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Lidocaine Attenuates an Induced Inflammatory Response

Stephanie Zack

Loyola University Chicago, szack1@luc.edu

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LIDOCAINE ATTENUATES AN INDUCED INFLAMMATORY RESPONSE

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

STEPHANIE R. ZACK

CHICAGO, ILLINOIS

AUGUST 2018
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing CARD</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium-release activated calcium channels</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger-associated molecular patterns</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>G</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>IBD</td>
<td>Irritable bowel disease</td>
</tr>
<tr>
<td>IC</td>
<td>Ion channel</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IM</td>
<td>Inflammatory mediators</td>
</tr>
<tr>
<td>JNK</td>
<td>JUN N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP kinase-2</td>
<td>Mitogen-activated protein kinase activated protein kinase-2</td>
</tr>
<tr>
<td>MAPKK</td>
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</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen-activated protein kinase-kinase-kinase</td>
</tr>
<tr>
<td>MAPK signaling</td>
<td>Mitogen-activated protein kinase-mediated signaling</td>
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<tr>
<td>MD2</td>
<td>Myeloid differentiation 2</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
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<tr>
<td>Nav</td>
<td>Voltage-gated sodium channels</td>
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<tr>
<td>NCX</td>
<td>Na⁺/Ca²⁺ exchanger</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NHE1</td>
<td>Sodium-hydrogen antiporter 1</td>
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<tr>
<td>NLRs</td>
<td>Nucleotide-binding oligomerization domain-like receptors</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR Family Pyrin Domain Containing 3</td>
</tr>
<tr>
<td>NNAV</td>
<td>Naja naja atra venom</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotoxin-Nna</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor β-activated kinase 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
</tbody>
</table>
TLR4  Toll-like receptor 4
TTP  Tristetraprolin
TTX  Tetrodotoxin
TNF-α  Tumor necrosis factor-α
VGSC  Voltage-gated sodium channel
CHAPTER ONE

OVERALL HYPOTHESIS AND EXPERIMENTAL GOALS

Inflammation is common to the pathogenesis of a variety of diseases. Persistent or excessive inflammation can lead to tissue damage and promote disease in the affected tissue. Therapeutics which can regulate and reduce damaging inflammation and subsequent disease development are therefore valuable in the treatment of inflammation and inflammatory diseases\(^1\).

One commonly used analgesic which has recently been identified as limiting inflammation is lidocaine, a known numbing agent that acts by inhibiting sodium ion channels\(^2\). Additionally, alternative, less toxic therapeutics like snake venom peptides, Zep 3 and 4, are thought to have anti-inflammatory effects\(^3\). Recent studies have shown that lidocaine can attenuate proinflammatory cytokine gene expression and subsequent release from microglial cells, the resident macrophages in the central nervous system\(^4\)–\(^14\). Snake venom peptides have also been shown to reduce cytokine release from activated macrophages in part due to reduced gene expression\(^15\)–\(^17\). Yet a well-defined mechanism or cellular target for lidocaine or Zep peptides’ anti-inflammatory effects has not been proposed.

The inflammatory response is mediated by the release of cytokines following the activation of macrophages or microglial cells. Expression and release of a proinflammatory cytokine like IL-1\(\beta\) requires two signals. The first priming signal
activates the toll-like receptor 4 (TLR4) signaling pathway, and the second signal leads to the formation of the inflammasome complex. TLR4 is activated by pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) or damage-associated molecular patterns (DAMPS) which result in a cascade of signaling through the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway\textsuperscript{18,19}. This activation leads to the translocation of the transcription factor, nuclear factor kappa B (NF-κB), into the nucleus resulting in the upregulation of the gene expression of proinflammatory cytokines such as IL-6 and proIL-1β\textsuperscript{18,19}. A second signal such as ATP initiates the assembly of the NLR family pyrin domain containing 3 (NLRP3) inflammasome complex that activates the protease, caspase-1. Caspase-1 then cleaves proIL-1β into its mature form allowing it to be secreted from the cell. This cytokine and others can then recruit other immune cells causing inflammation\textsuperscript{18}.

The canonical inflammatory pathway does not indicate a regulatory role for sodium ion channels. More broadly, lidocaine is known to non-selectively inhibit cation channels\textsuperscript{3,7}. In this context, lidocaine may inhibit ion channels to affect inflammatory signaling, reducing the release of IL-1β and other cytokines. For example, ATP-activated microglial cells have elevated levels of intracellular Ca\textsuperscript{2+} corresponding to increased cytokine release\textsuperscript{14,19}. Lidocaine treatment of the ATP-activated microglial cells significantly reduced intracellular Ca\textsuperscript{2+} concentrations\textsuperscript{14}. Perhaps lidocaine attenuates the inflammatory response by inhibiting an ion channel to minimize activation.
Another related pathway, the mitogen-activated protein kinase (MAPK) pathway, responds to stress stimuli to regulate gene expression. These signals include fluctuations in intracellular cation concentrations. For example, diminished intracellular 

[Ca^{2+}] signal the MAPK pathway to minimize the response to proinflammatory signals by downregulating cytokine gene transcription^{14,18}. Furthermore, NF-κB can also be activated by stress stimuli, so both MAPK signaling and NF-κB activation may contribute to reduced cytokine secretion^{19}.

Similarly, snake venom peptides have also been shown to have anti-inflammatory effects^{15,16}. Like lidocaine, they are classically recognized for their analgesic properties^{15,16}. For example, the Neurotoxin-Nna from the venom of Naja naja atra has been found to attenuate IL-1β release and inhibit activation of NF-κB^{15,16}. While not much is known about Zep peptides specifically, they may have anti-inflammatory effects comparable to Neurotoxin-Nna and lidocaine. The goal of this project is to understand how lidocaine and Zep peptides act as anti-inflammatory agents on activated macrophages.

Since lidocaine and snake venom peptides have been shown to have anti-inflammatory effects on inflammatory models through reduced proinflammatory cytokine secretion, we hypothesize that lidocaine and Zep peptides also are able to exert these effects on macrophages by (1) interacting with a cellular target to decrease intracellular cation concentrations and by (2) downregulating MAPK and
NF-κB signaling pathways to reduce proinflammatory cytokine expression and release.

Our first aim was to define how lidocaine attenuates proinflammatory cytokine release from macrophages. We hypothesized that lidocaine interacts with an ion channel to inhibit the MAPK and NF-κB signaling pathways to reduce proinflammatory cytokine release from activated macrophages. The goal was to understand the mechanism of how lidocaine reduces macrophage activation. Based on the literature, we believed that lidocaine interacts with the cell to inhibit the MAPK and NF-κB signaling pathways, reducing gene expression of the cytokines IL-1β and IL-6. Less gene expression would result in diminished cytokine secretion. The experiments described in Chapter 4 were aimed at determining the mechanism of how lidocaine attenuates macrophage activation and subsequent cytokine secretion.

Our second aim was to determine if Zep peptides exert an anti-inflammatory effect on activated macrophages. We hypothesized that Zep peptides interact with a cellular target to inhibit NF-κB activation, decreasing proinflammatory cytokine release. The goal was to assess how Zep peptides reduce macrophage activation to attenuate the release of inflammatory mediators. Recent literature suggested that snake venom peptides exert an anti-inflammatory effect by decreasing NF-κB activation. The experiments outlined in Chapter 5 were targeted towards determining if Zep peptides reduce macrophage activation by downregulating proinflammatory cytokine gene expression to decrease cytokine release.
Chapter 2 will consist of a review of the literature. It will provide an outline of the role of inflammation in disease and the disadvantages and advantages of current therapeutics to highlight the need for a better understanding of inflammatory pathways. The canonical inflammatory pathway will be detailed with special emphasis on activation of TLR4 signaling and NLRP3 inflammasome formation. Then lidocaine's analgesic mechanism will be outlined followed by an introduction to the role ion channels play in inflammation. It will be followed by a review of local anesthetics and inflammation with a focus on lidocaine and snake venom peptides' activity as an anti-inflammatory. Finally, a role for sodium, calcium, and potassium channels in regulation inflammation will be described.
CHAPTER TWO

REVIEW OF THE LITERATURE

Inflammation and Disease

**Acute and Chronic Inflammation.**

Inflammation is a host defense mechanism of protection against infection or injury. The goal of this defense is preventing the spread of a pathogen followed by its eradication and subsequent wound healing. First identified around 40 AD by Celsus, the cardinal signs of inflammation include swelling, heat, pain, and redness\(^{20,21}\). The inflammatory response is mediated by small proteins called cytokines. The IL-1 family cytokines are known to cause fever\(^{22}\).

Typically, acute inflammation resolves once the initial signal that triggered the response is cleared. However, when the signal persists, chronic inflammation is induced. Some signals that trigger chronic inflammatory responses include chronic infection, unrepaired tissue damage, persistent allergens, and indigestible crystals among others\(^{23}\). Chronic inflammation is increasingly a common cause of disease in industrialized countries. It is a risk factor that contributes to the cause or complications associated with obesity, type 2 diabetes, arthritis, cancer, and neurodegenerative diseases\(^{23–25}\). In some cases, the inflammatory response may be causing more damage than the initial cause of infection or signal that triggered the inflammatory response\(^{21}\).
There are many challenges associated with trying to develop drug therapies that aim to reduce the inflammatory response. New drug therapies need high specificity and high affinity for the target ligand, low toxicity, and high biostability\textsuperscript{23}. None of these ideal characteristics are easy to address within the context of inflammation and inflammatory diseases. However, they are essential to the development of both safe and effective drug therapies. The crux of the problem is that the immune response needs to be tightly regulated to ensure that the response is appropriate. Where to draw the line between an appropriate and an excessive response is exceedingly difficult.

**Surgery-Induced Inflammation.**

Not all inflammation is induced in response to an invading pathogen as shown in Figure 1. In the absence of an infection, the response is known as sterile inflammation. One example is surgery-induced inflammation. Prior to surgery, local anesthetics are usually applied topically or injected into the surgical site\textsuperscript{26}. Surgery itself induces a local inflammatory response at the site of tissue injury. Local anesthetics like lidocaine have been shown to reduce the release of cytokines and chemokines in the acute inflammatory response, yet there is still slow resolution of inflammation and the return to homeostasis\textsuperscript{26}. Within the context of surgery, local anesthetics may have an added benefit; first, they relieve pain at the site of surgery, and later, they may attenuate the induced inflammatory response.
Figure 1. Causes of Inflammation and Their Pathological Consequences. The three inflammatory triggers (infection, tissue injury, and tissue stress and malfunction) induce inflammation resulting in a response with potentially pathological consequences. Infection is the only activator of inflammation that induces an immune response\textsuperscript{27}.

**Treating Inflammatory Diseases**

Currently, many of the therapies that treat inflammatory diseases aim to minimize the symptoms, but they do not prevent progression of the disease\textsuperscript{28}. One drawback of these therapies is that there are typically severe side effects with long-term use\textsuperscript{28}. To address these disadvantages, a more comprehensive understanding of inflammatory pathways is imperative. This approach will fine-tune treatment to minimize side effects and directly resolve inflammation.
Protein Anti-Cytokine Drugs.

At present, most of the drugs that reduce the production of cytokines are proteins. There are many drawbacks to using protein drugs to treat inflammatory diseases. These drugs have poor stability, poor cellular penetration and cellular activity, have short-half lives, are rapidly metabolized, and are expensive to produce. Therefore, it is increasingly important to identify new drugs that have less disadvantages to better treat the wide range of inflammatory diseases.

Small Molecule Anti-Cytokine Drugs.

Newer therapies that are being identified or developed to target inflammatory pathways are small molecules. These drugs tend to be orally active and are successful at targeting proinflammatory signaling pathways in the cell to attenuate the release of proinflammatory cytokines without the disadvantages of protein agents. These small, organic molecule therapeutics are likely the most appropriate type to focus on to treat inflammatory diseases.

Canonical Inflammatory Pathway

The canonical inflammatory pathway consists of TLR4 signaling activation and formation of the NLRP3 inflammasome as shown in Figure 2. Innate immune cells such as macrophages respond to infection or tissue injury by releasing proinflammatory mediators that include cytokines and chemokines to attract circulating leukocytes to the affected tissues. To initiate the inflammatory response in macrophages, two signals are required. The first, priming signal activates TLR4, a transmembrane protein in the plasma membrane that responds to extracellular signals to upregulated proinflammatory
cytokine gene expression\textsuperscript{30}. The second signal leads to NLRP3 inflammasome formation allowing for the cytokine, proIL-1\(\beta\), to be cleaved into its mature form\textsuperscript{30}.

Cytokines are then released from the macrophage acting as the inflammatory mediators that bridge innate and adaptive immunity. The most common cytokines associated with the inflammatory response are TNF, IL-1, IL-6, IL-8, and IL-10\textsuperscript{23}. Cytokines such as IL-1\(\beta\) and IL-18 are unique because they don’t use the classic route of secretion through the endoplasmic reticulum-Golgi; instead, their immature, biologically inactive form that lacks a signal peptide needs to be cleaved for it to be released from the cell\textsuperscript{30,31}. Overall, cytokines elicit the inflammatory response as well as contribute to the immune response, cell growth, and cell migration\textsuperscript{23}. These small molecules facilitate various cellular functions by binding to a cellular receptor to initiate downstream signaling cascades\textsuperscript{23}.

