Characterization of *in vitro* and *in vivo* responses to double-stranded RNA in a mouse model of prenatal infection

by

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LIST OF ABBREVIATIONS

ANOVA; analysis of variance

AP-1; activator protein-1

APR; acute phase response

BBB; blood-brain barrier

BSA; bovine serum albumin

CDK; cyclin-dependent kinase

CNS; central nervous system

DAPI; 4',6-diamidino-2-phenylindole

DMSO; dimethyl sulfoxide

dsRNA; double-stranded RNA

eIF2-α; eukaryotic initiation factor 2-α

ELISA; enzyme-linked immunosorbent assay

GABA; γ-aminobutyric acid

GD; gestational day

H1N1; murine-adapted influenza virus

HEK; human embryonic kidney

HPA; hypothalamic-pituitary-adrenal

IL; interleukin

INF; interferon

IκB; Inhibitory κB-α

JAK2/STAT3; Janus tyrosine kinase-2/dignal transducer and activator of transcription-3

KO; knockout

LPS; lipopolysaccharide

MDA5; melanoma differentiation-associated gene 5

mGlu; metabotropic glutamate receptor

MIA; maternal immune activation

NF-κB; nuclear factor-kappa B

PBS; phosphate-buffered saline

PKR; dsRNA-activated protein kinase R

poly I:C; polyinosinic:polycytidylic acid

PPI; prepulse inhibition

RT-PCR; real-time polymerase chain reaction

TLR; toll-like receptor

TMEV; Theiler's murine encephalomyelitis virus

TNF; tumor-necrosis factor

WT; wild-type

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Rest in Peace my Brother

ABSTRACT

Prenatal infection increases the risk of developing schizophrenia in the affected offspring, although this association is poorly understood. Animal models have demonstrated that activation of the maternal immune system produces long-term effects on the behavior in offspring that are consistent with many rodent models of schizophrenia. The identification of factors that modulate the immune response during pregnancy may guide both clinical studies and therapeutic approaches for preventive care. The overarching goal of this dissertation was to evaluate potential mediators of immune activation in response to viral infection using the synthetic viral double-stranded RNA, poly I:C. Specifically, this series of studies examines the *in vitro* and *in vivo* production of the pro-inflammatory cytokine, IL-6, in response to poly I:C. The experiments in chapter one examine the effects of poly I:C on immortalized astrocyte- and macrophage-like cells. *In vitro*, poly I:C exposure elicited a time- and concentration-dependent immune response in both cell lines. This included activation of the canonical inflammatory NF-κB signaling pathway and the production of IL-6. However, poly I:C did not cause inhibition of protein synthesis, although this is a common cellular response to viral infection. Chapter two examines the *in vitro* effects of luteolin and C16, two potential anti-inflammatory agents, on the cellular

response to poly I:C. Luteolin and C16 both inhibited poly I:C-induced IL-6 protein and mRNA production, but failed to block activation of NF-κB signaling. These inhibitors also reduced protein synthesis, although they did not induce cytotoxicity. In chapter three, the effects of luteolin and C16 on poly I:C-induced IL-6 production were examined *in vivo.* Poly I:C elicited large increases in serum IL-6 levels in both non-pregnant and pregnant mice. In contrast to *in vitro* experiments, pretreatment with luteolin or C16 failed to inhibit poly I:C-induced IL-6 production. Surprisingly, pregnancy alone caused large increases in IL-6 production following poly I:C administration. Although these findings did not support an anti-inflammatory role for luteolin or C16 in response to poly I:C, they indicated that pregnancy alone may increase the inflammatory response to dsRNA. In order to further explore this pregnancy-specific effect, chapter four examines IL-6 production in mice lacking a functional TLR3. TLR3 is one of several proteins capable of binding dsRNA and initiating cytokine production. The TLR3 KO had no effect on poly I:C-induced IL-6 production in non-pregnant animals. However, in pregnant mice, the increase in poly I:C-induced IL-6 was attenuated in TLR3 KO animals. These findings indicate that inhibition of TLR3 signaling may represent a unique approach to decreasing the exaggerated inflammatory response to viral infection during

pregnancy. Identification of the mechanism by which TLR3 mediates the enhanced production of IL-6 may provide insight into novel approaches for the treatment of pregnancy-specific inflammatory diseases affecting both the mother and offspring.

INTRODUCTION

Brain development and maternal immune activation

Infection during pregnancy presents an immediate, and sometimes serious, threat to the health of the mother and developing fetus. In particular, prenatal infection increases the risk for preterm birth, organ damage to the fetus, and stillbirth (McClure & Goldenberg, 2009). Additionally, an association between prenatal infection and neuropsychiatric disease has been reported, including schizophrenia and autism (Meyer, Feldon, & Dammann, 2011). Clinical data regarding the incidence of prenatal infection and the subsequent effects on neurodevelopment in offspring are sparse due to ethical concerns regarding experimental intervention during pregnancy. Additional challenges are presented by the technical difficulties of accurately diagnosing infection and the organizational barriers of following a birth cohort across a long period of time. This is particularly relevant for determining associations between prenatal infection and schizophrenia, where the latter frequently does not present until early adulthood.

Although maternal infection during pregnancy has been associated with increased risk for the development of neuropsychiatric disorders in the affected offspring, this relationship remains controversial (Brown et al., 2004; Takei et al., 1996; Selten, Frissen, Lensvelt-Mulders, & Morgan, 2010). The vast majority of epidemiological studies have examined the frequency of psychiatric disorders in a birth cohort as a function of historical influenza rates in the general population. For example, Mednick et al. (1988) reported an increased risk for schizophrenia among the offspring of women exposed to the 1957 influenza pandemic in the first trimester (Mednick, Machon, Huttunen, &

Bonett, 1988). Similarly, Takei et al. (1996) reported that for every 100,000 cases of influenza in Denmark there was a 12% increase in schizophrenics born 4 months later (Takei et al., 1996). However, other studies employing similar methodologies have failed to find any association between influenza pandemics and timing of pregnancy among large populations of schizophrenic patients (Susser, Lin, Brown, Lumey, & Erlenmeyer-Kimling, 1994; Selten et al., 2010). The immediate cause of these discrepancies is unclear, but one major limitation of these studies is the reliance on correlations between the date of birth for psychiatric patients and the corresponding infection rates in the general population. Unfortunately, no large datasets exist that contain both the long term psychiatric status of offspring and detailed information regarding the prenatal environment, such as pathogen identification, duration of infection, or the use of anti-inflammatory and fever reducing drugs.

In order to clarify the relationship between prenatal infection and neurological development, several animal models have been established to examine the long term behavioral abnormalities in offspring following maternal immune activation (MIA) (Meyer, Feldon, Schedlowski, & Yee, 2006a; Shi, Fatemi, Sidwell, & Patterson, 2003; Smith, Li, Garbett, Mirnics, & Patterson, 2007). Animal models of prenatal infection benefit from control over genetic variability, timing of the infection, and the magnitude of the inflammatory response. In particular, the timing and magnitude of the inflammatory response appear to be critical mediators of the subsequent behavioral effects on offspring (Meyer, Feldon, Schedlowski, & Yee, 2005). Control over the timing and magnitude of the MIA is even more specific in rodent models of infection that employ synthetic compounds designed to mimic pathogen-associated molecular patterns

capable of eliciting strong immune responses in the absence of an infectious agent.

Immune activation and the central nervous system

Immune activation, both in the periphery and the central nervous system (CNS), alters neuronal activity and behavior. Infection typically generates an acute inflammatory response and generalized "sickness behavior" with symptoms including fever, fatigue, weight loss, and inflammation (Majde, 2000). Additionally, constitutive activation of immune pathways and chronic inflammation occurs in numerous neurodegenerative disorders, including multiple sclerosis, Alzheimer's disease, and stroke recovery (Okun et al., 2009). Acute and chronic inflammatory states are characterized by the upregulation of several protein families, most notably the production of pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines, including interleukins (IL), tumor-necrosis factor (TNF), and interferons (IFN), are the primary mediators of the inflammatory response to infection in the periphery and CNS. Importantly, peripheral administration of many pro-inflammatory cytokines can produce physiological and behavioral symptoms indistinguishable from those observed during infection (Capuron & Miller, 2011). Additionally, resident cells of the CNS, specifically astrocytes and microglia, are capable of cytokine production (Town, Jeng, Alexopoulou, Tan, & Flavell, 2006; Carpentier, Williams, & Miller, 2007; Park et al., 2006).

The blood brain barrier (BBB) protects the CNS from a wide variety of infection, yet glial cells posses the cellular mechanisms required for detection of both bacterial and viral pathogens. Of particular importance are the toll-like receptors (TLRs), a family of at least 13 pathogen recognition receptors. These transmembrane signaling proteins are

capable of binding, with high specificity, to pathogen-associated molecular patterns found in the nucleic acids, proteins, and cytoskeletal components of invading organisms (Okun et al., 2009). Upon ligand binding, TLRs initiate downstream signaling pathways resulting in antiviral, antibacterial, and inflammatory responses. For example, intracerebroventricular injection of the synthetic TLR3 agonist, polyinosinic:polycytidylic acid (poly I:C), induces the production of inflammatory cytokines within the CNS (Park et al., 2006). Additionally, intracerebroventricular injections of the pro-inflammatory cytokine, Interleukin-6 (IL-6), induce febrile responses and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Lenczowski et al., 1999). In the case of systemic injections, TLR agonists generate large increases in circulating levels of pro-inflammatory cytokines, but also increase cytokine production within the CNS, most notably increases in IL-6 (Cunningham, Campion, Teeling, Felton, & Perry, 2007). Systemic injections of IL-6 also alter CNS functions such as HPA activity and neurotransmitter metabolism. These results indicate that peripherally generated compounds, such as IL-6, can alter neuronal function in the absence of infection within the CNS (Wang & Dunn, 1998).

TLR agonists and the systemic immune response

Although members of the TLR family identify specific patterns associated with different pathogens (e.g. viral or bacterial), the systemic response following ligand binding to these receptors shares many common features (Takeda & Akira, 2004). Poly I:C is a synthetic double-stranded RNA (dsRNA) capable of eliciting a strong acute phase response (APR). Many viruses produce dsRNA, and poly I:C can elicit the production of

inflammatory cytokines through several pathways, although one prominent mechanism appears to be binding at the TLR3. A second molecule widely employed in animal models of the APR is lipopolysaccharide (LPS). LPS is a major component of the outer wall of gram-negative bacteria, and binding of LPS to TLR4 induces strong antibacterial and inflammatory responses. Systemic injections of poly I:C and LPS share many features, including the induction of febrile response and general sickness behavior. This is not surprising given that macrophages, primary initiators of the APR express both TLR3 and TLR4. In cultured RAW 264.7 macrophages, poly I:C and LPS stimulation initiates the production of a similar profile of cytokines, including IL-1, IFN-β, TNF-α, and IL-6 (Kimura et al., 1994).

Among the prominent mediators of immune response, notably IL-1β, TNF-α, and IL-6, there exists a large degree of functional overlap and regulatory feedback mechanisms. For example, both IL-1β and IL-6 potently induce febrile responses (Lenczowski et al., 1999; Fortier et al., 2004). In the case of poly I:C, co-administration of an IL-1 receptor antagonist inhibits the febrile response, but fails to alter the reduction in feeding, which is a characteristic symptom observed during sickness behavior. Although this indicates an important role for IL-1β in mediating the febrile response to poly I:C, the co-administration of the IL-1 receptor antagonist also drastically reduces the production of IL-6 following poly I:C administration, suggesting that IL-1 receptor activation can induce IL-6 production (Fortier et al., 2004). Additionally, intracerebroventricular IL-6 administration is capable of eliciting activation of the HPA axis and febrile response, but fails to alter social investigatory behavior or locomotor activity, both characteristic measures of sickness behavior. Likewise,

intracerebroventricular injections of IL-1β fail to alter these behaviors. However, co-administration of IL-6 and IL-1β strongly decreases social exploration and locomotor activity (Lenczowski et al., 1999). These examples indicate that pro-inflammatory cytokines produced following immune activation have both distinct and overlapping effects, but also share some positive feedback mechanisms.

Maternal immune activation during pregnancy

Epidemiological studies indicating an association between influenza and schizophrenia have generated a strong interest in modeling the acute and long-term behavioral effects of MIA in rodent models. Findings of behavioral abnormalities in offspring of rats and mice following MIA have been replicated across numerous labs, employing a variety of immune challenges, and examining a wide range of behaviors. Of particular interest is the observation that MIA during pregnancy produces long-term behavioral abnormalities in adult offspring that are consistent with many clinical features present in neuropsychiatric disorders, including schizophrenia and autism (Meyer et al., 2011). These effects are dependent upon the magnitude of the immune response and the timing at which this occurs relative to the age of the embryo/fetus, suggesting a specific mechanism involving immune activation and development (Meyer et al., 2005).

Animal models of influenza infection during pregnancy

Numerous studies have examined the acute and long-term effects of influenza infection during pregnancy. Results from these studies indicate that influenza infection during pregnancy leads to a range of genetic, neuroanatomical, and behavioral changes in

the affected offspring. Many of these alternations are consistent with clinical symptoms typically observed in schizophrenia, and several studies have demonstrated the efficacy of typical antipsychotics in normalizing the behavior of offspring. Collectively, these studies have provided a useful platform for examining the neurobiological effects of prenatal infection.

Long term changes in gene expression occur in offspring following infection of pregnant mice with the murine-adapted influenza A/WSN/33 (H1N1) virus. Fatemi et al. (1999) infected pregnant C57BL/6 mice with influenza (H1N1) at gestational day (GD) 9 *via* intranasal instillation. Prenatal infection reduced the thickness of the neocortex and decreased the number of Reelin positive cells in newborn pups, results consistent with postmortem observations in schizophrenics (Fatemi et al., 1999). Reelin is a large glycoprotein that controls synaptic migration during development and appears to be important for controlling synapse formation in adulthood, suggesting that prenatal poly I:C exposure can cause long-term effects on synaptic functioning (Forster et al., 2010). In Balb-c mice, influenza infection at GD 9 caused the upregulation of 21 genes and the downregulation of 18 genes in brain tissue at postnatal day 0 (Fatemi, Pearce, Brooks, & Sidwell, 2005). Even more striking are the lasting effects on gene expression observed following influenza infection at GD18 in C57BL/6 mice. Affymetrix microarrays revealed widespread and long-lasting alterations in gene expression that persisted through postnatal day 56 in the cerebellum, hippocampus, and frontal cortex (Fatemi et al., 2008). Similar results were found in a separate study of influenza infection at GD7. Altered gene expression in this model was also observed in the offspring as far out as postnatal day 56. Importantly, no expression of H1N1 viral-specific genes was observed in either the

placenta or fetal brain (Fatemi et al., 2011). Although failure to detect viral-specific genes does not fully rule out the presence of transient infection of the placenta or fetus, these finding suggest that direct viral infection of the fetus is not required for the induction of long-term changes in gene expression.

Prenatal influenza exposure also produces long-term changes in offspring behavior. Moreno et al. (2011) examined the effects of prenatal influenza exposure on behavioral responses to hallucinogenic and antipsychotic drugs. Mice exposed prenatally to influenza had reduced locomotor activity, increased head-twitch behavior, and increased cellular activation in response to hallucinogenic drugs. Additionally, prenatal influenza exposure reduced expression of the metabotropic glutamate receptor $(mGlu)_2$ in the frontal cortex and decreased the behavioral response to the mGlu agonist, LY379268. Finally, the authors measured viral titers in tissue homogenates from maternal lung and embryos at 3 and 6 days post-inoculation. While maternal lung displayed high levels of viral infection, no detectable virus was present in embryonic tissue (Moreno et al., 2011). These findings agree with the observations of Fatemi et al. (2011) indicating that direct infection of the fetus is not required for the generation of long-term behavioral abnormalities (Fatemi et al., 2011).

Shi et al. (2003) examined the effects of influenza infection during pregnancy in the offspring of both Balb/c and C57BL/6 mice (Shi et al., 2003). Offspring born to influenza infected dams showed significantly higher baseline measures of anxiety, as determined by the open-field and novel-object tests. The authors also examined prepulse inhibition (PPI) in the adult offspring. PPI measures the reduction in startle response to a loud tone when it is preceded by a weaker tone. Reduced PPI was initially identified as a

marker of impaired sensory motor gating in human schizophrenics, but has since been modified and employed in animal models of schizophrenia (Ouagazzal, Jenck, & Moreau, 2001). Adult offspring of prenatally infected mice displayed reduced prepulse inhibition (PPI) compared to sham infected animals. Additionally, administration of typical and atypical antipsychotics increased PPI in these offspring. These results are of particular importance considering that increases in PPI are associated with the clinical efficacy of many antipsychotic compounds (Ouagazzal et al., 2001).

Behavioral and neurobiological effects of prenatal poly I:C-induced MIA

In order to isolate the effects of MIA on offspring independently of viral infection, many animal models of prenatal infection have utilized the synthetic double-stranded RNA, poly I:C. The use of a direct TLR3 agonist has numerous advantages compared to viral infection, including greater control over dose, timing, and increased safety. In particular, several animal models have demonstrated that the acute inflammatory response during pregnancy is highly dependent upon gestational day. For example, the febrile response to LPS in rats decreases as the dams approach term (Fofie & Fewell, 2003; Fofie, Fewell, & Moore, 2005). Similarly, the behavioral effects of prenatal exposure to poly I:C on offspring are dependent upon both the timing of administration relative to the gestational age and the dose of poly I:C.

Meyer et al. (2005) injected pregnant C57BL/6 mice at GD9 with 0, 2.5, 5, and 10 mg/kg of poly I:C (Meyer et al., 2005). Injections of 5 and 10 mg/kg caused significant behavioral abnormalities in the affected offspring, including lower levels of PPI and enhanced locomotor activity in response to amphetamine. These results indicate that

prenatal exposure to poly I:C can dose-dependently alter dopamine function in offspring. Interestingly, the effects of prenatal poly I:C exposure are also partially dependent upon the gestational stage of pregnancy. While administration of 5 mg/kg poly I:C at GD9 reduces levels of PPI compared to controls, administration at GD17 had no effect on PPI in adult offspring. However, amphetamine induced locomotor activity was significantly increased in animals exposed to poly I:C at both GD9 and GD17 (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008). These finding suggest that the specific effect of poly I:C exposure on dopaminergic function may be dependent on either the developmental stage of the fetal brain or the characteristics of the immune response at different stages of pregnancy.

Several studies have explored the neurochemical and neurobiological effects in the offspring of mice following poly I:C-induced MIA. Acute injections of poly I:C (5 mg/kg) at GD9 in C57BL/6 mice reduced expression of the dopamine D1 receptor and decreased the number of Reelin positive cells in the medial prefrontal cortex (Meyer et al., 2008). In a similar study, poly I:C administration at GD9 also increased dopamine levels in the lateral globus pallidus and prefrontal cortex of adult offspring. Decreases in serotonin were also observed in several brain regions, while levels of γ-aminobutyric acid (GABA) and glutamate were unchanged by poly I:C administration (Winter et al., 2009). Similar to studies utilizing acute MIA, poly I:C (5 mg/kg) administration for 6 consecutive days, from GD 12-17, in BALB/c mice led to lower levels of PPI and an enhanced locomotor response to methamphetamine in adult offspring. These animals also displayed higher levels of dopamine metabolism in the striatum and, paradoxically, lower levels of D_2-like receptor expression in the striatum (Ozawa et al., 2006).

Collectively, these studies demonstrate substantial neurobiological and behavioral effects of prenatal poly I:C administration on adult offspring. The majority of these findings are consistent with alterations related to increased dopaminergic activity, particularly related to decreased PPI and increased sensitivity to the locomotor effects of drugs that directly and indirectly stimulate dopamine receptor activation.

Effects of pregnancy on immune function

The specific profile of the cytokine response and resulting physiological effects during pregnancy are highly dependent on the gestational stage for both the dam and fetus. For example, LPS administration in non-pregnant rats leads to an immediate and prolonged febrile response. However, in pregnant dams this response is altered throughout the gestational period. At GD10, 15, and 20 the administration of LPS leads to a bi-phasic febrile response with an initial hypothermic period followed by a sustained hyperthermic phase. Interestingly, the hypothermic response is lengthened and the hyperthermic response is shortened as dams get closer to term (Fofie et al., 2003). Furthermore, pregnant rats (GD20) administered LPS show reduced production of the pryrogenic cytokines IL-1β and IL-6 compared to non-pregnant controls. However, LPS administration in these animals did significantly increase levels of the antipyretic Interleukin-1 receptor antagonist (IL-1ra) which may account for the exaggerated hypothermic response to LPS during pregnancy (Fofie et al., 2005). These finding suggest that in addition to the absolute levels of cytokine induction, the relative expression of pro-inflammatory and anti-inflammatory signaling molecules may be critical in mediating the overall effects of MIA.

Poly I:C administration during pregnancy generates a strong APR, including the production of pro-inflammatory IL-1β, IL-6, and TNF-α. Poly I:C administration also induces the production of the anti-inflammatory cytokine IL-10 (Meyer et al., 2006a). The profile and duration of cytokine production is significantly altered by the gestational period in pregnant mice. Specifically, Meyer et al. (2006) examined cytokine expression following poly I:C administration (5 mg/kg) in pregnant C57BL/6 mice at GD9 and GD17. At 3 hours post injection, mice of both gestational age displayed significant increases in serum levels of IL-1β, IL-6, TNF-α, and IL-10. At 6 hours post-injection, serum levels of IL-6 and IL-10 remained elevated, while IL-1β and TNF-α levels had returned to baseline. Serum levels of IL-10 were significantly higher in GD9 than GD17 mice at 3 hours post-injection, but this pattern was reversed at 6 hours post injection with higher serum IL-10 in GD 17 dams. Importantly, levels of IL-6 in the fetus were elevated 3 hours post-injection in the GD9 fetal groups, but were suppressed in the GD17 fetal group. Conversely, levels of IL-10 were decreased in the GD9 fetal group and increased in the GD17 fetal group at 3-hours post injection. No differences in fetal levels of IL-6 or IL-10 were observed at 6 hours post-injection (Meyer et al., 2006b). Although prenatal poly I:C administration induces a wide range of cytokine responses, these findings indicate administration of poly I:C at GD9 results in an enhanced inflammatory IL-6 response and a decreased anti-inflammatory IL-10 response in the developing fetus when compared with administration at GD17. Thus, poly I:C administration at GD 9 presents an increase in both the inflammatory profile of the MIA and the subsequent behavioral deficits in the offspring. For this reason, *in vivo* experiments in this research project will focus on inhibition of poly I:C-induced MIA at GD9.

The role of Interlekin-6 in animal models of prenatal infection

Although prenatal MIA elevates numerous cytokines in the maternal serum and fetus, one of the most prominent responses is observed in the increased production of IL-6 (Meyer et al., 2006b). Smith et al. (2007) conducted a series of experiments designed to examine the specific role of IL-6 in mediating the behavioral changes in offspring following MIA (Smith et al., 2007). The authors briefly report the results of a pilot study in which pregnant C57BL/6 dams were injected on GD12.5 with either IL-6, IL-1α, TNF-α, or INF-γ. Lower levels of PPI were only observed in the adult offspring exposed prenatally to IL-6. In agreement with these findings, administration of an anti-IL-6 antibody prior to injections of poly I:C (20 mg/kg) on GD12.5 attenuated the behavioral effects of prenatal poly I:C exposure, including the reduction in PPI. Finally, administration of poly I:C under the same paradigm failed to alter PPI in the adult offspring of IL-6 knockout mice when compared with the offspring of IL-6 knockout mice receiving saline injections on GD12 (Smith et al., 2007). This series of studies indicates a key role of IL-6 in mediating the long-term behavioral effects observed following MIA. Therefore, production of IL-6 represents a primary outcome measure for both the *in vitro* and *in vivo* studies proposed in this project.

