Study of the Effect of *Leonurus Japonicus* Extract on Atopic Dermatitis

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Abstract

Leonurus Japonicus (LJ) is one of the many plants that constitute Chinese and Kampo traditional medicines, particularly known for its antioxidant and anti-inflammatory properties. Millions of people around the world suffer from inflamed sensitive skin and dryness, which is caused by many external factors such as temperature, pollution, and way of life, but also intrinsic factors linked to genetic disposition. For example, atopic dermatitis (AD) is one of the most common chronic skin diseases caused by genetic predisposition, affecting an important part of the human population. The purpose of this study was to investigate the ability of LJ extract to regulate key genes and protein expression involved in the initiation of AD. For this, an ex-vivo test using a cytokine cocktail was applied to induce AD on skin explants, as well as treatments with LJ 24 hours before the stress and during 24 hours of AD stress. LJ treatments was applied at three concentrations (0.01%, 0.02%, and 0.03%) along with cyclosporin (1µM), a positive control of inhibition of TSLP production, were investigated using quantitative PCR and immunohistochemistry (IHC). After an increase of TSLP in the epidermis induced by the cytokine cocktail, we provide evidence that the LJ extract represses the expression of TSLP at mRNA and protein levels. Moreover, we hypothesized that this active ingredient may also act on this condition by directly controlling key components of the inflammasome, whose dysregulation plays a major role in AD physiopathology. In conclusion, LJ may be a promising solution against AD.

Keywords: Leonurus Japonicus; Inflammation; Atopic Dermatitis; Active Ingredient

Introduction

Leonurus Japonicus (LJ) is one of the many plants that constitute Chinese and Kampo traditional medicines, and it is known for its cardioprotective activity, antioxidant, and anti-inflammatory properties [1]. The major components of interest in the aerial parts of the plant are flavones, diterpenes, and alkaloids [1], [2]. In the flavonoid family, one of the identified molecules is rutin, which has an important role in free radical scavenging that could help the skin to counteract harmful external factors [3]. Moreover, it exhibits an inhibitory effect on cyclooxygenase and lipoxygenase activity, PMNs (Polymorphonuclear neutrophils)

migration [4], and a diminution effect on several cytokines observed in mice models (IL-1, IL-4, IL-5, IL-13, TNF-α among others) [5], [6].

The skin is constantly exposed and provides efficient body protection against pathogens and different types of environmental insults. The epidermis mainly constituted with keratinocytes is the outermost of the three layers and was traditionally identified as a passive defensive barrier [7]. New findings highlighted that this epithelium actively coordinates the external signals in order to control the inflammatory response and maintain barrier homeostasis, by the activation of the inflammasome pathway, an innate immunity guardian [8]. Thus, environmental stimuli link to pattern recognition receptors (PRRs) from microbe-derived, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) activate the inflammasome pathways in epithelial cells. Then, this is conducted by caspase-1 activation which promotes the secretion of the proinflammatory cytokines IL-1β and IL-18 responsible for inflammatory response [9]. Aberrant regulation of this pathway contributes to the physiopathology of several skin diseases such as atopic dermatitis (AD), a common chronic eczema skin disorder characterized by severe pruritus, and inflamed and dry skin [10]. Indeed, the activation of inflammasomes by allergens or pathogens induces AD and IL-18 contributes to the spontaneous development of AD-like inflammatory skin lesions [11]. Promoting insight into AD disease is of interest because it significantly impacts the quality of life for 10-20% of children and nearly 1-3% of adults in industrialized countries [12].

The thymic stromal lymphopoietin protein (TSLP) is a cytokine highly produced in the epidermis by keratinocytes during AD [13], which can activate dendritic cells and can initiate inflammation [14]. It has been shown that TSLP has a crucial role in the initiation, development, and progression of AD [15]. The production of this protein is known to be induced by a cytokine cocktail composed of TNF-α, IL-4, IL-5, and IL-13 [16]. New findings highlighted that the epidermis actively coordinates the external signals to control the inflammatory response and maintain barrier homeostasis, by activating the inflammasome pathway, an innate immunity guardian [9], [17].

