

**MEDIATORS AND RECEPTORS OF CHRONIC ITCH
IN PRIMATES AND HUMANS**

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ABSTRACT

Chronic itch has a significant impact on quality of life for millions of patients worldwide, on a level comparable to that of chronic pain. Yet, although there are a host of effective drugs available for pain, there are no therapies that specifically target chronic itch. Current experimental approaches to investigate the pathogenesis of chronic pruritus and to test novel therapeutic agents are largely limited to rodent models. However, rodent models display significant dermatological, neurophysiological, and immunological differences from humans with chronic itch.

The disadvantages of the current rodent paradigms call for the design of a valid primate model of chronic itch. For four years, we have monitored scratching behavior in a primate colony (n=35) of Cynomolgus macaques (*Macaca fascicularis*) suffering from idiopathic chronic itch. By comparing molecular and genetic analyses of the primates' skin to their quantified scratching behavior, we attempted to characterize the underlying mechanisms of chronic itch in this model. Furthermore, the expression of itch-related proteins was examined in both the primate model and in humans with pruritic diseases.

The first aim of the study was to characterize the underlying molecular and genetic basis of chronic itch in the primate model. We were able to distinguish specific peripheral targets related to pruritus by correlating the genetic and protein expression results to the primates' scratching severity. In Aim 1a, RNA-sequencing was performed on skin biopsies from the primates to identify differentially expressed genes in pruritic, lichenified versus non-pruritic, non-lichenified skin. These results were then correlated to the quantified primate scratching behavior. This led to the identification of over 400

genes that were differentially expressed in the skin based on scratching intensity. Many of these differentially expressed transcripts were associated with sensory nerve fibers, keratinocytes, mast cells, or lymphocytes.

Selected genes that were overexpressed and correlated to itch intensity were then targeted for immunohistochemical and proteomic analysis in Aim 1b.

Immunohistochemical examination of the primate skin biopsies revealed that histamine levels were not elevated in primates that exhibited increased scratching behavior.

However, mast cells containing tryptase were significantly increased in the skin of primates with severe scratching as compared to primates with mild scratching. The increased levels of gastrin-releasing peptide and substance P in lichenified skin were also found to be correlated to the primates' scratching behavior. Of note, transient receptor potential channels V1, V3, and A1 were increased in the epidermis of primate skin, but the numbers of TRPV1+ and TRPA1+ nerve fibers were not significantly different between lichenified and non-lichenified skin. Transcriptome analysis of the opioid receptors and their ligands showed that primates with severe scratching behavior had a significant imbalance between the μ - and κ -opioid receptors and ligands. The μ -opioids had upregulated gene expression, while the κ -opioids were downregulated.

In Aim 2, to further characterize this primate model of chronic itch, we compared immunohistochemical results from the primate studies to human findings. Lesional and non-lesional skin biopsies from patients with atopic dermatitis, psoriasis, and cutaneous T-cell lymphoma underwent immunohistochemical analysis in order to reveal the similarities and differences between the primate model and different types of chronic itch in humans. As in the primate model, substance P was found to be increased in the skin of

lesional atopic and psoriasis skin. Additionally, similar to primate skin, human atopic and psoriatic skin had high levels of tryptase and its receptor in the epidermis. While IL-31 was only slightly elevated in primates, patients with cutaneous T-cell lymphoma or atopic dermatitis showed a significant correlation between itch severity and IL-31 levels.

In conclusion, our primate model displayed expression patterns of many endogenous pruritogens and receptors that were similar to those of humans with atopic dermatitis or psoriasis. While the primate model did not completely mimic these specific pruritic diseases, the overlap of pruritic components suggests a commonality of signaling pathways across several different chronic itch states. The similarity of this primate model to human disease offers the combined advantages of experimental modeling and long-term behavioral follow-up.

I dedicate my dissertation work to
all of my itches.

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TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
DEDICATION.....	vi
ACKNOWLEDGMENTS	vii
LIST OF TABLES.....	xi
LIST OF FIGURES	xii
CHAPTER 1	1
INTRODUCTION AND BACKGROUND	1
Pruritus.....	1
Pruritus in Specific Diseases.....	1
Pruritus Pathology.....	3
Primary Sensory Afferents in Pruritus.....	3
Receptors and Mediators Involved in Pruritus.....	5
Animal Models of Pruritus.....	25
Specific Aims.....	29
CHAPTER 2	31
OVER-EXPRESSION OF THE GASTRIN-RELEASING PEPTIDE IN CUTANEOUS NERVE FIBERS AND ITS RECEPTOR IN SPINAL CORD IN PRIMATES WITH CHRONIC ITCH.....	31
To the Editor	31
Supplemental.....	37

Materials and Methods.....	37
Behavioral.....	37
Histopathology.....	38
Immunohistochemistry.....	38
Quantification.....	40
Statistical Analysis.....	40
CHAPTER 3.....	41
RNA SEQUENCING REVEALS DIFFERENTIALLY EXPRESSED GENES IN A PRIMATE MODEL OF CHRONIC ITCH.....	41
Abstract.....	41
Introduction.....	42
Methods.....	44
Primate Behavior and Tissue.....	44
Human Subjects and Tissue.....	45
RNA Isolation and Sequencing.....	45
Immunohistochemistry.....	46
Results.....	48
Analysis of Primate DEGs.....	48
Immunohistochemistry of Selected DEG Products.....	52
Conclusions.....	60
CHAPTER 4.....	90
CUTANEOUS T-CELL LYMPHOMA AND PRURITUS: THE EXPRESSION OF IL-31 AND ITS RECEPTORS IN THE SKIN.....	90

Abstract.....	90
Introduction.....	91
Materials and Methods.....	92
Subjects.....	92
Histology and Immunohistochemistry.....	93
Quantification.....	94
Statistical Analyses.....	95
Results.....	95
CTCL Characteristics and Pruritus.....	95
IL-31 and Epidermal Innervation (PGP 9.5).....	96
IL-31RA and OSMR β	97
Conclusions.....	100
CHAPTER 5.....	102
CONCLUSION.....	102
Conclusion of Aim 1.....	103
Conclusion of Aim 2.....	106
Overall Conclusions.....	107
Future Directions.....	108
REFERENCES CITED.....	111

LIST OF TABLES

Table	Page
1.1 Cytokines involved in human pruritic diseases.....	19
1.2 Intradermal application of pruritogens in different species.....	28
3.1 Differentially expressed genes in the skin of primates with chronic itch.....	64
4.1 Cutaneous T-cell lymphoma. Sample demographic data.....	93

LIST OF FIGURES

Figure	Page
1.1 The International Forum for the Study of Itch (IFSI) classification of chronic pruritus.....	2
1.2 The pathway of itch transmission from the skin to the brain.....	5
1.3 Cells in the skin release various pruritogenic mediators that act on receptors expressed on sensory nerve terminals in the epidermis.....	6
1.4 Signaling pathways for pruritus in human peripheral nerve fibers.....	9
1.5 Controversy in the GRP/GRPR signaling pathway.....	16
2.1 Immunohistochemical staining of GRP ⁺ nerve fibers in the skin of primates with chronic itch.....	34
2.2 Immunohistochemical staining of GRPR ⁺ cells in the dorsal horn of primates with chronic itch.....	36
3.1 Primate skin and scratching behavior.....	50
3.2 Diagram of DEGs in severely scratched, lichenified primate skin.....	51
3.3 Immunohistochemistry of TRPV1 and TRPA1 expression in cutaneous nerves of primates and humans.....	54
3.4 Immunohistochemistry of TRPV1 and TRPA1 expression in the epidermis of primates and humans.....	55
3.5 Tryptase and PAR2 were elevated in lichenified skin of primates and lesional skin of patients with AD and PS.....	56
3.6 Substance P and its receptor NK-1R were elevated in primates skin and lesional skin of patients with AD and PS.....	58
3.7 Endothelin-1 and its receptor ETA were elevated in lichenified skin of primates and lesional skin of patients with AD and PS.....	59
4.1 Cutaneous T-cell lymphoma. Pruritic VAS rating and its correlation to CTCL stage.....	96
4.2 Cutaneous T-cell lymphoma. IL-31 is increased in patients with pruritic CTCL	98

4.3 Cutaneous T-cell lymphoma. IL-31RA and OSMR β are increased in patients with pruritic CTCL.....	99
5.1 Representative images of the GRP antibody cross-reactivity with SP in primate skin.....	105
5.2 Venn diagram illustrating itch-associated proteins common among the primate model, atopic dermatitis, and psoriasis.....	109

CHAPTER 1

INTRODUCTION AND BACKGROUND

Pruritus

Pruritus, or itch, is an unpleasant sensation that provokes the desire to scratch. This sensation can be acute (for a few seconds or days) or chronic (lasting more than six weeks) (Ständer et al, 2007). Chronic itch can range from a mild to an extremely disabling condition that can greatly affect the quality of life on a level comparable to chronic pain (Kini et al, 2011; Leader et al, 2015; Yosipovitch et al, 2007). Yet unlike pain, for which a host of effective drugs are available, chronic itch remains difficult to treat with current therapies (Kini et al, 2011; Patel and Yosipovitch, 2010; Reich et al, 2010; Yosipovitch and Bernhard, 2013; Yosipovitch et al, 2007; Yosipovitch et al, 2008; Wright et al 2013).

Recently, Mattered et al (2013) showed that one out of four people has experienced chronic itch during their lifetime. The same study also found that the incidence of chronic pruritus may be as high as 7% in the general population. While there are a number of studies evaluating the prevalence of pruritus in the general population, most research focuses on the epidemiology of pruritus in specific patient populations.

Pruritus in Specific Diseases

Many dermatological and systemic diseases are associated with chronic pruritus. These conditions fall into six categories: (1) dermatologic, (2) systemic, (3) neurologic, (4) psychogenic, (5) mixed (multifactorial origin), and (6) other/pruritus of undetermined origin (Ständer et al, 2007). This classification also considers the presence or absence of

skin changes, such as pruritus on diseased, inflamed skin; pruritus on non-diseased, non-inflamed skin; and secondary lesions from scratching (Figure 1.1).

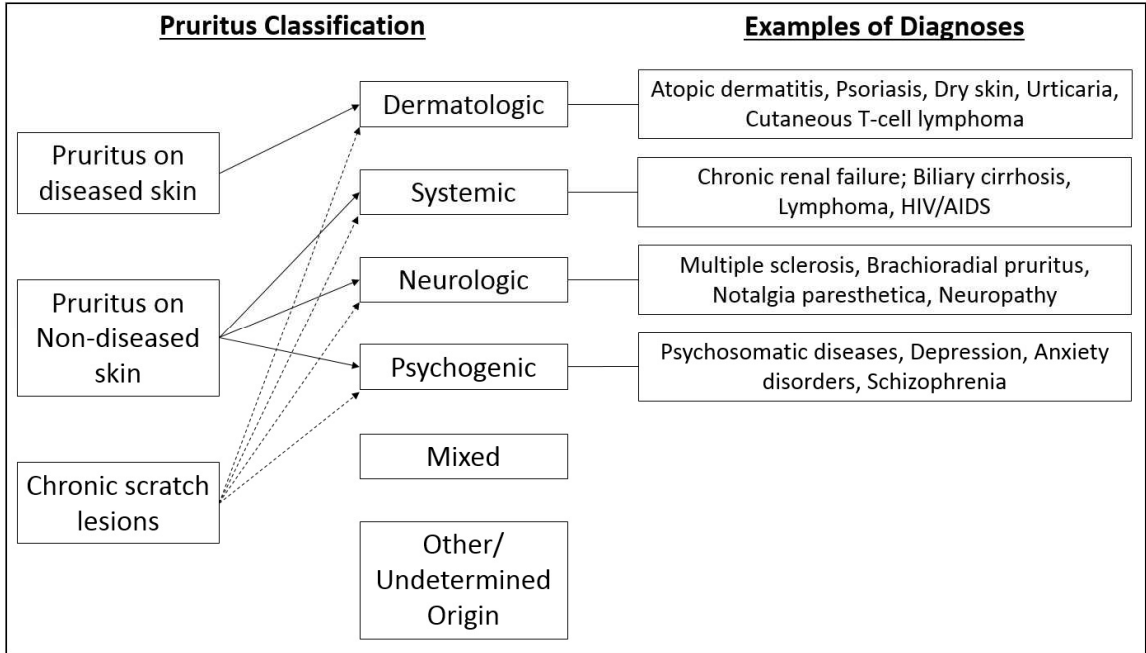


Figure 1.1. The International Forum for the Study of Itch (IFSI) classification of chronic pruritus. This classification scheme considers clinical and differential diagnostic criteria.

Adapted from Ständer et al, 2007; Grundmann and Ständer, 2011.

The majority of chronic itch patients have pruritus due to dermatoses (Weisshaar et al, 2006), with a high incidence of chronic itch in patients with atopic dermatitis, psoriasis, and cutaneous T-cell lymphoma (CTCL) (Ahern et al, 2012; Feramisco et al, 2010; Yosipovitch et al, 2002). Between 87 and 100% of atopic dermatitis patients report chronic itch, making it a defining feature of the disease (Dawn et al, 2009; Yosipovitch et al, 2002). In psoriasis patients, pruritus is the second-most (79%) reported symptom, following scaling (94%) (Yosipovitch et al, 2000). Surveys of patients with CTCL found

a prevalence of pruritus between 66 and 88% (Ahern et al, 2012; Vij et al, 2012; Wright et al, 2013).

Pruritus Pathophysiology

Despite the clinical importance of itch, its underlying neurobiological mechanisms are poorly understood. While histamine is the best-known pruritogen, and many antihistamine therapies are readily available, most of these treatments are ineffective for the treatment of chronic itch. This realization has led to the separation of histaminergic (histamine-dependent) and non-histaminergic (histamine-independent) itch mechanisms (Papoiu et al, 2012).

Primary Sensory Afferents in Pruritus

Itch is transmitted by subsets of spinal nociceptive neurons (Schmelz et al, 1997), including both unmyelinated C-fibers and thinly myelinated A δ -fibers with their cell bodies in the dorsal root ganglia (DRG). These fibers transmit the itch signal to the brain via the anterolateral spinothalamic tract (STT) in the spinal cord (Figure 1.2). Interestingly, both histaminergic and non-histaminergic signals are transmitted via the STT, but these two types of itch utilize distinct subsets of STT neurons (Davidson et al, 2007). Similarly, a common, core group of brain structures is involved in processing both types of itch, but these structures are selectively activated or deactivated for histaminergic versus non-histaminergic itch (Papoiu et al, 2012).

Histamine, which signals through four histamine receptors (H₁₋₄), was found to activate mechano-insensitive C-fibers (Huang et al, 2008; Schmelz et al, 1997). In contrast, the spicules from the plant cowhage (*Mucuna pruriens*) contain mucunain, a cysteine protease, which activates mechano-sensitive C-fibers in a histamine-independent

manner through the protease-activated receptors (PARs) (Johanek et al, 2008; Reddy et al 2008). A comparison of itch induction by these two substances revealed that specific populations of C-fibers are responsible for histamine-dependent and –independent transmission of itch. In contrast, A δ -fibers were found to respond to both histamine and cowhage (Ringkamp et al, 2011).

Primary afferent nerves innervate the skin to form complex neural networks. These fibers extend from the dermal-epidermal junction to the stratum granulosum in the epidermis and terminate in close proximity to skin cells (keratinocytes, fibroblasts, mast cells, epidermal immune cells, and Langerhans cells) (Zylka et al, 2005). This innervation network allows for both direct activation of nerve fibers and indirect communication between epidermal cells and nerve fibers in order to induce itch. For example, keratinocytes and immune cells can release pruritogens that act on sensory afferents, which in turn can release neuropeptides that target both of these epidermal cell types. Emerging data suggests that this multidirectional cell-to-cell signaling may be responsible for the maintenance of chronic itch and skin morphology changes (Bautista et al, 2014; Hilliges et al, 1995; Kabashima et al, 2013). Furthermore, when the epidermis is removed, the perception of itch (but not pain) is abolished (Orn et al, 1998).

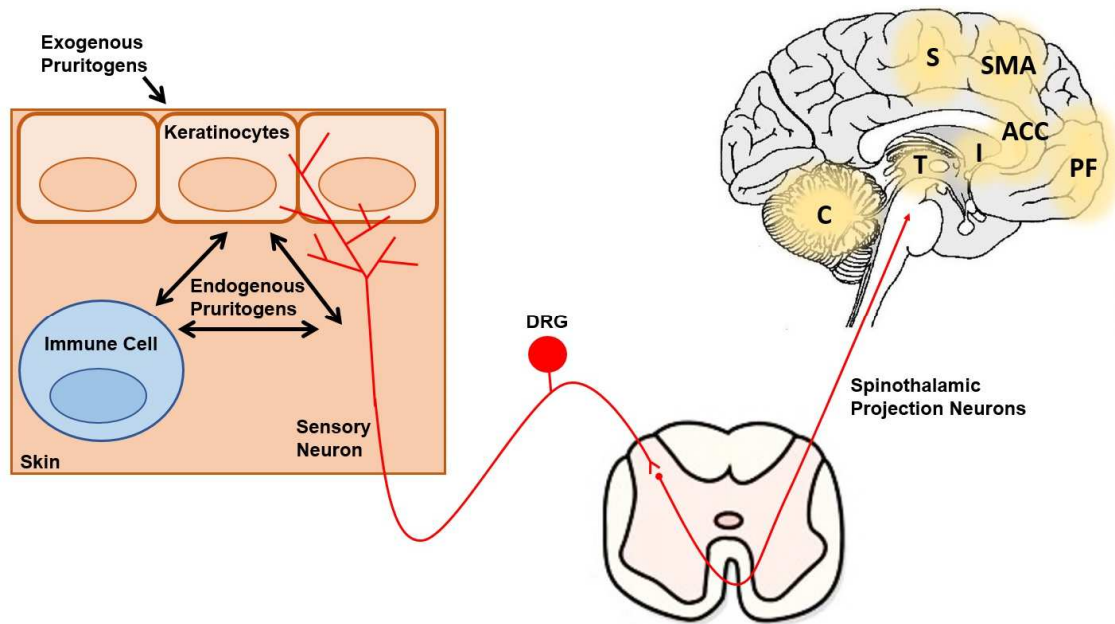


Figure 1.2. The pathway of itch transmission from the skin to the brain. Pruritogens in the skin directly or indirectly activate sensory nerve fibers that terminate in the superficial laminae of the dorsal spinal cord. These sensory nerves then activate secondary spinothalamic neurons that project to the brain. Brain structures (T=thalamus, I=insula, S= somatosensory cortex, ACC=anterior cingulate cortex) are then responsible for the perception of itch and motor activity involved in scratching (SMA=supplementary motor area, C=cerebellum, PF= prefrontal cortex). Adapted from Bautista et al, 2014 and Paus et al, 2006.

Receptors and Mediators Involved in Pruritus

Pruritogens that are endogenous to the body are capable of conducting the itch signal either directly through epidermal nerve terminals or indirectly through epidermal cells, which then signal to neurons (Figure 1.3).

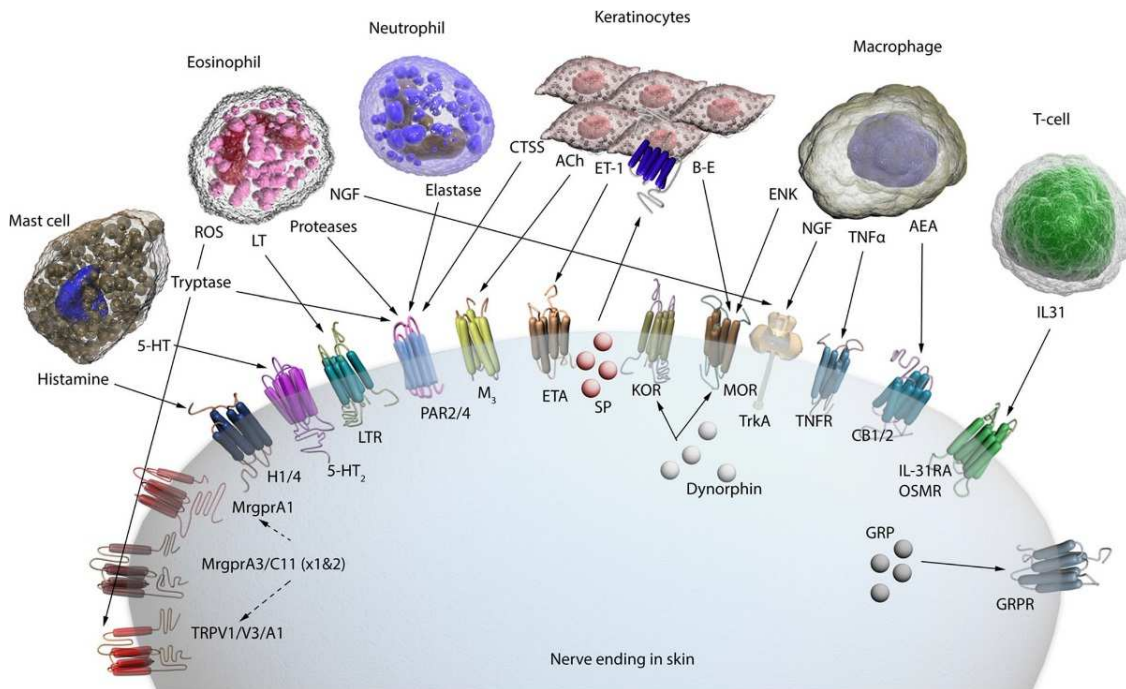


Figure 1.3. Cells in the skin release various pruritogenic mediators that act on receptors expressed on sensory nerve terminals in the epidermis. Abbreviations: TRPV1/V3/A1, transient receptor potential V1/V3/A1; MrgprA3/C11/A1 (X1&2), mas-related G protein-coupled receptor A3/C11/A1 (X1&2); H1/4, histamine receptor 1/4; 5-HT₂, serotonin receptor 2; LTR, leukotriene receptor; PAR2/4, protease-activated receptor 2/4; M₃, muscarinic acetylcholine receptor; ETA, endothelin-1 receptor A; SP, substance P; KOR, κ-opioid receptor; MOR, μ-opioid receptor; TNFR, tumor necrosis factor receptor; CB1/2, cannabinoid receptor 1/2; IL-31RA, interleukin-31 receptor A; OSMR, oncostatin receptor; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; 5-HT, serotonin; ROS, reactive oxygen species; LT, leukotriene; NGF, nerve growth factor; CTSS, cathepsin S; ACh, acetylcholine; ET-1, endothelin-1; B-E, β-endorphin; ENK, enkephalin; TNFα, tumor necrosis factor-α; AEA, anandamide; IL-31, interleukin-31. Used with permission from the authors, Mollanazar et al, 2015.

Transient receptor potential receptors.

Since itch sensation is transmitted through nociceptive nerve fibers, it is no surprise that itch and pain share many similar mediators and receptors (Figure 1.4). The transient receptor potential (TRP) cation channels vanilloid (V) 1 and ankyrin (A) 1 are well known to be involved in mediating thermal pain. There is now increasing evidence that these TRP channels are required to mediate itch signaling (Wilson et al, 2013). Furthermore, histaminergic itch has been shown to be dependent on TRPV1, and non-histaminergic itch relies on TRPA1 (Shim et al, 2007; Wilson et al, 2011).

TRPV1 mediates itch transmission by activating the G protein subunit G_{α} which causes calcium influx within C-fiber terminals, but the receptor can also be activated on keratinocytes, mast cells, and Langerhans cells (Ständer et al, 2004). Besides histamine, numerous other endogenous substances, such as bradykinin, prostaglandins, neurotrophins (NGF, NT3 and 4), and ATP, can activate TRPV1 (Lázár et al, 2004). Activation of TRPV1+ nerves is also thought to be responsible for the hallmark flare of histamine itch by releasing neuropeptides causing mast cell degranulation (Aubdool et al, 2011; Schmelz et al, 2000). TRPV1 channels were found to be overexpressed in keratinocytes and nerve fibers in pruritic skin of prurigo nodularis patients (Ständer et al, 2004). Interestingly, a recent randomized trial found that a topical TRPV1 inhibitor (SB705498) had no effect on pruritus induced by cowhage or histamine in healthy subjects (Gibson et al, 2014).

TRPV3 is a channel activated by innocuous warm temperatures and chemicals such as camphor (Chung et al, 2004). In humans, TRPV3 has been found to be expressed in the DRG and epithelium, whereas in mice it has been found in keratinocytes and not

the DRG (Peier et al, 2002; Smith et al, 2002). Mice with mutant TRPV3 develop pruritic atopic-like lesions (Asakawa et al, 2006; Yoshioka et al, 2009). Recently, TRPV3 was shown to be increased in the epidermis of pruritic burn scars (Yang et al, 2015).

TRPA1 is expressed on a subset of TRPV1-expressing C- and A δ -nerve fibers, as well as on epidermal keratinocytes (Atoyan et al, 2009; Wilson et al, 2011). It is bound to the G protein subunit G $\beta\gamma$, and activation of this channel results in calcium release from intracellular stores (Jeffrey et al, 2011). This channel is required for non-histaminergic itch, including the exacerbated itch of dry skin (Wilson et al, 2011; Wilson et al, 2013). It is a target for exogenous irritants, like allyl isothiocyanate and allicin, and also for endogenous inflammatory agents, such as reactive oxygen species, bradykinin, and arachidonic acid (Bautista et al, 2013). TRPA1 is found to be increased in lesional atopic dermatitis skin (Oh et al, 2013).

Like TRPA1, TRP melastatin (M) 8 is also activated by cold temperatures (McKemy et al, 2002). However, unlike TRPA1, TRPM8 activation can inhibit itch. Menthol, an exogenous activator of TRPM8, has been used to inhibit histamine-induced itch (Bromm et al, 1995). Additionally, cooling of atopic dermatitis skin relieves itch (Fruhstorfer et al, 1986). Although TRPA1 and TRPM8 are both activated by cold temperatures, it is postulated that only TRPM8 is responsible for innocuous cooling (Bharate and Bharate, 2012).

