*IFSCC 2025 full paper (**IFSCC2025-926**)*

"Virulent *staphylococcus aureus* triggers specific neurogenic inflammation responses in keratinocytes and in a new keratinocytes / sensory neurons model"

Juliane Robert [1], Renaud Barbier [1], Cécile Clavaud [1] and Samira Tamoutounour [1]

[1] Microbiome Domain, L'Oréal Advanced Research, Aulnay-sous-bois, France;

---

1. Introduction

Itch has a high burden on people suffering from skin conditions comprising dry skin, dandruff scalp, eczema, atopic dermatitis, psoriasis and prurigo nodularis. In Atopic Dermatitis (AD), 14% of patients suffer from depression, 17% from anxiety and up to 90% have sleep disturbance [1] [2]. Only new biologics treatments have shown efficacy on itch by completely abrogating lesions (e.g dupilumab) [3]. Therefore, there's a crucial need for a highly efficient dermocosmetic targeting itch for mild cases and between flares. In AD, they are several biological pathways implicated in this skin condition such as specific chemokines (e.g IL-31, IL-33, Thymic Stromal LymphoPoietin (TSLP)…) and skin microbes [4]. A recent study showed how a virulent methicillin-resistant S*taphylococcus Aureus* (MRSA) infection can induce neurogenic inflammation in mice [5]. This model does not recapitulate AD but demonstrates how a virulent *S.aureus* could induce neurogenic inflammation in AD. Indeed, S*.aureus* could use similar proteases to induce itch by directly activating a host receptor on sensory neurons in the skin. However, the complete role of this bacterium (and components) in neurogenic inflammation remains unclear, more specifically the epidermal communication between keratinocytes-neurons and micro-organism and the respective pathways involved [6]. Human *vitro* models recapitulating this interactions are challenging and the recently developed Dualink™ Ultra (NETRI (Lyon, France) chips could respond to this challenge [7] [8]. Those new chips allowed the development of neurons with axons in contact with keratinocytes, modelizing interactions between those cells in the skin. To compare cells behavior isolated or in network, we separately cultured keratinocytes (NHEK) and sensory neurons (derived from hiPSC) or co-cultured them using the Dualink™ Ultra (NETRI) chips. We then assessed the capacity of keratinocytes, sensory neurons, and co-cultures to respond to representative stimuli mimicking the pathogenic bacterial invasion: bacterial culture supernatant (containing toxins and proteases), Heat Killed (HK) bacteria, and Peptidoglycan PG (major component of bacterial Gram + cell wall). These first results suggested the importance of keratinocytes/sensory neurons sensing virulent

bacterial component, thus potentializing neurogenic inflammation and subsequent itch in Atopic Dermatitis.

2. Materials and Methods

Sensory neurons culture & differentiation

20k hiPSC (neural crest progenitors Axol, ax0055) were seeded in the neuron cellular body compartment of Dualink™ Ultra (NETRI) as described before [8]. 21 days after culture in supplemented sensory neuronal maintenance, neuronal differentiation is completed.

Keratinocytes culture

5000 NHEK were seeded in the microfluidic chips alone for 3 days in KGM medium complemented with the ROCK inhibitor Y-27632 (Focus Biomolecules, 10-2301) at 10µM. Cells were maintained for 3 days before functional assays. For co-cultures with sensory neurons, keratinocytes were seeded in compartment 3 at day 17 post seeding of hiPSC in compartment 1.

Immunofluorescence Staining

Cells were fixed 30 min at 37°C by a solution of 4% paraformaldehyde in the culture medium, then permeabilized 10 min at room temperature with triton X-100 at 0,1% and non-specific sites were blocked with PBS containing 3% of BSA for 30 min at room temperature. Keratinocytes or human sensory neurons or co-culture were stained with primary antibodies against Phalloidin and neurofilaments for 2h at room temperature, then washed 3 times with PBS/BSA 0,1%. Secondary antibodies coupled to FITC or Texas Red were added for 1h at room temperature, followed by nuclei were labeled by a Hoechst solution.

Bacterial strains: *S. epidermidis* and *S. aureus at* $1x10^8$

Bacterial extracts (filtered culture supernatants) and heat-inactivated bacteria were prepared from commensal *S. epidermidis* (s. e) strain ATCC 12228 and pathogenic *S. aureus* (s. a) strain ATCC 25904 in sensory neuron maintenance medium till exponential phase. Subsequently, cultures were split into two tubes; one was centrifuged (3,000 g, 10 min, 4°C), the supernatant was filter-sterilized (0.22 µm) and stored at -20°C, while the other tube was heat-inactivated (60°C for 1 hour) and stored at -20°C.