Cellular detection of dsRNA

The production of viral double-stranded RNA (dsRNA) and activation of dsRNA-dependent pathways occurs frequently during viral infection (Jacobs & Langland, 1996). The detection of dsRNA at the cellular level initiates strong immune and antiviral

responses (Daffis, Samuel, Suthar, Gale, Jr., & Diamond, 2008; Carpentier et al., 2007). Numerous pathways have been identified in the cellular response to dsRNA, including binding to the TLR3, the dsRNA-activated protein kinase R (PKR), and the melanoma differentiation-associated gene 5 (MDA5). Activation of these different pathways appear to be dependent on both the specific cell type and the source of the dsRNA, although the functional consequences regarding cytokine production and many overlapping downstream signaling pathways exist (Carpentier et al., 2007; De Miranda, Yaddanapudi, Hornig, & Lipkin, 2009)

Detection of poly I:C *via* TLR3

TLR3 expression across a wide range of cell types is responsible for initiating the response to dsRNA. The binding of dsRNA to TLR3 is highly dependent upon the size of the dsRNA, and reduction in dsRNA length to less than 48 base pairs drastically diminishes binding affinity (Leonard et al., 2008). Additionally, microscopy studies indicate that TLR3 is primarily located in endosomal compartments, and compounds that interfere with endosome acidification impair the cellular response to poly I:C (Leonard et al., 2008; Matsumoto et al., 2003). Alexopoulou et al. (2001) first identified dsRNA as a ligand for TRL3 by transfecting human embryonic kidney (HEK) cells with the wild-type human TLR3. In cells expressing TLR3, poly I:C caused a concentration dependent increase in cytokine production and activation of nuclear factor-kappa B (NF-κB), a canonical signaling intermediary for inflammatory responses (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Kawai & Akira, 2007). In order to examine the role of TLR3 *in vivo*, the authors generated mice expressing a mutant form of TLR3. These mice

had increased, but not total, resistance to the lethal effects of poly I:C-induced shock (Alexopoulou et al., 2001). These results provided convincing evidence for the prominent role of TLR3 in the cellular and systemic responses to the synthetic dsRNA, poly I:C.

Considerable evidence exists that TLR3 may modulate the response to dsRNA within the CNS. Studies using cells cultured from TLR3 knockout (KO) mice have shown reduced IL-6 production in response to poly I:C in microglia (Town et al., 2006) and astrocytes (Carpentier et al., 2007). Additionally, in primary cultures of human astrocytes prepared from 20- to 25-week old fetuses, poly I:C-induced IL-6 production is inhibited by pretreatment with an anti-TLR3 antibody (Kim et al., 2008). Finally, peripheral administration of poly I:C in mice leads to induction of IL-6 mRNA in the forebrain, brain stem, and cerebellum (Cunningham et al., 2007). These *in vitro* and *in vivo* studies indicate that poly I:C activation through TLR3 is capable of increasing IL-6 within the CNS.

Detection of dsRNA *via* PKR

The dsRNA-dependent protein kinase R (PKR) is a serine/threonine kinase capable of binding dsRNAs, including poly I:C (Su et al., 2006). PKR contains two binding motifs for dsRNA, and binding of dsRNA appears to initiate dimerization and autophosphorylation of PKR. Although PKR contains numerous phosphorylation sites, phosphorylation of the threonine-451 site appears to be critical for mediating the antiviral properties of PKR activation (Zhang et al., 2001). Binding of dsRNA by PKR during viral infection suppresses protein synthesis and represents a possible mechanism by which infected cells can reduce the viral replication that is dependent upon host cell

machinery (Lee et al., 1993). Suppression of viral replication relies on the ability of PKR to phosphorylate eukaryotic initiation factor 2-α (eIF2-α) leading to global inhibition of protein synthesis (Su et al., 2006). Additionally, PKR can generate inflammatory cytokine production in response to dsRNA, although the specific conditions under which poly I:C signals through this pathway are the subject of conflicting reports, several of which are outlined below.

Srivastava et al. (1998) employed a luciferase-based reporter assay to examine NF-κB activation in response to poly I:C in NIH3T3 cells and found that transfection of a dominant negative mutant of PKR abolished NF-κB activation in response to poly I:C (Srivastava, Kumar, & Kaufman, 1998). Similar results were obtained using a luciferase reporter assay and a dominant negative mutant of PKR in immortalized human astroglial cells (Auch et al., 2004). However, another report using dominant negative mutant forms of PKR found reduced IL-1 production following poly I:C but no effect on poly I:C-induced NF-κB activity in immortalized macrophage cells (Maggi, Jr. et al., 2000).

Several recent studies have indicated differential effects of intracellular and extracellular exposure to poly I:C or viral infection. Carpentier et al. (2007) reported that astrocytes cultured from PKR knockout mice showed no decrease in poly I:C induced IL-6 production, although these cells did show a significant reduction in IL-6 following infection with Theiler's murine encephalomyelitis virus (TMEV). When these same conditions were examined in astrocytes cultured from TLR3 KO mice, the exact opposite effect was observed with poly I:C activation being suppressed while the effects of TMEV infection were spared (Carpentier et al., 2007). The authors speculated that the effects may be related to differential accessibility of PKR and TLR3 to intracellular viral dsRNA

and extracellular synthetic poly I:C. Interestingly, differential signaling pathways do appear to be employed in cell culture depending on whether poly I:C exposure is exogenous (applied in the native dsRNA form) or introduced into the intracellular milieu using transfection agents, such as Lipofectamine^{TM} (De Miranda et al., 2009). Zhang and Samuel (2007) created PKR deficient HeLa cells using an RNA interference silencing strategy to examine the role of PKR in response to poly I:C. Cells were then transfected with poly I:C using Lipofectamine^{TM} and levels of phosphorylated eIF2-α and the amount of protein synthesis was measured using a luciferase-reporter assay. Poly I:C led to increased eIF2-α phosphorylation and decreased protein synthesis while poly I:C had no effect on PKR deficient HeLa cells (Zhang & Samuel, 2007). The experiments proposed in this research project will examine the effect of extracellular poly I:C on protein synthesis.

NF-κB signaling

Activation of the transcription factor NF-κB is common pathway in the cellular response to a wide variety of stressors, and translocation of this protein complex to the nucleus regulates transcription of approximately 400 different genes (Ahn & Aggarwal, 2005). Under basal conditions, NF-κB is restricted to the cytoplasmic compartment by the inhibitory protein Inhibitory κB-α (IκB). Stimulation by a wide range of cellular stressors causes IκB to undergo phosphorylation, ubiquitination, and rapid degradation, allowing for NF-κB translocation to the nucleus (Nakajima, Fujiwara, Furuichi, Tanaka, & Shimbara, 2008). Once in the nucleus, NF-κB can initiate the transcription of over 400 genes, including IL-6 and IκB. The activity of NF-κB in the nucleus is primarily

terminated by the production of new IκB which again inactivates NF-κB by shuttling it back to the cytoplasmic compartment (Newton, Adcock, & Barnes, 1996). Not surprisingly, activation of TLRs by poly I:C or LPS leads to rapid nuclear translocation and transcriptional activity of NF-κB (Alexopoulou et al., 2001). Finally, genetic and pharmacological manipulations that reduce NF-κB activation reduce IL-6 production in a number of inflammatory models (Alexopoulou et al., 2001; Chen, Peng, Tsai, & Hsu, 2007).

Inhibitors of dsRNA signaling

Inhibition of inflammatory processes has been the focus of a number of fields ranging from treatment of Type I diabetes to cancer. Many pharmacological strategies have been employed in the inhibition of the inflammatory response to poly I:C, for example: 1) TLR3 inhibitors: Agents such as bafilomycin A1 and chloroquine disrupt TLR3 pH-dependent recognition of dsRNA by inhibiting endosomal acidification, as indicated by reduced NFκB activation and IL-6 secretion following *in vitro* poly I:C stimulation (Liu et al., 2008; de Bouteiller et al., 2005). 2) NF-κB inhibition: GS143 suppresses the ubiquitination of IκB leading to inhibition of NF-κB translocation (Nakajima et al., 2008). Similarly, NSC 676914 represses phosphorylation of IκB, resulting in reduced nuclear translocation of NF-κB (Kang et al., 2009). 3) PKR inhibitors: Agents such as C16 (a novel oxindole/imidazole compound) and 2-aminopurine inhibit PKR autophosphorylation and block dsRNA-mediated translational inhibition (Jammi, Whitby, & Beal, 2003; Gusella, Musso, Rottschafer, Pulkki, & Varesio, 1995).

Inhibition of dsRNA signaling by flavonoids

Flavonoids are naturally occurring compounds found in a variety of food sources, including fruits, vegetables, wine, and tea. The most abundant groups of flavonoids can be categorized into flavonols, flavones, isoflavones, and flavanones (Kimira, Arai, Shimoi, & Watanabe, 1998). Although all classes of flavonoids appear to have some antioxidative and anti-inflammatory properties, the flavonol quercetin and the flavone luteolin have received considerable attention due to their superior potency in reducing inflammatory responses (Comalada et al., 2006). In particular, luteolin, is effective at reducing lethal toxicity and inflammation in a mouse model of septic shock and reduces IL-6 production in mice following administration of LPS (Kotanidou et al., 2002; Jang, Kelley, & Johnson, 2008). The exact mechanism by which luteolin inhibits LPS induced inflammatory responses has been the subject of numerous conflicting reports. Several studies have observed significant inhibition of LPS induced IκB degradation and NFκB activation in the presence of luteolin (Kim & Jobin, 2005; Chen et al., 2007). However, several studies have also reported no effect of luteolin on IκB degradation or NFκB activation, and instead indicated other targets involved in the inflammatory response, including activator protein-1 (AP1) and TRIF, an essential downstream signaling protein for TLR3 (Jang et al., 2008). Alternatively, numerous studies have reported that luteolin decreases proliferation of cultured cells, including bone-marrow-derived macrophages, immortalized human fibroblasts (HFK2), and prostate cancer (PC3) cells (Comalada et al., 2006; Fang, Zhou, Shi, & Jiang, 2007; Fotsis et al., 1997). Few studies examining the anti-inflammatory properties of luteolin have addressed potential effects on cell growth and division, and the interaction between the anti-proliferative and anti-inflammatory

effects of luteolin are not well described (Comalada et al., 2006). Experiments in this research project will examine the ability of luteolin to inhibit IκB degradation, IL-6 production, and cell growth in astrocyte-like and macrophage-like cells.

Inhibition of dsRNA signaling by PKR inhibitors

The apparent role of PKR in mediating inflammation and inhibiting protein synthesis has generated substantial interest in identifying pharmacological inhibitors of this kinase (Carpentier et al., 2007; Zhang et al., 2007; Jammi et al., 2003). Currently, the only compound commercially available as a specific inhibitor of PKR is the imidazolo-oxindole compound, C16. C16, was identified through a small-molecule screening for compounds capable of inhibiting PKR autophosphorylation, dsRNA binding, and suppression of protein synthesis (Jammi et al., 2003). Furthermore, *in vivo* dose-dependent effects of C16 have been reported to reduce phosphorylation of PKR and eIF2 (Ingrand et al., 2007). However, others have found no effect of C16 on PKR or eIF2-α phosphorylation and instead have demonstrated an inhibitory effect on cyclin-dependent kinases and cell cycle progression (Chen, Wang, & D'Mello, 2008). Importantly, no study to date has examined the effects of C16 following exposure to dsRNA. The experiments in this research project will examine the ability of C16 to inhibit IκB degradation, IL-6 production, and cell growth in astrocyte-like and macrophage-like cells.

Rationale

Pathogen recognition by macrophages or astrocytes initiates inflammatory responses in the periphery and central nervous system. In particular, the production of

pro-inflammatory cytokines during pregnancy adversely affects neurodevelopment in a manner consistent with changes observed in a number of neuropsychiatric disorders. Flavonoids appear to possess significant anti-inflammatory properties and are readily consumed by humans through a variety of foods. As a result, flavonoid consumption may be a significant mediator of the inflammatory response to viral infection. With respect to prenatal development, inhibition of the inflammatory response may extend to the developing fetus, with long-lasting consequences on gene expression and overall neurological functioning. Therefore, one of the primary aims of this study was to determine the *in vitro* and *in vivo* ability of luteolin to modulate the inflammatory response in a model of viral infection employing the synthetic dsRNA, poly I:C.

The activation of PKR in response to dsRNA remains poorly understood. The apparent ability of this kinase to generate inflammatory responses and inhibit protein synthesis suggests that activation of PKR could significantly alter the trajectory of fetal neurodevelopment. Therefore, a second aim of this study was to examine the ability of poly I:C to inhibit protein synthesis and examine the ability of the PKR inhibitor C16 to block this effect.

The ability of either luteolin or C16 to inhibit the *in vivo* pro-inflammatory responses to poly I:C remain completely untested. The possibility that one or both of these compounds could inhibit the increases in IL-6 during pregnancy could provide a useful model for delineating the potential contributions of PKR and NF-κB activation in mediating the effects of MIA on fetal neurodevelopment. Therefore, a third aim of this study is to test the ability of luteolin and C16 to inhibit poly I:C-mediated cytokine production in an animal model of prenatal infection.

The effects of pregnancy on immune function are substantial, yet few preclinical studies have directly examined the effects of pregnancy on TLR agonist-induced immune responses. Furthermore, the effects of TLR3 on poly I:C-induced immune function have not been examined, despite the prominent role of the receptor in mediating the response to dsRNA in many experimental models. Therefore, the fourth aim of this study was to examine the response to poly I:C in pregnant and non-pregnant mice expressing a mutant form of the TLR3.

Specific Aims

1) Examine the effects of poly I:C exposure on cytokine production, NFκB signaling, and protein synthesis in macrophage-like and astrocyte-like cells.

2) Examine the inhibitory effects of luteolin and C16 on poly I:C-induced cellular responses, including NF-κB signaling, protein synthesis, and cytokine production.

3) Test the ability of poly I:C to induce MIA and examine the *in vivo* effects of luteolin and C16 on poly I:C-treated pregnant and non-pregnant mice.

4) Determine the role of TLR3 in mediating the poly I:C-induced production of inflammatory cytokines during pregnancy.

Chapter 1: Poly I:C-induced cytokine production and NF-κB activation in macrophage-like and astrocyte-like immortalized cells

Introduction

Recognition of viral double-stranded RNA (dsRNA) constitutes a primary mechanism for the initiation of an immune response. Exposure to dsRNA both *in vitro* and *in vivo* increases cytokine production, inhibits protein synthesis, and can cause apoptosis (Jacobs et al., 1996). Several pathways are involved in the response to dsRNA, including recognition by the dsRNA-binding protein kinase R (PKR) and the toll-like receptor (TLR) 3 (Carpentier et al., 2007; Zhang et al., 2007). These pathways converge to initiate transcription *via* Nuclear Factor κB/p65 (NF-κB), which subsequently induces expression of genes regulating cell growth, differentiation, and immune function (Shishodia & Aggarwal, 2004; Mattson, 2005). Binding of dsRNA to PKR also phosphorylates eukaryotic initiation factor 2-α (eIF2) which slows viral replication by inhibiting protein synthesis (Lee, Green, Mathews, & Esteban, 1994). Polyinosinic:polycytidylic acid (poly I:C) is a synthetic dsRNA capable of eliciting strong inflammatory responses both *in vivo* and *in vitro* (Alexopoulou et al., 2001). Although the signaling pathways activated by poly I:C are often cell type specific, activation of NF-κB and the production of pro-inflammatory cytokines, such as interleukin (IL)-6, are common features of cellular and systemic responses to poly I:C (Jacobs et al., 1996).

NF-κB is restricted to the cytoplasmic compartment by the inhibitory protein, Inhibitory κB-α (IκB), and cellular stressors cause IκB to undergo phosphorylation,

ubiquitination, and rapid degradation. Following IκB degradation, the p65 subunit of NF-κB translocates to the nuclear compartment where it binds cofactors and initiates gene transcription (Nakajima et al., 2008). The activity of NF-κB in the nucleus is primarily terminated by the production of new IκB which shuttles NF-κB back to the cytoplasmic compartment.

Pathogen recognition by macrophages or astrocytes initiates inflammatory responses in the periphery and central nervous system. The current study examined the effects of poly I:C on cultured RAW 264.7 macrophage-like cells and C8-D1A type 1 astrocyte-like cells. We first analyzed changes in gene expression in RAW 264.7 cells in response to poly I:C using a microarray designed to identify changes in genes known to regulate immune function, neuronal activity, and cell growth and survival. Poly I:C, induced widespread changes in gene expression, including large increases in the the pro-inflammatory cytokine, IL-6. Subsequent experiments focused on the production of the pro-inflammatory cytokine IL-6, activation of the NF-κB pathway, and inhibition of protein synthesis in both RAW 264.7 and C8D1A cell lines. Our results indicate that poly I:C robustly induced IκB degradation, NFκB phosphorylation and nuclear translocation, and IL-6 production in both cell lines. Surprisingly, protein synthesis was not altered by poly I:C exposure, suggesting that activation of PKR is not a prominent feature of the cellular response to poly I:C under these conditions.

Materials and Methods

Materials and cell culture

Murine cell lines were obtained from ATCC (Manassas, VA). Raw 264.7

macrophages (#TIB-71) and type 1 C8-D1A astrocyte (#CRL-2541) cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, and 5 mg/L amphotericin B. All antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA) with the exception of the phospho-specific NF-κB antibody (Cell Signaling Technology, Danvers, MA). ^{3}[H]-leucine was purchased from American Radiolabeled Chemicals, St. Louis, MO. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, St. Louis, MO.

qRT-PCR array

Cells were grown until 75% confluent in 100mm tissue culture dishes and treated with either poly I:C or saline for 24 hours. Culture media was removed, cells were briefly rinsed with PBS, and then incubated at room temperature for 5 minutes RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA) followed by the addition of 200 μl chloroform per 1 ml of RNA-STAT 60. Samples were then shaken vigorously for 2-3 minutes, allowed to sit at room temperature for 2-3 minutes, and finally centrifuged at 12,000*g* for 15 minutes at 4^{o}C. The upper aqueous phase was transferred to a new tube and RNA was precipitated by the addition of 500 μl isopropanol, followed by incubation at room temperature for 10 minutes. The samples were then centrifuged at 12,000*g* for 15 minutes at 4^{o}C and the pellet was washed with 500 μl 75% ethanol, centrifuged at 7,500*g* for 5 minutes, and allowed to air dry at room temperature. The resulting pellet was resuspended in 20 μl of DEPC H_2O and contaminating DNA was removed through spin column purification, as per manufacturer instruction (Zymo Research, cat. R1015). RNA purity was confirmed by spectrophotometric analysis and the integrity of ribosomal RNA was confirmed by running samples on a 1% agarose gel followed by staining with

SYBRGold Nucleic Acid Stain (Molecular Probes, cat. S-11494). The purified RNA samples then underwent first-strand cDNA synthesis using q Script cDNA Supermix according to manufacturer instructions (Quanta Biosciences, cat. 95048-025). Real-time PCR for gene expression was performed by Bar Harbor Biotechnology (Trenton, ME, USA) using the Mouse Mood Disorder 384-well StellARay qPCR array. Significant changes in gene expression were identified by Bar Harbor Biotechnology using their Global Pattern Recognition algorithm (Akilesh, Shaffer, & Roopenian, 2003).

IL-6 production

Cells were plated in 96-well cell culture plates at 5000 cells/well. After 48 hours, plates were treated as indicated and cell culture media was collected. Samples were analyzed in duplicate using ELISA kits for murine IL-6 (R&D Systems, Minneapolis, MN).

Immunoblotting

For measurement of IκB degradation and phoshorylated NF-κB (pNF-κB), cells were treated with poly I:C (10 μg/ml) or saline control for the indicated time and cell lysates were obtained by washing 3 times with ice cold CMF-PBS followed by the addition of 1 ml ice cold buffer RIPA buffer containing 100 μM Na_3VO_4, 100 μM NaFl, and 1 μl/ml protease inhibitor cocktail (Calbiochem, San Diego, CA). Cell lysates were transferred to ice cold microcentrifuge tubes, incubated for 20 min at 4^{o}C, followed by centrifugation at 14,000*g* for 20 min at 4^{o}C. The supernatant was collected and stored at -20^{o}C. Protein content was quantified using a bicinchoninic acid (BCA) protein assay and samples were incubated in Laemmli's Sample Loading buffer for 1 hr at 37^{o}C. Following incubation, 20 μg of protein was separated on 10% SDS-PAGE gels at 100 V and

transferred to PVDF membrane at 30 V overnight at 4° C. For IκB immunoblotting, membranes were blocked for 1 hour at room temp in TBS-T containing 3% milk then incubated for 1 hr with IκB-α or actin antibodies (1:400) in TBS-T containing 3% milk. Membranes were washed three times for 10 minutes in TBS-T and incubated with either anti-rabbit or anti-goat IgG-AP (1:3000) in TBS-T containing 3% non-fat dry milk powder. Membranes were then washed three times for 10 minutes in TBS-T and incubated for 5 minutes with ECF™ substrate (GE Healthcare, Piscataway, NJ). For pNF-κB immunoblotting, membranes were blocked for 1 hour at room temp in TBS-T containing 1% BSA then incubated for overnight at 4°C with rabbit anti-pNF-κB (1:1000) in TBS-T containing 1% milk. Membranes were washed three times for 10 minutes in TBS-T and incubated with an alkaline phosphatase (AP) conjugated anti-rabbit antibody (1:3000) in TBS-T containing 1% BSA. Membranes were then washed three times for 10 minutes in TBS-T and incubated for 5 minutes with ECF™ substrate for imaging. Levels of pNF-κB were normalized to total NF-κB by stripping membranes and reprobing for total NF-κB as described above using rabbit anti-NFκB antibodies. Membranes were scanned using an Ultralum imaging system and bands were quantified using Ultraquant 6.0 software.

Confocal microscopy

For confocal microscopy, cells were grown on glass cover slips pretreated with poly D-lysine and placed in sterile twelve well culture plates. Plates were treated with poly I:C (10 μg/ml) or saline control for the indicated time periods. Following treatment, culture media was removed and cells were fixed at room temperature for 15 minutes in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, and washed 3 times

with PBS. Cells were then permeabilized for 15 minutes at room temperature in PBS containing 0.5% triton X-100, and washed 3 times with PBS. Samples were blocked for 1 hour at room temperature in PBS containing 0.1% triton X-100 and 5% goat serum. Following blocking, samples were incubated for two hours at room temperature in PBS (0.1% triton X-100) with anti-NF-κB p-65 antibody (1:100, Santa Cruz Biotechnology). Samples were then washed with PBS 3 times for 10 minutes each and incubated for 1 hour at room temperature with anti-rabbit IgG conjugated to Alexflour 568 (1:250, Invitrogen, Carlsbad, CA). Finally, samples were washed 3 times with PBS for 10 minutes and incubated for 5 minutes with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (300nM), washed three times with PBS and mounted onto glass slides using Prolong Gold^{TM} mounting medium (Invitrogen, Carlsbad, CA).

Slides were imaged using a Leica Sp5 confocal microscope at 40x optical magnification with 2.5x digital zoom and data were analyzed using the Leica Application Suite AF V2. Images for DAPI and NF-κB staining were acquired simultaneously at 4.8 micron intervals using a voxel height and width of 150 nm and a depth of 800 nm. In order to prevent spectral crosstalk between DAPI and NF-κB signal, emission bandwidths were filtered at 430nm-550nm and 577nm-670nm, respectively. Translocation of NF-κB was defined as the sum intensity of NF-κB colocalized with DAPI and normalized to the total DAPI signal.

Protein synthesis

Cells were plated in 96-well cell culture plates at 5000 cells/well. After 48 hours, media was replaced and 1.5 million counts of ^{3}[H]-leucine were added to each well. Plates were then treated with the indicated amount of drug or saline control for 16 hours.

Cell culture media was removed and cells were incubated at room temperature with gentle agitation for 10 minutes in PBS containing 0.1% trypsin and 0.02% Na-EDTA. Cells were then harvested onto glass fiber filters using a Tomtec Harvester 96 and radioactivity was measured with a Wallac 1205 Betaplate liquid scintillation counter.