To study the effect of LJ extract on AD and other inflammasome markers, skin explants of very dry skin with atopic tendency were induced in such states using a cocktail of cytokines (IL-5, IL-13, IL-4, and TNF-α). This model shows an over-expression of the TSLP cytokine at mRNA and protein levels but also the induction of IL-1β, TNF-α, and NOD2, an intracellular sensor for small peptides derived from the bacterial cell that may bind to inflammasome proteins such as PYCARD [13]. Thus, the cytotoxicity of the model should be first tested, followed by the effect of the active ingredient on the quantity of TSLP in the epidermis. Thus, the skin biopsies were treated with the active ingredients or cyclosporin 24h before the stress and for 24h during AD stress.

Materials and Methods

For this experiment, skin biopsies with no stretch marks, from the abdominal skin of a 59-year-old healthy Caucasian female were selected. First, the adipose tissues were removed and 10mm biopsy punches (Fisher scientific) were performed on the same explant. Each of the 10 mm biopsy punches was placed on a cell culture plate (Nunc). The dermal part of the skin tissue received medium nutrients; the epidermal surface was in direct contact with the air.

The cytokine cocktail composed of TNF-α, IL-4, IL-5, and IL-13 at a concentration of 200ng/mL was used to induce TSLP production and mimic AD inflammatory response and treatments with an active ingredient at 3 concentrations (0.01%, 0.02%, and 0.03%) were applied. Cyclosporin (1µM), an immunosuppressant drug, was used as a positive control of inhibition of TSLP production.

A lactate dehydrogenase (LDH) assay kit (Roche) was used to test the cytotoxicity, by measuring the release of LDH into the medium which occurs when tissues are stressed or damaged. The assay was performed according to the manufacturer's instructions. A negative control (Blank) test was done by measuring the fresh culture medium. Positive control for stressed biopsy was done on one skin explant in presence of 0,1% Triton-X100 in the culture medium. The tissue cytotoxicity was assessed for each condition using the skin biopsy culture medium at the end of the experiment.

The skin biopsies were cultured in the following six different conditions and each condition was performed in triplicates and processed separately following the experimental procedures found in Figure 1, and the culture medium was replaced every day. The effect of the active ingredient and cyclosporin was investigated by the modulation of AD key genes using quantitative PCR and by immunohistochemistry (IHC) of TSLP skin epidermis.

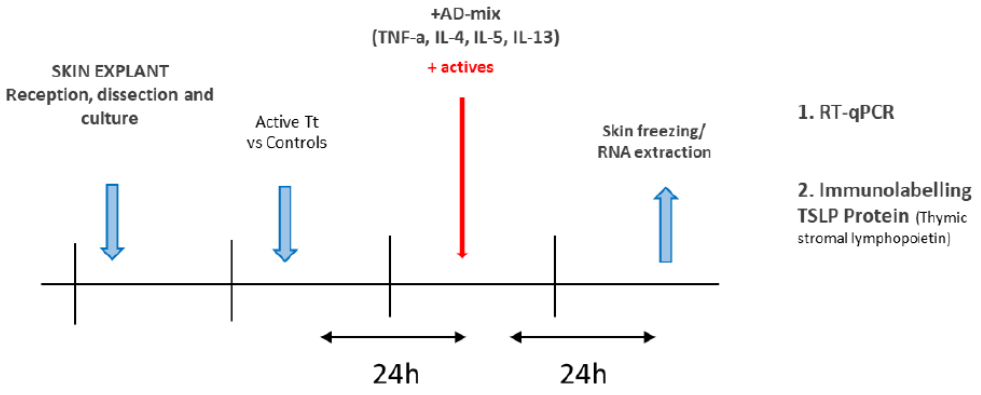


Figure 1: Experimental procedure for studying the effect of Leonurus Japonicus Extract on TSLP quantity in skin explants.