Quantification of Neuromediators release

Culture media were renewed with sensory neurons maintenance medium in compartment 1 and 2, compartment 3 of keratinocytes, or human sensory neurons or co-cultures were treated with 160µl of s.e or s.a supernatant, s.e or s.a HK or s.a peptidoglycan (PG, Sigma) (500µg/ml) or Lipoteichoic acid (LTA, Sigma) (500µg/ml) diluted in sensory neuron maintenance medium for 15 minutes. Medium or treatment solutions were used as negative control. Quantification of neuromediators release (Substance P, Neurotensin, β-Endorphin, Oxytocin) in the

supernatant of compartment 3 was performed immediately after centrifugation (5min, 1500rpm) within multiplex kit technology (Merck, HNPMAG-35K).

Real-Time cell calcium mobilization

Keratinocytes were seeded in 96 plates in KGM medium and cultured for 3 days. Then, cells are incubated first with a Ca^{2+} fluorescent probe Fura 2-AM (Fischer scientific 11524766) within 30 min, washed, recovered for 30 min, and treated with 50µl of Supernatants, HK or PG or LTA. Ca^{2+} mobilization is followed in real time with the fluorescent probe for each condition. Changes in intracellular Ca^{2+} are monitored for 3 min.

To identify nociceptor(s) involved in Ca^{2+} influx, specific antagonists of TRPV1 (Capsazepin Sigma, C191), PAR2 (AZ3451 Sigma, SML2050) or Histamine R (Astemizole Bio-Techne, 3489/50) were applied during recovery time, prior the different stimulations.

3. Results

3.1 Physical contacts between NHEK and axons from sensory neurons in Dualink™ Ultra (NETRI) chips for cell-cell communication

To disentangle the cellular communication between keratinocytes and sensory neurons, we established a protocol to culture them in microfluidic chips. hiPSC cells were seeded, 20 days prior in compartment 1, they differentiated in sensory neurons (Neurofilament positive cells) and projected axons through impervious compartment 2 till compartment 3 (Fig. 1). The specific design of compartment 2 allowed unidirectional prolongation of axons into compartment 3 while compartment 1 contains all cellular bodies of sensory neurons (HOECHST/Neurofilament positive) (Fig. 1(b)). We thus were able to reproduce the culture of neurons in the microfluidic chips [8]. We then established NHEK cultures in compartment 3 of the chips. 3 days after seeding, keratinocytes (Phalloidin positive) proliferated and colonized the compartment of the chip till confluence and covering the entirety of the channel surface (Fig. 2). This indicates that keratinocytes can be cultured in microfluidic chip.

Sensory neurons x10 *HOECHST/Neurofilament x10*

(a) (b)

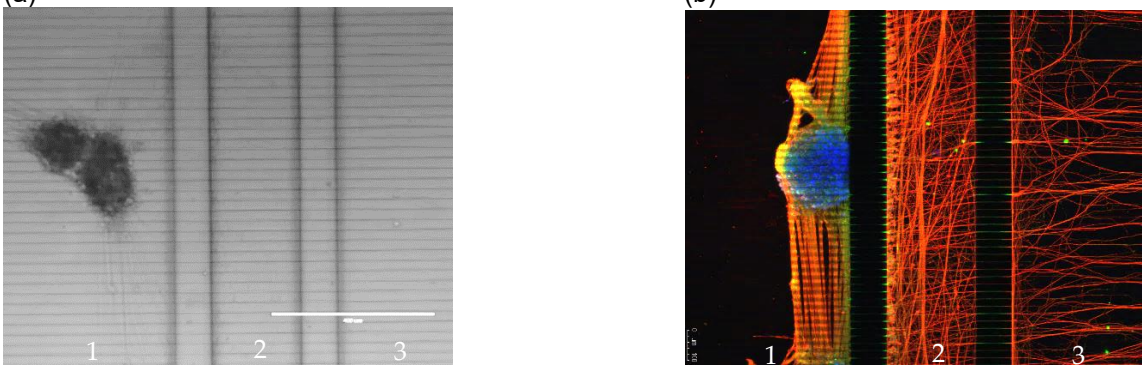
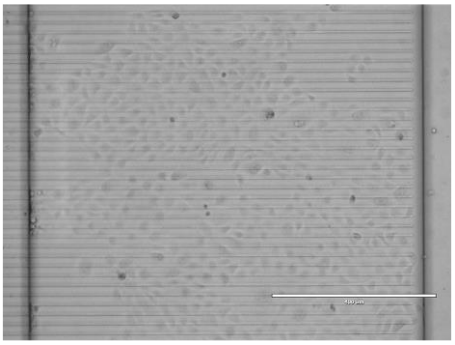


