected with GFP-tagged *N. meningitidis* serogroup B (MOI 10:1) for 24 h and 72 h (Slanina, Hebling et al. 2012). Following the incubation time, cells were rinsed in 1x HBSS and were fixed for 20 min in 4 % paraformaldehyde (PFA) in Dulbecco-Phosphate Buffered Saline (DPBS). After fixing, the cells were rinsed in DPBS 3 times for 5 min. Subsequently, nuclei were stained using DAPI for 5 min at room temperature. Followed by 2 times washes in DPBS for 3 min each, the cells were imaged using confocal microscopy (Olympus FluoView FV1000 microscope).

3.9 Generation and infection of floating liquid marble containing cells with *N. meningitidis* serogroup B

To study the nuclear alteration in three-dimensional (3D) trigeminal Schwann cell culture following the infection with *N. meningitidis* serogroup B, Naked Liquid Marbles (NLM) containing trigeminal Schwann cells were generated.

Our laboratory has developed a naked liquid marble (NLM) platform in which droplets of cell culture medium are incubated on a superhydrophobic coating (Australian Provisional Patent 2017904456). Inside the NLM platform, cells are free to interact with others, forming multiple 3D spheroids that are uniform in size and shape in less than 24h. For most types of cells, they form cell spheroids in NLM by cell-cell interactions and secreted extracellular matrix. The method for spheroid generation in NLM is similar to normal two dimensional cell culture but only needs optimizing specific cell seeding densities which is based on the cell types.

To generate liquid marbles, a micropipette was used to dispense the required volume of Schwann cell medium containing 385 cell/µL on the powder bed with volume of 20 µL. Thus, 7700 cells were added in each well. Then the cells were incubated overnight in 5 % CO_2 in air at 37 °C. Following the incubation, the spheroids were infected with GFP-tagged *N. meningitidis* (MOI10:1) for 24 h and 72 h as previously described. Following the infection the spheroids were rinsed in 1x HBSS and fixed for 20 min in 4 % paraformaldehyde (PFA) in DPBS. After fixation, the cells were rinsed in DPBS 3 times for 5 min. Hoechst were added to stain nuclei and subsequently the spheroids were visualized by confocal microscopy.

3.10 Quantitative Real Time PCR

Total RNA was extracted using the TRIzol method. Briefly, the primary Schwann cells were seeded and cultured at the density of 200,000 cells in 6 well plate, incubated and grown in Dulbeco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10 % Fetal Bovine Serum (GIBCO) and 1x G5 for 12 h in 5 % CO_2 at 37 °C in a humidified incubator. Following 12 h, cells were infected with *B. pseudomallei* MOI 75:1 and kept in incubator for 24 h. Following the incubation time, growth media were removed and 300 µL of TRIzol reagent directly added to the wells to lyse the cells. After several times of pipetting up and down to homogenise the lysate, the lysate was centrifuged at 5000 *x g* for 5 min to pellet the cells. Following the centrifugation, 150 µL of isopropanol were added to the aqueous phase and incubated for 10 min, followed by centrifugation for 10 min at 12,000 *x g*. The RNA formed a white gel-like pellet at the bottom of the tube. The RNA pellet was resuspended in 300 µL 75 % ethanol and then centrifuged for 5 min

at 7500 × g at 4 °C. The supernatant was discarded and then vacuumed for 5 min and finally resuspended in RNase free water and kept at -80 °C.

For zreal-time quantitative PCR (qRT-PCR), the SensiFAST Probe No-ROX One-Step Kit (BIO-76001) was used and samples were run in a Corbett Rotor Gene 6000. The cycling program was 45 °C for 10 min to synthesize cDNA followed by 95 °C for 3 min to activate polymerase and 40 cycles of 95 °C for 15 s and 59 °C for 15 s. Each experiment includes three independent biological assays and the results were normalized to the level of GAPDH. The data was analysed using Linreg software based on the delta-delta Ct method (Pfaffl 2001).

Table 3-1. The forward/reverse primer pairs

Primer	Sequence
Forward TLR-4	TGGCCCTACCAAGTCTCAGC
Reverse TLR-4	GTCTCAGGCTGTTTGTTCCCA
Forward IL-6r	CCCAGTGCAAGAATCCTCGT
Reverse IL-6r	GGAACCAGAAGGAAGGTCGG
Forward cox-2	CAAGCAGTGGCAAGGCCTCCA
Reverse cox-2	GGCACTTGCATTGATGGTGGCT
Forward MMP-2	ACAAGTGGTCCGCGTAAAGT
Reverse MMP-2	GTAAACAAGGCTTCATGGGGG
Forward HIF-1-α	GTCGGACAGCCTCACCAAACAG
Reverse HIF-1-α	TAGGTAGTGAGCCACCAGTGTCC
Forward LDHA	GGATGAGCTTGCCCTTGTTGA
Forward GLUT-1	CATCGCCCTGGCCCTGCAGGAGC
Reverse GLUT-1	GGCACCCCCCTGCCGGAAGCCGGA

Forward PDH α 1	GGGACGTCTGTTGAGAGAGC
Forward PDH α 1	TGTGTCCATGGTAGCGGTAA

3.11 Protein chemistry tools

Table 3-2 Standard solutions

Tris-buffer Saline(TBS)	20 mM Tris base (pH 7)138 mM, NaCl
TBS-Tween (TBST)	TBS, 0.1% Tween 20
1X Tris-glycine (running buffer)	28.8g glycine , 6.04 g Tris base, 2 g SDS, 1.8 L ddH2O
5X SDS sample buffer	0.310 M Tris-HCl (pH 6.8), 10 % SDS, bromophenol blue, 50 % glycerol
Blocking buffer	5 % Milk powder in TBST

3.12 Total cell lysate preparation

The primary Schwann cells were seeded and cultured at the density of 200,000 cells in 6-well plate, incubated and grown in Dulbeco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10 % Fetal Bovine Serum (Gibco) and 1x G5 for 12 h in 5 % CO_2 at 37 °C in a humidified incubator. Following 12 h, cells were infected with *B. pseudomallei* MOI 75:1 or *N. meningitidis* serogroup B MOI 10:1 (based on the experiment) and kept in the incubator for 24 h. Following the incubation time, the cells were trypsinized (using TrypleXpress from GIBCO) and harvested. The cell pellet was lysed in cold RIPA buffer (Sigma) supplemented with a cocktail of protease inhibitors (Sigma). After 15 min, the cell lysate was centrifuged for 20 min at 15,000 rpm (Eppendorf centrifuge 5804 R). The supernatant was collected into fresh tubes and kept at -80 °C. The protein concentration of total cell lysates was determined by using Bio-Rad DC protein assay kit.

3.13 Protein concentration estimation (DC Bio-Rad)

The assay was performed using DC™ Protein Assay Kit I (BIO RAD) as per the manufacturer's instructions. Briefly, the samples were diluted 1:10 and kept on ice, 5 µL of each sample dilution was used for measuring the concentration (triplicate). The reagent A (an alkaline copper tartrate solution), reagent B (a dilute folin reagent) and reagent S were provided with the assay kit. Reagent S and A were mixed in a ratio of 1:50 and 25 µL was added per sample. 200 µL of reagent B was added to each sample, mixed properly and incubated 15 min at room temperature and then absorbance was measured at 750 nm using a plate reader (Bio-Tek). Standards were plotted

against the Bovine Serum Albumin (BSA) and concentrations were calculated from the resultant curve.

3.14 SDS-PAGE

3.14.1 SDS-PAGE gel preparation

Polyacrylamide gels were prepared according to the standard protocol. Briefly, to make 10 % separating gel, 2.5 mL dd H_2O water, 1.25 mL 40 % acrylamide/bis stock, 1.25 ml 1.5 M Tris, pH 8.8, 50 µL 10 % ammonium persulfate, 5 µL TEMED were mixed thoroughly and pipetted the gel mix between the plates. Following the polymerization of separating gel , stacking gel including 3.13 mL dd H2O, 0.62 mL 40 % acrylamide/bis stock, 1.25 mL 1.5 M Tris, pH 6.8, 50 µL 10 % ammonium persulfate, 5 µL TEMED were added to the top of the separating gel and comb was placed.

3.14.2 Sample preparation

To prepare 30 µL of sample, 3 µL of 5x sample buffer, 6 µL glycerol, 18 µL protein sample and 3 µL 1M DTT were mixed and boiled for 5 min and then added to each wells. 10 µL of protein ladder (PAGE-ruler, Thermo Fischer Scientific, cat # 26616) was used to determine the molecular weight. After loading the samples, gels were run at 100v for 90 min in 1x running buffer.

3.15 Western Blot

Extracted proteins were separated on a 10 % SDS-PAGE and were transferred to a PVDF (Millipore) membrane. 5 % milk powder in Tris-Buffered Saline and Tween-20 (TBST) was used as a blocking solution to avoid any non-specific protein-antibody binding. The membranes were incubated overnight at 4 °C with primary antibodies listed in table 3.3 in blocking solution. The membrane was washed 3 x 5 min each and then incubated with HRP-conjugated secondary antibodies listed in table 3.3 for 45 min at room temperature. The membranes were then washed 3 x 5 min. Antigen-antibody reaction was detected by using Enhance chemiluminescence detection (ECL) following the instruction from the Millipore Immobilon kit (WBKLS0500) followed by exposure and imaging of the membranes using a Versadoc (Bio-Rad) imaging station.

Table 3-3 List of antibodies used for Western blots

Name of the antibody	The used concentration
TLR-4 (ab13867), Abcam	1:1000
IL-6r (GTX-37399), Genetex	1:1000
Cox-2 (sc-19999), Santa Cruz Biotechnology	1:1000
MMP-2 (sc-13595), Santa Cruz Biotechnology	1:1000
Goat anti-mouse IgG-HRP(sc-2005), Santa Cruz Biotechnology	1:500
Goat anti rabbit IgG-HRP (sc-2030), Santa Cruz Biotechnology	1:500

3.16 Densitometry Analysis

The western blot data was analysed by Quantity one software (Bio-Rad) to study the intensity of bands of the target proteins and were normalized against loading control.

3.17 SWATH-Proteomics

Proteomics is an important tool to clarify the differences in protein expression levels or any alterations in post-translational modifications. Recently mass spectra based proteomics has emerged as a powerful tool that enable a detection of most comprehensive proteome. The quantitative proteomics usually uses stable isotopes or label free method to measure the protein abundance.

SWATH-MS (sequential window acquisition of all theoretical mass spectra) is a tool for quantitative analysis of the protein expression in which data-independent acquisition (DIA) is coupled with peptide spectral library match.

In this research to study the changes in the protein expression and proteome alterations in the host mammalian cells after infection with *N. meningitidis* serogroup B, SWATH-Proteomics was performed following the infection in trigeminal Schwann cells.

After infection the Schwann cells with *N. meningitidis* serogroup BC311#3 cells (MOI 10:1 for 24 h and 72 h), infected Schwann cells were washed with cold PBS. Cells were harvested and lysed in 250ul 6M Guanidinium-HCl,50mM Tris-HCl pH8, 10 mM DTT. A final concentration of 25mM acrylamide was added to alkylate cysteines. Proteins were precipitated by addition of 1 mL of 1:1 methanol/acetone. Protein pellet was resuspended in 100 µL of 50 mM Tris-HCl pH 8 with 1 µg of trypsin and incubate overnight at 37C. Tryptic digested peptides were cleaned up with C18

ZipTips (Millipore). SWATH label free mass spectrometry analysis was performed as described previously by Ian Peak (Peak et al 2016). Briefly, 2µg of total peptide were desalted and separated on HPLC column (Vydac EVEREST reversed-phase C18). Separation used a gradient of the combination of 10 to 60 % of 80% acetonitrile and 0.1% formic acid with 1% acetonitrile and 0.1% formic acid for 45 min. The MS TOF scan was done from m/z of 350–1800 for 0.5 s followed by information dependent acquisition of MS/MS of the top 20 peptides from m/z 40–1800 for 0.05 s per spectrum, with automated CE selection. SWATH-MS (Sequential Window Acquisition of all THeoretical Mass Spectra) of three biological replicates was performed with MS-TOF scan from m/z of 350–1800 for 0.05 s, followed by high sensitivity information-independent acquisition with 26 m/z isolation windows with 1 m/z window overlap each for 0.1 s across an m/z range of 400–1250. The collision energy was captured and assigned by Analyst software (AB SCIEX) based on m/z window ranges. The proteins were identified by using ProteinPilot (AB SCIEX) and searching a database with all predicted mice proteins. The false discovery rate analysis was performed on all searches. ProteinPilot search results were used as ion libraries for SWATH analyses. The abundance of proteins was measured automatically using PeakView (AB SCIEX) with standard settings and the comparison relative abundance of protein was performed based on protein intensities. Proteins with greater than 30 % changes in abundance and with adjusted P-values < 0.05 were considered significant. All experiments were done in three biological replicates. By using Mass spectrometry in SWATH-MS proteomics and performing three biological replicates for each group, validation the results with western blotting is not necessary.

3.18 Statistical analysis

Statistical analysis was performed using two-sample t-test and P values of 0.05 or less were considered significant. Error bars indicate SEM in all figures. All data are described as the average ± SEM

Table3-4 List of buffers and media used in this study

TBS 10x	Add 87.7g of NaCl to 100ml Tris-Cl and volume into the 1L H_2O, adjust the pH to 7.5
TBST 10x	100ml TBS 10x, 900 ml distilled water and 1ml of Tween
1X Tris-glycine (running buffer)	28.8g glycine , 6.04 g Tris base, 2 g SDS, 1.8 L ddH2O
5X SDS sample buffer	0.310 M Tris-HCl (pH 6.8), 10 % SDS, bromophenol blue, 50 % glycerol
Blocking buffer 5 %	Skim milk powder in TBST
LB broth (Lysogeny broth)	Suspend 25g LB broth powder in 1000ml ultrapure water and autoclaving at 121^{o} c for 15 min.
BHI (Brain heart infusion)	Suspend 47g BHI agar in 1 litre of distilled water. Boil to dissolve the medium completely and autoclaving at 121^{o} c for 15 min.
4% PFA	Add 40 g of Paraformaldehyde powder to 1ml heated 1x PBS, once Paraformaldehyde is dissolved adjust the pH to 7.
DAPI (4′,6-diamidino-2-phenylindole) (stock solution)	Add 2ml of deionized H2O to the entire contents of DAPI vial (life sciences (Cat. No. D1306)) to make 5mg/ml DAPI stock solution
Transfer buffer	10% methanol, 24mM Tris, 194 mM glycine in 1L distilled water

Chapter 4: Nuclear Alterations

4 Nuclear Alterations

4.1 The formation of glial multinucleated cells after infection with *Burkholderia pseudomallei* and *Neisseria meningitidis* serogroup B

The link between bacterial infections and glioma is not known. Therefore, the central focus of this thesis was to investigate how pathogens that can invade the central nervous system affect glial cells. We focussed on the glial cells of the trigeminal and olfactory nerve (see Figure 1.1), since these two nerves constitute a characterised path by which the pathogens we investigated can enter the CNS (reviewed by Dando et al.,–(Dando, Mackay-Sim et al. 2014)). Specifically, we investigated whether infection with the pathogens *B. pseudomallei* and *N. meningitidis* induced cellular changes in glial cells potentially related to cancer, with a focus on multinucleation and atypical nuclei.

Distorted structure of cell nuclei is usually observed in tumour cells, including glioma cells, but the cause of this abnormality and the association with the carcinogenesis has remained unclear (Zink, Fischer et al. 2004). These alterations in nuclear structure include changes in nuclear size and shape, number of nuclei (multinucleation) and chromatin texture (Anderson 1991, Zink, Fischer et al. 2004). The most common nuclear alterations and shape changes associated with cancer are listed in table 4.1

Table 4-1 Nuclear structure in cancer cells

Nuclear characteristic	Cancer type
Changes in nuclear shape/ number	
Atypical nuclei with frequent crush artefacts	Glioma, Small-cell lung carcinoma (Sharma and Deb 2011)
Multinucleated cells	Glioma, Breast cancer (Ichijima, Kobashi et al. 1986, Maeda, Mizuno et al. 2003)
Grooves and long clefts	Papillary thyroid carcinoma, follicular lymphomas (Shim, Cho et al. 2008), (Chang, Lo et al. 2013)
Indentations, undulations, folds	Many types of cancers (Bussolati 2008)
Chromatin changes	
Spherical heterochromatin granules	Glioma (Tani, Ametani et al. 1972)
Asymmetric aggregates of heterochromatin	Wide variety of cancers including glioma (Saksouk, Simboeck et al. 2015)
Dispersed heterochromatin	Small-cell lung carcinoma (Zink, Fischer et al. 2004)

Loss of heterochromatin aggregates	Wide variety of cancers (Nalabothula and Carrier 2011)
Nucleolar alterations	
Enlarged nucleoli	Wide variety of cancers (Derenzini, Montanaro et al. 2009)
Inconspicuous nucleoli	small-cell anaplastic lung carcinoma (Zink, Fischer et al. 2004)
Marked cell to cell variation in numbers or sizes	Wide variety of cancers (Maciak and Michalak 2015)

Multinucleation is associated with neoplasms; however, the cellular and molecular mechanisms that cause multinucleation remain largely unclear. MNCs can arise from cell fusion and/or acytokinetic cell division (Hosaka, Hatori et al. 2004, Ariizumi, Ogose et al. 2009). Glioma cells in particular proliferate extensively and cells often undergo acytokinetic cell division resulting in MNC formation (Lewis and Petritsch 2013). Abnormalities in nuclear morphology, such as atypical nuclei with frequent crush artefacts, are frequently observed in neoplasms including gliomas (Sharma and Deb 2011). Although the exact mechanisms of atypical nuclei formation is not yet fully defined, nuclear atypias including nuclear blebs, chromatin strings, and micronuclei are formed by breakage-fusion-bridge cycles (mitotic disturbance mechanisms reflecting mitotic instability) (Gisselsson, Björk et al. 2001).

4.2 *Burkholderia pseudomallei* causes multinucleation of Schwann cells and olfactory ensheathing cells (OECs).

B. pseudomallei is a gram-negative bacterium which is unique in that it induces MNC formation by inducing cell-cell fusion of murine macrophages and human epithelial cells (Suparak, Kespichayawattana et al. 2005). Analysis of the *B. pseudomallei* genome followed by functional assays have demonstrated that the Type VI secretion system (T6SS), in particular the protein Burkholderia intracellular motility A (BimA), is responsible for multinucleation in infected murine macrophages (Galyov, Brett et al. 2010, Jani and Cotter 2010)

B. pseudomallei can invade the brain and spinal cord by infecting the nerves that innervate the nasal cavity (the olfactory and trigeminal nerves; (see Figure 1.1), resulting in neuronal death and open channels through which the bacteria penetrate the CNS (St John, Walkden et al. 2016). The death of the axons results in large amounts of cell debris which must be cleared by the glial cells of these nerves (olfactory ensheathing cells (OECs) or trigeminal Schwann cells). Thus, the glial

phagocytic machinery must handle both the presence of axon debris and the bacterial invasion. We hypothesised (1) that the combined stress of cell debris and bacterial load would decrease the ability for the glial cells to clear the bacteria, and (2) that OECs would be more resistant to the infection since they are continually removing axon debris in their normal environment (due to the constant turnover of olfactory sensory axons that occurs throughout life). We therefore examined the cellular response of OECs and trigeminal Schwann cells to *B. pseudomallei* in the absence and presence of axon debris.

Schwann cells and OECs were incubated with *B. pseudomallei* (MOI 75:1) and with/without cell debris for 24 h. Following the infection, the reaction of the cells to the challenges was examined immunohistochemically with anti-*B. pseudomallei* antibodies and nuclei stained with DAPI. The results showed that *B.pseudomallei* can induce multinucleation of both trigeminal Schwann cells and OECs (Figure 4.1 and 4.2), demonstrating for the first time that this pathogen can induce multinucleation of glial cells.