The first, priming signal is commonly a specific structural feature of a bacterium or virus classified as an extracellular pathogen-associated molecular patterns (PAMPs) or endogenous stress signals called danger-associated molecular patterns (DAMPs)\textsuperscript{23,24,30,32}. These signals interact with pathogen recognition receptors (PRRs) that include the Toll-like receptors to activate a signaling cascade\textsuperscript{23,24,30,32}. One common ligand for TLR4 is lipopolysaccharide (LPS), which is found on the surface of gram-negative bacteria. LPS treatment activates NF-\(\kappa\)B to upregulate gene expression of NLRP3 components and pro-IL-1\(\beta\)\textsuperscript{33}. 
Figure 2. Canonical Inflammatory Pathway. Two signals are required for upregulation and release of proinflammatory cytokines. The first priming signal (LPS binding to TLR4) upregulates gene expression of IL-1β and NLRP3. The priming signal deubiquitinates NLRP3 and linearly ubiquitinates and phosphorylates the adaptor protein ASC to initiate inflammasome assembly. A second signal is then required to activate the NLRP3 inflammasome. The four common stimuli for NLRP3 activation include relocalization of NLRP3 to the mitochondria, mitochondrial release of ROS, mtDNA, and cardiolipin, potassium efflux through the P2X7 receptor, or lysosomal destabilization leading to the release of cathepsin. The active NLRP3 inflammasome allows caspase-1 to cleave proIL-1β and proIL-18 into their mature forms that can then be secreted and mediate the inflammatory response.24

Upregulation of proinflammatory cytokine gene expression is dependent upon NF-κB activation through its canonical pathway. More generally, NF-κB is the
transcription factor that mediates upregulation of proinflammatory cytokine gene expression upon infection or injury, but it also plays a key role in resolution of the inflammatory response by upregulating anti-inflammatory genes and genes governing apoptosis\textsuperscript{23}. Specifically, the NF-κB subunits p65 and p50 are responsible for its role in the inflammatory response\textsuperscript{23}. Several kinases phosphorylate IκBα, the inhibitor of the NF-κB subunits p65/p50 that keeps the transcription factor sequestered in the cytoplasm\textsuperscript{23,34}. Phosphorylated IκBα is then polyubiquitinated and marked for degradation by the 26S proteasome\textsuperscript{23,34}. This step allows for NF-κB to translocate into the nucleus where it can bind to the DNA to upregulate inflammatory target genes among others\textsuperscript{23,25,34}.

A second signal is then required to initiate NLRP3 inflammasome formation. One potential signal such as ATP is required to activate the P2X\textsubscript{7} receptor, an ATP-gated ion channel expressed in monocytes and macrophages\textsuperscript{35}. Concentrations greater than 1 mM ATP are required for activation of the P2X\textsubscript{7} receptor leading to NLRP3 inflammasome activation and cleavage of proIL-1β into its mature, secreted form\textsuperscript{35}. IL-1β is then able to recruit other immune cells to initiate an inflammatory response.

Another cytokine of interest is IL-6. It has a unique role in recruiting neutrophils as part of the innate immune response\textsuperscript{28}. As the immune response shifts from innate to adaptive, this cytokine initially attracts monocytes and lymphocytes then it assists in the resolution of inflammation and tissue repair\textsuperscript{28}. Release of IL-6 is not dependent on inflammasome activation because it has a signal sequence that allows it to follow the conventional secretory pathway\textsuperscript{28}. There are nuances to the activation of NF-κB
subunits in the case of IL-6 that are worth noting\textsuperscript{36}. For example, transcription of IκBζ is induced with LPS activation to specifically bind to the p50 subunit of NF-κB that can then associate with the IL-6 promoter\textsuperscript{36}. Therefore, the canonical inflammatory pathway is complex, and that the diverse mechanisms that regulate these pathways still need to be well-defined.

**Toll-like Receptor 4 Signaling**

TLR4 is expressed on a variety of different cell types that include macrophages, cardiac myocytes, and cells in the CNS\textsuperscript{37}. It can be activated by a variety of well-characterized exogenous PAMPs and endogenous DAMPs\textsuperscript{37}. As shown in Figure 3, it most classically senses LPS, requiring the interaction between TLR4 and myeloid differentiation 2 (MD2)\textsuperscript{37}. There is homodimerization of TLR4 that leads to conformational changes that recruit the adaptor protein, myeloid differentiation primary response protein 88 (MyD88)\textsuperscript{37}. In the absence of MyD88, there is delayed activation of NF-κB and MAPK signaling pathways suggesting that the MyD88-dependent pathway is necessary for efficient upregulation of proinflammatory cytokine gene expression\textsuperscript{38}.

Downstream signaling through this pathway leads to activation of transforming growth factor β-activated kinase 1 (TAK1)\textsuperscript{37,39}. TAK1 then activates the mitogen-activated protein kinases (MAPKs), JUN N terminal kinase (JNK), p38, extracellular signal-regulated kinases (ERK1/2), and the IκB kinase complex (IKK) that activate the transcription factors, NF-κB and activator protein-1 (AP1)\textsuperscript{37,39}. TLR activation results in the upregulation of a large number of genes related to antimicrobial defense, metabolic changes, and tissue repair in macrophages as well as both pro- and anti-inflammatory
genes$^{40}$. The role that TLR activation plays in infections, inflammation, autoimmunity, cancer, and allograft rejection/inflammation is still minimally understood, but it is important to understand this pathway for the development of new therapeutics$^{41}$.

**Figure 3. TLR4 Signaling Pathway.** Binding of LPS stimulates homodimerization of TLR4. MyD88 is then recruited to the TIR domain of the TLR4 dimer. MyD88 recruits the IRAKs and TRAF6 to activate TAK1. TAK1 then initiates activation of the MKKs in the MAPK signaling pathway and IKK in the NF-κB signaling pathway. The inhibitors of the transcription factors, AP-1 and NF-κB, are marked for degradation allowing for translocation of the transcription factors into the nucleus. Then there is upregulation of proinflammatory cytokine gene expression. The MyD88-independent pathway leads to upregulation of Type 1 interferons, but that type of response is not of interest in this study$^{37}$. 


Activation of the NLRP3 Inflammasome

Mechanism of Activation.

Several PRRs such as nucleotide-binding domain, leucine-rich repeat containing proteins (NOD-like receptors, NLRs) play a critical role in inflammasome activation by sensing signals like DAMPs and PAMPs to upregulate NLRP3 expression and contribute to inflammasome activation via other poorly understood mechanisms\(^\text{24,42}\). Part of the response to the priming signal causes deubiquitination of NLRP3 which is a fast post-translational modification compared to the more commonly described changes in transcription\(^\text{43}\).

A variety of disparate but generally accepted mechanisms activate the NLRP3 inflammasome. These mechanisms include potassium efflux from the cell\(^\text{44}\), relocalization of NLRP3 to the mitochondria, mitochondrial release of ROS, mitochondrial DNA, cardiolipin, and cathepsin release from destabilized lysosomes\(^\text{24,45}\). Commonly accepted signals include ATP, pore-forming toxins, crystalline substances, nucleic acids, hyaluronan, and fungal, bacterial, and viral pathogens\(^\text{24,30,45}\). It is hypothesized that these diverse signals converge on a point in the signaling pathway to induce NLRP3 inflammasome activation\(^\text{24,45}\). There also is some evidence to suggest that an increase in intracellular calcium is associated with inflammasome activation\(^\text{24,45}\). Again, there is controversy regarding how all these signals can initiate inflammasome formation.

Inflammasomes are typically composed of a sensor protein (NLR), an adaptor protein (ASC), and a zymogen such as pro-caspase-1\(^\text{45}\). Pro-caspase-1 is constitutively
expressed in monocytes and macrophages, so its activity needs to be tightly regulated by the inflammasome\textsuperscript{44,46}. The NLRP3 inflammasome can be activated by whole pathogens, environmental irritants, or cell-derived signals that indicate cellular damage or stress\textsuperscript{46}. When activated, NLRs form a scaffold that activates caspase-1\textsuperscript{24}. Active, fully cleaved, caspase-1 which is a protease can then cleave interleukin-1 (IL-1) proteins into their mature form to be secreted from the cell\textsuperscript{24,47}. It also can induce pyroptosis, a form of inflammation-associated cell death\textsuperscript{24,47}. Pyroptosis occurs through swelling of the cytoplasm and rupture of the plasma membrane\textsuperscript{30}.

To ensure that there is not generally aberrant activation, there are a variety of negative regulators in the pathway\textsuperscript{48}. The canonical inflammatory pathways are generally mediated by protein kinases and ubiquitin ligases\textsuperscript{49}. Thus, it can be negatively regulated by phosphatases and de-ubiquitin ligases\textsuperscript{49}. It has recently been shown that among various NLRP3 agonists reducing cytosolic levels of K\textsuperscript{+} is necessary and sufficient to activate caspase-1\textsuperscript{50}. Furthermore, an increase in intracellular Na\textsuperscript{+} concentrations contribute to but are not required for inflammasome activation\textsuperscript{50}. As evidenced here, there are several examples of the inflammatory pathways being activated or regulated by changes in intracellular cation concentrations.

**Role in Disease.**

In neurological diseases, misfolded protein aggregates and excessive accumulation of metabolites act as endogenous DAMPs that lead to activation of the NLRP3 inflammasome\textsuperscript{24}. One type of systemic inflammation is caused by missense mutations in NLRP3 that lead to the overproduction of inflammatory cytokines\textsuperscript{51}. It
causes the following diseases: familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and neonatal-onset multisystem inflammatory disease. These diseases cause recurrent fever, rash, conjunctivitis, and arthralgia. A better understanding of the NLRP3 inflammasome will allow for the development of more effective treatments for these diseases.

**Lidocaine’s Classically Characterized Inhibition of Ion Channels**

**Voltage-Gated Sodium Channels.**

Lidocaine is currently used as a local anesthetic, and it prevents ventricular arrhythmias. Lidocaine has analgesic effects by inhibiting voltage-gated sodium channels (VGSCs) in neurons. It blocks $\text{Na}^+$ from passing through the channel pore. Thus, it prevents membrane depolarization in neurons to keep them from firing in response to pain stimuli. For lidocaine to interact with a VGSC in this manner, the drug needs to cross the cell membrane and enter the channel from the cytoplasmic side.

Perhaps lidocaine has the same type of interaction with VGSCs expressed in macrophages to mediate its anti-inflammatory properties.

One study analyzed the effect of lidocaine on chemokine release from intestinal epithelial cells. It was hypothesized that lidocaine reduces chemokine release by inhibiting VGSCs. To test this hypothesis, two VGSC agonists that open these channels were tested with lidocaine to determine whether the drug inhibits VGSCs in this context. This experimental condition did not affect lidocaine’s reduction in chemokine release. These results suggest that either lidocaine exerts anti-
inflammatory effect through a mechanism that doesn’t involve VGSCs or that lidocaine interacts with the channels in a different way than the two agonists that were used.

**Potassium Channels.**

In the literature, it has been shown that lidocaine also interacts with potassium channels. Predominantly expressed in immune cells, P2X receptors are a class of ligand-gated ion channels that are permeable to sodium, potassium, and calcium. Modeled using *Xenopus* oocytes, lidocaine was shown to inhibit current through ATP-activated purinergic P2X7. It was found that lidocaine noncompetitively inhibited the P2X7 receptor as evidenced by the ATP concentration-response curve. Additionally, the use of two specific agonists for P2X7 were unable to block the activity of lidcaine. Unique to this study, the positively-charged analog of lidocaine, QX-314, was either injected into the oocyte or was applied extracellularly to determine whether the target of lidocaine was localized on the plasma membrane or localized intracellularly. Interestingly, QX-314 applied extracellularly or intracellularly reduced ATP-induced currents. Thus, lidocaine may act at the plasma membrane in its charged form or it may cross the membrane in its uncharged form to also act intracellularly. There are still some open questions regarding how lidocaine interacts with ion channels and the effect these different interactions have on the cell.

**Ion Channels and Inflammation**

In general, ion gradients are essential to healthy cells, and ion fluxes contribute to activation of signaling pathways and enzymes. There is some evidence suggesting that ion channel expression or activity contributes to inflammation in the following
diseases: diarrheal illness, pyelonephritis, allergy, acute lung injury, and systemic inflammatory responses such as septic shock. The major ion channels that have been identified in this role include sodium, chloride, calcium, potassium, transient receptor potential, purinergic receptor, and acid-sensing ion channels. Recently, ion channels have become attractive drug targets because they play a critical role in a variety of cellular functions. Currently, approximately 15% of drugs target ion channels to ameliorate disease.

In the inflammatory response, when cells are activated by a second signal, ATP, there is a change in ionic intracellular concentrations from high K\(^+\), low Na\(^+\), and low Cl\(^-\) to low K\(^+\), high Na\(^+\), and high Cl\(^-\). Furthermore, an increase in intracellular Na\(^+\) coupled with a decrease in intracellular K\(^+\) is required for an unidentified step in P2X7 activation of caspase-1. It has also been shown that release of IL-1\(\beta\) corresponds to an increase in intracellular Ca\(^{2+}\) that was released from the ER in conjunction with the required ATP-induced K\(^+\) efflux for secretion. Thus, there are a variety of examples of changes in ion concentrations affecting activation of the inflammatory response.

