The contribution of MAPKs to microglial immune responses, and a pathogen type comparison study to probe maternal immune activation effects on the foetal brain

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Abstract

Schizophrenia is a psychiatric disorder, and despite a rapid increase in understanding of the disease both clinically and preclinically, the aetiology of the disease remains somewhat uncertain. Inflammation is increasingly correlated with schizophrenia, with evidence from patients' serum and post-mortem brain samples. Moreover, in the brain, microglia are the primary cells to respond to immune stimulation; thus, their cellular processes in neuroinflammation in terms of contribution of schizophrenia-related pathological changes should be investigated. Regarding microglial immune responses, this study looks specifically at the mitogen-activated protein kinase (MAPK) pathways, including c-jun N-terminal kinase (JNK), because the evidence from genome-wide genetic association studies suggests that some of the genetic risk factors for schizophrenia are related to this pathway. Furthermore, as suggested from the neurodevelopmental theory, maternal stress, or infection during pregnancy, is one risk factor in the development of schizophrenia in the offspring. Indeed, Maternal Immune Activation (MIA) animal studies have suggested that prenatal immune stress could negatively affect CNS development and offspring behaviours.

This thesis hypothesises that maternal immune activation caused by environmental challenges affects foetal microglial immunity via the MAPK pathway, and that differing responses are observed depending on the nature of the immune challenge. In order to examine the statement, this thesis introduces research to study the neurobiological functions of MAPKs in neuronal (primary mouse cortical cultured neurons) and microglial (SIM-A9 mouse cell line) cells, with preclinical studies of the impact of MIA in mice on immunological changes following the administration of different inflammatory agents.

Results in cultured primary mouse cortical neurons showed that two different TLR agonists, LPS (TLR4 agonist), and resiquimod (TLR7/8 agonist), did not cause any significant changes in the level of activated phospho-JNKs (pJNKs); however, poly I:C (TLR3 agonist) stimulation showed potential effects on JNK activation. Interestingly, chemokine stimulation, with CXCL10 in particular, affected the level of pJNKs. Continuing the *in vitro* studies, microglial immune responses were investigated. Resiquimod significantly increased the activation levels of MAPKs. LPS also increased the activation levels of MAPKs; however, it required a longer time than resiquimod. Interestingly, poly I:C did not significantly increase activation levels of any MAPKs. LPS and resiquimod initiated microglial immune responses (measured by RT-qPCR and ELISA); however, protein levels did not always reflect mRNA level changes. Besides, MAPK pathway contributions to the microglial immune reactions were suggested via MAPK inhibitor experiments, but in a stimulus-dependent manner. Interestingly, investigation of microglial markers, *Tspo*, *Aif-1*, and *Tmem119*, following TLR-mediated stimulations in microglial cells (SIM-A9) showed changes that did not necessarily reflect levels of microglial activity.

Alongside these *in vitro* studies, MIA models, induced by dsRNA (poly I:C) and ssRNA (resiquimod) virus mimetics, were investigated. Initial data suggested that the level of CXCL12 in

placental tissues was increased by maternal exposure to poly I:C, but placental CXCL10 was not affected, even though poly I:C administration meaningfully induced maternal inflammation.

The data from the main MIA study suggested that administration of a TLR7/8 (resiquimod), but not a TLR3 (poly I:C), agonist, induced the upregulation of cytokine and chemokine expression in embryo brains, even though the evidence of inflammation caused by both poly I:C and resiquimod was detected in maternal serum and placentae. These findings suggest that MIA has the potential to alter foetus brain immune status and that the response could be pathogen dependent, with single-stranded RNA (ssRNA) virus exposure potentially producing greater effects on the foetus. Corresponding with the *in vitro* microglia experiment, microglial markers in foetal brains were changed by MIA; these findings indicated these markers are changed by environmental conditions rather than remaining stable.

These data provide compelling evidence of the roles of MAPKs in microglial involved neuroinflammation, although the precise action of these signalling molecules on microglia-related pathology in schizophrenia remains unclear. In addition to previous works, a significant impact of maternal ssRNA virus (resiquimod) exposure on the developing foetal brain, but the double-stranded RNA (dsRNA) (poly I:C) virus did not. This means that resiquimod is potentially more effective than imiquimod or poly I:C in terms of producing an immune response; therefore, an MIA model using resiquimod may be a good model for the study of environmental contribution to psychiatric disease risk. Further work into the interaction between environmental and genetic factors in the MIA, and associated behaviour changes at later developmental ages, will provide insight into how maternal immune reactions and foetal CNS immune reactions are related to foetal brain development.

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Author’s Declaration

I declare that all work in this thesis in the result of my own work, except where explicit reference has been made to the contribution of others. None of the data included in this thesis has been submitted for any other degree, either at the University of Glasgow or at any other institution.

Signature…….

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Definitions/Abbreviations

4

4PL 4-parameter logistic regression

A

ASD Autism spectrum disorder

ASK1 Apoptosis signal-regulating kinase 1

B

BBB Blood-brain barrier

BDNF Brain-derived neurotrophic factor

BMDM Bone marrow derived macrophage

C

CAMs CNS-associated macrophages

CNS Central nervous system

CNV Copy number variant

CSF1 Colony-stimulating factor 1

CRF Central research facility

Ct Cycle threshold

D

DIV Day of *in vitro*

DLK Dual leucine zipper kinase

DRG Dorsal root ganglion

dsRNA Double-strand RNA

E

EAE Experimental autoimmune encephalomyelitis

ELISA Enzyme-linked immunosorbent assay

ERKs Extracellular signal-regulated kinases

F

FACS Fluorescence-activated cell sorting

FBS Foetal bovine serum

G

G-CSF Granulocyte colony-stimulating factor

GM-CSF Granulocyte-macrophage colony-stimulating factor

GWAS Genome-wide association studies

gDNA Genomic DNA

H

HCV Hepatitis C virus

HIV Human immunodeficiency virus

HSCs Haematopoietic stem cells

HSV-2 Herpes simples virus-2

I

IBA-1 Ionized calcium-binding adapter molecule 1

IHC Immunohistochemistry

IL Interleukin

IFNs Interferons

iPSCs induced pluripotent stem cells

J

JNKs c-Jun N-terminal kinases

L

LIF Leukaemia inhibitory factor

LPS Lipopolysaccharide

LTD Long-term depression

Ly6c Lymphocyte antigen 6C

M

M-CSF Macrophage colony-stimulating factor

MAPK Mitogen-activated protein kinase

MHC Major histocompatibility complex

MFI Mean fluorescence intensity

MIA Maternal immune activation

MIF Migration inhibitor factor

MOM mouse on mouse

MRI Magnetic resonance imaging

mPFC Medial prefrontal cortex

mGluR Metabotropic glutamate receptor

N

NDH Neurodevelopmental hypothesis

O

OPC Oligodendrocyte precursor cell

P

PAMPs Pathogen-associated molecular patterns

PDK 3-phosphoinositide-dependent protein kinase 1

PET Positron emission tomopraphy

PFA Paraformaldehyde

PFC Prefrontal cortex

Poly I:C Polyinosinic- polycytidylic acid

PRRs Pattern recognition receptors

PV Parvalbumin

pp38s Phospho-p38s

pERKs Phospho-ERKs

pJNKs Phospho-JNKs

R

RAF Rapidly accelerated fibrosarcoma

ROS reactive oxygen species

RT Reverse transcriptase

RT-qPCR Quantitative reverse transcription PCR

S

SCs Schwann cells

SGK1 Serum- and glucocorticoid-regulated kinase 1

STD Standard

ssRNA single-strand RNA

T

TAK1 Transforming growth factor-β-activated kinase 1

TLRs Toll-like receptors

TNF Tumour necrosis factor

TRAF6 Tumour necrosis factor receptor-associated factor 6

TSPO Translocator protein

V

VEGF Vascular endothelial growth factor

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Schizophrenia

Schizophrenia is a psychiatric disorder and its prevalence in the population is one in every hundred worldwide (Tandon et al., 2008a, Tandon et al., 2008b, Millier et al., 2014). Its symptoms are categorised into positive, negative, and cognitive symptoms (Tandon et al., 2009). Specific examples of each group are hallucinations and delusions as positive symptoms, anhedonia and amotivation as negative symptoms, and thought disorders and reduced cognition as cognitive deficits. The onset of these symptoms is generally in early adulthood. This disorder has been approached in many different ways to try to understand its origins; however these are still to a large extent mysterious. One example of this is sex differences (McCarthy et al., 2017). Males have 1.42 times higher prevalence than females, and male patients show earlier onset than females. In phenotypes, male patients tend to have more positive symptoms and language disturbance, while female patients show more affective symptoms. The data from magnetic resonance imaging (MRI) studies, morphological abnormalities, for instance, cortical asymmetry, the smaller volume of specific areas hippocampus and grey matter, have been found in people with schizophrenia and this pathological evidence is potentially connected to homeostatic signalling (Keshavan et al., 2008, Landek-Salgado et al., 2016, Roeske et al., 2020).

Currently, though medications are available, their effectiveness is not seen in all individuals or against all symptoms. The drugs are effective at reducing positive symptoms in most patients, although around 30% do not respond. However, they are largely ineffective against negative and cognitive symptoms. Examples include older drugs such as sulpiride or haloperidol, and slightly newer drugs such as clozapine, olanzapine, or aripiprazole. All are believed to exert their therapeutic effects by blocking D2 dopamine receptors, but none are completely selective for D2 receptors. Besides, side effects, e.g. extra-pyramidal movement disorders, hypotension, sedation, and weight gain, are serious issues (Tandon et al., 2010). Moreover, after the onset of the disorder, patients feel difficulties in carrying out daily life or work, although some symptoms can be improved after medical treatment. It affects not only patients themselves but also family and society negatively.

1.1.1 Aetiological studies in schizophrenia.

The neurodevelopmental hypothesis (NDH) of schizophrenia suggests that abnormal brain development during early developmental stages could be a reason for the disease in later life. The modern form of NDH of schizophrenia was established in the 1980s (Feinberg, 1982, Weinberger, 1987) developed from Dr Thomas Clouston (Clouston, 1891) and has been evaluated (Jaaro-Peled et al., 2009).

One of the recent studies shows a high correlation between abnormal development of white matter at a young age and risk of schizophrenia (Ahn et al., 2019). This finding supports the NDH of the

disease. Correlated to the NDH, Brown proposes environmental risk factors acting early in neurodevelopment as contributing to the cause of schizophrenia (Brown, 2011). Different influential factors that may contribute to having schizophrenia have been studied, such as rate of parental age, season of birth, and residential environment (Marcelis et al., 1998, Mortensen et al., 1999, Brown and Derkits, 2010, Gasse et al., 2019). Immune activation, such as resulting from maternal stress, or autoimmune diseases at or before illness development, is thought to be a risk factor for schizophrenia (Benros et al., 2011, Lipner et al., 2019). Interestingly, other epidemiological evidence also suggests that prenatal infection (or also known as maternal immune activation, MIA) can increase the risk of schizophrenia in offspring (Tandon et al., 2008a, Brown, 2012). However, conclusions from previous aetiological studies are sometimes challenged on the basis of methodological defects (Selten et al., 2010) and the medical records' sensitivity (Müller, 2017). Nonetheless, the correlation of prenatal infection hypothesis has been supported by many *in vivo* and *in vitro* studies (Patterson, 2009, Boksa, 2010, Knuesel et al., 2014, Marques et al., 2015, Blomstrom et al., 2016, Purves-Tyson et al., 2019). Thus, MIA can still be a considerable risk factor for psychiatric disorders.

1.1.2 MIA and environmental risk factors

Epidemiological evidence suggests that MIA, which is triggered by infection (e.g. bacteria, virus or parasite), can increase the risk of psychiatric disorders, for examples autism spectrum disorder (ASD) and schizophrenia (Brown and Derkits, 2010, Estes and McAllister, 2016, Careaga et al., 2017, al-Haddad et al., 2019a, Cattane et al., 2020, Cheslack-Postava and Brown, 2021). Also, environmental pollutant exposure, allergies, and autoimmunological disorder can lead to an abnormal immune condition in the progeny, and this condition is linked to increased risk of psychotic disorders. In particular, influenza, herpes simples virus type 2 (HSV-2), rubella, and *Toxoplasma gondii* are commonly associated with increased risk of the disease in epidemiologic studies (Brown et al., 2004a, Brown and Derkits, 2010, Brown, 2012, Cordeiro et al., 2015, Muller et al., 2015, Mahic et al., 2017, Pekala et al., 2020). Investigators have questioned whether different types of virus or pathogens show different effects on disease risk, and how the infections might act to increase the risk of schizophrenia (Patterson, 2009, Mueller and Schwarz, 2010). These studies frequently are integrated to indicate that infectious agents, virus, bacteria or parasite, activate immune responses in the mothers, and this atypical condition, e.g. high concentration of cytokines and chemokines entering the foetal compartment, can interrupt brain development in offspring, resulting in increasing possibility of suffering with schizophrenia in the children's later life (Buka et al., 2001b, Meyer et al., 2009b, Ellman et al., 2010, Meyer, 2019). One of the negative results of an increased level of inflammatory factors is that it may affect later activation of microglia, brain tissue-resident macrophages, in the neonatal brain. If the MIA offspring cannot have a well-established immune barrier, it would affect neuronal cell development, which can be a reason for having the psychiatric disorder in later life. Consequently, a deficit in the defence system in the brain can be considered to contribute to increasing the risk of schizophrenia. However, studies in human subjects and epidemiological findings are not enough to conclude this

hypothesis. Hence, animal models of MIA are necessary to investigate the hypothesis deeply (Zuckerman and Weiner, 2005, Meyer and Feldon, 2012, Bergdolt and Dunaevsky, 2019).

The most popular synthetic mimics of infectious agents, polyinosinic- polycytidylic acid (poly I:C), which acts like double-strand RNA (dsRNA), and lipopolysaccharide (LPS), a membrane protein of gram-negative bacteria, are used in animal models to activate their immune system. After such treatments, induced levels of inflammatory molecules, for example interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6, IL-10, CCL2, CCL5, are detected in MIA offspring (Urakubo et al., 2001, Garay et al., 2013). Correspondingly, evidence from many *in vivo* experiments, in mice or rats exposed to MIA, show behavioural and physiological abnormalities which are related to those shown in patients with schizophrenia (Meyer et al., 2005, Keshavan et al., 2008, Eßlinger et al., 2016, Talukdar et al., 2020). This suggests that an uncontrolled immune response, probably via altered levels of cytokines and chemokines, affects the brain not only in prenatal stages but also in neonatal, and potentially even in later developmental stages continuously. Above and beyond this, altered levels of inflammatory molecules have been observed alongside with deficits of neurotransmitters (Luchicchi et al., 2016) and interneurons in animal models (Dickerson et al., 2014, Fatemi et al., 2017, Zhang et al., 2017).

1.1.3 Genetic risk factors

As research techniques have been developed, scientists have been able to gain more knowledge about disease causes at the molecular level. Despite the fact that many seminal studies have shown that MIA or uncontrolled immunity can increase the risk of schizophrenia, the other major factor, the genetic risk factor, is required to contribute full risk of the illness. It is believed that maternal immune challenges, environmental stress and genetic contributions during pregnancy affect the brain development of offspring in the prenatal or neonatal period (Newschaffer et al., 2002, Knuesel et al., 2014, Morris and Pratt, 2014, Ratnayake et al., 2014, Ripke et al., 2014, Purcell et al., 2014).

Of the possible risk gene variants for schizophrenia, none of them is shared by all patients. Currently, it seems that schizophrenia is a “polygenetic disease” rather than a “unique genetic disorder”. For the majority of patients, genetic risk is conveyed by common sequence variations in a large number of genes, each variant individually conveying only a very small element of risk. For a small proportion of patients, genetic risk is conveyed by rare variants conveying much greater risk individually. Table 1 shows rare copy number genetic factors related to schizophrenia (Doherty et al., 2012, Grayton et al., 2012, Rethelyi et al., 2013, Rutkowski et al., 2017, Zhuo et al., 2017).

Table 1. Rare genetic copy number variant risk factors for schizophrenia

Genes	**Copy number variant (CNV)**	**Location**
34 genes	Deletions and duplications	1q21-22

NRXN1	Deletion	2p16.3
DLG1, PAK2 +17 others	Deletion	3q29
CYFIP1 + 3 others	Deletion	15q11.2
DOC2A, TAOK2, NDE1, MAPK3 (=ERK1) + ~ 25 others	Duplication (SCZ); Deletion (ASD)	16p11.2
HNF1B + 17 others	deletion	17q12
COMT, PRODH + ~ 33 others	Deletion (SCZ); Duplication (ASD)	22q11

For example, the 22q11.2 locus contains a number of genes regulating cortical circuit formation (Meechan et al., 2015) and the deletion at this region is associated with ~ 30x increased risk of schizophrenia, whereas the corresponding duplication is associated with ASD (Lin et al., 2017). Animal studies suggest that interneuron migration is significantly disrupted by 22q11.2 deletion (Meechan et al., 2012). One of the recent findings additionally suggests three risk gene variants related to methylation (Yu et al., 2020). This finding and other genome-wide association studies (GWAS) studies detecting common risk variants indicate that chromosome 22 mutations could be necessary for brain functions.

Another genetic risk factor is a common variant in the *MAP2K7* gene (encoding the protein MKK7), which conveys a relatively large increase in risk. Winchester and other researchers describe that in post-mortem studies from schizophrenia patients, MKK7/*MAP2K7* mRNA shows a decreased level (Winchester et al., 2012). This result corresponds with the level of mRNA of parvalbumin (PV), which shows a robust alteration in schizophrenia patients. Additionally, behavioural tests show that MKK7 heterozygous mice show poor performance in a working memory test (Openshaw et al., 2017). Recently, an animal study shows that low levels of MAPKs in PFC caused schizophrenia-like behaviours in rats (Deane et al., 2021); having difficulties in doing cognitive tasks is commonly shown by people with the disease.

A microduplication at the 16p11.2 region, which contains *ERK1* gene (*MAPK3*), is strongly correlated with schizophrenia (Ripke et al., 2020); on the other hand, microdeletion in the same region associated with ASD (Chang et al., 2017). Taken together, MAPK gene abnormalities could contribute to the causes of schizophrenia.

Interestingly, one of the most consistent genetics findings with schizophrenia risk is association of the 6p22.1 region (Ripke et al., 2014, Dennison et al., 2020). This region contains some genes that participate in immune functions, especially, the major histocompatibility complex (MHC) function

region. The gene responsible for the association signal is suggested to be Complement component C4 (Sekar et al., 2016). Hence, some of the schizophrenia-related genes are also clearly related to immunity. It is worth noting that while this gene comes up regularly as a risk gene for schizophrenia in European populations (Consortium and 2, 2012), this result is not replicated with GWAS of Asian populations (Lam et al., 2019). Consequently, genetic studies support abnormal immune reactions, which can be induced by genetic abnormalities or by MIA or a combination of both factors, are related to the onset of schizophrenia.

In order to understand the disease in-depth, many animal models have been introduced, and most of them involve rodents, although some researchers have questioned whether rodent models are reliable animal models to study psychiatric disease and mental health (Canetta and Kellendonk, 2018). Alternatively, non-human primates can be studied, which have closer functional and anatomical structural similarity to humans. Rose *et al*. showed that some cytokines and chemokines (e.g. IL-1β, IL-6 and TNF-α) are produced at higher levels in offspring after MIA in non-human primates. Also the alterations in inflammatory molecules are associated with behavioural abnormalities (stereotypies) (Rose et al., 2017).

Whatever techniques are used, understanding impacts of inflammatory molecules on neuronal and glial cells are important and how those induced maternal immune molecules or pathogen themselves affects prenatal brain developmental processes and neuronal and glial cells in the foetus brain is still doubtful. The possible ways how these immune molecules involved in the brain and the illness development will be discussed deeply in sections 1.4.

1.2 Immunity in schizophrenia

The immune system is very complex, and it involves the innate immune response and the adaptive immune response in mammals. These responses need cells of the immune system from the blood, to be appropriately positioned in tissues, interact with one another, and move to other tissues. In this section, general knowledge of the immune system, immune reactions (inflammation) in the peripheral system and the brain will be described.

1.2.1 The immune system

The immune system is the defensive system, and its primary function is to protect the body from pathogens for example, bacteria, viruses, and parasites. In animals, the immune system is divided into two parts, innate immunity and adaptive immunity (Lodish, 2008). Innate immunity is the first defence mechanism. This system involves epithelial barriers that prevent the entry of pathogens and chemicals. However, if these barriers are broken, then immune responses involving cellular innate defence and inflammation are activated. In addition, innate immunity contributes to adaptive responses that control the innate immune response and provide long-term protection and immune memory.

1.2.2 Immunological components

1.2.2.1 Toll-like receptors and their expression

When infectious agents come into the body, Toll-like receptors (TLRs) on the cell membrane recognise pathogen molecules to deliver information to activate further immune reactions. TLRs are the most well-known pattern recognition receptors (PRRs) and they detect various pathogen-associated molecular patterns (PAMPs) (Mallard, 2012). Thirteen different types of TLRs are identified in human and/or mouse (Kielian, 2006). Of these, TLR4 recognises bacterial membrane component, TLR3 recognises double-stranded RNA, while TLR7 and TLR8 recognise single-stranded viral RNA (Gay et al., 2014). The TLR expression profile is variable depending on the type of cells and the cells' activation status. Moreover, their expression is also regulated by stages of brain development.

Macrophages, in general express all types of TLRs (Mazaleuskaya et al., 2012, Fong et al., 2016), although their expression levels are diverse, and is altered by environmental challenges (Nhu et al., 2006). In neural cells, generally, TLR 2, 3, 4, and 8 are expressed on their membrane (Tang et al., 2007, Trudler et al., 2010, Hanke and Kielian, 2011). TLR2 and 4, when activated, can cause neural cell death by apoptosis (Tang et al., 2007). TLR3 works in growth cones, and roles of TLR8 are related to apoptosis, and also to growth of neurites (Trudler et al., 2010). On the other hand, microglia, the first immune barrier of infections of the brain, can express almost all types of TLRs (Olson and Miller, 2004, Ransohoff and Brown, 2012), and their expression levels are altered by developmental stages and also by bacterial pathogens (Crack and Bray, 2007). For example, Kielian and colleagues show that levels of the TLR1, 2, and 6 expression are increased after gram-positive bacteria exposure (Kielian et al., 2002). Correspondingly, other papers support enhanced TLR levels after an immune stimulus, e.g. gram negative bacteria and bacterial DNA (Dalpke et al., 2002, Rasley et al., 2002). Because of the diversity of combinations of TLRs, pathogens, and cell types, there is no easy way to predict TLR-mediated cellular mechanisms.

1.2.2.2 Cytokines and chemokines and their receptor expression

Cytokines are small signalling molecules. Small subgroups, chemokines, interferons (IFNs), interleukins (ILs), and tumour necrosis factors (TNFs) are included in the cytokine group. Cytokines can be categorised into pro- or anti-inflammatory cytokines. For example, IL-1β, IL-6, are the typical pro-inflammatory cytokines and IL-4 and IL-10 are generally considered anti-inflammatory cytokines.

Chemokines are a subgroup of small cytokines (7-12 kDa), which activate chemotaxis in responsive cells nearby. Over the decades, chemokines were named without fixed rules. In this paper, the nomenclature for chemokines and their receptors set by the Nomenclature Committee of the International Union of Pharmacology will be used (Bachelerie et al., 2014). Chemokines take roles in various immune functions, for example, leukocyte migration and inducing further inflammatory responses (Hughes and Nibbs, 2018). Histologically chemokines are studied in their

chemoattractant properties; however many papers show that chemokines are required for a wide variety of cell functions, for instance cellular proliferation, activation and differentiation, along with their immune functions (Bachelerie et al., 2014). Recent seminal studies have presented their roles in the central nervous system (CNS), including neurotransmission, neural migration, and neurodegeneration (Tran and Miller, 2003, Ambrosini and Aloisi, 2004, Ransohoff, 2009, Scovil Watson et al., 2020).

Microglia and astrocytes express most of the CC family and CXC family receptor and cytokine receptors, whereas neuronal cells show a more restricted expression profile, such as CXCL12 and CX3CL1, compared to glial cells (Bacon and Harrison, 2000, Lee et al., 2002, Stumm et al., 2002, Flynn et al., 2003, Verge et al., 2004, Stuart et al., 2015).

1.2.3 Immune reaction (inflammation)

Inflammation is the first immune defence system. Through this step, a body protects itself from external molecules or pathogens and the adaptive immune system is also developed. In peripheral organs, macrophages, which are the first immune cell to defend the body, track pathogens and remove them through phagocytosis.

1.2.3.1 Macrophages

Macrophages are differentiated from the yolk sack or through haematopoiesis in the foetal liver (Davies et al., 2013). To develop resident macrophages, the haematopoietic stem cells (HSCs) are exposed to many stimulating factors such as colony-stimulating factor 1 (CSF1; also known as M-CSF) and IL-34. Moreover, all tissues have their own tissue-resident population of macrophages where they are required. Macrophage phenotype and functions are highly heterogeneous and differentiated depending on the local tissue environment (Mosser and Edwards, 2008, Smigiel and Parks, 2018). The M1/M2 concept is broadly used to describe the macrophages population; however, it is largely established on *in vitro* macrophages. Therefore, applying this classification of macrophages to the *in vivo* condition is not straightforward. Anyhow, in a straightforward way, the M1 phenotype involves release of pro-inflammatory cytokines and production of reactive oxygen species (ROS). By contrast, the M2 phenotype may have anti-inflammatory and wound healing functions. Respectively, macrophages distinguished by differential expression of the Lymphocyte antigen 6C (Ly6c1/2) gene group - $Ly6C^{hi}$ and $Ly6C^{lo}$ macrophages - are considered to be the M1 and M2 type respectively (Saini et al., 2016). However, a recent publication suggests that the M1/M2 distinction is not specific enough to describe the distinct phenotypes and functions of macrophages *in vivo*. Furthermore, both M1 and M2 markers can be expressed simultaneously (Martinez and Gordon, 2014). Therefore, in this thesis, ‘pro-’ and ‘anti-inflammatory’ terms are used to describe the macrophages’ phenotype, rather than M1/M2. Pro-inflammatory macrophages dominantly express pro-inflammatory cytokines, such as IL-6, TNF-α, and IFN-γ. On the other hand, anti-inflammatory macrophages expressed mainly anti-inflammatory cytokines and growth factors, such as IL-10, TGF-β and IGF-1 (Martinez and Gordon, 2014).

1.2.3.2 Peripheral inflammation

In general, tissue resident macrophage populations are required for tissue repair stages, and the monocyte-derived macrophage population is recruited to an injured area from bone marrow. Phagocytic cells, such as neutrophils, macrophages, and dendritic cells, are vital cells in the innate immune system (Reece, 2011). They are recruited from the blood into damaged or infected tissues where they recognise pathogens and destroy them. This is an important part of the inflammatory responses.

At the injured site, mast cells and macrophages release signalling molecules, for instance histamines and cytokines and chemokines. Cytokines control immune responses and enhance the amount of blood supply to areas of injury. Released signalling molecules attract additional macrophages, phagocytic cells, and white blood cells to injured tissues, in order to engulf pathogens. Reduction of the macrophage population causes significant impairment of scar formation; indeed its outcomes are different depending on the timing of the depletion of the cell population (Smigiel and Parks, 2018).

As described above, cytokines and chemokines are key components that act to regulate the process of inflammation. IL-10 is important to return macrophages to an anti-inflammatory phenotype from a pro-inflammatory phenotype. Boehler and others present that IL-10 is important to regulate the macrophage phenotype, and it may occur via a reduction of the level of TNF-α (Boehler et al., 2014). Regarding chemokines, CCL2 is a key signalling molecule to recruit monocytes to where injury happens. Pro-inflammatory macrophages upregulate the production of CCL2 by neighbouring macrophages, which could mediate inflammation (Oishi and Manabe, 2018). These findings suggest that the balance of immune molecules is essential for macrophage phenotype and function regulation; indeed dysregulation of immune molecule signals disrupts this coordinated process and can lead to significant results.

1.2.4 Immune reaction in the brain (neuroinflammation)

1.2.4.1 Microglia

In the past, the brain was thought of as “an immune privileged organ”, but it is not entirely true. Microglia are the residence immune cells in the brain and their population is 10-15% of all brain cells (Carson et al., 2006, Boche et al., 2013). In the adult brain, microglia are more numerous in grey matter than white matter (Arcuri et al., 2017). Although microglia are also called ‘brain-resident macrophages’, they are derived from the yolk sac, and these microglia stem cells enter the brain around E7-9 in mice (Hoeffel et al., 2015). Broadly, microglia are considered as long-lived cells, and no new microglia are supplied from peripheral immune cells under healthy conditions. When they need to proliferate, or replace their population, the cells are produced from the stem cell pool, which is made during embryonic development (Askew et al., 2017). Even though the number of cells is not the largest population, they react very sensitively and actively to even minor pathological alterations. As a result, microglia secrete various functional molecules and free

radicals, which help to communicate with neighbouring cells. Those secreted molecules have to be regulated tightly in order to prevent adverse non-necessary influences.

Depending on which molecules activate resting microglia, their activation phenotypes are different (Nakagawa and Chiba, 2014). Although most of the researchers have used two terms, resting and activating, to describe microglial phases, a recent paper indicates that even resting or normal microglia are not actually resting at all (Li and Barres, 2017). They are continually checking their surrounding environment. In general, people prefer to use 'resting' when microglial processes look very long and developed (called ramified microglia), and 'activated' for when the cells look more round, with thicker, and shorter projections (also called ameboid microglia).

Another way to categorise microglial activation is into two groups: M1 and M2. This concept is taken from peripheral macrophages' activation status (see section 1.2.3.1). Although microglia do not match their properties to peripheral macrophages, this idea (M1/M2) is widely used to describe and categorise microglia activation phases in a simple way. Nonetheless, the diversity of microglial morphologies and uniqueness of microglial physiology distinguish them from peripheral macrophages, M1 and M2 are not acceptable terms to describe microglia's polarisation (Ransohoff, 2016).

In spite of the morphological differences, microglial morphological changes are not one or the other, but are more like a spectrum, and the morphological changes especially may not be very relevant for *in vitro* experiments to distinguish whether they are activated or not (Walker et al., 2014). In this thesis, with microglia, pro-inflammatory (instead of M1 or ameboid), or anti-inflammatory (instead of M2 or ramified), are the terms used.

To study microglia, many microglia cell lines have been introduced, such as BV-2 and N9. These cell lines are originally derived from primary microglial culture; however, oncogenic transformation was applied to generate the cell lines (Blasi et al., 1990, Bocchini et al., 1992). Due to the transformation, the cell line might have altered microglial characteristics (Stansley et al., 2012). For example, BV-2 is widely used to study murine microglial physiology but its sustainability has been questioned compared to primary microglial culture following LPS stimulation (Das et al., 2016, Sarkar et al., 2018) although there is evidence to show BV-2 is still valid to use for brain inflammation in some extent (Henn et al., 2009). The SIM-A9 cell line, which is a recently introduced murine microglial cell line, is isolated from mouse microglial culture, but in contrast to other cell lines, this cell line does not have any functional or exogenous genetic modification to achieve immortalisation (Nagamoto-Combs et al., 2014). Furthermore, the paper shows evidence that the cells maintain microglial characteristics, such as producing cytokines and NO over many passages (over 40). Although SIM-A9 is still a cell line, it is potentially closer to the nature of *in vivo* microglia due to free from the artificial transformation, as compared to other viral- or oncogene-transformed microglial cell lines.

1.2.4.2 Neuroinflammation

As mentioned above, microglia maintain their population through self-renewal during their lifetime without any contribution from peripheral immune cell types (Arcuri et al., 2017). In a normal physiological condition, the brain is firmly protected by the blood-brain barrier (BBB), and ingress of peripheral immune cells such as leukocytes, macrophages, and monocytes through the BBB is very limited. Therefore, under a resting condition without any damage, very few macrophages and inflamed microglia can be observed in the brain. Nonetheless, severe inflammation or infection can break the integrity of the BBB, resulting in infiltration of leukocytes and macrophages. Cytokines and chemokines participate in this disruption of the BBB. These proteins can be released from most types of brain cells, e.g. neurons, astrocytes and microglia and they are required not only for immune responses but also for maintaining homeostasis in the brain (Matcovitch-Natan et al., 2016). Hence, these proteins are essential in a normal physiological condition as well as in a pathological condition (Rostène et al., 2011).

Broadly, the term neuroinflammation is used to illustrate the basis of microglia-mediated inflammation. Even though microglia are the central immune cells in the brain, this does not mean that microglia are the only cell type involved in immune responses. Astrocytes and neurons can secrete cytokines and chemokines to communicate with peripheral immune cells, or through neurotransmitters they can talk to neighbouring cells. Especially, publications in psychiatric research often refer to neuroinflammation only in terms of microglial activation (Notter et al., 2017b). Thereby other aspects of inflammation in the brain are neglected. Consequently, the current definition among researchers is too narrow and biased. Despite the limitations and the bias of the definition of neuroinflammation, it can still be usefully used to illustrate microglia-involved inflammation.

Sex differences in development are a fascinating topic and increasing scientific evidence shows how much sex affects microglial development (Thion et al., 2018a). From experiments in mice, the number of microglia is similar in both males and females (E17); however, they show regional differences in the number of the cells at P7, for example there are more microglia in the hippocampus and hypothalamus in males than in females (McCarthy et al., 2017). From this point, we can hypothesise that these sex differences in microglial number can have functional significance in neuroimmune signalling for postnatal infections, and as a result can affect the risk of the disease.

1.2.5 Inflammation in schizophrenia

Not only MIA, but also other types of inflammation or immune dysfunction, are related to schizophrenia (Dickerson et al., 2016, Landek-Salgado et al., 2016). Moreover, the patients have shown a higher rate of having an autoimmune disease. This is, additionally, supported by experiments with anti-inflammatory drugs. For instance, the experimental results suggest that anti-inflammatory drugs, such as minocycline, can reduce negative symptoms or improve cognitive

deficits in human subjects and also schizophrenia-like symptoms in animal models (Levkovitz et al., 2010, Chaudhry et al., 2012, Dean et al., 2012, Cakici et al., 2019, Zhang et al., 2019). This means inflammation may be linked to schizophrenia symptoms; moreover these effects - improving cognitive functions - may be caused by the reduction of serum levels of cytokines such as IL-6 and IL-1β. On the other hand, a meta-analysis of human studies questions the efficiency of non-steroidal anti-inflammatory medications to reduce the illness (Nitta et al., 2013) and a well-controlled 2 centre trial recently found no efficacy for minocycline (Deakin et al., 2019). While the impact of inflammation on the mental illness has been being challenged, it still has to be considered the risk factor of schizophrenia (Rodrigues-Amorim et al., 2018). Therefore, in this section, evidence that explores the relationship between the illness and abnormal immune responses, in terms of cytokine and chemokine levels, in the blood and the brain, will be discussed.

1.2.5.1 Abnormal peripheral immunity in schizophrenia

The easiest way to check for immune activation is by measuring inflammatory molecules in the blood. Many papers consistently have reported abnormal (increased) levels of cytokines, e.g. IL-2, IL-6, TNF-α, and soluble IL-2 receptor, and chemokines, e.g. CCL2, CCL11, and CXCL8 (previously known as IL-8), in schizophrenia patients compared to normal health groups (Zhang et al., 2002, Potvin et al., 2008, Miller et al., 2011, Boerrigter et al., 2017, Hong et al., 2017, Dahan et al., 2018, Delaney et al., 2019). However, one recent paper shows that patients with treatment-resistant schizophrenia show decreased levels of *TNF-α* mRNA in their blood, compared to healthy controls (Mostaid et al., 2018). Moreover, IL-10, an anti-inflammatory cytokine, may be related to schizophrenia. The evidence shows the increased levels of IL-10 in patients' serum compared to controls (Maes et al., 2002, Kunz et al., 2011), but one contrast finding is reported (Xiu et al., 2014). Furthermore, Noto and others report that schizophrenia is related not only increased inflammatory cytokines and chemokines, but also it is associated with dysregulation of particular cytokines and chemokines; for instance IL-2, CCL11, and CXCL10 (Noto et al., 2015). Equally, Okazaki and co-workers report that levels of macrophage migration inhibitor factor (MIF), a potential biomarker for schizophrenia, in serum are positively correlated to the disease (Okazaki et al., 2018).

A few meta-analysis results suggest that upregulated levels of inflammatory molecules in serum, e.g. IL-6, TNF-α, and CXCL8, can be used as biomarkers of schizophrenia (Upthegrove et al., 2014, Goldsmith et al., 2016). However, Miller and colleagues argue that TNF-α is useful whereas IL-6 is not, as levels may depend on medications and patient's physical index (Miller et al., 2011). In addition, the meta-analysis result suggests that IL-6, one of the most robustly reported as showing increased levels in patients' serum, is difficult to use as a biomarker although it may be linked to clinical risk of psychosis compared to controls (Misiak et al., 2021).

An interesting point has been noted, that levels of cytokines and chemokines may explain why some patients are treatment-resistant but others are not (Kalmady et al., 2018). Therefore, altered levels of inflammatory molecules can be helpful not only to predict the illness but also to decide

what medications patients should be prescribed in the future although further studies are required to find stable biomarkers.

Other evidence from human samples that shows an abnormal immune system in schizophrenia patients compared to a healthy population is the quantity and quality of immune cells. A meta-analysis reports that patients have higher immune cell numbers than healthy controls (Mazza et al., 2020). In addition, patients in relapse show impaired monocytic activity (Uranova et al., 2017) or higher counts (Özdin and Böke, 2019) compared to healthy samples. Together with this finding, monocytes from patients may have functional deficits (Muller et al., 2012) and this may be correlated to altered levels of inflammatory molecules in serum noted above. In contrast, other evidence shows no significant difference between patients and healthy groups (Ormel et al., 2017).

Upregulated levels of immune molecules, such as IL-6 and TNF-α, have been frequently reported (Momtazmanesh et al., 2019, Zhou et al., 2021); however, how these molecules can affect the brain and the link with schizophrenia pathology are not well studied. Since IL-6 and TNF-α are produced by macrophages, and increased levels reported at time of birth (Buka et al., 2001b) possibly these two cytokines are used as a signal (via metabolic abnormality) to affect the brain functions in people with schizophrenia (Beumer et al., 2012). These excess inflammatory factors in patient serum could cause neuroinflammation via stimulating BBB permeability (Huang et al., 2020). Likewise, evidence from post-mortem tissues indicates that levels of mRNAs related to BBB structure maintenance are positively correlated with inflammatory status in disease groups compared to control (Cai et al., 2020).

To sum up, pro-inflammatory cytokines, such as TNF-α and IL-6, frequently showed abnormal levels in schizophrenia patients, although follow up studies are required to use them as the biomarkers. Moreover, a larger number of monocytes/macrophages are found in a higher inflammatory status in schizophrenia patients compared to controls. Indeed, peripheral inflammation affects brain vasculature, resulting in brain damage by microglia and/or infiltrating peripheral immune cells. Details of how CNS immunity relates to the illness will be discussed in the following section.

1.2.5.2 Abnormal CNS immunity in schizophrenia

Even though peripheral immune molecule concentrations show abnormal levels, it does not necessarily mean that the brain is exposed to the same conditions because of BBB protection. Even though the brain is located in the protected area, a great deal of research evidence indicates signs of neuroinflammation related to schizophrenia, mainly the abnormal levels of immune molecules in the CNS. For instance, in post-mortem samples, increased levels of IL-6, and TNF-α, and *IL-6,TNF-*α and *CXCL8* mRNAs (Fillman et al., 2013, Paterson et al., 2006), along with reduced levels of *IL-10* mRNAs (Pandey et al., 2018), have been detected compared with controls. Interestingly, there is evidence to show a positive correlation between complement transcripts with glial markers and schizophrenia (Purves-Tyson et al., 2020) is reported. Additionally, a meta-

analysis from schizophrenia patient data showed that *CX3CR1* was downregulated in the blood and the brain (Bergon et al., 2015). Equally, IL-6 and CXCL8 levels are significantly increased in cerebrospinal fluid (CSF) (Gallego et al., 2018). Likewise, *in vivo* evidence shows increased cytokines in patients compared to healthy cohorts. Along with this concept, abnormal levels of cytokines such as IL-1β can cause structural brain changes (Meisenzahl et al., 2001). However, one recent evidence shows that decreased levels of *IL-6, TNF-α,* and *IL-10* with a microglial marker (*CD68*) in elderly patients' post-mortem samples compared to controls (López-González et al., 2019). Although these pathological indications correspond with the epidemiological evidence suggesting an immune activation contribution to disease aetiology, however, they may be affected by duration of illness and medications.

1.2.5.3 Microglia studies in schizophrenia

Since neuroinflammation may be enhancing the risk of psychiatric disorders, microglia have received lots of attention (Mondelli et al., 2017). In terms of pathological evidence related to microglia from patients with schizophrenia, post-mortem studies and brain imaging have been employed. Firstly, findings from post-mortem studies have suggested increased microglial activities in specific parts of schizophrenia patients' brain (Bayer et al., 1999, Radewicz et al., 2000, Wierzba-Bobrowicz et al., 2004, Wierzba-Bobrowicz et al., 2005). The Rene Kahn research team produced the first *in vivo* imaging evidence to support the relationship between microglial activation and schizophrenia in the brain (van Berckel et al., 2008) and since then many papers have supported the idea (Doorduin et al., 2009, Takano et al., 2010, Bloomfield et al., 2016, Park et al., 2020). Furthermore, meta-analysis of post-mortem tissue studies indicates that several microglial specific genes (including *Cx3cr1* and *Tmem119*) are significantly downregulated in patients with schizophrenia, although microglial cell numbers and microglial morphological and quantitative changes are unchanged overall (Snijders et al., 2021).

To measure microglial activity *in vivo*, ligands for the mitochondrial 18 kDa translocator protein (TSPO) have generally been used, even though its reliability as a marker for inflammation or for microglial activity has been challenged (O'Donnell, 2017). Some imaging data show elevated TSPO levels in patients' brains (Doorduin et al., 2009, Bloomfield et al., 2016) and a meta-analysis detected a positive relationship (Bergon et al., 2015). However, in contrast to the former studies, a recent positron emission tomography (PET) study reported decreased TSPO binding in drug naïve patients in the early illness stage (Collste et al., 2017). Another paper reports that decreased level of TSPO binding in the prefrontal cortex (PFC) from mouse MIA offspring (Notter et al., 2017a) which is matched with human PET studies. Additionally, more recent meta-analyses claim that TSPO binding is negatively related to schizophrenia (Collste et al., 2017, Plavén-Sigray et al., 2018).

A possible reason for these contrasting findings regarding TSPO studies is that the level of TSPO is affected by inflammatory status. Bae and colleagues state that TSPO levels are negatively correlated to neuroinflammation (Bae et al., 2014). They report that when TSPO expression is

promoted in microglia, pro-inflammatory polarisation is suppressed, while with anti-inflammatory polarisation it is induced.

The other explanation, regarding TSPO reliability, is that TSPO may not be a good biological marker for measuring immune response in psychiatric patients (Notter et al., 2017b). Originally, Alzheimer's disease patients show clear TSPO changes with inflammation. This indicates that TSPO radioligands are able to measure microglial inflammation levels in the brain. However, evidence has shown that, as would be expected, TSPO is not expressed only on microglial mitochondria, but also in mitochondria in other brain cells, such as astrocytes and neurons (Sneeboer et al., 2019). Hence, TSPO binding in microglia will overlap with signals from other cells, result in ambiguous findings. Indeed, not only TSPO, but also other microglial markers tend to show the opposite results in neurodegenerative diseases and psychiatric diseases (Chiu et al., 2013, Olah et al., 2018, Snijders et al., 2021). This suggests that interpretation of the data with TSPO ligands should be re-evaluated, not only in terms of a microglial marker.

In conclusion, the findings from TSPO research in the brain may depend on the activation phases of microglia; however, microglial activity is also affected by medications, other diseases, and the illness stages. Therefore, a level change of TSPO binding cannot specifically reflect microglial inflammatory activity. Moreover, functions of TSPO in the brain are not clearly established yet. Hence, using TSPO binding as a marker for microglial immune activation is an approach that must be interpreted with great care.

Overall, cytokines and chemokines may play a role in increasing the risk of the mental illness. The data from recent studies indicate that some particular cytokines e.g. upregulation, in both serum and brain, of IL-1β, IL-6, TNF-α, and some chemokines, such as CCL2, CCL5, CCL11, and CXCL8 are highly related to increased risk of schizophrenia (Stuart et al., 2015, Hong et al., 2017). Although none of the studies has shown a direct interaction between one specific inflammatory molecule and the development of schizophrenia, there is some agreement that uncontrolled inflammatory conditions could increase the risk of the disease. Additionally, these abnormal levels of inflammatory factors may be more prominent in the medical treatment-resistant group. Furthermore, peripheral immunity affects BBB permeability which could ultimately promote neuroinflammation by microglia and/or allowing peripheral immune cell infiltration (Huang et al., 2020). Even though there are contradictory findings regarding microglia activity from live patient imaging data, the contribution of microglia to the illness has been suggested by many seminal papers (Calovi et al., 2020, Carrillo et al., 2020, Vidal and Pacheco, 2020). Moreover, activated microglia are consistently observed alongside MIA and schizophrenia related research. As a result, studies from human to mice or from mice to human have positively proposed that uncontrolled immune reactions in the brain may rise the risk of the psychiatric disorder. Hence, understanding its molecular mechanism is important.

1.3 The MAPK pathway and functions

When cells are activated by stress, they change their physical and physiological conditions, e.g. migration, proliferation, and alterations in the expression of cellular molecules, to respond to stress. Mitogen-activated kinase (MAPK) pathways are one of the common intracellular signalling pathways. Furthermore, findings from post-mortem and GWAS studies suggest that the MAPK pathway is important to cause schizophrenic pathophysiology. Additionally, this pathway takes essential roles to regulate immune functions. In this section, details of each MAPK, and the roles of MAPKs in the peripheral immune system and the CNS, will be described.

1.3.1 The MAPK pathway

MAPKs have been characterised in three major groups, p38s, extracellular-signal regulated kinases (ERKs), and c-JUN N-terminal kinases (JNKs), in mammals (Weston and Davis, 2007). Each MAPK is activated by phosphorylation which is mediated by MAPK-kinases (MAPKKs) that are themselves phosphorylated and activated by MAPKK-kinases (MAPKKKs).

There are four members of the p38 family (α,β,γ, and δ) in mammals, and they show about 60% amino acid sequence similarity (Cuenda and Rousseau, 2007). Although p38s are expressed in most cells types, γ and δ isoforms show region-dependent expression level changes (Mertens et al., 1996, Goedert et al., 1997). To study their biological purposes, individual null mice have generated. Although p38α null mice are embryonic-lethal as a result of faults of placental angiogenesis (Mudgett et al., 2000), other isoform KO mice models are available for research (Beardmore et al., 2005). The gene deletions not only impact on placental angiogenesis, but also p38s' biological contribution is essential for other functions such as myogenic differentiation during embryo development (Natale et al., 2004, de Angelis et al., 2005), cell differentiation (Perdiguero et al., 2007), and cell migration (Rousseau et al., 1997, Kim et al., 2003, Rousseau et al., 2006).

Another MAPK group, ERKs, have two different isoforms, 44kDa (ERK1), and 42kDa (ERK2); they show 80% amino acid sequence similarity (Chen et al., 2001). ERK pathways are induced by growth factors and cytokines respectively and result in modulating various cellular functions such as cell attachment and migration (Le et al., 2004, Ramos, 2008). The ERKs have a prominent role in oncogenesis and tumour formation (Deschênes-Simard et al., 2014). An *Erk1/2* KO rodent model has been generated to study the function of these genes; however the models are only partially successful: *Erk1*$^{-/-}$ is not embryonic-lethal, however *Erk2*$^{-/-}$ is embryonic-lethal by E8.5 (Fischer et al., 2005). In spite of their high amino-acid similarity, distinct biological functional differences for each isoform have been reported (Pagès et al., 1999, Saba-El-Leil et al., 2003).

The last of the main MAPK family is the JNKs. Three JNK genes and ten splice variants have been found. They are produced by alternative splicing and their sizes are 43kDa, 46kDa and 54kDa respectively. Even though *Jnk1* and *Jnk2* are expressed throughout the body, *Jnk3* is expressed in more limited tissues, such as the brain, heart, and testis (Davis, 2000). c-JUN is a transcription

factor activated by JNKs. The effects of c-JUN activation are variety, for example implicated in inflammation, apoptosis, and proliferation. Although JNKs' functions in neural cells have not been fully described, some of their roles in brain development, migration, and cell's life cycle are understood (Coffey, 2014). In order to study functions of JNK genes, one or more JNK gene deficient mice have been generated and studied (Yang et al., 1998, Sabapathy et al., 2001); however some of the mutant models are embryonic-lethal. For example, Jnk1 and Jnk2 double-knockout embryos show abnormal forebrain and hindbrain development (Kuan et al., 1999); thus this study suggests that these genes have critical roles in the formation of the CNS. Findings also indicate that JNKs mediate aspects of cell survival and death. Upstream of JNKs are MKK4 and MKK7, MKK7 is more specific to JNKs than MKK4 respectively (Wang et al., 2007). Indeed, Mkk4 KO and Mkk7 KO mice show differences in embryo development, indicating that these two kinases may have independent roles, as well as in regulation of the JNK pathway.

Table 2. Synonyms and gene names of MAPKs

Name	**Synonym / *gene name***
p38s	*MAPK11* (p38β), *MAPK12* (p38γ), *MAPK13* (p38δ), *MAPK14* (p38α, CSPB1, CSBP2)
ERK1	*MAPK3*, PRKM3, p44MAPK
ERK2	*MAPK1*, PRKM2, PRKM1, p42MAPK
JNK1	*MAPK8*, SAPK1, PRKM8,
JNK2	*MAPK9*, SAPK1a, PRKM9,
JNK3	*MAPK10*, SAPK1b, PRKM10
MKK4	*MAP2K4*, MAPKK4, PRKMK4, JNKK1, MEK4, SERK1, SEK1
MKK7	*MAP2K7*, MAPKK7, PRKM7, JNKK2, MEK7,

The studies about MAPKs clearly show that not only the MAPK pathways are important for diverse biological functions, but also their isoforms have distinctive biological functions. MAPKs signalling with respect to immune function will be described in the following sections.

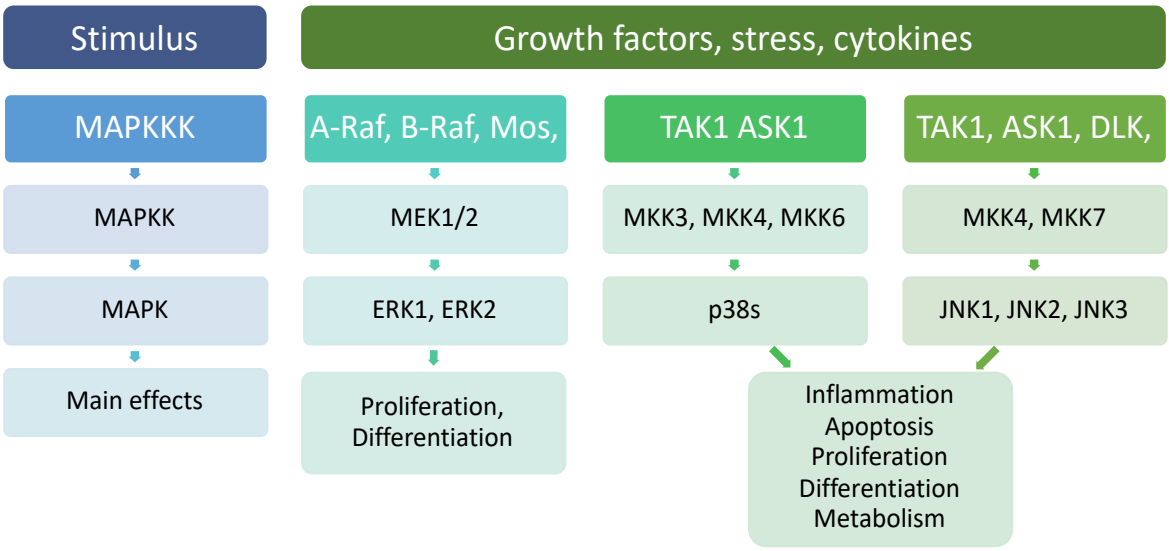

Figure 1. Simplified diagram of MAPK pathways in mammalian cells. ERKs, p38s, and JNKs are groups of the MAPK family. The MAPKs are activated via phosphorylation of the MAPKK group of protein kinases. The MAPKKs are activated, in turn, by phosphorylation by a group of MAPKKKs. The MAPK pathways cause many cellular effects such as proliferation, differentiation, and inflammation. (this diagram information is summarised from (Davis, 2000, Rose et al., 2010, Morrison, 2012, Coffey, 2014)

1.3.2 MAPKs functions in the peripheral immune system

Even though p38 family gene-deficient models have made, whole family null mice are unavailable. In spite of some limitations, there are many scientific observations to support how p38s participate in establishing and regulating the immune system. Beardmore and colleagues suggest that p38β may not be an essential kinase for the immune system development during embryogenesis, however p38α strongly takes that role (Beardmore et al., 2005). Furthermore, activation of p38s supports erythroid differentiation (Nagata and Todokoro, 1999), and p38α affects T helper 1 (Th1) cell differentiation through IFN- γ secretion (Berenson et al., 2006). Indeed, this pathway is also important for B lymphocyte proliferation (Craxton et al., 1998).

Although *Erk1* KO models do not show any physical abnormalities, many researchers suggest a relationship to immunity. For instance, the ERK pathway can have roles in T cells functions, with ERK1 mediating T cell differentiation (Pagès et al., 1999). Furthermore, the ERK pathway participates in the formation of macrophage colonies from HSCs (Hsu et al., 2007) and CD4 T cell maturation (Fischer et al., 2005) and wound healing (Leiper et al., 2006). In addition to immunological function, ERKs contribute early developmental stages. For instance, Saba and colleagues suggest that ERK2 is more important for trophoblast formation than ERK1 (Saba-El-Leil et al., 2003). ERK2 may also have a particular role in placental development (Hatano et al., 2003).

As with *Erk* KO mouse models, JNK gene deficiency is also difficult to study because some genes are essential for embryo development. In addition to knowledge about roles during development, JNKs have other biological functions. For instance, they are crucial for T cell development and responding to environmental stress in T cells (Nishina et al., 1997a, Nishina et al., 1997b) and for HSC development (Sasaki et al., 2001). JNK1 and 2 are required for IFN-γ production by T cells (Yang et al., 1998, Sabapathy et al., 2001). Furthermore, JNK signalling is required for not only immune functions but also other biological functions. For example, JNK signalling is critical for hepatoblast proliferation results in embryo lethality (Wada et al., 2004). Moreover, the over activated the JNK pathway contributes to heart failure (Petrich et al., 2004) and if it is controlled, it can prevent apoptosis and inflammation in the heart (Sadoshima et al., 2002).

1.3.3 MAPKs functions in the brain

Members of the p38 family take essential roles to regulate immune function as previously described. Indeed, they are also important for neuronal functions. Data from *in vitro* research show that p38s are required for neurite outgrowth (Morooka and Nishida, 1998). Also, illustrating the crosstalk between MAPK signalling pathways, p38 inhibition causes overexpression of ERKs. In astrocytes, p38s are involved in iNOS expression at the transcriptional level, whereas ERKs are not (Da Silva et al., 1997). These observations suggest that p38s are important for only brain cell physiology and closely link with the ERK pathway, depending on the environment.

The ERK pathway is involved in multiple functions in a body. Indeed, different biological functional differences for each isoform have been suggested. In the CNS, moreover, this pathway is necessary for learning and memory (Sun and Nan, 2017), although Mazzucchelli and others report that ERK1 deficiency enhanced striatum-dependent long-term memory (Mazzucchelli et al., 2002), and interestingly, ERK1 deficits may be compensated by upregulation of ERK2 signalling. In addition, unlike EKR2$^{-/-}$ mice, ERK1 null mice show hyperactivity in the open field test even though they do not show any developmental abnormalities (Selcher et al., 2001). These observations indicate that the complexity of the regulatory roles of the ERK pathway.

Depending on the types of tissues and cells, JNK isoforms can be expressed differently (Coffey et al., 2000, Davis, 2000, Hidding et al., 2002). Coffey and other researchers (Coffey et al., 2000, Coffey, 2014) report that neurons (primary and cell lines) express not only JNKs but also the corresponding MAPKKs, e.g. MKK4 and MKK7. Moreover, they describe effects on neural function and brain development due to JNK disruption.

Each of the MAPK families has been implicated in synaptic plasticity – a key process for learning and memory processes. Most forms of plasticity are sensitive to inhibition of ERKs (Sweatt, 2004, Maria Grazia Giovannini, 2006). JNK and p38 are involved in the suppression of hippocampal long-term potentiation (LTP) by LPS or IL-1β (Kelly et al., 2001, Kelly et al., 2003), and JNK and p38 mediate the suppressive effects of amyloid beta peptides (Minogue et al., 2003, Costello and Herron, 2004, Wang et al., 2004, Minogue et al., 2007). For instance, p38 mediates aspects of

metabotropic glutamate receptor (mGluR)-induced long-term depression (LTD) in the hippocampus (Moult et al., 2008).

Similarly, some microglial immune functions are mediated through the JNK pathway (Waetzig et al., 2005). Correspondingly, Hidding and colleagues propose that microglia produce all three isoforms of JNKs and they are highly involved in immunological responses (Hidding et al., 2002). Compared to untreated microglia, LPS treated microglia have larger amounts of phosphorylated JNK and c-JUN, and the release of pro-inflammatory cytokines is decreased by indirect JNK inhibition. Taken together, the JNK signalling pathway seems to be clearly involved in cytokine responses after immune challenges. The MAP kinases, JNK and p38 in particular, are also involved in microglial structural changes (Nakamichi et al., 2007). The evidence shows that microglial function is regulated via morphological changes; besides, the JNK pathway is essential for this process. Although microglia have a very dynamic morphology that may be partially independent of their activation status, morphological changes are still essentially connected to cellular activity changes.

Besides, uncontrolled JNK pathway activity can be a cause of diseases, for instance neurodegenerative disease, stroke, and epilepsy. One of the pathways in the brain is the apoptosis pathway (Huntwork-Rodriguez et al., 2013) and this pathway is thought as a cause of neurodegenerative diseases when it goes wrong (Antoniou et al., 2011, Ahmed et al., 2020). With regard to psychiatric disease, MKK7, which lies upstream of JNKs, is potentially involved in the development of schizophrenia via genetic association, and via altered protein phosphorylation (Funk et al., 2012, Winchester et al., 2012, McGuire et al., 2017). In addition to pathological findings, recently, MKK7 is one of the genes significantly affected by the presence of macrophages to suppress microglial inflammation (Greenhalgh et al., 2018). This indicates that the JNK pathway is important for modulating functions of microglia and macrophages following pathological events.

