European Journal of Pharmaceutical Sciences 200 (2024) 106846

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Development of Imiquimod-induced HaCaT-THP-1 co-culture for modeling of psoriasis

Judit Váradi [a], Boglárka Oláh [a], Dominik Hosszú [a], Ferenc Fenyvesi [e], Judit Remenyik [c], Judit Homoki [c], Béla Nagy Jr. [d], Zsolt Fejes [d], Ildikó Bácskay [a,b], Ágnes Klusóczki [b,*]

[a] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Debrecen, Nagyerdei körút 98., Debrecen H-4032, Hungary
[b] Institute of Healthcare Industry, University of Debrecen, Nagyerdei körút 98., Debrecen H-4032, Hungary
[c] Institute of Food Technology, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, Egyetem tér 1., Debrecen H-4032, Hungary
[d] Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Nagyerdei körút 98., Debrecen H-4032, Hungary
[e] Department of Molecular and Nanopharmaceutics, Faculty of Pharmacy, University of Debrecen, Nagyerdei körút 98., Debrecen H-4032, Hungary

ARTICLE INFO

Keywords:
HaCaT
THP-1
Co-culture
Psoriasis
Imiquimod
Chloroquine
Anthocyanin

ABSTRACT

Psoriasis is one of the most prevalent and chronic inflammatory disease of the skin, associated with disrupted barrier function. Currently, a widely accepted, generally usable cell culture model has not been developed yet. In the present work, we aimed to establish a co-culture model with human keratinocyte (HaCaT) and human monocyte cells (THP-1) induced by Imiquimod (IMQ), which acts on the TLR7 receptor. The role of TLR7 expressed on THP-1 cells was confirmed by immunofluorescence staining of NF-κB activation. Chloroquine (CH) was used as a receptor inhibitor, in the presence or absence of which the NF-κB pathway was activated. We determined the most effective proliferation-stimulating IMQ concentration by RTCA method and the hyperproliferative effect was investigated by wound-healing test. The effect of IMQ was compared with the effects of the anthocyanin (AC) components from the anti-inflammatory sour cherry extract that we have already studied. We found that IMQ significantly increased the migration rate however, the combined treatment resulted in a decreased migration rate compared to the IMQ treatment alone. Inflammatory cytokines were measured from the supernatant of co-culture by ELISA. During the development of the co-culture intended to model psoriasis, we confirmed the induction effect of IMQ and in the case of AC treatment, we supported the stabilizing effect of the barrier.

1. Introduction

Psoriasis (PS) is a complex, immune-mediated, chronic inflammatory skin disease characterized by excessive growth of epidermal keratinocytes, which can affect skin and nails (Albanesi et al., 2018; Smith et al., 1993). Environment, genetic, and immunological factors play a crucial role in the pathogenesis. The molecular genetic basis of psoriasis is complex, and evidence suggests that multiple genes are involved (Langley et al., 2005). It is difficult to confirm the susceptibility loci of psoriasis because of the heterogeneity among the different populations. In addition to genetic factors, the contribution of the immune system is also important during pathogenesis, as skin cells, immunocytes, and numerous biological signaling molecules interact, which are triggered by environmental stimuli. The disease process is a result of a network of cell types, including T cells, dendritic cells, and keratinocytes that, with the production of different cytokines generate chronic inflammation.

Psoriasis is mainly dendritic cells (DCs) and T-cell-mediated disease. DCs are thought to be responsible for that Type 17 (Th17) and T type 1 (Th1) T cells are activated by IL-23 and IL-12 which are secreted by the antigen-presenting cells (APCs) in the skin (Hugh et al., 2018). Crosstalk between the adaptive immune system and the innate immune system is mediated by cytokines including TNF-α (Elder et al., 2010), which induce chronic inflammation and enhance epidermal proliferation, differentiation, and apoptosis. However, cytokines can also be produced by epithelial cells, such as the keratinocytes of the skin. This mixture of cellular response secretes cytokines and leads to a cascade of events involving keratinocytes, endothelial cells, fibroblasts, and neutrophils which create the cutaneous lesions seen in psoriasis (Di Cesare et al.,

* Corresponding author.
E-mail address: klusoczki.agnes@euipar.unideb.hu (Á. Klusóczki).

https://doi.org/10.1016/j.ejps.2024.106846
Received 9 April 2024; Received in revised form 25 June 2024; Accepted 4 July 2024
Available online 5 July 2024

2009). If the balance between immunogenic APCs and housekeeping T cells is upset, inflammatory conditions such as psoriasis can result, and for the disease to become chronic, persistent dysfunction of T cells is necessary (Christophers et al., 2014).

Many in vivo models exist for designing human psoriasis including the following animal models: spontaneous mutations, transgenic rodents, knockout mice, topical modulation of skin environment, T-cell transfer, and xenotransplantation model. Extensive comparisons between these models have been presented (Van der Fits et al., 2009; Schön et al., 2021; Gudjonsson et al., 2007; Boehncke et al., 2007). Imiquimod (IMQ) is used as a potent immune activator on TLR7 and TLR8 in most animal models. Topical application of IMQ induces migration of Langerhans cells, stimulates the maturation of dendritic cells and can induce intensive Th1 responses and increased cytokine production was detected by stimulation of keratinocytes (Van der Fits et al., 2009).

Nowadays, drug development is increasingly carried out on in vitro cell models, which models are also used to study psoriasis. Keratinocyte monolayer cultures are widely used for biological and pharmacological studies, as well as for screening drugs for the treatment of psoriasis because they provide an easily reproducible first-step model system. Initial attempts to create an in vitro model of psoriasis were made using skin cells from freshly isolated, lesional psoriatic skin. The addition of cytokines was required to maintain the psoriatic phenotype. However, given the lack of reproducibility and the difficulties associated with obtaining isolated lesional psoriatic skins, this approach is inefficient and does not meet the needs of a reliable, accessible preclinical model (Bocheńska et al., 2017). To minimize these problems, Colombo et al. started to use the HaCaT human keratinocyte cell line spontaneously immortalized from adult skin to create the model and study keratinocyte functions. HaCaT is an immortalized human keratinocyte cell line that provides long-term growth, availability, and persistence. In addition, it has morphogenesis similar to mature adult skin cells and produces all the important surface markers and functional activities of isolated keratinocytes. Another important feature is that it can differentiate under stimulation. The use of this model is limited by the cytokine spectrum, as the two types of cytokines used are not sufficient to represent a real pro-inflammatory environment (Colombo et al., 2017). The further development of the HaCaT model required a substance that increases the in vivo equivalence of the model and increases the complexity of cell responses. The choice fell on the immune-stimulant substance called Imiquimod (IMQ). IMQ was registered in 1997 and is still used in cream form to treat basal cell tumors and genital warts (Skinner, 2003). It was determined that the HaCaT cell type does not express the TLR receptors necessary for the development of the effect of IMQ, yet it was proved that a small activation also occurs in the immortalized keratinocyte cell line as a result of the treatments (Schön and Schön, 2007). Preclinical in vitro and in vivo results demonstrated that IMQ directly induces immunomodulatory cytokines via TLRs from monocytes, macrophages, and DCs. These immunomodulatory cytokines have been shown to potentiate the activity of T-helper 1 immune cells (Richwald, 1991). To increase the in vivo equivalence of the psoriasis model, it is worth developing a co-culture. By combining human keratinocytes with immune cells, it becomes possible to perform tests on a more complex model that better correlates with in vivo conditions (Thélu et al., 2020). HaCaT-THP-1 co-culture (COCAT) has been experimentally demonstrated to be effective in the treatment of skin sensitization symptoms (Hennen et al., 2011; Hennen and Blömeke, 2017). It was found that in the case of cells that have been used separately until now, when used in co-culture, the reaction of the cell model to sensitizing chemicals can be increased and the in vitro COCAT method was also shown to be able to identify the potency of skin sensitizers (Eskes et al., 2019).