Figure 1: human induced Pluripotent Stem Cells (hiPSC) derived into sensory neurons after 20 days of culture in microfluidics chip. (a) Bright field image of sensory neurons, (b) HOECHST (blue) / Neurofilament (red) immunofluorescent staining performed on a monolayer culture of sensory neurons. 1, 2 and 3 are the different compartment of the chip. Scale bar 400µM.

Keratinocytes x10

(a)



HOECHST/Phalloidin *x10*

(b)

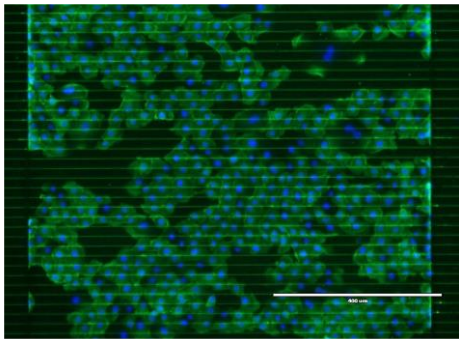
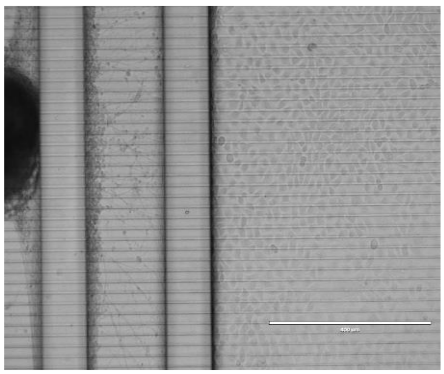


Figure 2: Human primary keratinocytes after 3 days of culture in microfluidic chip. Repartition and morphological aspect. (a) Bright field image of the keratinocytes compartment (b) HOECHST (blue) / Phalloidin (green) immunofluorescent staining performed on a keratinocytes culture. Scale bar 400µM.

As the first isolated cultures of keratinocytes and neurons were successful, we then co-cultured them to modelize the interaction between neuronal axon and keratinocytes in the basal layer of the epidermis. hiPSC cells were seeded in compartment 1, differentiated into neurons (neurofilament positive) for 17 days. As previously described, the neurons grew axons through compartment 2 till compartment 3 (Fig. 3 (a) and (b)). At day 17, NHEK (Phalloidin positive), were seeded in compartment 3 and by day 20 of total culture of the neurons, were covering the entirity of the compartment. As observed previously, cellular bodies of neurons (HOECHST neurofilament positive) remained in compartment 1, keratinocytes (Phalloidin positive) in compartment 3 and couldn't pass into the impervious compartment 2 (Fig. 3 (b)). Talagas et al. in previously described connection between keratinocytes and neurons in classical 2D cultures and potential synapses [9]. In compartment 3 of our co-cultures, we could observe connections between neuronal axons and keratinocytes at zoom 40 (Fig. 3 (c) and (d)). Formation of synapses between keratinocytes and sensory neurons has to be further investigated. Furthermore, the keratinocytes proliferation did not injure axons, as we observed their uninterrupted course through the keratinocytes cell layer at the bottom of the compartment. We thus successfully co-cultured keratinocytes and neurons in the chips. Connections between keratinocytes and axons of the sensory neurons were observed, suggesting a potential communication between.