Non-infected OECs, or OECs incubated only with cell debris without bacteria, rarely formed MNCs. In contrast, when OECs incubated with *B. pseudomallei* alone or in presence with debris, numerous MNC cells were detected, with some MNCs having many nuclei (Fig 4.1). Similarly, trigeminal Schwann cells exhibited a similar response; untreated cells or cells incubated with debris alone were rarely multinucleated, whereas incubation with *B. pseudomallei* bacteria alone or together with axon debris led to the formation of numerous multinucleated Schwann cells (Fig 4.2). Whilst it was clear that *B. pseudomallei* caused multinucleation of cells, Immunofluorescence microscopy results did not show any evidence of atypical nuclei after infection with this pathogen

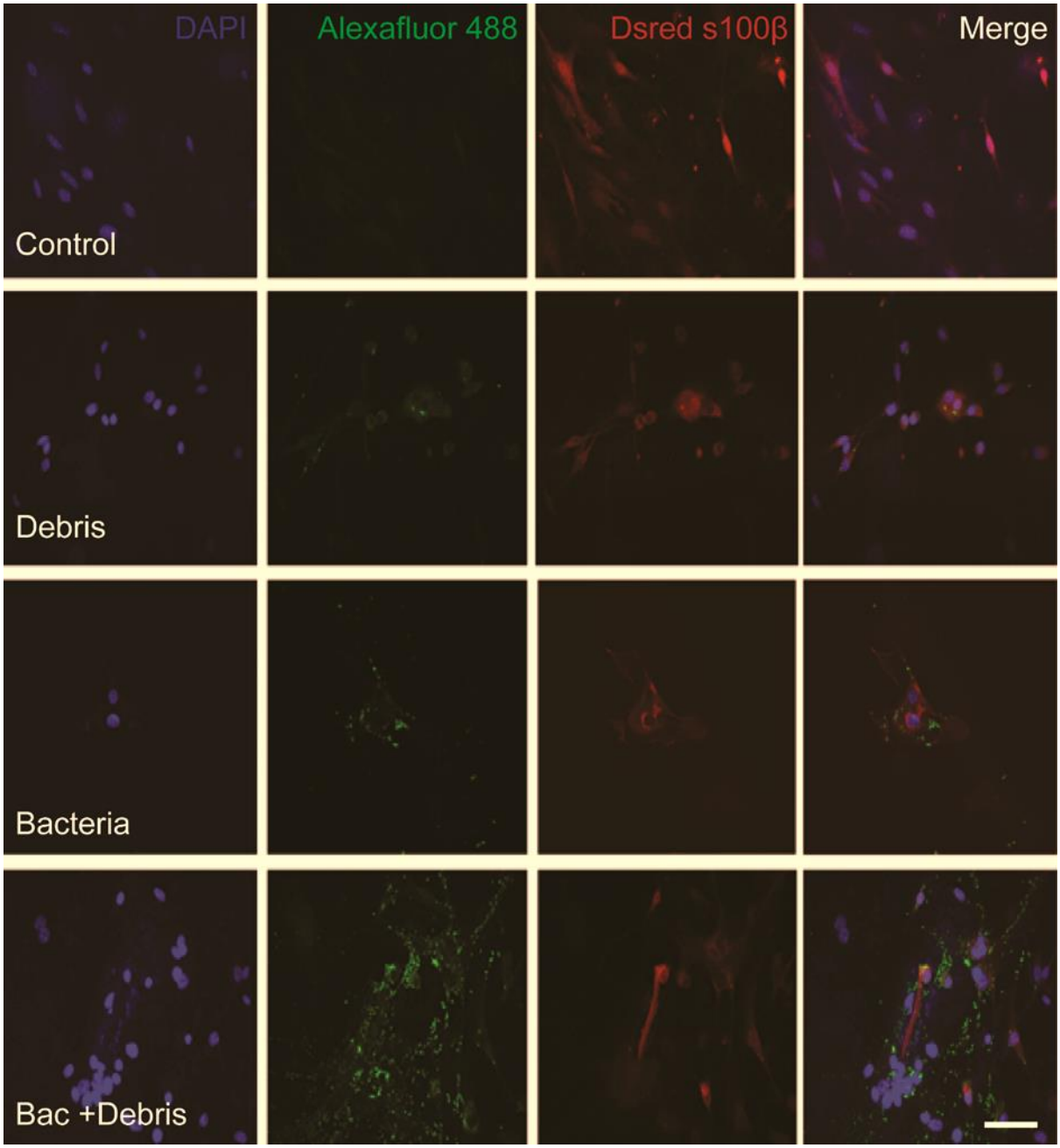


Figure 4-1. B. pseudomallei induces multinucleation of OECs *both in the absence and presence of axonal debris. The panels show from top to bottom cultured OECs (1) incubated for 24 h in the absence of bacteria/debris (control), (2) with debris alone (debris), (3) with B. pseudomallei (MOI 75:1) (Bacteria), and (4) with a combination of B. pseudomallei and debris (Bac + Debris). After 24 h of incubation, the cells were fixed with 4% PFA and stained/immunolabelled for visualisation under the confocal microscope (Olympus Fluoview FV1000 microscope). Blue fluorescence: DAPI (nuclear stain), green fluorescence: immunolabelling for B. pseudomallei (Wt), red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice). Scale bar: 40 µm*

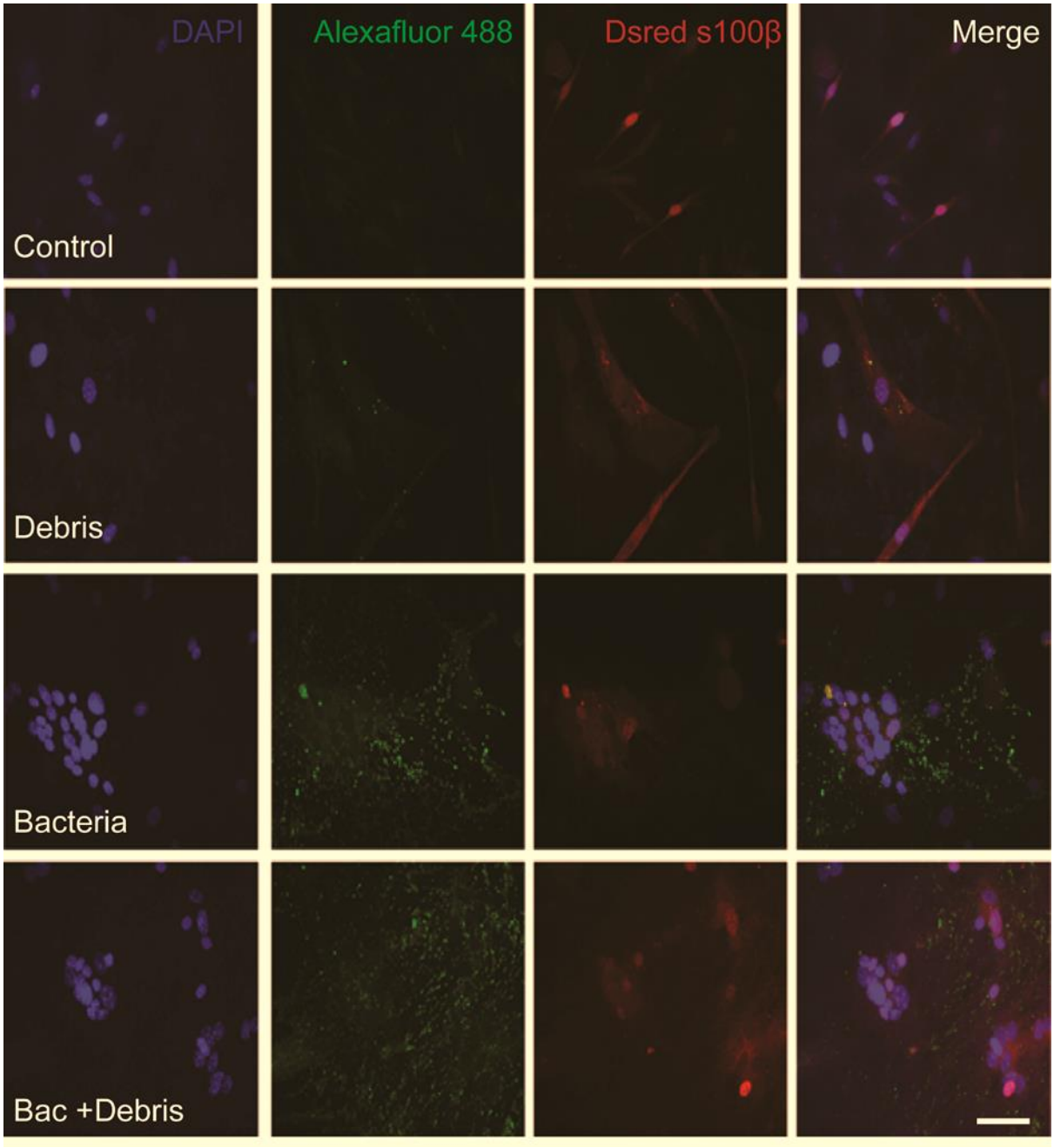


Figure 4-2. B. pseudomallei induces multinucleation of trigeminal Schwann cells *alone and in combination with axonal debris. The panels show from top to bottom cultured trigeminal Schwann cells (1) incubated for 24 h in the absence of bacteria/debris (control), (2) with debris alone (debris), (3) with B. pseudomallei (MOI 75:1) (Bacteria), and (4) with a combination of B. pseudomallei and debris (Bac + Debris). Blue fluorescence: DAPI (nuclear stain), green fluorescence,), red fluorescence: the DsRed protein in the glial cells. Scale bar: 40 µm*

4.3 *B.pseudomallei*-induced MNC is more prominent in trigeminal Schwann cells than in OECs, and axon debris does not significantly affect multinucleation.

The percentage of MNCs in each treatment was calculated by quantifying number of MNCs in five randomly selected fields of view (using the x20 objective of an Olympus Fluoview FV1000 confocal microscope), typically comprising approximately ~50-70 cells, times three biological replicates. For both OECs and Schwann cells cultured in control medium or with debris alone, only ~ 1% of cells were multi-nucleated (Fig 4.3). When OECs were incubated with bacteria, there were significantly more MNCs than in the control/debris alone conditions, with about 7% of cells exhibiting more than one nucleus. The addition of cell debris slightly increased the proportion of multi-nucleated OECs. However, it was not significantly different with bacteria only. The response of trigeminal Schwann cells was considerably more dramatic, with 22% of Schwann cells being multi-nucleated after bacterial infection which was significantly different from control/debris alone. When bacteria and debris were combined, 26% of cells had more than one nucleus. However, the combination of cell debris and bacteria did not change significantly the quantity of MNCs in comparison with bacteria only.

These results demonstrate that both OECs and Schwann cells can become multi-nucleated after incubation with *B. pseudomallei*, however, the formation of MNCs in Schwann cells was considerably more pronounced compared to OECs. The addition of cell debris slightly and not significantly increased the formation of MNCs in both cell types.

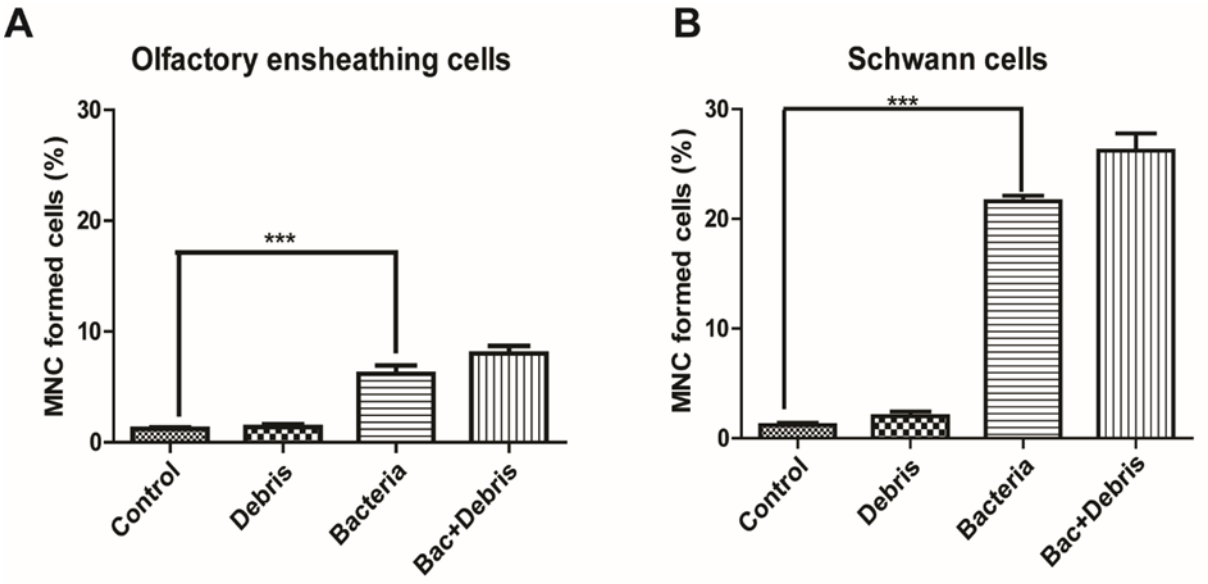


Figure 4-3. Quantification of the formation of multinucleated cells in OEC/Schwann cell cultures in the absence and presence of axon debris*. Bar graphs show the percentage of MNCs under the different conditions (control, debris, bacteria and bacteria + debris). A: OECs, B: Schwann cells. For both glial types, there was a significant difference in the number of MNCs between control and bacteria-incubated culture (p<0.0001). n = 3 biological repeats of 5 randomly selected fields of view comprising 50-70 cells.*

Since the overall aim of this study was to investigate potential cancer-related changes in glia induced by bacteria, rather than the pathogenesis of known diseases caused by the pathogens (melioidosis and meningitis), we decided that the following experiments would be conducted with trigeminal Schwann cells rather than OECs. We also excluded the use of cell debris as the addition of cell debris did not contribute a large increase in the formation of MNCs.

4.4 *B. pseudomallei*-induced multinucleation of Schwann cells is rapid with maximal number of MNCs detected 24 h post infection.

To determine how the percentage of multinucleated Schwann cells changed over time following infection with *B. pseudomallei*, the percentage of MNCs was determined at 6 h, 12 h, 18 h, and 24 h post infection (MOI: 75:1), again by counting five different randomly selected fields of view (three biological replicates). After only 6 h of incubation, MNCs were detected in the Schwann cell cultures (Fig 4.4) with 8% of cells having more than one nucleus (Fig 4.5) this demonstrates that the multi-nucleation occurs rapidly. With increasing time, more MNCs were detected with one-quarter of cells having more than one nucleus at 24 h (Fig 4.5). However, the highest proportion of MNCs was observed at 24 h of incubation with *B. pseudomallei*, while continued incubation time further than 24 hours led to reduce cell survival. Therefore 24 h of incubation time was used for the following experiments.

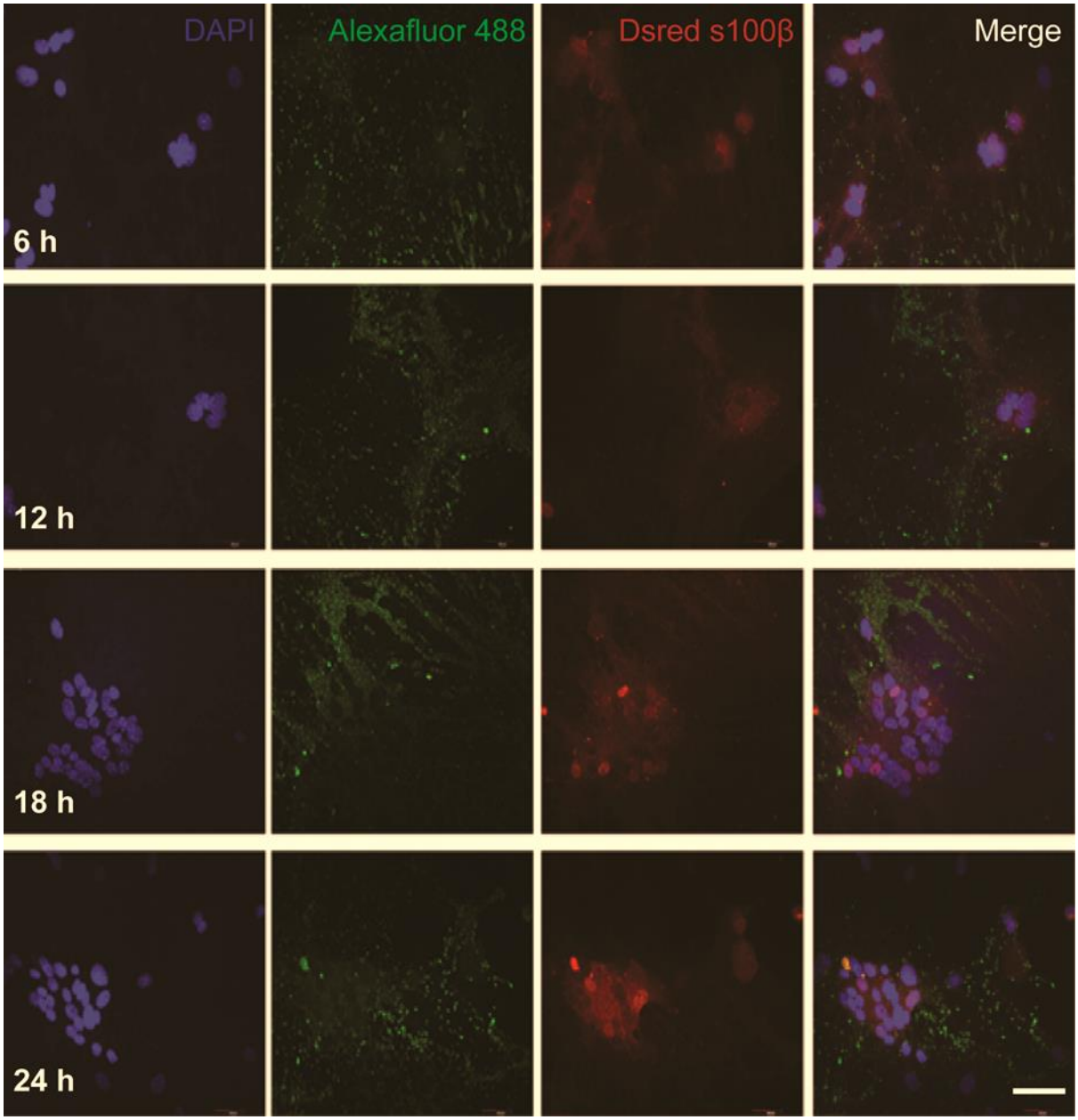


Figure 4-4. Typical examples of Schwann cell appearance at different time-points following B. pseudomallei infection. *6h, 12h, 18h and 24h post infection of Schwann cells with B.pseudomallei. Blue fluorescence: DAPI (nuclear stain), green fluorescence: immunolabelling for B. pseudomallei (wt), red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice). Scale bar: 40 μm*

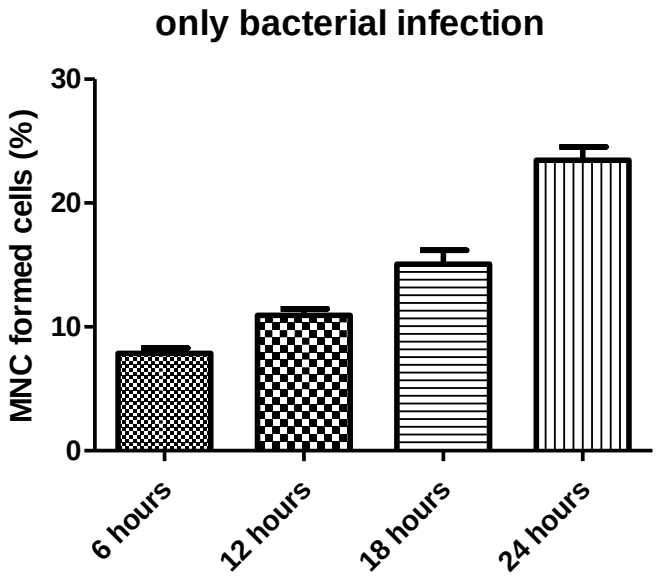


Figure 4-5. Percentage of Schwann cells containing more than one nucleus *at different time-points after B. pseudomallei infection. This figure shows the percentage of Schwann cells containing more than one nucleus after infection with B. pseudomallei at 6, 12, 18 and 24 h post infection.*

4.5 The number of nuclei in multinucleated *B. pseudomallei*-infected Schwann cells increases with time post-infection.

The number of nuclei in multinucleated Schwann cells was highly variable. The number of nuclei in a cell is directly proportional to (1) the number of cells that have fused to form one cell, and/or (2) how many times the cell has undergone incomplete cell division. *B. pseudomallei* is thought to primarily induce the formation of MNCs via fusion (Jani and Cotter 2010; Galyo, et al. 2010). Therefore, to quantify the degree of multinucleation, it is not enough to count the number of multinucleated cells; the number of nuclei within these cells must also be taken into account. To quantify the number of nuclei that formed in MNC Schwann cells at different time points post infection, the number of nuclei within each cell was counted and binned into groups of (i) two nuclei, (ii) three nuclei, or (iii) four or more nuclei. At 6 hours post infection, most of the cells with more than one nucleus were binucleated. With increasing time, the proportion of cells having four or more nuclei increased so that by 24 h each category constituted 5-6% of cells (Fig 4.6) . Overall, these results demonstrate that multi-nucleation of Schwann cells is rapidly induced after infection with *B. pseudomallei*. Increasing the incubation time led to continued formation of MNCs and the formation of MNCs with a higher number of nuclei. It is expected that further incubation time would lead to continued increases in MNC formation but on other hand, decreases the cell viability; thus, for the purposes of the following experiments we considered that 24 h incubation was sufficient.

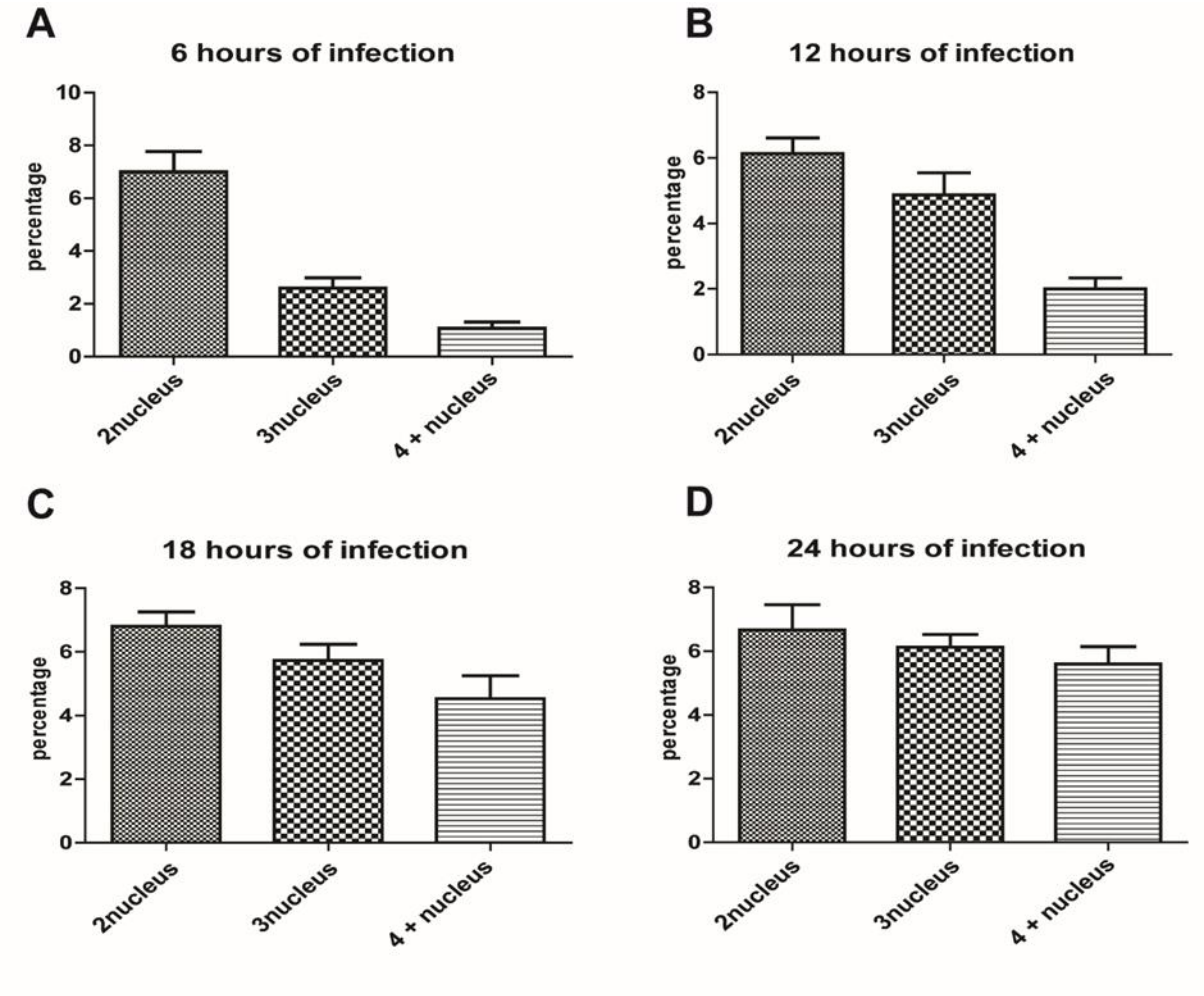


Figure 4-6. The number of nuclei in B. pseudomallei- infected multinucleated Schwann cells increased over time. *Bar graphs show the percentage of cells exhibiting two, three and four or more nuclei at different time-points post infection.*

4.6 The bacterial protein BimA is critical for *B.pseudomallei*-induced formation of multinucleated Schwann cells.

Although the exact molecular mechanism of MNC formation induced by *B. pseudomallei* is not yet fully elucidated, this phenomenon has been suggested to be due to the Bim-A-mediated actin-based motility (Galyov, Brett et al. 2010). The protein BimA, which mimics a eukaryotic actin polymerase to mobilise a tail of host cell actin leading to bacterial motility, cell-cell dissemination and cell-cell fusion in murine macrophages (Stevens, Ulrich et al. 2005, Benanti, Nguyen et al. 2015). To study the role of Bim-A in formation of multinucleated cells in trigeminal Schwann cells, we infected the cells with *B. pseudomallei* in which BimA had been deleted (*B. pseudomallei* ΔBimA) (MOI 75:1 for 24 hours). In stark contrast to Schwann cells infected with wild-type *B. pseudomallei*, cells incubated with *B. pseudomallei* ΔBimA rarely formed MNCs in comparison with wild type *B. pseudomallei.*

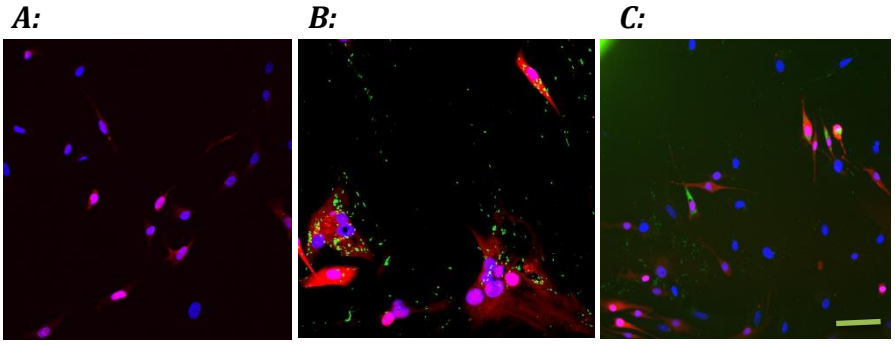


Figure 4-7. BimA has a critical role in multinucleation*. A) non-infected trigeminal Schwann cells. B) trigeminal Schwann cells infected with B.pseudomallei* wild type C) *B. pseudomallei* (*Δ BimA*) . Red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice)*. Blue fluorescence: DAPI (nuclear stain), green fluorescence: immunolabelling for B. pseudomallei wt and mutant. Scale bar: 40 µm*

4.7 *Burkholderia pseudomallei* causes multinucleation in low MOI and long-term infection in trigeminal Schwann cells

To study the long term infection effect of *B.pseudomallei* on trigeminal Schwann cells, the cells were infected with low MOI (MOI 10:1) for 72 h. The immunofluorescence microscopy results showed that 72 h of infection can induce multinucleation in trigeminal Schwann cells with 17% of cells being multinucleated.