To verify the effectiveness of IMQ induction on the co-culture, we used anthocyanin extract which has been proven to have anti-inflammatory activity and, according to our assumption, is suitable for mitigating the effects of increasing migration, proliferation, and cytokine secretion.

During our previous tests, we were convinced of the anti-inflammatory effect of the anthocyanin-enriched extract produced from sour cherries (Le Phoung Nguyen et al., 2018; Remenyik et al., 2022; Klusóczki et al., 2023).

It is well characterized that the TLR7 receptors are present on the THP-1 cell membrane and as a next step their role was investigated by immunofluorescence staining (Zheng et al., 2021). In this experiment, the goal was to learn more about the development of the inflammatory process, which we wanted to confirm by activating the NF-κB signal pathway by immunofluorescence following the translocation of the p65 subunit to the cell nuclei after different treatments. Chloroquine, a commonly used TLR inhibitor, was applied to test the effects of IMQ on NF-κB activation in THP-1 cells (Zhou et al., 2015, 2021).

Despite our increasing knowledge of psoriasis, there have been several attempts to reproduce the disease in vitro and in vivo models to reach more detailed insights into the pathogenesis, as well as therapeutic options. The present study aimed to investigate and optimize the best conditions to use HaCaT and THP-1 cell cultures as a reliable in vitro model of psoriasis.

2. Materials and methods

2.1. Materials

All chemicals and reagents were obtained from Sigma-Aldrich (Budapest, Hungary) unless stated otherwise. Pure sour cherry anthocyanin extract (AC) was provided by the Department of Feed and Food Biotechnology, University of Debrecen (Hungary), as described previously (Homoki et al., 2016).

2.2. Cell culture

HaCaT cells, spontaneously immortalized human keratinocyte cells were purchased from Cell Lines Service, CLS, Heidelberg, Germany. HaCaT cells were cultured in calcium-free DMEM media supplemented with 10% fetal bovine serum (FBS). HaCaT cells were incubated at 37 °C in an atmosphere of 5% CO_2. In the case of the MTT test, wound healing assay, and NF-κB investigation, when the cells had completely grown over the well's membrane or near the 100 % confluent stage, the experiments were ready to perform.

THP-1 is a human leukemia monocytic cell line, which has been widely used to study monocyte/macrophage functions. THP-1 cells were cultured in T-75 flasks in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS, and 1% penicillin/streptomycin. THP-1 cells were incubated at 37 °C in a 95% humidified atmosphere and 5% CO2.

We performed co-culture with HaCaT and THP-1 cells to establish a cell model. The co-culture was grown in calcium-free DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 95% humidified atmosphere and 5% CO_2. On the first day, HaCaT cells were seeded. The next day, when they were attached to the surface, THP-1 cells were added to make a co-culture at least for 24 h.

2.3. HaCaT cell viability test

The MTT assay was used to investigate the cytotoxicity and proliferation-stimulating effect of IMQ. HaCaT cells were seeded in a 96-well plate at a final concentration of 2×10^4 cells/well. When the cells were fully grown over the well's membrane, the experiment was ready to perform. Monolayers were washed with HBSS and treated with 1 nM – 500 μM concentration range of IMQ-containing medium. The 1 % TX-100 was used as a positive control. The treatment was stopped 72 h later, and the treatment medium was replaced with a 5 mg/mL MTT solution made with PBS. The plates were then incubated for 3 h at 37 C°. At the end of the incubation, the MTT solution was removed, and the formazan crystals were dissolved with a 25:1 mixture of 0.1 ml of

isopropanol-HCl. Then, the absorbance of the solution was measured at 570 nm and 690 nm at a reference wavelength on a Ther-mo-Fisher Multiskan Go (Thermo Fischer, Budapest) microplate reader. The absorbance was corrected for individual variations of the plate with the values measured at the reference wavelength. Cell viability was measured as a percentage of untreated control cultures.

2.4. Investigation of the morphology of HaCaT-thp-1 co-culture after IMQ treatment

Microscopic images were taken from HaCaT-THP-1 co-culture. The cells were spread on 12 well plates, HaCaT at the concentration of 1×10^5 cells/well and THP-1 cells at the concentration of 1×10^4 cells/well. 24 h after co-culture formation, 1 μM IMQ or 100 μM IMQ treatments were applied. Untreated cells served as control. We incubated the cultures for 72 h and then stopped the test by removing the medium. The samples were washed with PBS and then fixed with 4 % paraformaldehyde solution for 20 min. The images were taken by Olympus CKX 41 4x phase contrast objective and we examined the morphology of the cells.

2.5. Real-Time monitoring of cell index (RTCA) measurements on HaCaT-THP-1 co-culture induced by IMQ

At the beginning of the study, our first step was to determine the most effective concentration of Imiquimod (IMQ). The effects of Imiquimod (IMQ) (Cat. No. 1338313) on cell proliferation were monitored by the xCELLigence system, which is a label-free, dynamic monitoring of cell proliferation and viability in real-time. Plates are coated by gold sensor arrays to measure electrical impedance. 2×10^4 HaCaT cells per well were seeded into 100 μl of DMEM media into E-plate 16 in duplicates and maintained at 37 °C with 5 % CO2. After HaCaT cells were attached to the surface (approximately 6 h after the seeding), 2×10^3 THP-1 cells were added to the E-plates to make a co-culture. 24 h later, different concentrations (0.01 μM, 1 μM, 100 μM, 500 μM) of IMQ solutions were used for the treatments and their cell induction effects were compared. After 72 h of testing, we stopped our experiment and evaluated our results.

2.6. Real-Time monitoring of cell index (RTCA) measurements on HaCaT cells induced by the effective IMQ and AC concentrations

2×10^4 number of HaCaT cells per well was seeded into 100 μl of DMEM media into E-plate 16 in duplicates and maintained at 37 °C with 5 % CO_2. Approximately 24 h after seeding, when the cells were in the log growth phase, they were treated with 100 μl media containing 1 μM IMQ; 100 μM AC; 1 μM IMQ + 100 μM AC. Cell culturing medium was the negative control. The proliferation of the cells was monitored every 15 min for 72 h. Results were expressed as a normalized cell index calculated by the software of the instrument.

2.7. Wound healing assay

We also examined cell proliferation and cell migration parameters on the model, for which we performed the wound healing test.

To create a wound, we used a 24-well-plate containing a special 0.5 mm wound forming insert (ABS Bio Wound Healing Assay Kit, Cat. No. K040–24, Advanced BioReagents), which is a ready to use culture-inserts for wound healing studies. HaCaT cells were plated at a concentration of 3×10^5 cells / well into two compartments of the insert. After 6 h when the HaCaT cells were attached to the surface, 3×10^4 THP-1 cells were added to HaCaT cells and they were also divided into two compartments. The co-culture was incubated for 24 h at 37 °C, then the insert was removed and the medium was changed for the treatment solutions. Treatments were performed with 1 μM IMQ solution and 100 μM AC solution. The shape of the wound was observed for 12 h in 3 periods. After 12 h, the experiment was stopped, and cells were fixed with a fixing solution. The samples were stained with Trypan Blue dye (Cat. No. 93595) and pictures were taken with a Zeiss Axioscope A1 (Carl Zeiss AG, Germany) microscope.

