Pruritic Activity and Neurogenic Inflammation with *S. aureus* δ-toxin in Atopic Dermatitis

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Abstract

This study investigates how toxins secreted from *Staphylococcus aureus*, such as δ-toxin, can produce pruritis and initiate neurogenic inflammation to help characterize the cutaneous neural reaction to *S. aureus* colonization in atopic dermatitis. Determining this process could identify targets to help mitigate itch and neurogenic inflammation and its averse immune effects in atopic dermatitis patients. The pruritic activity of δ-toxin was confirmed using behavior tests with mice, since δ-toxin treatment elicits significantly more scratching bouts than the control. ELISA kits were used to determine the extracellular concentrations of CGRP and Substance P after treatment with δ-toxin, PSMα3, PSMα2, and α-toxin of cultured DRG cells. This showed that all toxins triggered significant release of both peptides, indicating that they are sufficient to activate DRG neurons and initiate neurogenic inflammation. The behavior test show that δ-toxin can activate pruriceptors however since the whole DRG were cultured, the results of the ELISA tests are not necessarily directly from the itch receptors.

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Introduction

Atopic dermatitis (AD) is a chronic skin condition that affects approximately 20% of children and 3% of adults (Avena-Woods, 2017). One of the main symptoms in this disease is pruritis (itch), especially in lesional skin, which can affect a patient's quality of life (Rudikoff & Lebwohl, 1998). There are known genetic factors that put individuals at greater risk of developing this disorder given that certain environment factors are encountered (Rudikoff & Lebwohl, 1998). The pathology governing the progression of this disorder has been shown to be a disruption of the immune system acting in the skin, causing aberrant allergic reactions and disruption of the skin barrier (Rudikoff & Lebwohl, 1998). As a result of the skin barrier abnormality, bacterial and fungal infection is a common complication (Rudikoff & Lebwohl, 1998).

Notably, *Staphylococcus aureus (S. aureus)* often colonizes the skin of atopic dermatitis patients (Byrd et al., 2017; Matsui, Nishikawa, Suto, Tsuboi, & Ogawa, 2000; Totté et al., 2016). According to Totté et al. (2016) significantly more AD patients are infected by *S. aureus* compared to healthy patients. More specifically, Matsui et al. (2000) showed that lesional skin tends to contain a higher cell count of *S. aureus* within atopic dermatitis. In contribution to this, it has been shown that δ-toxin, which is secreted by *S. aureus*, induces degranulation of mast cells, triggering the aberrant allergic response attributed to AD (Nakamura et al., 2013). It is also well established that cutaneous nociceptors and prurpriceptors participate in neurogenic inflammation by modulating the immune system to induce inflammation through release of peptides such as Calcitonin gene-related protein (CGRP) and Substance P, like that seen in lesional skin of AD patients (I. M. Chiu et al., 2013; Dong & Dong, 2018; Han et al., 2013). The prevalence of this infection in lesional skin along with its ability to cause inflammation suggests that *S. aureus* contributes to the pruritis seen in AD.

To produce pruritis, *S. aureus* must activate sensory neurons, which will be confirmed in this study. The most studied sensory modality evoked by *S. aureus* is nociception (pain). Specifically, pore forming toxins increase cation permeability and therefore depolarization of nociceptive neurons (Blake et al., 2018; Isaac M. Chiu, Pinho-Ribeiro, & Woolf, 2016) and receptors detect toxins and lipopolysaccharides (Yang & Chiu, 2017). However, there is not much known about how *S. aureus* may mediate pruritic activity and subsequent inflammatory peptide release. This study will look at the roles of δ-toxin in itch and neurogenic inflammation. To accomplish this, CGRP and SubP concentration will be measured using ELISA kits following toxin treatment of DRG cell culture. Behavior tests will also be used to observe the effect of δ-toxin on itch sensation. This will be able to show if these toxins affect itch receptors and subsequently modulate the immune system and contribute to neurogenic inflammation.

