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### Publication Date

2014

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Spinal Role of Toll-Like Receptors in Mediating Histamine-dependent and Independent  
Pruritus

A Thesis submitted in partial satisfaction for the requirements for the degree  
Master of Science

in

Biology

by

James Christian Frederick Skahen

Committee in charge:

Professor Tony L Yaksh, Chair  
Professor Michael David, Co-Chair  
Professor Elina I Zuniga

2014

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The Thesis of James Christian Frederick Skahen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2014

## DEDICATION

This is dedicated to the most wonderful Sara K., my incredibly supportive parents, and also to the good people of the UCSD Anesthesia Research Lab. You all made this process a splendid experience.

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## ACKNOWLEDGMENTS

I would like to acknowledge Dr. Tony L. Yaksh for giving me the opportunity to work in the UCSD Anesthesia Research Lab and earn a Master's degree. I would also like to thank him for all his generous support and feedback over my course of study.

I would also like to acknowledge Shelle Malkmus and Kelly Edinger for guiding me through and helping me with my animal experiments, answering my many questions, helping me revise methods, and for their patience and kindness.

I would also like to acknowledge Joanne Steinauer for her incredibly patient direction and advice regarding the immunohistochemistry employed in this project.

I would also like to acknowledge Dr. Roshni Ramachandran for her kindness and instruction on the proper methods for collecting and preparing tissue for analysis.

I also would like to thank Dr. Maripat Corr for very kindly providing a large portion of the animals used in the following experiments.

I would also like to thank Sara Katleman for acting as scribe when medical complications arose from prolonged time spent at a keyboard.

I would like to acknowledge and thank Dr. Linda Sorkin for her helpful discussions, and also for reading and providing feedback for this manuscript.

I would also like to express my deep appreciation for Marc Marino. Without Marc's attitude, knowledge, advice, patience, incredible kindness, and generous assistance this project may not have been successful.

ABSTRACT OF THE THESIS

A Spinal Role of Toll-Like Receptors in Mediating Histamine-dependent and Independent  
Pruritus

by

James Christian Frederick Skahen

Master of Science in Biology

University of California, San Diego, 2014

Professor Tony L Yaksh, Chair

Professor Michael David, Co-Chair

Pruritus is the sensation that induces the desire or act of scratching. Pruritus is of broad clinical significance, but its etiology is poorly understood. Most pruritus is insensitive to traditional antihistamine-treatment, and there is great need of new

mechanisms and treatments of itch. Toll-like Receptors are found to play a role in pruritus. In this work I developed a detection system to analyze scratching induced by intradermal injection of 48/80 or chloroquine (CQ). Roles of TLR3,4,7, and 9 and their adaptors in scratching produced by 48/80 and CQ were examined. 48/80 and CQ initiated a robust scratching in wild type (WT) mice. 48/80-initiated scratching was significantly reduced by TLR3 deficiency but not TLR4,7,9. Adaptor MyD88-knockout reduced 48/80 scratching, while TRIF and MyD88-TRIF deficiency *increased* scratching. TLR3,4,7,9, MyD88,TRIF, or MyD88-TRIF deficiency decreased CQ scratching. Intrathecal TLR4-antagonist LPS-RS did not effect 48/80 scratching, but reduced CQ; 48/80 and CQ significantly increased cFOS expression in ipsilateral, not contralateral, dorsal horn. LPS-RS reduced CQ-induced cFOS, but not 48/80. Dorsal horn GFAP and IBA1 glial-staining revealed no effect of side by 48/80 or CQ. These results support 48/80 and chloroquine acting differentially through several TLRs and adaptors. TLR3,4,7,9, MyD88 and TRIF play a role in histamine-independent CQ-induced pruritus. Only TLR3 and TRIF were required for histamine-dependent 48/80-induced pruritus. Increased scratching in TRIF KO after 48/80, not CQ, suggests a regulatory feedback system. Spinal TLR4 antagonism emphasize that for chloroquine, but not 48/80, a component mediating the role of TLR4 receptors lies within the neuraxis.

## Introduction

Pruritus is the skin localized sensation that leads to the desire to scratch or, in the absence of a verbal declaration, the act of scratching. Widely known as “itching”, pruritus can be induced by a wide range of agents, and symptoms can vary from a momentary, to a life-impacting chronic pruritus. Studies have shown chronic pruritus to affect between 8 and 18 % of the US / world population (Matterne et al, 2013; Dalgard et al, 2004). Some determinants of pruritus include various skin disorders such as atopic dermatitis and eczema, nearly every disease of the liver, diabetes, and even high body mass (Matterne et al, 2013). Pregnancy, allergic reactions, and fungal or bacterial infections are often associated with intense pruritic sensations (Bergman et al, 2013; Pfaar et al, 2009; Siddiqi et al, 2008; Sonnex, 2004). In addition, a variety of chemical entities can also lead to pruritus; including various venoms (e.g. bee stings), chloroquine (used as an antimalarial), opiates (morphine) and a variety of endogenous agents such as histamine (Karppinen et al 2002; Sowunmi et al, 1989; Chaney, 1995; Simone, 1991), bradykinin (Hosogi et al, 2006, Costa et al, 2010), various proteases (Costa et al, 2008), and growth factors (Raap et al 2011). Itching may also be of a psychogenic origin (Yosipovitch, 2003). While pruritus is of great import to humans, pruritus in companion animals (cats and dogs) is an important challenge to the veterinarian– manifesting commonly in the feline and canine populations (Olivry et al, 2013; Scott et al, 2013).

While itching may affect many people every day, the pathophysiology of pruritus is poorly understood. Though often considered to be associated with pain, pruritic stimuli, unlike painful stimuli, evoke not a withdrawal, but an active effort to stimulate (scratch) the affected skin region. Due to the clinical significance of pruritus and its significant impact on the population, it is important that effective treatments be developed, because currently there is no comprehensive treatment of itch (Greaves, 1997; Jeffery et al,

2011). Pruritis has classically been mechanistically divided into two components: histamine-dependent and histamine-independent.

### *Histamine-dependent pruritus*

Common management for pruritus involves use of agents that block the histamine H-1 receptor, reflecting the classic importance of histamine (Davidson *et al.*, 2010; Han *et al.*, 2013). It has long been known that mast cells release histamine when tissues become inflamed or irritated (Benditt *et al.*, 1955; Rowley & Benditt, 1956). Compound 48/80 is often used as a pruritogen in animal models of pruritus as it initiates the release of histamine from mast cells (Koibuchi *et al.*, 1985; Kuraishi *et al.*, 1995). Importantly, histamine is not the sole product released after the activation of mast cells (Theoharides *et al.*, 2007; Rukwied *et al.*, 2000). Mast cell activation is controlled by the wide variety of receptors on the cell surface. The classic mechanism for mast cell activation is in allergic and anaphylactic reactions, wherein the immunoglobulin E (IgE) receptor (FcεRI) leads to exocytosis initiates the degranulation cascade during which the granule contents are released (Theoharides *et al.*, 2007). It is now appreciated that a variety of receptors are expressed by the mast cell, such as neurokinin 1 receptor for substance P (Church *et al.*, 1989; Harvima *et al.*, 2010). Neurotrophin receptors such as NT-3 (Metz *et al.*, 2004), neuropeptides including vasoactive intestinal peptide, calcitonin gene-related peptide, substance P, and endothelin-1 (Metz *et al.*, 2006) can also lead to mast cell activation (Metz *et al.*, 2004, 2006).

With activation, mast cells will release a large number of preformed mediators stored in granules, e.g. amines (histamine and serotonin), cytokines (interleukin-31), peptides (bradykinin), growth factors (NGF, BDNF), proteoglycans (heparin), proteases (trypsin), as well as newly formed mediators, e.g. prostaglandins, leukotrienes,

thromboxanes, chemokines (platelet-activating factor) (Oldford & Marshall, 2014 ). In many instances, the products released can be shown to produce scratching after local cutaneous delivery, including: bradykinin (through BK1/BK2 receptors) (Hosogi et al, 2006, Costa et al, 2010); trypsin through proteinase activated receptors (PAR) (Costa et al, 2008); and Nerve Growth Factor (NGF) (Raap et al, 2011). Needless to say, the myriad contents released by mast cells in response to different stimuli have made further study difficult, and that is why most studies have focused on the direct activation of sensory afferents via pruritogens (Patel & Dong, 2011).

Further complicating interpretation of the contribution of mast cells is that mast cells are not always necessary for compound 48/80 induced pruritus, lending credence to a hypothesized direct effect of Compound 48/80 on sensory afferents. (Dunford et al, 2007; Inagaki et al, 2002).

Studies have shown that histamine induced itch is triggered by the excitation of a subset of small, slowly conducting, unmyelinated C fibers through Histamine 1 receptor (H1R) and Histamine 4 receptors present on the C fiber terminal (Tani et al, 1990; Schmelz et al, 1997; Dunford et al, 2007). H1R activation leads to an influx of calcium ions through transient receptor potential subtype vanilloid 1 (TRPV1) via intermediate actions by phospholipases PLC- $\beta$ 3 and PLA2 (Han et al, 2006; Shim et al, 2007). These histamine-sensitive C fibers project into the superficial dorsal horn where they have been shown to activate dorsal horn neurons (Schmelz et al, 1997, 2003).

#### *Mast Cell / Histamine-independent pruritus*

Histamine-dependent pruritus, e.g. sensitive to histamine receptor antagonists, while common, accounts for only a portion of the observed pruritic responses (Jeffrey et al, 2011; Liu et al, 2012a). Examples of histamine-independent pruritus are: post-

operative recovery, renal disease, and certain cancers, such as Hodgkin lymphoma (Waxler et al, 2005; Mattered et al, 2013). There are many models of histamine-independent itch as defined by a lack of responsiveness to histamine receptor antagonists. A classic non-histaminergic pruritogen in murines and humans is the anti-malarial drug chloroquine (Jimenez-Alonso et al, 1998; Onibogi et al, 2000; Liu et al, 2012a; Sowunmi et al, 1989). The effects of chloroquine on mast cells are unclear. Early work suggests that chloroquine caused mast cell degranulation or that metabolites of chloroquine might trigger release of mast cell mediators such as IgE (Green & Lim 1989; Nosal et al, 1991; Aghahowa et al 2010), but chloroquine induced pruritus is *not* responsive to H-R antagonist treatments (Green & Lim 1989, Liu et al, 2009).

Chloroquine has been shown to excite DRG neurons in a manner dependent on Murine gastrin releasing peptide receptors (Mrgpr) in mice. Interestingly, sensory neurons expressing MrgA3 (the Mrgpr necessary for chloroquine induced itch in mice) were found to be afferent C fibers (Liu et al 2009, 2012a). In addition, DRG neurons expressing MrgA3 also have been shown to express receptors for histamine (H1), and TRPV1 (Sun & Chen, 2007).

Recently, it was shown that chloroquine directly activates MrgprA3 which in turn is coupled to TRP channels (Liu, 2009; Imamachi et al, 2009; Patel & Dong, 2011; Wilson et al 2011). This TRP channel in mice was identified to be transient receptor potential subtype ankyrin 1 (TRPA1) in the case of chloroquine and other histamine-independent pruritogens, including Bovine adrenal medulla peptide 8-22 (BAM8-22), and SLIGRL-NH<sub>2</sub> (Wilson et al, 2011; Liu et al, 2010, 2012, 2012a; Han, 2013).

It should be noted that studies have found TRPV1 positive afferent C-fibers required for histamine-independent pruritic response mediated by TRPA1, but not necessarily TRPV1 itself (Liu et al, 2009; Han et al, 2012). In addition, researchers have



identified populations of histamine sensitive TRPV1 positive neurons also expressed MrgprA3, leading some to suspect a population of itch sensitive neurons (proposed “pruriceptors”) that could respond to multiple pruritogens to evoke scratching (Imamachi et al, 2009; Mishra et al, 2011; Han et al, 2012).

### *Toll-Like receptor signaling*

An interesting advance in our understanding of pruritus is the finding that Toll Like Receptors (TLRs) are expressed both on afferent C fibers and on neurons in the spinal dorsal horn; and these spinal TLRs have been implicated in modulating pruritic responses to both histamine-dependent and histamine-independent pruritogens (Liu et al, 2012, 2012a; Kim et al, 2011).

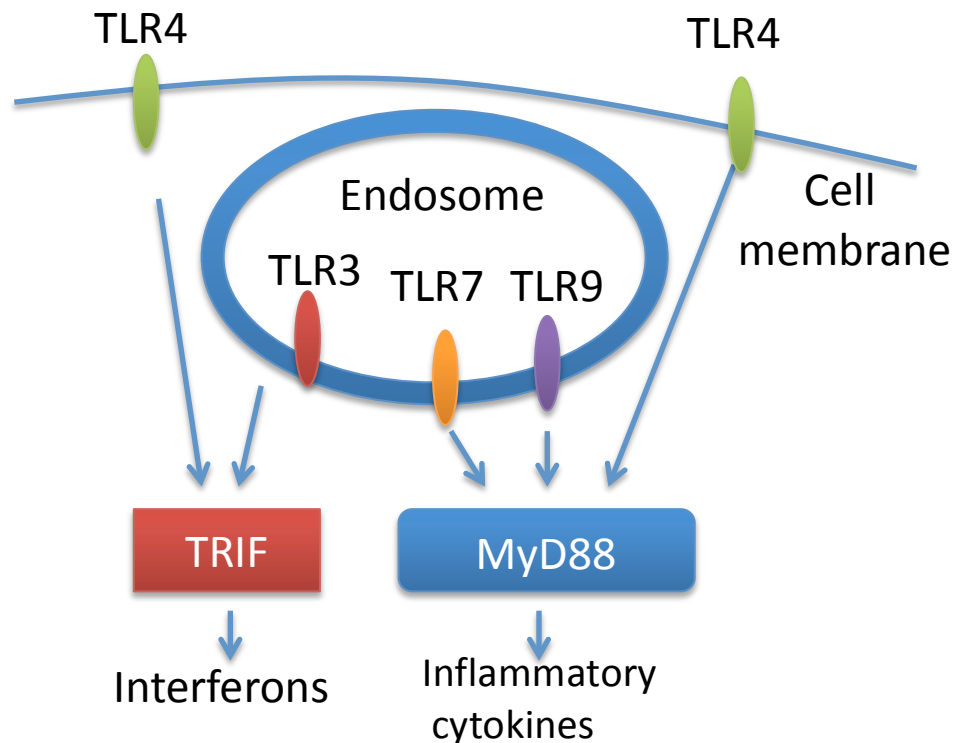
TLRs are key recognition structures fundamental to the innate immune response by sensing pathogen-associated-molecular-patterns (PAMPS) like those of invading microbes, and also damage-associated-molecular-patterns (DAMPS) which are endogenously produced during tissue damage (see Table 1.1) (Akira et al 2006; Kawai et al, 2007). There are 11 human and 13 mouse TLRs that are known, that when coupled with their respective adaptor molecules (which aid downstream signaling after initial TLR recognition of its unique ligand), function in the innate immune system’s ability to recognize a wide variety of potential threats to an organism and coordinate a sufficient response (Kawai et al, 2007). Broadly speaking, the TLRs can be divided into those localized to the cell surface and others on endosomes. These respectively signal through several cascades characterized by specific adaptor proteins (See Figure 1.1). The two prominent pathways being dependent on:

i) The myeloid differentiation factor 88 (MyD88). The MyD88 activation pathway, common to all TLRs except TLR3, serves to activate NF- $\kappa$ B, leading to pro-inflammatory

cytokines such as TNF and IL-1 $\beta$  (Akira et al, 2004).

ii) The TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF). The Trif pathway is employed in both TLR3 and TLR4 signaling, and leads initially to type I interferon production but also serves in a delayed fashion to activate NF- $\kappa$ B (Kiwai & Akira, 2006).

TLR activation, through these two pathways, can variously yield a wide-range of cellular actions.



**Figure 1.1 TLR pathway and key adaptor proteins of interest.**

Depicted are the TLR pathways and adaptor proteins relevant to this body of work. TLRs 3,7,and 9 are all localized to the endosome, while TLR 4 is located on the cell surface. All TLR responses are mediated by MyD88 except for TLR3 which signals primarily through the TRIF pathway it shares with TLR4.TLR activation through MyD88 leads to the expression of proinflammatory cytokines while signaling through TRIF leads to the production of TNF and Type I IFN.