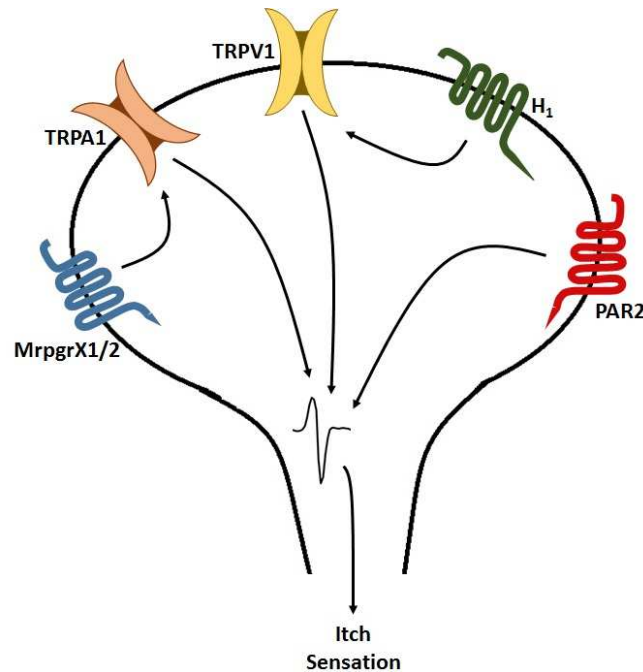


Figure 1.4. Signaling pathways for pruritus in human peripheral nerve fibers. Pruritus can be induced by direct activation of PARs and TRPs. Additionally, G protein-coupled receptors, such as Mas-related G protein-coupled receptors (Mrgprs) and histamine receptors can indirectly activate TRP channels to transmit pruritus.

Histamine and its receptors.

The neurotransmitter histamine is one of the most studied itch mediators. When applied superficially, histamine causes itch accompanied by characteristic wheal and flare skin reactions (Lewis et al, 1927). However, when applied subcutaneously, histamine causes pain (Rosenthal et al, 1977). Histamine is released by mast cells to activate the H₁ and H₄ receptors (Inagaki et al, 1999; Rosshach et al, 2009; Simons et al, 2011). While histamine receptor antagonists suppress histamine-induced itch, they are usually ineffective at relieving itch in chronic itch conditions (Ikoma et al, 2006).

The H₁ receptor has been the most studied and the target for many therapeutic drugs. This receptor is coupled with a G_q subunit and activates TRPV1 through the phospholipase A2 and 12-lipoxygenase pathways, which in turn leads to calcium influx (Jeffrey et al, 2011; Kajihara et al, 2010; Kim et al, 2004; Shim et al, 2007). The H₄ receptor is largely involved in the immune response and is expressed not only on nerve fibers, but also on keratinocytes, mast cells, and Th₂ T-cells (Dijkstra et al, 2008). In atopic dermatitis, the H₄ receptor was found to be highly expressed on keratinocytes and Th₂ T-cells (Glatzer et al, 2013; Gutzmer et al, 2009). The H₂ and H₃ receptors have shown limited involvement in pruritus (Jeffrey et al, 2011). Agonists for the H₂ receptor did not induce pruritus, and an antagonist for this receptor did not reduce histamine-induced pruritus (Bell et al, 2004). However, blockade of the H₃ receptor significantly increases the incidence of pruritus in mice (Sugimoto et al, 2004).

Proteases and protease-activated receptors.

PARs, members of the G protein-coupled receptor (GPCR) family, have four subtypes and are activated by the proteolytic cleavage of their extracellular N-termini (Macfarlane et al, 2001). Endogenous or exogenous proteases activate PAR1, PAR2, and PAR4 to cause itch; however, PAR2 is the only PAR found to be involved in non-histaminergic itch in humans (Tsuji et al, 2008). Activation of both PAR2 and 4 causes calcium release, activation of the phospholipase C pathway, and activation of the TRPA1 channel by the G protein subunit G_q (Jeffrey et al, 2011). Cowhage spicules that contain mucunain activate PAR2 and cause a strong, non-histaminergic pruritus sensation without the wheal and flare response (Reddy et al, 2008). Interestingly, the use of a PAR2

agonist in PAR2 knockout mice caused more scratching than in the wild type mice (Liu et al, 2011).

Increased levels of endogenous proteases can contribute to inflammation accompanied by chronic itch. The serine protease tryptase, which activates PAR2, is the most abundant protease released from mast cells. It was found to be increased in the serum of hemodialysis patients and the skin of atopic dermatitis (Dugas-Breit et al, 2005; Steinhoff et al, 2003). Furthermore, PAR2 and tryptase are increased in the nerve fibers and keratinocytes of atopic dermatitis (Buddenkotte et al, 2005; Steinhoff et al, 1999; Steinhoff et al, 2003). Additionally, treatment with an antihistamine reduced plasma levels of tryptase in atopic dermatitis, and this reduction correlated with decreased pruritus (Kawakami et al, 2006). Cathepsin S (CTSS), another serine protease, is released from macrophages and dendritic cells to induce itch by activating PAR2 (Liu and Spero, 2004; Reddy et al, 2010). Recently, CTSS was found to be increased on the scalps of patients with seborrheic dermatitis and correlated to itch severity (Viodé et al, 2014). Other serine proteases, such as chymase, trypsin, and kallikrein, have also been used to induce itch by activating PAR2 (Hägermark et al, 1972; Hägermark et al, 1974; Rajka 1969; Stefansson et al, 2008). Kallikrein is responsible for the liberation of bradykinin, which can cause a painful itch by activating the bradykinin B₂ receptor on nerve fibers (Cormia and Dougherty, 1960; Hägermark, 1974).

Mas-related G protein-coupled receptors.

Mrgprs, orphan GPCRs, comprise more than 50 members in the murine genome, but only 10 members in the human genome (Dong et al, 2001; Lembo et al, 2002). These receptors are specifically expressed in sensory neurons in the dorsal root and trigeminal

ganglia and have recently emerged as novel non-histaminergic receptors. Human Mrgprs, also known as sensory neuron-specific receptors (SNSRs), share some mouse orthologs. For instance, mouse MrgprA3 responds to chloroquine, and mouse MrgprC11 responds to bovine adrenal medulla peptide (BAM) 8-22 while the human MrgprX1 responds to both (Liu et al, 2009; Zylka et al, 2003). Endogenous opioids, like the opioid precursor proenkephalin A, also activate MrgprX1 (Lembo et al, 2002). Like the mouse MrgprC11, the human MrgprX2 responds to SLIGKV, a PAR2 agonist (Liu et al, 2011). These findings suggest that other pruritogens may transduce itch through Mrgprs along with their presumed receptors.

Voltage-gated sodium channels.

Voltage-gated sodium (Na_v) channels are responsible for the rising phase of action potentials in neurons. Humans have nine subtypes of Na_v channels, with four of these subtypes (Na_v 1.3, 1.7, 1.8, and 1.9) implicated in pain. Recently, Lee et al (2014b) reported that Na_v 1.7 also plays a role in itch. The authors showed that blocking the Na_v 1.7 channel in mice resulted in inhibition not only of pain, but also of acute and chronic itch.

Neuropeptides.

Neuropeptides like tachykinins and peptide hormones have been extensively implicated in pruritogenesis accompanied by neurogenic inflammation. Tachykinins are a family of neuropeptides that include neurokinin A, neurokinin B, and substance P (SP). While neurokinin A was found to induce itch and histamine release, neurokinin B did not (Krumins et al, 1992; Thomsen et al, 2002). SP was found to cause itch in healthy controls, atopic dermatitis, and psoriasis (Amatya et al, 2010; Hägermark et al, 1978;

Heyer et al, 1991). SP is released from A δ - and C-fibers and then binds to the neurokinin 1 receptor (NK-1R) on mast cells to release histamine and on other skin cells to release non-histaminergic itch mediators (Andoh et al, 1996; Cappugi et al, 1992; Staniek et al, 1999). SP was found to be elevated in the nerves of atopic dermatitis, prurigo nodularis, and psoriasis patients (Amatya et al, 2011; Ciannetti et al, 1992; Järvikallio et al, 2003; Pincelli et al, 1990; Tobin et al, 1992). However, one study found that SP levels did not correlate with itch in psoriasis (Remröd et al, 2007). The NK-1R antagonist aprepitant shows clinical promise as its oral administration was found to reduce pruritus levels in chronic pruritus patients in non-controlled studies (Ally et al, 2013; Borja-Consigliere et al, 2014; Ständer et al, 2010).

Another neuropeptide, calcitonin gene related peptide (CGRP), was found to be increased in peripheral nerve fibers of atopic dermatitis, prurigo nodularis, and nummular eczema (Järvikallio et al, 2003; Liang et al, 2000). Both forms of CGRP, α and β , are found in nerve fibers and Langerhans cells, while keratinocytes only express β CGRP (Hou et al, 2011; Torii et al, 1997). Unlike SP, CGRP administration does not directly cause itch or histamine release, but can sustain itch caused by SP (Ekblom et al, 1993; Steinhoff et al, 2006; Wallengren and Hakanson, 1987). Furthermore, the CGRP antagonist telcagepant did not inhibit histamine-induced itch (Wallengren and Edvinsson, 2014).

Endothelin-1 (ET-1) is a well-known vasoconstrictor peptide that is released from endothelial cells, keratinocytes, and mast cells (Gandhi et al, 1994; Hirobe 2005). Similar to SP, ET-1 causes a burning itch and histamine release (Hans et al, 2007; Katugampola et al, 2000; Namer et al, 2008). It binds endothelin-A receptor (ET_A) on nerves,

keratinocytes, and mast cells to cause itch (Liang et al, 2010; McQueen et al, 2007; Metz et al, 2006; Vellani et al, 2011). Furthermore, it was shown that ET-1 does not require TRPV1 or TRPA1 for pruritic signaling (Liang et al, 2010; Liang et al, 2011). ET-1 is increased in atopic dermatitis skin and was recently shown to be elevated in peripheral nerves of prurigo nodularis, along with ET_A and ET-converting enzyme 1 (Aktar et al, 2015; Kido-Nakahara et al, 2014). Additionally, increased plasma levels of ET-1 were highly correlated to itch severity during exacerbation of atopic dermatitis (Tsybikov et al, 2015).

Neurotensin (NT), secretin, somatostatin, and vasoactive intestinal polypeptide (VIP) are hormone neuropeptides that cause histamine release (Fjellner and Hägermark, 1981; Lowman et al, 1988; Steinhoff et al, 2006). Both NT and VIP cause itch in healthy and atopic dermatitis subjects (Giannetti and Girolomoni, 1989; Giannetti et al, 1992; Rukwied and Heyer, 1998). Somatostatin and VIP were found to be decreased in atopic dermatitis nerve fibers (Giannetti et al, 1992; Pincelli et al, 1990). However, VIP was increased in the plasma of atopic dermatitis, and its levels correlated to itch intensity (Teresiak-Mikołajczak et al, 2013).

Acetylcholine (ACh) is a neurotransmitter that causes itch, but only in atopic dermatitis lesional skin (Heyer et al, 1997; Vogelsang et al, 1995). In healthy controls, ACh only causes pain. It is thought to transmit itch indirectly via muscarinic receptor M3 on keratinocytes (Miyamoto et al, 2002a). Botulinum neurotoxin A inhibits the release of ACh and reduces histamine-induced itch (Gazerani et al, 2009).

Serotonin or 5-hydroxytryptamine (5-HT) causes itch and pain in healthy and atopic dermatitis patients (Weisshaar et al, 1999). 5-HT was shown to cause itch with a

reduced intensity and shorter duration than histamine-induced itch (Rausl et al, 2013). It is released from mast cells and is thought to activate receptors 5-HT₁ and 5-HT₂ on nerve fibers to induce itch (Imamachi et al, 2009; Jinks and Carstens, 2002; Sommer, 2004; Yamaguchi et al, 1999). The 5-HT₃ antagonist ondansetron showed little to no effect in reducing pruritus in several conditions (Balaskas, et al, 1998; Jones et al, 2007; Müller et al, 1998; Murphy et al, 2003; O'Donohue et al, 2005). However, selective serotonin reuptake inhibitors have been shown to have antipruritic effects in atopic dermatitis, lymphoma, and chronic liver diseases (Hundley and Yosipovitch, 2004; Mayo et al, 2007; Ständer et al, 2009; Zyllicz et al, 2003).

In the spinal cord, several neuropeptides have been indicated to be involved in transmitting itch signals. A major role of gastrin-releasing peptide (GRP) and its receptor (GRPR) as an itch-specific neural pathway was discovered in 2007 (Sun and Chen, 2007; Sun et al, 2009). In mice, GRP was found in a subset of unmyelinated DRG neurons, while GRPR was expressed in lamina I of the dorsal horn of the spinal cord and played a vital role in itch, but not pain signaling. Constitutively activated B-Raf, a serine/threonine kinase that activates the extracellular signal-regulated kinase (ERK) cascade, enhanced the expression of GRP and MrgprA3 in TRPV1 nociceptive neurons in mice (Zhao et al, 2013). When these signals were blocked, itch was attenuated.

Recently, B-type natriuretic peptide (BNP), also called natriuretic polypeptide b (nppb), was also shown to cause itch in mice. When its receptor (Npra, natriuretic peptide receptor A) was ablated, the scratching response was impaired (Goswami et al, 2014; Mishra and Hoon, 2013). It is postulated that BNP from primary afferents triggers the release of GRP by spinal interneurons to act on GRPR to transmit itch (Figure 1.5).

However, contradictory evidence revealed that BNP-induced scratching was not affected by the blockage of GRP-GRPR signaling in mice (Liu et al, 2014).

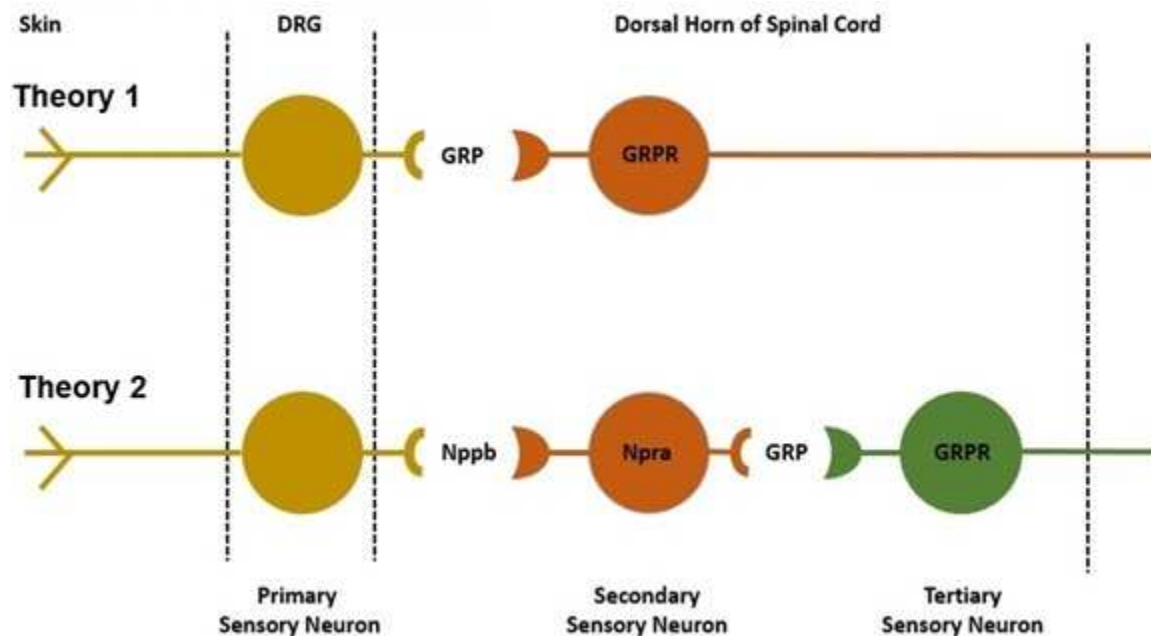


Figure 1.5. Controversy in the GRP/GRPR signaling pathway. Originally, GRP was thought to be released by primary sensory neurons (Theory 1). However, a recent study postulated that Nppb and not GRP is what is released from primary sensory neurons (Theory 2). Nppb would then trigger the release of GRP by spinal interneurons expressing its receptor Npra, and then GRP would bind to GRPR to transmit the itch signal. Adapted from Bautista et al, 2014.

Neurotrophins.

Neurotrophins are proteins that induce the differentiation, survival, and maintenance of neurons and have been implicated to indirectly mediate itch. Nerve growth factor (NGF) from keratinocytes and mast cells is responsible for cutaneous

innervation density, which may promote hyperinnervation (Hassankhani et al, 1995). Histological studies have shown increased epidermal nerve densities in atopic dermatitis and xerosis (Ikoma et al, 2006). However, many other studies have shown patients with lichen amyloidosis, nummular dermatitis, keloids, prurigo nodularis, psoriasis, and neuropathic itch to have a decrease in epidermal nerve fibers (Maddison et al, 2008; Maddison et al, 2011; Schuhknecht et al, 2011; Taneda et al, 2011; Tey et al, 2012; Wallengren et al, 2002). These alterations are thought to be due to imbalances between the nerve elongation promoter NGF and the nerve repulsion factor Sema3A (Kou et al, 2012; Tominaga and Takamori, 2010; Tominaga et al, 2008).

NGF was found to sensitize TRPV1+nerves, cause mast cell degranulation, and promote the upregulation of SP and CGRP (Basbaum et al, 2009). It also enhances cowhage-induced itch, but not histamine-induced itch (Rukwied et al, 2013). Several studies found NGF and its main receptor, tropomyosin receptor kinase A (TrkA), to be increased in the serum and skin of atopic dermatitis, psoriasis, prurigo nodularis, and CTCL (Dou et al, 2006; Groneberg et al, 2005; Johansson et al, 2002; Suga et al, 2013; Teresiak-Mikolagczak et al, 2013; Toyoda et al, 2002) Recently, a topical TrkA inhibitor reduced pruritus scores in psoriasis patients (Roblin et al, 2015). Other neurotrophins are also thought to be involved in chronic itch diseases. For instance, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) are increased in atopic dermatitis (Grewe et al, 2000; Hon et al, 2007; Raap et al, 2006; Yamaguchi et al, 2009).

Cytokines and chemokines.

Almost all cells in the skin can produce cytokines and chemokines and express receptors for many of them (Luger et al, 1981). Normally associated with inflammation

and pain, cytokines and chemokines have recently been under examination for their role in itch. Out of the 36 known interleukins (ILs), 23 have been shown to be involved in pruritus or pruritic diseases. Emerging evidence suggests that many ILs and other cytokines are involved in several chronic pruritic diseases (Table 1.1). Several cytokines involved in these diseases, including IL-2 and IL-31, have even been found to induce itch in healthy and pruritic subjects (Hawro et al, 2014; Wahlgren et al, 1995). Two other cytokines, IL-8 and TNF- α , were found not to induce itch when intradermally injected (Darsow et al, 1997; Lippert et al, 1998).

Chemokines, a family of small soluble cytokines, have also been investigated in pruritic diseases. The expression of the α -chemokine CXCL8 (also known as IL-8), is elevated in psoriatic lesions (Nomura et al, 2003). Itch severity in CTCL patients has been correlated to the expression levels of β -chemokines CCL1 and CCL26, but not CCL17 or CCL27 (Suga et al, 2013). Additionally, T-cells expressing the chemokine receptor CCR4 were found to be the main source of IL-31 in CTCL (Cendeno-Laurent et al, 2015). β -chemokines CCL1, CCL17, CCL18, and CCL27, δ -chemokine CX₃CL1, and its receptor CX₃CR1 are upregulated in atopic dermatitis (Gombert et al, 2005; Lee and Yu, 2011; Staumont-Sallé et al, 2014). Furthermore, CCL18 was found to correlate with atopic dermatitis pruritus (Hon et al, 2011).

Table 1.1. Cytokines involved in human pruritic diseases.

Cytokine	Disease				References
	Atopic Dermatitis	Psoriasis	CTCL	Prurigo Nodularis	
IL-2	induces itch	+ skin			Darsow et al, 1997; Nakamura et al, 2003; Wahlgren et al, 1995
IL-4	+ skin	n.s. skin	+ T-cells, skin		Dummer et al, 1996; Jeong et al, 2003; Miyagaki et al, 2011; Miyagaki et al, 2013; Vowels et al, 1994
IL-5	+ skin		+ T-cells, skin		Dummer et al, 1996; Jeong et al, 2003; Vowels et al, 1994
IL-6	+ cutaneous nerves		+ T-cells	+ cutaneous nerves	Dummer et al, 1996; Nordlind et al, 1996
IL-8	+ skin, plasma, PBMC does not induce itch	+ skin	n.s. skin		Hatano et al, 1999; Kimata et al, 1994; Lippert et al, 1998; Miyagaki et al, 2011; Miyagaki et al, 2013; Nomura et al, 2003; Ständer and Steinhof, 2002; Sticherling et al, 1992
IL-9	+ skin n.s. serum				Sismanopoulos et al, 2012
IL-10		-/n.s. skin	+ T-cells, skin, serum		Asadullah et al, 1998a; Asadullah et al, 1998b; Dummer et al, 1996; Lessin et al, 1995; Miyagaki et al, 2011; Miyagaki et al, 2013
IL-12	+ skin, serum	+ skin			Hamid et al, 1996; Piancatelli et al, 2008; Yawalkar et al, 1998
IL-13	+ skin, serum				Hamid et al, 1996; Jeong et al, 2003; Metwally et al, 2004

(+)=increase; (-)=decrease; (n.s.)=no significant change

Table 1.1. Continued.

Cytokine	Disease				References
	Atopic Dermatitis	Psoriasis	CTCL	Prurigo Nodularis	
IL-17		+ skin (A,C,F) - skin (B,D)	n.s. skin (A,F)		Johansen et al, 2009; Miyagaki et al, 2011
IL-21	+ skin				Jin et al, 2009
IL-22	+ skin, serum	+ skin, serum	+ skin, serum		Gittler et al, 2012; Hao, 2014; Hayashida et al, 2011; Kanda et al, 2012; Miyagaki et al, 2011; Nograles et al, 2009; Wolk et al, 2004
IL-23		+ skin	n.s. skin		Fitch et al, 2007; Miyagaki et al, 2011
IL-31	+ skin, serum induces itch	n.s. skin	+PBMC, serum	+ skin	Bilsborough et al, 2006; Hawro et al, 2014; Kato et al, 2014; Malek et al, 2015; Miyagaki et al, 2013; Nobbe et al, 2012; Neis et al, 2006; Ohmatsu et al, 2012; Raap et al, 2008; Singer et al, 2013; Sonkoly et al, 2006
IL-33	+ skin				Oboki et al, 2010
IL-36	+/n.s. skin (α,γ)	+ skin, serum (γ)			Carrier et al, 2011; D'Erme et al, 2015; Suárez-Fariñas et al, 2015
OSM	+ skin				Boniface et al, 2007
INF-γ	+ skin	+ skin	- PBMC		Barker et al, 1991; Grewe et al, 1994; Vowels et al, 1992
TNF-α		+ skin			Kristensen et al, 1993
TSLP	+ skin, serum	+ skin			Jariwala et al, 2011; Volpe et al, 2014; Wilson et al, 2013

(+)=increase; (-)=decrease; (n.s.)=no significant change

Opioids.

Although itch and pain share many common elements, they do not share all their mechanisms and pathways. Opioid analgesics are used to treat chronic pain, but their use can often induce itch (Szarvas et al, 2003). It was discovered that activation of the μ -opioid receptor (MOR) causes pruritus, and activation of the κ -opioid receptor (KOR) suppresses itch (Inui, 2012; Jones and Bergasa, 1999; Kumagai et al, 2012; Taneda et al, 2011). It was suggested that chronic itch could be related to an imbalance of overexpression of MOR and downregulation of KOR (Phan et al, 2012). MOR is found in keratinocytes, fibroblasts, and cutaneous nerves (Cheng et al, 2008; Ständer et al, 2002). Expression of this receptor was originally reported to be decreased in the skin of atopic dermatitis, but a later study found no difference of expression in atopic dermatitis or psoriasis patients when compared to healthy controls (Bigliardi-Qi et al, 2005; Taneda et al, 2011; Tominaga et al, 2007). While KOR is also expressed on keratinocytes, fibroblasts, and nerves, its expression was found to be decreased in atopic dermatitis skin (Cheng et al, 2008; Salemi et al, 2005; Tominaga et al, 2007).

MOR antagonists, like nalmefene and naltrexone, have been used to treat chronic itch in uremic, atopic dermatitis, and biliary cirrhosis patients (Bigliardi et al, 2007; Miller and Hagemann, 2011; Peer et al, 1996; Thornton and Losowsky, 1988a). However, MOR antagonists are frequently accompanied by adverse side effects, such as dependence, respiratory depression, and nausea (Phan et al, 2010). Butorphanol and nalbuphine act both as KOR agonists and MOR antagonists and demonstrate great potential for treatment of chronic pruritus with less dependence and fewer central side effects than pure MOR antagonists. Butorphanol has been shown to reduce idiopathic

chronic itch and histamine-induced itch, while nalbuphine reduces itch in patients on hemodialysis and morphine-induced itch after surgeries (Cowan et al, 2015; Dawn and Yosipovitch, 2006; Hawi et al, 2015; Papoiu et al, 2015). Nalfurafine, a KOR agonist, has been approved for the treatment of end stage renal disease pruritus in Japan (Inui, 2015; Kumagahi et al, 2010; Nakao and Mochizuki, 2009). Asimadoline, which also activates KOR, is currently in clinical trials to test its antipruritic effects (Cowan et al, 2015).