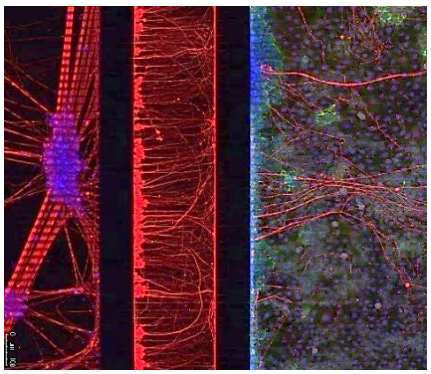
Co-culture x10

(a)



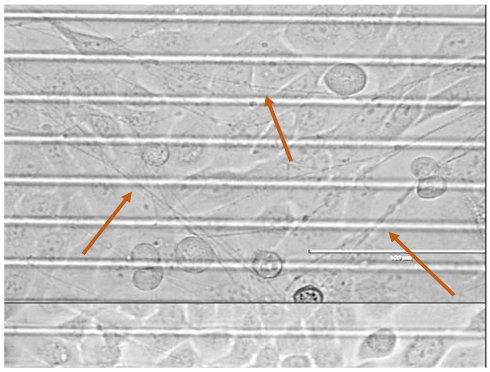
HOECHST/Phalloidin / Neurofilament x10

(b)



Co-culture x40

(c)



HOECHST/Phalloidin / Neurofilament x40

(d)

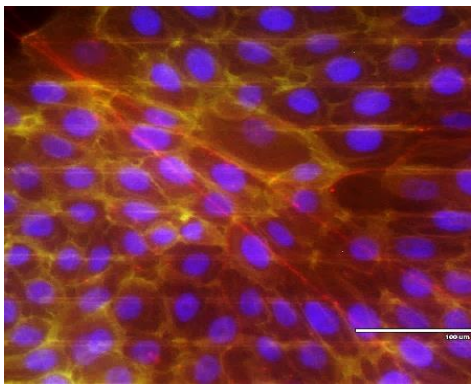


Figure 3: Co-culture of keratinocytes and sensory neurons in microfluidics chip after 3 days. (a) Bright field image of keratinocytes/sensory neurons co-culture, (b) HOECHST (blue) / Phalloidin (green) / Neurofilament (red) immunofluorescent staining, (c) 40X Zoom Bright field image of neuronal axons in contact with keratinocytes (red arrows), (d) 40X Zoom HOECHST (blue) / Phalloidin (green) / Neurofilament (red) immunofluorescent staining performed 3 days after co-culture. Scale bar 400µM.

3.2 Release of Substance P and other neuromediators by co-cultured keratinocytes and sensory neurons specific to virulent Staphylococcus aureus

To study keratinocytes and sensory neurons function in response to microbial components, we first stimulated them with specific nociceptors agonists. Isolated keratinocytes and neurons, as well as co-cultures, responded to the different specifics triggers (data not shown). Immediate release of neuromediators was only observed in compartment 3, where keratinocytes and neuronal axons were located (data not shown). We then proceeded with stimulating the cells with different skin bacteria potentially beneficial or harmful for the host. We selected a strain of beneficial skin bacteria from the skin *S. epidermidis* 12228 (s.e) and a well described virulent strain of *S. aureus* 25904 (s.a) with high potential to induce neurogenic inflammation. We prepared two stimulatory fractions of bacteria: bacterial culture supernatants, potentially containing toxins and proteases, and Heat Killed (HK) bacteria, mainly containing bacterial cells and their wall components. We first evaluated Substance P (SP) release for its major role in neurogenic inflammation leading to itch through sensory neurons [10]. We observed a significant release of SP by keratinocytes alone 2 times higher than control with s.a supernatant and around 10 times higher than control for HK s.a (Fig. 4 (a)). However, s.e supernatants nor HK s.e induced a specific release of SP. Sensory neurons (SN) and co-cultures released SP only in response to HK s.a (Fig. 4 (a)). Co-culture condition showed 10 times higher release of SP compared to SN alone, showing possibly a potentialization of SP release in the co-culture model. Oxytocin and β-endorphin were mainly significantly released by keratinocytes in control non-stimulated condition. Their release was 2 to 3 times higher with s.a supernatant and 10 times higher with HK s.a (Fig. 4 (b) and (c)). This response was also observed in the co-culture condition. Neurotensin, regulating dopamine activity, was specifically released by NS and co-cultures and by both HK s.a and s.e (Fig. 4 (d)). Here, we could show specific release of

neuromediators and specifically SP in response to the virulent s.a and not by beneficial to s.e. We could observe a possible potentialization of responses when keratinocytes were co-cultured and touching neuronal axons. In conclusion, those data highlighted potential cellular communication/interaction between keratinocytes and SN and synergy potentially leading to local neurogenic inflammation. Both supernatants and HK bacteria (10 times higher) were driving the specific release of neuromediators, this suggested that toxins, proteases contained in supernatants as well as cell wall component from total bacteria could induce the specific release of neuromediators. We decided to further investigate the the role cell wall components of s.a in the induction of neuromediators release.