**P2X7 Receptor.**

P2X receptors are ion channels found on the plasma membrane that are activated by extracellular ATP. When ATP binds, the P2X pore switches from a closed to open conformation that allows for ions to pass through the channel. When ATP is present for long periods of time, there is generation of a large pore in the plasma membrane that allows for hydrophilic molecules of up to 900 Da to pass through the
channel. One study found that pharmacological inhibition of the P2X7 receptor attenuated IL-1β release from LPS-activated spinal microglial cells.

More recent evidence suggests that NLRP3 activation is connected to fluxes in calcium concentration. When there are elevated extracellular concentrations or movement of intracellular reservoirs of calcium into the cytosol, NLRP3 is activated by various agonists. When there is a decrease in extracellular Ca\(^{2+}\), there is a 90% reduction in IL-1β secretion, but this change has no effect on cleavage of proIL-1β into its mature form by caspase-1. Specifically, the role between a decrease in intracellular potassium concentration and an increase in intracellular calcium concentrations in NLRP3 inflammasome activation has been controversial. One recent study found that an increase in intracellular [Ca\(^{2+}\)] was not necessary or required to activate the inflammasome in macrophages. However, many studies were reviewed and showed that calcium ion flux does contribute to NLRP3 activation, so it is likely that more work needs to be done to address these discrepancies.

Furthermore, bone-marrow derived macrophages (BMDMs) cultured with LPS, ATP, and either extracellular Ca\(^{2+}\) or K\(^{+}\) reduced IL-1β secretion. When LPS-only stimulated BMDMs were cultured with Ca\(^{2+}\), IL-1β was secreted in an NLRP3 inflammasome dependent manner. NRLP3 activation caused by extracellular ATP has been shown to be affected by intracellular Ca\(^{2+}\) signaling. Furthermore, LPS-stimulated macrophages secreted fewer cytokines when the large-conductance Ca\(^{2+}\)-activated potassium channel MaxiK was blocked. Blockade of this potassium channel selectively inhibited NF-κB and MAPK p38 signaling. Currently, there is some
evidence in the literature showing that inhibition of ion channels has an anti-inflammatory effect on cells.

**Mitogen-activated protein-kinase signaling pathway.**

![Figure 4. Model of MAPK Signaling Pathway Regulating Ion Channel Expression.](image)

Inflammatory mediators (IM) activate G-protein coupled receptors on the cell surface to initiate a phosphorylation cascade of kinases. Mitogen-activated protein kinase activated protein kinase-2 (MAPKAP kinase-2) phosphorylates to inactivate tristetraprolin (TTP). TTP then destabilizes ion channel (IC) mRNA by binding to AU-rich sequences. MAPKAP kinase-2 can also stabilize TTP mRNA to further contribute to degradation of IC mRNA. MAPK can also phosphorylate and activate NF-κB to increase IC gene expression\(^2\).

One key pathway that links VGSC and the inflammatory response is the mitogen-activated protein-kinase signaling pathway (MAPK)\(^2\). Intracellular and extracellular
signals including peptide growth factors, cytokines, hormones, and cellular stressors are responsible for activating MAPK signaling\(^73\). A MAP3K, apoptosis signal-regulating kinase 1 (ASK1), is a mediator in both the p38 and JNK signaling pathways\(^73\). Interestingly, ASK1 is activated by several cellular stressors that include reactive oxygen species (ROS), LPS, endoplasmic reticulum stress, and Ca\(^{2+}\) influx\(^73\). This pathway is known to mediate changes in gene expression and mRNA stability of many of the ion channels mentioned as shown in Figure 4\(^20\). It is also known that p38 MAPK signaling contributes to the transcription of TNF-\(\alpha\), IL-1, IL-6, and IL-8\(^28\). One study found that in LPS and ATP stimulated dorsal horn microglial cells there was widespread phosphorylation of the p38 MAPK signaling cascade that was associated with IL-1\(\beta\) release\(^61\). A second study found that by selectively inhibiting the ERK 1/2, JNK, and p38 MAPK signaling pathways, there was a reduction in IL-1\(\beta\) release from activated cells, and the degree of the inhibitor's effect on cytokine secretion depended on the activating signal\(^74\). These results suggest that the p38 MAPK signaling pathway is contributing to some degree to IL-1\(\beta\) release from activated microglia\(^61\). Additionally, when monocytes had a 30 min pretreatment with a MAPK inhibitor before LPS activation, there was less IL-1\(\beta\) protein present primarily due to less gene transcription\(^75\). Additionally, it has been shown in the literature that there is phosphorylation in the p38 MAPK signaling pathway resulting from activation of both TLR4 and the P2X7 receptor\(^61\). Specifically, TLR4 signaling results in phosphorylation of p38 which contributes to stabilization of the mRNA transcripts of various cytokines\(^61\). It is likely that any anti-inflammatory drug that
reduces the transcription of proinflammatory cytokines has some effect on the MAPK signaling pathway.

**Local Anesthetics and Inflammation**

Local anesthetics are typically composed of a tertiary amine attached via an ester or amide linkage to a substituted aromatic ring\textsuperscript{76}. The classic mechanism of action for local anesthetics is preventing Na\textsuperscript{+} from entering the cell\textsuperscript{1}. This blockade prevents membrane depolarization in neurons to prevent them from firing in response to pain stimuli\textsuperscript{1}. Local anesthetics act on Na\textsuperscript{+} channels, K\textsuperscript{+} channels, Ca\textsuperscript{2+} channels, transport enzymes and phospholipases in excitable cells\textsuperscript{77–82}. One study showed that lidocaine had less affinity for Ca\textsuperscript{2+} channels compared to Na\textsuperscript{+} channels\textsuperscript{77}. Interestingly, one study showed that 1 mM lidocaine blocked the K\textsuperscript{+} channel TASK-2 from the tandem pore domain K\textsuperscript{+} channels (2P K\textsuperscript{+} Channels), but the positively charged analog, QX-314 had no effect\textsuperscript{76}. The 2P K\textsuperscript{+} channels are expressed in the CNS, and they regulate the resting membrane potential and firing of excitable cells\textsuperscript{76}. The results from these studies suggest that lidocaine can interact with a variety of channels but with different affinities. This insight will be valuable in our investigation of how lidocaine has anti-inflammatory effects on activated macrophages.

There are two types of anti-inflammatory drugs that are currently available\textsuperscript{26}. Classic examples act as enzyme inhibitors or receptor antagonists with more recent drugs acting as resolution agonists\textsuperscript{26}. It has been reported that local anesthetics have anti-inflammatory properties in several contexts including reducing inflammation in
muscle cells and cardiac tissues. In the future, local anesthetics may be repurposed to treat inflammatory diseases.

**Lidocaine as an Anti-Inflammatory Agent**

The literature suggests that local anesthetics such as lidocaine can reduce pro-inflammatory responses in activated microglia. In general, results from multiple studies indicate that lidocaine pretreatment or use as a topical agent has the most anti-inflammatory effects and improvement in disease models. One study addressed whether lidocaine can reduce inflammation associated with LPS-induced sepsis in a rat model. They found that there was a reduction in TLR4 and NF-κB expression and IL-6 release. These data specifically suggest that lidocaine inhibits the TLR4 MyD88-dependent signaling pathway by reducing NF-κB translocation into the nucleus.

A few in vitro studies have analyzed how lidocaine treatment affects stimulated cultured rat or mouse microglial cells. Several types of stimulatory molecules have been used. In LPS-only activated cells, the data showed that lidocaine pretreatment reduced p38 MAPK phosphorylation, NF-κB inhibitor, IκBα, phosphorylation, and NF-κB translocation into the nucleus. An in vitro model using RAW264.7 murine macrophages found a decrease in IL-1β secretion and mRNA expression in LPS and lidocaine treated cells. In TNF-α only activated intestinal epithelial cells, there was a marked reduction in the transcription of the inhibitor of NF-κB, IκB, decrease in the degradation of IκBα, and a decrease in the translocation of NF-κB into the nucleus. These results suggest that lidocaine may be acting on a target or pathway that is upstream of the activity of the inhibitor of NF-κB. Microglial cells stimulated with LPS and Interferon γ exhibit signs of
cell injury. This treatment followed by lidocaine reduced pro-inflammatory cytokine release. Interestingly, a permanently charged analog of lidocaine exhibited a degree of reduction of cytokine production compared to lidocaine, suggesting that lidocaine may be interacting with the cell at the plasma membrane. At physiological pH, lidocaine is in both its neutral and positively charged forms, so it can cross the cell membrane or act on the cell’s extracellular plasma membrane surface. It is still unclear what cellular target lidocaine has in this context.

Another study treated rat microglial cells with ATP only. ATP induces formation of the NLRP3 inflammasome complex which allows for proIL-1β to be cleaved into its mature form in the classical inflammatory pathway, but ATP is also known to induce elevated intracellular concentrations of calcium. In this study, lidocaine treated cells had lower concentrations of ATP-induced intracellular calcium as well as decreased levels of phosphorylated p38 MAPK and decreased mRNA and protein levels of IL-1β and IL-6. Overall, the anti-inflammatory effect of lidocaine has been tested in several different cell types and disease models.

Most of the data in the literature reproducibly shows that lidocaine attenuates proinflammatory cytokine release by either downregulation or inhibition of the NF-κB signaling pathway in activated microglia or macrophages as well as other cell types. Concentrations of lidocaine ranging from 0.2 mM to 20 mM have been shown to reduce the secretion of IL-1β (comparable to 50 μg/mL to 5 mg/mL). However, there is some evidence to suggest that lidocaine treatment of LPS-activated macrophages also affects inflammasome formation leading to reduced activation of caspase-1. Wen et al.
also showed that lidocaine reduces the occurrence of pyroptosis which is a proinflammatory form of cell death\textsuperscript{33}.

The current literature consistently demonstrates that lidocaine inhibits p38 MAPK phosphorylation and NF-κB translocation into the nucleus and that it attenuates pro-inflammatory cytokine production\textsuperscript{4–6,14,86}. These studies are starting to show pieces of lidocaine’s anti-inflammatory effect, but there still needs to be more consensus in the field and the proposal of a well-defined mechanism of action.

**Snake Venom Peptides as Anti-Inflammatory Agents**

Snake venoms have been used as a form of alternative anti-inflammatory medicine in China for centuries\textsuperscript{15,87}. This venom contains a diverse set of molecules that include toxins, enzymes, and other bioactive factors that have been found to have anti-tumor, anti-inflammatory, anti-stroke, and analgesic effects\textsuperscript{15,87}. There are several studies in the literature that are examining whether these molecules derived from snake venom have anti-inflammatory properties and their mechanisms of action\textsuperscript{15–17}. One study modeled pulmonary fibrosis in mice using LPS-induced inflammation\textsuperscript{15}. Prior to this study, it had been shown that naja naja atra venom (NNAV) or some component of it had anti-inflammatory effects in a rheumatoid arthritis mouse model\textsuperscript{15}. NNAV consists of several different molecules including a neurotoxin, a cardiotoxin, nerve growth factor, phospholipase A2, and cobra venom factor\textsuperscript{15}. Thus, it is difficult to know which specific factor is responsible for the anti-inflammatory effects. There was a significant reduction in IL-1β and TNF-α found in the serum of animals treated with NNAV compared to control only\textsuperscript{15}. Additionally, NF-κB activation was measured. With LPS treatment, the
p65 subunit was translocated from the cytoplasm to the nucleus, yet with NNAV treatment there was a significant reduction in NF-κB translocation into the nucleus\textsuperscript{15}. It is worth noting that the dose of NNAV used in this study was very small compared to the dose injected by a snake bite\textsuperscript{15}. More adverse effects are seen with the dose of a snake bite, but significantly lower therapeutic doses can potentially be beneficial with reduced side effects\textsuperscript{15}.

A second study analyzed the anti-inflammatory effects of a specific peptide isolated from Naja naja atra venom. The peptide, neurotoxin-Nna (NT), had previously been characterized as an analgesic that binds to the nicotinic acetylcholine receptor to block the transmission of the nerve impulse\textsuperscript{16}. This study treated with NT for 1 hr. before inducing inflammation with carrageenan, a linear, sulfated polysaccharide found on seaweed\textsuperscript{16}. Pretreatment with NT before carrageenan attenuated IL-1β and TNF-α release at least in part by a reduction in NF-κB activation\textsuperscript{16}.

Another study found that a small peptide isolated from sea snakes has antimicrobial and anti-inflammatory properties\textsuperscript{17}. They inhibit LPS-induced production of TNF-α, IL-1β, and IL-6\textsuperscript{17}. It has been found that they act by either directly binding and neutralizing LPS or by binding to the TLR4/MD2 complex to prevent homodimerization and downstream signaling\textsuperscript{17}. Furthermore, in a murine colitis model, mice were treated with a peptide derived from Hydrophis cyanocinctus venom\textsuperscript{88}. Treated mice had reduced phosphorylation of IκB and NF-κB activation accompanied by reduced phosphorylation of p38, JNK, and ERK1/2 in the MAPK pathway\textsuperscript{88}. It was also found that a disintegrin from the venom of Trimeresurus mucrosquamatus inhibits the release
of proinflammatory cytokines from activated macrophages\textsuperscript{89}. In treated cells, there was decreased MAPK phosphorylation and inhibition of NF-κB activation\textsuperscript{89}.

