

A new competent re-innervated human skin model mimicking sensitive skin

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Abstract

Sensitive skin is a syndrome characterized by unpleasant sensations in response to chemical or physical stimuli that do not induce similar perceptions in normal skin. Different studies have demonstrated that SS is characterized by a defective intra-epidermal nerve fibers network, resulting in hyperreactive skin with neurogenic inflammation. Few experimental approaches, mainly based on the use of rodent neurons, are available to evaluate this process.

In this study, we have developed a reinnervated human skin explant model mimicking SS using sensory neurons differentiated from human skin-derived precursor (SKPs). To study the functionality of this model, re-innervated skin explants were treated with lactic acid (LA). The molecular profile was characterized by transcriptomic analysis and immuno-histochemistry. LA induces a specific neurogenic inflammation, provoking the release of pro-inflammatory mediators affecting potentially peripheral nerve integrity. To determine the soothing activity of a mucilage-rich polysaccharide extracted from the seeds of *Plantago lanceolata* L, reinnervated human skin explants were topically treated with this vegetal compound upon LA exposure. These findings suggest that *Plantago lanceolata* seed mucilage could be a promising active for treatment and prevention of sensitive skin.

Introduction

In recent years, the prevalence of sensitive skin has been increasing worldwide, driven by worsening environmental pollution and rising work-related stress. Sensitive skin (SS) is a syndrome defined as “the occurrence of unpleasant sensations (stinging, burning, pain, pruritus, and tingling sensations) in response to various stimuli that normally should not provoke such sensations”. An increasing body of scientific evidence supports the hypothesis that SS represents a form of small-fiber neuropathy characterized by reduced intraepidermal nerve fiber density, abnormalities in quantitative sensory testing, and associations with conditions such as irritable bowel syndrome and ocular sensitivity [1].

Moreover, SS is related to a sensory neurons (SN)-mediated neurogenic inflammation triggered by the release of neuropeptides such as substance P (SP) and calcitonin gene related protein (CGRP). Moreover, skin cells themselves, such as keratinocytes, can also release neurotrophic factors affecting nerve fiber density, morphology, axon growth, and neuropeptide levels.

To date several models are available for evaluating the soothing efficacy of actives or dermo-cosmetic products intended for sensitive skin. These models are mainly based on *in vitro* cultured human SNs, or a co-culture of human primary keratinocytes and neurons.

These models lack the tridimensional architecture and cellular diversity proper to human skin. Overcoming these limits, the development of a more complex model, including *ex vivo* re-innervated living human skin explants is a key need in dermatological research [2].

The first attempts to develop such model were reached few years ago with the set-up of a re-innervated human skin explant using primary SNs from the dorsal root ganglia of rat [3]. However, this model could not be used in the cosmetic field due to regulatory constraints. The

aim of the present research was to develop a fully human re-innervated *ex vivo* skin model, integrating SN of human origin. The response of this model to LA has been deeply characterized using different approaches, allowing us to mimic a sensitive skin-like molecular profile characterized by neurogenic inflammation [4].

Finally, to determine the reversibility of this LA induced neurogenic inflammation, reinnervated skin explants were treated with seed mucilage from *Plantago lanceolata* (PML).

PML, commonly known as ribwort plantain, is a perennial species from the Plantaginaceae family with a worldwide distribution, including widespread presence in European flora. Well recognized for its medicinal properties, *P. lanceolata* exhibits among other important anti-inflammatory activities, seems to lie in the richness in mannose, a polysaccharide known for anti-inflammatory properties.

We showed that PML can reverse the LA-induced inflammation and increase epidermal nerve fiber density, suggesting that PML is a promising molecule for the treatment and prevention of sensitive skin.

Materials and methods

Isolation and Cultivation of Human Skin-derived precursor cells

Small pieces of skin were obtained from abdomen of healthy donors who had undergone plastic surgery. Epidermis and dermis were mechanically separated in thermolysin (Sigma-Aldrich, ref: T7902), incubated in collagenase IV (Sigma-Aldrich, ref: C1889) and finally dissociated by trypsin/EDTA (Lonza, ref: BE17-161E). Obtained cells were maintained in DMEM/F12 mixture (DMEM and DMEM/F12; Lonza, ref: BE12-604F) with B27 (without vitamin A; Gibco, Thermo Fisher Scientific, ref: 12587-001), LIF (Leukemia Inhibitory Factor; Santa Cruz Biotechnology, ref: sc-4377,), EGF (Epidermal growth factor; Sigma-Aldrich, ref: E9644) and FGF (Fibroblast growth factor; Sigma-Aldrich, ref: F0291). After 1 month, some SKPs were adherent [5].