**Table 1.1 TLR ligands.** TLRs play a key role in the immune system, via recognition of multiple pathogen-associated molecular patterns. TLRs sense pathogens and provoke adaptive response, recognizing structurally conserved molecules derived from: damage products, microbes, viruses, bacteria cell walls, flagella, etc.

Receptor	Exogenous Ligands	Endogenous Ligands
<b>TLR3</b>	Viral, dsRNA (Poly(I:C))	mRNA
<b>TLR4</b>	Gram- bacteria (LPS)	HSP-22,-60,-70; HMGB1, fibronectin, defensin 2, oxLDL
<b>TLR7</b>	Viral, ssRNA	selfRNA,miRNA
<b>TLR9</b>	CpG-DNA, microbial	selfDNA, HMGB1

Systematic examination of TLR expression has shown that TLRs are expressed in spinal microglia and astrocytes. Microglia express most TLRs, while astrocytes predominately express TLR3 and less so TLR2 (Bowman, et al, 2003; Bsibsi et al, 2002; Jack, et al, 2005). The functionality of these spinal glial TLRs is emphasized by *in vitro* studies of a robust TNF release following the addition of TLR1/2, 2, 4, 5, and 2/6 ligands to spinal microglial cultures while primary astrocyte cultures showed TNF release following TLR 2, 4, and 5 ligands. TLR3 ligands conversely increased  $\text{INF}\beta$  release (Stokes et al, 2013). *In vivo* spinal glia play an important role in small afferent processing though the release of pro excitatory mediators (Hua et al, 2005; Mika et al, 2007). In addition, TLR3 is expressed by neurons in the trigeminal ganglia (Lafon et al, 2006; Prehaud et al, 2005) and TLRs 3, 4, 7 and 9 in dorsal root ganglia (DRG) (Barajon et al, 2009; Qi et al, 2011; Wadachi & Hargreaves, 2006). Murine DRGs exposed to TLR ligands have been found to increase expression of proinflammatory chemokines and cytokines, as well as expression of TRPV1 receptors (Barajon et al, 2009).

### *Potential role of TLRs in pruritus*

An evolving literature suggests that, when examined, at least some TLRs play a key role in the mediation of pruritus. A reduction in scratching in response to both histamine and chloroquine was shown in TLR3 deficient mice, and in response to chloroquine in TLR7 deficient mice (Liu et al, 2010, 2012a). Because of the increasing evidence that some TLRs, like TLR3 and 7, are expressed in primary sensory neurons, (dorsal root ganglia and trigeminal ganglion neurons) which carry signals from sensory organs (chemical, mechanical) in the periphery to the brain, there may be other TLRs or their downstream effector molecules that might be possible targets for new treatments of pruritus, as they may be crucial in the signal transduction pathway initiated by various pruritogens (Liu et al, 2012a; Kim et al, 2011).

Though some TLRs have been implicated in mediating histamine-dependent and independent pruritus, the role of the other TLRs and which of their extensive downstream adaptor molecules are actually involved in the response to specific pruritogens is not clear (Ji, 2012). TLR4 has been found in TRPV1 positive neurons, and more recent studies have shown the TLR4 agonist Lipopolysaccharide (LPS) binds trigeminal neurons (TGs) and increases TRPV1 activity (Wadachi & Hargreaves, 2006; Diogenes et al, 2011). TLR 4 is also co-expressed in DRGs with calcitonin gene related peptide (CGRP, a neuropeptide reportedly involved in itch hypersensitivity (Steinhoff et al, 2003). LPS exposure enhanced CGRP release in a TRPV1-dependent manner (Ferraz et al, 2011). However, despite potential connections drawn between TRPV1, CGRP and itch, the role of TLR4 in pruritus remains unclear and given its ubiquitous distribution in the periphery and within the spinal cord, the site of TLR4 function contributing to the pruritic response is not known (Liu & Ji, 2014).

### *Preclinical evaluation of pruritic behavior*

Development of new treatments often involves preclinical (animal surrogate) models for a particular syndrome, and it is well known that animals demonstrate scratching in response to common pruritogens delivered into the skin. Thus, mice that receive either an intradermal injection of histamine or chloroquine in the skin on the lateral side of the back of their neck will scratch with the hind paw on the same side as the injection. The assessment of this behavior involves the counting of the incidence of these ipsilateral scratches. A common methodology for this assessment has been visual counting. Given the rapidity of the observed behavior and the fatigue associated with following the behavior for intervals typically up to an hour, the behavior of the animal has been analyzed by being recorded using a video camera and scratch counting undertaken by a observer without knowledge as to treatment (see LaMotte *et al.*, 2011). While video recording has important advantages, it still requires a labor-intensive analysis, which characteristically will vary over time due to observer fatigue. Further, such observational analysis requires considerable training and cross validation of each observer to permit comparison of scoring in a single study by multiple observers— raising the issues of intra-observer stability over time, and inter-observer reliability between animals. Such variables, though not often addressed in many published papers, make this visual method an approach that has intrinsic variability, thereby increasing the requirement for group size to achieve sufficient power. A variety of “automated” techniques have been employed such as observer counting from video recordings, motion-tracking software to quantify the behavior of animals in response to various treatments has been developed (see Marino *et al.*, 2012).

One project of the UCSD Anesthesiology Research Laboratory was the development of a system to count homotopic scratching of the back with the hind paw. My colleagues and I have developed a Paw Motion Detector (PMD) machine which can show the homo-laterality and site-specific of scratching behavior in response to the pruritogenic compounds 48/80 (histamine-dependent mechanism) and chloroquine (histamine-independent mechanism) (Marino et al, 2012). Using the PMD it is possible to quantify scratching initiated by such intradermal injections. I employed this technology in the present series of studies to determine the role of the TLRs on the scratching behavior initiated by intradermal 48/80 and chloroquine to characterize their role in histamine dependent and histamine independent pruritus.

The above comments emphasize several points. i) There is a lack of understanding as to the role of TLR receptors other than TLR3 and TLR7 in the pruritic response. ii) While limited work has pointed to several TLRs, there is less work assessing the contribution of the associated adaptor proteins. In recent work by the Yaksh laboratory, for example, it was found that KO of the TLR4 and MyD88 diminished pain initiated by the spinal delivery of a TLR4 agonist (LPS). Similarly, KO of TLR3 and TRIF reduced the effects of TLR3 activation by poly IC. However, quite unexpectedly, the TRIF KO actually led to an enhancement of pain states generated by TLR4 activation (Stokes, et al 2013). iii) As chloroquine and 48/80 appear to initiate scratching behavior through distinguishable pathways, it would be important to determine the differential effects of the several TLRs and their adaptor proteins on the pruritus initiated by these two classes of pruritic stimuli. iv) Finally, the majority of work involving the TLR receptors has employed KO mice. Such data, while important, are confounded by the

fact that the KO influences both central and peripheral TLR expression and there is the potential issue of adaptive changes that may occur during development of the mutant mouse. Accordingly, I sought to determine if the role of one of the TLRs (TLR4) could be demonstrated to be associated with an action within the spinal dorsal horn by the use of spinally delivered TLR4 antagonism. Importantly, in that work I sought to determine if the pruritic response initiated by ID chloroquine and 48/80 was acutely associated with activation of dorsal horn neurons and glia, and, if so, was it affected by spinally delivered TLR4 antagonist?

My overarching hypothesis in this work is that Toll like receptors and associated downstream signaling adaptors TRIF and MyD88 play a role in spinal processing of both histamine-dependent and independent pruritus. To test this hypothesis, I employed the PMD model of mouse hind limb scratching in response to histamine-dependent and - independent pruritogens in various TLR and associated adaptor protein deficient animals. I tested our hypothesis as described in the following specific aims:

1. To determine the effects of 48/80 and chloroquine on scratching behavior in WT mice as reported by PMD.
2. To examine the roles of TLR3, TLR4, TLR7, TLR9, MyD88, or TRIF, 48/80 and chloroquine induced scratch was studied using PMD.
3. To test whether TLR4 is mediating pruritus spinally, the effects of intrathecal TLR4-antagonism on scratching behavior induced by 48/80 and chloroquine were assessed via PMD.
4. To test whether changes in pruritic responses in KO animals could be assessed at the neuronal level, c Fos, microglia, and astrocyte activation was studied via immunohistochemistry.

## Materials and Methods

### Animals

Animal experiments were carried out in accordance with the protocols and regulations approved by the University of California San Diego Institutional Animal Care and Use Committee. Male C57Bl/6 mice (25-30g) were purchased from Harlan (Indianapolis, IN, USA) and used as Wild Type (WT) in this study. *Tlr3<sup>-/-</sup>*, *Tlr4<sup>-/-</sup>*, *Tlr7<sup>-/-</sup>*, *Tlr9<sup>-/-</sup>*, *Myd88<sup>-/-</sup>*, and *Myd88/Trif<sup>#ps2</sup>* mice were a gift from Dr. M. Corr (University of California, San Diego) and contained the C57BL/6 background (see Table 2.1 for knockout animal genotypes). Animals were received and allowed a minimum 7-day period of acclimation prior to being admitted into the study. Animals were housed in groups of 2-4 in standard rodent cages, maintained in a 12-hour light/dark cycle environment and allowed *ad libitum* access to water and standard rodent chow. All studies were carried out between 10:00 h and 16:00 h of the light cycle.

**Table 2.1: Summary of mouse strains.**

This table shows the non-wild type mouse strains used in this paper, and the nomenclature they are referred to by. In addition to genotype, this table lists the extent of each deficiency.

Protein	Genotype nomenclature	Type of Deficiency
<b>TLR3</b>	<i>TLR3<sup>-/-</sup></i>	TLR3 whole-body knockout
<b>TLR4</b>	<i>TLR4<sup>-/-</sup></i>	TLR4 whole-body knockout
<b>TLR7</b>	<i>TLR7<sup>-/-</sup></i>	TLR7 whole-body knockout
<b>TLR9</b>	<i>TLR9<sup>-/-</sup></i>	TLR9 whole-body knockout
<b>MyD88</b>	<i>MyD88<sup>-/-</sup></i>	MyD88 whole-body knockout
<b>MyD88 and TRIF</b>	<i>MyD88-TRIF<sup>-/-</sup></i>	MyD88 whole-body knockout and TRIF point-mutation on <i>ticam1</i> gene
<b>TRIF</b>	<i>TRIF<sup>#ps2</sup><sup>-/-</sup></i>	TRIF adaptor protein point-mutation on the <i>ticam1</i> gene



### *Drugs*

In the present study, intradermal treatment volume was 0.05ml. Drugs used were Compound 48/80 (48/80 [0.5mg/ml]) and Chloroquine (CQ [4mg/ml]) prepared for delivery in saline (0.9%) (Sigma-Aldrich, St Louis). Normal saline was used as a control in initial scratch experiments to determine efficacy of 48/80 and CQ. TLR 4 antagonist LPS-RS (Lipopolysaccharide *Rhodobacter sphaeroides*) was purchased from Invivogen (San Diego, CA) and dissolved in saline (0.9%). LPS-RS [2µg/µl] and saline (0.9%) were delivered intrathecally in a volume of 5µl. All drug doses are consistent with previous studies.

### *Animal Preparation*

A lightweight metal band was temporarily affixed to the hind paw ipsilateral to the future site of injection using a drop of cyanoacrylate. The animals were then acclimated to the testing chambers for 40 minutes. Before inducing scratching behavior, the animal was first lightly sedated (Isoflurane 2% in Air, until loss of righting response). The dorsolateral aspect of the neck and upper shoulder was then shaved on the side ipsilateral to the detection band. In addition, if mice were to receive intrathecal pretreatment, the lower back was shaved to help visualize the lumbar region.

### *Analysis of Intradermal Pruritogen on hind limb scratching*

WT male C57Bl/6 mice were prepared for intradermal injection as above and ID injections of saline, chloroquine [4mg/ml], or compound 48/80 [0.5mg/ml] were administered to the dorsolateral aspect of the neck in volumes of 0.05ml using a 30-gauges needle while the animal remained lightly sedated. The animals were then

returned to the PMD testing chamber for scratch analysis. The animals were ambulatory within 1-2 minutes. Each experiment was of a 40 minutes duration.

*Analysis of the role of TLR and associated-adaptor proteins in pruritogenic itch*

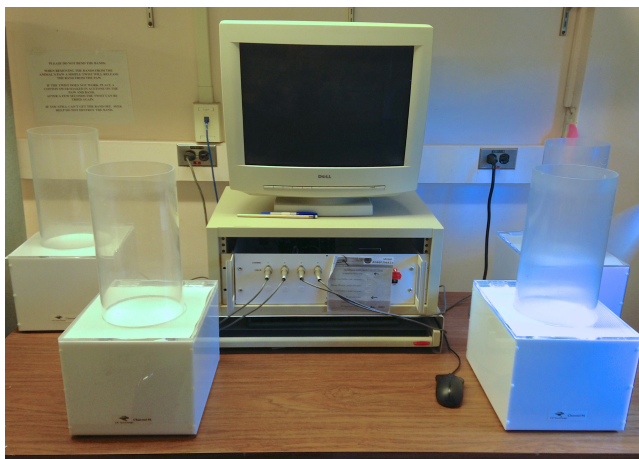
To assess the role played by certain TLRs and related adaptors, knockout (KO) mice were prepared for intradermal injection as above. Knockout mice received ID 48/80 [0.5mg/ml] or ID Chloroquine [4mg/ml] into the nape of the neck just above the shoulder under light sedation. Mice were then transferred to the test chamber for 40 minute scratch assessments. Animals were ambulatory within 1-2 minutes.

*Intrathecal TLR 4 antagonist pretreatment on induced itch*

If animals were to receive IT pretreatment, animals were prepared as above. In lightly sedated animals, intrathecal needle placement for the IT Saline and IT LPS-RS pretreatment was performed as previously described (Hylden, 1980). A 1" 30-gauge needle attached to a 50 $\mu$ L Hamilton syringe was inserted between the L5 and L6 vertebrae, evoking a flick of the tail. Randomly assorted animals either received IT saline or IT LPS-RS (2 $\mu$ g/ $\mu$ l) in a volume of 5 $\mu$ l. Mice were then removed from 2% isoflurane in air and placed back in their cages once ambulatory. 1 hour after IT pretreatment, animals were again briefly induced with isoflurane until loss of righting reflex (approximately 3 minutes). Sedated animals were then injected in the dorsolateral aspect of the neck intradermally with compound 48/80 [0.5mg/ml] or chloroquine [4mg/ml] in a volume of 0.05ml using a 30 gauge needle. Mice were then transferred back to the PMD test chamber for scratch analysis. Animals were ambulatory within 1-2 minutes.

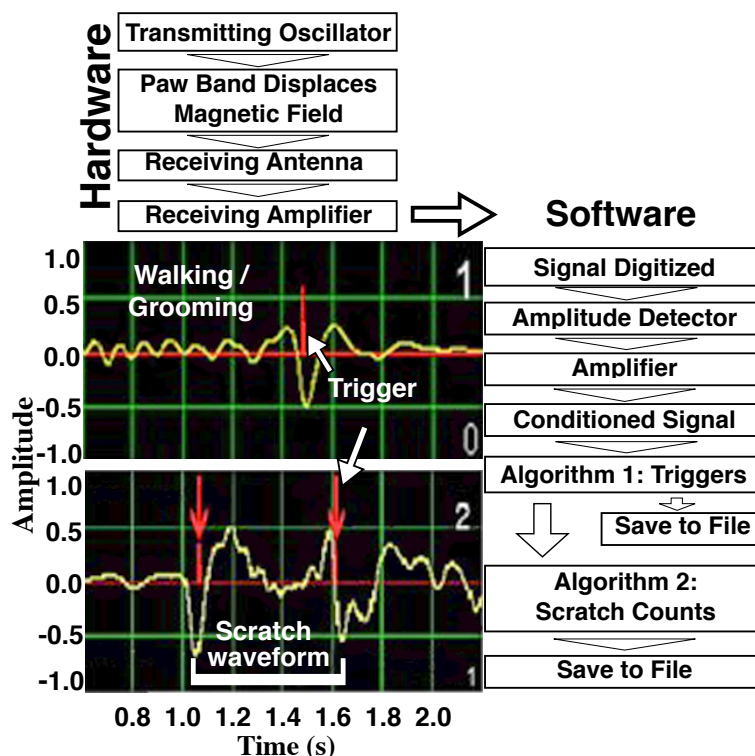
### *Automated Scratch Quantification*

Image 2.1 shows the Paw Motion Detector (PMD). The detection system employs transmitting coils under the Plexiglas test cylinder (15 cm diameter, 35 cm high), which emit a 5-8 mW, 6-8 KHz sinusoidal electromagnetic field (Marino et al, 2012). Movement of a small metal band temporarily affixed to the hind paw of the animal subject perturbs the standing wave in the electromagnetic field thereby inducing a signal within the detection coil. See (Yaksh *et al.*, 2001) for details of construction of the detection systems, the paw band and the test chamber. The time varying analog signal produced by the detection coil is passed to a National Instruments signal acquisition system (PCI-MIO-16Xe or USB610e) driven by a LabView 2012-based 4-channel input-processing and output-cataloguing application (National Instruments Corporation, Austin, TX, USA). The signal is amplified and digitized during this process (1000Hz sample rate, 12 bit resolution) (Yaksh *et al.*, 2001). The amplified and digitized “raw waveform” produced by the motion of the band on the paw of the rat can be saved for later analysis or immediately processed over the course of an experiment. Signal processing of the raw waveform by the LabView modules includes smoothing the raw data and specification of the frequency of motion associated with an animal scratching. Processed waveforms are then subject to a controllable peak detector, which counts the number of wave-peaks (Figure 2.1). These peaks correspond to the number of scratch counts or motions of interest. Visual confirmation and prior validation of the PMD in mice using the currently employed pruritogens confirmed the ability of the PMD to accurately report on scratch criteria previously described (Kuraishi et al, 1995). Once processed, the results for all channels are exported to a spreadsheet in 1-minute intervals to allow for subsequent analysis. (see Marino et al, 2012 for further details on PMD).