The endogenous opioids endorphin and enkephalin all cause itch in humans (Casale et al, 1984). β -endorphin and met-enkephalin activate MOR and can enhance histamine-induced itch (Fjellner and Hägermark, 1982). Furthermore, these endogenous opioids have been implicated to be involved in the itch of several chronic pruritic diseases. Serum and plasma levels of β -endorphin are increased in atopic dermatitis, psoriasis, and patients on hemodialysis, with the majority of studies finding no correlation with itch intensity (Georgala et al, 1994; Glinski et al, 1994a; Glinski et al, 1994b; Lee et al, 2006; Lee et al, 2012; Mettang et al, 1998). β -endorphin was not found to be increased in atopic dermatitis or psoriasis skin biopsies (Taneda et al, 2011; Urashima and Mihara, 1998). Met-enkephalin serum levels were shown to be elevated in uremic and biliary cirrhosis patients (Danno et al, 1995; Odou et al, 2001; Spivey et al, 1994; Thornton and Losowsky, 1988b). However, these studies found no correlation between serum levels and itch severity. Dynorphin A, which activates KOR, was found to be decreased in the skin of psoriasis patients (Taneda et al, 2011). It was also found to inhibit itch caused by intrathecal injection of β -endorphin and GRP into awake primates (Lee and Ko, 2015).

Eicosanoids.

Eicosanoids are signaling molecules made by the oxidation of fatty acids and are found throughout the skin. The eicosanoid subfamily of prostaglandins is produced by arachidonic acid being oxidized by cyclooxygenases (COX)-1 and -2 (Williams and DuBois, 1996). Intradermal injection of prostaglandin (PG) H₂ and PGE₂, which is further processed from PGH₂ by prostaglandin E₂ synthase, induces weak pruritus and potentiates histamine and serotonin pruritus (Hägermark and Strandberg, 1977; Hägermark et al, 1977; Neisius et al, 2002). Treatment with antihistamines alleviates the pruritus caused by PGE₂, suggesting that prostaglandin-induced pruritus is mediated by histamine release (Hägermark and Strandberg, 1977). Furthermore, NSAIDs or COX inhibitors did little to alleviate itch in chronic itch patients (Daly and Shuster, 1986). Interestingly, PGE₁ does not cause itch, but still potentiates histamine itch (Greaves et al, 1973). However, this potentiation is not abolished by antihistamines (Boss and Burton, 1981). Unlike other prostaglandins, PGD₂ was shown to have antipruritic effects by suppressing histamine release from mast cells in mice (Hashimoto et al, 2005).

Thromboxane A₂ (TXA₂) is produced from PGH₂ by thromboxane synthase and can be spontaneously changed into its inactive form TXB₂ (Needleman et al, 1976). This inactive form is increased in the serum of pruritic diseases such as urticaria and psoriasis and in hemodialysis patients with chronic itch (Marks et al, 1991; Mysliwiec et al, 1985; Veale et al, 1994). It is unclear if administration of thromboxanes can cause itch in humans, but thromboxane receptors are expressed on keratinocytes and primary afferents (Andoh et al, 2007).

Another subfamily of eicosanoids, leukotrienes, are also produced from arachidonic acid by lipoxygenase. Leukotrienes, including leukotriene B₄ (LTB₄), do not cause itch in healthy subjects, but do induce pruritus in mice (Andoh and Kuraishi, 1998; Camp et al, 1983; Soter et al, 1983). Levels of LTB₄ were found to be increased in lesional skin of psoriatic and atopic patients, but were not found to be correlated with the increased itch in hemodialysis patients (Brain et al; 1984; Kanai et al, 1995; Ruzicka et al, 1986). However, the leukotriene receptor antagonist, montelukast, reduced pruritus by 35% in hemodialysis patients (Nasrollahi et al, 2007). Also, treatments that target synthesis of leukotrienes reduce pruritus in atopic dermatitis and Sjögren-Larsson ichthyosis (Willemsen et al, 2001; Woodmansee and Simon, 1999).

Endocannabinoids.

Endocannabinoids are neuromodulatory lipids that are derived from arachidonic acid and are thought to be released from keratinocytes (Maccarrone et al, 2003). The enzymes responsible for their synthesis and degradation, such as fatty acid amide hydrolase (FAAH), are also found in the skin, especially in keratinocytes (Maccarrone et al, 2003). Furthermore, the cannabinoid receptors CB1 and CB2 are expressed on peripheral nerve fibers, keratinocytes, mast cells, hair follicles, and sweat glands (Ständer et al, 2005). Although the endocannabinoid system has not extensively been examined in the skin of pruritic diseases, several topical treatments that contain cannabinoid agonists have had significant antipruritic effects. A topical cream containing the endocannabinoid anandamide (AEA) and the non-cannabimimetic palmitoylethanolamide (PEA) significantly reduced itch in subjects with uremic pruritus and even completely eliminated itch in 38% of the subjects (Szepietowski et al, 2005). Topical treatments

containing just AEA reduced pruritus in atopic dermatitis and dry skin patients (Eberlein et al, 2008; Ständer et al, 2006). The locally applied synthetic CB1 agonist HU210 reduced histamine-induced itch in healthy subjects (Dvorak et al, 2003).

Animal Models of Pruritus

The use of animal models has led to an ongoing revolution in the understanding of the pathophysiology of itch (Garibyan et al, 2013). In most animal models of itch, pruritus is induced by injection of pruritogens or induction of various dermatoses (Akiyama and Carstens, 2013; Kuraishi et al, 2013). In rodent models, hind paw scratching is used to measure itch (Kuraishi et al, 1995). However, distinguishing itch-related scratching from non-itch-related scratching (i.e., grooming, response to pain) can be difficult. Furthermore, rodents also scratch due to the application of non-pruritic substances, like ointment bases.

Acute itch is mainly induced by intradermal injections of itch mediators into the rostral back/nape of the neck in mice and rats. This method elicits “bouts” of hind paw scratching, which are defined as 0.5 to 2 seconds of back and forth movements (Nojima and Carstens, 2003). A cheek model is used to compare itch versus pain responses by measuring hind paw scratching versus fore paw wiping (LaMotte et al, 2011; Shimada and LaMotte, 2008). However, the cheek has been shown to be less sensitive than the rostral back for some pruritogens (Spradley et al, 2012). Itch can also be generated by the induction of cutaneous diseases.

Chronic dermatitis mimicking atopic dermatitis can be produced in mite-infested NC mice. This inbred strain of fancy mice from Japan develop spontaneous skin lesions associated with hyperkeratosis, acanthosis, and skin barrier dysfunction similar to

changes seen in atopic dermatitis skin (Suto et al, 1999). Additionally, these mice develop spontaneous and persistent scratching of the face, ears, and back (Yamaguchi et al, 2001). Atopic dermatitis-like lesions and itch can also be induced in dogs. Dog models utilize fleas, mites, and food allergies to produced allergen sensitization and atopic-like lesions in laboratory beagles (Olivry and Bäumer, 2015).

Pruritic dry skin can be modeled in mice by a repeated topical application of a mixture of acetone and diethylether followed by water (AEW) twice daily over a period of five days (Akiyama et al, 2010; Miyamoto et al, 2002b). This treatment causes spontaneous scratching and skin barrier disruption. Chemical manipulation in rats can model chronic itch associated with systemic disease (Bautista et al, 2014). Cholestasis, which presents with pruritus as the primary symptom, can be induced in rats with ethnylestradiol, an estradiol derivative. Injection of quisqualate, a glutamate receptor agonist, into the dorsal horn of the spinal cord can induce neuropathic itch. Also, the toxin streptozotocin is used to kill insulin-producing cells to causes diabetes, which is associated with localized pruritus.

Rodent models offer advantages to studying itch, including the relative ease of measuring scratching behavior and the ability to study pruritogenesis by genetic manipulation (LaMotte et al, 2011). Disadvantages to using mouse models include marked anatomical and physiological differences between mice and humans, such as skin structure and immunological and neurophysiological responses and regulation (Ghosh et al, 2000; Mestas and Hughes, 2004; Wong et al, 2011). Specifically, human skin has a thicker epidermis with more cell layers than mice skin (Pasparakis et al, 2014). Furthermore, mice have a prominent population of dendritic T-cells that serve as

regulators of immune responses; these cells are absent from the human epidermis. Most importantly, rodents exhibit different pruritoceptive responses in comparison to humans (Akiyama and Carstens, 2013; Jankowski and Koerber, 2010). Specifically, some pruritogenic compounds (e.g., TNF- α , LTB₄, and ACh) elicit itching in mice but not in humans, while on the other hand certain compounds (e.g., SP and histamine) induce itching in humans, but not in rats or all strains of mice (Table 1.2).

The pitfalls of the current rodent paradigms call for the design of a valid primate model of chronic itch. From a comparative biology perspective, the neurosensory system in non-human primates is the closest to that of humans, anatomically and physiologically. While only a limited number of pruritogens have been tested in primates, it has been shown that administration of histamine and cowhage in monkeys is pruritogenic, eliciting scratching responses (Cremins et al, 2012; Davidson et al, 2007; Davidson et al, 2009). Although non-human primates are expensive to house and difficult to handle, they offer a unique opportunity to investigate pathomechanisms that might not be present in other animal models. Therefore, it is of primary importance to analyze the expression of mediators and receptors that play a role in histaminergic and non-histaminergic itch transmission in primates with chronic itch.

Table 1.2. Intradermal application of pruritogens in different species.

Pruritogen	Human	Dog	Rat	Mice	References
Interleukin-2	+	-			Carr et al, 2009; Darsow et al, 1997; Wahlgren et al, 1995
Interleukin-8	-				Ständer and Steinhoff, 2002
Interleukin-31	+	+		+	Arai et al, 2013; Arai et al, 2015; Dillon et al, 2004; Gonzales et al, 2013; Hawro et al, 2014
Tumor Necrosis Factor-α	-			+	Bae et al, 2004; Darsow et al, 1997
Prostaglandin E₂	+			-	Andoh and Kuraishi, 1998 ; Neisius et al, 2002
Thromboxan A₂				+	Andoh et al, 2007
Leukotriene B₄	-	-		+	Andoh and Kuraishi, 1998; Camp et al, 1983; Soter et al, 1983
Substance P	+/-	-	-	+	Andoh et al, 1998; Carr et al, 2009; Hägermark et al, 1978; Marsella and Nicklin, 2001
Calcitonin Gene Related Peptide	-			-	Averbeck and Reeh, 2001; Ekblom et al, 1993;
Endothelin-1	+		+	+	Gomes et al, 2012; Katugampola et al, 2000; Namer et al, 2008; Trentin et al, 2006
Histamine	+	-	-	+/-	Carr et al, 2009; Han et al, 2006; Inagaki et al, 1999; Inagaki et al, 2001; Lewis, 1927
Acetylcholine	+/-*			+	Miyamoto et al, 2002a; Rukwied and Heyer, 1999
Serotonin	+	-	+	+	Carr et al, 2009; Thomsen et al, 2001; Weisshaar et al, 2004; Yamaguchi et al, 1999
Nerve Growth Factor	-		-		Rukwied et al, 2013
Tryptase	+	-		+	Carr et al, 2009; Ui et al, 2006

(+)=causes itch; (-)=does not cause itch; *Itch induced in atopic dermatitis skin, but not in healthy controls

SPECIFIC AIMS

Aim 1: To characterize the underlying molecular and structural basis of chronic itch in a primate model. For four years, the scratching behavior in a colony of adult, female *Cynomolgus* macaques (*Macaca fascicularis*) suffering from idiopathic chronic itch was monitored and recorded.

Aim 1a: Itch-specific pathways previously discovered in mice were examined in the primate model. Immunohistochemistry was used to examine the expression of itch-related ligands in the primate skin and their receptors in the spinal cord.

Aim 1b: Gene expression levels in lichenified and non-lichenified skin of primates with varying scratching intensities were measured using RNA sequencing. These gene expression profiles were then correlated to the animals' scratching severity to identify genes specifically related to itch.

Aim 1c: Based on Aim 1a findings, selected genes correlating to itch were targeted for immunohistochemical and proteomic analysis. The presence of these mediators and receptors was examined for co-localization with cutaneous nerve fibers.

Aim 2: To compare the molecular and structural findings from primate skin to those of human skin biopsies. In order to reveal the similarities and differences between the primate model and human chronic itch, lesional and non-lesional skin biopsies from patients with different chronic pruritic skin diseases (atopic dermatitis, psoriasis, and CTCL) and healthy controls underwent immunohistochemical analysis along with the primate tissue. The expression levels and anatomical distribution of the itch-related proteins were analyzed from lesional and non-lesional skin sections and then correlated to

itch severity ratings taken at the time of biopsy. The results from the human skin biopsies were then compared to the findings from the primate skin to compare the mechanisms behind chronic itch of different origins.

CHAPTER 2

**OVER-EXPRESSION OF THE GASTRIN-RELEASING PEPTIDE IN
CUTANEOUS NERVE FIBERS AND ITS RECEPTOR IN SPINAL CORD IN
PRIMATES WITH CHRONIC ITCH***

*This work was accepted in Journal of Invest Dermatol (Nattkemper et al, 2013)

To the Editor

Chronic pruritus affects millions of patients worldwide and has a significant impact on quality of life similar to that of chronic pain (Kini et al, 20011; Stander et al, 2007). Significant advances have been made in the last five years to elucidate the molecular pathways of acute itch (Liu et al, 2009; Sun and Chen, 2007). However, experimental approaches investigating the pathogenesis of pruritus and the ability to test novel therapeutic agents are largely limited to rodent models. Although these models offer some advantages, their translational potential to human disease remains to be established (Jeffry et al, 2011; Seok et al, 2013). Furthermore, most animal models focus on acute itch, which displays significant pathophysiological differences in comparison with chronic itch (Yosipovitch et al, 2007). Therefore, there is an unmet need for better animal models for chronic itch research.

Recently, gastrin-releasing peptide (GRP) and its receptor (GRPR) were discovered to play a key role in itch transmission, but not in nociception (Sun and Chen, 2007). GRP was found in a subset of unmyelinated dorsal root ganglion (DRG) neurons, while GRPR was expressed in lamina I of the dorsal horn of the spinal cord in mice. In

the skin, GRP is present in primary afferent nerve fibers and found to be increased in mice with chronic dermatitis (Tominaga et al, 2009). The GRP/GRPR signaling pathway is considered to be the first molecular pathway specific to itch transmission, but there is no evidence that this pathway has an analogous role in primates or humans.

We identified a subgroup of adult female *Cynomolgus* macaques (*Macaca fascicularis*) suffering from idiopathic chronic itch and observed their scratching behaviors twice every week for 10 minutes for four years using a focal observation technique (Altmann, 1974). The population pattern of GRP and GRPR expression in the skin and spinal cord were assessed in a blinded manner, respectively. The expression of GRP and GRPR was quantified by immunohistochemistry and analyzed with a 1-way ANOVA and Bonferroni post hoc test, while an Unpaired t-test was used to compare the amount of PGP9.5⁺ nerve fibers between groups. The scratching behavior of each animal was then analyzed for potential correlations with GRP and GRPR expression levels using a 2-tailed Spearman correlation test and linear regression.

The frequency (number of scratching episodes per hour) and duration (percent time of scratching during focal observation) of scratching were recorded over four years, totaling 68 hours of observation per animal. The animals were euthanized and histological sections from six randomly selected animals with different itch severities were collected. For each primate, the total count of nerve fibers expressing GRP in lichenified (chronically damaged skin lesion) and non-lichenified skin from thoracic dermatomes were correlated with itch severity. Skin cryosections (20 μm thickness) were double stained with antibodies for GRP (Immunostar, Hudson, WI) and the neuronal marker Product Gene Protein 9.5 (PGP9.5; Neuromics, Edina, MN) to examine the

amount of co-localization at the dermal-epidermal junction. Although fibers were found in both the epidermis and dermis, they were mainly present in the dermal-epidermal junction so all quantification was focused on this area. The population of GRPR expressing cells in the dorsal horn of the thoracic spinal cord of the same animals was also analyzed for a potential correlation to itch intensity by staining the spinal cord cryosections with the GRPR antibody (MBL International, Woburn, MA). For a detailed methods section, see supplementary information.

Primates that exhibited a comparatively higher severity of itching consistently displayed an increased percentage of GRP expressing nerve fibers at the dermal-epidermal junction of lichenified skin ($p=0.03$; Figure 2.1A and E). The mean percentage of GRP⁺/ PGP9.5⁺ fibers was 4.8 fold higher in primates exhibiting the highest itch intensity than in primates with the lowest intensity. The frequency (Figure 2.1B) and duration (Figure 2.1C) of scratching were significantly correlated with the percentage of GRP in lichenified skin ($r=0.94$, $p=0.02$; for both frequency and duration), but not in non-lichenified skin ($r=0.77$, $p=0.10$; for both frequency and duration). The innervation pattern of PGP9.5⁺ fibers in the skin show no significant difference of fibers in animals with severe itch compared to those with mild/moderate itch (Figure 2.1D).

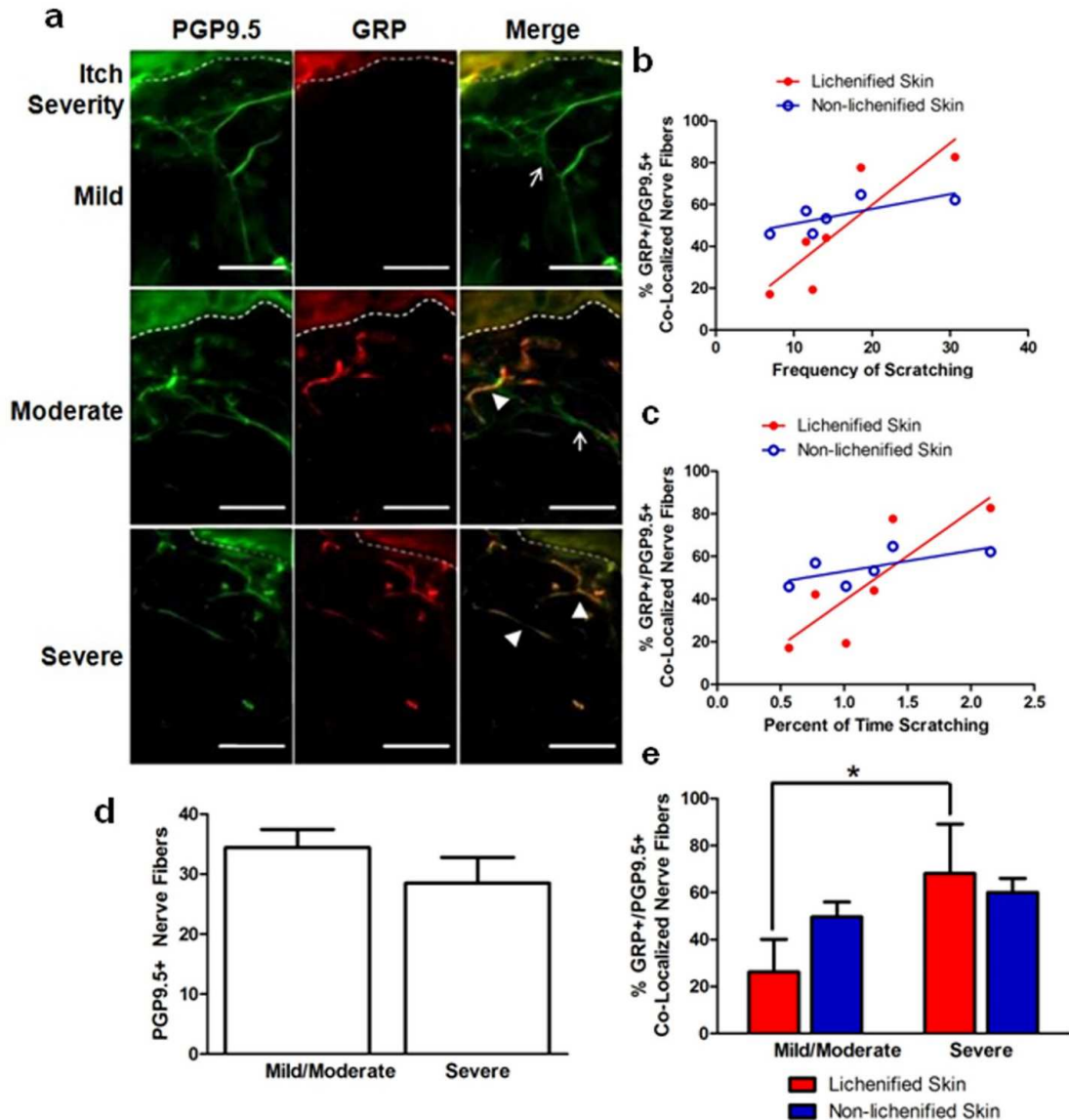


Figure 2.1. Immunohistochemical staining of GRP⁺ nerve fibers in the skin of primates with chronic itch. (a) Double-labeling of PGP9.5 (green) and GRP (red) in lichenified skin of primates representing mild, moderate, and severe itch intensities. Primates with higher scratching severity showed an increase of co-localization (yellow; arrowhead) of GRP in PGP9.5⁺ fibers at the dermal-epidermal junction compared to primates with lower scratching severity (not co-localized; arrow). The border between the epidermis and dermis is indicated with a dashed line (- -). Scale bars = 56 μ m. The mean percent of

double labeled GRP⁺/PGP9.5⁺ fibers at the dermal-epidermal junction in lichenified skin but not in non-lichenified skin significantly correlate with the frequency of scratching (b) and the percent time spent scratching (c). The amount of PGP9.5⁺ nerve fibers do not change between itch severities (d), while the expression of GRP significantly increases with high itch severity when compared to mild/moderate severity in lichenified skin (e) (n=3 per group; *p<0.05).

Additionally, an increased amount GRPR expressing cells was observed in the dorsal horn of the spinal cord of primates exhibiting a higher itch severity compared to animals with lower itch severity (p=0.002; Figure 2.2A and D). The frequency (Figure 2.2B) and duration (Figure 2.2C) of scratching was also found to be significantly correlated with the expression of GRPR in the superficial lamina I & II (r=0.94, p=0.02; for both frequency and duration), but only trended toward significance in the deep lamina III-V (r=0.83, p=0.06; for both frequency and duration) of the dorsal horn of the thoracic spinal cord. The number of GRP⁺ fibers in lichenified skin also significantly correlated to the amount of staining of GRPR in the superficial (r=0.94, p=0.01) and deep (r=0.89, p=0.03) lamina of the dorsal horn of each primate (Figure 2.2E).

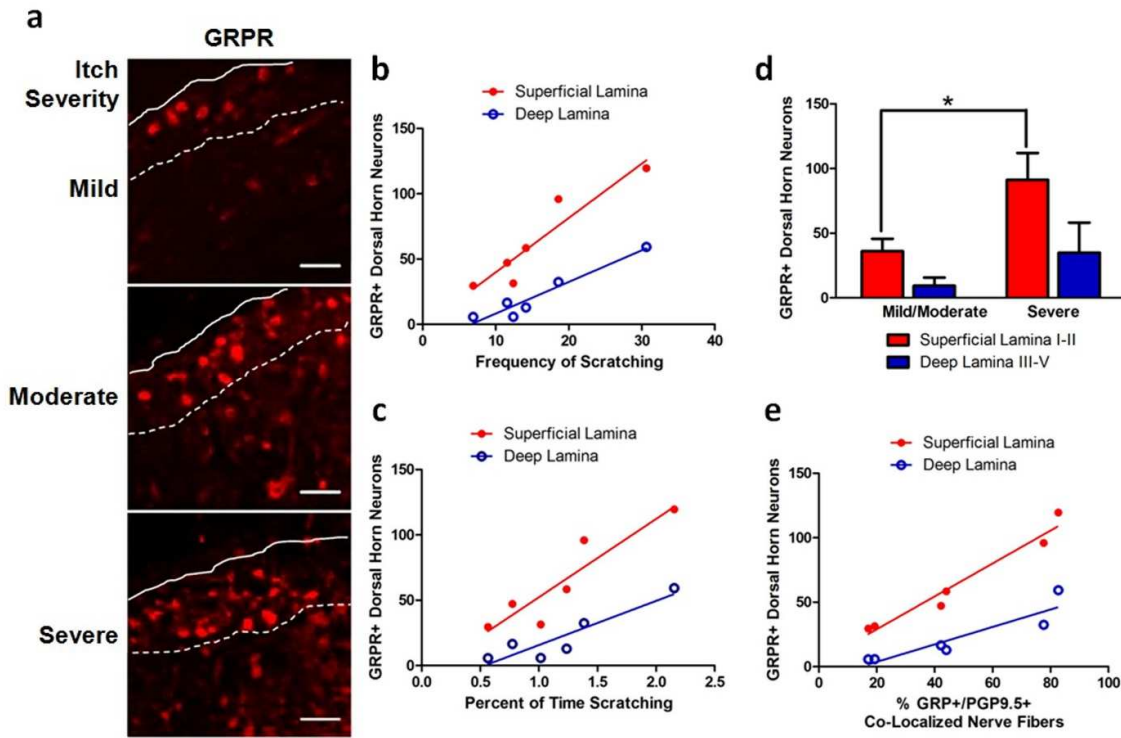


Figure 2.2. Immunohistochemical staining of GRPR⁺ cells in the dorsal horn of primates with chronic itch. (a) Labeling of GRPR⁺ (red) cells in the dorsal horn of the spinal cord of primates representing mild, moderate, and severe itch severities. A significant increase of GRPR⁺ cells was found in the superficial lamina but not in the deep lamina of primates exhibiting severe scratching. Superficial and deep lamina are separated with a dashed line (- -) and the dorsal horn surface is indicated with a solid line (-). Scale bars = 56 μ m. The number of labeled GRPR⁺ cells in the superficial lamina of the dorsal horn correlate with the frequency of scratching (b) and the percent time spent scratching (c). (d) The number of GRPR⁺ neurons significantly increases with severe itch intensity compared to mild/moderate intensity in superficial lamina (n=3 per group; *p<0.05). (e) The percentage of GRP⁺ fibers in lichenified skin significantly correlated to the amount of GRPR⁺ cells in the dorsal horn for each primate.