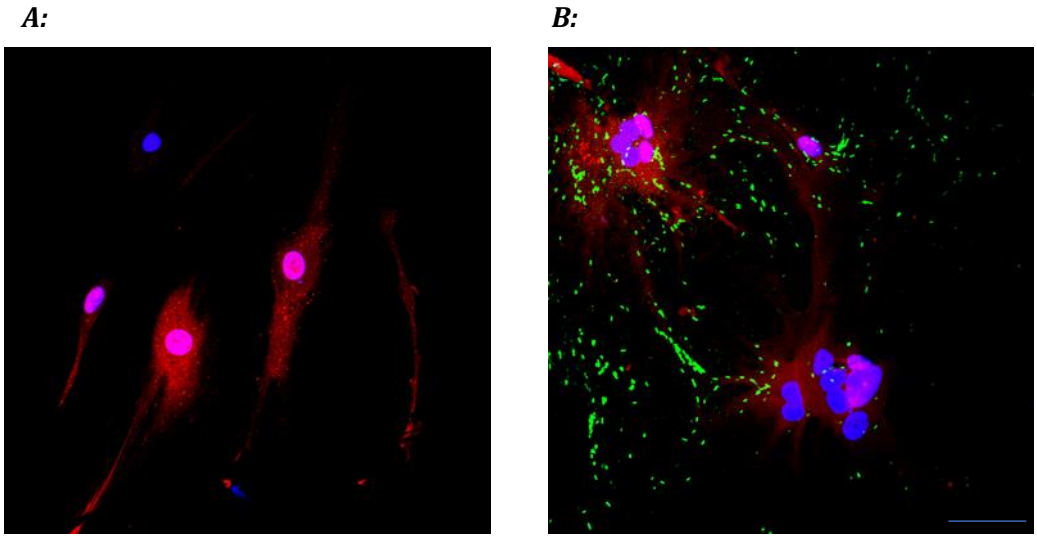


Figure 4-8. B.pseudomallei induces multincleation in trigeminal Schwann cells in lower MOI and higher infection time A) non-infected trigeminal Schwann cells B) infected trigeminal Schwann cells with *B.pseudomallei* (MOI 10:1) for 72 h. DAPI: nuclei, Green : Bacteria, red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice) Scale bar: 40 µm .

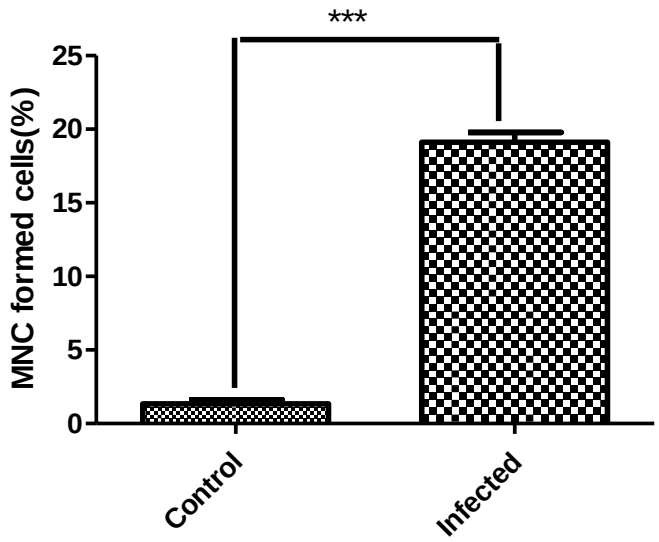


Figure 4-9. Percentage of MNC trigeminal Schwann cells after long-term infection (72 h) with B. pseudomallei (MOI 10:1) . There was a significant difference in the number of MNCs between control and bacteria-incubated culture ($p<0.0001$). n = 3 biological repeats of 3 randomly selected fields of view comprising 40-70 cells.

4.8 *N. meningitidis* serogroup B infection causes nuclear abnormalities and multinucleation of trigeminal Schwann cells

N. meningitidis is a gram-negative coccus and a common inhabitant of the human nasopharynx. It has been shown that following intranasal infection of mice, *N. meningitidis* is able to pass directly from nasopharynx to the brain through the olfactory nerve (Sjolinder and Jonsson 2010). While it has not yet been shown that the trigeminal nerve can become infected by *N. meningitidis*, we suggest it is highly likely since this nerve is so susceptible to infection by *B. pseudomallei*. *N. meningitidis* is a more common cause of CNS infection than *B. pseudomallei*, however, the relationship between *N. meningitidis* infection of the CNS and nervous system sequelae remains unknown. *N. meningitidis* can survive intracellularly (Nikulin, Panzner et al. 2006), but to date has not been shown to induce atypical nuclei or formation of MNCs in any cell type, and does not contain any known protein such as BimA capable of inducing cell-cell fusion. Since the effects of *N. meningitidis* infection of glia in the nerves innervating the nasal cavity has never been characterised before, we investigated how this pathogen affected trigeminal Schwann cells and OECs. We focussed particularly on whether the bacteria could induce potential cancer-related cell changes in the cells.

Trigeminal Schwann cells and OECs were incubated with *N. meningitidis* serogroup B (MOI 10:1) (Sigurlasdottir, Engman et al. 2017) for 24 h and 72 h. Our results indicated that following the

infection of OECs with *N.meningitidis* serogroup B (MOI 10:1) for 24 h and 72 h, the cells did not change their nuclear morphology (Fig 4-10). Thus, we decided to exclude OECs from our research.

Unlike *B. pseudomallei* infection, trigeminal Schwann cells infected with *N. meningitidis* serogroup B could survive for 72 hours. Thus, we included this time point in our research.

Following the incubation time, the reaction of the cells to the infection was examined using immunofluorescence microscopy as described earlier for *B. pseudomallei*. Our results showed that *N. meningitidis* induced bi-nucleation, multinucleation and nuclear atypia such as horseshoe nuclei, pleomorphic nuclei and budding nuclei in these cells; this is the first time *N. meningitidis* has been shown to (1) infect trigeminal Schwann cells and (2) induce potential cancer-related changes in any glial cell type (Fig4.12).

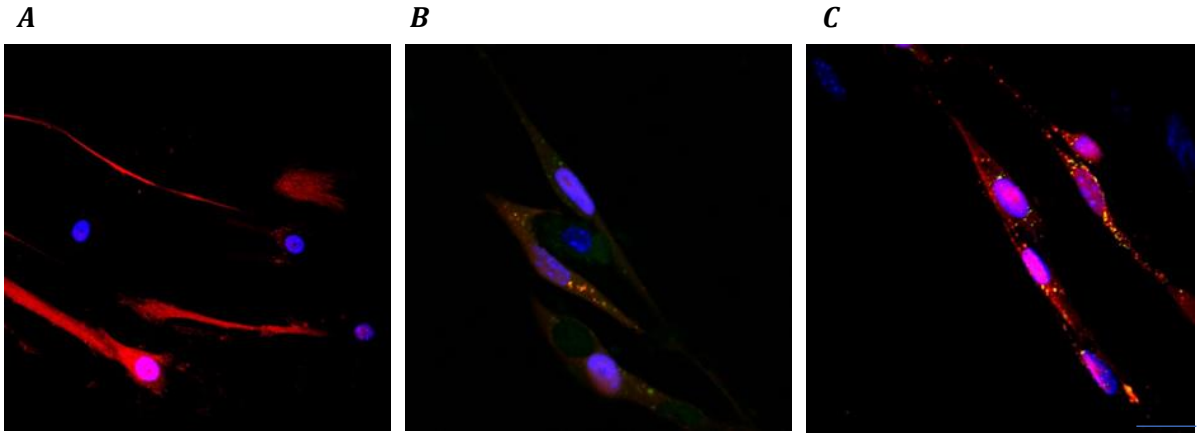


Figure 4-10. Infection of OECs with N. meningitidis serogroup B. *A) Non-infected OECs, B) Infected OECs with MOI 10:1 for 24 h C) Infected OECs with MOI 10:1 for 72 h. . Blue fluorescence: DAPI (nuclear stain), green fluorescence: GFP-tag N. meningitidis serogroup B, red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice) Scale bar: 40 µm*

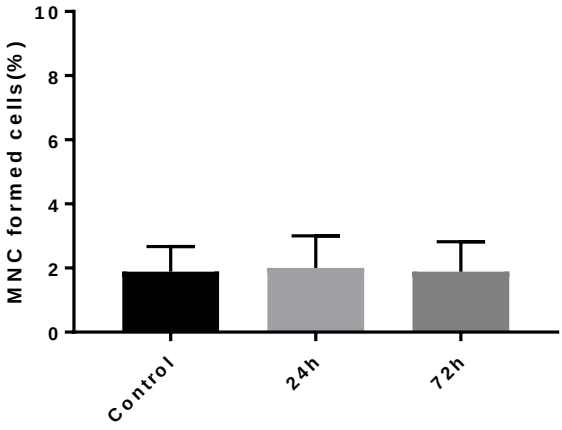


Figure 4-11. Percentage of multinucleated OECs after infection with N. meningitidis serogroup B*. There was no significant differences in the number of MNCs between control and bacteria-incubated culture after 24 h and 72 h of infection. n=3, one-way Anova, Tukey's post hoc test was done between each group.*

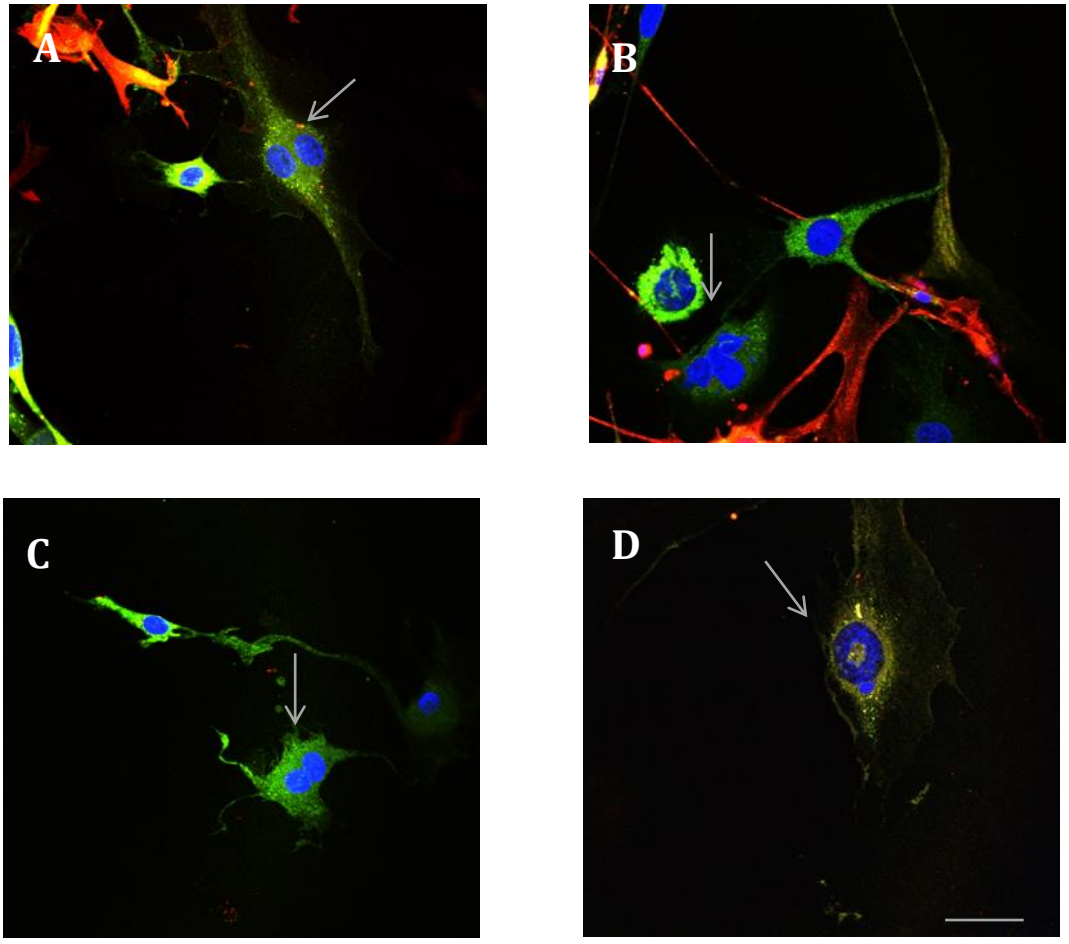


Figure 4-12. N. meningitidis serogroup B induces nuclear alteration of trigeminal Schwann cells *(A) binucleation after 24 h of infection, (B) nuclear atypia after 24 h of infection, (C) binucleation after 72 h of infection , (D) nuclearatypia after 72 h of infection. Blue fluorescence: DAPI (nuclear stain), green fluorescence: GFP-tag N. meningitidis serogroup B, red fluorescence: the DsRed protein in the glial cells (from S100β-DsRed transgenic mice) Scale bar: 40 µm*

N. meningitidis-mediated bi/multinucleation of Schwann cells occurs faster than the induction of nuclear abnormalities.

We quantified the percentage of the infected Schwann cells that (1) were bi/multinucleated and (2) exhibited atypical nuclei after 24 and 72 h. When the cells were infected with *N. meningitidis* (MOI 10:1) for 24 h, approximately 30% of the cells were bi- or multinucleated, whereas about 10% showed atypical nuclei. With increasing the incubation to 72 h, the percentage of bi- and multinucleated cells decreased to ~25% whereas the portion of atypical nuclei significantly increased to 23% (Fig 4.13). These results show that whilst bi/multinucleation as induced by *N. meningitidis* occurs rapidly, the induction of atypical nuclei is slower.

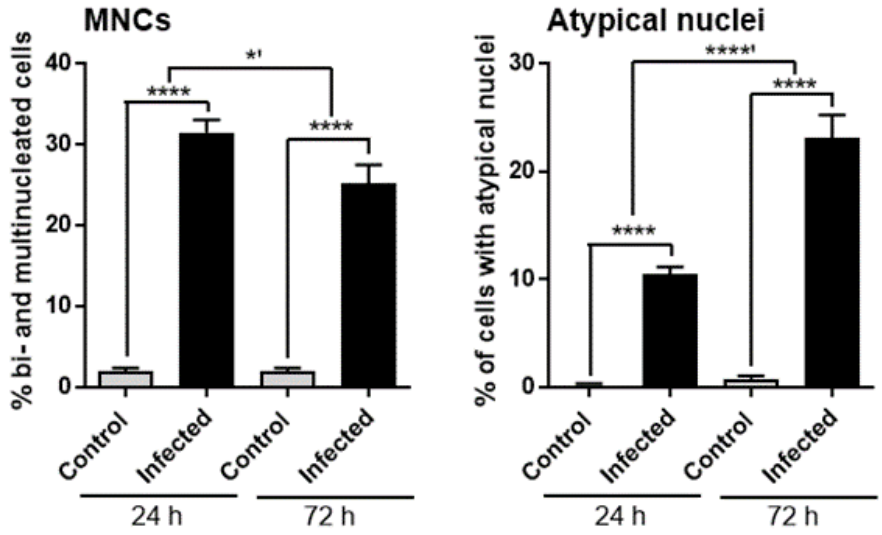


Figure 4-13. ***Quantification of Schwann cell multinucleation and appearance of atypical nuclei at different time-points following infection with N. meningitidis serogroup B.*** *Bar graphs show the percentage of infected Schwann cells that exhibited more than one nucleus (left) or atypical nuclei (right) at 24 and 72 h post infection. MOI was 10:1 n=3 (three biological replicates) of 5 randomly selected fields of view comprising 40-70 cells. There was a significant increase in the number of bi/multinucleated cells, and cells with atypical nuclei, after infection with N. meningitidis at both time-points. **** p<0.0001 (one-way Anova, Tukey's post hoc test). The percentage of cells with more than one nucleus was significantly higher at 24 h than at 72 h, and conversely, the percentage of cells that had abnormal nuclei was higher at 72 h than at 24 h. *' and ****': Analysis of the difference in percentage of multinucleated and atypical nuclei between the two time-points (T-test); p<0.05 and p<0.0001, respectively*.

4.9 *N. meningitidis* serogroup B induces nuclear alteration in 3D spheroids

To study the interaction of *N. meningitidis* serogroup B with trigeminal Schwann cells and to bridge a gap between *in vitro* and *in vivo* models and mimic tissue and organ specific microarchitecture 3D culture of trigeminal Schwann cells was performed. In 3D culture, floating liquid marble containing trigeminal Schwann cells were generated and infected with *N. meningitidis* serogroup B (MOI 10:1) for 24 h and 72 h..

Following the incubation time, the reaction of spheroids to the infection was examined using confocal microscopy. Our results showed that *N. meningitidis* serogroup B induces binucleation in spheroids (Figs 4.14, 4.15, 4.16).