Induction of SNs Differentiation from human SKPs

Adherent cells were plated in precoated with poly-L-ornithine (Sigma-Aldrich, ref: P3655)/laminin (Sigma-Aldrich, ref: L2020)/fibronectin (Miltenyi Biotech, ref: 130-109-393) dishes and expanded in the maintenance medium. To initiate differentiation into SNs, the maintenance medium was replaced with DMEM/F12/Neurobasal (Gibco, Thermo Fisher Scientific, ref: 21103-049) that contained NeuroBrew-21 (Miltenyi Biotech, ref: 130-093-566), N2 (Gibco, Thermo Fisher Scientific, ref: 17502-048), CHIR99021 (Sigma-Aldrich, ref: SML1046), purmorphamin (Sigma-Aldrich, ref: SML0868), and ascorbic acid (Sigma-Aldrich, ref: PHR1008). Then purmorphamin and ascorbic acid were removed from the medium. BMP4 (Sigma-Aldrich, ref: SRP3016) was added to the medium. For maturation of SNs, the medium was composed of different neurotrophic factors such as NGF (Nerve Growth Factor), GDNF (Glial cell-derived neurotrophic factor), BDNF (Brain-Derived Neurotrophic Factor), and dibutyryl cyclic AMP (dbcAMP; Sigma-Aldrich, ref: D0260) (maturation medium).

Human skin explants and SNs co-culture

SNs obtained from SKPs were seeded in precoated poly-L-ornithine/laminin/fibronectin inserts to obtain a neuronal network in maturation medium. Full-thickness human skin biopsies were obtained from abdomen of healthy female donor who had undergone plastic surgery, in accordance with the Declaration of Helsinki. Skin biopsies were maintained in survival in BIO-EC's Explants Medium (BEM) at 37°C in 5% CO₂ and they were put in contact with the obtained neuronal network and co-cultured under classical cell culture conditions (37°C in 5% CO₂) in BEM supplemented with NGF for several days to obtain a re-innervated tissue.

Re-innervated skin explants treatment

A part of re-innervated skin explants was treated four times in a preventive manner with a vehicle +/- PLM extract (2 mg/cm²) to characterize the shooting activity of this product. When required, a part of re-innervated skin explants was topically stimulated by 10% LA (Sigma-Aldrich, L1750) and systemically with 0.1% LA 24 hours (h) before ending the experiments. Control explants receiving no treatment. Fifteen minutes (min) after LA stimulation, the supernatants were frozen with anti-proteases at -20°C for biochemical analyzes. At the end of the experiment, skin explants were processed as following. A third was fixed in buffered formalin solution for 24 h then impregnated with paraffin and embedded (Leica PEARL and EG 1160) for evaluation of the skin morphology and immunostainings. A third was preserved in RNAProtect (Qiagen, ref: R76106) for the transcriptomic study. A third was put in 4% paraformaldehyde (ThermoFisher, ref: J61899) for 24 h, then after rinsing in 10% sucrose for an equivalent time, it was quickly frozen at -80°C being used subsequently for specific PGP9.5 immunostaining.

Induction of SNs Differentiation from hiPS

For *in vitro* experimentation performed Neuron Experts, sensory neurons were derived from hiPS cells (human induced Pluripotent Stem cells) obtained from human fibroblasts. hiPS were plated in precoated Matrigel® (Corning, ref: 354277) plate in a differentiation medium. This medium consisted of DMEM-F12 (Panbiotech, ref: P04-41450) supplemented with KnockOut Serum Replacement (KSR, Life Technologies, ref: 10828028), retinoic acid, EPO (Erythropoietin; Sigma, ref: R4643) and a cocktail of central differentiation pathway inhibitors. Cells were maintained in culture for 6 days at 37°C and 5% CO₂. Under these conditions, hiPS cells differentiated into human sensory neurons.

Co-culture of SNs with human keratinocytes

Keratinocytes derived from an adult donor were seeded on top of the neurons in a culture medium composed of 2/3 maturation medium for sensory neurons and 1/3 keratinocyte growth medium. The maturation medium consisted of DMEM-F12 supplemented with different neurotrophic factors such as N2 supplement, BDNF, GDNF, NGF and NT3 (Neurotrophin-3). After 8 days of co-culture, the medium was replaced with a medium composed of two-thirds sensory neuron maturation medium without growth factors and one-third keratinocyte growth medium, supplemented or not with PLM extract at 1% and 0.3%. The co-culture was maintained for 4 additional days. For this study, co-cultures were established in 6 replicate wells for each experimental condition.

Microscopy

For SNs derived from SKPs analysis, cells were fixed and frozen. After drying, cells were permeabilized with 0.1% Triton X-100 and saturated with 10% goat serum and 1% bovine serum albumin (BSA) (Sigma-Aldrich, ref: PK-7200). Cells were incubated with primary antibodies anti- β-tubulin III (Abcam, ref: ab78078), anti-neurofilament (Abcam, ref: ab8135) and anti-peripherin (Abcam, ref: ab4666). Then, cells were incubated with the secondary antibody (Life Technologies, ref: A11008 / A11001). Microscopical observations were realized using a BX63 or BX43 Olympus microscope. Pictures were digitized with an Olympus DP camera and the Cell Sens data storing software.