2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were performed on HaCaT-THP-1 co-culture. A transwell system with 12 inserts was used for the experiments, which are permeable polycarbonate filters with 0.4 μM pore size. HaCaT cells were seeded in the apical (upper) chamber at a density of 3×10^5 cells/chamber. The basal (lower) chamber contained 1 ml medium. 6 h later THP-1 cells (10 %) were added to the HaCaT cells to perform a co-culture. When the cells fully grew the membrane, culture media was removed and cells were incubated with the following: 1 μM Imiquimod (IMQ), 100 μM anthocyanin (AC), and combined treatment with IMQ + AC. Untreated culture medium served as a control. Invitrogen human ELISA kits were used. TNF-α (Cat. No. BMS223–4, Invitrogen), IL-17A (Cat. No. BMS2017, Invitrogen), IFN-γ (Cat. No. BMS228, Invitrogen) and IL-8 (Cat. No. KHC0081, Invitrogen) were investigated from supernatant according to the manufacturer's instructions.

2.9. Investigation of the NF-κB pathway

The NF-κB pathway activation in THP-1 cells was investigated via p65 nuclear immunofluorescence staining. Briefly, cells (50,000 cells/slide) were seeded on sterile glass coverslips placed into 12 well plates, and cultured in DMEM. When the cells reached the appropriate confluence, media was renewed, and cells were incubated with 1 μM IMQ (Cat. No. 1338313) / 10 μM Chloroquine (Cat. No. C6628) or the combination of them (IMQ+CH). Then, cells were washed twice with HBSS and fixed with ice-cold methanol-acetone (50–50 %) for 10 min and non-specific binding sides were blocked with fe-tal-bovine-serum (FBS) for 15 min. After this, cells were washed with HBSS and were incubated with 2 μg/ml Alexa-Fluor 488 conjugated NF-κBp65 (F-6) antibody (Cat. No. sc-8008 AF488) for 1 h at 37 °C, followed by washing with Hanks Balanced Salt Solution (HBSS) three times. Bis-benzimide dye (Hoechst 33s342) was used to stain cell nuclei for 10 min at 37 °C. After this, cells were washed once with HBSS, and the glass coverslips were glued to microscope slides. Fluorescence microscopy measurements were carried out by a Zeiss Axioscope A1 (Carl Zeiss AG, Jena, Germany) fluorescent microscope. Images were analyzed by ZEN 2012. v.1.1.0.0. software (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

3. Results

3.1. Cytotoxic effect of IMQ on HaCaT cells

Cytotoxicity of IMQ on HaCaT cells was determined by the MTT test (Fig. 1). Cell viability in the lower concentration range (0.1–25 μM) increased by over 100%. However, at higher concentrations above 100 μM, it significantly decreased (below 75%). The viability of HaCaT cells drastically decreased below 50 % at 250 μM. The curve was edited with the GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). The IC50 value of IMQ on HaCaT cells was determined also GraphPad Prism 8.0 software, the value was 124 μM.

3.2. The morphology of HaCaT cells in co-culture after IMQ treatment

Microscopic images were taken 72 h after the treatment of the co-culture. Fig.2.A shows the untreated co-culture cells, compared to Fig. 2.B, where we can see an increased proliferation state of the HaCaT cells as a result of 1 μM IMQ treatment. The HaCaT cells overgrew the available space without any residue, the cell density is more increased compared to the untreated sample. On the other hand, in the samples

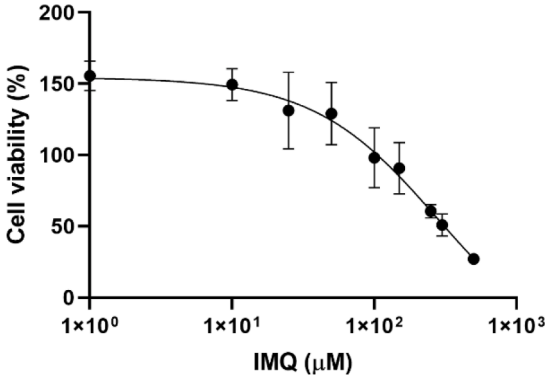


Fig. 1. Cytotoxicity of IMQ on HaCaT cell in 0.1–500 μM concentration interval. Cell viability was expressed as the percentage of untreated control. The concentration of IMQ was represented on a logarithmic scale. Values presented are means ±SD, $n = 6$, $p < 0.05$.

treated with 100 μM IMQ in Fig. 2.C, the cell density is significantly lower, and cell groups were formed after adhesion and treatment.

3.3. Determination of the effective IMQ and AC concentration by RTCA proliferation test

The informative curve obtained by the RTCA software clearly shows that a maximum cell index value was obtained for each treated sample group. The data were plotted in the period following the treatments. After the treatments, the maximum of the cell index appeared approximately 40–45 h later. The most intense proliferative effect was observed with the 1 μM IMQ solution, which is shown by the black curve in our diagram (Fig. 3.A).

The RTCA software was used to calculate the slope of the linear sections, the value of which represents the initial rate of proliferation. These data were plotted on the bar diagram shown bottom, on which we marked the significance values compared to the control. It is also clearly visible here that the most intense proliferative effect was produced by the 1 μM IMQ solution (Fig. 3.B). Therefore, treatment with a concentration of 1 μM IMQ was applied in our further studies.

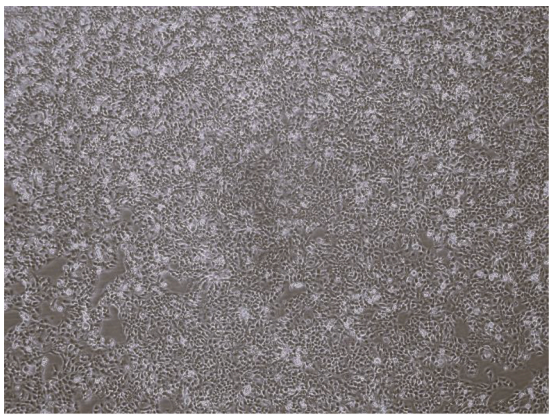
The 25 and 100 μM AC concentrations reduced the increase in cell proliferation induced by 1 μM IMQ, which is illustrated by the bar graph of the proliferation rate (Fig. 4). Compared to the control, the IMQ-treated samples had the highest proliferation rate, however in the presence of AC post-treatment, the proliferation rate decreased close to the control in the case of 100 μM AC treatment. The concentration-dependent anti-proliferative effect of the AC extract was observed, so the treatment with a higher concentration (100 μM) was found to be suitable for further studies.

3.4. Wound healing test on HaCaT cells induced by IMQ and AC solutions

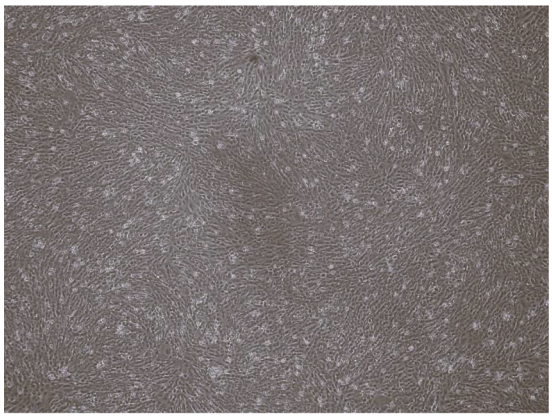
Migration of the HaCaT cells was investigated. The different treatments (IMQ/AC/AC+IMQ) resulted in different migration distances between the cell fronts (see Fig. 5.A). The distances were measured, and the migration rate was calculated from the obtained data. The bar chart clearly shows that the IMQ treatment significantly increased it compared to the control, while the combination treatment (AC+IMQ) significantly decreased the migration rate compared to the IMQ-treated sample. AC treatment alone did not result in a significant difference compared to the control sample (Fig. 5.B).