Data analysis

Data for time- and concentration-dependent effects were analyzed using non-linear fit for sigmoidal curves. Data for IκB degradation and NF-κB translocation were analyzed using one-way ANOVA with post hoc testing using Dunnett's multiple comparison test. All statistical analyses were conducted using GraphPad Prism 4. Statistical significance was defined as $p<0.05$ for all tests.

Results

Poly I:C-induced gene expression in RAW 264.7 macrophage-like cells

RAW 264.7 cells were treated with 10 μg/ml poly I:C for 24 hours and mRNA expression for 384 genes of interest were analyzed using a qRT-PCR array. The genes expressing the highest levels of upregulation and down-regulation are listed in Table 1.1. In particular, poly I:C generated large increases in expression of the pro-inflammatory cytokines IL6, IL-1α, and IL-1β. Additionally, poly I:C exposure led to the suppression of a number of genes associated with anti-inflammatory processes, such as the chemokine receptor, Cx3cr1 (Yu et al., 2007).

Characterization of poly I:C induced IL-6 production

Due to the large increase in IL-6 mRNA, we sought to characterize the ability of poly I:C to initiate protein production of this inflammatory cytokine in cultured C8-D1A and

Table 1.1. Poly I:C-induced changes in gene expression. RAW 264.7 cells were treated for 24 hours with 10 μg/ml poly I:C. Changes in gene expression were quantified using a qRT-PCR array for 384 genes of interest. Gene targets were selected for broad-based analysis of signaling pathways related to inflammatory processes and neuronal function. The expression levels of approximately 100 genes were altered by poly I:C treatment. The table below contains the 10 genes with the highest fold change for induction and the 10 genes with the highest fold change for suppression.

Gene Name	p-value	Fold Change
Csf3	**0.000043**	**3029.26754**
Cxcl2	**0.000121**	**622.614328**
Il1b	**0.00013**	**531.964228**
Lif	**0.000396**	**162.035082**
Il1a	**0.000211**	**157.429657**
Ptgs2	**0.000427**	**144.527648**
Il6	**0.000229**	**108.516804**
Plaur	**0.001056**	**76.41604**
Il1rn	**0.000894**	**66.745631**
Ccl3	**0.000907**	**64.426061**
Icam2	**0.003945**	**-4.279384**
Ctla4	**0.03584**	**-4.718303**
IgD-C1v1	**0.012183**	**-4.752959**
Il18r1	**0.030062**	**-5.088803**
Il16	**0.005434**	**-5.25603**
Cd24a	**0.004274**	**-7.506401**
Lifr	**0.00063**	**-18.926005**
Fcer2a	**0.013823**	**-21.987037**
Itga4	**0.000763**	**-24.193167**
Cx3cr1	**0.000764**	**-26.840108**

Figure 1.1. Poly I:C-induces time- and concentration-dependent IL-6 production in C8-D1A and RAW 264.7 cells. C8-D1A astrocyte-like cells (closed squares) and RAW 264.7 macrophage-like cells (open squares) were plated at 5000 cells/well in 96-well culture plates and treated 48 hours later with poly I:C as indicated. Cell culture media was collected and analyzed for IL-6 using ELISA. (A) Cells were treated with 10 μg/ml poly I:C over the course of 24 hours and media was collected at the indicated time points and assayed for IL-6. (B) Cells were treated for 16 hours with the indicated concentration of poly I:C and media was collected and assayed for IL-6. Error bars represent the SEM from 3 independent experiments conducted in duplicate.

Figure 1.1

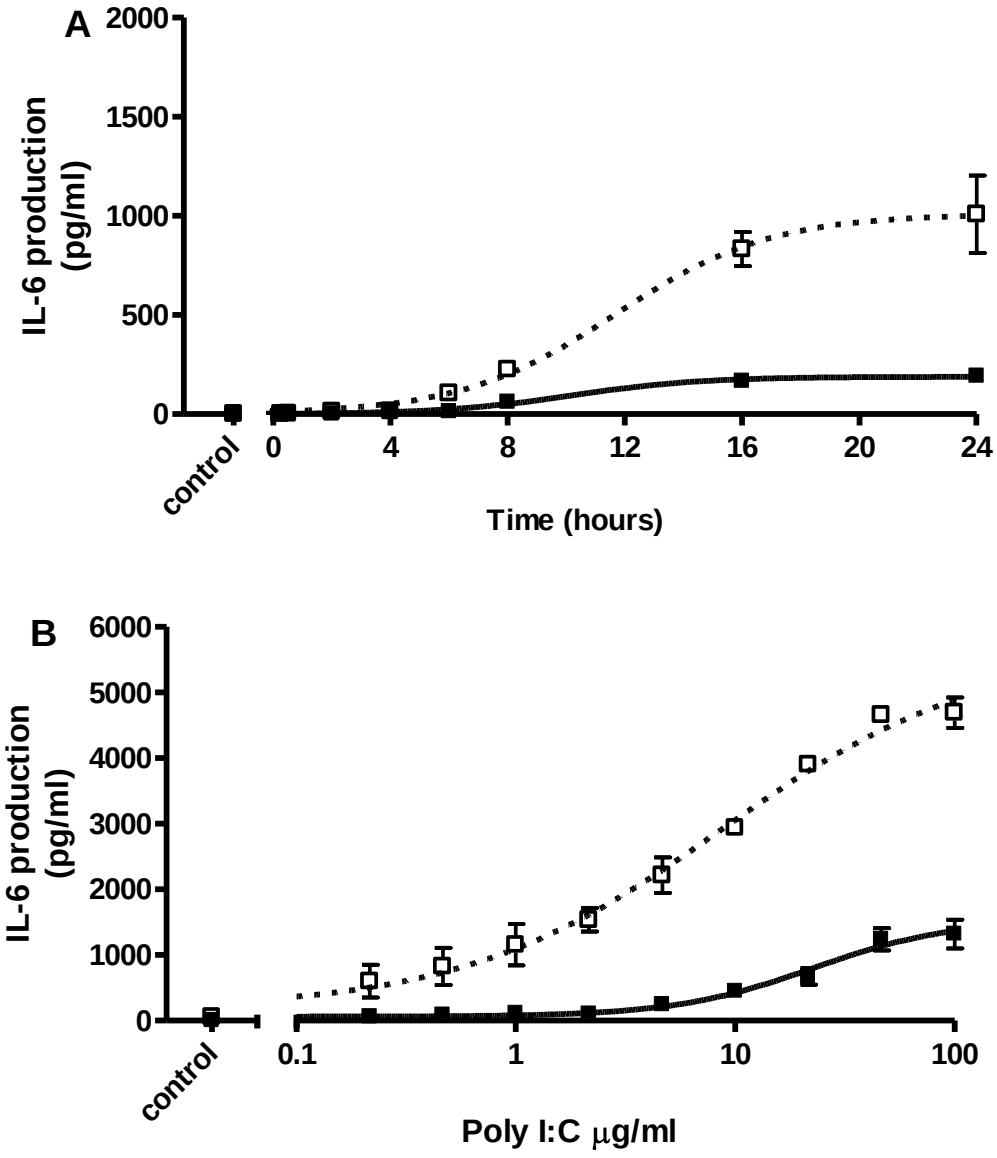
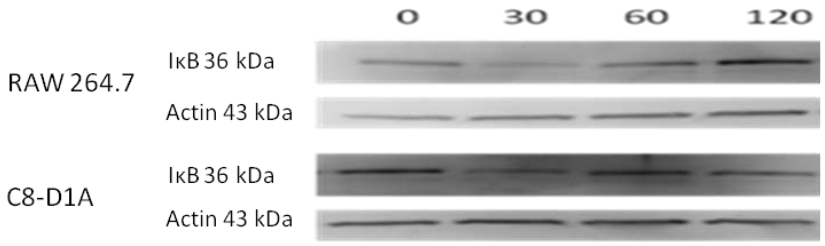

A
2000
1500
1000
500
0
IL-6 production (pg/ml)
control
0
4
8
12
16
20
24
Time (hours)
B
6000
5000
4000
3000
2000
1000
0
IL-6 production (pg/ml)
control
0.1
1
10
100
Poly I:C μg/ml

Figure 1.2. Poly I:C induces transient IκB degradation in C8-D1A and RAW 264.7 cells. C8-D1A (closed squares) and RAW 264.7 (open squares) cells were grown until 75% confluent in 100 mm tissue culture dishes and treated with 10 μg/ml poly I:C for the indicated times. Cell lysates were then collected and subjected to immunoblotting for IκB. (A) Representative immunoblots for IκB and actin in C8-D1A and RAW 264.7 cells following treatment with poly I:C. Data were normalized to actin. (B) Results for IκB degradation in C8-D1A and RAW 264.7 cells. Data were normalized to percent control. Error bars represent the SEM from independent experiments in C8-D1A (n=4) and RAW 264.7 cells (n=3). (*p<0.05)



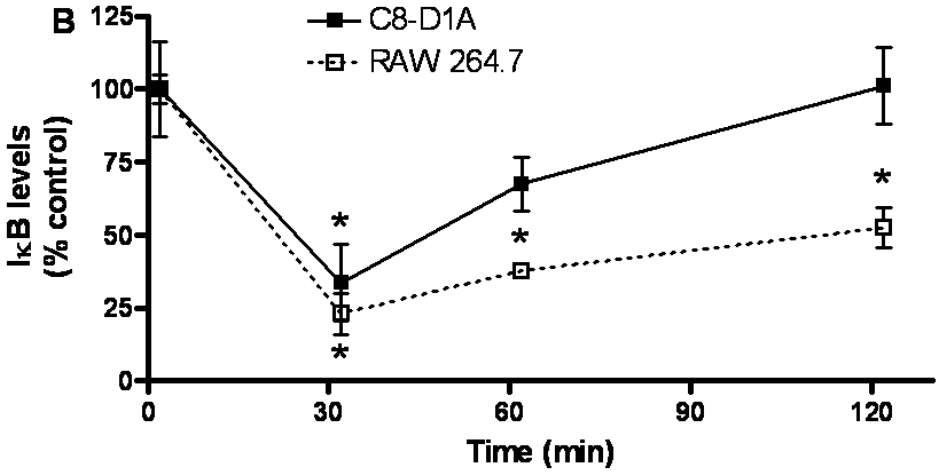


Figure 1.3. Poly-I:C induced phosphorylation of NF-κB. C8-D1A (closed squares) and RAW 264.7 (open squares) cells were grown until 75% confluent in 100 mm tissue culture dishes and treated with 10 μg/ml poly I:C for the indicated times. Cell lysates were then collected and subjected to immunoblotting for pNF-κB. (A) Representative immunoblots for pNFκB and NFκB in C8-D1A and RAW 264.7 cells following treatment with poly I:C. Data were normalized to NFκB. (B) Results for pNFκB in C8-D1A and RAW 264.7 cells. Data were normalized to percent control. Error bars represent the SEM from independent experiments in C8-D1A (n=3) and RAW 264.7 cells (n=3). Treatment with poly I:C led to a significant increase in phosphorylation in C8-D1A cells at 15, 30, 60 and 90 minutes. Poly I:C also significantly increased levels of pNFκB in RAW 264.7 cells at 30, 60, and 90 minutes post-treatment. ($p<0.05$)

Figure 1.3

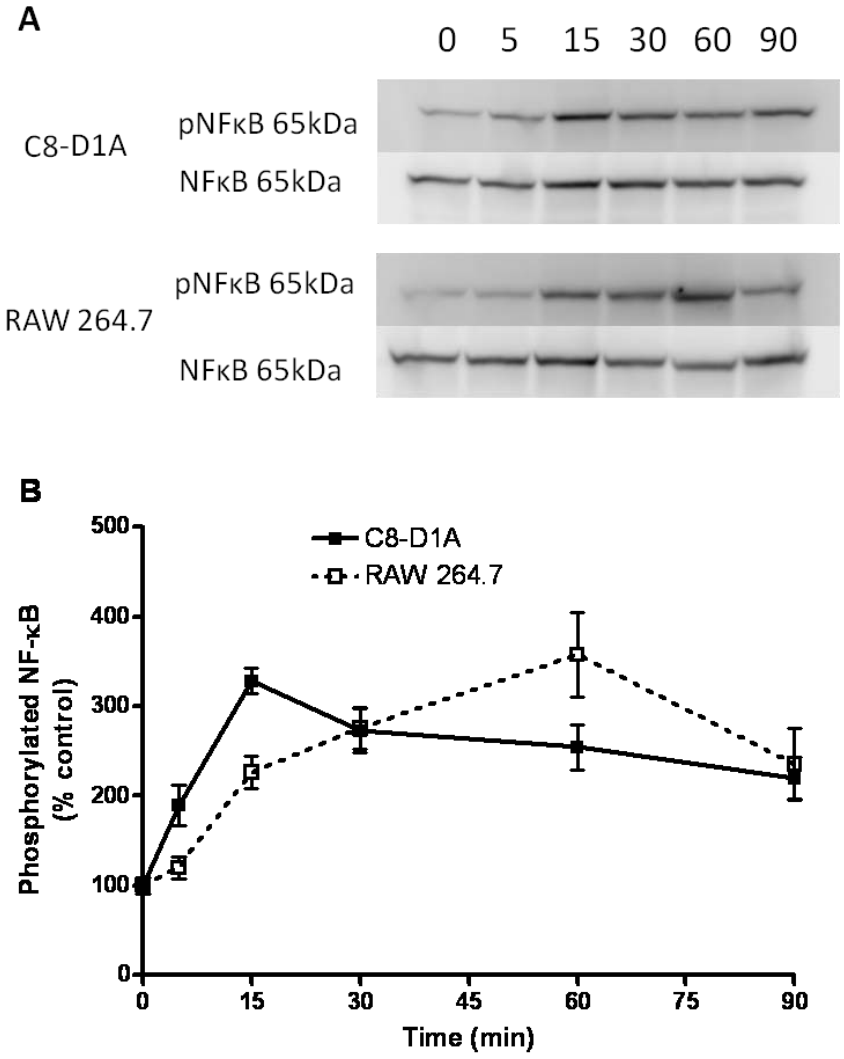
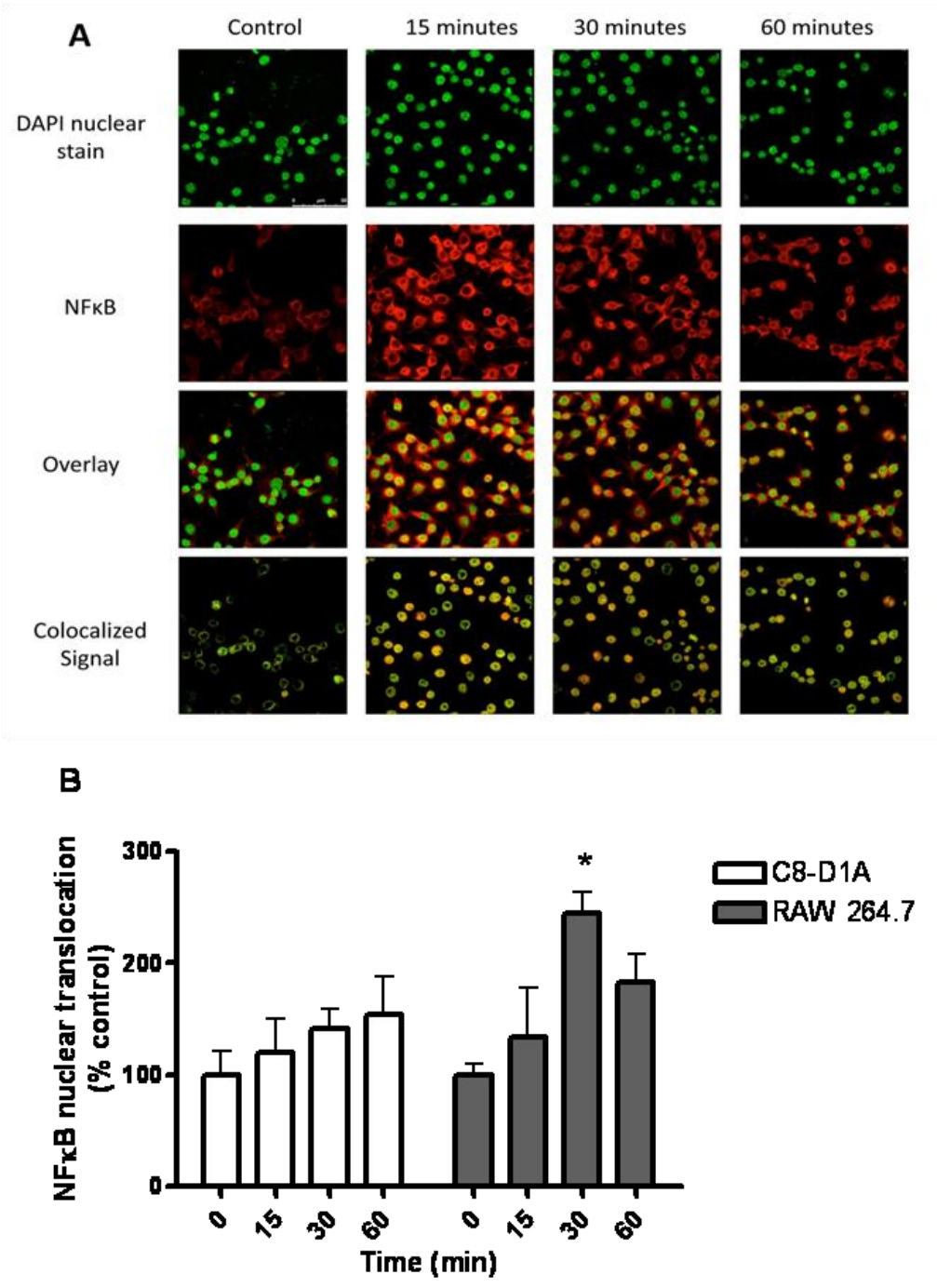

A
0 5 15 30 60 90
C8-D1A
pNFκB 65kDa
NFκB 65kDa
RAW 264.7
pNFκB 65kDa
NFκB 65kDa
B
C8-D1A
RAW 264.7
Phosphorylated NF-κB (% control)
500
400
300
200
100
0
0 15 30 45 60 75 90
Time (min)

Figure 1.4. Poly I:C induces NF-κB nuclear translocation in C8-D1A and RAW 264.7 cells. Cells were treated with poly I:C (10 μg/ml) and nuclear translocation of NF-κB was examined by immunohistochemistry. Green fluorescence indicates DAPI staining of the nucleus. Red fluorescence is immunostaining of NFκB. The yellow "overlay" is the staining of both, and the colocalized signal represents pixels that are positive for both NFκB and DAPI. Cells were imaged using a 40x lens with 2.5x digital zoom. (A) Representative figures for RAW 264.7 cells stained for NF-κB at the indicated time points. (B) Results for NF-κB translocation in C8-D1A and RAW 264.7 cells. Translocation of NF-κB was defined as the sum intensity of NF-κB colocalized with DAPI and normalized to the total DAPI signal. No significant differences were observed in total NF-κB signal at any time point. Error bars represent the SEM from three independent experiments. (*$p<0.05$)

Figure 1.4


A
Control
15 minutes
30 minutes
60 minutes
DAPI nuclear stain
NFκB
Overlay
Colocalized Signal
B
NFκB nuclear translocation (% control)
300
200
100
0
*
C8-D1A
RAW 264.7
0
15
30
60
0
15
30
60
Time (min)

RAW 264.7 cells. Cells were exposed to poly I:C over the course of 24 hours and the cell culture media was collected and assayed for IL-6. Poly I:C led to a time-dependent increase in IL-6 production in both cell lines. IL-6 production peaked near 16 hours (Fig. 1.1 A). Poly I:C-induced IL-6 production was concentration-dependent in both cell lines with EC_{50} values of 22.6 μg/ml (95% CI of 11.2 to 45.5) in C8-D1A and 8.7 μg/ml (95% CI of 4.4 to 17.2) in RAW 264.7 cells (Fig. 1.1 B).

Poly I:C-induced NF-κB signaling

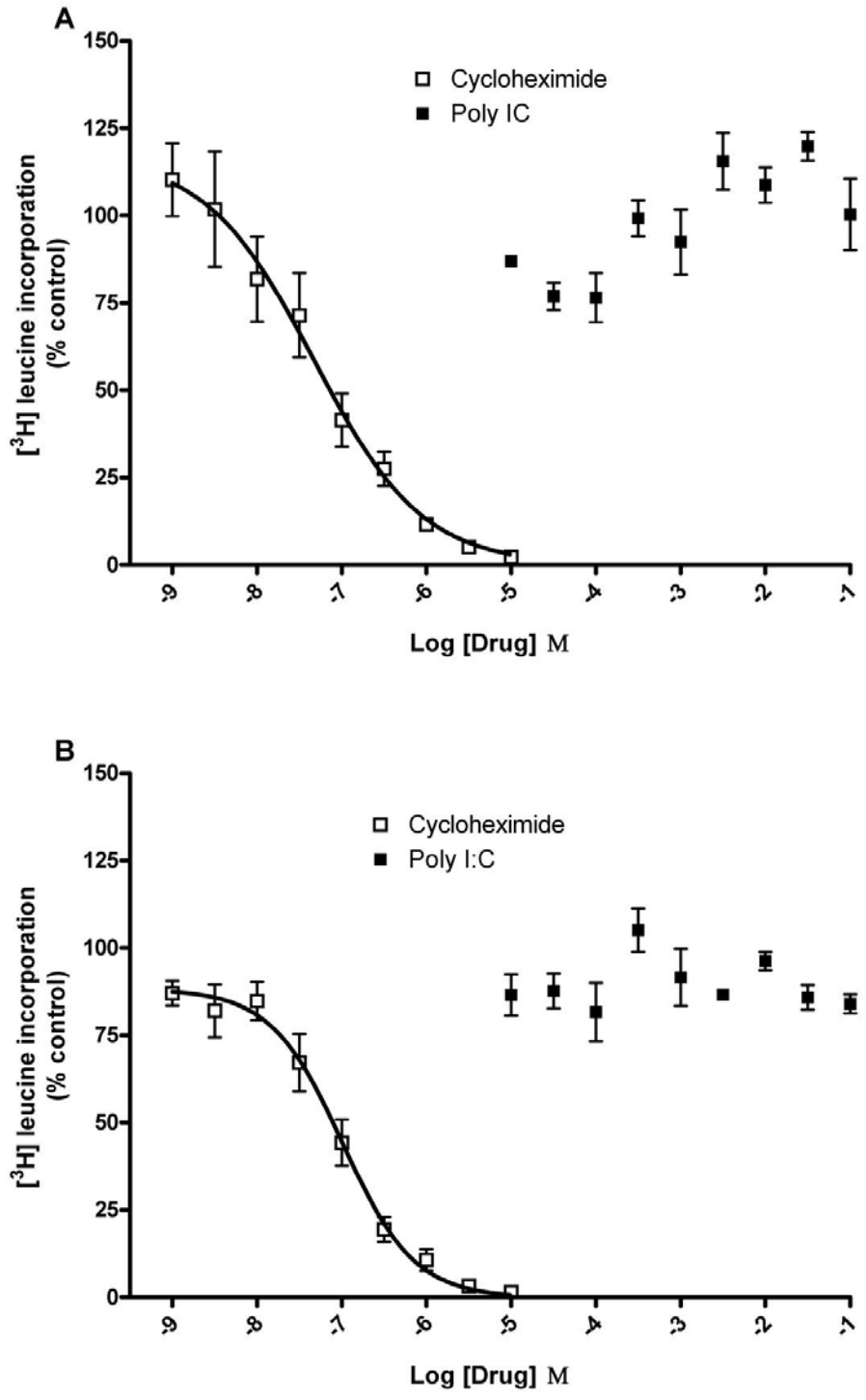
Degradation of IκB is a necessary precursor of NF-κB nuclear translocation. Poly I:C (10 μg/ml) led to a transient degradation of IκB in C8-D1A and RAW 264.7 cells (Fig. 1.2 A), reflecting rapid activation of NF-κB. Highest degradation of IκB in both cell lines occurred approximately 30 minutes after exposure to poly I:C. The levels of IκB in C8-D1A cells had completely recovered by 120 minutes while IκB in RAW 264.7 cells remained significantly depressed (Fig. 1.2 B). Poly I:C (10 μg/ml) also caused a rapid and sustained increase in phosphorylation of NF-κB in both C8-D1A and RAW 264.7 cells (Fig. 1.3AB)

Nuclear translocation of NF-κB

In order to confirm that nuclear translocation of NF-κB was consistent with degradation of IκB and phosphorylation of NF-κB, cells were treated with poly I:C and the amount of NF-κB within the nucleus was quantified. Cells were double-labeled using anti-NF-κB antibody and DAPI staining of the nuclear compartment. Cells were imaged for colocalized NF-κB and DAPI nuclear stain and normalized to untreated controls (Fig. 1.4 A). Poly I:C treatment resulted in a time-dependent increase in nuclear translocation of NF-κB in RAW 264.7 cells, but translocation in C8-D1A failed to reach statistical

Figure 1.5. Inhibition of protein synthesis. C8-D1A (A) and RAW 264.7 (B) were plated at 5000 cells/well in 96-well culture plates. After 48 hours 1.5 million counts of [^{3}H] leucine were added to each well and cells were incubated for 16 hours with the indicated concentrations of poly I:C (μg/ml) or cycloheximide. Data were normalized to vehicle treated controls. Error bars represent the SEM from 3 independent experiments conducted in duplicate.