While there is little known about the Zep peptides that were used in this study, the literature suggests some potential mechanisms of action for the anti-inflammatory activity of characterized snake-derived peptides. Finally, recent work demonstrates that snake venom peptides can attenuate cytokine release, but it fails to propose a specific mechanism for their action or test the effects of peptides on more broadly relevant models like human macrophage cell lines.

**Potential Role for Sodium Channels**

**Voltage-Gated Sodium Channels.**

As stated earlier, within the context of pain relief, lidocaine acts on VGSCs to prevent sodium ions from passing through the channel\textsuperscript{2}. Lidocaine and other VGSC blockers prefer to interact with the ion channel in its inactive state specifically at the α subunit\textsuperscript{52}. Sodium channels are expressed in several non-excitable cell types including macrophages to contribute to many of the cell’s common functions\textsuperscript{90,91}. There are nine different voltage-gated sodium channels (Nav1.1-Nav1.9) that share homologous structural elements, but they each have distinct interactions with pharmacological agents\textsuperscript{90,91}. Macrophages specifically express TTX-S current, Nav1.5, and Nav1.6\textsuperscript{90}. There is evidence of upregulation of the sodium channels Nav1.5 and Nav1.6 in various disease states in microglial cells\textsuperscript{91}. The use of TTX and phenytoin, toxins that specifically bind VGSC, reduce the release of cytokines and chemokines from activated microglial cells\textsuperscript{91}. Additionally, in microglial cells treated with a combination of lidocaine
and VGSC inhibitors then LPS, there was a reduction in activation of TLR-4, NF-κB, and MAPK. When exploring lidocaine’s anti-inflammatory activity on macrophages, voltage-gated sodium channels are unlikely to first come to mind as lidocaine’s most likely cellular target. There have been few studies that have first asked the question of whether the same ion channels are expressed in neurons as well as macrophages and microglia and second whether blocking these specific channels influences the inflammatory response.

In 2006, Roselli et al. reviewed the role of VGSC blockers as immunomodulators. Electrophysiological recordings have revealed the presence of VGSC in microglial cells and macrophages. Expression of VGSC in non-excitable cells fluctuates depending on the state of the cell. Additionally, some studies have shown that VGSC specific drugs have reduced the amount of proinflammatory cytokines released from microglia. It has been shown that in microglial cells Na⁺ flux is necessary for their activation because Na⁺ free media or VGSC blockers inhibited their activation. Additionally, this diminished activation is proposed to be due to decreased intracellular Na⁺ concentrations which will affect the exchange of ions through the Na⁺/Ca²⁺ exchanger. If this ion channel has diminished activity, then there will be reduced levels of intracellular Ca²⁺ concentrations which would be associated with decreased activation of microglial cells and macrophages by extension. As stated, stress stimuli such as changes in intracellular Ca²⁺ concentrations affect the MAPK signaling pathway to decrease proinflammatory cytokine gene expression. Figure 5 depicts how sodium channel activity also affects intracellular calcium concentrations.
Ion channel activity in cells is complex, and it has diverse effects on the function of cells. Further study will need to be done to understand how inhibition of ion channels affects the inflammatory response.

**Figure 5. Schematic Depicting the Contribution of Sodium Channels to Intracellular Ca\(^{2+}\) Concentrations.** Depolarization of the microglial cell membrane activates the voltage-gated sodium channel (Nav) allowing Na\(^+\) to enter the cell. Increased intracellular Na\(^+\) concentrations reverses activity of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) to increase intracellular calcium concentrations. This increase contributes to effector functions of the cell. Blockage of Nav with TTX and NCX with KB-R7943 decreases intracellular calcium concentrations\(^{91}\). [Modified from Persson et al. (2014)].

**Sodium-Hydrogen Antiporter 1.**

The sodium-hydrogen antiporter 1 (NHE1) is known to be expressed in multiple cell types, and there is data to suggest that it contributes to inflammatory responses\(^{92}\). It has been shown that inhibition of NHE1 inhibits NF-κB activation and IL-6 production in ischemia-reperfusion injury models\(^{92}\). Inhibition of the antiporter also reduced proinflammatory cytokine levels in the serum of septic rats, and it attenuated secretion
of IL-1β and TNF-α from LPS-activated alveolar epithelium by decreased phosphorylation of the IκB-α92. Furthermore, in intestinal tract epithelial cells, there was a reduction in proinflammatory cytokine release with specific inhibition of NHE1 to minimize NF-κB and p38 MAPK activation92. It is possible that lidocaine affects leukocytes by changing the intracellular pH by inhibiting the Na+/H+ antiporter93. Here is further evidence that another sodium transporter has been connected to the inflammatory response, and inhibition of NHE1 attenuates proinflammatory cytokine release.

Potential Role for Calcium Channels

Store-Operated Calcium Entry.

Increases in intracellular calcium concentrations activate lymphocytes specifically microglial cells19,91. These changes also contribute to the effector functions of microglial cells that include migration and release of cytokines and chemokines to mediate the inflammatory response91. Influx of Ca2+ into lymphocytes is primarily mediated by store-operated calcium entry (SOCE) and calcium-release activated calcium channels (CRAC)19. There is constant seepage of calcium ions from the ER that are returned via the SOCE mechanism94. Effector functions of microglial cells such as phagocytosis and cytokine secretion that are induced by activation with LPS require sustained high intracellular concentrations of Ca2+19.

Additionally, one study conducted by Heo et al. showed that the use of pharmacological inhibitors and knockdown of these calcium channels resulted in a decrease in the amount of TNF-α and IL-6 that were released from activated microglial
Under regular cellular conditions, activity of calcium channels needs to be regulated because sustained high intracellular concentrations of Ca\textsuperscript{2+} cause cell death\textsuperscript{19}. Data from this study showed that SOCE activity is required for the release of cytokines especially IL-6 from activated microglia\textsuperscript{19}. Overall, the literature suggests that calcium channels contribute to activation of the inflammatory response and release of proinflammatory cytokines. Here is another potential target for drug anti-inflammatory activity.

**Potential Role for Potassium Channels**

Several studies have found that amide local anesthetics including lidocaine interact with K\textsuperscript{+} channels\textsuperscript{10}. It has been shown that blocking of potassium channels inhibits NF-κB activation\textsuperscript{10}. One study found that in myelinated neurons lidocaine decreased K\textsuperscript{+} channel activity by reducing the probability that the channel was in its open state\textsuperscript{95}. Lidocaine most likely blocks K\textsuperscript{+} channels from inside the cell because adding the positively charged analog of lidocaine, QX-314, intracellularly blocks channel activity to the same extent as lidocaine\textsuperscript{95}. QX-314 applied extracellularly is unable to block the pore suggesting that it is necessary for lidocaine to be able to cross the cell membrane to act on an intracellular target\textsuperscript{95}. Computer simulations have also found that snake venom derived polypeptides can bind to the outer portion of a K\textsuperscript{+} to block ion flow\textsuperscript{3}. Potassium channel blockade is another potential mechanism that anti-inflammatory drugs may to decrease cytokine release from activated macrophages or microglia.
Conclusion

Recently, therapeutics have been identified for their anti-inflammatory properties. These drugs include lidocaine and Zep peptides 3/4. In our model, we plan to test whether lidocaine and Zep peptides attenuate proinflammatory cytokine release from LPS and ATP activated human differentiated THP-1s. If they do attenuate cytokine release, then the primary question will be what is their mechanism of action? There is plenty of evidence in the literature that sodium, calcium, and potassium channel activity all contribute to some degree to the activation of inflammation. Which channels are involved in the anti-inflammatory effect of lidocaine or Zep peptides? Overall, the literature lacks a well-defined hypothesis for the mechanism for how these newly identified drugs attenuate the inflammatory response. In the following chapters, we will address these questions to enrich our understanding of inflammatory pathways: how they are regulated and what cellular targets are implicated in this response.
CHAPTER THREE
MATERIALS AND METHODS

Lidocaine Attenuates Proinflammatory Cytokine Release from Activated Macrophages

Cell Culture.

THP-1s, a human monocyte cell line, were obtained from American Type Culture Collection (ATCC). The THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 10% characterized fetal bovine serum (FBS) from Gibco, 100 IU/mL penicillin, 1 mg/mL streptomycin, and 10 ug/mL ciprofloxacin hydrochloride. Characterized FBS was used because it has lower levels of endotoxins compared to standard FBS to reduce background activation in the following assays. The cells were cultured at 37° C with 5% carbon dioxide.

THP-1 cells were differentiated into macrophage-like cells by treating with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 24 or 48 hrs. The cells rested for 48-72 hrs before treatment to reduce background activation from PMA differentiation of the proinflammatory genes of interest.

Quantification of IL-1β Secretion by ELISA.

THP-1 cells were plated at 500,000 cells per well in a 24-well plate with 100 ng/mL PMA for 24 hrs to differentiate the monocytes into macrophages. After approximately 36 hrs, the differentiated macrophages were pretreated with 1 mM
lidocaine hydrochloride for 16 hrs (Sigma-Aldrich). At the end of the pretreatment, some wells had a media change, were treated again with 1 mM lidocaine, or previously untreated wells were treated with 1 mM lidocaine. Then to activate TLR4 signaling, 10 ng of LPS was added for 4 hrs. A media change was done then 5 mM ATP was added to initiate NLRP3 inflammasome formation. After 3.5 hrs, the supernatant was collected from each sample. This experiment was repeated at least three times unless otherwise noted.

In a second set of experiments following the same basic protocol as described above, the treatments differed in the following ways: the cells were either pretreated for 30 minutes with 1 mM lidocaine before the addition of 10 ng LPS, were pretreated for 16 hrs with 1 mM lidocaine then treated with 1 mM lidocaine before the addition of 10 ng LPS or were treated with 5 mM ATP only as a control. LPS treatment was for 4 hrs., and ATP treatment was for 3.5 hrs. Then the supernatant was collected. This experiment was repeated at least three times unless otherwise noted.

The samples were analyzed following the manufacturer’s instructions for the R&D Systems Human IL-1 beta/IL-1F2 DuoSet ELISA. The plate was read using the Gen 5 Plate Reader software. The standard curve was fit with a 4-parameter logistic curve. The fit equation was generated and the sample concentrations of IL-1β were predicted using the Gen 5 Plate Reader software.
RT-PCR Analysis of Proinflammatory Cytokine Gene Expression.

THP-1 cells were plated at a density of 500,000 cells/well in a 24 well plate with 100 ng/mL PMA and RPMI media to differentiate the monocytes into macrophages. After 24 hrs, the RPMI media was replenished, and the cells rested for 36 hrs. Then the cells were treated with 1 mM of lidocaine hydrochloride for 16 hrs (Sigma-Aldrich). Some wells had a media change, then 1 mM lidocaine was added immediately prior to the addition of 10 ng of LPS in the appropriate wells. After 4 hrs, the media was changed, and 5 mM ATP was added in the indicated wells. After 3.5 hrs, the cell lysate was collected.

The RNA was isolated following the manufacturer’s protocol for the Nucleospin RNA Plus kit (Macherey-Nagel). The RNA was then reverse transcribed to the complementary DNA (cDNA) sequence using an oligodeoxythymidylic acid primer following the manufacturer's protocol (Promega). Real-time PCR (RT-PCR) was performed on the cDNA with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). PCR was performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds then 50°C for 1 minute. GAPDH was the housekeeping gene for normalization. The primer set used to amplify GAPDH was 5’-GCACCCTCAAGGCTGAGAAC-3’ for the 5’ primer and 5’-GCCTTCTCCATGGTGTTGA-3’ for the 3’ primer. The primer set used to amplify IL-6 was 5’-GGAGACTTGGCCTGGTGAAA-3’ for the 5’ primer and 5’ATCTGAGGTGCCCATGCTAC-3’ for the 3’ primer. The primer set used to amplify proIL-1β was 5’-AATCTGTACCTCTGCCTAGT-3’ for the 5’ primer and 5’-
TGGGTATAATTTTGATCTACTACTCT-3' for the 3' primer. The mRNA levels of both IL-6 and proIL-1β were normalized to the GAPDH mRNA levels. Fold change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method was used.

**Statistical Analyses.**

GraphPad Prism software (GraphPad Software, San Diego, CA) was used to conduct the statistical analyses. In most cases a one-way ANOVA was performed using the Tukey multiple comparison test (the mean of each group was compared to the mean of every other group). All the reported values are reported as mean ± SEM. Each experiment was performed at least three times unless otherwise noted.

**Zep 3 and Zep 4 Peptides Attenuate Proinflammatory Cytokine Release from Activated Macrophages**

**Cell Culture.**

THP-1s, a human monocyte cell line, were obtained ATCC. The THP-1 cells were cultured in RPMI 1640 media, supplemented with 10% characterized FBS from Gibco, 100 IU/mL penicillin, 1 mg/mL streptomycin, and 10 ug/mL ciprofloxacin hydrochloride. Characterized FBS was used for the reasons described above. The cells were cultured at 37°C with 5% CO₂.