1.4 The maternal-foetal interface

As described above, many studies support the concept that malfunctional inflammation in the brain early in development is strongly related to a cause of psychiatric disorders. MIA is a key risk factor, therefore understanding its biological mechanism becomes important (Brown, 2011, Bergdolt and Dunaevsky, 2019, al-Haddad et al., 2019b). Many papers have shown that induced maternal (immune) stress disturbs embryonic peripheral immune system development; furthermore the foetal immune system is essential for CNS development (Schonkeren et al., 2011, Babenko et al., 2015, Filiano et al., 2015, Svensson-Arvelund and Ernerudh, 2015, Mori et al., 2016). In this section, details about how maternal changes can affect the foetus's development, and how these impacts may increase the risk of schizophrenia in later life.

1.4.1 Placenta

The placenta is structured by trophoblast cells, the primary cell types of the placenta, and other cell types (fibroblasts, immune and vascular cells), and the barrier allows the exchange of oxygen, nutrients, and waste products between a mother and a foetus (Rossant and Cross, 2001). Interestingly, placental development and gene expression is affected by foetal sex and birth weight ratios (Sood et al., 2006, Watkins et al., 2015). Although this physical barrier has many essential roles, one of the main purposes is to protect the foetus from the mother's immune system, thus preventing rejection (Hsiao and Patterson, 2012, Sailasree et al., 2017). Under normal physiological conditions, the placenta secretes anti-inflammatory molecules with unique immunological aspects throughout pregnancy (Mor et al., 2017). In the event of infections or stress, placenta inflammation is initiated as well as maternal systematic inflammation, and this is associated with increased risk of obesity and gestational diabetes (Challier et al., 2008). Placental inflammation disrupts its functions, including nutrient transport (Howerton and Bale, 2012). Equally, induced levels of CCL5 (indicating a level of inflammation) affect placental development (Bae et al., 2020). All these findings suggest that placental development and function are very complex and depend upon the link between mother and baby.

Interrupted placental function, via maternal systemic or placental inflammation, therefore affects foetal development, increasing risk for multiple diseases (e.g. metabolic or psychiatric) diseases in the future for the offspring.

1.4.2 Three possible ways whereby MIA may affect the foetus

MIA can significantly affect foetal development, resulting in increased risk for the offspring developing psychiatric disease later in life (see section 1.1). Indeed, the recent pandemic situation raises lots of concerns, especially about how to deal with viral infections during pregnancy; unfortunately most of them are unresolved (Dashraath et al., 2020). For example, maternal COVID-19 infection is similar to H1N1 influenza infection (Cavalcante et al., 2020) and one of the most common symptoms in severe patients, the cytokine storm, could harmfully impact on the baby. Understanding how MIA affects foetal brain development would help not only understand the causes of schizophrenia, but also help predict the long-term consequences of the current situation.

Several hypotheses can be proposed as to how MIA affects the embryonic brain, which is generally considered as an environment protected by the placenta (summarised in Figure 2).

One potential mechanism is that the pathogen acts directly on the foetus. When a mother is infected by bacteria or viruses, these pathogens may cross the placenta and reach the foetal body and the brain, and as a result they can induce immune activation or disrupt immune system development in a foetus. There is evidence that many pathogens can pass through the placenta (also known as vertical transmission), such as Hepatitis C virus (HCV) (Ward and Holtzman, 2018),

human immunodeficiency virus (HIV) (del Rio and Curran, 2015), Rubella (Santis et al., 2006, Arora et al., 2017) and *Toxoplasma gondii* (Robbins et al., 2012, Ville and Leruez, 2015). During the recent pandemic, vertical transmission becomes a serious issue. Kotlyar and colleagues suggested that most SARS-COV-2 infections happening in late pregnancy are negative for virus presence in neonates, but they point out that early pregnancy infection could increase risk of vertical transmission based on medical case reports (Kotlyar et al., 2021). Additionally, clinical evidence suggests transplacental transmission of SARS-COV-2 during late pregnancy can happen (Vivanti et al., 2020). Nonetheless, conflicting evidence is also reported (Shi et al., 2005, Ashdown et al., 2006, Racicot et al., 2017, Karimi-Zarchi et al., 2020). Therefore, this hypothesis is almost certainly pathogen-dependent, and has to be studied more.

Another potential mechanism involves direct effects of maternal immune molecules. Maternal infection increases the levels of cytokines and chemokines, particularly pro-inflammatory cytokines and chemokines, in maternal serum, e.g. IL-6, TNF-α, and CXCL8 (Brown et al., 2004b, Meyer et al., 2009b, Brown and Derkits, 2010, Selten et al., 2010, Garay et al., 2013, Scola and Duong, 2017), and these maternally-derived molecules may impact on neuroimmunological and neurodevelopmental processes in the foetal brain (Gilmore and Fredrik Jarskog, 1997, Boksa, 2010, Ellman et al., 2010, Fineberg and Ellman, 2013, Filiano et al., 2015). Consistently, these observations have been demonstrated through animal studies. Additionally, at least in the case of IL-6, these maternal immune molecules could actively transport to the foetus from maternal blood rather than from placental production (Zaretsky et al., 2004). The evidence from rat shows that maternal IL-6 penetrates into the foetal compartment has seen (Dahlgren et al., 2006). Moreover, because this permeability is gestation dependent, the early period of pregnancy allows more cytokines across the foetus than the late period (Dahlgren et al., 2006). The resulting cytokines in foetal circulation can then alter the foetal immune system development. It is commonly known that the foetal BBB is more delicate and immature compared to adults, making it easy for these cytokines to then cross into the foetal brain (Saunders et al., 2012). For instance, peripheral inflammation (induced by LPS) caused loosen the BBB, which means peripheral inflammatory factors infiltrate and promote inappropriate neuroinflammation (Jaeger et al., 2009, Takeda et al., 2013).

The last potential mechanism is that the key effects on the foetus are due to effects of immune molecules released from the placenta. Usually, trophoblasts work as a barrier between a mother and a foetus, and they selectively allow molecules and nutrition to cross the cell barrier. When MIA stimulates the trophoblasts, they are likely to secrete cytokines and chemokines toward the foetus, resulting in altered foetal microglial activation and sensitive (or primed) immunity. The possibility that placentally-derived immune molecules mediate increased schizophrenia risk in offspring is supported by clear evidence that preeclampsia (inflammation of the placenta) is associated with elevated disease risk (Mary Cannon et al., 2002, Byrne et al., 2007, Dachew et al., 2018). Experimental evidence has suggested that the secretion of immune mediators by the placenta would be selective for specific chemokines and cytokines. There is evidence from rodent models that many of the effects of MIA on the foetus (i.e. brain IL-6 and Cxcl10 induction) are mediated by

maternal IL-6 acting on the placenta, which ten produces secondary, as yet uncharacterised mediators that act on the foetus (Hsiao and Patterson, 2012, Wu et al., 2017). In mice, IL-17α mRNA expression in placenta was strongly induced by maternal poly I:C injection (an effect apparently dependent on maternal IL-6) (Choi et al., 2016). Besides, pretreating the dams with IL-17α antibodies seemed to prevent the abnormal brain gene expression and autism-related behavioural phenotypes induced in the offspring by the MIA, suggesting that IL-17α might be a placentally-derived mediator of MIA effects on the foetus, although further independent studies are needed to confirm the central role of placental IL-17α in MIA.

The three suggestions are very simplified; thus MIA could affect offspring development via more complicated systems.

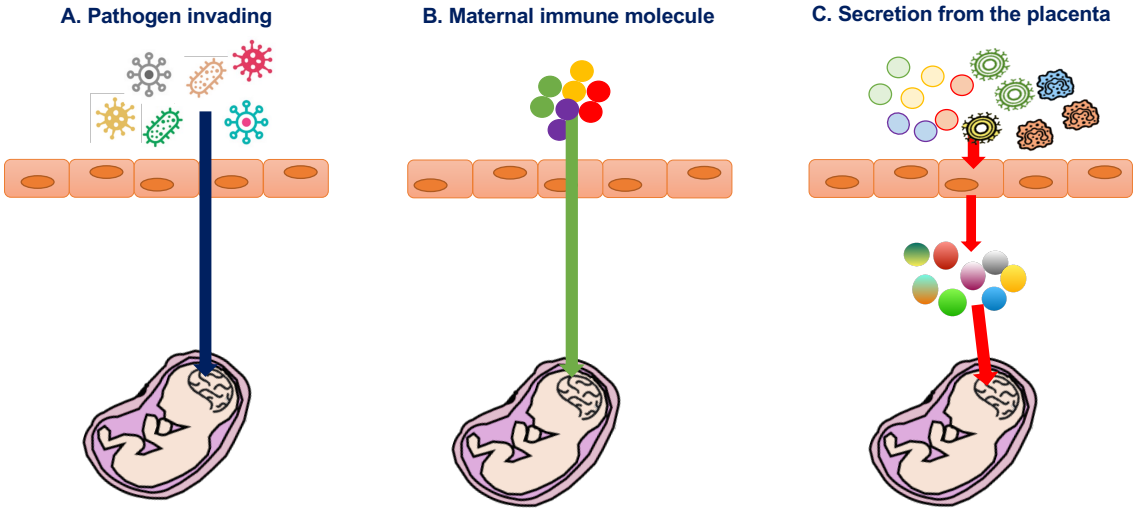

Figure 2. Three possible maternal-foetal interference. The simplified diagram of how maternal immune activation (MIA) can affect the foetus brain development in three different ways. **(A)** Infectious agents or pathogens invade the foetal compartment. **(B)** Maternal immune molecules pass through the placenta and affect the foetus. **(C)** Either Placental inflammation secretes immune molecules to the foetal side and these interrupts the developmental process.

1.5 Aims of the project

From the current understanding, the hypothesis that MIA could enhance the risk of psychiatric diseases has been established. Aetiological and epidemiological studies support this idea, but the precise mechanisms are unclear, and further studies at molecular levels are required. When MIA activates the maternal immune system, the innate immune system cells produce signalling molecules, for instance cytokines and chemokines, to trigger the adaptive immune system. At this moment, the altered microenvironment in a mother may affect foetal brain development directly or indirectly. Since JNKs are implicated in many of these effects, the role of the JNK pathway in TLR-induced immunity and production of inflammatory molecules is relevant in relation to schizophrenia.

Thus, the central hypotheses are as follows:

1. Resiquimod (TLR7/8) will induces a different microglial immune reaction (cytokine and chemokine production) via the MAPK pathway compared to poly I:C (TLR3) and LPS (TLR4) and that this will involve differential actions via the MAPK pathway.

    a. That the MAPK pathways have roles in chemokine and cytokine production in the innate immune response, and that these will differ according to the nature of the immune stimulus. Considering the evidence specifically implicating prenatal single-stranded virus infection in schizophrenia risk, the two prototypical immune stimuli used in neuroscience research - LPS, mimicking bacterial infection, and poly I:C mimicking infection with double-stranded viruses, will be compared with a less well-characterised stimulus, resiquimod, to mimic single-stranded virus infection.

2. Resiquimod (TLR7/8) will induce a different pattern of maternal immune activation as compared to poly I:C (TLR3).

    a. That the nature of the immune stimulus to which the mother is exposed during pregnancy affects not only maternal aspects of the innate immune response, but also the response in foetal brain tissues. The prototypical agent used in MIA studies in mice (poly I:C) will be compared with resiquimod, for induction of cytokines and chemokines, in both maternal and foetal tissues.

To test these hypotheses, various research techniques, mainly Western blot, RT-qPCR, ELISA, IHC are used. For *in vitro* tests, primary neurons and microglia (mouse SIM-A9 cell line) are used to see how these cells react with stimuli and immune mediators. Besides, specific MAPK inhibitors are introduced to test their importance after immune mimetic exposure.

For *in vivo* studies, poly I:C or resiquimod are administered to pregnant mice at E12.5, and tissues studied *ex vivo* 4h post-administration

The specific aims of the experimental studies were :

1. To assess the extent of MAPK pathway responses to different immune challenges(poly I:C, LPS, resiquimod, mimicking infection with dsRNA viruses, bacteria and ssRNA viruses respectively) in primary cortical neurons, and also to assess the effects of downstream immune mediators (Cxcl10, Cxcl12) (Chapter 3).

2. To assess the extent of MAPK pathway responses to different immune challenges (poly I:C, LPS, resiquimod) in microglial (SIM-A9) cells, and the role of the MAPK pathways in the production of cytokines and chemokines (Chapter 4).

3. To compare the prenatal immune responses (chemokine and cytokine induction) in maternal blood, placenta and foetal brain to maternal administration of 2 different immune challenges (poly I:C, resiquimod) (Chapter 5).

Chapter2

Material and Methods

Chapter 2 Material and Methods

2.1 Statistical analysis

All statistical analysis was performed in Minitab (Minitab® 19 Statistical Software) and graph making was performed in GraphPad 9 (GraphPad Software, La Jolla California USA). Whenever the data were not normally distributed, the data were log transformed or Box-Cox transformed, and analysis of variance (ANOVA) with post hoc Tukey or Bonferroni multiple tests was conducted. Details of ANOVA F values and p values are provided in Appendix9 (Table 9-Table 11)

2.2 Cell culture methods

2.2.1 Cell line culture media

The media and supplements were required differently depending on the cell lines. In Table 3 are summarised the details of media and supplements of each cell line. More details of each supplements will be mentioned in appropriate sections below.

Table 3. The cell culture medium and supplements

Main cell culture medium				
Cell line (species)	Purpose	Medium	Company (Cat. No.)	Supplements (final concentration)
NG108 (mouse)	Growth	DMEM	Gibco (21885-025)	Serum (10%) P/S (1%)
	Differentiate	DMEM	Gibco (21885-025)	Serum (0.5%) P/S (1%)
SIM-A9 (mouse)	Maintenance	DMEM:F12	Gibco (302006) 11320033	Serum (5%) P/S (1%)
	Experiment	DMEM:F12	Gibco (302006)	P/S (1%)
Primary neuron (mouse)	Maintenance	Neurobasal	Gibco (21103-049)	B27 P/S(1%) Glutamax (1%)
Bone marrow-derived Macrophage (BMDM) (mouse)	Maintenance/ Experiment	G-MEM	Gibco (11710-035)	Serum (10%) L929 conditioned media (15%) P/S (1%) Glutamax (1%) Non-essential amino acid (1%) Sodium pyruvate (1%) β-merceptoethanol (0.1 mM)
Neonatal microglia	Culture	DMEM	Gibco11995-065	Serum (10%) P/S (1%)

(mouse)	Enrich	DMEM	Gibco11995-065	Serum (10%) P/S (1%) GM-CSF (5ng/ml)
Additional medium				
Purpose	Cell line	Medium	Company (Cat. No.)	Supplements (final concentration)
Thawing medium	NG-108	DMEM	Gibco (21885-025)	Serum (10%) + P/S (1%)
	SIM-A9	DMEM:F12	Gibco (302006)	Serum (15%) + P/S (1%)
Freezing medium	Any	RecoveryTM-cell culture freezing medium	Gibco (12648-010)	0% or 10% serum
	BMDM	Horse serum	Gibco (26050-088)	10% DMSO

*p/s: Penicillin-Streptomycin

2.2.2 Thawing cells from frozen stocks

The cells (SIM-A9, purchased from ATCC, CRL3265) or frozen cell stocks were thawed from freezing condition by putting them into a 37°C water bath for 1 minute. The cells were moved to a centrifuge tube. The prepared medium was dropped carefully into the tube until the final volume reached to 6-7 ml. The cells were centrifuged (200g, 5minutes, 4°C). The supernatant was removed and the pellet was mixed with the fresh media for each cell line. The cells were split into two culture flasks. Required volume of fresh media was added and the cells grew in 5% CO_2, at 37°C for 2-3 days. The cells were observed under inverted microscope every day. Once the cells fully recovered and then started to fill media, the cells were passaged.

2.2.3 Cryopreservation of the cells

The cells and media were collected and then centrifuged (200g, 5 minutes, 4°C). The pellets were re-suspended in freezing media with required supplements (Table 3). 1 ml of aliquots were stored in a freezer (-80°C) for a short term storage or a liquid nitrogen tank (-180°C) for a long term storage.

2.2.4 Cell line maintenance *in vitro*

2.2.4.1 Passage of adherent cells (NG-108)

Media were discarded and Trypsin/EDTA (Gibco 25300-054) solution was added to detach cells (3ml for 25 cm^2 flask). After incubation for 30 seconds to 1 minute at room temperate, the cells were observed using an inverted microscope to ensure detachment. If cells were not completely detached after 1 minute, the cells were re-suspended by gentle pipetting. Trypsinisation was stopped by the addition of serum containing media (equal volume of trypsin/EDTA solution). The

suspension was centrifuged (405g, 5 minutes, 4°C) and supernatant was discarded. The pellet was re-suspended in an appropriate volume of fresh culture media contained 5% horse serum (Gibco 26050-088) and transferred to a new flask. Normally, cells were diluted between 1:20 and 1:40. Cells were regularly passaged every 3-4 days.

2.2.4.2 Passage of semi-adherent cells (SIM-A9)

Media were collected in a 15ml centrifuge tube and trypsin/EDTA was added for detachment (3ml for 25 cm^2 flask, less than 1 minute in room temperature). Gentle pipetting was needed during incubation time. Detachment of the cells examined using inverted microscope. The cells were collected and move to the centrifuge tube which had previous media. The suspension was centrifuged (200g, 5 minutes, 4°C) and the supernatant was removed. The cells were re-suspended in fresh culture media and transferred to a new flask. Approximately the cells were diluted between 1:100 and 1:200 every 4 days.

2.2.5 Primary neuronal cell culture

Mice were bred and maintained at the University of Glasgow. To collect embryonic brain tissues, pregnant mice were taken at 17-18 days after the plug date. Only cerebral cortex (including PFC) in embryonic brains was collected and transferred to ice-cold HBSS (Gibco 14175-053). The collected tissues were chopped in fine pieces. The brain tissues were washed by pre-chilled HPSS twice and transferred into a 15ml centrifuge tube which was pre-heated 3ml of trypsin/EDTA (Gibco 25300-054) for 10 mins. 3ml of DMEM (Gibco 21885-025) containing 1% Penicillin-Streptomycin (Sigma P0781) and 10 % horse serum (Gibco 26050-088) was added to inactive trypsin. The tissues were centrifuged (405g, 5 minutes, 4°C) and the supernatant was discarded. The tissues were re-suspended in 8ml of DMEM containing 10% horse serum (Gibco 26050-088) 1% Penicillin-Streptomycin (Sigma P0781) and triturated with fine tip Pasteur pipette. The medium was transferred into a 50ml centrifuge tube and the maintenance medium (Table 3) was added to give the required final volume (9ml for each embryo). The cells were seeded on a plate which had been pre-coated with 4µg/ml poly-D-Lysine (Sigma P6407) and 6µg/ml Laminin (Gibco 23017-015). The maintenance medium had to be pre-added to give 50% dilution of the cell suspension. After 24 hours, 50% of the medium was changed and further medium changes (50% of total medium in a well) were required every 3-4 days.

2.2.6 Bone marrow derived macrophage (BMDM) culture

Mice were maintained in the Central Research Facility (CRF) at the University of Glasgow, and were killed by a Schedule 1 method. To collect bone marrow, two hind legs were dissected, all surrounding tissues were removed as much as possible and the legs were kept in the ice-cold double concentration of antibiotic conditioned media or PBS. Collected legs' bones were kept in 70% ethanol for 30 seconds and then transferred into the GMEM (Gibco 11710-035) contained 10% Foetal bovine serum (FBS, Gibco10270106), 15% L929 cell containing media (kindly offered

from Dr. Robin Bartolini in Chemokine Research Group), 1% non-essential amino acid (Gibco, 11140-050), 1% Glutamax (Gibco 35050-061), 1% sodium pyruvate (Gibco, 11360-070), 0.1mM β-merceptoethanol (Gibco 31350-010), 1 %Penicillin-Streptomycin (Sigma P0781)(called conditioned medium below). On a clear plastic dish, the ends of the bones were cut, and the bone marrow was flushed using a 23G needle (Henke Sass wolf, 4710006030) with the conditioned medium. To make single cell suspension and remove unnecessary parts, the collected bone marrow containing medium was filtered (70μm, Greiner Bio-one, 542070). The dissection dish was washed by 5ml of the fresh conditioned medium and the medium was filtered by the 70μm filter. The cell suspension was centrifuged (300g, 5 minutes, room temperature). The supernatant was discarded, and the pellet was re-suspended in 1ml of ACK lysis buffer (Gibco A10492) in 1 minute to remove red blood cells. 9 ml of the conditioned medium was added to stop lysis, and this was centrifuged then (300g, 5 minutes, room temperature). The pellet was re-suspended in the required volume of the conditioned medium, depending on the number of the cells, and then suspended cells were cultured in a petri dish (10 million cells in a dish, 10ml of the conditioned medium in a dish). After 3 days, the conditioned medium was removed from the dish, followed by gentle shaking to remove all unattached cells which are monocytes and dendritic cells. 10ml of the medium was added on a dish. On day 5 or 6, the cells were detached by TrypLE (Gibco, A12177-01) and centrifuged (300g, 5 minutes, room temperature). The pellet was re-suspended in the conditioned medium to acquire 1 million cells/ml. The re-suspended cells were seeded on a culture plate depending on experimental conditions, and, after a few hours to sit the cells down, the medium was completely removed. The required volume of the new fresh conditioned medium for the experiment was then added.

2.2.7 Neonatal microglia culture

Mice were bred and maintained at the CRF. All mice had a C57Bl/6J genetic background, and were killed by a Schedule 1 method. To gain microglia, neonatal pups (P2-3) were used. Ice cold culture medium (Gibco 11995-065) containing 10% FBS (Gibco 10270106) and 1% Penicillin-Streptomycin (Sigma P0781) was prepared before the dissection. The litters were kept on ice while dissection was carried out. Brains were removed from the skulls and meninges removed as for as possible. Once unnecessary parts were all removed, the brains were kept in the ice-cold medium. Brains were chopped into small pieces, placed into fresh media (20ml for whole 4 brains), and aspirated vigorously to make a single cell suspension by a 10 ml serological pipette. The suspension was filtered through a filter (40μm, Greiner Bio-one, 542040) and centrifuged at 300g for 5 minutes in room temperature. The pellet was resuspended in a total volume of 40ml of the medium per T-175 flask (4 whole brains are maximum per flask). The cells were left for 1 week to settle down. On following week, the medium was discarded and an enrich medium containing 10% FBS (Gibco 10270106), 1% Penicillin-Streptomycin (Sigma P0781) and GM-CSF (final concentration 5ng/ml, R&D system 415-ML-005/CF) were added and left for a week. The microglia grew on top of the confluent layer and the astrocyte layer were bottom. 14 days since from the dissection was carried out, microglia were detached by gentle shaking in an orbital shaker incubator (37°C, overnight, 100 rev per min, NO CO_2). HEPES (final concentration 50mM) was

added prior to shaking off. Following overnight shaking, the supernatant was collected from the flask and centrifuged (300g, 10 minutes). The pellet was re-suspended in the medium containing 10% FBS (Gibco 10270106) and 1% Penicillin-Streptomycin (Sigma P0781) and plated (density: 3-5 x 10^5/ml). If the cells were plated on the glass coverslips, the coverslips should be coated with 10 μg/ml of poly-D-lysine and the coated coverslips could be stored at 4°C for a month. The cells were ready to use the next day.

2.3 *In vivo* studies

2.3.1 Inflammatory effects on mouse brain

WT mice (C57Bl/6J, wk 12-13) were mated from a breeding colony at the University of Strathclyde. The mice were housed under standard conditions. To induce the immune activation, resiquimod (Tocris 828; 1mg/kg in saline) was injected Intraperitoneally and saline was used as vehicle. The mice were culled after 24 hours. All animal procedures were conducted by Dr. Rebecca Openshaw under license and using UK Home Office regulations. The sections were used for immunohistochemistry (IHC) studies. Details of brains collections (section 2.3.2) and the technique (section 2.10) are described below.

2.3.2 Brain tissue dissections

To collect fresh frozen brains, mice were culled by cervical dislocation and the brains were collected within 1 minute of death. Brains were placed in 4% PFA overnight in fridge and then placed into 30% of sucrose. After immersion fixation, the brains were snap-frozen and stored at -80°C until further used. Brains were then sectioned (10μm thickness) in a cryostat. The first area (Bregma 1.34~0.62mm) containing the medial prefrontal cortex (mPFC) and the second area (Bregma -0.82~-2.18mm) containing the hippocampus and thalamus were collected. Before storage at -20°C, the sections were dried at room temperature at least two hours after the sectioning.

2.4 Cell stimulation

For experiments, the cells were grown in specific media. The cells were seeded on culture plates and left until they differentiated enough to be used. The cells were stimulated and incubated in the required condition in the culture incubator (37°C, 5% CO_2) unless specific conditions are otherwise mentioned. Cell growing time and specific conditions of stimulation are detailed in Table 4.

Table 4. Experimental conditions

Experimental conditions of the cells		
Cell line	**Growing time**	**Medium change**

NG 108	5 days	Not required		
SIM-A9	1 day	Not required		
Primary neuron	14 days	Every 3-4 days		
BMDM	5-6 days	Day 3 and 5		
Stimulation condition				
Stimulus	**Company**	**Final Concentration**	**Solvent**	**Incubation time**
LPS	Sigma (L-8274)	50ng/ml	dH_2O	15 minutes
Poly I:C	Invivogen (LMW, tlrl-picw)	100ng/ml	dH_2O	
Resiquimod	Tocris (R848)	3μM	DMSO	
CXCL10	R&D systems (466-CR)	10nM	0.1% BSA	
CXCL12	R&D systems (460-SD)	10nM	0.1% BSA	
Inhibition condition				
Inhibitor (Target)	**Company**	**Final Concentration**	**Solvent**	**Incubation time**
5Z-7 (TAK1)	Tocris	300nM	dH_2O	30 minutes
NQDI (ASK1)	Tocris	30μM	DMSO	
JNK-IN-8 (JNK)	Sigma (SML1246-5mg)	1μM	DMSO	3 hours
SP600125 (JNK)	Enzo	5μM	DMSO	30 minutes
PD98059 (ERK)	Calbiochem (513000)	40μM	DMSO	30 minutes
SB203580 (p38)	Calbiochem (559389)	5μM	DMSO	30 minutes
BMS345541 (IκK)	CAY16667-1mg	10μM	PBS	1 hour

The experimental conditions used in this research are chosen based either on evidence from previous publications or on previous findings in the lab.

The poly I:C concentration used in here was reported in other *in vitro* studies to be sufficient to activate p38 and JNK signalling in microglial culture (rat) (de Oliveira et al., 2016) and to induce

inflammation (enhancement of cytokine release in microglia) (Remels et al., 1990, Ribes et al., 2010, Peltier et al., 2010, Lehmann et al., 2012). The LPS concentration used by other groups varies considerably from 5 ng/ml to 10μg/ml to induce inflammation in microglia (Lehnardt et al., 2003, Kremlev et al., 2004, Le et al., 2004, Jack et al., 2005, Horvath et al., 2008); thus, the concentration used here is towards the lower end of that range, but still sufficient to induce inflammatory reaction in microglia, according to published research (Waetzig et al., 2005, Huang et al., 2015). Similarly, the concentration of resiquimod used was selected towards the lower end of the range used by other groups, but still reportedly sufficient to produce a clear immune response in microglia or macrophages (Michaelis et al., 2019, Adzavon et al., 2017). CXLCL10 and CXCL12 conditions were used according to the supplying company's recommendation.

There is also some variation in the literature concerning the appropriate concentrations to be used for the kinase inhibitors. However, for these agents, the appropriate concentration to achieve inhibition without loss of selectivity can be estimated from published IC50 values (ERK inhibitor; PD98058 (Alessi et al., 1995, Dudley et al., 1995), p38 inhibitor; SB203580 (Lali et al., 2000), JNK inhibitor; SP600125 (Bennett et al., 2001, Bain et al., 2007), JNK inhibitor; JNK-IN-8 (Zhang et al., 2012), ASK1 inhibitor; NQDI (Volynets et al., 2011), TAK1 inhibitor; 5Z-7 (Ninomiya-Tsuji et al., 2003)), and this was the strategy employed here.

2.5 Western blot

2.5.1 Protein extraction from cultured cells and Bradford assay

After stimulation, the plates were placed on ice and media were discarded. Each well was washed by ice-cold PBS (100μl for 12 well plate, 200μl for 6 well plate) for a few seconds, and ice-cold lysis buffer (with added protease inhibitor cocktail (Sigma, P8340) was added to cells (80μl for 12 well plate, 160μl for 6 well plate). The cells were left between 30 minutes and 1 hour on ice. The lysate was collected from the plate using a pipette and transferred to a 1.5ml microcentrifuge tube. Samples were centrifuged (Sigma laboratory centrifuge 2K15, 10621g, 10 minutes 4°C). Pellets were discarded and supernatant was stored at -20°C.

To measure protein concentration, the Bradford assay was used. 6 standard concentration samples were serially diluted from BSA stock (0.02mg/ml). Bradford assay reagent (Bio-RAD, Cat.No. 500-0006) was diluted (1:5) by distilled water and extracted protein from the cells was diluted (1:100) by 400μl of distilled water. 200μl of diluted Bradford assay reagent was mixed with 400μl of prepared samples and 200μl of mixtures was load in duplicate onto a 96 well plate. The plate was read by a spectrometer (Multiskan® Spectrum; Thermo ScientificTM, 450nm).

2.5.2 Electrophoresis

Proteins were mixed with the 4x sample buffer (NuPAGE®, Invitrogen, NP0007) and the reducing agent (NuPAGE®, Invitrogen, 1769410) and heated on a heating block (80°C, 10 minutes). 20-25μl of preheated samples and 10μl of protein ladder (Bio-Rad, 161-0375) were loaded onto a pre-cast gel (10% Bis-Tris gel, NuPAGE®, Invitrogen), in an electrophoresis tank (xCell sureLock™, Invitrogen) filled with diluted NuPAGE running buffer (1:20). 500μl of Antioxidant (NuPage™, Invitrogen, NP0005) was added. Electrophoresis was run for 90-100 minutes at 150V until sufficient separation had been achieved (as assessed from the ladder).

2.5.3 Western blotting

The protein on the gels was transferred onto a pre-methanol/diluted NuPAGE transfer buffer soaked PVDF membrane (0.45μm pore size, Novex, LC2005) at 30V for 1 hour. The transferred membrane was washed twice by distilled water and blocked in 5% milk/TTBS for 30 minutes at room temperature. After blocking, the membrane was incubated with primary antibody overnight at 4°C with gentle shaking. If primary antibodies are mixed with signal booster (Calbiochem, 407207) or 1% TTBS/milk, the membrane had to be washed several times to remove blocking solution completely. Following primary antibody incubation, the membrane was wash 3 times for 10 minutes each in TTBS and incubated with secondary antibody for 2 hours at room temperature. Blots were developed via ECL (Milipore, WBKLS0100; CST, 6883S). The images of the membrane were captured by a gel imager (Syngene PXi).

Table 5. Western blot antibody details

Primary antibodies						
Target protein	Concentration	Company	Cat. No.	Base solution	Incubation time	Incubation temperature
pJNK	1:10000	Abcam	ab76572	Signalboost	Overnight	4°C
pERK	1:5000	CST	4377	1% milk/TTBS	Overnight	4°C
pp38	1:4000	CST	4511	Signalboost	Overnight	4°C
pTAK1	1:14000	CST	4508	Signalboost	Overnight	4°C
pASK1	1:4000	GTX	50229	Signlaboost	Overnight	4°C
GAPDH	1:20000	GTX	GTX627408	1% milk/TTBS	1 hour	Room temperature
ß-Actin	1:20000	Sigma	A3854	1% milk/TTBS	1 hour	Room temperature
Secondary antibodies						
Anti-Rabbit	1:10000	Merck	12-348	1% milk/TTBS	2 hour	Room temperature

All steps from 2.5.1 to 2.5.3 were replicated with light modifications in Chapter 3 and Chapter 4 to measure level changes of phospho-MAPKs. A list of all used antibodies and suppliers are detailed in Table 5.

2.6 Morphological analysis

The cells were seeded on sterile coverslips and left for 24 hours. The cells were then stimulated by LPS or vehicle for 24 or 8 hours. The media was removed, the coverslips were fixed by 4 % PFA for 10 minutes on ice. After fixation, the coverslips (22 x 50 mm, MENZEL-GLASER) were gently washed by PBS 3 times for 3 minutes. 0.2% Cresyl violet solution (40 µl for 24 well plates) was added and left for 20-30 minutes in room temperature. The coverslips were taken from the plate and washed in dH_2O, 70% EtOH, 90% EtOH, 100% EtOH for a few seconds. During washing steps, staining colour had to be constantly observed under a bright microscope to avoid the loss of too much stain. After 100% EtOH washing, the coverslips were left for approximately one minute to complete drying. After drying, the coverslips were immersed in Histoclear for 10 minutes and put on histomount onto microscope slides. The coverslips had to be allowed to dry overnight before taking images.

2.7 RT-qPCR

2.7.1 RNA extraction

RNA can be extracted from cells or tissue directly after treatment or the sample can be stored at -80°C for 6 months for later extraction using RNAlater (Invitrogen, AM7020) treatment. Under RNase-free conditions, RNA was extracted using an RNeasy mini kit (Qiagen, 74104) with additional DNase I (Qiagen, 79254) as per manufacturer's instruction. Briefly, if a sample was stored in RNAlater, it has to be removed by high speed centrifugation after mixing with 5 times higher volume of PBS to reduce its density. If a sample was used directly after stimulation, lysis buffer (1ml of RLT buffer with 10µl of β-merceptoethanol) was added on each well (350µl for 12 well plate) and the plate was left for 5 minutes to lyse cells at room temperature. 70% ethanol (the same volume as the lysis buffer) was added and gently mixed with pipetting. The mixture was then transferred to a spin column. When RNA was extracted from RNAlater-stored frozen tissue samples, the samples were thawed at room temperature and RNA later debris removed as much as possible. If tissue was big, it was chopped into smaller pieces (max, 20mg). In a 2ml RNase-free tube, one steel bead (5mm steel beads, Qiagen, 69989) and prepared tissues samples were mixed with 700µl of lysis buffer. The tubes were loaded on Tissuelyser LT (Qiagen) and set for 50 oscillation for 10 minutes. To remove any insoluble material, only the lysates was transferred to a new 1.5ml RNase free tube and centrifuged for 10 minutes at max speed. Only supernatant was taken and equal volume of 70% of ethanol was added and gently mixed. The mixture was transferred to a spin column. The mixture of 70% ethanol with cell or tissue lysate loaded spin column was spun down for 15 seconds at 13,000g, flowthrough was discarded, and washed once with RW1 buffer. To inhibit DNases, DNase I was added on a column membrane directly and the

column was incubated for 15 minutes at room temperature. After a series of washes were completed and the column dried, Nuclease free water (Qiagen 129115; 50µl for cell lysates, 200µl of tissue samples) was added to elute RNA. RNA quality was confirmed before cDNA synthesis using a Nanodrop (DeNovix DS-11+Spectrophotometer). If A260/280 is between 1.8 and 2.2, the RNA was considered as "pure enough" for cDNA synthesis.

2.7.2 cDNA synthesis

Under RNase-free conditions, cDNA was synthesised using a High-capacity RNA-to-cDNA™ kit (Applied Biosystems, 4387406) as per manufacture's instruction. Briefly, the extracted RNA samples were normalised to a maximum concentration of 2000ng/20ul reaction volume, or the maximum possible amount from the sample with the lowest concentration. To make a master mix, the required volumes of 2X RT buffer and 20X RT enzyme mix were mixed depending of the number of samples. Appropriate volumes of normalised RNA and RNase free water (total volume is 9µl) were mixed with 11µl of the master mix (20µl overall reaction volume). –RT, omission of reverse transcriptase, was also generated with one of the RNA samples to confirm lack of genomic DNA (gDNA) contamination. The tubes were quickly spun down and kept on ice until a machine was ready to start the reverse transcription reaction. The PCR machine (AB Applied Biosystems, Veriti™ 96-Well Thermal Cycler) was set for the thermal cycles (incubation; 37°C for 1 hour, stop reaction; 95°C for 5 minutes, holding; 4°C for infinite). The generated cDNA was diluted with RNase free water (1:5) and stored at -20°C for short-term storage or -80°C for long-term storage.

2.7.3 Primer design

Primers were designed using the software, Primer3 (Untergasser et al., 2012). Details of conditions for designing primers were described below.

- Size: 18 - 23 base-pair (bp) (20 bp optimal)
- GC content: 40-65% (50% optimal)
- Annealing temperature (Tm): 59.5 - 61°C (60°C optimal)
- Amplicon size: 50 – 150 bp
- Max self-complementarity: 2
- Max 3' self-complementarity: 1
- No stretches of G or C >4

Furthermore, if it is possible, primers were designed to be intron-spanning, to avoid producing amplicons from gDNA, using sequence information from NCBI reference sequences.

Specificity of primers were examined by using Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for species Mus Musculus (taxid 10090). Standard (STD) primers were designed using the same software, Primer3, according to the following conditions.

- Adding 20 bp at both the 3' and 5' ends. Regions have to include where primers amplify.
- Size of amplicon was extended to 100 – 1000 bp

STD primers were checked their specificity by Primer blast as per the above specification. Details of all used primers were mentioned in Table 6.

Table 6. Targets and their standards sequences

Target	Forward primers	Reverse primers	STD forward primers	STD reverse primers
Tlr3	CAG GCG TCC TTG GAC TTG AA	TAG CTT GCT GAA CTG CGT GA		
Tlr4	GCT TGA ATC CCT GCA TAG AGG TAG	TGT CAT CAG GGA CTT TGC TGA G		
Tlr7	AGA AAG ATG TCC TTG GCT CCC	CCG TGT CCA CAT CGA AAA CAC		
Tlr8	TCT GGT CCA GCT ATA GAG CAC A	AAT CCA TGA CTG AGG GGG CA		
Il-6	CGG CCT TCC CTA CTT CAC AA	TCA TTT CCA CGA TTT CCC AGA GA	GGA GCC CAC CAA GAA CGA T	TGG TCC TTA GCC ACT CCT TCT
Il-10	CAG AGA AGC ATG GCC CAG AA	GCT CCA CTG CCT TGC TCT TA	TGC TAA CCG ACT CCT TAA TGC A	GGC CTT GTA GAC ACC TTG GT
Tnf-α	CAC CAC CAT CAA GGA CTC AA	GAG GCA ACC TGA CCA CTC TC	TCT GTG AAG GGA ATG GGT GT	GGC TGG CTC TGT GAG GAA
Ccl2	CTC ACC TGC TGC TAC TCA TTC A	CCA TTC CTT CTT GGG GTC A	CAC CAG CAC CAG CCA ACT	GCA TCA CAG TCC GAG TCA CA
Ccl5	CTG CTG CTT TGC CTA CCT CT	ACA CAC TTG GCG GTT CCT T	CCC TCA CCA TCA TCC TCA CT	TCA GAA TCA AGA GGC CCT CTA TCC
Ccl11	GCT CAC CCA GGC TCC ATC	TCT CTT TGC CCA ACC TGG TC	CCA CCC ACT CTG CTC CCT AT	GGC ATC CTG GAC CCA CTT C
Cxcl1	TGC ACC CAA ACC GAA GTC AT	TGG GGA CAC CTT TTA GCA TCT	ACA CTC CAA CAC AGC ACC AT	AAA CAC AGC CTC CCA CAC AT
Cxcl10	GCT CAA GTG GCT GGG ATG	GAG GAC AAG GAG GGT GTG G	CGA TGG ATG GAC AGC AGA GAG CCT	GAC AAG GAG GGT GTG GGG AGC A
Cxcl12	TGC ATC AGT GAC GGT AAA CCA	GCG ATG TGG CTC TCG AAG AA	GTC CTC TTG CTG TCC AGC TCT	CTT CAG CCG TGC AAC AAT CTG

Ccr2	CTT GGA ATG ACA CAC TGC TGC	AGC TCA CTC GAT CTG CTG TC	CCA CAC CCT GTT TCG CTG TA	GCC TCA TGC CCT CCT TTC TT
Cx3cr1	GCT CAC GAC TGC CTT CTT CT	TGC ACT GTC CGG TTG TTC AT	CGT TCG GTC TGG TGG GAA AT	CCC ATC TCC CTC GCT TGT G
Ly6c2	ACC CGT CAG TGC CTT TCT TT	CAG TGG GAA CTG CTG CAT TG	GCC TGC AAC CTT GTC TGA GA	TGG ACT CAA CAG GGG TCA TTG
Tmem119	TGC ACC CCA GGA AAC ATC TC	AGT GGT GCG TTA GGG TGA AG	GAG GGA GCA AAG CCT GTG AA	GCA GAG TGA CAG CGA CAT TG
Tspo	CCA TCT GGG GCA CAC TGT ATT	GAA ACC TCC CAG CTC TTT CCA	GCG GGG CTG TGG ATC TTT	GGC ACT GAC CAC CCT AAC C
Bdnf	CAC TCC ACT GCC CAT GAT GT	GGA GGA GGG AGG GAA AGA GT	TCA AGG TGC TGT TGT CAT TGC	TCC CCA CCT CCA TCC TAG AC
Xist	TCC TCG GTT CCA CAC ATT GC	AGA GCA TCC CTC TGC TTT CC	GGC ATG CTT GGT AGA GTG GA	GGA GCC AAG TGA AGG CCT AA
Gapdh	AAT GTG TCC GTC GTG GAT CT	AGA CAA CCT GGT CCT CAG TG	GCA TTG TGG AAG GGC TCA TG	GGC ATC GAA GGT GGA AGA GT
Henmt1	AGG CAT CTC GTG TGA AAA GGA	TCA GAA TCA GCG GCA ACT CA	TGA CCC AAA ACC GAC ACC TT	GCC ACC AAC CAC ACT ATT GC
Tbp	TGC TGT TGG TGA TTG TTG GT	AAC TGG CTT GTG TGG GAA AG	GAG TTG CTT GCT CTG TGC TG	ATA CTG GGA AGG CGG AAT GT

2.7.4 Primer validation

In order to use the primers for RT-qPCR, the primers had to be validated for their specificity. The primers were reconstituted by RNase free water to make up 100 µM final concentration. Each tube contained 1µl of cDNA sample, 5 µl of SybrGreen (Applied Biosystem, Fast SYBR™ green master mix, 4385612), 0.3µl of forward primer, 0.3µl of reverse primer, and 3.4µl of RNase free water. Due to the purpose of the validation, cDNA sample had to be chosen from the sample which expressed the tested mRNA so that it could be amplified by the primers. The tubes were thoroughly mixed and spun down. The PCR machine (AB Applied Biosystems, Veriti™ 96-Well Thermal Cycler) was set for the thermal cycles: 95°C for 3 minutes for initial denaturation, followed by 40 cycles of 95°C for 3 seconds for denaturation and 60°C for 30 seconds for annealing, and 95°C for 1 minute for extension, and holding at 4°C for infinite. While the reaction was going, an ethidium bromide containing 1.5% agarose gel was prepared. To make the gel, 100 ml of TAE buffer was mixed with 1.5 mg of agarose powder in a flask and the mixture was heated by microwave for roughly 2 minutes (every 30 seconds, the flask had to be checked to avoid boiling). Once the mixture showed clear colour with no granules, 5µl of ethidium bromide were added to the mixture and swirled quickly to mix everything thoroughly. The gel was poured into tray with a comb, and allowed to sit for 45 minutes at room temperature for solidification.

When the reaction cycles were done, the loading dye (Biolabs, B7025S) was mixed thoroughly with the samples. The solidified gel was placed into an electrophoresis tank filled with TAE buffer and the comb was gently removed. The 10µl of the samples and 5µl of 100 bp DNA ladder was loaded onto the gel. The gel was running at 120V for 45-50 minutes. To image the gel, the gel was placed in UV lamp machine (Alpha Innotech, AlphaImage™).

2.7.5 Standard curve generation

Once primers were validated for producing a single amplicon of the predicted size, their standards could be generated. To make the standards, Q5® High-Fidelity DNA polymerase (M0491) was used and all procedures were performed as per manufacturer's instruction. The required volumes of enzymes, STD primers and a positive cDNA sample were mixed and the machine was programmed. Optimal Tms had to be checked using new England biolab (NEB) Tm calculator (http://tmcalculator.neb.com/#!/main). The full volume of products was separated by gel electrophoresis. If products did not show non-specific products, the total PCR products were cut and the specific bands extracted by QIAquick® Gel extraction Kit (Qiagen, 28704) as per manufacturer's instruction.

After a standard product was generated, it is quantified using nanodrop readings. To obtain an estimate of number of DNA molecules present, using OligoCalc (Kibbe, 2007) and the following formular to calculate an estimate of the number of molecules present in the raw STD per µl.

$$\frac{concentration\ (g/\mu l)}{molecular\ weight\ (g/mol)} \times Avogadro'sconstant$$

The raw standard was diluted 100-fold to generate a 10^{-2} stock prior to storage at -20°C. The first point on the STD curve was 10^{-4} and 10-fold serial dilution (10^{-4} to 10^{-9}) was made to generate STD curves. The concentration (outcome from the formula) was multiplied by 10^{-4} for input into the machine.

2.7.6 RT-qPCR

RT-qPCR was performed using QuantaStudio7 (Thermo Fisher Scientist). Fast SYBR™ green master mix (Applied biosystem, 4385612) was used:

- Template (cDNA): 1µl (1-2µg)
- Sybr green: 5µl
- Nuclease free water: 3.85µl
- Primer pair 0.15µl (final concentration: 0.15µM)

cDNA samples were run in triplicate on 384 well qPCR plates (Applied Biosystems, 4309849) and calibrated using a standard curve for absolute quantification. From 10^{-4} dilution, 6 serial dilutions were made (1:10 dilution, final point of 10^{-9}). Thermal cycles for the machine was programmed for 95°C for 20 seconds for initial denaturation, followed by PCR cycles (40 cycles, 95°C for 1 seconds and 60°C for 20 seconds) and 95 °C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds.

Cycle threshold (Ct) values and quantities were exported from the RT-qPCR machine software after confirming that samples had amplified, sufficient efficiency (over 90%) from standard curves, lacking contamination from –RT and negative controls (NTC), and melting curves were checked for one specific amplicon product. All data were analysed in Excel 2016 (Microsoft) and statistical analysis was performed using Minitab® 19 Statistical Software (Minitab, LLC).

To perform quality control, the coefficients of variation of Ct triplicates were checked. If any variation of triplicates was over 2.5%, an outlier was excluded (over 1.5 from median values). If any background levels were detected from –RT and NTC, the values were subtracted from the samples' values. The levels of target genes were normalised to the geometric mean of two reference genes, which were not significantly differently different within treatment groups (data were analysed in Thermo Fisher Connect™ https://apps.thermofisher.com/apps/spa/#/dashboard), and relative differences in target gene expression were determined using the absolute quantification method. Absolute quantification was presented as cDNA copies/house keeping gene cDNA copies (HKGs). Only for *Tlr* expression analysis on SIM-A9 cells (Figure 5), the relative quantification method (2^-ΔΔCT) was used. The arbitrary value relative to *Gapdh* was compared to the average of all control (no RT) values as an index of relative expression.

From 2.7 to 2.7.6 were used in Chapter 4 and Chapter 5 to measure levels of mRNAs in cell or tissue samples. Apart from types of samples and own experimental conditions, RT-qPCR steps described in this section were remained as much as possible. If any changes were made, it will be written in a allocated section.

2.8 Griess assay

To assess iNOS levels, the Griess assay (Enzo Life Sciences, ALX-400-004-L050) was performed. If absolute quantification was required, 6 standard concentration samples of sodium nitrite ($NaNO_2$) were serially diluted in culture medium without serum from the starting concentration (100μM). After the experiment was done, duplicates or triplicates of 50μl of cell culture media were transferred into a 96 well plate. An equal volume of Griess reagent was gently mixed with the culture media. After 10-15 minutes at room temperature in the dark, measurements were taken (Multiskan® Spectrum; Thermo Scientific™, 540nm), operated via the SkanItTM software (Thermo Scientific™). 650nm absorbance readings was taken for reference. This assay was assessed every time after the SIM-A9 cells were thawed to valid the condition of the cells' immune reactivity (checking a stock variability).

2.9 ELISA

To measure secreted cyto-/chemokine levels, ELISA (enzyme-linked immunosorbent assay) was employed. The assay was performed as per manufacturer's instructions. Briefly, an assay plate (Corning 9018, 96 well, high binding affinity, flat bottom) was coated with reconstituted capture antibodies (100μl/ well, working concentration as recommended) and the plate was left at 4°C for overnight. The next day, antibodies were aspirated and the plate was washed 3 times with washing buffer (0.05% Tween 20 in PBS). The plate was incubated with the reagent diluent (200μl/ well) for 2 hours at room temperature to reduce the background signals and aspirated and washed 3 times. A 2-fold serial dilution was made to generate STDs and samples (100μl/ well), and all of the samples and STDs were assayed in duplicate. The STDs and the samples were incubated at 4°C overnight for maximal sensitivity. On the 3rd day, after the aspirating and washing steps, reconstituted detection antibodies were added and incubated for 2 hours (room temperature, dark). After aspirating and washing 3 times, reconstituted streptavidin-HRP (100μl/ well) was added, and the plate was incubated (30 minutes, room temperature, dark). After repeating washing 5 times, the substrate solution was added (100μl/ well) and the plate was left for 15 to 20 minutes in the dark. However the reaction time is variable. In order to stop the reaction, the required volume of stop solution (2N H_2SO_4, Fisher chemical, 12933634) was added and the plate was read within 5 minutes. Measurements were taken (Tecan Sunrise v6.6, 450nm), operated via the megellan6 software. 620nm absorbance was taken for the reference. ELISAs were accepted if the STDs fitted a 4-parameter logistic regression (4PL) curve ($R^2 \geq 0.99$, see Appendix7 Figure 38), and duplicate samples varied (CoV) less than 15%.

The list of the kits used is below:

- IL-6 (eBiosciece™, Ready-SET-GO™, 12364003)
- TNF-α (eBiosciece™, Ready-SET-GO™, 155501117)
- CCL2 (eBiosciece™, Ready-SET-GO™, 15561137)
- CCL5/RANTES (R&D system, DuoSet®, DY478-05)
- CXCL10/IP-10/CRG-2 (Invitrogen, BMS6018MST)

ELISA was used in Chapter 4 to measure levels of proteins in cell culture media. Apart from types of samples and own experimental conditions, steps described in this section were very brief. Each essay protocol respects the company's instruction as much as possible.

2.10 Immunohistochemistry

The frozen sections were dried on a pre-heated slide drier (60°C) for 10 minutes and then wax barriers were drawn around the sections. After the wax barriers were completely dry, the sections were soaked into PBS for 5 minutes, 2 times for rehydration. 100μl of blocking solution (10% of host serum with 0.5% Triton X-100 PBS) was added to each section and incubated for 1 hour at room temperature. While blocking was progressing, primary antibody or (antibody mixtures), at the required dilution, with 1% of host serum with 0.5% Triton X-100 PBS, were prepared. Blocking serum was discarded and 100μl of the prepared primary antibody solution was added to each section. Sections were then incubated in the dark (4°C, overnight). Slides were washed 3 times in 1X PBS for 5 minutes with gentle shaking. 100 μl of the required secondary antibody solution, with 1% of host serum with 0.5% Triton X-100 PBS, was added onto each section and incubated for 2 hours in the dark at room temperature. The slides were washed by 1XPBS with gentle shaking (5 minutes, 5 times) and during the last washing, Mowiol (Calbiochem 4705904) containing DAPI (1:2000) was prepared and kept in the dark until the sections were ready. Once the sections were washed, they were rinsed 3-5 times in cold tap water. 15μl Mowiol was added onto each section and coverslips applied. The sections were stored at 4°C in the dark and could be used for up to a month. The stained section was viewed using an inverted spinning disk confocal microscope (AxioImager M2), and images captured by its integral software.

2.11 Immunocytochemistry

The cell cultures on coverslips were fixed with 4% PFA for 7 minutes at room temperature (1ml per dish or 50μl per individual coverslip) and washed with PBS 3 times. 50μl of permeabilisation solution (0.5% Triton X-100 PBS) was added to each coverslip and incubated for 15 minutes at room temperature. During the permeabilisation, the blocking solution (10% of host serum with 0.5% Triton X-100 PBS) was made. After washing the coverslips with PBS 3 times, and tapping off excess onto blue roll by holding onto coverslip with fine forceps, 50μl of prepared blocking solution was applied to each coverslip (30 minutes, room temperature). While blocking was progressing, primary antibody or (antibody mixtures), at the required dilution, with 1% of host serum with 0.5% Triton X-100 PBS, were prepared. Blocking serum was discarded and 100μl of the prepared primary antibody solution was added to each coverslip. Coverslips were then incubated in the dark (45 minutes at room temperature or 4°C, overnight). Coverslips were washed 3 times in 1X PBS and excess tapped off onto blue roll. 50μl of the required secondary antibody solution, with 1% of host serum with 0.5% Triton X-100 PBS, was added onto each coverslip and incubated for 20 minutes in the dark at room temperature during the incubation, Mowiol (Calbiochem 4705904) containing DAPI (1:2000) was prepared and kept in the dark until the coverslips were ready. Coverslips were washed 3 times by 1XPBS and tapped off excess onto blue roll. 10μl Mowiol was added onto a slide and then the coverslip placed on it (making sure the cell culture slide was facing mounting media). The coverslips were stored at 4°C in the dark and could be used for up to a

month. The stained coverslips were viewed using an inverted spinning disk confocal microscope (AxioImager M2), and images captured by its integral software.

Chapter 3
Activation of MAPKs after immune-related challenges in primary cortical neurons

Chapter 3 Activation of MAPKs after immune-related challenges in primary cortical neurons

3.1 Introduction

Prior works have indicated that abnormal neuronal function is related to pathological features of schizophrenia (Meyer and Feldon, 2009, Ramamoorthi and Lin, 2011, Glausier and Lewis, 2017, Roeske et al., 2020). Furthermore, the *MKK7*, the upstream of JNKs, and other MAPKs, e.g. *ERK1* (*MAPK3*), are considered genetic risks factor for schizophrenia (Winchester et al., 2012, Ripke et al., 2020). Therefore, it is worth studying how these genes contribute neuronal reactions after various environmental stimulus.

In addition to the genetic risk, MIA impacts foetal brain development via neuronal cell migration and microglial activity, but also MIA is linked with intercellular communication via neurotransmitters or growth factors. This is also a mechanism to affect brain function. There is established evidence to suggest that cytokines and chemokines have roles beyond the immune response, with involvement in neurotransmitter function, e.g. GABA and glutamate, modulation (Murray et al., 1997, Loscher et al., 2000, Costello and Lynch, 2013, Borroto-Escuela et al., 2017). Additionally, there is evidence that offspring levels of serotonin receptors as well as glutamate receptors are affected by MIA (Holloway et al., 2013). Such neurotransmitter receptors are also expressed in microglia, so there is the possibility for reciprocal communication (Pocock and Kettenmann, 2007). Equally, chemokines affect the production of neurotransmitters (Giovannelli et al., 1998, Cho and Miller, 2002).

Moreover, Immune molecule impacts on neuronal and glial cells have reported. For example, CCL5 (also known as RANTES) induced dorsal root ganglion (DRG) neuronal migration (Bolin et al., 1998) and CXCL8 and CXCL1 administration increases primary neuronal culture viability (Araujo and Cotman, 1993, Horuk et al., 1997, Meucci et al., 2000). MIA or elevated levels of immune molecules could also affect neuronal maturation. Poly I:C exposure during pregnancy could cause immature hippocampal development; indeed this may be related to upregulated cytokines in mice (Giovanoli et al., 2016). In addition, increased maternal inflammatory cytokines suppress dendrite and cortical neuron development in rats (Gilmore et al., 2004). Furthermore, Hein and colleague report that the increasing the level of IL-1β in the hippocampus of adult mice produces behavioural abnormalities (Hein et al., 2010). Unlike inflammatory cytokines and chemokines, one particular chemokine, CXCL12 (also known as SDF-1α) and its two receptors, CXCR4 and ACKR3 (also known as CXCR7), contribute to brain development differently. They are widely expressed in developing and developed CNS (Stumm et al., 2007). Since CXCL12 regulates neuronal stem cell migration and neuronal cell differentiation, which are not typical properties of chemokines, researchers think CXCL12-CXCR4-ACKR3 interactions have some other functions in CNS development rather than immunity (Rostène et al., 2011). Besides, results also indicate a relationship between GABAergic interneuron migration and CXCL12 release (Bajetto et al., 1999, Stumm et al., 2007). A recent paper shows elevated expression of *Cxcr4* and *Cxcr7/Ackr3* mRNAs

in post-mortem PFC from patients with schizophrenia, and an inverse correlation between these levels and the expression the mRNAs encoding PV/GAD67 (Volk et al., 2015). CXCR4 and CXCL12 are required for GABAergic neuron migration during developmental stages (Stumm and Höllt, 2007). In KO mice experiments, lack of *Cxcl12* or *Cxcr4* produces abnormal brain morphology. All these remarkable outcomes suggest that the relationship between chemokines and their receptors is important for the development of schizophrenia.

Chemokines and cytokines are not only participating in developmental processes, but also contribute to cellular communication. For example, CXCL10 is one of the inflammatory chemokines that plays as a initiation signal to microglia (Biber et al., 2007). Especially under viral infection, this chemokine is released from neurons and works as an initiation signal to microglia (Klein et al., 2005). Regarding astroglial cells, another type of glial cells, these cells take an important part in maintaining homeostasis and possibly involved in immune reactions. Additionally, they may have a link with schizophrenia, still supporting evidence is somewhat controversial (Radewicz et al., 2000, Rothermundt et al., 2004).

All these strands of research evidence indicate how important the interaction between maternal and foetal compartments is during progeny. Even though the foetus is physically separated from the mother by the placenta, they are very dependent on each other, in terms of keeping their body systems functioning as normally as possible, and for optimising the growth and development of the foetus. Many scientific discoveries show that MIA negatively affects foetal development, especially brain development, resulting in increased risk of having a mental illness in the later life of offspring.