Figure 1.5


A
Cycloheximide
Poly IC
[3H] leucine incorporation (% control)
150
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
-1
Log [Drug] M
B
Cycloheximide
Poly I:C
[3H] leucine incorporation (% control)
150
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
-1
Log [Drug] M

significance (Fig. 1.4 B). Total NF-κB expression levels and DAPI signal were not significantly altered by poly I:C treatment (data not shown).

Inhibition of protein synthesis

dsRNA is widely reported to inhibit protein synthesis as a function of the cellular response to viral infection (Clemens, 1997). However, there have been no reports of poly I:C-induced inhibition of protein synthesis assessed utilizing actual protein synthesis. Therefore, we examined the ability of poly I:C to inhibit protein synthesis using a leucine incorporation assay to quantify the amount of radiolabeled leucine incorporated into cellular proteins over 16 hours. Cycloheximide was used as a positive control (Schneider-Poetsch et al., 2010). Surprisingly, poly I:C had no effect on protein synthesis, even at concentrations as high as 100 μg/ml. Cycloheximide potently inhibited leucine incorporation in C8-D1A (IC_{50} = 46.8 nM, 95% CI of 16.7 to 130.9) and RAW 264.7 (IC_{50} = 102.3 nM, 95% CI of 71.4 to 146.5) cells (Fig 1.5AB).

Discussion

Exposure to poly I:C induced significant upregulation and downregulation of genes related to immune response, including large increases in IL-6 (Table 1.1). Consequently, we examined the time- and concentration-dependent secretion of IL-6 in immortalized murine C8-D1A and RAW 264.7 cells in response to poly I:C (Fig. 1.1). Both C8-D1A and RAW 264.7 cells generated large amounts of IL-6 in a time- and concentration-dependent manner. Additionally, poly I:C led to NF-κB activation as indicated by the transient degradation of the inhibitory protein IκB (Fig. 1.2), phosphorylation of NF-κB (Fig. 1.3AB), and the subsequent nuclear translocation of NF-

κB (Fig. 1.4AB). Contrary to our hypothesis, poly I:C failed to have any effect on protein synthesis despite numerous reports of viral dsRNA inhibiting the activation of eIF2-α, a protein required for initiating translation (Zhang et al., 2007; Nakatsu, Takeda, Ohno, Koga, & Yanagi, 2006). Additionally, we were unable to detect any increase in phosphorylation of PKR or eIF2-α following poly I:C exposure (data not shown), consistent with a failure to activate pathways capable of inhibiting protein synthesis. This lack of effect on PKR/eIF2-α activity and protein synthesis is in agreement with another recent report by Carpentier et al. (Carpentier et al., 2007) indicating that extracellular exposure to poly I:C primarily signals through TLR 3, while cytoplasmic production of dsRNA by viral infection activates the PKR signaling pathway. These data suggest that although poly I:C administration may adequately model the inflammatory response to viral infection, it may be inadequate for modeling the inhibition of translation observed during viral infection. Given that inhibition of protein synthesis could significantly impact development, these findings may be of particular relevance for animal models employing poly I:C to examine the effects of maternal immune activation on neural development and offspring behavior (Meyer & Feldon, 2010; Watanabe, Someya, & Nawa, 2010).

Chapter 2: The effects of luteolin and the PKR inhibitor, C16, on poly I:C-induced IL-6 production and protein synthesis

Introduction

In mouse models of septic shock, the naturally occurring flavonoid luteolin reduces lethal toxicity and pro-inflammatory cytokine production (Kotanidou et al., 2002). Activation of NF-κB comprises a key step in the canonical pathway for initiating the production of pro-inflammatory cytokines, including IL-6. Although the cellular mechanisms underlying the anti-inflammatory properties of luteolin are frequently attributed to a blockade of NF-κB activation, others have reported anti-inflammatory effects in the absence of an inhibitory effect on the NF-κB signaling pathway (Chen et al., 2007; Rezai-Zadeh et al., 2008; Kim et al., 2005). *In vivo*, luteolin decreases IL-6 production in response to the inflammatory bacterial component lipopolysaccharide (LPS), however few data exist regarding the ability of luteolin to reduce NF-κB activation and inflammatory cytokine production in response to viral infection or to the dsRNA, poly I:C (Jang et al., 2008; Lee et al., 2009).

The role of PKR in mediating the cellular responses to poly I:C in cell culture remains unclear. Several reports indicate that activation of NF-κB or pro-inflammatory cytokine production in response to poly I:C is abolished by expression of dominant negative mutant forms of PKR (Srivastava et al., 1998; Auch et al., 2004). However, data from primary cell cultures obtained from PKR knockout mice found no contribution of PKR to poly I:C mediated inflammatory responses, although PKR was capable of mediating the response to actual viral infection (Carpentier et al., 2007). The reason for

these conflicting reports may result from cell-type specific responses or substantial differences in methodology. The current study examines the effects of a novel PKR inhibitor on the response to poly I:C in immortalized cell lines. The imidazolo-oxindole derivative C16 was identified by *in vitro* screening for compounds capable of inhibiting phosphorylation of PKR in response to poly I:C (Jammi et al., 2003). I*n vivo,* C16 reduced phosphorylation of PKR and eIF2-α by approximately 50% in rat brain (Ingrand et al., 2007). Surprisingly, there are no reports concerning the functional effects of poly I:C or C16 on protein synthesis.

We hypothesized that luteolin would reduce poly I:C-induced IL-6 production by decreasing NF-κB activation through inhibition of IκB degradation. Furthermore, we hypothesized that C16 would be effective in reducing NFκB activation and IκB degradation due to upstream inhibition of PKR phosphorylation. We found that although luteolin and C16 decreased the production of IL-6 in response to poly I:C, neither C16 nor luteolin reduced the degradation of IκB, suggesting that these compounds do not interfere with poly I:C effects via the NFκB pathway. Furthermore, in the course of conducting experiments regarding the effects of poly I:C in protein synthesis (see Fig. 1.5) we observed an inhibitory effect of luteolin and C16 on the incorporation of [^{3}H] leucine. This observation prompted us to fully examine the effects of luteolin and C16 on protein synthesis. The results indicated that both compounds inhibit protein synthesis. In order to determine whether these drugs were toxic at concentrations inhibiting IL-6 production we conducted trypan blue staining for cell viability. Luteolin and C16 led to a reduction in the number of cells, but no toxic effects on membrane permeability were observed. In order to examine the effects of luteolin and C16 on IL-6 production

independently of protein synthesis, we quantified production of IL-6 mRNA in response to poly I:C following pretreatment with luteolin or C16. These results showed inhibition of IL-6 mRNA production by both luteolin and C16 in RAW 264.7 cells. These data suggest that although luteolin and C16 reduce inflammatory cytokine production, they are also capable of inhibiting protein production and possibly cellular proliferation in immortalized astrocyte-like and macrophage-like cells at concentrations typically employed *in vitro.*

Materials and Methods

Materials and cell culture

Murine cell lines were obtained from ATCC (Manassas, VA). Raw 264.7 macrophage (#TIB-71) and type 1 C8-D1A astrocyte (#CRL-2541) cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, and 5 mg/L amphotericin B. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. ^{3}[H]-leucine was purchased from American Radiolabeled Chemicals, St. Louis, MO. PKR inhibitor C16 was obtained from Calbiochem, San Diego, CA (Cat# 527-450). Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich, St. Louis, MO.

IL-6 production

Cells were plated in 96-well cell culture plates at 5000 cells/well. After 48 hours, plates were treated with the indicated amount of luteolin, C16, or cycloheximide for 15 minutes prior to the addition of 10 μg/ml poly I:C and media was collected 16 hours later. Samples were analyzed in duplicate using ELISA kits for murine IL-6 (R&D Systems,

Minneapolis, MN). Data were normalized to vehicle treated controls (0.1% DMSO for luteolin and C16, saline for cycloheximide).

Immunoblotting

Cells were grown until 75% confluent in 12-well cell culture plates. Luteolin, C16, or vehicle (0.1% DMSO) were added at the indicated concentrations one hour prior to the addition of poly I:C (10μg/ml). Cells were harvested 30 minutes after the addition of poly I:C and IκB levels were determined according to the immunoblotting procedures described for the methods in Chapter 1. Levels of IκB degradation were normalized to saline treated controls.

Protein synthesis

Cells were plated in 96-well cell culture plates at 5000 cells/well. After 48 hours, media was replaced and 1.5 million counts of ^{3}[H]-leucine were added to each well. Cells were treated with the indicated amount of drug or vehicle control (0.1% DMSO) for 16 hours. Cell culture media was removed and cells were incubated at room temperature with gentle agitation for 10 minutes in PBS containing 0.1% trypsin and 0.02% Na-EDTA. Cells were then harvested onto glass fiber filters using a Tomtec Harvester 96 and radioactivity was measured with a Wallac 1205 Betaplate liquid scintillation counter.

Trypan blue staining for cell viability

Cells were plated in 12-well cell culture plates at 150,000 cells per well. After 24 hours, cells were treated for 16 hours with the indicated amount of drug or vehicle (0.1% DMSO). Culture media was removed and cells were detached by incubation with phosphate-buffered saline containing 0.1% trypsin and 0.02% Na-EDTA for 20 minutes at room temperature. Cell suspensions were then centrifuged at 500*g* for 5 min,

resuspended in 500 µl of PBS containing 0.4% trypan blue, and incubated at room temperature for 5 minutes. Cell counts were determined using a light microscope and a standard hemocytometer for cell counting (Strober, 2001).

Real-time quantitative RT-PCR

Cells were plated in 6-well cell culture plates at 400,000 and 100,000 per well for C8-D1A and RAW 264.7 cells, respectively. After 48 hours, cells were pretreated for 1 hour with luteolin, luteolin vehicle (0.1% DMSO), C16, or C16 vehicle (0.01% DMSO) prior to the addition of poly I:C (10 µg/ml). RNA was extracted using an RNeasy Minikit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA purity and quantity was determined by spectrophotometric analysis. IL-6 mRNA levels were determined by one-step RT-PCR using the QuantiTect SYBR Green I RT-PCR Kit (Qiagen) in accordance with the manufacturer's instructions. Reactions contained 20 ng of RNA in a final volume of 25 µl and mRNA levels were quantified using murine IL-6 or actin primers from Qiagen (Cat#QT00098875 and QT01136772). Samples were run in triplicate using an iCycler iQ^{TM} real-time PCR thermocycler (Bio-Rad Laboratories, Hercules, CA) with the iQ Real-Time analysis module. RT-PCR data were normalized to total RNA as determined using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Carlsbad, CA) according to the methods of Hashimoto et al. (Hashimoto, Beadles-Bohling, & Wiren, 2004). All data are expressed as fold-change relative to the indicated controls.

Data analysis

Time- and concentration-dependent effects were analyzed using non-linear fit for sigmoidal curves. In instances of single mean comparisons, *t* tests were used to test for

significant differences. For multiple mean comparisons, data were analyzed using one-way or two-way ANOVA, as indicated, with post hoc testing using Bonferroni correction. All statistical analyses were conducted using GraphPad Prism 4. Statistical significance was defined as $p<0.05$ for all tests.

Results

Inhibition of IL-6 protein expression

First, we determined the ability of luteolin and C16 to inhibit IL-6 production in response to poly I:C. Luteolin has been reported to inhibit NF-κB activation (Chen et al., 2007), while C16 reportedly inhibits PKR activity (Eley, McDonald, Russell, & Tisdale, 2009). Furthermore, activation of NF-κB by poly I:C is attenuated by mutations of PKR (Auch et al., 2004). Therefore, we hypothesized that both compounds would reduce IL-6 production. Both luteolin and C16 inhibited IL-6 production in a concentration-dependent manner. In C8-D1A cells (Fig. 2.1 A), the IC_{50} value for luteolin was 25.7 μM (95% CI of 19.5 to 33.9) and the IC_{50} value for C16 was 2.5 μM (95% CI of 1.1 to 5.9). In RAW 264.7 cells (Fig. 2.1 B), the IC_{50} value for luteolin was 24.1 μM (95% CI of 19.2 to 30.3) and the IC_{50} value for C16 was 1.2 μM (95% CI of 1.1 to 1.3).

Inhibition of NF-κB signaling

We next sought to determine if the reduction in poly I:C-induced IL-6 production by luteolin and C16 was associated with a reduction in NF-κB signaling. Cells were pretreated with the indicated concentrations of luteolin, C16, or vehicle for 1 hour and then treated with poly I:C (10μg/ml) for 30 minutes (Fig. 2.2). This time point was selected because it represented the maximal effect of poly I:C on IκB degradation (Fig.

Figure 2.1. Luteolin and C16 inhibit poly I:C induced IL-6 production. C8-D1A (A) and RAW 264.7 cells (B) were plated at 5000 cells/well in 96-well culture plates and treated 48 hours later with poly I:C (10 μg/ml) for 16 hours with the indicated concentrations of luteolin (closed circles) or C16 (open circles). Data were normalized to cells treated with poly I:C and vehicle controls. Error bars represent the SEM from 3 independent experiments each conducted in duplicate.

Figure 2.1

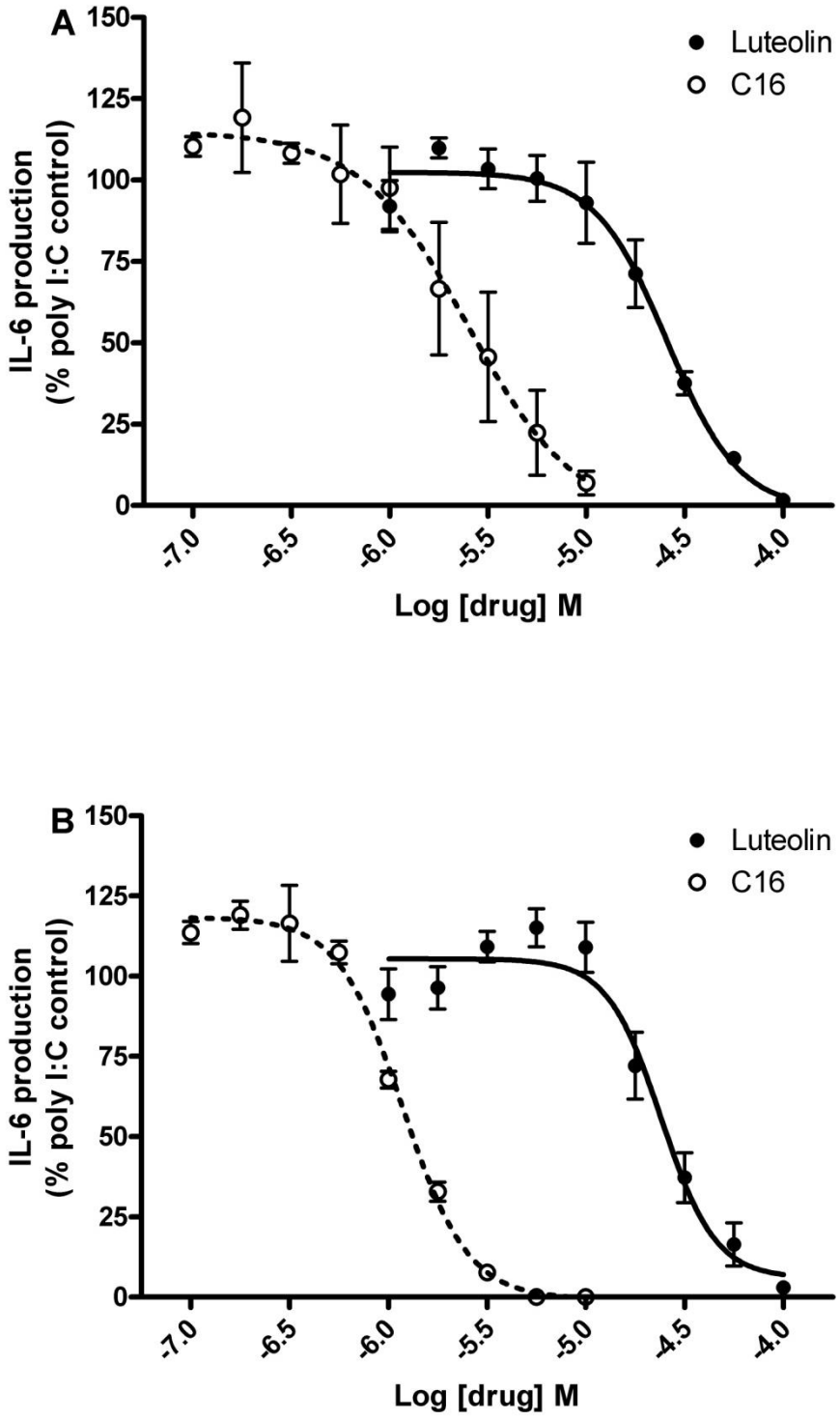
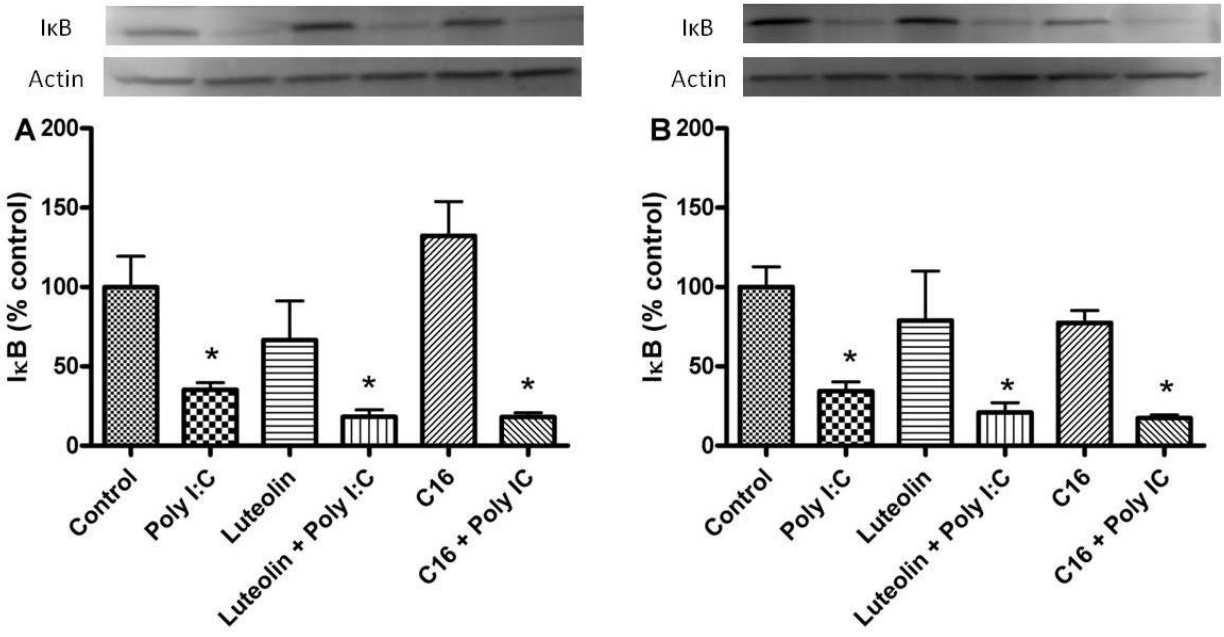

A
B
Luteolin
C16
IL-6 production
(% poly I:C control)
150
125
100
75
50
25
0
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
Log [drug] M

Figure 2.2. Luteolin and C16 do not inhibit poly I:C-induced IκB degradation. C8-D1A (A) and RAW 264.7 cells (B) were pretreated for one hour with luteolin (25 μM) or C16 (10 μM) and stimulated with poly I:C (10 μg/ml) for 30 minutes. Cell lysates were then collected and subjected to immunoblotting for IκB. Data were normalized to untreated control cells. Error bars represent the SEM from 4 independent experiments. (* $p<0.05$ compared to control)



1.2). Pretreatment with luteolin and C16 had no effect on poly I:C-induced IκB degradation in C8-D1A (Fig. 2.2 A) or RAW 264.7 cells (Fig. 2.2 B).

Inhibition of protein synthesis

During the course of examining whether poly I:C could reduce protein synthesis we observed substantial inhibition of [^{3}H] lecine incorporation in response to luteolin and C16 (data not shown). As a result, we examined whether luteolin or C16 could inhibit [^{3}H] leucine incorporation in concentration-dependent manner. Luteolin and C16 were both capable of completely inhibiting protein synthesis. Nonlinear analyses revealed inhibition of leucine incorporation in C8-D1A cells (Fig. 2.3 A) with IC_{50} values for C16 1.2 μM (95% CI of 0.8 to 1.9) and luteolin 46.1 μM (95% CI of 34.2 to 62.1). In RAW 264.7 cells (Fig. 2.3 B), inhibition of leucine incorporation was also observed with the following IC_{50} values for C16 1.7 μM (95% CI of 1.5 to 1.8) and luteolin 6.8 μM (95% CI of 4.1 to 11.4). These results suggest that both luteolin and C16 potently reduce protein synthesis at concentrations typically employed for *in vitro* studies (Chen et al., 2007; Chen et al., 2008; Kim et al., 2005; Ito, Onuki, Bando, Tohyama, & Sugiyama, 2007).

Effects of cycloheximide on poly I:C-induced IL-6 production

The finding that luteolin and C16 were able to inhibit leucine incorporation prompted us to examine the effects of the well characterized protein synthesis inhibitor, cycloheximide, on poly I:C induced IL-6 production. Pretreatment of cells with cycloheximide prior to poly I:C generated a biphasic response for IL-6 production that was concentration dependent in both cell lines (Fig. 2.4). IL-6 levels at each concentration of cycloheximide were analyzed using one-way analysis of variance with

Figure 2.3. Inhibition of protein synthesis by luteolin and C16. C8-D1A (A) and RAW 264.7 cells (B) were plated at 5000 cells/well in 96-well culture plates. After 48 hours 1.5 million counts of [^{3}H] leucine were added to each well and cells were incubated for 16 hours with the indicated concentrations of luteolin or C16. Data were normalized to vehicle treated controls. Error bars represent the SEM from 3 independent experiments conducted in duplicate.