**Image 2.1: Paw Motion Detector.**

The Paw Motion Detector (PMD) is capable of automatically assessing the scratching behavior of four animals at once. Detection coils are located beneath test chambers.



**Figure 2.1: Raw waveform digitization and analysis by the Paw Motion Detector.**

The complete sequence of events by which scratches are identified and catalogued by the automated Paw Motion Detector starting with the initial motion of a test animal's banded limb to the logging of scratches in a specified database. Digitized animal behavior waveforms are subjected to a number of user-specified algorithm parameters designed to select for certain phenotypes of motion (See Marino et al 2012 for processing specifics and validation).

*c Fos Immunoreactivity*

To determine in part whether there was neuronal activation and whether itch was being mediated peripherally or centrally, c Fos expression in the cervical superficial dorsal horn was examined in mice pretreated with IT saline or IT LPS-RS. Following ID treatment at t=0, mice were deeply anesthetized with beuthanasia (0.04mL) and perfused intracardially with 0.9% saline immediately before 4% paraformaldehyde (t=2 hours). The two experimental groups and the two control pretreatment groups contained 4 mice each for a total of 16 animals. The cervical spinal cord was removed and post-fixed in 4% paraformaldehyde over-night before being transferred to 30% sucrose after 24 hours for cryoprotection. Cervical sections (C2–C4) of the spinal cord were cut as free-floating sections (30 $\mu$ m) and placed in antifreeze. Identification of level of spinal cord innervation mouse was determined through prior analysis tissue sections from C1-T2 in CQ and 48/80 treated mice to determine the level of spinal cord in which c FOS expression was most pronounced (Data not shown). Cervical tissue sections were processed for c Fos immunoreactivity using the avidin-biotin complex method described by Brailoiu et al (2005). Sections were first washed with PBS to remove antifreeze. Next, endogenous peroxidase activity was quenched by washing sections with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes, and then washed again in PBS before being blocked with normal goat serum (10%) for 1 hour. Sections were then incubated with rabbit c Fos antibody for 24 hours (1:20,000; Calbiochem, Germany), and washed with PBS. Goat anti-rabbit secondary antibody was then applied for 1 hour at room temperature (1:1000; Calbiochem, Germany) and then rinsed in PBS. Avidin-biotin complex was then applied for 1 hour after which sections were rinsed with PBS. Vector DAB staining kit was then used to stain sections for 90 seconds after which sections were immediately placed in dH<sub>2</sub>O to stop the reaction. Sections were then dried and mounted. c Fos positive cell counts were

then recorded from each section in laminae 1 and 2 of the superficial dorsal horn (4-6 sections/animal).

#### *Microglia (Iba1) and Astrocyte (GFAP) evaluation*

Mice were deeply anesthetized with beuthanasia (0.04mL) and perfused intracardially with 0.9% saline immediately before 4% paraformaldehyde. The cervical spinal cord was removed, post-fixed in 4% paraformaldehyde over-night and transferred to 30% sucrose after 24 hours for cryoprotection. Cervical sections (C2–C4) of the spinal cord were cut as free-floating sections (30 $\mu$ m). Cervical tissue sections were incubated with anti-GFAP antibody made in rabbit (1:2000, Sigma-Aldrich), and anti-Iba1 antibody made in goat (1:2000, Abcam plc., Cambridge, MA). Secondary donkey-anti-rabbit antibodies conjugated with fluoro-Alexa-488 and Alexa-594 respectively were used to visualize binding sites (1:1000, Molecular Probes, Eugene, OR). Images were captured using an Olympus BX51 with Olympus GH043916-H MagnaFire-SP medical research camera (Olympus, America) and quantified using ImageJ software (NIH, Bethesda, MD). Glia reactivity was characterized by an increase in the quantity of cells and in the cells and morphological changes of these cells (thickening of processes and rounding out of the cell bodies) which lead to an increase in labeling as glia reactivity increased. Microglia (Iba1) and astrocyte (GFAP) staining was quantified by measuring the total optical density area of pixels divided by the total number of pixels in a standardized area of the dorsal horn (background). Images were individually normalized to a per-picture background threshold to account for differences in staining between sections. Staining intensity was examined in laminae 1 and 2 of the superficial dorsal horn with four to six sections analyzed per animal with 8 animals per experimental group. Microglia and astrocyte reactivity was assessed by an increase in the optical density/pixel area for Iba1

or GFAP staining. All data are presented as intensity scores on a scale of 0-255 (255 being the least intense reactivity) after normalization. Statistics were performed on raw data values of intensity. The investigator was blinded to experimental conditions during the quantification.

### *Statistics*

Cumulative Scratch counts are presented by median and interquartile range when one variable is under examination (e.g. WT v. KO). Differences between groups were analyzed using Mann-Whitney U test as data sets were assumed to be non-parametric. Scratch time course curves are plotted by mean  $\pm$  SEM of scratch counts summed per minute over 40 minutes. Due to the variability between animals, and the small bin sizes, statistical analyses of Time Courses were not carried out. In addition, there was no observable periodicity of either chloroquine or compound 48/80 in early experiments (see Marino et al, 2012). Effect of IT pretreatments on microglia and astrocytes were determined by ipsilateral immunohistochemistry via 2-way non-repeated measures ANOVA. In addition, differences between sides (ipsilateral v. contralateral) were also assessed using 2-way repeated measures ANOVA. cFos data was represented by the means of the average c Fos positive cells/section from each animal, and treatments were compared using 2-way non-repeated measures ANOVA. Linear regressions were carried out between mean ipsilateral c Fos positive cells (per each section and animal) and the corresponding animal's cumulative scratch counts for both IT pretreatments and ID treatments. Significance threshold:  $p < 0.05$ . All analyses were carried out using Prism statistical software (Graphpad, CA, USA).

## Results

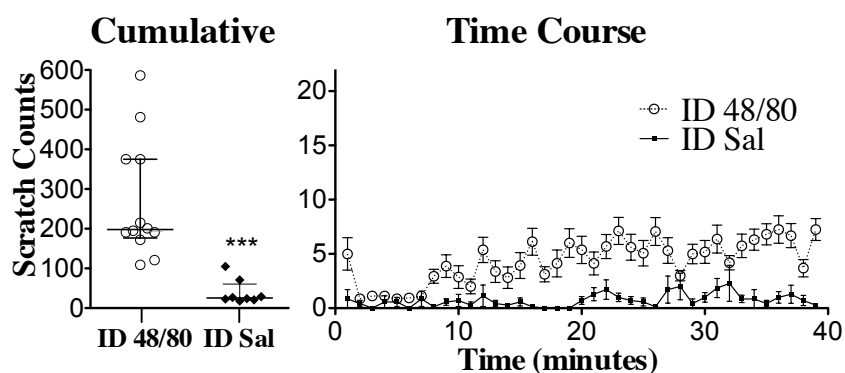
### *Effect of Intradermal Pruritogens on Hind Limb Scratching*

Two pruritogens were examined for their ability to induce scratching in Male C57Bl/6 mice. The intradermal injection of Chloroquine or Compound 48/80 elicited ipsilateral hind limb scratching verified by visual inspection and quantified by the Paw Motion Detector. Upon injection, animals displayed with a short latency, the onset of vigorous bursts of scratching with the hind paw ipsilateral to the intradermal injection of either pruritogen. This scratching lasted through 40 minutes.

Figure 3.1 shows intradermal Compound 48/80 induced a significant increase in cumulative scratching over 40 minutes beginning immediately after ID injection compared to Saline treated animals (Mann-Whitney  $U=0.000$ ,  $n=12,8$ ,  $median_{WT-4880}=198$ ,  $median_{WT-Sal}=25.5$ ,  $p=0.0002$ ). After injection at  $t=0$ , the rate of scratching appears to increase and is sustained beginning around 8 min. Following experiment conclusion, all animals treated with 48/80 showed normal behavior, and absence of scratching and normal weight gain (data not shown).

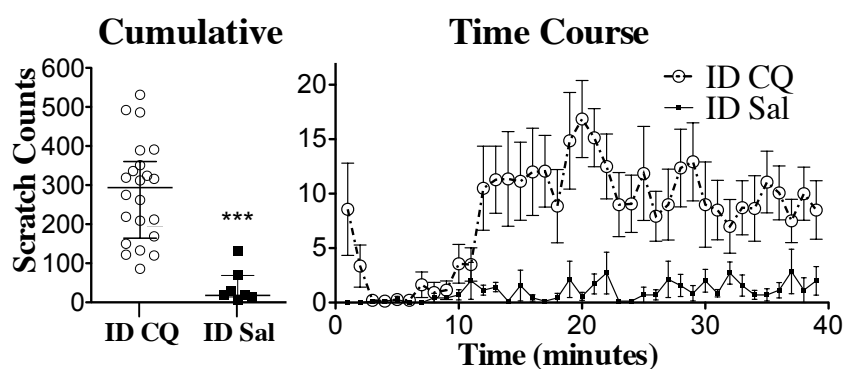
Figure 3.2 shows that mice treated with ID Chloroquine ( $n=22$ ) exhibit a significantly increased scratch count as compared to ID Saline ( $n=8$ ) treated animals (Mann-Whitney  $U=3.00$ ,  $p=0.0002$ ,  $median_{WT-CQ}= 293.5$ ;  $median_{WT-Sal}=18.00$ ). In addition, Figure 3.2 shows that the rate of scratching in Chloroquine treated mice rose by around 10min, and remained relatively sustained. After the experiment was concluded, all animals following chloroquine treatment showed normal behavior, an absence of scratching and normal weight gain (data not shown).





**Figure 3.1: Compound 48/80 induces significant scratching**

ID 48/80 induced a significant increase in cumulative scratching compared to ID Saline induced scratch. (median and interquartile range, mean  $\pm$  SEM, \*\*\*=p<0.001).



**Figure 3.2: Chloroquine induced significant scratching**

ID Chloroquine significantly increased cumulative scratch counts compared to ID Saline (Median and interquartile range, mean  $\pm$  SEM, \*\*\*=p<0.001)

#### *Histamine-dependent Scratching in TLR- and Associated-Adaptor KO Animals*

Following intradermal injection of Compound 48/80 at t=0, hind limb scratching by various TLR deficient animals was examined via PMD over 40 minutes, and compared against wild type animals under the same treatment by the Mann-Whitney U Test (see Fig. 3.1: n=12, median<sub>wt-4880</sub>=195.0).

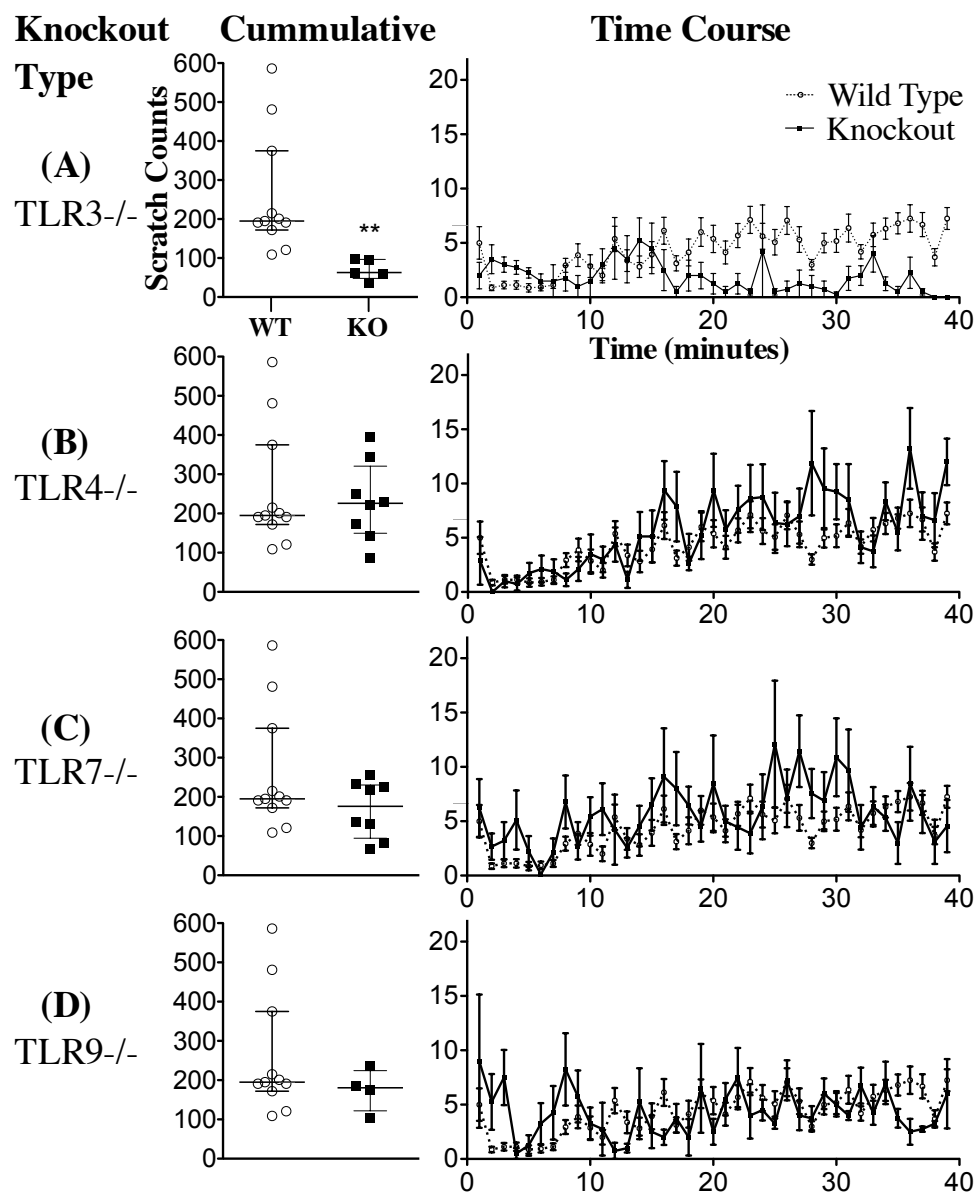
Figure 3.3A illustrates that TLR 3 deficiency significantly reduced scratching compared to WT (Mann-Whitney U=0.000, n=5, p=0.0022, median<sub>TLR3-</sub>=63).

Figure 3.3B shows that there was no difference in scratching exhibited by WT and TLR4<sup>-/-</sup> animals after intradermal 48/80 (Mann-Whitney U=42.50, n=8, p=0.9341, median<sub>TLR4<sup>-/-</sup></sub>=226).

Figure 3.3C shows no significant difference in ID 48/80 treated TLR7<sup>-/-</sup> scratch counts (Mann-Whitney U= 36.00, n=8, p=0.5355, median<sub>TLR7<sup>-/-</sup></sub>=176)

Figure 3.3D similarly shows that TLR 9 deficient animals showed no difference in scratching induced by ID 48/80 compared to WT animals (U= 14, n=4, p=0.3271, median<sub>TLR9<sup>-/-</sup></sub>= 180.3)

Associated-adaptor deficient animals were also injected with ID Compound 48/80 and scratching behavior was assessed for 40 minutes via PMD. Scratch counts were then compared to wild type animals under same treatment ((see Fig. 3.1, n=12, median<sub>wt-4880</sub>=195.0).