For SNs derived from hiPS analysis, cells were fixed and permeabilized. Then, cells were saturated with 0.1% saponin (Sigma-Aldrich, ref: S7900), 1% BSA (Sigma Aldrich, ref: A2153), and 1% goat serum (Life Technologies, ref: 16210072). Cells were incubated with an anti-β-tubulin antibody (Sigma Aldrich, ref: T8660). Then, cells were incubated with the secondary antibody (Life Technologies, ref: A32723). Microscopical observations were realized using an automated microscope InCell 2200 (GE Healthcare). Sensory neuron cell bodies were counted, and the length of neurite extensions was measured and normalized to the number of neuronal cell bodies stained.

For skin explants analysis, formol-fixed paraffin-embedded sections (5 μm) were saturated in horse serum (Vector, ref: PK-7200). Skin sections were incubated with primary antibodies anti-

TSLP (Abcam, ref: ab188766), anti-COX-2 (Santa Cruz Biotechnology, ref: sc-376861), anti-TRPV-1 (Abcam, ref: ab3487) and anti-IL-6 (LSBio, ref: LS-A9692). After that, sections were incubated with the secondary biotinylated antibody (Vector, ref: PK-7200). After drying, frozen explant skin sections (30 µm) were saturated with 10% BSA and then incubated with the primary antibody anti-PGP9.5 (Abcam, ref: ab27053). Successively, sections were incubated with the secondary antibody (Lifetechnologies, ref: A11008). The microscopical observations were realized using a BX63 or BX43 Olympus microscope. Pictures were digitized with an Olympus DP camera and the Cell Sens data storing software. Image analysis were performed with the cellSens data storing software.

Biochemical analysis

Substance P ELISA was realized according to manufacturer's instructions (Cayman, ref: 583751).

Transcriptomic analysis

Total RNAs were extracted using the ReliaPrep Tissue Miniprep system from Promega (Z6111) and used for reverse transcription, amplification and cRNA synthesis with Cy3 labeling. All cRNAs were hybridized to human whole genome oligo microarrays (Agilent Technologies V3 AMADID072363) and normalized with R tools. Induced (fold change (FC) ≥ 1.5) or repressed genes ($FC \leq 0.65$) between (I vs NI or ALI vs I) conditions were selected. An enrichment analysis was performed by Metascape [6], to identify the significant keys words associated with induced genes.

***Plantago lanceolata* seed mucilage extraction**

An aqueous extraction solution is used. Whole *Plantago lanceolata* seeds, free of any debris or foreign matter, are introduced into the extraction reactor at a temperature of 70°C (1 kg of seeds + 10 kg aqueous solution). The seeds are extracted for 2 h at 70°C with vigorous stirring using counter-blades. The extraction mixture is then hot-filtered several times to remove the majority of the seeds. The resulting extract is reintroduced into a stirred tank and heat-treated at 90°C for 1 h to reduce the microbial load. A preservative system is introduced during the cooling phase between 80-90°C with vigorous stirring. This preservative system is chosen from hexanediol in a proportion of 3.5% in the final mixture. The resulting extract is filtered one last time while hot (at least 70-80°C). Finally, the resulting product is returned to a reactor for homogenization while hot at 50°C for 30 min to obtain the final product.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis or Student's t-test. A p-value of <0.05 was considered statistically significant; *: $p < 0.05$ and **: $p < 0.01$.

Results and discussion

Validation of re-innervated human skin explant model with SNs

The aim of the first part of the project was to characterize and validate the functionality of the re-innervated ex vivo skin model. To this purpose, we firstly confirm that SKPs differentiated into sensory neurons (SNs). Cell cultures were immunostained with different neuronal markers (Figure 1a-b-c). The obtained cells were positive for β -tubulin III and neurofilaments, specific neuronal markers, and for peripherin, a SN marker. Moreover, microscopical observations of the obtained sensitive neurons prior to co-culture with skin explants shows the establishment of a dense neuronal network (Figure 1d).

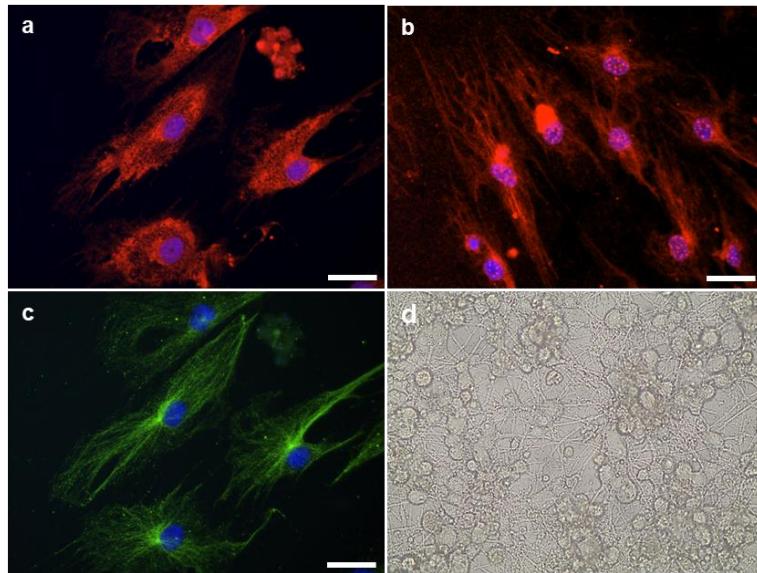


Figure 1. Establishment of a SN network. Immunofluorescence staining of β -tubulin III (a), neurofilaments (b) and peripherin (c) of differentiated SKPs into SNs during the maturation step. The cells were counterstained by DAPI (blue). Scale bar = 20 μ m. Images of obtained neuronal network just before co-culture with skin explant using phase-contrast microscopy (d). Scale bar = 20 μ m.