To study the relationship between how the activity of MAPKs might be changed in neurons after immune challenges, cultured mouse primary cortical neurons were used. Although cell lines, such as NG108-15 neuronal cells, may be a more convenient tool for studying, they may not be ideal for some experiments (Allen and Kershaw, 1989). Besides, relatively, primary cultures create a closer *in vivo* environment compared to transformed cell lines. In spite of prior evidence (Lehnardt et al., 2003, Gilmore et al., 2004, Lafon et al., 2006, Leow-Dyke et al., 2012), the neuronal cellular responses after various environmental challenges in neurons are still not clear. This led us to test the following hypothesis in this chapter: levels of pMAPKs are used as a main signalling pathway in primary mouse cultured cortical neurons after immune challenge, and that the nature of the response differs for different immune stimuli. The aim was to assess the extent of MAPK pathway responses to different immune challenges(poly I:C, LPS, resiquimod, mimicking infection with dsRNA viruses, bacteria and ssRNA viruses respectively) in primary cortical neurons, and further to determine if neurones were relatively more responsive to downstream immune mediators (Cxcl10, Cxcl12).

3.2 Methods

The methods used in this Chapter have been described in detail in Chapter 2

3.3 Results

3.2.1 Primary cortical neurons may not use JNKs as main signalling molecules to respond to TLR-mediated signalling

As noted above, primary cortical cultured neurons are a better research tool than the cell line in certain aspects. Previously TLR expression in neurons, including cortical neurones, is well established (Acosta and Davies, 2008, Mishra et al., 2006, Tang et al., 2007, Hung et al., 2018, Ritchie et al., 2018). Indeed, since the *MAP2K7* gene is a genetic risk factor for schizophrenia, in this project, we started to investigate JNK pathway in primary cultured neurons. This primary cultured cortical neuron population was defined (via IHC) that 80-85% were neurons and the rest of the population was astrocytes (these experimental data were carried and collected by Ashleigh Willis and kindly shared during personal discussion).

In order to test the hypothesis, the cultured cortical neurons were exposed to one of three different experimental conditions (details in Table 4). In addition to the treatments, analysing isoform expression is important because of different biological functions and expression profile of MAPKs' isoforms (see section 1.3). Thus, in this study, individual isoform expression levels after the treatments are considered seriously. JNK isoforms were not significantly affected by all three TLR mimetics.

The levels of pJNKs were significantly upregulated with poly I:C (vehicle vs. poly I:C, p=0.045, Two-way ANOVA, Tukey post-hoc test), but not with LPS or resiquimod (Figure 3). However, after Grubb's test, the level of pJNK was not significantly induced by any of the treatments ($F(3,91)=2.128$, p=0.1021, p=0.1463, two-way ANOVA, Tukey). Thus, the significance we reported may be caused by one outstanding sample.

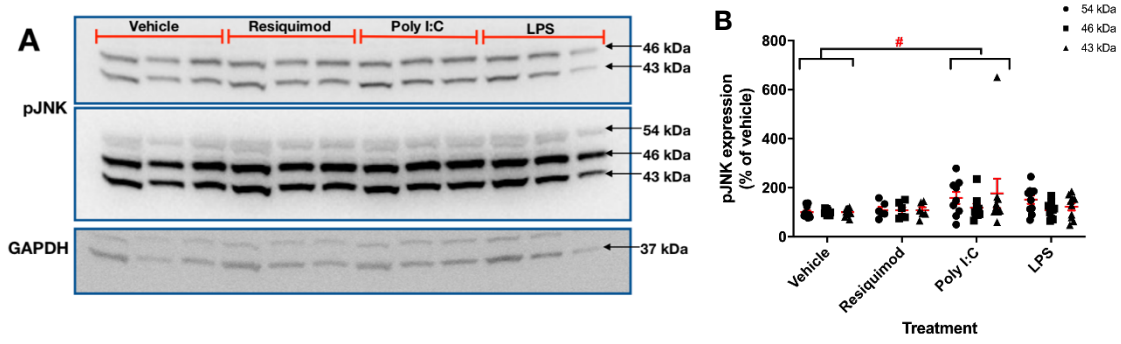

Figure 3. Primary neuronal cells do not use JNKs as the main Kinase after TLR-mediated signalling. The embryonic brains (E17-18) were taken and cultured for two weeks meanwhile the medium was changed every 3-4 days. The cells were treated with vehicle, or stimulated by one of three different immune mimetics, LPS (50ng/ml), poly I:C (100ng/ml), Resiquimod (3μM) for 15 minutes. **(A)** One representative gel image out of two independent experiment images is illustrated to show pJNK isoforms. **(B)** The increased levels of pJNK with poly I:C stimulation were significant compared to vehicle. Individual dots show expression relative to vehicle ± SEM (n=6-10). The data were log transformed and analysed by two-way ANOVA. (#p≤0.05, Post-hoc Tukey comparisons) Details of ANOVA F values and p values are provided in Table 9.

The data suggest that even though there is a hint of a relationship between the JNK pathway and poly I:C, the JNK pathway may not be the main signalling pathway following TLR-induced signalling in primary cortical neurons. Possibly our experimental conditions were not optimal to observe changes in the cells, or possibly activation of the JNK pathway in cortical cultured neurons may be pathogen type-dependent, and involve TLR ligands which are not tested in this studied, such as, Gram-positive bacteria.

Even though no overall significant changes in the levels of pJNKs after environmental stimuli were detected, neurons also respond to immune molecules, cytokines and chemokines. Although neurons are not immune-functional cell types, they can produce immune molecules for various reasons, such as communicating with neighbour cells. Therefore, primary mouse cortical neuronal cultures were exposed to immune molecules, CXCL10 or CXCL12

3.2.2 Effect of chemokines on MAP kinase activity in primary neuronal cultures

Cytokines and chemokines are immune molecules that have the potential to stimulate the neuronal cells; as a result, the cellular functions could be disrupted if they are unregulated. Moreover, abnormal levels of the immune molecules have been reported pathological features of schizophrenia (see section 1.2.5). Among all chemokines, two specific chemokines, CXCL10 and CXCL12, were used to assess whether neuronal cells respond through the MAPK pathway. These two chemokines are not only relevant to psychiatric disorders (Toritsuka et al., 2013, Noto et al., 2015, Cash-Padgett et al., 2016, Misiak et al., 2020), but also are known to regulate neurological functions (Nelson and Gruol, 2004, Cho et al., 2009, Watson et al., 2020). In this study, two other types of MAPKs, ERKs and p38, in addition to the pJNKs, were also investigated, based on emerging evidence that shows that these two kinases are used for immune signalling (see section 1.3.2 and 1.3.3). Thus, it is worth testing all three different MAPKs for activation following exposure to CXCL10 and CXCL12.

14 DIV primary cortical cultured neurons, and the protein extraction was done following exposure to vehicle, or chemokines CXCL10 (10nM), or CXCL12 (10nM), for 15 minutes. Even though this study does not investigate CXCR3 (CXCL10 receptor) and CXCR4 (CXCL12 receptor) expression, there is clear evidence to support their expression on neurons via *in situ* hybridisation (Tran et al., 2007, Jiang et al., 2017). Hence, these two chemokines are presumably able to stimulate culture neuronal cells. The quantity of pMAPK protein was measured by Western blot.

The experimental data showed that overall, pJNK was decreased by CXCL10 compared to vehicle (Figure 4B-1). pp38 was not changed by CXCL12 and CXCL10 stimulation (Figure 4B-3). Levels of pERKs were not significantly changed by any chemokines, although overall isoform effects were seen (Figure 4B-2). Like pJNK, this may be argued that the significance reported here in Figure 4B-2 is from the one outstanding data point; however a statistical analysis after removing the outlier

(following Grubb's test), isoform effect still showed significant (F(1,65)=5.985, p=0.0171, p=0.0321, two-away ANOVA, Bonferroni).

This experiment reproduced the findings of earlier experiment with environmental and immunological factors with minor but significant changes in pJNKs (Figure 3 and Figure 4).

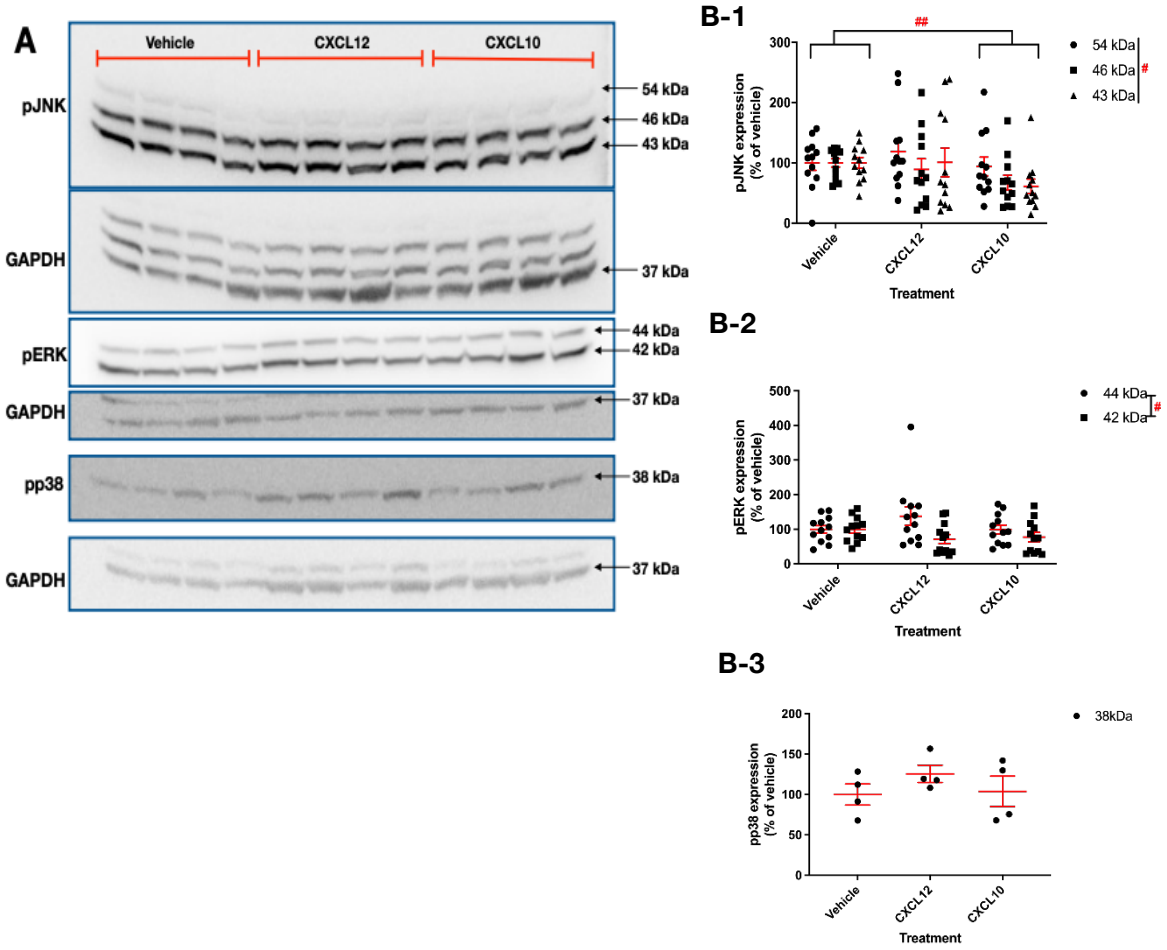

Figure 4. Chemokines slightly affect MAPK phosphorylation. The embryonic brains (E17-18) were taken and cultured for two weeks meanwhile the medium was changed every 3-4 days. The cells were stimulated by vehicle or by one of two chemokines, CXCL10 (10nM), or CXCL12 (10nM) for 15 minutes. **(A)** Chemokine stimulation showed minor but significant change in pMAPKs. **(A, B-1)** pJNKs levels were reduced with CXCL10; **(A, B-2)** pERKs did not show treatment effects, but showed isoform's effects **(A, B-3)** the level of of pp38 was not significantly affected by both chemokine stimulations. One representative blot (A) out of two independent experiment images is shown for B-1 and B-2; one blot of experiment image for B-3. Individual dots show expression relative to vehicle ± SEM (n=12 for pJNKs and pEKRs, n=4 for pp38). The data were log transformed and two-way ANOVA with Tukey comparisons (pJNKs and pERKs) or with one-way ANOVA with Tukey comparisons (pp38). **p≤0.05, Post-hoc Tukey comparisons; $^{\#}$p≤0.05, $^{\#\#}$p≤0.005 Post-hoc Tukey comparisons) Details of ANOVA F values and p values are provided in Table 9.

3.3 Discussion

3.3.1 Summary of results

The work showed in this chapter has investigated how levels of pMAPKs are changed in primary cultured cortical neurons after different stimulations. The observations show that, under these conditions, pJNKs are potentially upregulated by TLR3 (poly I:C) stimulation, and not by TLR4 or TLR7/8 stimulation. Furthermore, CXCL10 reduces pJNKs; however pERK and p38 are not significantly changed. These findings suggest that activation of the MAPK pathways in primary cultured cortical neurons is stimulation type dependent.

3.3.2 TLR3 stimulation potentially induces the levels of pJNKs

The mouse primary cultured cortical neurones showed induced levels of pJNKs after TLR3 (poly I:C) stimulation, but this was not observed with TLR4 (LPS) and TLR7/8 (resiquimod) although poly I:C effect might be seen because of an outstanding sample (Figure 3). This finding is similar to a previous report that primary neuronal culture did not show any pMAPKs changes after LPS (10ng/ml) in 1 hour, even IFN is applied to enhance the effects of LPS (Xie et al., 2004). The other recent finding equally shows that no pJNKs and p38 changes after LPS (100ng/ml) and poly I:C (10μg/ml) in 4 hours (Chistyakov et al., 2018). Note that Ma and others detected TLR8 but not TLR4 (TLR3 not tested) in 5 DIV mouse cortical neurones (Ma et al., 2006) as well as Lehnardt and colleagues presented the similar PCR data: an absence of TLR4 expression in cultured neurons, however detected in cultured microglia (Lehnardt et al., 2003). Although further research, such as different concentrations or longer exposure times, has to be carried to conclude, the current experimental condition in this study may not be sufficient enough to observe the level changes of MAPKs in primary cortical neurons following TLR-mediated stimulations.

Because of the relevance to schizophrenia (Winchester et al., 2012, Openshaw et al., 2017, Ripke et al., 2020, Deane et al., 2021), only JNKs were measured in this study after immune challenges. However, many seminal studies show the importance of other MAPKs. One of the papers reports corresponding data, where there is no pMAPK induction in primary cultured cortical neurons, even the cells are treated with a harsher condition (resiquimod, 100μM) used here (Ma et al., 2006). Intriguingly, they also observed a negative effect on neurite growth after resiquimod stimulation, but this was not observed with LPS. This may of course reflect the very high, possibly damaging, concentration of resiquimod that they used.

In contrast, the level of JNK is reportedly upregulated via LPS in primary neuronal culture, this is however when experimental conditions are different: 10ng/ml, 30 minutes (Leow-Dyke et al., 2012), or 100ng/ml, 24h (Barry et al., 2005). Indeed, the data from KO mice support the idea that JNKs are essential for TLR4 mediated signalling to prevent neuronal apoptosis (Tang et al., 2007). Correspondingly, the upregulated levels of pMAPKs after LPS stimulation are reported (Huang et al., 2015), but this was with mixed neurone/glia cultures after 180 minutes exposure..

Data similar to ours have been reported from neuron-like cells, the SH-SY5Y cell line. The results showed that LPS (100ng/ml) does not induce phosphorylation of ERKs and p38, but poly I:C (50μg/ml) increases the level of pp38 after 30 minutes (Lawrimore and Crews, 2017). Other evidence also showed the induction of pJNKs and pp38 after poly I:C administration on SH-SY5Y cells (NESSA et al., 2006). pERKs' changes are reported in 6 days experiment with LPS (1μg/ml) on SH-SY5Y (Das et al., 2012). SH-SY5Y is one of the most widely used neuron-like cell lines and is of human blastoma origin (Biedler et al., 1978, Påhlman et al., 1984). Most cell lines are modified to gain immortality or easy to handle in the laboratory. However, because of these modifications, cell lines lose their originality (Gordon et al., 2013). Correspond to this argument, Mielke and others report the different expression levels of JNKs between three common neuron-like cells after various stress factors (Mielke et al., 2000) and it clearly shows valid differences between the cell lines. Thus, to overcome these issues, primary culture is often used. It is interesting to discuss how the results are different. It is worth noting, in general, the experimental conditions used for cell lines tend to be harsher e.g. higher concentration or longer exposure time, than the conditions with primary neuronal cultures.

Of particular note, the MAPK activation can be different depending on culture conditions and cell types also, in this case cell types. Lundberg and others point out that different poly I:C effects can be seen depending on the cell type (Lundberg et al., 2007). In addition, Hassan and colleagues present evidence that neuroblastoma cells do activate JNKs and p38 following LPS stimulation, indeed with longer exposure times (30 and 60 minutes) (Hassan et al., 2006) whereas the data present the importance of JNKs, not ERKs and p38, for TLR4 mediated reaction in neurons (Tang et al., 2007, Leow-Dyke et al., 2012). Furthermore, recombinant gp120 protein activates ERK and JNK in cultured neurons, although it is time-dependent (Lannuzel et al., 1997). These findings indicate that outcomes of the signalling pathway are cell-type dependent even if cells are stimulated by the same pathogen type. Although *in vitro* LPS administration did not alter the levels of pJNKs in this project, other evidence emphasises the importance of JNKs for TLR4 mediated signalling reported (Leow-Dyke et al., 2012, Huang et al., 2015). In addition, there is strong evidence that JNKs are activated in the CNS *in vivo* following systemic LPS administration (Lynch et al., 2004, Barry et al., 2005), although in the *in vivo* situation the cell type that is the direct target of LPS is not clear.

Overall, it appears that JNKs and other MAPKs are used downstream of TLR signalling, and these effects may differ according to the types of cells.

3.3.3 Chemokines alter the levels of pMAPKs in primary cortical cultured neurons

The cultured mouse cortical neurons showed minor but interesting changes after exposure to either of the two chemokines, CXCL10 and CXCL12. pJNKs were only affected by CXCL10 whereas pERKs and pp38 were not shown any significant changes except overall iso-form effect of pERKs (Figure 4).

Interactions between CXCL12 and neurons are critical, especially during embryonic development, because of stem cell migration (Guyon, 2014). Expression of the CXCL12 receptor CXCR4 is high at late embryonic and early postnatal stages in the mouse (Lu et al., 2002) suggesting that its function is important at the time when studied in our experiments. The data showed very slight changes in the levels of pERKs induced by CXCL12, and not by CXCL10, however the effects were not of large magnitude. Xia and colleagues show that CXCL10 (25nM) induces pERK (Xia et al., 2000) in primary cortical neurons, and chronic CXCL10 exposure upregulates levels of ERKs (Bajova et al., 2008). Other research supports the importance of the CXCL12/ERK signalling pathway (Luo et al., 2008). In astrocytes, which are an essential cellular population for maintaining environmental homeostasis, ERKs are significantly important for transducing their proliferative signals that are CXCL12-dependent (Bajetto et al., 2001, Odemis et al., 2010). The evidence suggests that the CXCL12-induced ERK pathway is also important for oligodendrocyte precursor cell (OPC) migration (Tian et al., 2018). Besides, the induced levels of pERKs by CXCL12 are reported in immune cells, such as microglia (Lu et al., 2009). Indirectly, this result indicates that the CXCL12/ERK pathway is important not only for neuronal functions but also for keeping the environment for other cellular functions.

This study showed no pp38 level changes following CXCL10 and CXCL12 stimulations (Figure 4). Not many studies show the direct relationship between the CXCL12/p38 pathway in neurons (Kaul et al., 2007); most of the studies related to CXCL12 and p38 are immune function related (Honczarenko et al., 2002, Zhao et al., 2013, Zhang et al., 2016b, Shi et al., 2017). However, some of the studies showed non-immune related functions. For example, in OPC, CXCL12 treatment downregulates levels of pp38, but with no significant changes in pJNKs (Yuan et al., 2018) corresponding to the observation in this research. Our own observations with the NG108 cell line showed CXCL12 significantly downregulated the levels of pERKs and pp38 but with no changes in pJNKs (Figure 33). In contrast, other evidence shows upregulated levels of pp38 in cerebrocortical neurons (mixed with glial population) (Sanchez et al., 2016). In cultured schwann cells (SCs), CXCL12 treatment positively affects pp38 (Gao et al., 2018).

The changes of pp38 are primary culture and the neural cell line (NG108) are slightly different in this study, therefore, the functional significance of regulation of p38 activity by CXCL12 is difficult to make. However, elevated p38 activity has been linked with impaired synaptic plasticity (Vereker et al., 2000, Wang et al., 2004), and CXCL12 has been linked with modulation of neurotransmission in the cerebellum (Limatola et al., 2000, Ragozzino et al., 2002). We speculate that the effect of this specific regulation of p38 activity may be to facilitate plasticity in these developing neurones.

3.3.4 Summary and conclusion

In this chapter, the primary mouse cortical neuronal responses following various environmental stimuli were studied, and the pMAPKs contribution in these processes was studied. The levels of phosphorylation of JNK were possibly increased by poly I:C stimulation; whereas the stimulations via TLR4 by LPS and TLR7/8 by resiquimod had no effect on the levels of pJNKs. Surprisingly, CXCL10 stimulation affected the levels of pJNKs in primary cortical neurons, whereas CXCL12

affected pERKs and pp38 in NG108 cells, not in primary cortical neurons. These findings suggest that the JNK pathway may not be used to respond to LPS and resiquimod stimulations in primary cultured neurons, at least at this developmental stage, and that chemokine stimulation tends to reduce neuronal MAPK activity. Since the neuronal population is not the main immune cells in the brain, it is worth investigating these pathogen mimetic effects on microglia. In addition, how the MAPK pathway regulates microglial reaction after immune challenges will be of interest, leading to the experiments described in Chapter 4.

Chapter 4

A contribution of MAPK pathways to control of microglial immune response

Chapter 4 A contribution of MAPK pathways to control of microglial immune response

4.1 Introduction

Microglia are the primary immune cells in the brain and they have many roles, including immune reactions also maintaining homeostasis with astrocytes. Previous work, and the findings presented so far, have suggested that microglia are an important cell population in the brain, as much as neurons, even though they represent 10-15% of a total brain cell population in the adult brain.

Pro-inflammatory microglial cells (activation status) are usually considered as mediating inflammation by secreting pro-inflammatory cytokines e.g. IL-1, IL-6 and TNF-α, along with iNOS and ROS, while anti-inflammatory microglial cells inhibit inflammatory reactions and restore homeostasis by releasing anti-inflammatory cytokines such as IL-4 and IL-10. In the case of schizophrenia patients, it has been proposed that they exhibit an unbalanced pro- and anti-inflammatory microglial population (Nakagawa and Chiba, 2014). Dysregulation of pro- and anti-inflammatory microglia polarisation can affect the development of other brain cells (Nakagawa and Chiba, 2014). Also, mitochondrial dysfunction in microglia can inhibit the anti-inflammatory component (Ferger et al., 2010), and compromise cortical interneuron development, while evidence also suggests mitochondrial abnormalities in schizophrenia (Park et al., 2020). Furthermore, other pathological evidence suggests that microglia are involved in psychiatric disorders (van Berckel et al., 2008, Bloomfield et al., 2016).These findings imply that regulation of microglia polarisation is important for maintaining immunological homeostasis and reducing the risk of the disease. Nevertheless, details of the cellular mechanisms are unknown.

Because MAPKs, especially ERK1 and MAP2K7, are considered as risk factors for schizophrenia (see 1.1.3), the MAPK pathway would participate microglial immune reaction to the disease development in later life. After cytokine and chemokine release, activation of MAPKs in various cell types has been reported. First of all, among the three MAPK families, ERKs are regulated following stimulation by CXCR1/2 via CXCL8 (Venkatakrishnan et al., 2000), and CXCR3 (Bonacchi et al., 2001). CCL5 stimulation induces the phosphorylation of ERKs in monocytes (Sato et al., 2001). Corresponding to Huang and colleagues' findings, stimulation of CXCR4 via CXCL12 induces activation of both ERKs and NF-$\varkappa$B proteins (Ganju et al., 1998). Another MAPK, p38, takes part in immune reactions, such as CXCL8 and iNOS production, in microglia (Stirling et al., 2005) and chondrocytes (Badger et al., 1998). CXCL10 production in human monocytes is engaged by the p38 pathway (Zhao et al., 2017). JNK phosphorylation is increased in monocytes within 15 minutes following CXCL8 exposure (Yang et al., 2001), and following CCL5 exposure (Sato et al., 2001).

Furthermore, these MAPKs level induction is observed in the brain. ERKs and p38s are involved in iNOS and TNF-α gene production in microglia and astrocytes in culture (Bhat et al., 1998). However, Lee and others show that p38 participates in the regulation of TNF-α mRNA production in human microglia, but not in astrocytes, following LPS stimulation (Lee et al., 2000). Moreover,

eosinophils show clear changes of activation of ERKs and p38 following stimulation with CCL11 (a ligand of CCR1 and CCR3) (Kampen et al., 2000). A ligand of CXCR3, CXCL10, activates ERKs in mouse cortical neurons (Xia et al., 2000, Xia and Hyman, 2002). In addition, JNK phosphorylation upregulation is observed in a hippocampal neuronal call line (HT-22) following TNF-α exposure (Xiao et al., 2020). The levels of all three MAPK phosphorylation are induced following IL-1β and TNF-α exposure (30 minutes) in rat astrocytes (Thompson and Van Eldik, 2009). However, a human astrocyte cell line (CRT-MG) shows the upregulation of pJNK and pERK in a time-dependent manner after poly I:C stimulation (Park et al., 2006).

Macrophages and microglia produce various cytokines and chemokines to challenge environmental changes. However, MAPK roles in this response in microglia are not fully understood. As with Huang's paper (Huang et al., 2015) and other observations previously described, MAPKs are used to respond to LPS, resulting in increases in several inflammatory molecules, e.g. ROS, NO, however evidence is still not enough to conclude whether MAPKs are 'the main' kinases compared to other signalling molecules responsible for initiating immune reactions. Despite the fact that seminal studies report the links between activation of MAPKs and immune responses, more details of signalling pathways following pathological challenges are needed. This is a particular issue for studies with microglia, where, as noted above, the main cell lines used have been immortalised via prolonged MAPK pathway activation (Righi et al., 1989, Blasi et al., 1990). Hence their MAPK response to experimental manipulation may not be representative of native microglial responses.

Although, results of microglial immune reaction vary; but because of our specific interest, the MAPK pathway contribution to microglial immune reaction to cytokine and chemokine production will only considered in this study.

From previous experiments, TLR stimulations on primary cortical mouse neuronal culture did not show many changes in pMAPKs. However, microglia are the first cells to respond to environmental changes, not neurons, therefore microglia cells are possibly more sensitive and reactive for keeping environmental homeostasis (Li and Barres, 2017, Deczkowska et al., 2018).

In vitro tools for microglia study have been developed such as BV-2 or N9, and the SIM-A9 cell line is one of them (Nagamoto-Combs et al., 2014). This cell line is different from previous mouse microglial cell lines; the cells are isolated from primary mouse microglia culture. Indeed, the cells maintain microglial characteristics over many passages. The SIM-A9 cells may hence represent a particularly good model for testing hypotheses concerning microglial activation in disease conditions. Therefore various techniques will be used to test the following hypothesis: microglial cells (SIM-A9) regulate immune responses (production of cytokines and chemokines) via the MAPK pathways. The specific aim was to assess the extent of MAPK pathway responses to different immune challenges (poly I:C, LPS, resiquimod) in microglial (SIM-A9) cells, and their role of the MAPK pathways in the production of cytokines and chemokines.

4.2 Methods

The methods used in this Chapter have been described in detail in Chapter 2

4.3 Results

4.3.1 SIM-A9 cells express *Tlr*s

As noted above, SIM-A9 microglia cells are potentially closer to primary microglia cultures compare to other microglial cell lines. Even though SIM-A9 cells were characterised at certain levels such as morphology analysis after LPS stimulation by IHC and cytokine production by ELISA (Nagamoto-Combs et al., 2014), TLRs' expression levels have not been confirmed. Moreover, previous publications have studied the expression levels in cells which are maintained in serum, and thus may be in a partially-activated state (Bsibsi et al., 2002, Olson and Miller, 2004, Trudler et al., 2010). Thus, prior to the main experiments, TLR3, TLR4, TLR7, and TLR8 expression levels were assessed in a resting condition.

TLR mRNAs were amplified from SIM-A9 cells (Figure 5). At the mRNA level, *Tlr4* and *Tlr7* were highly expressed compared to *Tlr3* and *Tlr8* mRNAs. Though mRNA levels do not 100% reflect protein levels, the data suggest that resiquimod (TLR7/8 agonist) may affect the cells dominantly through TLR7, as compared to TLR8. These data confirm that SIM-A9 cells satisfy the basal condition: expression of TLRs for three different immune stimuli, and additionally give an idea of the amount of TLR expression on the cell membrane in a resting condition. Because now we confirmed the (SIM-A9) cells' TLR expression, we could move on to an experiment examining the effects of immune stimulation.

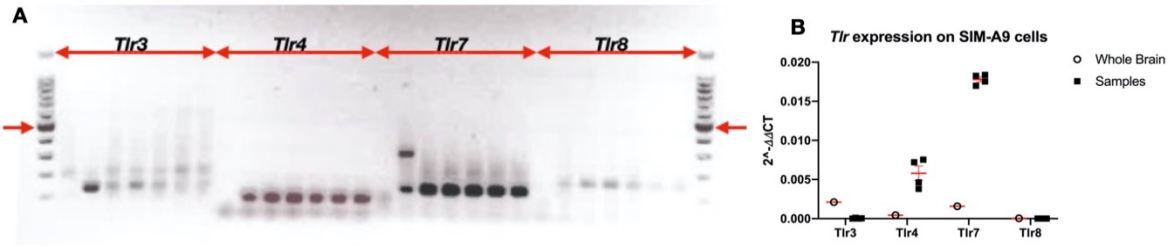

Figure 5. *Tlr* expression in SIM-A9 cells. The cells were plated on a culture plate under serum-free conditions and left overnight. Without stimulation, RNAs were extracted from the cells and then cDNA was generated. **(A)** A 100bp DNA ladder was shown at left and right (red arrow indicates 500bp). Loading order, negative control, positive control (cDNA from whole mouse brain), and five independent SIM-A9 cell samples; loading order was repeated for each TLR. **(B)** Relative quantification, as compared to *Gapdh*, of *Tlr* expression on SIM-A9 cell samples; whole brain cDNA was used as a positive control.

4.3.2 Confirmation of usage of pMAPKs after TLRs mediated signalling pathways

After checking TLR expression on SIM-A9 cells, the cells were tested to assess whether pMAPKs are used after environmental challenges. The cells were stimulated with one of three different types of pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), or resiquimod (3μM), for 15 minutes, and pMAPKs were then quantified by western blotting.

Resiquimod (TLR7/8 agonist) significantly induced pMAPKs at 15 minutes stimulation time, however poly I:C (TLR3 agonist) and LPS (TLR4 agonist) did not show any significant changes (Figure 6). No changes were detected in 15 minutes exposure with LPS or poly I:C, poly I:C could be explainable from the low level of *Tlr3* mRNA. However, the lack of a LPS response was a bit surprising to us. Therefore, to confirm the ability of these two mimetic's stimulation on SIM-A9 cells to change levels of pMAPKs, an extended time course experiment was done. Longer incubations e.g. 30 minutes and 60 minutes, with LPS and poly I:C on the cells were tested. After 30 minutes, LPS meaningfully upregulated pMAPKs. Poly I:C did not show any clear increases, although pp38 was decreased at 30 minutes (Figure 7 and Figure 8).

In addition, because MAPK isoforms have different biological functions (see section 1.3), it is worth to consider their isoform separately rather than together. In short exposure time (15 minutes), resiquimod stimulation significantly upregulated both pERKs' isoforms (Figure 6D), whereas p54kDa pJNK was significantly upregulated (Figure 6C). With LPS stimulation, isoforms of pERKs (46kDa, and 44kDa) and pJNKs (46kDa, and 43kDa) were significantly affected after 30 minutes, however 54kDa of pJNKs were not affected (Figure 7).

This observation suggests that in the microglial cells, MAPK signalling pathways are activated downstream of TLR4 and TLR7/8 mediated intracellular reactions; however, these reactions can be time dependent.

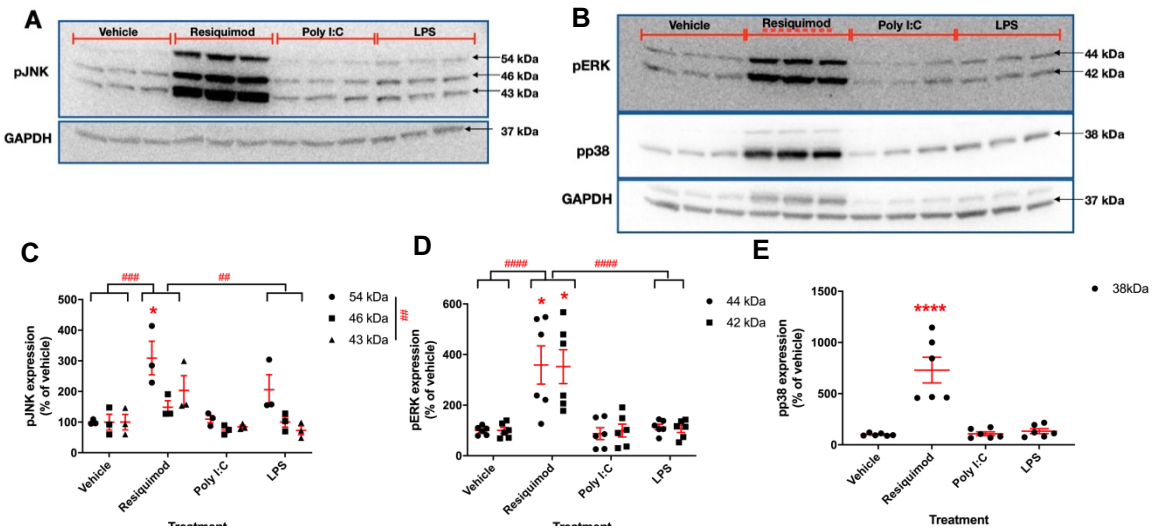

Figure 6. Resiquimod induces MAPKs phosphorylation in microglial cells. The microglial cells were cultured in serum-free medium overnight, and treated with LPS (50ng/ml), poly I:C (100ng/ml), or resiquimod (3μM) for 15 minutes. **(A, C)** The increased level of pJNK with resiquimod is significant compared to vehicle. **(B, D)** Resiquimod, but not poly I:C or LPS, increased pERK and pp38 levels significantly. **(B, E)** Resiquimod induced pp38 levels significantly. One blot for (pJNK) and one representative blot out of two independent experiment images (pERKs and pp38) are shown for A and B. Individual data points are shown along with mean ± SEM (n=3 for pJNKs, n=6 for pERKs and pp38). The data were analysed by two-way ANOVA, Tukey comparison (pJNK, pERK) and by one-way ANOVA, Bonferroni comparison (pp38) $^{*}p \leq 0.05$, $^{****}p \leq 0.0001$ vs. same size of isoform in a vehicle group; $^{\#\#}p \leq 0.005$, $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$ Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

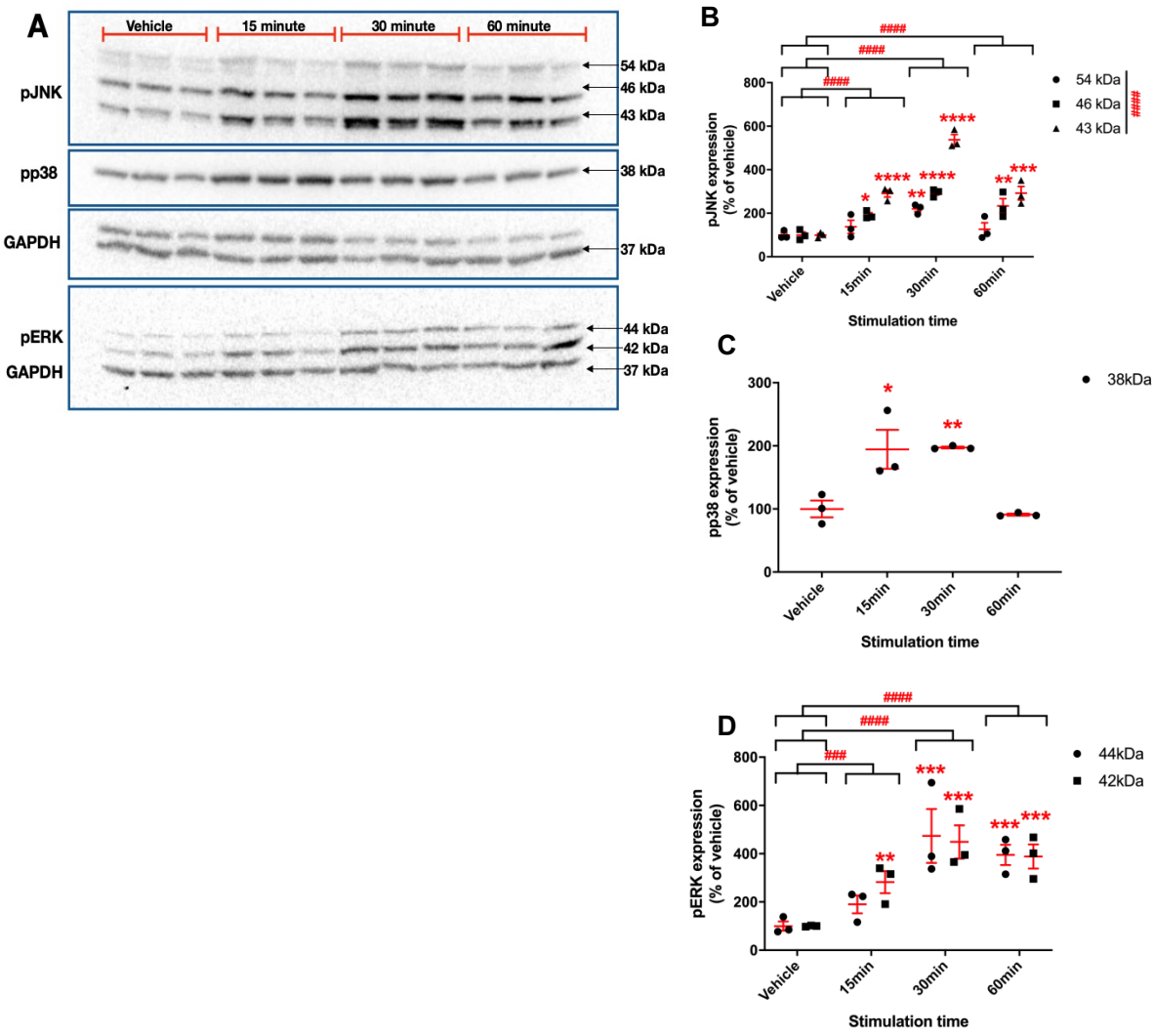

Figure 7. LPS requires longer exposure time. The microglial cells were cultured in serum-free medium overnight before treatment. The cells were culture with LPS (50ng/ml) for 15 minutes, 30 minutes and 60 minutes. Because of the size of proteins, pMAPKs were measured in two separate membranes, pJNKs and pp38 were in the same membrane, pERKs were observed in the other membrane. **(A)** After 30 minutes stimulation, all MAPKs, JNKs, ERKs, and p38 were phosphorylated more than vehicle. The upper bands in the GAPDH image were pp38 bands. **(B-D)** The increased level of pMAPK in 30 minutes was significant, although 15 min stimulation might change significantly depending on isoforms. Individual data points show expression relative to vehicle ± SEM for three independent experiments. One blot image is shown for A. Individual data points are shown along with mean ± SEM (n=3). The data were analysed by two-way ANOVA, Tukey comparison (pJNKs, pERKs) and by one-way ANOVA, Bonferroni comparison (pp38) (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$, ****$p \leq 0.0001$ vs. same size of isoform in a vehicle group; $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$ Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

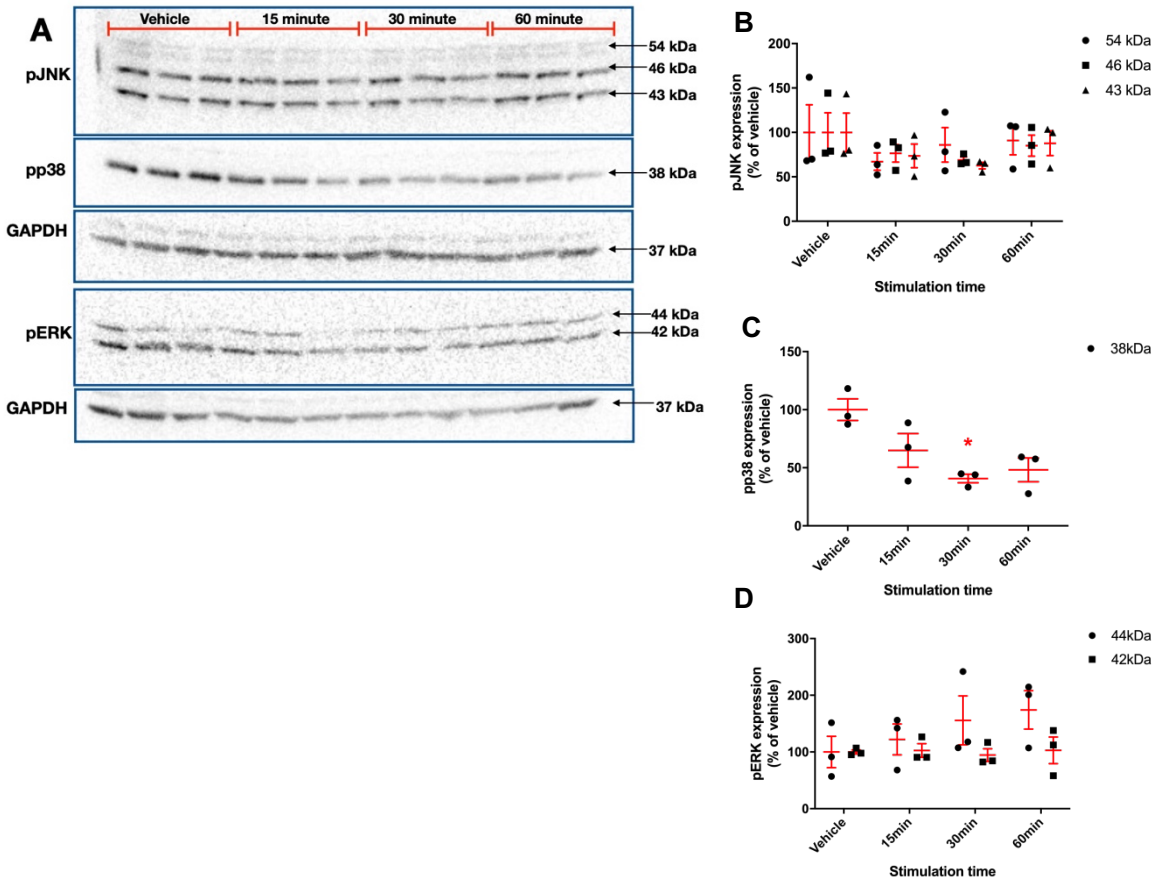

Figure 8. Poly I:C time course experiment. The microglial cells were cultured in serum-free medium overnight before treatment. The cells were treated by poly I:C (100ng/ml) for 15 minute, 30 minute and 60 minute. Depending on the size of proteins, pMAPKs were measured in two separate membranes, pJNK and pp38 were in a same membrane, pERKs were observed in the other membrane. **(A)** Poly I:C did not change the levels of pJNKs and pERKs, but the phosphorylation of p38 was suppressed by the treatment. **(B-D)** The level of pp38 was considerably reduced at 30 minute exposure, while pJNKs and pERKs were not statistically significant different from the vehicle condition over all exposure time. One blot image is shown for A. Individual data points are shown along with mean ± SEM (n=3). The data were analysed by two-way ANOVA, Tukey comparison (pJNKs, pERKs) and by one-way ANOVA, Bonferroni comparison (pp38) (*$p \leq 0.05$ vs. same size of isoform in a vehicle group). Details of ANOVA F values and p values are provided in Table 10.

The previous data suggest that the MAPK pathway is activated following TLR-mediated stimulation, but it may be time- and type-dependent. As JNKs, ERKs and p38 are the kinases which are at relatively late steps of the MAPK signalling pathway, effects of the upstream components on MAPK phosphorylation regulation are useful to understand a bigger picture of the signalling pathway. Although there are different MAPK3Ks (also called MAPKKK) (Figure 1), TAK1 and ASK1 are the main upstream components of the MAPK pathway in immune responses (Arthur and Ley, 2013). Therefore, measuring pMAPKs levels with TAK1 or ASK1 inhibition could give us an idea about the extended TLR-activated MAPK pathways in microglia.

Resiquimod was chosen for the stimulus because the microglia responded to resiquimod more sensitively than other immune stimuli (Figure 6). The microglial cells were prepared in the serum-free condition and stimulated with or without inhibitors, a TAK1 inhibitor (5Z-7)(Ninomiya-Tsuji et al., 2003) and a ASK1 inhibitor (NQDI)(Volynets et al., 2011) for 30 minutes, followed by resiquimod (3μM) for 15 minute. The cells were lysed, and the extractions were analysed by Western blot.

Clearly, resiquimod significantly induced the levels of pJNKs and pp38 ($p=0.000$, overall treatment effect Tukey post-hoc) which corresponded to the previous findings (Figure 9B,C). Certainly, pMAPKs were undetectable under the TAK1 inhibited condition. In addition, this observation was not changed by resiquimod stimulation, while the ASK1 inhibition did not cause any significant changes compared to the vehicle condition (overall inhibitor effect Tukey post-hoc test details are given in Table 10. Unfortunately, due to quality with the pERKs membrane and small sample size ($n=2$), statistical analysis could not be done in this experiment (Figure 9D). Despite of absence of statistical analysis, it was clear that pMAPK signals were undetectable after treatment with the TAK1 inhibitor.

In this experiment, GAPDH, which was used for the loading control in the previous experiments, was unable to be the loading control under the TAK1 and ASK1 inhibited conditions, as the signal was not detectable; thus the level of actin protein was used for new loading control. Moreover, morphological phenotypes, with TAK1 inhibition, suggested that the cells were in a “sick” condition; with shortened cell body and dark/black colour nucleus. This suggests that the cells are beyond the activation stage. Moreover. the absence of GAPDH signal suggests that the function of the cells is severely compromised. These data indicate that TAK1 is essential for microglial physiology, and potentially it is a main upstream compartment of the MAPK signalling pathways. Because TAK1 inhibition was essential to microglial survival, it is difficult to characterise the whole MAPK pathway as aimed. But, at least the previous investigation showed that SIM-A9 cells used MAPK pathways for TLR mediated signalling, and the results suggest that the cells definitely respond to pathogen-mimetic stimuli, even though there is some degree of sensitivity difference between pathogen types.

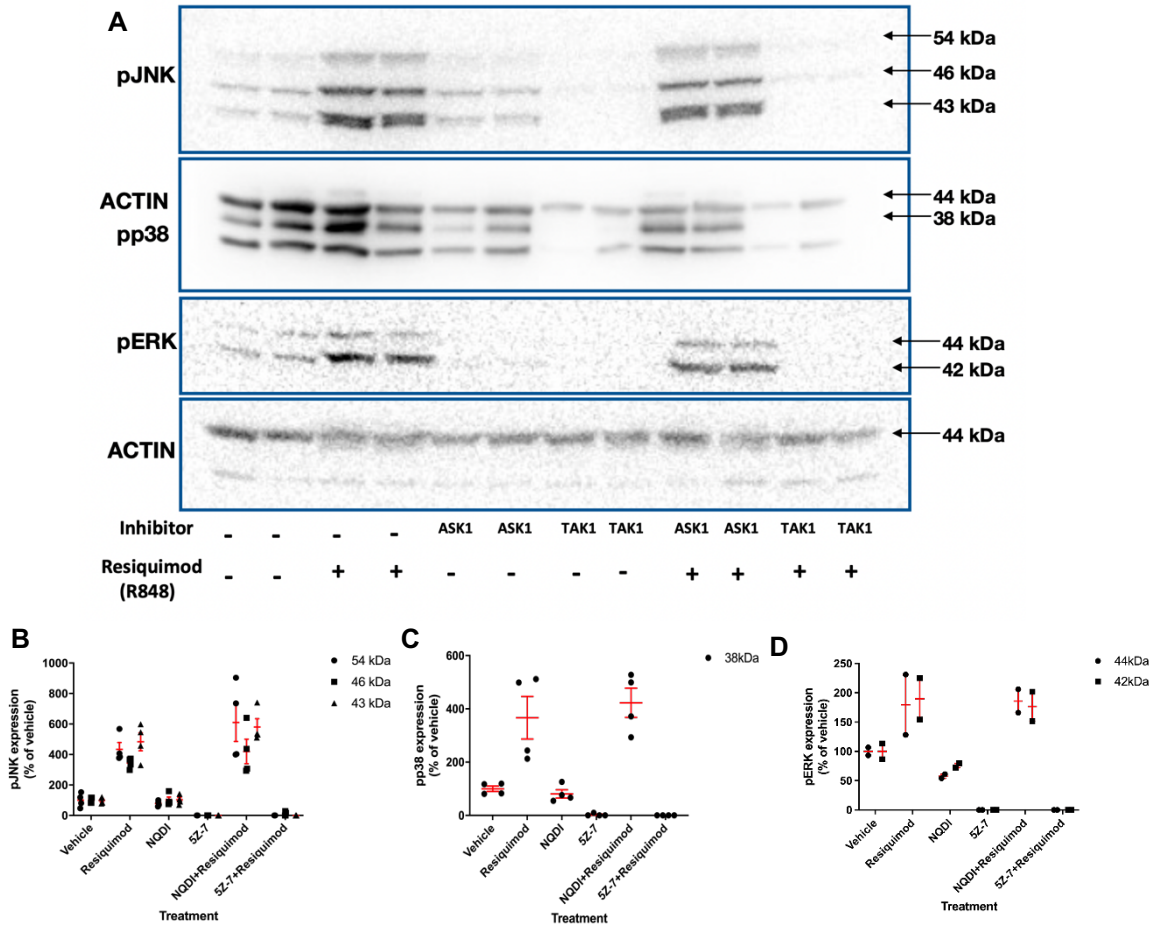

Figure 9. TAK1 activity is required for MAPK signalling in microglial cells. The microglial cells were cultured in serum-free medium overnight and treated with or without inhibitors for 5Z-7 (TAK1 inhibitor, 300nM) and NQDI (ASK1 inhibitor, 30μM) for 30 minutes, followed by vehicle or resiquimod (3μM) for 15 minutes. Depending on the size of proteins, pMAPKs were measured in two separated membranes, pJNKs and pp38 were on the same membrane, pERKs were observed in the other membrane. **(A)** The TAK1 inhibitor completely blocked phosphorylation of all three MAPKs, JNKs, ERKs, and p38 **(B-D)** pMAPKs were totally suppressed under the TAK1 inhibition. One representative blot out of two independent experiment images is shown for A. Individual data points are shown along with mean ± SEM (n=4 for pJNKs and pp38, n=2 for pERKs). Statistical details of total treatment and inhibitor effects of pJNKs and pp38 were given in Table 10. Because of the number of the samples, the level of pERKs could not be statistically analysed.

To further explore impacts of the immune molecules on SIM-A9's, the cells were stimulated with two chemokines, CXCL10 (10nM) and CXCL12 (10nM), for 15 minutes and pMAPKs were quantified. This experiment reproduced the findings of the primary cortical mouse neuronal culture with minor but significant changes in only the pJNKs (Figure 3 and Figure 10).

Initial investigation of pMAPKs activation after various environmental challenges on microglial cells was confirmed, although the cells did not respond to some of the conditions through the signalling pathways (Figure 6, Figure 8 and Figure 10). The cell responsivity might be pathogen-type dependent (Figure 6 and Figure 7). Additionally, TAK1 appears to be essential for microglial physiology (Figure 9).

These findings suggest that stimuli do induce MAPK signalling pathways, at least *in vitro* conditions (microglia). Because of this finding, with the MAPK pathway potentially participating after chemokine stimulation in microglial, we aimed to research further how deeply the MAPK pathway is involved in cytokine and chemokine production after the immune stimuli. In order to answer this, we should know initially how microglia respond (producing cytokines and chemokines) to immune stimuli (experimental condition), at various time points. We therefore exposed the cells to three different stimuli and measured cytokine and chemokine levels.

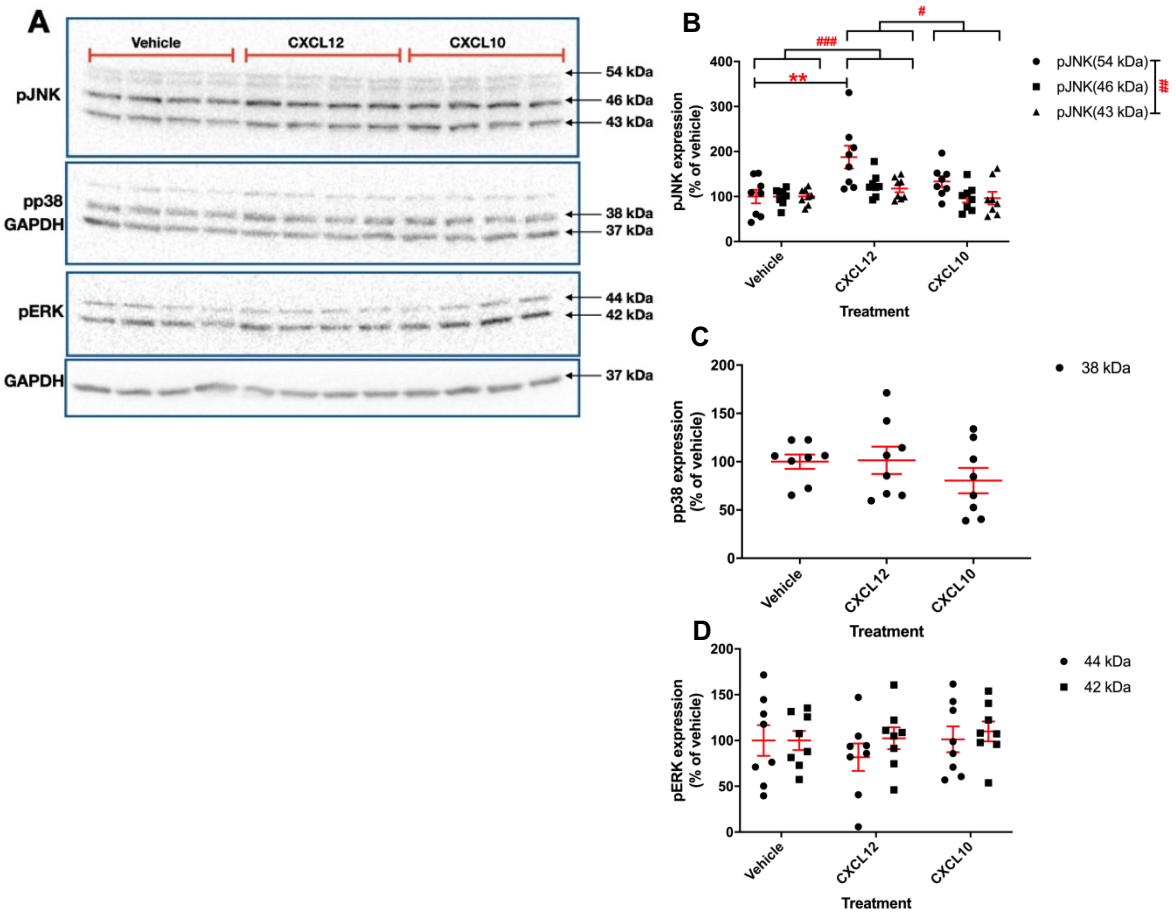

Figure 10. Chemokines do not induce MAPKs phosphorylation dramatically. The microglial cells were cultured in serum-free medium overnight, and treated with CXCL10 (10nM), and CXCL12 (10nM) for 15 minutes. Because of the size of proteins, pMAPKs were measured in two separated membranes, pJNKs and pp38 were in a same membrane, pERKs were observed in the other membrane. **(A, B)** The increased level of pJNKs with CXCL12 was significant compared to vehicle. **(A, C, D)** The levels of pERKs and pp38 levels were not changed by chemokine stimulation. One representative blot out of two independent experiment images is shown for A and B. Individual data points are shown along with mean ± SEM (n=8). The data were analysed by two-way ANOVA, Tukey comparison (pJNKs, pERKs) and by one-way ANOVA, Bonferroni comparison (pp38) $^{**}p \leq 0.005$ vs. same size of isoform in a vehicle group; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.005$, $^{\#\#\#}p \leq 0.001$, Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

4.3.3 Microglia show rapid pro-inflammatory cytokine changes

Cytokines and chemokines are immune molecules and as microglia are the primary immune cell type in the brain, they are potentially the major producer of immune molecules in order to defend any immune challenges and to maintain homeostasis (Gogoleva et al., 2019). As noted above, microglia are primary immune cells in the brain, producing cytokine and chemokine would be first considering their defence mechanism, therefore the levels of immune molecule changes could suggest directly and indirectly levels of inflammation and microglial activation.

To define cytokines' mRNA and protein level changes over time, the cells were stimulated with one of three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), at three time points, 0.5,8, and 24 hours in serum-free conditions. Two pro-inflammatory cytokines, *Il-6* (IL-6)*, Tnf-a* (TNF-α), which are thought as highly related to increasing the risk of schizophrenia (see section 1.2.5), were measured by RT-qPCR for mRNA and ELISA for protein quantifications in culture medium.

Overall, with LPS and resiquimod, not poly I:C, two pro-inflammatory cytokines, *Il-6,* and *Tnf-a* mRNA levels were increased compared to the control (Figure 11A,C). Interestingly, their trends were very different. *Il-6* mRNA level was gradually induced over time, nonetheless *Tnf-α* mRNA level was the highest at 0.5 hour stimulation, after its level was gradually decreased. IL-6 protein was detectable after 8 hour (0.5 hour levels were below the quantification limit, data were not shown) (Figure 11B), whereas *Il-6* mRNA was increased from 0.5 hour (Figure 11A). TNF-α protein was significantly induced from 8 hour with both LPS and resiquimod stimulation but not with poly I:C (Figure 11D). The data suggest that both *Il-6* and *Tnf-a* mRNAs are produced after TLR mediated stimulations, although their *Il-6* levels continuously increase whereas *Tnf-a* levels decline over the measured time points. The levels of IL-6 and TNF-α proteins released from the cells are increased regardless of level changes in mRNA over time.