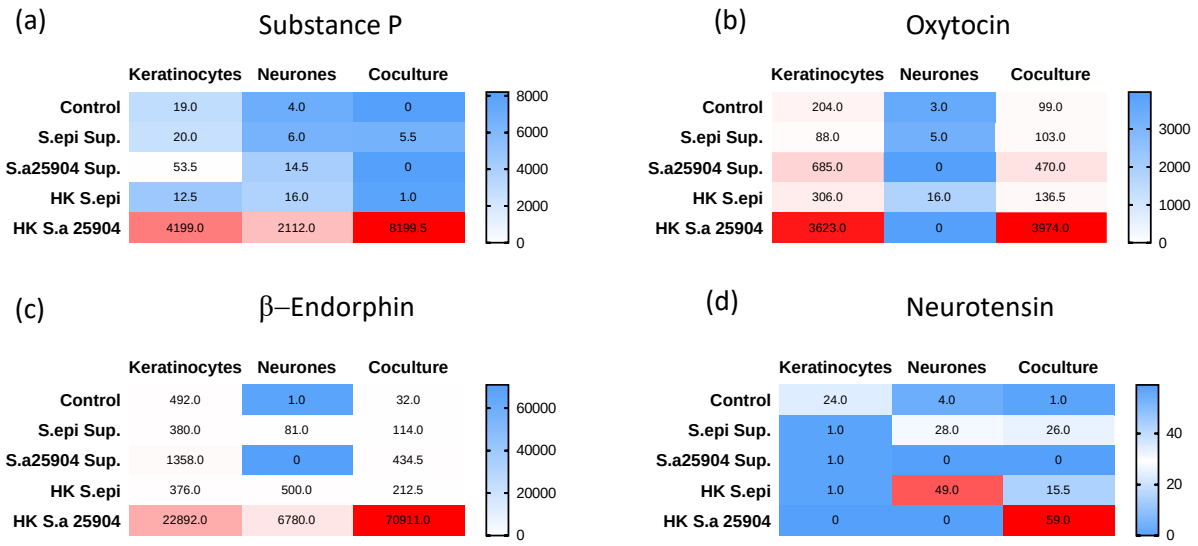


Figure 4: SP, Oxytocin, β-Endorphin and Neurotensin release from keratinocytes, sensory neurons or co-culture treated with s.e or s.a supernatant,1x10^8 Heat Killed (HK) s.e or s.a for 15 min compared to control.

3.3 Substance P and neuromediators release in co-cultured keratinocytes and sensory neurons in response to component cell wall peptidoglycan

We previously showed that HK s.a were driving the strong and specific release of neuromeditors such as SP, Oxytocin, β–endorphin (Fig. 4 and 5). We hypothetized that cell wall component could be generating the specific release of neuromediators and subsequent neurogenic inflammation. Peptidoglycans (PG) and Lipoteichoic acid (LTA) are major cell wall components of gram-positive bacteria such as S*taphylococcus aureus* and drivers of inflammation [11]. We observed that PG and not LTA could induce the release of SP in SN and co-cultures with keratinocytes (Fig. 5 (a)). This SP release by PG was 10 times lower than HK s.a, however 2 times higher in the PG co-cultures than isolated keratinocytes and neurons. We could still observe a potential potentialization of in the co-culture of SN and keratinocytes compared to the different cell types alone. Both PG and LTA could induce the release of Oxytocin and β–endorphin (Fig. 5 (b) and (c)) and at similar concentrations. We could not conclude on the specificity of release of Neurotensin in response to PG and LTA (Fig. 5 (d)). In conclusion, based

on the release of SP, PG showed a potential specific induction of neurogenic inflammation and potentialization in co-cultured SN and keratinocytes. The PG used in those experiments is from *staphylococcus aureus* and we have yet to compare its response to PG from other bacteria such as S*taphylococcus epidermidis.*