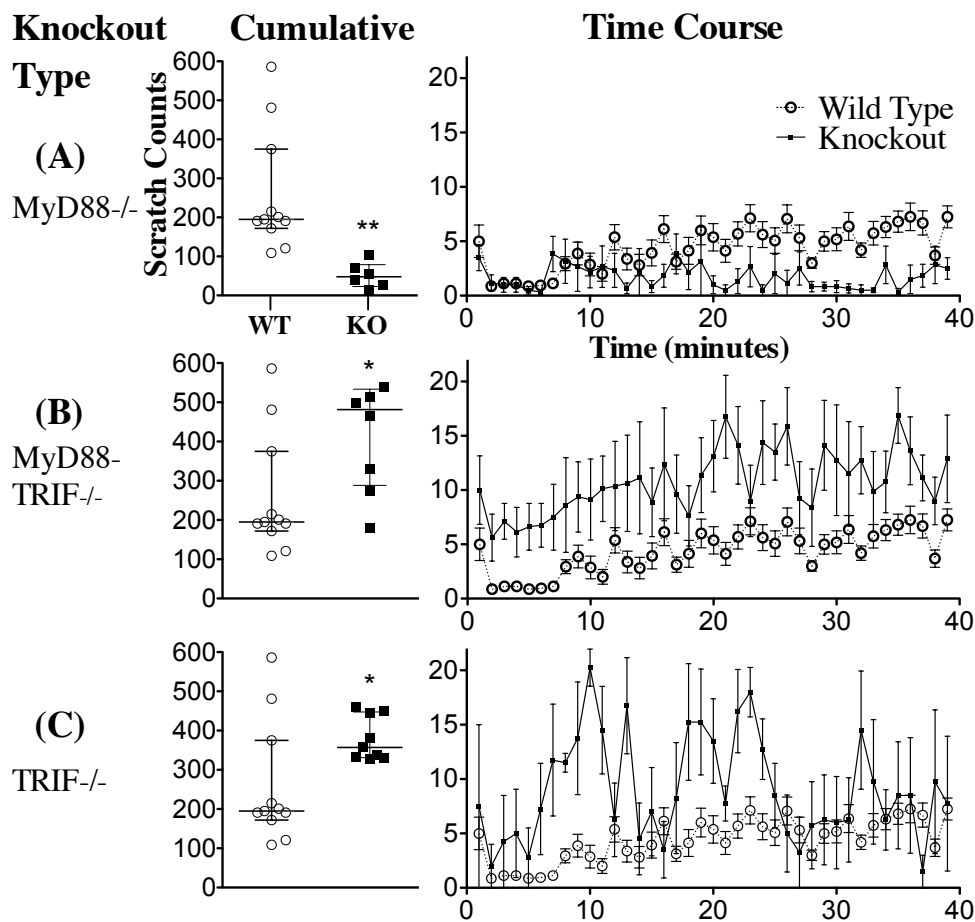
**Figure 3.3: TLR KO Scratch counts post ID 48/80.**

Following ID injection of Compound 48/80 (0.5mg/ml, 0.05ml), male wild Type (WT) and TLR-Knockout (KO) mouse scratching behavior was assessed using the Paw Motion Detector (A) TLR3<sup>-/-</sup> animals exhibited a significant decrease in cumulative scratching compared to WT. (B) TLR4<sup>-/-</sup> animals showed no significant change in scratching. (C) TLR7 deficient animals also showed no significant reductions in scratching. (D) There was no change in CQ induced scratching in TLR9<sup>-/-</sup> animals compared to WT (Median and Interquartile range; Mean  $\pm$  SEM; \*\*= $p < 0.01$ ).

Figure 3.4A shows that MyD88<sup>-/-</sup> animals exhibited significantly reduced scratching after treatment with ID 48/80 (Mann-Whitney U= 0.000, n= 6,  $p=0.0011$ ).

Figure 3.4B shows that animals deficient in both MyD88 and TRIF displayed significantly higher scratch counts after ID 48/80 (Mann-Whitney U= 19.00, n=8, p= 0.0430). In addition, their scratching behavior appears to increase more rapidly as compared to WT mice.

Figure 3.4.C shows that TRIF knockout animals also scratched significantly more than WT ID 48/80 treated animals (Mann-Whitney U= 23.00, n=9, p=0.0482). As in the case of MyD88-TRIF<sup>-/-</sup> animals, TRIF<sup>-/-</sup> mice displayed a unique scratch time course compared to WT animals.



**Figure 3.4 TLR-associated adaptor knockout scratch counts post ID 48/80.**

Following ID 48/80, WT and MyD88, MyD88-TRIF, or TRIF, knockout animal scratching behavior was assessed via PMD. (A) MyD88<sup>-/-</sup> animals shows a significant decrease in scratching compared to. (B) MyD88-TRIF<sup>-/-</sup> and (C) TRIF<sup>-/-</sup> animals also showed a significant decrease in scratching compared to WT (Median and Interquartile range; Mean  $\pm$  SEM; \* $p$ <0.05, \*\* $p$ <0.01).

*Non Histamine-dependent Scratching in TLR- and Adaptor- KO Animals*

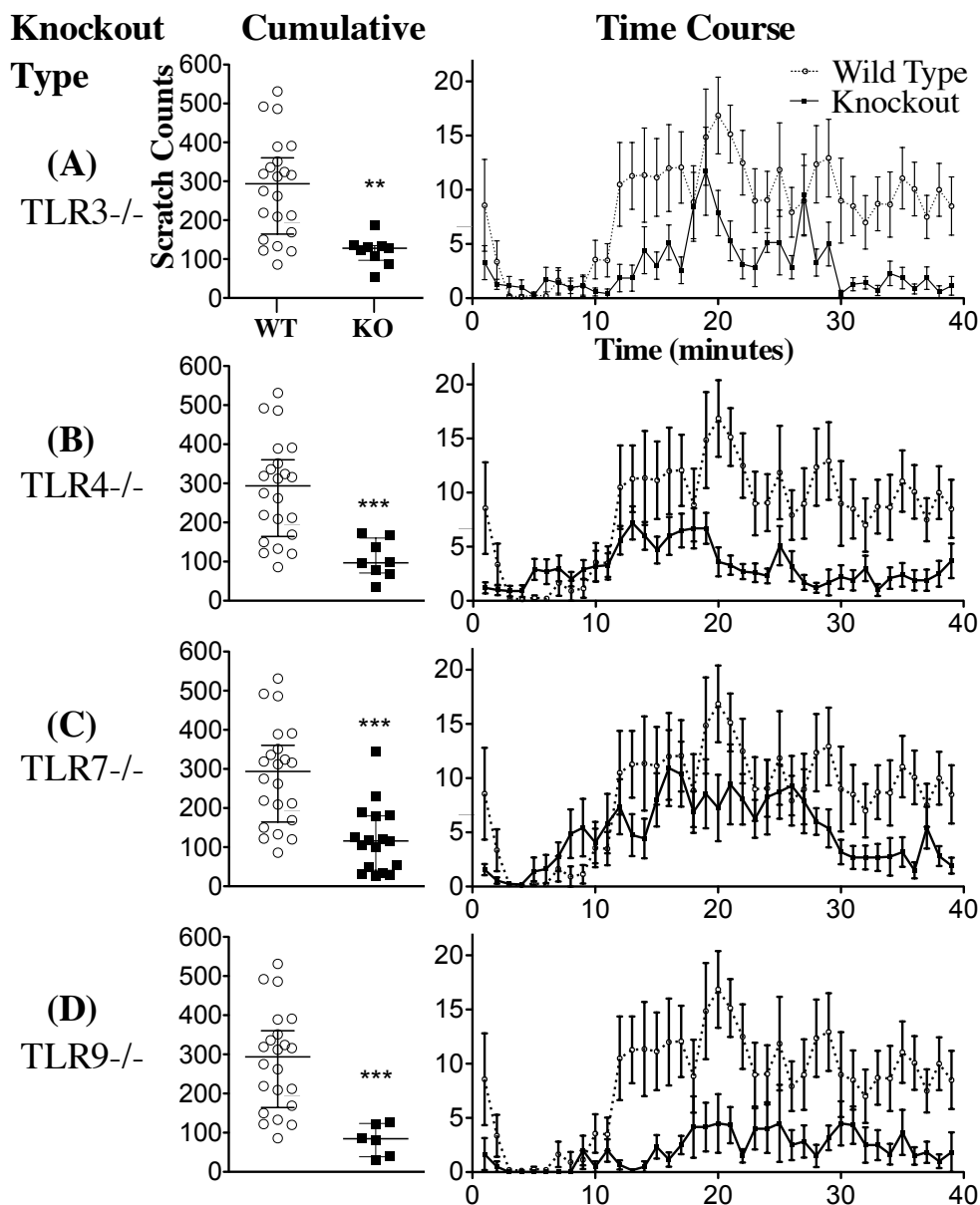
Intradermal injection of Chloroquine- (t=0) induced hind limb scratching was quantified by PMD in various TLR-lacking animals was quantified by via PMD over 40 minutes, and compared to CQ induced scratch in wild type animals by the Mann-Whitney U Test (see Fig. 3.2, n=22, median<sub>WT-CQ</sub>= 293.5).

Figure 3.5A displays the significant reduction in ID Chloroquine induced scratching in TLR 3 deficient mice compared to WT (Mann-Whitney U=250.00, n=9, p=0.0014, median<sub>TLR3-</sub>=120.7).

Figure 3.5B shows that TLR 4 deficient mice also exhibited reduced scratching after ID CQ (Mann-Whitney U= 17.00, n=8, p=0.0009, median<sub>TLR4-</sub>=97.00).

Figure 3.5C illustrates that TLR 7 deficiency also significantly reduces scratching compared to WT animals (Mann-Whitney U= 51.50, n=17, p=0.0001, median<sub>TLR7-</sub>=116.00).

Figure 3.5D shows that TLR9<sup>-/-</sup> presented a significant reduction in scratching after ID Chloroquine (Mann-Whitney U=6.5000, n=6, p=0.0010, median<sub>TLR9-</sub>= 84.5).



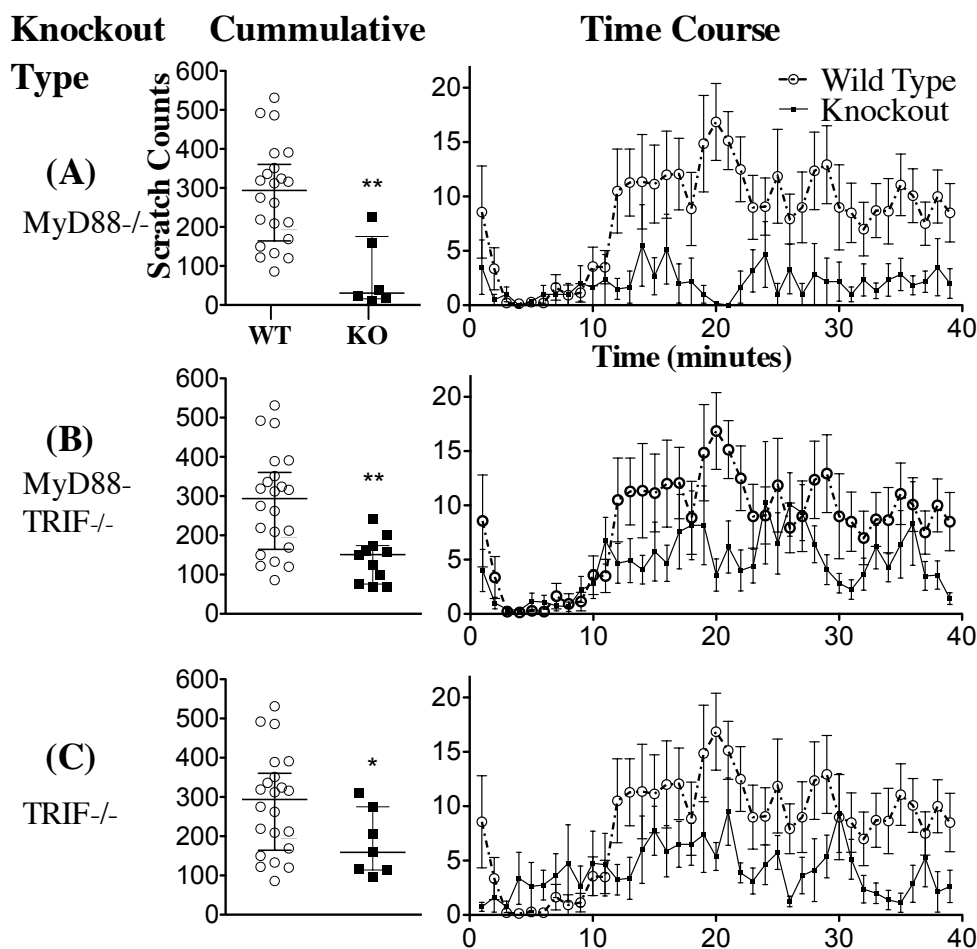
**Figure 3.5: TLR KO Scratch counts post ID chloroquine.**

Following ID Chloroquine, WT and TLR-Knockout mouse scratching behavior was assessed using the PMD. (A) TLR 3<sup>-/-</sup> animals showed a significant decrease in scratching compared to WT. (B) TLR4<sup>-/-</sup> animals exhibited a significant reduction in scratching. (C) TLR 7 deficient animals also showed significant reductions in scratching. (D) CQ evoked a significant reduction in scratching in TLR 9<sup>-/-</sup> (Median and Interquartile range; Mean  $\pm$  SEM; \*\*\*=p<0.001).

Associated-adaptor Knockout animals were also assessed for their sensitivity to ID Chloroquine via scratching quantified by PMD. After injection at t=0, hind limb scratches were recorded over 40 minutes, and compared to wild type animals also treated with chloroquine using the Mann-Whitney U Test (See Fig. 3.2, n=22, median<sub>WT-CQ</sub>= 293.5).

Figure 3.6A illustrates the significantly reduced scratching elicited from MyD88<sup>-/-</sup> animals by ID CQ (Mann-Whitney U=14.00, n= 6, p=0.0039, median<sub>MyD88<sup>-/-</sup></sub>=30.50; median<sub>WT</sub>=293.5).

Figure 3.6B shows that MyD88-TRIF<sup>-/-</sup> deficient animals displayed significantly reduced scratching after ID CQ (Mann-Whitney U=40.00, n=11, p=0.0021, median<sub>MyD88-TRIF<sup>-/-</sup></sub>=151.0). Figure 3.6C shows TRIF KO reduced scratch compared to WT after treatment with chloroquine (Mann-Whitney U=35.50, n=7, p=0.0366, median<sub>TRIF<sup>-/-</sup></sub>=159.0).



**Figure 3.6: TLR-associated adaptor Knockout Scratch post ID CQ.**

Following ID CQ, WT and MyD88<sup>-/-</sup>, MyD88-TRIF<sup>-/-</sup>, TRIF<sup>-/-</sup> KO scratching behavior was assessed via PMD over 40 minutes. (A) MyD88<sup>-/-</sup> animals show a significant decrease in scratching compared to. (B) MyD88-TRIF<sup>-/-</sup> deficient animals exhibited a significant decrease in. (C) TRIF<sup>-/-</sup> animals showed a significant decrease in scratching compared to (Median and Interquartile range; Mean  $\pm$  SEM; \* $p$ <0.05, \*\* $p$ <0.01).