THP-1 cells were differentiated into macrophage-like cells by treating with 100 ng/mL PMA for 24 or 48 hrs. The cells rested for 48-72 hrs before treatment to reduce background activation from PMA differentiation of the proinflammatory genes of interest.
Quantification of IL-1β Secretion by ELISA.

THP-1 cells were plated at 500,000 cells per well in a 24-well plate with 100 ng/mL PMA for 24 hrs to differentiate the monocytes into macrophages. After approximately 36 hrs, the differentiated macrophages were pretreated with 12 ug/mL Zep 3 or 50 ug/mL Zep 4 for 16 hrs (Dr. Bruce Gaynes, Loyola University Chicago). At the end of the pretreatment, some wells had a media change, were treated again with 12 ug/mL Zep 3 or 50 ug/mL Zep 4, or previously untreated wells were treated with 12 ug/mL Zep 3 or 50 ug/mL Zep 4. Then to activate TLR4 signaling, 10 ng of LPS was added for 4 hrs. A media change was done then 5 mM ATP was added to initiate NLRP3 inflammasome formation. After 3.5 hrs, the supernatant was collected from each sample. The samples were analyzed following the manufacturer’s instructions for the R&D Systems Human IL-1 beta/IL-1F2 DuoSet ELISA. The plate was read using the Gen 5 Plate Reader software. The standard curve was fit with a 4-parameter logistic curve. The fit equation was generated and the sample concentrations of IL-1β were predicted using the Gen 5 Plate Reader software.

RT-PCR Analysis of Proinflammatory Cytokine Gene Expression.

THP-1 cells were plated at a density of 500,000 cells/well in a 24 well plate with 100 ng/mL PMA and RPMI media to differentiate the monocytes into macrophages. After 24 hrs, the RPMI media was replenished, and the cells rested for 36 hrs. Then the cells were treated with 12 ug/mL Zep 3 or 50 ug/mL Zep 4 for 16 hrs (Dr. Bruce Gaynes, Loyola University Chicago). Some wells had a media change, then 12 ug/mL Zep 3 or 50 ug/mL Zep 4 was added immediately prior to the addition of 10 ng of LPS in the
appropriate wells. After 4 hrs, the media was changed, and 5 mM ATP was added in the indicated wells. After 3.5 hrs, the cell lysate was collected.

The RNA was isolated following the manufacturer’s protocol for the Nucleospin RNA Plus kit (Macherey-Nagel). The RNA was then reverse transcribed to the complementary DNA (cDNA) sequence using an oligodeoxythymidylic acid primer following the manufacturer’s protocol (Promega). Real-time PCR (RT-PCR) was performed on the cDNA with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). PCR was performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds then 50°C for 1 minute. GAPDH was the housekeeping gene for normalization. The primer set used to amplify GAPDH was 5’-GCACCGTCAAGGCTGAGAAC-3’ for the 5’ primer and 5’-GCCTTCTCCATGGTGTTGAA-3’ for the 3’ primer. The primer set used to amplify IL-6 was 5’-GGAGACTTGCTGCTGAAAA-3’ for the 5’ primer and 5’ATCTGAGGTGCCCATGCTAC-3’ for the 3’ primer. The primer set used to amplify proIL-1β was 5’-AATCTGTACCTGTCTGCGTT-3’ for the 5’ primer and 5’-TGGGTAGGTGGATCTACACTCT-3’ for the 3’ primer. The mRNA levels of both IL-6 and proIL-1β were normalized to the GAPDH mRNA levels. Fold change in gene expression was calculated using the 2-ΔΔCt method was used.

Optimized Differentiation of THP-1s into Human Macrophage-like Cells

Cell Culture.

THP-1s, a human monocyte cell line, were obtained ATCC. The THP-1 cells were cultured in RPMI 1640 media, supplemented with 10% characterized FBS from
Gibco, 100 IU/mL penicillin, 1 mg/mL streptomycin, and 10 ug/mL ciprofloxacin hydrochloride. Characterized FBS was used for the reasons described above. The cells were cultured at 37° C with 5% CO₂.

THP-1 cells were differentiated into macrophage-like cells by treating with 100 ng/mL PMA for 24 or 48 hrs. The cells rested for either 24 hrs, 48 hrs, 72 hrs, or 96 hrs before treatment to find the optimal time when background activation from PMA differentiation is at its lowest.

**Cell Imaging.**

The cells were imaged after 24 hrs. or 48 hrs. of PMA differentiation. The cells were again imaged after 24 hrs., 48 hrs., 72 hrs., and at 96 hrs. of rest following differentiation. The changes in morphology at each of these time points was noted. The cells were imaged using a confocal microscope.

**RT-PCR Analysis of Proinflammatory Cytokine Gene Expression.**

THP-1 cells were plated at a density of 500,000 cells/well in a 24 well plate with 100 ng/mL PMA and RPMI media to differentiate the monocytes into macrophages. After 24 hrs. or 48 hrs., the RPMI media was replenished, and the cells rested for 24 hrs., 48 hrs., 72 hrs., or 96 hrs. The appropriate wells were then treated with 10 ng of LPS to upregulate proinflammatory cytokine gene expression. After 4 hrs, the cell lysate was collected.

The RNA was isolated following the manufacturer’s protocol for the Nucleospin RNA Plus kit (Macherey-Nagel). The RNA was then reverse transcribed to the complementary DNA (cDNA) sequence using an oligodeoxynthymidylic acid primer
following the manufacturer's protocol (Promega). Real-time PCR (RT-PCR) was performed on the cDNA with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). PCR was performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds then 50°C for 1 minute. GAPDH was the housekeeping gene for normalization. The primer set used to amplify GAPDH was 5’-GCACCGTCAAGGCTGAGAAC-3’ for the 5’ primer and 5’-GCCTTCTCCATGCTGGTGAA-3’ for the 3’ primer. The primer set used to amplify IL-6 was 5’-GGAGACTTGCTGGTGAA-3’ for the 5’ primer and 5’-ATCTGAGGTGCCCATGCTAC-3’ for the 3’ primer. The primer set used to amplify proIL-1β was 5’-AATCTGTACCTGCTCGTGGT-3’ for the 5’ primer and 5’-TGTTATTTTTTGGATCTACACTCT-3’ for the 3’ primer. The mRNA levels of both IL-6 and proIL-1β were normalized to the GAPDH mRNA levels. Fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method was used.
CHAPTER FOUR
LIDOCAINE ATTENUATES PROINFLAMMATORY CYTOKINE RELEASE FROM ACTIVATED MACROPHAGES

Introduction: IL-1β Secretion from Lidocaine Treated Macrophages

Recently, local anesthetics such as lidocaine have been identified for their potential anti-inflammatory effects on activated macrophages or microglial cells. Within the context of pain relief, lidocaine acts on voltage-gated sodium channels\(^2\). However, the canonical inflammatory pathways including TLR4 signaling and NLRP3 inflammasome activation do not include a defined role for ion channels aside from potassium efflux that initiates inflammasome formation upon ATP activation of the P2X7 receptor.

Briefly, several studies have shown that lidocaine attenuates proinflammatory cytokine release from macrophages and other cell types in different inflammatory disease models\(^{4-14}\). In the literature, various inflammatory stimuli were used, and lidocaine treatment reduced cytokine release to different degrees\(^{4-14}\). In one study, mouse microglial cells were treated with LPS and IFN-γ to cause endotoxemia and subsequent inflammation\(^6\). A positively charged lidocaine analog that cannot cross the plasma membrane was used\(^6\). The data showed a slight decrease in IL-1β release but no change in TNF-α from the lidocaine treated cells compared to the positive control\(^6\). In contrast, another study showed a more significant decrease in TNF-α and IL-1β release...
from LPS-activated and lidocaine treated rat microglial cells⁴. Additionally, rat microglial cells were stimulated with 1 mM ATP only for 2 hrs and lidocaine¹⁴. With lidocaine treatment, there was a dose-dependent decrease in TNF-α, IL-1β, and IL-6¹⁴. In general, there is some discrepancy in the literature regarding the degree of lidocaine’s anti-inflammatory effect on proinflammatory cytokine release.

Overall, several studies have been done to understand the effect lidocaine has on microglial cells and other cell types that have been stimulated by different combinations of inflammatory signals. Generally, these studies showed that lidocaine treatment attenuated the release of IL-6, TNF-α, and IL-1β. However, none of these studies have used a more broadly applicable model human cell line nor have the cells been stimulated by both LPS and ATP which are commonly used stimuli of the canonical TLR4 signaling and NLRP3 inflammasome activation.

Based on the literature and some preliminary experiments, we hypothesized that if lidocaine has an anti-inflammatory effect on activated macrophages then there will be attenuated release of the proinflammatory cytokine, IL-1β, from these cells. To expand upon what is currently known about lidocaine’s anti-inflammatory effect, we used differentiated THP-1s which are an immortalized human macrophage-like cell line. Additionally, we used both LPS to initially prime the TLR4 MyD88-dependent signaling pathway to upregulate the gene expression of proIL-1β. Then ATP was used as the second signal to trigger inflammasome complex formation and cleavage of proIL-1β into its mature, secreted form. It has been shown that monocytes that were only activated
with a priming signal released less than 10% of the IL-1β that had accumulated in the cytosol\textsuperscript{22}. In our experiments, we measured IL-1β released in the supernatant after two activating signals which gave us higher concentrations of the cytokine release compared to other studies.

The differentiated THP-1s cells were either pretreated with lidocaine for 16 hrs. before activation and/or a lidocaine treatment at the time of LPS activation. These different timepoints of treatment allow for an understanding of whether there are temporal restraints on lidocaine’s anti-inflammatory effect on these cells. If lidocaine pretreatment reduces IL-1β release, then the effect will not be directly due to ion flux, suggesting a mechanism that affects cellular signaling. In contrast, if lidocaine treatment at the time of LPS attenuates IL-1β release, then lidocaine has a rapid, direct anti-inflammatory effect comparable to its rapid analgesic response. Again, if lidocaine diminishes proinflammatory cytokine release, then with lidocaine treatment we would expect significantly reduced levels of IL-1β compared to LPS and ATP treatment only.

**Experimental Design: Quantification of IL-1β Release**

WT THP-1s were plated at a cell density of 500,000 cells/well in a 24 well plate with 100 ng/mL PMA to differentiate the cells for 24 or 48 hrs. At this time, the media was replaced with fresh RPMI containing Characterized FBS. The cells were then allowed to rest for 36 hrs (after 24 hrs PMA differentiation) or 60 hrs (after 48 hrs PMA differentiation). Lidocaine (1 mM) was added for the 16 hr pretreatment. After the pretreatment, the media was changed in the indicated wells. Then lidocaine (1 mM) was added immediately prior to the addition of 10 ng LPS. After 4 hrs, the media was
changed, and 5 mM of ATP was added to the appropriate wells for 3.5 hrs. Then the supernatant was collected to quantify the amount of IL-1β that was released using a sandwich ELISA (R&D Systems Human IL-1 beta/IL-1F2 DuoSet ELISA). This experiment was repeated at least three times unless otherwise noted. In a second set of experiments following the same basic protocol as described above, the treatments differed in the following ways: the cells were either pretreated for 30 minutes with 1 mM lidocaine before the addition of 10 ng LPS, were pretreated for 16 hrs with 1 mM lidocaine then treated with 1 mM lidocaine before the addition of 10 ng LPS or were treated with 5 mM ATP only as a control. This experiment was repeated at least three times unless otherwise noted.

**Results: Quantification of IL-1β Release**

To establish if lidocaine has anti-inflammatory effects on activated macrophages, we chose to use an overnight pretreatment and a lidocaine treatment at the time of LPS addition. This treatment plan allowed for an understanding of whether a pretreatment was required or if lidocaine had an immediate effect. When lidocaine acts as a numbing agent, its effects are more immediate. In that context, lidocaine directly blocks the voltage-gated sodium channels in neurons. This blockade prevents the neuron from firing and signaling pain. The question here was whether lidocaine initiates a cascade of signaling to minimize an induced inflammatory response over time or does lidocaine’s interaction with a cellular target immediately reduce release of proinflammatory cytokines. We hypothesized that the drug more likely initiates a change in signaling to reduce an inflammatory response because inflammation is activated and regulated by
several signaling pathways. In contrast, the pain response is mediated directly by ion channel activity as evidenced by lidocaine’s known blockade of sodium ion flux.

As expected, no treatment of differentiated THP-1s resulted in minimal release of IL-1β. LPS and ATP treatment caused a significant increase in IL-1β release (Figure 6). All lidocaine pretreatments attenuated IL-1β release by approximately a 3-fold reduction. Interestingly, the lidocaine pretreatment with a media change before LPS stimulation also reduced release of the cytokine. These results suggested that lidocaine causes changes in the cell that are still altered at the time of treatment with the proinflammatory signals to minimize the response to those stimuli. It was unexpected that a lidocaine pretreatment then media change would still affect the responsiveness of the cell to the inflammatory signals. A possible explanation is that lidocaine is acting intracellularly and was unaffected by a media change. This explanation is also reasonable because at physiological pH lidocaine is in both its charged and uncharged forms. Its uncharged form can pass the plasma membrane and act intracellularly in either its positive or uncharged form or the charged form can target the cell surface.