These findings suggest that although IL-6 and TNF-α are both "pro-inflammatory cytokines", they may have sequential temporal roles: e.g. IL-6 mediates later pro-inflammatory signalling than TNF-α. Thus, pro-inflammatory cytokines may work in sequence over a longer time, rather than an acute effect in a short period.

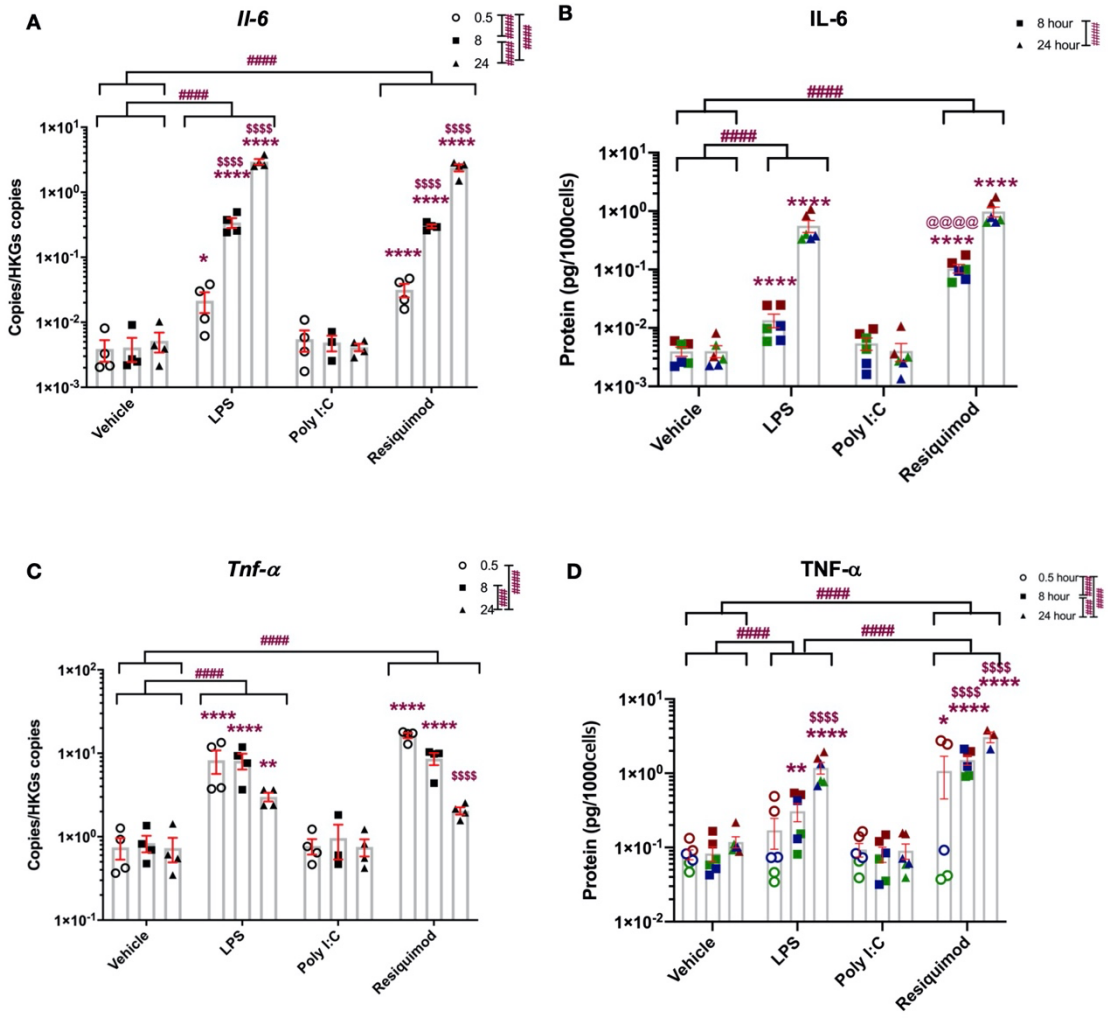

Figure 11. *Il-6 and Tnf-a* are induced in microglia after LPS and resiquimod stimulation. The cells were stimulated by one of three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), for the required time and RNA was extracted. **(A)** *Il-6* mRNA showed meaningful changes from 8 hour stimulation with LPS and resiquimod but not with poly I:C. **(B)** IL-6 release increased significantly from 8 and 24 hour stimulation with LPS and resiquimod. 0.5 hour stimulation data were below quantification limit. **(C)** *Tnf-α* mRNA was maximally increased at 0.5 hour after stimulation with LPS or resiquimod. **(D)** TNF-a release was significantly increased from 8 hour and this level continued to increase. Among all tested three different pro-inflammatory chemokines with stimulation time groups, poly I:C did not show any detectable changes in all conditions. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. Total amount of protein (per 1000 cells) was presented on graphs and its amount was measured by ELISA. The individual data points are shown along with mean ± SEM. The colour represents passages of the cells. The data were analysed by two-way ANOVA (RT-qPCR); or three-way ANOVA (ELISA), Tukey post-hoc test (n=4-6 independent samples; $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ vs. vehicle control at same stimulation time; $^{\$\$\$\$}p \leq 0.0001$ vs. 0.5 hour stimulation within the same treatment group; $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$ Tukey comparisons; $^{@@@@}p \leq 0.0001$ LPS vs. resiquimod in a same stimulation time Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

When inflammation occurs, anti-inflammatory cytokines are released to dampen pro-inflammatory cytokines' influences around the area. If their regulation does not function properly, further damage on cells or tissues can be caused, and this can contribute to development of diseases. IL-10 is one of the well-known anti-inflammatory cytokines, and its clinical evidence to schizophrenia has been suggested (see 4.1). As a prototypical anti-inflammatory cytokine, IL-10 is chosen to be examined in this study.

An anti-inflammatory cytokine*, Il-10,* mRNA was significantly increased at the 8 hour point with LPS (Figure 12). Overall, resiquimod also affected *Il-10* mRNA levels. This result suggests that, like pro-inflammatory cytokines, anti-inflammatory cytokine production could also rely on pathogen type, even though the differences may not be as noticeable as pro-inflammatory cytokines in early time points (before 24 hours) in microglia.

The data of cytokine production in mRNA and protein levels indicate that LPS and resiquimod had clear effects, but poly I:C did not. This was possibly linked to the low levels we observed for *Tlr3* mRNA expressions (Figure 5). Furthermore, protein levels were not exactly reflected by cytokine mRNA levels in SIM-A9 cells after immune stimuli administration. In line with these findings, we examined changes of another type of immune molecule, inflammatory chemokines, at mRNA and protein levels in 0.5, 8, and 24 hour points.

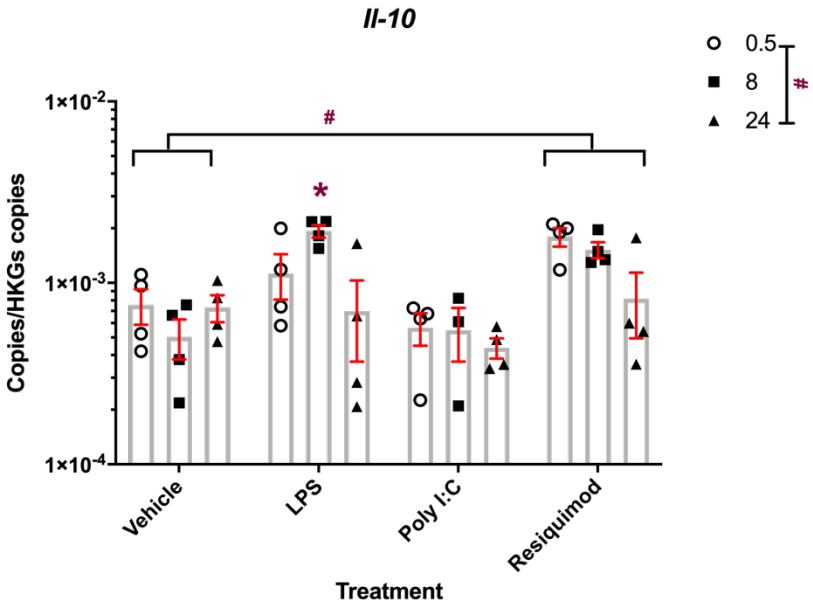

Figure 12. *Il-10* mRNA level is induced in microglia after resiquimod stimulation. The cells were stimulated by one of three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), for the required time and RNA was extracted. *Il-10* mRNA was up-regulated at 8 hour with LPS compared to vehicle. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. The individual data points are shown along with mean ± SEM. The data was analysed by two-way ANOVA, Tukey post-hoc test (n=4 independent samples; $^{*}p \leq 0.05$, vs. vehicle control at same stimulation time; $^{\#}p \leq 0.05$, Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

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4.3.4 Microglial cells show induced levels of inflammatory chemokines after pathogen mimetic stimulation

Chemokines are one of the subgroups of cytokines, originally known as chemoattractants. Recently their diverse functions have been reported, and hence many researchers are looking for their clinical applications. Three chemokines, *Ccl2, Ccl5,* and *Cxcl10,* were chosen for study, due to their relevance to schizophrenia, based on previous publications (see section 1.2.5). The microglial cells were stimulated with one of the three pathogen mimetics, and the samples were analysed at three time points via RT-qPCR and ELISA.

In contrast to the pro-inflammatory cytokines (Figure 11), the changes of these chemokines over time were almost consistent. *Ccl2, Ccl5* and *Cxcl10* mRNA levels were significantly induced from 8 hour with LPS and resiquimod and the induced levels of mRNAs from 8 hour were roughly remained until the 24 hour point. (Figure 13A, C, and E). In 0.5 hour stimulation, *Cxcl10* with resiquimod showed meaningful changes, but LPS did not (Figure 13E). Even though the cells responded to LPS more slowly than resiquimod to produce *Cxcl10* mRNA, they showed much higher levels at 8 hour and 24 hour. LPS and resiquimod induced the levels of mRNAs of chemokines significantly, however, consistent with the pro-inflammatory cytokine data, poly I:C did not affect the time course of changes in transcriptional chemokine expression (Figure 13A, C, and E).

Regardless of these significant changes in the levels of mRNAs over time, CCL5 and CXCL10 protein did not show any changes over time (Figure **13**D and F). Compared to the *Ccl2* mRNA levels, CCL2 protein showed its changes at a delayed time point (24 hour); however these roughly reflected the observed mRNA changes (Figure **13**B). Like TNF-α protein, CCL2 and CCL5 proteins showed statistical significances differences between LPS and resiquimod treatments while CXCL10 and IL-6 did not (Figure 11B and D, Figure **13**B, D and F). These findings indicate further that the results of immune stimulation, producing immune molecules, will vary according to the pathogen.

Consistently, the findings showed weak responses to poly I:C and this could be linked to the very low expression of *Tlr3* (Figure 5). A level of mRNA does not mean that the cells will produce a similar amount of proteins. In order to solve this question, time course experiments were performed (Figure 8, Figure 11, Figure 12 and Figure 13); if an mRNA level is low, more likely, a protein level will be low. Therefore, SIM-A9 cells hypothetically have a lower expression level of TLR3 compare to TLR4 and TLR7, and this could explain the constant findings of lack of response to poly I:C. Moreover, the findings emphasise that protein level does not always reflect mRNA level. However, these data give confidence that LPS and resiquimod were strong enough to induce elevated levelsof mRNA and protein of the cytokines and chemokines we measured. According to the Aims of the study, we determined the contribution of the MAPK to production of cytokines and chemokines, 0.5 and 8 hours were chosen to assess mRNA levels, and 8 hour for protein levels. Furthermore, at this stage, poly I:C was excluded because of consistent weak responses over a

series of experiments, suggesting a very limited role for Tlr3 signalling in microglial responses under these conditions.

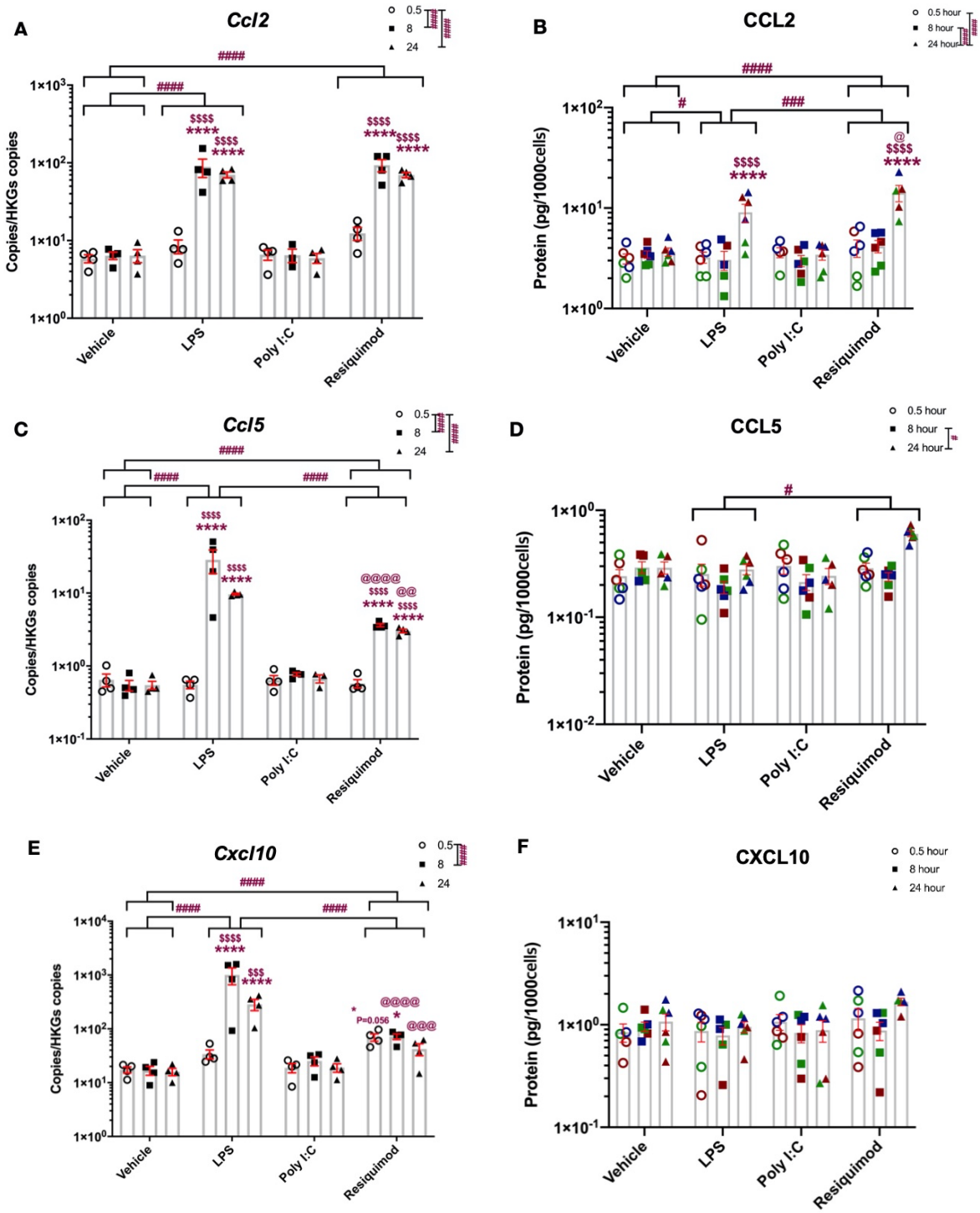


Figure 13. *Ccl2, Ccl5,* and *Cxcl10* are induced in microglia after LPS and resiquimod stimulation. The cells were cultured in serum-free conditions and left overnight. The cells were stimulated by one of three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), for the required time, and RNA was extracted. (A, B) *Ccl2* mRNA levels were increased by LPS and resiquimod after 8 hour stimulation. CCL2 protein level showed meaningful changes with LPS and resiquimod stimulation at 24 hour. (C, D) *Ccl5* mRNA showed considerable changes after 8 hour resiquimod stimulation and its level remained increased till 24 hours. CCL5 protein levels did not change. (E, F) *Cxcl10* mRNA increased from 8 hour after stimulation with LPS. Resiquimod showed a lesser induction from 0.5 hour and its level was no different from control levels by 24 hour. CXCL10 protein levels did not change at any timepoints with any treatments. Poly I:C treatment did not cause any changes in measured chemokines at any timepoint. Absolute

quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. Total amount of protein (per 1000 cells) was presented on graphs and its amount was measured by ELISA. The individual data points are shown along with mean ± SEM The data were analysed by two-way ANOVA (RT-qPCR); three-way ANOVA (ELISA), Tukey post-hoc test (n=4-6 independent samples; $^{*}p \leq 0.05$, $^{****}p \leq 0.0001$ vs. vehicle control at same stimulation time; $^{\$\$\$}p \leq 0.001$, $^{\$\$\$\$}p \leq 0.0001$ vs. 0.5 hour stimulation within the same treatment group; $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$ vs. Tukey comparisons; $^{@}p \leq 0.05$, $^{@@}p \leq 0.005$, $^{@@@}p \leq 0.001$, $^{@@@@}p \leq 0.0001$ LPS vs. resiquimod in a same stimulation time Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

4.3.5 MAPK effects on immune molecule production

The data have shown that immune molecules are generated from the microglial cells, but to understand whether MAP kinases are involved in this process still needs further studies. To determine their role, the cells were treated with vehicle, LPS or resiquimod, stimuli which showed significant changes from past experiments, for 0.5 and 8 hours, with MAP kinase inhibitors, JNK-IN-8 (JNKs), PD98059 (ERKs) or SB203580 (p38s), added prior to stimulus. The details of the inhibitors used, and the experimental conditions, were mentioned in Table 4 and Table 5. The samples were prepared, and mRNA levels were measured by RT-qPCR and protein levels were measured by ELISA. Because of the short exposure time, and observations from time course data, protein quantification at the 0.5 hour time point with MAPK inhibitors was not investigated; thus, the data of protein levels in cell culture medium (ELISA) analysis comes from 8 hour stimulation samples.

4.3.5.1 Cytokine mRNA production is affected by MAPK inhibition over time

At 0.5 hours, LPS and resiquimod treatment upregulated *Il-6* mRNA levels compared to vehicle (* indicates vs. vehicle under the same inhibition). In addition, overall effects showed that LPS and resiquimod were significantly different to the vehicle condition (Figure 14A).

Regarding MAPK inhibitor effects, no inhibitor effects were detected at 0.5 hour by 2 way ANOVA ($ indicated vs. stimulation within the same treatment group) (Figure 14A). In a 1 way ANOVA restricted to the resiquimod-stimulated samples, *Il-6* mRNA was increased by JNK inhibition with resiquimod (ǂ; $F(3,18)=13.07$, $P<0.0001$; resiquimod vs. resiquimod + JNK-IN-8, $p=0.0097$, Bonferroni post-hoc).

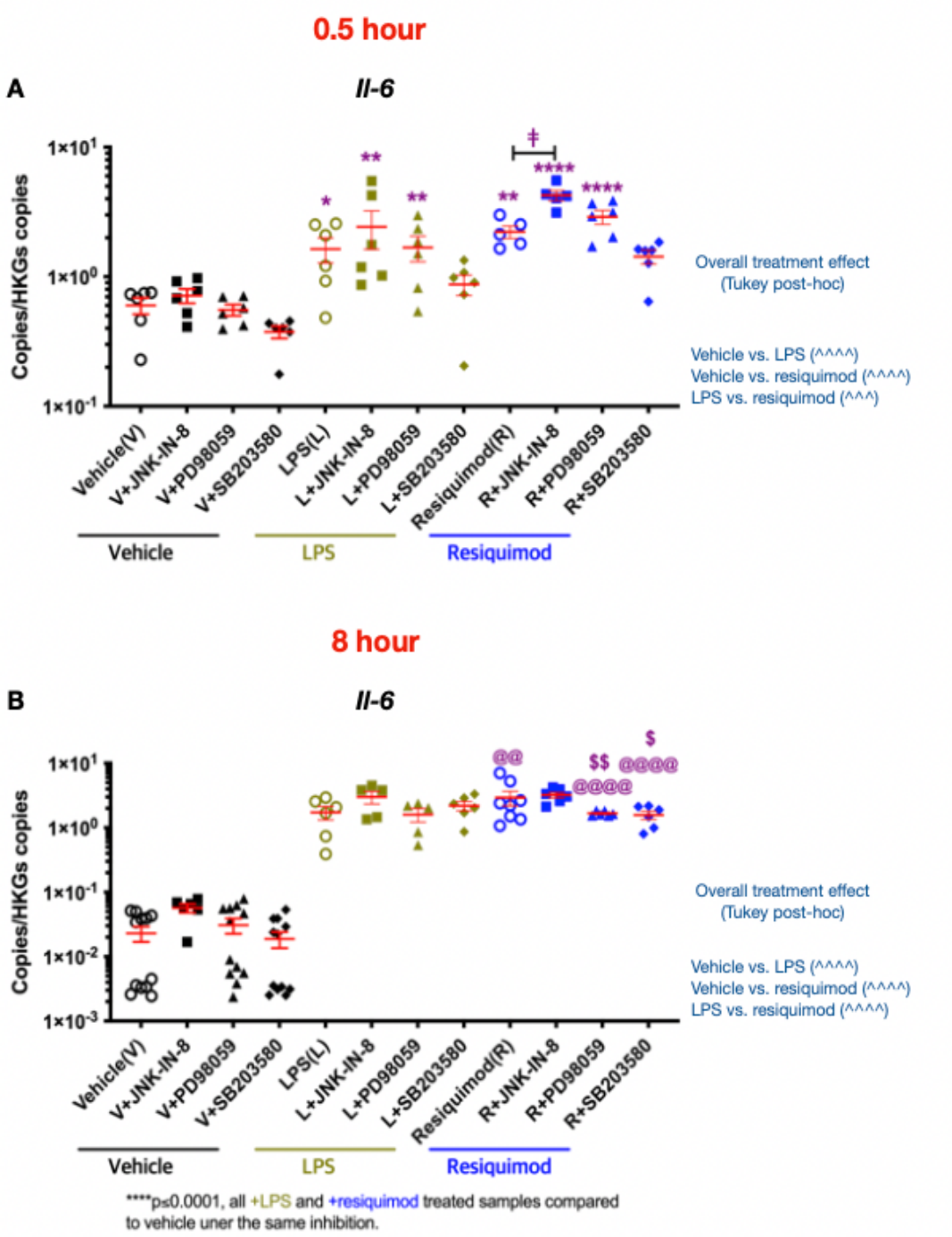


Figure 14. MAPK inhibition effects on *Il-6* mRNA production are detected under resiquimod, not LPS. The cells were cultured in serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), were added before the mimetics. **(A)** *Il-6* mRNA at 0.5 hour **(B)** *Il-6* mRNA at 8 hour. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA (RT-qPCR) Tukey post-hoc test (n=5-12 independent samples; $^{*}p\leq0.05$, $^{**}p\leq0.005$, $^{****}p\leq0.0001$ vs. vehicle under a same inhibitor; $^{\$}p\leq0.05$, $^{\$\$}p\leq0.005$ vs. vehicle alone within the same immune mimetic group; $^{\wedge\wedge\wedge}p\leq0.001$, $^{\wedge\wedge\wedge}p\leq0.0001$ overall treatment effects Tukey comparisons; $^{@@}p\leq0.005$, $^{@@@@}p\leq0.0001$ LPS vs. resiquimod in a same inhibitor Tukey comparisons; $^{\ddagger}P\leq0.05$ resiquimod vs. resiquimod + JNK-IN-8 within resiquimod treatment one way ANOVA Bonferroni post-hoc test). Details of ANOVA F values and p values are provided in Table 10.

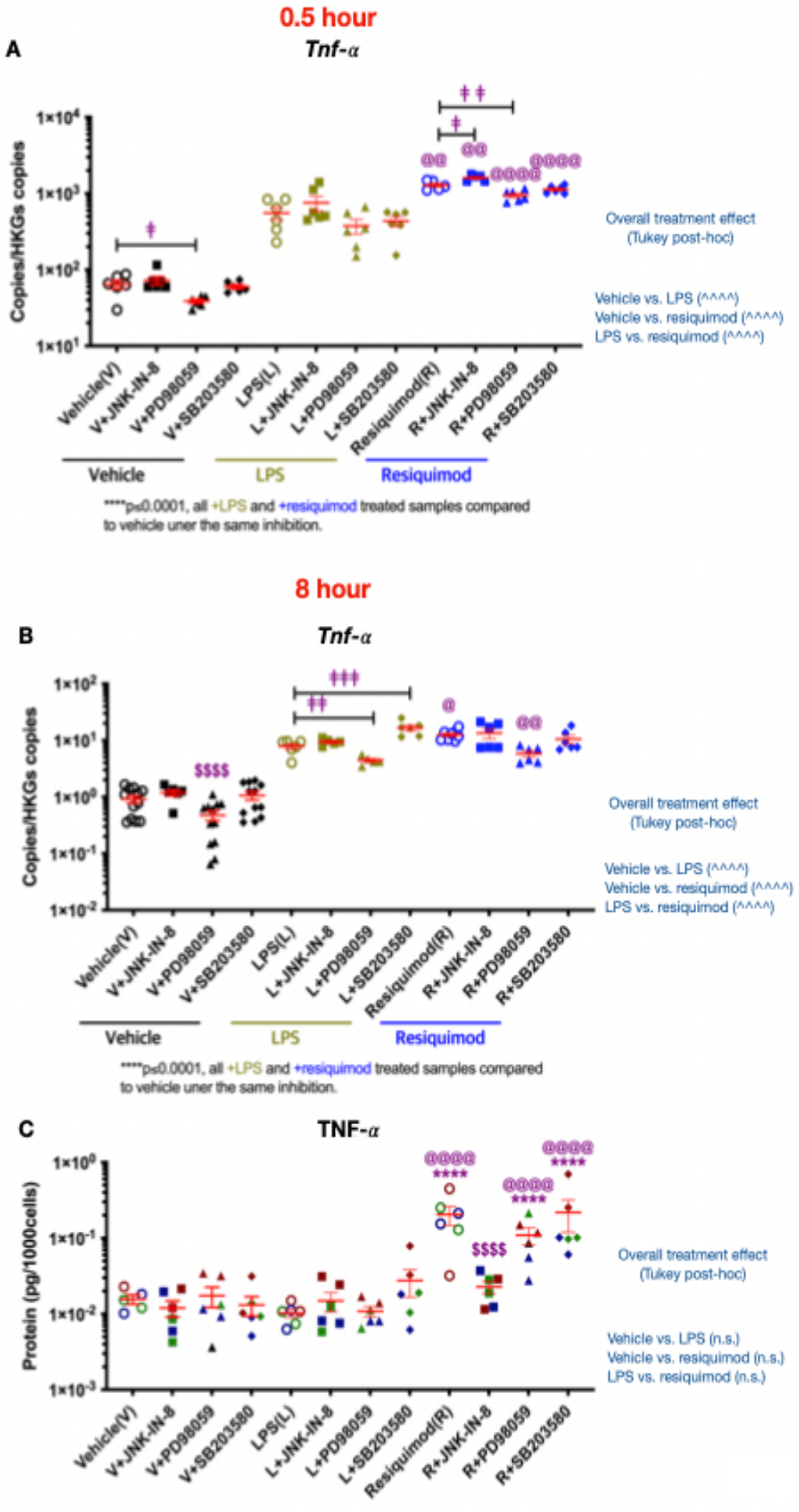
8 hours after stimulation, *Il-6* mRNA was induced by LPS and resiquimod stimulation (* indicates vs. vehicle under the same inhibition) (Figure 14B). In addition, overall effects showed that LPS and resiquimod were significantly different to the vehicle condition. MAPK inhibition effects were not seen after exposure to vehicle or LPS ($ indicated vs. stimulation within the same treatment group). It was only shown with resiquimod, with minor suppression of *Il-6* mRNA induction by inhibition of ERK or p38. IL-6 protein under these experimental settings was below the quantification limit (data not shown).

Since LPS and resiquimod are stimulating cells via different TLRs, it is worth investigating whether pathogen type-dependent effects on production of immune molecules are present. There were no differences between LPS and resiquimod at 0.5 hour (@ indicates LPS vs. resiquimod in a same inhibitor) (Figure 14A). However, at 8 hour stimulation, the levels of *Il-6* mRNA in the resiquimod alone condition was significantly different to those with LPS alone (more production); however, in combination with MAPK inhibitors, ERK and p38, there was significantly less production of *Il-6* mRNA level (Figure 14B).

At 0.5 hour, *Tnf-*α mRNA was significantly upregulated by both resiquimod and LPS compared to vehicle (Figure 15A). Regarding MAPK inhibitor effects, at 0.5 hour, ERK inhibition downregulated the level of *Tnf-*α mRNA level under the vehicle condition (ǂ; $F(3,20)=6.216$, $p=0.0037$; vehicle vs. vehicle + PD98059, $p=0.0153$) (Figure 15A). Moreover, with resiquimod, JNK inhibition upregulated the level of *Tnf-*α mRNA (ǂ; $F(3,18)=15.98$, $P<0.0001$; resiquimod vs. resiquimod + JNK-IN-8, $p=0.0394$, Bonferroni post-hoc); on the hand, ERK inhibition downregulated *Tnf-*α mRNA level (ǂ; $F(3,18)=15.98$, $P<0.0001$; resiquimod vs. resiquimod + PD98059, $p=0.0034$, Bonferroni post-hoc).

At 8 hours, *Tnf-*α mRNA was significantly upregulated by both resiquimod and LPS compared to vehicle; however, TNF-α protein level was only significantly upregulated under the resiquimod condition, except in combination with the JNK inhibitor (* indicates vs. vehicle under the same inhibition) (Figure 15B).

At 8 hours, *Tnf-*α mRNA was significantly downregulated by ERK inhibition compared to vehicle ($ indicated vs. vehicle within the same treatment group) (Figure 15B). Under the LPS condition, ERK inhibition significantly suppressed the level of *Tnf-*α mRNA, (ǂ; $F(3,18)=24.33$, $p<0.0001$; LPS vs. LPS+PD98059, $p=0.0079$, Bonferroni post-hoc), but p38 inhibition caused a significant upregulation of *Tnf-*α mRNA production (ǂ; $F(3,18)=24.33$, $p<0.0001$; LPS vs, LPS + SB203580, $p=0.0002$, Bonferroni post-hoc).


0.5 hour
A
Tnf-α
Copies/HKGs copies
1×10⁴
1×10³
1×10²
1×10¹
Vehicle(V)
V+JNK-IN-8
V+PD98059
V+SB203580
LPS(L)
L+JNK-IN-8
L+PD98059
L+SB203580
Resiquimod(R)
R+JNK-IN-8
R+PD98059
R+SB203580
Vehicle
LPS
Resiquimod
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (^^^^)
Vehicle vs. resiquimod (^^^^)
LPS vs. resiquimod (^^^^)
****p≤0.0001, all +LPS and +resiquimod treated samples compared
to vehicle uner the same inhibition.
8 hour
B
Tnf-α
Copies/HKGs copies
1×10²
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Vehicle(V)
V+JNK-IN-8
V+PD98059
V+SB203580
LPS(L)
L+JNK-IN-8
L+PD98059
L+SB203580
Resiquimod(R)
R+JNK-IN-8
R+PD98059
R+SB203580
Vehicle
LPS
Resiquimod
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (^^^^)
Vehicle vs. resiquimod (^^^^)
LPS vs. resiquimod (^^^^)
****p≤0.0001, all +LPS and +resiquimod treated samples compared
to vehicle uner the same inhibition.
C
TNF-α
Protein (pg/1000cells)
1×10⁰
1×10⁻¹
1×10⁻²
1×10⁻³
Vehicle(V)
V+JNK-IN-8
V+PD98059
V+SB203580
LPS(L)
L+JNK-IN-8
L+PD98059
L+SB203580
Resiquimod(R)
R+JNK-IN-8
R+PD98059
R+SB203580
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (n.s.)
Vehicle vs. resiquimod (n.s.)
LPS vs. resiquimod (n.s.)

Figure 15. MAPK inhibition effects on *Tnf-α* mRNA but not on protein levels. The cells were cultured in serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), were added before the mimetics. **(A)** *Tnf-α* mRNA at 0.5 hour **(B)** *Tnf-α* mRNA at 8 hour **(C)** TNF-α protein at 8 hour. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. Total amount of protein (per 1000 cells) was presented on graphs and its amount was measured by ELISA. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA (RT-qPCR); three-way ANOVA (ELISA), Tukey post-hoc test (n=5-12 independent samples; $^{****}p \leq 0.0001$ vs. vehicle under a same inhibitor; $^{\$\$\$\$}p \leq 0.0001$ vs. vehicle alone within the same immune mimetic group; $^{\wedge\wedge\wedge}p \leq 0.0001$ overall treatment effects Tukey comparisons; $^{@}p \leq 0.05$, $^{@@}p \leq 0.005$, $^{@@@@}p \leq 0.0001$ LPS vs. resiquimod in a same inhibitor Tukey comparisons; $^{\dagger}P \leq 0.05$,$^{\ddagger}p \leq 0.005$, $^{\ddagger\ddagger}p \leq 0.001$ Vehicle vs. Vehicle + PD98059 within Vehicle treatment, LPS vs. LPS + PD98059, LPS vs. LPS + SB203580 within LPS treatment, resiquimod vs. resiquimod + JNK-IN-8 within resiquimod treatment group one way ANOVA Bonferroni post-hoc test). Details of ANOVA F values and p values are provided in Table 10.

Under vehicle and LPS-stimulated conditions, TNF-α protein did not show any MAPK inhibitor effects ($ indicated vs. stimulation within the same treatment group) (Figure 15C). After resiquimod treatment, JNK inhibition significantly reduced TNF-α levels, but this change was not detected in mRNA.

At 0.5 hour, resiquimod treatment significantly upregulated *Il-10* mRNA levels compared to vehicle (* indicates vs. vehicle under the same inhibition). LPS treatment also up-regulated *Il-10* mRNA production. Minor effects of MAPK inhibition (ERK inhibition), further enhancing the effect of the stimulus, were detected under the resiquimod condition ($ indicated vs. stimulation within the same treatment group); however no significant MAPK inhibition effects were detected under LPS and vehicle conditions (Figure 16A).

At the 8 hour time point, *Il-10* mRNA levels were increased by resiquimod, but LPS did not significantly alter *Il-10* mRNA (Figure 16B). Moreover, significant effects of MAPK inhibition effects were not generally observed, except for p38 inhibition elevating *Il-10* mRNA levels after resiquimod application. Because of sample limitations, protein analysis is not available.

In short, SIM-A9 microglial cells show greater responses to resiquimod than LPS, at least at the doses used, in terms of producing *Il-10* mRNA and TNF-α protein. Additionally, MAPK inhibitions show different regulatory effects on inflammatory molecule production. For example, ERK inhibition downregulated *Tnf-α* mRNA levels at both 0.5 and 8 hours under the vehicle condition; however, ERK inhibition effects under resiquimod are only detected at 0.5 hour, and indeed there was no effect with LPS. Even though these findings suggest that MAPKs regulate cytokine production, this signalling does not affect cytokine production powerfully as imagined. Indeed, the regulation process is more complex than expected. This indicates that microglial cytokine production after TLR stimulation may be managed by other signalling pathway e.g. NF-κB pathway dominantly. Though the MAPK pathway seems that it is not used as a primary signalling pathway in microglial

immune reactions, it may be different for chemokine production. We therefore exposed the cells to same conditions but measured chemokine mRNAs and proteins instead.

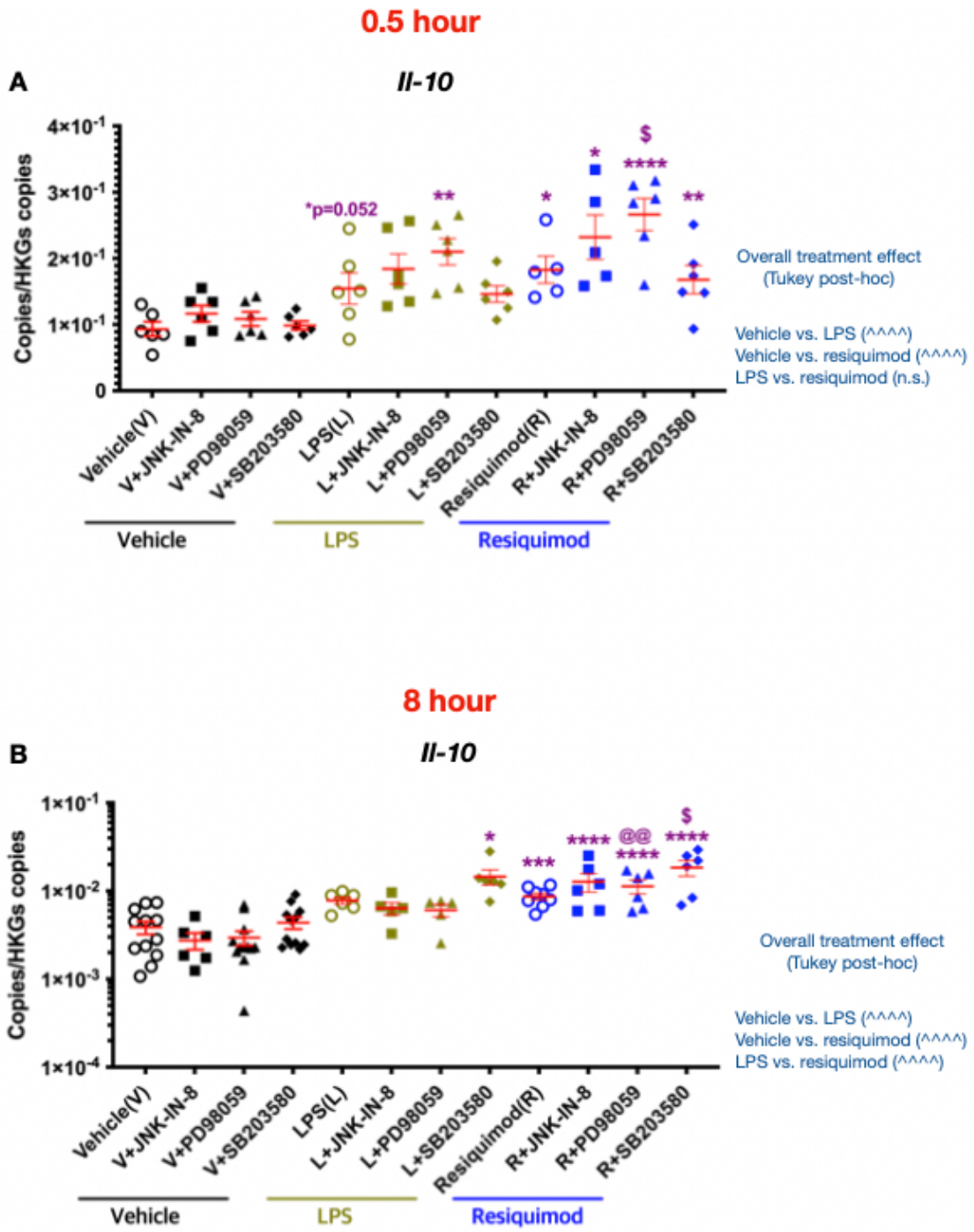

Figure 16. Effect of MAPK inhibition on *Il-10* mRNA are only detected under resiquimod. The cells were cultured in serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), were added before the mimetics. **(A)** *Il-10* mRNA was up-regulated by ERK inhibition with resiquimod compared to resiquimod alone condition. **(B)** *Il-10* mRNA was significantly up-regulated by resiquimod. Within the resiquimod condition, p38 inhibition caused up-regulation of *Il-10* mRNA compared to resiquimod alone condition. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA (RT-qPCR) Tukey post-hoc test (n=5-12 independent samples; $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ vs. vehicle under a same inhibitor; $^{\$}p \leq 0.05$ vs. vehicle alone within the same immune mimetic group; $^{@@}p \leq 0.005$ vs. LPS+ the same inhibitor; $^{\wedge\wedge\wedge\wedge}p \leq 0.00001$ overall treatment effects Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

4.3.5.2 Chemokine production is less significantly affected by MAPK inhibition over time

Considering the cytokine data, it is interesting to see how some selected chemokines, CCL2, CCL5 and CXCL10 relevant to psychiatric diseases are regulated under the same conditions.

At 0.5 hours, *Ccl2* mRNA level was not affected by LPS, except in combination with JNK inhibition, where an increase in expression was observed. Resiquimod exposure did increase *Ccl2* mRNA levels (* indicates vs. vehicle under the same inhibition) (Figure 17A), and here there was also a further enhancement with pretreatment with JNK-IN-8, (ǂ ; $F(3,18)=13.44$, $P<0.0001$; resiquimod vs. resiquimod + JNK-IN-8, $p=0.0133$, Bonferroni post-hoc) (Figure 17A).

At 8 hours, *Ccl2* mRNA was significantly upregulated by both resiquimod and LPS compared to vehicle; however, CCL2 protein level was not significantly changed by any treatments (* indicates vs. vehicle under the same inhibition) (Figure 17B,C).

Under the vehicle condition, ERK inhibition reduced *Ccl2* mRNA levels ($ indicated vs. stimulation within the same treatment group) (Figure 17B). Under the LPS condition, *Ccl2* mRNA was not changed by ERK inhibition, but p38 inhibition did upregulate mRNA levels. No significant MAPK inhibitor effects were detected with resiquimod on *Ccl2* mRNA.

With CCL2 protein, MAPK inhibition effects were not detected under the vehicle condition in CCL2 protein. Under LPS condition, CCL2 protein level was significantly increased by JNK inhibition ($ indicated vs. stimulation within the same treatment group). No significant MAPK inhibitor effects were detected with resiquimod on CCL2 protein. (Figure 17C).

It is worth noting that *Ccl2* mRNA production showed a clear LPS and resiquimod difference. There were no differences between LPS and resiquimod at 0.5 hour (@ indicates LPS vs. resiquimod in a same inhibitor) (Figure 17A). However, at 8 hour stimulation, the level of *Ccl2* mRNA in the resiquimod alone condition was significantly different to LPS alone (more production) (Figure 17B).

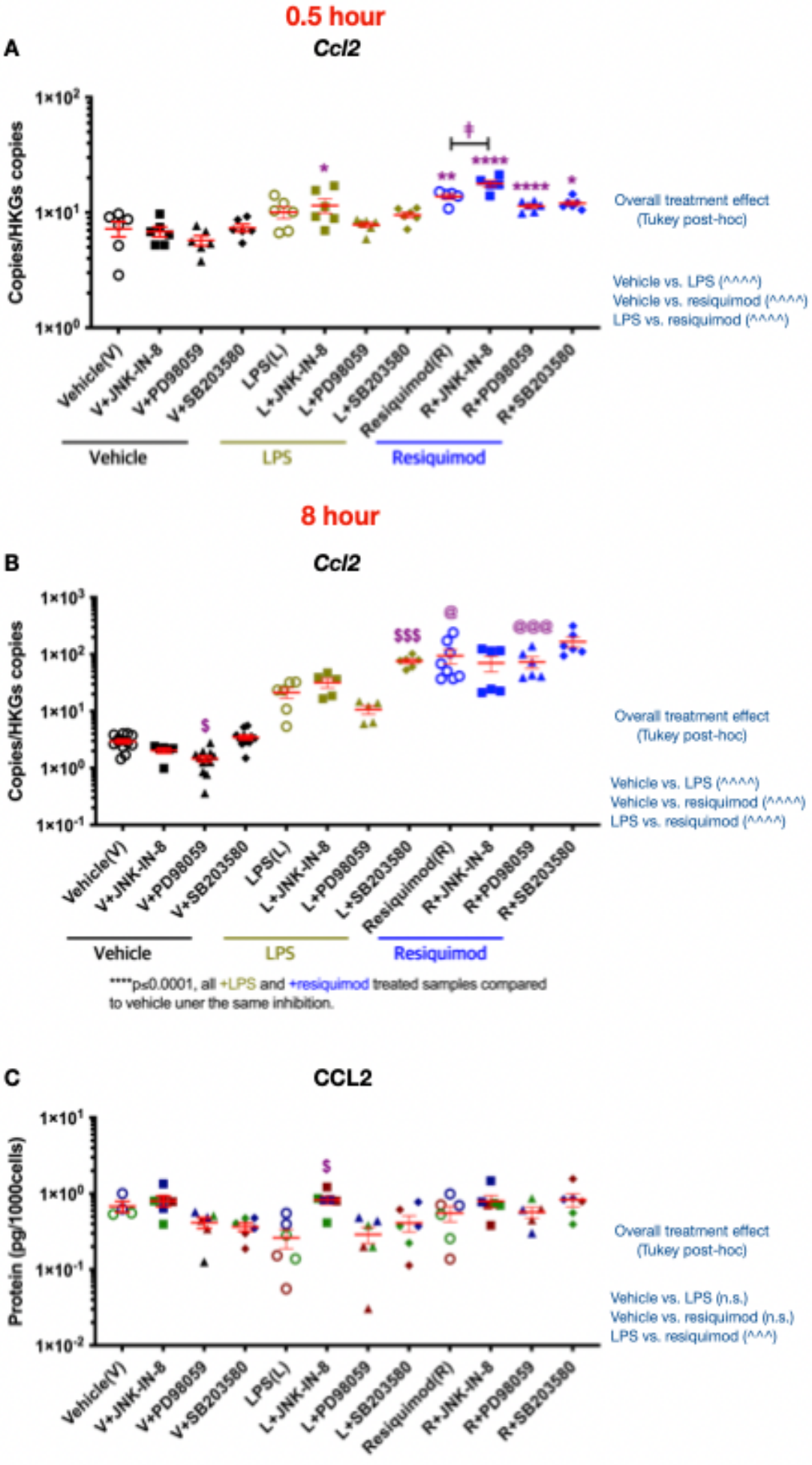
0.5 hour
A
Ccl2
Copies/HKGs copies
1×10²
1×10¹
1×10⁰
Vehicle(V)
V+JNK-IN-8
V+PD98059
V+SB203580
LPS(L)
L+JNK-IN-8
L+PD98059
L+SB203580
Resiquimod(R)
R+JNK-IN-8
R+PD98059
R+SB203580
Vehicle
LPS
Resiquimod
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (^^^^)
Vehicle vs. resiquimod (^^^^)
LPS vs. resiquimod (^^^^)
8 hour
B
Ccl2
Copies/HKGs copies
1×10³
1×10²
1×10¹
1×10⁰
1×10⁻¹
Vehicle
LPS
Resiquimod
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (^^^^)
Vehicle vs. resiquimod (^^^^)
LPS vs. resiquimod (^^^^)
****ps0.0001, all +LPS and +resiquimod treated samples compared
to vehicle uner the same inhibition.
C
CCL2
Protein (pg/1000cells)
1×10¹
1×10⁰
1×10⁻¹
1×10⁻²
Vehicle
LPS
Resiquimod
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (n.s.)
Vehicle vs. resiquimod (n.s.)
LPS vs. resiquimod (^^^)

Figure 17. MAPK inhibitors regulate *Ccl2* mRNA and protein production, but time- and pathogen dependent. The microglial cells were cultured in serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), or vehicle, were added before the mimetics. **(A)** *Ccl2* mRNA at 0.5 hour **(B)** *Ccl2* mRNA at 8 hour **(C)** CCL2 protein at 8 hour. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. Total amount of protein (per 1000 cells) was presented on graphs and its amount was measured by ELISA. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA (RT-qPCR); three-way ANOVA (ELISA), Tukey post-hoc test (n=5-12 independent samples; $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{****}p \leq 0.0001$ vs. vehicle under a same inhibitor; $^{\$}p \leq 0.05$, $^{\$\$\$}p \leq 0.001$ vs. vehicle alone within the same immune mimetic group; $^{@}p \leq 0.05$, $^{@@@}p \leq 0.001$ vs. LPS+ the same inhibitor; $^{\wedge\wedge\wedge}p \leq 0.001$, $^{\wedge\wedge\wedge\wedge}p \leq 0.0001$ overall treatment effects Tukey comparisons; $^{ł}P \leq 0.05$ resiquimod vs. resiquimod + JNK-IN-8 within resiquimod treatment group one way ANOVA Bonferroni post-hoc test). Details of ANOVA F values and p values are provided in Table 10.

Ccl5 mRNA was not upregulated by any treatments, and there were no MAPK inhibition effects on *Ccl5* mRNA, at 0.5 hours (Figure 18A).

At 8 hours, *Ccl5* mRNA was significantly upregulated by both resiquimod and LPS compared to vehicle; however, CCL5 protein level was not significantly changed by any treatments (* indicates vs. vehicle under the same inhibition) (Figure 18B). It is important that, whereas stimulation of *Ccl2* mRNA at 8 hours was greater with resiquimod than LPS, stimulation of *Ccl2* mRNA at 8 hours was greater with LPS than resiquimod. This implies that the differences in magnitude of effects are not simply due to non-corresponding doses of the two stimulants, but rather reflect differences in the effects of TLR4 vs TLR7 activation.

At 8 hours, MAPK inhibition effects were not detected under vehicle and LPS conditions in *Ccl5* mRNA levels (Figure 18B). With exposure to resiquimod, p38 inhibition showed that p38 could regulate *Ccl5* mRNA (upregulation) ($ indicated vs. stimulation within the same treatment group).

With CCL5 protein, MAPK inhibition effects were not detected under vehicle and resiquimod conditions (Figure 18C). LPS stimulation with JNK inhibition enhanced CCL5 protein release (ł; $F(3,16)=7.917$, $p=0.0018$; LPS vs. LPS+JNK-IN-8, $p=0.0014$; Bonferroni post- hoc).

The *Cxcl10* mRNA level was significantly affected by both LPS and resiquimod at 0.5 hours, and to a similar extent by both stimulants (* indicates vs. vehicle under the same inhibition, no overall significant difference between LPS and resiquimod groups) (Figure 19A).

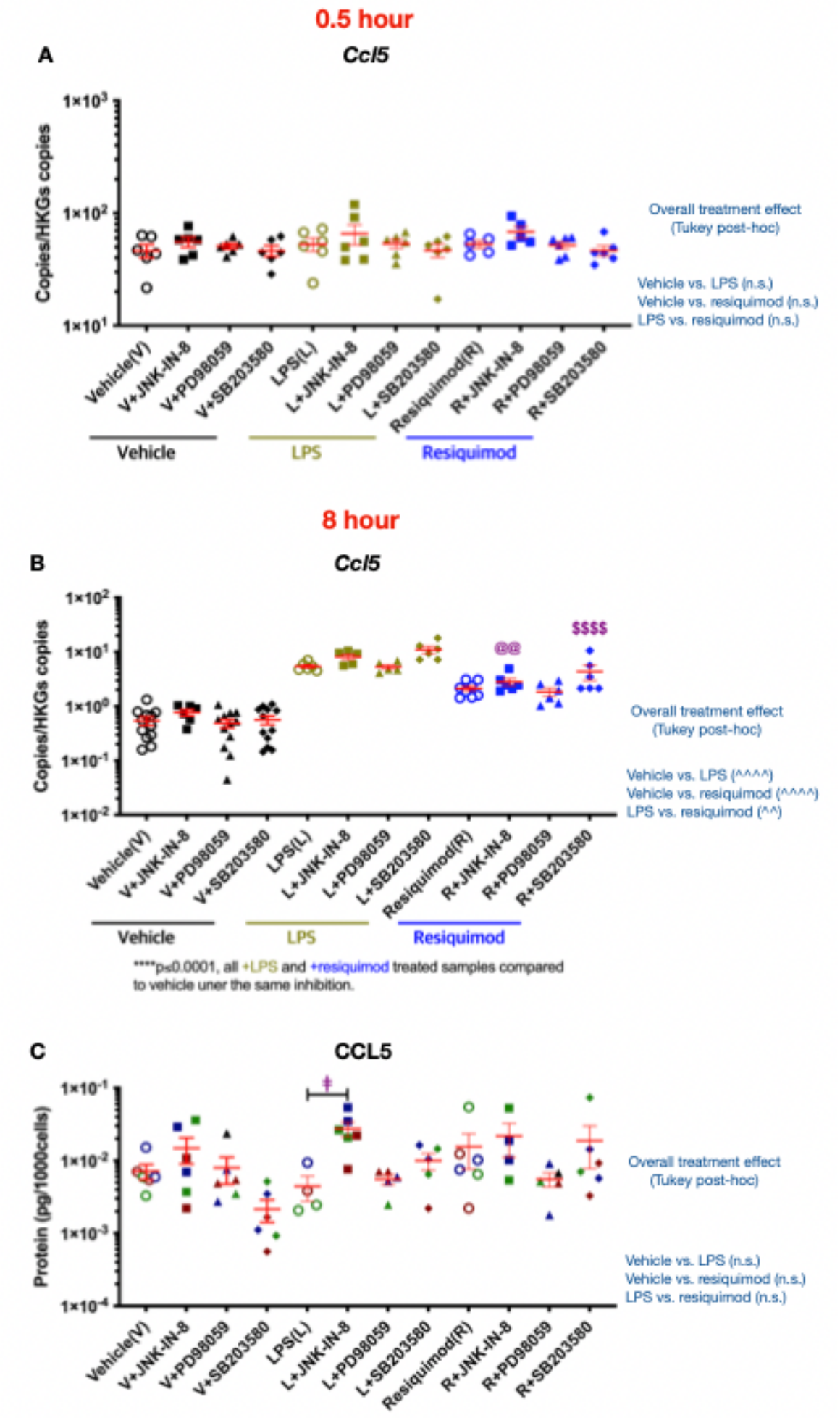
0.5 hour
A
Ccl5
Copies/HKGs copies
1×10³
1×10²
1×10¹
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (n.s.)
Vehicle vs. resiquimod (n.s.)
LPS vs. resiquimod (n.s.)
Vehicle(V)
V+JNK-IN-8
V+PD98059
V+SB203580
LPS(L)
L+JNK-IN-8
L+PD98059
L+SB203580
Resiquimod(R)
R+JNK-IN-8
R+PD98059
R+SB203580
Vehicle
LPS
Resiquimod
8 hour
B
Ccl5
Copies/HKGs copies
1×10²
1×10¹
1×10⁰
1×10⁻¹
1×10⁻²
$$$$
@@
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (^^^^)
Vehicle vs. resiquimod (^^^^)
LPS vs. resiquimod (^^)
Vehicle
LPS
Resiquimod
****p≤0.0001, all +LPS and +resiquimod treated samples compared
to vehicle uner the same inhibition.
C
CCL5
Protein (pg/1000cells)
1×10⁻¹
1×10⁻²
1×10⁻³
1×10⁻⁴
‡
Overall treatment effect
(Tukey post-hoc)
Vehicle vs. LPS (n.s.)
Vehicle vs. resiquimod (n.s.)
LPS vs. resiquimod (n.s.)

Figure 18. MAPK inhibitors regulate *Ccl5* mRNA and CCL5 protein in microglia 8 hours after LPS or resiquimod. The microglial cells were cultured in serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), or vehicle, were added before the mimetics. **(A)** *Ccl5* mRNA at 0.5 hour **(B)** *Ccl5* mRNA at 8 hour **(C)** CCL5 protein at 8 hour. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. Total amount of protein (per 1000 cells) was presented on graphs and its amount was measured by ELISA. The individual data points are shown along with mean ± SEM The data were analysed by two-way ANOVA (RT-qPCR); three-way ANOVA (ELISA), Tukey post-hoc test (n=5-12 independent samples; $^{\$\$\$\$}p\leq0.0001$ vs. vehicle alone within the same immune mimetic group; $^{@@}p\leq0.005$ vs. LPS+ the same inhibitor; $^{\wedge\wedge}p\leq0.005$, $^{\wedge\wedge\wedge\wedge}p\leq0.0001$ overall treatment effects Tukey comparisons; $^{\text{I}}P\leq0.05$ LPS vs. LPS + JNK-IN-8 within LPS treatment group one way ANOVA Bonferroni post-hoc test). Details of ANOVA F values and p values are provided in Table 10.

A lack of MAPK effects was detected, except again for JNK inhibition with resiquimod, where the presence of the inhibitor again resulted in greater mRNA induction $F(3,18)=5.5952$, $P=0.0053$; resiquimod vs. resiquimod + JNK-IN-8, $p=0.0294$, Bonferroni post-hoc) (Figure 19A).

At 8 hours, *Cxcl10* mRNA was significantly upregulated by both resiquimod and LPS compared to vehicle (* indicates vs. vehicle under the same inhibition) (Figure 19B).

At 8 hours, there was no evidence of MAPK effects on *Cxcl10* mRNA (Figure 19B). Like IL-6 protein, CXCL10 protein levels were below the quantification limit (data not shown).

It is worth noting that *Cxcl10* mRNA production showed a difference between LPS and resiquimod exposure. At 8 hour stimulation, the levels of *Cxcl10* mRNA after resiquimod were significantly less compared to those after LPS (@ indicates LPS vs. resiquimod in a same inhibitor) (Figure 19B).

Overall, the results show significant upregulation of the levels of cytokines and chemokines at 0.5 and 8 hours (^ indicates overall treatment effects), This indicates that LPS and resiquimod induced inflammation, which corresponds with the previous time course data. However, protein level did not reflect these changes. These results suggest that pro-inflammatory cytokine protein changes do not reflect mRNA changes at these stimulation times.