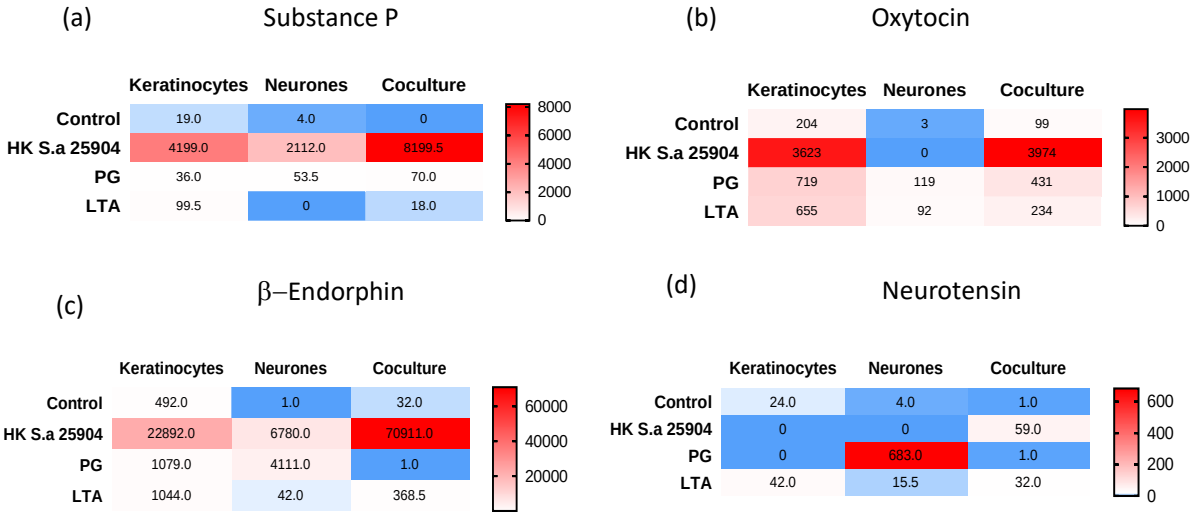


Figure 5: SP, Oxytocin, and β-Endorphin release from keratinocytes, sensory neurons or co-cultures treated with s.a peptidoglycan (PG 500µg/ml) or Lipoteichoic acid (LTA 500µg/ml), $1x10^8$ Heat Killed (HK) s.e or s.a for 15 min compared to control.

3.4 Heat Killed S.a and membrane component peptidoglycans induce keratinocytes activation potentially through TRPV1

We previously showed that s.a, and component cell walls from gram-positive bacteria, such as PG and LTA, could induce a certain release of neuromediators by keratinocytes, SN and co-cultures of both cell types. Immediate release of neuromediators can be mediated by nociceptors and activation of such nociceptors in keratinocytes can be measured by cell calcium entry [12]. We thus measured cell calcium entry in keratinocytes in response to s.a supernatants, HK s.a, PG from *S. aureus* and LTA. PG and HK s.a induced respectively a 7 and 15 times significant calcium flux compared to LTA and s.a supernatants (Fig. 6). Interestingly, HK s.a induced only a transient calcium flux in the cells and amounting to only half the calcium flux from PG. As for neuromediators release, we are unable to estimate the amount of PG in the HK bacteria and in the incapacity to do a fair comparison to conclude on higher activation of nociceptors in keratinocytes by PG compare to HK s.a. However, we can conclude that HK s.a and PG from *S. aureus* can activate keratinocytes through nociceptors.

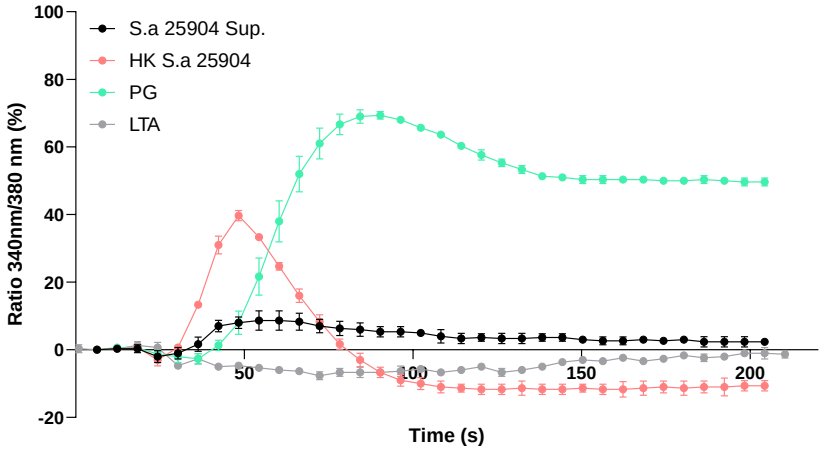


Figure 6: Quantification of calcium mobilization in keratinocytes in response to s.a supernatant, HK s.a or s.a PG.