Figure 2.3

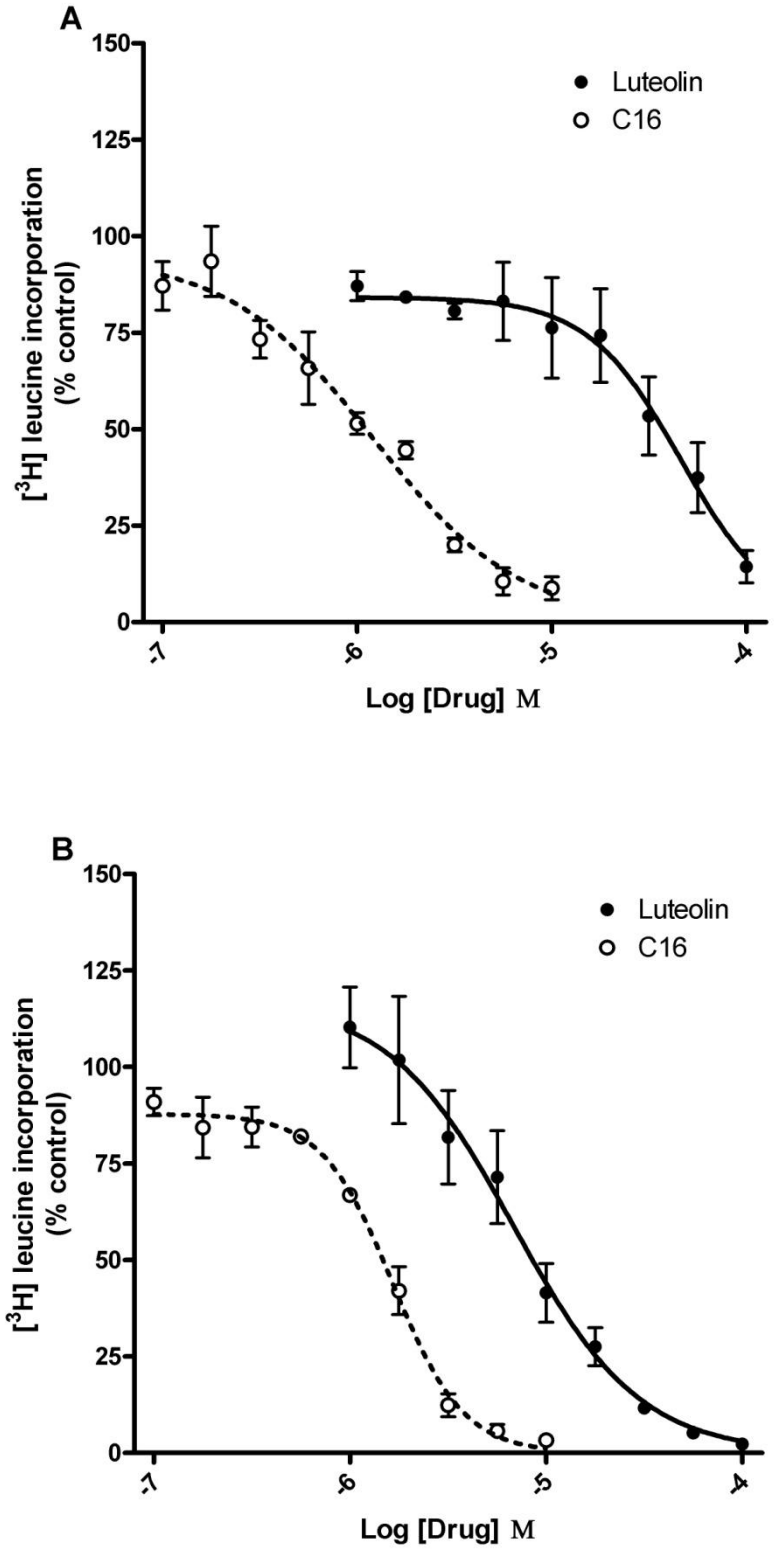
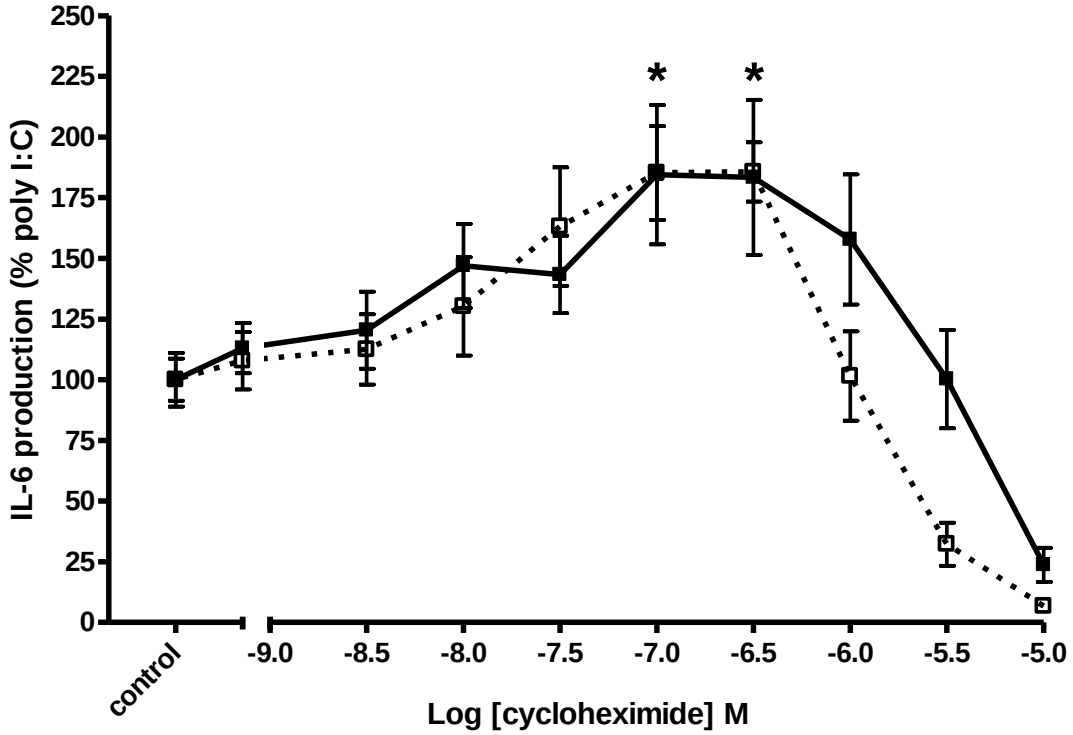

A
150
125
100
75
50
25
0
[3H] leucine incorporation (% control)
Luteolin
C16
-7
-6
-5
-4
Log [Drug] M
B
150
125
100
75
50
25
0
[3H] leucine incorporation (% control)
Luteolin
C16
-7
-6
-5
-4
Log [Drug] M

Figure 2.4. Cycloheximide induces superinduction of poly I:C mediated IL-6 production. C8-D1A (closed squares) and RAW 264.7 cells (open squares) were plated at 5000 cells/well in 96-well culture plates and treated 48 hours later with poly I:C (10 μg/ml) for 16 hours with the indicated concentrations of cycloheximide. Data were normalized to cells treated with poly I:C and vehicle controls. Error bars represent the SEM from 3 independent experiments each conducted in duplicate (*$p < 0.05$).



post-hoc testing using Bonferroni correction. Concentrations of 100 nM cycloheximide significantly increased IL-6 production following stimulation with poly (I:C) in both C8-D1A and RAW 264.7 cells, despite the fact that this concentration suppressed general protein synthesis by approximately 90% (Figure 1.5AB). Furthermore, this was followed by a sharp decrease in IL-6 production at higher concentrations, with nearly 100% suppression of poly I:C mediated IL-6 production in the presence of 10 μM cycloheximide. Cycloheximide alone did not induce IL-6 production at any concentration (data not shown).

Trypan blue staining for cell viability

In order to determine whether luteolin and C16 may be toxic to these cell lines, we conducted trypan blue staining to examine cell proliferation and viability. Trypan blue stain is impermeable to the membranes of living cells but crosses the membranes of dead cells. After staining, the number of living and dead cells was determined by counting cells that were impermeable or permeable to the dye, respectively. Data from C8-D1A and RAW 264.7 cells were analyzed using two-way ANOVA (treatment x viability). Significant decreases in cell numbers were observed following treatment with either luteolin or C16, as compared to vehicle, in both C8-D1A (Fig. 2.5 A) and RAW 264.7 cells (Fig. 2.5 B). No differences were observed in the number of dead cells under any treatment condition. Consistent with the results from the leucine incorporation assay, poly I:C had no effect on cell number compared to saline treated controls (data not shown). Importantly, despite the decrease in cell number, neither luteolin nor C16 caused a significant increase in cell death (Fig. 2.5AB).

Figure 2.5. Luteolin and C16 do not decrease cell viability. C8-D1A (A) and RAW 264.7 cells (B) were plated at 150,000 cells per well in 12-well culture plates. After 24 hours, cells were incubated with the indicated compounds or vehicle for 16 hours and cells were counted. Cell viability (alive or dead) was determined using trypan blue staining. Data were analyzed using two-way ANOVA (treatment x viability). No significant differences were observed in vehicle treated or untreated control groups at 16 hours and these groups were collapsed into a single control group for each cell type. Error bars represent the SEM from 4 independent experiments conducted in duplicate. Post-hoc testing was conducted for differences from control ($*p < 0.05$).

Figure 2.5

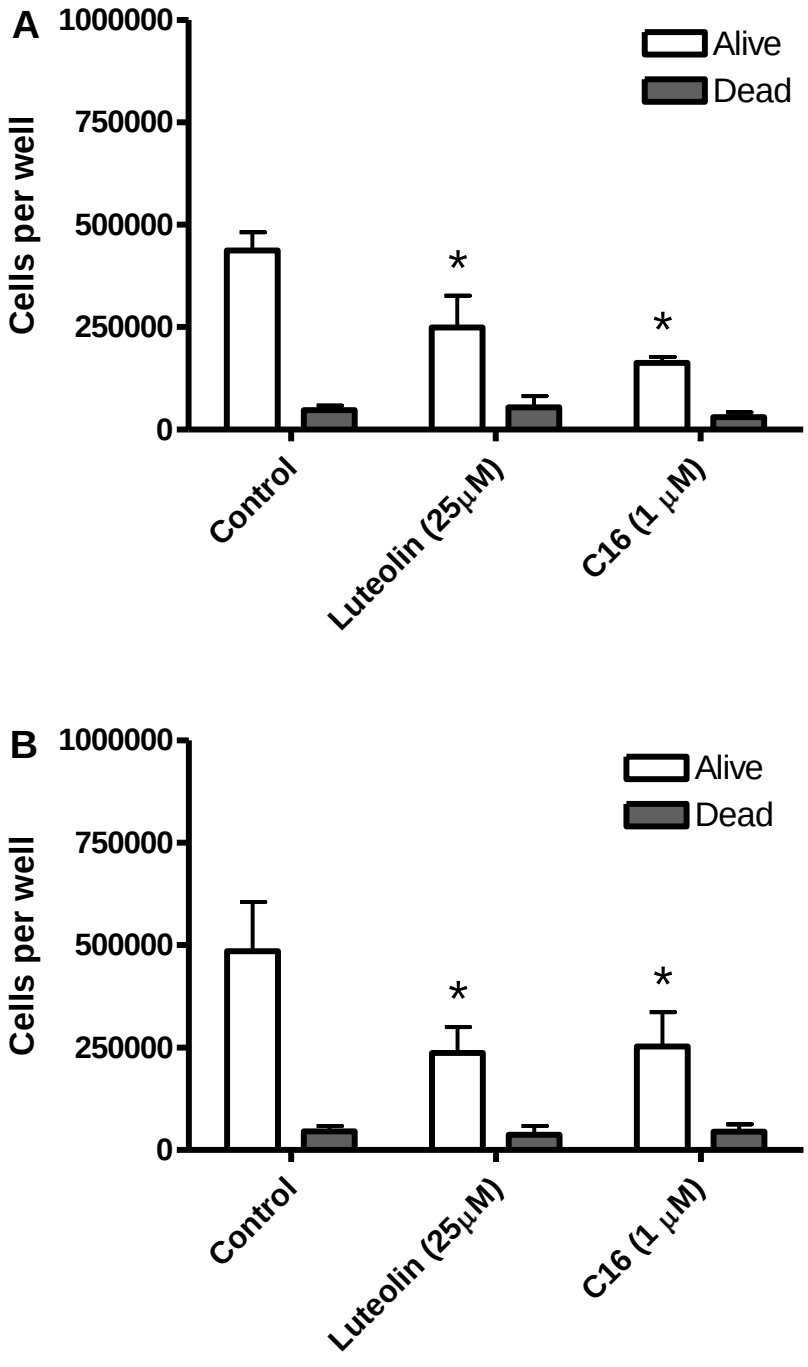

A
1000000
750000
500000
250000
0
Cells per well
Alive
Dead
*
*
Control
Luteolin (25μM)
C16 (1 μM)
B
1000000
750000
500000
250000
0
Cells per well
Alive
Dead
*
*
Control
Luteolin (25μM)
C16 (1 μM)

Figure 2.6. Luteolin and C16 inhibit poly I:C-induced production of IL-6 mRNA. RAW 264.7 cells were plated at 100,000 cells per well in 6-well plates and grown for 48 hours. (A) Cells were then treated with poly I:C for the indicated amount of time and IL-6 mRNA was measured using real-time quantitative RT-PCR. (B) The effects of luteolin and C16 on IL-6 mRNA production were determined by pretreating cells for one hour with luteolin, C16, or vehicle control followed by poly I:C (10 μg/ml) treatment for 4 hours. Post-hoc testing was conducted for differences from the zero time point (A) and the untreated control (B). Error bars represent the SEM from 3 independent experiments. (*$p<0.05$)

Figure 2.6

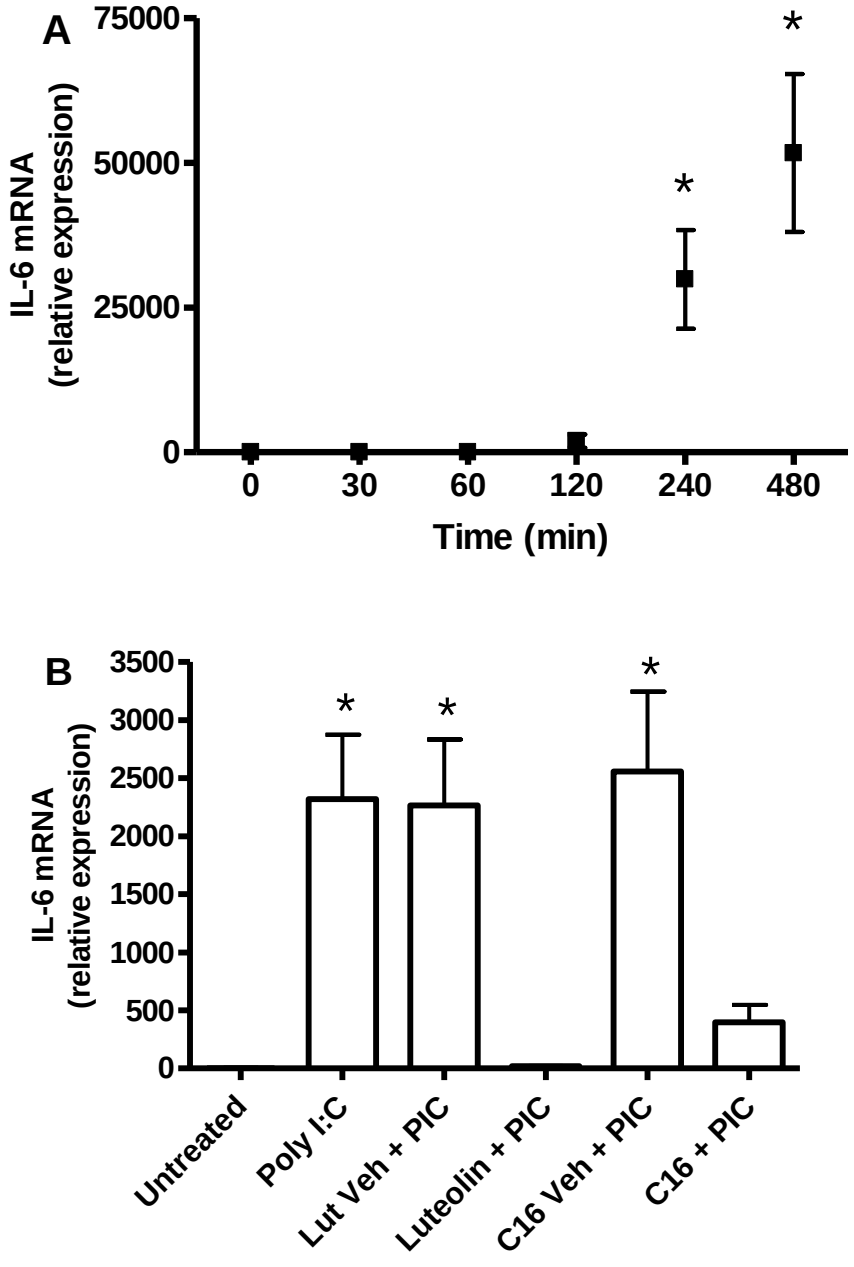
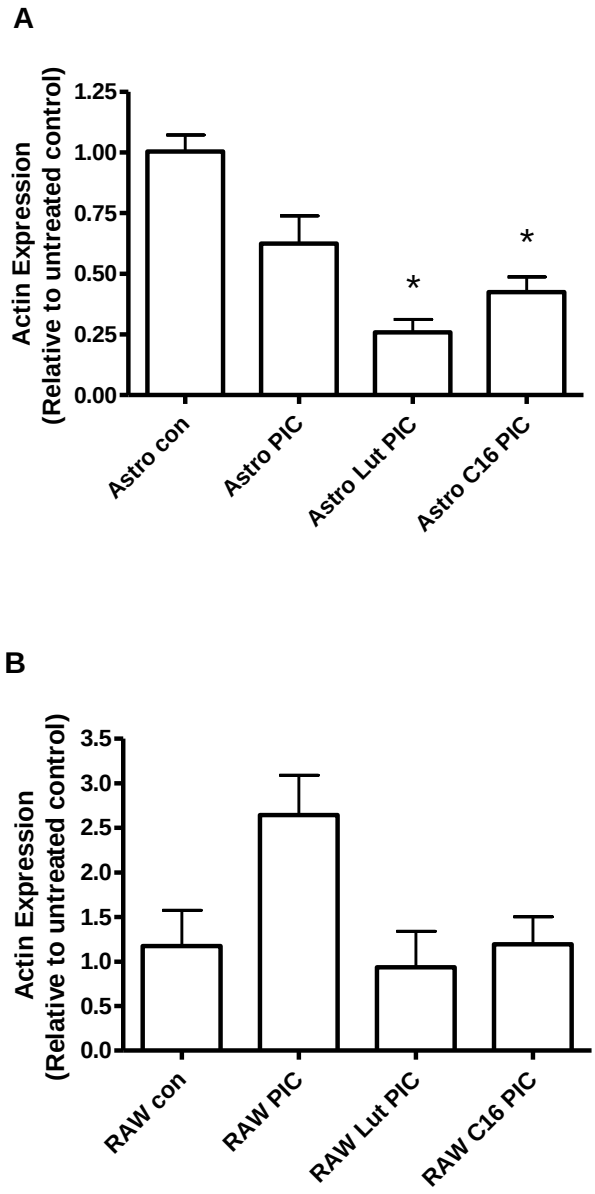

A
IL-6 mRNA
(relative expression)
75000
50000
25000
0
0
30
60
120
240
480
Time (min)
*
*
B
IL-6 mRNA
(relative expression)
3500
3000
2500
2000
1500
1000
500
0
*
*
*
Untreated
Poly I:C
Lut Veh + PIC
Luteolin + PIC
C16 Veh + PIC
C16 + PIC

Figure 2.7. Pretreatement with luteolin and C16 decrease actin mRNA following poly I:C treatment in C8-D1A astrocyte-like cells. To determine whether pretreatment with luteolin or poly I:C caused a general inhibition of transcription, actin mRNA levels were quantified. Treatment with vehicle controls did not significantly alter expression of actin in response to poly I:C, and these groups were combined for further analysis. Pretreatment of C8-D1A cells with luteolin or C16 followed by poly I:C (Fig 3.7A) caused a significant decrease in actin expression compared to untreated controls (n=3). However, pretreatment with luteolin or C16 followed by poly I:C did not cause a significant reduction in actin mRNA in RAW 264.7 cells when compared to either untreated controls or poly I:C alone (n=2) (Fig 3.7B). (*$p<0.05$)

Figure 2.7


A
Actin Expression
(Relative to untreated control)
1.25
1.00
0.75
0.50
0.25
0.00
*
*
Astro con
Astro PIC
Astro Lut PIC
Astro C16 PIC
B
Actin Expression
(Relative to untreated control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RAW con
RAW PIC
RAW Lut PIC
RAW C16 PIC

Inhibition of IL-6 mRNA production

In order to determine the effects of luteolin and C16 independently of cell proliferation and protein production, we conducted real-time quantitative RT-PCR for IL-6 mRNA. Treatment of RAW 264.7 cells with poly I:C led to a robust increase in IL-6 mRNA over the course of 6 hours (Fig. 2.6 A). Pretreatment for one hour with luteolin or C16 significantly inhibited IL-6 production following four hours of poly I:C exposure (Fig. 2.6 B). C8-D1A did not produce statistically significant increases in IL-6 mRNA at 4 hours following poly I:C exposure (data not shown). In order to rule out non-specific effects on transcription, levels of actin mRNA were quantified. The results indicated that treatment with luteolin and poly I:C or C16 and poly I:C caused significant reductions in actin mRNA in C8-D1A cells compared to controls (Fig 2.7A). This effect did not reach significance in RAW 264.7 cells pretreated with luteolin or C16 (Fig 2.7B).

Discussion

Activation of PKR occurs over a wide range of inflammatory conditions, and inhibition of PKR by pharmacological or genetic manipulations has been reported to prevent apoptosis and reduce NF-κB activation (Wang, Fan, Wang, Luo, & Ke, 2007; Russell, Eley, & Tisdale, 2007). Interestingly, the ability of poly I:C to activate PKR is the subject of conflicting reports. While Auch et al. (Auch et al., 2004) reported that PKR was required for poly I:C-induced NF-κB activation in astroglia, Carpentier et al. (Carpentier et al., 2007) found that PKR was not required for poly I:C-induced inflammatory responses in C8-D1A cells, including the induction of type 1 interferons and IL-6. Likewise, while several studies have reported the inhibition of PKR phosphorylation by

C16 (Eley et al., 2009; Ingrand et al., 2007), others have found no effect of C16 on PKR activity (Chen et al., 2008). Furthermore, several studies have indicated that C16 reduces tumor growth (Eley et al., 2009) and proliferation of cultured cells (Chen et al., 2008). In the current study, we did not observe PKR activation in response to poly I:C (data not shown). Nevertheless, C16 potently reduced the production of IL-6 protein (Fig. 2.1) and mRNA (Fig. 2.6) following poly I:C exposure. Additionally, C16 had no effect on poly I:C-induced NF-κB activation (Fig. 2.2), which compromises a key step in the canonical pathway for initiation of IL-6 production (Kato et al., 2006). In agreement with previous reports of C16 reducing tumor growth (Eley et al., 2009) and cell proliferation (Chen et al., 2008), we observed a concentration-dependent reduction in the rate of protein synthesis (Fig. 2.3) in response to C16. This effect was further confirmed through reduced cell numbers in the absence of cytotoxicity or compromised membrane integrity, as measured by trypan blue staining (Fig. 2.5). Furthermore, C16 reduced thymidine incorporation with a similar potency as that observed for inhibition of leucine incorporation (IC_{50}=1.1 μM, unpublished observation). The exact mechanism for these effects remains unclear, but a recent report demonstrated that C16 is a potent inhibitor of several cyclin-dependent kinases (CDKs), and suggested that the reported neuroprotective effects of C16 *in vitro* may be attributable to the inhibition of CDK mediated proliferation (Chen et al., 2008). The current data are consistent with C16 potently inhibiting cell growth *in vitro.* It is worth noting that the cell lines tested in the current study are tumor-derived lines, and the effects of C16 on growth and division of non-malignant cell lines remains to be determined.