3.5. Determination of cytokine secretion on HaCaT and THP-1 co-culture

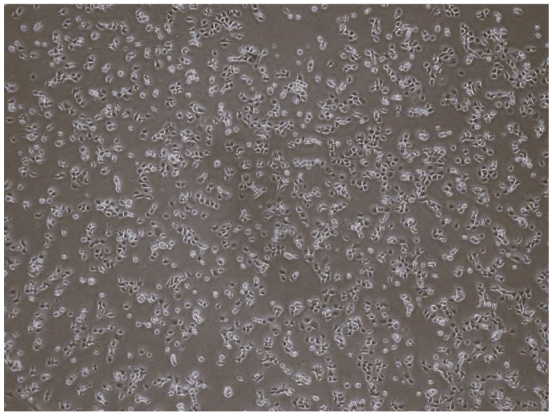
Cytokine secretion of co-culture cells was measured according to the following groups: untreated control, 1 μM IMQ, 100 μM AC, and their combination (AC+IMQ). The concentration of TNF-α, IFN-γ, IL-17A, and IL-6 was determined in the medium, and the percentages of the production of cytokines were displayed on the diagrams (Fig. 6). The presence of IMQ compared to the control sample tended to elevate with the different significance of the TNF-α, IFN-γ, IL-17A, and IL-6 release in the media of HaCaT-THP-1 co-culture. As a result of the combined treatment, following IMQ induction, AC moderated the cytokine



(A)



(B)



(C)

Fig. 2. Representative phase contrast microscopic images of HaCaT-THP-1 co-culture morphology. The images were taken 72 h after the co-culture was assembled. The (**A**) was recorded from the untreated control, (**B**) the 1 μM IMQ-treated, and (**C**) the 100 μM IMQ-treated co-culture. The IMQ treatment was performed 24 h after the co-culture was assembled. The images were taken by Olympus CKX 41 4x phase contrast objective.

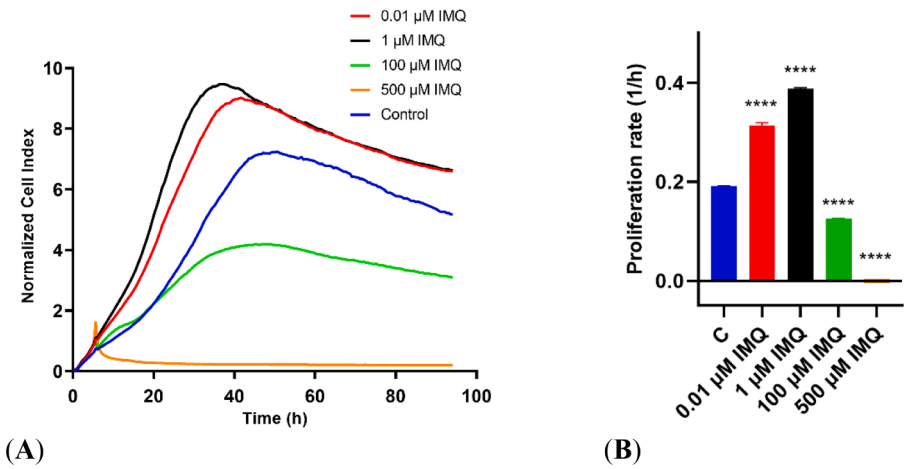


Fig. 3. Investigation of the proliferation effect of IMQ by RTCA, XCelligence system. Kinetics of co-culture (HaCaT+THP-1 cells) in response to treatment was monitored using cell index (CI) (**A**). The initial rate of proliferation was calculated by XCelligence software. (**B**). Results are presented as means ± SDs, $n = 4$. Differences were considered significant at $p < 0.05$; **** (0.01 μM/1 μM/100 μM/500 μM IMQ vs. C) $p < 0.0001$.

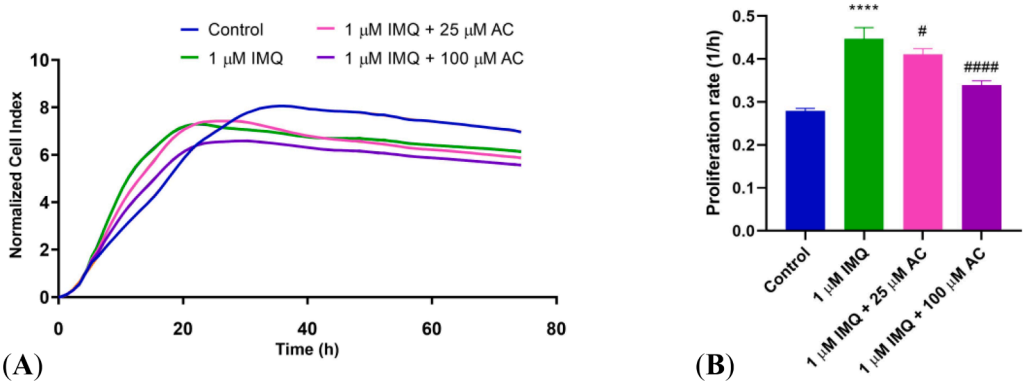


Fig. 4. Proliferative changes on HaCaT cells as a result of IMQ and AC treatment. The proliferation effect was monitored in the cell index on an RTCA device (**A**). The initial rate of proliferation was calculated by XCelligence software (**B**). Results are presented as means ± SDs, $n = 3$. Differences were considered significant at $p < 0.05$; **** (1 μM IMQ, vs. C) $p < 0.0001$, # (1 μM IMQ +25 μM AC vs. 1 μM IMQ) $p < 0.05$, #### (1 μM IMQ +100 μM AC vs. 1 μM IMQ) $p < 0.0001$.

production in every case. We did not experience any significant differences in cytokine concentration in AC-treated samples compared to the control.

3.6. Activation of NF-κB pathway on THP-1cells induced by IMQ and CH

During the experiment, in addition to the control samples, Imiquimod (IMQ) solution with a concentration of 1 μM, Chloroquine (CH) solution with a concentration of 10 μM, and their combination (IMQ + CH) were used. In our recordings, the cell nuclei were marked and made visible in blue, while the p65 proteins were shown in green. Strongly induced p65 protein translocation was established into the nucleus as a result of IMQ treatment compared to the control. Chloroquine alone did not result in a significant difference than in controls. During combined treatment (IMQ + CH), CH significantly reduced the inducing effect of IMQ compared to the results of samples treated only with IMQ. This was further proved by calculating the nuclei/cytosol intensity. The same trend was observed, we found a higher expression of the intensity of nuclei/cytosol as a result of IMQ treatment however, it is also demonstrated that the combination cocktail of IMQ + CH resulted in a materially reduced expression of the abovementioned intensity compared to IMQ-treated samples.