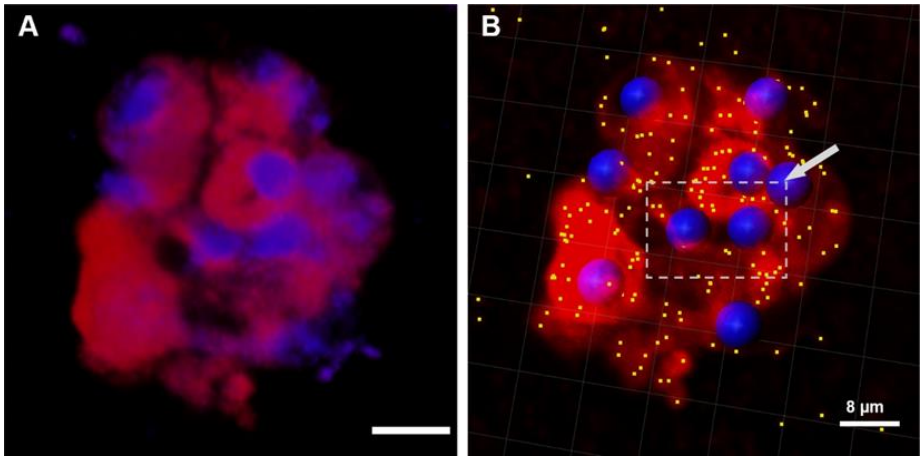


Figure 4-14. Schwann cells in 3D cell culture (non-infected). *A, Schwann cells stained with Hoechst (nucleus, blue) and CellMask (cell membrane, red). B, Nucleus were recognized by DAPI stain and size. Yellow dots are cell boundaries that were recognized by intensity. Scale bar is 8 µm. Image captured by Nikon AR1+. Image analysis by Imaris 9.0.*

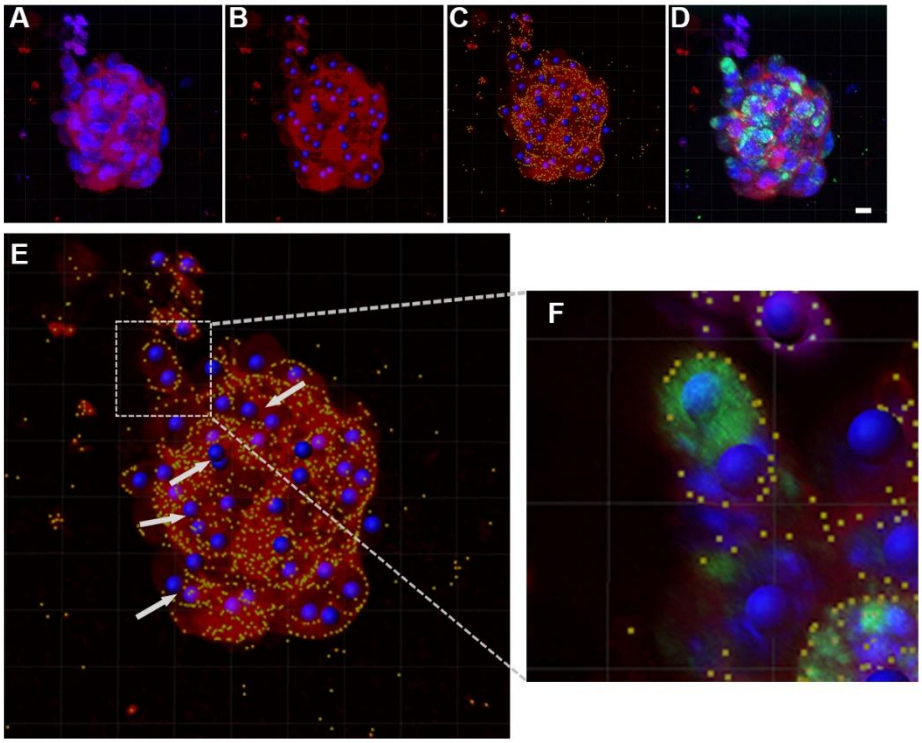


Figure 4-15. Infected Schwann cells in 3D cell culture*. Schwann cells form cell spheroids, then infected with N. meningitidis serogroup B for 24 h. A, Schwann cells stained with Hoechst (nucleus, blue) and CellMask (cell membrane, red). B, Nuclei were recognized by DAPI stain and size. C, yellow dots are cell membrane boundaries that were recognized by intensity. D, GFP-tagged N. meningitidis serogroup B. E, multinucleated cells in 3D. F, Multinucleated cells and N. meningitidis serogroup E is high power of C and and F is high power of E Scale bar, 10 µm. Image captured by Nikon AR1+. Image analysis by Imaris 9.0.*

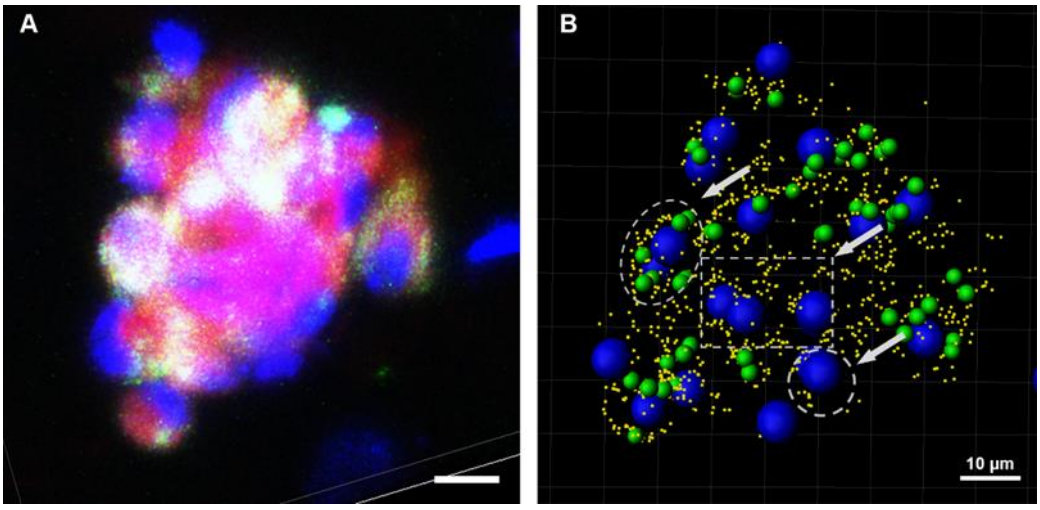


Figure 4-16. Infected Schwann cells in 3D cell culture*. Schwann cells formed cell spheroids, after which they were infected with N. meningitidis serogroup B for 72 h. A, Schwann cells stained with Hoechst (nucleus, blue) and CellMask (cell membrane, red). B, Nucleus were recognized by size. Cell membrane boundaries were recognized by intensity. Green: GFP-tag N. meningitidis serogroup B. Scale bar, 10 µm. Image captured by Nikon AR1+. Image analysis by Imaris 9.0.*

4.10 Discussion

In this study, for the first time we investigated infection of glial cells from the olfactory/trigeminal nerves by the gram-negative intracellular neuroinvasive bacteria *B. pseudomallei* and *N. meningitides* (Deuble, Aquilina et al. 2013). We showed that both these pathogens caused cellular changes that may potentially be associated with cancer/glioma, in particular *N. meningitidis* which caused both atypical nuclear changes and the formation of multinuclear cells (MNCs) of trigeminal Schwann cells.

Previous studies by our group showed that *B. pseudomallei* is able to travel along the trigeminal (St John, Walkden et al. 2016)and olfactory (St John, Ekberg et al. 2014) nerve fascicles and reach the brain and spinal cord within 24 hours of intranasal exposure. These pathways constitute two previously largely uncharacterised mechanisms for bacterial infection of the CNS and can have implications for many other diseases ((St John, Walkden et al. 2016); reviewed by (Dando, Ipe et al. 2016)). Spread along these nerves could due to both movements of bacteria through empty channels resulting from neuronal death, as suggested for *B. pseudomallei* invasion of the olfactory nerve, and/or by cell-to-cell dissemination between glial cells (St John, Walkden et al. 2016). A bacterial strategy for cell-cell dissemination is the ability to induce cell-cell fusion/multinucleation, which may lead to detrimental downstream effects in the glial cells even potentially related to glioma.

In the current study, for *B. pseudomallei*, the experiments were designed to study the formation of multinucleated cells in trigeminal Schwann cells and olfactory ensheathing glia (OECs). The results showed that *B. pseudomallei* induced multinucleation of both cell types, but that trigeminal Schwann cells were significantly more prone to multinucleation than OECs. Based on these results we focused on trigeminal Schwann cells for the remainder of the experiments. We found that multinucleation occurred rapidly (within 6 h) and was optimal for experimental purposes (pronounced multinucleation but low cell death) at 24 h post infection. Thus, we selected this time point for further experiments. Further, at 24 h, we observed mostly multinucleated rather than binucleated cells, many with four or more nuclei. *B. pseudomallei* infection did not cause any visible nuclear anomalies except for multinucleation; we did not observe any nuclei of atypical appearance.

The actin-mobilising *B. pseudomallei* protein BimA has been suggested to be crucial for multinucleation of other cell types (Stevens, Stevens et al. 2005, Benanti, Nguyen et al. 2015). To determine whether BimA was also crucial for multinucleation of Schwann cells, the cells were infected with *B. pseudomallei* in which the BimA protein had been deleted (ΔBimA). The immunofluorescence microscopy results showed that the Schwann cells rarely formed MNCs

after the infection. Thus, we suggest that BimA is likely to be the main mediator of multinucleation also in this cell type.

Previous studies showed that *N. meningitidis*, an obligatory human pathogen whose principal habitat is the nasopharynx mucosa, can rapidly penetrate the CNS via the olfactory nerve (Sjölinder and Jonsson 2010). We hypothesise that this bacterium can also enter the brain via the trigeminal nerve as our laboratory has shown the intranasal branches of this nerve to be even more sensitive to bacterial infections than the olfactory nerve (St John, Walkden et al. 2016); St John, Beacham, Batzloff & Ekberg, unpublished data). Thus, the interaction of trigeminal Schwann cells as one of the major glial cells in nasopharyngeal region with *N. meningitidis* was examined (*In vitro*) by immunofluorescence microscopy. The trigeminal Schwann cells were infected with *N. meningitidis* serogroup B MOI 10:1 for 24 hours and 72 hours. As the immunofluorescence microscopy results show, after 24 hours of infection the cells formed multinucleated and atypical nuclei with different ratios of 30% and 10% respectively while after 72 hours of infection the number of multinucleated cells reduced to 25% and atypical nuclei increased to 23% of total cells. According to these results, we suggest that long term infection with *N. meningitidis* serogroup B can increase the nuclear atypia which is consistent with one of the significant features of gliomagenesis.

In order to study the cellular responses to the infection in a setting resemble *in vivo* environment, we infected the 3D culture of trigeminal Schwann cells with *N. meningitidis* serogroup B. our results demonstrated that *N. meningitidis* serogroup B were phagocytosed and induced nuclear alteration in spheroid containing trigeminal Schwann cells. It is important to consider the reaction of cells in 3D cultures compared to two-dimensional cultures. In 2D cultures all cells are exposed to the bacteria, whereas in 3D cultures it is only the cells on the exterior of the 3D spheroid that are initially exposed. For the external cells, the potential trophic and cell-cell contact with interior cells may confer resistance to the changes induced by bacteria. However, multinucleated cells were detected in the 3D cultures with *N. meningitidis* similar to the 2D cultures, suggesting that the morphological changes are consistent across the different culture formats.

4.10.1 Conclusion

Overall, these results demonstrate that bacterial infection of OECs and trigeminal Schwann cells can lead to perturbed nucleation of the cells. As multi-nucleation and atypical nuclear morphology are hallmarks of gliomas, it raises the question of whether markers associated with gliomagenesis are also altered in these cells after bacterial infection. This question is addressed in the following chapters.

Chapter 5: Expression of gliomagenesis Markers

5 Gliomagenesis Markers Expression

5.1 *B. pseudomallei* upregulates the expression of TLR-4, IL-6R, COX-2 and MMP-2 in trigeminal Schwann cells

As described in Chapter 4, *B. pseudomallei* and *N. meningitidis can* readily induce the formation of multinucleated cells (MNCs) in glial cell cultures. As discussed previously, MNCs are a key characteristic of glioma tumours (Winger, Macdonald et al. 1989). We were therefore interested in investigating whether the induction of MNC formation was accompanied by any other cellular changes indicative of cancer. Glioma cells, like other neoplasms, show altered genome expression, and there are key genetic changes that define tumours as gliomas (Louis, Holland et al. 2001). We chose to study the expression of four proteins (TLR-4, IL-6r, COX-2 and MMP-2) which are usually upregulated in glioma cells and are also classified as key regulatory markers in carcinogenesis and gliomagenesis (Wang, Wang et al. 2003, Xu, Wang et al. 2014, Shan, He et al. 2015, Zeuner, Kruger et al. 2016).

5.1.1 TLR-4

Toll-like receptors (TLRs), a family of pattern recognition receptors, are important regulators of tumour biology. TLR-4, the most extensively studied TLR, recognises bacterial lipopolysaccharide (LPS). In addition to immune cells, TLR-4 is constitutively expressed within most cell types of the central nervous system and peripheral nervous system including neural stem cells, microglia, neurons, astrocytes, Schwann cells and neural stem cells (Harmey, Bucana et al. 2002, Rolls, Shechter et al. 2007, Hao, Peduzzi-Nelson et al. 2009, Okun, Griffioen et al. 2011, Zeuner, Kruger et al. 2016). The role of TLR-4 in tumorigenesis came from reports demonstrating that TLR-4 ligands increase proliferation, migration, invasion, angiogenesis and inhibition of apoptosis in tumour cells (Harmey, Bucana et al. 2002). The tumorigenesis mechanism of TLR-4 may be due to stimulation of tumour necrosis factor (TNF) production, resulting in the upregulation of nuclear factor κB (NF-κB)-regulated anti-apoptotic agents, such as B-cell lymphoma extra-large (BCL-XL) as well as cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2) (Maeda, Mizuno et al. 2003). Other studies have demonstrated that TLR-4 has a crucial role in development of tumours via NF-κB-dependent production of inflammatory mediators such as interleukin 6 (IL-6) (Naugler, Sakurai et al. 2007).

5.1.2 IL-6R

IL-6 is a cytokine participating in the immunoregulation and inflammatory responses. The IL-6 pathway plays a significant role in various malignant tumours including glioma (Sun, Mao et al.

2014, Wang, Ye et al. 2014). It has been shown that IL-6 and its receptor (IL-6R) expression are both upregulated in glioma cells and their expressions are in direct relationship with glioma grades (Hotfilder, Knupfer et al. 2000, Li, Li et al. 2010). Binding of IL-6 to the IL-6Rα increases the phosphorylation of signal transducer and activator of transcription 3 (STAT-3), resulting in increased proliferation and anti-apoptotic responses (Shan, He et al. 2015). Moreover, this binding promotes angiogenesis in tumours by regulating the expression of vascular endothelial growth factor (VEGF) and also promoting tumour invasiveness via epithelial mesenchymal transition (EMT) in glioma cells(Piperi, Samaras et al. 2011).

5.1.3 MMP-2

EMT is a biological process causing transformation of polarized adhesive epithelial cells to mobile mesenchymal cells, and is also applicable to other cell types than epithelial cells (Kalluri and Neilson 2003). This process has been identified as a key initiator mechanism of invasion and metastasis in subset of cancer cells (Thiery, Acloque et al. 2009). Matrix metalloproteinase 2 (MMP-2) or gelatinase A, which specifically degrades type IV collagen, is one of the key markers of EMT and the progression of malignant tumours (Zeng, Cohen et al. 1999). MMP-2 has been implicated in many steps of malignancy, including primary tumour growth, angiogenesis, invasion of the basement membrane, and metastatic progression (Egeblad and Werb 2002, Lafleur, Handsley et al. 2003) (Nelson, Fingleton et al. 2000). MMP-2 plays an important role in gliomagenesis and invasiveness in glioma cells by mediating the degradation of the extracellular matrix (ECM) and angiogenesis (Wang, Wang et al. 2003). Bacteria, in particular gram-negative species, upregulate MMP-2 in a variety of cell types (reviewed by (Elkington, O'Kane et al. 2005, Vanlaere and Libert 2009). Intracellular pathogens, in particular mycobacteria, can cause persistent upregulation of MMP-2 (Chang, Wysocki et al. 1996, Quiding-Jarbrink, Smith et al. 2001). Thus far, the long-term implications of this up-regulation remains unknown, however, the link between *Mycobacterium tuberculosis* and cancer is well known (Kuo, Hu et al. 2013, Silva, Valentini Jr et al. 2013).

5.1.4 COX-2

Cyclooxygenase 2 (COX-2), also known as prostaglandin-endoperoxide synthase (PTGS), is an inducible isoform of cyclooxygenases expressed in many cell types including Schwann cells (Takahashi, Kawaguchi et al. 2004) that catalyses conversion of arachidonic acid to prostaglandins and other eicosanoids (Sawada, Sano et al. 2007). It is well understood that the overexpression of COX-2 in cancerous cells contributes to tumour development and progression, angiogenesis, tumour invasion and resistance to apoptosis (Frances, Ingaramo et al. 2013),(Liu,

Qu et al. 2015). It has been recently shown that COX-2 has direct transforming activity by increasing the malignancy of glioma cells in both *in vitro* and *in vivo*, along with inhibition of apoptosis and differentiation (Rahaman, Harbor et al. 2002). Human papilloma virus (Song, Lee et al. 2006), Epstein-Barr virus (Wakisaka and Pagano 2003) and human cytomegalovirus (Baryawno, Rahbar et al. 2011) (Maussang, Langemeijer et al. 2009) are known to induce cancer in different cell types via up-regulation of COX-2.

To determine whether infection with bacteria that cause multinucleation of glia leads to altered expression of these key markers, we infected trigeminal Schwann cells with *B. pseudomallei*. We chose trigeminal Schwann cells since these cells displayed higher degree of multinucleation than OECs after infection. mRNA for these markers were quantified by using qRT-PCR (specific primers are shown in Table 3.1) 24 h after infection with *B. pseudomallei* (MOI 75:1), accompanied by detection of protein amounts with immunoblotting analysis of Schwann cells total lysates. All the data presented in the following Figures are from three independent experiments and Student's T-test was performed to determine if any changes in transcription or translation level were significantly different.

5.2 Study the expression of TLR-4, IL-6, Cox-2 and MMP-2 after infection with *B.pseudomallei*

The endogenous expression of mRNA for TLR-4, IL-6R, COX-2 and MMP-2 after 24 h infection with *B. pseudomallei* was compared with control (cells that were not infected) and was significantly upregulated for all markers as a result of infection (Figure 5.1). Protein expression of these markers was next examined using immunoblots and densitometry quantification. The housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control to normalise the expression of the target genes during the analysis. Consistent with the mRNA results, the expression of all markers TLR-4, IL-6R, COX-2 and MMP-2 were strongly upregulated following 24 h of infection with *B. pseudomallei* (Figure 5.1).

As the results show, the infection with *B. pseudomallei* not only initiated the formation of MNCs but, importantly, reprogrammed the Schwann cell gene expression machinery such that TLR-4, IL-6R, COX-2 and MMP-2 expression was highly significantly and strongly upregulated at both transcriptional and translational levels.

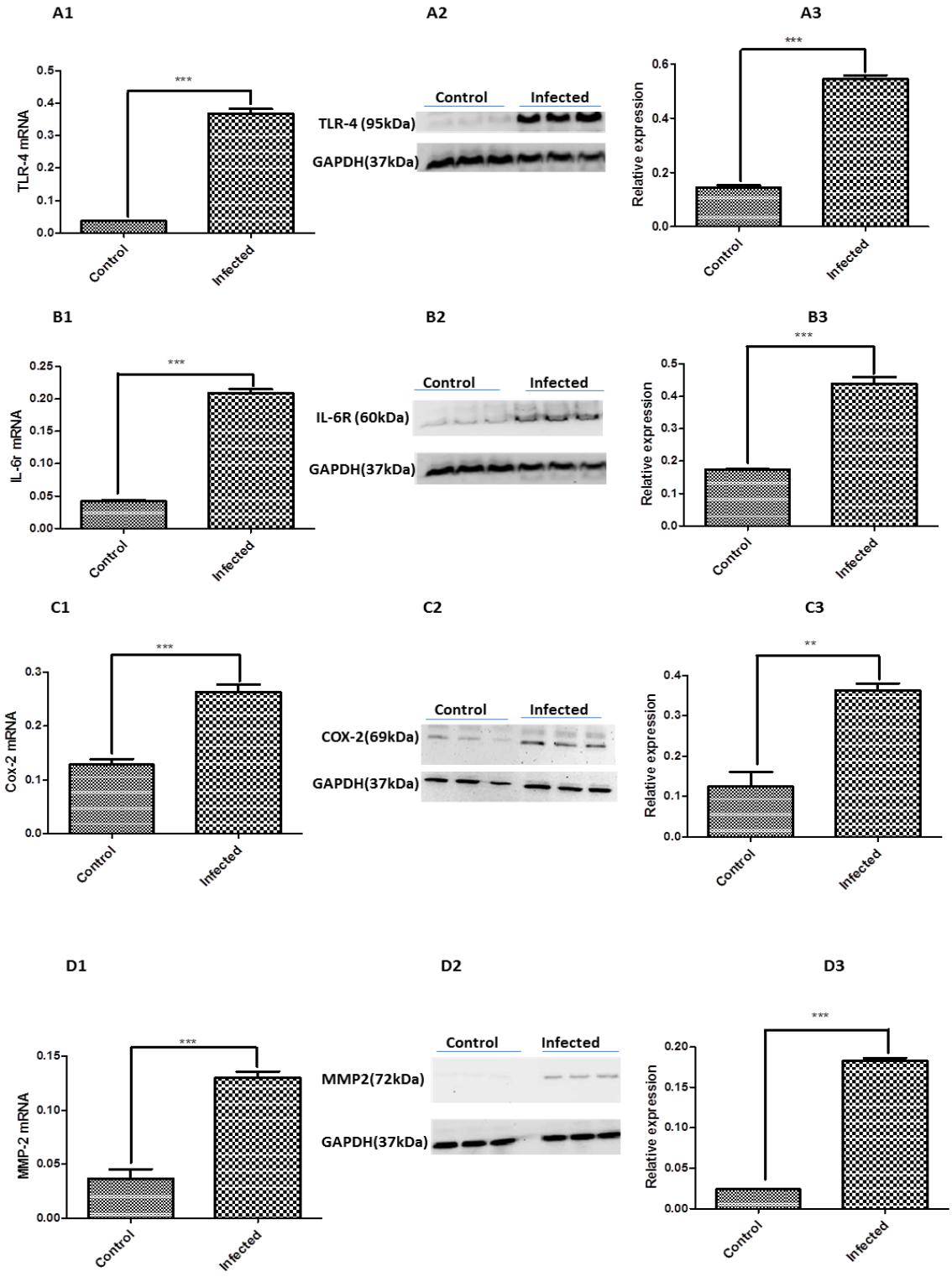


Figure 5-1. The expression of TLR-4, IL-6R, Cox-2 and MMP2, *both at transcriptional and translational levels. A1,B1,C1,D1 show the mRNA expression of abovementioned genes after infection with B. pseudomallei (MOI 75:1) for 24 h compared with control. n=3, ***p<0.001. A2, B2, C2, D2 show the immunoblot for the expression of abovementioned genes after infection with B. pseudomallei for 24 h in comparison with control. A3, B3, C3, D3 show the densitometry data from immunoblotting for expression of abovementioned genes after infection with B. pseudomallei compared to control. n=3, **p<0.01; ***p<0.001.*

5.3 Bim A-mediated multinucleation is crucial for upregulation of MMP-2

Since MMP-2 is the marker amongst the tested ones that is directly linked to epithelial-mesenchymal transition and cancer, we examined whether the formation of MNCs was crucial for upregulation of this particular marker. We infected the cells with the deletion mutant of *B. pseudomallei* (ΔBimA) that lacks the BimA protein and the ability to induce cell-cell fusion (MOI 75:1, 24 h) and analysed the endogenous protein expression of the MMP-2 protein using immunoblotting. The results demonstrated that, in contrast to Schwann cells infected with wild-type *B. pseudomallei*, MMP-2 was not up-regulated in cells infected with *B. pseudomallei* Δ BimA (Fig. 5.2). This result demonstrates that multinucleation as induced by BimA is crucial for up-regulation of MMP-2.