Literature Review

Atopic dermatitis is a skin condition that is characterized by chronic itch (pruritis) and dermatitis (Rudikoff & Lebwohl, 1998). These symptoms manifest due to an abnormality in the innate immune system which resides in the skin and participates in allergic reactions (Rudikoff & Lebwohl, 1998). Because of these deficiencies, a common complication is an infection by bacteria (Byrd et al., 2017; Matsui et al., 2000; Rudikoff & Lebwohl, 1998). It has been proven that the most prevalent bacterial infection is *S. aureus* with limited strain diversity due to clonal replication (Byrd et al., 2017; Matsui et al., 2000; Totté et al., 2016). Lesional skin is typical in atopic dermatitis and is accompanied by severe pruritis and disruption of the skin barrier (Rudikoff & Lebwohl, 1998). Not only has *S. aureus* been shown to be prevalent in atopic dermatitis, but it is also specifically linked to lesional skin and therefore immune abnormalities and itch (Matsui et al., 2000; Totté et al., 2016). It has also been indicated that factors such as δ-toxin secreted by *S. aureus* can induce changes in the immune system and interact with sensory neurons innervating the skin (Blake et al., 2018; Nakamura et al., 2013; Yang & Chiu, 2017).

The most relevant sensory innervation for bacterial infection is the C-fibers which transduce pain and itch in distinct subpopulations (Han & Dong, 2014; Han et al., 2013). The mechanisms that δ-toxin utilizes to mediate itch are unknown but there are many known processes of itch we can look at to inspire theory. One of the first processes of itch discovered is the role of histamine, which is released by mast cells (Han & Dong, 2014; Han et al., 2013). Given that δ-toxin has been proven to trigger mast cell degranulation, attributing to skin barrier dysfunction, it is unlikely that δ-toxin acts through the histaminergic pathway (Han et al., 2013; Nakamura et al., 2013). Non-histaminergic itch is mediated by a very diverse set of receptors, including Mas-related G-protein-coupled receptors (Mrgprs), Toll-like receptors (TLRs), and protease-activated receptors (PARs), which are expressed in a distinct population from the population expressing histamine receptors H1R and H4R (Han & Dong, 2014). Not all of the receptors are homogeneously expressed, but MrgprA3 has been shown to be a biological marker for neurons mediating non-histaminergic itch, therefore looking at the effect of δ-toxin on these neurons can inform part of its mechanism (Han & Dong, 2014; Han et al., 2013).

Interestingly, some sensory neurons imitate the bacterial detection devices used in the innate immune system as well as modulate the immune system in the skin (Dong & Dong, 2018; Lim, Choi, Choi, & Hwang, 2016). Often in neurogenic inflammation, CGRP and Substance P are released from the sensory neurons to activate the present innate immune cells and recruit different adaptive immune cells, initiating allergic inflammation (I. M. Chiu, von Hehn, & Woolf, 2012; Richardson & Vasko, 2002; Talbot et al., 2015). Therefore, the activation of the immune system by *S. aureus* could be partially attributed to the stimulation of itch receptors. However it is unclear whether this is an advantageous adaptation for the host or the bacteria since sensory neurons can also be activated by cytokines released by the immune system to release more peptides, forming a feed forward loop promoting neurogenic inflammation and it has been shown that blocking this activity can help reduce the allergic reaction and bacterial

clearance (Pinho-Ribeiro et al., 2018; Talbot et al., 2015). Since they often share receptors, examining the known detection strategies used by the immune system for *S. aureus* could suggest a mechanism to explore for itch.

Neurons that detect pain and itch are very similar, so mechanisms bacteria use to activate pain receptors can be looked at to guide potential itch transduction mechanisms. *S. aureus* in particular secretes toxins that insert into the cellular membrane to form pores, allowing cations to flow into the cell to transduce a neural signal (Blake et al., 2018; I. M. Chiu et al., 2013; Isaac M. Chiu et al., 2016). Although these toxins were studied in nociceptors, they could act on the membrane of any neuron. The other mechanisms such as that of α-toxin through ADAM10 and lipopolysaccharides (LPSs) activating TRPA1 in nociceptors can be looked at as examples (Yang & Chiu, 2017). It may also be prudent to consider indirect methods such as sensitization as seen with TRPV1 in nociceptors (Blake et al., 2018). TRPV1 is specific to nociceptors but a highly related channel TRPA1 and could play a similar role. Similar to pruriceptors, nociceptors are capable of modulating the immune cell response to bacteria, inhibiting inflammation which might increase the effectiveness of bacterial infection (I. M. Chiu et al., 2013).