The upper view of a human skin explant co-cultured with SNs shows several fibers reaching the tissue (Figure 2a). To confirm the presence of sensory nerve fibers in the epidermis of human skin explant which has undergone a co-culture phase, specific PGP9.5 immunostaining was performed on skin sections (Figure 2b). On not-reinnervated skin explants, just some residual sensory nerve fibers have been detected. After several days of contact with the neuronal network derived from SKPs, the number of sensory nerve fibers was significantly increased (Figure 2c) by 147% ($p<0.01$) (Figure 2c).

Moreover, transcriptomic analysis by microarray reveals a specific gene expression profile in re-innervated condition. Human skin explant co-cultured with SNs shows over-expression of VGF (FC=71), a gene encoding for a neuropeptide selectively distributed in neurons and neuroendocrine tissues which plays a pivotal role in synaptic plasticity. The gene SNAP25 is also overexpressed (FC=40) upon re-innervated condition. SNAP25 is a component of the SNARE protein complex, which is involved in neurotransmitter trafficking by regulating the exocytotic release of neurotransmitters during synaptic transmission [7].

To investigate the functionality of the model developed in the present project, we have transposed on our ex vivo skin model, the LA stinging test (LAST), which is considered the gold *in vivo* method to discriminate between non-sensitive and sensitive skin. The stimulation of re-innervated skin explants with LA results in an increase of the neuropeptide substance P (SP) release in the culture medium compared to the untreated condition (Figure 2d). Mainly secreted from the skin by nerve endings, SP plays a major role in itching and neuroinflammation. It binds to keratinocytes or mast cells, and it induces successively the

release of pro-inflammatory cytokines. SP is involved in vasodilation, activation of B lymphocytes and proliferation of keratinocytes and fibroblasts [8].

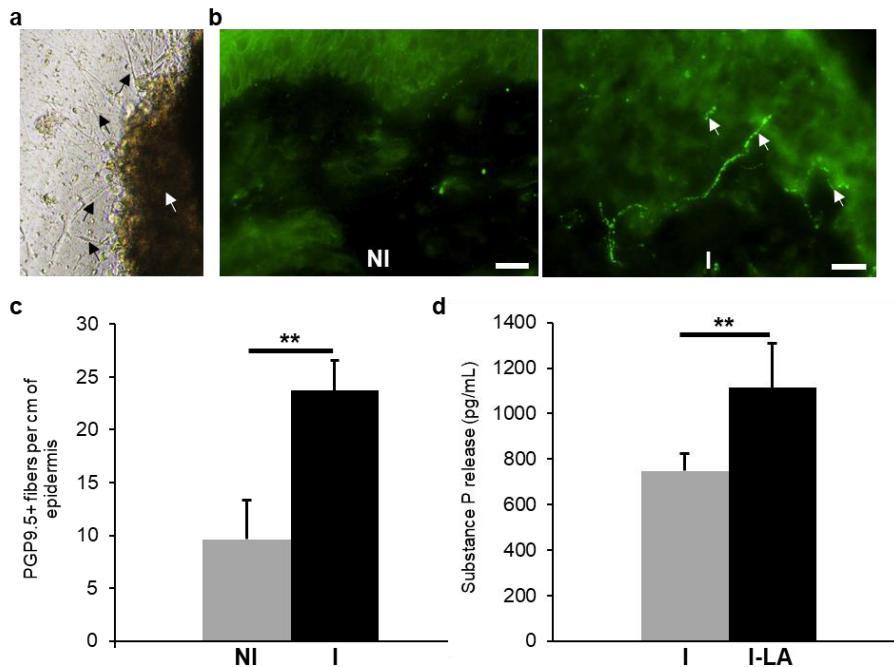


Figure 2. Re-innervation of the human skin explants. (a) Top view of a part of skin explant (white arrow) co-cultured with sensory neurons (black arrows) which reach the tissue (b). Immunostaining of PGP9.5 in non-re-innervated skin explant (NI) and in skin explant co-cultured with SNs (I) with the detection of nerve fibers in the epidermis and dermis (arrows). Scale bars: 20 μ m. In (c), quantification of PGP9.5 positive nerve fibers per centimeter of epidermis which reach the dermal-epidermal junction in non-re-innervated skin explant (NI) and in re-innervated skin explant (I). In (d), Substance P release in the culture medium of re-innervated skin explant in normal conditions (I) or upon LA stimulation (I-LA). Graphics represent the mean +/- SEM, **p < 0.01. n=3 explants per condition

Moreover, among 39 induced genes (I vs NI), several genes were associated with biological pathways such as neuron projection development (GO:0031175: NCAM1, NPY, SNAP25, STMN2, PACSIN1, TET1, STMN4, GREM1) or synaptic vesicle cycle (GO:0099504: SH3GL3, SNAP25, SNAP91, PACSIN1, GREM1, KIF1A, RREEP2, CHCHD10, CCDC136, Figure 3a), suggesting that co-cultured of skin explants with SNs cells promotes neuronal biological processes.