The dietary flavonoid, luteolin, can inhibit the production of IL-6 in response to a

wide range of inflammatory stimuli, including LPS and interleukin-1β (Chen et al., 2007; Sharma et al., 2007). However, the mechanism for the anti-inflammatory effects remains unclear, although it has often been attributed to a reduction in IκB degradation and NF-κB activation (Chen et al., 2007; Kim et al., 2005). In the current study, we demonstrated that luteolin is also capable of reducing poly I:C-induced production of IL-6 protein (Fig. 2.1) and mRNA (Fig. 2.6). These results are in agreement with the recent findings of Lee et al. (Lee et al., 2009) showing that luteolin suppresses poly I:C-induced mRNA production of several inflammatory cytokines, including IL-6. Surprisingly, luteolin did not inhibit poly I:C-induced IκB degradation (Fig. 2.2). Other studies have shown an inconsistent effect of luteolin on NF-κB signaling, with some reports suggesting a mechanism involving NF-κB signaling while others have indicated alternative targets, including transcription initiating proteins such as activator protein-1 (AP-1), the signal transducer and activator of transcription (STAT-1), and TBK1 (Jang et al., 2008; Rezai-Zadeh et al., 2008; Lee et al., 2009). In particular, the current results are in agreement with the findings of Lee et al. (Lee et al., 2009) who also observed no inhibition of poly I:C induced IκB degradation by luteolin in RAW 264.7 cells. In addition, the results of the current study demonstrate that luteolin inhibits protein synthesis (Fig. 2.3) and cell proliferation without inducing apoptosis, as indicated by a lack of effect on trypan blue staining (Fig. 2.5). Luteolin also reduced thymidine incorporation at a similar concentration (IC_{50}=32.5 μM, unpublished observation). In agreement with these findings, Comalada et al. (Comalada et al., 2006) reported decreased cell proliferation in bone marrow-derived macrophages in response to luteolin without a decrease in cellular viability. Previous reports have also characterized the anti-cancer properties of luteolin

and other flavonoids as a function of their ability to suppress tumor growth and proliferation of malignant cell lines (Fotsis et al., 1997; Fang et al., 2007).

The mechanisms by which luteolin and C16 reduce protein synthesis and cell numbers in culture are unclear. In the current study, cycloheximide, a well characterized inhibitor of protein synthesis, generated superinduction of IL-6 protein (Fig 2.4). Although the phenomenon of superinduction in the presence of cycloheximide is complex, considering that synthesis of new IκB is required to terminate NF-κB signaling one potential mechanism for this effect could be translational inhibition of IκB production following NF-κB activation (Newton et al., 1996). Furthermore, the observed superinduction of IL-6 (Fig 2.4) occurred at concentrations at or near the IC_{50} for cycloheximide induced inhibition of protein synthesis (Fig 1.4), suggesting that inhibition of protein synthesis may be the mechanism underlying the increased IL-6 production. Conversely, the potency of luteolin and C16 in inhibiting IL-6 production and protein synthesis were similar (Fig 2.1 and 2.3), although luteolin was several times more potent at inhibiting protein synthesis in RAW 264.7 cells (Figure 2.3). These findings suggest that the inhibition of IL-6 production and the inhibition of protein synthesis may occur through common mechanisms. However, both luteolin and C16 reduced IL-6 mRNA production in response to poly I:C (Fig 2.6) suggesting that the inhibition of poly I:C-induced IL-6 production can occur independently of any effects on protein synthesis. One possible explanation for these findings is a general inhibition of transcriptional activity. In order to address this possibility, we examined mRNA levels of the highly expressed cytoskeletal protein β-actin. Our results indicate that in C8-D1A cells combined treatment with poly I:C and luteolin or C16 significantly reduced β-actin expression, suggesting a

that these compounds may cause widespread inhibition of translation in this cell line (2.7 A). However, in RAW 264.7 cells, changes in β-actin mRNA levels did not reach significance when cells were pretreated with luteolin or C16 followed by poly I:C (Fig. 2.7 B). These results suggest that the inhibition of transcriptional activity may be cell type specific. Future research examining the effects of luteolin and C16 in other cell lines may help to clarify the effects of these compounds on transcription.

The results of the current study confirm the ability of luteolin to reduce IL-6 production in response to activation of TLR signaling pathways. Furthermore, this is the first report of the effects of C16 on inhibition of a dsRNA initiated response. However, in addition to inhibition of IL-6 protein and mRNA production, we also observed potent inhibition of protein synthesis and cell numbers in culture at concentrations similar to those producing anti-inflammatory effects. Therefore, it seems likely that *in vivo* use of luteolin and C16 for anti-inflammatory purposes may be limited by adverse effects on protein synthesis and cell growth.

Chapter 3: Effects of *in vivo* administration of poly I:C, luteolin, and C16 on IL-6 production in pregnant C5BL6/J mice

Introduction

Infection during pregnancy causes an immediate, and sometimes serious, threat to the health of the mother and developing fetus. In addition to immediate threats to physical health and organ function, there may also be an association between prenatal infection and neuropsychiatric diseases such as schizophrenia and autism (Meyer et al., 2011). Awareness of a potential association between maternal infection and subsequent schizophrenia arose following observation of increased occurrences of schizophrenia among cohorts born after influenza pandemics. Unfortunately, few data exist that contain both the long term psychiatric status of offspring and detailed information regarding the prenatal environment, such as pathogen identification, duration of infection, or the use of anti-inflammatory and fever reducing drugs. As a result, the vast majority of studies have relied on correlations between the date of birth for psychiatric patients and the corresponding infection rates in the general population. For example, Takei et al. (1996) examined the relationship between date of birth and influenza infection rates in approximately 8,000 schizophrenic patients. Based on influenza rates in Denmark from 1914 to 1970, the authors found that for every 100,000 cases of influenza there was a 12% increase in schizophrenics born 4 months later (Takei et al., 1996; Selten et al., 2010). Although these findings are intriguing, the lack of predictive data at the individual level precludes any analysis of interacting variables, particularly those that may be related to environmental or genetic factors mediating susceptibility to the development of

psychiatric disorders following prenatal infection.

Although epidemiological data have important limitations regarding an association between influenza and schizophrenia, many animal models have directly demonstrated that maternal immune activation (MIA) - from infectious agents, or by other mechanisms - can lead to long term behavioral abnormalities in the offspring. (Meyer et al., 2006a; Shi et al., 2003; Smith et al., 2007). These models have obvious advantages over epidemiological approaches, including strict control over genetic and environmental factors, standardized timing of infection, and uniform pathogen exposure (Meyer et al., 2011). These studies indicate that activation of the maternal immune system, and the subsequent production of pro-inflammatory cytokines, significantly alters behavior in the exposed offspring (Meyer et al., 2006b; Smith et al., 2007). A number of these alterations are observed in behavioral tests widely considered to model some clinical component of schizophrenia, such as decreased prepulse inhibition or increased sensitivity to the stimulating effects of amphetamines (Meyer, Feldon, & Yee, 2009). However, the effects observed in these models occur indiscriminately. In other words, the majority of exposed animals show neurodevelopmental and behavioral deficits. Considering that the infection rate during the 1957 influenza epidemic has been estimated at approximately 50%, the corresponding effects should be widespread if prenatal infection alone is a sufficient cause of neuropsychiatric disorders (Glass, Glass, Beyeler, & Min, 2006; Selten et al., 2010). The relatively small increases in the relative risk suggests that, if prenatal infection does contribute causally to the development of psychiatric disorders, there are other factors accounting for a substantial portion of individual variance.

Surprisingly few studies have examined the specific effects of pregnancy on immune function, and the mechanisms by which the maternal immune system adapts to allow for implantation and growth of the fetus remain poorly understood. It has been hypothesized that alterations in immune function during pregnancy involve differential regulation of the innate and adaptive arms of the immune response (Luppi, 2003). The adaptive arm of the immune system involves recognition of specific antigens and the subsequent production of antibodies, a highly specific but relatively slow response to infection. Alternatively, the innate immune response occurs rapidly through activation of signaling pathways capable of recognizing generalized properties of invading pathogens (Bhat & Steinman, 2009). In particular, Toll-like receptors (TLRs), are capable of recognizing a wide range of pathogen-associated molecular patterns and appear to be important in mediating the innate response to infection. Interestingly, pregnancy seems to be associated with activation of the innate arm of the immune system and suppression of the adaptive arm (Luppi, 2003).

Several clinical studies examining preeclampsia have demonstrated increased activity of the innate immune system during pregnancy. Preeclampsia is a pregnancy-specific disorder typically characterized by hypertension and proneturia, which can endanger the health of both the mother and fetus (Luppi, 2003). Although the specific etiology of preeclampsia is poorly understood, there is evidence that this disorder is associated with increased markers of innate immune system activation, including increased monocyte activation and elevated levels of circulating IL-6 (Luppi et al., 2006). Furthermore, administration of IL-6 to pregnant rats causes a pregnancy-specific increase in systolic blood pressure (Orshal & Khalil, 2004). Finally, administration of the TLR3

agonist, poly I:C, produces pregnancy-specific hypertension and proneturia in pregnant rats, and has been proposed as a putative animal model of preeclampsia (Tinsley, Chiasson, Mahajan, Young, & Mitchell, 2009). Surprisingly, despite the potentially convergent role of viral infection and enhanced IL-6 production contributing to this pregnancy-specific disorder, no studies to date have examined the effects of pregnancy on IL-6 production in response to poly I:C.

The production of IL-6 also appears to be necessary for the emergence of behavioral differences in offspring following MIA. Smith et al. (2007) co-administered an IL-6 antibody with poly I:C in C57BL6/J mice at gestational day 12.5 (Smith et al., 2007). Administration of the IL-6 antibody prevented behavioral abnormalities in offspring, including decreases in prepulse inhibition, latent inhibition, exploratory behavior, and social interaction. Similar results were observed when IL-6 knockout animals were injected with poly I:C, further supporting the critical role of this cytokine in mediating the effects of prenatal infection (Smith et al., 2007). Additionally, Parker-Athill et al. (2009) examined the effects of inhibition of the Janus tyrosine kinase-2/signal transducer and activator of transcription-3 (JAK2/STAT3) pathway on the effects of IL-6 in pregnant mice and the effected offspring (Parker-Athill et al., 2009). Their results indicate that exposure to IL-6 leads to increased activation of the JAK2/STAT3 pathway in both cultured neurons and the brains of newborn mice following maternal injections of IL-6. Furthermore, treatment of pregnant mice with diosmin, a structural analog of the flavonoid luteolin, decreased IL-6 induced activation of the JAK2/STAT3 pathway and blocked IL-6 induced changes in offspring behavior (Parker-Athill et al., 2009). Flavonoids are present in a wide range of food sources readily consumed by humans,

suggesting a possible interaction between dietary compounds and MIA-induced behavioral abnormalities. Collectively, these studies indicate that the effects of pathogen exposure on MIA and offspring behaviors are capable of being modulated by both environmental and genetic factors.

Flavonoids have been reported to induce a wide range of cellular effects, including inhibition of the production of pro-inflammatory cytokines, increased production of anti-inflammatory cytokines, and decreased cell proliferation (Comalada et al., 2006). In particular, the flavonoid luteolin, can reduce the production of pro-inflammatory IL-6 and increase production of the anti-inflammatory IL-10 (Jang et al., 2008; Comalada et al., 2006). Numerous studies have reported that luteolin reduces activation of the nuclear factor-kappa B (NF-κB) pathway, a convergent mechanism for numerous inflammatory responses (Chen et al., 2007; Kim et al., 2005). Additionally, luteolin has been reported to reduce IL-6 production and NF κ-B activation *in vivo* following administration of LPS (Jang et al., 2008; Kim et al., 2005). The apparent anti-inflammatory actions of this commonly consumed flavonoid makes luteolin an interesting candidate for mediating the maternal and prenatal immune response to infection.

Binding of viral double-stranded RNA and synthetic compounds, such as poly I:C, to the toll-like receptor 3 (TLR3) receptor is an important pathway for initiating inflammatory responses *via* NF κ-B, including the production of IL-6 (Alexopoulou et al., 2001). However, other pathways exist for recognition of dsRNA that can induce NF-κB activation and other antiviral responses. In particular, the dsRNA-dependent protein kinase R (PKR) can stimulate NF-κB activity in response to dsRNA (Srivastava et al., 1998). Additionally, PKR can inhibit protein synthesis during viral infection (Zhang et

al., 2007). Jammi et al. (2003) identified a potential inhibitor of PKR, termed C16, that was capable of rescuing PKR mediated inhibition of protein translation in response to dsRNA (Jammi et al., 2003). This compound also reduces the *in vivo* phosphorylation of PKR, although no studies have evaluated the potential anti-inflammatory effects of C16 in response to poly I:C *in vivo* (Ingrand et al., 2007).

The objective of the current study was to evaluate the anti-inflammatory effects of luteolin and C16 on poly I:C induced IL-6 production in pregnant and non-pregnant C57BL6/J mice. Mice were injected on with either luteolin or C16, then injected with poly I:C and serum protein IL-6 levels were measured. Pregnant mice received injections on gestational day 9.5 and fetal levels of IL-6 mRNA were also evaluated. Serum IL-6 levels were below detection in saline injected animals. Poly I:C induced high levels of serum IL-6, however, at gestational day 9.5 no detectable increases in IL-6 were found in fetal mice. Neither luteolin nor C16 caused a significant decrease in poly I:C-induced IL-6 production. Surprisingly, poly I:C administration in pregnant mice produced significantly higher serum levels of IL-6 compared to the levels in non-pregnant animals. This is the first report comparing the effects of poly I:C-induced IL-6 production in pregnant and non-pregnant animals.

Materials and Methods

Subjects

Adult male and female C57BL6/J mice were obtained at 6-8 weeks of age from Jackson Laboratory (Sacramento, CA, USA) and allowed to acclimate for at least one week prior to breeding. Animals were group housed three or four to a cage on a 12:12–

hour light:dark cycle with ad libitum access to food and water. For timed breeding, female mice were fed a high fat diet (Purina Mouse Diet 9F^{TM}, 9% fat content) and exposed to male bedding 48 hours prior to mating. For mating, male and female mice were pair housed overnight, and the following day was designated as gestational day 0.5. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Portland VA Medical Center's Institutional Animal Care and Use Committee.

Drug injections

Non-pregnant and pregnant (gestational day 9.5) female mice were moved to a procedure room and allowed to acclimate for at least one hour. Animals were then given an intraperitoneal (i.p.) injection of ether luteolin (0.2 mg/kg, volume of 10ml/kg), luteolin vehicle (1% DMSO in PBS, volume 10ml/kg), C16 (20 μg/kg, volume of 10ml/kg), or C16 vehicle (20% DMSO, volume 10ml/kg). One hour after i.p. injections, mice received an intravenous (i.v.) tail vein injection of either saline (vehicle, volume of 5ml/kg) or poly I:C (5 mg/kg, volume of 5 ml/kg). Three hours after i.v. injections, mice were euthanized using carbon dioxide gas. Trunk blood was collected immediately, followed by dissection of the uterine horns which were immediately placed on dry ice. Tissue samples were stored at -80 °C until the time of analysis. Trunk blood was stored at 4 °C for 48 hours. Samples were then centrifuged at 2,000 *g* for 20 minutes before serum was removed and stored at -80 °C until the time of analysis.

Quantification of fetal IL-6 mRNA

Fetal mice were dissected from the uterine horn according to the method of Shea and Geijsen (2007) with slight modifications (Shea & Geijsen, 2007). Briefly, individual

fetal mice were isolated from the uterine horn in between the implant sites and thawed in ice cold saline. A small incision was made in the uterine lining and the fetus was gently extracted by applying pressure to the muscular layer of the uterine horn. The placenta was detached by blunt dissection and the isolated fetus was homogenized in cell lysis buffer and RNA was extracted using an RNeasy Minikit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA purity and quantity was determined by spectrophotometric analysis. IL-6 mRNA levels were determined by one-step RT-PCR using the QuantiTect SYBR Green I RT-PCR Kit (Qiagen) in accordance with the manufacturer's instructions. Reactions contained 100 ng of RNA in a final volume of 25 μl and IL-6 was quantified using murine IL-6 primers from Quiagen (Cat#QT00098875). Samples were run in triplicate using an iCycler iQ^{TM} real-time PCR thermocycler (Bio-Rad Laboratories, Hercules, CA) with the iQ Real-Time analysis module. RT-PCR data were normalized to total RNA as determined using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Carlsbad, CA) according to the methods of Hashimoto et al. (Hashimoto et al., 2004).

IL-6 measurements

Serum levels of IL-6 were measured in duplicate using ELISA kits for murine IL-6 (R&D Systems, Minneapolis, MN). Samples were diluted 1:50 for analysis and the limit of detection was 15.6 pg/ml.

Data analysis

In instances of single mean comparisons, *t* tests were used to test for significant differences. For multiple mean comparisons, data were analyzed using one-way or two-way ANOVA, as indicated, with post hoc testing using Bonferroni correction. Data for

standard curves were analyzed using linear regression analysis. All statistical analyses were conducted using GraphPad Prism 4. Statistical significance was defined as $p<0.05$ for all tests.

Results

Descriptive Characteristics for female C57BL6/J mice

Female mice (N=80) were bred for timed pregnancy. Successful mating occurred in approximately 50% of breeding pairs. Pregnancy led to a significant increase in weight, with pregnant females weighing 2.3g more, on average, than non-pregnant females ($p<0.05$). Among pregnant females, the average litter size was 7.1 pups per litter.

Poly I:C-induced production of IL-6 in maternal serum

Serum IL-6 levels for poly I:C-injected mice are presented in Table 3.1. Among saline injected animals, IL-6 content in serum was below the level of detection (i.e. less than 15.6 pg/ml). As a result, these animals were excluded from further analysis. We had initially hypothesized that luteolin and C16 would decrease the IL-6 response to poly I:C in pregnant dams. However, because data were collected for pregnant and non-pregnant animals, two analyses were conducted to determine significant effects. Initially, all mice were included in a two-way analysis of variance (treatment condition x pregnancy status) in order to test for potential interactions between pregnancy and treatment condition. Post hoc testing was done to compare each treatment group to the poly I:C + saline injected animals. Pretreatment with luteolin, luteolin vehicle, C16, or C16 vehicle did not significantly affect serum levels of IL-6 in response to poly I:C. However, there was a

Table 3.1. Serum IL-6 levels in female C57BL6/J mice. Serum levels in female mice three hours after intravenous injection of poly I:C (5 mg/kg) as determined by ELISA for murine IL-6. Serum IL-6 levels in saline treated control animals were below the limit of detection (15.6 pg/ml). Values are expressed as mean ± SEM. Two-way analysis of variance showed a significant main effect of pregnancy. Post-hoc testing using Bonferroni correction was used to compare serum IL-6 levels in pregnant and non-pregnant mice. (*p<0.05, **p<0.01; compared to non-pregnant mice)

Treatment Group	Pregnant	Non-pregnant
Poly I:C + Saline	10409 ± 2442 * (n=4)	4162 ± 893 (n=7)
Poly I:C + Luteolin	12811 ± 2462 ** (n=5)	4889 ± 2016 (n=4)
Poly I:C + Luteolin vehicle	7577 ± 2537 (n=4)	3306 ± 713 (n=8)
Poly I:C + C16	7968 ± 1056 * (n=7)	3393 ± 835 (n=9)
Poly I:C + C16 vehicle	11793 ± 1158 ** (n=2)	1506 ± 392 (n=4)

Figure 3.1. Serum IL-6 levels in pregnant and non-pregnant C57BL6/J mice following treatment with poly I:C. Pregnant and non-pregnant mice received intraperitoneal injections of vehicle, luteolin (0.2 mg/kg), or C16 (20 μg/kg). One hour later animals received an intravenous tail vein injection of 5 mg/kg poly I:C. Three hours after polyI:C injections animals were euthanized and trunk blood was collected for analysis of serum IL-6 levels using ELISA. Data were analyzed using two-way analysis of variance with Bonferroni correction for post-hoc testing. (n= at least 4 per group) (*p<0.05, **p<0.01, ***p<0.001; compared to non-pregnant mice)

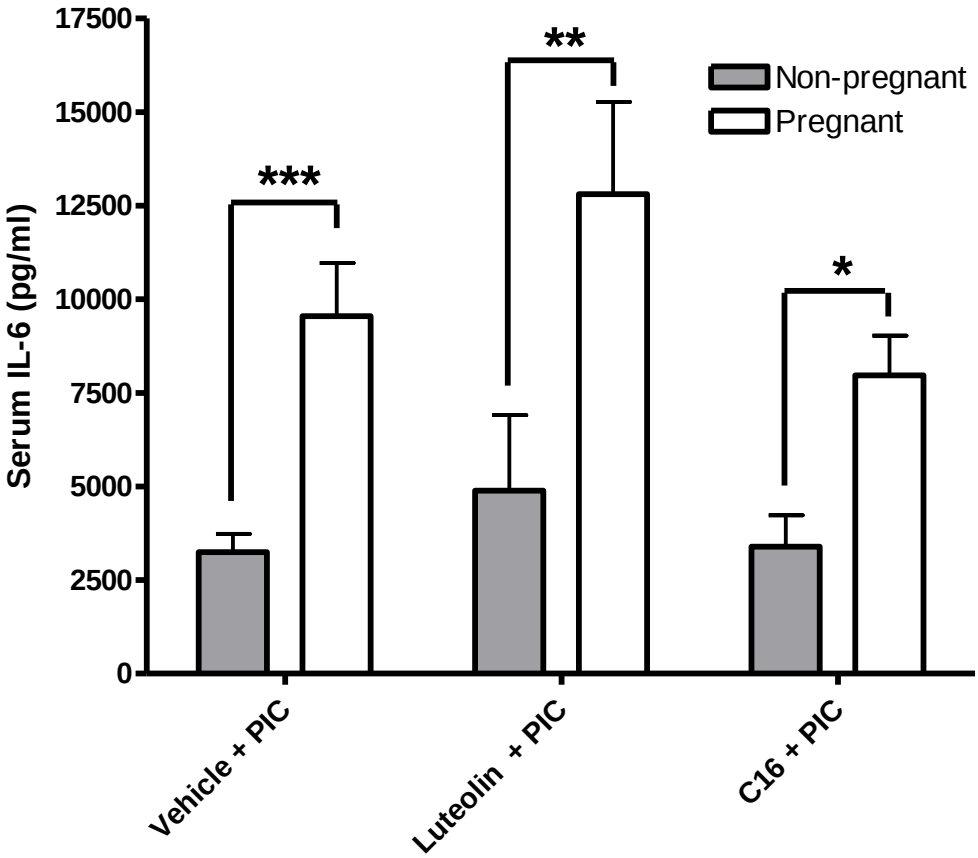


Figure 3.2. Pregnancy increases the IL-6 response to poly I:C. Administration of poly I:C led to a large increase in serum IL-6 levels in pregnant (n=10) and non-pregnant (n=19) C57BL6 mice. For ease of interpretation only data from pregnant mice in the vehicle pretreatment groups are shown. Serum IL-6 levels in pregnant mice were significantly higher at three hours following poly I:C administration. (***p<0.001)