Since bacterial infection can evoke both pain and itch, it is important to be able to distinguish between them in mouse models. Traditionally to model AD, allergens were injected into the skin to initiate the immune disruption that characterizes atopic dermatitis (Han & Dong, 2014). Based on the placement of the injection, neck or cheek, there are limited behaviors available to the mouse to react to the nociceptive or pruritic stimulus (Shimada & LaMotte, 2008; Yamanoi, Kittaka, & Tominaga, 2019). Using cheek injection, mice will wipe the affected cheek using their forepaw and scratch using their hind-paw to respond to pain and itch, respectively, making it easy to distinguish between the two in behavior tests (Shimada & LaMotte, 2008; Yamanoi et al., 2019).

The extreme pruritis associated with atopic dermatitis can be uncomfortable for those affected, especially with the chronic nature of the disease. To help ease the discomfort patients feel, it first needs to be understood how itch is transduced in the skin. Prevalence of *S. aureus* infection in the lesional skin of atopic dermatitis patients suggests that this bacterium could play a major role in the induction of pruritis. One way bacteria influence the host after infection is the secretion of peptides such as CGRP and Sub P in a process called neurogenic inflammation, *S. aureus* releases many toxins one of which is δ-toxin. Little is known about how δ-toxin affect itch receptors and the subsequent immune modulation, but there are many tools available to aid in the investigation. What is known about itch sensation and bacteria activating cutaneous neurons can be referred to as models of possible mechanisms. Also, given that δ-toxin is known to trigger pain sensation, cheek injection is a powerful tool to discriminate the induction of itch specifically.

Methodology

Behavior Test

Two to four months old wildtype C57BL/6J mice were acclimated to the environment for 30 min the day before testing. They were then reacclimated for ten minutes before injection with saline or δ-toxin (0.5 mM) in the cheek. All mice were recorded for 30 minutes and the videos were later analyzed to count the number of scratching bouts. A one tailed t-test was used to determine significance.

DRG Culture for ELISA

Dorsal root ganglion (DRG) were collected form the spines of 3- to 4-week-old wildtype C57BL/6J mice and suspended in DH10 media with dissociation enzyme. Cells were then centrifuged, resuspended in media, and plated in 96 well plates. The plates were incubated at 37 °C for 2 days and treatment added then incubated for 30 min. Samples were collected for the ELISA tests from the supernatant following plate centrifugation.

CGRP ELISA

The Bertin Bioreagent CGRP ELISA kit was used following the methodology provided. DRG were cultured in a 96 well plate and treated with toxins. Capsaicin and KCl treatment were used as positive controls for CGRP release. Student's t-test was used to determine significance between the nonstandard binding (NSB) and test conditions.

Sub P ELISA

The Cayman Chemical Substance P ELISA kit was used following the methodology provided. DRG were cultured in a 96 well plate and treated with toxins. Capsaicin and KCl treatments were used as positive controls for Sub P release. Student's t-test was used to determine significance between the maximum binding (B0) and test conditions.