Total RNA extraction and real-time quantitative PCR: Total RNAs from samples were homogenized in Trizol reagent (Thermo Fisher Scientific: TRIzol Plus RNA purification kit) in presence of liquid nitrogen. RNA was extracted following the manufacturer's instructions. Quality controls were performed using Agilent RNA Nano kit with Analysis Agilent 2100 bioanalyzer. The total RNA quantitation was performed using a Nanodrop spectrophotometer (Appendix II). For target quantitation, 250ng of total good quality RNA (RIN~6) was reverse transcribed into cDNA with Superscript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed with a Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen) according to the manufacturer's instructions using the CFX system (Bio-Rad). PCR primers were computationally designed and bought from our knowledgeable suppliers. PCR test was performed in duplicates for each biological sample. Each condition was done from the beginning in 3 biological samples, so, we obtained about 3 mean values for each condition. The results were normalized against HPRT1 housekeeping gene expression as an endogenous control. Furthermore, the results were presented as ratios of gene expression for active ingredients stressed or not against untreated and unstressed controls (NT_NS).

Skin punch biopsy processing for Immunohistochemistry: Molds were filled with an Optimum Cutting Temperature medium (Thermo Fisher, Shandon Cryomatrix, 6769006) for cryopreservation and the samples were immersed inside with the required orientation, and then frozen on a dry-ice/96% Ethanol bath. Once the freezing temperature was achieved, the samples were kept at -80°C until the procedure for sectioning began. Each biopsy was then placed on a sample block within a cryostat cabinet (Leica CM3050 S) set at a -25ºC. The frozen block (containing the biopsy) was sectioned at a thickness of 8µm and sections were mounted on poly-lysine super frost slides (Thermo Fisher Superfrost Plus). The slides were stored at -80°C before immunostaining.

Immunohistochemistry of TSLP: Slides were thawed for 5 minutes and then fixed in Formalin for 10 minutes. They were incubated in TBS-T 5% BSA at room temperature for 1 hour to prevent nonspecific binding of the primary antibody. Next, the slides were incubated overnight at 4°C with TSLP primary antibody (ab188766, Abcam). Following this, the slides were incubated with secondary antibody anti-rabbit Cyanine 5 (Jackson Lab) for 1h at room temperature in the dark. Finally, the slides were incubated with Hoechst® 33342 1/5000 for 10 minutes and preserved in mounting medium Fluoromount-G (Thermo Fisher Scientific, 00-4958-02). Each step was rinced three times with TBS-T for 5 minutes. The images were then taken with an epifluorescence microscope (Zeiss, Axio Imager Z1, ApoTome, Zen2 blue edition software) equipped with a Cyanine 5 channel and analyzed on ImageJ / FIJI software. To study the effect of the active ingredient, an Image J macro was designed to quantify TSLP in the cytoplasm of the cells. Images were selected to have around 200 nuclei and have minimal holes or damage in the epidermis. 12 images minimum were analyzed per condition. The first step consisted to select the epidermis, the region of expression of TSLP, and

excluding the dermis and the stratum corneum that can contain non-specific staining. The macro used was then able to measure the Cy-5 mean intensity fluorescence in the cytoplasm of the cells. The mean fluorescence intensity for each image taken was then calculated.

Statistical test: The data represent the mean + SD. The differences between the control and the treated groups were analyzed using a Student t-test. Groups of each condition were carried out in triplicates. The statistical significance was considered at * *p-value <0,05*.

Results

Firstly, cytotoxicity tests assessing the production of Lactate dehydrogenase (LDH) by the cells show that the active ingredient does not induce any proven toxicity at those levels (See Figure 2).