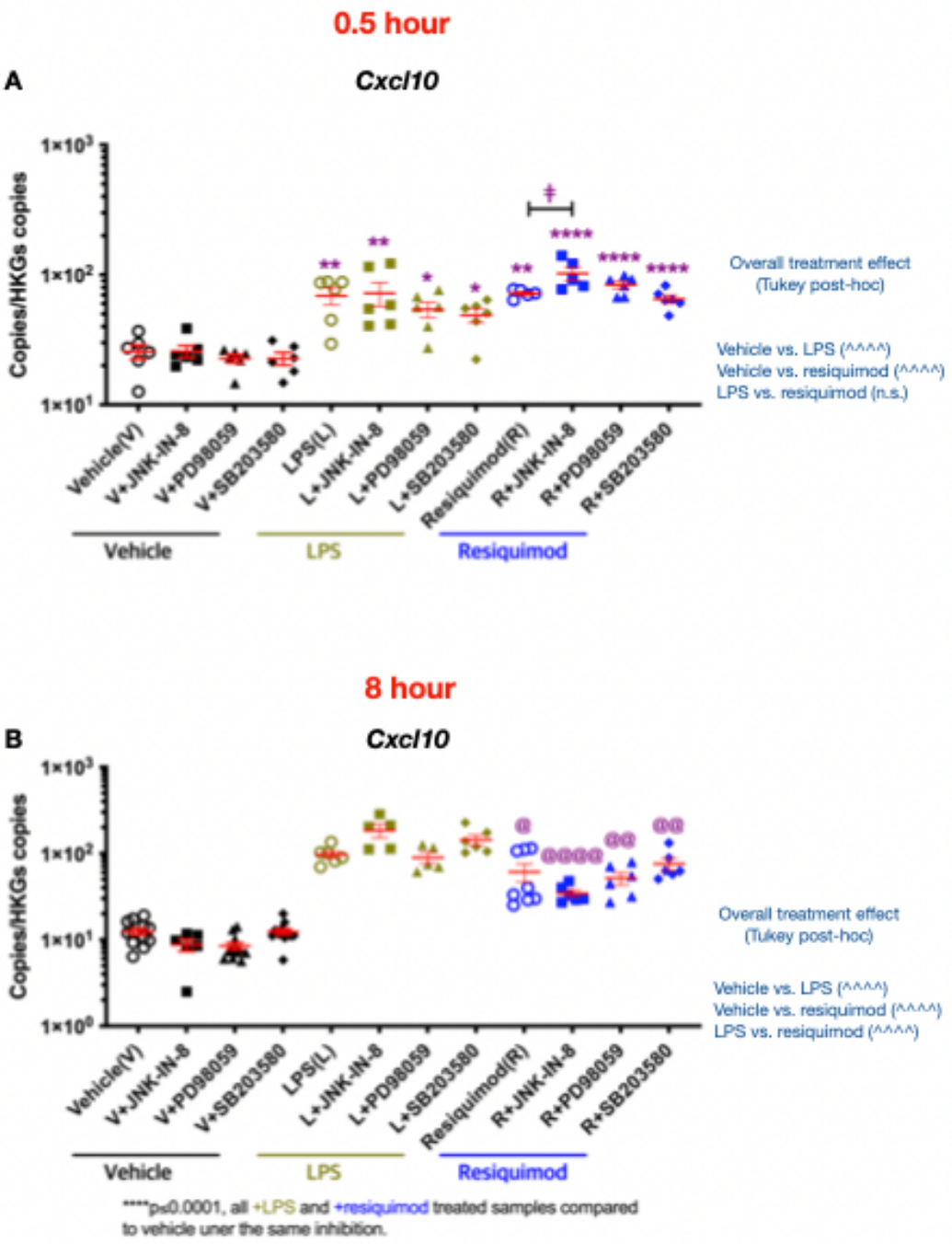

Figure 19. JNK inhibition affects *Cxcl0 10* mRNA levels at 0.5 hour after resiquimod, but LPS. The microglial cells were cultured in the serum-free medium and left overnight. The cells were stimulated by LPS (50ng/ml) or resiquimod (3μM) for 0.5 or 8 hour and MAPK inhibitors, JNK-IN-8 (JNK inhibitor, 1μM), PD98059 (ERK inhibitor, 40μM), or SB203580 (p38 inhibitor, 5μM), or vehicle, were added before the mimetics. **(A)** *Cxcl10* mRNA at 0.5 hour **(B)** *Cxcl10* mRNA at 8 hour. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA, Tukey post-hoc test (n=5-12 independent samples; $^{*}p\leq0.05$, $^{**}p\leq0.005$, $^{****}p\leq0.0001$ vs. vehicle under a same inhibitor; $^{@}p\leq0.05$, $^{@@}p\leq0.005$, $^{@@@@}p\leq0.0001$ vs. LPS+ the same inhibitor; $^{\wedge\wedge\wedge\wedge}p\leq0.00001$ overall treatment effects Tukey comparisons; $^{\dagger}P\leq0.05$ resiquimod vs. resiquimod + JNK-IN-8 within LPS treatment group one way ANOVA Bonferroni post-hoc test). Details of ANOVA F values and p values are provided in Table 10.

Additionally, MAPK inhibitions show different regulatory effects on inflammatory molecule production. For example, ERK inhibition downregulates the levels of *Tnf-α* and *Ccl2* mRNA; however, JNK (under resiquimod) and p38 inhibitions (under LPS) upregulate both genes' expression level. Together with previous findings, the data suggest that MAPKs participate in immune molecule induction as a part of microglial immune responses, but in a modulatory capacity, rather than as major regulators of the immune response.

4.3.6 Testing reliability of three microglial markers over time

Even though induction and release of cytokines and chemokines are good indicators to show inflammatory status, they do not show a microglia-specific situation. While a variety of evidence supports the importance of microglia for many aspects of CNS function, including inflammatory and immune responses, various research tools have been proposed over the decades for their study. For example, a few generations of radioligands, including those that bind to one of the mitochondrial membrane proteins named TSPO, have been introduced detect microglia *in vivo* (Mondelli et al., 2017). However, TSPO ligand binds to a site on mitochondria, and this site is not only expressed microglial mitochondria but also other cell types such astrocytes and neurons (Barresi et al., 2021). Not surprisingly, research results using TSPO as a microglial marker are not consistent (see section 1.2.5.3). Hence the reliability and accuracy of TSPO as a ligand to detect microglia specifically have been questioned recently (Notter et al., 2017b, Sneeboer et al., 2019). Another example is ionized calcium-binding adapter molecule 1 (IBA-1) protein (gene name: *Aif1*), used to detect microglia *ex vivo* and *in vitro*. This marker is widely used to measure levels of microglial activation in tissue (Ribeiro et al., 2013, Elmore et al., 2014, Bruttger et al., 2015). However, it is well known that IBA-1 is also expressed by macrophages. Using an IBA-1 antibody on cell culture may not be a big issue, because it is very unlikely that macrophage-lineage cell contamination will have occurred; otherwise, it could be a potentially big issue. Thus, IBA-1-positive cell populations always have the possibility to contain macrophages along with microglia, even though macrophages may not be a huge population in the brain. Additionally, TMEM119 is recently reported as a microglial-specific marker (Bennett et al., 2016), however its homeostatic functions are poorly studied.

As noted at the beginning of the chapter, SIM-A9 cells are possibly the closest cell line to *in vivo* microglia, and they are a pure microglial cell population; therefore, there are no interferential signals from other cell types. Thus, it is worth testing these microglial markers' reliability, regardless of the limitations of *in vitro* experimental settings. In order to confirm its changes over time especially after immune challenges, *Tmem119* mRNA levels were measured, together with *Tspo* and *Aif1* mRNAs. The cells were stimulated with one of the three pathogen mimetics for 0.5, 8, and 24 hours and cDNA was generated from RNAs and the sample analysis performed via RT-qPCR.

Under the experimental conditions, *Tspo* mRNA levels were not altered by any stimulus (Figure 20). On the other hand, *Aif1* mRNA level showed detectable changes (decreases) after 24 hour exposure to LPS or resiquimod. Compared to *Aif1* mRNA, *Tmem119* mRNA level was significantly

decreased from 8 hour and, after which its expression showed an even lower level by 24 hour after stimulation. Again, no evidence of altered expression under poly I:C stimulation was detected.

From the previous investigation, the microglial cells were surely activated, increasing the expression of pro-inflammatory cytokines and chemokines. However, the levels of none of these three microglial markers increased. Rather, expression of the marker not specific for immune cells (*Tspo*) was unchanged, and the expression of the two markers employed to label microglia was decreased.

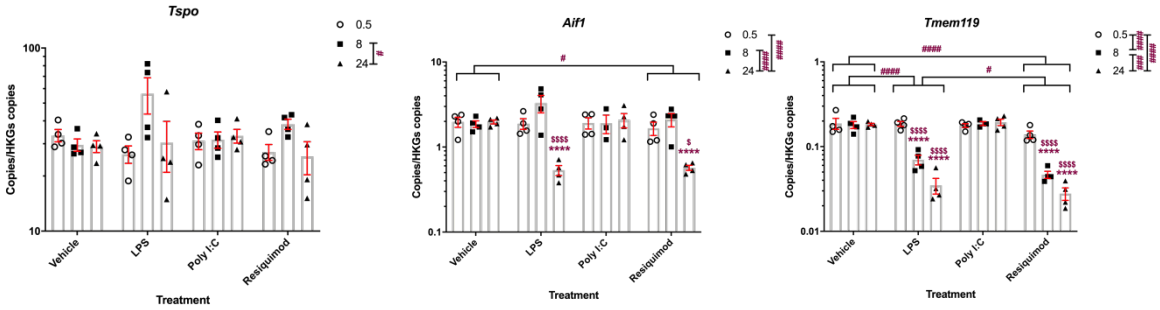

Figure 20. *Tmem119* mRNA level is changed by immune challenged. The cells were stimulated by three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), for required time and RNA was extracted. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Henmt1*. The individual data points are shown along with mean ± SEM. The data were analysed by two-way ANOVA, Tukey post-hoc test (n=4 independent samples; $^{****}p \leq 0.0001$ vs. vehicle control at same stimulation time; $^{\$}p \leq 0.05$, $^{\$\$\$\$}p \leq 0.0001$ vs. 0.5 hour stimulation within the same treatment group; $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$ Tukey comparisons). Details of ANOVA F values and p values are provided in Table 10.

4.4 Discussion

4.4.1 Summary of results

The work presented in this chapter has investigated how the MAPK pathways regulate microglial immune responses after TLR-mediated stimulation. The data indicate that SIM-A9 microglial cells respond to TLR4 (LPS) and TLR7/8 (resiquimod) agonists; however TLR3 agonist (poly I:C) administration did not initiate any immune reactions. Furthermore, the observations suggest a complex contribution of MAPK pathways to the regulation of the microglial immune response.

4.4.2 MAPK phosphorylation is increased by TLR mediated stimulation

The mouse microglial SIM-A9 cells showed different levels of pMAPKs after the stimulation. In spite of rapid responses to resiquimod, LPS stimulation requires a longer time to phosphorylate MAPKs, especially pERKs. These findings are similar to a previous report that primary microglial cells show a peak pERK levels in 20-30 minutes after TLR4 mediated stimulation (Bhat et al., 1998, Leow-Dyke et al., 2012). Conversely, viral-transduction immortalised microglial cell lines, such as BV-2 and N9, report delayed detection or very low levels of pERKs; indeed the cells are treated with much higher concentrations (1 and 5μg/ml) of LPS for longer exposer times (24 and 48 hours) (Watters et al., 2002, Le et al., 2004, Horvath et al., 2008). But these cell lines also show that induction of pJNKs and pp38 is relatively faster. This is consistent with the idea that SIM-A9 have closer cellular characteristics to primary microglia rather other previously used microglia cell lines.

In contrast to TLR stimulations, chemokine stimulations in general did not affect the levels of pMAPKs, though CXCL12 showed slight changes in pJNKs. pERKs are increased by CXCL12 in BV-2 cells, but it is time-dependent (Lu et al., 2009). In astrocytes, CXCL12 increases pERKs, not pp38 and pJNKs (Bajetto et al., 2001, Odemis et al., 2010). CXCL10 activates the p38 pathway in monocytes (Zhao et al., 2017), and the pERK pathway in neurons (Xia et al., 2000). Together with observations from this study and other publications, these probably indicate that pMAPK levels following chemokine stimulation are highly cell type- and time- dependent.

In order to have a bigger picture of the MAPK pathways in microglia, and in particular upstream of MAPKs, TAK1 and ASK1 were inhibited. However, the microglial cells showed shrink and dark cell body morphologies, which indicate close to death, especially under TAK1 inhibition. Even though morphological change is noticeable, the undetectable GAPDH protein is also the other evidence to tell how much TAK1 is vital to maintain microglial physiological homeostasis. This may not be surprising results because the importance of TAK1 has reported (Tang et al., 2008, Karin and Gallagher, 2009, Bettermann et al., 2010). But there is a contrasting finding from *in vivo* experiments that suggests that TAK1 expression may not be essential for microglia health (Goldmann et al., 2013). The finding in this study may be more dramatic because of the *in vitro* condition, which means that they are an almost pure microglial population and do not have any other cellular contributions, whereas the *in vivo* system could be supported by surrounding cells.

4.4.3 Immune responses after TLR mediated environmental challenges.

4.4.3.1 Consideration of current issues of Poly I:C

Compared to poly I:C and LPS, resiquimod is less studied in microglia. From this research, resiquimod was a strong stimulant of cytokine and chemokine release, consistent with its strong effects on MAPK phosphorylation. The expression level of *Tlr8,* was relatively low, suggesting that findings followed by resiquimod stimulation are mediated via TLR7 rather than TLR8. TLR4, a receptor for LPS, is expressed by other cells types in the brain, but microglia and neurons are the dominant cell populations to respond TLR4 mediated stimulation (Leow-Dyke et al., 2012), and this experiment supports microglial sensitivity to TLR4.

Previous findings consistently show low poly I:C effects on MAPK phosphorylation and immune molecule production. However, the lack of poly I:C response is highly unexpected. Many previous publications have used poly I:C as a stimulant, and see clear changes in rat microglial cultures (de Oliveira et al., 2016), neurons (Lafon et al., 2006, Peltier et al., 2010), and astrocytes (Kim et al., 2008).

This investigation reported relative low expression levels of *Tlr3* on microglial cells, compared to *Tlr4* and *Tlr7*. Microglia isolated from primate brain also express very low levels of TLR3 (Zuiderwijk-Sick et al., 2007). In addition, many scientific reports have shown that the level of TLR3 expression is dependent on the history of prior activation (Olson and Miller, 2004, Monguió-Tortajada et al., 2018). Together with the low expression level of TLR3, the concentration of poly I:C used could be raised as an issued. However, the concentration used here (100ng/ml) has previously been reported to significantly activate pMAPK signalling (de Oliveira et al., 2016), and increasing immune molecule expression/release (Ribes et al., 2010, Lehmann et al., 2012) in primary microglial culture. In addition, previous observations in our laboratory shows substantial *in vivo* systemic immune responses to this batch of poly I:C in mice (Openshaw et al., 2019). Therefore, the concentration and batch of poly I:C used here is unlikely to be an issue.

Even though many papers have reported poly I:C effects on MAPK activation or immune molecule secretion, they usually expose the cells under extreme conditions, for instance very high concentrations (50-100μg/ml) (Lafon et al., 2006, Town et al., 2006, Steelman and Li, 2011), with serum (Nakamichi et al., 2007), or very long exposure times (e.g. 48 hour) (Blasi et al., 1990). In fact, one study has reported poly I:C effects on microglia without extreme conditions (Das et al., 2016), but endotoxin contamination is now known to be a possible issue with the source of poly I:C used (Kowash et al., 2019), and hence TLR4 rather than TLR3 may be involved in the effects.

Hence, since the SIM-A9 cells are under these experimental conditions are showing low levels of TLR3 expression, this could be the main reason for the consistent lack of poly I:C responses throughout the project.

4.4.3.2 Microglial immune responses over time

It was clear from the time course experiment (Figures 7-9), that the microglial cells responded strongly to TLR 4 and TLR7/8 stimulation, in terms of pro-inflammatory cytokine release. Overall, although alteration of protein levels showed slight delays, compared to mRNA levels, as expected, some chemokine mRNA and protein level changes did not reflect the mRNA changes.

Il-6 mRNA and IL-6 protein continuously built their levels, but slowly, over time. In contrast, *Tnf-*α mRNA levels were at a peak in 0.5 hour, and TNF-a protein induction developed over time but faster than IL-6. This observation corresponded with data from mouse macrophages (Pauls et al., 2013). This different temporal release profile is interesting to note; however this finding is not unique to microglia for example, serum TNF is rise faster than IL-6 by endotoxin administration (Michie et al., 1988, Fong et al., 1989, Eskay et al., 1990).

Among the chemokines, *Ccl2* and *Ccl5* mRNA were induced from 8 hours and the raised levels are detected at 24 hour point. Though CCL5 protein levels did not mirror the changes in mRNA levels, CCL2 protein changes appeared by 24 hours, which reflects *Ccl2* mRNA changes to some extent. *Cxcl10* mRNA was upregulated from 8 hour under LPS stimulation, in contrast, with resiquimod the rapid changes were detected from 0.5 hours until 24 hours, although the upregulation of *Cxcl10* mRNA, compared to vehicle, achieved were less than those observed following LPS application. The CXCL10 protein did not alter. This rapid *Cxcl10* mRNA change caused by TLR7/8 stimulation is not surprising, because CXCL10 has an important role in antiviral responses (Trifilo et al., 2004, Melchjorsen et al., 2006, Skinner et al., 2018). In addition, similar effects have been reported *in vivo* from exposure to *Toxoplasma Gondii,* where *Cxcl10* mRNA expression is relatively higher and faster than that of *Ccl2 and Ccl2* mRNAs (Khan et al., 2000).

Il-10 mRNA was affected by LPS and resiquimod stimulation, but not by poly I:C. Among many anti-inflammatory cytokines, IL-10 was specially chosen in this study because meta-analysis showed that IL-10 is potentially related to schizophrenia (Maes et al., 2002, Kunz et al., 2011, Xiu et al., 2014). However, other anti-inflammatory cytokines, such as IL-4 and IL-13, could also give meaningful data because these molecules are involved in maintaining brain homeostasis (Shin et al., 2004, Zhao et al., 2015, Deczkowska et al., 2018).

Furthermore, one of the findings from the time course experiment is that pathogen types affect microglial immune responses differently, in terms of secretion of immune molecules. This is supported by other reports (Hemmi et al., 2002, Heil et al., 2004, Olson and Miller, 2004). For example, there is evidence that IL-6 and CCL2 proteins are less changed via ssRNA (TLR 7 agonist) stimulation compared to LPS, while TNF-α is highly induced in primary mouse microglia (probably in the presence of serum) (Lee et al., 2016). In addition, in terms of secretion of IL-6 and TNF-α from PBMC following exposure to resiquimod, the level of TNF-α is higher than that of IL-6 (Heil et al., 2004).

The data presented here suggest that the levels of induction of immune molecules by TLR4 and TLR7/8 stimulations are roughly equivalent, at the concentrations of immune mimetics used. But, there are interesting points to notice that *Tnf-α , Ccl2, Il-10* and *Cxcl10* mRNA levels are produced more with TLR7/8 stimulation whereas *Il-6* and *Ccl5* mRNA levels are produced more with TLR4 stimulation (Figure 11 - Figure **13**). This suggests recruitment of distinct signalling pathways downstream of TLR4 and TLR7 in microglia.

4.4.3.3 MAPK inhibitor effects on microglia immune responses

The MAPK inhibitor effects on microglial immune activation are very complex, moreover it appears that distinct pathways are activated in microglial cells by TLR4 and TLR7/8 stimulation.

Generally, the effects seen in the MAPK inhibitor experiment with cells exposed to vehicle treatment (rather than any of the inhibitors), at 0.5 and 8 hours, replicated the results obtained in the time-course experiment. Exceptions were the significant induction of *Cxcl10* mRNA by LPS, and the significant induction of *Ccl2* mRNA with resiquimod, detected at 0.5h in the inhibitor experiment. This almost certainly reflects the increased statistical power in the latter experiment.

ERK inhibition suppressed *Ccl2* mRNA expression and TNF-α protein levels in the absence of stimulation (vehicle-treated group), while JNK inhibition increased CCL2 and CCL5 protein levels after resiquimod treatment. ERK inhibition, but not JNK inhibition, also down-regulated *Tnf*-a mRNA production following both LPS and resiquimod administration. Again, this supports the idea that pathogen type could affect the sensitivity of the microglial reaction to stimulation. Even though p38 inhibition did not alter any microglial immune responses at 0.5 hours, at a longer exposure time (8 hours) it suppressed *Il-6 mRNA,* and enhanced *Tnf-α, Ccl2* and *Ccl5* mRNA levels.

No reduction in *Tnf-α* mRNA with p38 inhibition was found. In fact, mRNA levels were increased with p38 inhibition 8 hours after LPS, but not resiquimod, treatment (Figure 15). However TNF-α protein levels after TLR4 mediated stimulation is significantly reduced by p38 inhibition in mouse BMDMs, human monocytes and human microglia have reported (Lee et al., 2000, Rutault et al., 2001, Tessaro et al., 2017). However, there is some evidence to show no p38 inhibition effects on *Tnf-α* mRNA in primary rat glial culture (Bhat et al., 1998), and in monocytes (Scherle et al., 1998). Indeed recently, a positive relationship between p38 inhibition and *Tnf-α* mRNA has reported (Shah et al., 2016). The fact is noted above that the enhancing effect of p38 inhibition on *Tnf-α* mRNA is also seen with *Ccl2* and *Ccl5* mRNAs in longer exposure time (8 hour). This may suggest that some commonality in the regulation of this group of genes.

In contrast to *Tnf-α* mRNA, *Il-6* mRNA showed less significant changes caused by MAPK inhibition, and the release of IL-6 and CXCL10 from microglia after TLR4 and TRL7/8 stimulation could not be presented, as IL-6 and CXCL10 protein levels were below the quantification limit. This may be influenced by the time points selected, or the experimental conditions (e.g. adding DMSO). A possible reason is that DMSO, which is used for reconstituting MAPK inhibitors, can suppress

protein levels. Negative effects of DMSO on cytokine and chemokine production on various cell types have been reported (Kelly et al., 1994, Elisia et al., 2016, de Abreu Costa et al., 2017). Compared to other cytokines and chemokines, IL-6 and CXCL10 have shown particularly low levels at 8 hour even without MAPK inhibitors. Therefore, if DMSO negatively affects protein production, possibly their levels are too low to be quantified.

To understand the facilitatory role of JNK inhibition on *Il-6, Tnf-α, Ccl2* and *Cxcl10* mRNA expression, following TLR7, but not TLR4, stimulation, it is worth noting tumour necrosis factor receptor-associated factor 6 (TRAF6). This is an adaptor protein of TLR-mediated signalling pathways and this may contribute to signalling by TLR7 but not TLR4, and is involved in mediating JNK activation in various cell types (Kobayashi et al., 2001, Wan et al., 2004, Loniewski et al., 2007).

ERK inhibition suppressed *Tnf-α* mRNA (at both 0.5 and 8 hours) in the absence of any stimulation, and also after resiquimod; however its impact was not detectable in protein levels (8 hour). In contrast to the observations, ERK inhibition would decrease TNF-α protein in mouse BMDMs (16hour) and in human monocytes (6 hours) (Rutault et al., 2001, Tessaro et al., 2017). Conversely, ERK inhibition would increase *Ccl5* and *Cxcl10* mRNA levels in response to LPS (6 hour), while suppressing *Ccl2* mRNA (Bandow et al., 2012). In human primary microglia, ERKs and p38 are involved in CXCL10 and CCL2 production, but neither pathway is involved for CCL5 (Aversa et al., 2004). However in mouse microglial cell line, p38 inhibition suppresses CCL5 and CXCL10 protein production, where ERK inhibition positively affects CCL5 but suppresses CXCL10 release (Nakamichi et al., 2005). These pieces of evidence suggest that outcomes of MAPK inhibition are strongly dependent on the cell types involved.

The effects of JNK inhibition were relatively clear at 0.5 hour, especially with resiquimod stimulation, however only isolated effects were detectable 8 hours after stimulation. The lack of *Tnf-α* mRNA and TNF-α protein alterations by inhibition of JNK pathways, following exposure to LPS, is completely unexpected, just as with p38. Actually, JNK inhibition suppressed the TNF-α protein response to resiquimod, however this effect was not detected at the mRNA level. Consistent with a complex role for JNK in response to TLR4 stimulation, primary microglia from JNK1 knockout mice reportedly show an increased release of TNF-α protein following TLR4 agonist exposure (Hidding et al., 2002). Furthermore, IL-6 and TNF-α proteins from BV-2 microglial cell line and mouse BMDMs are significantly affected by JNK inhibition (SP600125, 25μM) following exposure to TLR4 stimulation (Sánchez-Tilló et al., 2007, Wang et al., 2010). However, Waetzig and colleagues show that JNK inhibition, caused by SP600125 with various concentration, in response to LPS did not affect IL-6 and TNF-α protein production from primary rat microglial culture (Waetzig et al., 2005). These findings suggest that the JNK pathway has a modulatory role, but may not be the main pathway in terms of regulating immune molecule production.

Among the chemokines, regulatory effects of JNK signalling were detected. At the early time point, *Ccl2* and *Cxcl10* mRNAs were elevated by JNK inhibition with resiquimod, but not with LPS. In

contrast, after longer stimulation, CCL2 and CCL5 proteins were enhanced by JNK inhibition with LPS, but not with resiquimod. This result is corresponded with the findings from primary microglia which show CCL2 is induced after the JNK inhibitor (SP600125) application with LPS (Waetzig et al., 2005). As noted above, CCL5 and CXCL10 protein levels are not changed by JNK inhibition (SP600125) following by viral infection in mouse microglial cells (Nakamichi et al., 2005). Again, this reflects the distinct TLR-dependent signalling pathways recruited by different stimulators in order to initiate microglial immune responses.

The mixed messages regarding the effects of JNK inhibition on the immune response may be partly because of the use of SP600125 to inhibit JNKs. This compound has been known as a selective JNK inhibitor, but recently it has been reported that SP600125 is not selective for JNKs, and that JNK-IN-8 has much better selectivity (Bain et al., 2007, Zhang et al., 2012). In our own investigation, SP600125 and JNK-IN-8 showed meaningful differences in terms of cytokine mRNA secretion (Figure 35), such as *Il-10* and *Cxcl10* mRNAs reduction by SP600125 which was not observed under JNK-IN-8 compared to control. This is almost certainly because SP600125 is blocking not only JNKs, but also other signalling molecules such as serum- and glucocorticoid-regulated kinase 1 (SGK1) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Bain et al., 2007).This observation suggests that (lack of) inhibitor selectivity most likely contributes to the variable results obtained in other studies.

The NF-κB pathway is well known to be a part of immune signalling in macrophage lineage cells. Because this study shows that the MAPK pathway affects, but not as the main pathway, microglial immune responses (cytokines and chemokines); thus, as a positive condition, to check that the microglial cells' immune responses are regulated largely as expected, in one experiment the NF-κB pathway was blocked by a IKKβ inhibitor (BMS345541). However, measurement of cytokine and chemokine induction was not possible, and the experiment had to be terminated because the cells died. (Figure 34). The longest time that could be achieved after inhibitor application, with the cells maintaining some viability (from morphological appearance) was 3 hours, which is too short for the experiment. Therefore, the IKKβ inhibitor (BMS345541) cannot be used as a positive control condition. This observation suggests that the NF-κB pathway is not only critical for immune responses, but also microglia viability. Since the NF-κB pathway is a downstream branch of TAK1 signalling, this likely is connected to the similar effects seen with TAK1 inhibition.

Moreover, throughout the study, cell passage affects cell reactivity even though it is within a healthy range. According to characterisation data from Nagamoto-Combs and others, SIM-A9 cells maintain their cellular properties, such as cytokine secretion and membrane protein expression, after 40 passages (Nagamoto-Combs et al., 2014). All samples in this study were collected within in 3 passages (14 – 17 passages) so that they are considered maintaining SIM-A9 cellular properties; however, our own observation showed that cell passage number significantly affected microglial immunity. Although evidence from bone marrow cells suggests that they keep their characteristics from 4 to 24 passages (Kwist et al., 2016), which may suggest before 20 passage is still a young age to use, immune cells may be very different. A possible reason to raise is genomic

instability (Li et al., 2019a). Recently Ben-David and others report a surprisingly high percentage of non-silent single nucleotide variations (SNV) in rapidly proliferating cells, furthermore these modifications can affect sensitivity to drugs (Ben-David et al., 2018). We generally consider that a cell line passes its characteristics to the next generation with almost no changes. However, it may not be true. Thus, in our experimental condition, the cell passage number is still young, nevertheless there may be more factors to affect cellular reactivity.

Throughout the studies, mRNA and protein levels do not reflect each other as much as we expected. It is possible that the time points we measured are not long or frequent enough to detect their corresponding changes. However, there is another explanation that can be suggested: that post-transcriptional/translational control mechanisms are very important. Vogel and Marcotte have proposed that a more comprehensive dynamic range of regulation is generally observed for protein as compared to mRNA and this is probably a reason of differing effects on mRNA vs. protein in prior observation (Vogel and Marcotte, 2012).

To sum up, microglial cells (SIM-A9) strongly responded to TLR 4 and TLR7/8 stimulation in terms of pro-inflammatory cytokine release. At a short exposure time, JNK and ERK inhibition, but not p38 inhibition, affect mRNA production from microglial cells after TLR4 and TRL7/8 stimulation; indeed MAPK inhibition effects are stronger with TLR 7/8 than with TLR4 stimulation. On the other hand, at a longer exposure time, p38 and JNK inhibition influence microglial immune responses, the former acting on mRNA, the latter on protein, but ERK inhibition effects have disappeared. The evidence suggests that MAPKs are involved in the regulation of microglial immune reactions, but the regulation is complex, and depends on the stimulus.

4.4.3.4 Microglial markers

In order to study microglia, a few *in vivo* and *in vitro* markers have been introduced. However, these markers have certain limitations, such cell type specificity, and expression level stability across developmental stages. In this study, three makers, *Tspo, Aif1, and Tmem119* mRNA were measured. Even though this study was done using an *in vitro* system, an advantage is eliminating interfering signals from other cell populations which are the main concerns with TSPO and IBA-1. Moreover, SIM-A9 cells could have a closer characteristic to primary microglia than other microglial cell lines. Therefore, any changes of these microglial makers are purely reflecting microglia activation level depending on exposure conditions.

Tspo mRNA levels were not changed by any TLR stimulations. TLR4 stimulation reportedly induces TSPO protein levels in BV-2 cells (Bae et al., 2014) and primary mouse microglia (Owen et al., 2017). Lee and colleagues have reported that in primary mouse microglia culture, TSPO protein levels were affected by LPS, but not by an ssRNA mimetic (Lee et al., 2016).

In contrast to *Tspo*, *Aif1* mRNA level was suppressed at 24 hour by both LPS and resiquimod stimulation, but not by poly I:C. This suggests that *Aif1* mRNA level does not really mirror microglial activation changes; moreover its level may be time- and environment- dependent. Regarding

TSPO and IBA-1 (Gene name: *Aif1*), the main problem of using these markers is that they are not microglial specific. Although TSPO is dominantly expressed by glial cell population in the brain, it is meaningfully expression on other cell population (Notter et al., 2017a, Sneeboer et al., 2019). Furthermore, a detected signal within the glial cell population could be mainly from astrocytes, not from microglia, and its strength is depending on the background conditions (Lee et al., 2016). This is a possible explanation of inconsistency result of across TSPO PET studies (see section 1.2.5.3). In the same way, IBA-1 is also expressed by not only microglia but also periphery macrophage population (Imai et al., 1996, Tanaka et al., 2003). In spite of its specificity issue, lots of papers have referred to IBA-1 positive cells as microglia in the brain.

Recently, a new microglial marker, called TMEM119, which known to be the most specific to the microglial population, has been introduced (Bennett et al., 2016). Even though it is a microglia-specific marker, its biological functions in microglial cells are not fully studied yet. The data from this SIM-A9 experiment have suggested that *Tmem119* mRNA was significantly reduced by LPS and resiquimod at 8 and 24 hours. The level of reduction is greater with resiquimod stimulation than LPS stimulation (at the doses used). A recent paper has reported that TMEM119 is involved in microglial homeostatic function, and its mRNA is down-regulated in pathological conditions (Masuda et al., 2019) which corresponds with the findings from this research. Furthermore, Shemer and colleague have shown that *Aif1* and *Tmem119* are down-regulated by LPS stimulation (Shemer et al., 2020); again, this supports the changes presented in Figure 20.

These markers are generally used in IHC (measuring protein levels); however, in this study, because of time and sample limitations only RT-qPCR analysis is available. As discussed above, mRNA and protein levels do not always reflect each other's levels. Therefore, protein levels of each marker may be different, however our observation (mRNA levels) contributes to ideas as to how these "microglial" markers are changed over time in the conditions.

4.4.4 Summary

In this chapter, the microglial immune response following various TLR-mediated stimulations was studied over time, and the complexity of the MAPK pathway involvement to regulate microglia immune reactions was investigated.

Based on the findings, the following main points can be addressed:

1. MAPKs are activated following TLR-mediated stimulation

2. Poly I:C stimulation does not upregulate pMAPKs even at a longer exposure time

3. Immune molecule stimulation (CXCL12) induces pJNKs, but not pERKs and p38s

4. Microglia immune response is initiated strongly by TLR4 and TRL7/8 stimulation, not by TLR3

5. Protein levels do not reflect mRNA level changes as much as might be expected

6. MAPK inhibitor experiments suggest that the MAPK pathway is involved in microglial immune reactions; but in a stimulus-dependent manner

These observations emphasise distinct effects of TLR3, TLR4 and TLR7/8 stimulation on microglia. Although the data in this chapter were gathered from in vitro experiments, these pathogen mimetics are widely used in the maternal immune activation (MIA) model - TLR 3 and 4 dominantly. Because the data clearly suggest TLR7/8 effects on microglial immune responses, it is worth studying this pathogen mimetic effects on the maternal immune system and foetal development, brain development in particular. In addition, possibly, the effects caused via different pathogens could be different, based on the in vitro observations, and this is interesting to investigate further.

Chapter 5
Maternal Immune Activation (MIA)

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5.1 Introduction

Maternal infection during pregnancy can increase the risk of psychiatric disorders, such as major depressive disorder (Brown and Meyer, 2018, al-Haddad et al., 2019b) and schizophrenia (Brown et al., 2001, Brown, 2006, Brown, 2012). Moreover, aetiological studies suggest that pathogens may directly affect the developing foetal CNS (Brown et al., 2001, Brown, 2006, Mortensen et al., 2007, Brown, 2012). Furthermore, microglia affect brain development (Thion et al., 2018a), and these changes are potentially linked to schizophrenia (Mondelli et al., 2017).

Previously, three potential pathways of MIA were described (see 1.4.2), involving immune factors released from maternal or foetal tissues through TLR activation by stimuli such as LPS and poly I:C (Jack et al., 2005, Ueta et al., 2005). As described earlier, cytokines and chemokines have crucial roles in immune functions and cell migrations, for example interneuron migration (Meechan et al., 2012). Thus, if their gradient is not properly regulated during pregnancy, it could misguide stem cell migration. Because of these reasons, cytokines and chemokines can affect foetal brain development. They can be generated in a mother or a foetus. These two classes of immune molecules have important roles in activating, developing, and regulating immune responses. Thus, their concentration has to be controlled carefully. If not, the foetal development might be impaired. For example, an abnormal alteration of cytokine and chemokine levels in maternal serum after MIA and its influence on the foetus brain (Buka et al., 2001a, Buka et al., 2001b, Brown et al., 2004a, Miller et al., 2011, Brown, 2012) and spine (Pekala et al., 2020) development have been elucidated from MIA animal models. For instance, enhanced pro-inflammatory cytokine levels in serum from mothers can be associated with altered neurodevelopment (Ashdown et al., 2006, Garay et al., 2013). Likewise, behavioural data show deficits in MIA offspring (Smith et al., 2007, Shi et al., 2003, Ozaki et al., 2020, Talukdar et al., 2020). However, the details behind the relationship between immune molecules and CNS development are not clear.

As mentioned earlier, the involvement of microglial cells with MIA in schizophrenia has been supported by many papers. For example, one recent report suggests a co-relationship between an animal MIA model and schizophrenia (post mortem samples), by measuring IL-1β, IL-6, and TNF-α, alongside microglia and astrocyte markers (Purves-Tyson et al., 2019). Additionally, a recent human PET study suggests that there is a systemic inflammation induced by LPS administration, occurring not only in microglial activity changes but also in pro-inflammatory cytokine level changes in the blood (Sandiego et al., 2015). It should be noted, though, that microglial activation was inferred from increased TSPO binding, with all the inherent limitations discussed above. Excitingly, a research group showed that rat pups exposed to prenatal environmental stress showed increased microglial pro-inflammatory molecules, e.g. IL-6, TNF-α, CXCL12, and abnormal microglial morphologies (Ślusarczyk et al., 2015). Furthermore, MIA mouse pups have shown a reduced microglial proliferation rate and abnormal behaviours (Ben-Yehuda et al., 2019) and transiently increased placental cytokine levels and foetal brain microglial activation has been

detected after MIA in pigs (Antonson et al., 2019). These findings indicate that systemic maternal inflammation is able to affect microglia in the foetal brain even though the pathogens are not directly invading the foetal compartment. Additionally, systemic inflammation affects BBB permeability, which is able to initiate inflammation in the brain (Takeda et al., 2013). Due to dysregulated peripheral and central immunity in MIA offspring, the microglial microenvironment will be perturbed. These activated microglia will influence neighbouring neuronal cell functions; as a result, whole brain function can be disrupted.

Beyond immune abnormality specifically, another possible way that MIA can affect CNS function is by interrupting an interaction between microglia and neurons. The two cell types continuously communicate in various ways, for instance via neurotransmitters, chemokines, and nitric oxide. The neural connection is very flexible, and it is spontaneously reconstructed by learning (experience and memory) and physiological environment changes (diseases). Indeed during foetal brain developmental stages, microglia have essential roles in neuronal network establishment (Xavier et al., 2014, Thion et al., 2018a).

Microglia affect neuronal network foundation. For example, *Cx3cr1* KO mice data show that microglia are linked to synaptic pruning during development (Paolicelli et al., 2011, Schafer et al., 2012). Additionally, related to schizophrenia, microglia depletion affects the intensity of perineuronal nets (which surround PV neurones), and the activity of excitatory and PV neurons (Liu et al., 2020). In addition, MIA (poly I:C model) offspring show schizophrenia-like behaviour with GABAergic impairment; however, the behavioural abnormality is diminished with administration of minocycline (Xia et al., 2020), which is suggested to be a potent microglia activation inhibitor (Tikka et al., 2001). From these papers, it can be suggested how important microglia are for constructing a neural circuit. Therefore, when microglia lose their function via MIA, it could cause a misformed neuronal network. Consequently, offspring brain development may proceed abnormally. This is consistent with evidence that cortical interneuron metabolic function in primary culture is significantly disrupted by the existence of activated microglia (Park et al., 2020). Indeed, this disruption is exacerbated in cortical interneurons derived from schizophrenia patient induced pluripotent stem cells (iPSCs), compared to those from healthy control subjects. Therefore, this accumulating evidence suggests that microglial activation contributes to the development of pathological changes in schizophrenia.

A further possible way that MIA can affect CNS function via microglia is through inappropriate microglial priming. Microglia were thought of as “long-lived cells”; however recent evidence clearly demonstrates that, at an individual level, microglial cells show significant proliferating and turnover rate without any peripheral immune cells’ input in unchallenged conditions throughout the whole lifetime (Amit et al., 2016, Askew et al., 2017, Holtman et al., 2017, Prinz et al., 2019). Additionally, because of the active renewal, microglia priming (a heightened response to a subsequent stimulus on account of exposure to an earlier stimulus) may be a critical event for later neuroinflammation and sub-optimal maintenance of brain cell networks. Equally, innate immune challenges can change microglial activity in the long-term (Wendeln et al., 2018). Thus, microglia in offspring with

MIA may have an altered priming memory that can affect brain development stages during prenatal and postnatal times, resulting in an increased risk of schizophrenia.

An additional way that MIA can affect CNS function via microglia is by producing an abnormal balance between pro- and anti-inflammatory microglial populations (Calcia et al., 2016). If pro-inflammatory microglia are not sufficiently suppressed by products from anti-inflammatory microglia, or if the pro-inflammatory microglia's population remaining is too small, a disrupted balance of microglia can affect brain functions. Therefore, the balance of polarisation of microglia in the brain is important to maintain the healthy condition.

Not only does MIA impact foetal brain development via neuronal cell migration and microglial activity, but also MIA is linked with intercellular communication via neurotransmitters or growth factors. This is also a mechanism to affect brain function. A key observation related to growth factors concerns brain-derived neurotrophic factor (BDNF). BDNF is a growth factor to help the growth and differentiation of new neurons and synapses. It is especially very important to synaptic plasticity, learning, memory and higher cognitive function. In schizophrenia patients, compared to a healthy population, a decreased BDNF serum level is detected (Green et al., 2011, Fernandes et al., 2015, Kumar et al., 2020, Singh et al., 2020). Furthermore, Zhang and colleague report that lower BDNF levels correlate with higher levels of cytokines (IL-2, IL-8) in chronic schizophrenia patients (Zhang et al., 2016a). Additionally, BDNF acts as an inactivation signal to microglia (Biber et al., 2007). Since MIA is reported to affect foetal BDNF expression (Gilmore et al., 2003, Gilmore et al., 2005), this may be an important mechanisms for schizophrenia aetiology.

From previous experiments, SIM-A9 microglial cells showed substantially increased levels of cytokine and chemokine production after exposure to resiquimod. In addition, the MAPK pathway regulated the microglial immune response.

Among MIA models, the most commonly used employ LPS or poly I:C to cause systemic maternal immune activation (Bergdolt and Dunaevsky, 2019). The effects of maternal immune infection with ss-virus mimetics such as resiquimod or imiquimod, stimulating TLR7/8 (Hemmi et al., 2002, Isobe et al., 2006), are very poorly studied, although ss-virus types are most strongly linked to schizophrenia (rubella virus and influenza virus). The evidence also suggests that *Toxoplasma Gondii*, which is one of the most relevant infectious agents, induces an immune reaction via TLR7; therefore, TLR7/8 mediated MIA is important to consider. Furthermore, in the previous chapter, the data demonstrated how microglial immunity could differ depending on the infectious agents. We therefore extended this work into the corresponding in vivo situation, and tested the following hypothesis via MIA models: MIA induced by resiquimod (ssRNA virus mimetic) would cause a stronger foetal brain immune reaction (cytokine and chemokine production) compared to poly I:C (ds RNA virus mimetic). These specific aim was therefore to compare the prenatal immune responses (chemokine and cytokine induction), in maternal blood, placenta and foetal brain, to maternal administration of the 2 different prototypical immune challenges (poly I:C, resiquimod) .

5.2 Methods

5.2.1 Ethical and legislative approval for experimental animal works

In vivo experiments were approved by the University of Glasgow Animal Welfare Ethical Review Board, and conducted according to Home Office legislation under an appropriate licence.

5.2.2 Establish MIA model

In order to establish the MIA model, two main factors have to be considered: the gestational time and the stimuli (with dose). From previous publications, of the gestational times that are used, the earliest is E4 to the latest E21 (Bilbo and Schwarz, 2009, Boksa, 2010, Smolders et al., 2015, Pekala et al., 2020), however generally, E9, E12.5, or E17 are used. Since E12.5 in mice is argued to correspond to a gestation time in humans that, with respect to brain development and brain gene expression (Otis and Brent, 1954, O'Rahilly, 1979, Clancy et al., 2001, Liscovitch and Chechik, 2013, Richardson et al., 2013), corresponds to the period the most associated with increased incidence of schizophrenia (Brown, 2011), therefore E12.5 is used in this study.

Alongside LPS, poly I:C is commonly used in mice to build the MIA model. To decide the dose of poly I:C, the most commonly used dose is 20mg/kg (i.p.or s.c.) with the E12.5 time point (Smith et al., 2007, Bergdolt and Dunaevsky, 2019, Talukdar et al., 2020).

Additionally, resiquimod is added here as a new pathogen mimetic in the second MIA model. Previous investigation suggests that resiquimod is a powerful tool for studying microglial immune reactions (see Chapter 4). Moreover, it has been used to induce a systemic immune reaction in mice at doses between 1 and 10 mg/kg (Su et al., 2005, Baenziger et al., 2009, Adzavon et al., 2017, Michaelis et al., 2019). An intermediate dose of 2mg/kg was selected for study here.

5.2.3 MIA model (the second model)

C57/BL6J WT female mice (aged wk 4) were purchased from Envigo and kept for a minimum of period (7 days) within the CRF to allow for acclimatisation prior to the procedures. They were bred and maintained at the CRF at the University of Glasgow. All experiments were reviewed by local ethical committees and were performed under the authority of a UK Home Office License. Mice were mated and separated the following day. If the females (aged between wk 8-11) were pregnant, the day was given as E0. To induce MIA, all treatments, vehicle (PBS, 2ml/kg, Gibco 14190144), poly I:C (20mg/kg of a 10 mg/ml solution in PBS, LMW, Invivogen tlrl-picw), resiquimod (2mg/kg of a 1 mg/ ml solution in PBS, Invivogen tlrl-r848), were administered subcutaneously between 9:00-11:00 am, and the condition of the mice was monitored to ensure that there was no sign of any severe sickness symptoms or abnormalities. Mice were culled by a Schedule 1 method (CO_2 euthanasia) with cutting of the right femoral artery for the terminal procedure. The Schedule1 was performed by trained CRF staff and blood was collected after. The mothers' and embryos'

tissues were collected and processed for further experiments. The half hemisphere of mother's brain was dissected as region specific, mPFC, cerebellum, hypothalamus, and hippocampus and kept in RNAlater (Invitrogen AM7020) and the other half hemisphere was stored in 4% ice cold paraformaldehyde (PFA) for fixation. The brains were removed from embryos for RT-qPCR and stored in RNAlater, otherwise whole embryos were fixed in 4% PFA for histology. Mother's plasma, spleen, placentae were also collected and stored for further use. More details of sample preparation procedures are described in separate sections.

5.2.4 Multiplex / Luminex

To measure a range of secreted cytokine and chemokine levels in plasma, the Multiplex/Luminex system (Merck, MCYTMAG-70K-PX32) was employed. The assay was performed as per manufactural instruction. Briefly, an assay plate washed with gentle shaking for 10 minutes at room temperature. Meantime, quality control samples, serum master mix, and STDs were reconstituted (working concentration as recommended) and left at room temperature for 15 minutes. A 5-fold serial dilution was made to generate STDs, then quality control samples, assay buffer (only sample wells), serum matrix solution and samples (25μl/ well) were added and the samples and all of the samples and STDs were duplicated. The premixed beads were added to each well (25μl/ well). The beads had to be sonicated for 30 seconds in the bottle with vortex mixing for 1 minute prior to use. The STDs and the samples were incubated at 4°C overnight with agitation for maximal sensitivity. The next day, the plate was sat on the magnetic plate washer (QP0701) and left for 1 min to allow the beads to bind to the magnet. The well contents were gently removed, and the plate washed twice (200μl wash buffer/ well, 30 seconds each time). After the aspiration and washing steps, detection antibodies were added and incubated for 1 hour with agitation (room temperature, dark). Streptavidin-phycoerythrin (25μl/ well) was added, and the plate was incubated with agitation (30 minutes, room temperature, dark). Washing was repeated twice with the magnetic plate washer. In order to stop the reaction, the Sheath Fluid was added (150μl/ well) and the plate was left for 5 minutes with agitation in the dark. Measurements were taken by the LUMINEX 200® system, operated via Bio-Rad's Bio-Plex software version 6.1™. The beads were read determining the mean fluorescence intensity (MFI) (Breen et al., 2015, Breen et al., 2016, Richter et al., 2017) and the data were accepted if the duplicated samples varied less than 15%.

5.2.5 RT-qPCR

The RT-qPCR methods used in this Chapter have been described in detail in Chapter 2

5.3 Results

5.3.1 The first MIA model: an environmental factor (dsRNA maternal viral infection: poly I:C) with a genetic factor (*Map2k7*)

As described above, for the first MIA model, poly I:C (20mg/kg) was used to induce inflammation, and saline was used as control. The drugs were administered subcutaneously, and the mice were culled 6 hours following the administrations. In total, 8 WT male mice, 8 WT female mice, 8 *Map2k7*$^{+/-}$ male mice and 8 *Map2k7*$^{+/-}$ female mice were used in the experiment. In this (first) MIA model, a genetic factor (*Map2k7)* is considered with MIA because a decreased level of *MAP2K7* gene in post-mortem tissues from schizophrenia patients is reported (Winchester et al., 2012); furthermore a reduced level of *Map2k7* expression in the CNS is correlated to poor behaviour performance in mice (*Map2k7*$^{+/-}$*)* (Openshaw et al., 2017). All animal procedures were conducted by Dr. Rebecca Openshaw under license and using UK Home Office regulations, and the placental tissues were kindly offered by her for this study. Levels of CXCL10 and CXCL12 were monitored by specific ELISA.

The data showed that the level of placental CXCL10 in WT mice was not changed by poly I:C administration, however the maternal genotype was important, as an effect of poly I:C was observed in *Map2k7*$^{+/-}$ female dams, irrespective of pup genotype (Figure 21A). Similarly, the level of CXCL12 was also increased by poly I:C, dependent on maternal but not foetal genotypes;

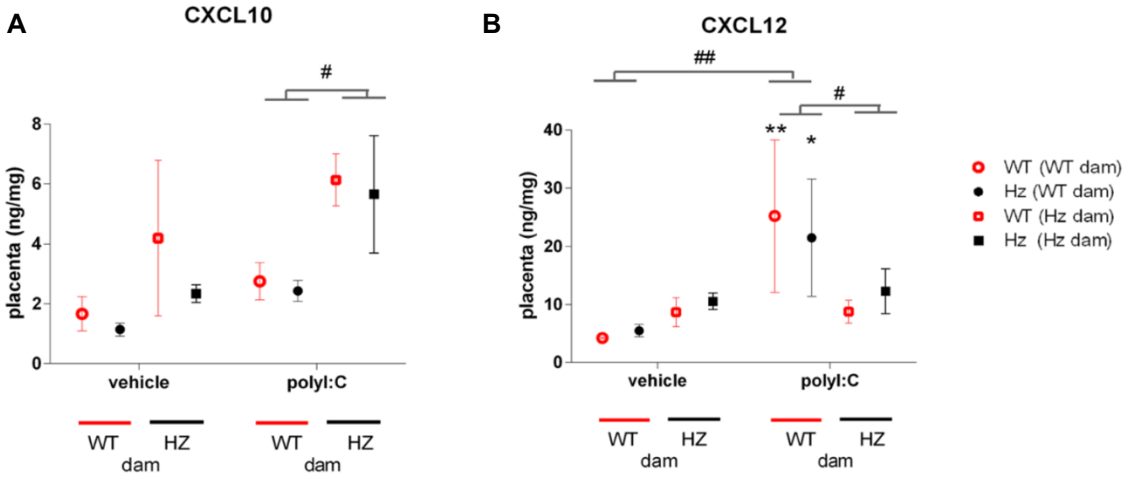


Figure 21. CXCL10 and CXCL12 levels in placental tissues after poly I:C administration. The placental tissues were collected 6 hours after MIA (PBS, poly I:C (20mg/kg, LMW)) and stored at -80 °C until further use. The proteins in the tissues were measured via ELISA as per manufacturer's instruction. **(A)** The level of CXCL10 was significantly induced by maternal genotypes within poly I:C treatment, regardless of embryo genotypes **(B)** The poly I:C effect was shown in WT mothers, but not in HZ mothers. The results are shown as mean ± SEM. The data were box-cox transformed and analysed by one way-ANOVA, Fisher LSD post-hoc test (n=4 -7 independent samples in each group; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001 vs vehicle- treated group, same dam, and embryo genotype; $^{\#}$p≤0.05, $^{\#\#}$p≤0.005, $^{\#\#\#}$p≤0.001, $^{\#\#\#\#}$p ≤0.000 as shown). Details of ANOVA F values and p values are provided in Table 11.

however, with CXCL12 the effect was observed with WT mothers. (Figure 21 is published (Openshaw et al., 2019))

5.3.2 The second MIA Model: dsRNA (poly I:C) vs. ssRNA virus (resiquimod) mimetics

5.3.2.1 New MIA conditions are sufficient to induce maternal inflammation

From the first MIA model (looking at interaction between genotype x MIA), the experimental data suggest that poly I:C administration significantly induces the level of placental CXCL12 but not CXCL10 in WT dams (vs. within same dam and embryo genotype). Although the data are not presented here the levels of immune molecules in maternal plasma are also upregulated following poly I:C injection compared to saline (Openshaw et al., 2019). Additionally, the findings from the *in vitro* microglia (SIM-A9 cells) experiments (see section 4.2) indicate that resiquimod could be a powerful immune stimulus in the CNS. Furthermore, the microglial activity could be pathogen type dependent. In the new MIA model, the different mimetics were chosen, poly I:C, which is a double-strand RNA virus mimetic commonly used to establish the MIA model, and resiquimod which is a single-stranded RNA virus mimetic and induces the most robust microglial immune responses (see section 4.4). Furthermore, ssRNA viruses (e.g. rubella virus and influenza virus) are the most strongly linked to schizophrenia risk, but evidence in MIA related to ssRNA mimetic such as resiquimod or imiquimod is rare (Hemmi et al., 2002, Isobe et al., 2006). Equally, ds-viruses such as Herpes simplex II are not robustly linked to schizophrenia risk (Brown et al., 2006). Thus, it is interesting to carry out further investigation on how two different pathogen mimetics affect differently maternal and foetal systems.

To induce immune reactions in the maternal system, poly I:C (20mg/kg), resiquimod (2mg/kg) and PBS as control were subcutaneously administered, and the maternal plasma samples were collected after 4 hours. The levels of 32 different types of immune related molecules were quantified via Luminex (Figure 22-Figure 25).

Of the 9 pro-inflammatory cytokines that were detectable in maternal plasma from mice, typical pro-inflammatory cytokines such as IL-6 and TNF-α were significantly elevated following poly I:C and resiquimod administration compared to PBS; however, IL-1α, IL-12p40, and IL-12p70 were only induced by resiquimod admiration. Other cytokines, such as IL-1β, IL-3, IL-7, and IL-15 were not significantly changed by either poly I:C or resiquimod, compared to PBS (Figure 22).

9 anti-inflammatory cytokines were measured in maternal plasma from mice. Similar to the pro-inflammatory cytokines, resiquimod and poly I:C significantly upregulated the levels of anti-inflammatory cytokines compared to PBS: IL-10, IL-13, and LIF (Leukaemia Inhibitory Factor), however IFN-γ was only induced by resiquimod. The other anti-inflammatory cytokines, such as IL-2, IL-4, IL-5, and IL-9, were not affected by MIA, at least at this time point (Figure 23).

Of the 10 chemokines that were measured in maternal plasma after MIA, all were meaningfully upregulated following resiquimod administration compared to PBS: CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, CXC2, CXCL9, and CXCL10; however, poly I:C administration only affected the levels of CCL2, CCL5, CXCL9 and CXCL10 compared to PBS (Figure 24).

Along with the 28 cytokines and chemokines, 4 different growth factors especially related to immune functions were also monitored.

Granulocyte colony-stimulating factor (G-CSF) regulates haematopoiesis, and drives and regulates inflammation (Roberts, 2005). Furthermore, G-CSF shows neuroprotective functions (Schneider et al., 2005, Pitzer et al., 2008, Peng, 2017). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has multiple biological functions, such as co-ordinating macrophage development from progenitor cells (Hamilton, 2019). Moreover, it is used to stimulate microglial proliferation in culture (Lee et al., 1994, Koshida et al., 2015). Macrophage colony-stimulating factor (M-CSF) regulates proliferation, differentiation of monocytes, macrophages, and bone marrow progenitor cells (Stanley et al., 1997, Ushach and Zlotnik, 2016). Vascular endothelial growth factor (VEGF) is essential for angiogenesis in embryonic development and homeostasis (Ferrara, 2004). It is also important for monocyte/macrophage recruitment (Breen, 2007) and neuronal development (Verena and Carsten, 2018). Taken together, the findings suggest that these growth factors are crucial for immune system development and so worth investigating.

All 4 measured growth factors were significantly upregulated by resiquimod administration compared to PBS: G-CSF, GM-CSF, M-CSF, and VEGF; but, poly I:C administration only significantly affected two growth factors compared to PBS: M-CSF, and VEGF (Figure 25).