This is the first study to demonstrate that the GRPR and its ligand GRP are highly expressed in the spinal cord and skin of primates with chronic itch. It is consistent with previous finding in non-chronic itch mice models (Andoh et al, 2011; Sun and Chen, 2007; Sun et al, 2009). Therefore, the GRP/GRPR system is a promising drug target for the treatment of chronic pruritus in humans. Furthermore, this subset of female Cynomolgus macaques offers a novel model of itch that could better represent chronic itch in humans. This would allow for long term follow up studies assessing other mediators involved in chronic itch.

Supplemental

Materials and Methods

Behavioral

All animal experiments were approved by the Wake Forest University Animal Care and Use Committee. The frequency of scratching and the amount of time spent scratching was documented for adult female Cynomolgus macaques of reproductive age (n=6) over a four year period at similar times during the day (early afternoon). Scratching was defined as moving the fingertips repeatedly across the same skin area for duration longer than one second and is distinct from grooming behaviours. All observers were trained in assessing behaviors of monkeys. The frequency and duration of scratching was recorded twice every week for 10 minutes using a validated focal animal observation technique (Altmann, 1974) throughout the four year experimental period and expressed as frequency per session and percentage of time duration over all. Thus the data reported here represents 68 hours of observation per animal. Variance for each animal's behavior

throughout the four years was minimal (frequency $\sigma^2=0.03$; duration $\sigma^2=3.08$). Animals that scratched more than 15 times during the 10 minute observational period were designated as severe scratchers (n=3), while those that scratch less were designated as mild/moderate (n=3). Before data collection and semi-annually thereafter, inter-observer reliability tests were conducted with a criterion of $r \geq 0.92$ for reliability. For all behavioral observations, animals were not identified for their scratching severities and several observers conducted focal observations of each animal to avoid selection bias. The behavioral data was then analyzed by a non observer after all observations were completed for 4 years

Histopathology

The animals were anesthetized with an overdose of sodium pentobarbital (60-100 mg/kg, IV). Lichenified (regions of chronically damaged skin) and non-lichenified skin sections were collected from thoracic dermatomes corresponding to T10-T12. Lichenified skin was identified postmortem and then confirmed by observing hypertrophied epidermis. After the animals were perfused via transcardiac perfusion with Lactate Ringers solution, thoracic spinal cord at T-10-12 were isolated. All tissue was immersion fixed in 4% paraformaldehyde in PBS overnight at 4°C. Tissue was processed for immunohistochemistry by cryoprotection in 30% sucrose and then frozen embedded in OCT compound. Skin tissue was sectioned at 20 μm onto slides for staining and spinal cord tissue was sectioned at 20 μm and placed in PBS for free floating staining.

Immunohistochemistry

The researchers were blinded to the behavioral condition of all animal tissues assessed and the results were only decoded after the analysis was fully performed.

Skin cryo-sections were double stained with anti-protein gene product 9.5 (PGP9.5, 1:500; Neuromics, Edina, MN) to detect nerve fibers and anti-gastrin releasing peptide (GRP, 1:400; Immunostar, Hudson, WI) primary antibodies as described previously (Lui et al, 2009; Tominaga et al, 2009). Sections were blocked with 5% normal goat serum and 0.2% Triton x-100 in PBS for 2 hrs and then incubated with the primary antibodies overnight at 4°C. Alexa fluor (488 & 594, 1:300; Molecular Probes, Eugene, OR) secondary antibodies were used for detection. Sections treated without any primary antibodies were used as negative controls. Furthermore, the specificity of the GRP antibody was confirmed by pre-absorbing full length Bombesin and GRP peptides (50 µg/ml; American Peptide Company, Sunnyvale, CA) in blocking solution with the GRP antibody (1:400) O/N at 4°C with gentle agitation. Solutions were centrifuged at high speed (~16,000xg) for ten minutes and the supernatant was used for immunohistochemistry as described above, which resulted in blocking of GRP-nerve fiber immunoreactivity.

Spinal cord cryo-sections were stained with anti-gastrin releasing peptide receptor (GRPR, 1:4000; MBL International, Woburn, MA) primary antibody as previously described (Sun and Chen, 2007). Free floating sections were blocked with 2% normal donkey serum and 0.3% Triton x-100 in PBS for 1 hr and then incubated with the primary antibody overnight at 4°C. Cyanine 3 (Cy3, 1:1000; Jackson ImmunoResearch, Toronto, ON) secondary antibody was used for detection. Sections treated without any primary antibody were used as negative controls.

Quantification

Fluorescent labeled images were obtained with Olympus microscopes (Center Valley, PA) and ImageJ software was used for analysis. All quantification was performed in a blinded manner, where the behavioral condition of each animal was unknown to the researchers. In skin sections, the number of PGP9.5+ nerve fibers and the percentage of PGP9.5+/GRP+ fibers were hand counted in 21 serially selected sections. Although fibers were found in both epidermis and dermis, they were mainly present in the epidermal-dermal junction, as previously described (Tominaga et al, 2009), so all quantification was focused to this area. In at least 15 serially selected spinal cord sections, only neurons with clearly visible nucleoli were counted as previously described (Sun and Chen, 2007). The substantia gelatinosa was used to differentiate between superficial (I-II) and deep lamina (III-V).

Statistical Analyses

A nonparametric two-tailed Spearman correlation and linear regression were used for statistical analyses to correlate the immunohistochemical findings and the complete four years of behavioral data. 1-way ANOVAs with Bonferroni pos hoc. tests and an Unpaired t-test was used to analyze the differences in immunohistochemical findings between groups; significance was set at $p < 0.05$ (GraphPad; La Jolla, CA).

CHAPTER 3

RNA SEQUENCING REVEALS DIFFERENTIALLY EXPRESSED GENES IN A PRIMATE MODEL OF CHRONIC ITCH

Abstract

The genetic profile of chronic pruritus at the peripheral level is currently under-investigated. In this study, we used RNA sequencing to analyze the complete transcriptome of skin from a primate model of chronic itch. Paired lichenified and non-lichenified skin biopsies were collected from 35 *Cynomolgus* macaques with idiopathic chronic itch. RNA sequencing was performed to identify differentially expressed genes (>2.0 fold change; <0.05 false detection rate) in pruritic, lichenified versus non-pruritic, non-lichenified skin, generating an average of ~60 million paired-end 100-bp reads per sample. The RNA sequencing data were correlated to quantified primate scratching behavior. Over 2,264 genes were differentially expressed in the lichenified primate skin, with ~400 genes correlating to scratching behavior. Many of these differentially expressed transcripts were associated with sensory nerve fibers, keratinocytes, mast cells, neutrophils, or lymphocytes. To confirm the RNA sequencing findings, selected gene products were quantified in the primate skin using immunohistochemistry. Staining patterns from the primate skin were then compared to human atopic dermatitis, psoriasis, and healthy control skin. These results have led to an increased understanding of the molecular mechanisms involved in different pruritic conditions and may provide novel targets for treatment.

Introduction

Transcriptome analyses using microarrays and RNA sequencing (RNA-seq) allow for the characterization of global changes of gene expression associated with pruritic diseases. Studies using RNA-seq in subjects with psoriasis have revealed many differentially expressed genes (DEGs) when comparing lesional skin to non-lesional or healthy skin. A small study (n=3) found 2,629 DEGs in lesional versus non-lesional skin, while a larger study (n=174) found 3,577 DEGs in lesional versus healthy skin (Jabbari et al, 2012; Li et al, 2014). These enriched DEGs were commonly involved in inflammatory response, cytokine-receptor interaction, cell division, and keratinization pathways. The larger study specifically found increased expression of several cytokines (interleukin (IL)-6, IL-12B, IL-17A/F, IL-21, IL-22, IL-24, IL-26, interferon (IFN)- γ , and IFN- ϵ), cytokine receptors (IL-21R and IL-23R), and transcription regulators [signal transducer and activator of transcription (STAT) 1, STAT3, CCAAT/enhancer binding protein (C/EBP) β , and nuclear factor kappa-light-chain-enhancer of B cells (NF- κ B)]. This study also found IL-34 to be significantly downregulated in lesional skin. Although these studies examined lesional psoriatic skin, they did not specifically focus on itchy plaques. Another study examined 908 DEGs in lesional versus non-lesional skin of patients with atopic dermatitis (Suárez-Fariñas et al, 2015). These DEGs consisted of inflammatory mediators [S100 calcium binding protein (S100) A7/A8/A9, chemokine (C-C motif) ligand (CCL) 2, CCL3, IL-36 α/γ , IL-36RN, triggering receptor expressed on myeloid cells (TREM) 1] and skin barrier proteins [marker of proliferation Ki67 (MKi67) and keratin 16].

Transcriptome analysis has also been applied to animal models of itch. A microarray study found several genes that were differentially expressed in the trigeminal nerves and skin of a mouse model of dry skin with chronic itch (Wilson et al, 2013). These genes included itch-related receptors, channels, and proteins, such as protease-activated receptor 2 (PAR2), mas-related G-protein coupled receptor (Mrgpr) A3, aquaporin 3, filaggrin, IL-33, IL-31RA, and keratin 6. An RNA-seq study was used to localize the expression of itch-associated mediators in sensory ganglia and the spinal cord of mice, rats, and humans without pruritus (Goswami et al, 2014). This study found that natriuretic precursor peptide B (NPPB) was expressed in sensory ganglia, while gastrin-releasing peptide (GRP) was found in the spinal cord. Recently, a single-cell RNA-seq technique was used to differentiate eleven types of sensory neurons in mouse dorsal root ganglia, and three types were suggested to be itch-sensitive based on their gene expression profiles (Usoskin et al, 2015).

To further elucidate pruritic mechanisms, we used RNA-seq to examine peripheral gene expression in a colony of Cynomolgus macaques (*Macaca fascicularis*) with idiopathic chronic itch. Cynomolgus macaques, the most common nonhuman primate animal model, have annotated transcriptome assemblies available for reference (Ebeling et al, 2011; Lee et al, 2014a). These primates have similar thresholds for detecting sensory and pruritic stimuli compared to humans (Davidson et al, 2007). In our study, we examined DEGs in severely scratched, lichenified (chronically damaged) versus mildly/moderately scratched, non-lichenified skin of primates. Over 2,264 genes were differentially expressed in the lichenified primate skin, with ~400 genes correlating to severe scratching behavior. To confirm the expression patterns revealed by the RNA-

seq, select genes were targeted for immunohistochemical analysis in the primate skin and compared to findings in human pruritic diseased skin.

Methods

Primate Behavior and Tissue

All animal experiments were approved by the Wake Forest University Animal Care and Use Committee. The frequency of scratching and the amount of time spent scratching were documented for adult female *Cynomolgus* macaques (*Macaca fascicularis*) of reproductive age (n=35) over a four-year period. Scratching was defined as moving the fingertips repeatedly across the same skin area for a duration longer than one second and was distinct from grooming behaviours. All observers were trained in assessing these behaviors. The frequency and duration of scratching was recorded twice every week for ten minutes in the early afternoon using a validated focal animal observation technique (Altmann et al, 1974) throughout the experimental period. Thus, the data reported here represent 68 hours of observation per animal. Variance for each animal's behavior throughout the four years was minimal (frequency $\sigma^2=0.03$; duration $\sigma^2=3.08$). Animals that scratched on average more than 15 times per ten-minute observational period were designated as severe scratchers (n=14), and those that scratched fewer than 15 times were designated as mild/moderate (n=21). Before data collection and semi-annually thereafter, inter-observer reliability tests were conducted with a criterion of $r \geq 0.92$. For all behavioral observations, animals were not identified for their scratching severities, and several observers conducted focal observations of each animal to avoid selection bias. The behavioral data were then analyzed by a non-observer after all observations were completed.

The animals were anesthetized with an overdose of sodium pentobarbital (60-100 mg/kg, IV). Lichenified and non-lichenified skin sections were collected from thoracic dermatomes corresponding to T10-T12. Lichenified skin was identified postmortem by excoriations and then confirmed with hematoxylin and eosin (H&E) staining to observe hypertrophied epidermis (Figure 3.1A and B). Tissue for RNA and protein isolation was immediately frozen and stored at -80°C until processed. Tissue for immunohistochemical analysis was immersion-fixed in 4% paraformaldehyde in PBS overnight at 4°C and then embedded into paraffin. 5- μ m thick sections of paraffin-embedded skin tissue were processed for standard H&E staining.

Human Subjects and Tissue

Skin biopsies were obtained from healthy controls (n=8) and patients with atopic dermatitis (AD, n=8) and psoriasis (PS, n=8) in accordance with the Declaration of Helsinki and with Temple University Institutional Review Board approval. Punch biopsies were obtained from pruritic lesional and non-pruritic non-lesional skin of patients, while age-, gender-, and site-matched skin was obtained from healthy controls. Tissue was immersion-fixed in 10% neutral buffered formalin overnight at 4°C and then embedded into paraffin. Pruritus severity was measured at the time of the biopsy using a visual analog scale (VAS) from 0 (“no itch”) to 10 (“unbearable itch”). The Eczema Area and Severity Index (EASI) or Psoriasis Area and Severity Index (PASI) score was also assessed at time of biopsy.

RNA Isolation and Sequencing

Primate skin was homogenized using an SK-200X freeze-crush apparatus (Diagnocine, Hackensack, NJ). Tissue was incubated in TRIzol reagent (Life

Technologies, Carlsbad, CA), and the RNeasy kit (Qiagen Inc, Valencia, CA) was used to isolate RNA following the manufacturer's protocol. Purified RNA was quantified with 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Nanodrop ND 1000 spectrophotometer (Fisher Scientific, Pittsburg, PA).

Polyadenylated mRNA libraries were prepared using TruSeq Stranded mRNA with Ribo-Zero (Illumina, San Diego, CA). cDNA was fragmented to ~250-bp and 15 cycles of amplification were performed. RNA-seq was performed with the Illumina HiSeq2000 (Illumina, San Diego, CA) at a depth of 200 million paired-end reads of 100-bp. Quality control was performed on all raw sequence data using FastQC. Using TopHat, reads were aligned to the reference genome (GCA_000364345.1) and the de novo transcriptome assembly, produced by Trinity and annotated by BLAST+ (Pipes et al, 2013; Grabherr et al, 2011; Camacho et al, 2009). Read counts were normalized to the number of reads per kilobase per million mapped reads (RPKM) (Mortazavi et al, 2008; Garber et al, 2011). A Wilcoxon rank-sum test with a Bonferroni corrected p-value ($p < 0.000001$) was used to identify differentially expressed genes (based on a false detection rate less than 0.05 and a fold change (FC) greater than 2.0) in severely scratched, lichenified primate skin versus mildly/moderately scratched, non-lichenified skin.

Immunohistochemistry

Researchers were blinded to the identity of the biopsies, and the results were only decoded after the immunohistochemical analysis was fully performed. 5- μ m thick sections of paraffin-embedded skin tissue were double-stained from each human and primate biopsy. Sections were deparaffinized and then underwent antigen retrieval using

Target Retrieval Solutions (DAKO, Glostrup, Denmark) heated in a humidified oven overnight at 60°C, then washed in PBS. Sections were blocked with 5% normal donkey serum and 0.2% Triton X-100 in PBS for 2 hours and then incubated with primary antibodies overnight at 4°C.

Primary antibody combinations were: anti-substance P (SP; 1:1000; Abcam, Cambridge, MA) and anti-neurokinin 1 receptor (NK-1R; 1:750; Pierce Thermo Scientific, Rockford, IL); anti-tryptase (1:100; Abcam, Cambridge, MA) and anti-protease-activated receptor 2 (PAR2; 1:100; Santa Cruz, Dallas, TX); anti-endothelin 1 (ET-1; 1:200; GeneTex, Inc., Irvine, CA) and anti-endothelin receptor A (ETA; 1:200; Pierce Thermo Scientific, Rockford, IL); anti-met-enkephalin (1:100; Abcam, Cambridge, MA) and anti- β -endorphin (1:2000; Abcam, Cambridge, MA); anti-dynorphin A (5 μ g/ml; Abcam, Cambridge, MA) and anti-histamine (1:1000; EMD Millipore, Billerica, MA); anti-TRPV1 (1:200; Abcam, Cambridge, MA); anti-TRP ankyrin 1 (TRPA1; 1:500; Abcam, Cambridge, MA); and anti- β -tubulin III (1:300; Neuromics, Edina, MN) and anti-protein gene product (PGP) 9.5 (1:1000; Ultracclone Limited, Wellow, Isle of Wight, UK).

Alexa Fluor (488 & 594, 1:300; Molecular Probes, Eugene, OR) secondary antibodies were used for detection. When double staining with two rabbit primary antibodies was performed (e.g., ET-1/ETA and TRPV1/TRPA1), the Zenon Rabbit IgG Labeling kit (488 & 594; Molecular Probes, Eugene, OR) was used for detection. All slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and imaged under a fluorescence microscope. Sections treated without any primary antibodies were used as negative controls. Furthermore, specificity of each primary

antibody was confirmed by pre-absorption with its respective peptide in blocking solutions overnight at 4°C with gentle agitation. Solutions were centrifuged, and the supernatant was used for immunohistochemistry as described above. In each case, this process resulted in blocking of the primary antibody's immunoreactivity.

Three fields (20X objective magnification) were measured for every section. The total field and selected field (epidermis) fluorescence areas (in μm^2) were measured and normalized to background staining using ImageJ Software (NIH, Bethesda, MD). Data are presented as mean epidermal fluorescence normalized to mean total field fluorescence. Mast cell (tryptase+ cells) or nerve (β -tubulin III+ or PGP9.5+ nerves) counts were also performed using ImageJ Software and normalized to epidermal length as previously described (McArther et al, 1998). All data are reported as mean \pm SD. One-way ANOVAs with Bonferroni post hoc tests were used to compare the differences between primates and human tissue. Statistical significance was set at $p < 0.05$ (GraphPad Prism, La Jolla, CA).

Results

Analysis of Primate DEGs

Primates in the severe group significantly ($p < 0.0001$) scratched more than primates in the mild/moderate group (Figure 3.1C and D).

The RNA sequencing identified 2,264 DEGs between severely scratched, lichenified and mildly/moderately scratched, non-lichenified primate skin, with ~400 genes previously known to be implicated in itch pathways (Table 3.1). These include receptor channels (TRPV3, PAR2, voltage gated sodium channel (NA_v) 1.7, and MrgprX2), proteases (cathepsin S (CTSS), kallikrein (KLK) 5, and tryptase),

neuropeptides (SP, proopiomelanocortin (POMC), ET-1, and S100A7), and cytokines and chemokines (INF- γ , IL-36, IL-17, chemokine (C-X-C motif) ligand (CXCL) 3, and CCL1) (Figure 3.2).

The largest FC of 9.1 was seen in the gene PLA2G4D, which encodes the enzyme phospholipase A2 (group IVD) that is part of the arachidonic acid pathway to produce eicosanoids. The eicosanoid leukotriene-B4 (LTB4) receptor 2 was also found to be upregulated, with an FC of 4.04. Many neuropeptide ligands and their receptors had high FCs. SP, a pruritogen, and its receptor NK-1R had FCs of 7.87 and 4.51, respectively. ET-1 (FC 3.99) and its receptor ETA (FC 5.25) were also increased. Additionally, the met-enkephalin and β -endorphin precursor POMC (FC 3.29), and the μ -opioid receptor (MOR, FC 7.11) showed increased expression. However, the κ -opioid receptor (KOR, FC 0.16) and the dynorphin A precursor prodynorphin (PDYN, FC 0.06) was downregulated. Trypsin (FC 4.32), a serine protease, and its receptor PAR2 (FC 4.71) were also upregulated. Other channels known to be involved in itch and/or pain were increased in severely scratched, lichenified skin as follows: TRPV1 (FC 4.91), TRPV3 (FC 5.67), MrgprX2 (FC 3.31), and Nav1.3 (FC 3.29) and 1.7 (FC 3.43). The sequencing revealed many cytokine and chemokine DEGs, including CCL1 (FC 3.17), CCL18 (FC 4.39), IL-36 α (FC 3.42), IL-36 γ (FC 4.04), IL-36RN (FC 3.37), IL-23 α (FC 3.50), IL23- β (FC 3.13), IL-17A (FC 3.83), IL-17F (FC 3.88), IL-9 (FC 3.75), and IL-6 (FC 3.42).

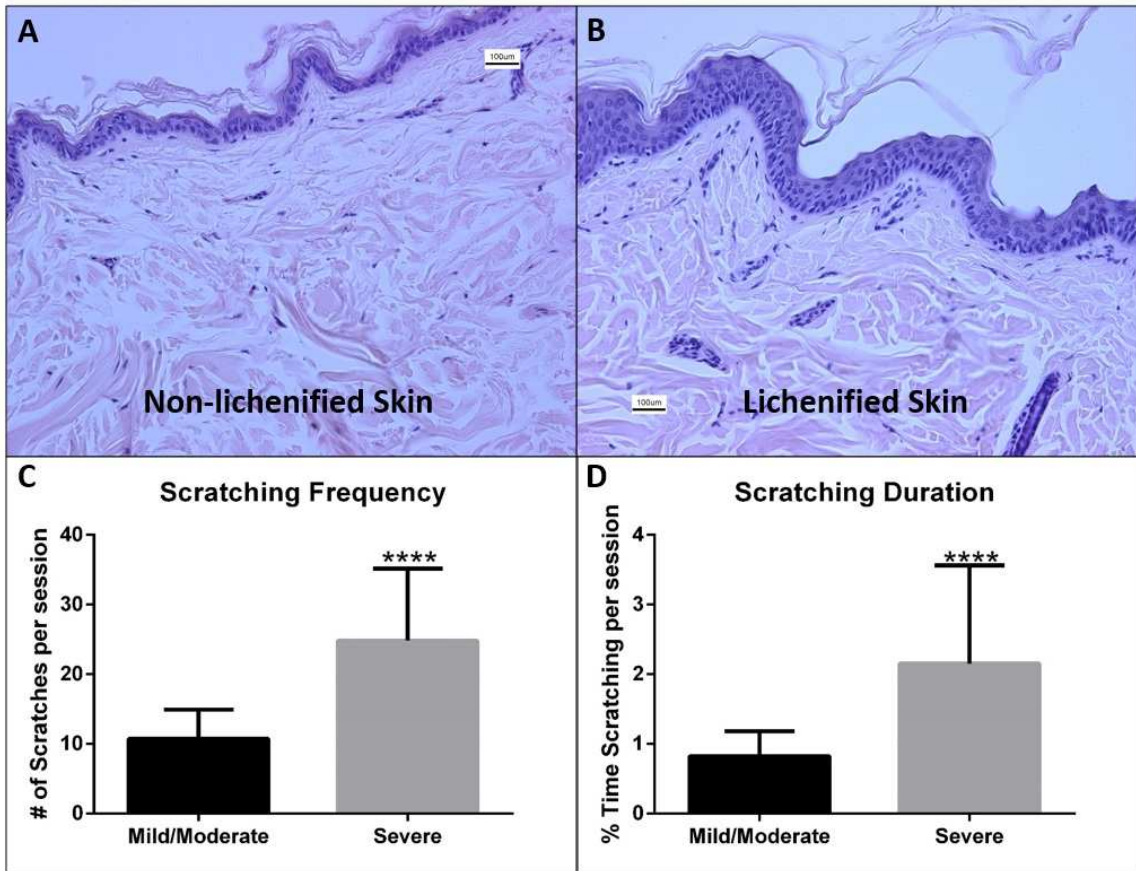


Figure 3.1. Primate skin and scratching behavior. Representative images of non-lichenified (A) and lichenified (B) primate skin. Average primate scratching behavior per ten-minute observation session. Primates in the severe scratching group (n=14) scratched significantly more often (C) and for a longer duration (D) than primates in the mild/moderate group (n=21). ****; $p < 0.0001$.

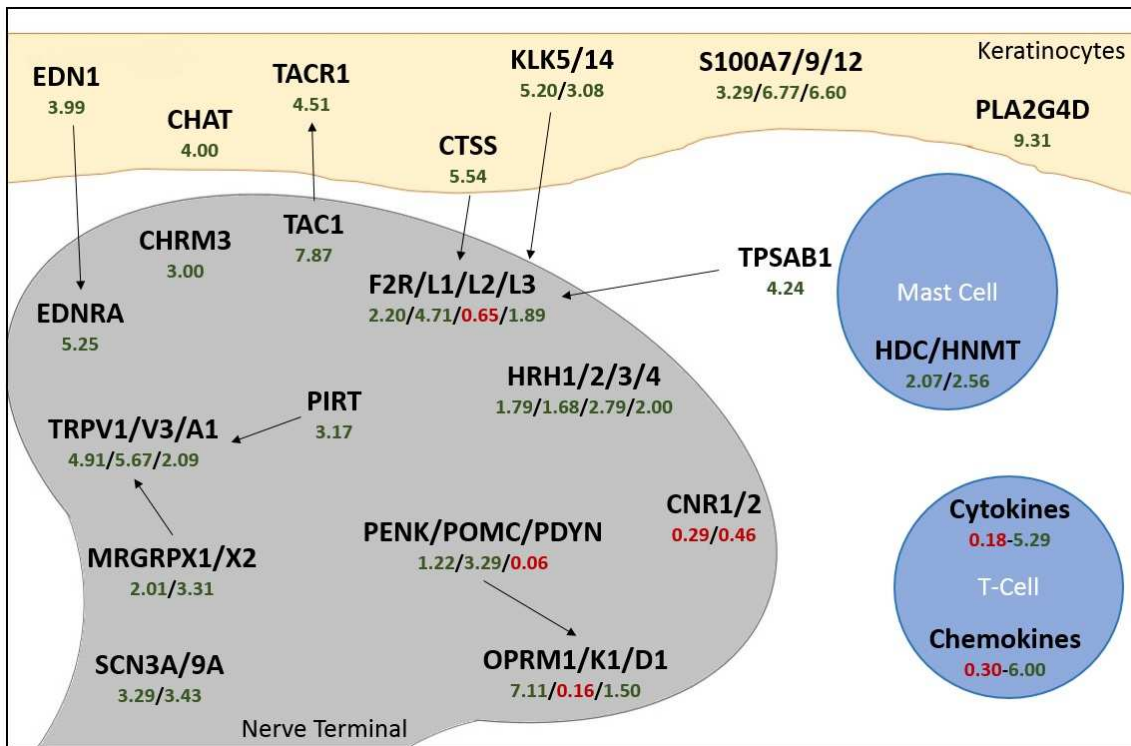


Figure 3.2. Diagram of DEGs in severely scratched, lichenified primate skin. DEGs included receptor channels, neuropeptides, and cytokines. Interactions of gene products are designated with arrows. The fold change (FC) between lichenified and non-lichenified skin is listed under each gene name (green = upregulated, red = downregulated). For cytokines and chemokines, a range of FCs are listed. Gene symbols/names: SCN3A/9A, voltage-gated sodium channel 1.3/1.7; MRGPRX1/X2, mas-related G protein-coupled receptor X1/X2; TRPV1/V3/A1; transient receptor potential cation channel V1/V3/A1; PIRT, phosphoinositide-interacting regulator of TRPs; EDN1, endothelin-1; EDNRA, endothelin receptor A; CHAT, choline O-acetyltransferase; CHR3, cholinergic receptor muscarinic 3; TAC1, substance P; TACR1, tachykinin receptor 1; CTSS, cathepsin S; KLK5/14, kallikrein 5/14; TPSAB1, tryptase $\alpha\beta$ -1; F2R/L1/L2/L3, protease-activated receptor 1/2/3/4; HRH1/2/3/4, histamine

receptor 1/2/3/4; CNR1/2, cannabinoid receptor 1/2; PENK/POMC/PDYN, proenkephalin/proopiomelanocortin/prodynorphin; OPRM1/K1/D1, opioid receptor $\mu/\kappa/\delta$; S100A7/9/12, S100 calcium binding protein A7/9/12; PLA2G4D, phospholipase A2 group IVD; HDC/HNMT, histamine decarboxylase/histamine N-methyltransferase.