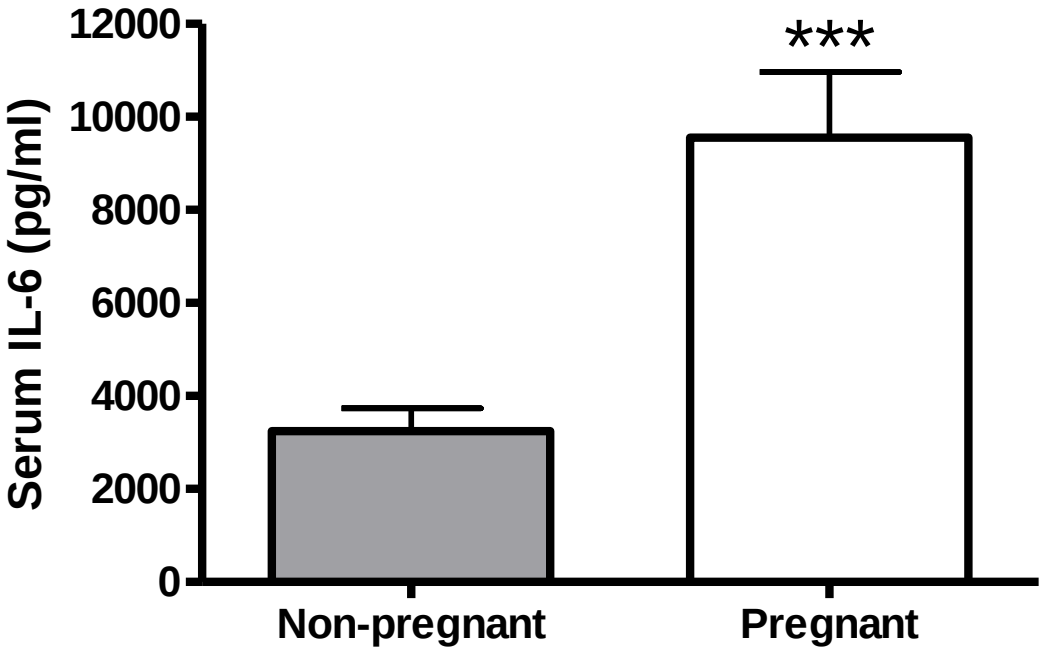
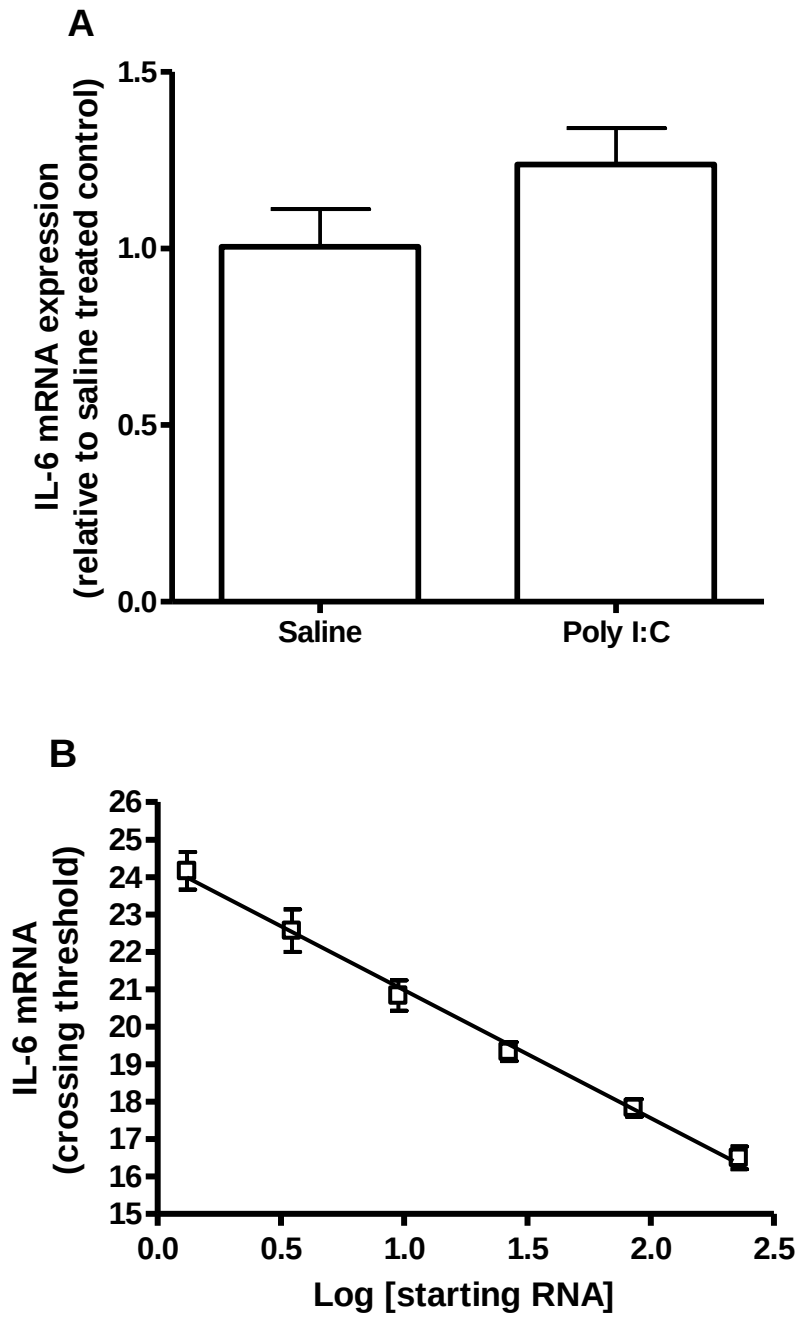


Figure 3.3. IL-6 mRNA expression in fetal brain following poly I:C administration. Fetal mRNA was isolated from poly I:C treated and saline treated C57BL6/J mice. No significant differences in expression of IL-6 mRNA was observed between saline (n=8) and poly I:C treated (n=18) fetal mice (Fig 4.3A). Standard curves were generated using mRNA extracted from poly I:C treated RAW 264.7 macrophage-like cells (Fig 4.3B). Samples from fetal mice were run at a concentration of 100 ng/rxn.

Fig 3.3


A
IL-6 mRNA expression
(relative to saline treated control)
1.5
1.0
0.5
0.0
Saline
Poly I:C
B
IL-6 mRNA
(crossing threshold)
26
25
24
23
22
21
20
19
18
17
16
15
0.0
0.5
1.0
1.5
2.0
2.5
Log [starting RNA]

significant main effect for pregnancy, $F_{(1,44)}$=45.51, p<0.05, which accounted for 44.37% of the variance (Table 3.1). In order to simplify further analyses, saline and vehicle pretreatment groups were compared using separate one-way analyses of variance for pregnant or non-pregnant mice. Because no significant differences were observed in animals that were pretreated with saline, luteolin vehicle, or C16 vehicle these groups were collapsed into a single control group for further analyses. Data for animals pretreated with vehicle, luteolin, or C16 were then compared using two-way analysis of variance for pregnant or non-pregnant animals (Fig. 3.1). Luteolin and C16 did not significantly alter the poly I:C-induced IL-6 response in either the pregnant or non-pregnant group, however a main effect of pregnancy was apparent, $F_{(1,48)}$=37.46, p<0.0001. Finally, pregnant and non-pregnant animals pretreated with vehicle followed by poly I:C were compared directly (Fig. 3.2) in order to simplify interpretation. Again, pregnancy significantly increased poly I:C-induced IL-6 production (p<0.001).

IL-6 mRNA in fetal mice

RT-PCR was conducted to quantify IL-6 mRNA production in fetal mice following maternal poly I:C injections. RT-PCR was run for 36 cycles and a standard curve was generated using mRNA extracted from poly I:C-treated RAW 264.7 cells as described in Chapter 2 (Fig. 2.5). The mRNA samples from fetal mice treated with either saline or poly I:C were run at 100 ng/rxn. There was no significant difference in IL-6 mRNA between animals treated with saline (C_t= 28.1±1.8, n=7) or poly I:C (C_t= 27.8±1.3, n=7) at three hours post injection (Fig 3.3A). Linear regression analysis for the standard curve showed low variability (r^2=0.98) and good efficiency (108.4%) for the RT-PCR reaction (Fig 4.3).

Discussion

Administration of the synthetic dsRNA, poly I:C, has been widely employed in animal models of infection due to the ability of this compound to induce a large acute phase response, including the production of pro-inflammatory cytokines. Consistent with previous findings, in the current study we observed a large increase in serum levels of pro-inflammatory IL-6 three hours after poly IL:C administration in both pregnant and non-pregnant mice. Several studies have reported both *in vitro* and *in vivo* inhibition of TLR-mediated immune activation by the flavonoid luteolin (Lee et al., 2009; Kim et al., 2005). In the current study, we did not observe an inhibitory effect of luteolin on serum levels of IL-6 following poly I:C injections. Likewise, although activation of the dsRNA-binding protein, PKR, appears to mediate the immune response to poly I:C under certain conditions, we observed no inhibitory effect of a commercially available PKR inhibitor on poly I:C-induced IL-6 production (Srivastava et al., 1998; Auch et al., 2004). Interestingly, we observed that pregnancy alone was capable of significantly altering IL-6 production following poly I:C administration. This finding may be of considerable importance given that elevated IL-6 levels during pregnancy appear to present a substantial risk to the health of both the mother and the developing fetus (Luppi et al., 2006).

Although we did not observe any decrease in poly I:C-induced IL-6 production as a result of luteolin pretreatment, others have reported that luteolin can inhibit NF-κB activation and IL-6 production in response to LPS administration. Kim and Jobin (2005) observed a reduction in NF-κB activity in peripheral blood mononuclear cells following pretreatment with luteolin (0.2 mg/kg, i.p.), although this effect was observed 18 hours

after mice were challenged with LPS (Kim et al., 2005). Similarly, Kotanidou et al. (2002) found that the same dose of luteolin modestly decreased LPS-induced IL-6 serum levels 60 minutes after LPS challenge in mice, although this effect was no longer present 180 minutes post-injection (Kotanidou et al., 2002). Finally, Jang et al. (2008) reported that luteolin reduced plasma IL-6 levels by approximately 75% four hours after LPS administration in mice. However, this study administered luteolin in drinking water for 21 days at 5 mg/ml prior to challenge with LPS (Jang et al., 2008). The current study is the first to examine the effects of luteolin on the poly I:C-induced IL-6 production *in vivo*. It is possible that the lack of effect observed following poly I:C administration is due to differences in the potency of luteolin to inhibit TLR3 or TLR4 signaling, however the *in vitro* potency for the inhibitory effects of luteolinin on IL-6 production appear to be similar for LPS and poly I:C (Jang et al., 2008) (Lee et al., 2009). It is difficult to estimate the bioavailability of luteolin in these two paradigms, but mice receiving luteolin in drinking water ingested an average of 19.5 mg per day, which may lead to far higher systemic concentrations than a single i.p. injection of 0.2 mg/kg. Although the current study does not support a role for luteolin in mediating *in vivo* poly I:C-induced IL-6 production in pregnant or non-pregnant female mice, future research examining higher doses or alternative methods of administration may be warranted.

Studies indicating a role for PKR in mediating the immune response to poly I:C have been almost entirely limited to cell culture, and the *in vivo* importance of this protein kinase remains largely unknown (Srivastava et al., 1998; Auch et al., 2004). Currently, there is only one report examining the PKR inhibitor, C16, *in vivo*. Ingrand et al. (2007) found significant inhibition of PKR phosphorylation in the brain of 7-day-old

rats, which displayed high constitutive levels of phosphorylated PKR. The authors reported a dose-dependent reduction in PKR phosphorylation with a maximal effect of approximately 50% inhibition at doses higher than 3.35 µg/kg (Ingrand et al., 2007). Although the role of PKR in activation of NF-κB and cytokine production remains controversial, several reports indicate that the expression of mutant forms of PKR can inhibit activity of NF-κB and block poly I:C-induced phosphorylation of eIF2-α (Srivastava et al., 1998; Auch et al., 2004). In the current study we did not observe any effects of C16 on poly I:C-induced IL-6 production *in vivo*. Although levels of phosphorylated PKR were not measured, the doses administered (20 µg/kg) were well within the range reported to maximally inhibit PKR phosphorylation (Ingrand et al., 2007). With respect to the current experimental paradigm, these results do not support an anti-inflammatory role for C16 following poly I:C administration.

The presence and origin of IL-6 in the fetus following immune activation appears to be dependent upon a number of factors, including the nature of the infectious agent, route of administration, and timing of the insult. Systemic administration of IL-6 in pregnant mice is capable of generating long-term abnormalities in offspring behavior, suggesting that the maternal-fetal interface is permeable to IL-6 (Smith et al., 2007; Parker-Athill et al., 2009). Furthermore, in C57BL6/J mice at gestational day 9.5, administration of poly I:C generates increases in detectable IL-6 protein in fetal brain in the absence of any detectable changes of IL-6 mRNA, suggesting a maternal origin of IL-6 (Meyer et al., 2006b). The results of the current study further support the theory that generation of IL-6 by the fetus may not be a prominent feature of prenatal poly I:C administration.

Although pregnancy increases the risk of complications during infection, including mortality, relatively few studies have examined the specific effects of pregnancy on the immune response (Mortimer, 2006). In humans, pregnancy leads to suppression of the adaptive arm of the immune system, presumably to allow for implantation and growth of the fetus (Luppi, 2003). Conversely, there appears to be a substantial increase in the activation of the innate arm of the immune system, including increased monocyte activation (Luppi et al., 2002). Furthermore, in certain pathological conditions, such as the pregnancy-specific hypertensive disorder, preeclampsia, increased activation of NF-κB and elevated levels of circulating IL-6 have been reported in humans (Luppi et al., 2006). Evidence for an exacerbated immune response during pregnancy has been further supported by an animal model of pre-eclampsia examining the hypertensive response to poly I:C in pregnant rats. Administration of poly I:C beginning at GD10 caused elevated systolic blood pressure and decreased endothelial function at GD18. This effect was specific to pregnant rats, and pregnancy also elevated levels of the pro-inflammatory cytokine IFNγ in response to poly I:C (Tinsley et al., 2009). These studies indicate that although implantation and growth of the fetus may require suppression of the adaptive arm of the immune system, mechanisms mediating the APR (i.e. the innate arm) may be more responsive to invading pathogens during pregnancy.

The results of the current study suggest that the pro-inflammatory response to poly I:C is enhanced during pregnancy. IL-6 levels in response to poly I:C were elevated approximately 3-fold in pregnant mice compared to non-pregnant females. To date, there are no other reports comparing the inflammatory response to poly I:C in pregnant and non-pregnant mice. Maternal IL-6 production appears to be a key factor in mediating the

long-term behavioral effects of poly I:C exposure on offspring, and the effects of poly I:C in these models are dose-dependent (Smith et al., 2007; Meyer et al., 2006a). The large increases in IL-6 production observed during pregnancy suggest that the specific mechanism underlying this phenomenon may be of particular importance in mediating the effects of prenatal infection on the developing fetus. Currently, the most well characterized pathway for the induction of pro-inflammatory cytokine production by poly I:C is signaling through the TLR3 (Alexopoulou et al., 2001; Carpentier et al., 2007). Although no reports have examined changes in TLR3 expression in macrophages during pregnancy, Gonzalez et al. reported that pregnancy in mice led to a significant increase in TLR3 mRNA expression in the uterus (Gonzalez, Xu, Ofori, & Elovitz, 2007). These findings suggest that increased expression of TLR3 may underlie the exacerbated the inflammatory response observed during pregnancy.

The current study examined the effects of the bioflavonoid, luteolin, and the PKR inhibitor, C16, on poly I:C-mediated IL-6 production in pregnant and non-pregnant mice. Luteolin has been reported to reduce inflammatory cytokine production during sepsis, and the common occurrence of luteolin in a variety of food sources makes it an interesting candidate for environmental modulation of the inflammatory response during prenatal infection. The results of the current study do not support the hypothesis that luteolin is an important modulator of pro-inflammatory cytokine production in response to dsRNA. Likewise, the novel PKR inhibitor, C16, did not significantly attenuate the production of pro-inflammatory IL-6 following poly I:C administration. Surprisingly, pregnancy alone caused a significant increase in the IL-6 response to poly I:C. These findings may be of particular relevance for animal models of prenatal infection related to

preeclampsia and fetal development, as the production of IL-6 appears to be a critical mediator of the maternal and fetal response to dsRNA. Future research examining the pregnancy-specific elevations in IL-6 following dsRNA exposure may help elucidate causal mechanisms and identify potential targets for ameliorating the adverse effects of prenatal infection.

Chapter 4: Poly I:C mediated IL-6 production in TLR3 KO mice

Introduction

Exposure to the synthetic dsRNA, poly I:C, generates a strong immune response and has been extensively employed in models of viral infection. Most viruses produce dsRNA at some point in the replication cycle, and a number of proteins are capable of binding dsRNA (Majde, 2000). Collectively, these proteins, and the signaling pathways they activate, represent the initial response to infection and are thought to be the primary mediators of the acute phase response (Bhat et al., 2009). The acute phase response to infection generates a wide range of behavioral and physiological responses, including fatigue, loss of appetite, and fever. These effects are primarily mediated through the rapid production of pro-inflammatory cytokines in response to pathogen-associated molecular patterns found in the nucleic acids, proteins, and cytoskeletal components of invading pathogens. With respect to dsRNA, several key proteins have been identified in the initiation of the acute phase response, including TLR3, MDA5, and PKR (Jacobs et al., 1996). Although both TLR3 and MDA5 can directly bind dsRNA, TLR3 is primarily located in endosomal compartments while MDA5 is a cytosolic protein. Additionally, many conflicting reports exist regarding the necessity of TLR3 and MDA5 in the initiation of the acute phase response to dsRNA, both *in vitro* and *in vivo* (Carpentier et al., 2007; Gitlin et al., 2006).

TLR3 was first identified as a potential extracellular or endosomal target of dsRNA-mediated cytokine production by Alexopoulou et al. using a mutant form of the TLR3 (Alexopoulou et al., 2001). Cells transfected with the dominant negative mutant

for TLR3 failed to activate NF-κB in response to poly I:C and showed reduced production of pro-inflammatory cytokines, including IL-6. Furthermore, mice expressing the mutant receptor were resistant to poly I:C induced shock, although this effect was overcome by higher doses (Alexopoulou et al., 2001). Additional evidence for the necessity of TLR3 in mediating the pro-inflammatory cytokine response to poly I:C has been observed in primary cultures of microglia and astrocytes isolated from mice expressing the mutant form of the receptor (Town et al., 2006; Carpentier et al., 2007; De Miranda et al., 2009). However, several conflicting reports exist regarding the ability of TLR3 mutants to attenuate cytokine production in response to poly I:C *in vivo*. Bunting et al. measured IL-6 levels in female TLR3 KO and WT mice following poly I:C administration (50 µg). Mice expressing the mutant form of TLR3 had decreased serum IL-6 production in response to poly I:C at 1 hour post injection (Bunting et al., 2011). Similarly, Gowen et al. examined the response to either 10 µg or 100 µg of poly I:C in TLR3 KO and WT male and female mice. Mice expressing the mutant TLR3 had decreased levels of IL-6 three hours after administration of the low dose of poly I:C, but this effect was not observed at the higher dose. The authors did not report whether there was a main effect of sex and it was unclear whether this variable was included in the analyses (Gowen et al., 2007). Finally, Gitlin et al. examined the response to poly I:C in mice deficient for either TLR3 or MDA5. Three hours following administration of 100 µg poly I:C, serum levels of IL-6 were measured. Mice expressing the mutant form of TLR3 or WT had similar induction of IL-6, while animals expressing the mutant form of MDA5 failed to produce IL-6 following poly I:C administration (Gitlin et al., 2006). Collectively, these studies suggest that there are multiple pathways for initiating

production of IL-6 following poly I:C exposure, and that the specific pathways activated in response to poly I:C may be dose-dependent.

During pregnancy, the innate arm of the immune system appears to be upregulated, although the specific mechanisms for elevated inflammatory signaling remain poorly understood (Luppi et al., 2006; Luppi, 2003). Few studies have examined the specific effects of pregnancy on the inflammatory response to poly I:C, although Tinsley et al. reported that pregnancy in rats significantly increased the pro-inflammatory/anti-inflammatory cytokine ratio (IFNγ/IL-10) in response to poly I:C (Tinsley et al., 2009). Similarly, we have observed an increase in poly I:C mediated IL-6 production in pregnant mice compared to non-pregnant animals (Section IV). Surprisingly, no studies to date have examined the role of TLR3 or MDA5 in mediating the poly I:C-induced production of IL-6 during pregnancy.

The goal of the current study was to determine the role of TLR3 in mediating the IL-6 response to poly I:C in pregnant mice. Pregnant and non-pregnant TLR3 KO mice were injected with poly I:C and serum IL-6 levels were measured. Poly I:C administration lead to significant increased in IL-6 in both pregnant and non-pregnant animals compared to saline injected controls. Non-pregnant TLR3 KO mice had similar levels of IL-6 induction compared to non-pregnant WT animals. In agreement with our previous findings in C57BL6/J mice, poly I:C administration generated significantly higher levels of IL-6 production in pregnant WT mice compared to non-pregnant WT animals. However, the enhanced IL-6 production in seen during pregnancy in WT animals was absent in pregnant TLR3 KO mice. Additionally, we observed that the overall induction of IL-6 in both WT and TLR3 KO mice, which were hybrid

B6129SF2/J strains, was substantially lower than previously observed values for C57BL6/J female mice. Therefore, we examined poly I:C induced IL-6 production in TLR3 KO, TLR3 WT, and C57BL6/J males. In agreement with our previous results from non-pregnant females, the induction of IL-6 following administration of poly I:C was similar in TLR3 KO and WT mice. Furthermore, male B6129SF2/J mice had significantly lower levels of poly I:C induced IL-6 compared to male C57BL6/J mice. These findings provide further support for increased activation of the innate immune system during pregnancy, and suggest that activation of the TLR3 may be important in mediating this effect.

Materials and Methods

Subjects

Adult male and female TLR3 KO, TLR3 WT, and C57BL6/J mice were obtained at 6-8 weeks of age from Jackson Laboratory (Sacramento, CA, USA) and allowed to acclimate for at least one week. TLR3 KO mice (Cat# 005217) and WT mice (Cat# 101045) were generated as hybrid strains on a mixed B6129SF2/J background. Animals were group housed three or four to a cage on a 12:12–hour light:dark cycle with ad libitum access to food and water. For timed breeding, female mice were fed a high fat diet (Purina Mouse Diet 9F^{TM}, 9% fat content) and exposed to male bedding 48 hours prior to mating. For mating, male and female mice were pair housed overnight, and the following day was designated as gestational day 0.5. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Portland VA Medical Center's Institutional

Animal Care and Use Committee.

Poly I:C administration in female TLR3 KO and WT mice

On gestational day 9.5 female mice were moved to a procedure room and allowed to acclimate for at least one hour. Mice then received an intravenous (i.v.) tail vein injection of either saline (vehicle, volume of 5ml/kg) or poly I:C (5 mg/kg, volume of 5 ml/kg). Three hours after i.v. injections, mice were euthanized using carbon dioxide gas. Trunk blood was collected immediately and stored at 4 °C for 48 hours. Samples were then centrifuged at 2,000 *g* for 20 minutes before serum was removed and stored at -80 °C until the time of analysis.

Poly I:C administration in male TLR3 KO, TLR3 WT, and C57BL6/J mice

Male mice were moved to a procedure room and allowed to acclimate for at least one hour before receiving an intravenous (i.v.) tail vein injection of either saline (vehicle, volume of 5ml/kg) or poly I:C (5 mg/kg, volume of 5 ml/kg). Three hours after i.v. injections, mice were euthanized using carbon dioxide gas. Trunk blood was collected immediately and stored at 4 °C for 48 hours. Samples were then centrifuged at 2,000 *g* for 20 minutes before serum was removed and stored at -80 °C until the time of analysis.

IL-6 measurements

Serum levels of IL-6 were measured in duplicate using ELISA kits for murine IL-6 (R&D Systems, Minneapolis, MN). Samples were diluted 1:50 for analysis and the limit of detection was 15.6 pg/ml.

Data analysis

For multiple mean comparisons, data were analyzed by two-way ANOVA, as indicated, with post hoc testing using Bonferroni correction. In instances of single mean

comparisons, *t* tests were used to test for significant differences. All statistical analyses were conducted using GraphPad Prism 4. Statistical significance was defined as $p<0.05$ for all tests.