The major nociceptors implicated in itch are TRPV1, PAR2 and Histamine Receptor [13]. We preincubated keratinocytes with antagonists capsazepin for TRPV1, AZ3451 for PAR2 and astemizole for Histamine Receptor 1 (Hr1) and then stimulated the cells with HK s.a and PG. Antagonizing TRPV1 with capsazepin resulted in the total abrogation of the calcium flux in keratinocytes stimulated with HK s.a or PG (Fig. 7 (a) et (b)). Hr1 antagonizm with astemizole didn't affect the response to PG and partially reduced the calcium flux of 30% in response to HK s.a. Antagonizing PAR2 with AZ3451 resulted in a reverve phenotype with no reduction and 30% decrease of the signal after stimulation with respectively HK s.a and PG. In conclusion, induction of calcium flux resulting in the activation of keratinocytes is dependant on nociceptor TRPV1 for both HK s.a and PG from *Staphylococcus aureus.* TRPV1 is a known final integrator of nociceptors such as PAR2 and Hr1 [14]. HK s.a and PG were partially dependant for their signal on distinct nociceptors, respectively on PAR2 and Hr1. Thus, a distinct stimulatory components from PG in HK s.a might be responsible for the activation of keratinocytes and the release of neuromediators we previously described.

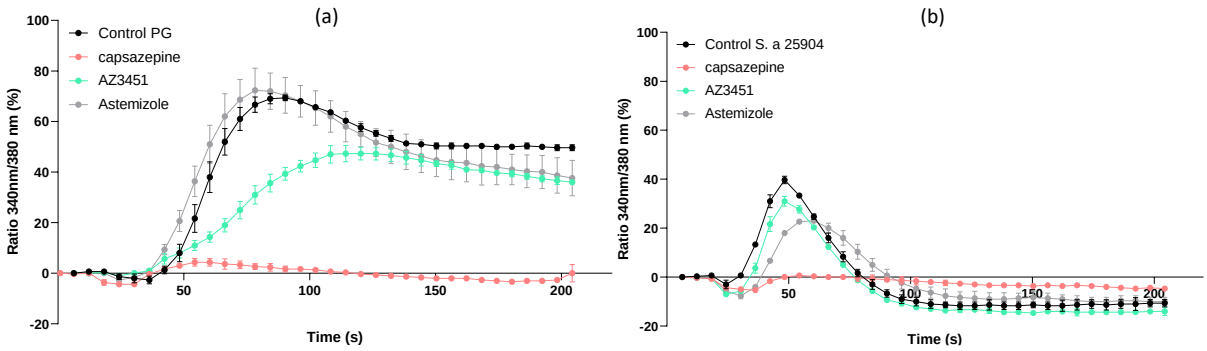


Figure 7: Inhibition of calcium flux by TRPV1, PAR2 and Histamine R antagonists in response to HK s.a or s.a PG. Quantification of calcium mobilization in keratinocytes pretreated for 30 min with CPZ 50µM or 1µM AZ3451 or Astemizole 1µg/ml and then treated with HK S.a or S.a PG.

4. Discussion

This study explores the potential connection between *S. aureus* and neurogenic inflammation, focusing on the interaction between keratinocytes and sensory neurons. We successfully co-cultured these two cell types in a newly available NETRI chips that allow modelization of epidermal interaction between epithelium and the nervous system. We observed potential communication between keratinocytes and sensory neurons, as they were physically in contact in the confocal microscopic imaging. Such contacts were observed in other co-cultures set-up [9] and we need to confirm if actual synapses can form between keratinocytes and sensory neurons in our system. We could demonstrate that cells cultured in those NETRI chips were functional as releasing neuromediators specific to non-virulent beneficial *S. epidermidis* and virulent *S. aureus*. Indeed, virulent *S. aureus* specifically induced the release of Substance P and up to 10 times more Oxytocin and β-endorphin compare to beneficial commensal *S. epidermidis.* This response seemed linked to cell body components rather than secreted toxins and proteases as the response to heat-killed *S. aureus* was 10 times higher than with the supernatant. We didn't characterize the supernatants and need further investigation to determine if we were in optimal concentrations of proteases and toxins to have a significant response. We further tested cell wall components and PG from *S. aureus* rather than LTA were able to stimulate the release of neuromediators. Both HK *S. aureus* and PG release of neuromediators was significantly amplified when keratinocytes and sensory neurons were co-cultured, pointing to a synergistic relationship in neurogenic inflammation.
We then investigated the possible role of nociceptors like PAR2, H1R (Histamine Receptor 1), and TRPV1 in the neurogenic inflammation to HK s*.aureus* and PG. PG appeared to signal partially through PAR2, while HK s*.aureus* signaled partially through H1R, with TRPV1 acting as a downstream integrator of these signals. This suggested that distinct components of *S. aureus* and its PG activate different pathways, ultimately converging on neurogenic inflammation.