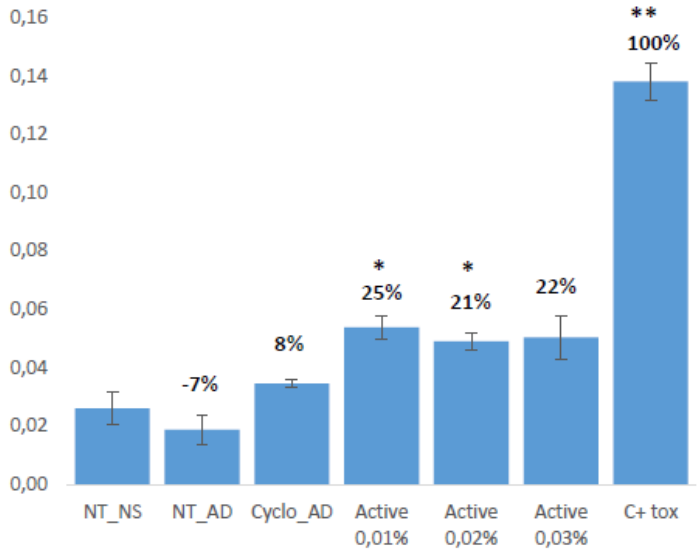


Figure 2: Effect of active ingredients on LDH production by skin explants. *Explants were either left in the medium (NT_NS), treated with a cytokine cocktail diluted in the media (NT_AD), and then treated with the 3 concentrations of the active ingredient (0.01%, 0.02%, and 0.03%) or Cyclosporin (Cyclo_AD). Triton 0.1% was used as a positive control for damaged tissues cytotoxicity (C+ tox). A toxicity of about 25% is considered acceptable. The statistical analysis was performed using two-tailed unpaired T-test vs NT_NS (* p-value <0.05 - ** p-value<0.01-*** p-value<0.001).*

Then, the cytokine cocktail was used to induce AD and significantly increase the amount of TSLP (marker of AD), IL-1β and TNF-α (2 genes of the inflammatory response) and NOD2, an inflammasome protein, in the epidermis, as shown in Figure 3.

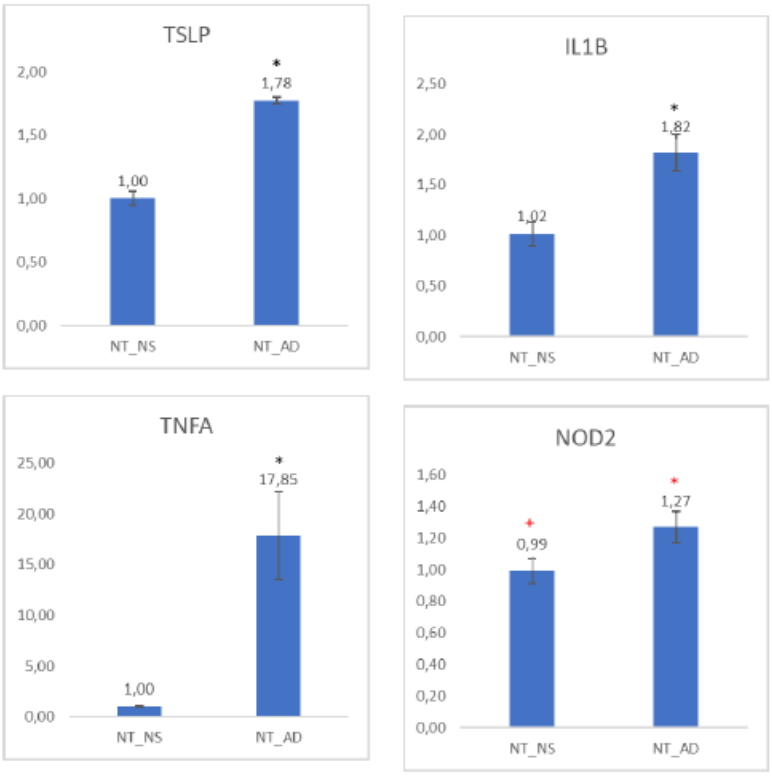
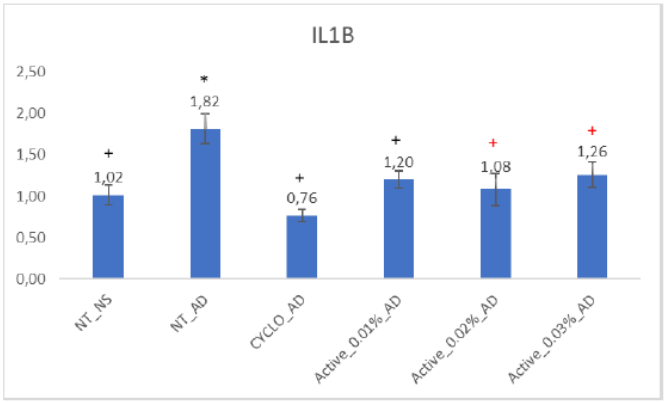
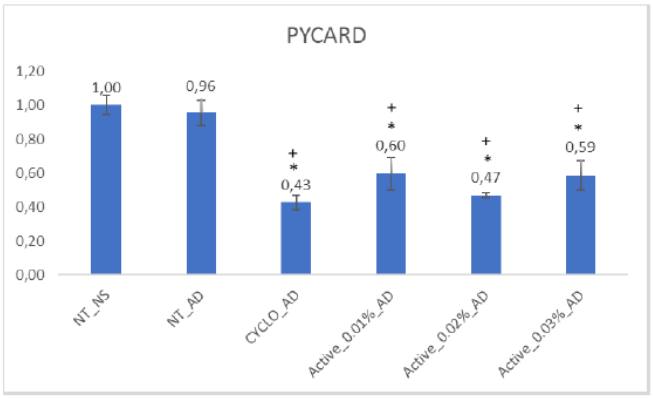