4. Discussion

The goal of the study was to establish a cell culture model for psoriasis in which two cell types increase the in vivo cell equivalence and use the keratinocyte-immune cell relationship. The first cell type is the HaCaT cell line, which models the function of human keratinocytes. The second cell type is the THP-1 cell line, which plays the role of immune cells. THP-1 is a human leukemia monocyte cell line widely used as an in vitro model of human monocytes and macrophages. The THP-1 cell line is used to model monocyte/macrophage signaling pathways and inflammatory mechanisms, to study the immunomodulatory effect of compounds, and to study drug delivery (Chanput et al., 2014). The characteristic of the immortalized cell line is that it can be cultured in vitro at maxi-mum Passage 25 with no change in cell sensitivity and activity which is an advantage in the cell culture model. This is why we decided on the THP-1 cell type, on the one hand, because it forms the basis of an officially accepted model cell culture (Eskes et al., 2019). On the other hand, the choice was justified by our previous knowledge of the communication between immune cells experienced during psoriasis. The dominant role of T cells in the pathomechanism of psoriasis is known, by communicating with macrophages and monocytes the keratinocyte inflammation increases. This reinforcing communication has been explored in recent years. Recent studies have shown that removing macrophages moderates the severity of psoriasis, improving clinical symptoms and reducing Th1 cytokine levels. Moreover, it has been

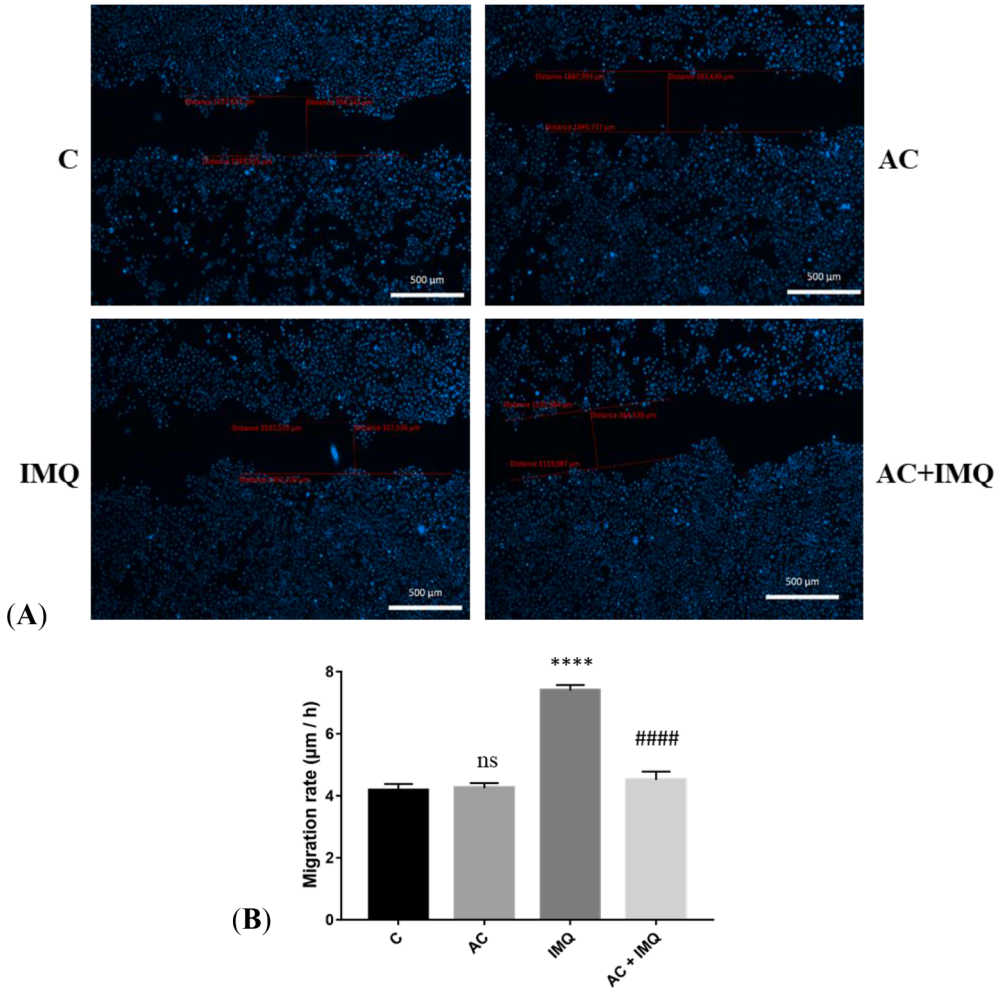


Fig. 5. Migration of HaCaT cells. Representative pictures of images acquired at 24 h after treatments in wound healing assay (**A**). Wounds were treated with 100 µM AC, 1 µM IMQ, and a combination treatment of 100 µM AC and 1 µM IMQ. Then, the migration rate (µm/h) was calculated in each treatment (**B**). Results are presented as means ± SDs, $n = 6$. Differences were considered significant at $p < 0.05$; **** (IMQ vs. C) $p < 0.0001$, #### (AC+IMQ vs. IMQ) $p < 0.0001$.

proven that psoriasis can be alleviated by inhibiting the inflammation of macrophages. The use of biological agents that inhibit tumor necrosis factor α (TNF-α) significantly reduced the proportion of macrophages and M1 macrophages in the skin lesions of patients with psoriasis.

In our model, IMQ plays the role of an inflammatory inducer, which exerts its effect on the TLR7 receptor. The TLR7 receptor is endosomal present in THP-1 cells (Petes et al., 2017) and its expression indicates that it is crucial in mediating inflammatory processes in autoimmune diseases (Eng et al., 2018). However, it was previously proven that HaCaT cells do not express TLR7 receptors (Köllisch et al., 2005; Wang et al., 2023; Lebre et al., 2007).

No results have been published yet on how the viability of HaCaT cells changes as a result of IMQ treatment, thus an MTT was carried out. The IC50 value of IMQ was 124 µM, therefore, for the co-culture studies, we determined the appropriate dose of IMQ that results in the greatest proliferation below 100 µM. The proliferation test on RTCA was performed in a co-culture, in the concentration range of 0.01 µM-500 µM. The highest concentration of 500 µM confirmed the cytotoxicity also observed in the monoculture, the proliferation effect was evaluated in the range of 0.01 µM-100 µM. In the RTCA test, by increasing the concentration of IMQ, the proliferation effect increased up to a concentration of 1 µM, after which a further increase in concentration already inhibited proliferation. To verify these experiences, a morphological examination was performed, and agreeing with the RTCA results it was established, that the co-culture treated with 1 µM IMQ concentration showed the strongest proliferation effect.

The apoptosis and autophagy-inducing effect of IMQ on THP-1 cells were investigated by several research groups and in most cases IMQ was used at a concentration of 10 µg/ml (41.6 µM). In a publication (Yu et al., 2014) the cytotoxicity of IMQ was tested in the concentration range of 0.1–10 µg/ml and it was established that at a concentration of 10 µg/ml, the viability of the cells decreased to around 50 %. At a concentration of 1 µg/ml (4.16 µM), which is in the same order of magnitude as in this study, IMQ reduced the viability to a value of around 80 %, while at this concentration the activation 14 of TLR7 was confirmed by the induction of IL-6 and MCP-1 secretion. Therefore, we can assume that the 1 µM treatment used in this study will activate THP-1 cells, which induced further psoriasiform changes in the HaCaT culture.

During the second test performed on RTCA, we monitored the effect of different AC concentrations applied with 1 µM IMQ induction on co-culture. These observations agreed with our previous results that the application of 100 µM AC significantly reduced the increase in proliferation induced by different agents (Remenyik et al., 2022; Klusóczki et al., 2023). Then 100 µM AC treatment was applied in our further tests.