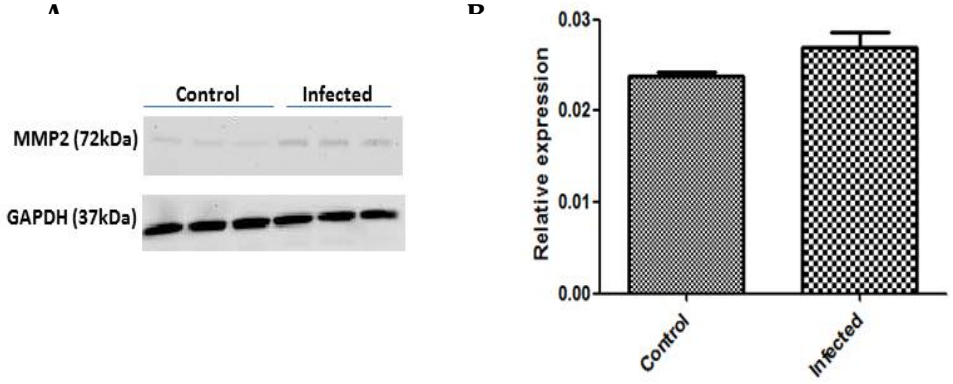
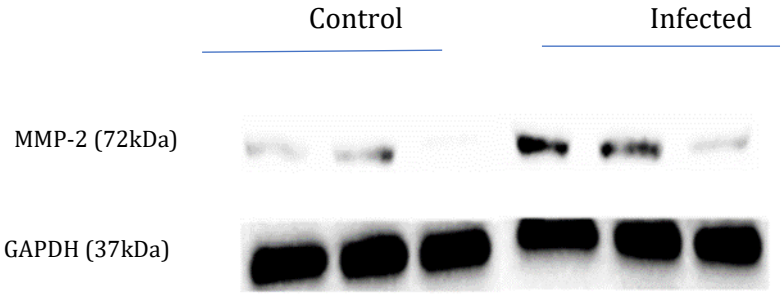


Figure 5-2. Multinucleation of Schwann cells mediated by the B. pseudomallei protein BimA is crucial for upregulation of MMP-2*. A, shows the immunoblot for the expression of MMP2 after infection with B. pseudomallei ΔBimA mutant for 24 h in comparison with control (non-infected). B, shows the densitometry data from immunoblotting for expression of MMP2 after infection with B. pseudomallei ΔBimA mutant compared to the control (non-infected). n=3, p=0.115.*

5.4 Long-term infection of trigeminal Schwann cells with *B.pseudomallei* upregulates the expression of MMP-2

To study whether long-term infection can alternate the expression of MMP-2 as the main EMT marker in trigeminal Schwann cells, we infected the cells with low MOI (10:1) and higher incubation time (72h). The immunoblotting results demonstrated that endogenous protein expression of MMP-2 was significantly upregulated following long-term infection with *B.pseudomallei.*

A



B

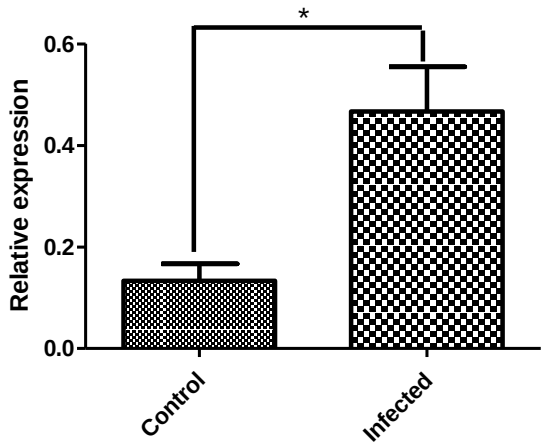


Figure 5-3. Expression of MMP-2 after infection with B.pseudomallei MOI 10:1 for 72 h. *A) The immunoblot for the expression of MMP-2 after infection with B.pseudomallei B) the densitometry data from immunoblotting for expression of MMP-2 after infection with B.pseudomallei MOI 10:1 for 72h compare to control. n=3, P<0.05*

5.5 Infection with *N. meningitidis* serogroup B causes the alteration in gliomagenesis markers

For the first time, our results showed that *N. meningitidis* serogroup B can induce multinucleation and nuclear atypia in trigeminal Schwann cells (Chapter 4). The molecular mechanisms, the genes and the pathways involved in multinucleation are not known yet. To study whether nuclear alteration following infection with *N. meningitidis* serogroup B regulates the expression of gliomagenesis genes, we performed quantitative SWATH-MS proteomics (see 3.15). Based on our results, out of 930 studied genes 281genes (~30%) showed significant changes in their expression following the infection (see appendix). Some of the main regulated proteins involving in gliomagenesis are listed in Table 5.1.

Table 5-1 *some of the significantly regulated proteins involved in gliomagenesis after infection with N. meningitidis serogroup B*

Protein name (regulation following infection)	Role in gliomagenesis
PAI2 or SerpinB2 (Upregulated ~ 2 fold) **P<0.001**	Retinoblastoma protein (Rb) plays a pivotal role in regulation of cell proliferation and sensitivity to apoptosis. Loss of Retinoblastoma in response to stress or inflammatory cytokines can leads to increasing apoptosis by eliminating Rb-mediated repression of proapoptotic gene transcription. SerpinB2 is cytoprotective retinoblastoma-binding protein and can protect retinoblastoma from cleavage by calpain. Thus, upregulation of retinoblastoma level results in increasing cell survival (Ekberg and St John 2015).
VPs35 or Vacuolar protein sorting-associated protein 35 (Downregulated ~1.14 fold) **P<0.001**	This protein is an essential retromer subunit which is required in wnt signalling pathway. The aberrant wnt signalling pathway is seen in many cancers. Thus, loss of VPs35 results in downregulation of wnt signalling pathway and leads to carcinogenesis and gliomagenesis (Nazareth, Lineburg et al. 2015).
PGAM1 or Phosphoglycerate mutase 1 (Downregulated ~1.4 fold) **P<0.001**	PGAM1 is usually downregulated in glioma cells compare with normal brain cells (Rodriguez, Folpe et al. 2012).
PROF1 or Profilin 1 (Downregulated ~1.15 fold)	This protein is a putative tumour suppressor and has been shown to inhibit the tumour cell growth and metastasis in several cancers. Thus,

P<0.001	downregulation of Profilin1 leads to decrease of tumour suppression and elevation of tumorigenesis (Casadei, Scheithauer et al. 1995, de Martel, Ferlay et al. 2012).
FinC or FibronectinC (Downregulated ~ 1.6 fold) **P<0.001**	Loss of fibronectin leads to loss of contact inhibition of cell movement and proliferation and allows cells to facilitate the invasion of neighbouring tissues and metastasis to remote organs and initiate carcinogenesis especially in head and neck cancers (Ryu, An et al. 2008).
ANXA1 or AnnexinA1 (Downregulated ~1.1 folds) **P<0.001**	This protein has anti proliferative function by downregulating the COX-2 expression. Thus, loss of Annexin 1 leads to the overexpression of Cox-2 (Stone, DeShazer et al. 2014).
VINC or Vinculin (Downregulated ~1.15 folds) **P<0.05**	Vinculin as a key adhesive protein can regulate metastasis in several tumours by losing cell-cell adhesion. Loss of Vinculin can promote the EMT and metastasis (Gong, Lai et al. 2015).
MIF or Macrophage Migration inhibitory factor (Upregulated ~1.14 folds) **P<0.05**	MIF as an inflammatory cytokine is usually overexpressed in many tumours and promotes EMT by decreasing E-cadherin (Gong, Cullinane et al. 2011).
LMNα or laminin alpha (Upregulated ~ 1.14 folds) **P<0.001**	This protein is one of the major components of extracellular matrix and is associated with cancerous cells metastasis and EMT. Interaction of laminin and its receptor can increase the invasion and migration of the tumour cells. The upregulation of laminin in the trigeminal Schwann cells following the infection with *N. meningitidis* can increase the cellular migration capacity(Ryu, An et al. 2008).

5.6 Discussion

Glioma encompasses all tumours that originate from glial cells (Zong, Verhaak et al. 2012). As discussed earlier in this Thesis, pathogens constitute significant but not well known risk factors in brain cancer (Alibek, Kakpenova et al. 2013). Glioma tumours are characterized by certain unique gene expression and biological pathways (Holland 2001) which are used by pathologists for diagnosis. Here, we examined the regulation of four key inflammatory markers that are also markers of gliomagenesis (TLR-4, IL-6R, COX-2 and MMP-2) in trigeminal Schwann cells after infection with *B. pseudomallei*. The results clearly demonstrate that the Schwann cells reprogram their gene expression machinery after the infection to upregulate the expression of these four genes. When infected with *N. meningitidis* serogroup B, trigeminal Schwann cells also reprogrammed their gene and altered numerous gliomagenesis and carcinogenesis related proteins.

TLRs are usually expressed on tumour cells and participate in a variety of tumour-promoting activities such as cell growth, proliferation, invasion, migration, and even stem cell maintenance (Deng, Zhu et al. 2014). Like other neoplasms, glioma cells alter their TLR-4 expression both at transcriptional and translational levels (Persson, Petritsch et al. 2010, Samarakoon, Weerasekera et al. 2012). Our *In vitro* results demonstrated that trigeminal Schwann cells alter significantly TLR-4 expression following the 24 h of infection with *B. pseudomallei* both at transcriptional (~9-fold increase) and translational (~3-fold increase) levels. These results show that 24 h of infection with *B. pseudomallei* is enough to cause alteration in TLR-4 gene expression, resulting in increased amounts of the TLR-4 protein. Up-regulation of TLR-4 may be involved in the natural anti-bacterial response of Schwann cells to pathogens; exposure to LPS results in up-regulation of TLR-4 in many cell types, including peripheral nerve Schwann cells (Hao, Peduzzi-Nelson et al. 2009). However, if the expression of TLR-4 is persistent over time, which may occur due to long-term intracellular survival of the bacteria, this molecular change may drive the cells towards a pathological state. Persistent bacterial infections may cause cancer via induction of chronic inflammation (reviewed by (Parsonnet 1995, Mager 2006), which involves up-regulation of proteins such as TLR-4 (Multhoff, Molls et al. 2011, Yeh, Huang et al. 2016). Indeed, modulation of TLR-4 expression is now considered as a possible future anti-cancer immunotherapy (Oblak and Jerala 2011, Awasthi 2014).

IL-6R, the receptor for IL-6, is another protein that is often expressed at high levels in glioma cells. The IL-6 pathway is one of the key regulatory pathways in oncogenesis and tumour growth (Trikha, Corringham et al. 2003). IL-6, as one of the major players in inflammatory responses, can modulate several oncogenic and tumour suppressor genes during inflammation (Braconi,

Huang et al. 2010). Thus, this cytokine can connect inflammation to carcinogenesis by altering gene expression. Upregulation of the IL-6R and IL-6 both at transcriptional and translational levels has been detected in several glial and brain tumours (Goswami, Gupta et al. 1998). Knockdown of the IL-6R has been shown to significantly reduce growth of glioblastoma multiforme, along with apoptosis of cancer cells (Wagner and Myers 1996). The interaction between IL-6 and its receptor leads to activation of the JAK/STAT, ERK, and PI3K signalling pathways, resulting in STAT3 activation (Sengupta, Talbot et al. 1998). STAT3 activation can increase the cell proliferation and inhibition of apoptosis (Scheller, Chalaris et al. 2011). Our results showed that *B. pseudomallei* infection in trigeminal Schwann cells leads to upregulated expression of IL-6R both at the transcriptional and the translational levels. In Schwann cells, IL-6R is thought to be involved in the transformation of the cells towards a phagocytic phenotype during Wallerian degeneration (Lee, Seo et al. 2009). Thus, up-regulation of this receptor, like the increase in TLR-4 expression, may be part of the natural defence against pathogens; however, it may also suggest that the cells are undergoing pathological changes initiated by the bacteria related to chronic inflammation and potentially cancer.

We also examined the expression of an inflammatory mediator that is also one of the key enzymes in tumour formation, COX-2. COX-2 expression is stimulated in a number of malignancies including glioma and brain cancers. It plays as a one of the key tumorigenesis factors by stimulating angiogenesis, enhancing cell invasiveness and inhibiting apoptosis in several cancers (Giercksky 2001, Singh and Lucci 2002). Our results showed that *B. pseudomallei* can up regulate the expression of COX-2 in glial cells. Although, at this point we cannot explain the exact molecular mechanism, we hypothesize that *B. pseudomallei* upregulates the Cox-2 mRNA by increasing the prostaglandin E2 (PGE2) production in trigeminal Schwann cells (Rahaman, Harbor et al. 2002).

The other marker that we studied following the infection with *B. pseudomallei* is MMP-2, a key modulator of EMT. Out of the proteins tested, MMP-2 is the one most closely linked to cancer, and the one that is consistently upregulated in many different types of cancer (Brehmer, Biesterfeld et al. 0000) including glioma (Beliveau, Delbecchi et al. 1999, Hagemann, Anacker et al. 2012, Ramachandran, Sorensen et al. 2017, Turunen, Tatti-Bugaeva et al. 2017).

Our results strongly showed that MMP-2 was upregulated significantly due to the infection with *B. pseudomallei*. We also demonstrated that infection with the *B. pseudomallei* ΔBim-A mutant, which lacks the ability to form MNCs, did not alter the expression of MMP-2, suggesting that the formation of multinucleated cells is directly related to and required for the enhanced MMP-2 expression. Thus, it is possible that multinucleation is a crucial process in EMT of Schwann cells.

It has previously been shown that the intracellular pathogen *Mycobacterium leprae*, which causes leprosy, upregulates the expression of MMP-2 in human peripheral nerves *in vivo*, and in cultured Schwann cells (Oliveira, Antunes et al. 2010). Interestingly, leprosy has been shown to be associated with increased incidence of head and neck cancer (however, no specific data exist specifically regarding glioma) (John, Roul et al. 1994); leprosy has recently been considered a model of cancer immunosurveillance (Park, Rendini et al. 2016), and *M. leprae* infections of the brain, though extremely rare, can mimic glioma tumours (Lee, Moon et al. 2014). Together, these findings raise the possibility that certain intracellular bacteria may cause cellular changes relating to cancer.

Our SWATH-MS proteomics data clearly showed that following the infection with *N. meningitidis* serogroup B, trigeminal Schwann cells reprogram their gene expression machinery. Our proteomics profiling strongly revealed that *N. meningitidis* infection can affect the expression of gliomagenesis and carcinogenesis related proteins (Table 5.1 and Apendix1). Based on the SWATH-MS proteomics data, numbers of proteins involved in cell survival, EMT and proliferation are upregulated and proteins involved in tumour suppression and apoptosis downregulated following the infection with *N. meningitidis.*

One of the disadvantage of SWATH-MS proteomics is the limitation of binding the targeted protein to spotted probes on forward and reverse microarray. Thus, synthesis of many different probes is necessary which limits detection of all proteins targeted by the probes. Thus, it may be a reason that the SWATH-MS proteomics didn't detect the four before-mentioned proteins.

Chapter 6: Metabolic Shift

6 *B. pseudomallei* infection induces a metabolic shift resembling the Warburg effect in trigeminal Schwann cells

6.1 Glucose metabolism and regulation of expression of key glycolysis related enzymes in trigeminal Schwann cells infected with *B. pseudomallei*

Since 1924 when Otto Warburg for first time described the Warburg effect in cancerous cells, many question remains unresolved. The main question is *why exactly the cancerous cells "prefer'' to use highly inefficient method to metabolize glucose?* Although several hypotheses have been suggested, to date there is no clear explanation of this rather unusual effect. It is well assumed that the cellular switch to aerobic glycolysis may represent the very early point in time when a normal cell becomes cancerous (Devic 2016).

The full oxidation of one glucose molecule by oxidative phosphorylation (OXPHOS) in the presence of oxygen produces 38 molecules of ATP whereas glycolysis produces only 2 molecules of ATP, resulting in the production of 2 molecules of pyruvate. To address the earlier question about the reason of utilizing glycolysis instead of OXPHOS, one possibility is that insufficient ATP production is only a problem when the resources are scarce but in proliferating mammalian cells, which are exposed to a continual supply of nutrient in circulating blood, this is not the case. It has been shown that in actively proliferating cells, ATP production may never be limited and the cells exhibit a high ratio of ATP/ADP and $NADH/NAD^+$ (Christofk, Vander Heiden et al. 2008, DeBerardinis, Lum et al. 2008). The other possibility is that proliferating cells have important metabolic requirements that can be derived from glycolysis (explained in details in the literature review chapter, chapter 2).

Glioma cells, like most cancerous cells, possess the unique metabolic state known as the Warburg effect, where they utilize aerobic glycolysis as the main supplier of ATP by primarily metabolizing glucose (Warburg 1956, Poteet, Choudhury et al. 2013). Glucose transporter-1 (GLUT-1), a plasma membrane glucose transporter that transports glucose into the cytosol, plays critical roles in Warburg effect, progression and development of cancers including glioma (Han, Lee et al. 2012). Although, the relationship between GLUT-1 and the Warburg effect is not yet fully understood, it has been shown that inhibition of GLUT-1 expression can reverse the Warburg effect and induce apoptosis (Zhang, Zhao et al. 2015),(Han, Lee et al. 2012). The precise mechanisms of GLUT-1 overexpression in cancerous cells is not fully understood but numerous

studies have shown that HIF-1α plays a vital role in metabolic reprogramming and changes in expression of key glycolytic genes such as GLUT-1 (Chen, Pore et al. 2001).

Lactate dehydrogenase A (LDH-A), another key glycolytic enzyme, is overexpressed in cancer cells including glioma cells (Valvona, Fillmore et al. 2016). The enhanced expression of HIf-1α is associated with LDH-A overexpression and poor survival in brain cancer, including glioma (Miao, Sheng et al. 2013). Several studies, including *in vivo* investigations, prove the importance of LDH-A in tumour cell growth and survival through protection from reactive oxygen species (ROS) via inhibition of OXPHOS (Fantin, St-Pierre et al. 2006). Moreover, increased NAD^+ production by upregulated LDH-A activity in cancerous cells can affect the homeostasis of ROS by altering the concentration of reducing equivalents in the mitochondria (Locasale and Cantley 2011)

HIF-1α is an important oxygen-sensitive transcription factor in all nuclear cells that allows the adaptation to hypoxic environments. This factor is an important mediator of the cellular response to stress e.g. metabolic, hypoxic, or inflammatory (Shay and Celeste Simon 2012). In particular, enhanced HIF-1α expression has been detected in the majority of primary brain tumours, including glioma, and correlates with poor survival in those tumours (Joseph, Conroy et al. 2015). Although hypoxia in cancerous cells accounts for HIF-1α stabilization, many studies reveal that high pyruvate concentrations also result in HIF1α stabilization independently of hypoxia (Lu, Forbes et al. 2002)

Under normal conditions pyruvate is predominantly oxidized in mitochondria by the pyruvate dehydrogenase-1α (PDH-1α) resulting in acetyl-CoA production which enters into the Krebs cycle. PDH-1α is known as a gatekeeper directing the carbon flux into the mitochondria. Downregulation of PDH expression may plays a central role in switch from OXPHOS to glycolysis (Gray, Tompkins et al. 2014). Moreover, in non-hypoxia condition downregulation of PDH-1α may be an important factor for HIF1α stabilization and shifting to the Warburg effect (Koukourakis, Giatromanolaki et al. 2005).

Thus, in summary, the Warburg effect is characterised by increased expression of GLUT-1, LDH-α and HIF-1α, accompanied by decreased expression of PDH-α1 (reviewed by (Mahase, Rattenni et al. 2017, Strickland and Stoll 2017). To study the regulation of key involving enzymes in Warburg effect after infection with *B. pseudomallei*, we therefore analysed the expression of GLUT-1, LDH-A, HIF-1α and PDH-1α (at the transcriptional level), and compared the mRNA levels of these Warburg markers between control cells and infected cells.

Endogenous mRNA expression of GLUT-1, LDH-A, HIF-1α and PDH-1α was studied by performing qRT-PCR using specific primers (**Table3.1)** after infection with *B. pseudomallei* (MOI 75:1) for 24 hours. All the data presented in the following Figures are from three independent experiments and a Student's t-test was performed to determine if any changes in transcription level were significantly different.

6.2 Upregulation of facilitative glucose transporter-1 (GLUT-1)

The first crucial step in glucose metabolism is the capture and transportation of glucose into the cells. We examined the mRNA levels of GLUT-1 as the main expressed glucose transporter in brain and glial cells following the infection with *B. pseudomallei*. The results showed that following 24 h of infection, the GLUT-1 transcript increased ~1.4-fold. (Fig 6.1)

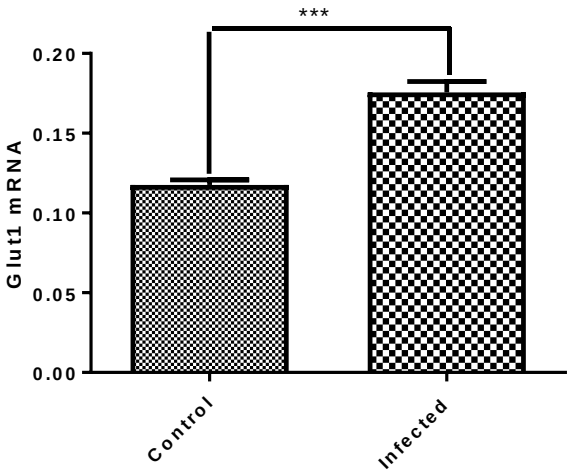


Figure 6-1. B. pseudomallei infection causes a ~1.5 fold up-regulation of the GLUT-1 mRNA in trigeminal Schwann cells. *qRT-PCR analysis demonstrated that the mRNA levels were significantly higher in infected cells than control cells. Error bars show +/-SEM. ***p ≤ 0.0001.*

6.3 Upregulation of lactate dehydrogenase -A (LDH-A)

Next, we analysed the mRNA level of LDH-A. To sustain glycolysis, pyruvate must be converted into lactate and (NAD) H to (NAD+), crucial for redox homeostasis, by LDH-A. Our results suggested that LDH-A transcript increased ~1.2-fold following 24 h of infection with *B. pseudomallei*.

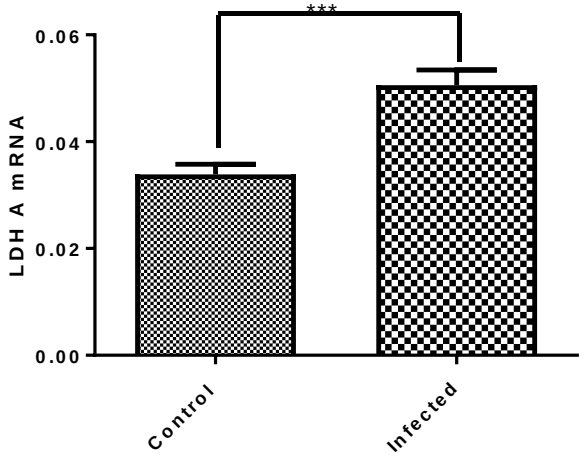


Figure 6-2. Infection with B. pseudomallei infection causes a ~1.2 fold up-regulation of the LDH-A mRNA level. *Error bars show +/-SEM. ***p=0.0002.*

6.4 Upregulation of Hypoxia inducible factor-1α (HIF-1α)

A growing body of evidence suggests that the transcription factor HIF-1α induces genes encoding glycolytic enzymes, glucose transporters and lactate export (O'Neill and Hardie 2013). Moreover, it has been shown that HIF-1α is activated during various infection with human pathogens such as *Mycobacteria tuberculosis* (Werth, Beerlage et al. 2010, Shi, Salamon et al. 2015). We therefore investigated whether infection with *B. pseudomallei* resulted in altered levels of HIF-1α mRNA. Our results show that following 24 h of infection, HIF-1α transcript increased ~2.5-fold.