Figure 3: Effect of AD cytokines cocktail on inflammatory genes expression. *Skin explants were either left unstressed or stressed with cytokines cocktail. The experiment was performed in triplicates for each condition. Total RNA was extracted using Trizol (Thermo Fisher Scientific). The genomic target expression levels after treatments were measured by RT-qPCR and compared to untreated cells. The results were normalized to endogenous control HPRT1 expression. The SEM bars are represented in black lines on each histogram. The statistical analysis was performed using t-test (*stat NTNS p<0.05).*

Figure 4 shows the effect of LJ on the modulation of IL-1β and PYCARD (coding for ASC protein), two key genes of the inflammasome pathway, and NOD2. Therefore, this active ingredient is a promising molecule against inflammation and specifically AD. In Figure 5, we can see the effect of LJ at 3 concentrations (0.01%, 0.02% and 0.03%) on the expression of 2 genes tightly associated to Atopic Dermatitis: TSLP and SPINK5. In this study, we showed that the active ingredient at 3 concentrations decreased the SPINK5 gene expression like with the cyclosporin. Moreover, the histogram showed that the active ingredient at 0.03% decreased the expression of TSLP gene expression. The other concentrations did not have an effect on the modulation of TSLP like cyclosporin. It is observed that using LJ at 0.03% in a final product would be able to repress the TSLP expression at the mRNA level (see Figure 5).