Garay et al. investigated the correlation between the migration and proliferation of cells with different embryonic origins (Garay et al., 2013). Their studies found a positive correlation between cell proliferation and migration in melanoma cell lines. In the case of HaCaT cells, the results obtained in RTCA proliferation studies, and the wound

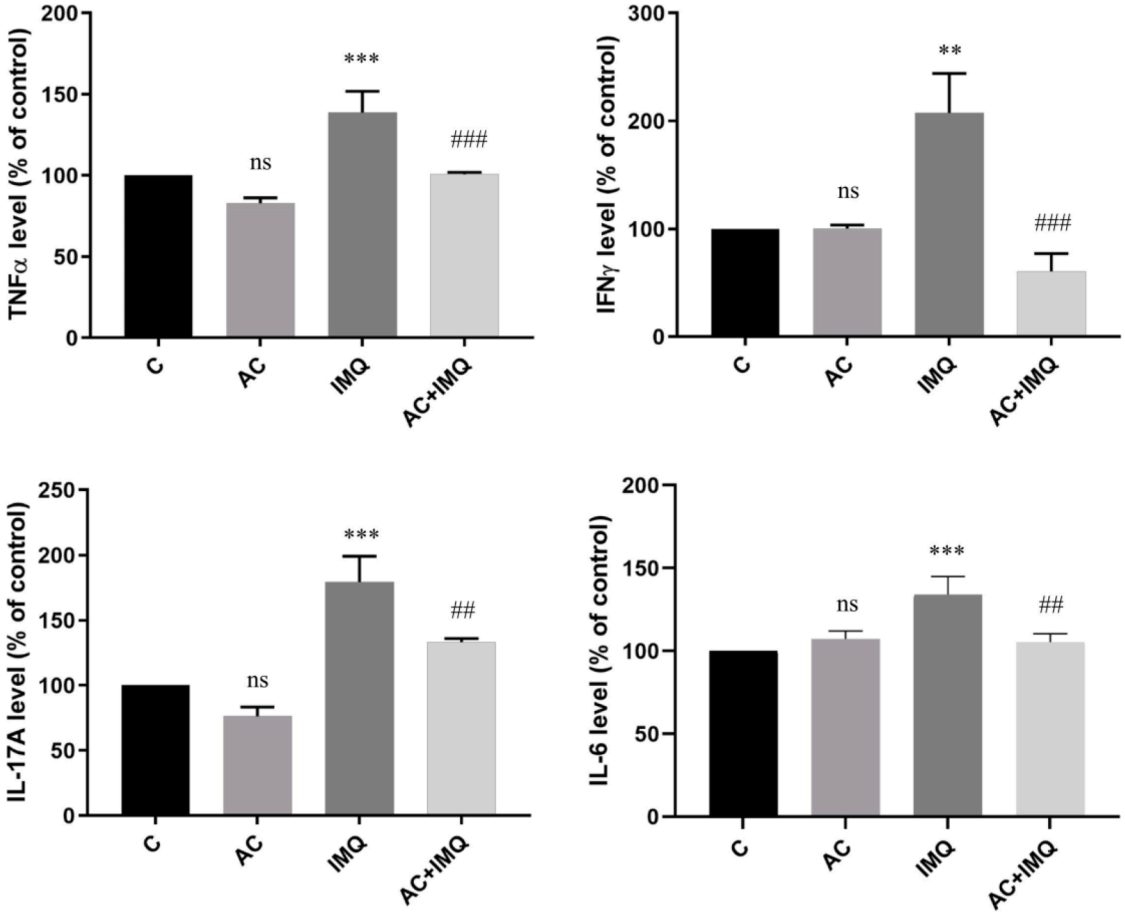


Fig. 6. The secretion of TNF-α, IFN-γ, IL-17A, and IL-6 in HaCaT-THP-1 co-culture. Cells treated with 1 μM IMQ and 100 μM AC. Treated culture medium samples were collected and cytokine secretions of the supernatants were measured. The control group (C) received an untreated culture medium. Results are presented as means ± SDs, $n = 3$. Differences were considered significant at $p < 0.05$; ** (IMQ vs. C) $p < 0.01$; *** (IMQ vs. C) $p < 0.001$; ## (AC+IMQ vs. IMQ) $p < 0.01$; ### (AC+IMQ vs. IMQ) $p < 0.001$.

healing test correlated. In addition to the proliferation effect of IMQ, we also observed the effect of increasing migration in the wound healing test. In contrast, AC showed a mitigating effect on proliferation and migration. The psoriasiform-inducing effect of IMQ has already been used in numerous animal experiments, but it has not yet been investigated in the HaCaT cell culture due to the lack of the TLR7 receptor.

The induction of THP-1 cells via the TLR7 receptor through the NF-κB pathway results in the expression of chemokines, inflammatory mediators, and cytokines, which induce inflammatory signaling pathways in HaCaT cells and induce further cytokine secretion. Additionally, activated HaCaT cells also affect THP-1 cells, which was confirmed in previous studies (Eskes et al., 2019). Communication is established between HaCaT-THP-1 cells in the co-culture, which triggered an increased response in THP-1 cells after treatment with sensitizing chemicals (Schellenberger et al., 2019). In addition, IMQ is also able to stimulate keratinocyte cytokine production independently of TLR7 (Schön and Schön, 2007), probably through inhibition of adenylyl cyclase and adenosine receptor signaling (Schön et al., 2006).

The increased secretion of cytokines characteristic of psoriasis in the co-culture was investigated by measuring TNF-α, IFN-γ, IL-6, and IL-17. In our measurements, we confirmed not only the increased cytokine expression but also the mitigating effect caused by AC. Chloroquine was applied to confirm IMQ-induced NF-κB activation in THP-1 cells. Based on our results, while IMQ activated the NF-κB pathway in THP-1 cells, in the presence of chloroquine, IMQ did not result in a beneficial level of activation. However, it should be noted that chloroquine is an antagonist of TLR and not an inhibitor, therefore we did not obtain a full inhibition in this experiment. To sum up, we confirmed the activation of the NF-κB pathway by IMQ.

While our co-culture model has numerous benefits for modeling psoriasis, it also has shortcomings. Psoriasis involves complex interactions of the innate and the adaptive immune system, including T-cells, neutrophils, dendritic cells, and others; however, our co-culture involves only keratinocytes and monocytes. It means the lack of the complete immune system during the experiments, also missing the extracellular matrix components and vascular components (which are also important for cellular behavior). We also need to face the limitations of the cell line, as THP-1 cells differentiate excellently up to passage number 25. Moreover, HaCaT cells are immortalized, which may not fully mimic the behavior of primary human keratinocytes. Furthermore, cell lines do not express the genetic and epigenetic diversity found in the human population.

5. Conclusions

In this study, we established a co-culture model of HaCaT and THP-1 cells. Our goal is to imitate a pathological process and induce symptoms approaching physiological ones and preserve the 15 hyperproliferation of keratinocytes as a result of the induction with IMQ. The top advantage of our model is that both cell lines are derived from human cells. This co-culture system mimics the interactions between the human keratinocytes and human immune cells, which plays a crucial role in psoriasis pathology. These interactions can result in the production of inflammatory cytokines, which was confirmed by ELISA methods, as well as