This experiment also demonstrated that lidocaine treatment at the time of LPS addition does not have an anti-inflammatory effect on these cells as evidenced by no difference between the amount of IL-1β released from this lidocaine treatment compared to the LPS and ATP only control. These results do not support the idea that lidocaine has an immediate effect perhaps through direct changes in ion flux to attenuate the inflammatory response. The data suggested that changes in ion flux are not directly responsible for reducing differentiated THP-1 response to LPS and ATP. In
the case where cells were treated with both a lidocaine pretreatment and lidocaine at the time of signal 1, there was release of IL-1β comparable to the amount of IL-1β released from cells only pretreated with lidocaine. Taken together, the effect on the twice lidocaine treated differentiated THP-1s was likely due primarily to the lidocaine pretreatment since the lidocaine treatment at the time of LPS did not result in reduced cytokine secretion.

**Figure 6. Lidocaine Pretreatment Reduces IL-1β Release from LPS and ATP Activated Macrophages.** Differentiated THP-1s were treated with 1 mM lidocaine 16 hrs. prior to LPS activation and/or at the time of LPS activation. After 4 hrs., the media was changed, and 5 mM ATP was added to the appropriate wells. After 3.5 hrs., the supernatant was collected, and an ELISA was performed to measure the amount of IL-1β secreted. This image was representative of three independent experiments.

One challenge of working with THP-1s is that there can be a lot of variability in responsiveness to stimuli and drug treatment between cell cultures and experiments. To address these differences, Figure 7 shows the average of 10 independent lidocaine
experiments with either the 16 hrs. lidocaine pretreatment and treatment at the time of LPS or lidocaine treatment at the time of LPS only. Furthermore, the raw maximum concentration of IL-1β released from can vary greatly, so here the data are represented as fold change in IL-1β secretion. Across experiments, there is a significant reduction in IL-1β release from lidocaine pretreated cells, and there is a slight decrease in IL-1β release from lidocaine treated cells at the time of LPS. Even with the variability associated with THP-1s (discussed further in Chapter 7), lidocaine pretreatment significantly attenuated the release of IL-1β to levels comparable to no treatment. Even lidocaine treatment at the time of LPS slightly reduces cytokine release which is not as evident when looking at the results from only one experiment. Overall, the lidocaine pretreatment consistently reduced the amount of IL-1β released to approximately $17 \pm 7\%$ of the amount released by LPS and ATP only.
Figure 7. Lidocaine Treatment Significantly Reduced the Average Fold Change in IL-1β Secretion from LPS and ATP Activated Differentiated THP-1s. Differentiated THP-1s were treated with 1 mM lidocaine 16 hrs. prior to LPS activation and/or at the time of LPS activation. After 4 hrs., the media was changed, and 5 mM ATP was added to the appropriate wells. After 3.5 hrs., the supernatant was collected, and an ELISA was performed to measure the amount of IL-1β secreted. This graph was representative of ten independent experiments. Data were analyzed by one-way ANOVA with Tukey's post-hoc test. Error bars indicated standard error of the mean.

The 16 hrs. pretreatment raised the question of whether it was lidocaine itself that had the anti-inflammatory effect or if it could potentially be a metabolite or some other factor that caused reduction in cytokine release. To address this question, cells were pretreated with lidocaine for 30 mins. before the addition of LPS and later ATP. This timepoint was chosen because in the clinical setting patients receive a lidocaine injection approximately 30 mins. before a procedure when numbing is required\textsuperscript{26}. If lidocaine has an anti-inflammatory effect with a 30 mins. pretreatment before initiation of
an inflammatory response, then patients who received a lidocaine injection before treatment may also have the added benefit of less inflammation as part of the healing process and less inflammation-associated pain. Lidocaine may have a dual function of providing immediate pain relief during surgery, but also limiting later inflammation caused by the tissue injury.

The second challenge to be addressed was that proIL-1β is highly expressed in untreated differentiated THP-1s. As THP-1s are in culture, we noticed that they have increased expression of proinflammatory genes over time. We think these changes relate to how sometimes with lidocaine treatment the amount of IL-1β released is more comparable to no treatment levels but in other experiments the release is not as attenuated. An ATP control only allowed us to appreciate the quantity of IL-1β cleaved by ATP that solely depends on basal expression of proIL-1β.

The data showed that a 30 min lidocaine pretreatment attenuated IL-1β release from activated macrophages to approximately the same degree as the 16 hrs. lidocaine pretreatment with a second lidocaine treatment at the time of LPS (Figure 8). Moreover, the differentiated THP-1s treated with ATP only released a quantity of IL-1β that was not very different from the lidocaine treatment. These results suggested that with a clinically relevant lidocaine pretreatment of 30 mins. there were decreased levels of IL-1β released from activated macrophages.
Figure 8. A Clinically Relevant Lidocaine Pretreatment and an ATP Only Control Reduced Release of IL-1β Compared to LPS and ATP Activated Differentiated THP-1s. Differentiated THP-1s were treated with lidocaine 30 min. before LPS or treated with lidocaine overnight and at the time of LPS. After 4 hrs., the cells were additionally treated with ATP or ATP only as a control. After 3.5 hrs., the supernatant was collected, and an ELISA was performed to measure IL-1β release. Data were analyzed by one-way ANOVA with Tukey’s post-hoc test. Error bars indicated standard error of the mean.

Introduction: Quantification of Proinflammatory Cytokine Gene Expression

To begin to understand the mechanism through which lidocaine attenuated release of the proinflammatory cytokine, IL-1β, we decided to quantify the fold change in gene expression of IL-6 and proIL-1β. ProIL-1β requires two signals for it to be released from the cell. Release of IL-1β requires upregulation of its expression and activation of the NLRP3 inflammasome to cleave it into its mature, secreted form. In contrast, IL-6 release does not require a second signal for its release from activated macrophages.\textsuperscript{28}
It contains a signal sequence that marks it for conventional secretion\(^2\). As mentioned earlier, proIL-1\(\beta\) is highly expressed in untreated differentiated THP-1s, meaning that upon activation with LPS there was a smaller increase in gene expression of this cytokine. In contrast, IL-6 is minimally expressed in untreated macrophage-like THP-1s, so upon LPS treatment, there is a significant increase in IL-6 gene expression. In these experiments, we used two signals to activate these pathways to establish a model in our lab that can be used to test a variety of potential anti-inflammatory drugs. Activating the differentiated THP-1s with both LPS and ATP will allow us to begin to elucidate lidocaine’s mechanism of action.

In the literature, a decrease in proinflammatory cytokine gene expression with lidocaine treatment accounts for at least part of the reduction in secretion of TNF-\(\alpha\), proIL-1\(\beta\), and IL-6\(^8,10\). In rat microglial cells stimulated with ATP only, there was a dose-dependent decrease in gene expression of these cytokines with lidocaine treatment\(^14\). A second study used LPS only to activate TLR4 signaling, and the data showed that there was a decrease in gene expression of TNF-\(\alpha\) and proIL-1\(\beta\) with lidocaine treatment\(^4\). Even though there is some evidence in the literature showing that lidocaine reduces proinflammatory cytokine gene expression, the extent of this reduction is rather minimal. As a result, perhaps the reduction in gene expression is not the only factor contributing to reduced proinflammatory cytokine release.

Furthermore, in the literature, there are two main pathways that are commonly studied in relation to gene expression of proinflammatory cytokines\(^4,14\). In the canonical inflammatory pathway, activation of TLR4 signaling leads to the translocation of the
transcription factor, NF-κB, into the nucleus where it binds to DNA and upregulates gene expression related to several cellular processes. Additionally, the MAPK signaling pathway responds to stress stimuli such as changes in intracellular cation concentrations to activate downstream transcription factors that also regulate gene expression. If lidocaine acts through a mechanism that inhibits or downregulates these pathways, then we would expect reduced gene expression of proIL-1β and IL-6.

**Experimental Design: Quantification of Proinflammatory Cytokine Gene Expression**

We will again use THP-1s plated at a density of 500,000 cells/well in a 24 well plate. They were differentiated with 100 ng/mL of PMA for 24 hrs then allowed to rest for 36 hrs in fresh RPMI medium. The cells were either pretreated with 1 mM lidocaine for 16 hrs and/or with 1 mM lidocaine at the time of LPS treatment. The cells were treated with 10 ng of LPS for 4 hours then with 5 mM of ATP for 3.5 hrs. The cell lysate was collected. Then RNA isolation, cDNA conversion, and RT-PCR were performed on each of the samples. These RT-PCR analyses were performed on the same treated cells whose supernatant was collected for the previously described ELISA experiments. This experimental approach allowed for comparisons to be made between changes in gene expression and the amount of protein released. Each experimental condition was repeated at least 3 times unless otherwise noted.

In a second set of experiments, the protocol above was followed, but the treatments were different in the following ways: the differentiated THP-1s were pretreated for 30 mins. with 1 mM lidocaine before the addition of 10 ng LPS, the cells
were pretreated for 16 hrs with 1 mM lidocaine and again with 1 mM lidocaine at the
time of 10 ng LPS treatment or the cells were treated with 5 mM ATP only for 3.5 hrs.
Each experimental condition was repeated at least 3 times unless otherwise noted.

**Results: Quantification of Proinflammatory Cytokine Gene Expression**

Our first step towards understanding how lidocaine attenuated proinflammatory
cytokine release was to measure gene expression for proIL-1β and IL-6. If there was
less expression of these genes in lidocaine treated cells, we would begin to understand
one of the contributing factors that accounted for lidocaine’s anti-inflammatory effect on
human macrophage-like cells. We would expect the fold change in cytokine gene
expression of lidocaine treated cells to be comparable to no treatment if lidocaine
inhibited or downregulated the NF-κB and MAPK signaling pathways.

The fold change in gene expression was normalized to the no treatment
expression of each of the proinflammatory cytokine genes. There was significant
upregulation of gene expression of proIL-1β and IL-6 when the differentiated THP-1s
were treated with both LPS and ATP (Figure 9 A. & B.). There was far greater fold
increase in IL-6 expression with LPS and ATP treatment compared to proIL-1β. This
difference was likely due to there being high expression of proIL-1β in untreated
differentiated THP-1s compared to relatively low expression of IL-6 in these cells. Upon
activation, IL-6 was more upregulated than proIL-1β due to differences in their basal
expression.

In the case of proIL-1β, a 16 hrs. lidocaine pretreatment followed by a media
change before either a second lidocaine treatment or the addition of LPS resulted in a
decrease in gene expression comparable to no treatment. With the other lidocaine pretreatments, there was also a downregulation in proIL-1β, but not to the same extent (Figure 9. A.). These results suggested that the reduction in IL-1β release from activated macrophages was at least in part due to a downregulation of the cytokine’s gene expression. With the lidocaine treatment at the time of LPS, there was no significant change in gene expression between that treatment and the LPS and ATP only treatment (Figure 9. A.). These data suggested that the changes in IL-1β release from activated macrophages was perhaps dependent on lidocaine having time to reach its target to initiate downregulation signaling of proinflammatory cytokine gene expression.

Similar trends were seen with IL-6 gene expression (Figure 9. B.). With the lidocaine pretreatments consisting of a 16 hrs. pretreatment and/or a second lidocaine treatment at the time of LPS addition, there was a reduction in IL-6 gene expression that was comparable across all those treatment groups. Again, lidocaine treatment at the time of LPS did not result in a change in IL-6 gene expression compared to the LPS and ATP only control. For both genes analyzed by RT-PCR, there was a reduction in gene expression with lidocaine pretreatment, but no change in gene expression when lidocaine was added at the time of the initiation of the inflammatory response.
Figure 9. Lidocaine Pretreatment Reduces Gene Expression of proIL-1β and IL-6 from LPS and ATP Activated Macrophages. Differentiated THP-1s were pretreated with lidocaine for 16 hrs and/or were treated with lidocaine at the time of LPS treatment. The macrophage-like cells were treated with LPS for 4 hrs. then ATP for 3.5 hrs. The cell lysate was collected. The RNA was isolated, cDNA was made, and RT-PCR was performed for each of the samples. These are representative images from at least three independent experiments. A. Fold change in gene expression of proIL-1β. B. Fold change in gene expression of IL-6.
Figure 10. Lidocaine Pretreatment Significantly Reduced the Average Fold Change in IL-1β Gene Expression from LPS and ATP Activated Differentiated THP-1s. Differentiated THP-1s were pretreated with lidocaine for 16 hrs and/or were treated with lidocaine at the time of LPS treatment. The macrophage-like cells were treated with LPS for 4 hrs. then ATP for 3.5 hrs. The cell lysate was collected. The RNA was isolated, cDNA was made, and RT-PCR was performed for each of the samples. This graph was representative of ten independent experiments. Data were analyzed by one-way ANOVA with Tukey’s post-hoc test. Error bars indicated standard error of the mean.