The enrichment analysis results using acid lactic-induced genes compared to I condition highlighted the induction of several biological process including inflammation, dermatologic disorders and brain ischemia (Figure 3b). Indeed, LA stimulation of re-innervated human skin explant led to an over-expression of genes involved in neurogenic inflammation, driven mainly by *IL-6* ($FC=3.44$), *IL-20* ($FC=6.10$), *CSF3* ($FC=8.32$) and *PTGS2* ($FC=1.85$) (Figure 3c). In the epidermis, the release from the nerve fibers of neuropeptides such as SP, stimulates keratinocytes to produce proinflammatory cytokines including IL-6 or IL-20. It has been shown that IL-20 is implicated in peripheral (skin-derived) itch and in intercellular neuron-epidermal communication. *PTGS2* encodes the enzyme Cyclooxygenase-2 (COX-2) which synthesizes the proinflammatory mediator prostaglandins which in turn plays a regulatory role in neuroinflammation [9]. These results suggest that LA stimulation on re-innervated skin explants can be used to mimic a sensitive skin-like molecular profile which is characterized by neurogenic inflammation.

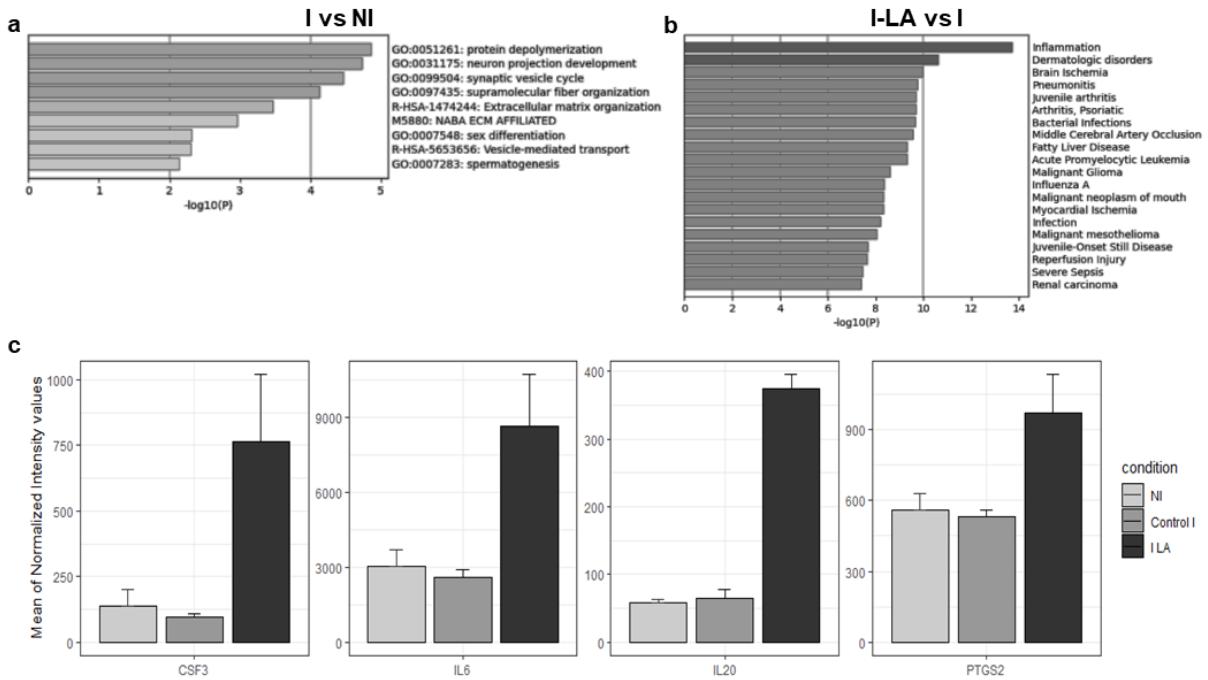


Figure 3. Effects of re-innervation and LA stimulation on transcriptome of skin explants model.
 (a) Enriched biological key words associated with induced gene (FC ≥ 1.5) lists upon re-innervation compared to non-reinnervated samples and (b) upon LA stimulation on re-innervated explants, compared to innervated ones. (c) Barplots representing microarray normalized intensity value mean (arbitrary units) for CSF3, IL6, IL20 and PTGS2 genes, in non-reinnervated skin explants (NI), re-innervated explants (Control I), or re-innervated explants upon LA stimulation (I-LA). Graphics represent the mean \pm SEM. n=3 explants per condition.