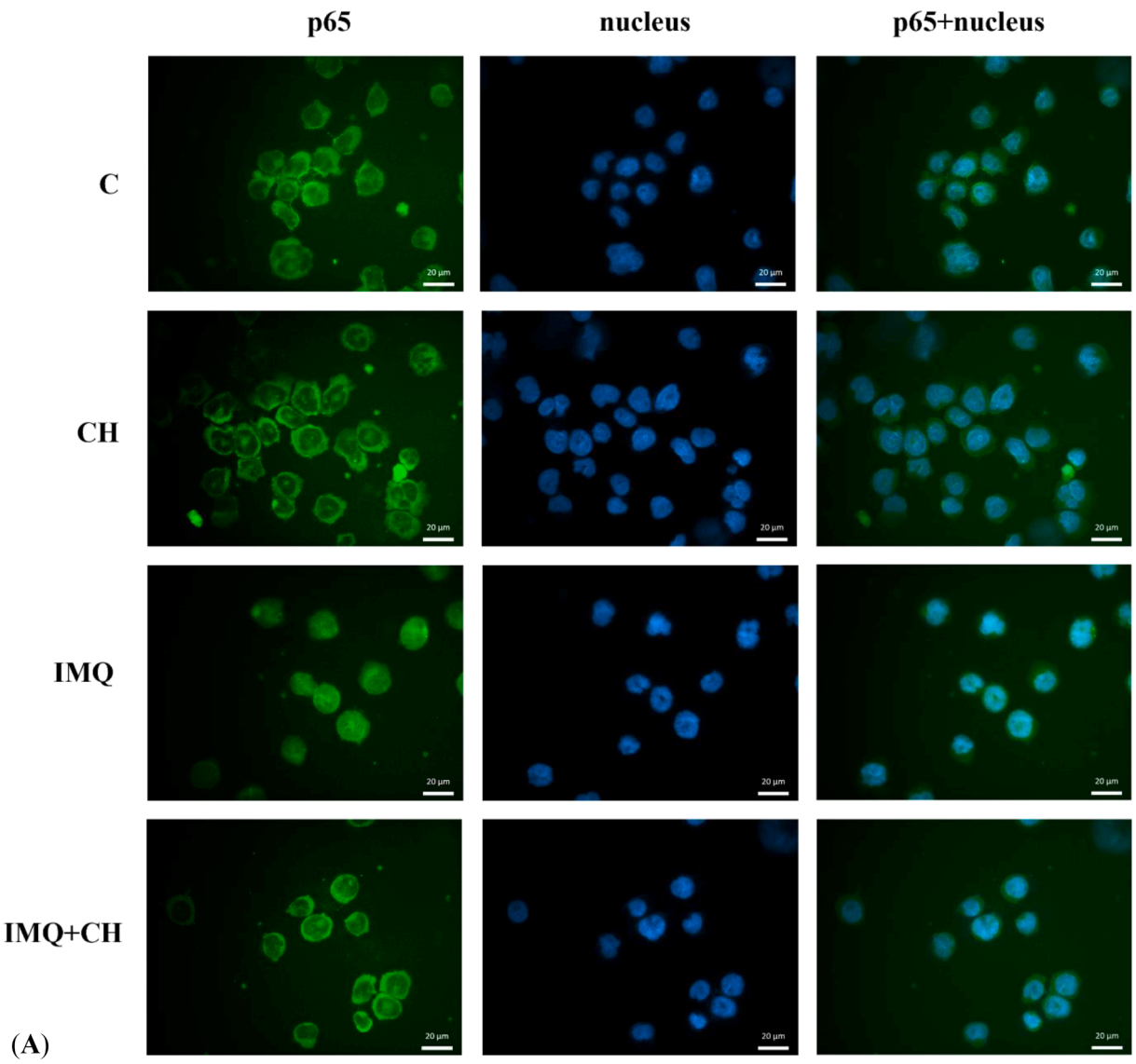


Fig. 7. Fluorescence microscopy investigation of the activation of the NF-κB pathway in THP-1 cells. THP-1 cells were treated with Imiquimod (IMQ) in a concentration of 1 µM, Chloroquine (CH) with a 13 concentration of 10 µM, and their combination (IMQ+CH) was used. One hour of incubation was applied. Nuclear localization of the p65 subunit was monitored by immunostaining. Green pixels: p65 subunit, blue pixels: cell nuclei. The scale bar is 20 µm (Fig. 7.A). Nucleus/cytosol intensity means the ratio of the fluorescence intensity of the NF-κB immunostaining in the cell nuclei and cytoplasm (Fig. 7.B). Results are presented as means ±SD. $n = 8–10$. Differences were considered significant at $p < 0.05$; *(IMQ + CH vs. C) $p < 0.05$, **** (IMQ vs. C) $p < 0.0001$; # (IMQ + CH vs. IMQ) $p < 0.05$.

the anti-inflammatory and barrier-stabilizing effect of AC. RTCA analysis shows that the induction effect of 1 μM IMQ was considered adequate. As compared to animal models, our model system is more cost-effective and it reduces the need for animal testing and the ethical issues are less. Furthermore, we can control our experimental conditions in vitro circumstances. To sum up, we propose a unique co-culture model for testing excipients of externally applied medicinal products and examination of the components of nutrients and cosmetics in psoriasis, but further validations are required for reliable application.

CRediT authorship contribution statement

Judit Váradi: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Boglárka Oláh:** Visualization, Investigation. **Dominik Hosszú:** Investigation. **Ferenc Fenyvesi:** Supervision. **Judit Remenyik:** Methodology. **Judit Homoki:** Investigation. **Béla Nagy:** Conceptualization. **Zsolt Fejes:** Investigation. **Ildikó Bácskay:** Funding acquisition. **Ágnes Klusóczki:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation.

Declaration of competing interest

We wish to confirm that there is no conflict of interest associated with this publication.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors would like to thank the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund. Project no. TKP2021-EGA-18 has been implemented with the support, and financed under the TKP2021-EGA funding scheme. Furthermore, the work/publication is supported by the GINOP-2.3.4–15–2016–00002 project, we would like to thank them for their financial support. The European Union and the European Regional Development Fund co-financed the project.

References

Albanesi, C., Madonna, S., Gisondi, P., Girolomoni, G., 2018. The interplay between keratinocytes and immune cells in the pathogenesis of psoriasis. Front. Immunol. 9 https://doi.org/10.3389/fimmu.2018.01549.

Bocheńska, K., Smolińska, E., Moskot, M., Jakóbkiewicz-Banecka, J., Gabig-Cimińska, M., 2017. Models in the research process of psoriasis. Int. J. Mol. Sci. 18 (12), 2514. https://doi.org/10.3390/ijms18122514.

Boehncke, W.-H., Schön, M.P., 2007. Animal models of psoriasis. Clinics in Dermatology 25 (6), 596–605. https://doi.org/10.1016/j.clindermatol.2007.08.014.

Chanput, W., Mes, J.J., Wichers, H.J., 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. Int. Immunopharmacol. 23 (1), 37–45. https://doi.org/10.1016/j.intimp.2014.08.002.

Christophers, E., Metzler, G., Röcken, M., 2014. Bimodal immune activation in psoriasis. Br. J. Dermatol. 170 (1), 59–65. https://doi.org/10.1111/bjd.12631.

Colombo, I., Sangiovanni, E., Maggio, R., Mattozzi, C., Zava, S., Corbett, Y., Fumagalli, M., Carlino, C., Corsetto, P.A., Scaccabarozzi, D., Calvieri, S., Gismondi, A., Taramelli, D., Dell'Agli, M., 2017. HaCaT cells as a reliable in vitro differentiation model to dissect the inflammatory/repair response of human keratinocytes. Mediators Inflamm. 2017, 1–12. https://doi.org/10.1155/2017/7435621.

di Cesare, A., di Meglio, P., Nestle, F.O., 2009. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. J. Investig. Dermatol. 129 (6), 1339–1350. https://doi.org/10.1038/jid.2009.59.

Elder, J.T., Bruce, A.T., Gudjonsson, J.E., Johnston, A., Stuart, P.E., Tejasvi, T., Voorhees, J.J., Abecasis, G.R., Nair, R.P., 2010. Molecular dissection of psoriasis: integrating genetics and biology. J. Investig. Dermatol. 130 (5), 1213–1226. https://doi.org/10.1038/jid.2009.319.