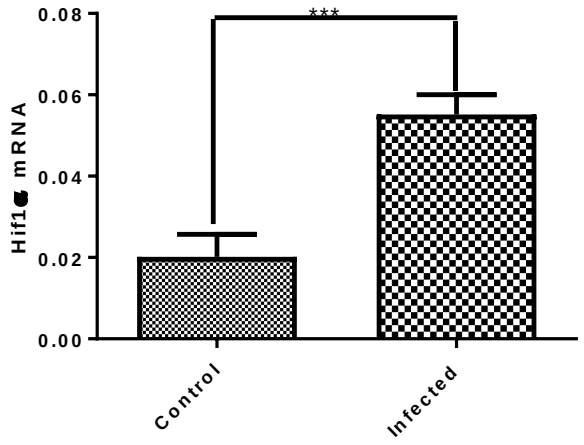


Figure 6-3. Infection with B. pseudomallei infection causes a ~2.5 fold up-regulation of the mRNA level for HIF-1α. *Error bars show +/-SEM. ***p≤ 0.0001.*

6.5 Downregulation of Pyruvate dehydrogenase α 1 (PDH-α1)

PDH-α1 is a part of the pyruvate dehydrogenase complex catalysing the oxidative decarboxylation of pyruvate, leading to the formation of CO_2 NADH(H) and acetyl-CoA which subsequently enters into the Krebs cycle. This enzyme is the key connector of glycolysis in the cytosol with the Krebs cycle in mitochondria. The results showed that following 24 hours of infection with *B. pseudomallei*, the PDH-α1 transcript decreased ~1.1 fold indicative of decreased carbon flux through pyruvate oxidation into the mitochondria.

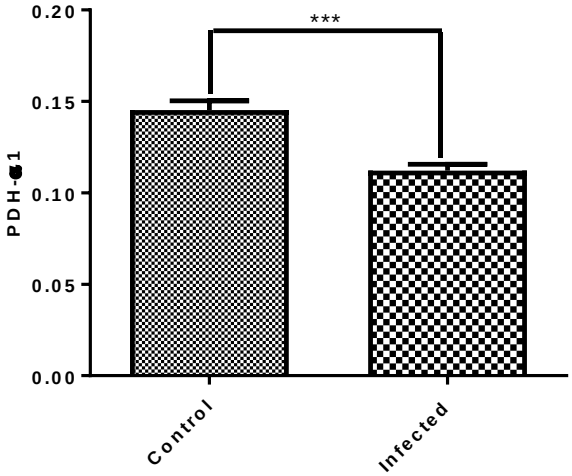


Figure 6-4. Infection with B. pseudomallei infection causes a ~1.1 fold down-regulation of the PDH-α1 mRNA level*. Error bars show +/-SEM. ***p≤ 0.0001.*

6.6 Discussion

The observed transcriptional changes of host central metabolism genes suggest that infection of trigeminal Schwann cells with *B. pseudomallei* is accompanied by a metabolic shift from OXPHOS towards enhanced glycolysis and formation of lactic acid, which ultimately decreases the pH of the cell. This metabolic signature known as the Warburg effect is typically associated with cancer metabolism and is usually regulated by HIF-1α.

Here, for first time we showed that bacterial infection (*B. pseudomallei*) in glial cells can increase the expression of HIF-1α as a key regulatory Warburg effect enzyme. The levels of active HIF-1α are altered during the Warburg effect due to changes in both at the protein and mRNA levels. Under normoxic conditions, the HIF-1α protein is present at relatively low levels. On the posttranslational level, HIF-1α protein in the cell is regulated by ubiquitination and proteasomal degradation, mediated by a tumour suppressor, von Hippel-Lindau protein (pVHL) and an E3

ubiquitin ligase. In hypoxia, however, HIF-1α is dephosphorylated at two key proline residues, resulting in this degradation pathway being inhibited and the cellular levels of HIF-1α increasing (Kamura, Sato et al. 2000). The mRNA level of HIF-1α also increases in hypoxia, but the mechanism is unknown (Roy, Volgin et al. 2004). The level of iron within the cell is known to affect the HIF-1α mRNA level with low iron level corresponding to high level of HIF-1α mRNA (Roy, Volgin et al. 2004) (Kempf, Lebiedziejewski et al. 2005). Infection with certain intracellular bacteria can alter iron levels in host cells, and *B. pseudomallei* is known to exhibit an iron-acquisition system (Kvitko, Goodyear et al. 2012) which could potentially trigger HIF-1α upregulation and activation (Kempf, Lebiedziejewski et al. 2005). Increased HIF-1α levels may also be dependent on TLR-4 expression; TLR-4 activation has been shown to cause upregulation of HIF-1α mRNA and protein (Zhou, Zhu et al. 2014). Thus, the upregulation of HIF-1α mRNA may occur secondary to the increase in TLR-4 that resulted from the *B. pseudomallei* infection.

GLUT-1 is the critical regulator of proliferation, glucose uptake and aerobic glycolysis in most cancer cells (Young, Lewis et al. 2011). Increased expression of GLUT-1 in numerous cancer cells is mediated via activation of the mTOR pathway (Bhattacharya, Low et al. 2014). Previous studies showed that *B. pseudomallei* infection can upregulate the mTOR pathway in lung epithelial cells (Vellasamy, Mariappan et al. 2016). This result is consistent with our finding of increased GLUT-1 mRNA levels (~1.5-fold) following the infection with *B. pseudomallei* in Schwann cells.

The upregulation of HIF-1α promotes the expression of LDH-A by binding to specific binding sites of the LDH-A promotor (Cui, Han et al. 2017). However, the exact mechanisms underlying this interaction remain unclear. Our results suggest that the upregulation of LDH-A (~1.2 fold) at transcription level may be due to the increased expression of HIF-1α following the infection with *B. pseudomallei* as a key regulatory transcription factor and glycolytic enzyme. Interestingly, the BimA expression in *B. pseudomallei* is activated by acidic pH (Chen, Wong et al. 2011), suggesting that cell-cell fusion may be dependent on and occurs downstream of the bacteria-induced metabolic shift. Thus, the ability to lower host cell pH may be an adaptive mechanism critical for *B. pseudomallei* invasiveness and/or intracellular survival. For the first time we found that bacterial infection (*B. pseudomallei*) can reduce the mRNA level for the mitochondrial enzyme PDH-α1 in glial cells (~1.1 fold). PDH-α1 acts as a gatekeeper of OXPHOS and connect glycolysis to mitochondrial respiration by converting pyruvate to acetyl-CoA which directly enters the TCA cycle. As this conversion is irreversible, this represents a critical regulatory enzymatic function in cellular energy metabolism. It has been shown that HIF-1α, as a master regulatory transcription factor involved in controlling glycolytic gene expression, can downregulate the expression of PDH-α1 at the transcriptional level (Kim, Tchernyshyov et al. 2006). Thus, we

postulate that HIF-1α overexpression following the infection (*in vitro*) may reprogram the mitochondrial respiration in Schwann cells by downregulating PDH-α1, resulting in a shift from OXPHOS to glycolysis even in the presence of oxygen, similar to the Warburg effect.

6.7 Conclusion

Overall these results demonstrate that *B. pseudomallei* infection of trigeminal Schwann cells results in alteration of expression of GLUT-1, LDH-A, HIF-1α and PDH-1α which are involved in the Warburg effect of altered glucose metabolism. As the Warburg effect is considered the early time point when cells become cancerous, these results provide further evidence that bacterial (*B. pseudomallei*) infection initiates cancer-like changes in Schwann cells.

Chapter 7: Discussion

7 Discussion

The main question in this study is whether bacteria, in particular intracellular species known to invade the nervous system such as *Burkholderia pseudomallei* and *Neisseria meningitidis*, can initiate glioma-like responses in glial cells. Despite significant breakthroughs in the understanding, prevention, and treatment of cancer during the last decades, the role of bacterial infections in carcinogenesis is not yet fully understood and requires further studies. Despite our, as yet, incomplete knowledge of carcinogenesis, infection has been already established as one of the major causative agents of human cancer and over 15% of all malignancies are attributed to infections (Kuper, Adami et al. 2000). The link between viral infections and cancer is now well established (White, Pagano et al. 2014, Luo and Ou 2015), reflected in the Australian breakthrough discovery by Ian Frazer that resulted in the human papilloma virus (HPV)vaccine as a cervical cancer prophylactic (Frazer and Levin 2011). The link between bacteria and cancer is also emerging strongly; for example, *Helicobacter pylori* is a recognized cause of gastric cancer (Ishaq and Nunn 2015), whilst *Streptococcus* species and *Chlamydia pneumoniae* are linked to colon (Abdulamir, Hafidh et al. 2011) and lung (Chaturvedi, Gaydos et al. 2010, Zhan, Suo et al. 2011) cancer, respectively. The key mechanisms implicated in bacteria-induced oncogenesis are chronic/persistent infection, immune invasion and immune suppression(Kuper, Adami et al. 2000), as well as the production of mutagenic compounds by bacterial metabolism (Parsonnet 1995). For instance, *Helicobacter pylori,* the first established carcinogen bacteria causing gastric adenocarcinoma, can induce long life inflammation which in turn induces cell proliferation and production of N-nitroso compounds (human carcinogens) and mutagenic free radicals (Handa, Naito et al. 2010). Moreover, it has been shown that oral bacteria such as *Porphyromonas gingivalis* may be responsible for the p53 mutation known to cause pancreatic cancer by producing peptidyl arginine deaminase (PAD) enzymes (Ogrendik 2015). It is therefore necessary that we more fully understand how bacteria can alter the growth and metabolism of mammalian cells so that potential links with cancer can be identified.

Glioma as the most common type of malignant brain tumours comprise ~80% of all brain tumours (Goodenberger and Jenkins 2012). Most of gliomas are associated with poor survival. Thus, many epidemiological studies have been focused on identifying the gliomagenesis risk factors. To date, only two risk factors have so far been conclusively demonstrated to affect gliomagenesis; 1) exposure to therapeutic or high-dose radiation (Ohgaki and Kleihues 2005), (Schwartzbaum, Fisher et al. 2006) and 2) genetic syndromes such as Li–Fraumeni syndrome, and Turcot's syndrome (Ichimura, Ohgaki et al. 2004, Schwartzbaum, Fisher et al. 2006). The role of bacterial infection in brain cancer and gliomagenesis has been under investigation during last decades but until now there has been no firm association between glioma/brain cancer and

bacteria. The only link shown so far is evidence of bacterial infection with *Mycoplasma* and *Brucella* species, which have been found in some brain cancer tissues including glioma (Huang, Li et al. 2001).

In this research, for the first time we studied whether the gram negative facultative intracellular bacillus *B. pseudomallei* and gram negative diplococcus *N. meningitidis* (serogroup B), which are the etiological agents of melioidosis and bacterial meningitis, respectively, initiate changes in Schwann cells consistent with gliomagenesis. Our *in vitro* experiments clearly demonstrated that *B. pseudomallei* and *N. meningitidis* infection of trigeminal Schwann cells can initiate multinucleation of glia. Further, we showed *N. meningitidis* induced nuclear atypia. Multinucleation and the presence of atypical nuclei are two key characteristic features of glioma cells.

Our group showed that *B. pseudomallei* is able to travel along the trigeminal nerve and reach the brain, spinal cord and cranial cavity within 24 hours of intranasal exposure. This pathway constitutes a largely uncharacterised mechanism for bacterial infection of the CNS and can have implications for many other diseases (St John, Ekberg et al. 2014, St John, Walkden et al. 2016) (Fig 1.1). The bacterial strategy of cell-cell dissemination is due to their ability to induce cell-cell fusion/multinucleation (Benanti, Nguyen et al. 2015), which may lead to detrimental downstream effects in the infected cells. While we have previously shown that the related pathogen *Burkholderia thailandensis* can infect and survive within trigeminal Schwann cells (Panni, Ferguson et al. 2013), the resultant effects on the glia by *Burkholderia* species were, until the current study, unknown.

To understand how the incubation time with *B. pseudomallei* affects the formation of MNCs, the trigeminal Schwann cells were incubated for different time points. While MNCs were detected with short incubation times (6 h), increasing the incubation time to 24 h significantly increased the number of MNCs in the culture, with many of the multinucleated trigeminal Schwann cells having four or more nuclei.

The formation of multinucleated cells is dependent on the *B. pseudomallei* T6SS protein BimA, which has been shown to induce cell-cell fusion in other types of cells (murine macrophages) (Kespichayawattana, Rattanachetkul et al. 2000). To study the role of BimA in Schwann cells multinucleation, we infected the Schwann cells with a ΔBim A mutant. Our immunofluorescence microscopy results showed that Schwann cells infected with *B. pseudomallei* (ΔBimA) rarely formed MNCs in comparison with *B. pseudomallei* (wt) infection. Thus, we postulate that the multinucleated cells formed via cell-cell fusion. Live cell imaging studies will answer this

question, however we were not able to perform these due to the potential risk factor of handling *B. pseudomallei*, it can only be used within PC3 standard laboratories and our facilities currently do not have equipment capable of live cell imaging.

Multinucleation is a characteristic feature of cancerous glioma cells, and we therefore investigated whether *B. pseudomallei* induced expression of key markers relating to cancer. We found that TLR-4, the IL-6R, COX-2 and MMP-2 were all upregulated following infection. TLRs are expressed on tumour cells and participate in a variety of tumour promoting activities such as cell growth, proliferation, invasion, migration, and even stem cell maintenance (Deng, Zhu et al. 2014). In particular, TLR4 expression is upregulated and elevated in glioma cells at both the transcriptional and the translation level (Samarakoon, et al. 2012; Persson, et al. 2010). In Schwann cells, TLR4 can be upregulated in response to bacterial lipopolysaccharides (LPS) (Hao, Peduzzi-Nelson et al. 2009), but the downstream effects relating to potential cancer-related changes remain largely unknown.

IL-6R, the receptor for IL-6, is another marker that is often overexpressed in glioma cells. Upon IL-6-induced receptor activation, intracellular signalling is mediated by the JAK family (JAK1-3), leading to activation of STAT3 transcription factors. STAT-3 activation induces invasiveness and migration in glioma cells (Machairiotis et al. 2013) and inhibits apoptosis (Rahaman, et al. 2002; Alibek, et al. 2013). Knockdown of IL-6R has been shown to significantly reduce growth of glioblastoma multiforme, along with increased apoptosis (Wagner and Myers 1996). In Schwann cells, the IL-6R is thought to be involved in the transformation of the cells towards a phagocytic phenotype during Wallerian degeneration (Jung, Cai et al. 2011).

COX-2 is an inducible isoform of cyclooxygenases expressed in many cell types including Schwann cells (Takahashi and Miyazawa 2012) that catalyses conversion of arachidonic acid to prostaglandins and other eicosanoids (Sawada et al. 2007). It is well understood that the overexpression of COX-2 in cancerous cells contributes to tumour development and progression, angiogenesis, tumour invasion and resistance to apoptosis (Frances et al. 2013) Liu, Qu et al. 2015). It has been recently shown that COX-2 has direct transforming activity by increasing the malignancy of glioma cells in both *in vitro* and *in vivo*, along with inhibition of apoptosis and differentiation (Rahama, et al. 2002). Human papilloma virus (Song, Lee et al. 2006), Epstein-Barr virus (Wakisaka and Pagano 2003)and human cytomegalovirus (Baryawno et al. 2011; Maussang et al. 2009) are known to induce oncogenesis in different cell types via upregulation of COX-2.

Whilst increased expression of TLR-4, IL-6R and COX-2 is seen in cancer cells, this response may also be part of the normal immune/inflammatory response of Schwann cells to pathogens. We

therefore examined whether *B. pseudomallei* also induced elevated expression of MMP-2, which is overexpressed in Schwannoma (Schwann cell tumour) cells. This protease has been specifically shown to be involved in infections by pathogens that are linked to cancer (reviewed by (Elkington, O'Kane et al. 2005). MMP-2, which promotes migration of cancerous cells resulting in metastasis, is thought to be a key factor involved in epithelial to mesenchymal transition (EMT). EMT is comprised of several steps including loss of cell-cell cohesion between individual cells in the epithelium and the stimulation of cell motility (Xu, Wang et al. 2014). Here, MMP-2 specifically degrades type IV collagen. High activation and upregulated expression of MMP2 has been shown in some types of glioma, including glioblastoma multiforme which suggest that MMP-2 might be an important factor in invasion of malignant gliomas (Wang, Wang et al. 2003). The upregulation we observed following infection of trigeminal Schwann cells with *B. pseudomallei* may be due to the LPS-induced activation of TLR4, MAPK and the PI3K/Akt signalling pathway, which leads to elevated MMP-2 gene expression (Cho, Kang et al. 2014).

Infection with the *B. pseudomallei* ΔBimA mutant did not cause upregulation of MMP-2, suggesting that the formation of multinucleated cells is directly related to and required for the enhanced MMP-2 expression. Thus, it is possible that multinucleation is a crucial process in EMT of Schwann cells.

Toll-like receptor activation is known to cause a metabolic shift in innate immune cells towards oxidative glycolysis and formation of lactic acid, which ultimately decreases the pH. This cascade of events closely resembles the Warburg effect in cancer cells (reviewed by(Labak, Wang et al. 2016)). Oxidative glycolysis, as well as the Warburg effect, is characterised by increased expression of GLUT-1, LDH-A and HIF-1α, and decreased expression of PDH-α1 (reviewed by (Strickland and Stoll 2017) We found that *B. pseudomallei* infection of trigeminal Schwann cells caused changes in the expression levels of these markers indicative of oxidative glycolysis or the Warburg effect.

The first crucial step in oxidative glucose metabolism is the capture and transportation of glucose into the cells, mediated by GLUT-1, which is one of the two glucose transporters expressed in Schwann cells (Magnani, Cherian et al. 1996). GLUT-1 upregulation which has been seen in numerous cancer cells is associated with the activation of mTOR pathway (Bhattacharya et al. 2014). Previous studies showed that *B. pseudomallei* infection can upregulate the mTOR pathway in lung epithelial cells (Vellasamy et al. 2016) which is consistent with our results in the sense of increasing the GLUT-1 expression.

To sustain glycolysis, pyruvate must be converted into lactate, and (NAD) H to (NAD+), for redox homeostasis by lactate dehydrogenase A (LDH-A). *B. pseudomallei* infection caused an upregulation of both GLUT-1 and LDH-A, as well as of suggesting that the cells were undergoing a metabolic shift towards oxidative glycolysis. Increased LDH-A expression causes a significant decrease in intracellular pH. Interestingly, the BimA gene in *B. pseudomallei* is activated by acidic pH (Chen et al. 2011), raising the possibility that cell-cell fusion may be dependent on and occurs downstream of the bacteria-induced metabolic shift.

An increase in LDH-A expression has also been shown in mouse lung following infection with *Mycobacterium tuberculosis*, as well as in cancer cells and activated immune cells (Shi et al. 2015 ; Herling et al. 2011; Pan, Beverley et al. 1991). One recent study, however, showed that *Mycobacterium leprae* does not induce the Warburg effect in Schwann cells, and instead caused down-regulation of LDH, suggesting that different pathogens may have strikingly different and cell-type specific effects (Medeiros, Girardi et al. 2016).

Pyruvate dehydrogenase α 1 (PDH-α1) is part of the pyruvate dehydrogenase complex catalysing the oxidative decarboxylation of pyruvate with the formation of CO_2, NADH(H) and acetyl-CoA which subsequently enters into the Krebs cycle. This enzyme is the key connector of glycolysis in the cytosol and the Krebs cycle in mitochondria, a critical regulatory point in cellular energy metabolism (Modak et al. 2002). The results showed that following *B. pseudomallei* infection, the PDH-α1 transcript levels decreased, which is indicative of decreased carbon flux through pyruvate oxidation into the mitochondria, another classical feature of the Warburg effect.

Induction of expression of GLUT-1 and LDH (O'Neill and Hardie 2013 ; Palsson-McDermott and O'Neill 2013 ; Cui et al. 2017), as well as inhibition of PDH-α1 expression (Kim, Tchernyshyov et al. 2006) are thought to be mediated by the transcription factor HIF-1α. HIF-1α has been shown to be upregulated after infection with *M. tuberculosis* (Werth, Beerlage et al. 2010). Here, for first time we showed that bacterial infection in Schwann cells can increase the expression of HIF-1α. HIF-1α is usually upregulated under hypoxic conditions, here potentially a result of the *B. pseudomallei*-induced metabolic shift, and induces expression of a large variety of genes involved in promoting cell survival after hypoxia (Semenza 2003, Kaelin and Ratcliffe 2008) (Hartmann, Eltzschig et al. 2008). It is also possible that *B. pseudomallei* bacteria compete with the host cell for intracellular iron can trigger the HIF-1α upregulation and activation (Peyssonnaux, Cejudo-Martin et al. 2007). Upregulation of HIF-1α may also be dependent on TLR-4 expression (Zinkernagel, Hruz et al. 2012). The upregulated expression of TLR-4 following *B. pseudomallei* infection may have in turn increased the expression of HIF-1α.

Overall, the findings presented here suggest that *B. pseudomallei* induces cellular and metabolic changes in trigeminal Schwann cells that mimic those in cancer: the formation of multinucleated cells accompanied by upregulation of key markers associated with cancer. The origin of multinucleated cells in glioma is not yet understood; it has been postulated that these cells remain in the early mitotic phase, undergoing neither fusion nor degeneration (Gladfelter, Hungerbuehler et al. 2006), however, it has not been ruled out that cell-cell fusion can also occur in glioma MNC formation. Thus, pathogens that can cause cell-cell fusion may indeed contribute to gliomagenesis. Thus far, there are no reports of brain cancer in patients who have survived neuromelioidosis, However, follow-up studies of these patients are often lacking; further, melioidosis is considered highly under-reported (Limmathurotsakul, Golding et al. 2016). We have previously shown that minor nasal infections can lead to *B. pseudomallei* bacteria entering the brain without eliciting an immune response, opening up the possibility that chronic neuromelioidosis with low-level infection may go unnoticed for long periods of time (St John, Walkden et al. 2016). Thus, the connection between the infection and potential sequelae may not be clear.

In this research, we observed a metabolic shift from oxidative phosphorylation toward enhanced glucose uptake, glycolysis and lactate formation, along with upregulation of the regulator transcription factor HIF-1α. These events constitute the hallmarks of the Warburg effect, the metabolic shift associated with cancer. Again, it is possible that these changes are crucial cellular strategies for rapid generation of ATP (Werth et al. 2010), however, they may also be driving the cells towards a pathological state such as glioma.

After we showed that *B. pseudomallei* induced a range of potential carcinogenesis-related features in trigeminal Schwann cells, we decided to investigate another pathogen that is also intracellular and "neuroinvasive" but much more common world-wide than *B. pseudomallei*: *N. meningitidis*. *N. meningitidis* causes meningitis, meningo-encephalitis and sepsis ("meningococcal disease"); Serogroup B is the predominant cause of disease and mortality(Harrison 2010). Despite the fact that these diseases are common and serious, very little is known about how the pathogen affects cells within the nervous system.