As described earlier, working with THP-1s can be challenging because there are differences in how responsive the cells are between experiments and between cell cultures. These challenges were again addressed by averaging the gene expression of proIL-1β for the corresponding 10 independent lidocaine experiments that were shown in Figure 7. The differentiated THP-1s were treated with either a 16 hrs. lidocaine pretreatment and treatment at the time of LPS or lidocaine treatment at the time of LPS only. Between experiments, there was a significant reduction in proIL-1β gene expression from lidocaine pretreated cells, but there was no significant decrease in
proIL-1β gene expression from cells treated with lidocaine at the time of LPS (Figure 10). Overall, the data suggested that lidocaine pretreatment attenuated IL-1β release at least in part by reducing this cytokine’s gene expression.

Moreover, corresponding to the ELISA data where a 30 mins. lidocaine pretreatment and an ATP only control were tested, the cell lysate from those experiments was analyzed for changes in proinflammatory gene expression (Figure 11. A. & B.). Again, the fold change was normalized for all treatment groups against no treatment. With LPS and ATP treatment, there was upregulation of gene expression of proIL-1β and IL-6 to roughly the same extent as in the previous set of experiments. However, there was no reduction in gene expression of either proIL-1β or IL-6 for the 30 mins. lidocaine pretreatment or the 16 hrs. lidocaine pretreatment with a second lidocaine treatment at the time of LPS addition. These results were somewhat surprising, but they suggested that the reduction of IL-1β from lidocaine pretreated differentiated THP-1s were likely not solely due to a reduction in cytokine gene expression. As expected, there was only minimal upregulation of proIL-1β and IL-6 gene expression for the ATP only treatment. These results were consistent with the results of the earlier Su et al. study that showed there was marginal upregulation of proinflammatory cytokine gene expression in ATP treated cells\textsuperscript{5}. Taken together, these data suggested that the reduction in IL-1β release from LPS and ATP activated macrophages was partially due to a decrease in proinflammatory cytokine gene expression.
Differentiated THP-1s were pretreated with lidocaine for 30 mins. or were pretreated for 16hrs and again at the time of LPS. The cells were then activated with LPS for 4 hrs. and ATP for 3.5 hrs. In this experiment, there was also an ATP only control. The cell lysate was collected. The RNA was isolated, cDNA was made, and RT-PCR was performed on all samples. This is representative data from one experiment that was performed at least 3 times for all treatment groups. A. Fold change in gene expression of proIL-1β. B. Fold change in gene expression of IL-6.
Discussion

Our goal was to determine if lidocaine could attenuate proinflammatory cytokine release from human macrophage-like cells. Several previous studies have analyzed cytokine release from predominantly in vitro or in vivo mouse or rat models\textsuperscript{4–14}. We wanted to use a more relevant model in our studies, so we chose the human monocyte cell line, THP-1s. We activated differentiated THP-1s with both LPS and ATP to provide the two signals necessary for TLR4 signaling and NLRP3 inflammasome formation. The use of these two signals in the same experiment was unique to our study. It allowed us to begin to determine if lidocaine treatment affected activation of one or both signaling pathways. To identify whether lidocaine attenuated proinflammatory cytokine release from differentiated THP-1s, we measured IL-1\(\beta\) secretion by ELISA. Additionally, we aimed to elucidate the mechanism of lidocaine’s anti-inflammatory effects. We measured fold change in gene expression of proIL-1\(\beta\) and IL-6 by RT-PCR to determine if lidocaine treatment downregulated cytokine gene expression. Overall, we hypothesized that if lidocaine has anti-inflammatory effects on LPS and ATP activated human macrophage-like cells, then it would attenuate proinflammatory cytokine release by (1) inhibiting a cellular target to decrease intracellular cation concentrations and by (2) reducing upregulation of proinflammatory cytokine gene expression mediated by NF-\(\kappa\)B and MAPK signaling.

Our data showed that lidocaine pretreatment was required for the most significant decrease in IL-1\(\beta\) release compared to LPS and ATP activated cells. Lidocaine treatment at the time of LPS did reduce cytokine release but to a minimal
degree in comparison. The corresponding results for gene expression showed that proIL-1β expression was downregulated closer to no treatment levels. Also, between experiments, IL-6 expression for lidocaine pretreated cells was downregulated but to a lesser extent than proIL-1β. Perhaps there were differences in the degree of the effect of lidocaine treatment between genes because proIL-1β is highly expressed in these cells so activation by LPS has a lesser effect than it does for IL-6. Furthermore, the literature shows that IL-6 expression is regulated by a specific subunit of NF-κB that differs from proIL-1β gene upregulation\textsuperscript{36}. Lidocaine pretreatment may not affect transcription of these cytokines in the same way.

Since our pretreatment was for 16 hrs., the question arose of whether lidocaine was being metabolized in that time frame resulting in a metabolite being responsible for the effect we saw. We tested a 30 min. lidocaine pretreatment to address this question. This length of time is comparable to the amount of time lidocaine is applied before surgery\textsuperscript{26}. The results showed that a 30 min. pretreatment was sufficient to attenuate cytokine release, but it had no significant effect on cytokine gene transcription. The data suggested that downregulation of cytokine gene transcription was only one part of a more complex mechanism that contributes to decreased cytokine secretion. Our results showed that a clinically relevant pretreatment reduced the production of IL-1β, suggesting that patients may experience less surgery-induced inflammation. Further studies would be required to identify other ways that lidocaine reduces inflammatory activation in our model.
In the second set of experiments, an ATP only control was used to gain a sense of how much released IL-1β came from the high basal expression level of proIL-1β. The amount of the cytokine found in the supernatant from ATP only treated cells was slightly less than the amount released from lidocaine, LPS, and ATP treated cells. The data suggested that inflammasome activation and cleavage may still contribute significantly to the amount of IL-1β released from lidocaine treated cells. Further experiments focusing on NLRP3 inflammasome activation specifically would need to be done to identify what effect lidocaine has on inflammasome formation.

One caveat of these experiments is their variability. Between experiments, lidocaine has varying degrees of effect on the amount of IL-1β that is released from these activated cells. One possible explanation is that there may be variability in the differentiation of the WT THP-1s. Even though the procedure followed is the same each time, perhaps the cells from different cultures and from cells with more passages in cultures may respond differently to PMA. There is some suggestion in the literature that less differentiated cells have a reduced number of ion channels. If as we hypothesize that lidocaine has an ion channel target, then perhaps in less differentiated cells, there are fewer targets for lidocaine diminishing its effect. In experiments where we only see a marginal effect with lidocaine treatment, those cells may have been less differentiated and expressed fewer ion channels to reduce lidocaine’s anti-inflammatory effect.

A second possibility is that over time in culture these cells express more proinflammatory cytokines at the basal level. As the cells become more proinflammatory, stimulation with LPS has a smaller effect on transcription. Thus, the
fold change in gene expression between the no treatment and LPS and ATP narrowed. Perhaps under these conditions, lidocaine has less of an anti-inflammatory effect. There was more fluctuation between experiments in terms of cytokine gene expression, but that was likely related to changes in basal expression. Additionally, it was likely that lidocaine’s anti-inflammatory effect resulted from other mechanisms besides downregulation in proinflammatory cytokine gene expression. Nevertheless, as evidenced by the average results from ten experiments, consistent results were seen in how much IL-1β secretion decreased from lidocaine treated cells.

Lidocaine treatment at the time of LPS may not allow sufficient time for lidocaine to access its target to reduce cytokine expression and secretion. Okura et al. found that when oocytes were treated with lidocaine and ATP at the same time followed by a second treatment of lidocaine after 5 minutes there was greater inhibition with the second treatment suggesting that there was more time for lidocaine to access its target\(^{53}\). The same may be true in our case where treatment of lidocaine immediately before LPS may not allow enough time for lidocaine to reach its site of activity before inflammation was induced.

From these experiments, we have shown that lidocaine pretreatment of short and long duration attenuated release of IL-1β from LPS and ATP activated differentiated THP-1s. This anti-inflammatory effect was partially due to downregulation of proinflammatory cytokine gene expression, but it also was likely that other mechanisms contributed to this effect.
Future Directions

The next goal towards understanding lidocaine’s anti-inflammatory activity would be to identify the cellular target of lidocaine. The inhibition of several ion channels has been shown to reduce inflammatory responses as outlined in Chapter 2. To identify the cellular target of lidocaine in this context, we have decided to use RNA-Seq. It is an unbiased, global technique that detects all the genes that are either upregulated or downregulated by lidocaine treatment compared to the LPS and ATP control. The results can be analyzed by organizing the genes in their cellular pathways. The data can generate hypotheses about lidocaine’s target and anti-inflammatory mechanism of action.
CHAPTER FIVE
ZEP 3 and ZEP 4 PEPTIDES ATTENUATE PROINFLAMMATORY CYTOKINE
RELEASE FROM ACTIVATED MACROPHAGES

Introduction: IL-1β Secretion from Zep Peptide Treated Macrophages

In the literature, it has been shown that whole snake venom and peptides isolated from the venom have analgesic and anti-inflammatory properties that need to be better understood\textsuperscript{15–17,87}. In several in vivo models, there was less IL-1β and TNF-α in the serum of animals treated with NNAV or NT before activation of an inflammatory response\textsuperscript{15,16}. Peptides derived from snake venom have been used as alternative medicines for centuries, but little is known still about how they have anti-tumor, anti-inflammatory, anti-stroke, and analgesic effects\textsuperscript{15,87}.

Here, we used Zep peptides derived from snake venom obtained from Dr. Bruce Gaynes. We assessed whether the peptides have anti-inflammatory effects on differentiated THP-1s activated with both LPS and ATP. We hypothesized that if Zep peptides have anti-inflammatory properties like other characterized snake venom peptides, then activated macrophages treated with Zep peptides would release less IL-1β compared to LPS and ATP stimulated macrophages. We followed the same general protocol that we used for the lidocaine experiments. Here, differentiated THP-1s were pretreated with either Zep 3 or Zep 4 overnight and at the time of LPS or at the time of LPS only. The cells were primed with LPS then activated with ATP. The cell supernatant
was collected, so that the amount of IL-1β released from these cells could be quantified. If Zep 3 or Zep 4 has an anti-inflammatory effect on differentiated THP-1s, then we would expect to measure less IL-1β release from peptide treated cells compared to LPS and ATP only.

**Experimental Design: Quantification of IL-1β Release**

THP-1s were plated 500,000 cells/well in a 24 well plate. They were differentiated with 100 ng/mL PMA for 24 hrs. After 24 hrs., the media was changed, and the cells rested for 36 hrs. The appropriate wells were pretreated with either 12 µg/mL Zep 3 or 50 µg/mL Zep 4 for 16 hrs. Some of the wells were again treated with either Zep 3 or Zep 4 before 10 ng LPS treatment. After 4 hrs., the differentiated THP-1s were treated with 5 mM ATP. After 3.5 hrs, the supernatant was collected to measure the concentration of IL-1β that was released. A sandwich ELISA was used to quantify the amount of IL-1β that was secreted (R&D Systems Human IL-1 beta/IL-1F2 DuoSet ELISA). These experiments were repeated more than three times unless otherwise noted.

**Results: Quantification of IL-1β Release**

To determine if Zep peptides have anti-inflammatory effects on activated macrophages, we first measured the amount of IL-1β released from treated differentiated THP-1s. If Zep peptides have an anti-inflammatory effect on activated macrophages, then there will be reduction in the amount of IL-1β released from Zep peptide treated cells.
With LPS and ATP activation, there was a robust increase in the amount of IL-1β released compared to no treatment. The differentiated THP-1s treated with Zep 3 or Zep 4 overnight for 16 hrs. and with a second Zep treatment at the time of LPS significantly attenuated the release of IL-1β. For cells that were treated with Zep peptides only at the time of LPS, there was no difference in the amount of IL-1β released compared to the LPS and ATP only control. These results suggested that Zep peptide pretreatment does have an anti-inflammatory effect on activated human macrophage-like cells as seen by a significant decrease in the amount of IL-1β that was secreted.

**Figure 12. Quantification of IL-1β Released from Zep 3 and Zep 4 Treated Activated Differentiated THP-1s.** Differentiated THP-1s were pretreated with Zep 3 or Zep 4 for 16 hrs. and/or at the time of LPS stimulation. The cells were then stimulated with both LPS and ATP. A sandwich ELISA was used to quantify the amount of IL-1β that was released from each condition. This image is representative of at least three independent experiments.
Introduction: Quantification of Proinflammatory Cytokine Gene Expression

Since little was known about these Zep peptides, our experimental plan was exploratory. We followed the same approach we took with lidocaine to better understand how these peptides attenuated proinflammatory cytokine release. In the literature, naja naja atra venom and neurotoxin-nna isolated from the venom have been shown to inhibit NF-κB activation\textsuperscript{15,16}. Based on these earlier studies, it would be reasonable to hypothesize that Zep peptides may also inhibit NF-κB activation. To test this hypothesis, we collected the cell lysate from the earlier ELISA experiments for comparison. The RNA was isolated, cDNA was made, and RT-PCR was performed to measure changes in proinflammatory cytokine gene expression between Zep peptide treated activated cells and LPS and ATP only treated cells. Again, there was a 16 hrs. Zep peptide pretreatment with a second treatment at the time of LPS or the cells were treated with Zep peptides at the time of LPS only before ATP treatment. Based on the literature, if Zep peptides attenuated IL-1β release by inhibiting NF-κB, then we would expect to measure downregulation of proinflammatory cytokine gene expression with Zep peptide treatment.