The neuroinflammatory profile induced by LA stimulation was confirmed at the protein level by immunostaining (Figure 4). After LA stimulation, the expression of IL-6, IL-20 and COX-2 was respectively increased by 64% ($p<0.01$), 20% ($p<0.05$) and 96% ($p<0.01$) (Figure 5a-b-c). Moreover, complementary immunostaining analysis have shown that LA stimulation is associated with an increase of TSLP (Thymic Stromal Lymphopoietin) expression by 33% ($p<0.01$) and NMB (Neuromedin B) expression by 128% ($p<0.01$) (Figure 5d-e). TSLP is a cytokine highly expressed by keratinocytes and it is considered as a primary pruritogen that can directly activate neurons and induced pruritus. NMB is a neuropeptide released by SNs and it is selectively required for transmitting pruritus [10].

Taken together these results demonstrate that the fully human re-innervated skin model developed can mimic sensitive skin with the induction of a significant neurogenic inflammation and pruritus.

Evaluation of the soothing properties of PLM extract

Seed mucilage from *Plantago lanceolata* (PLM) is a type of polysaccharide mucilage obtained from the Plantaginaceae family plant. Originally native to Eurasia, *Plantago lanceolata* has successfully spread around the globe. PLM consists of an arabinoxylomannan-type polysaccharide rich in mannose. Since mannose has well-known anti-inflammatory properties, PLM could represent a new solution for the treatment and prevention of sensitive skin.

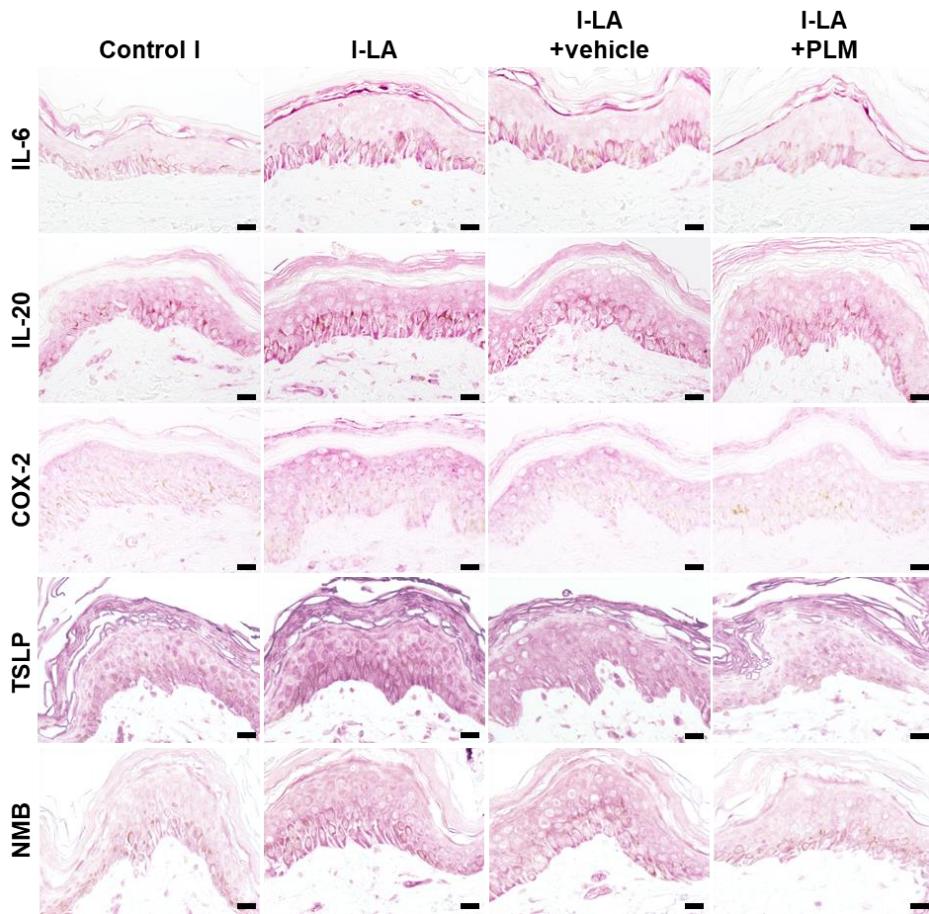


Figure 4. Re-innervation of the human skin explants. Immunostaining of IL-6, IL-20, COX-2, TSLP and NMB on re-innervated skin explant (Control I), re-innervated skin explant stimulated with LA (I-LA), re-innervated skin explant pre-treated with the basic formula and stimulated with LA (I-LA+vehicle) and re-innervated skin explant pre-treated with PLM and stimulated with LA (I-LA+PLM). Scale bar = 20 μ m.

In the present study, the ability of a cosmetic formula incorporating PML to reduce cutaneous neurogenic inflammation was evaluated using the re-innervated skin model previously characterized. First, histological hematoxylin-eosin staining revealed that all the skin samples stimulated with LA and treated or not with PLM extract, shows a tissular morphology and cellular organization similar to untreated samples (data not shown) proving that LA stimulation or PLM extract treatment does not affect the viability of the tissue.