Immunohistochemistry of Selected DEG Products

Immunohistochemistry was performed on pruritic, lesional and non-pruritic, non-lesional human and primate skin to validate selected findings from the RNA-seq. The mean ages of AD (n=8, 4 male and 4 female; mean age 40 ± 13), PS (n=8, 4 male and 4 female; mean age 41 ± 13), and healthy (n=8, 4 male and 4 female; mean age 40 ± 14) subjects did not significantly differ from one another. For AD subjects, the average EASI was 42 ± 3 , while the average itch intensity at the site of lesional biopsy was 8.4 ± 1.5 . For PS subjects, the average PASI score was 21 ± 16.7 , and the itch intensity at the site of lesional biopsy was 8.5 ± 1.8 . Healthy controls reported no itch at the site of biopsy.

Although the sequencing found the histamine-producing enzyme histamine decarboxylase (HDC, FC 2.07) to be upregulated, histamine was not significantly increased in lesional skin of primates, AD skin, or PS skin (data not shown). This lack of elevation may be due to the increased expression of histamine N-methyltransferase (HNMT, FC 2.56), a major histamine-degrading enzyme in the skin. TRPV1 and TRPA1 mRNA levels were shown to be increased, but the numbers of TRPV1+ and TRPA1+ cutaneous nerve fibers were not significantly different between groups (Figure 3.3 A and B). However, the fluorescence intensity for TRPV1 was increased ($p < 0.0001$) throughout

the epidermis in lichenified (712795 ± 84253) and non-lichenified (621250 ± 175798) primate skin (Figure 3.4A). TRPA1 had higher expression ($p < 0.0001$) in keratinocytes of the basal membrane in primate skin (lichenified: 203695 ± 15851 ; non-lichenified: 180784 ± 29762) and lesional AD skin (168415 ± 31842), but not in PS skin (lesional: 91613 ± 21231 ; non-lesional: 80443 ± 15641) (Figure 3.4B)

Tryptase and its receptor PAR2 were found to be elevated ($p < 0.0001$) in primates that exhibited a higher severity of scratching and in lesional AD and PS skin. Tryptase was detected in numerous mast cells scattered within the dermis of all skin types. However, most tryptase+ mast cells were located in the papillary dermis at the dermal-epidermal junction in AD, PS, and primate tissue (Figure 3.5A). Tryptase+ mast cells were significantly increased in lesional AD (162 ± 24), lesional (241 ± 31) and non-lesional (129 ± 38) PS, and lichenified primate (132 ± 48) skin when compared to healthy (67 ± 21) skin. The largest increases of PAR2 expression were found throughout in the epidermis of AD (lesional: 14557490 ± 1196560 ; non-lesional: 1281754 ± 1859510) and primate skin (lichenified: 10138560 ± 1384608 ; non-lichenified: 8275493 ± 403408) (Figure 3.5B). The receptor PAR2 was predominantly found in keratinocytes of the granular layer in healthy skin with a fluorescence intensity of 3817303 ± 550011 . This area had increased expression of PAR2 in PS skin (lesional: 6726425 ± 347537 ; non-lesional: 4837010 ± 2236953).

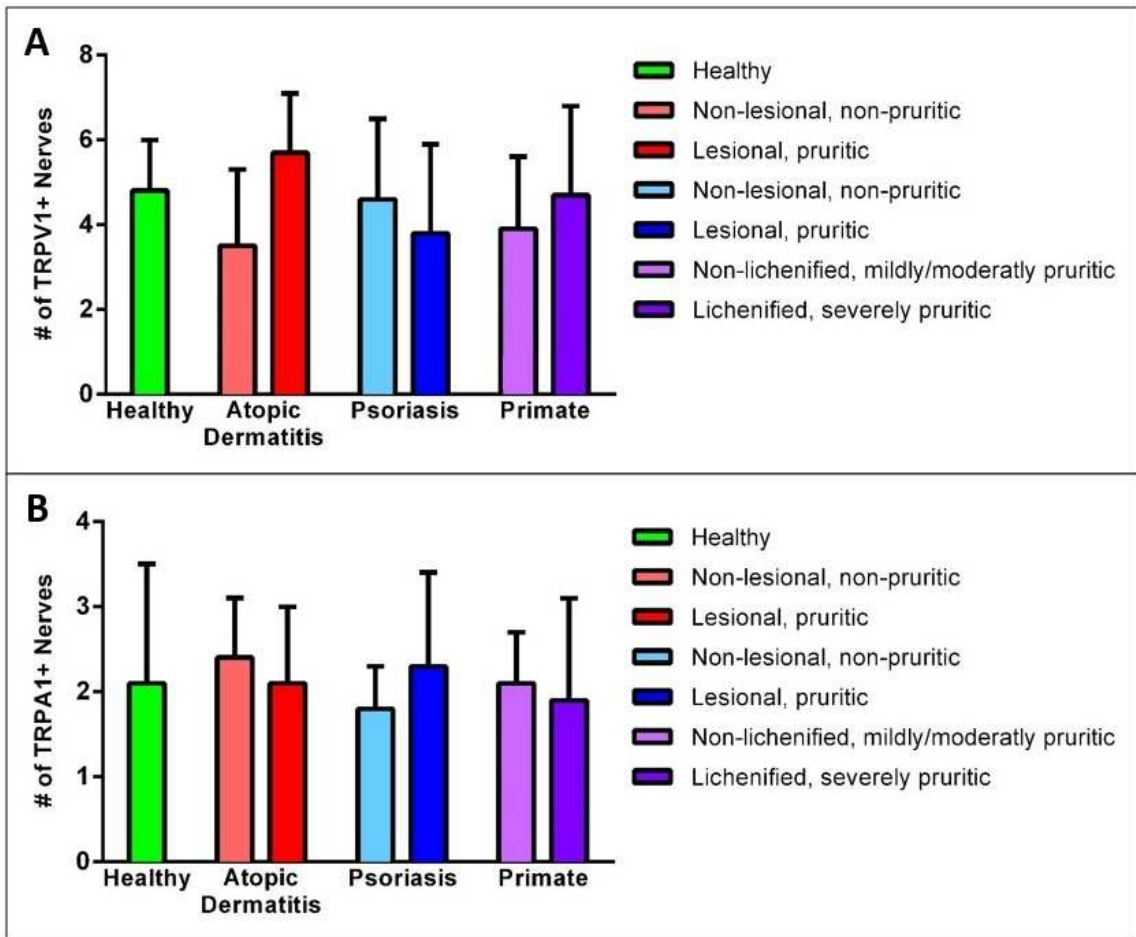


Figure 3.3. Immunohistochemistry of TRPV1 and TRPA1 expression in cutaneous nerves of primates and humans. The numbers of TRPV1+ (A) and TRPA1+ (B) nerves were not significantly different between groups.

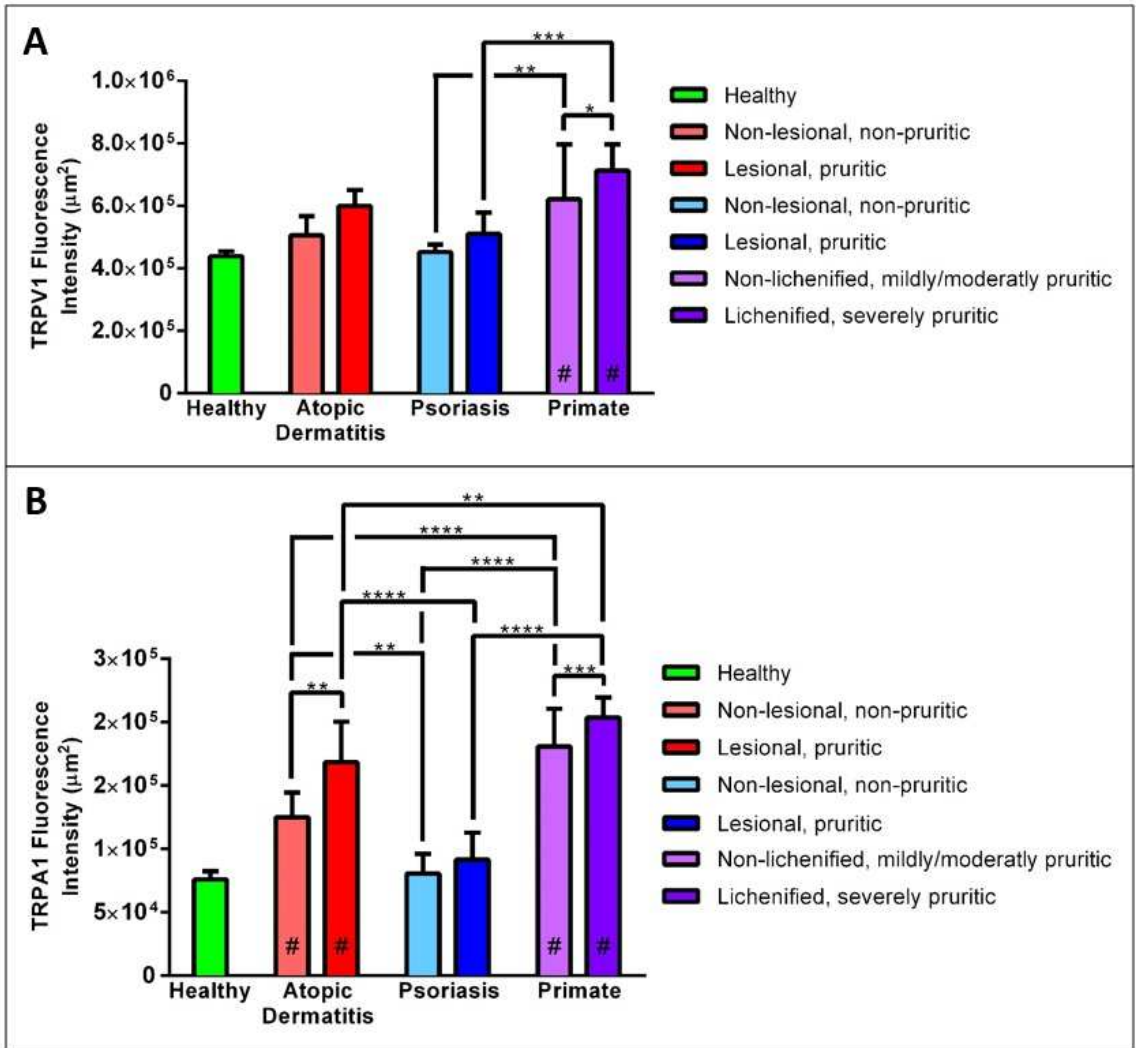


Figure 3.4. Immunohistochemistry of TRPV1 and TRPA1 expression in the epidermis of primates and humans. TRPV1 (A) was significantly increased in primate skin, while TRPA1 (B) was significantly increased in primate and AD epidermis (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). #; significant difference when compared to healthy skin, $p < 0.01$.

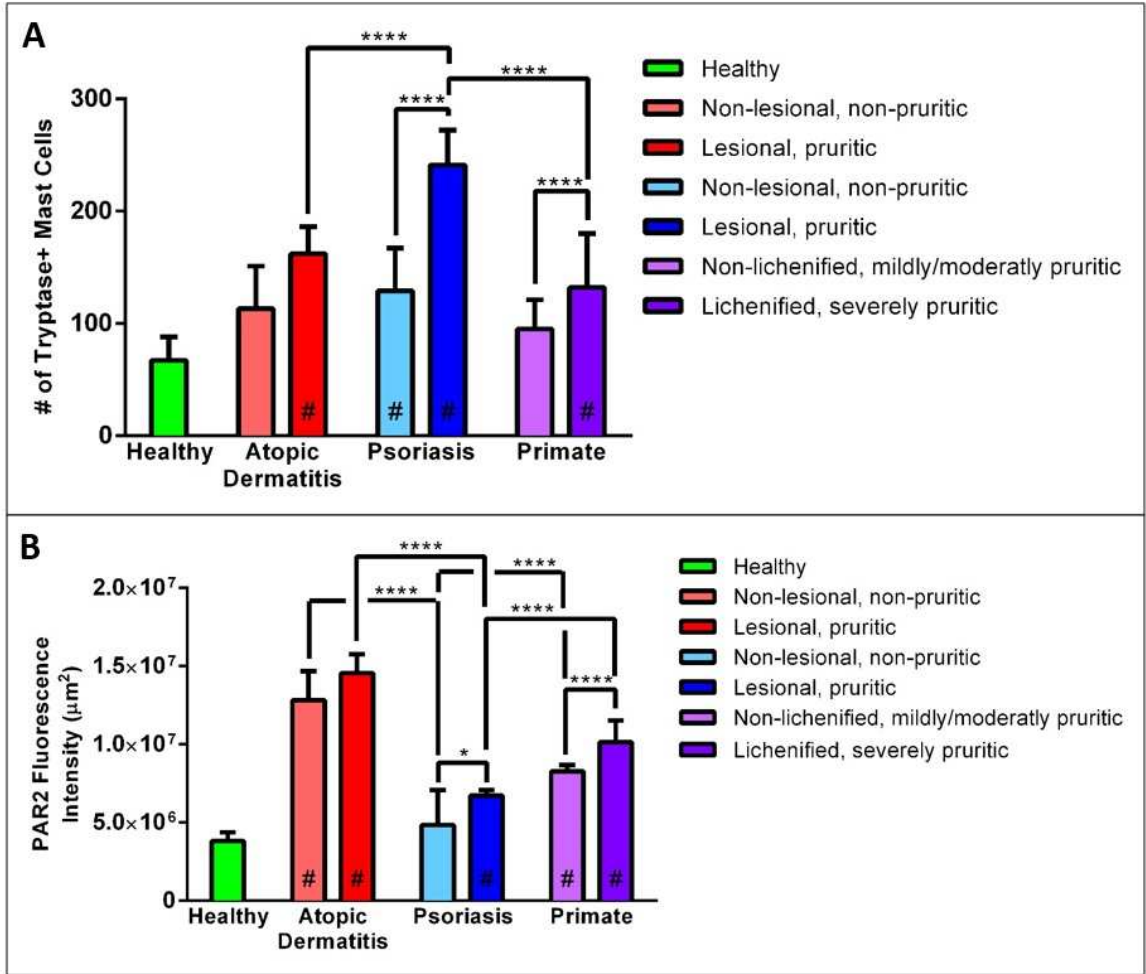


Figure 3.5. Tryptase and PAR2 were elevated in lichenified skin of primates and lesional skin of patients with AD and PS. Tryptase+ mast cells (A) were highly expressed in the papillary dermis of lesional and lichenified skin. The PAR2 receptor (B) was increased within the epidermis of AD, PS, and primate skin (* $p < 0.05$, **** $p < 0.0001$). #; significant difference when compared to healthy skin, $p < 0.05$.

SP transcripts were increased over 7 fold in lichenified primate skin.

Immunohistochemical analysis showed that SP+ nerve fibers were significantly increased ($p < 0.0001$) in the lichenified skin (13.2 ± 4.3) of primates when compared to non-lichenified skin (6.4 ± 1.7) (Figure 3.6A). Additionally, SP+ nerve fibers were increased in lesional skin of AD (7.8 ± 1.8) and PS (8.6 ± 2.7). This increase of SP was usually seen in nerve fibers in close proximity to the dermal-epidermal junction. Moreover, the receptor NK-1R was overexpressed ($p < 0.0001$) within the epidermis of all lesional skin (AD: 6030723 ± 430722 ; PS: 9100092 ± 739480 ; primate: 9520411 ± 732769) when compared to healthy skin (30723 ± 307242) (Figure 3.6B). Non-lesional PS skin (4150718 ± 914518) had similar NK-1R levels compared to healthy controls.

Another neuropeptide that the RNA-seq showed to be upregulated was ET-1, along with its receptor ETA (Figure 3.7A and B). ET-1 was significantly elevated ($p < 0.0001$) in the epidermis of primate skin (lichenified: 536727 ± 81365 ; non-lichenified: 394496 ± 46283) and lesional AD (484552 ± 93492) and lesional PS skin (389067 ± 129025). ET-1 expression was not increased in non-lesional AD (236692 ± 39492) and non-lesional PS skin (214658 ± 34850). ETA was only significantly elevated ($p < 0.0001$) in lichenified primate skin (4720379 ± 1690428) and lesional AD skin (4150718 ± 14518).

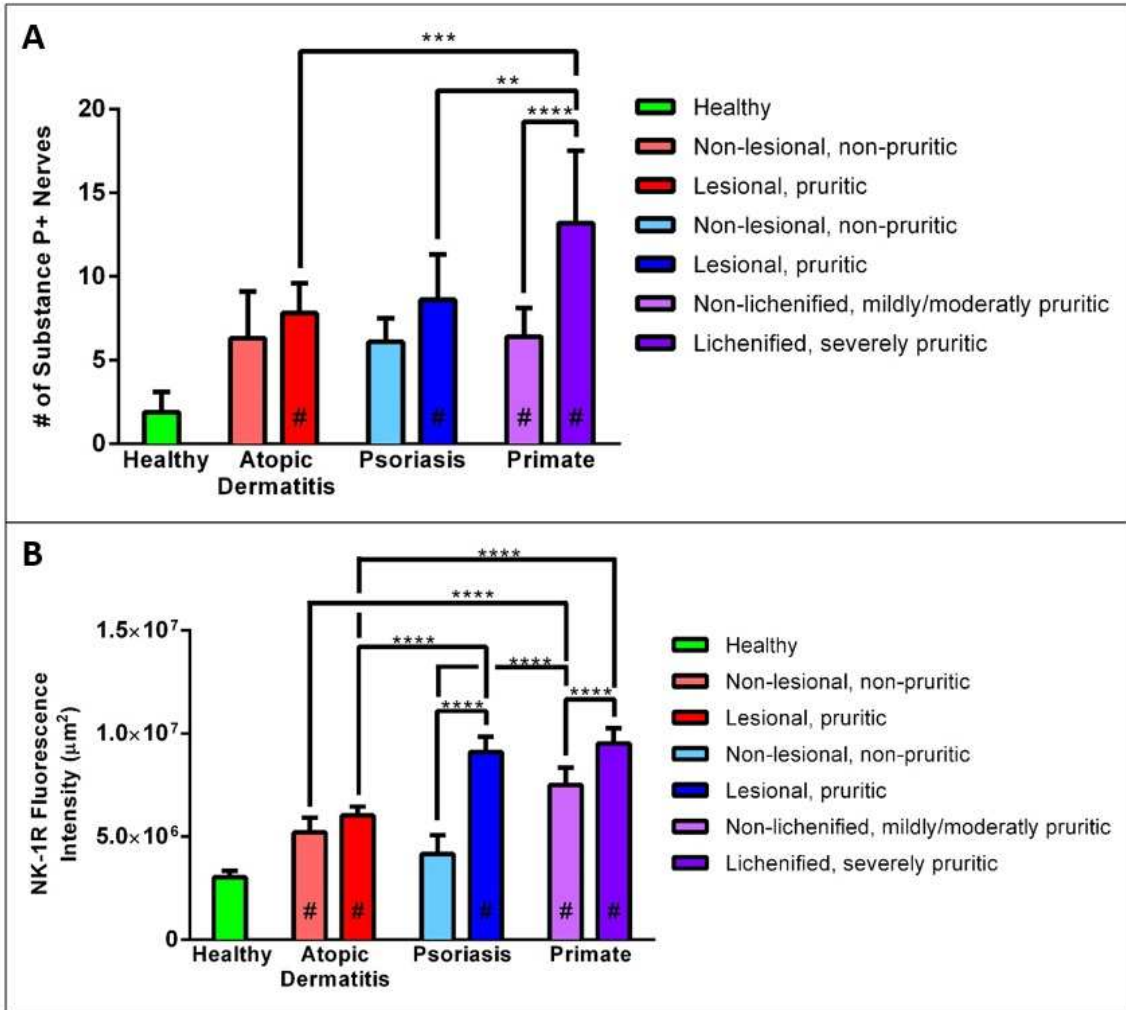


Figure 3.6. Substance P and its receptor NK-1R were elevated in primate skin and lesional skin of patients with AD and PS. SP+ nerves (A), epidermal NK-1R (B), were increased in lichenified primate, and lesional AD and PS skin (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). #; significant difference when compared to healthy skin, $p < 0.01$.

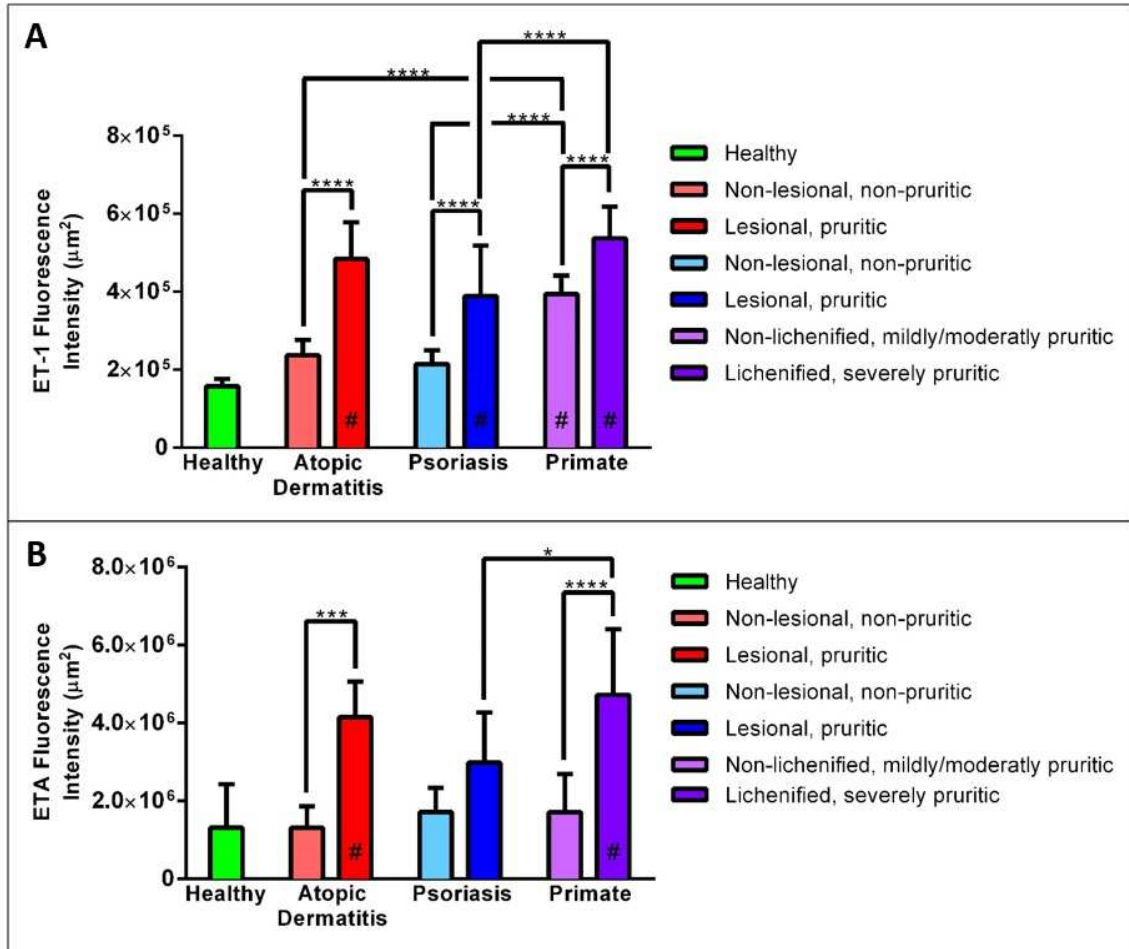


Figure 3.7. Endothelin-1 and its receptor ETA were elevated in lichenified skin of primates and lesional skin of patients with AD and PS. ET-1 (A) was increased in primate skin, and lesional AD and PS skin, while ETA (B) was only elevated in lichenified primate and lesional AD skin (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).. #; significant difference when compared to healthy skin, $p < 0.001$.