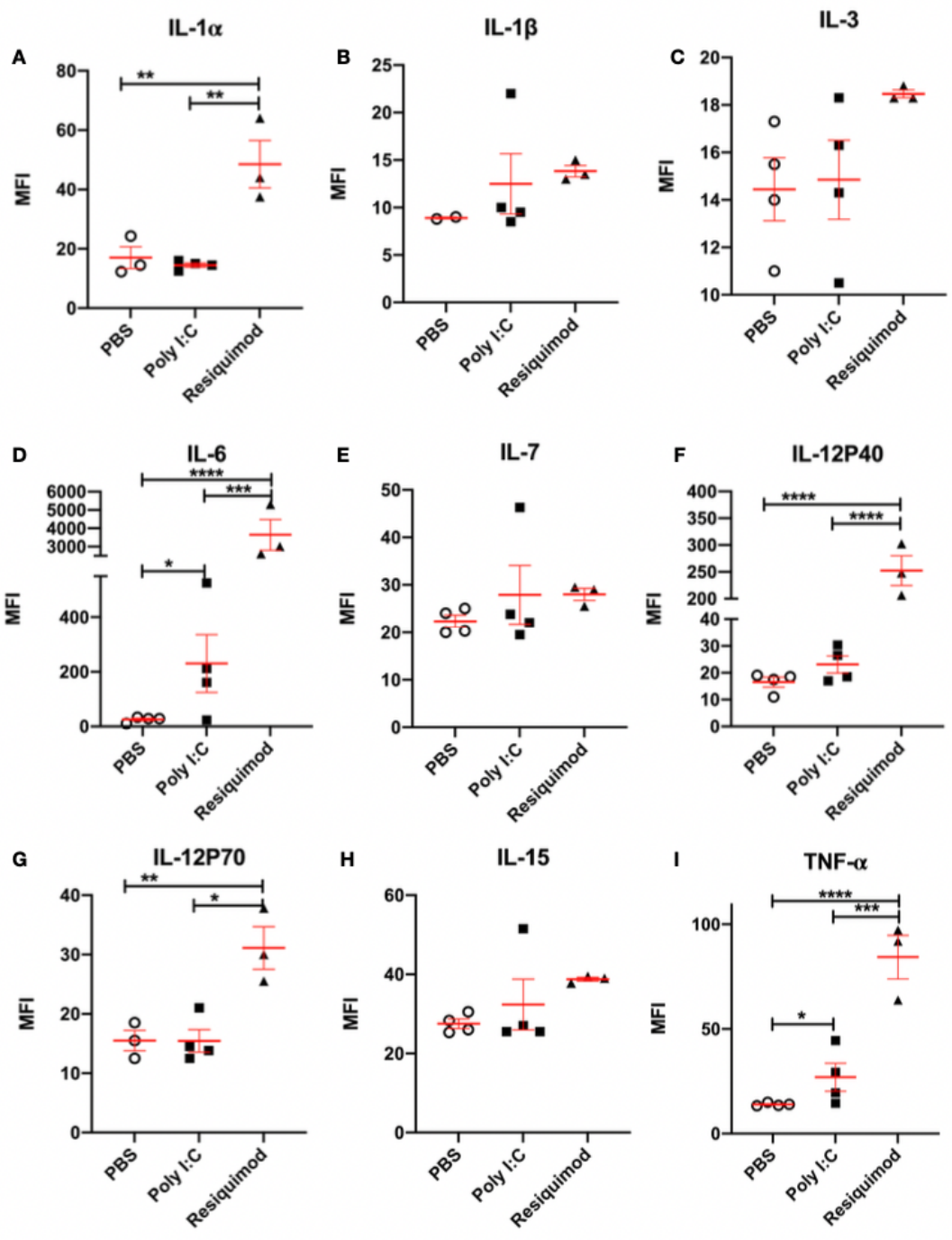


Figure 22. The levels of pro-inflammatory cytokines after MIA in maternal plasma. The maternal plasma samples were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored until further use. The proteins in plasma were measured via Luminex as per manufacturer's instruction. Typical pro-inflammatory cytokines, IL-6 and TNF-α, were upregulated in the plasma of mothers following both resiquimod and poly I:C compared to control (PBS). IL-1α, IL-12p40, and IL-12p70 were only upregulated by resiquimod. IL-1β, IL-7, and IL-15 were not affected by either treatment. The individual data points are shown along with mean ± SEM. The data were box-cox transformed and analysed by one way-ANOVA, Fisher LSD post-hoc test (n=3-4 independent samples in each condition, as some data points were excluded if they did not pass the QC); *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

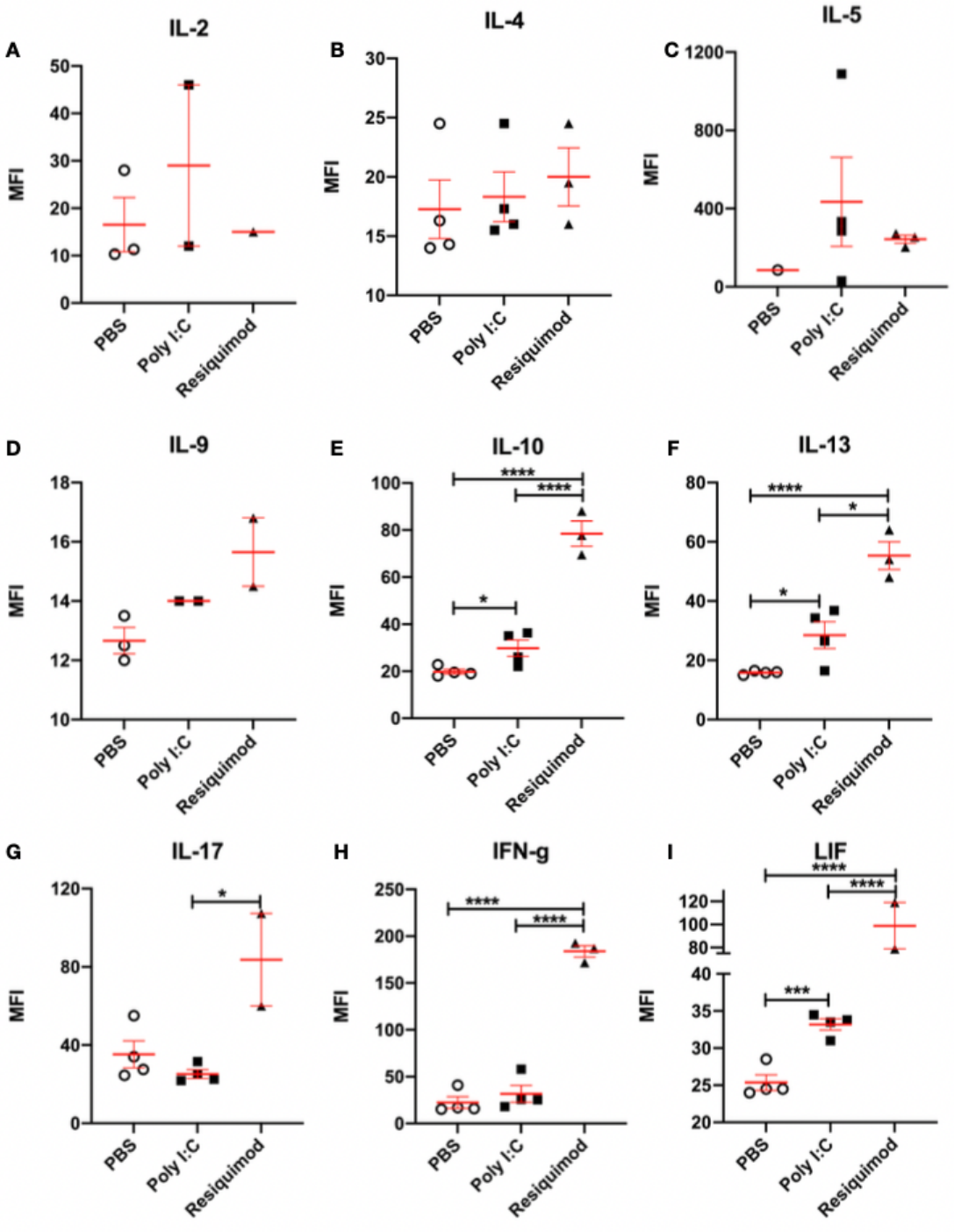


Figure 23. The levels of anti-inflammatory cytokines after MIA in maternal plasma. The maternal plasma samples were collected after 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored until further use. The proteins in plasma were measured via Luminex as per manufacturer's instruction. Anti-inflammatory cytokines, IL-10, IL-13, and LIF, were upregulated in the plasma of mothers following both resiquimod and poly I:C compared to control (PBS). IFN-γ was only upregulated by resiquimod. IL-2, IL-4, IL-5, and IL-9 were not affected by either treatment. The individual data points are shown along with mean ± SEM. The data were box-cox transformed and analysed by one way-ANOVA, Fisher LSD post-hoc test (n=3-4 independent samples in each condition, as some data points were excluded if they did not pass the QC); *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

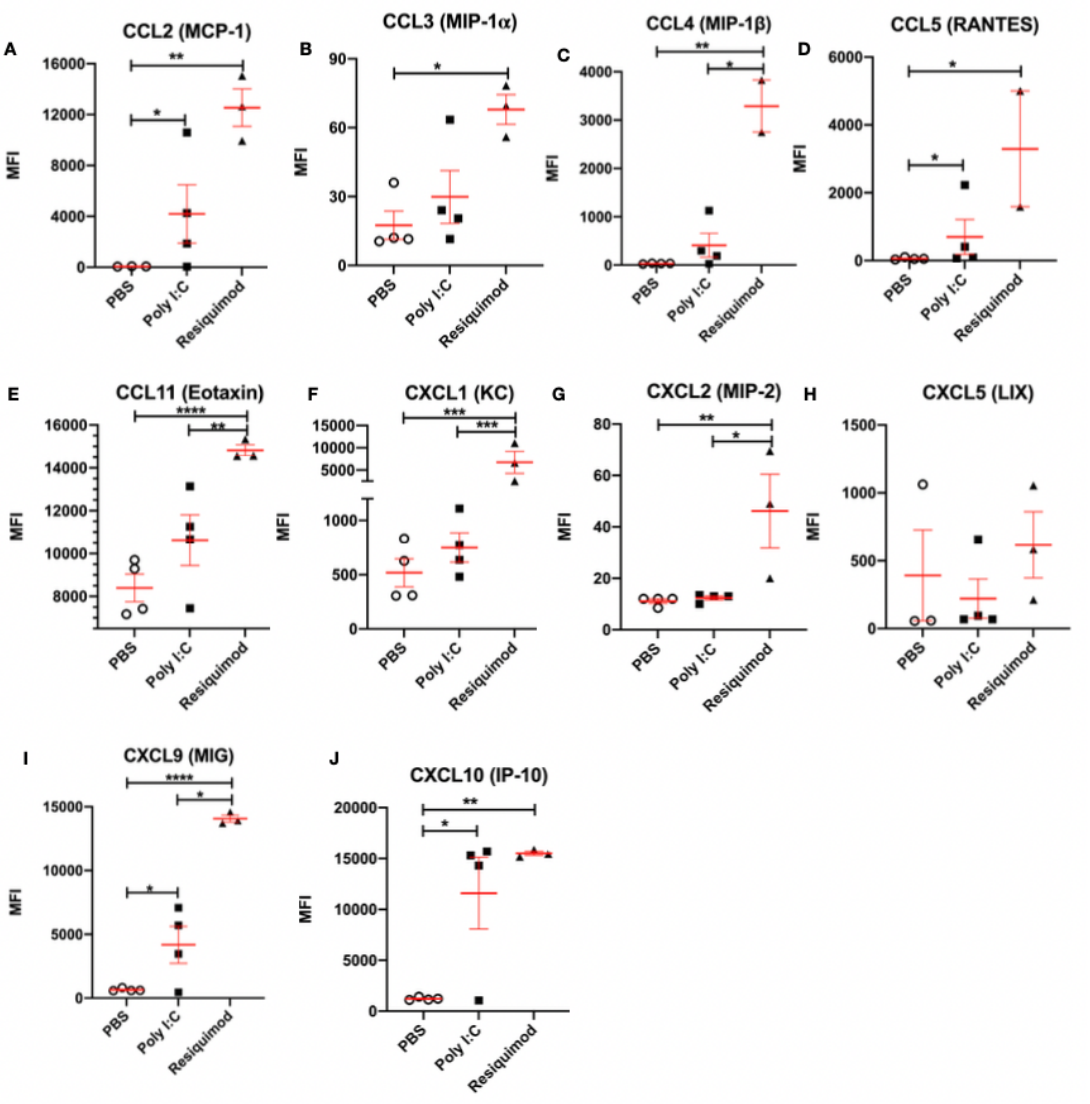


Figure 24. The levels of chemokines after MIA in maternal plasma. The maternal plasma samples were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored until further use. The proteins in plasma were measured via Luminex as per manufacturer's instruction. Measured chemokines, CCL2, CCL5, CXCL9, and CXCL10 were upregulated in the plasma of mothers following both resiquimod and poly I:C compared to control (PBS). CCL3, CCL4, CCL11, CXCL1, CXCL2, were upregulated by resiquimod, not by poly I:C, compared to PBS. CXCL5 was not affected by either treatment. The individual data points are shown along with mean ± SEM. The data were box-cox transformed and analysed by one way-ANOVA, Fisher LSD post-hoc test (n=3-4 independent samples in each condition, as some data points were excluded if they did not pass the QC); *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

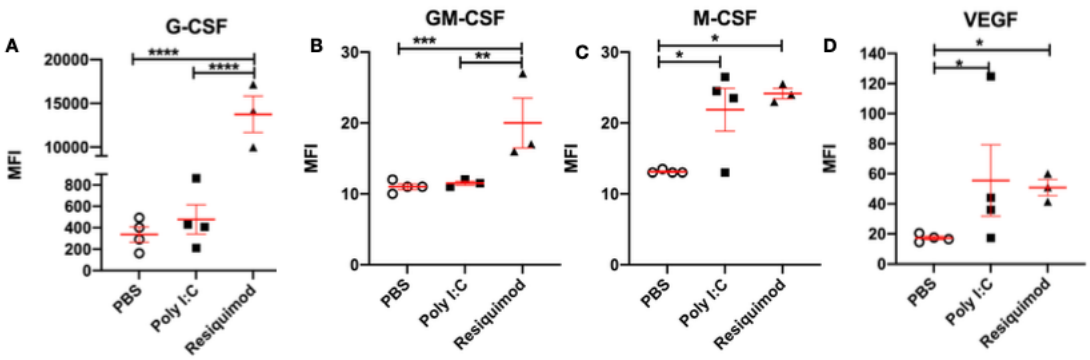


Figure 25. The levels of growth factors after MIA in maternal plasma. The maternal plasma samples were collected after 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored until further use. The proteins in plasma were measured via Luminex as per manufacturer's instruction. Resiquimod significantly induced all measured growth factors, G-CSF, GM-CSF, M-CSF, and VEGF compared to PBS; however, poly I:C significantly induced the levels of M-CSF, and VEGF. The individual data points are shown along with mean ± SEM. The data were box-cox transformed and analysed by one way-ANOVA, Fisher LSD post-hoc test (n=3-4 independent samples in each condition, as some data points were excluded if they did not pass the QC); *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

5.3.2.2 Effects of poly I:C are more limited in response compared to resiquimod in placental tissues

In summary, maternal plasma from mice injected with resiquimod showed significantly upregulated levels of immune molecules compared to those injected with PBS. Although poly I:C caused many signs of inflammation in mothers, the changes in some of the levels of inflammatory molecules did not reach statistical significance. Nevertheless, it can be concluded that the current MIA conditions established by poly I:C and resiquimod were sufficient to induce inflammation in the maternal system. Although MIA by poly I:C and resiquimod, as used under these conditions, was confirmed, it is important also to check the condition of placental tissue, because the placenta works as a barrier between a mother and a foetus, and can contribute to immune responses during pregnancy. We considered that the placenta will be possibly inflamed by MIA, as indeed noted in the first MIA model, and that the nature of the response may be different depending on pathogen types. Thus, cytokine and chemokine mRNA levels were measured in placental tissues via RT-qPCR.

To determine how placental tissues respond to immune stimuli, the levels of mRNA rather than protein were measured, so that the origin of the molecules could be clearly ascribed to the placenta.

Resiquimod administration significantly elevated most inflammatory cytokine and chemokine mRNAs such as *Tnf-α, Ccl5, Ccl11* and *Cxcl1*, but notably without any changes in the mRNA levels of *Ccl2* and *Cxcl12* after the injections (Figure 26). Poly I:C induced *Il-6* (p=0.053)*, Il-10,* and *Cxcl10* mRNA levels, but this was not observed in any other genes (Figure 26A, C, and H). CCL2

in maternal plasma was significantly induced by both poly I:C and resiquimod injection (Figure 24A); however, *Ccl2* mRNA in placenta was not changed by any MIA conditions compared to PBS (Figure 26D). In placenta samples, there are differences in the level of inflammation observed between mothers, except with *Tnf-α, ccl2*, and *Ccl11*. This indicates that biological differences could be detected even though experimental conditions, such as age, strains, and time for the experiment are regulated.

Overall, the results suggest that the levels of cytokine and chemokine mRNA in the placenta induced by MIA reflect the changes in maternal plasma, but poly I:C effects on placental tissues are much more restricted compared those of resiquimod. These data suggest that ssRNA virus types possibly cause inflammatory reactions more noticeably in placenta tissues than ds RNA virus types. This indicates that MIA caused by ssRNA virus types may affect the foetus more seriously than ds RNA virus types. It therefore became important to assess the effects on foetal tissue directly. Thus, levels of inflammatory molecules' mRNA levels were measured in foetal brains, to observe any differential pathogen type effects.

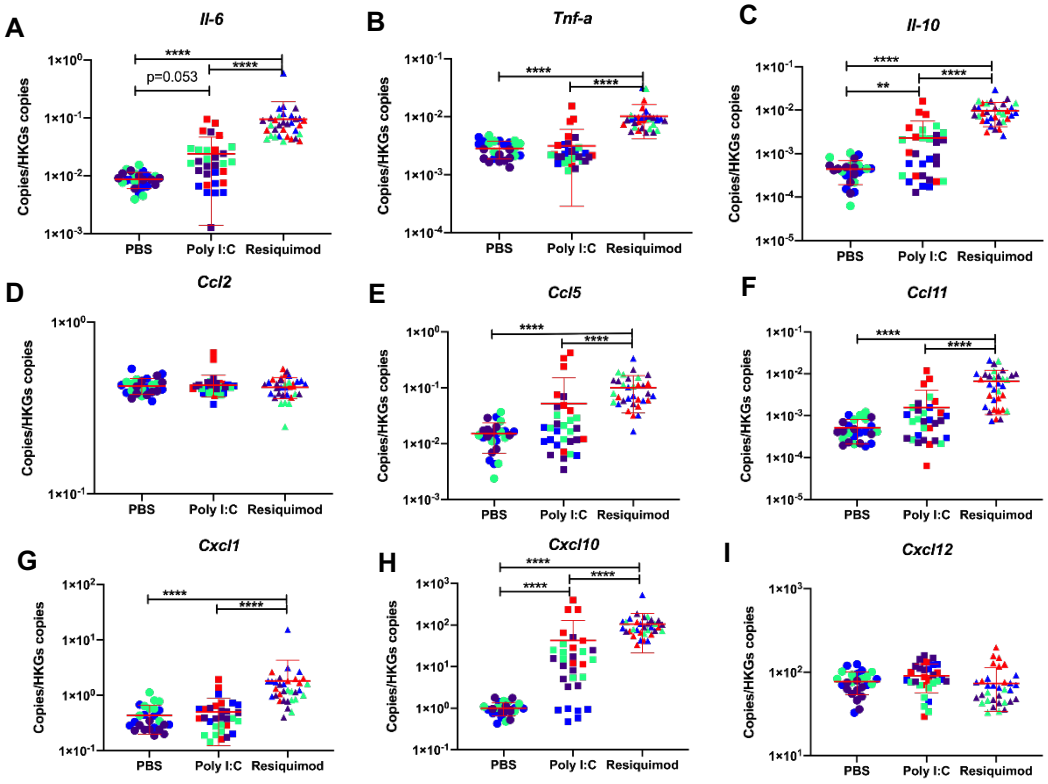


Figure 26. *Il-6, Il-10* and *Cxcl10* mRNA are significantly induced by MIA in placentae. The placentae tissues were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored in RNAlater until further use. RNA extraction and cDNA synthesis were performed as per manufacturer's instructions. **(A, C, H)** *Il-6, Il-10,* and *Cxcl10* mRNA levels were significantly upregulated by poly I:C and resiquimod compared to PBS. **(B, E, F, G)** *Tnf-a, Ccl5, Ccl11,* and *Cxcl1* mRNA levels were induced by resiquimod, but not by poly I:C compared to PBS. **(D, I)** *CC2* and *Cxcl12* mRNA levels were not changed by MIA. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Tbp*. The individual data points are shown along with mean ± SEM. Colour indicates dams within a single treatment (same colour means "same dam"). The data were log transformed and analysed by two way-ANOVA, Tukey post-hoc test (n=26-33 independent samples; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

5.3.2.3 Only resiquimod induces immune reactions in foetal brains

The previous observations suggest that maternal inflammation induced by exposure to poly I:C or resiquimod affects placental tissues, which show induced levels of cytokine and chemokine mRNA. The same cytokine and chemokine mRNAs in foetal brains were measured, to compare with maternal plasma and placenta expression. Resiquimod administration caused increased levels of various mRNAs of cytokines and chemokines compared to those injected with PBS (Figure 27). Nevertheless, poly I:C did not alter any immune molecule gene expression in foetal brain compared to PBS (Figure 27), despite showing clear evidence of maternal plasma inflammation (Figure 22). CXCL12 is notable among the various genes measured because it is highly involved in embryo development (Rostène et al., 2011). This chemokine plays a key role in stem cell migration; therefore, if its level is changed, the cell migration is likely to be compromised. The data suggested that *Cxcl12* in the embryo brain was not changed by maternal resiquimod administration, but was slightly decreased by poly I:C administration (Figure 27I); however, this significance in *Cxcl12* may be because of one outlying sample (marked in pink), as when this data point is excluded, significance is lost : Grubb's test, followed by one way ANOVA ($F(2,43)=1.716$, $p=0.1919$, $p=0.1940$ PBS vs. poly I:C, Tukey post-hoc). The one odd sample data point (in pink) has not been excluded throughout, as although it is a clear outlier in some genes, e.g. *Ccl2, Cxcl1* and *Cxcl12*, it is sitting within the cluster of data points in some genes e.g. *Il-6*, *Ccl5*, and *Cxcl10*. Indeed, there is no experimental reason, such as foetal developmental abnormality or contamination, to remove it; therefore, this sample is presented rather than removed.

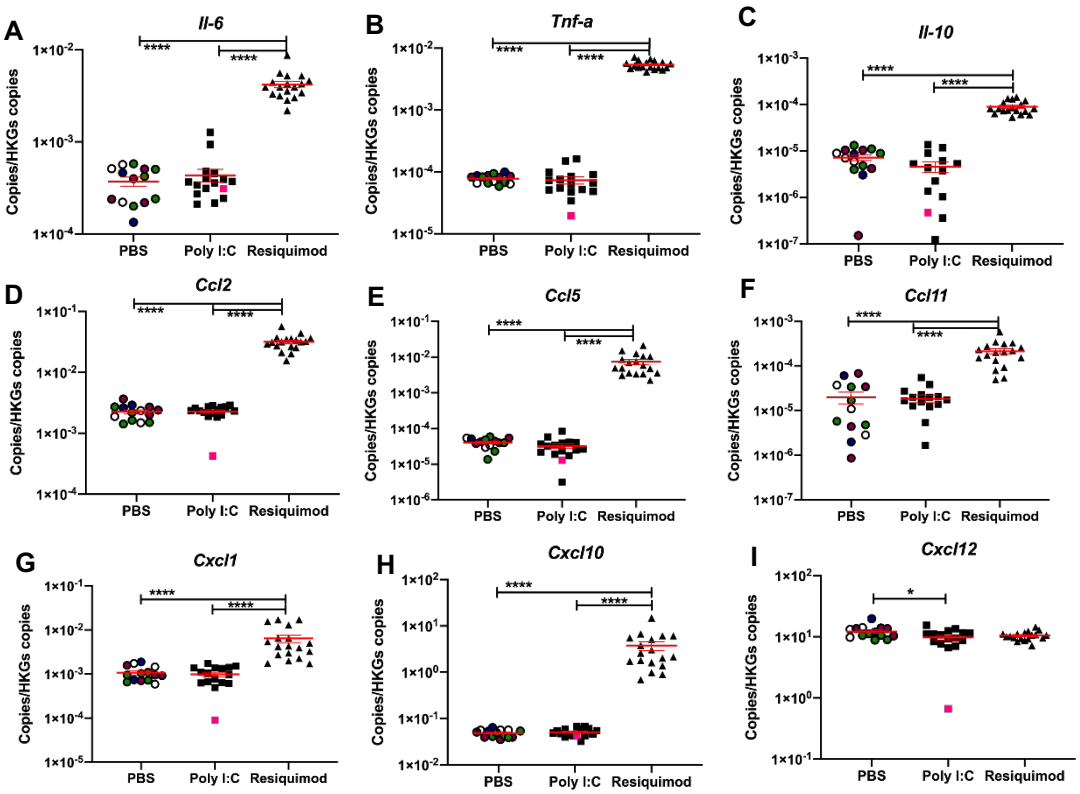


Figure 27. Cytokines and chemokines are significantly induced by resiquimod, not by poly I:C. The foetal brain tissues were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored in RNAlater until further use. RNA extraction and cDNA synthesis were performed as per manufacturer's instruction. **(A-H)** All the measured cytokines and chemokines mRNAs were significantly induced by resiquimod but not poly I:C compared to PBS. **(I)** *Cxcl12* mRNA was slightly decreased by poly I:C. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Tbp*. The individual data points are shown along with mean ± SEM. The data were log transformed and analysed by two way-ANOVA, Tukey post-hoc test (n=14-18 independent samples; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

5.3.2.4 Microglial markers in foetal brains are altered by MIA

These data from the foetal brain suggest that MIA with resiquimod, but not with poly I:C, elevates inflammatory cytokines and chemokines; however, this reflects overall changes only in the brain.

As described earlier, microglia are the primary cellular source to defend immune challenges in the brain, and changes in this cell population are implicated in schizophrenia (see section 1.2.5). Based on previous publications, 5 different markers were chosen to quantify microglial and macrophage activity changes after MIA. Iba1 (gene name: *Aif1*) is widely used as a microglia marker (Ribeiro et al., 2013, Elmore et al., 2014, Bruttger et al., 2015); however, it is also expressed by macrophages, and hence is not totally microglia-specific (Imai et al., 1996, Tanaka et al., 2003). TMEM119 is recently discovered, and reported to be a microglia-specific marker (Bennett et al., 2016). Another marker, CX3CR1, is also widely used as a microglial marker (Harrison et al., 1998, Ginhoux et al., 2010), although like Iba1, it is also expressed by peripheral monocytes (Lee et al., 2018). CCR2 is expressed by monocytes and macrophages, but is widely believed not to be expressed in the microglial population throughout mice developmental stages (Prinz et al., 2011, Mizutani et al., 2012, Greter et al., 2015). The last marker, Ly6C2, is argued to be specific to monocyte-derived macrophages, and not to be expressed by microglia (Jordão et al., 2019, Monaghan et al., 2019). Although these markers are generally used in histology or flow cytometry analysis, transcriptional measurements could also give meaningful clues to distinguish between these populations.

The results indicated that *Tmem119* and *Cx3cr1* mRNA levels were down regulated by resiquimod, but *Aif1* mRNA levels were significantly elevated (Figure 28A-C). *Ccr2* mRNA was decreased by MIA with both poly I:C and resiquimod, although resiquimod's impact was greater than poly I:C's; however, *Ly6c2* mRNA was not affected by MIA (Figure 28D,E). Poly I:C significantly reduced the level of *Tmem119* mRNA, but *Aif1* and *Cxcr1* were not changed. The results indirectly suggest microglial activation via downregulation of *Tmem119* by poly I:C and resiquimod and *Cx3cr1* by resiquimod. A more detailed dissection will follow in section 5.4.6

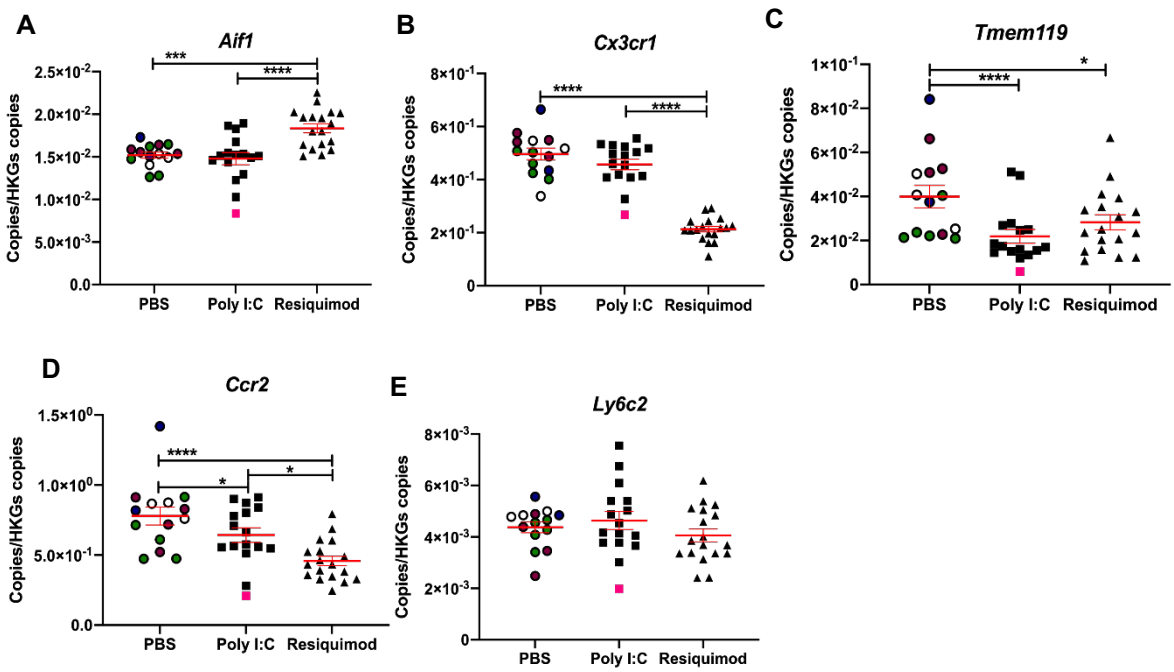


Figure 28. Microglial markers after MIA in the foetal brain. The foetal brain tissues were collected after 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored in RNAlater until further use. RNA extraction and cDNA synthesis were performed as per manufacturer's instruction. **(A)** *Aif1* gene expression was induced by resiquimod compared to PBS **(B,C)** *Tmem119* gene expression was downregulated by both poly I:C and resiquimod whereas *Cx3cr1* gene expression was only reduced by resiquimod compared to PBS **(D,E)** *Ccr2* gene expression was downregulated by both poly I:C and resiquimod whereas *Ly6c2* gene was not changed by MIA compared to PBS. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Tbp*. The individual dots are shown along with mean ± SEM. The data were log transformed and analysed by two way-ANOVA, Tukey post-hoc test (n=14-18 independent samples; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

5.4 Discussion

5.4.1 Summary of results

The data presented in this chapter demonstrates the placental and foetal brain immune response to MIA. The data show that dsRNA (poly I:C) and ssRNA (resiquimod) virus mimetic administration can induce meaningful maternal systemic inflammation, and also that, at least at the doses used, resiquimod has more potent effects as the immune agent. The findings also show that there is a significant impact of maternal ssRNA virus exposure on the developing foetal brain.

5.4.2 MIA models

There are many MIA models that have been tested; however, the data are not consistent in terms of immune changes (measuring microglial activity changes) in the foetus (Smolders et al., 2018). It may be because the selected time-window of the administration varies. Meyer and Feldon argue that the timing of inducing prenatal infection significantly affects the results of experiments (Meyer and Feldon, 2012). In the current study, the time point, E12.5, was chosen based on aetiological evidence from human studies (see section 5.2.2). But, if the time point is chosen to study foetal CNS immune disruption in-depth, either E8-9 when cell migration is happening from yolk sac, or E14-15 when microglial cells have matured, could be suggested based on microglial developmental stages. This means that the selection of time window is important. In this study, E12.5 is chosen to maximise disease-relevance.

In this study, poly I:C, the dsRNA virus mimetic, was selected, as it is widely used for MIA models. The results with this mimetic in the literature are variable, and it may be because of possible endotoxin contamination and batch variation (Kowash et al., 2019, Mueller et al., 2019). The concerns related to poly I:C are thoroughly discussed in (see section 4.4.3.1). Equally, selection of the time-window is also partly potentially responsible for variability of its effects (Boksa, 2010, Meyer and Feldon, 2012).

Alongside poly I:C, resiquimod as the ssRNA virus mimetic was used here. There is another ssRNA mimetic, imiquimod, available. In spite of the relative benefits of imiquimod clinically, due to actions unrelated to TLR7/8 stimulation (Schön and Schön, 2008), resiquimod was chosen in this study for various reasons. First, resiquimod is more potent than imiquimod in terms of producing an immune response (Dockrell and Kinghorn, 2001). Although both imiquimod and resiquimod are able to stimulate an immune response (Tomai et al., 2000), resiquimod tends to require lower concentrations than imiquimod to elevate cytokine levels (Wagner et al., 1999).

Second, while recent studies using imiquimod to assess the effect on CNS function of ssRNA virus infection (Thomson et al., 2014, McColl et al., 2016), and to assess the behavioural effects of MIA (Missig et al., 2020), imiquimod is selective for TLR7, whereas resiquimod activates both TLR7 and TLR8 (Patinote et al., 2020). Besides, previous findings from this study (see section 4.4.1) show

that resiquimod is a potent of inducer of microglial immune response. Furthermore, epidemiological studies suggest that psychiatric disorder risk correlates to ssRNA viral infection (Fatemi and Folsom, 2009) without discriminating between TLR7 and TLR8 mediation. Butch and others show that TLR7 or TLR7/8 mediated immune responses are different (Butchi et al., 2008). Therefore, resiquimod seems to be the better choice to maximise immune response and test environmental contribution to psychiatric disease risk.

5.4.3 Maternal plasma

It was clear from the data from maternal plasma (Figure 22-Figure 25) measurements by Luminex that inflammation is caused in mothers in response to both poly I:C and resiquimod. Induction of pro-inflammatory cytokines (e.g. IL-6 and TNF-α), anti-inflammatory cytokines (e.g. IL-10 and LIF), chemokines (e.g. CCL2, CCL5, and CXCL10), and growth factors (e.g. M-CSF, and VEGF).

IL-6 is one of the cytokines most commonly shown to be linked to schizophrenia (Kunz et al., 2011, Boerrigter et al., 2017, Dahan et al., 2018, Delaney et al., 2019). Maternal IL-6 possibly disrupts placental function, resulting in disruption of foetal development (Hsiao and Patterson, 2011, Wu et al., 2017). Indeed, there is the possibility that IL-6 could cross the placental barrier (Zaretsky et al., 2004); therefore, maternally derived IL-6 could act on the placenta or directly affect the foetus.

Anti-inflammatory cytokines were induced by immune mimetic administration; indeed, the two molecules, IL-10 and LIF, are potentially linked to schizophrenia. Abnormal levels of IL-10 in patients' serum have been reported (Maes et al., 2002, Kunz et al., 2011, Xiu et al., 2014). It is known that LIF is linked to foetal neurogenesis (Wright et al., 2006, Simamura et al., 2010) is induced as part of a range of inflammatory reactions (Yue et al., 2015). It is worth noting that, in a previous report, MIA in mice (4mg/kg or 20mg/kg poly I:C at E12.5) caused elevation (4mg/kg) or reduction (20mg/kg) of the levels of LIF, according to dose of poly I:C, resulting in decreasing neurogenesis in the cerebrum; moreover, this may be linked to level of IL-6 in maternal serum (Tsukada et al., 2015). In addition, foetal LIF is required to respond to elevated maternal glucocorticoids (Ware et al., 2003). Unlike IL-10, there is no clear and direct evidence to make a link between LIF and schizophrenia, but previous evidence suggests that induction of LIF in maternal serum may be critical for foetal brain development; therefore, it could be a risk of psychiatric disorders.

Among the chemokines, except CXCL5, all of the measured chemokines were significantly induced by resiquimod; however, poly I:C administration significantly upregulated the levels of CCL2, CCL5, CXCL9, and CXCL10 compared to PBS. CXCL10 upregulation is not surprising because of its biological function (antiviral response) (Trifilo et al., 2004, Melchjorsen et al., 2006, Skinner et al., 2018), but CXCL1 is not significantly induced by poly I:C, which is in contrast to previous reports (Nair et al., 2009, Ballendine et al., 2015, Lins et al., 2018). Overproduction of CCL2, CCL5 and CCL11 are known to be induced by MIA (Arrode-Bruses and Bruses, 2012) or by inflammatory or pathological conditions (Aviles et al., 2008, Mendonça et al., 2010, Bosmann et al., 2012, Chao et al., 2012). Dysregulation of CCL2, CCL5 and CCL11 in schizophrenia patients is reported (Reale et

al., 2011, Noto et al., 2015, Hong et al., 2017), besides they could damage the brain (Roberts et al., 2012, Guo et al., 2020).

Resiquimod induced all of the growth factors that were measured: G-CSF, GM-CSF, M-CSF, and VEGF. However, poly I:C significantly induced only M-CSF and VEGF. Similar to our data, the poly I:C MIA model shows that the levels of CSFs and VEGF are induced by MIA in foetal brain tissues (Arrode-Bruses and Bruses, 2012).

Although growth factors are generally categorised into neither typical inflammatory cytokines nor chemokines, they are essential for immune cell development (Roberts, 2005, Ushach and Zlotnik, 2016, Hamilton, 2019).

3 colony-stimulating factor (CSF) molecules (G-CSF, GM-CSF, and M-CSF) are known to be essential for embryo implantation (Robertson, 2007, Rahmati et al., 2015); thus, their levels should be tightly regulated throughout pregnancy. GM-CSF is essential for placental growth (Athanassakis et al., 1987), regulating immune trophoblast immunity (Litwin et al., 2005, Moldenhauer et al., 2010), and foetal development (Robertson et al., 2001, Michael et al., 2006). In addition, these molecules positively affect CNS cells. For example, GM-CSF stimulates dose-dependent neuronal differentiation *in vitro* (Krüger et al., 2007). G-CSF inhibits neuronal loss via apoptosis (Yata et al., 2007). M-CSF is essential for microglial migration during embryogenesis (Ginhoux et al., 2010) and induces microglial proliferation in the spinal cord (Okubo et al., 2016). However, there is also evidence to show that a high-induced maternal level of G-CSF causes inflammatory status in the foetus (Sezik et al., 2019).

VEGF is required for angiogenesis in embryonic development (Ferrara, 2004), and modulates neurogenesis and neuronal migration in the developing CNS (Rosenstein et al., 2010). Plasma levels are increased by inflammation (Bien et al., 2015). Our data also showed that the level of VEGF in maternal serum was upregulated by both poly I:C and resiquimod. This outcome may not be surprising because the early steps of inflammation, where mast cells are participating, are closely linked with VEGF (Shaik-Dasthagirisaheb et al., 2013). In addition, MIA (using LPS) increased the level of VEGF mRNA in the foetal brain (Liverman et al., 2006). Overall, while it is not clear that these growth factors can penetrate the placenta and access the foetal compartment, the altered maternal plasma levels are likely to influence the health of the maternal-foetal interaction and may leave a long-last impact on foetal brain functions.

5.4.4 Placenta

In previous MIA studies, LPS administration (0.05 or 2.5mg/kg, 2-8hours) significantly induces the levels of IL-6 and TNF-α in placental tissues, but these effects are dose- and time-dependent (Urakubo et al., 2001, Ashdown et al., 2006). Poly I:C administration (10mg/kg, 6hours) increased *Il-6* and *Tnf-*α mRNAs (McColl and Piquette-Miller, 2019), and increased *Il-6* and *Tnf-*α mRNAs (20mg/kg, 3 hours) after poly I:C administration (Hsiao and Patterson, 2011). A gestational stress

model, using environmental noise stress, increases *Il-6, Ccl5*, and *Cxcl10* mRNAs (Bronson and Bale, 2014).

The first MIA model data indicated that when poly I:C administration (environmental factor) was combined with a genetic factor, *Map2k7* hemizygosity, CXCL12 was significantly upregulated by poly I:C administration (without the genetic factor) after 6 hours (Figure 21, the data are published)(Openshaw et al., 2019). In addition, the observations from the second MIA model suggested that the most of measured mRNAs including *Il-6, Tnf-α, Il-10*, *Ccl5*, *Ccl11*, and *Cxcl10* were significantly induced by resiquimod; however, *Tnf-α, Ccl5*, and *Ccl11* were not affected by poly I:C compared to PBS (Figure 26). The data indicate that resiquimod (TLR7/8 agonist) can be a stronger immune reaction initiator in placental tissue in mice.

5.4.5 Foetal brains

In foetal brain samples, resiquimod significantly induced the levels of all measured inflammatory molecules except *Cxcl12,* whereas poly I:C only affected *Cxcl12* despite the effects seen in maternal plasma and the placenta. However, the significant poly I:C effect on foetal *Cxcl12* mRNA may be seen because of one outstanding sample.

LPS administration elicited expression of various immune molecules, but its changes are time- or dose-dependent (Cai et al., 2000, Urakubo et al., 2001, Liverman et al., 2006, Oskvig et al., 2012, Simões et al., 2018).

Some studies demonstrate that poly I:C administration can induce immune reactions in embryo brains (Meyer et al., 2006, Meyer et al., 2009a, Wu et al., 2017); nonetheless, there is also evidence to show no changes after poly I:C administration (Abazyan et al., 2010), or even reduction of *Tnf-α* mRNA is reported (Ratnayake et al., 2014).

From own observations, poly I:C effects (upregulation of inflammatory molecules) were clearly seen in maternal plasma (proteins) and partly in placental tissues (mRNAs), but these effects were rarely observed in foetal brains (mRNAs). On the other hand, immune responses are elicited by resiquimod in all samples; maternal plasma, placental tissues, and foetal brains.

Current concerns about the reproducibility of poly I:C administration have been raised (Ozaki et al., 2020, Kowash et al., 2019, Mueller et al., 2019, Murray et al., 2019). For examples, the increased levels of IL-6 in maternal plasma after poly I:C administration in rats are highly variable (Kowash et al., 2019, Murray et al., 2019). In our study, minimal changes in some of the measured cytokines and chemokines were detected in maternal plasma following poly I:C MIA. Also, even less-pronounced poly I:C effects were observed in placental tissues regarding immune molecule transcriptional changes after MIA. As noted above, many papers have reported elevated levels of cytokines and chemokines by poly I:C in the placenta (Wu et al., 2017, Mueller et al., 2019) and embryo brains (Meyer et al., 2006, Meyer et al., 2009a), nonetheless Ratnayake and others report

no significant *Il-6* and *Tnf-α* mRNAs changes in placenta (Ratnayake et al., 2014) and the foetal brain (Abazyan et al., 2010). Together with previous evidence, our observations highlight the variability of the maternal immune response to poly I:C in mice and rats.

There is a concern that can be discussed related to elevated levels of immune molecules in foetal brains, and that is whether they come from maternal plasma. There is the evidence to support the idea that LPS (Kohmura et al., 2000) can potentially cross the placental tissues, although there appears to be no evidence for a similar capacity for poly I:C.

One possibility to consider is that resiquimod can pass the placental barrier like LPS, although no experimental evidence exists for this at this moment. Because other pathogens, such as Zika virus (Zanluca et al., 2018), HIV (del Rio and Curran, 2015), and *Rubella* and *Toxoplasma Gondii* (Robbins et al., 2012, Ville and Leruez, 2015) can show vertical transmission, therefore, there is clinical correlate for the potential direct effect of resiquimod on the foetal brain. Alongside any direct effect of the pathogen, maternal immune molecules can pass through the placenta.

As noted above, poly I:C upregulated *Il-6* and *Il-10* mRNA expression in the placenta, but not in the brain, whereas resiquimod upregulated these cytokines in both compartments. There is evidence to support that the effects of MIA on the foetus (i.e. brain *Il-6* and *Cxcl10* induction) are mediated by maternal IL-6 (Hsiao and Patterson, 2012, Wu et al., 2017). Besides, IL-6 may cross placental tissues (Zaretsky et al., 2004, Dahlgren et al., 2006), and thereby affects the foetal brain. However, despite the increases in maternal plasma IL-6, and placental *Il-6* mRNA induced by poly I:C, only resiquimod evoked increases in immune molecule gene expression in the foetal brain. This suggests that other mechanisms apart from maternal IL-6 are also involved in the foetal brain changes (in other words, increased maternal IL-6 may be necessary but not sufficient for foetal effects).

There is an interesting contrast between the effects in foetal brain and the placenta in terms of *Ccl2* mRNA expression. *Ccl2* mRNA expression was elevated by maternal resiquimod administration in the foetal brain, whereas not in the placenta.

The placenta is an immunologically highly active organ during pregnancy (Zourbas et al., 2001, Bowen et al., 2002, Ostojic et al., 2003). Although trophoblasts express CCL2 (Renaud et al., 2009, Du et al., 2014, Pavličev et al., 2017), there is evidence to show that it may be challenging for CCL2 to pass the placenta because of the expression of ACKR1 and 2, which are scavenging receptors for CCL2 (Borroni et al., 2008, Wessels et al., 2011, Teoh et al., 2014, Lee et al., 2019). It is possible that the MIA condition could increase permeability to allow maternal CCL2 to pass into the foetal compartment. Indeed, the data suggested that *Ccl2* was not produced by placental tissues (Figure 26). Therefore, resiquimod can induce *Ccl2* gene expression in foetal brain but not in placenta.

To be specific, the cellular source of *Ccl2* in the CNS is predominantly astrocytes (Barna et al., 1994, Glabinski et al., 1996) and microglia (Hanisch, 2002). CCL2 secretion via TLR7 from astrocytes and microglia has been reported (Lewis et al., 2008, Butchi et al., 2010); however, there is evidence to suggest that CCL2 production in the murine brain is from astrocytes, not from microglia (Hayashi et al., 1995, Peterson et al., 2004). Therefore, the *Ccl2* mRNA induction may be demonstrating a direct or indirect action of resiquimod on foetal astrocytes.

In addition, our own correlation analysis was conducted to determine whether the degree of induction of immune molecules might be linked between the tissues. 5 different immune molecules, IL-6, TNF-α, IL-10, CCL2, CCL5, and CXCL10, were selected based on relevance to schizophrenia. Overall, the analysis suggested that the observations for each marker from each tissue were likely independent (no significant correlation) effects (Figure 41).

As informative additional measurements apart from immune molecules, *bdnf* mRNA expression in foetal brains, and the effect of foetal sex on microglial immune responses were checked. Firstly, the levels of *Bdnf* mRNA were not changed in the foetal brain, but resiquimod exposure decreased *Bdnf* mRNA levels in the placenta (Figure 40). A reason for the importance of looking at this gene is its frequent link to environmental risk for psychiatric disease (see section 5.1)&(Zhang et al., 2016a, Kumar et al., 2020). Furthermore, developing evidence suggests that foetal microglial development is affected by sex (Thion et al., 2018b, Gildawie et al., 2020). In order to investigate foetal sex influence on foetal immune response in the brain, their sex was determined by RT-qPCR, using the sex-specific imprinted *Xist* gene (Cheung et al., 2017). High *Xist* expression indicated that tissue was of female origin and low expression indicated male origin (Figure 39). The data showed that the samples within a treatment group were well-balanced for sex. However, the interaction of sex with the effect of MIA was not statistically significant for any of the markers.

Altogether, under normal physiological conditions, a foetus is tightly protected from the maternal side's inflammatory molecules by the placenta; indeed, the placenta selectively exchanges the molecules helpful to the foetus. However, our data suggest that extreme conditions, like MIA during pregnancy, induce not only maternal immune reactions (Figure 22-Figure 25), but also clearly result in a substantial immune response in the foetal brain, at what is potentially a key stage of brain development (Figure 27). This may occur via disruption of placental immune regulation (Figure 26). During development, infiltrating maternal molecules, especially cytokines and chemokines, are possibly linked to atypical immunity and abnormal foetal brain development (Ashdown et al., 2006, Garay et al., 2013) consequently may particularly be relevant to schizophrenia. Thus, malfunction of the placenta could potentially be a key aspect affecting foetus development. Furthermore, it is clear from our observations that the level of immune response is pathogen-dependent: at least at the doses used, resiquimod induces a much greater immune reaction than poly I:C in general, and in the foetal brain in particular.

5.4.6 Microglial markers

Since one of our main interests is how the foetal immune reaction in the brain is changed by MIA, examining the microglial population is relevant. Additionally, the BBB is still immature at E12.5 in mice (Delaney and Campbell, 2017, Langen et al., 2019); therefore, potentially peripheral immune cell infiltration into the foetal brain could be induced by MIA. Typically, to distinguish different cell populations, IHC and Fluorescence-activated cell sorting (FACS) are used; however, because of the limitation of samples, RT-qPCR analysis was used in this study. Despite technical limitations, 5 different genes, *Aif1, Tmem119, Cx3cr1, Ccr2,* and *Ly6c2,* were measured to give an idea of how much microglial and peripheral immune cell populations are changed in foetal brains after MIA.

As introduced previously, in spite of the lack of specificity, many studies have used IBA-1 antibodies to identify the microglial population. IBA-1 (gene name: *Aif1*) cannot separate microglia from any infiltrated periphery macrophage population. Under a normal physiological condition, the microglia population is maintained without any peripheral macrophage contribution (Waisman et al., 2015, Askew et al., 2017); however, this is not the absolute condition; under pathological conditions, leukocyte infiltration can happen, and these cell largely contribute to microglial populations (Hickey and Kimura, 1988, Greter et al., 2005). Therefore, inflammation in the brain under pathological conditions, like MIA, could have a potential peripheral immune cell contribution.

The data showed that maternal resiquimod administration increased *Aif1* gene expression, but not poly I:C. The cytokine and chemokine RT-qPCR data also suggested that poly I:C did not affect transcriptional levels, as compared to resiquimod. Unfortunately, our date could not discriminate which populations contribute to the increase in *Aif1* mRNA because of technical limitations.

Two markers, *Tmem119, Cx3cr1* are characteristic of microglia in the CNS, and TMEM119 is believed to be a microglial-specific marker, not expressed peripheral myeloid cells. The findings suggested that, in both cases, their mRNA levels are downregulated by resiquimod compared to PBS. Interestingly, *Tmem119* was also significantly changed by poly I:C; however, *Cx3cr1* was not. One recent paper has shown that *Tmem119* is suppressed in a pathological inflammatory condition, which may be correlated with demyelination (Masuda et al., 2019). Moreover, the data from single cell analysis showed that the level of *Tmem119* mRNA is reduced when microglia are activated compared to resting status (Ronning et al., 2019). Additional evidence, the reduction of *Tmem119* mRNA (Krasemann et al., 2017) and protein (van Wageningen et al., 2019, Young et al., 2020) expressions after a pathological event, has consistently been reported.

Although TMEM119 is believed to be a microglial-specific marker, its expression may not be microglial-specific (Grassivaro et al., 2020). Moreover, its expression level has been tested from E17 by IHC (Bennett et al., 2016) and but our MIA model is E12.5; therefore, the stability of the expression in an earlier developmental stage still remains to be studied.

Compared to TMEM119, CX3CR1 is now known not to be a microglial-specific marker; there is evidence to show that it is expressed during development by hemopoietic stem cell lineage cells

(Grassivaro et al., 2020). Similar to our findings, CX3CR1 is down-regulated in AD patients samples, where the levels of activated microglia are increased (Keren-Shaul et al., 2017).

Since both *Tmem119* and *Cx3cr1* have homeostatic functions in microglia, together with our own findings and previous building observations, it can be proposed that pathological conditions and MIA could cause microglial transcriptional changes (decreased *Tmem119* and *Cx3cr1* mRNA expression) in order to adapt to environmental changes.

Ccr2 gene is widely-believed not to be expressed by both resting and activating microglia; however, monocyte-related cell populations abundantly express the gene (Prinz et al., 2011, Mizutani et al., 2012, Greter et al., 2015, Ronning et al., 2019). Recent findings illustrate that the expression level of *Ccr2* mRNA in the brain is noticeably affected by developmental stages (Kierdorf et al., 2013, Chen et al., 2020); thus, it could be proposed that the *Ccr2* signal in the foetal brain at E12.5 more likely from embryonic microglia than from infiltrating macrophages/monocytes. In general, like Ronning *et al*'s observation, *Ccr2* mRNA is likely going up under when peripheral cells are infiltrating neural tissue. However, our observation showed the opposite to this: *Ccr2* mRNA level was downregulated by MIA. However, there is evidence to show that *Ccr2* is significantly downregulated in microglia and macrophages in pathological inflammatory conditions (Li et al., 2019b). Correspondingly, *Ccr2* mRNA levels are negatively correlated to experimental autoimmune encephalomyelitis (EAE) scores (Lewis et al., 2014). Equally, CCR2 expression is decreased after LPS admiration in monocytes (Sica et al., 1997, Weber et al., 1999, Parker et al., 2004, Heesen et al., 2006, Sousa et al., 2018).

Like *Ccr2*, *Ly6c2* is considered as a specific monocyte/macrophage marker, not present in microglia (Butovsky et al., 2014, Bowman et al., 2016, DePaula-Silva et al., 2019, Jordão et al., 2019, Ronning et al., 2019); therefore its level is less likely to be changed in the foetal brain after immune challenges (Jordão et al., 2019, Ronning et al., 2019). The data showed that in contrast to *Ccr2* gene, *Ly6c2* expression was not significantly changed by MIA. In the mouse, the BBB starts to form around E13 (Daneman et al., 2010) at the time that myelopoiesis is happening (Prinz et al., 2017), but the microglia derived from yolk sac starts at E8.5 (Hoeffel et al., 2015). Taken together, in E12.5 developing brain, the immune cell population is predominantly microglia, and monocyte infiltration is less likely to happen in the foetal brain at this time.

In conclusion, the data illustrated that microglial, and monocytic markers were changed by MIA (Figure 28). Consistent with previous responses (Figure 22-Figure 26), most markers were more altered by resiquimod than poly I:C. However, there is direct and indirect evidence to show microglial activation in the foetal brain can be induced by MIA with poly I:C (E12 or E15, 10-20mg/kg, i.p.) although in these studies the tissues are collected a few days after the administration or giving double injections (Pratt et al., 2013, Smolders et al., 2015, Ozaki et al., 2020). Thus, this less overt change may be caused by the current experimental design (for example the time point studied).

5.4.7 Summary

In this chapter, the impact of maternal immune activation (MIA) on the immune response in the placenta and foetal brain was studied, and changes of microglial/monocyte markers following MIA in the foetal brain were investigated.

Based on the findings, the following main points can be summarised:

1. Poly I:C and resiquimod both induced cytokines (e.g. Il-6, TNF-α, IL-10, LIF) chemokines (e.g. CCL2, CCL5, CXCL10), and growth factors (e.g. (M-CSF, VEGF) in maternal plasma, but resiquimod was more potent in inducing an immune reaction compared to poly I:C, at the doses used.

2. The levels of immune molecules, such as I*l-6*, *Il-10*, and *Cxcl10*, in placental tissues were induced by MIA, but poly I:C induced fewer immune responses than resiquimod.

3. CCL2 protein in maternal plasma and *Ccl2* mRNA in the foetal brain was significantly induced, however its mRNA levels were not altered in the placenta.

4. Resiquimod caused immune response (producing cytokines and chemokines) in the foetal brain; however, poly I:C did not.

5. Microglial and monocytic markers were changed by MIA.

    a. Because of the current experimental setting (E12.5, 4hr administration), monocyte infiltration is less likely to happen at least in this model.

    b. Both homeostatic functions related genes (*Tmem119* and *Cx3cr1*) in microglia were significantly downregulated by MIA and these results are potential indirect evidence of microglia activation.

These observations emphasise the distinct effects of MIA on foetal brain immunity. Because of the time limits, other samples collected for analysis, such as embryo brain histology (microglial markers), and maternal brain transcriptomic analysis, could not be done. These samples will give much thorough information on how MIA affects CNS inflammation during pregnancy, and affects foetal development depending on pathogen types. As discussed above, because of lack of evidence to support vertical pathogen transmission, it would be of value to investigate whether poly I:C and resiquimod are able to cross the placental. This vertical transmission study is planned, but because of the current COVID-19 restriction and time limit, it could not be done yet. Although the data presented here only show the changes at a short exposure time, it would be worth studying the effects on later behaviours of MIA offspring. In addition, how ssRNA viral infection (resiquimod administration) or even bacterial infection (e.g. LPS administration) combine with genetic factors

related to schizophrenia would be interesting to investigate. Furthermore, how different types of gestational environmental challenge, such as dietary restrictions or non-infectious stress, differ from infectious MIA to affect foetal brain immune development would also be very interesting to study.

Chapter 6
Discussion

Chapter 6 Discussion

6.1 Summary of findings

This thesis initially introduced the interaction between aetiological evidence of MIA relevant to schizophrenia (Brown, 2011, Meyer, 2019) and abnormal immune molecule levels in the peripheral system (Potvin et al., 2008, Miller et al., 2011, Hong et al., 2017, Delaney et al., 2019) and the CNS (Fillman et al., 2013, Gallego et al., 2018, Pandey et al., 2018), possibly suggesting involvement of microglia in these processes (Bloomfield et al., 2016, Doorduin et al., 2009). Investigations in this field have suggested a putative mechanism; however, details of the underlying biology are unknown. As cytokines and chemokines appear to be important molecules in the immune system and the CNS (Bacon and Harrison, 2000, Ambrosini and Aloisi, 2004, Hughes and Nibbs, 2018), a few specific cytokines and chemokine were selected, because of their relation to schizophrenia, for further investigation. From this point of view, this thesis addressed the hypothesis that maternal immune activation caused by environmental challenges affects foetal microglial immunity via the MAPK pathway. More specifically, resiquimod induces stronger microglial immune reaction (cytokine and chemokine production) via the MAPK pathway compared to other pathological mimetics. As a result of induced microglial activity, maternal immune activation will disrupt foetal brain development. Our observation shows strong potency of resiquimod as a MIA model inflammation inducer. Furthermore, evidence reported in this thesis suggested that the MAPK pathways were used in microglial immune reactions (cytokine and chemokine reactions) although this signalling pathway may not be a main pathway to regulate the reactions. Lastly, a new resiquimod MIA model induced much stronger immune reactions in foetal brains compared to poly I:C MIA. Even though we could not do comparison test at further time points to test how negative the impact is on foetal brain development, however these observations contribute to establishing a resiquimod MIA model for further studies.

Previous work has shown that neuronal cells show an immune reaction following various immune/inflammatory stimulations (Lehnardt et al., 2003, Lafon et al., 2006, Leow-Dyke et al., 2012); however, the details of the signalling pathways, and particularly the importance of the MAPK pathways, need further study. Mouse primary cortical cultured neurons showed significantly increased JNK phosphorylation level following poly I:C. Because neurons also actively respond to immune molecules, although their reactivity may be limited to glial population, MAPK phosphorylation levels were measured after CXCL10 and CXCL12 stimulation. CXCL10 stimulation affected the level of pJNKs (down-regulation), but not pERKs and pp38, whereas CXCL12 stimulation affected the levels of pERKs (down-regulation, isoform-dependent) and pp38 (down-regulation), but not pJNKs.

A role for microglial cells in the aetiology of schizophrenia has been proposed (Volk, 2017, Marques et al., 2019, Birnbaum and Weinberger, 2020). Moreover, a genetic risk factor for schizophrenia is linked to MAPKs (Winchester et al., 2012). However, how these signalling molecules affect microglial immune functions is somewhat unclear. To address this point, MAPKs'

contribution to the microglial immune reaction was studied following various stimuli. Microglial cells (SIM-A9) showed significantly upregulated levels of MAPKs phosphorylation after TLR activation; however, it was pathogen type- and time- dependent. Besides, cytokine and chemokine production suggested similar pathogen- and time-dependent responses. Indeed, MAPKs' contribution to cytokine and chemokine induction was much more complex For example, JNK inhibition upregulated the levels of immune molecule mRNA production after resiquimod exposure (except for *Ccl5* and *Il-10* at 0.5 hour); however, its effect was to diminish the resiquimod response at the 8 hour point. Intriguingly, our observation showed that protein and mRNA levels do not reflect each other, although it is difficult to compare directly.

Previous work within our laboratory showed that the poly I:C MIA model significantly affects embryo development potentially via an elevated level of CXCL10 (Openshaw et al., 2019). Experiments were performed to establish and test a new MIA model with a new pathogen mimetic, resiquimod, selected on the basis of epidemiological evidence linking maternal ssRNA virus exposure to schizophrenia risk. In contrast to the previous SIM-A9 experiment, poly I:C noticeably induced maternal systemic inflammation, similar to resiquimod. for the data from the placental tissues, the *Il-6, Il-10,* and *Cxcl10* mRNA inductions were detected after both poly I:C and resiquimod, but some of the genes e.g. *Ccl2* and *Cxcl12* did not show any significant changes. mRNA levels in embryo brain were only induced by resiquimod, not by poly I:C. The work suggests that resiquimod is a strong immune stimulus to initiate a microglial immune reaction.

6.2 Discussion

6.2.1 Inflammation and schizophrenia

The relationship between inflammation and psychosis is difficult to study, even though they are many studies that show indirect evidence to support it. For example, minocycline, an anti-bacterial medication, is widely believed to suppress microglial activation. In terms of schizophrenia and minocycline treatment, contrasting findings exist, e.g. with positive (Zhang et al., 2019) and negative (Deakin et al., 2019) treatment response results.