Results

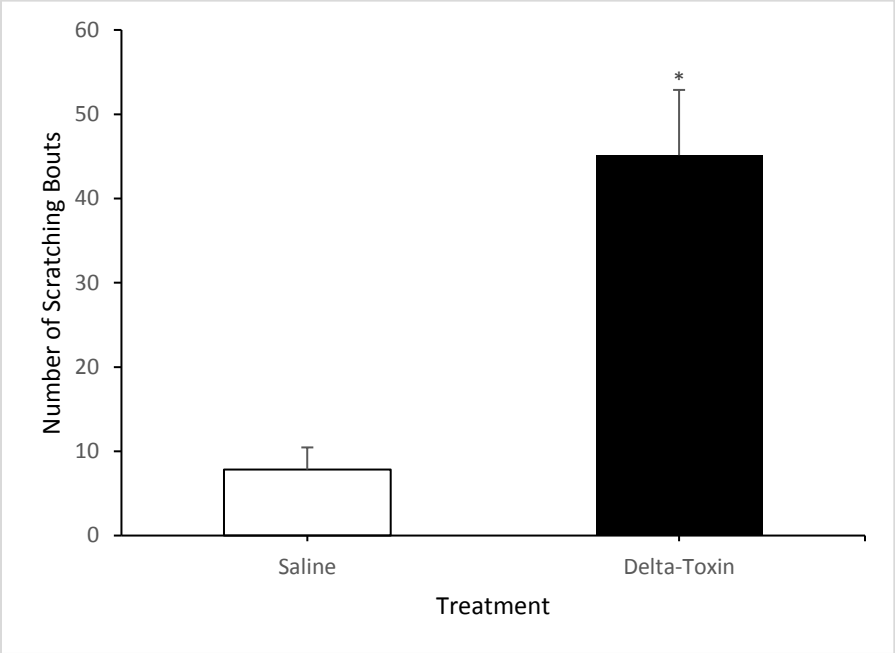

Figure 1. Scratching behavior increased with δ-toxin. Behavior test were ran with Saline (n=6) or δ-toxin (0.5mM, n=7) injected into the cheek of the mouse. * A one tailed t-test showed that the average number of scratching bouts with δ-toxin is significantly higher than Saline with a p-value = 0.00115.

Behavior test videos were utilized to quantify itch sensation by counting the number of scratching bouts. Figure 1 shows that significantly more scratching bouts with δ-toxin treatment than with the negative saline control. This indicates that δ-toxin induces pruritis but does not specify the mechanism through which it acts.

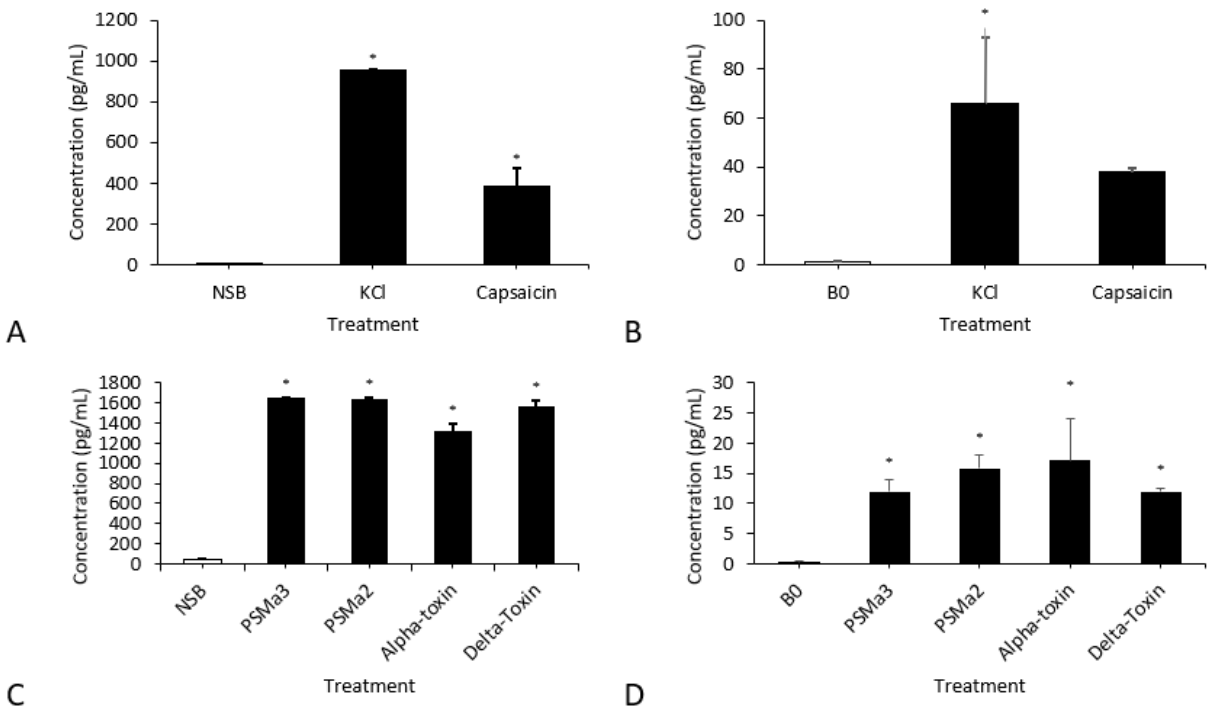

Figure 2. Toxins trigger CGRP and Sub P release. An ELISA kit was used to measure the concentration of A) CGRP with positive control treatments, B) Substance P with positive control treatments, C) CGRP with toxin treatments, and D) Substance P with toxin treatments. * Student's t-tests showed statistical significance compared to the control (NSB or B0) with a p-value<0.05. Duplicates were used for the A and B and triplicate for C and D. Error bars represent SEM.