Conclusions

Inspired by gene expression findings in a pruritic dry skin mouse model (Wilson et al, 2013), we performed RNA-seq on a colony of *Cynomolgus* macaques with idiopathic chronic itch. As part of the non-human primate reference transcriptome resource (NHPRTR) study, *Cynomolgus* macaques have undergone deep RNA-seq to develop references for gene expression profiling (Peng et al, 2015). To date, twelve tissue types have been sequenced, with the pooled sequences aligning to 93% of the human reference sequences. Our study is the first study to sequence RNA transcripts in *Cynomolgus* skin. Furthermore, this is the first RNA-seq study to correlate gene expression to scratching behavior. This correlation allowed for examination of genes specifically involved in itch signaling.

We have defined an RNA-seq transcriptome for active, chronic pruritus in primates by comparing gene expression differences in lichenified versus non-lichenified skin. We found that a transcript for phospholipase A2 had the highest FC. This gene was previously found to be upregulated in psoriatic skin and leads to the production of arachidonic acid after histamine receptor 1 (H₁) activation of TRPV1 (Leurs et al, 1994; Shim et al, 2007). Indeed, both H₁ and TRPV1 transcripts were also found to be increased in lichenified primate skin. We also showed an increase in expression of TRPA1 in the keratinocytes of our primate model and subjects with AD. This channel has been implicated in non-histaminergic itch transmission (Wilson et al, 2013).

Additionally, our sequencing showed that TRPV3 was overexpressed by five fold in lichenified primate skin. Recently, this channel was shown to be involved in itch processing and was increased in the epidermis of pruritic burn scars (Steinhoff and Bíró,

2009; Yang et al, 2015). Furthermore, the RNA-seq study by Li et al revealed that TRPV3 was the most elevated TRP channel in psoriatic skin (2014). A gain-of-function mutation in this gene causes the development of atopic-like skin lesions in mice and the pruritic Olmsted syndrome in humans (Lin et al, 2012; Yoshioka et al, 2009).

PAR2, another receptor involved in non-histaminergic itch transmission, was upregulated in primate skin (FC 4.71), but was previously found not to be differentially expressed in psoriatic skin (Li et al, 2014). We showed epidermal expression of PAR2 was increased in primate, AD, and PS skin, while its ligand tryptase was overexpressed in lichenified primate skin and lesional AD and PS skin, with the highest expression patterns seen in the upper dermis, in close contact with the epidermis. Similarly, SP+ nerves were found to be increased at the dermal-epidermal junction of lichenified and lesional skin, while the NK-1R was found to be expressed throughout the epidermis of all skin types. Another neuropeptide, ET-1 was also found to be elevated in the epidermis of lichenified and lesional skin. However, its receptor ETA was only increased in lichenified primate skin and lesional AD skin.

Interestingly, this study and the previous RNA-seq studies of AD and PS skin revealed a common set of S100 calcium binding proteins that were upregulated in pruritic skin conditions (Li et al, 2014; Suárez-Fariñas et al, 2015). S100A7, or psoriasin, which is released by keratinocytes during wound healing, was increased in lichenified primate skin (FC 3.29) as it was in lesional AD (FC 3.91) and PS skin (FC 247.11). Similarly, S100A9 and S100A12, both molecules with pro-inflammatory properties, were increased in all three groups. Previous studies have found increased expression of these S100 proteins in AD and PS skin and serum, suggesting they play a role in dermatosis

susceptibility (Aochi et al, 2011; Foell et al. 2003; Jin et al, 2014; Mimohammadsedegh et al, 2000; Semprini et al, 2002).

The sequencing also revealed the involvement of several cytokines and chemokines in pruritic primate skin. Pro-inflammatory cytokines IL-1, IL-6, IL-17, IL-23, and IL-36 were upregulated in the primate model, similar to their elevation in lesional AD and PS skin (Li et al, 2014; Suárez-Fariñas et al, 2015). IL-10, a cytokine found to be involved in skin inflammation of AD, PS, and CTCL, was also increased in primate skin. Chemokines (CCL7, CCL25, CCL26, CXCL3, CXCL5, CXCL6) that are responsible for the recruitment of immune cells, including T-cells, monocytes, macrophages, eosinophils, and neutrophils, had increased FCs in lichenified primate skin. These mediators have also been reported to be involved in inflammation processes in the skin of AD, PS, and CTCL (Li et al, 2014; Hon et al, 2011; Suga et al, 2013).

Only 44 DEGs were found to be downregulated in pruritic primate skin. Of note, the gene corresponding to the precursor of dynorphin A, with an FC of 0.06, was one of the most downregulated transcripts. The receptor for dynorphin A, κ -opioid receptor (KOR), was also significantly reduced in lichenified primate skin. KOR was previously shown to be decreased in AD skin (Tominaga et al, 2007). However, the precursor protein for β -endorphin and the μ -opioid receptor (MOR) were both significantly upregulated, with MOR having an FC of 7.11. This imbalance of κ - and μ -opioids may play a significant role in the propagation of chronic itch (Cowan et al, 2015).

Our RNA-seq analysis and immunohistochemical validation provide valuable insight into the pathomechanisms of chronic pruritus. Our primate model displayed differential gene expression in lichenified versus non-lichenified skin, and many of these

genes correlated to the intensity of the primates' scratching behavior. While the idiopathic chronic itch of the primate model was not clinically identical to either AD or PS, the primates and human subjects shared altered expression of several itch-related mediators. These results suggest that *Cynomolgus* macaques are an ideal model system for the study of itch due to their similar molecular profile to human pruritic disease. Future studies could further examine itch-associated transcripts that are not involved in the itch-scratch cycle by comparing paired lichenified and non-lichenified skin from the severely scratching primates. Further studies should also continue this comparison in other tissues, such as DRG, spinal cord, and brain, to identify targets for treatment.

Table 3.1. Differentially expressed genes in the skin of primates with chronic itch.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
Transient Receptor Potential Receptors				
TRPA1	Transient receptor potential cation channel A1	1.21	0.58	2.09
LOC102139829	Transient receptor potential cation channel A1-like	1.07	0.77	1.39
TRPC1	Transient receptor potential cation channel C1	0.23	0.41	0.56
TRPC3	Transient receptor potential cation channel C3	0.02	0.06	0.33
TRPC4	Transient receptor potential cation channel C4	0.03	0.07	0.43
TRPC5	Transient receptor potential cation channel C5	0.01	0.04	0.25
TRPC6	Transient receptor potential cation channel C6	0.82	1.00	0.82
TRPC7	Transient receptor potential cation channel C7	0.01	0.01	1.00
TRPM1	Transient receptor potential cation channel M1	6.82	3.28	2.08
TRPM2	Transient receptor potential cation channel M2	0.45	0.24	1.88
TRPM3	Transient receptor potential cation channel M3	0.08	0.05	1.60
TRPM4	Transient receptor potential cation channel M4	1.56	1.74	0.90
TRPM5	Transient receptor potential cation channel M5	0.26	0.18	1.44
TRPM6	Transient receptor potential cation channel M6	0.38	0.84	0.45
TRPM7	Transient receptor potential cation channel M7	1.42	1.01	1.41

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
TRPV1	Transient receptor potential cation channel V1	4.81	0.98	4.91
TRPV2	Transient receptor potential cation channel V2	2.82	2.34	1.21
TRPV3	Transient receptor potential cation channel V3	1.02	0.18	5.67
TRPV4	Transient receptor potential cation channel V4	0.57	2.25	0.25
TRPV5	Transient receptor potential cation channel V5	1.10	1.84	0.60
TRPV6	Transient receptor potential cation channel V6	1.24	1.57	0.79
PIRT	Phosphoinositide-interacting regulator of TRPs	2.76	0.87	3.17
Histamine				
HRH1	Histamine receptor 1	3.84	2.15	1.79
HRH2	Histamine receptor 2	1.65	0.98	1.68
HRH3	Histamine receptor 3	0.95	0.34	2.79
HRH4	Histamine receptor 4	0.16	0.08	2.00
HDC	Histamine decarboxylase	6.18	2.98	2.07
HNMT	Histamine N-methyltransferase	8.98	3.51	2.56
Proteases				
TPSAB1	Tryptase α/β -1	4.88	1.15	4.24

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
TPSB2	Tryptase β -2	17.54	6.88	2.55
TPSG1	Tryptase γ -1	0.25	0.11	2.27
GZMK	Granzyme K	1.57	0.92	1.71
CTSB	Cathepsin B	0.99	0.47	2.11
CTSC	Cathepsin C	15.47	6.17	2.51
CTSD	Cathepsin D	1.26	1.58	0.80
CTSE	Cathepsin E	0.09	0.18	0.50
CTSF	Cathepsin F	0.67	0.38	1.76
CTSG	Cathepsin G	10.69	1.84	2.78
CTSH	Cathepsin H	0.25	0.15	1.67
CTSK	Cathepsin K	1.69	1.55	1.09
CTSL	Cathepsin L	0.28	0.34	0.82
CTSO	Cathepsin O	0.14	0.08	1.75
CTSS	Cathepsin S	28.23	5.10	5.54
CTSV	Cathepsin V	0.84	0.62	1.35
CTSW	Cathepsin W	1.49	1.08	1.38

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CTSZ	Cathepsin Z	1.36	0.59	2.31
ELANE	Neutrophilic elastase	9.14	11.36	2.57
CELA1	Chymotrypsin-like elastase family, member 1	0.48	0.36	1.33
CELA2A	Chymotrypsin-like elastase family, member 2A	0.64	0.55	1.16
CELA2B	Chymotrypsin-like elastase family, member 2B	0.35	0.46	0.76
CELA3A	Chymotrypsin-like elastase family, member 3A	0.74	0.94	0.79
CELA3B	Chymotrypsin-like elastase family, member 3B	0.94	0.71	1.32
MMP12	Matrix metalloproteinase 12	1.47	0.62	3.98
LOC101865786	Leukocyte elastase inhibitor; SerpinB1	0.49	0.65	0.75
KLK1	Kallikrein-related peptide 1	2.28	3.58	0.63
KLK2	Kallikrein-related peptide 2	0.84	0.59	1.42
KLK3	Kallikrein-related peptide 3	0.26	0.48	0.54
KLK4	Kallikrein-related peptide 4	0.24	0.13	1.85
KLK5	Kallikrein-related peptide 5	20.14	3.87	5.20
KLK6	Kallikrein-related peptide 6	3.59	3.21	1.12
LOC102134711	Kallikrein-related peptide 7-like	7.78	3.18	2.47

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
KLK8	Kallikrein-related peptide 8	3.99	2.40	1.66
KLK9	Kallikrein-related peptide 9	4.94	2.66	1.86
KLK10	Kallikrein-related peptide 10	1.01	0.57	1.77
KLK11	Kallikrein-related peptide 11	3.87	2.89	1.34
KLK12	Kallikrein-related peptide 12	0.48	0.38	1.26
KLK13	Kallikrein-related peptide 13	6.44	4.41	1.46
KLK14	Kallikrein-related peptide 14	4.56	1.48	3.08
KLK15	Kallikrein-related peptide 15	0.84	0.64	1.31
F2R	Protease-activated receptor 1	9.26	4.20	2.20
F2RL1	Protease-activated receptor 2	34.18	7.26	4.71
F2RL2	Protease-activated receptor 3	0.26	0.40	0.65
F2RL3	Protease-activated receptor 4	1.59	0.84	1.89
BDKRB2	Bradykinin receptor 2	5.15	3.85	1.34
KNG1	Kininogen 1	0.48	0.56	0.86
Mas-related G-Protein Receptors				
MRGPRX1	Mas-related G protein-coupled receptor X1	2.51	1.25	2.01

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
MGRPRX2	Mas-related G protein-coupled receptor X2	3.21	0.97	3.31
MRGPRX3	Mas-related G protein-coupled receptor X3	0.82	0.92	0.89
MRGPRD	Mas-related G protein-coupled receptor D	0.52	0.42	1.24
MRGPRE	Mas-related G protein-coupled receptor E	0.72	0.61	1.18
MRGPRG	Mas-related G protein-coupled receptor G	0.09	0.07	1.29
Sodium Channels				
SCN2A	Voltage-gated sodium channel II α ; Na _v 1.2	0.38	0.20	1.90
SCN3A	Voltage-gated sodium channel III α ; Na _v 1.3	0.69	0.21	3.29
SCN4A	Voltage-gated sodium channel IV α ; Na _v 1.4	0.02	0.02	1.00
SCN5A	Voltage-gated sodium channel V α ; Na _v 1.5	0.02	0.03	0.67
SCN7A	Voltage-gated sodium channel VII α ; Na _v x	5.82	3.48	1.67
SCN8A	Voltage-gated sodium channel VIII α ; Na _v 1.6	0.01	0.03	0.33
SCN9A	Voltage-gated sodium channel IX α ; Na _v 1.7	0.24	0.07	3.43
SCN10A	Voltage-gated sodium channel X α ; Na _v 1.8	0.04	0.06	0.66
SCN11A	Voltage-gated sodium channel XI α ; Na _v 1.9	0.14	0.06	2.33
SCN1B	Voltage-gated sodium channel I β ; Na _v β 1	0.01	0.08	0.13

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
SCN2B	Voltage-gated sodium channel II β ; Na ν β 2	0.21	0.56	0.38
SCN3B	Voltage-gated sodium channel III β ; Na ν β 3	0.53	0.34	1.56
SCN4B	Voltage-gated sodium channel IV β ; Na ν β 4	0.04	0.12	0.33
Neuropeptides				
TAC1	Tachykinin precursor 1; neurokinin A/substance P	26.84	3.41	7.87
TAC3	Tachykinin precursor 3; neurokinin B	0.51	0.85	0.60
TACR1	Tachykinin receptor 1; NK-1R	6.81	1.51	4.51
TACR2	Tachykinin receptor 2; NK-2	0.06	0.04	1.50
TACR3	Tachykinin receptor 3; NK-3	0.41	0.14	2.93
CALCA	Calcitonin gene-related peptide α	3.52	2.11	1.67
CALCB	Calcitonin gene-related peptide β	4.26	3.84	1.11
SST	Somatostatin	1.28	0.55	2.33
SSTR1	Somatostatin receptor 1	0.10	0.13	0.77
SSTR2	Somatostatin receptor 2	0.05	0.08	0.63
SSTR3	Somatostatin receptor 3	0.16	0.22	0.73
SSTR4	Somatostatin receptor 4	0.08	0.21	0.38

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
SSTR5	Somatostatin receptor 5	0.01	0.01	1.00
NTS	Neurotensin	1.24	1.02	1.22
NTSR1	Neurotensin receptor 1	0.61	0.54	1.13
NTSR2	Neurotensin receptor 2	0.26	0.15	1.73
VIP	Vasoactive intestinal polypeptide	0.09	0.04	2.25
GRPR	Gastrin-releasing peptide receptor	0.00	0.00	0.00
GRP	Gastrin-releasing peptide	0.00	0.00	0.00
EDN1	Endothelin 1	8.14	2.04	3.99
EDN2	Endothelin 2	0.84	0.77	1.09
EDN3	Endothelin 3	1.05	0.97	1.08
EDNRA	Endothelin receptor A	6.25	1.19	5.25
ECEL1	Endothelin-converting enzyme 1	0.48	0.17	2.82
ACHE	Acetylcholinesterase	0.15	0.08	1.88
CHAT	Choline o-acetyltransferase	0.08	0.02	4.00
CHRM1	Cholinergic receptor muscarinic 1	1.03	2.51	0.41
CHRM2	Cholinergic receptor muscarinic 2	0.04	0.14	0.29

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CHRM3	Cholinergic receptor muscarinic 3	1.95	0.65	3.00
CHRM4	Cholinergic receptor muscarinic 4	0.11	0.85	0.13
CHRM5	Cholinergic receptor muscarinic 5	0.05	0.07	0.71
CHRA1	Cholinergic receptor nicotinic α 1	0.01	0.03	0.33
CHRNA2	Cholinergic receptor nicotinic α 2	0.02	0.04	0.50
CHRNA3	Cholinergic receptor nicotinic α 3	0.85	0.41	2.07
CHRNA4	Cholinergic receptor nicotinic α 4	0.01	0.01	1.00
CHRNA5	Cholinergic receptor nicotinic α 5	0.31	0.24	1.29
CHRA6	Cholinergic receptor nicotinic α 6	0.02	0.07	0.29
CHRA7	Cholinergic receptor nicotinic α 7	0.34	0.57	0.60
CHRA9	Cholinergic receptor nicotinic α 9	0.29	0.16	1.81
CHRNA10	Cholinergic receptor nicotinic α 10	0.01	0.02	0.50
CHRB1	Cholinergic receptor nicotinic β 1	0.08	0.67	0.09
CHRN3	Cholinergic receptor nicotinic β 3	0.26	0.09	2.89
CHRN4	Cholinergic receptor nicotinic β 4	0.07	0.04	1.75
CHRND	Cholinergic receptor nicotinic δ	0.07	0.04	1.75

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CHRNG	Cholinergic receptor nicotinic γ	0.02	0.03	0.67
TPH1	Tryptophan hydroxylase 1	0.26	0.08	3.25
TPH2	Tryptophan hydroxylase 2	0.18	0.16	1.13
DDC	Dopa decarboxylase	0.56	0.81	0.69
HTR1A	Serotonin receptor 1A	1.04	0.94	1.11
HTR1B	Serotonin receptor 1B	1.23	0.65	1.89
HTR1D	Serotonin receptor 1D	0.14	0.26	0.54
HTR1E	Serotonin receptor 1E	0.07	0.40	0.18
HTR1F	Serotonin receptor 1F	0.09	0.15	0.60
HTR2B	Serotonin receptor 2B	0.50	0.26	1.92
HTR2C	Serotonin receptor 2C	0.13	0.08	1.63
HTR3A	Serotonin receptor 3A	0.34	0.37	0.92
HTR3B	Serotonin receptor 3B	0.01	0.04	0.25
HTR3C	Serotonin receptor 3C	0.26	0.13	2.00
HTR3E	Serotonin receptor 3E	0.68	0.31	2.19
HTR4	Serotonin receptor 4	0.01	0.03	0.33

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
HTR5A	Serotonin receptor 5A	0.15	0.11	1.36
HTR6	Serotonin receptor 6	0.01	0.02	0.50
HTR7	Serotonin receptor 7	0.22	0.08	2.75
LOC102142547	S100 calcium binding protein A1-like	3.64	4.08	0.89
S100A2	S100 calcium binding protein A2	4.21	1.57	2.68
S100A3	S100 calcium binding protein A3	1.88	2.07	0.91
S100A4	S100 calcium binding protein A4	2.32	2.11	1.10
S100A5	S100 calcium binding protein A5	1.05	1.25	0.84
S100A6	S100 calcium binding protein A6	3.94	4.21	0.94
S100A7	S100 calcium binding protein A7	14.04	4.27	3.29
S100A8	S100 calcium binding protein A8	2.19	2.52	0.97
S100A9	S100 calcium binding protein A9	27.82	4.11	6.77
S100A10	S100 calcium binding protein A10	15.21	14.07	1.08
S100A12	S100 calcium binding protein A12	14.19	2.15	6.60
S100A13	S100 calcium binding protein A13	2.82	3.18	0.89
S100A14	S100 calcium binding protein A14	6.80	3.94	1.73

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
LOC102120158	S100 calcium binding protein A15 like	1.25	0.41	3.05
S100A16	S100 calcium binding protein A16	0.82	0.44	1.86
S100G	S100 calcium binding protein G	0.31	0.11	2.82
S100P	S100 calcium binding protein P	2.62	1.27	2.06
S100Z	S100 calcium binding protein Z	0.84	0.28	3.00
FLG	Filaggrin	5.15	8.20	0.63
NPPA	Natriuretic peptide A; NPA	0.24	0.18	1.33
NPPB	Natriuretic peptide B; NPB	0.85	0.60	1.42
NPPC	Natriuretic peptide C; NPC	0.01	0.02	0.50
NPR1	Natriuretic peptide receptor A	1.18	1.18	1.00
NPR2	Natriuretic peptide receptor B	2.85	6.15	0.46
NPR3	Natriuretic peptide receptor C	3.19	5.48	0.58
SLC17A6	Vesicular glutamate transporter 2; VGLUT2	9.29	2.15	4.32
SLC17A8	Vesicular glutamate transporter 3; VGLUT3	1.24	1.51	0.82
Neurotrophins				
NGF	Nerve growth factor	0.48	0.36	1.33

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
NTF3	Neurotrophin 3	0.24	0.09	2.67
NTF4	Neurotrophin 4	2.47	1.13	2.19
BDNF	Brain-derived neurotrophic factor	0.84	0.55	1.53
NTRK1	Tyrosine receptor kinase type 1, TrkA	1.06	0.31	3.42
NTRK2	Tyrosine receptor kinase type 2, TrkB	1.55	0.97	1.60
NTRK3	Tyrosine receptor kinase type 3, TrkC	0.59	0.24	2.46
NGFR	NGF receptor; p75	2.34	3.14	0.75
SEMA3A	Semaphorin-3A	0.58	0.17	3.41
Cytokines and Chemokines				
IL1B	Interleukin-1 β	2.04	1.52	1.34
IL1RN	Interleukin-1 receptor	9.20	3.18	2.89
IL1R1	Interleukin-1 receptor type 1	8.18	5.92	1.38
IL1R2	Interleukin-1 receptor type 2	5.25	6.14	0.86
IL1RL1	Interleukin-1 receptor-like 1	0.01	0.01	1.00
IL1RL2	Interleukin-1 receptor-like 2	0.02	0.01	2.00
IL2	Interleukin-2	0.54	0.34	1.59

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
IL2RA	Interleukin-2 receptor α	0.48	0.12	4.00
IL2RB	Interleukin-2 receptor β	1.07	0.87	1.23
IL2RG	Interleukin-2 receptor γ	0.18	0.04	4.50
IL3	Interleukin-3	0.78	1.04	0.75
LOC12138639	Interleukin-3 receptor α	0.45	1.85	0.24
IL4I1	Interleukin-4 induced 1	2.57	1.88	1.37
IL4R	Interleukin-4 receptor	4.86	1.67	2.91
IL5	Interleukin-5	0.24	0.12	2.00
IL5RA	Interleukin-5 receptor α	0.18	0.14	1.29
IL6	Interleukin-6	1.30	0.38	3.42
IL6R	Interleukin-6 receptor	0.69	0.22	3.14
IL7	Interleukin-7	1.85	1.52	1.22
IL7R	Interleukin-7 receptor	1.20	1.25	0.96
IL-8	Interleukin-8	8.48	3.99	2.13
IL9	Interleukin-9	0.15	0.04	3.75
IL9R	Interleukin-9 receptor	0.30	0.12	2.50

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
IL10	Interleukin-10	2.22	0.42	5.29
IL10RA	Interleukin-10 receptor α	6.72	1.89	3.56
IL10RB	Interleukin-10 receptor β	5.03	2.58	1.95
LOC102128313	Interleukin-11-like	0.47	0.37	1.27
IL11RA	Interleukin-11 receptor α	0.87	0.74	1.18
IL12A	Interleukin-12A	0.40	0.15	2.67
IL12B	Interleukin-12B	0.04	0.09	0.44
IL12RB1	Interleukin-12 receptor β 1	1.52	0.97	1.57
IL12RB2	Interleukin-12 receptor β 2	14.27	5.18	2.75
IL13	Interleukin-13	0.87	0.91	0.96
IL13RA1	Interleukin-13 receptor α 1	3.14	1.81	1.73
IL13RA2	Interleukin-13 receptor α 2	1.58	1.14	1.39
IL15	Interleukin-15	1.67	1.02	1.64
IL15RA	Interleukin-15 receptor α	1.90	1.22	1.56
IL16	Interleukin-16	2.57	4.84	0.53
IL17A	Interleukin-17A	5.67	1.48	3.83

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
IL17B	Interleukin-17B	1.45	1.27	1.14
IL17C	Interleukin-17C	3.24	1.64	1.98
IL17F	Interleukin-17F	4.81	1.24	3.88
IL17RB	Interleukin-17 receptor B	0.61	0.27	2.26
IL17RC	Interleukin-17 receptor C	1.67	1.01	1.65
IL17RD	Interleukin-17 receptor D	0.74	0.48	1.54
IL17RE	Interleukin-17 receptor E	0.33	0.47	0.70
IL17REL	Interleukin-17 receptor E-like	0.87	0.98	0.89
IL18	Interleukin-18	0.69	3.84	0.18
IL18R1	Interleukin-18 receptor 1	1.08	1.67	0.65
IL19	Interleukin-19	0.86	0.72	1.19
IL20	Interleukin-20	0.11	0.07	1.57
IL20RA	Interleukin-20 receptor α	0.07	0.04	1.75
IL20RB	Interleukin-20 receptor β	0.19	0.16	1.19
IL21	Interleukin-21	0.65	0.84	0.77
IL21R	Interleukin-21 receptor	0.08	0.11	0.73

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
IL22	Interleukin-22	3.27	1.90	1.72
IL22RA1	Interleukin-22 receptor α 1	2.04	0.82	2.49
IL22RA2	Interleukin-22 receptor α 2	1.13	0.67	1.69
IL23A	Interleukin-23A	0.28	0.08	3.50
IL23B	Interleukin-23B	0.47	0.15	3.13
IL23R	Interleukin-23 receptor	0.17	0.08	2.13
IL24	Interleukin-24	1.04	1.28	0.81
IL25	Interleukin-25	1.81	1.56	1.16
IL26	Interleukin-26	2.40	1.92	1.25
IL27	Interleukin-27	1.24	1.92	0.65
IL27RA	Interleukin-27 receptor α	1.47	1.66	0.89
IL31	Interleukin-31	0.18	0.09	2.00
IL31RA	Interleukin-31 receptor α	0.08	0.04	2.00
IL33	Interleukin-33	1.25	1.07	1.17
IL34	Interleukin-34	0.21	0.64	0.33
IL36A	Interleukin-36 α	1.54	0.45	3.42