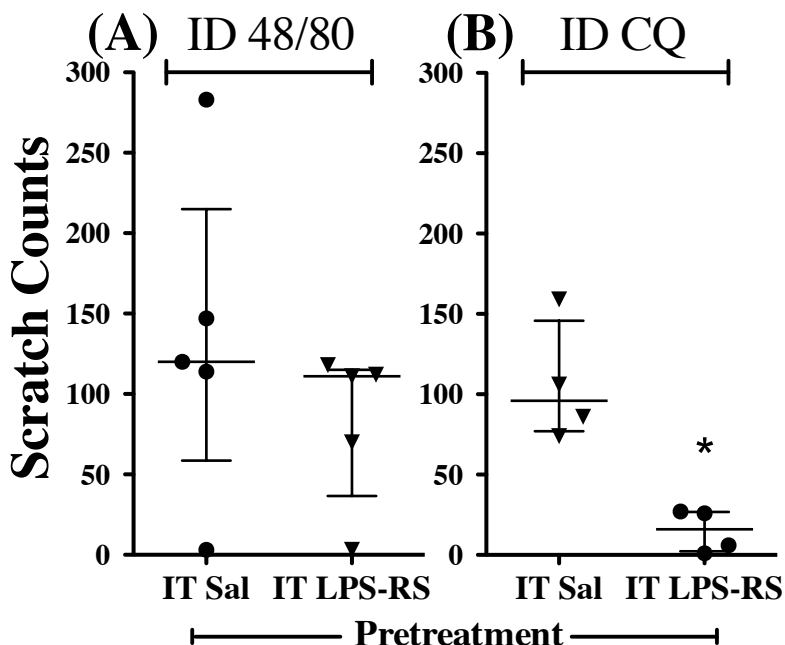
*Effect of Spinal TLR 4 Antagonist LPS-RS on Intradermal Pruritogen induced Scratching*

To further assess the role of TLR 4, the intrathecal delivery of the TLR 4 antagonist LPS-RS was used to confirm previous results and also to determine whether the role of TLR4 in itch is centrally or peripherally mediated. The data presented in Figure 3.7 is from animals for which immunohistochemistry was also performed.

Figure 3.7 shows the effect on scratching of pretreatment with IT LPS-RS 1 hour before treatment. Figure 3.7A shows that animals pretreated with IT LPS-RS then ID 48/80 exhibited scratching that was not significantly different from IT Sal pretreated animals (Mann-Whitney  $U= 1.00$ ,  $n=4,4$ ,  $p=0.0571$ ,  $\text{median}_{\text{LPSRS-4880}}= 111.5$ ,  $\text{median}_{\text{Sal-4880}}=133.5$ ).

However, Figure 3.7B shows IT LPS-RS significantly reduced ID Chloroquine induced scratching compared with IT Saline pretreatment ( $U=0.00$ ,  $n=4,4$ ,  $p=0.0286$ ,  $\text{median}_{\text{LPSRS-CQ}}= 16.00$ ,  $\text{median}_{\text{Sal-CQ}}=96.00$ ).





**Figure 3.7: Effect of TLR-4 antagonist pretreatment 1hr before ID CQ or 48/80.**

Mice pretreated with either IT LPS-RS or IT Saline were treated with ID Chloroquine or ID 48/80. Scratch behavior was recorded for 40 minutes. IT LPS-RS did not significantly reduce scratching in 48/80 treated mice. (B) LPS-RS did significantly reducing CQ induced scratch (Median and interquartile range;  $*=p<0.05$ ).

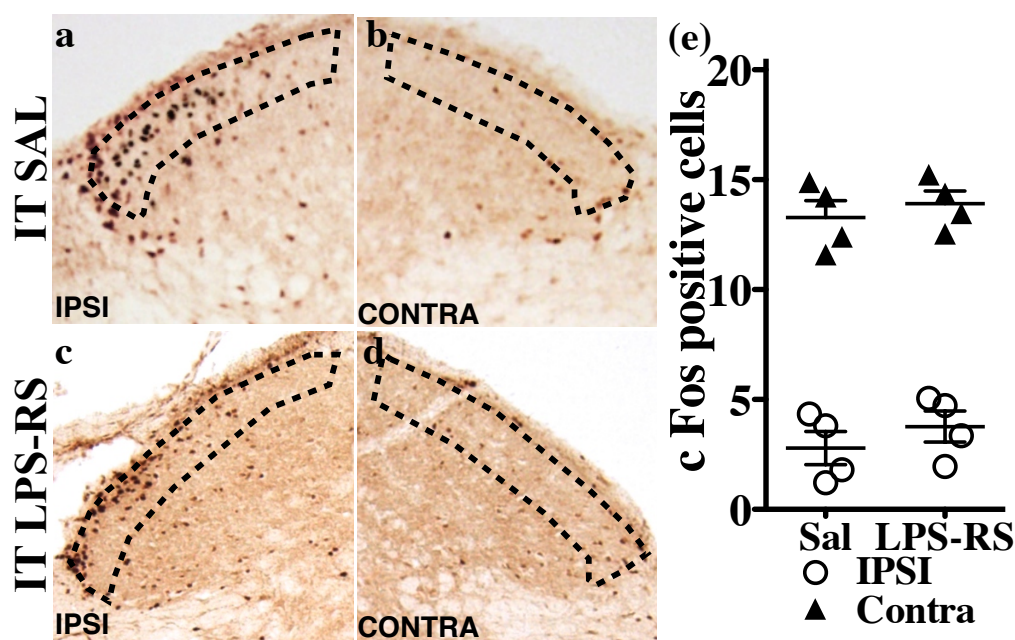
#### *Effect of Spinal TLR 4 Antagonist LPS-RS on ID Pruritogen induced c Fos expression*

In addition to examining scratch in IT LPS-RS 1 hour pretreated animals, cervical spinal cord c Fos expression was also quantified and compared to IT Saline pretreated animals (scratch data presented in figure 3.7). Tissue was harvested 2 hours post ID treatment (3 hours after IT pretreatment) and c Fos positive cells were tallied from Laminae 1 and 2 of the dorsal horn between the spinal levels of C2-C4, and analyzed with a 2-way repeated measures ANOVA.

Figure 3.8 shows typical images of c Fos-stained dorsal horns from 1hour- IT Saline pretreated animals (a,b), and from 1 hour IT LPS-RS pretreated animals (c,d). 3.8e shows that ID 48/80-treated ipsilateral c Fos expression in IT LPS-RS pretreated animals was not significantly changed from IT saline pretreated animals ( $F(1,12)=12$ ,

$p=0.2763$ ;  $\text{mean} \pm \text{sem}_{\text{LPSRS-48/80ipsi}} = 27.80 \pm 04.747$  ;  $\text{mean} \pm \text{sem}_{\text{SAL-48/80}} = 29.56 \pm 4.334$ ; 2-way non-repeated measures ANOVA). 48/80 c Fos expression was, however, significantly different between ipsilateral and contralateral sides within each pretreatment group ( $F(1,6)=260.86$ , 2-way repeated measure ANOVA,  $p<0.000$ ;  $n=5-7$  sections/ mouse, with 4 mice/group;  $\text{mean} \pm \text{SEM}$  plotted).

### ID 48/80-induced cFos post IT Pretreatment

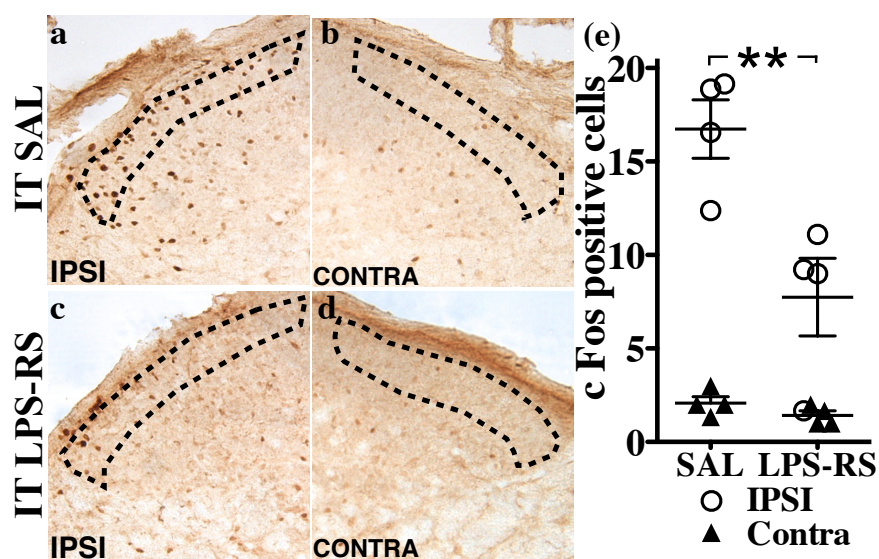


**Figure 3.8 Effect of TLR-4 antagonist 1 hr pretreatment on ID 48/80 induced cFos.** WT mice pretreated with (c,d) IT LPS-RS or (a,b) IT Saline were treated with ID 48/80 after 1 hour. Scratch behavior was recorded for 40 minutes (see figure 3.7), and then cervical spinal cord was collected 2 hours after ID injection. c Fos expression was analyzed in Laminae 1 and 2 of the dorsal horn. (e) Ipsilateral c Fos expression from (c) IT LPS-RS pretreated CQ injected animals was not significantly different than in (a) IT Saline pretreated animals. c Fos expression was significantly different between ipsilateral and contralateral sides for both pretreatments ( $p<0.001$ ) (Images representative of typical immunoreactivity;  $\text{Mean} \pm \text{SEM}$ ).

Figure 3.9 depicts the typical expression of c Fos positive cells (dark-brown) in the ipsilateral superficial dorsal horn from animals pretreated with IT Saline (panels a,b) or IT LPS-RS (c,d) 1 hour before treatment ID CQ. Figure 3.9e illustrates a significant

reduction in ipsilateral c Fos positive immunoreactivity after pretreatment with IT LPS-RS compared with IT Saline before ID CQ treatment ( $F(1,12)=13.39$ ,  $p=0.0033$ ;  $\text{mean} \pm \text{sem}_{\text{LPSRS-CQipsi}}=7.747 \pm 2.081$ ;  $\text{mean} \pm \text{sem}_{\text{SAL-CQ}}=16.74 \pm 1.566$ ; 2-way non-repeated measures ANOVA). CQ induced c Fos expression across sides (ipsilateral v. contralateral) in both pretreatment groups was significantly different:  $F(1,6)=58$ ,  $n=5-7$  sections/mouse, with 4 mice/group; 2-way repeated measure ANOVA,  $p=0.0003$ ;  $\text{mean} \pm \text{SEM}$ ).

### ID CQ-induced cFos post IT Pretreatment



plotted).

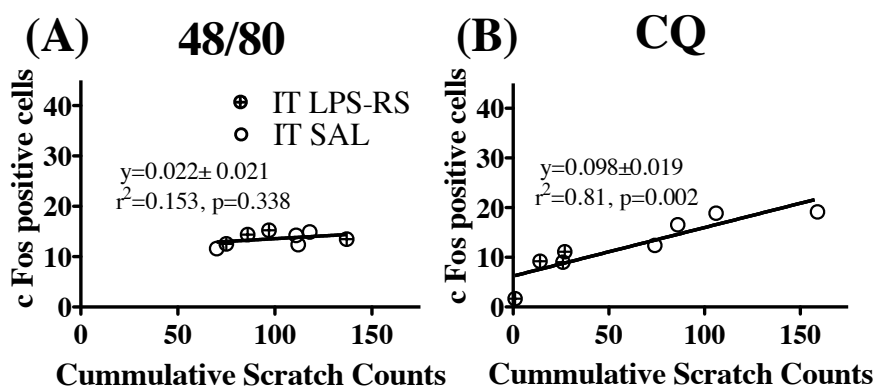
**Figure 3.9: Effect of TLR-4 antagonist 1hr pretreatment on ID CQ induced c Fos.** C57Bl/6 Male mice pretreated with (c,d) IT LPS-RS or (a,b) IT Saline were treated with ID Chloroquine (CQ) after 1 hour. Scratch behavior was recorded for 40 minutes, then cervical spinal cord was collected 2 hours after ID injection, and c Fos expression was analyzed in Laminae 1 and 2 of the dorsal horn(outlined). (e) Ipsilateral c Fos expression from (c) IT LPS-RS pretreated CQ injected animals was significantly reduced compared to (a) IT Saline pretreatment. In addition, there was a significant difference of c Fos expression between ipsilateral and contralateral sides for both pretreatments ( $p<0.001$ ) Images representative of typical immunoreactivity;  $\text{Mean} \pm \text{SEM}$ ;  $**=p<0.01$ )

*Relationship between ID pruritogen induced c Fos expression and scratching*

In order to examine the association between c Fos expression and scratch counts, a linear regression of ipsilateral c Fos positive cells versus ipsilateral scratch counts was performed using data from animals pretreated with IT LPS-RS or IT Saline, and treated with ID CQ or ID 48/80 (see figures 3.7-3.9).

Figure 3.10A shows the slope of a linear regression between the mean c Fos positive cell counts/animal and 40 minutes cumulative scratch in animals treated with ID 48/80 after IT Saline or IT LPS-RS pretreatment (both pretreatments represented by best fit line). The slope of this regression was not found to be significantly different from 0 ( $n=4,4$ ,  $y=0.022\pm 0.021$ ,  $r^2=0.153$ ,  $p=0.338$ .)

Figure 3.10B shows the regression between mean c Fos positive cell counts per animal versus 40 minute cumulative scratching after ID Chloroquine and IT Saline or IT LPS-RS 1 hour pretreatment (both pretreatments represented by best fit line). The slope of this regression was significantly greater than 0 ( $y=0.098\pm 0.019$ ,  $r^2=0.81$ ,  $p=0.002$ ).



**Figure 3.10: Linear Regression of c Fos positive Cells versus Scratch.**

Mice pretreated with either IT LPS-RS or IT Saline were treated with ID Chloroquine (4mg/ml, 0.05ml) or ID 48/80 (0.5mg/ml, 0.05ml). Scratch behavior was recorded for 40 minutes, and cervical spinal cord was collected after 2 hrs and c-Fos expression was analyzed. Slopes represent relationship between mean ipsilateral c Fos expression from each animal and scratch counts regardless of pretreatment. (A) The slope produced by 48/80 was not significantly different from 0. (B) Regression of CQ induced scratch and c FOS counts yielded a slope significantly different from 0.

*Microglial and Astrocytic activity after ID pruritogen and TLR 4 antagonism*

Tissues harvested from animals in previous IT LPS-RS/ IT Saline pretreatment, ID Pruritogen (See Figures 3.7-3.10) experiments were also assessed for spinal Microglial (Iba1) and Astrocytic (GFAP) immunoreactivity. Activation 2 hours post ID injection and 3 hours post IT pretreatment as measured by optical density (OD) is plotted showing mean OD for each animal, after which mean  $\pm$  SEM was calculated for each group.

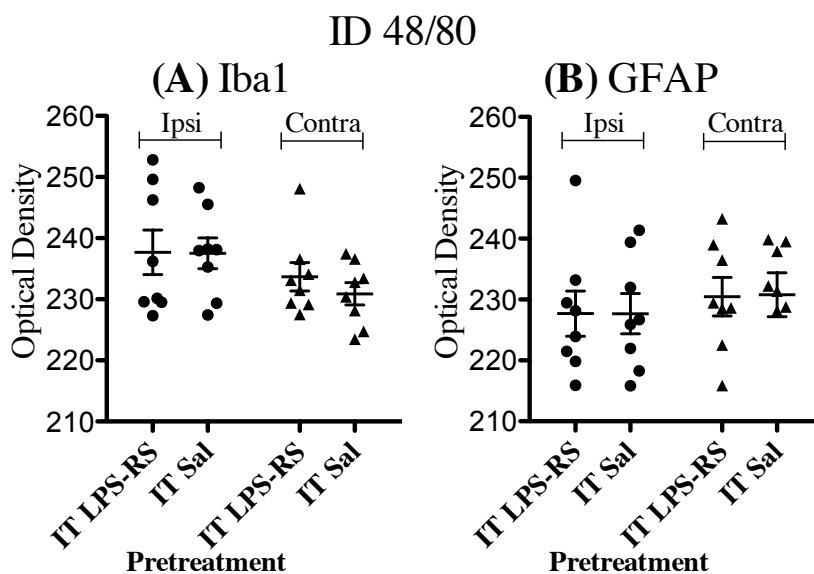
Figure 3.11 shows OD of laminae 1 and 2 of the dorsal horn in tissue sections incubated with antibodies against Iba1 (A), and GFAP (B) after IT Saline or LPS-RS pretreatment followed by ID 48/80.

Figure 3.11A shows that there is no difference in 48/80 microglial activation between IT LPS-RS and IT Saline pretreatment ( $F(1,28)=0.31$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.5826$ ; 2-way non repeated measures ANOVA; mean  $\pm$  SEM). Fig. 3.11A also shows that there was no difference between ipsilateral and contralateral Iba1 staining within pretreatments ( $F(1,14)=0.07$ ;  $n=4-6$  sections/ mouse, with 4 mice/group  $p=0.0567$ ; 2-way non-repeated measures ANOVA, mean  $\pm$  SEM).