Experimental Design: Quantification of Proinflammatory Cytokine Gene Expression

THP-1s were plated at a density of 500,000 cells/well in a 24 well plate. They were differentiated with 100 ng/mL of PMA for 24 hrs then allowed to rest for 36 hrs in fresh RPMI medium. The appropriate wells were pretreated with either 12 ug/mL Zep 3 or 50 ug/mL Zep 4 for 16 hrs. Some of the wells were again treated with either Zep 3 or
Zep 4 before 10 ng LPS treatment. After 4 hrs., the differentiated THP-1s were treated with 5 mM ATP. The cell lysate was collected. Then RNA isolation, cDNA conversion, and RT-PCR were performed on each of the samples. These RT-PCR analyses were performed on the same treated cells whose supernatant was collected for the previously described ELISA experiments. This experimental approach allowed for comparisons to be made between changes in gene expression and the amount of protein released. Each experimental condition was repeated at least 3 times unless otherwise noted.

**Results: Quantification of Proinflammatory Cytokine Gene Expression**

To begin to elucidate the anti-inflammatory mechanism of Zep peptides, we measured the changes in proinflammatory cytokine gene expression. If Zep peptides reduced IL-1β release from activated macrophages by inhibiting NF-κB or other relevant transcription factors, then we would expect to measure a decrease in proinflammatory cytokine gene expression from Zep peptide treated cells.

Similar to the lidocaine results, pretreatment with Zep peptides reduced the expression of proIL-1β and IL-6. Zep peptide treatment at the time of LPS addition did not significantly affect gene expression, and the cytokine expression levels were comparable to the LPS and ATP control. Again, Zep peptide pretreatment had a more significant effect on proIL-1β expression compared to IL-6. With Zep peptide pretreatment, proIL-1β expression was comparable to no treatment whereas there was only a marginal reduction in IL-6 expression. Here, the relative fold change in IL-6 expression from the positive control compared to no treatment was not in the same range as other experiments. These data corresponded to a higher basal expression of
proinflammatory cytokines. This variability observed between experiments will be addressed further in the next chapter. The data suggested that Zep peptides attenuated IL-1β release from activated macrophages by downregulating proinflammatory cytokine gene expression.

Figure 13. Zep 3/4 Pretreatment Reduced Gene Expression of proIL-1β and IL-6 from LPS and ATP Activated Macrophages. Differentiated THP-1s were pretreated with Zep 3/4 for 16 hrs. and/or were treated with Zep 3/4 at the time of LPS treatment. The macrophage-like cells were treated with LPS for 4 hrs. then ATP for 3.5 hrs. The cell lysate was collected. The RNA was isolated, cDNA was made, and RT-PCR was performed for each of the samples. This is a representative image from at least three independent experiments.

Discussion

A review of the literature showed that snake venom peptides have analgesic and anti-inflammatory properties, but well-defined mechanisms of action for these peptides have not been identified. In several in vivo models, reduced amounts of IL-1β and
TNF-α were measured in the serum of animals treated with NNAV or NT before activation of an inflammatory response\textsuperscript{15,16}. In these same studies, NNAV and NT were found to inhibit NF-κB activation\textsuperscript{15,16}. It is difficult to draw comparisons between these prior studies and our work with Zep peptides. Little prior work has been done with these peptides. Specifically, we are not aware of their regular functions. For example, they could be enzymes, toxins, or other bioactive molecules which are all commonly found in snake venom\textsuperscript{15,87}. Furthermore, it would be difficult to identify a cellular target without knowing more about its structure and function. In the literature, it has been shown previously that peptides derived from animal venoms inhibited cation channels\textsuperscript{3}. They bind extracellularly to the channel and insert a side chain into the selectivity filter to prevent ions from passing through the channel\textsuperscript{3}. Perhaps Zep peptides could also interact with ion channels to minimize the inflammatory response.

In our experiments, we found that Zep peptide pretreatment before LPS and ATP activation attenuated IL-1β release, but Zep peptide treatment at the time of LPS had no effect. Additionally, we found that Zep peptide pretreatment downregulated expression of proIL-1β and IL-6, but Zep peptide treatment at the time of LPS did not affect gene expression. The data indicated that Zep peptides were able to reduce an induced inflammatory response when applied 16 hrs. prior to LPS and ATP. Less IL-1β was secreted due to reduced gene expression. It is possible that other mechanisms contributed to Zep peptides’ anti-inflammatory activity. Our studies did not assess the effect that Zep peptides have on NLRP3 inflammasome formation or identify potential cellular targets for the peptides. These are both possible future directions that would
offer a more complete understanding of how Zep peptides diminish inflammatory activation in macrophage-like cells.
CHAPTER SIX
OPTIMIZED DIFFERENTIATION OF THP-1s INTO MACROPHAGE-LIKE CELLS

Introduction: Optimized Differentiation of THP-1s into Macrophage-Like Cells

One of the challenges we faced was variability between experiments resulting in differences in the degree of the effect lidocaine had on activated differentiated THP-1s. We observed varying results between THP-1s in different cultures and differences as the cells were in culture for longer periods of time. We noted that based on our qPCR data the cells became more proinflammatory over time which corresponded to them being less responsive to activation by LPS and ATP. Additionally, we used PMA to differentiate the THP-1s into macrophages. PMA activates proinflammatory genes, so it was important to allow the cells to rest following differentiation. Longer periods of rest allowed for activation by PMA to subside. Thus, we wanted to identify the time point when there was a minimum level of background inflammation resulting in a better response to LPS and ATP activation.

A second factor that was noted was how the morphology of the differentiated THP-1s varied depending on how long the cells were treated with PMA and how long they rested. We wanted to identify the ideal length of differentiation and rest before treating the cells. These conditions should give the most consistently differentiated THP-1s to reduce variability between experiments. We were looking for the time point when the cells had the most characteristic morphology of macrophage-like cells. The
cells should be adherent to the plate with a more triangular, pointed morphology compared to when they are circular monocytes. Therefore, we aimed to minimize differences observed in differentiated THP-1s by optimizing the differentiation process to identify when the cells were most responsive to activation by LPS and ATP.

**Experimental Design: Optimized Differentiation of THP-1s into Macrophage-like Cells**

WT THP-1s were seeded at a density of 500,000 cells/well in a 24-well plate. The cells were differentiated with 100 ng/mL PMA. After 24 or 48 hrs., the media was replaced with fresh RPMI. The differentiated cells rested for 24 hrs., 48 hrs., 72 hrs., and 96 hrs. At each time point, pictures were taken of the differentiated cells to record how their morphology changed over time. After each of the designated periods of rest, the cells were treated with 10 ng LPS for 4 hrs. Then the cell lysate was collected. Samples were collected for no treatment and LPS treatment for each time point of rest. After all samples were collected, the RNA was isolated, cDNA was made, and RT-PCR was performed on all the samples. These protocols were executed as described in the Materials and Methods section.

**Results: Optimized Differentiation of THP-1s into Macrophage-like Cells**

First, we wanted to understand how the morphology of differentiated THP-1s changed over time after either 24 hrs. or 48 hrs. of PMA differentiation. Characteristically, when monocytes are differentiated into macrophages, they first have a more circular shape when they first adhere to the plate and then over time they have a more triangular and varied morphology. After 48 hrs. of PMA treatment, the THP-1s
appeared to be more firmly attached to the plate than after only 24 hrs. of PMA differentiation (Figure 14). Following 24 hrs. of rest for either group, the cells started to have more varied and triangular shape. Based on morphology alone, the cells that were differentiated for 48 hrs. before a media change had the most consistent, characteristic morphology after 72 hrs. of rest. For both differentiation groups, by 96 hrs. of rest most of the cells were floating after a 4 hr. LPS treatment. Again, based on morphology alone the cells appeared to have the most characteristic macrophage-like shape after 48 hrs. of PMA differentiation followed by 72 hrs. of rest.

Next, we measured fold change in gene expression for the proinflammatory cytokines, IL-1β and IL-6. In general, cells that were differentiated for 48 hrs. were more responsive to LPS treatment as evidenced by greater fold changes in cytokine expression compared to no treatment. Furthermore, across conditions, 72 hrs. of rest resulted in the greatest upregulation of gene expression with LPS treatment. It is worth noting that between 48 hrs. and 72 hrs. of rest the C_t values for proIL-1β increased by several cycles after that additional 24 hrs. of rest, meaning that there was less basal expression of proIL-1β after more rest. This observation was particularly important in our study because we wanted to find conditions that minimized basal expression of proinflammatory genes. Overall, the optimal differentiation protocol for THP-1s was 48 hrs. of PMA differentiation then 72 hrs. of rest before treatment. These conditions produced cells that had characteristic macrophage morphology and were most responsive to activation with LPS.
Figure 14. Morphology of 24 or 48 hrs. PMA Differentiated THP-1s. THP-1s were differentiated with PMA for either A.) 24 or B.) 48 hrs. Cell images were taken after 24 or 48 hrs. differentiation (labeled day 0), then after 24 hrs, 48 hrs, 72 hrs, or 96 hrs. of rest.
Figure 15. Fold Change in Proinflammatory Cytokine Gene Expression of 24 or 48 hrs. PMA Differentiated THP-1s. THP-1s were differentiated with PMA for 24 or 48 hrs. The cells were then allowed to rest for 24 hrs., 48 hrs., 72 hrs., or 96 hrs. They were activated by LPS for 4 hrs. Then the cell lysate was collected for RNA isolation. cDNA was made and RT-PCR was performed for each of the samples. A.) 24 hrs. PMA differentiation fold change in IL-1β gene expression. B.) 24 hrs. PMA differentiation fold change in IL-6 gene expression. C.) 48 hrs. PMA differentiation fold change in IL-1β gene expression. D.) 48 hrs. PMA differentiation fold change in IL-6 gene expression.
CHAPTER SEVEN

CONCLUSIONS

Inflammation is commonly associated with the development and progression of disease. Chronic or unnecessary inflammation can cause tissue damage which contributes to the pathogenesis of disease. Effective therapeutics that target inflammatory pathways to minimize excessive inflammation will be valuable in the treatment of disease.¹

Since lidocaine and snake venom peptides have been shown to have anti-inflammatory effects on inflammatory models through reduced proinflammatory cytokine secretion, we hypothesized that lidocaine and Zep peptides also were able to exert these effects on macrophages by (1) interacting with a cellular target to decrease intracellular cation concentrations and by (2) downregulating MAPK and NF-κB signaling pathways to reduce proinflammatory cytokine expression and release.

The literature has shown that lidocaine and snake venom peptides attenuated proinflammatory cytokine release from stimulated macrophages, microglial cells, and other cell types.⁴,⁵,¹⁴–¹⁷,⁶–¹³. In our study, we sought to determine whether lidocaine and Zep peptides reduced release of IL-1β from LPS and ATP activated human macrophage-like cells. Our work has found that pretreatment with lidocaine and Zep peptides attenuated the release of IL-1β from activated differentiated THP-1s. We then
aimed to begin to characterize the mechanism of lidocaine and Zep peptides’ anti-inflammatory activity. It has been shown in the literature that lidocaine and snake venom peptides inhibit NF-κB and MAPK activation among other mechanisms. We hypothesized that these anti-inflammatory agents reduced IL-1β release by downregulating proinflammatory cytokine gene expression. We found that pretreatment with lidocaine and Zep peptides decreased gene expression of IL-6 and proIL-1β. Our data suggested that lidocaine and Zep peptides decreased release of inflammatory mediators in part due to a decrease in proinflammatory cytokine gene expression (Figure 16). Further work needs to be done to identify cellular targets of these therapeutics and to further characterize their anti-inflammatory mechanisms of action.
Figure 16. Model of Lidocaine and Zep peptides’ Anti-inflammatory Activity. LPS primes TLR4 to upregulate proinflammatory cytokine and NLRP3 gene expression. ATP is the second signal that activates the P2X7 receptor to initiate NLRP3 inflammasome formation. Caspase-1 then cleaves proIL-1β into its mature, secreted form that mediates the inflammatory response. Pretreatment with lidocaine and Zep peptides downregulated expression of proIL-1β to attenuate IL-1β release. Cellular targets and other contributing factors to their anti-inflammatory mechanism of action still need to be identified.
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VITA

Stephanie Zack was born in Darien, Illinois, on June 12, 1994 to Steven and Linda Zack. She earned her Bachelor of Arts degree in Chemistry from Carthage College in Kenosha, Wisconsin, in May 2016. After graduation, Stephanie then matriculated into the Loyola University Chicago Stritch School Infectious Disease and Immunology Graduate Program under the mentorship of Dr. Edward Campbell.

Stephanie’s thesis work focused on understanding how lidocaine and Zep peptides attenuate an induced inflammatory response. After completion of her Master of Science degree, Stephanie will begin her Ph.D. training in the Integrated Program in Biomedical Science at Loyola University Chicago.