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
IL36G	Interleukin-36 γ	15.15	3.75	4.04
IL36RN	Interleukin-36 receptor	3.27	0.97	3.37
IL37	Interleukin-37	1.21	0.87	1.39
XCL1	Chemokine (C motif) ligand 1	1.27	2.14	0.59
XCR1	Chemokine (C motif) receptor 1	1.99	1.58	1.26
CCL1	Chemokine (C-C motif) ligand 1	1.84	0.58	3.17
CCL2	Chemokine (C-C motif) ligand 2	17.28	6.40	2.70
CCL3	Chemokine (C-C motif) ligand 3	1.12	0.92	1.22
CCL4	Chemokine (C-C motif) ligand 4	2.38	1.94	1.23
CCL7	Chemokine (C-C motif) ligand 7	0.27	0.06	4.50
CCL11	Chemokine (C-C motif) ligand 11	0.85	0.67	1.27
CCL13	Chemokine (C-C motif) ligand 13	6.71	5.17	1.30
CCL14	Chemokine (C-C motif) ligand 14	15.25	21.20	0.72
CCL15	Chemokine (C-C motif) ligand 15	1.25	2.15	0.58
CCL16	Chemokine (C-C motif) ligand 16	0.65	0.84	0.77
CCL17	Chemokine (C-C motif) ligand 17	1.28	0.47	2.72

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CCL18	Chemokine (C-C motif) ligand 18	2.68	0.61	4.39
CCL19	Chemokine (C-C motif) ligand 19	29.57	17.27	1.71
CCL20	Chemokine (C-C motif) ligand 20	2.27	1.58	1.44
CCL21	Chemokine (C-C motif) ligand 21	11.47	15.24	0.75
CCL22	Chemokine (C-C motif) ligand 22	5.81	4.12	1.41
CCL23	Chemokine (C-C motif) ligand 23	0.58	0.91	0.64
CCL24	Chemokine (C-C motif) ligand 24	0.94	1.05	0.90
CCL25	Chemokine (C-C motif) ligand 25	0.04	0.01	4.00
CCL26	Chemokine (C-C motif) ligand 26	0.87	0.28	3.11
CCL27	Chemokine (C-C motif) ligand 27	3.59	1.65	2.18
CCL28	Chemokine (C-C motif) ligand 28	2.28	3.59	0.64
CCR1	Chemokine (C-C motif) receptor 1	2.65	2.87	0.92
CCR2	Chemokine (C-C motif) receptor 2	1.64	1.47	1.12
CCR3	Chemokine (C-C motif) receptor 3	0.99	1.25	0.79
CCR4	Chemokine (C-C motif) receptor 4	1.25	0.95	1.32
CCR5	Chemokine (C-C motif) receptor 5	1.18	1.38	0.86

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CCR6	Chemokine (C-C motif) receptor 6	0.28	0.92	0.30
CCR7	Chemokine (C-C motif) receptor 7	3.14	4.68	0.67
CCR8	Chemokine (C-C motif) receptor 8	2.18	1.81	1.20
CCR9	Chemokine (C-C motif) receptor 9	1.62	1.80	0.90
CCR10	Chemokine (C-C motif) receptor 10	1.68	1.05	1.60
CXCL1	Chemokine (C-X-C motif) ligand 1	5.40	2.38	2.27
CXCL2	Chemokine (C-X-C motif) ligand 2	1.27	1.55	0.82
CXCL3	Chemokine (C-X-C motif) ligand 3	0.54	0.09	6.00
CXCL5	Chemokine (C-X-C motif) ligand 5	0.04	0.01	4.00
CXCL6	Chemokine (C-X-C motif) ligand 6	0.54	0.15	3.60
CXCL9	Chemokine (C-X-C motif) ligand 9	3.48	1.02	3.40
CXCL10	Chemokine (C-X-C motif) ligand 10	4.98	2.16	2.30
CXCL11	Chemokine (C-X-C motif) ligand 11	1.22	0.87	1.40
CXCL12	Chemokine (C-X-C motif) ligand 12	4.91	15.05	0.33
CXCL13	Chemokine (C-X-C motif) ligand 13	2.51	1.25	2.01
CXCL16	Chemokine (C-X-C motif) ligand 16	9.28	6.17	1.50

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
CXCL17	Chemokine (C-X-C motif) ligand 17	1.58	1.82	0.87
CXCR1	Chemokine (C-X-C motif) receptor 1	1.57	1.05	1.50
CXCR2	Chemokine (C-X-C motif) receptor 2	1.33	0.98	1.36
CXCR3	Chemokine (C-X-C motif) receptor 3	0.84	0.54	1.56
CXCR4	Chemokine (C-X-C motif) receptor 4	0.59	0.31	1.90
CXCR5	Chemokine (C-X-C motif) receptor 5	1.57	1.11	1.41
CXCR6	Chemokine (C-X-C motif) receptor 6	1.46	0.95	1.54
CX3CL1	Chemokine (C-X3-C motif) ligand 1	5.16	6.67	0.77
OSM	Oncostatin M	1.57	0.97	1.62
OSMR	Oncostatin M receptor	7.85	4.10	1.91
TSLP	Thymic stromal lymphopoietin	0.44	0.27	1.63
TNF	Tumor necrosis factor	1.88	1.04	1.81
INFA1	Interferon α 1	2.54	1.52	1.67
INFA2	Interferon α 2	1.20	1.25	0.96
INFA4	Interferon α 4	1.44	1.87	0.77
INFA6	Interferon α 6	0.97	0.84	1.15

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
INFA8	Interferon α 8	1.54	1.36	1.13
INFA13	Interferon α 13	1.48	2.28	0.65
INFA14	Interferon α 14	0.67	1.05	0.64
LOC102147079	Interferon α 17-like	1.14	1.82	0.63
INFA21	Interferon α 21	1.57	1.67	0.94
INFB1	Interferon β 1	1.57	1.22	1.29
IFNW1	Interferon ω 1	1.36	1.68	0.81
INFG	Interferon γ	15.21	5.18	2.94
INFK	Interferon κ	1.84	1.44	1.28
INKE	Interferon ϵ	1.25	1.87	0.67
INFL1	Interferon λ 1	0.57	0.81	0.70
INFL2	Interferon λ 2	0.03	0.04	0.75
INFL3	Interferon λ 3	0.56	0.78	0.72
INFL4	Interferon λ 4	1.25	1.55	0.81
INFLR1	Interferon λ receptor 1	0.97	1.05	0.92
IFNAR2	Interferon α receptor 2	0.64	0.41	1.56

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
Opioids				
PENK	Proenkephalin	0.58	0.44	1.22
POMC	Proopiomelanocortin	6.15	1.87	3.29
PDYN	Prodynorphin	0.27	4.42	0.06
OPRM1	μ -opioid receptor	1.28	0.18	7.11
OPRK1	K-opioid receptor	0.09	0.55	0.16
OPRD1	δ -opioid receptor	0.06	0.04	1.50
OPCML	Opioid binding protein/cell adhesion	0.08	0.06	1.33
Eicosanoids				
PTGS1	Prostaglandin-endoperoxide synthase 1 (COX1)	21.84	16.11	1.36
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX2)	2.18	1.54	1.42
PTGES	Prostaglandin E synthase	11.27	9.45	1.19
PTGES2	Prostaglandin E synthase 2	7.19	5.44	1.32
PTGES3	Prostaglandin E synthase 3	3.89	4.10	0.95
PTGER1	Prostaglandin E receptor 1	0.16	0.80	0.20
PTGER2	Prostaglandin E receptor 2	1.05	1.24	0.85

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
PTGDR	Prostaglandin D2 receptor	0.15	0.06	2.50
PTGDR2	Prostaglandin D2 receptor 2	0.28	0.94	0.30
PTGR1	Prostaglandin reductase 1	4.57	1.58	2.89
HPGDS	Hematopoietic D synthase	2.89	4.18	0.69
TBXAS1	Thromboxane A synthase 1	1.26	1.98	0.64
TBXA2R	Thromboxane A2 receptor	0.54	0.41	0.01
LOC102130440	Leukotriene-B4 omega-hydroxylase 1	11.48	17.17	0.67
LOC102120579	Leukotriene-B4 omega-hydroxylase 2	10.51	16.84	0.62
LTB4R	Leukotriene-B4 receptor	15.24	6.48	2.35
LTB4R2	Leukotriene-B4 receptor 2	18.02	4.46	4.04
Cannabinoid				
FAAH	Fatty acid amide hydrolase	3.45	2.22	1.55
FAAH2	Fatty acid amide hydrolase 2	2.84	1.98	1.43
CNR1	Cannabinoid receptor 1	0.26	0.89	0.29
CNR2	Cannabinoid receptor 2	0.10	0.22	0.46
GPR55	G protein-coupled receptor 55	0.14	0.13	1.08

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
GPR18	G protein-coupled receptor 18	0.45	0.31	1.45
PLA2G1B	Phospholipase A2, group IB	0.74	0.55	1.35
PLA2G2A	Phospholipase A2, group IIA	17.67	4.84	3.65
PLA2G2C	Phospholipase A2, group IIC	2.11	1.72	1.23
PLA2G2D	Phospholipase A2, group IID	1.61	0.88	1.83
PLA2G2E	Phospholipase A2, group IIE	1.13	0.64	1.77
PLA2G2F	Phospholipase A2, group IIF	16.47	8.21	2.01
PLA2G3	Phospholipase A2, group III	10.04	9.17	1.09
PLA2G4A	Phospholipase A2, group IVA	3.67	4.82	0.76
PLA2G4B	Phospholipase A2, group IVB	11.35	3.46	3.28
PLA2G4D	Phospholipase A2, group IVD	7.66	2.97	9.31
PLA2G4E	Phospholipase A2, group IVE	15.34	4.85	3.16
PLA2G4F	Phospholipase A2, group IVF	20.08	19.42	1.03
PLA2G5	Phospholipase A2, group V	0.41	0.78	0.53
PLA2G7	Phospholipase A2, group VII	1.44	1.63	0.88
PLA2G10	Phospholipase A2, group X	0.09	0.24	0.38

(green)=upregulated; (red)=downregulated

Table 3.1 Continued.

Gene Symbol	Gene Name	Lesional Skin RPKM	Non-lesional Skin RPKM	FC
PLA2G12A	Phospholipase A2, group XIIA	4.81	5.42	0.89
PLA2G12B	Phospholipase A2, group XIIB	3.10	1.85	1.68
PLA2G16	Phospholipase A2, group XVI	8.57	17.22	0.50
PLA2R1	Phospholipase A2 receptor 1	6.23	11.49	0.54
PLCB1	Phospholipase C β 1	0.90	1.62	0.56
PLCB2	Phospholipase C β 2	1.94	1.38	1.41
PLCB3	Phospholipase C β 3	9.15	7.68	1.19
PLCG1	Phospholipase C γ 1	11.67	11.94	0.98
PLCG2	Phospholipase C γ 2	2.94	3.45	0.85
PLCD3	Phospholipase C δ 3	16.24	14.81	1.10
PLCD4	Phospholipase C δ 4	1.74	3.56	0.50
PLCE1	Phospholipase C ϵ 1	0.08	0.11	0.73
PLCZ1	Phospholipase C ζ 1	2.48	5.33	0.47
PLCH1	Phospholipase C η	0.03	0.26	0.12
PLCH2	Phospholipase C η 2	0.54	0.71	0.76

(green)=upregulated; (red)=downregulated

CHAPTER 4

CUTANEOUS T-CELL LYMPHOMA AND PRURITUS: THE EXPRESSION OF IL-31 AND ITS RECEPTORS IN THE SKIN*

Abstract

Background: Approximately 88% of cutaneous T-cell lymphoma (CTCL) patients are affected by pruritus that responds poorly to current antipruritic therapies. Interleukin-31 (IL-31), a Th2 cytokine, has been found to be increased in the serum of CTCL patients and to correlate with itch severity.

Objective: To investigate the role of IL-31 and its receptors (IL-31RA and OSMR β) in the skin of CTCL patients with mild versus moderate/severe pruritus.

Methods: Expression levels of IL-31, IL-31RA, and OSMR β in the skin were measured using immunohistochemistry and correlated to pruritus severity and disease stage.

Results: In CTCL patients with moderate/severe pruritus (compared to CTCL patients with mild pruritus and to healthy controls), IL-31 was significantly elevated in the keratinocytes of the epidermis and dermal infiltrate, while IL-31RA and OSMR β were significantly elevated only in the keratinocytes. Furthermore, epidermal IL-31 levels correlated to itch severity.

Limitations: This study was limited by a low number of CTCL patients without pruritus and in early stages of the disease.

Conclusion: These results show that IL-31 may play a role in CTCL pruritus by exerting indirect effects on sensory nerves through keratinocytes to transmit itch.

Introduction

Pruritus significantly impacts the lives of patients with cutaneous T-cell lymphoma (CTCL), the most common forms of which are mycosis fungoides (MF) and Sézary syndrome (SS) (Ahern et al, 2012; Demierre et al, 2006; Sampogna et al, 2008; Vij & Duvic, 2012; Wright et al, 2013). Surveys of patients with CTCL have found a prevalence of pruritus between 66% and 88% (Demierre et al, 2006; Vij & Duvic, 2012; Wright et al, 2013). Because pruritus of CTCL is non-histaminergic, it is difficult to treat and responds poorly to antihistamines (Ahern et al, 2012). The need for a more complete understanding of the pathophysiology of CTCL pruritus has led to an investigation into potential pruritic mediators.

Cytokines may play an important role in the pruritus of CTCL. CTCL skin lesions typically express Th2 cytokines, such as interleukins (IL)-4, 5, 6, 9, 10, and 13. Th2 expression increases as the disease progresses, which may explain why pruritus is more severe at later stages of the disease (Kim et al, 2005). Studies in other pruritic disorders have found elevated levels of IL-31, a member of the IL-6 family of cytokines, in atopic dermatitis, prurigo nodularis, and allergic contact dermatitis (Kato et al, 2014; Neis et al, 2006; Nobbe et al, 2012; Sonkoly et al, 2006). IL-31 acts through a heterodimeric receptor composed of IL-31 receptor alpha (IL-31RA) and oncostatin M receptor beta (OSMR β) (Cornelissen et al, 2011). IL-31 is produced by a variety of sources, including Th2 T-cells, dendritic cells, mast cells, and keratinocytes. IL-31RA and OSMR β are expressed on keratinocytes and dorsal root ganglia, which conduct the afferent sensation of pruritus (Cornelissen et al, 2011).

Recently, Singer et al. found that the malignant T-cell population produced IL-31 and that the increased levels of this cytokine in serum and peripheral blood mononuclear cells correlated with pruritus severity (Singer et al, 2013). In another study, IL-31 serum levels were elevated in CTCL patients but did not correlate with pruritus severity (Malek et al, 2015). However, these observed differences could be due to differences in the patients' stages of CTCL. Given the potential association between IL-31 and pruritus, we investigated the expression of IL-31 and its receptors in patients with CTCL and correlated our findings with both the clinical stage of each patient and the severity of their pruritus.

We discovered that expression levels of IL-31, IL-31RA, and OSMR β were elevated in affected CTCL skin keratinocytes and that IL-31 expression was significantly correlated with itch severity. These results offer insight into the pathophysiology of pruritus in CTCL.

Materials and Methods

Subjects

Skin biopsies were obtained from CTCL patients and healthy subjects at the dermatology clinics of Temple University, Northwestern University, and the University of Pennsylvania in accordance with the Declaration of Helsinki and with approval by each institution's IRB.

Patients were diagnosed based on clinical, histopathological, and immunohistological criteria and staged using the Tumor-Node-Metastasis-Blood (TNMB) 2007 International Society for Cutaneous Lymphomas (ISCL) and European Organization of Research and Treatment of Cancer (EORTC) revised classification

system (Murphy, 1988; Olsen et al, 2007). Patient characteristics are described in Table 4.1. Patients with MF had patch lesions biopsied for stage IB and plaque lesions biopsied for stage IIB. Tumor lesions were biopsied for patients with stage IIB folliculotropic MF. SS patients had patch lesion biopsies performed and the patient with PCGDTCL was biopsied in a plaque lesion.

Table 4.1. Cutaneous T-cell lymphoma. Sample demographic data.

		Moderately/ Severely Pruritic CTCL	Mildly Pruritic CTCL	Healthy Controls
N		15	8	8
Age (mean ± SD)		65 ± 13.9	69.4 ± 12.6	66.9 ± 9.7
Sex (M:F)		7:8	4:4	4:4
VAS rating		7.5 ± 1.2	2.5 ± 1.5	0
Diagnosis	MF ^a	3 (IB) ^b , 1 (IIB)	1 (IB)	
	Folliculotropic MF	3 (IIB)	2 (IIB)	
	Sézary Syndrome	7 (IVA)	5 (IVA)	
	PCGDTCL ^c	1 (T3N2M0)	0	

^aMycosis Fungoides; ^bStage (TNMB); ^cPrimary cutaneous gamma delta T-cell lymphoma

Pruritus severity was measured at the time of the biopsy using a visual analog scale (VAS). Subjects were grouped into the following categories: mildly pruritic CTCL (VAS 1-5; n=8), moderately/severely pruritic CTCL (VAS 6-10; n=15), and healthy controls (VAS 0; n=8). We did not recruit CTCL patients without pruritus.

Histology and Immunohistochemistry

Researchers were blinded to the identity of the biopsies and the results were only decoded after the analysis was fully performed. For histology to confirm disease stage, 5-

μm thick sections of paraffin-embedded skin tissue were processed for standard hematoxylin and eosin (H&E) staining.

For immunohistochemistry, a total of twelve 20- μm thick sections of paraffin-embedded skin tissue were double stained from each biopsy. Sections were deparaffinized and then underwent antigen retrieval using Target Retrieval Solutions (DAKO, Glostrup, Denmark) heated in a humidified oven overnight at 60°C, then washed in PBS. Sections were blocked with 5% normal donkey serum and 0.2% Triton X-100 in PBS for 2 hours and then incubated with primary antibodies overnight at 4°C. Primary antibody combinations were: anti-PGP9.5 (1:50; Abcam, Cambridge, MA) and anti-IL-31 (1:100; Abcam, Cambridge, MA); anti-IL-31RA (1:200, Abcam, Cambridge, MA) and anti-OSMR β (1:100; Santa Cruz, Dallas, Texas). Alexa Fluor (488 & 594, 1:300; Molecular Probes, Eugene, OR) secondary antibodies were used for detection. The slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and imaged under a fluorescence microscope.

Sections treated without any primary antibodies were used as negative controls. Furthermore, specificity of the IL-31 and IL-31RA antibody was confirmed by pre-absorbing the full length IL-31 and IL-31RA peptides (20 $\mu\text{g}/\text{ml}$; Abcam, Cambridge, MA) in blocking solutions with their respective antibodies overnight at 4°C with gentle agitation. Solutions were centrifuged, and the supernatant was used for IHC as described above, which resulted in blocking the IL-31 and IL-31RA immunoreactivity.

Quantification

Three fields (20X objective magnification) were measured for every section. The total field and selected field (epidermis) fluorescence area (in μm^2) were measured and

normalized to background staining using ImageJ Software. Data is presented as the mean epidermal fluorescence and mean dermal infiltrate fluorescence, which was calculated as the total field – selected field fluorescence. Inter-epidermal nerve fibers were counted and normalized to tissue length as previously described (McArther et al, 1998).

Statistical Analyses

Statistical analysis was carried out using one-way ANOVAs with Bonferroni post hoc tests and nonparametric two-tailed Spearman correlations with linear regression; significance was set at $p < 0.05$ (GraphPad Prism; La Jolla, CA).

Results

CTCL Characteristics and Pruritus

The mean ages of the three groups (Table 4.1) did not significantly differ from one another. There was a statistically significant difference ($p < 0.0001$) in pruritus VAS ratings among healthy (VAS 0), mildly pruritic CTCL (VAS 2.5 ± 1.5), and moderately/severely pruritic CTCL (VAS 7.5 ± 1.2) subjects (Figure 4.1A). No correlation was found between age and VAS ratings. The stage of CTCL correlated to VAS ratings ($r = 0.85$, $p = 0.04$) in mildly pruritic CTCL subjects only (Figure 4.1B).

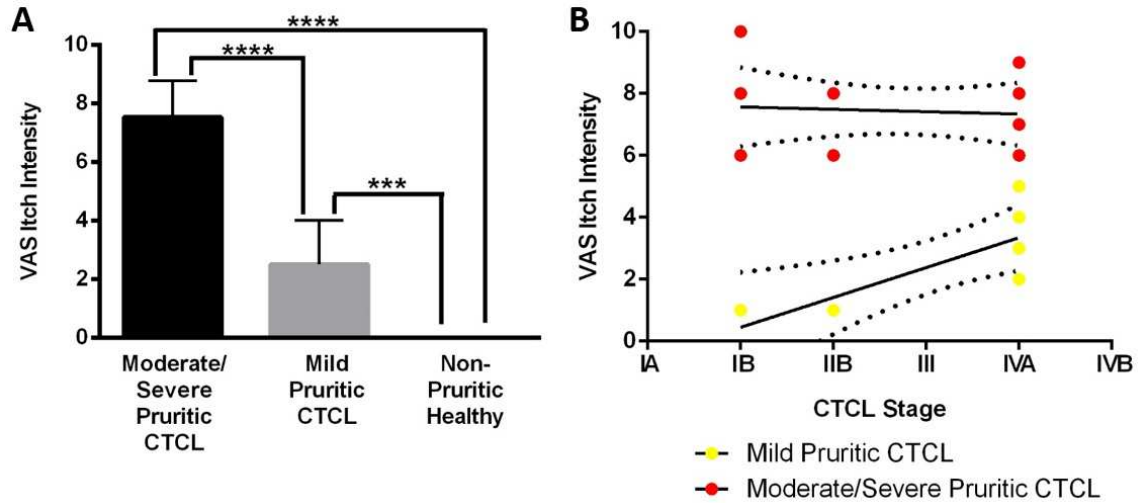


Figure 4.1. Cutaneous T-Cell lymphoma. Pruritic VAS rating and its correlation to CTCL stage. (A) The pruritic VAS rating was significantly higher in CTCL patients when compared to healthy controls (** $p < 0.001$, **** $p < 0.0001$). (B) The pruritus ratings correlated to CTCL disease stage in the mildly pruritic CTCL group only.

IL-31 and Epidermal Innervation (PGP9.5)

IL-31 was significantly ($p < 0.0001$) increased in the epidermis and lymphocytic infiltrate of CTCL subjects, with the moderately/severely pruritic CTCL subjects having the highest levels of IL-31 in the skin (Figure 4.2A). The mean \pm SD IL-31 fluorescence levels in the epidermis of moderately/severely pruritic CTCL subjects were 1859 ± 271 , while the infiltrate levels were 1049 ± 164 . In mildly pruritic CTCL subjects, the IL-31 levels were 1489 ± 123 in the epidermis and 625 ± 123 in the infiltrate. Healthy controls did present with some constitutive IL-31 in the epidermis, with levels of 239 ± 72 , but little to none in the dermis (5 ± 15). Interestingly, in all subjects, IL-31 expression was limited to the lymphocytic infiltrate in the dermis and keratinocytes in the epidermis

(representative image: Figure 4.2B). No significant difference among groups was found in the number of epidermal nerve fibers, and IL-31 did not appear to co-localize to any afferent fibers. Epidermal IL-31 levels significantly correlated ($r=0.94$; $p<0.0001$) with VAS ratings (Figure 4.2C), but not with CTCL stage ($r=0.04$; $p=0.86$; Figure 4.2D).

IL-31RA and OSMR β

IL-31RA was significantly ($p<0.0001$) elevated in only the epidermis of CTCL subjects (Figure 4.3A; representative image: Figure 4.3B). The mean \pm SD IL-31RA fluorescence levels were highest in moderately/severely pruritic subjects, with levels of 1394 ± 183 in the epidermis and 50 ± 12 in the lymphocytic infiltrate. Mildly pruritic CTCL subjects had expression levels of 620 ± 42 in the epidermis and 21 ± 29 in the infiltrate, while healthy controls had levels of only 377 ± 56 in the epidermis and 12 ± 3 in the dermis.

Furthermore, OSMR β was also significantly ($p<0.0001$) higher in the epidermis of CTCL subjects (Figure 4.3C; representative image: Figure 4.3D). In moderately/severely pruritic CTCL subjects, the mean \pm SD OSMR β fluorescence levels were 693 ± 184 in the epidermis and 142 ± 93 in the lymphatic infiltrate. Mildly pruritic CTCL subjects had levels of 421 ± 83 in epidermis and 174 ± 123 in the lymphocytic infiltrate. The lowest levels of OSMR β were observed in healthy controls (epidermis 183 ± 26 ; dermis 78 ± 15).