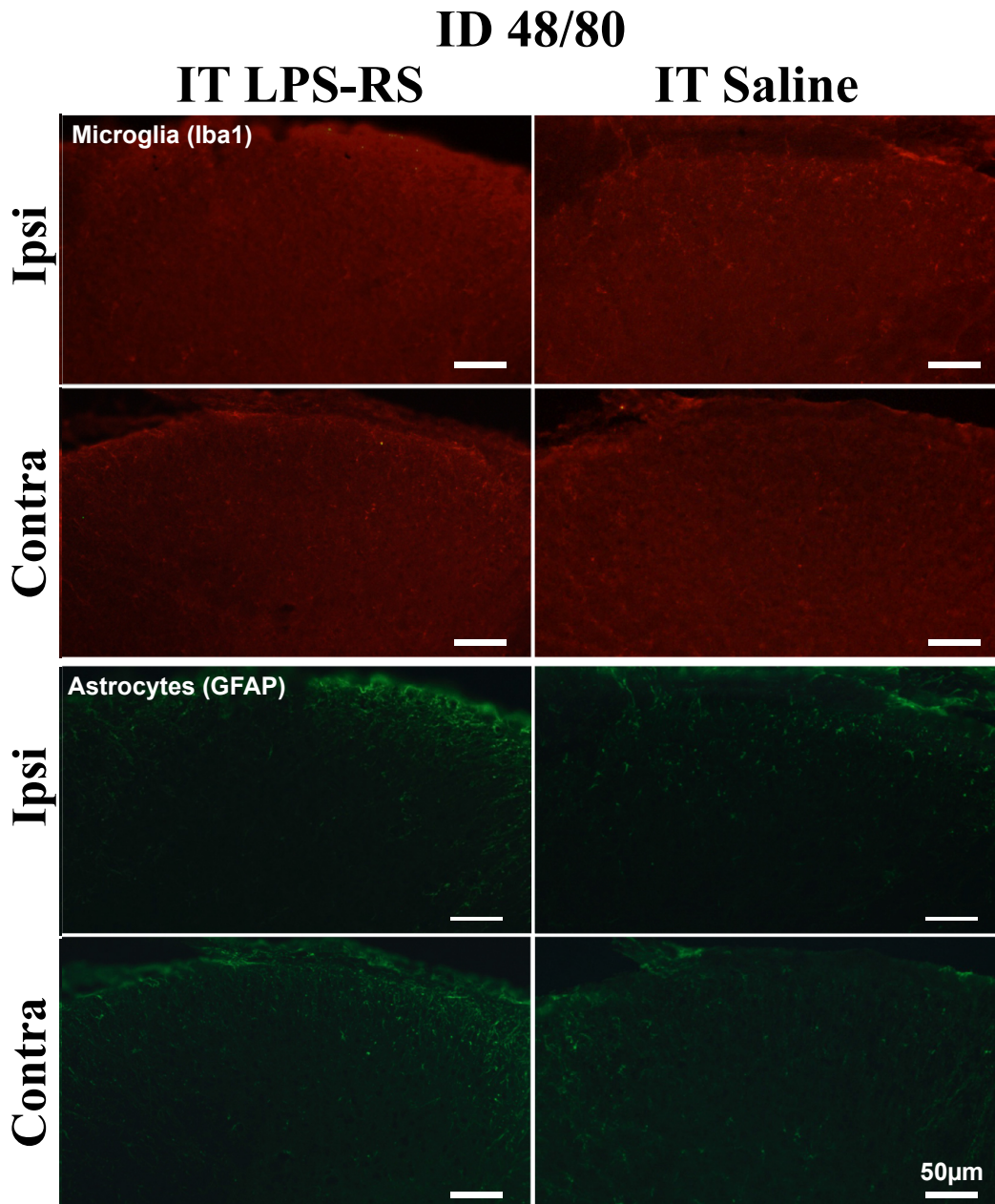
Figure 3.11B shows that there was no difference in ipsilateral astrocyte proliferation in IT LPS-RS pretreatment compared to IT saline ( $F(1,28)=0.00$ ;  $n=4-6$  sections/mouse, 4 mice/group;  $p=0.9636$ ; 2-way non-repeated measures ANOVA, mean  $\pm$  SEM). In addition, Fig. 3.11B shows that there was no difference between ipsilateral and contralateral GFAP staining within pretreatments ( $F(1,14)=1.64$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.221$ ; 2-way repeated measures ANOVA, mean  $\pm$  SEM).

Figure 3.12 presents images typical of microglial and astrocytic activation in the dorsal horn from sections of the cervical spinal cord harvested 2 hours post ID 48/80 following intrathecal pretreatment. Images of both ipsilateral and contralateral Iba1 and GFAP staining from a single IT LPS-RS pretreated animal are shown on the left. Images

from an IT Saline pretreated animal are depicted on the right. Images were quantified by optical density via ImageJ, and the numerical results are shown in Figure 3.11.



**Figure 3.11: Spinal Microglial- (Iba1) and astrocytic- (GFAP) activation 2 hrs post ID 48/80 following IT LPS-RS Pretreatment.** Two hours post ID 48/80, the cervical region of the spinal cord was harvested and incubated with antibodies against Iba-1 and GFAP. Neither (A) Iba-1 nor (B) GFAP ipsilateral immunoreactivity was elevated compared to contralateral in the dorsal horn within each group as quantified by densitometry of Lamina 1,2 of the dorsal horn. No differences were found across IT pretreatments or ID pruritogen treatment. Immunoreactivity was quantified by densitometry of Laminae 1,2 of the dorsal horn using ImageJ (n=5-7 sections/mouse, with 4 mice/group, effect of pretreatment: 2-way non-repeated measures ANOVA; side: 2-way repeated measures ANOVA Mean ± SEM)



**Figure 3.12: Dorsal horn spinal microglial- (Iba1) and astrocytic- (GFAP) activation 2 hrs post ID pruritogen following IT LPS-RS pretreatment.** Neither (A) Iba-1 nor (B) GFAP ipsilateral immunoreactivity was elevated compared to contralateral in the dorsal horn within each group 2 hours post ID 48/80 with IT LPS-RS or IT Sal pretreatment. Images quantified by densitometry of Laminae 1,2 of the dorsal horn (see Fig. 3.11, Scale bars= 50µm).

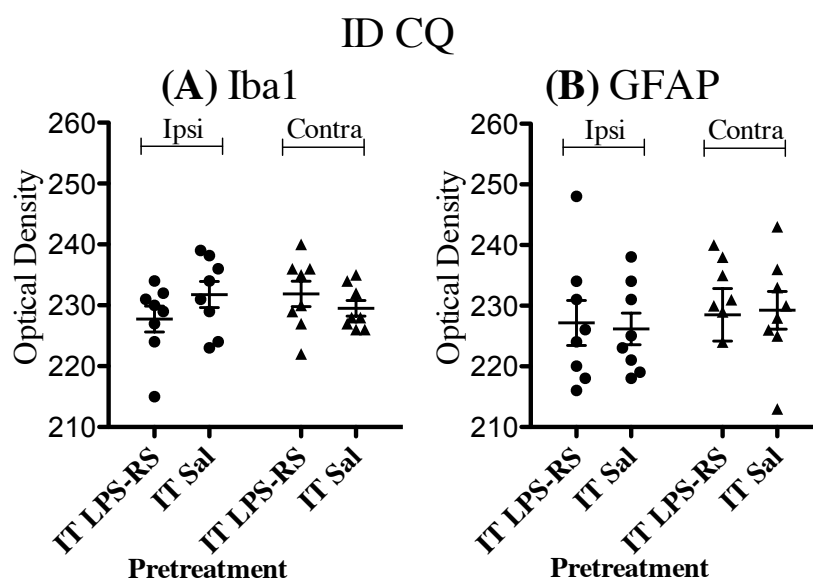
Figure 3.13 shows OD of laminae 1 and 2 of the dorsal horn in tissue sections incubated with antibodies against Iba1 (A), and GFAP (B) (t=2hr) after IT Saline or LPS-RS pretreatment followed by ID Chloroquine (t=0).

Figure 3.13A shows that there is no difference in microglia between IT LPS-RS and IT Saline pretreatment ( $F(1,28) = 0.18$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.6755$ , 2-way non-repeated measures ANOVA, mean  $\pm$  SEM). In addition, Fig. 3.13A shows that there was no difference between ipsilateral and contralateral Iba1 staining within pretreatments ( $F(1,14) = 0.6755$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.6375$ ; 2-way repeated measures ANOVA, mean  $\pm$  SEM).

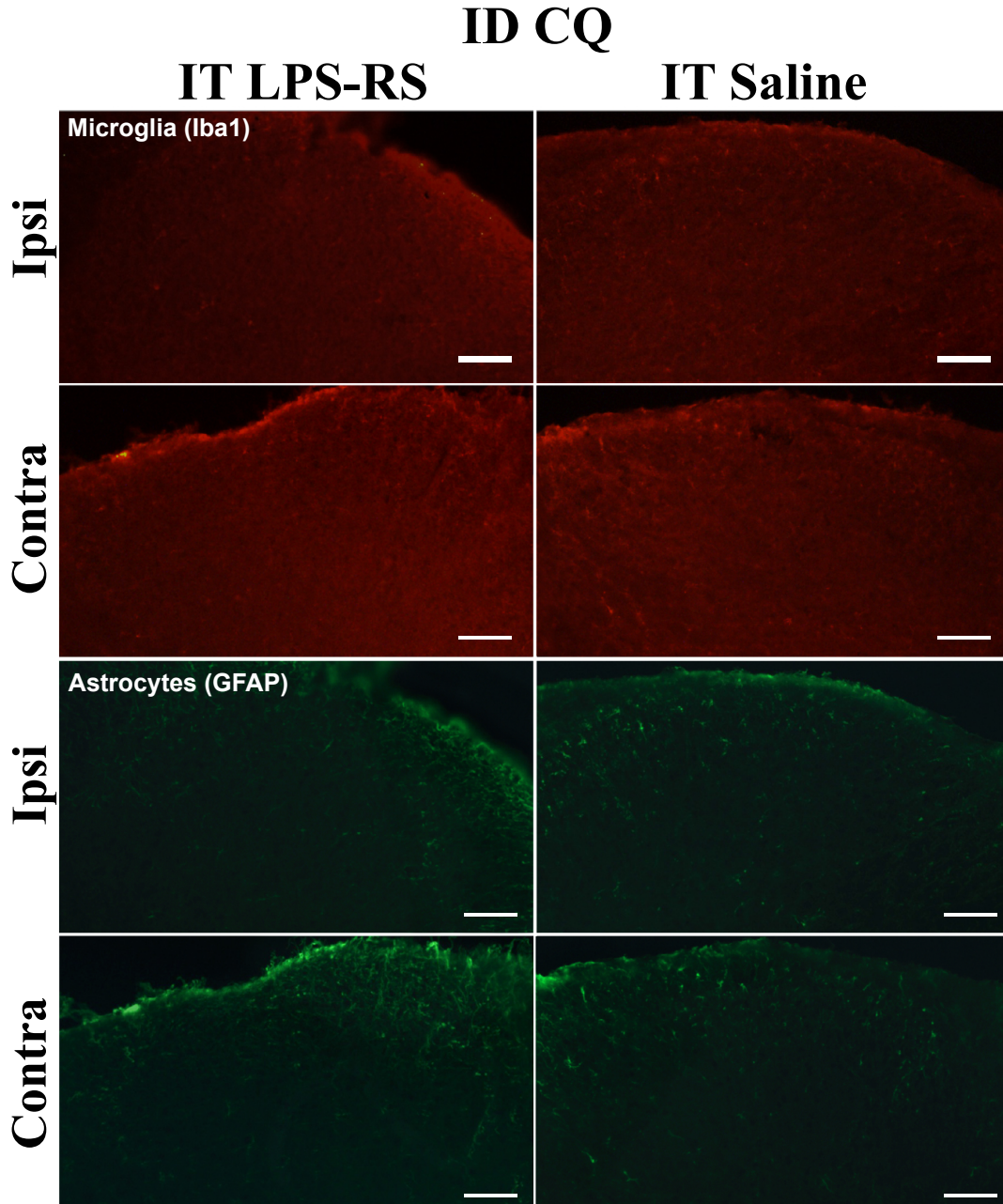
Figure 3.13B shows that there was no difference in ipsilateral astrocyte activity in IT LPS-RS pretreatment compared to IT saline ( $F(1,28)=0.00$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.9717$ ; 2-way non-repeated measures; mean  $\pm$  SEM). In addition, Fig. 3.13B shows that there was no difference between ipsilateral and contralateral GFAP staining within either pretreatment ( $F(1,28) = 0.67$ ;  $n=4-6$  sections/ mouse, 4 mice/group;  $p=0.5253$ ; 2-way repeated measures ANOVA, mean  $\pm$  SEM).

Figure 3.14 presents typical images of microglia and astrocyte activity in the dorsal horn from cervical spinal cord sections harvested 2 hours post ID Chloroquine following IT pretreatment. Images of both ipsilateral and contralateral Iba1 and GFAP staining from a single IT LPS-RS pretreated animal are shown on the left. Images from an IT Saline pretreated animal are depicted in the right column. Images were quantified via optical density using ImageJ, and the numerical results are shown in Figure 3.13. As indicated, there was no change in glial markers after unilateral intradermal pruritogen when given alone or after IR-LPS-RS.





**3.13: Spinal microglial- (Iba1) and astrocytic- (GFAP) activation 2 hrs post ID CQ following 1 hour IT LPS-RS pretreatment.** Two hours post ID Chloroquine, the cervical region of the spinal cord from mice was harvested 3 hours post IT pretreatment and incubated with antibodies against Iba-1 and GFAP. Neither (A) Iba-1 nor (B) GFAP ipsilateral immunoreactivity was elevated in LPS-RS pretreated animals compared to Saline pretreated animals. In addition, no differences were found across sides within a pretreatment, or between contralateral groups. Immunoreactivity was quantified by densitometry of Laminae 1,2 of the dorsal horn using ImageJ (n=5-7 sections/ mouse, with 4 mice/group, effect of pretreatment: 2-way non-repeated measures ANOVA; side: 2-way repeated measure ANOVA, Mean  $\pm$  SEM).



**Figure 3.14: Dorsal horn spinal microglial- (Iba1) and astrocytic- (GFAP) activation 2 hrs post ID CQ following IT LPS-RS pretreatment.** Neither (A) Iba-1 nor (B) GFAP ipsilateral immunoreactivity was elevated compared to contralateral in the dorsal horn within each group. Images quantified by densitometry of Lamina 1,2 of the dorsal horn (see Fig 3.13, Images are representative of typical immunoreactivity. Scale bars= 50 $\mu$ m.)

## Discussion

In the present studies, I used a murine model that utilizes a focused scratching to investigate the role of Toll-like Receptors and associated adaptor proteins in pruritus. Our hypothesis was that Toll-like Receptors, and their associated downstream adaptors mediate both histaminergic, and histamine-independent pruritus.

### *Murine pruritic model*

Intradermal injection of the pruritogens (48/80 / Chloroquine) but not vehicle (saline) initiated a robust homotopic scratching with the hind paw of the neck region into which the pruritogen was injected. The scratching characteristically appeared as short high frequency bursts punctuated by brief periods of inactivity. Our group has shown in mice (Marino et al, 2012) and rats (Skahen et al, 2014) that the movement of the banded paw was efficiently detected by the automated paw motion detector (PMD). Systematic comparison of the scratching as assessed by human observers and by the detection system revealed that the algorithm employed to define scratching showed a significant covariance over a range of scratching frequencies when studied in the mouse and rat (Marino et al, 2012; Skahen et al, 2014). Further the system was able to detect scratching that was ipsilateral to ID pruritogen and showed little response to scratching that was contralateral to the banded paw (Marino et al, 2012; Skahen et al, 2014). There is always the possibility of a confound based on the fact that the mice were recovering from the anesthetic. The time of onset of peak scratching was ~6 minutes (48/80), and ~8 minutes (CQ) (Fig. 3.1-10). This onset, however, may be earlier given there is a possibility that the animals had to be lightly anesthetized and this might have obscured immediacy of the pruritogen's action. However, at least one other study has reported similar times of onset (Zhang et al, 2014).