5. Conclusion

Several important avenues for future research emerge from this study. Firstly, the precise nature of the communication between keratinocytes and sensory neurons, including the formation of synapses, requires further investigation. Secondly, comparing the effects of PG from *S. aureus* to PG from other skin-resident bacteria like *S. epidermidis* will help determine the specificity of these interactions. Finally, a deeper understanding of the molecular mechanisms involved in the activation of nociceptors and the subsequent release of neuromediators will be crucial for developing targeted strategies to decrease neurogenic inflammation from skin microbes. This research provides a foundation for future investigations into the complex interplay between skin bacteria, keratinocytes, and sensory neurons in the context of inflammatory skin conditions. Specifically, this model opened the path to investigate more extensively the role of

the microbiome in itch and will help to develop new technologies to manage itch in skin inflammatory conditions.

6. Acknowledgments

NETRI team who provided us technical expertise for chip use and cell culture in microfluidic Dualink™ Ultra chips.

References

1. Schonmann, Y., et al., *Atopic Eczema in Adulthood and Risk of Depression and Anxiety: A Population-Based Cohort Study.* J Allergy Clin Immunol Pract, 2020. **8**(1): p. 248-257 e16.
2. Bawany, F., et al., *Sleep Disturbances and Atopic Dermatitis: Relationships, Methods for Assessment, and Therapies.* J Allergy Clin Immunol Pract, 2021. **9**(4): p. 1488-1500.
3. Kishi, R., et al., *Effects of Dupilumab on Itch-Related Events in Atopic Dermatitis: Implications for Assessing Treatment Efficacy in Clinical Practice.* Cells, 2023{Kishi, 2023 #297}. **12**(2).
4. Dainichi, T., et al., *The epithelial immune microenvironment (EIME) in atopic dermatitis and psoriasis.* Nat Immunol, 2018. **19**(12): p. 1286-1298.
5. Deng, L., et al., *S. aureus drives itch and scratch-induced skin damage through a V8 protease-PAR1 axis.* Cell, 2023. **186**(24): p. 5375-5393 e25.
6. Deng, L., et al., *Sensory neurons: An integrated component of innate immunity.* Immunity, 2024. **57**(4): p. 815-831.
7. Maisonneuve, B.G., et al., *Rapid mask prototyping for microfluidics.* Biomicrofluidics, 2016. **10**(2): p. 024103.
8. Miny, L., et al., *Modeling Neurodegenerative Diseases Using In Vitro Compartmentalized Microfluidic Devices.* Front Bioeng Biotechnol, 2022. **10**: p. 919646.
9. Talagas, M., N. Lebonvallet, and L. Misery, *Intraepidermal nerve fibres are not the exclusive tranducers of nociception.* J Neurosci Methods, 2018. **306**: p. 92-93.
10. Azimi, E., et al., *Substance P activates Mas-related G protein-coupled receptors to induce itch.* J Allergy Clin Immunol, 2017. **140**(2): p. 447-453 e3.
11. Wang, J.E., et al., *Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism.* Shock, 2003. **20**(5): p. 402-14.
12. Marek-Jozefowicz, L., et al., *Molecular Mechanisms of Neurogenic Inflammation of the Skin.* Int J Mol Sci, 2023. **24**(5).
13. Yosipovitch, G., T. Berger, and M.S. Fassett, *Neuroimmune interactions in chronic itch of atopic dermatitis.* J Eur Acad Dermatol Venereol, 2020. **34**(2): p. 239-250.
14. Yosipovitch, G., J.D. Rosen, and T. Hashimoto, *Itch: From mechanism to (novel) therapeutic approaches.* J Allergy Clin Immunol, 2018. **142**(5): p. 1375-1390.