After that, the neurogenic inflammatory environment of the model upon LA stimulation and the anti-inflammatory properties of PLM were characterized by microscopy. We showed that after the preventive treatment with PLM extract in a neuroinflammatory context, IL-6, IL-20 and COX-2 expression was respectively decreased by 17% ($p<0.05$), 28% ($p<0.05$) and 67% ($p<0.01$) (Figure 5a-b-c). PLM application was also associated with a decrease of pruritus associated markers. More specifically, TSLP and NMB expression was significantly decreased by 37% ($p<0.01$) and 45% ($p<0.01$) respectively, compared to the vehicle application (Figure 5d-e). Moreover, the preventive application of PLM can increase the number of sensory nerve fibers by 185% ($p<0.01$) in comparison with the vehicle application (Figure 5f). SS is characterized by a decrease in term of number and density of intra-epidermal end fibers [1], and in this context, we also tested the ability of PML to modulate these parameters. Using 2D based model, we have demonstrated that PML also contributes to activate the neuritic growth of sensory neurons. Indeed, co-culture model of sensory neurons and keratinocytes shows that PLM treatment at 3% induces an increase of neurite length by 25%# (Figure 6a-b).

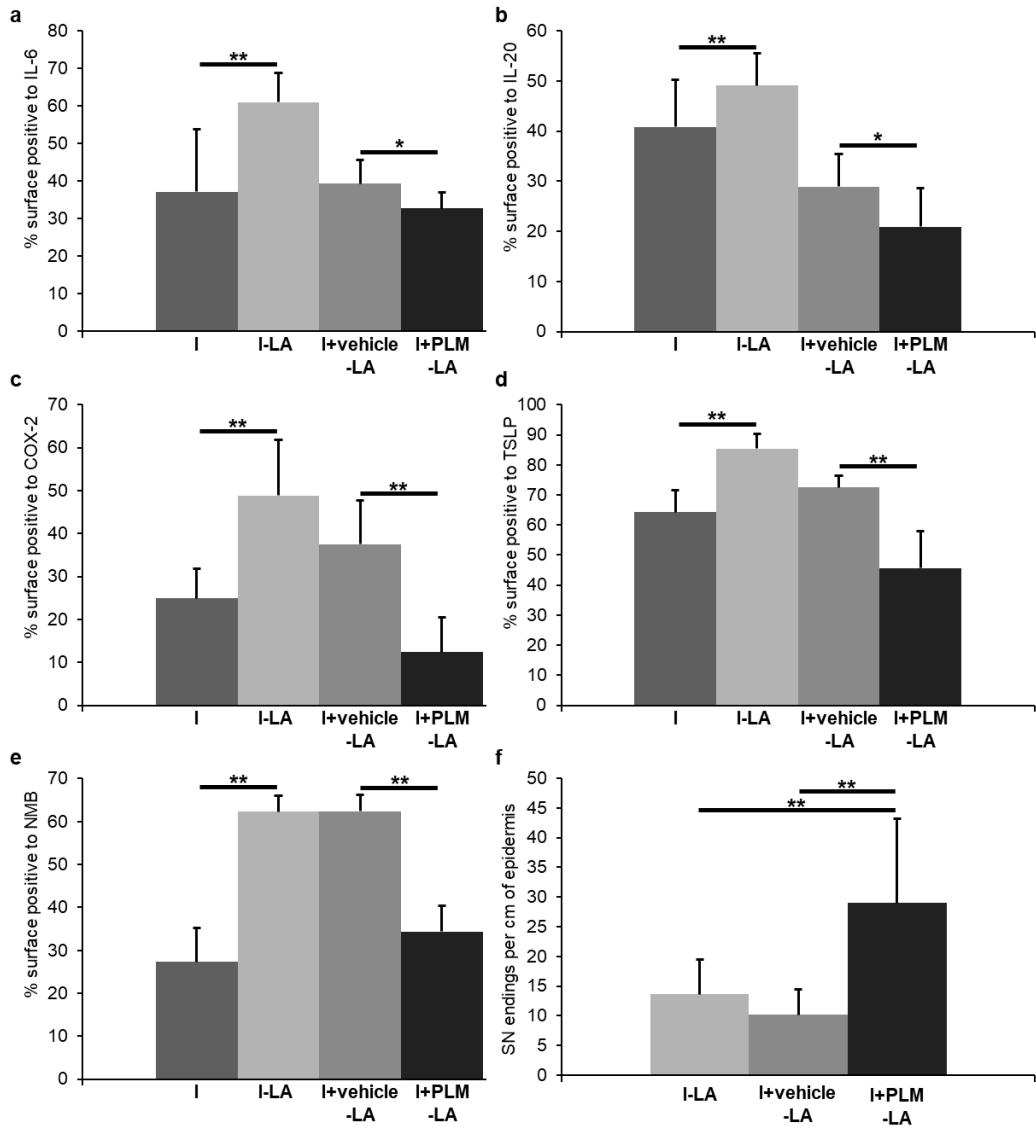


Figure 5. Soothing effect of PLM extract. Image analysis of epidermal surface percentage positive to IL-6 (a), IL-20 (b), COX-2 (c), TSLP (d) and NMB (e) immunostaining on re-innervated skin explant (Control I), re-innervated skin explant stimulated with LA (I-LA), re-innervated skin explant pre-treated with the basic formula and stimulated with LA (I-LA+vehicle) and re-innervated skin explant pre-treated with PLM and stimulated with LA (I-LA+PLM) samples. (f) Counting of sensory nerve endings per cm of epidermis after PGP9.5 immunostaining. Graphics represent the mean +/- SEM, *p < 0.05, **p < 0.01.