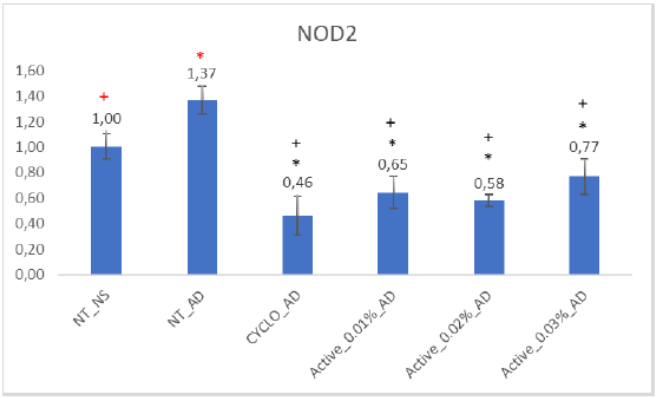


Figure 4: Decrease expression of inflammasome genes. *Skin explants were either treated for 24h with the active ingredient or Cyclosporin or not. Skin explants were then stressed with the AD cytokines cocktail with or without active ingredient (0.01%, 0.02%, or 0.03%) or untreated (NT_NS). The experiment was performed in triplicates for each condition. Total RNA was extracted using Trizol (Thermo Fisher Scientific). IL-1β, NOD2, and PYCARD expression levels after treatments were measured by RT-qPCR and compared to untreated cells. The results were normalized to endogenous control HPRT1 expression. The SEM bars are represented in black lines on each histogram. The statistical analysis was performed using a t-test (*stat NTNS $p<0.05$). NT-NS: Non-treated explants, NT-AD Explants treated with cytokines cocktail, CYCLO-AD: Cyclosporine Control.*

Figure 5: Effect of the active ingredient on TSLP and SMINK5 at gene-level expression. *Explants were either left in the medium (NT_NS), or treated with a cytokine cocktail in the media (NT_AD), and then treated with the active ingredient (0.01%, 0.02%, and 0.03%) or with the positive control Cyclosporin (CYCLO_AD). The statistical analysis was performed using a two-tailed unpaired T-test vs NT_NS (* p-value <0.05).*

As LJ showed an effect on TSLP gene expression, we also investigated the effect of the active ingredient on TSLP expression by immunostaining the protein in the human skin explants (See Figure 6). The capacity of the active ingredient to reduce the production of TSLP after induction by the cytokine cocktail was then assessed by immunohistochemistry.