N. meningitidis can invade the brain in the absence of blood-borne infection (Sjolinder and Jonsson 2010). The suggested alternative routes of invasion are via the olfactory (Sjolinder and Jonsson 2010) and/or trigeminal nerves (Dando, Mackay-Sim et al. 2014). Prior to this study, infection of the glia of these nerves with this pathogen had never been shown; thus, we investigated whether *N. meningitidis* (serogroup B) infected trigeminal Schwann cells, and how infection altered cell morphology. Our results show that *N. meningitidis* serogroup B readily

infected the Schwann cells, resulting in the formation of MNCs. Further, we found that *N. meningitidis* not only caused multinucleation, but also the appearance of atypical nuclei. As nuclear atypia is one of the significant features of gliomagenesis these results are consistent with the bacteria initiating the gliomagenesis pathway.

SWATH-MS proteomics is a powerful tool to examine unbiased changes in protein expression. Comparison of the trigeminal Schwann cells proteome between infected cells and control cells clearly showed that *N. meningitidis* can alter the expression of carcinogenesis-related genes. *N. meningitidis* infection down-regulated the expression of proteins responsible for tumour suppression such as profilin 1, and up-regulated the anti-apoptotic protein SerpinB2. Further, our results suggest that the infection with *N. meningitidis* may cause EMT via downregulation of E-cadherin via MIF-1 overexpression (Funamizu, Hu et al. 2013). The proteomics results also suggest that *N. meningitidis* increases the cellular proliferation capacity and cellular invasiveness by downregulation of annexin1 (Cox-2 inhibitor) and fibronectin C, respectively (discussed in more detail in Chapter 5). Overall, these changes in molecular expression together with the morphological changes are consistent with *N. meningitidis* initiating the transformation of trigeminal Schwann cells into Schwannoma cells.

Despite significant breakthroughs in the understanding, prevention, and treatment of glioma during the last decades, the role of bacterial infection in carcinogenesis is largely unknown. Glioma encompasses all tumours that originate from glial cells (Zong et al. 2012). The etiology of gliomas implicates a complex of known and unknown risk factors including environmental, genetics, immunological and microbial factors that ultimately result in malignant transformation (Kyritsis, Bondy et al. 2010). Infectious agents constitute under-appreciated but significant risk factors in gliomagenesis and other types of brain cancer (Alibek et al. 2013). For instance, viruses such as with human cytomegalovirus, and the protozoa *Toxoplasma gondii* may contribute to glioblastoma multiforme formation by provoking inflammation and via inhibition of apoptosis (Baryawno et al. 2011; Thomas et al. 2012). To date, little is known regarding the association of microbes, in particular bacteria, and brain cancer, except for a few species. Evidence of *Mycoplasma* species infection has been found to be implicated in certain brain cancer tissues including glioma(Huang, Li et al. 2001), and certain *Brucella* species in medulloblastomas (Zhang et al. 2011). Most pathogens cannot in fact invade the central nervous system (CNS) due to the blood-brain and blood-cerebrospinal fluid barriers, as well as the astrocytic glia limitans layer which constitutes an immune barrier between the CNS and the peripheral nervous system (PNS). However, some pathogens are capable of traversing these barriers for example by invading the peripheral nerves that innervate the nasal cavity (reviewed by Dando et al. 2016). Both *B.*

pseudomallei and *N. meningitidis* are part of the short list of bacteria that are "neuroinvasive"; they can invade the CNS via the olfactory and/or trigeminal nerves (Sjolinder and Jonsson 2010, St John, Ekberg et al. 2014, St John, Walkden et al. 2016). All previous studies focused on brain infection and the route of entry of the bacteria but in this thesis, the interaction and the behaviour of glial cells, as the most abundant cell type in central nervous system, with the infection was studied. The results of our study suggest that the triggering of inflammatory responses in glial cells may be a first step in initiation of carcinogenesis cascades. Regardless of lack of previous evidences, for first time the outcomes of the current study suggest that these bacterial species generate molecular and cellular responses in the glia, that are consistent with inflammation and/or the initiation of glioma.

In conclusion, in this study for first time we showed that infection with facultative intracellular bacteria such as *B. pseudomallei* and *N. meningitidis* can cause molecular and cellular changes indicative of inflammation and/or carcinogenesis in glial cells: (1) the formation of MNCs, (2) alteration of the nuclear morphology and (3) mis-regulation of gene/protein expression. Specifically, infection with these pathogens can mis-regulate the expression of genes responsible for cellular haemostasis, proliferation, and invasion, and transit the cells into the mesenchymal phase by upregulating the expression of EMT-related genes. We also showed that bacterial infection (*B. pseudomallei*) can initiate cancer-like changes in Schwann cells by regulating the Warburg effect-related genes and shift the cellular metabolism from OXPHOS into the aerobic glycolysis which is known as an initiating factor of carcinogenesis. Taken together, these data suggest that infection with *B. pseudomallei* and *N. meningitidis* can result in glia pathologies that have characteristics of inflammation and glioma.

7.1 Future Direction

In this study, we have shown that *B. pseudomallei* and *N. meningitidis* can infect trigeminal Schwann cells leading to cellular and molecular changes consistent with inflammation and/or gliomagenesis. The following aspects of the research in particular can be expanded upon as suggested:

Mechanism of MNC formation: In order to study the mechanisms involved in cell fusion and giant multinucleated cell formation, live cell microscopy should be performed following the infection with *B. pseudomallei* (wt and ΔBim A). These experiments would provide conclusive evidence that MNC formation and cellular fusion are due to cellular fusion mediated by the T6SS Bim A protein. Due to the requirement to handle *B. pseudomallei* within a PC3 laboratory, these experiments can only be performed in PC3 facilities equipped with a live-cell imaging apparatus. Such equipment is not currently available in our laboratory or in Australia.

Behaviour of infected cells in 3D: Further, we showed that *N. meningitidis* can infect and alter the nuclear morphology of three-dimensional cultures of trigeminal Schwann cells. As 3D cultures better mimic the *in vivo* environment, the interaction of multinucleated Schwann cells with other types of glial cells could be further explored in 3D cultures. As glioma is often characterised by containing a heterogeneous mixture of different cells, some of which are multinucleated, the 3D co-cultures could reveal the MNC Schwann cells interact with other cells to initiate the longer term glioma formation.

***In vivo* experiments:** Our *in vitro* results clearly showed that *B. pseudomallei* and *N. meningitidis* infection can alter the regulation of carcinogenesis and gliomagenesis markers in trigeminal Schwann cells and we suggest that this misregulation in cellular gene expression machinery may transform the Schwann cells into the Schwannoma cells. In order to study the gene expression *in vivo*, intranasal bacterial infection could be performed to allow the bacteria to penetrate the trigeminal nerve. Analysis of tissue sections collected from the heads of the infected mice could then be analysed using immunohistochemistry to determine whether the expression of gliomagenesis markers are upregulated *in vivo*. However, as we have shown previously, the bacteria can penetrate at such low levels that an immune response is not elicited and therefore there may not be detectable changes in cancer-related markers in the short-medium term (24-48 h). Indeed, it may take extended periods of time before the subthreshold bacterial infection triggers the changes *in vivo* that are observed *in vitro.* In order to study the role of LPS and inflammatory responses in Warburg effect, LPS mutant can be used to infect the cells. In a big picture, to study the role of gram negative bacteria in inducing Warburg effect, outer membrane

vesicles containing LPS can be a good tool to infect the cells. Also *in vivo* studies can reveal the effect of acute infection on Warburg effect and LDH production

Other pathogens: Rather than pursuing *in vivo* confirmation of a bacterial cause of gliomagenesis at this stage, it may be more worthwhile determining what other bacterial species can also initiate morphological and molecular changes in glial cells. In particular, the commensal bacteria that normally populate the nasopharynx such as *Staphylococcus aureus* could be examined for the potential to alter Schwann cell health.

Clinical patient follow-up studies: Both *B. pseudomallei* and *N. meningitidis* exhibit risks of morbidity and mortality. *B. pseudomallei* has a high mortality even when treated (10-15 % in Australia, 40 % in Thailand, and overall 90 % when fulminating and septicemic) (Chaowagul, White et al. 1989). *B. pseudomallei* is endemic in 45 countries, but is predicted to be endemic in at least 40 % of the countries in the world and is considered highly underreported (Limmathurotsakul, Golding et al. 2016). In Australia, 1 in 100 000 people becomes infected with *N. meningitidis* every year(Lawrence, Wang et al. 2016), whilst in the so-called meningitis belt in Africa, the figures are much higher: 1 in 1000 to 1 in 100(Agier, Martiny et al. 2017). Serogroup B is associated with high severity of disease and mortality(Harrison 2010) and until recently, no vaccine existed for this serogroup. Recently, a vaccine has been developed and has been released for use Europe and the United States (Gandhi, Balmer et al. 2016, Donald, Hawkins et al. 2017, Toneatto, Pizza et al. 2017). However, there have been recent reports of adverse events with this vaccine(Fiorito, Baird et al. 2017), showing that we have not found a way to prevent the most serious type of meningitis, the one caused by *N. meningitidis* serogroup B. To date, studies have been focussed on treating or preventing acute disease, and virtually nothing is known regarding the long-term implications of infections with *B. pseudomallei* and *N. meningitidis*. Here, we suggest for the first time that these pathogens induce pathological changes in glial cells that may be associated with cancer. No studies exist regarding the correlation between neuromelioidosis or *N. meningitidis*-caused CNS infection and brain cancer. The outcomes of this study suggest that a clinical investigation regarding brain cancer frequency in patients who have survived these diseases is highly warranted.

Overall, this thesis has identified a potential bacterial cause of cancer-related changes in glial cells and it now raises the questions of what other bacteria can also initiate such changes, and which bacteria are responsible for gliomagenesis in humans.

Appendix

	Protein	Label	log2FC	SE	Tvalue	DF	pvalue
451	sp|P61750|ARF4_MOUSE	SC_Nm_24-SC_24	-1.69953	0.121843	-13.9485	39	0
135	sp|P10126|EF1A1_MOUSE	SC_Nm_24-SC_24	-0.45944	0.048926	-9.39041	348	0
1121	sp|Q9DCV7|K2C7_MOUSE	SC_Nm_24-SC_24	-0.36522	0.019957	-18.3005	44	0
577	sp|P68369|TBA1A_MOUSE	SC_Nm_24-SC_24	-0.22862	0.025195	-9.07388	298	0
84	sp|P02089|HBB2_MOUSE	SC_Nm_72-SC_72	0.346812	0.020803	16.67099	33	0
647	sp|Q04447|KCRB_MOUSE	SC_Nm_24-SC_24	-0.26649	0.030632	-8.6999	298	2.22E-16
201	sp|P17182|ENOA_MOUSE	SC_Nm_24-SC_24	-0.33393	0.039024	-8.55707	316	4.44E-16
133	sp|P10107|ANXA1_MOUSE	SC_Nm_24-SC_24	-0.19101	0.02372	-8.05275	279	2.35E-14
195	sp|P16858|G3P_MOUSE	SC_Nm_24-SC_24	-0.21042	0.026898	-7.8228	339	6.62E-14
435	sp|P60843|IF4A1_MOUSE	SC_Nm_24-SC_24	-0.36858	0.047247	-7.80117	196	3.57E-13
134	sp|P10107|ANXA1_MOUSE	SC_Nm_72-SC_72	-0.18471	0.023804	-7.75972	279	1.61E-13
83	sp|P02089|HBB2_MOUSE	SC_Nm_24-SC_24	0.229352	0.020803	11.02476	33	1.33E-12
397	sp|P52480|KPYM_MOUSE	SC_Nm_24-SC_24	-0.27338	0.037654	-7.26029	347	2.56E-12
235	sp|P20152|VIME_MOUSE	SC_Nm_24-SC_24	-0.14924	0.021232	-7.02907	341	1.14E-11
213	sp|P18760|COF1_MOUSE	SC_Nm_24-SC_24	-0.21107	0.031056	-6.79641	352	4.60E-11
261	sp|P26041|MOES_MOUSE	SC_Nm_24-SC_24	-0.44231	0.064342	-6.87438	240	5.34E-11
243	sp|P23927|CRYAB_MOUSE	SC_Nm_24-SC_24	-0.67098	0.100048	-6.70658	252	1.30E-10
559	sp|P63260|ACTG_MOUSE	SC_Nm_24-SC_24	-0.16936	0.026161	-6.47373	363	3.11E-10
147	sp|P11499|HS90B_MOUSE	SC_Nm_24-SC_24	-0.30816	0.04785	-6.44014	353	3.91E-10
255	sp|P25444|RS2_MOUSE	SC_Nm_24-SC_24	-0.31162	0.051504	-6.05047	136	1.32E-08
129	sp|P09411|PGK1_MOUSE	SC_Nm_24-SC_24	-0.26791	0.045768	-5.85372	271	1.39E-08
455	sp|P61982|1433G_MOUSE	SC_Nm_24-SC_24	-0.74616	0.126493	-5.89879	192	1.63E-08
873	sp|Q8CGP2|H2B1P_MOUSE	SC_Nm_24-SC_24	-0.19332	0.031652	-6.10783	103	1.81E-08
153	sp|P12388|PAI2_MOUSE	SC_Nm_24-SC_24	1.052445	0.156931	6.706416	49	1.89E-08
625	sp|P99024|TBB5_MOUSE	SC_Nm_24-SC_24	-0.1662	0.028915	-5.74793	365	1.91E-08

539	sp|P62962|PROF1_MOUSE	SC_Nm_24-SC_24	-0.23818	0.04139	-5.75456	283	2.25E-08
57	sp|O88342|WDR1_MOUSE	SC_Nm_24-SC_24	-0.59271	0.101637	-5.83168	105	6.12E-08
716	sp|Q61207|SAP_MOUSE	SC_Nm_72-SC_72	0.330529	0.051187	6.457323	63	1.75E-08
1131	sp|Q9EQH3|VPS35_MOUSE	SC_Nm_24-SC_24	-1.86823	0.297106	-6.28808	39	2.06E-07
323	sp|P40124|CAP1_MOUSE	SC_Nm_24-SC_24	-0.62511	0.114694	-5.45027	145	2.11E-07
211	sp|P18242|CATD_MOUSE	SC_Nm_24-SC_24	-0.62993	0.115304	-5.46326	133	2.22E-07
607	sp|P84228|H32_MOUSE	SC_Nm_24-SC_24	-1.91785	0.285884	-6.70851	28	2.78E-07
307	sp|P35700|PRDX1_MOUSE	SC_Nm_24-SC_24	-0.18102	0.034976	-5.17571	263	4.51E-07
874	sp|Q8CGP2|H2B1P_MOUSE	SC_Nm_72-SC_72	0.190664	0.032899	5.795521	103	7.49E-08
612	sp|P97298|PEDF_MOUSE	SC_Nm_72-SC_72	0.401285	0.064703	6.201918	53	8.63E-08
144	sp|P11276|FINC_MOUSE	SC_Nm_72-SC_72	-0.78695	0.143325	-5.49068	195	1.24E-07
1107	sp|Q9DBJ1|PGAM1_MOUSE	SC_Nm_24-SC_24	-0.54321	0.10613	-5.11833	284	5.69E-07
363	sp|P48678|LMNA_MOUSE	SC_Nm_24-SC_24	-0.36679	0.071042	-5.16301	163	7.00E-07
573	sp|P68254|1433T_MOUSE	SC_Nm_24-SC_24	-0.30265	0.056432	-5.36316	75	8.73E-07
639	sp|Q01853|TERA_MOUSE	SC_Nm_24-SC_24	-0.23368	0.047414	-4.92849	225	1.61E-06
591	sp|P80313|TCPH_MOUSE	SC_Nm_24-SC_24	-0.76187	0.15312	-4.97562	117	2.26E-06
99	sp|P07091|S10A4_MOUSE	SC_Nm_24-SC_24	-0.44573	0.073577	-6.05804	25	2.49E-06
351	sp|P47757|CAPZB_MOUSE	SC_Nm_24-SC_24	-1.10116	0.186123	-5.91628	27	2.64E-06
281	sp|P29341|PABP1_MOUSE	SC_Nm_24-SC_24	-0.67911	0.141425	-4.80189	124	4.44E-06
341	sp|P45591|COF2_MOUSE	SC_Nm_24-SC_24	-1.51958	0.286231	-5.30895	39	4.71E-06
25	sp|O35343|IMA3_MOUSE	SC_Nm_24-SC_24	-0.8424	0.163244	-5.16041	39	7.54E-06
191	sp|P16460|ASSY_MOUSE	SC_Nm_24-SC_24	-1.1166	0.221109	-5.05003	38	1.14E-05
103	sp|P07356|ANXA2_MOUSE	SC_Nm_24-SC_24	-0.29019	0.065433	-4.43485	370	1.22E-05
987	sp|Q99LX0|PARK7_MOUSE	SC_Nm_24-SC_24	-1.16134	0.245291	-4.73452	65	1.23E-05
627	sp|P99027|RLA2_MOUSE	SC_Nm_24-SC_24	-0.31296	0.069851	-4.48044	196	1.26E-05
531	sp|P62908|RS3_MOUSE	SC_Nm_24-SC_24	-0.61367	0.131248	-4.67567	69	1.41E-05
779	sp|Q64522|H2A2B_MOUSE	SC_Nm_24-SC_24	-0.3512	0.080783	-4.34742	263	1.97E-05

1081	sp|Q9D6R2|IDH3A_MOUSE	SC_Nm_24-SC_24	-0.73345	0.144607	-5.07205	29	2.08E-05
125	sp|P09103|PDIA1_MOUSE	SC_Nm_24-SC_24	-0.31163	0.072556	-4.29498	304	2.35E-05
1122	sp|Q9DCV7|K2C7_MOUSE	SC_Nm_72-SC_72	-0.10516	0.019957	-5.26949	44	3.94E-06
452	sp|P61750|ARF4_MOUSE	SC_Nm_72-SC_72	-0.63171	0.118907	-5.31264	39	4.65E-06
81	sp|P01942|HBA_MOUSE	SC_Nm_24-SC_24	0.314839	0.075565	4.166456	159	5.06E-05
273	sp|P27659|RL3_MOUSE	SC_Nm_24-SC_24	-0.59049	0.135919	-4.34445	60	5.47E-05
751	sp|Q62167|DDX3X_MOUSE	SC_Nm_24-SC_24	-0.4244	0.102123	-4.15577	120	6.11E-05
780	sp|Q64522|H2A2B_MOUSE	SC_Nm_72-SC_72	0.301451	0.067864	4.441977	263	1.31E-05
897	sp|Q8VDM4|PSMD2_MOUSE	SC_Nm_24-SC_24	-0.38134	0.092546	-4.12058	96	8.02E-05
381	sp|P50543|S10AB_MOUSE	SC_Nm_24-SC_24	-0.22815	0.055164	-4.13587	88	8.06E-05
1129	sp|Q9EPL8|IPO7_MOUSE	SC_Nm_24-SC_24	-1.33712	0.262096	-5.10164	17	8.86E-05
105	sp|P07724|ALBU_MOUSE	SC_Nm_24-SC_24	0.367441	0.091511	4.015278	159	9.13E-05
247	sp|P24369|PPIB_MOUSE	SC_Nm_24-SC_24	-0.64836	0.122657	-5.28597	15	9.15E-05
504	sp|P62806|H4_MOUSE	SC_Nm_72-SC_72	0.196598	0.046648	4.214495	297	3.33E-05
367	sp|P49312|ROA1_MOUSE	SC_Nm_24-SC_24	-0.25212	0.065882	-3.82679	191	0.000176
945	sp|Q922F4|TBB6_MOUSE	SC_Nm_24-SC_24	-0.23574	0.061478	-3.83456	166	0.000178
1079	sp|Q9D3D9|ATPD_MOUSE	SC_Nm_24-SC_24	-1.70576	0.331027	-5.15292	13	0.000186
68	sp|O88783|FA5_MOUSE	SC_Nm_72-SC_72	-0.66028	0.155711	-4.24044	135	4.11E-05
161	sp|P14069|S10A6_MOUSE	SC_Nm_24-SC_24	-0.66692	0.172502	-3.86614	91	0.000207
360	sp|P47963|RL13_MOUSE	SC_Nm_72-SC_72	0.277754	0.062209	4.464827	48	4.85E-05
805	sp|Q6ZWN5|RS9_MOUSE	SC_Nm_24-SC_24	-0.36327	0.095535	-3.80243	111	0.000235
143	sp|P11276|FINC_MOUSE	SC_Nm_24-SC_24	-0.38185	0.102003	-3.74353	195	0.000239
637	sp|Q01768|NDKB_MOUSE	SC_Nm_24-SC_24	-0.23545	0.062636	-3.75906	145	0.000247
545	sp|P63017|HSP7C_MOUSE	SC_Nm_24-SC_24	-0.1488	0.040205	-3.70104	325	0.000252
776	sp|Q64433|CH10_MOUSE	SC_Nm_72-SC_72	0.251432	0.060269	4.171836	111	6.02E-05
339	sp|P45377|ALD2_MOUSE	SC_Nm_24-SC_24	-0.66243	0.17228	-3.84509	62	0.000287
137	sp|P10605|CATB_MOUSE	SC_Nm_24-SC_24	-0.45675	0.115072	-3.96927	40	0.000292

841	sp|Q8BGQ7|SYAC_MOUSE	SC_Nm_24-SC_24	-0.62113	0.157998	-3.93124	44	0.000295
223	sp|P19324|SERPH_MOUSE	SC_Nm_24-SC_24	-0.31328	0.085515	-3.66352	274	0.000299
354	sp|P47911|RL6_MOUSE	SC_Nm_72-SC_72	-0.27779	0.06836	-4.06359	190	7.07E-05
427	sp|P58252|EF2_MOUSE	SC_Nm_24-SC_24	-0.19165	0.052403	-3.65727	244	0.000312
333	sp|P43024|CX6A1_MOUSE	SC_Nm_24-SC_24	-0.79066	0.183613	-4.30616	21	0.000312
165	sp|P14131|RS16_MOUSE	SC_Nm_24-SC_24	-0.28019	0.068881	-4.06767	26	0.000392
167	sp|P14148|RL7_MOUSE	SC_Nm_24-SC_24	-0.49754	0.13604	-3.65729	100	0.000409
991	sp|Q99P72|RTN4_MOUSE	SC_Nm_24-SC_24	-0.19941	0.055198	-3.6127	131	0.000431
903	sp|Q8VEK3|HNRPU_MOUSE	SC_Nm_24-SC_24	-0.9726	0.181273	-5.3654	9	0.000453
695	sp|Q60854|SPB6_MOUSE	SC_Nm_24-SC_24	-0.35362	0.100101	-3.53263	135	0.000564
308	sp|P35700|PRDX1_MOUSE	SC_Nm_72-SC_72	-0.13092	0.033764	-3.87762	263	0.000133
857	sp|Q8BMS1|ECHA_MOUSE	SC_Nm_24-SC_24	-0.61031	0.17269	-3.53416	108	0.000603
505	sp|P62814|VATB2_MOUSE	SC_Nm_24-SC_24	-1.6219	0.343452	-4.72235	11	0.000627
361	sp|P48036|ANXA5_MOUSE	SC_Nm_24-SC_24	-0.1537	0.044635	-3.44338	338	0.000647
599	sp|P80317|TCPZ_MOUSE	SC_Nm_24-SC_24	-0.43371	0.125044	-3.46849	161	0.000672
1215	sp|Q9WVA4|TAGL2_MOUSE	SC_Nm_24-SC_24	-0.19572	0.057233	-3.41969	248	0.000733
175	sp|P14602|HSPB1_MOUSE	SC_Nm_24-SC_24	-0.2734	0.079237	-3.45049	114	0.000786
887	sp|Q8R1F1|NIBL1_MOUSE	SC_Nm_24-SC_24	-0.3356	0.09633	-3.48387	78	0.000814
1191	sp|Q9R0P5|DEST_MOUSE	SC_Nm_24-SC_24	-0.18824	0.055567	-3.38761	169	0.000877
728	sp|Q61703|ITIH2_MOUSE	SC_Nm_72-SC_72	0.24778	0.064916	3.816952	157	0.000194
35	sp|O35887|CALU_MOUSE	SC_Nm_24-SC_24	-0.32408	0.095373	-3.39806	127	0.000907
1045	sp|Q9CXW4|RL11_MOUSE	SC_Nm_24-SC_24	-0.37925	0.103454	-3.66587	29	0.000983
785	sp|Q68FD5|CLH1_MOUSE	SC_Nm_24-SC_24	-0.25231	0.075851	-3.32634	191	0.001055
429	sp|P58771|TPM1_MOUSE	SC_Nm_24-SC_24	-0.17123	0.051668	-3.31405	238	0.001063
215	sp|P19096|FAS_MOUSE	SC_Nm_24-SC_24	-0.51313	0.152572	-3.36322	104	0.001079
1199	sp|Q9R0Y5|KAD1_MOUSE	SC_Nm_24-SC_24	-0.35008	0.105383	-3.32201	166	0.001099
296	sp|P32261|ANT3_MOUSE	SC_Nm_72-SC_72	0.349043	0.091688	3.806852	86	0.000263