Despite the contrasting effects of minocycline in patients, in laboratory and pre-clinical levels, its effects are still significant. For example, minocycline reportedly rescues the behavioural and neural network abnormalities of MIA offspring (Hou et al., 2016, Mattei et al., 2017, Ji et al., 2020, Xia et al., 2020). It has been argued that the minocycline effects are due to diminished inflammation. Indeed, a study shows that microglia depletion and minocycline administration show similar data (Zhang et al., 2021).

In addition, Vitamin E, an antioxidant, was considered as to whether it helps attenuate microglial immune reactions. The reason for choosing vitamin E is that it may be one of the potential metabolic biomarkers of schizophrenia: lower levels of vitamin E in serum have been reported in patients compared to healthy groups (Group, 2000, Davison et al., 2018). Data not presented in

this thesis showed that vitamin E treatment on SIM-A9 cell did not produce any morphological changes, indicative of either microglial activation or ramification. However, other published data showed a significantly increased microglial proliferation rate in rat primary microglia culture with vitamin E exposure (Flanary and Streit, 2006).

This suggests that while down-regulation of microglial activity (or inflammation levels in the brain) is almost certainly important to brain function, the relevance for schizophrenia remains unclear.

6.2.2 Microglial responses to resiquimod stimulation

Even though SIM-A9 cells have advantages compared to other previous microglial cell lines in terms of being free of external modification, it does not mean that they demonstrate 100% of *in vivo* microglial physiology. Moreover, evidence reports that microglia rapidly lose their characteristics under *ex vivo* conditions compared to *in vivo* (Gosselin et al., 2014, Bohlen et al., 2017). Therefore, it is better to investigate tissue microglia (*in vivo*) following stimulations, and, when using cell lines, to remember their limitations.

During this study, commercial antibodies for TMEM119, a microglial specific marker, are available, and this marker is more informative when co-staining with IBA-1 to confirm microglia structure rather than using alone, since TMEM119 antibodies do not stain the full cell morphology [Maria Suessmilch, personal communication]. Unfortunately, a good antibody for IBA-1 (019-19741) and all currently available products for TMEM119 are raised from rabbit; therefore, they cannot be used together. There is one mouse anti IBA-1 (NCNP24; 012-26723); thereby, it is a good alternative. The product was tested with a primary microglial culture, which has much closer microglial characteristic than cell lines and brain tissues.

I attempted to confirm microglial characteristics in SIM-A9 cells using Iba-1 staining, and primary microglial culture staining was also compared to mixed brain cell culture samples (a positive control), which were kindly gifted from Maria Suessmilch. I also prepared to conduct Iba-1 immunohistochemistry in brain tissue samples collected from MKK7$^{+/-}$ mice. As the data, especially from brain tissues, showed clear non-specific IgG staining (Figure 36, Figure 37), and because of time and resource limitations, I was not able to optimise the staining protocol; however, a few suggestions can be made. Since for the Iba-1 antibody I was attempting mouse on mouse (MOM) staining, the blocking step has to be more careful. However, because of this blocking step, it may affect signal intensity. To overcome losing intensity, commercial kits or biotin conjugation can be used. Had further time been available, I would have continued this immunohistochemical/immunocytochemical approach.

6.2.3 Evaluating the MIA model

The data from the SIM-A9 experiment showed that microglial cells sensitively respond to resiquimod stimulation. Furthermore, MAPKs' contributions were suggested. Since *MAP2K7* sequence variations are a genetic risk factor of schizophrenia, resiquimod effects on microglia from

Map2k7 $^{+/-}$ mice' would have been worth investigating prior to making the MIA model with *Map2k7* $^{+/-}$ mice.

The importance of obtaining data from these brain tissues will show a direct relationship between microglia and the JNK pathway in the *in vivo* condition. In our laboratory, a previous study showed that MKK7 is intriguingly related to schizophrenia-like behaviour in mice (Openshaw et al., 2017); moreover, the poly I:C MIA model shows potent genotype effects on the embryonic immune response (Openshaw et al., 2019). In this regard, the resiquimod MIA model with MKK7$^{+/-}$ mice is worth investigating, in terms of understanding the link between genetic and environmental factors.

Like *MAP2K7* sequence variants, another genetic risk factor for schizophrenia is the microduplication in 16p11.2 region, which contains the *MAPK3* (ERK1) gene. Since microglia participate in CNS immune responses, macrophages contribute to peripheral immune responses, and because the ERK pathway is important to immune function, collecting macrophages and microglia from DUP mice and observing their cellular responses following maternal stimulations, such as LPS, poly I:C or resiquimod, was planned. Our laboratory has 16p11.2 DUP mice to study the functions of this gene on schizophrenia-relevant parameters such as GABAergic interneuron function or behaviour changes. However, no prior data exists with primary macrophages or microglia from 16p11.2 DUP mice, and the contribution of ERK to immune responses. Therefore I had done a pilot experiment with BMDMs (Figure 42). Because of variation between cultures (experiments), it is difficult to interpret the data (with resiquimod); however, the data suggest that the JNK and ERK inhibitions do not significantly affect the production of iNOS following LPS stimulation. Although the observations do not show significant changes (treatment*inhibitor interaction, $F(4, 85)=0.90$, $p=0.471$), the results still need further confirmation. Even though the results from pilot macrophage experiments (from WT mice) show weak evidence to support the importance of the ERK pathway following TLR stimulation, macrophages from DUP mice possibly have different reactivity to TLR agonists because of developmental effects or an altered basal immune abnormality. Further research with the MIA model, with MKK7$^{+/-}$ and DUP mice, will give a better understanding of MAPKs effects on embryo immune development following prenatal immune challenge.

6.2.4 Microglial heterogeneity

Macrophage and monocyte abnormalities have been reported in schizophrenia patients' samples (blood) (Özdin and Böke, 2019, Mazza et al., 2020) and these are likely related to a high-inflammation status e.g. judging from the upregulated levels of cytokines and chemokines. Now we know the origins of microglia and peripheral macrophages are different; indeed, microglial heterogeneity is also important. Building evidence suggests that microglia and CNS-associated macrophages (CAMs) show partially shared, but also have a unique transcriptomic profile (Van Hove et al., 2019); indeed, unlike other CAMs, one of the CAM populations, choroid plexus macrophages, regularly exchanged their population with peripheral immune cells during the lifetime (Kierdorf et al., 2019).

While some interesting findings concerning microglial markers have been presented here, further studies are still needed, e.g. measuring protein levels, FACs analysis, so as not to draw any false conclusions.

However, building evidence from single cell studies shows that microglial marker expression level is very dynamic, so their expression levels are not as stable as what was previously believed. The first example is *Tspo*. Our data from SIM-A9 cells showed that *Tspo* mRNA levels did not significantly change over time after stimuli administration. Bader and others show that after LPS stimulation, TSPO protein levels (immunoreactivity) are induced within 24 hours, in BV-2 cells (Bader et al., 2019). In our observation, only LPS stimulation showed a hint of induction of *Tspo* mRNA at the 8 hour time point, although no statistical significance was not observed. As introduced at the beginning of this thesis, TSPO ligands are widely believed to reflect the inflammation status in the brain; however, results are not consistent, and its reliability is questionable (see section 1.2.5.3). In addition, another publication claims that TSPO inflammation activation is species dependent. They show different TSPO gene level changes upon pro-inflammatory stimulation, and these changes are different between human and rodent (Owen et al., 2017). The next example, expression of *Cx3cr1* and *Iba1* is shared by microglia and CAMs as well as microglia, while *Tmem119* and *P2ry12* are enriched in microglia (Goldmann et al., 2016, Jordão et al., 2019). Under unchallenged conditions, microglial transcriptional levels remain stable, but exposure to immune or inflammatory stimuli, or inflammatory disease conditions, can change them. For instance, the *Tmem119* level is decreased following LPS stimulation (Sousa et al., 2018), as we observed. In addition, with the EAE condition, *Tmem119* and *P2ry12* are significantly downregulated (Jordão et al., 2019). Recent evidence shows that expression levels of these markers, e.g. *CX3CR1, TMEM119* and *P2RY12* mRNAs, are decreased in patients with schizophrenia (Snijders et al., 2021). Our findings from SIM-A9 cells and MIA models are neither EAE nor psychiatric diseases. In broad terms, the samples were exposed to a higher inflammatory condition; thus, alteration of the markers can be considered as reasonably reflecting the changes under inflammation.

Of course, this alteration may differ in what population of microglia and when developmental stages are taken. Taken together, these data suggest the microglial complexity and importance of using the markers in the context experiment.

6.3 Conclusions

An unexpected theme that emerges from the work in this thesis is the relatively mild responses to TLR3 stimulation. The microglial cells consistently showed strong responses to LPS and resiquimod, but not poly I:C in terms of production of cytokines and chemokines. The findings from the MIA study showed both poly I:C and resiquimod caused a strong maternal immune reaction; however, only resiquimod effects were detected in foetus brains. Although the findings with resiquimod administration are intriguing, however it is required further study to confirm its

accessibility to the placenta and brain parenchyma via mass spectrometry investigations. Nonetheless, the study suggested that resiquimod is a strong immune agent for future research.

The clear response in maternal plasma shows that there is no issue with the biological activity of the poly I:C. Also, interestingly, while poly I:C did not affect any MAPK phosphorylation in SIM-A9 cells, this was different to what we saw in cortical primary neuronal culture, where there was a response. The work in this thesis suggests that the routine adoption of poly I:C, as a stimulus to study effects of immune stimuli on the brain, should be re-examined.

Equally, overlapping findings from SIM-A9 cells and MIA foetal brains concerning microglia markers are worth noting that microglial-specific markers such as *Tmem119* and *Cx3cr1* mRNAs were down-regulated by inflammation. All these markers are generally involved in microglial homeostatic functions; therefore, it may be not a surprising point. However, these observations propose that the experimental conditions must be considered when using these microglial markers, even though these findings are better confirmed in protein levels.

Overall, these data strongly suggest that, for the investigation of microglia function in the brain in the context of schizophrenia and neuroinflammation, resiquimod has strong potential as the immune stimuli, for the study of MIA or neuroinflammation in general. Throughout this study, the data propose the complexity of microglia (immune responses); therefore, we still need more knowledge to understand microglia.

6.4 Future directions

The main value of this project is in confirming that resiquimod (ssRNA virus mimetic) is a powerful tool for inducing microglial immune activation, and this reaction happens (partially) through the MAPK pathways. Furthermore, through this project, the evidence obtained shows that resiquimod is a considerable pathogen mimetic to induce MIA compared to poly I:C (another widely used viral mimetic).

Because of the current COVID-19 situation, one important experiment that was planned could not be done, which is checking for vertical transmission of poly I:C and resiquimod in the current (second) MIA model. The next thing would be collecting protein level data. As I draw the point from SIM-A9 results, protein levels do not always reflect mRNA levels. However, the data presented in the thesis are mostly mRNA data only (especially MIA results). Therefore, further analysis in protein level would be better to give deeper insights into what happens in each tissue after MIA. The last, the current (second) MIA model could not test genetic factor impacts on maternal and foetal systems. Thus, as a next step, because through this project the validation with resiquimod MIA was done, an advanced MIA model, a combination of environmental factor and genetic factor, can now be run.

Chapter 7 Appendices

7.1 General solutions and consumables

7.1.1 Details of chemical solutions

All general chemical solutions are mentioned in Table 7. The table describes the recipes of stock solutions and their concentration and special solutions will be mentioned in individual sections later if required.

Table 7. Recipes of chemical solutions

Solution	Recipes	Storage condition
10x PBS	80g NaCl + 2g KCl + 14.4g Na_2HPO_4 + 2.4g KH_2PO_4 + 1L dH_2O (pH 7.5) *PBS on method meant 1x PBS (diluted from 10x PBS) unless it was specified.	Room temperature
RIPA buffer	10mM Tris-HCl (pH 7.4) + 150mM NaCl + 1mM EDTA + 1%(v/v) Triton x-100 + 0.1%(w/v) SDS + 0.5% (w/v) sodium deoxcholate + dH_2O	4℃
Sodium Orthovanadate (200mM)	3.68g + 100ml dH_2O	Room temperature, dark
Sodium Fluoride (1M)	20.995g + 500ml dH_2O	Room temperature
Sodium Pyrophosphate (100mM)	22.3g + 500ml dH_2O	Room temperature
Lysis buffer	1mM Sodium Orthovanadate + 2.5mM Sodium Pyrophosphate + 25mM Sodium Fluoride + 1% (v/v) protease inhibitor cocktail + 1ml RIPA buffer	
20X running buffer	50mM MOPS + 50mM Tris-Base + 0.1% SDS + 1mM EDTA (pH 7.7)	Room temperature
20X transfer buffer	25mM Bicine + 25mM Bis-Tris (free base) + 1mM EDTA (pH 7.2)	Room temperature
Electrophoresis running buffer	40ml 20x running buffer + 760ml dH_2O	Room temperature
Transfer buffer	50ml 20x transfer buffer + 850ml dH_2O + 100ml methanol + 1ml antioxidant	Room temperature
10X TBS	24g Tris-HCl + 5.6g Tris-Base + 88g NaCl + 900ml dH_2O	Room temperature
1X TTBS	100ml 10x TBS +0.5ml Tween-20 + 900ml dH_2O	Room temperature
50X TAE buffer	252g Tris-base (2M) + 57.1ml Acetic acid + 100ml 0.5M EDTA (pH 8.0, 50mM) + 1650ml dH_2O	Room temperature
10X TAE buffer	100ml 50X TAE buffer + 4900ml dH_2O	Room temperature

7.1.2 Consumables

All general consumables are mentioned in Table 8. Special consumables will be mentioned individual sections if required.

Table 8. General consumables and their details

Item	Company	Cat. No.
6 well culture plate	Corning	3516
12 well culture plate	Corning	3513
24 well culture plate	Corning	3524
96 well culture plate	Corning	3596
25cm^2 Culture flask	Corning	430639
75cm^2 Culture flask	Corning	430641U
175cm^2 Culture flask	Corning	431080
1.5 ml microcentrifuge tube (RNase free)	Greiner Bio-one	616-201
2 ml microcentrifuge tube (RNase free)	Starlab	S1620-2700
500 µl microcentrifuge tube	Griener	667201
200 µl PCR tube	Starlab	I1402-8100
15 ml centrifuge tube	Coring	430790
50 ml centrifuge tube	Coring	430828
384 well qPCR plate	Applied Biosystems	4309849
Sterile alexa mixing kwills/Needles	Henleys medical	MN5
5 ml syringe	BD plastipak™	302187
20 ml syringe	BD plastipak™	300613
5 ml serological pipette	Corning	4051
10 ml serological pipette	Corning	4101
25 ml serological pipette	Cellstar®	760-180
Supercharged slides	VWR	631-0108

7.2 SIM-A9 culture condition optimisation

After receiving the cell (SIM-A9) vial from the ATCC company (CRL3265) culture condition was followed exactly manufactural set up at the beginning except FBS to horse serum. Since no one has experience with this cell line in Prof. Morris's lab, I had tested various factors which potentially could affect cell culture to optimise cell culture protocols for future experiments.

Factor 1. Serum concentration (5% vs. 10% vs. 15%)

Even though 15% serum concentration was recommended to use (Nagamoto-Combs et al., 2014), the growth speed of the cells was unable to control. Even if 1:100000 dilution was made for splitting, next day splitting was required (lemon serum colour indicates that the medium has to be changed). Too often splitting is increasing stress the cell; therefore, growth speed has to be managed in certain levels. Some of cell types require very high levels of serum, however some of them do not. Thus, once the cells were fully recovered from frozen status, the cells were cultured in 3 flasks and three different concentrations were designated in each flask: 5%, 10% and 15%. The serum concentration was gradually decreased until reach the final concentration to reduce stress on the cell in 2 weeks. When serum concentration was finalised, the cells were continuously exposed with their final concentration for 2 passages (minimum duration to expose for changing cell physiology by serum concentration) and then the cells were moved into no serum contained medium (called experimental medium) left them overnight. To their reactivity, the cells were stimulated with LPS (100ng/ml), poly I:C (100ng/mg) and resiquimod (3μM) for 24 hr at 37 °C in 5% CO_2 and the culture medium were collected for Griess assay. Results showed that different serum concentration did not affect iNOS productions (results were not shown). Therefore, in a future experiment, 5% serum is used because it makes that the growth speed is under control and reducing stress from splitting.

Factor2. Cell density

From previous cell culture experience, certain cell types (e.g. leukocytes and primary macrophages) required a certain level of cell-cell interaction to help to maintain their health and proliferation's ability. If the cell density is too low or too high, the cells do not grow well. Since I decided to reduce serum levels, keeping the cell-cell interaction (i.e. cell density) may become an important factor to keep the cells. Once 5% serum became to final growth medium serum concentration, three different dilution factors were tested: 1:100, 1:1000, and 1:2000. Among three dilutions, 1:100 dilution factor gave a decent amount of the cells for experiments and next passages. Thus, 1:100 (approximately 1.5-2.0*10^3 cells in a 25cm^2 flask) becomes a standard dilution factor unless there are special needs.

SIM-A9 cells have to be cultured in serum-free condition for an experiment. Serum-contain medium (called growth medium) help proliferation, however serum-free medium (call experimental medium) help differentiation. Therefore, on a culture plate, higher cell density is required to gain enough

number of experimental samples, because the cells cannot proliferate where the absence of the serum. Under the experimental condition, three different cell densities were tested: $6*10^5$/ml, $3*10^5$/ml and $1.5*10^5$/ml. In order to gain enough number of experimental samples, $3*10^5$/ml seeding density (12 well culture plate) is ideal to use unless there are special requirements.

Factor3. Size of Flask ($25cm^2$ vs. $75\ cm^2$)

Same concept as the cell density, cell culture flask size could affect. SIM-A9 cells are semi-adherent cells, they require their own territory to attach. Two different size of culture flasks, $25cm^2$ and $75\ cm^2$, which are commonly used, were tested. The results indicate that SIM-A9 was not very sensitive to the cell-cell interaction to keep their health. However, they tend to proliferate slightly faster if they are close to other cells (no scientific data, only observation).

Factor4. Material (plastic vs. non-coating glass vs. coating glass)

Some types of cells have to grow specific types of material, for example primary cortical mouse neurons have to culture on coated material. Likewise, primary mixed brain cell cultures have to culture on coated material, additionally they prefer glass than plasticware (Maria Suessmilch per comm). Because no one offers this information, I have tested different culture materials. The results showed that SIM-A9 did not have any preferrable material to grow and extra coating was not required (no helps for keeping cells). Therefore, the cells maintained un-coated plasticware or glass if it is required.

Factor5. Collecting cell population (Floating cell vs. Attached cells)

Although SIM-A9 cells are semi-adherence cells, floating sometimes indicates unhealthy statues of the cells. Especially, SIM-A9 cells are very easily detached even with gentle tapping if they are cultured in the growth medium. Therefore, separately collecting the cells into two populations, floating and attached population. The floating population was collected with previous culture medium, and the attached population was collected after a trypsin detachment step. Between the population collection, a culture flask was gently washed by cold-DPSB to remove. The collected cells were cultured to see how the cells grow further.

Regardless of their origins, the cells showed both floating and attach types in both culture flasks. Under a microscope, both the floating and the attached cells showed clear cell body and no morphological differences (results were not shown). Additionally, as long as if the seeding density is similar, their growth speed is similar. Therefore, in a future experiment, all cells are collected regardless of their adherent levels unless there is no contamination or sicken cells are found.

Factor6. Keeping the culture longer

In order to run a future planned experiment, I would like to keep the cell longer then 1 week under the experimental medium. Three different ways were tested, changing medium half, complete medium changing and no medium changes (control). Idea behind of half medium changing is from primary cortical mouse neuronal culture. Half medium change could reduce stress from cell medium changes also give more natural and rich physiological components which may not be offered by artificial culture medium. Potentially, because the experimental medium does not contain any serum, these processed biological components from the cells may increase their viability. Unfortunately, none of conditions kept the cells longer than 1 week which is maximum duration I managed to maintain the cells under the experimental medium. Though serum-contained condition was tried, this condition could not be used because of these reasons: 1. The cells proliferate, not differentiate (even the serum concentration was less than 5%). 2. It does not match to experimental condition (the serum-free condition). One publication was successfully kept SIM-A9 cells longer than 1 week (Farrell et al., 2016). Their method is completing medium change and keep the cells under experimental conditions (i.e. 0% serum) all along. But, experiments I tested showed that the cells could not survive longer than 1 week without the serum nevertheless the ways of changing methods of the culture media.

Factor7. pH and temperature

The cell culture media generally contain phenol to show their pH levels. Clear red colour is good pH to use, and when it becomes to pink colour, it means the pH goes up (basic). When SIM-A9 cells cultured straight into basic pH medium, either cell did not growth cell or even die. Even the cells transferred into the fresh medium, recovery rate was not good. In contrast, resilience to acid is not bad. This observation indicates that the cells are sensitive to basic condition. Therefore, if the culture medium become pink coulure, an aliquot of media leave in the incubator before use.

In terms of temperature, SIM-A9 cells do not sensitive for temperature. Warming up the culture medium is recommended; however it does not affect cell culture conditions.

Factor8. Frozen medium test

Once the cells were fully recovered from the frozen status, the cells started to proliferate. When the cells are fully recovered and before the serum concentration goes down, the frozen stocks have to be made for future experiments.

In order to make were freezing stocks, following four different conditions: w/wo 10% serum + two different types of freezing medium were tested. 10% serum contained with the freezing medium showed best. The cell pallets were resuspended with conditioned freezing medium and freeze in -80 °C (Thermorfisher, Mr.Frosty™ 11315674) for 24hr. Following day, the cells were transfer to a liquid nitrogen tank for long term storage. Once the optimised cell culture protocol was set, the cells were thawed and grown exactly how it was described (section 2.1). Compared between 10%

serum- and none-serum contain freezing stocks, 10% serum -contained frozen stock showed better viability.

7.3 RT-qPCR trouble shooting story

Cxcl1, *Cxcl12* and *Ccl11* old primer stocks showed multiple melting (MT) curves with SIM-A9 samples, although these primer pairs were already validated and actively used by colleagues without problems.

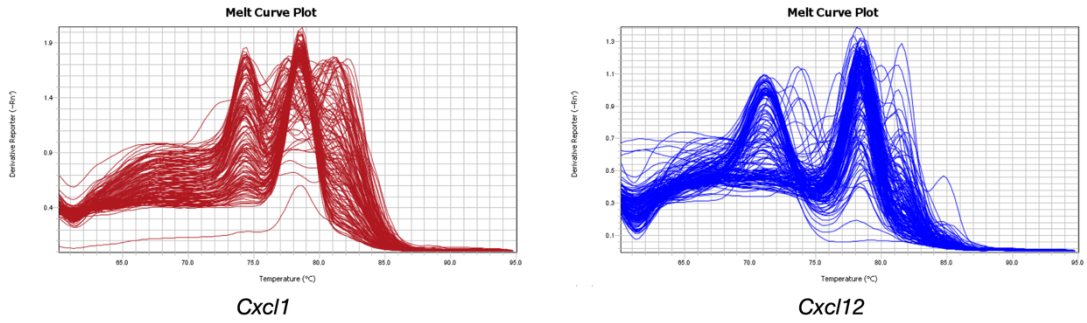


Figure 29. Multiple MT curves with SIM-A9 sample. Multiple MT curves were shown with SIM-A9 samples amplifed by *Cxcl1* and *Cxcl12* primer pairs.

In order to understand why it happens, I ran qPCR and conventional PCR products on a gel; however, no distinguishable amplicons were detectable on a gel. Thus, I tried to change single factors, e.g. sample types, Sybr green, STD, and then compared the results to find reasons of multiple MT curves.

Testing Sybr green result showed Sybr green may has issues with these primers (Figure 30), however other test results looked different. For example, an examination of sample type (Figure 31) results suggest that testing Sybr and the comparison Sybr showed one specific MT curve. Indeed, testing with STDs of *Cxcl1* and *Cxcl12* suggested testing Sybr may not be vital reason of multiple MT curves (Figure 32). Besides, A testing Sybr worked nicely with other primer and STDs, such as *CCl5*, *Cxcl10*, and *Gapdh.* Furthermore, the samples's purity measured by nanodrop was in an acceptable range.

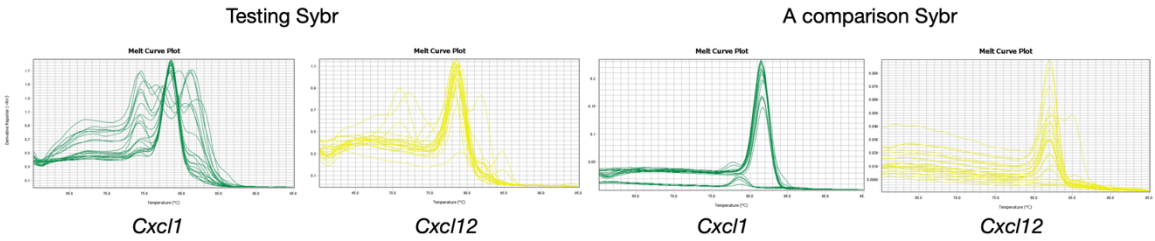


Figure 30. A testing Sybr looks worse than a comparison.A testing Sybr showed multiple MT curves.

From all these trouble shooting processes, it looked like old primer pair did not work well with SIM-A9 samples even though they are fine with brain tissue samples. One possible thing I could try to avoid multiple MT curves is design new primer pair include exon junctions (intron spanning) if it is possible. Intron spanning is believed to increase primer specificity of position and splicing variants. With the new *Cxcl1* and *Cxcl12* primer and STD pairs, multiple MT curves did not happened with SIM-A9 samples.

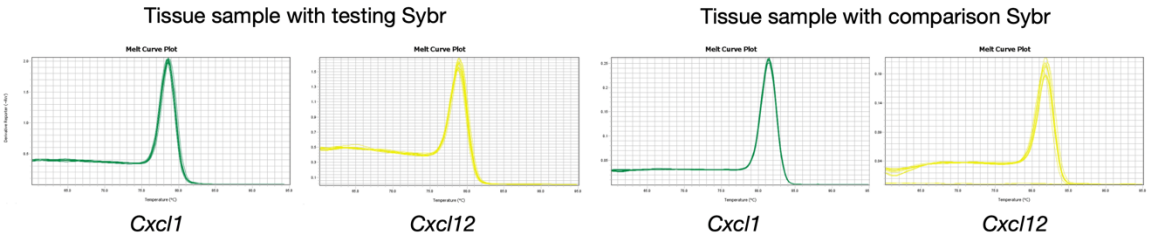


Figure 31. The brain tissue sample does not show multiple MT curves with both Sybr.

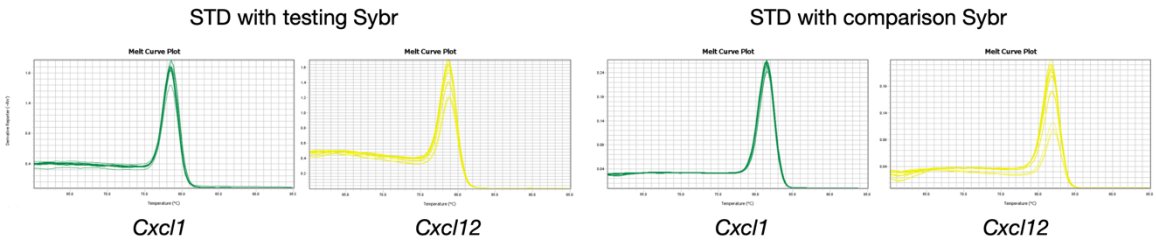


Figure 32. Multiple MT curves do not appear with STDs.

7.4 Chemokine stimulation on NG108 cells

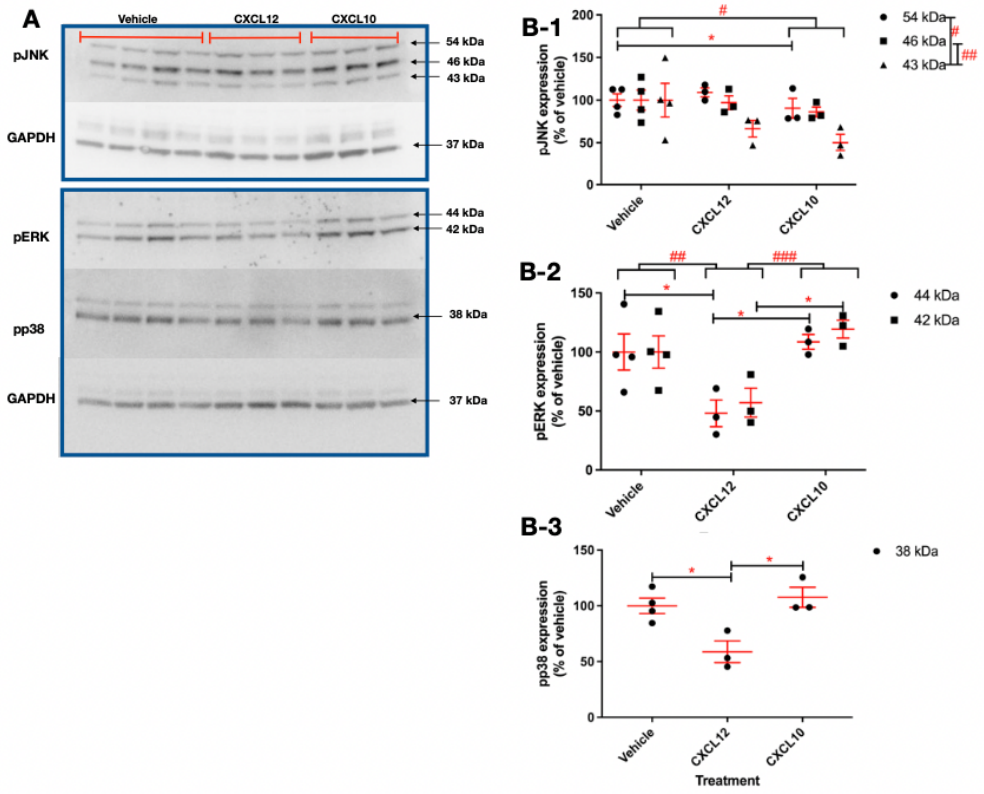
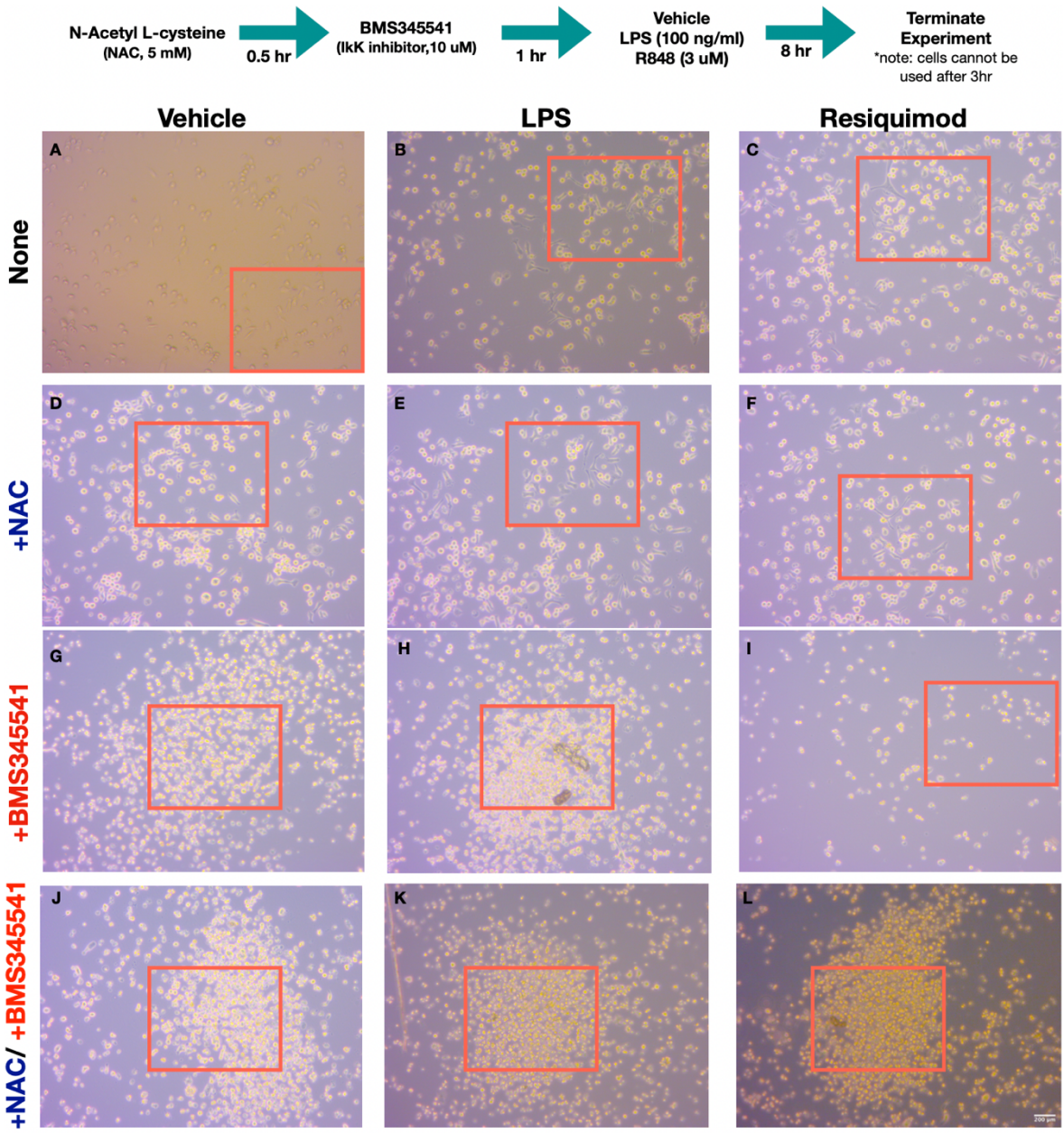


Figure 33. CXCL12 downregulated ERKs and p38, but not JNK phosphorylation. The NG108 cells were cultured in 0.5% serum contained medium for 5 days and stimulated by vehicle or by one of two chemokines, CXCL10 (10nM), or CXCL12 (10nM) for 15 minutes. **(A)** Chemokine stimulation showed minor changes in pMAPKs. **(A, B-1)** pJNKs levels were not changed with CXCL10 and CXCL12; **(A, B-2)** pERKs was down-regulated by CXCL12, not by CXCL10 compared to vehicle. **(A, B-3)** CXCL12 downregulated levels of pp38. One blot experiment image is shown for A and B. Individual dots show expression relative to vehicle ± SEM (n=3-4). Individual dots show expression relative to vehicle ± SEM (n=3-4). The data were log transformed and two-way ANOVA with Tukey comparisons (pJNKs and pERKs) or with one-way ANOVA with Tukey comparisons (pp38). (**p≤0.05, Post-hoc Tukey comparisons; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.005$, $^{\#\#\#}p \leq 0.0001$ Post-hoc Tukey comparisons)

7.5 IKK inhibitor experiment (+/- NAC) for 8hr stimulation



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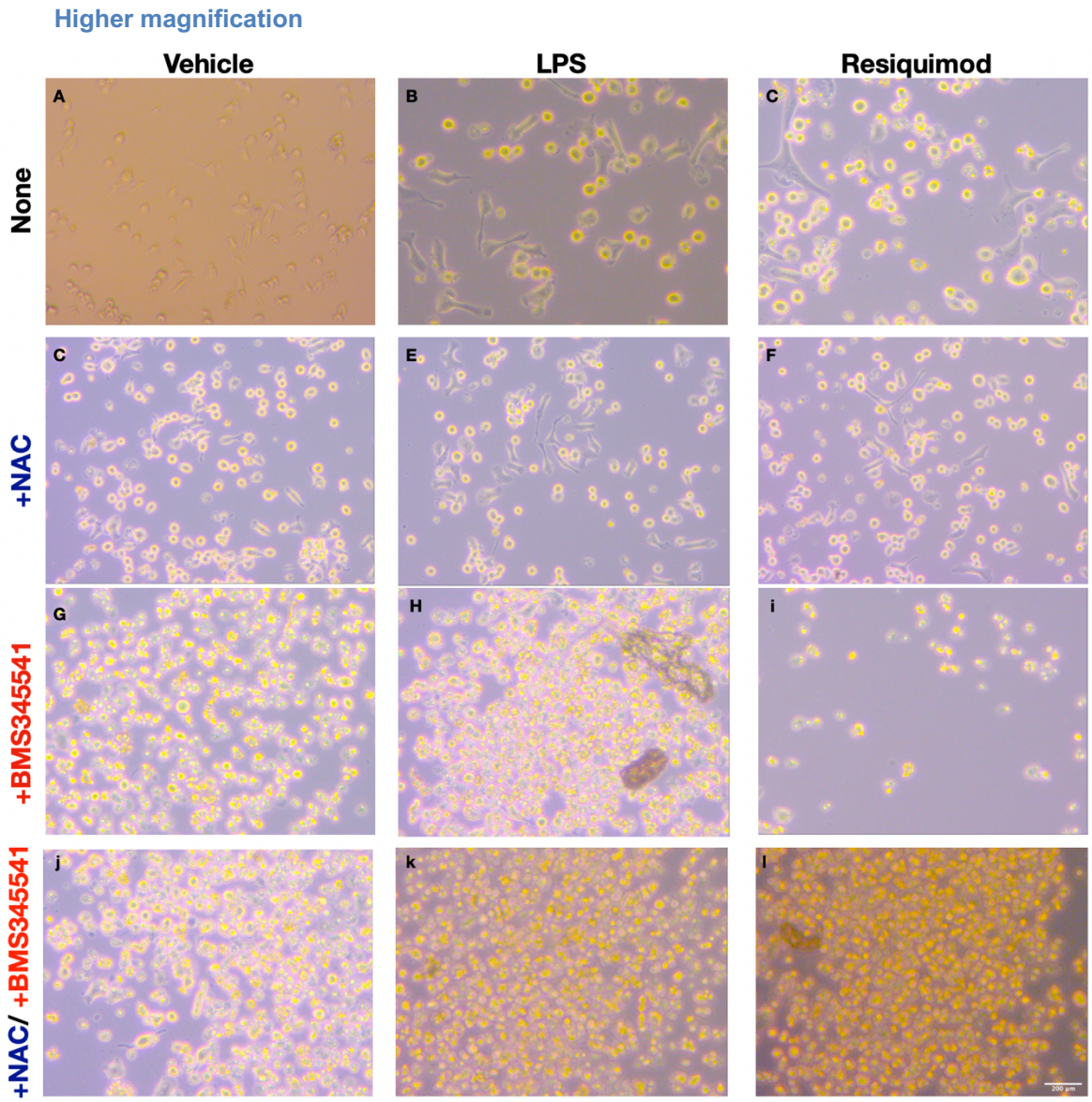

Figure 34. The microglial cells cannot survive under IκK inhibition for 8 hour. The microglial cells were cultured in serum- free medium and left overnight. The cells were stimulated by LPS (100ng/ml) and resiquimod (3μM) for 8 hr. NAC (5mM) and BMS345541 (10uM, IKK inhibitor inhibitor) were added prior to mimetics. **(A-C)** Although the cells activated but the cells were still healthy. **(D-F)** NAC solo treatment did not cause any additional effects on the cells in terms of morphological changes. **(G-I)** IKK Inhibition caused the cell death thus the cells made a mass of death cells and looked like crumbs which indicated the death or sick of the cells. **(J-L)** The combination of NAC and IKK inhibition did not help of surviving. **(a-l)** Higher magnification images (red selected area in images in A-L). The cells could not survive under the IKK inhibition. (Scale bars at right bottom corner represent 200μm in both original and higher magnification images)

7.6 JNK inhibitor comparison

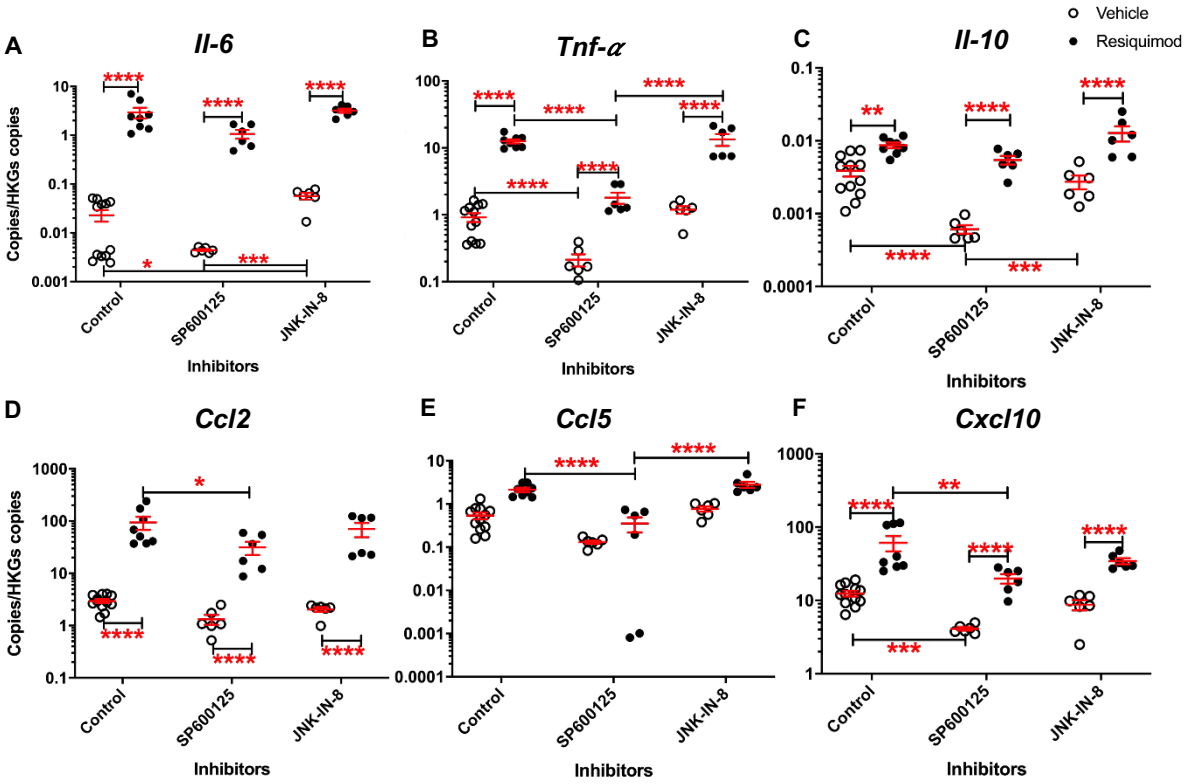

Figure 35. SP600125 inhibits not only JNKs but also other signalling kinases. The microglial cells were cultured in the serum-free medium and left overnight. The cells were stimulated by resiquimod (3μM) for 8 hour and two types of JNK inhibitors, JNK-IN-8 (Sigma, 1μM, 3hours ahead), or SP600125 (Enzo, 5μM, 0.5hours ahead) were added before resiquimod. **(A)** *Il-6* mRNA was downregulated by SP600125 and upregulated by JNK-IN-8 compared to control (without resiquimod) **(B)** *Tnf-α* mRNA was not affected by JNK-IN-8, but SP600125 decreasing mRNA levels in both, vehicle and resiquimod conditions **(C)** SP600125 down-regulated *Il-10* mRNA under vehicle although SP600125 did not affect *Il- 10* mRNA level under resiquimod condition **(D-E)** SP600125 administration downregulated *Ccl2* mRNA in only resiquimod condition; however, *Ccl5* mRNA levels were not significantly altered by both inhibitions **(F)** *Cxcl10* mRNA was not affected by JNK-IN-8, but SP600125 suppressed *Cxcl10* mRNA in both conditions compared to control. The individual data points are shown along with mean ± SEM. The data were log transformed and analysed by two-way ANOVA, Tukey post-hoc test (n=5-6 independent samples; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001 Tukey comparison)

7.7 IBA1 antibody testing

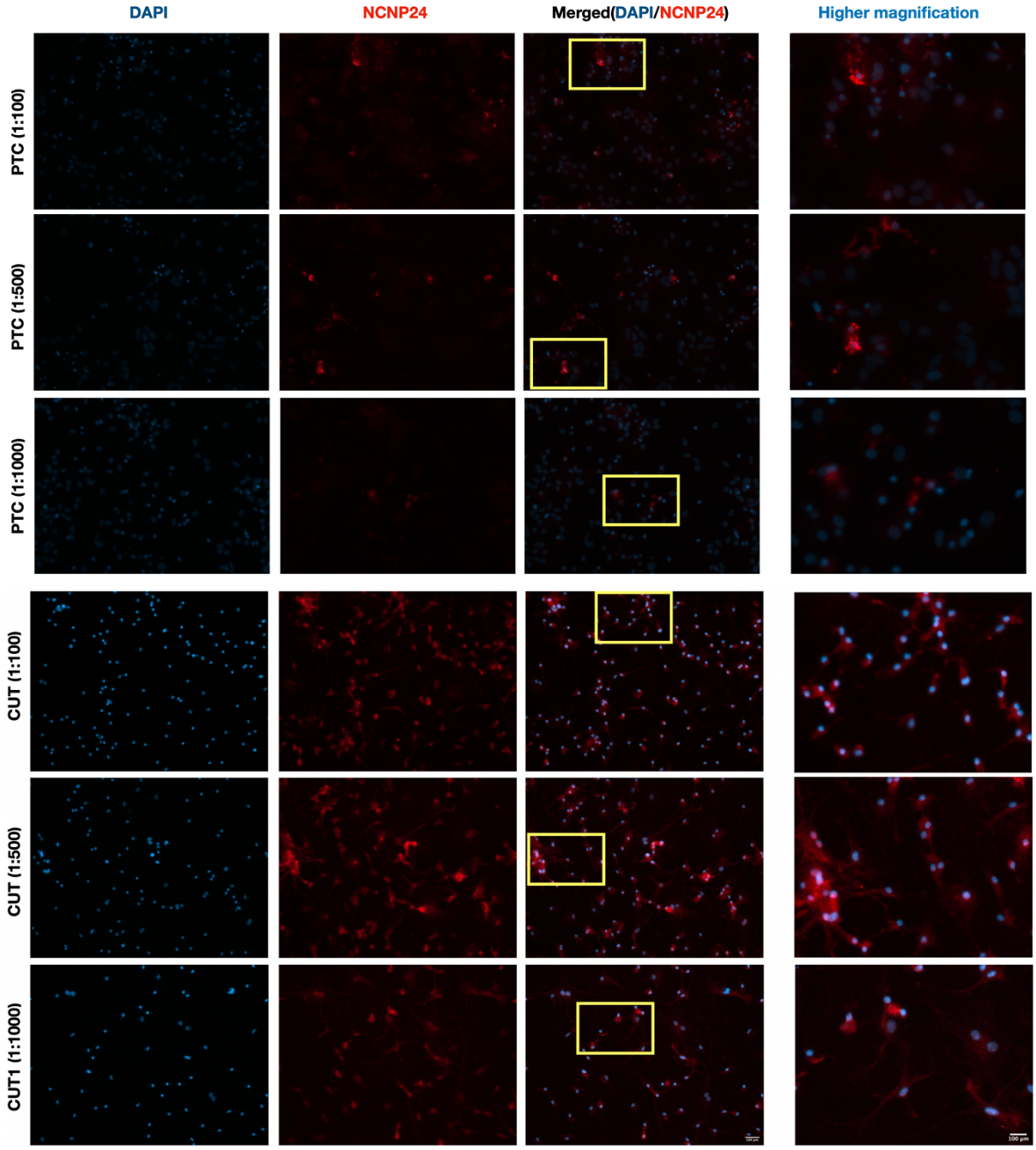

Figure 36. NCNP24 antibody optimisation on primary microglia culture. The primary microglial cells isolated from p2-3 aged-pups and and cultured for two weeks. When the cells were mature enough, they were detached and plated on coated coverslips. The cells were used stained with NCNP24 after 4% PFA fixation and fluorescently labelled secondary antibodies (red). All pictures are taken by inverted spinning disk confocal microscope (x20). To test antibody binding affinity, also optimised concentration to use, three different concentrations (1:100, 1:500 and 1:1000) were tested. Fluorescence signals from both positive controls (PTC, mixed brain cell culture) and primary microglial cultures (CUT) were detected. (Scale bars at right bottom corner represent 100μm in both original and higher magnification images)

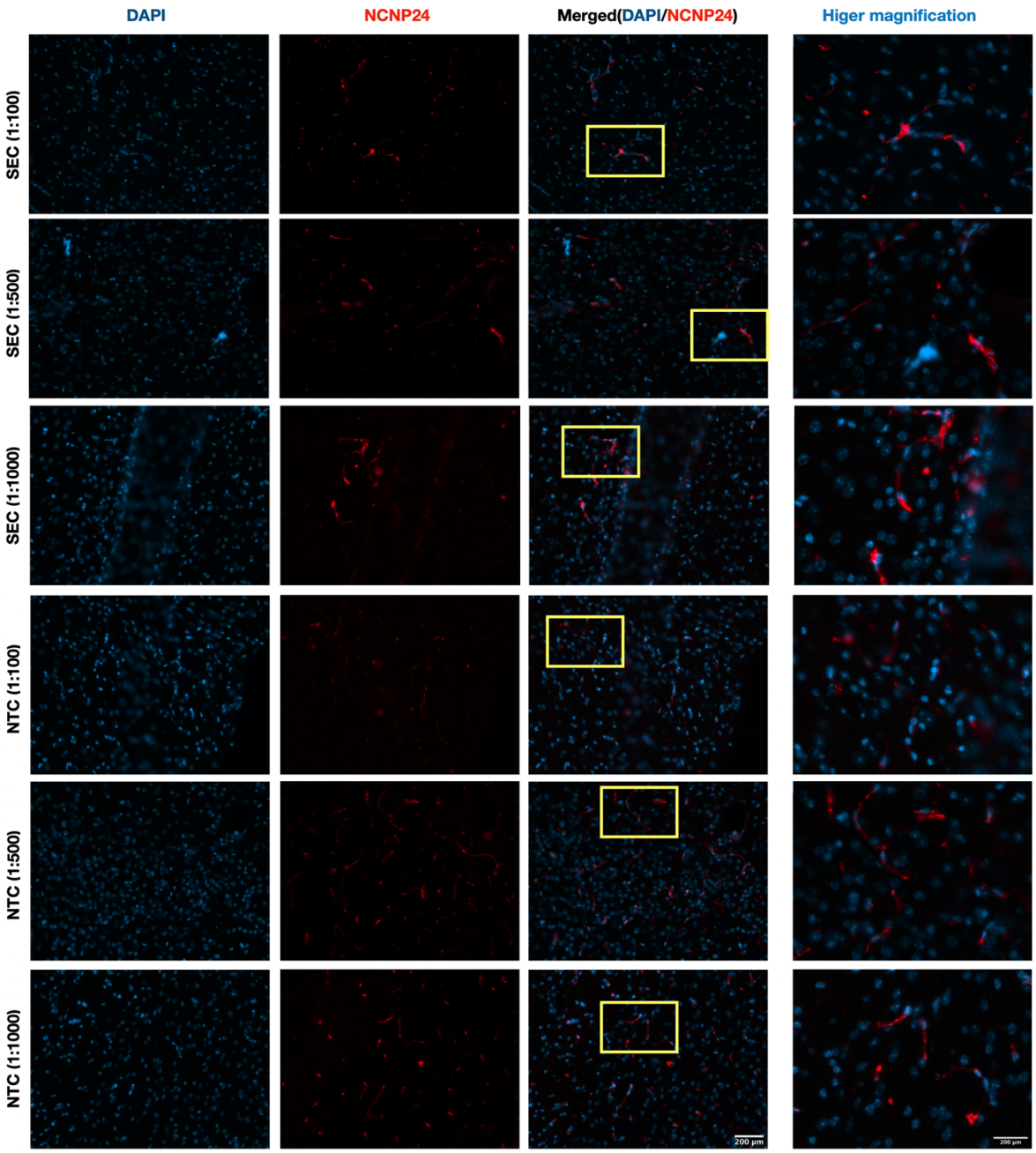

Figure 37. NCNP24 antibody optimisation on brain tissues. The brains were obtained from resiquimod injected mice and the sections were used stained with NCNP24 after 4% PFA fixation and fluorescently labelled secondary antibodies (red). All pictures are taken by inverted spinning disk confocal microscope (x20). In order to test antibody binding affinity, also optimised concentration to use, three different concentrations (1:100, 1:500 and 1:1000) were tested. Fluorescence signals from both the brain sections (SEC) and the negative controls (NTC) were detected. (Scale bars at right bottom corner represent 200μm in both original and higher magnification images)

7.8 ELISA dilution experiment

In order to find proper dilution factor, several dilution factors were made and tested.

- Experiment design for dilution factor checking

0.5 and 24hr stimulation with LPS, poly I:C and resiquimod

The shortest and the longest stimulation time of an actual experiment

Vehicle condition in same stimulation time is also tested

Presumably the lowest condition of each time point

Test protein: IL-6, TNF-a, CCL2, CCL5, CXCL10

- Protocol

Exact volume from the kit was used

STDs: 7 STD points and 0 concentration (total 8 points)

Cell culture medium only condition (as blank condition) was added on each plate

Tested dilution factors: 0(=no dilution), 1:2, 1:5, 1:10

- Duplication quality control : CoV - less than 10%
- Data analysis : 4PL (prism)

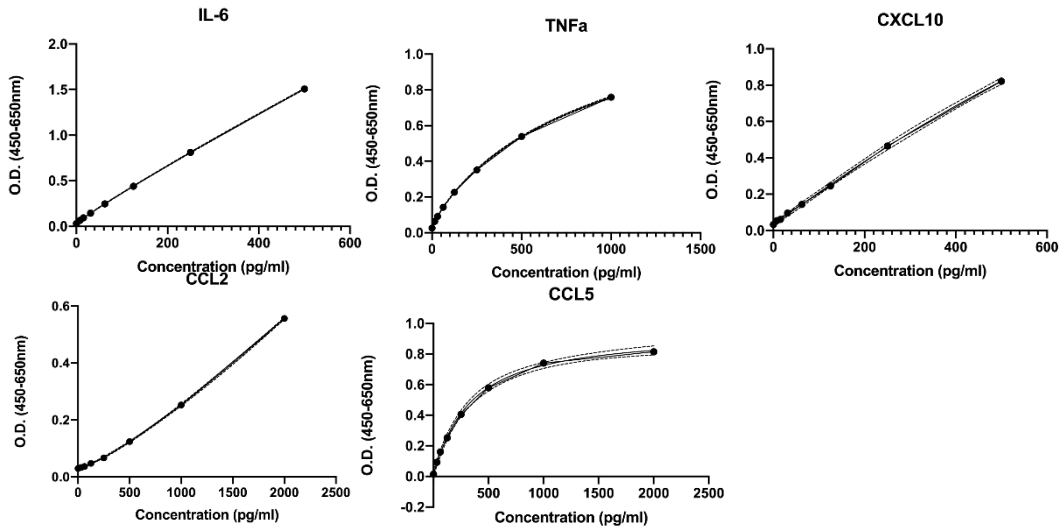

Figure 38. STD curve quality checking. The cells were cultured in the serum-free medium and left overnight. The cells were stimulated by three pathogen mimetics, LPS (50ng/ml), poly I:C (100ng/ml), resiquimod (3μM), for required time and culture medium were collected and stored at -80°C. All of tested STD curved showed over 0.99 R^2 values therefore, the quality of STDs are fine to use to calculate

- Results and Conclusion

0.5hr stimulation was not enough to make IL-6 above the detection. Even zero dilution was below the quantification limit. Therefore, only 24hr data were considered to find dilution factors. Zero and 1:2 dilution factors (only for the resiquimod treated samples) were selected.

TNF-a was fine with 1:5 dilution and other dilution factors were out of quantification ranges either too low (1:10) or too high concentration (1:2 and no dilution).

CCL2: Zero, 1:2, and 1:5 dilutions were not high enough dilute the sample to make within the quantification range. 1:10 dilution was satisfied both 0.5hr and 24hr.

CCL5: Except 1:10 (below the quantification limit), the other dilution factors were fine to use. Since 1:5 dilution with vehicle (0.5hr), which was expecting the lowest level of CCL5, was sitting middle of STDs points, 1;5 dilution factors was used

CXCL10: CXCL10 was detected with all dilution factors. Vehicle (0.5hr), which was expecting the lowest level of CXCL10, was sitting middle of STDs, 1:10 was taken for the experiment.

7.9 MIA additional data

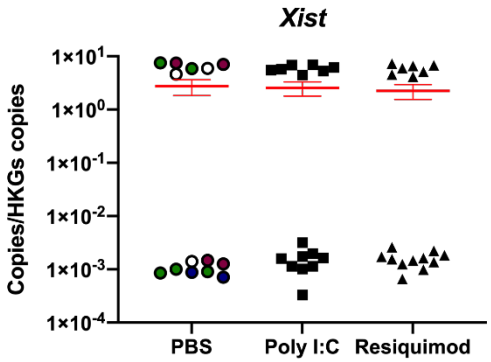

Figure 39. *Xist* could determine sex in foetal brain by RT-qPCR. The foetal brains were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored in RNAlater until further use. RNA extraction and cDNA synthesis had done as per manufacturer's instruction. *Xist* mRNAs could be used to group the samples by sex in foetal brain via RT-qPCR. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Tbp*. The individual data points are shown along with mean ± SEM. The data were log transformed and analysed by twoway-ANOVA, Tukey post-hoc test (n=14-18 (foetal brain)).

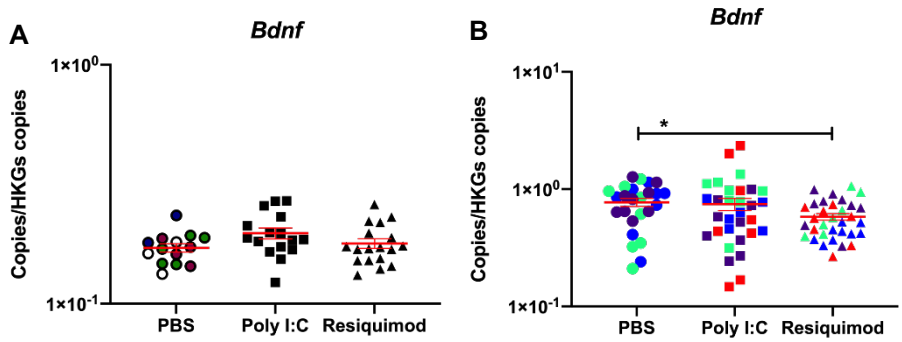

Figure 40. *Bdnf* mRNA was not altered in foetal brain. The foetal brain and placental tissues were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored in RNAlater until further use. RNA extraction and cDNA synthesis had done as per manufacturer's instruction. **(A)** *Bdnf* mRNA levels were not changed by MIA compared to PBS. **(B)** *Bdnf* mRNA levels were decreased by resiquimod, but not by poly I:C compared to PBS. Absolute quantification was performed via RT-qPCR and the data were normalised to *Gapdh* and *Tbp*. The individual data points are shown along with mean ± SEM. The data were log transformed and analysed by twoway-ANOVA, Tukey post-hoc test (n=14-18 (foetal brain), n=26-33 (placenta) independent samples; *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001). Details of ANOVA F values and p values are provided in Table 11.