### *Compound 48/80 and Chloroquine evoked scratching*

The present study, in agreement with previous work, demonstrated that both ID 48/80 and ID chloroquine, delivered in the lateral aspect of the neck, initiated a robust dose-dependent homolateral scratching. Assessment of the pharmacology of this scratching induced by the two pruritogens demonstrated that the scratching initiated by 48/80 was prevented in a dose dependent fashion by diphenhydramine, an H1 receptor antagonist. While some previous work has suggested that chloroquine may act, in part through mast cell degranulation (Nosál et al, 1991), our group and others have shown that doses of diphenhydramine, which blocked the effects of 48/80, had no effect upon the scratching initiated by chloroquine (Mnyika & Kihamia, 1991; Adebayo et al, 1997). While not excluding completely a role of mast cells in the chloroquine effect, these observations emphasize the distinct actions of these two pruritogens.

### *Role of TLR signaling in 48/80 and chloroquine evoked scratching*

As reviewed, and shown in Figure 1.1, the present work examined the role of membrane (TLR 4) and endosomal TLRs (TLR 3,7,9). As noted in Figure 1.1, TLR 3, 7, and 9 are not cell surface receptors. TLR 7 and 9 signal through MyD88 to induction of NF- $\kappa$ B or IRF7 to largely elicit the expression of proinflammatory cytokines (Akira, 2008). TLR3 signals exclusively through the TRIF adaptor and can also initiate production of proinflammatory cytokine via activation of NF- $\kappa$ B (Alexopoulou et al, 2001). In addition, TLR3 signals in a TRIF dependent activation of Type 1 IFN production that is slightly delayed (Akira et al, 2006). In contrast, TLR 4 *must* signal through both MyD88 and TRIF in order to induce the production of inflammatory cytokines, though the reason for this has not been elucidated (Rowe et al, 2006; Tanimura et al 2008). MyD88 is robustly linked to NF- $\kappa$ B and cytokine production (Akira et al, 2006). As will be seen, the

overlapping signaling pathways made possible by multiple adaptor protein linkages make simple associations with any one TLR-mediated specific signaling cascade impractical.




With regard to the role of TLR signaling, Table 4.1 summarizes the observed effects of the TLR-KOs. Several points were evident in the present work with regard to 48/80 and chloroquine evoked scratching:

i) 48/80 and chloroquine evoked scratching showed distinct differences in the face of TLR KO. ID 48/80 induced scratching was reduced with the loss of TLR3, but not TLR 4, 7, or 9. In contrast, ID chloroquine induced scratching was decreased in all TLR KOs examined, e.g. in TLR 3, 4, 7, or 9 deficient animals. This emphasizes the heterogeneity of the systems that process the histamine dependent and independent stimuli leading to the scratching phenotype.

ii) Given this apparent role of the endosomal TLR3 in 48/80 effects, I hypothesized that scratching would be attenuated by TRIF, but not Myd88 KO. In fact, ID 48/80 in MyD88 deficient mice exhibited *decreased* scratching, while TRIF and MyD88-TRIF KO animals showed an *increase* in scratching.

iii) For ID chloroquine-induced scratching, the broad contribution of the several TLRs was associated with a reduction of scratching in MyD88-, TRIF- or MyD88-TRIF-KO mice. In contrast to the effects reviewed for ID 4880, no evidence of augmentation was noted.

**Table 4.1: Summary of effects of TLR and adaptor protein KO on evoked scratch.**  
Scratching was induced by ID 4880 (top) or chloroquine (bottom).

<b>Pruritogen</b>	<b>Knockout</b>	<b>Effect</b>
<b>48/80</b>	<b>TLR MyD88</b>	 <b>Scratching</b>
	<b>TRIF</b>	 <b>Scratching</b>
<b>Chloroquine</b>	<b>TLR</b>	 <b>Scratching</b>
	<b>MyD88</b>	
	<b>TRIF</b>	

To determine whether TLR4 was involved in spinal processing of pruritus, the TLR4 antagonist LPS-RS was delivered spinally. IT LPS-RS did not reduce ID 48/80 scratching or dorsal horn c Fos expression. IT LPS-RS, however, reduced ID CQ induced scratching and c Fos expression in the dorsal horn. This action was consistent with the discriminating effects observed with the TLR-4 KO mice.

#### *TLRs and spinal glia*

As reviewed, the association of functional TLRs with spinal glia, both astrocytes and microglia (Clark et al, 2013 Stokes, et al 2013), along with the defined effects of glia in facilitating neuraxial processing led to the mechanistic hypothesis that the spinal TLRs related to the changes in the response to pruritogens would be reflected by glial activation. Assessment of dorsal horn GFAP (astrocyte) and Iba1 (Microglia) staining

revealed no difference between ipsilateral and contralateral effect of ID 48/80 or CQ. However, I would note that while increases in these epitopes would argue for their activation, however, absent these change in GFAP or iba1 does not necessarily exclude the possibility that they are playing a role. Thus, moderate activation of glia is often acutely associated with phosphorylation of MAP-Kinases (such as p38, ERK, JNK). In previous work our group has shown that activation of C fibers leading to a facilitated pain state was associated with an acute increase in activated (phosphorylated) P38 MAPK (pP38MAPK) in microglia and this facilitated state was prevented by P38 MAPK inhibition. Importantly this rapid increase in pP38 MAPK was not associated with changes in OX42 (a marker of microglia activation) with no change in epitope expression. Thus the absence of changes in GFAP or Iba1 in this study may not fully elucidate the activity of glia in the dorsal horn in this model of pruritus. However, I would note that while increases in these epitopes would argue for their activation, however, absent no change in GFAP or Iba1 does not necessarily exclude the possibility that they are playing a role. Thus, moderate activation of glia is often acutely associated with phosphorylation of MAP-Kinases (such as p38, ERK, JNK). As in the case of recent work, our group has shown that activation of C fibers leads to a facilitated pain state was associated with act through glia may lead to an acute increase in activated (phosphorylated) P38 MAPK (pP38MAPK) increase in microglia and microglial phospho-p38- this facilitated state was prevented by P38 MAPK inhibition. Importantly this rapid increase in pP38 MAPK was not associated with changes in OX42 (a marker of microglia activation) with no change in epitope expression. Thus the absence of changes in examined in the present study. Thus the limited staining for GFAP or Iba1 in this study may not fully elucidate the activity of glia in the dorsal horn in this model of pruritus. Similarly, a previous study showed chloroquine did not significantly

increase GFAP staining in rat cerebral cortex and hippocampus, and the researchers hypothesized that Chloroquine may inhibit glial modification by inhibiting glial replication of DNA and post translational alteration of new proteins (Zhang et al, 2005). In addition, histamine has been suggested to attenuate inflamed microglial responses and act as an anti-inflammatory via binding Histamine Receptor 4 (H4R) in exacerbated cases of microglia inflammation (Ferreira et al, 2012). These mechanisms however, may not play an immediate role in the present work where the pruritogens were delivered peripherally. The lack of glial activation in response to either 48/80 or CQ is not an implausible finding, and it may be that simply the other markers of glial activation would prove more useful in understanding glial activation after 48/80 and CQ.

These findings present evidence that compound 48/80 and chloroquine induce scratching behavior through mechanisms that act through multiple Toll-like receptors and associated adaptor proteins. These studies must be viewed in terms of the two *caveats* characterizing the use of mutant animal models: i) they represent animals that have deficiencies present throughout development and may thus be confounded by adaptive changes secondary to reactions of the target protein absence and ii) the KO reflects whole body target deletions.

#### *Histamine-Dependent Scratching*

Decreased compound 48/80-dependent scratching in TLR3 deficient mice suggests that histamine-dependent pruritus is at least in part not MyD88-dependent as TLR3 has been shown to signal exclusively through TRIF (Kiwai & Akira, 2010). However, in this study, MyD88 deficient mice were also less sensitive to ID 48/80 and show reductions in scratch. This duality is consistent with previous work indicating that parallel pathways indeed mediate histamine-dependent itch, and the fact that TLR3 can



also induce activation of NF- $\kappa$ B through a TRIF dependent pathway (Liu et al, 2012a; Takeuchi & Akira, 2010).

On the other hand, mice deficient in TRIF, a crucial downstream signaling molecule for TLR3, exhibited *increases* in scratching compared to WT. These findings are different from previous work where mice were treated intrathecally with a membrane-peptide TRIF inhibitor showed reductions in scratching induced by both 48/80 and CQ (Liu et al, 2012a). This difference may be due to the fact that our model of TRIF deficiency is a whole body knockout of the adaptor, and not just a spinal change in TRIF dependent signaling. However, these results with the TRIF deficient mouse are consistent with previous work from the Yaksh group, wherein it was shown that TRIF KO mice showed an enhanced pain behavior secondary to nerve injury and intrathecal LPS (Stokes et al, 2013). In those studies, the enhanced pain behavior was thought to be mediated by a loss of regulatory feedback on Myd88 / NF- $\kappa$ B mediated signaling and proinflammatory cytokine release. It was not clear if these were a peripheral or spinally mediated mechanisms.

In recent clinical studies of sulfur mustard induced Atopic Dermatitis in humans, subcutaneous injection of IFN- $\gamma$  significantly decreases symptoms of pruritus (Panahi et al, 2012). IFN- $\gamma$  production has been shown to be induced by LPS (a TLR4 agonist) in the presence of IL-2 in macrophages in conditions that would otherwise lead to IFN $\alpha/\beta$  (Blanchard DK et al, 1986). In addition, TLR3 agonist Poly(I:C) induces IFN- $\gamma$  production in NK cells in a IL-12 dependent mechanism (Gerosa et al, 2005). This pattern of cytokine-induced production of IFN- $\gamma$  is seen across many immune cell types, and IFN- $\gamma$  is widely seen as an anti-inflammatory agent (see Schoeborn & Wilson, 2007 for

review). IFN- $\gamma$  production is dependent on TRIF but is independent of MyD88 (Weighardt et al, 2006). A study in TRIF KO mice found that TRIF plays a role in suppressing the effects (such as increases in production of IL-2 IL-6, IL12, and IFN- $\gamma$ ) of the immunomodulator rEA- (Eimeria tenella derived antigen that modulates immune responses against several pathogens) a TLR agonist (Seregin et al, 2011). Taken together, these findings suggest a mechanism of negative feedback by which TRIF might negatively regulate TLR signaling, perhaps via production of interferons. It seems reasonable that the present work points to a facilitative role of TLR3 in 48/80 induced pruritus, a finding consistent with the ability of TLR3 to signal through NF- $\kappa$ B and at the same time to initiate regulatory effects upon the facilitative effects that are revealed when TRIF function is deleted. If taken at face value, this would suggest that TRIF plays a role in attenuating scratching, or at least participates in crosstalk to regulate the pruritic response induced by ID 48/80.

Similarly, mice deficient in both MyD88-TRIF scratched significantly more than WT mice in response to ID 48/80, which could provide more support to the attenuating role of TRIF. This might beg the question: if MyD88, which appears necessary for histamine-dependent scratching, is knocked out, then “what is mediating the itch signal?” It may be that TLRs and their associated adaptors are merely aiding in sensitizing the neurons that actually respond to pruritic ligands. This speculation is supported by observations wherein scratching is initiated by more direct activation of TRPV1 positive neurons that also express TLRs (Shim et al, 2007; Imamachi et al, 2009; Liu et al, 2010). It may be that lack of attenuation via a TRIF dependent mechanism hypersensitizes these *pruriceptive* neurons.

Interestingly, although TLR 4, 7, and 9 signal at least in part through MyD88, it appears that a KO in any one of these TLRs is not sufficient to significantly reduce ID 48/80 scratching (Akira et al, 2006). Other researchers have examined the role of TLR7 in histamine-dependent pruritus and while they do not exclude the possibility that TLR7 mediates histaminergic scratching, their data does not support this idea (Liu et al, 2010). Some studies have suggested that MAPK pathways down stream of the TNF-associated receptor factor-6 (TRAF6, which links MyD88 and TRIF to NF- $\kappa$ B activation) may play a role in the response to noxious stimuli sensed by TLRs (Kashiwada et al, 1998; Takeuchi & Akira, 2010; Troutman et al, 2012; Zhang et al, 2014). It was recently found that extracellular signal-regulated kinase (ERK) activation is critical for histamine-dependent scratching induced by intradermal histamine and 48/80 (Zhang et al, 2014). ERK1/2 is activated via TRAF6, and like other MAPK pathways, it leads to the production of the AP-1 family of transcription factors which are believed to regulate proinflammatory mediators (Xu et al, 2005). This additional seemingly histamine dependent pathway regulated at least in part by TLRs may serve as an additional method by which itch is regulated.

#### *Histamine-Independent Scratching*

Intradermal chloroquine was significantly reduced by deficiency in TLR 3,4,7, or 9. This suggests, that TLR3 plays a similar role in histamine-independent pruritus as well. This finding is in line with other research in the field (Liu, 2012a). Unlike, TLR3, TLR4 KOs showed a different response to ID CQ compared to 48/80. The scratching reduction in TLR4 KO animals after ID CQ, was also found in TLR7, and TLR9 knockouts. TLR7 KO animals have previously shown reduced scratching behavior in response to ID CQ, while TLR 4, and 9 have not been studied in relation to ID CQ

induced pruritus (Liu et al, 2010). Interestingly, chloroquine has been reported to be an inhibitor of TLR 3, and 9 signaling (Kuzniak et al, 2011). It has been shown that CQ can directly induce itch by acting on MrgprA3, which then activates TRPA1 on sensory afferents to induce scratching (Liu et al, 2009). This of course only confounds the role of TLR3 in CQ induced pruritus, as it would seem that a TLR3 knockout should be no different than a WT treated with chloroquine. One speculative possibility is that the TLR effects are mediated in large part with the dorsal root ganglion and/or neuraxis, and not in the periphery. At the time and dose of chloroquine examined (8mg/kg) there could potentially be no central action and therefore these effects may not have been present. Accordingly, CQ had no effect upon the centrally mediated TLR actions. If chloroquine did act to block TLR3 function, in future work it would be interesting to determine, whether IT chloroquine was able to block ID 48/80 induced scratching, as suggested by the efficacy of TLR3 KO on 48/80 evoked scratching.

Unlike compound 48/80, there appears to be a more straightforward role for the associated adaptor proteins in ID chloroquine induced scratching. The findings that mice deficient in MyD88, TRIF or both MyD88-TRIF all exhibit reduced scratching induced by chloroquine are in direct relation to their upstream TLRs, which appear to be required for full response to CQ. The finding that TRIF KO exhibit reduced scratch is in line with the findings of other researchers, and contrasts with the role played by TRIF in 48/80 evoked scratching (Liu et al, 2009). In short, the first series of studies emphasized that chloroquine and 48/80 evoked scratching served to act through multiple TLR signaling pathways and enable different down stream signaling through shared types of adaptor proteins.

### *Spinal actions of TLR4 in scratching behavior*

In the second series of studies, this work focused on addressing whether one could identify a central action for one of the signaling cascades identified in the first series of KO studies. Here I chose to consider the central effects of transiently reversing the actions of spinal TLR4 by the intrathecal delivery of a defined TLR 4 antagonist. This choice was made for a number of reasons: i) LPS-RS has been shown to be an effective inhibitor of TLR4 activation by a variety of known TLR4 ligands (Hutchinson et al, 2010; Stevens et al, 2013). ii) The Yaksh lab has previously characterized the antagonism by LPS-RS given by intrathecal delivery in the mouse in an arthritis model in which TLR4 KO diminished the associated hyperalgesia (but not inflammatory reaction) (Christiansen, et al 2011) iii). The TLR4 KO work in this study suggested that TLR4 was required for expression of chloroquine-induced scratching.

In the present studies, the intrathecal delivery of LPS-RS at a dose which had previously been shown to be effective in the TLR4 dependent murine arthritis model was observed to significantly reduce the scratching response produced by chloroquine, but had no effect upon the scratching behavior produced by 48/80. These results, using the intrathecally delivered TLR4 antagonist with an action limited to the spinal cord, recapitulated the results observed with the differential effects of TLR4 KO on ID 48/80 and ID CQ-induced scratching. These results confirm that our findings in TLR KO animals were not merely due to some developmental defect as a result of the genotype, but were instead due to the lack of a functional TLR4 at the spinal level. In addition, this finding supports that TLR4 is acting to mediate histamine-independent, but not histamine-dependent scratching, at the spinal level. Importantly, the differential effect of IT LPS-RS on the two scratching stimuli emphasizes that the effects of LPS-RS represented “non specific” suppression of behavior. Thus, while it does not exclude a

peripheral role of TLR4 in the pruritic response, it clearly emphasizes a central (spinal) mechanism for the chloroquine-induced scratching. (Koibuchi et al, 1985; Antohe et al, 1988; Green & Lim, 1989; Nosal et al; 1991).