964	sp|Q99JX4|EIF3M_MOUSE	SC_Nm_72-SC_72	-1.2244	0.296232	-4.13323	29	0.000278
795	sp|Q6P5E4|UGGG1_MOUSE	SC_Nm_24-SC_24	1.309764	0.32672	4.008831	14	0.001293
1149	sp|Q9JII6|AK1A1_MOUSE	SC_Nm_24-SC_24	-0.22923	0.070345	-3.25867	180	0.001338
663	sp|Q0GNC1|INF2_MOUSE	SC_Nm_24-SC_24	-0.41708	0.119928	-3.47774	35	0.001371
337	sp|P45376|ALDR_MOUSE	SC_Nm_24-SC_24	-0.43932	0.133178	-3.29872	91	0.001387
969	sp|Q99K48|NONO_MOUSE	SC_Nm_24-SC_24	-0.88703	0.25454	-3.48485	30	0.001537
993	sp|Q99PT1|GDIR1_MOUSE	SC_Nm_24-SC_24	-0.19767	0.061877	-3.19464	188	0.001642
1011	sp|Q9CQ92|FIS1_MOUSE	SC_Nm_24-SC_24	0.397101	0.11703	3.393164	35	0.00173
309	sp|P35762|CD81_MOUSE	SC_Nm_24-SC_24	0.645834	0.172283	3.748682	16	0.001753
832	sp|Q80X90|FLNB_MOUSE	SC_Nm_72-SC_72	-0.14857	0.041445	-3.58484	268	0.000401
1082	sp|Q9D6R2|IDH3A_MOUSE	SC_Nm_72-SC_72	-0.54223	0.13664	-3.96832	29	0.000436
480	sp|P62301|RS13_MOUSE	SC_Nm_72-SC_72	0.337703	0.094684	3.56662	172	0.000469
188	sp|P16045|LEG1_MOUSE	SC_Nm_72-SC_72	-0.22538	0.063723	-3.5368	268	0.000477
771	sp|Q64152|BTF3_MOUSE	SC_Nm_24-SC_24	-0.35937	0.102759	-3.49718	22	0.002038
513	sp|P62843|RS15_MOUSE	SC_Nm_24-SC_24	-0.35898	0.113025	-3.17613	90	0.002045
981	sp|Q99LC5|ETFA_MOUSE	SC_Nm_24-SC_24	-1.04982	0.308854	-3.3991	26	0.00219
1127	sp|Q9EPC1|PARVA_MOUSE	SC_Nm_24-SC_24	-1.5886	0.432367	-3.6742	15	0.002255
537	sp|P62960|YBOX1_MOUSE	SC_Nm_24-SC_24	0.229721	0.072408	3.172576	63	0.002336
1007	sp|Q9CQ60|6PGL_MOUSE	SC_Nm_24-SC_24	-0.71654	0.218955	-3.27252	36	0.002356
238	sp|P21107|TPM3_MOUSE	SC_Nm_72-SC_72	0.756774	0.208712	3.625922	53	0.000647
292	sp|P31786|ACBP_MOUSE	SC_Nm_72-SC_72	0.308736	0.088675	3.481645	137	0.000669
417	sp|P56480|ATPB_MOUSE	SC_Nm_24-SC_24	-0.10504	0.034585	-3.03716	333	0.002577
635	sp|Q01730|RSU1_MOUSE	SC_Nm_24-SC_24	-0.55883	0.173074	-3.22886	37	0.002607
1019	sp|Q9CQI6|COTL1_MOUSE	SC_Nm_24-SC_24	-0.81029	0.237196	-3.41614	20	0.002738
41	sp|O54962|BAF_MOUSE	SC_Nm_24-SC_24	-0.36866	0.11762	-3.13432	54	0.002784
1165	sp|Q9JM14|NT5C_MOUSE	SC_Nm_24-SC_24	-0.53566	0.170138	-3.14839	45	0.002913
503	sp|P62806|H4_MOUSE	SC_Nm_24-SC_24	-0.14432	0.048179	-2.99554	297	0.00297

579	sp|P68372|TBB4B_MOUSE	SC_Nm_24-SC_24	-0.48959	0.16119	-3.03734	85	0.003169
1005	sp|Q9CQ19|MYL9_MOUSE	SC_Nm_24-SC_24	-0.19001	0.063575	-2.98875	164	0.003232
1024	sp|Q9CQM9|GLRX3_MOUSE	SC_Nm_72-SC_72	0.964873	0.215355	4.48038	11	0.000931
1023	sp|Q9CQM9|GLRX3_MOUSE	SC_Nm_24-SC_24	0.719001	0.196071	3.667051	11	0.003709
463	sp|P62204|CALM_MOUSE	SC_Nm_24-SC_24	-0.31076	0.103734	-2.99572	73	0.003738
163	sp|P14115|RL27A_MOUSE	SC_Nm_24-SC_24	-0.24095	0.079276	-3.03933	44	0.003982
633	sp|Q01405|SC23A_MOUSE	SC_Nm_24-SC_24	-0.69413	0.220995	-3.14092	27	0.004056
789	sp|Q6IRU2|TPM4_MOUSE	SC_Nm_24-SC_24	-0.13156	0.045341	-2.90149	185	0.004165
557	sp|P63242|IF5A1_MOUSE	SC_Nm_24-SC_24	-0.35648	0.123547	-2.88539	175	0.004401
645	sp|Q03265|ATPA_MOUSE	SC_Nm_24-SC_24	-0.12857	0.044883	-2.86453	341	0.004435
449	sp|P61514|RL37A_MOUSE	SC_Nm_24-SC_24	0.575948	0.170212	3.383701	14	0.004455
900	sp|Q8VDN2|AT1A1_MOUSE	SC_Nm_72-SC_72	1.390645	0.387738	3.586556	30	0.001173
549	sp|P63038|CH60_MOUSE	SC_Nm_24-SC_24	-0.18138	0.063908	-2.8382	254	0.004903
1143	sp|Q9JHJ0|TMOD3_MOUSE	SC_Nm_24-SC_24	-0.83879	0.283555	-2.95811	43	0.005014
173	sp|P14211|CALR_MOUSE	SC_Nm_24-SC_24	-0.15459	0.054515	-2.83576	201	0.005039
837	sp|Q8BG05|ROA3_MOUSE	SC_Nm_24-SC_24	-0.42163	0.146383	-2.8803	84	0.00504
1207	sp|Q9WTQ5|AKA12_MOUSE	SC_Nm_24-SC_24	-0.27887	0.097147	-2.87065	94	0.005061
725	sp|Q61598|GDIB_MOUSE	SC_Nm_24-SC_24	-0.21895	0.0774	-2.82887	208	0.005128
622	sp|P97461|RS5_MOUSE	SC_Nm_72-SC_72	-0.4022	0.112112	-3.58747	26	0.001357
174	sp|P14211|CALR_MOUSE	SC_Nm_72-SC_72	-0.20547	0.06344	-3.23881	201	0.001404
629	sp|P99029|PRDX5_MOUSE	SC_Nm_24-SC_24	-0.16467	0.058663	-2.80713	178	0.005556
91	sp|P05202|AATM_MOUSE	SC_Nm_24-SC_24	-0.19024	0.067791	-2.80628	129	0.005789
530	sp|P62900|RL31_MOUSE	SC_Nm_72-SC_72	0.337856	0.096526	3.50014	28	0.001576
1041	sp|Q9CX86|ROA0_MOUSE	SC_Nm_24-SC_24	-0.81609	0.236042	-3.45738	10	0.00615
407	sp|P54775|PRS6B_MOUSE	SC_Nm_24-SC_24	-0.89391	0.279212	-3.20154	14	0.006401
949	sp|Q922R8|PDIA6_MOUSE	SC_Nm_24-SC_24	-0.27671	0.100622	-2.74995	136	0.006772
586	sp|P70296|PEBP1_MOUSE	SC_Nm_72-SC_72	0.199874	0.062905	3.177389	148	0.001809

1059	sp|Q9CZX8|RS19_MOUSE	SC_Nm_24-SC_24	-0.41896	0.147631	-2.83786	35	0.007508
203	sp|P17225|PTBP1_MOUSE	SC_Nm_24-SC_24	-0.5515	0.189061	-2.91704	23	0.007758
229	sp|P20029|GRP78_MOUSE	SC_Nm_24-SC_24	-0.15393	0.05786	-2.66041	244	0.008322
183	sp|P15379|CD44_MOUSE	SC_Nm_24-SC_24	-0.15515	0.055781	-2.7815	37	0.008463
593	sp|P80314|TCPB_MOUSE	SC_Nm_24-SC_24	-0.31981	0.118302	-2.70336	56	0.009072
911	sp|Q8VI75|IPO4_MOUSE	SC_Nm_24-SC_24	0.485253	0.17616	2.754611	32	0.009611
389	sp|P51410|RL9_MOUSE	SC_Nm_24-SC_24	-0.38193	0.143513	-2.66131	61	0.009935
271	sp|P27546|MAP4_MOUSE	SC_Nm_24-SC_24	-0.28612	0.10914	-2.62162	108	0.010014
743	sp|Q61990|PCBP2_MOUSE	SC_Nm_24-SC_24	-0.36505	0.126895	-2.87675	18	0.010036
902	sp|Q8VED5|K2C79_MOUSE	SC_Nm_72-SC_72	0.286898	0.088472	3.24282	35	0.002602
772	sp|Q64152|BTF3_MOUSE	SC_Nm_72-SC_72	-0.39089	0.116518	-3.35476	22	0.002864
943	sp|Q921H8|THIKA_MOUSE	SC_Nm_24-SC_24	-0.5453	0.206183	-2.64473	47	0.01108
89	sp|P05064|ALDOA_MOUSE	SC_Nm_24-SC_24	-0.14721	0.057714	-2.55069	305	0.011239
275	sp|P27773|PDIA3_MOUSE	SC_Nm_24-SC_24	-0.11729	0.046017	-2.54894	320	0.011272
1227	sp|Q9Z1Q5|CLIC1_MOUSE	SC_Nm_24-SC_24	-0.38071	0.136593	-2.78717	20	0.011375
595	sp|P80315|TCPD_MOUSE	SC_Nm_24-SC_24	-0.51185	0.196575	-2.60387	54	0.011879
359	sp|P47963|RL13_MOUSE	SC_Nm_24-SC_24	-0.15057	0.057816	-2.60422	48	0.01222
415	sp|P56399|UBP5_MOUSE	SC_Nm_24-SC_24	-0.30073	0.113556	-2.64832	30	0.012773
114	sp|P08113|ENPL_MOUSE	SC_Nm_72-SC_72	-0.15887	0.053705	-2.95817	295	0.003345
853	sp|Q8BMJ2|SYLC_MOUSE	SC_Nm_24-SC_24	-0.67234	0.204775	-3.28329	7	0.013426
1017	sp|Q9CQE8|CN166_MOUSE	SC_Nm_24-SC_24	-0.93224	0.33801	-2.75803	17	0.01344
509	sp|P62827|RAN_MOUSE	SC_Nm_24-SC_24	-0.23407	0.093764	-2.4964	83	0.014524
457	sp|P62082|RS7_MOUSE	SC_Nm_24-SC_24	-0.15192	0.061643	-2.46446	182	0.01465
87	sp|P04104|K2C1_MOUSE	SC_Nm_24-SC_24	0.678244	0.266731	2.542804	43	0.014678
93	sp|P06151|LDHA_MOUSE	SC_Nm_24-SC_24	-0.22868	0.093064	-2.45728	219	0.014776
159	sp|P13020|GELS_MOUSE	SC_Nm_24-SC_24	-0.14676	0.059808	-2.4539	212	0.014938
923	sp|Q91VI7|RINI_MOUSE	SC_Nm_24-SC_24	-0.58632	0.231607	-2.53152	35	0.016002

1070	sp|Q9D1D4|TMEDA_MOUSE	SC_Nm_72-SC_72	-0.95604	0.27003	-3.5405	12	0.004067
145	sp|P11438|LAMP1_MOUSE	SC_Nm_24-SC_24	0.295346	0.117757	2.508104	38	0.016527
1141	sp|Q9JHH6|CBPB2_MOUSE	SC_Nm_24-SC_24	0.401593	0.158591	2.53226	29	0.017005
1231	sp|Q9Z204|HNRPC_MOUSE	SC_Nm_24-SC_24	-0.49261	0.187366	-2.62915	18	0.017022
56	sp|O70475|UGDH_MOUSE	SC_Nm_72-SC_72	0.933899	0.306556	3.04642	32	0.004612
194	sp|P16546|SPTN1_MOUSE	SC_Nm_72-SC_72	-0.56134	0.188717	-2.97449	46	0.004662
177	sp|P14824|ANXA6_MOUSE	SC_Nm_24-SC_24	-0.14488	0.060935	-2.37752	265	0.018139
7	sp|O08553|DPYL2_MOUSE	SC_Nm_24-SC_24	-0.19822	0.083915	-2.36217	200	0.019129
955	sp|Q93092|TALDO_MOUSE	SC_Nm_24-SC_24	-0.35227	0.144895	-2.4312	42	0.019396
733	sp|Q61753|SERA_MOUSE	SC_Nm_24-SC_24	-0.23858	0.100912	-2.36423	93	0.020147
601	sp|P80318|TCPG_MOUSE	SC_Nm_24-SC_24	-0.35791	0.144098	-2.48377	23	0.020722
1099	sp|Q9DB05|SNAA_MOUSE	SC_Nm_24-SC_24	-0.29358	0.117439	-2.49981	19	0.021749
747	sp|Q62048|PEA15_MOUSE	SC_Nm_24-SC_24	-0.30855	0.131105	-2.35348	61	0.021837
1167	sp|Q9JM58|CRLF1_MOUSE	SC_Nm_24-SC_24	-0.80724	0.319032	-2.53028	16	0.022273
864	sp|Q8BP92|RCN2_MOUSE	SC_Nm_72-SC_72	-1.06331	0.357666	-2.97291	30	0.005769
763	sp|Q62465|VAT1_MOUSE	SC_Nm_24-SC_24	-0.32143	0.14008	-2.2946	111	0.023637
107	sp|P07901|HS90A_MOUSE	SC_Nm_24-SC_24	-0.14707	0.06448	-2.28085	158	0.023894
157	sp|P12970|RL7A_MOUSE	SC_Nm_24-SC_24	-0.19735	0.086655	-2.27738	137	0.024312
29	sp|O35639|ANXA3_MOUSE	SC_Nm_24-SC_24	-0.12704	0.056211	-2.26003	270	0.024616
547	sp|P63028|TCTP_MOUSE	SC_Nm_24-SC_24	-0.27703	0.122151	-2.26796	162	0.024653
699	sp|Q60930|VDAC2_MOUSE	SC_Nm_24-SC_24	-0.12205	0.054014	-2.25966	241	0.024736
783	sp|Q64727|VINC_MOUSE	SC_Nm_24-SC_24	-0.24391	0.108116	-2.25603	183	0.025252
604	sp|P84078|ARF1_MOUSE	SC_Nm_72-SC_72	0.586354	0.21025	2.788846	85	0.006527
453	sp|P61979|HNRPK_MOUSE	SC_Nm_24-SC_24	-0.23809	0.106216	-2.24159	120	0.026826
325	sp|P40142|TKT_MOUSE	SC_Nm_24-SC_24	-0.26963	0.121054	-2.22731	191	0.027094
602	sp|P80318|TCPG_MOUSE	SC_Nm_72-SC_72	-1.11365	0.377338	-2.95132	23	0.007162
546	sp|P63017|HSP7C_MOUSE	SC_Nm_72-SC_72	0.110929	0.041111	2.698291	325	0.007334

16	sp|O09061|PSB1_MOUSE	SC_Nm_72-SC_72	0.525344	0.178989	2.935066	23	0.007439
558	sp|P63242|IF5A1_MOUSE	SC_Nm_72-SC_72	-0.25084	0.093113	-2.69395	175	0.007748
696	sp|Q60854|SPB6_MOUSE	SC_Nm_72-SC_72	-0.29447	0.109172	-2.69733	135	0.007881
1012	sp|Q9CQ92|FIS1_MOUSE	SC_Nm_72-SC_72	0.36779	0.130843	2.81092	35	0.008038
1030	sp|Q9CQV8|1433B_MOUSE	SC_Nm_72-SC_72	0.399545	0.14751	2.708589	93	0.008043
1179	sp|Q9QXS1|PLEC_MOUSE	SC_Nm_24-SC_24	-0.18929	0.085345	-2.21791	240	0.027498
1089	sp|Q9D8E6|RL4_MOUSE	SC_Nm_24-SC_24	-0.16078	0.072523	-2.21692	186	0.02784
23	sp|O35129|PHB2_MOUSE	SC_Nm_24-SC_24	-0.24444	0.109931	-2.22359	111	0.028201
257	sp|P26039|TLN1_MOUSE	SC_Nm_24-SC_24	-0.23356	0.105404	-2.21581	101	0.028949
450	sp|P61514|RL37A_MOUSE	SC_Nm_72-SC_72	0.49499	0.162471	3.046629	14	0.008708
399	sp|P52927|HMGA2_MOUSE	SC_Nm_24-SC_24	0.355854	0.151678	2.346116	19	0.029971
1057	sp|Q9CZU6|CISY_MOUSE	SC_Nm_24-SC_24	-0.23285	0.10641	-2.18824	134	0.030385
251	sp|P24527|LKHA4_MOUSE	SC_Nm_24-SC_24	0.483863	0.214979	2.250743	32	0.031404
555	sp|P63101|1433Z_MOUSE	SC_Nm_24-SC_24	-0.12579	0.058245	-2.15963	274	0.031669
206	sp|P17710|HXK1_MOUSE	SC_Nm_72-SC_72	-0.6538	0.237567	-2.75205	33	0.009547
952	sp|Q922U2|K2C5_MOUSE	SC_Nm_72-SC_72	0.294188	0.10771	2.731299	36	0.009709
364	sp|P48678|LMNA_MOUSE	SC_Nm_72-SC_72	0.20195	0.07739	2.609526	163	0.009911
931	sp|Q91WK2|EIF3H_MOUSE	SC_Nm_24-SC_24	-0.54889	0.24333	-2.25576	27	0.032394
1181	sp|Q9QXS6|DREB_MOUSE	SC_Nm_24-SC_24	-0.33564	0.142499	-2.3554	15	0.032535
19	sp|O09167|RL21_MOUSE	SC_Nm_24-SC_24	0.519099	0.201853	2.571673	8	0.033041
67	sp|O88783|FA5_MOUSE	SC_Nm_24-SC_24	0.317801	0.147889	2.148918	135	0.033425
553	sp|P63087|PP1G_MOUSE	SC_Nm_24-SC_24	-0.37735	0.16953	-2.22586	30	0.033685
115	sp|P08207|S10AA_MOUSE	SC_Nm_24-SC_24	0.229224	0.100664	2.277122	19	0.034531
469	sp|P62259|1433E_MOUSE	SC_Nm_24-SC_24	-0.11858	0.055727	-2.12792	155	0.034925
571	sp|P68040|GBLP_MOUSE	SC_Nm_24-SC_24	0.382465	0.177401	2.155937	59	0.035177
218	sp|P19157|GSTP1_MOUSE	SC_Nm_72-SC_72	0.162581	0.06323	2.571263	89	0.011792
302	sp|P35441|TSP1_MOUSE	SC_Nm_72-SC_72	0.210704	0.08088	2.605144	48	0.012191

618	sp|P97371|PSME1_MOUSE	SC_Nm_72-SC_72	-0.66135	0.250794	-2.63703	34	0.012518
97	sp|P06801|MAOX_MOUSE	SC_Nm_24-SC_24	-0.32296	0.155587	-2.07573	81	0.041091
542	sp|P62984|RL40_MOUSE	SC_Nm_72-SC_72	-0.3427	0.135366	-2.53164	88	0.013129
540	sp|P62962|PROF1_MOUSE	SC_Nm_72-SC_72	0.115945	0.046545	2.491038	283	0.01331
373	sp|P49945|FRIL2_MOUSE	SC_Nm_24-SC_24	0.39565	0.18517	2.13668	28	0.041502
563	sp|P63325|RS10_MOUSE	SC_Nm_24-SC_24	-0.12397	0.059977	-2.06695	76	0.042144
1115	sp|Q9DCH4|EIF3F_MOUSE	SC_Nm_24-SC_24	-0.31524	0.151144	-2.08566	44	0.042841
471	sp|P62264|RS14_MOUSE	SC_Nm_24-SC_24	-0.34021	0.159602	-2.13159	24	0.04348
754	sp|Q62188|DPYL3_MOUSE	SC_Nm_72-SC_72	0.413634	0.165405	2.500734	83	0.01436
418	sp|P56480|ATPB_MOUSE	SC_Nm_72-SC_72	-0.09111	0.037349	-2.43931	333	0.015237
999	sp|Q9CPU0|LGUL_MOUSE	SC_Nm_24-SC_24	0.303546	0.148268	2.047284	41	0.047074

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