Results

The effects of TLR3 on poly I:C induced IL-6 production during pregnancy

Administration of poly I:C induced IL-6 production in pregnant and non-pregnant mice compared to saline injected controls, which did not have detectable levels of serum IL-6. Two-way analysis of variance revealed a significant interaction between pregnancy status and genotype, $F_{(1,27)}=45.51$, $p<0.05$, with a significant main effect of both pregnancy, $F_{(1,27)}=45.51$, $p<0.05$, and genotype $F_{(1,27)}=45.51$, $p<0.01$, for serum IL-6 levels following administration of poly I:C (Figure 4.1). Post-hoc testing using Bonferroni correction indicated that the response to poly I:C was significantly higher in pregnant TLR3 WT mice compared to non-pregnant TLR3 WT mice ($p<0.001$). In contrast to this finding, pregnancy failed to significantly increase poly I:C-induced IL-6 production in TLR3 KO mice.

Poly I:C induced IL-6 in TLR3 KO, TLR3 WT, and C57BL6/J male mice

Male TLR3 KO and WT mice received intravenous injections of poly I:C or saline and serum IL-6 levels were examined 3 hours post-injection. Saline injected mice did not have detectable levels of serum IL-6. Administration of poly I:C generated similar induction of IL-6 in both TLR3 KO and WT mice (Figure 4.2). Both TLR3 KO and WT mice were a hybrid B6129SF2/J strain. Previous observations had indicated that female hybrid B6129SF2/J mice may have lower poly I:C-induced IL-6 production compared to

Figure 4.1. Poly I:C induced IL-6 production in WT and TLR3 KO female mice. Administration of poly I:C increased serum IL-6 levels in WT non-pregnant (n=9), WT pregnant (n=7), KO non-pregnant (n=4), KO and pregnant (n=10) mice compared to saline injected controls (IL-6 not detectable). Pregnant WT animals showed increased poly I:C-induced IL-6 production as a result of pregnancy. This increase in IL-6 production was not observed in pregnant TLR3 KO animals. ($p<0.001$)

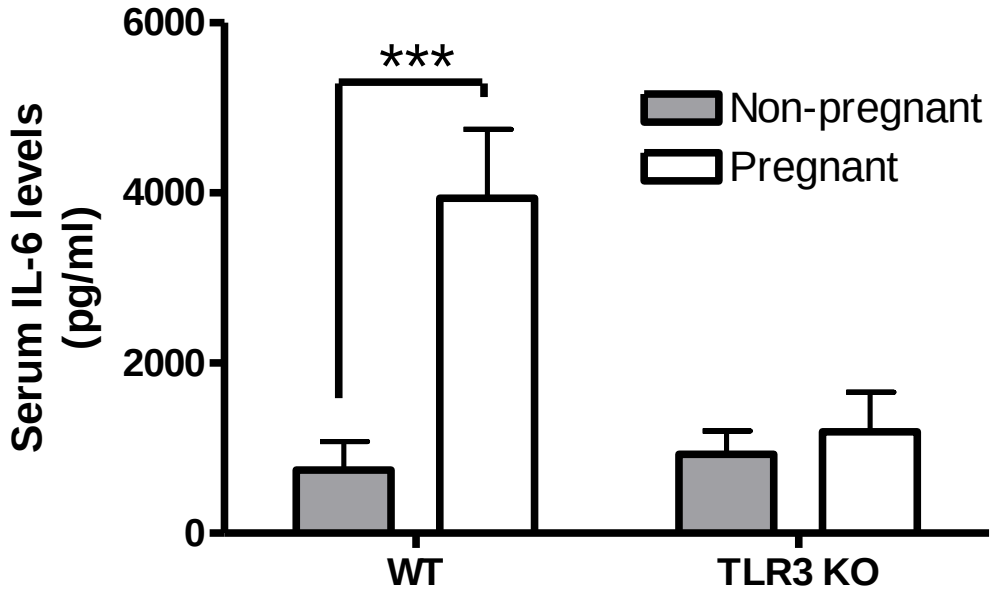


Figure 4.2. Poly I:C-induced IL-6 production in TLR3 KO and WT male mice. Administration of poly I:C increased serum IL-6 levels in both wild type (n=7) and TLR3 KO (n=5) mice compared to saline injected controls (IL-6 not detectable). No significant differences were observed in poly I:C-induced serum IL-6 production.

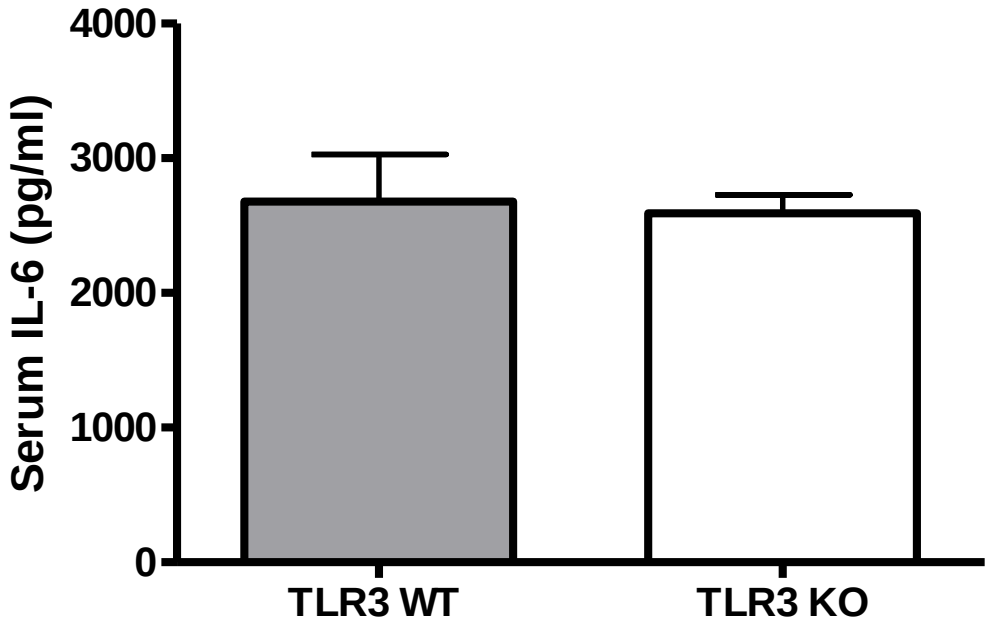
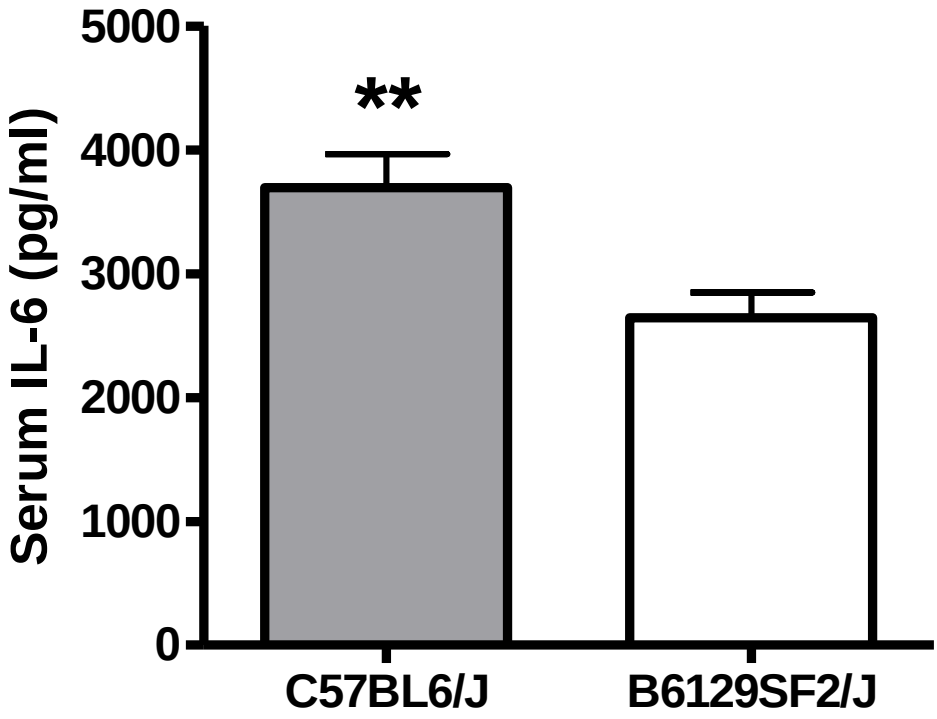


Figure 4.3. Poly I:C-induced IL-6 production in C57BL6/J and B6129SF2/J males. Administration of poly I:C increased serum IL-6 levels in both C57BL6 (n=8) and B6129SF2/J (n=12) mice compared to saline injected controls (IL-6 not detectable). IL-6 production following poly I:C administration was significantly higher in C57BL6/J males (**p<0.01)



C57BL6/J mice. In order to test this hypothesis, male c57BL6/J mice were injected with 5 mg/kg poly I:C or saline. Saline injected male C57BL6/J mice did not have any detectable levels of serum IL-6. Poly I:C induced significantly higher levels of serum IL-6 in male C57BL6/J mice compared to male B6129SF2/J mice (Figure 4.3).

Discussion

Pregnancy induces a series of complex changes in the immune system in order to allow for proper fetal growth and development. Infection during pregnancy is associated with a number of adverse outcomes, including still birth, preeclampsia, and neuropsychiatric disorders in the affected offspring. However, the mechanisms by which activation of the immune system can lead to such a wide range of immediate and long-term complications remain poorly understood. Likewise, the specific mechanisms underlying alterations in the immune response during pregnancy, including tolerance of the maternal immune system to the fetal allograft, are not well characterized. Several studies have indicated that pregnancy is associated with increased activation of the innate immune system and production of pro-inflammatory cytokines in response to infection, although the pathways mediating these effects have yet to be elucidated (Luppi et al., 2006; Tinsley et al., 2009).

The current study examined the role of the TLR3 in mediating the pro-inflammatory response to the viral mimetic poly I:C during pregnancy. Our results in WT pregnant and non-pregnant mice confirm previous findings that pregnancy exacerbates the IL-6 response to poly I:C. Furthermore, this effect appears to be mediated by TLR3 since pregnant mice expressing a null mutant form of this receptor failed to show

increased IL-6 compared to non-pregnant poly I:C injected TLR3 KO mice. This is the first report identifying a potential candidate for the increased inflammatory response observed during pregnancy. Elevated levels of IL-6 following immune activation or administration of recombinant IL-6 during pregnancy have been associated with a number of adverse outcomes for the mother and developing fetus (Smith et al., 2007; Tinsley et al., 2009; Orshal et al., 2004). Considering that these effects are dose-dependent, the mechanisms underlying elevated IL-6 production during pregnancy may serve as potential therapeutic targets for prevention of adverse outcomes during prenatal infection. Our results indicate that activation of TLR3 by dsRNA may account for a large portion of IL-6 production in pregnant mice. Additionally, the finding that IL-6 production in non-pregnant females was similar in both TLR3 KO and WT mice indicates that other pathways for detecting dsRNA may normally mediate IL-6 production.

The role of TLR3 in the generation of the acute phase response to dsRNA *in vivo* has been the subject of conflicting reports. Overall, the current evidence seems to indicate that at low doses of poly I:C (≤50 μg), TLR3 KO mice show reduced poly I:C-mediated IL-6 production (Bunting et al., 2011; Gowen et al., 2007). However, at higher doses (100 μg), the IL-6 response to poly I:C is unaffected by TLR3 mutations (Gowen et al., 2007; Gitlin et al., 2006). In the current study, we did not observe any decreases in poly I:C-mediated IL-6 production in non-pregnant TLR KO mice. Doses for the current study were calculated based on body weight, and the corresponding average weights per treatment group were 23.1g for non-pregnant females, 24.6g for pregnant females, and 28.6g for male mice. Therefore, the corresponding average doses for treatment groups in

the current study were 116 µg in non-pregnant females, 123 µg in pregnant females, and 143 µg in male mice. Unfortunately, previous studies injecting fixed amounts of poly I:C did not report average weights, making it difficult to directly compare the current data. However, the current dosing regimen appears to be in excess of previously reported high doses of poly I:C, which failed to show a significant effect of TLR3 mutations on poly I:C-induced IL-6 production. Specifically, Gitlin et al. (2006) reported that in mice expressing null mutant receptors for either MDA5 or TLR3, IL-6 production in response to poly I:C (100 µg) was dependent on MDA5 rather than TLR3. The current results support the hypothesis that pathways other than TLR3 are capable of fully mediating the inflammatory response to higher levels of dsRNA exposure.

The mechanism underlying the differential response of TLR3 mutant mice to low and high doses of poly I:C has not been clearly identified. It has been speculated that the dominant role of MDA5 over TLR3 in mediating the inflammatory response to high-doses of poly I:C may be related to differences in tissue or cellular expression of these proteins as a function of the pharmacokinetics of different doses of poly I:C. However, one additional hypothesis is that the TLR3 pathway may become saturated following high doses of poly I:C. This theory seems to be supported by the *in vitro* results of Alexopoulou et al. (2001) examining poly I:C-induced IL-6 production in bone-marrow-derived macrophages from TLR3 KO and wild-type mice. Macrophages isolated from TLR3 KO mice were able to produce IL-6 in response to poly I:C, but the potency of poly I:C was reduced by several orders of magnitude. Similar results were reported for the *in vivo* effects of the TLR3 KO on poly I:C-induced shock, where the maximal effects of the TLR3 KO were observed at the lowest doses of poly I:C (Alexopoulou et al.,

2001). The notion that the TLR3 system may become easily saturated following poly I:C administration is particularly interesting given the large differences in the IL-6 response to poly I:C in pregnant TLR3 KO and WT mice. These findings suggest that TLR3 may play a unique role in modulating the immune response to dsRNA during pregnancy, perhaps through increased activation of TLR3 expressing cells or increased expression of TLR3. Alternatively, increased suppression of anti-inflammatory processes through TLR3 may serve to enhance dsRNA-induced cytokine production through other pro-inflammatory pathways. Future studies examining the cell-specific regulation of TLR3 throughout pregnancy may help to clarify the mechanisms mediating this effect. Finally, our results comparing the IL-6 response in hybrid B6129SF2/J males and C57BL6/J males indicate that the magnitude of the response to poly I:C may be strain-dependent. Although no studies to date have extensively compared the response to poly I:C across several strains of mice, this approach may prove useful for identifying new candidate genes capable of modulating cytokine production in response to dsRNA.

SUMMARY AND CONCLUSIONS

Over the past decade tremendous progress has been made in the understanding of mechanisms responsible for initiating the acute phase response to infection. Primarily, identification of the Toll-like Receptor family and the importance of these transmembrane proteins in the recognition of pathogen-associated molecular patterns has provided a new set of genetic tools for examining the cellular and systemic responses to pathogen exposure. However, perhaps the most surprising finding for the field of neuroscience is that many members of the TLR family are highly expressed within the central nervous system. TLR agonists and pro-inflammatory cytokines have clear effects neuronal function, behavior, and development. Although the clinical relevance of TLR activation and cytokine production in the formation of neuropsychiatric disorders remains unclear, animals models of TLR activation developed over the past decade have the potential to identify causal mechanisms that may direct clinical and epidemiological research in fruitful new directions.

The recent focus on the possible neurodevelopmental origins of psychiatric disorders presents a new avenue for understanding the etiologies of complex disorders. However, the challenges of identifying specific prenatal factors associated with neurodevelopmental abnormalities remain daunting. In fact, identifying the prevalence and specific risk factors for well-characterized neurodevelopmental disorders with known causes, such as fetal alcohol spectrum disorder, remains difficult (May & Gossage, 2001). As a result, establishing firm clinical evidence for the long-term impacts of transient environmental insults, such as influenza, would likely require an extremely large effect

size. Furthermore, it is probable that many genetic and environmental factors are capable of mediating both the maternal and fetal response to infection. Future refinement of theories regarding the causative role of prenatal infection and the development of neuropsychiatric disorders in offspring will require a better understanding of both the causal mechanisms and the potential environmental and genetic factors mediating abnormal brain development. The primary focus of this dissertation was to identify factors that may modulate the inflammatory response to viral infection during pregnancy.

Few factors have been identified that may increase or decrease the risk of abnormal brain development following prenatal infection. One major challenge still lies in understanding the specific changes in the immune system that occur during pregnancy. Alterations of the maternal immune response throughout gestation are substantial, and the interaction of the immune response with the specific stage of fetal development appears to be a critical mediator of the long-term consequences of prenatal infection (Fofie et al., 2003; Meyer et al., 2006a). With respect to animal models, the use of inbred strains, timed pregnancy, and synthetic TLR agonists has proven useful in isolating these variables and the effects of MIA on adult offspring in rodents have been replicated in a number of independent laboratories (Meyer et al., 2006b; Smith et al., 2007; Shi et al., 2003; Ozawa et al., 2006). While these findings will no doubt be useful in guiding future epidemiological research, the identification of genetic or environmental factors that contribute substantially to the variance in neurodevelopment will likely be the key to identifying vulnerable populations and further clarifying the true risk of prenatal infection on offspring.

Although a great deal of progress has been made in the identification of TLR

ligands capable of generating large increases in pro-inflammatory cytokine production, the identification of compounds capable of inhibiting these pathways has been slow. To date, no specific chemical inhibitor of TLR3 signaling has been identified, although a recent report suggests that administration of a monoclonal anti-TLR3 antibody can attenuate the *in vivo* pro-inflammatory cytokine production in response to poly I:C (Bunting et al., 2011). However, many studies have examined downstream inhibitors of the TLR3 pathway, particularly with respect to NF-κB signaling. As of 2006, a review of the literature revealed over 750 reported inhibitors of the NF-κB pathway. This exhaustive list includes a wide range of proteins, peptides, synthetic compounds, and naturally occurring products (Gilmore & Herscovitch, 2006). Although the *in vivo* success rate for inhibition of NF-κB signaling remains remarkably low, the plethora of substances capable of regulating NF-κB activation and the subsequent production of pro-inflammatory cytokines suggests that many potential environmental and biological processes may converge to modulate cytokine production in response to TLR agonists. In accordance with this notion, one of the primary aims of the current study was the evaluation of the potential anti-inflammatory role of the flavonoid luteolin and the PKR inhibitor C16 in mediating poly I:C induced cytokine production.

The *in vivo* and *in vitro* effects of luteolin

The results of the current set of studies support the findings of numerous reports suggesting that luteolin is capable of reducing the production of pro-inflammatory cytokines in response to TLR activation. Furthermore, the calculated IC_{50} for luteolin was approximately 25 μM (Fig 2.1), which is in very close agreement with previously

reported values for the inhibition LPS-induced IL-6 mRNA production in primary murine microglia and BV-2 cells (Jang et al., 2008). Similar values were also obtained for the inhibition of TNFα production in response to LPS in bone marrow-derived macrophages (Comalada et al., 2006). Likewise, Lee et al. (2009) found that 50 μM luteolin pretreatment blocked poly I:C-induced IL-6 mRNA production in bone marrow-derived macrophages. I observed a significant decrease in poly I:C-induced IL-6 mRNA with pretreatment of 25 μM luteolin in RAW 264.7 macrophage-like cells. Although these results are consistent with previous findings for an anti-inflammatory role of luteolin, I also observed strong inhibition of protein synthesis, decreased expression of actin mRNA, and a reduction in cell number following luteolin treatments. Few studies reporting the anti-inflammatory effects of luteolin have considered the potential impact of the compound on cell growth and survival. Li et al. (2008) compared the antioxidative and cytotoxic profiles of 20 flavonoids, including luteolin, in normal human liver cells (L-02) and human hepatoma cells (HepG2). For all flavonoids tested, the potency for the antioxidative effects was similar to the potency that induced cytotoxicity, as determined by MTT assay for cell viability (Li, Liu, Zhang, & Yu, 2008). I obtained similar results for the potency of luteolin in reducing IL-6 production and general protein synthesis. Furthermore, I did not find any *in vivo* efficacy for luteolin in reducing poly I:C-mediated IL-6 production. Collectively, the results of the current study indicate that luteolin does not have a favorable profile for reducing inflammatory response to dsRNA.

The *in vivo* and *in vitro* effects of the PKR inhibitor C16

The antiviral response to infection is characterized by the production of pro-

inflammatory cytokines and inhibition of protein synthesis (Jacobs et al., 1996). The role of the dsRNA dependent kinase R (PKR) in mediating the response to dsRNA has been the subject of numerous conflicting reports (Auch et al., 2004; Carpentier et al., 2007). Although many pathways exist for the production of inflammatory cytokines following exposure to dsRNA, PKR is unique in that it can phosphorylate the alpha-subunit of eukaryotic initiation factor (eIF), thereby inhibiting protein synthesis and impairing viral replication (Su et al., 2006). In the current study I examined the effects of a PKR inhibitor, C16, on poly I:C induced IL-6 production and inhibition of protein synthesis. In cultured macrophage-like and astrocyte-like cells poly I:C had no effect on the rate of protein synthesis, but did induce large increases in the pro-inflammatory cytokine, IL-6. Furthermore, I was unable to detect any increase in phosphorylation of PKR or eIF2-α. These data suggest that PKR is not involved in the response to poly I:C, an observation that has been reported by other investigators (Carpentier et al., 2007). However, I did find that C16 potently reduced production of IL-6 protein and mRNA. C16 did not inhibit poly I:C induced degradation of IκB, and the mechanism for reducing IL-6 production remains unclear. Furthermore, I found that C16 potently reduces protein synthesis at concentrations similar to those capable of reducing IL-6 production. Likewise, treatment with C16 over a 16 hour period at half-maximal concentrations for inhibition of IL-6 production reduced the number of cells present in culture. These findings would seem to agree with a previous report that C16 reduces cell growth and proliferation due to inhibition of cyclin-dependent kinases (Chen et al., 2008). Additionally, when the effects of C16 on poly I:C-induced IL-6 production were investigated *in vivo* at a dose previously shown to reduce PKR phosphorylation, no inhibition of IL-6 production was

observed. Overall, the findings in this report indicate that C16 is not a good candidate for reducing the inflammatory response to dsRNA.

The effects of pregnancy on immune function

Although pregnancy substantially alters functioning of the immune system, there is a surprising lack of data regarding the specific differences in immune responses between pregnant and non-pregnant animals in preclinical models of prenatal infection. Recent clinical studies suggest that the classical view of pregnancy as an immunosuppressive state may oversimplify the complex modulation of the adaptive and innate arms of the immune system during gestation (Luppi, 2003). In particular, evidence from both human and animal studies suggests that pregnancy enhances the response of the innate immune system to invading pathogens (Luppi et al., 2006; Gonzalez et al., 2007; Tinsley et al., 2009).

In this dissertation, I examined the response to poly I:C in pregnant and non-pregnant female mice. The results of the current study suggest that pregnancy enhances the response of the innate immune system to viral infection. Pregnancy increased the IL-6 response to poly I:C by almost 3-fold compared to non-pregnant animals. Furthermore, I found that this effect is dependent upon function of the TLR3, as this enhanced response was absent in pregnant mice expressing a mutant form of this receptor. This is the first report examining the effects of pregnancy on poly I:C-induced IL-6 production, and these findings may be important for future investigations of prenatal infection. IL-6 is a key mediator of both the long-term behavioral effects on offspring and the acute hypertensive effects of infection in pregnancy (Smith et al., 2007; Orshal et al., 2004). The dependence

of this response on TLR3 suggests that factors modulating the TLR3 pathway could substantially alter the adverse effects of viral infection on the mother and developing fetus. Furthermore, some studies have indicated that TLR3 is not a key mediator of the response to dsRNA *in vivo* (Gitlin et al., 2006; Gowen et al., 2007). Our results from male and female TLR3 KO mice support the conclusion that other pathways are capable of mediating the response to dsRNA, however the results also indicate that TLR3 may be an important mediator of the response to dsRNA during pregnancy. Others have reported enhanced responses to dsRNA in pregnant rats, compared to non-pregnant animals, and there is evidence that pregnancy leads to an upregulation of the TLR family. Future research examining the regulation of TLR3 and the inflammatory response throughout pregnancy will be required to identify the specific mechanisms responsible for the TLR3-dependent increase in poly I:C-induced cytokine production during pregnancy.

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