#### *Spinal TLR4s and pruritogen evoked spinal c Fos expression*

The primary end point in these studies of scratching has been the assessment of behavior. While this is a critical measure, as there are no ex vivo surrogate markers of pruritus, e.g. agents which do not produce scratching in the unanesthetized animal with normal motor function. However, it is reasonable to assert that this input must serve to activate second order dorsal horn neurons, given the organization of the peripheral nervous system. A robust index of such activation is the assessment of the change in the neuronal expression of the immediate early gene cFOS (Hoffman et al, 1993; Coggeshall, 2005).

In accordance, with our behavioral observations, analysis of c Fos immunohistochemistry revealed that both compound 48/80 and chloroquine were able to induce a significant increase in c Fos positive dorsal horn cells ipsilateral to the side of treatments at the cervical spinal level. The lack of significant contralateral expression of c Fos positive cells served as an indicator of normal c Fos expression (Inan, 2009, Ayikama, 2009).

This work undertook to determine if the spinal blockade of TLR4, which suppressed chloroquine, but not 48/80 scratching would have corresponding effects upon ipsilateral dorsal horn c Fos expression. As indicated, IT LPS-RS indeed resulted in a significant suppression of chloroquine-evoked c Fos expression, but had no effect upon the 48/80-evoked c FOS expression.

Through comparing matched c Fos expression and scratch counts induced by ID 48/80 and chloroquine from TLR4 antagonist pretreated mice, a relationship between neuronal activation and scratching was established in ID chloroquine treated animals. This relationship has also been shown in histamine-dependent models of itch (Yao et al, 2012). Interestingly, one study noted that there was a significant decrease in c Fos positive cells in the dorsal horn of Histamine-treated animals, if the treated site was not allowed to be scratched. The resulting c Fos expression was still significantly higher than control (Yao et al, 1992).

#### *Microglia (Iba1) and Astrocyte (GFAP) activation after ID Pruritogen*

The current literature on TLRs has focused in various models of pain and inflammation as they are expressed by microglia and astrocytes. In the pruritus model, however, this author found no increase in dorsal horn microglia or astrocyte immunoreactivity two hours after ID treatment (Olson & Miller, 2004; Bowman et al, 2003). The intraplantar injection of formalin, a well defined algogen, has been shown to lead to increased expression of glial markers in the dorsal horn as early as 1 hour after intraplantar delivery (Wang, et al, 2012). These changes are considered to reflect the effects of the activation by nociceptive C fibers of dorsal horn glia. Glial activation has been considered to be an important mediator of the facilitated state initiated by injury leading to repetitive small afferent input (Bradesi, 2010; Nakagawa & Kaneko, 2010). In the present studies, neither ID chloroquine nor 48/80 led to evidence of glial activation at doses producing a robust scratching response. Given their apparent association with the TLR family in general and TLR4 in particular, the lack of a glial response was unexpected. While it may be argued that the two hour tissue harvest precluded seeing changes in spinal glial epitopes, as noted above, intraplantar delivery of the algogen

formalin indeed resulted in an increase in glial markers within the hour. In any case, the observation of no change after the pruritogen emphasizes the distinction of pain and itch.

### *Endogenous TLR ligands and pruritus*

The present studies with TLR4 and the effects of IT TLR4 antagonism emphasizes role for these systems in 48/80 and chloroquine -evoked scratching. Though beyond the scope of these studies, the present results raise the interesting issue as to what normally activates these TLR cascades in the face of the pruritogenic stimuli. As indicated in Table 1.1, the TLRs have a series of identified endogenous ligands. Perhaps the best studies of these ligands in their relation to pain has been the TLR4-activating HMGB1 (High Mobility Group Box 1)– a nuclear protein involved in transcriptional regulation. HMGB1 has been identified in spinal cord; found to be released by afferent input; and has been shown through a TLR4 KO to mediate a hyperpathic state (Maeda, et al 2013; Feldman, et al 2012). While HMGB1 is ubiquitously distributed, its translocation after nerve injury has been largely found in neurons and not glia. Induction of HMGB1 has been shown in DRG neurons and satellite (astrocyte-like) cells (Shibasaki, et al, 2010). In addition, TLR signaling in mast cells leads to the release of cytokines, chemokines, and lipid mediators which may potentiate further TLR signaling in other immune, skin or neural cells (for review see Sandig & Bolfone-Paus, 2012). It has been shown that exogenous ligands of TLR3 (poly (I:C), viral components) and TLR7 (imiquod, viral RNA) have been shown to elicit scratching when introduced experimentally to itch-sensitive neurons (Liu et al, 2010, 2012a; Han et al 2012). In relation to skin diseases, clinical cases of extremely dry skin associated pruritus, epidermal cells such as keratinocytes and Langerhans cells have been shown to release



a proinflammatory cytokines such as IL-6, TNF $\alpha$  which cause the release of other TLR activating ligands into the skin, where it is likely these effects are then propagated by other TLR containing cells (Hari et al, 2010). In addition, clinical presentation of pruritus associated with many skin diseases has shown high levels of TLR ligands (Hari et al, 2010). Interestingly, patients presenting high levels of microbial products in epidermal bacterial infection- associated pruritus– (which is an exogenous ligand for TLR1 and 2) have been found to be deficient or contain polymorphisms for TLR 1 and 2 (Petry & Gaspari, 2009; Hari et al, 2010). This suggests that in addition to inducing pruritus, TLRs can also attenuate it– feedback that may be illustrated by my findings in TRIF knockout animals exposed to 48/80.

#### *Hypothesized mechanisms of TLR modulation of the evoked scratching response*

Currently, very little is known as to which endogenous ligands might be activating TLR signaling in response to various pruritogens. In the case of compound 48/80, it is commonly held that histamine released from mast cells by compound 48/80 directly activates sensory neurons by binding histamine receptors (H1/H4R) and induces pruritus via activation of TRPV1 via PLC3 $\beta$  and PLA2 (Shim et al 2007; Han et al, 2006). LPS has been shown to sensitize TRPV1 in rat sensory neurons in a TLR4-dependent manner (Diogenes et al, 2011). Also, it has been shown that ligands of TLR 3, 7 and 9 significantly up-regulated TRPV1 expression by DRG neurons, which results in heightened Ca<sup>2+</sup> influx in DRGs (Qi et al, 2011). Additionally, TLR3, 7 and 9 ligands increased the release of proinflammatory cytokines and chemokines in cultures of DRG neurons (Qi et al, 2011).

In our study, the lack of reduction in 48/80 induced scratching and c Fos expression in TLR4 KO mice leads one to speculate that TLR4 is unlikely to mediate

histamine-dependent scratch. This hypothesis fits in with known literature about TLR4. Despite a lack of TLR4 mediated sensitization of TRPV1 (in TLR4 KO animals), the pruritic scratching response to 48/80 was intact, whereas one might expect it to be decreased in KO animals if TLR4 activation sensitized TRPV1 in response to 48/80. This indicates that histamine-dependent TRPV1 sensitization may be controlled by other TLRs, such as TLR3.

The primary known ligand for chloroquine induced scratching is chloroquine itself, and it directly activates MrgprA3 on sensory neurons (Liu, 2009). In certain populations of itch-sensitive neurons, MrgprA3 will then activate TRPA1, which is required for neuronal activation and pruritus induced by histamine-independent pruritogens (Imamachi; 2009; Wilson et al, 2011; Liu et al, 2010, 2012a; Han, 2013; Than et al, 2013). Very interestingly, recent work showed that CQ was capable of exciting TRPA1 in sensory fibers and inducing itch (Than et al, 2013). Interestingly, TLR 3,4,7 and 9 have been localized in TRPA1 positive sensory afferents (Liu et al, 2012; Han et al 2012). LPS has been shown to directly sensitize nociceptors via excitation of TRPA1 in an inflammation and pain model (Meseguer et al, 2014). Together, these findings present a possible mechanism of itch sensitization dependent on TLR signaling to excite nociceptors responsive to histamine-independent pruritogens. Our findings in both whole-body TLR4 and spinal TLR4 antagonist treated mice are consistent with the proposed mechanism, as these TLR4 deficient mice exhibited reduced scratching after treatment with ID chloroquine. This indicates that the lack of sensitization was specifically due to a lack of *direct* TLR4 mediated pruritus.

### *Indirect chloroquine mediated effects*

An important aspect of TLR signaling in pruritus is the identity of the endogenous ligands involved. Pruritus through compound 48/80 is believed to be mediated via histamine as it is sensitive to H1/4R antagonists (Dunford et al, 2007; Shim & Oh, 2008). The greater importance of the role played by mast cells, from which compound 48/80 is thought to elicit histamine release, in pruritus is unclear and requires further examination. Once activated, mast cells are known to release an overwhelming assortment of different mediators including cytokines, peptides prostaglandins, and proteases (Oldford & Marshall, 2014). Chloroquine may have similar effect regarding the amount of intracellular contents released into the extracellular fluid. These products released into the skin might have diverse effects, and their impact is unclear in pruritus, though a number of these mast cell mediators are endogenous TLR ligands (see Table 1.1). Chloroquine is well known for its ability to inhibit autophagy via increasing endosomal pH (Kuznik et al, 2011). As an antimalarial, chloroquine works to inhibit parasitic cells by preventing degradation of heme inside the lysosome of RBCs, resulting in an excess of heme, which CQ then binds forming a highly toxic complex that disrupts membrane function and leading to cell lysis and digestion (Hemphelmann, 2007). In the case of CQ induced pruritus, the result of an increase in necrotic cells may be a rise in cellular components leaking into the skin similar to mast cell contents. It is likely, in any case, that these mediators at least help potentiate/propagate the pruritic response perhaps by acting on TLRs which could induce production of proinflammatory cytokines and Type I IFN, while simultaneously sensitizing pruritic nociceptors via activation of TLRs co-localized with TRPA1 or TRPV1. This indirect action of chloroquine induced pruritus, may be secondary to direct stimulation of pruriceptors.

### *Indirect mechanism of TLR mediating Pruritus*

There is much to suggest that this sensitization may not only have a direct component. TLRs expression has been observed in a wide variety of cell types. Mast cells express TLR 1-4, and 6-9 (McCurdy et al, 2001). Epidermis keratinocytes express TLR1-6, 9, 10 and have been implicated in chronic itch (Baker et al, 2003; Miller, 2008; Emertcan et al 2011) Microglia express TLR 1-9 (Mallard et al, 2009). Astrocytes express TLR 1-5 and 6-8, however this expression by astrocytes is very subdued (compared to microglia) (Bsibsi et al, 2006; Carpenter et al, 2005; Kim et al, 2008). As indicated before, TLR 3,4,7,9 have been found on Primary sensory afferents (DRG and TG neurons) (Liu et al, 2012a). This wide expression of TLRs leads one to the conclusion that signals which may stimulate neuronal TLRs must also be able to activate TLRs elsewhere (e.g. in the skin). As noted, pruritogens likely activate the release of endogenous TLR ligands through the activation of mast cell mediators (apart from histamine). It may even be the case that CQ elicits the release of some unknown mast cell mediator that the TLR signaling cascade is sensitive to. Figure 4.1 depicts an additional two components to the hypothesized mechanism of TLR control over pruritogen induced scratch: Skin TLR-presenting mediators, and Glial TLR-presenting spinal mediators. It is likely that during an initial reaction to a pruritogen, different endogenous TLR ligands are released from a number of skin cell types (as they are in direct contact with the presenting pruritogen). This may cause the excitation of TLRs on sensory "pruriceptive" afferents necessary to transduce the itch. In addition, central terminals of the sensory afferents might be secreting TLR products or ligands which might have the effect of sensitizing glial TLRs. If this is the case, it is also likely that these glial cells will feedback on the central terminal, thus potentiating the response to

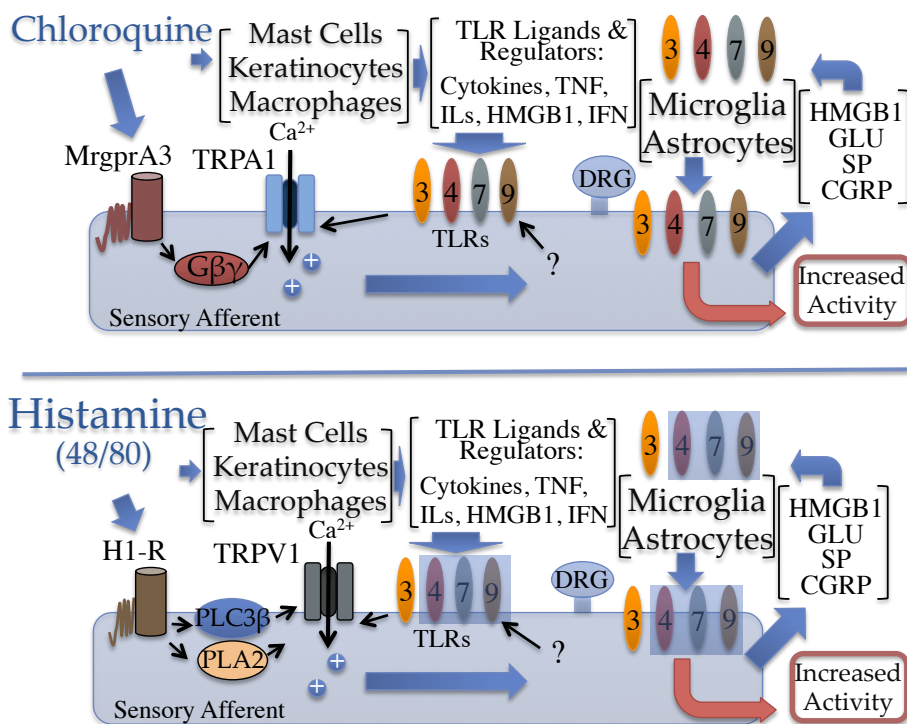
the pruritogen. In the case of 48/80 induced pruritus, though knockouts of TLR4,7, and 9 did not appear to have an effect on scratching, it may be that this was due to a compensatory activation by one of the other TLRs, and it is for this reason they are pictured. In the case of chloroquine-induced pruritus, which was sensitive to any one knockout out (TLR or Adaptor), the effects may be due to a synergistic method of neural sensitization by these TLRs in histamine-independent pruritus, wherein all TLRs are required for proper function.

### *Summary*

In summary, TLR 3, 4, 7, 9, MyD88 and TRIF play a role in mediating CQ-produced histamine-independent itch, but only TLR3 and TRIF are critical to mediating histamine-dependent 48/80 induced pruritus. The increase in scratching in TRIF KO in 48/80 but not CQ induced scratch suggests a regulatory feedback mechanism resembling systems revealed in pain processing. The effects of spinal TLR4 antagonism emphasize that at least for chloroquine, but not for 48/80, an important component mediating the role of TLR4 lies within the neuraxis. The present findings provide evidence that 48/80 and chloroquine initiate scratching behavior through differentially acting systems utilizing a number of TLRs and their downstream adaptors.

Unfortunately, due to limited availability of viable pharmacological TLR or adaptor inhibition, more TLRs and adaptors were not studied for their spinal specific role in mediating itch. In addition, due to the parallel mechanisms of itch described above, it seems apparent that in the case of some KOs there was essentially a rescue of scratching phenotype perhaps by other TLRs or adaptors acting in a parallel pathway of itch mediation. It is for this reason, future studies would be well advised to secure more

double-knockout models of studying TLR and associated adaptor proteins mediating pruritus induced by ID 48/80 and ID chloroquine. Clearly, the sensation of pruritus and the role of the TLRs in the encoding of these stimuli constitute a complicated process.



**Figure 4.1: Proposed mechanisms of TLR sensitization of primary afferents.**

This diagram depicts histamine-sensitive and -insensitive (Chloroquine sensitive) neurons characterized by the H1-R or MrgprA3 receptor cascade, respectively. In addition it follows the role TLRs may play in sensitizing pruriceptors. Following exposure to either Chloroquine or Histamine, there is the possibility of indirect and direct sensitization of the neurons by TLRs on the neuron itself or perhaps on immune or glial cells. The end result of either direct or indirect sensitization would be an increase in transmitter release (activity) from pruriceptors. Blue shaded TLRs were not individually shown to be required to mediate histamine dependent itch, though there may be a compensatory action by other TLRs in their absence, where as they might normally contribute to the pruritic response. “?” indicates lack of information regarding localization of TLR9 on these potential pruriceptors.

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