7.10 Correlation analysis

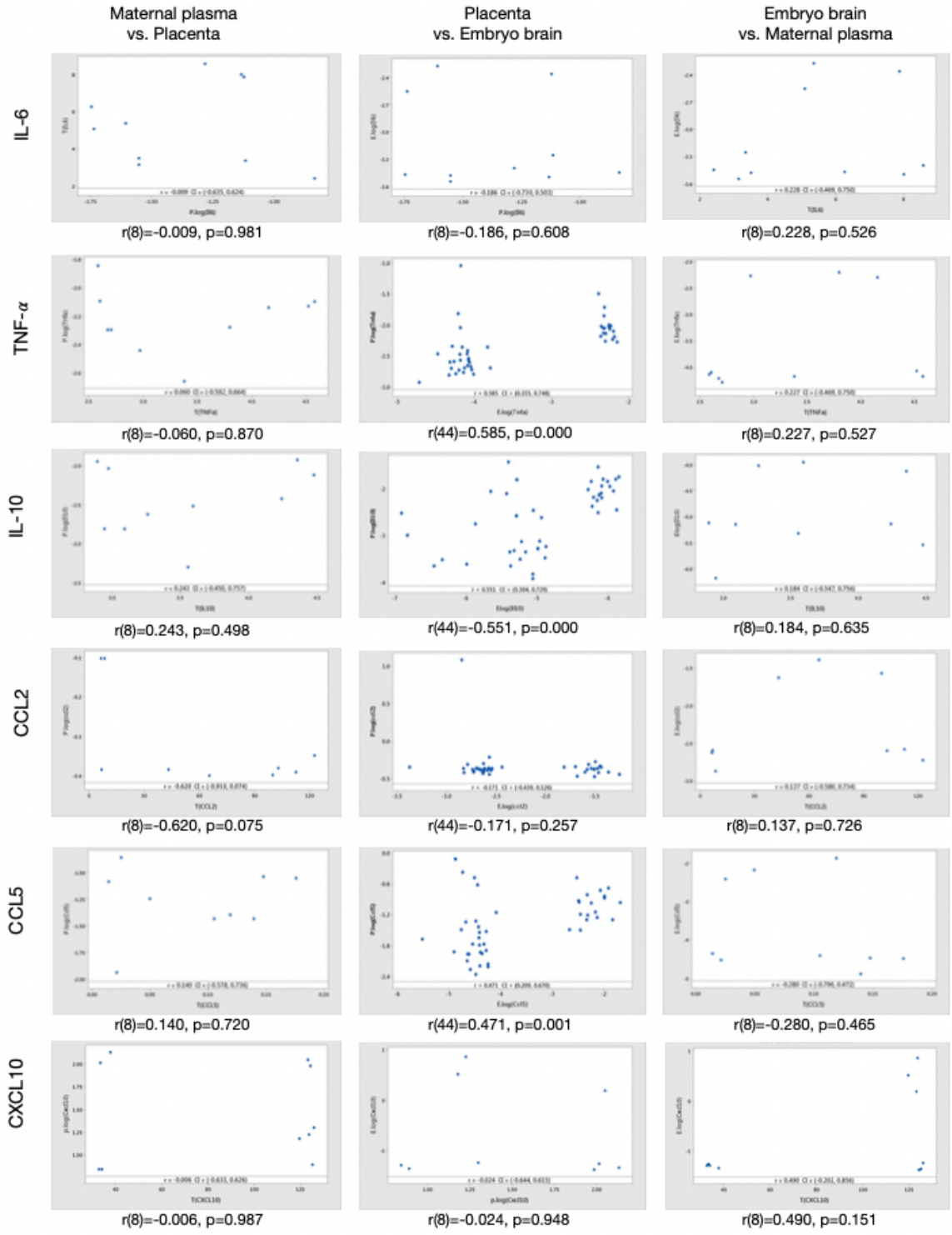

Figure 41. Correlation analysis. Maternal plasma, placental tissues and the foetal brains were collected 4 hours after MIA (PBS, poly I:C (20mg/kg, LMW), resiquimod (2mg/kg)) and stored until further use. Correlation between Maternal plasma (Y axis) and placenta (X axis) (left), placenta (Y axis) and embryo brain (X axis) (middle), Embryo brain (Y axis) and maternal plasma (X axis) (right) levels of immune molecules (labelled in left of the figure). Details of Pearson's correlation analysis are provided under each graph.

7.11 MAPK inhibitors on Macrophage cultures

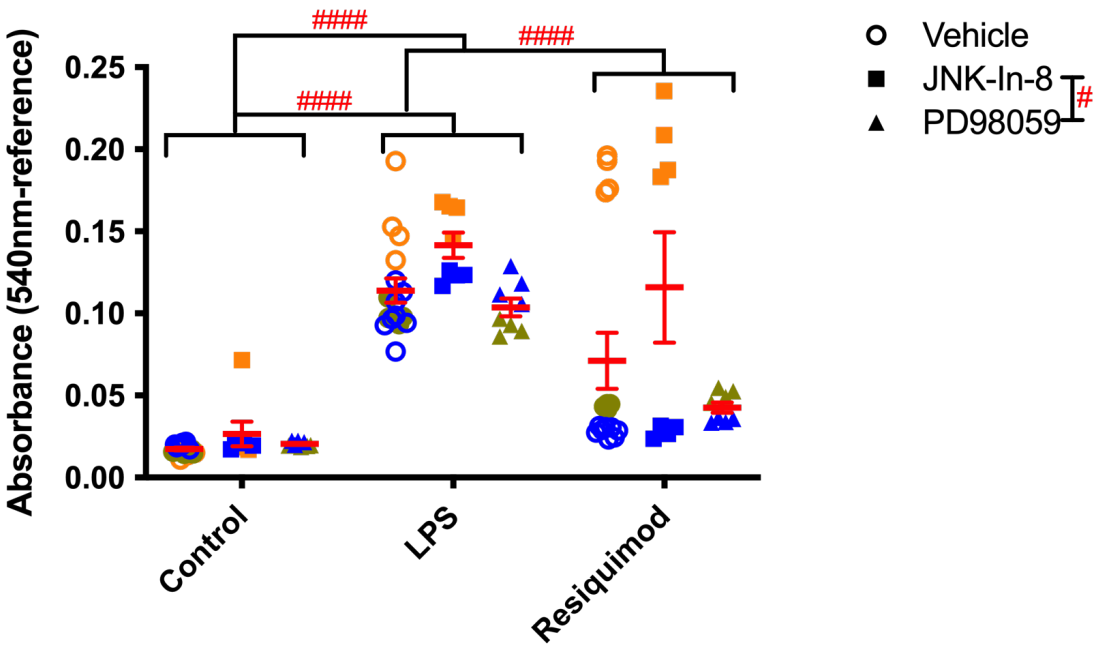

Figure 42. BMDMs do not show inhibitions effects following LPS and resiquimod administrations. The BMDMs were culture in the conditioned medium and used at day 5. The cells were stimulated by two pathogen mimetics, LPS (100ng/ml), Resiquimod (3μM) for 18 hours, followed by two inhibitors, JNK-IN-8 (1μM) and PD98059 (40μM). **BMDMs showed significantly increased levels of iNOS following LPS and resiquimod stimulations.** The data were analysed by two way ANOVA, Tukey post-hoc test (n=16 of none-inhibitor conditions, n=8 of inhibitor conditions, independent samples; $^{\#}p \leq 0.05$, $^{\#\#} \leq 0.005$, $^{\#\#\#}p \leq 0.001$, $^{\#\#\#\#}p \leq 0.0001$; colours indicate different culture/mice).

7.12 Details of statistical analysis

Table 9. Details of statistical analysis outputs, related figures in Chapter 3

Figure (Protein)	**Source**	**F and p values & Post-hoc comparisons**
Figure 3B(pJNK)	Treatment	F (3, 93) = 2.820, P=0.0432
	Vehicle vs. Resiquimod (p=0.9563) Vehicle vs. poly I:C (p=0.0415) Vehicle vs. LPS (p=0.2504)	
	Isoform	F (2, 93) = 1.122, P=0.3301
	Treatment x Isoform	F (6, 93) =0.5335, P=0.7815
Figure 4B-1(pJNK)	Treatment	F (2, 98) = 5.912, P=0.0038
	Vehicle vs. CXCL10 (p=0.0026) Vehicle vs. CXCL12 (p=0.3007) CXCL10 vs. CXCL12 (p=0.1298)	
	Isoform	F (2, 98) = 3.201, P=0.0450
	Treatment x Isoform	F (4, 98) = 0.4337, P=0.7840
Figure 4B-2(pERK)	Treatment	F (2, 65) = 1.077, P=0.3465
	Isoform	F (1, 65) = 5.985, P=0.0171
	Treatment x Isoform	F (2, 65) = 1.846, P=1.660
Figure 4B-3(pp38)	Between groups	F (2, 9) = 0.6969, P=0.5231

Table 10. Details of statistical analysis outputs, related Figures in Chapter 4

Figure (Protein/*gene*)	**Source**	**F and p values** **Post-hoc comparisons**
Figure 6C(JNK)	Treatment	F(3,24)=13.54, p<0.000
	Vehicle vs. resiquimod (p=0.0002) Vehicle vs. poly I:C (p=0.9328) Vehicle vs. LPS (p=0.7715) LPS vs. resiquimod (p=0.0017)	
	Isoform	F(2,24)8.602, p=0.0015
	54 kDa vs. 46 kDa (p=0.0032) 54 kDa vs. 43 kDa (p=0.0052) 46 kDa vs. 43 kDa (p=0.9793)	
	Treatment x Isoform	F(6,24)=1.735, p=0.1560
	Vehicle (54 kDa) vs. resiquimod (54 kDa) (p=0.0096)	
Figure 6D(ERK)	Treatment	F(3,40)=18.19, p<0.0001
	Vehicle vs. resiquimod (p=0.000) Vehicle vs. poly I:C (p=0.6068) Vehicle vs. LPS (p=0.9827) LPS vs. resiquimod (p=0.0000)	
	Isoform	F(1,40)=0.009870, p=0.9214
	Treatment x Isoform	F(3,40)=0.1271, p=0.9435
	Vehicle (44 kDa) vs. resiquimod (44 kDa) (p=0.0084) Vehicle (42 kDa) vs. resiquimod (42 kDa) (p=0.0056)	
Figure 6E(p38)	Between groups	F(3,20)=37.00, p=0.000
Figure 7B(pJNK)	Time	F(3,24)=49.22, p<0.0001
	Vehicle vs. 15min (p<0.0001) Vehicle vs. 30min (p<0.0001) Vehicle vs. 60min (p<0.0001)	
	Isoform	F(2,24)=28.23, p<0.0001
	54 kDa vs. 46 kDa (p=0.0023) 54 kDa vs. 43 kDa (p<0.0001) 46 kDa vs. 43 kDa (p=0.0031)	
	Time x Isoform	F(6,24)=3.631, p=0.0105
Figure 7C(pp38)	Between groups	F(3,8)=15.47, p=0.0009
	Vehicle vs. 15min (p=0.0055) Vehicle vs. 30min (p=0.0039) Vehicle vs. 60min (p>0.9999)	
Figure 7D(pERK)	Time	F(3,16)=34.64, p<0.0001
	Vehicle vs. 15min (p=0.0007) Vehicle vs. 30min (p<0.0001) Vehicle vs. 60min (p<0.0001)	
	Isoform	F(1,16)=0.7298, p=0.4056
	Vehicle(44kDa) vs. Vehicle (42kDa) (p>0.9999) 15min(44kDa) vs. 15min(42kDa) (p=0.3889) 30min(44kDa) vs. 30min(42kDa) (p>0.9999) 60min(44kDa) vs. 60min(42kDa) (p>0.9999)	
	Time x Isoform	F(3,16)=0.8050, p=0.5093
Figure 8B(pJNK)	Time	F(3,24)=1.963, p=0.1464

	Vehicle vs. 15min (p=0.2221) Vehicle vs. 30min (p=0.2195) Vehicle vs. 60min (p=0.9044)	
	Isoform	F(2,24)=0.06345, p=0.9387
	54 kDa vs. 46 kDa (p=0.9952) 54 kDa vs. 43 kDa (p<0.9369) 46 kDa vs. 43 kDa (p=0.9659)	
	Time x Isoform	F(6,24)=0.2315, p=0.9622
Figure 8(pp38)	Between groups	F(3,8)=4.597, p=0.0375
	Vehicle vs. 15min (p=0.3212) Vehicle vs. 30min (p=0.0279) Vehicle vs. 60min (p=0.0557)	
Figure 8(pERK)	Time	F(3,16)=0.6222, p=0.6109
	Vehicle vs. 15min (p=0.9463) Vehicle vs. 30min (p=0.8011) Vehicle vs. 60min (p=0.5678)	
	Isoform	F(1,16)=2.989, p=0.1031
	Vehicle(44kDa) vs. Vehicle (42kDa) (p>0.9999) 15min(44kDa) vs. 15min(42kDa) (p>0.9999) 30min(44kDa) vs. 30min(42kDa) (p=0.6335) 60min(44kDa) vs. 60min(42kDa) (p=0.3493)	
	Time x Isoform	F(3,16)=0.9192, p=0.4539
Figure 9B(pJNK)	Treatment	F(1,42)=354.06, p=0.000
	Vehicle vs. resiquimod (p=0.000)	
	Isoform	F(2,42)=1.46, p=0.243
	54 kDa vs. 46 kDa (p=0.788) 54 kDa vs. 43 kDa (p=0.588) 46 kDa vs. 43 kDa (p=0.218)	
	Inhibitor	F(2,42)=73.81, p=0.0000
	Control vs. 5Z-7 (p=0.000) NADI vs. 5Z-7 (p=0.000) Control vs. NQDI (p=0.623)	
Figure 9C(pp38)	Treatment	F(1,13)=70.24, p=0.000
	Vehicle vs. resiquimod (p=0.000)	
	Inhibitor	F(2,13)=17.44, p=0.000
	Control vs. 5Z-7 (p=0.000) NQDI vs. 5Z-7 (p=0.000) Control vs. NQDI (p=0.987)	
Figure 10B(pJNK)	Treatment	F (2, 63) = 8.932, P=0.0004
	Vehicle vs. CXCL10 (p=0.7063) Vehicle vs. CXCL12 (p=0.0005) CXCL10 vs. CXCL12 (p=0.0061)	
	Isoform	F (2, 63) = 4.277, P=0.0181
	Treatment x isoform	F (4, 63) = 1.982, P=0.1081
Figure 10C(pp38)	Between group	F(2,21)=1.449, p=0.2573
	Vehicle vs. CXCL12 (p>0.9999) Vehicle vs. CXCL10 (p=0.4104) CXCL12 vs. CXCL10 (p=0.5385)	
Figure 10D(pERK)	Treatment	F(2,42)=0.8718, p=0.4256

	Vehicle vs. CXCL12 (p=0.6436) Vehicle vs. CXCL10 (p=0.9207) CXCL12 vs. CXCL10 (0.4103)	
	Isoform	F(1,42)=1.714, p=0.1975
	Vehicle(44kDa) vs. Vehicle (42kDa) (p>0.9999) CXCL12(44kDa) vs. CXCL12 (42kDa) (p=0.3472) CXCL10(44kDa) vs. CXCL10(42kDa) (p=0>0.9999)	
	Treatment x Isoform	F(2,42)=0.5471, p=0.5827
Figure 11A(*Il-6*)	Treatment	F(3,35)=328.58, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.885) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.987)	
	Time	F(2,35)=82.68, p=0.000
	24hr vs. 8hr (p=0.000) 8hr vs. 0.5hr (p=0.000) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,35)=26.01, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.006) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.000) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.000) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.954) LPS (8hr) vs. resiquimod (8hr) (p=1.000) LPS (24hr) vs. resiquimod (24hr) (p=1.000)	
Figure 11B(IL-6)	Treatment	F(3,24)=559.90, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.970) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.987)	
	Passage	F(2,24)=38.96, p=0.000
	1 vs. 2 (p=0.000) 1 vs. 3 (p=0.000 2 vs. 3 (p=0.052)	
	Time	F(1,24)=228.88, p=0.000
	24hr vs. 8hr (p=0.000)	
	Treatment x Time	F(3,24)=108.74, p=0.000
	Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (8hr) vs. LPS (24hr) (p=0.000) Resiquimod (8hr) vs. resiquimod (24hr) (p=0.000) LPS (8hr) vs. resiquimod (8hr) (p=0.000) LPS (24hr) vs. resiquimod (24hr) (p=0.065)	
	Treatment	F(3,35)=76.18, p=0.000

Figure 11C(*Tnf-α*)	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.982) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.774)	
	Time	F(2,35)=11.79, p=0.000
	24hr vs. 8hr (p=0.001) 8hr vs. 0.5hr (p=0.877) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,35)=4.27, p=0.003
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.000) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.003) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.000) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.062) LPS (0.5hr) vs. LPS (8hr) (p=1.000) LPS (0.5hr) vs. LPS (24hr) (p=0.352) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.673) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.367) LPS (8hr) vs. resiquimod (8hr) (p=1.000) LPS (24hr) vs. resiquimod (24hr) (p=0.992)	
Figure 11D(TNF-α)	Treatment	F(3,44)=83.78, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.909) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Passage	F(2,44)=49.65, p=0.000
	1 vs. 2 (p=0.000) 1 vs. 3 (p=0.000) 2 vs. 3 (p=0.012)	
	Time	F(2,44)=30.59, p=0.000
	24hr vs. 8hr (p=0.001) 8hr vs. 0.5hr (p=0.000) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,44)=11.85, p=0.000
	LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.158) Vehicle (8hr) vs. LPS (8hr) (p=0.003) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.009) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.104) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.158) LPS (8hr) vs. resiquimod (8hr) (p=0.000) LPS (24hr) vs. resiquimod (24hr) (p=0.994)	
Figure 12(*Il-10*)	Treatment	F(3,35)=8.10, p=0.000
	Vehicle vs. LPS (p=0.111) Vehicle vs. poly I:C (p=0.664) Vehicle vs. resiquimod (p=0.012) LPS vs. resiquimod (p=0.773)	
	Time	F(2,35)=4.01, p=0.027

	24hr vs. 8hr (p=0.056) 8hr vs. 0.5hr (p=0.999) 24hr vs. 0.5hr (p=0.045)	
	Treatment x Time	F(6,35)=2.51, p=0.040
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.997) Vehicle (8hr) vs. LPS (8hr) (p=0.016) Vehicle (24hr) vs. LPS (24hr) (p=0.999) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.364) Vehicle (8hr) vs. resiquimod (8hr) (p=0.082) Vehicle (24hr) vs. resiquimod (24hr) (p=1.000) LPS (0.5hr) vs. LPS (8hr) (p=0.830) LPS (0.5hr) vs. LPS (24hr) (p=0.759) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=1.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.313) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.925) LPS (8hr) vs. resiquimod (8hr) (p=1.000) LPS (24hr) vs. resiquimod (24hr) (p=1.000)	
Figure 13A(*Ccl2*)	Treatment	F(3,35)=112.39, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=1.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.796)	
	Time	F(2,35)=51.59, p=0.000
	24hr vs. 8hr (p=0.573) 8hr vs. 0.5hr (p=0.000) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,35)=16.85, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.960) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.345) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle(24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.000) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.988) LPS (8hr) vs. resiquimod (8hr) (p=1.000) LPS (24hr) vs. resiquimod (24hr) (p=1.000)	
Figure 13B(CCL2)	Treatment	F(3,33)=25.62, p=0.000
	Vehicle vs. LPS (p=0.031) Vehicle vs. poly I:C (p=0.862) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.001)	
	Passage	F(2,33)=48.79, p=0.000
	1 vs. 2 (p=0.000) 1 vs. 3 (p=0.605) 2 vs. 3 (p=0.000)	
	Time	F(2,33)=55.17, p=0.000
	24hr vs. 8hr (p=0.000) 8hr vs. 0.5hr (p=0.988) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,33)=2.17, p=0.072

	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.998) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.975) Vehicle (8hr) vs. resiquimod (8hr) (p=0.990) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=1.000) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=1.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.992) LPS (8hr) vs. resiquimod (8hr) (p=0.690) LPS (24hr) vs. resiquimod (24hr) (p=0.010)	
Figure 13C(*Ccl5*)	Treatment	F(3,36)=80.20, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.497) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Time	F(2,36)=60.30, p=0.000
	24hr vs. 8hr (p=0.155) 8hr vs. 0.5hr (p=0.000) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,36)=23.67, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=1.000) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.000) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=1.000) LPS (8hr) vs. resiquimod (8hr) (p=0.000) LPS (24hr) vs. resiquimod (24hr) (p=0.006)	
Figure 13D(CCL5)	Treatment	F(3,33)=4.01, p=0.015
	Vehicle vs. LPS (p=0.737) Vehicle vs. poly I:C (p=0.902) Vehicle vs. resiquimod (p=0.214) LPS vs. resiquimod (p=0.016)	
	Passage	F(2,33)=9.04, p=0.001
	1 vs. 2 (p=0.334) 1 vs. 3 (p=0.124) 2 vs. 3 (p=0.785)	
	Time	F(2,33)=4.71, p=0.016
	24hr vs. 8hr (p=0.012) 8hr vs. 0.5hr (p=0.465) 24hr vs. 0.5hr (p=0.145)	
	Treatment x Time	F(6,33)=2.17, p=0.071

	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.893) Vehicle (24hr) vs. LPS (24hr) (p=1.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.999) Vehicle (8hr) vs. resiquimod (8hr) (p=1.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.110) LPS (0.5hr) vs. LPS (8hr) (p=0.999) LPS (0.5hr) vs. LPS (24hr) (p=0.999) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=1.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.078) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.997) LPS (8hr) vs. resiquimod (8hr) (p=0.994) LPS (24hr) vs. resiquimod (24hr) (p=0.058)	
Figure 13E(*Cxcl10*)	Treatment	F(3,36)=45.24, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.821) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Time	F(2,36)=8.49, p=0.001
	24hr vs. 8hr (p=0.060) 8hr vs. 0.5hr (p=0.001) 24hr vs. 0.5hr (p=0.202)	
	Treatment x Time	F(6,36)=7.61, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.885) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.056) Vehicle (8hr) vs. resiquimod (8hr) (p=0.030) Vehicle (24hr) vs. resiquimod (24hr) (p=0.562) LPS (0.5hr) vs. LPS (8hr) (p=0.000) LPS (0.5hr) vs. LPS (24hr) (p=0.001) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=1.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.936) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.802) LPS (8hr) vs. resiquimod (8hr) (p=0.000) LPS (24hr) vs. resiquimod (24hr) (p=0.001)	
Figure 13F(CXCL10)	Treatment	F(3,33)=1.29, p=0.294
	Vehicle vs. LPS (p=0.707) Vehicle vs. poly I:C (p=0.948) Vehicle vs. resiquimod (p=0.851) LPS vs. resiquimod (p=0.256)	
	Passage	F(2,33)=9.04, p=0.001
	1 vs. 2 (p=0.027) 1 vs. 3 (p=0.001) 2 vs. 3 (p=0.259)	
	Time	F(2,33)=1.65, p=0.207
	24hr vs. 8hr (p=0.179) 8hr vs. 0.5hr (p=0.641) 24hr vs. 0.5hr (p=0.635)	
	Treatment x Time	F(6,33)=0.88, p=0.521
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.965) Vehicle (24hr) vs. LPS (24hr) (p=1.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=1.000) Vehicle (8hr) vs. resiquimod (8hr) (p=1.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.952)	

	LPS (0.5hr) vs. LPS (8hr) (p=0.999) LPS (0.5hr) vs. LPS (24hr) (p=1.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.999) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.953) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.998) LPS (8hr) vs. resiquimod (8hr) (p=1.000) LPS (24hr) vs. resiquimod (24hr) (p=0.898)	
Figure 14A(*Il-6*)	Treatment	F(2,36)=66.12, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.001)	
	Inhibitor	F(3,36)=11.00 p=0.000
	Control vs. JNK-IN-8 (p=0.070) Control vs. PD98059 (p=0.641) Control vs. SB203580 (p=0.026)	
	Passage	F(2,36)=22.05, p=0.000
	Treatment x Inhibitor	F(6,36)=0.58, p=0.741
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.999) Vehicle, none vs. vehicle, PD98059 (p=1.000) Vehicle, none vs. vehicle, SB203580 (p=0.816) LPS, none vs. LPS, JNK-IN- 8 (p=0.986) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.389) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.451) Resiquimod, none vs. resiquimod, PD98059 (p=0.670) Resiquimod, none vs. resiquimod, SB203580 (p=0.999) Vehicle, none vs. LPS, none (p=0.027) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.011) Vehicle, PD98059 vs. LPS, PD98059 (p=0.014) Vehicle, SB203580 vs. LPS, SB203580 (p=0.134) Vehicle, none vs. resiquimod, none (p=0.003) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=1.000) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.734) LPS, PD98059 vs. resiquimod, PD98059 (p=0.279) LPS, SB203580 vs. resiquimod, SB203580 (p=0.453)	
Figure 14B(*Il-6*)	Treatment	F(2,66)=1116.36, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,66)=5.14, p=0.003
	Control vs. JNK-IN-8 (p=0.790) Control vs. PD98059 (p=0.003) Control vs. SB203580 (p=0.022)	
	Passage	F(2,66)=3.57, p=0.034
	Treatment x Inhibitor	F(6,66)=9.31, p=0.000
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.714) Vehicle, none vs vehicle, PD98059 (p=0.723) Vehicle, none vs. vehicle, SB203580 (p=0.999)	

	LPS, none vs. LPS, JNK-IN- 8 (p=0.749) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.968) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.413) Resiquimod, none vs. resiquimod, PD98059 (p=0.002) Resiquimod, none vs. resiquimod, SB203580 (p=0.012) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.002) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) LPS, PD98059 vs. resiquimod, PD98059 (p=0.000) LPS, SB203580 vs. resiquimod, SB203580 (p=0.000)	
Figure 15A(*Tnf-*α)	Treatment	F(2,36)=708.88, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,36)=12.60, p=0.000
	Control vs. JNK-IN-8 (p=0.064) Control vs. PD98059 (p=0.006) Control vs. SB203580 (p=0.917)	
	Passage	F(2,36)=10.83, p=0.000
	Treatment x Inhibitor	F(6,36)=0.96, p=0.466
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.999) Vehicle, none vs vehicle, PD98059 (p=0.204) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.761) LPS, none vs. LPS, PD98059 (p=0.339) LPS none, vs. LPS, SB203580 (p=0.963) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.827) Resiquimod, none vs. resiquimod, PD98059 (p=1.000) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.003) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.004) LPS, PD98059 vs. resiquimod, PD98059 (p=0.000) LPS, SB203580 vs. resiquimod, SB203580 (p=0.000)	
Figure 15B(*Tnf-*α)	Treatment	F(2,66)=350.46, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	

	Inhibitor	F(3,66)=17.36, p=0.000
	Control vs. JNK-IN-8 (p=0.760) Control vs. PD98059 (p=0.002) Control vs. SB203580 (p=0.007)	
	Passage	F(2,66)=0.11, p=0.894
	Treatment x Inhibitor	F(6,66)=2.41, p=0.036
	Vehicle, none vs. vehicle, JNK-IN-8 (p=1.000) Vehicle, none vs vehicle, PD98059 (p=0.000) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=1.000) LPS, none vs. LPS, PD98059 (p=0.639) LPS none, vs. LPS, SB203580 (p=0.125) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.875) Resiquimod, none vs. resiquimod, PD98059 (p=1.000) Resiquimod, none vs. resiquimod, SB203580 (p=0.825) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.041) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.968) LPS, PD98059 vs. resiquimod, PD98059 (p=0.003) LPS, SB203580 vs. resiquimod, SB203580 (p=0.767)	
Figure 15C (TNF-α)	Treatment	F(2,35)=86.72, p=0.000
	Vehicle vs. LPS (p=1.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,35)=7.97. p=0.003
	Control vs. JNK-IN-8 (p=0.002) Control vs. PD98059 (p=0.611) Control vs. SB203580 (p=0.970)	
	Passage	F(2,35)=12.18. p=0.003
	1 vs. 2 (p=0.001) 1 vs. 3 (p=0.000) 2 vs. 3 (p=0.705)	
	Treatment x Inhibitor	F(6,35)=5.65, p=0.000
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.972) Vehicle, none vs vehicle, PD98059 (p=1.000) Vehicle, none vs. vehicle, SB203580 (p=0.995) LPS, none vs. LPS, JNK-IN- 8 (p=1.000) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.620) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.000) Resiquimod, none vs. resiquimod, PD98059 (p=0.842) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.956) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=1.000)	

	Vehicle, PD98059 vs. LPS, PD98059 (p=0.999) Vehicle, SB203580 vs. LPS, SB203580 (p=0.839) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.565) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.000) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.889) LPS, PD98059 vs. resiquimod, PD98059 (p=0.000) LPS, SB203580 vs. resiquimod, SB203580 (p=0.000)	
Figure 16A(*Il-10*)	Treatment	F(2,36)=45.07, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.172)	
	Inhibitor	F(3,36)=7.73, p=0.000
	Control vs. JNK-IN-8 (p=0.070) Control vs. PD98059 (p=0.001) Control vs. SB203580 (p=0.986)	
	Passage	F(2,36)=7.72, p=0.002
	Treatment x Inhibitor	F(6,36)=0.94, p=0.479
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.871) Vehicle, none vs vehicle, PD98059 (p=0.986) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.944) LPS, none vs. LPS, PD98059 (p=0.373) LPS none, vs. LPS, SB203580 (p=1.000) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.976) Resiquimod, none vs. resiquimod, PD98059 (p=0.043) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.052) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.086) Vehicle, PD98059 vs. LPS, PD98059 (p=0.002) Vehicle, SB203580 vs. LPS, SB203580 (p=0.225) Vehicle, none vs. resiquimod, none (p=0.010) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.026) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.037) LPS, none vs. resiquimod, none (p=1.000) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) LPS, PD98059 vs. resiquimod, PD98059 (p=0.852) LPS, SB203580 vs. resiquimod, SB203580 (p=0.999)	
Figure 16B (*Il-10*)	Treatment	F(2,66)=10.30, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,66)=12.78, p=0.000
	Control vs. JNK-IN-8 (p=0.098) Control vs. PD98059 (p=1.000) Control vs. SB203580 (p=0.000)	
	Passage	F(2,66)=0.67, p=0.543
	Treatment x Inhibitor	F(6,66)=1.86, p=0.101

	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.300) Vehicle, none vs vehicle, PD98059 (p=0.931) Vehicle, none vs. vehicle, SB203580 (p=0.997) LPS, none vs. LPS, JNK-IN- 8 (p=0.985) LPS, none vs. LPS, PD98059 (p=0.992) LPS none, vs. LPS, SB203580 (p=0.615) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=1.000) Resiquimod, none vs. resiquimod, PD98059 (p=0.557) Resiquimod, none vs. resiquimod, SB203580 (p=0.011) Vehicle, none vs. LPS, none (p=0.671) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.351) Vehicle, PD98059 vs. LPS, PD98059 (p=0.834) Vehicle, SB203580 vs. LPS, SB203580 (p=0.031) Vehicle, none vs. resiquimod, none (p=0.001) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.712) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.256) LPS, PD98059 vs. resiquimod, PD98059 (p=0.002) LPS, SB203580 vs. resiquimod, SB203580 (p=0.064)	
Figure 17A(*Ccl2*)	Treatment	F(2,36)=55.22, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.001)	
	Inhibitor	F(3,36)=5.09, p=0.005
	Control vs. JNK-IN-8 (p=0.417) Control vs. PD98059 (p=0.167) Control vs. SB203580 (p=0.997)	
	Passage	F(2,36)=7.94, p=0.001
	Treatment x Inhibitor	F(6,36)=0.86, p=0.535
	Vehicle, none vs. vehicle, JNK-IN-8 (p=1.000) Vehicle, none vs. vehicle, PD98059 (p=0.956) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.998) LPS, none vs. LPS, PD98059 (p=0.841) LPS none, vs. LPS, SB203580 (p=1.000) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.617) Resiquimod, none vs. resiquimod, PD98059 (p=1.000) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.181) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.018) Vehicle, PD98059 vs. LPS, PD98059 (p=0.330) Vehicle, SB203580 vs. LPS, SB203580 (p=0.654) Vehicle, none vs. resiquimod, none (p=0.002) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.016) LPS, none vs. resiquimod, none (p=0.819) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.175) LPS, PD98059 vs. resiquimod, PD98059 (p=0.158) LPS, SB203580 vs. resiquimod, SB203580 (p=0.767)	
Figure 17B(*Ccl2*)	Treatment	F(2,66)=360.50, p=0.000

	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,66)=20.84, p=0.000
	Control vs. JNK-IN-8 (p=0.998) Control vs. PD98059 (p=0.023) Control vs. SB203580 (p=0.000)	
	Passage	F(2,66)=1.83, p=0.168
	Treatment x Inhibitor	F(6,66)=2.85, p=0.016
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.998) Vehicle, none vs vehicle, PD98059 (p=0.031) Vehicle, none vs. vehicle, SB203580 (p=0.999) LPS, none vs. LPS, JNK-IN- 8 (p=0.976) LPS, none vs. LPS, PD98059 (p=0.800) LPS none, vs. LPS, SB203580 (p=0.001) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.999) Resiquimod, none vs. resiquimod, PD98059 (p=1.000) Resiquimod, none vs. resiquimod, SB203580 (p=0.340) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.010) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.953) LPS, PD98059 vs. resiquimod, PD98059 (p=0.001) LPS, SB203580 vs. resiquimod, SB203580 (p=0.891)	
Figure 17C(CCL2)	Treatment	F(2,45)=7.22, p=0.002
	Vehicle vs. LPS (p=0.096) Vehicle vs. resiquimod (p=0.313) Resiquimod vs. LPS (p=0.001)	
	Inhibitor	F(3,45)=6.97, p=0.001
	Control vs. JNK-IN-8 (p=0.003) Control vs. PD98059 (p=0.999) Control vs. SB203580 (p=0.700)	
	Passage	F(2,45)=6.73, p=0.003

	1 vs. 2 (p=0.632) 1 vs. 3 (p=0.003) 2 vs. 3 (p=0.028)	
	Treatment x Inhibitor	F(6,45)=1.69, p=0.147
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.997) Vehicle, none vs vehicle, PD98059 (p=1.000) Vehicle, none vs. vehicle, SB203580 (p=0.998) LPS, none vs. LPS, JNK-IN- 8 (p=0.006) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.888) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.935) Resiquimod, none vs. resiquimod, PD98059 (p=1.000) Resiquimod, none vs. resiquimod, SB203580 (p=0.902) Vehicle, none vs. LPS, none (p=0.463) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=1.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.842) Vehicle, SB203580 vs. LPS, SB203580 (p=1.000) Vehicle, none vs. resiquimod, none (p=1.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.995) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.401) LPS, none vs. resiquimod, none (p=0.354) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) LPS, PD98059 vs. resiquimod, PD98059 (p=0.257) LPS, SB203580 vs. resiquimod, SB203580 (p=0.376)	
Figure 18A(*Ccl5*)	Treatment	F(2,36)=0.31, p=0.736
	Vehicle vs. LPS (p=0.732) Vehicle vs. resiquimod (p=0.980) LPS vs. resiquimod (p=0.841)	
	Inhibitor	F(3,36)=4.09, p=0.039
	Control vs. JNK-IN-8 (p=0.089) Control vs. PD98059 (p=0.556) Control vs. SB203580 (p=0.995)	
	Passage	F(2,36)=24.71, p=0.000
	Treatment x Inhibitor	F(6,36)=0.16, p=0.985
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.962) Vehicle, none vs vehicle, PD98059 (p=0.998) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.989) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.999) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.865) Resiquimod, none vs. resiquimod, PD98059 (p=0.995) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.999) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=1.000)	

	Vehicle, PD98059 vs. LPS, PD98059 (p=1.000) Vehicle, SB203580 vs. LPS, SB203580 (p=1.000) Vehicle, none vs. resiquimod, none (p=1.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=1.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=1.000) LPS, none vs. resiquimod, none (p=0.999) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) LPS, PD98059 vs. resiquimod, PD98059 (p=1.000) LPS, SB203580 vs. resiquimod, SB203580 (p=1.000)	
Figure 18B (*Ccl5*)	Treatment	F(2,66)=331.79, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.005)	
	Inhibitor	F(3,66)=15.06. p=0.000
	Control vs. JNK-IN-8 (p=0.715) Control vs. PD98059 (p=0.674) Control vs. SB203580 (p=0.000)	
	Passage	F(2,66)=4.13, p=0.020
	Treatment x Inhibitor	F(6,66)=6.62, p=0.000
	Vehicle, none vs. vehicle, JNK-IN-8 (p=1.000) Vehicle, none vs vehicle, PD98059 (p=0.948) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.946) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.056) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=1.000) Resiquimod, none vs. resiquimod, PD98059 (p=0.137) Resiquimod, none vs. resiquimod, SB203580 (p=0.000) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.054) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.005) LPS, PD98059 vs. resiquimod, PD98059 (p=1.000) LPS, SB203580 vs. resiquimod, SB203580 (p=1.000)	
Figure 18C (CCL5)	Treatment	F(2,41)=2.85, p=0.069
	Vehicle vs. LPS (p=0.168) Vehicle vs. resiquimod (p=0.092) LPS vs. resiquimod (p=0.941)	
	Inhibitor	F(3,41)=4.65, p=0.008
	Control vs. JNK-IN-8 (p=0.086) Control vs. PD98059 (p=0.908) Control vs. SB203580 (p=0.820)	
	Passage	F(2,41)=3.32, p=0.046
	1 vs. 2 (p=0.095) 1 vs. 3 (p=0.053) 2 vs. 3 (p=0.959)	

	Treatment x Inhibitor	F(6,41)=3.00, p=0.016
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.999) Vehicle, none vs vehicle, PD98059 (p=1.000) Vehicle, none vs. vehicle, SB203580 (p=0.139) LPS, none vs. LPS, JNK-IN- 8 (p=0.065) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.996) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=1.000) Resiquimod, none vs. resiquimod, PD98059 (p=0.958) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.999) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.671) Vehicle, PD98059 vs. LPS, PD98059 (p=1.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.119) Vehicle, none vs. resiquimod, none (p=0.999) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=1.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=1.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.009) LPS, none vs. resiquimod, none (p=0.896) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.936) LPS, PD98059 vs. resiquimod, PD98059 (p=1.000) LPS, SB203580 vs. resiquimod, SB203580 (p=1.000)	
Figure 19A (*Cxcl10*)	Treatment	F(2,36)=67.53, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.061)	
	Inhibitor	F(3,36)=1.40, p=0.258
	Control vs. JNK-IN-8 (p=0.690) Control vs. PD98059 (p=1.000) Control vs. SB203580 (p=0.781)	
	Passage	F(2,36)=5.59, p=0.008
	Treatment x Inhibitor	F(6,36)=0.82, p=0.559
	Vehicle, none vs. vehicle, JNK-IN-8 (p=1.000) Vehicle, none vs vehicle, PD98059 (p=1.000) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=1.000) LPS, none vs. LPS, PD98059 (p=0.993) LPS none, vs. LPS, SB203580 (p=0.903) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.870) Resiquimod, none vs. resiquimod, PD98059 (p=0.860) Resiquimod, none vs. resiquimod, SB203580 (p=1.000) Vehicle, none vs. LPS, none (p=0.002) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.002) Vehicle, PD98059 vs. LPS, PD98059 (p=0.009) Vehicle, SB203580 vs. LPS, SB203580 (p=0.029) Vehicle, none vs. resiquimod, none (p=0.005) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=1.000) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.989)	

	LPS, PD98059 vs. resiquimod, PD98059 (p=0.455) LPS, SB203580 vs. resiquimod, SB203580 (p=0.894)	
Figure 19C(*Cxcl10*)	Treatment	F(2,66)=333.93, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.000)	
	Inhibitor	F(3,66)=4.91, p=0.004
	Control vs. JNK-IN-8 (p=0.994) Control vs. PD98059 (p=0.159) Control vs. SB203580 (p=0.322)	
	Passage	F(2,66)=0.92, p=0.404
	Treatment x Inhibitor	F(6,66)=1,21, p=0.312
	Vehicle, none vs. vehicle, JNK-IN-8 (p=0.998) Vehicle, none vs vehicle, PD98059 (p=0.339) Vehicle, none vs. vehicle, SB203580 (p=1.000) LPS, none vs. LPS, JNK-IN- 8 (p=0.605) LPS, none vs. LPS, PD98059 (p=1.000) LPS none, vs. LPS, SB203580 (p=0.777) Resiquimod, none, vs. resiquimod, JNK-IN-8 (p=0.996) Resiquimod, none vs. resiquimod, PD98059 (p=0.994) Resiquimod, none vs. resiquimod, SB203580 (p=0.999) Vehicle, none vs. LPS, none (p=0.000) Vehicle, JNK-IN-8 vs. LPS, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. LPS, PD98059 (p=0.000) Vehicle, SB203580 vs. LPS, SB203580 (p=0.000) Vehicle, none vs. resiquimod, none (p=0.000) Vehicle, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) Vehicle, PD98059 vs. resiquimod, PD98059 (p=0.000) Vehicle, SB203580 vs. resiquimod, SB203580 (p=0.000) LPS, none vs. resiquimod, none (p=0.008) LPS, JNK-IN-8 vs. resiquimod, JNK-IN-8 (p=0.000) LPS, PD98059 vs. resiquimod, PD98059 (p=0.015) LPS, SB203580 vs. resiquimod, SB203580 (p=0.002)	
Figure 20(*Tspo*)	Treatment	F(3,36)=0.43, p=0.736
	Vehicle vs. LPS (p=0.895) Vehicle vs. poly I:C (p=0.989) Vehicle vs. resiquimod (p=0.981) LPS vs. resiquimod (p=0.700)	
	Time	F(2,36)=4.26, p=0.022
	24hr vs. 8hr (p=0.029) 8hr vs. 0.5hr (p=0.063) 24hr vs. 0.5hr (p=0.936)	
	Treatment x Time	F(6,36)=2.10, p=0.077
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=0.985) Vehicle (8hr) vs. LPS (8hr) (p=0.229) Vehicle (24hr) vs. LPS (24hr) (p=1.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.995) Vehicle (8hr) vs. resiquimod (8hr) (p=0.976) Vehicle (24hr) vs. resiquimod (24hr) (p=0.999) LPS (0.5hr) vs. LPS (8hr) (p=0.060) LPS (0.5hr) vs. LPS (24hr) (p=1.000)	

	Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.833) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=1.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=1.000) LPS (8hr) vs. resiquimod (8hr) (p=0.931) LPS (24hr) vs. resiquimod (24hr) (p=1.000)	
Figure 20(*Aif1*)	Treatment	F(3,35)=5.08, p=0.005
	Vehicle vs. LPS (p=0.150) Vehicle vs. poly I:C (p=0.999) Vehicle vs. resiquimod (p=0.011) LPS vs. resiquimod (p=0.673)	
	Time	F(2,35)=19.00, p=0.000
	24hr vs. 8hr (p=0.000) 8hr vs. 0.5hr (p=0.404) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,35)=8.09, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.691) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=1.000) Vehicle (8hr) vs. resiquimod (8hr) (p=1.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.677) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.999) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.006) LPS (0.5hr) vs. resiquimod (0.5hr) (p=1.000) LPS (8hr) vs. resiquimod (8hr) (p=0.850) LPS (24hr) vs. resiquimod (24hr) (p=1.000)	
Figure 20(*Tmem119*)	Treatment	F(3,35)=95.52, p=0.000
	Vehicle vs. LPS (p=0.000) Vehicle vs. poly I:C (p=0.996) Vehicle vs. resiquimod (p=0.000) LPS vs. resiquimod (p=0.011)	
	Time	F(2,35)=55.58, p=0.000
	24hr vs. 8hr (p=0.001) 8hr vs. 0.5hr (p=0.000) 24hr vs. 0.5hr (p=0.000)	
	Treatment x Time	F(6,35)=20.50, p=0.000
	Vehicle (0.5hr) vs. LPS (0.5hr) (p=1.000) Vehicle (8hr) vs. LPS (8hr) (p=0.000) Vehicle (24hr) vs. LPS (24hr) (p=0.000) Vehicle (0.5hr) vs. resiquimod (0.5hr) (p=0.781) Vehicle (8hr) vs. resiquimod (8hr) (p=0.000) Vehicle (24hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. LPS (8hr) (p=0.000) LPS (0.5hr) vs. LPS (24hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (8hr) (p=0.000) Resiquimod (0.5hr) vs. resiquimod (24hr) (p=0.000) LPS (0.5hr) vs. resiquimod (0.5hr) (p=0.747) LPS (8hr) vs. resiquimod (8hr) (p=0.374) LPS (24hr) vs. resiquimod (24hr) (p=0.970)	

Table 11. Details of statistical analysis outputs, related figures in Chapter 5.

Figure (Protein/*gene*)	**Source**	**F and P values** **Post-hoc comparisons**
Figure 21A(CXCL10)	Embryonic WT vs. HZ	P=0.332
	Maternal WT vs. HZ	F(1,34)=4.88, p=0.043
	(Drug x Maternal Genotype interaction)	P=0.375
Figure 21B(CXCL12)	Embryonic WT vs. HZ	P=0.803
	Maternal WT vs. HZ	F(1,31)=8,66, p=0.010
	(Drug x Maternal Genotype interaction)	F(1,31)=7.13, p=0.017
Figure 22A(IL-1α)	Mother injection	F(2,9)=18.59, p=0.002
	Poly I:C vs. PBS (p=0.798) Resiquimod vs. PBS (p=0.005) Resiquimod vs. poly I:C (p=0.002)	
Figure 22B(IL-1β)	Mother injection	F(2,8)=3.21, p=0.112
	Poly I:C vs. PBS (p=0.533) Resiquimod vs. PBS (p=0.106) Resiquimod vs. poly I:C (p=0.288)	
Figure 22C(IL-3)	Mother injection	F(2,10)=4.54, p=0.048
	Poly I:C vs. PBS (p=0.899) Resiquimod vs. PBS (p=0.051) Resiquimod vs. poly I:C (p=0.093)	
Figure 22D(IL-6)	Mother injection	F(2,10)=27.61, p=0.000
	Poly I:C vs. PBS (p=0.020) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.001)	
Figure 22E(IL-7)	Mother injection	F(2,10)=1.43, p=0.295
	Poly I:C vs. PBS (p=0.805) Resiquimod vs. PBS (p=0.269) Resiquimod vs. poly I:C (p=0.544)	
Figure 22F(IL-12p40)	Mother injection	F(2,10)=116.42, p=0.000
	Poly I:C vs. PBS (p=0.213) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
Figure 22G(IL-12p70)	Mother injection	F(2,9)=11.71, p=0.006
	Poly I:C vs. PBS (p=0.997) Resiquimod vs. PBS (p=0.012) Resiquimod vs. poly I:C (p=0.008)	
Figure 22H(IL-15)	Mother injection	F(2,10)=3.38, p=0.086
	Poly I:C vs. PBS (p=0.918) Resiquimod vs. PBS (p=0.089) Resiquimod vs. poly I:C (p=0.152)	
Figure 22I(TNF-α)	Mother injection	F(2,10)=26.79, p=0.000
	Poly I:C vs. PBS (p=0.037) Resiquimod vs. PBS (p=0.000)	

	Resiquimod vs. poly I:C (p=0.001)	
Figure 23A(IL-2)	Mother injection	F(2,5)=0.20, p=0.830
	Poly I:C vs. PBS (p=0.816) Resiquimod vs. PBS (p=0.985) Resiquimod vs. poly I:C (p=0.949)	
Figure 23B(IL-4)	Mother injection	F(2,10)=0.78, p=0.491
	Poly I:C vs. PBS (p=0.741) Resiquimod vs. PBS (p=0.469) Resiquimod vs. poly I:C (p=0.857)	
Figure 23C(IL-5)	Mother injection	F(2,7)=0.34, p=0.726
	Poly I:C vs. PBS (p=0.728) Resiquimod vs. PBS (p=0.733) Resiquimod vs. poly I:C (p=1.000)	
Figure 23D(IL-9)	Mother injection	F(2,6)=6.13, p=0.060
	Poly I:C vs. PBS (p=0.251) Resiquimod vs. PBS (p=0.055) Resiquimod vs. poly I:C (p=0.418)	
Figure 23E(IL-10)	Mother injection	F(2,10)=57.42, p=0.000
	Poly I:C vs. PBS (p=0.012) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
Figure 23F(IL-13)	Mother injection	F(2,10)=24.05, p=0.000
	Poly I:C vs. PBS (p=0.027) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.011)	
Figure 23G(IL-17)	Mother injection	F(2,9)=8.03, p=0.015
	Poly I:C vs. PBS (p=0.284) Resiquimod vs. PBS (p=0.075) Resiquimod vs. poly I:C (p=0.012)	
Figure 23H(IFNg)	Mother injection	F(2,10)=130.22, p=0.000
	Poly I:C vs. PBS (p=0.638) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
Figure 23I(LIF)	Mother injection	F(2,9)=91.63, p=0.000
	Poly I:C vs. PBS (p=0.001) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
Figure 24A(CCL2)	Mother injection	F(2,9)=8.50, p=0.013
	Poly I:C vs. PBS (p=0.036) Resiquimod vs. PBS (p=0.005) Resiquimod vs. poly I:C (p=0.116)	
Figure 24B(CCL3)	Mother injection	F(2,10)=6.02, p=0.025
	Poly I:C vs. PBS (p=0.485) Resiquimod vs. PBS (p=0.021) Resiquimod vs. poly I:C (p=0.108)	
Figure 24C(CCL4)	Mother injection	F(2,9)=14.79, p=0.003
	Poly I:C vs. PBS (p=0.066) Resiquimod vs. PBS (p=0.002) Resiquimod vs. poly I:C (p=0.037)	
Figure 24D(CCL5)	Mother injection	F(2,9)=7.93, p=0.016
	Poly I:C vs. PBS (p=0.036) Resiquimod vs. PBS (p=0.007) Resiquimod vs. poly I:C (p=0.132)	
	Mother injection	F(2,10)=23.09, p=0.000

Figure 24E(CCL11)	Poly I:C vs. PBS (p=0.090) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.001)	
Figure 24F(CXCL1)	Mother injection	F(2,9)=17.81, p=0.002
	Poly I:C vs. PBS (p=0.524) Resiquimod vs. PBS (p=0.001) Resiquimod vs. poly I:C (p=0.001)	
Figure 24G(CXCL2)	Mother injection	F(2,10)=15.06, p=0.002
	Poly I:C vs. PBS (p=0.668) Resiquimod vs. PBS (p=0.002) Resiquimod vs. poly I:C (p=0.006)	
Figure 24H(CXCL5)	Mother injection	F(2,9)=1.52, p=0.283
	Poly I:C vs. PBS (p=0.997) Resiquimod vs. PBS (p=0.342) Resiquimod vs. poly I:C (p=0.330)	
Figure 24I(CXCL9)	Mother injection	F(2,10)=25.52, p=0.000
	Poly I:C vs. PBS (p=0.050) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.005)	
Figure 24J(CXCL10)	Mother injection	F(2,10)=10.12, p=0.006
	Poly I:C vs. PBS (p=0.010) Resiquimod vs. PBS (p=0.003) Resiquimod vs. poly I:C (p=0.286)	
Figure 25A(G-CSF)	Mother injection	F(2,10)=62.01, p=0.000
	Poly I:C vs. PBS (p=0.655) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
Figure 25B(GM-CSF)	Mother injection	F(2,9)=21.25, p=0.001
	Poly I:C vs. PBS (p=0.665) Resiquimod vs. PBS (p=0.001) Resiquimod vs. poly I:C (p=0.004)	
Figure 25C(M-CSF)	Mother injection	F(2,10)=9.06, p=0.009
	Poly I:C vs. PBS (p=0.026) Resiquimod vs. PBS (p=0.011) Resiquimod vs. poly I:C (p=0.692)	
Figure 25D(VEGF)	Mother injection	F(2,10)=7.79, p=0.013
	Poly I:C vs. PBS (p=0.039) Resiquimod vs. PBS (p=0.016) Resiquimod vs. poly I:C (p=0.689)	
Figure 26A(*Il-6*)	Mother injection	F(2,78)=73.86, p=0.000
	Poly I:C vs. PBS (p=0.053) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=3.22, p=0.002
Figure 26B(*Tnf-α*)	Mother injection	F(2,78)=46.38, p=0.000
	Poly I:C vs. PBS (p=0.519) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=1.73, p=0.097
Figure 26C(*Il-10*)	Mother injection	F(2,78)=69.85, p=0.000

	Poly I:C vs. PBS (p=0.019) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=2.12, p=0.038
Figure 26D(*Ccl2*)	Mother injection	F(2,78)=2.07, p=0.133
	Poly I:C vs. PBS (p=0.245) Resiquimod vs. PBS (p=0.136) Resiquimod vs. poly I:C (p=0.949)	
	Mother ID coding (MIA injection)	F(9,78)=1.36, p=0.219
Figure 26E(*Ccl5*)	Mother injection	F(2,78)=30.83, p=0.000
	Poly I:C vs. PBS (p=0.466) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=2.88, p=0.005
Figure 26F(*Ccl11*)	Mother injection	F(2,78)=33.23, p=0.000
	Poly I:C vs. PBS (p=0.417) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=1.29, p=0.257
Figure 26G(*Cxcl1*)	Mother injection	F(2,78)=33.24, p=0.000
	Poly I:C vs. PBS (p=0.984) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=2.81, p=0.007
Figure 26H(*Cxcl10*)	Mother injection	F(2,78)=225.77, p=0.000
	Poly I:C vs. PBS (p=0.000) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,78)=13.31, p=0.000
Figure 26I(*Cxcl12*)	Mother injection	F(2,78)=2.93, p=0.059
	Poly I:C vs. PBS (p=0.999) Resiquimod vs. PBS (p=0.112) Resiquimod vs. poly I:C (p=0.096)	
	Mother ID coding (MIA injection)	F(2,78)=5.37, p=0.000
Figure 27A(*Il-6*)	Mother injection	F(2,36)=205.80, p=0.000
	Poly I:C vs. PBS (p=0.737) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	

	Mother ID coding (MIA injection)	F(9,36)=1.71, p=0.122
Figure 27B(*Tnf-α*)	Mother injection	F(2,36)=897.83, p=0.000
	Poly I:C vs. PBS (p=0.247) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=0.75, p=0.664
Figure 27C(*Il-10)*	Mother injection	F(2,34)=65.17, p=0.000
	Poly I:C vs. PBS (p=0.121) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,34)=2.06, p=0.062
Figure 27D(*Ccl2*)	Mother injection	F(2,36)=419.15, p=0.000
	Poly I:C vs. PBS (p=0.888) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=2.92, p=0.011
Figure 27E(*Ccl5*)	Mother injection	F(2,36)=635.87, p=0.000
	Poly I:C vs. PBS (p=0.092) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=3.93, p=0.001
Figure 27F(*Ccl11*)	Mother injection	F(2,34)=36.10, p=0.000
	Poly I:C vs. PBS (p=0.689) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,34)=0.57, p=0.811
Figure 27G(*Cxcl1*)	Mother injection	F(2,36)=54.05, p=0.000
	Poly I:C vs. PBS (p=0.475) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=4.14, p=0.001
Figure 27H(*Cxcl10*)	Mother injection	F(2,36)=907.89, p=0.000
	Poly I:C vs. PBS (p=0.999) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=10.70, p=0.000
Figure 27I(*Cxcl12*)	Mother injection	F(2,36)=3.43, p=0.043
	Poly I:C vs. PBS (p=0.034)	

	Resiquimod vs. PBS (p=0.408) Resiquimod vs. poly I:C (p=0.312)	
	Mother ID coding (MIA injection)	F(9,36)=2.60, p=0.020
Figure 28A(*Aif1*)	Mother injection	F(2,36)=18.05, p=0.000
	Poly I:C vs. PBS (p=0.469) Resiquimod vs. PBS (p=0.001) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=4.96, p=0.000
Figure 28B(*Cx3cr1*)	Mother injection	F(2,36)=117.14, p=0.000
	Poly I:C vs. PBS (p=0.208) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.000)	
	Mother ID coding (MIA injection)	F(9,36)=2.91, p=0.011
Figure 28C(*Tmem119*)	Mother injection	F(2,36)=11.25, p=0.000
	Poly I:C vs. PBS (p=0.000) Resiquimod vs. PBS (p=0.008) Resiquimod vs. poly I:C (p=0.200)	
	Mother ID coding (MIA injection)	F(9,36)=4.49, p=0.001
Figure 28D(*Ccr2*)	Mother injection	F(2,36)=19.89, p=0.000
	Poly I:C vs. PBS (p=0.008) Resiquimod vs. PBS (p=0.000) Resiquimod vs. poly I:C (p=0.009)	
	Mother ID coding (MIA injection)	F(9,36)=5.47, p=0.000
Figure 28E(*Ly6c2*)	Mother injection	F(2,33)=1.20, p=0.315
	Poly I:C vs. PBS (p=0.956) Resiquimod vs. PBS (p=0.348) Resiquimod vs. poly I:C (p=0.461)	
	Mother ID coding (MIA injection)	F(9,33)=3.43, p=0.004
Figure 40A(*Bdnf)*	Mother injection	F(2,36)=1.96, p=0.156
	Poly I:C vs. PBS (p=0.181) Resiquimod vs. PBS (p=0.923) Resiquimod vs. poly I:C (p=0.268)	
	Mother ID coding (MIA injection)	F(9,36)=6.18, p=0.000
Figure 40B(*Bdnf)*	Mother injection	F(2,78)=3.87, p=0.025
	Poly I:C vs. PBS (p=0.119) Resiquimod vs. PBS (p=0.022) Resiquimod vs. poly I:C (p=0.746)	
	Mother ID coding (MIA injection)	F(9,78)=1.62, p=0.124

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