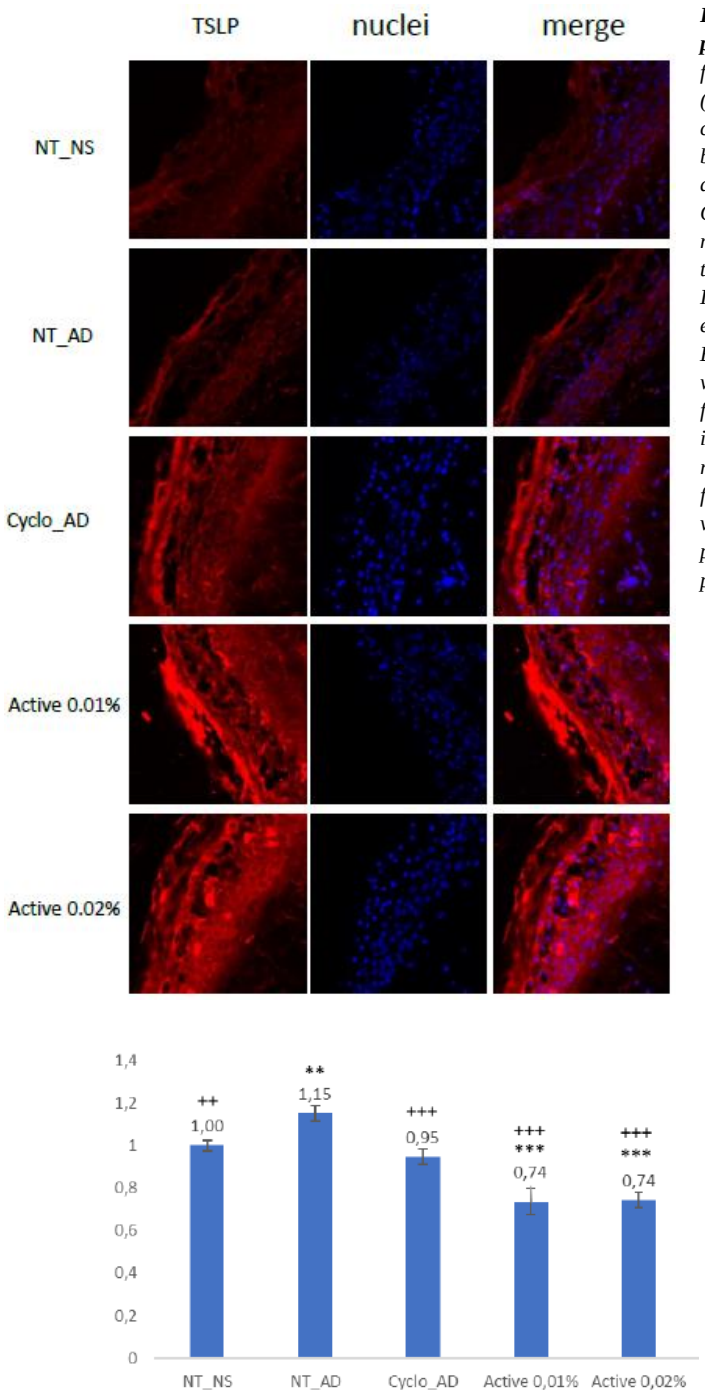


Figure 6: Effect of the active ingredient on TSLP at protein expression. *Skin explants were either treated for 24h with the active ingredient/Cyclosporin or not (NT_NS) and then stressed with an AD cytokines cocktail with or without active ingredients. Punch biopsy cuts were incubated with a specific antibody against TSLP and a secondary antibody paired to Cy5. After Hoechst 33342 dyeing, slides were mounted with Fluoromount-G medium. Pictures were taken with an epifluorescence microscope (Zeiss, Axio Imager Z1, ApoTome, Zen2 blue edition software) equipped with a Cy5 channel (Excitation 663 nm / Emission 691 nm) and analyzed on ImageJ. A macro was used to measure the mean intensity of fluorescence of TSLP in the epidermis. IHC of TSLP is represented in red and nuclei staining is represented in blue. The results are expressed as fluorescence mean intensity normalized by NT_NS value using a two-tailed unpaired T-test vs NT_NS (** p-value <0.01, *** p-value<0.001) or vs NT_AD (++ p-value<0.01, +++ p-value<0.001).*

As shown in Figure 6, TSLP protein expression increased after AD stress. Adding Cyclosporin reduced TSLP expression as expected. Finally, treating with LJ at a concentration of 0.01% or 0.02% also reduced TSLP expression after AD stress.

Discussion

In this study, we provide a new model for mimicking AD disease on explant skin cells. To do this, a skin explant is stimulated with a cocktail of cytokines (IL-5, IL-4, IL-13, and TNF-α) in order to mimic chronic inflammation that occurs in AD. We showed that LJ can decrease the dependent induction of IL-1β and NOD2 gene expressions. Additionally, LJ represses the SPINK5 and PYCARD gene expressions. TSLP mRNA expression was also reduced by LJ active ingredient and this decrease in TSLP expression was confirmed at the protein level from IHC labelling.

Therefore, the active ingredient seems to control the AD physiopathology by repressing inflammasome-related proteins IL-1β, PYCARD (coding for ASC protein), and NOD2, an intracellular sensor for small peptides derived from the bacterial cell that may bind to inflammasome proteins such as PYCARD and induce the IL-1β [18]. Moreover, our study highlights that LJ represses the SPINK5 gene named LEKTI. LEKTI is a specific repressor of KLK5 and KLK7 [19]. It's important to note that the serine protease KLKs play a major role in the regulation of skin desquamation and inflammation and their dysregulation is crucial in AD. Finally, we noticed that LJ represses the triggering factor in AD, TSLP cytokine, at mRNA and protein levels.

Conclusion

Taken together, LJ may be a promising molecule against AD. It has also been hypothesized that LJ may also act on AD by directly controlling the key component of the inflammasome and KLKs, as the dysregulation of the latter plays a major role in the pathophysiology of AD.

Conflict of Interest Statement

None.

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