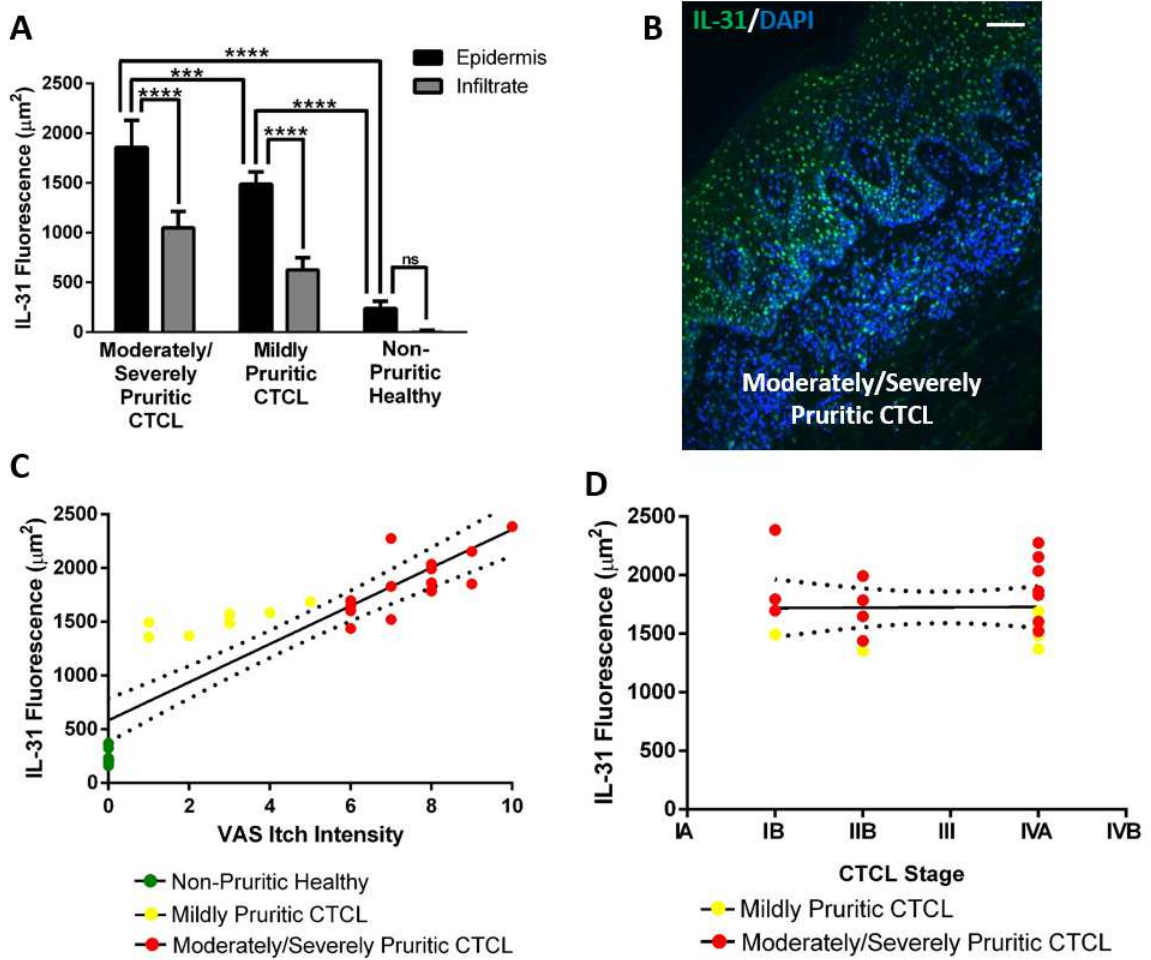


Figure 4.2. Cutaneous T-Cell Lymphoma. IL-31 is increased in patients with pruritic CTCL. (A) IL-31 was elevated in the epidermis and lymphocytic infiltrate of CTCL patients ($***p < 0.001$, $****p < 0.0001$), with the highest expression in the epidermis of the moderately/severely pruritic CTCL group (B). Scale bar = $1000\mu\text{m}$. (C) Epidermal IL-31 expression correlated to pruritic VAS ratings, but not with CTCL stage (D).

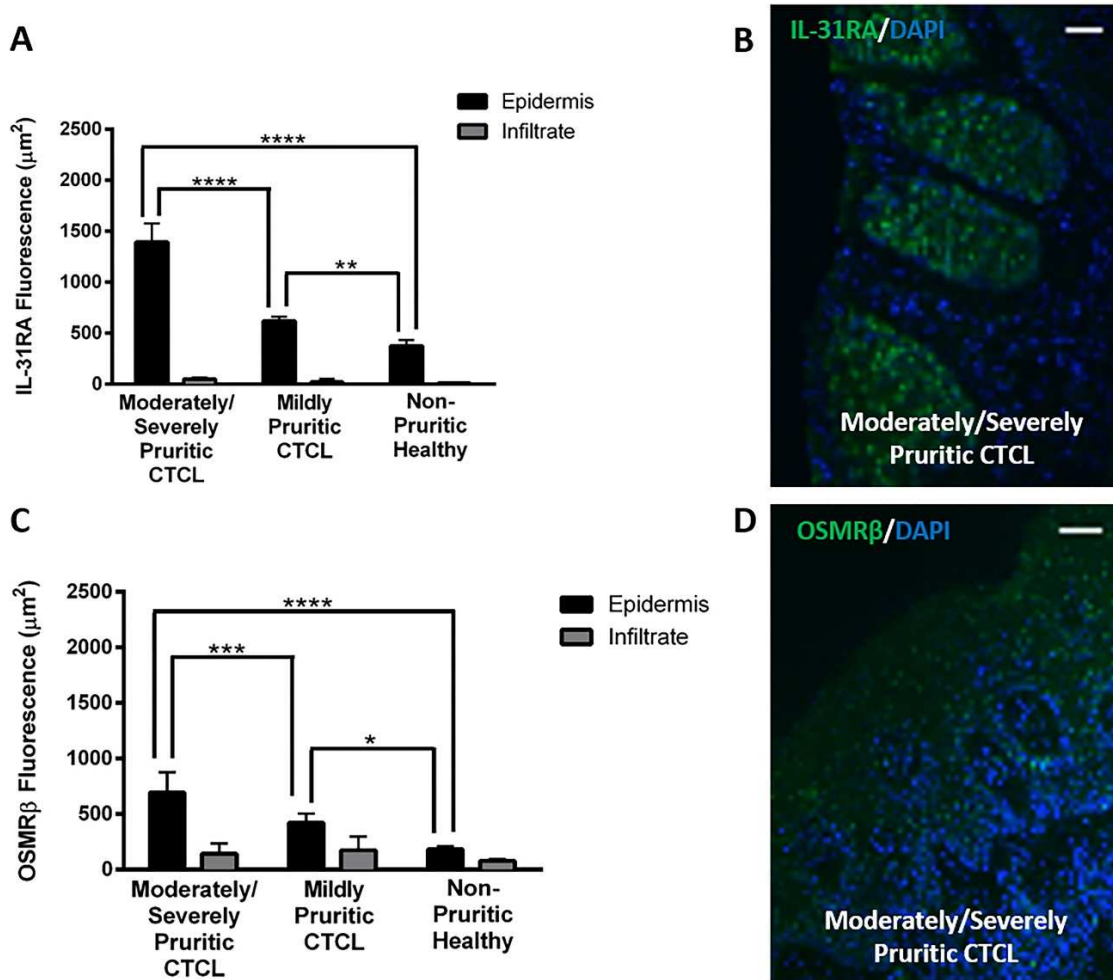


Figure 4.3. Cutaneous T-Cell Lymphoma. IL-31RA and OSMR β are increased in patients with pruritic CTCL. IL-31 receptors (A) IL-31RA and (C) OSMR β are elevated in the epidermis of CTCL patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), with the highest levels found in moderately/severely pruritic CTCL group (B and D). Scale bar = 1000 μm .

Conclusions

Although several recent studies have found increased levels of IL-31 in CTCL, these studies largely focus on serum analysis and do not address the crucial question of IL-31's role in the skin (Malek et al, 2015; Möbs et al, 2014; Singer et al, 2013). This is the first study to show the expression levels and patterns of IL-31 and both of its receptors within the skin of CTCL subjects with pruritus. Similar to most of the previous serum studies, this study shows that elevated IL-31 is positively correlated to itch intensity (Möbs et al, 2014; Singer et al, 2013). We also report that IL-31 was not correlated to disease stage, suggesting that IL-31 does not play an essential role in the pathogenesis of CTCL. However, our study was limited by the low number of early stage CTCL patients and by the lack of CTCL patients without itch.

This study also found increased expression of the IL-31 receptors, IL-31RA and OSMR β . The previous findings for these receptors in CTCL is limited to one serum and cancer cell line study, which did not find evidence of significant elevation of these receptors, possibly due to their focus on the blood compartment of CTCL cells (Möbs et al, 2014). The IL-31RA and OSMR β receptors are known to be located on keratinocytes, rather than serum, making this study better suited to assess the role of IL-31 signaling in CTCL. Interestingly, a recent study in mice showed that repeated IL-31 exposure caused elevated expression of IL-31RA and OSMR β in the dorsal root ganglia (Arai et al, 2015). This mechanism could explain the increase in receptor density we found in the epidermis. In another study, a single intradermal exposure of IL-31 did not cause pruritus in healthy subjects (Hawro et al, 2014). Therefore, the increase in IL-31RA and OSMR β expression

after chronic exposure to IL-31 may be necessary in order for a subsequent exposure to IL-31 to induce pruritus.

Our study showed elevated expression of IL-31 in both keratinocytes and the lymphocytic infiltrate. This result is similar to studies performed in atopic dermatitis subjects, which localized IL-31 to lymphocytes infiltrating the skin (Kato et al, 2014), and in lichen planus, which found overexpression of IL-31 in the epidermis. However, IL-31 expression in lichen planus did not correlate to pruritus ratings (Welz-Kubiak et al, 2015). It is thought that IL-31 can induce keratinocytes and infiltrating cells to release additional mediators involved in pruritus. Furthermore, IL-31 has also been shown to cause the release of pro-inflammatory cytokines from eosinophils, monocytes, and macrophages (Cheung et al, 2010; Kasraie et al, 2010). It would be of great interest to investigate the role of other pruritic mediators and cell types involved in CTCL pruritus.

The finding of elevated IL-31 and its receptors in the skin of pruritic CTCL subjects adds the missing link to previous studies investigating the role of IL-31 in CTCL pruritus. As pruritus treatments begin to focus on reducing IL-31 in CTCL patients, it will be interesting to correlate the therapeutic response with epidermal IL-31, as well as possible changes in the expression of IL-31 receptors (Cedeno-Laurent et al, 2015; Kasutani et al, 2014; Singer et al, 2013).

CHAPTER 5

CONCLUSION

Many skin diseases, such as atopic dermatitis and psoriasis, cause pruritus that results in an impaired quality of life. In America alone, it is estimated that \$29.13 billion is spent annually in direct medical costs for these diseases, with \$9.7 billion spent on prescription and over-the-counter treatments (Bickers et al, 2006). However, development of effective treatment modalities for pruritus has proven difficult. Because itch involves a complex interaction among skin cells, immune cells, secreted factors, and cutaneous neural networks, there is no one specific cause of chronic itch. Furthermore, these components may interact differently in various pruritic disease states. Identifying the elements that are unique to each disease state and those shared across multiple pruritic diseases is crucial for the development of itch-specific drug therapies.

A thorough understanding of the underlying pathologies of pruritic diseases is restricted by the currently available experimental models. Most experimental studies use rodent models, created by the injection of pruritogens or induction of various dermatoses. Although they offer the advantage of genetic manipulation and are cost efficient, rodents exhibit different pruritoceptive responses from humans. Additionally, though chronic pruritus in humans is defined as lasting more than six weeks, “chronic itch” in rodent models lasts only on the order of days. The disadvantages of current rodent models highlight the need to design a valid primate model of chronic itch.

A non-human primate model can provide key genetic, physiological, neurological, and behavioral similarities to humans (Ogawa and Vallender, 2014). It has been shown

that monkeys have similar thresholds for detecting pruritic stimuli. Unlike in rodents, histamine induces acute itch in primates, and morphine induces intense, long-lasting itch (Ko and Naughton, 2000). Lee and Ko further showed that using an established non-human primate model is imperative to identifying specific ligand-receptor systems involved in pruritus and testing pharmacological treatments in awake, behaving animals (2015). Given that non-human primates are well suited and established as translational models for drug testing, it was of the utmost importance to examine a primate colony with chronic itch.

Conclusion of Aim 1

For four years, the scratching behavior of a *Cynomolgus* macaque colony with idiopathic chronic itch was monitored and recorded. To better understand the mechanisms underlying itch in these animals, tissue was harvested when the primates were euthanized. Since chronic itch involves a multifactorial and complex process, we originally focused our investigation on a recently discovered itch-specific pathway. This work is presented in Chapter 2 (Nattkemper et al, 2013).

Gastrin-releasing peptide (GRP) and its receptor (GRPR) were shown to play a key role in itch, but not pain, transmission in mice (Sun and Chen, 2007). Therefore, we hypothesized that this ligand-receptor system would be analogous in the primates with chronic itch. Expression of GRP in the skin and GRPR in the spinal cord was quantified by immunohistochemistry in primates with varying scratching behavior (n=6). We found that primates with severe scratching displayed an increased number of GRP+ cutaneous nerve fibers and that these expression levels correlated with the frequency and duration of scratching. Furthermore, these animals showed an increase in the number of GRPR+ cells

within the dorsal horn of the spinal cord. Based on these results, we concluded that GRP was a peripheral mediator that signaled through spinal GRPR to transmit itch.

However, there has been continuing controversy as to whether GRP is expressed in primary afferents. Recently, Solorzano et al revealed that the most commonly available primary antibody for GRP, the same antibody used in our study, had high cross-reactivity with substance P (SP) at the recommended concentrations and was blocked by pre-absorption of the SP protein (2015). Therefore, we reexamined our primate skin with the same anti-GRP antibody (1:400; Immunostar, Hudson, WI) while pre-absorbed with an SP peptide (10 μ g/ml; Tocris Bioscience, Bristol, UK) or double-stained with anti-SP (1:1000; Abcam, Cambridge, MA). Consistent with Solorzano's findings, the GRP immunostaining was significantly reduced by pre-absorption of SP, and double staining revealed an almost complete overlap of GRP immunoreactivity with SP immunoreactivity (Figure 5.1). These results were found in both pruritic, lichenified and non-pruritic, non-lichenified primate skin. Therefore, since the anti-GRP antibody cross-reacted with SP, we cannot be certain that GRP is expressed within the primate skin.

In order to positively identify itch-associated transcripts with higher selectivity, we turned to RNA sequencing (RNA-seq) to study our primate model of chronic itch. RNA-seq allows for precise gene identification and quantification of gene expression, even when genes are expressed at low levels (Wang et al, 2009). This work is presented in Chapter 3.

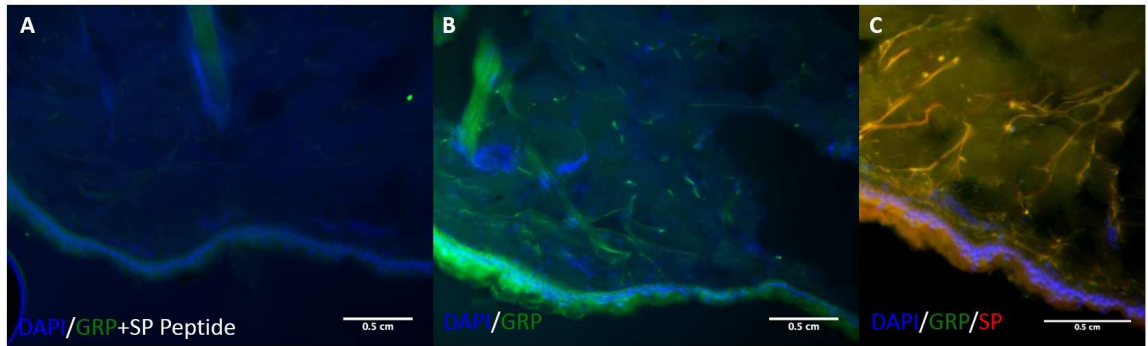


Figure 5.1. Representative images of the GRP antibody cross-reactivity with SP in primate skin. The GRP antibody pre-absorption with SP (A) shows significantly reduced immunostaining compared to the un-absorbed antibody (B). GRP and SP were co-localized (C).

RNA-seq was performed on paired lichenified and non-lichenified skin biopsies of primates with chronic itch (n=35). To identify differentially expressed genes (DEGs) involved in itch, transcriptome reads were compared between lichenified skin of severely scratching primates (n=14) and non-lichenified skin of mildly/moderately scratching primates (n=21). This sequencing uncovered 2,264 genes that were differentially expressed in the lichenified versus non-lichenified primate skin, with ~400 genes correlating to scratching behavior. Many of these DEGs included receptor channels (transient receptor potential [TRP] V1 and V3, protease-activated receptor 2 [PAR2], voltage-gated sodium channels [Na_v] 1.3 and 1.9, and mas-related G-coupled protein X2 [MrgprX2]), proteases (cathepsin S [CTSS], kallikreins [KLK], and tryptase), neuropeptides (SP, proopiomelanocortin [POMC], endothelin-1 [ET-1], and S100 proteins), and cytokines and chemokines (interferon [INF]- γ , interleukin [IL]-2/6/17/36,

chemokine (C-C motif) ligand [CCL] 1/7/17/25/26 and chemokine (C-X-C motif) ligand [CXCL] 3/5/6/9/10). To confirm the RNA-seq findings, selected gene products were quantified in the primate skin using immunohistochemistry. This analysis confirmed that TRPV1, TRPA1, tryptase and its receptor PAR2, SP and its receptor neurokinin 1 (NK-1R), and ET-1 and its receptor endothelin A (ETA) were significantly increased in lichenified skin of severely scratching primates versus non-lichenified skin of mildly/moderately scratching primates.

Conclusion of Aim 2

In order to examine the similarities and differences between the primate model and human chronic itch, lesional and non-lesional skin biopsies from patients with atopic dermatitis, psoriasis, and cutaneous T-cell lymphoma (CTCL) underwent immunohistochemical analysis alongside the primate tissue. Expression levels of the selected itch-associated proteins were compared among all groups. This work is presented in Chapters 2 and 3.

Our studies revealed that pruritic, lichenified primate skin shared several common itch-associated proteins with pruritic lesional skin of atopic dermatitis and psoriasis. Tryptase, SP, NK-1R, and ET-1 were all found to be increased in primate and human pruritic skin when compared to healthy controls. PAR2 was found to be increased in all skin types, except in non-lesional psoriatic skin. SP and ET-1 were also increased in non-lichenified primate skin, while NK-1R was increased in non-lesional atopic skin and tryptase was increased in non-lesional psoriatic skin. ETA was found to be elevated in pruritic skin of primates and atopic dermatitis, while TRPA1 was increased in all skin

types of primates and atopic dermatitis. Interestingly, TRPV1 was only increased in primate skin, in both lichenified and non-lichenified skin.

We also showed that IL-31 and its receptors (IL-31RA and OSMR β) were significantly more highly expressed in lesional skin of CTCL patients with itch when compared to CTCL patients without itch and healthy controls. Lesional pruritic primate skin also had a slight, but significant, increase in IL-31 and its receptors. IL-31 and IL-31RA were increased in atopic skin, but not in psoriatic skin.

Overall Conclusions

Our primate model offers an ideal system for the study of chronic itch because its anatomical and physiological similarity to humans. These parallels also extend to the molecular level, with *Cynomolgus* macaques sharing 93% of the human transcriptome. Using RNA-seq we identified 2,264 DEGs when comparing transcriptome read counts from lichenified versus non-lichenified primate skin. Of these, itch-specific transcripts were distinguished by comparing lichenified skin of severely scratching primates to non-lichenified skin of mildly/moderately scratching primates. This analysis revealed over 400 DEGs, with ~110 genes to be upregulated (fold change >2.0) and ~40 genes to be downregulated (fold change <0.5) in pruritic primate skin. By considering these proteins in the context of previous literature and our own immunohistochemistry studies, we were able to identify a fingerprint of itch-associated mediators and receptors for our idiopathic primate model, human atopic dermatitis, and psoriasis (Figure 5.2). Although we found distinct proteins associated with each group, a common core of components was revealed to be involved in all of the studied pruritic disease states. These proteins are connected to all aspects of itch transmission at the peripheral level and are expressed by skin cells,

immune cells, and nerves. Treatments targeting these common elements could provide itch relief that is effective across multiple disease states.

Future Directions

Further studies should continue the RNA-seq analysis of DEGs related to chronic itch in other tissues, such as DRG, spinal cord, and brain, within a primate model. These results should next be confirmed at the proteomic level. It would also be of great interest to use RNA-seq to examine DEGs correlated to itch severity in human pruritic disease states, such as atopic dermatitis, psoriasis, CTCL, and prurigo nodularis. Most importantly, drugs targeting the common core components should be tested in several of these disease states. Aprepitant, an inhibitor of NK-1R, and the MOR antagonists butorphanol and nalbuphine have already shown to be efficacious in multiple chronic pruritus conditions (Ally et al, 2013; Borja-Consigliere et al, 2014; Cowan et al, 2015; Dawn and Yosipovitch, 2006; Hawi et al, 2015; Jiménez et al, 2014; Papoiu et al, 2015; Santini et al, 2012; Ständer et al, 2010). Future studies should examine the effect of these treatments on expression of the targeted protein within lesional skin and correlate changes in expression to itch relief. Finally, a primate model of chronic itch would be a suitable system to test new therapies that target these common proteins.

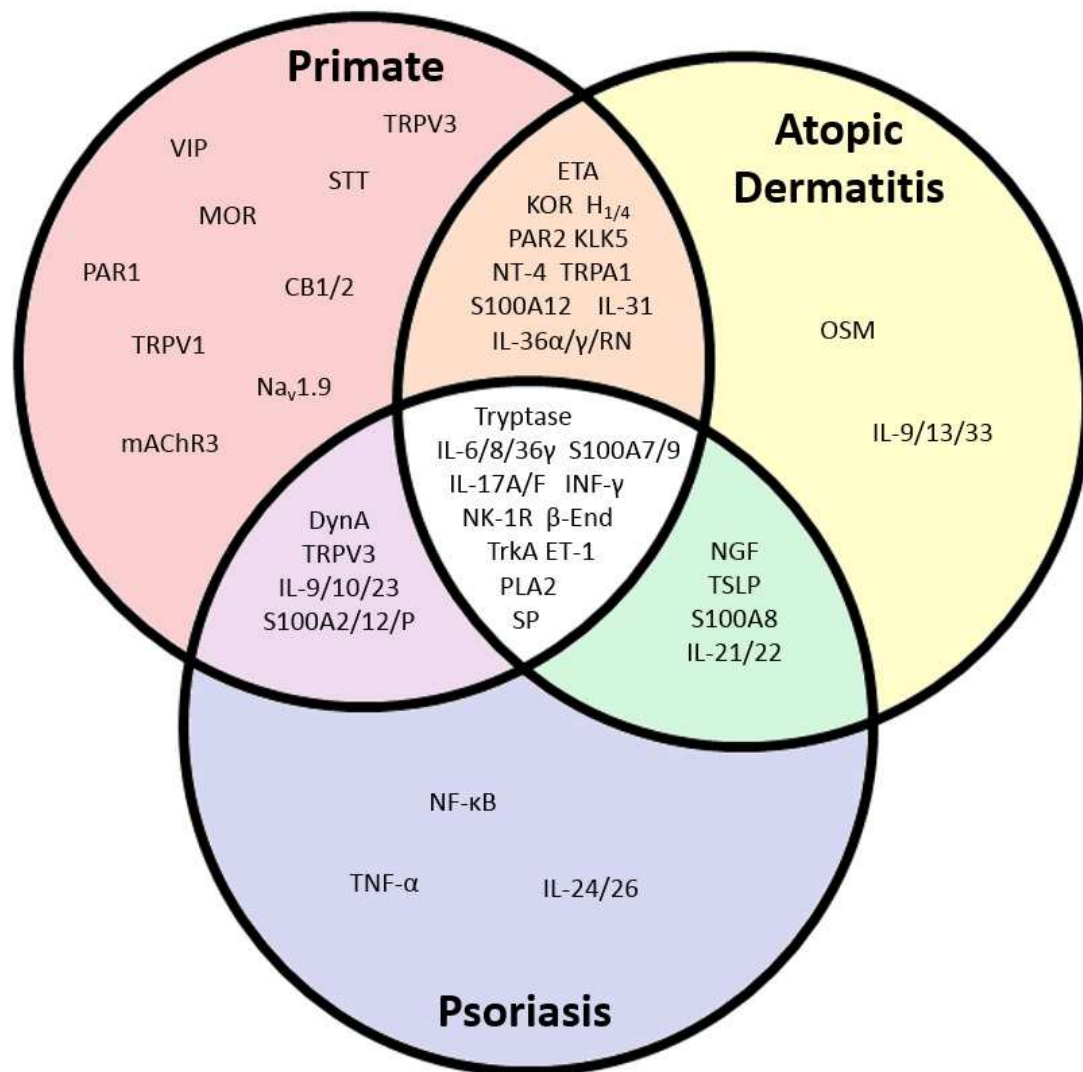


Figure 5.2. Venn diagram illustrating itch-associated proteins common among the primate model, atopic dermatitis, and psoriasis. TRPV3= transient receptor potential vanilloid 3; VIP=vasoactive intestinal peptide; STT=somatostatin; MOR=μ-opioid receptor; PAR1=protease-activated receptor 1; CB1/2=cannabinoid receptor 1/2; TRPV1=transient receptor potential vanilloid 1; Na_v1.9=voltage-gated sodium channel 1.9; mAChR3=muscarinic acetylcholine receptor 3; ETA=endothelin receptor A; KOR=κ-opioid receptor; H_{1/4}=histamine receptor 1/4; PAR2=protease-activated receptor 2; KLK5=kallikrein 5; NT-4=neurotrophin-4; TRPA1=transient receptor potential

ankyrin 1; S100A12=S100 calcium binding protein A12; IL-31=interleukin-31; IL-36 α / γ /RN=interleukin-36 α / γ /receptor; OSM=oncostatin M; IL-9/13/33=interleukin-9/13/33; DynA=dynorphin A; TRPV3=transient receptor potential vanilloid 3; IL-9/10/23=interleukin-9/10/23; S100A2/12/P=S100 calcium binding protein A2/12/P; IL-6/8/36 γ =interleukin-6/8/36 γ ; S100A7/9=S100 calcium binding protein 7/9; IL-17A/F=interleukin-17A/F; INF- γ =interferon- γ ; NK-1R=neurokinin 1 receptor; β -End= β -endorphin, TrkA=tropomyosin receptor kinase A; ET-1=endothelin-1; PLA2=phospholipase A2; SP=substance P; NGF=nerve growth factor; TSLP=thymic stromal lymphopoietin; S100A8=S100 calcium binding protein A8; IL-21/22=interleukin 21/22; IL-24/26=interleukin-24/26; NF- κ B=nuclear factor kappa-light-chain-enhancer of activated B-cells; TNF- α =tumor necrosis factor- α .

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