The CGRP and Sub P ELISA kits were used to determine the concentration of each peptide outside of the cells after culture and treatment. The CGRP ELISA was non-competitive, so the non-specific binding (NSB) was used as the negative control since that is the most extreme case when no CGRP is present. And since the Substance P Elisa test was competitive the maximum binding (B0) represents when the maximum amount of tracer was bound without the presence of Sub P, making it the negative control for this test. Ditionally any negative vlues for the controls were assumed to be 0. Figure 2 A/B shows that the positive controls produce a significantly higher peptide concentration than the negative controls, establishing the validity of the test. The toxin treatments shown in Figure 2 C/D establish that all toxin tested from *S. aureus* initiate a significant release of CGRP and Sub P. This suggests that these toxins contribute to neurogenic inflammation.

Discussion

Overall, δ-toxin elicits pruritis and neurogenic inflammation. The behavior tests show that scratching is observed more in mice injected with δ-toxin than saline and therefore δ-toxin initiates itch sensation. ELISA tests show a significant extracellular concentration of CGRP and Sub P with toxin treatment, suggesting that this interaction contributes to neurogenic inflammation. These results implies that δ-toxin activates pruriceptors to produce itch sensation and triggers subsequent release of peptides associated with neurogenic inflammation.

A challenge with behavior test is to ensure that what is measured is what is expected, to accomplish this in pruritis, the cheek injection model was used to distinguish between itch and pain (Shimada & LaMotte, 2008; Yamanoi et al., 2019). This showed that δ-toxin treatment led to significantly greater number of scratching bouts compared to the saline control. Therefore, proving that δ-toxin can induce itch sensation, although this does not indicate the mechanism. It is known that other toxins such as α-toxin and other bacterial identifiers such as lipopolysaccharides are detected by receptors on neurons directly binding to the ligand an initiating an internal cascade to affect excitation and peptide release (Yang & Chiu, 2017). It is then likely that δ-toxin utilizes a similar mechanism, although the receptor is unknown. The behavior test results make it clear that either directly or indirectly δ-toxin causes excitation of pruriceptors to produce itch sensation, although it is also not confirmed that δ-toxin acts only on pruriceptors so similar effects could be seen in other populations of DRG neurons. This activation can also be further investigated to determine a deeper involvement in neurogenic inflammation.

One of the major responses involved in neurogenic inflammation is the release of peptides such as CGRP and Sub P. ELISA kits were used to measure the concentration of each peptide extracellularly after treatment with four different toxins secreted by *S. aureus*. Positive controls KCl and Capsaicin were used to establish the validity of the test by showing that they elicited a significantly higher peptide release than the negative control. This means that the results showing that the toxin treatments produce a significantly high peptide concentrations can be upheld. In neurogenic inflammation, the neurons innervating the skin such as nociceptors or pruriceptors are activated by ligand of specialized danger receptors, then an internal cascade can directly release the peptides or sensitize the neurons to release peptides (I. M. Chiu et al., 2012; Richardson & Vasko, 2002). Given that there was no other stimulation, it can be inferred that these toxins activate neurons from the DRG to induce CGRP and Sub P release directly instead of sensitizing them.

Conclusions

Firstly, it was confirmed that δ-toxin elicits scratching by producing itch sensation, although the mechanism is still unknown. Following toxin induced activation of DRG neurons, there was a significant release of CGRP and Sub P, showing that all the toxins tested from S. *aureus* are involved in triggering neurogenic inflammation. However, there are still many holes in knowledge of this system that further research will be needed to fill.

To characterize how δ-toxin activates pruriceptors and possibly other neurons, first the receptor or receptors used by the neurons must be identified and it must be determined whether it binds δ-toxin as the ligand or if the effect is more indirect. One potential receptor is formyl peptide receptor 1 and 2 since they are involved in the immune response, which neurons have been known to mimic(Dong & Dong, 2018; Lim et al., 2016). The expression pattern of these receptors in neurons could also indicate which populations of neurons contribute to neurogenic inflammation. Secondly, after receptor activation, the internal signaling cascade leading to peptide release. Previously, calcium dependent internal cascades have been studied in neurons for triggering neurogenic inflammation associated release of peptides (Richardson & Vasko, 2002). Although, it was speculated that the peptide release was triggered directly, this claim should be substantiated by looking a peptide release over time with and without stimulation.

Determining, this mechanism that contributes to the extreme itch and poor clearance of the bacteria could identify targets to intervene and improve the lives of patients afflicted with atopic dermatitis.

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