These results suggest that PLM could reestablish contact between sensory nerves and keratinocytes in a sensitive skin context and prevents skin neuroinflammation and pruritus induction.

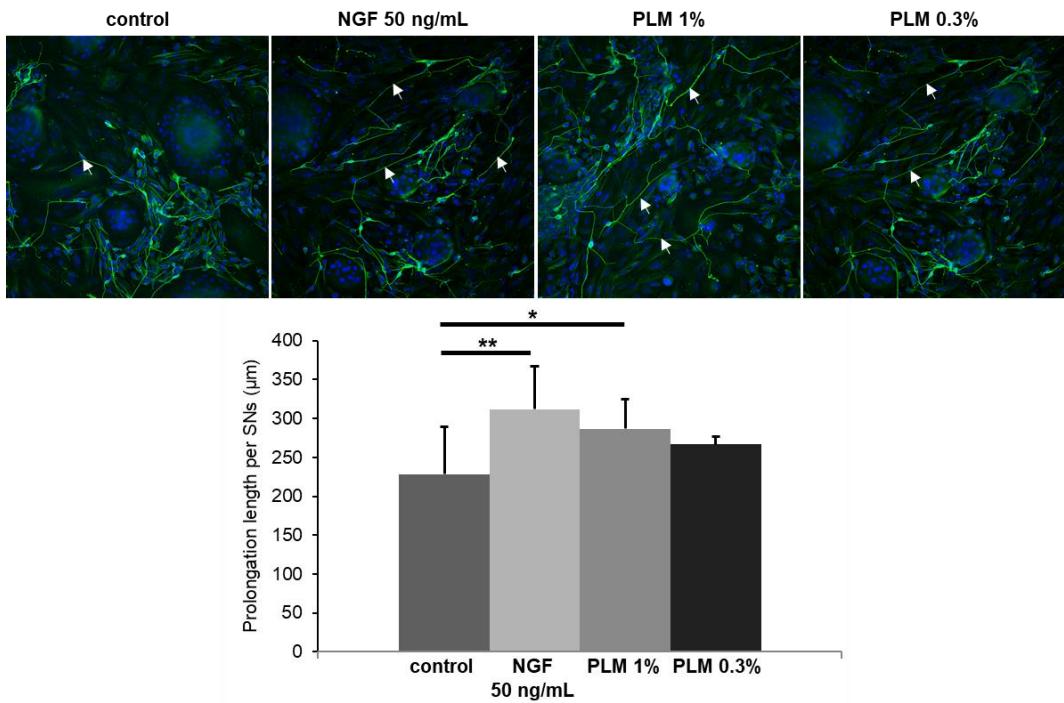


Figure 6. Effect of PLM extract on neurite growth. (a) Immunofluorescence staining of β -tubulin on human keratinocyte and SN co-culture in control condition (without any treatment), treated with NGF 50 ng/mL, PLM extract 1% or PLM extract 0.3%. The cells were counterstained by Hoechst (blue). White arrows indicate long neurite extensions. (b) Evaluation of neurite growth by calculation of the length of neurites (μm) divided by the number of SNs in control condition (without any treatment), after treatment with NGF 50 ng/mL, PLM extract 1% or PLM extract 0.3%. Graphic represents the mean \pm SEM, * p < 0.05, ** p < 0.01.

Discussion and conclusion

SS is an unpleasant condition of the skin extremely impacting the quality of life of the concerned subjects. The development of models allowing to one side to mimic SS and to other side to facilitate the development of molecules with soothing activity is a real societal need. So, we have developed a fully human skin model integrating a functional intra-epidermal nerve fibers network. To validate the functionality of this model, reinnervated skin explants were treated with LA, whose molecular effects on the skin have been poorly studied although it is routinely used *in vivo* as inducer of erythematous reaction in subjects with SS.

We demonstrated that LA induces a specific neurogenic inflammation, provoking the release of pro-inflammatory mediators affecting potentially peripheral nerve integrity. To conclude, we investigated the soothing effect of a seed mucilage-rich polysaccharide demonstrating that this model can be easily used for the development of new compounds for SS treatment.

Taken together these results demonstrate that the model developed in the present project is a robust and useful tool for the cosmetic industry allowing, potentially, the characterization of active ingredients and end dermo-cosmetic products aimed to treat sensitive skin.

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