Eng, H.-L., Hsu, Y.-Y., Lin, T.-M., 2018. Differences in TLR7/8 activation between monocytes and macrophages. Biochem. Biophys. Res. Commun. 497 (1), 319–325. https://doi.org/10.1016/j.bbrc.2018.02.079.

Eskes, C., 2019. The HaCaT/THP-1 Cocultured Activation Test (COCAT) for skin sensitization: a study of intra-lab reproducibility and predictivity. ALTEX. https://doi.org/10.14573/altex.1905031.

Garay, T., Juhász, É., Molnár, E., Eisenbauer, M., Czirók, A., Dekan, B., László, V., Hoda, M.A., Döme, B., Tímár, J., Klepetko, W., Berger, W., Hegedűs, B., 2013. Cell migration or cytokinesis and proliferation? – revisiting the "go or grow" hypothesis in cancer cells in vitro. Exp. Cell Res. 319 (20), 3094–3103. https://doi.org/10.1016/j.yexcr.2013.08.018.

Gudjonsson, J.E., Johnston, A., Dyson, M., Valdimarsson, H., Elder, J.T., 2007. Mouse models of psoriasis. In: Journal of Investigative Dermatology, 127. Elsevier BV, pp. 1292–1308. https://doi.org/10.1038/sj.jid.5700807.

Hennen, J., 2017. Keratinocytes improve prediction of sensitization potential and potency of chemicals with THP-1 cells. ALTEX. 279–288. https://doi.org/10.14573/altex.1606171.

Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M., Blömeke, B., 2011. Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization. Toxicol. Sci. 123 (2), 501–510. https://doi.org/10.1093/toxsci/kfr174.

Homoki, J.R., Nemes, A., Fazekas, E., Gyémánt, G., Balogh, P., Gál, F., Al-Asri, J., Mortier, J., Wolber, G., Babinszky, L., Remenyik, J., 2016. Anthocyanin composition, antioxidant efficiency, and α-amylase inhibitor activity of different Hungarian sour cherry varieties (Prunus cerasus L.). Food Chem. 194, 222–229. https://doi.org/10.1016/j.foodchem.2015.07.130.

Hugh, J.M., Weinberg, J.M., 2018. Update on the pathophysiology of psoriasis. Cutis 102 (5S), 6–12.

Klusóczki, Á., Oláh, B., Hosszú, D., Fenyvesi, F., Remenyik, J., Homoki, J., Gyöngyösi, A., Bácskay, I., Váradi, J., 2023. Effectiveness of anthocyanin-rich sour cherry extract on gliadin-induced Caco-2 barrier damage. Nutrients. 15 (18), 4022. https://doi.org/10.3390/nu15184022.

Köllisch, G., Kalali, B.N., Voelcker, V., Wallich, R., Behrendt, H., Ring, J., Bauer, S., Jakob, T., Mempel, M., Ollert, M., 2005. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology 114 (4), 531–541. https://doi.org/10.1111/j.1365-2567.2005.02122.x.

Langley, R.G.B., 2005. Psoriasis: epidemiology, clinical features, and quality of life. Ann. Rheum. Dis. 64 (suppl_2), ii18–ii23. https://doi.org/10.1136/ard.2004.033217.

le Phuong Nguyen, T., Fenyvesi, F., Remenyik, J., Homoki, J.R., Gogolák, P., Bácskay, I., Fehér, P., Ujhelyi, Z., Vasvári, G., Vecsernyés, M., Váradi, J., 2018. Protective effect of pure sour cherry anthocyanin extract on cytokine-induced inflammatory Caco-2 monolayers. Nutrients 10 (7), 861. https://doi.org/10.3390/nu10070861.

Lebre, M.C., van der Aar, A.M.G., van Baarsen, L., van Capel, T.M.M., Schuitemaker, J.H.N., Kapsenberg, M.L., de Jong, E.C., 2007. Human keratinocytes express functional toll-like receptor 3, 4, 5, and 9. J. Investig. Dermatol. 127 (2), 331–341. https://doi.org/10.1038/sj.jid.5700530.

Petes, C., Odoardi, N., Gee, K., 2017. The toll for trafficking: toll-like receptor 7 delivery to the endosome. Front. Immunol. 8 https://doi.org/10.3389/fimmu.2017.01075.

Remenyik, J., Biró, A., Klusóczki, Á., Juhász, K.Z., Szendi-Szatmári, T., Kenesei, Á., Szőllősi, E., Vasvári, G., Stündl, L., Fenyvesi, F., Váradi, J., Markovics, A., 2022. Comparison of the modulating effect of anthocyanin-rich sour cherry extract on Occludin and ZO-1 on Caco-2 and HUVEC cultures. Int. J. Mol. Sci. 23 (16), 9036. https://doi.org/10.3390/ijms23169036.

Richwald, G.A., 1999. Imiquimod. Drugs Today 35 (7), 497. https://doi.org/10.1358/dot.1999.35.7.548263.

Schellenberger, M.T., Bock, U., Hennen, J., Groeber-Becker, F., Walles, H., Blömeke, B., 2019. A coculture system composed of THP-1 cells and 3D reconstructed human epidermis to assess activation of dendritic cells by sensitizing chemicals after topical exposure. Toxicol. Vitro 57, 62–66. https://doi.org/10.1016/j.tiv.2019.02.002.

Schön, M.P., Schön, M., 2007. Imiquimod: mode of action. Br. J. Dermatol. 157, 8–13. https://doi.org/10.1111/j.1365-2133.2007.08265.x.

Schön, M.P., Schön, M., Klotz, K.-N., 2006. The small antitumoral immune response modifier imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion. J. Investig. Dermatol. 126 (6), 1338–1347. https://doi.org/10.1038/sj.jid.5700286.

Schön, M.P., Manzke, V., Erpenbeck, L., 2021. Animal models of psoriasis—highlights and drawbacks. J. Allergy Clin. Immunol. 147 (2), 439–455. https://doi.org/10.1016/j.jaci.2020.04.034.

Skinner, R.B., 2003. Imiquimod. Dermatol. Clin. 21 (2), 291–300. https://doi.org/10.1016/S0733-8635(02)00094-3.

Smith, A.E., Kassab, J.Y., Payne, R., Beer, W.E., 1993. Bimodality in age of onset of psoriasis, in both patients and their relatives. Dermatology 186 (3), 181–186. https://doi.org/10.1159/000247341.

Thélu, A., Catoire, S., Kerdine-Römer, S., 2020. Immune-competent in vitro co-culture models as an approach for skin sensitisation assessment. Toxicol. Vitro 62, 104691. https://doi.org/10.1016/j.tiv.2019.104691.

Van Der Fits, L., Mourits, S., Voerman, J.S., Kant, M., Boon, L., Laman, J.D., Lubberts, E., 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J. Immunol. 182 (9), 5836–5845. https://doi.org/10.4049/jimmunol.0802999.

Wang, Z., Feng, Y., Hu, Q., Wang, X., Zhang, L., Liu, T., Zhang, J., Yang, X., Fu, Q., Fu, D., Hu, J., Liu, T., 2023. Keratinocyte TLR2 and TLR7 contribute to chronic itch through pruritic cytokines and chemokines in mice. J. Cell. Physiol. 238 (1), 257–273. https://doi.org/10.1002/jcp.30923.

YU, X., WANG, Y., ZHAO, W., ZHOU, H., YANG, W., GUAN, X., 2014. Toll-like receptor 7 promotes the apoptosis of THP-1-derived macrophages through the CHOP-dependent pathway. Int. J. Mol. Med. 34 (3), 886–893. https://doi.org/10.3892/ijmm.2014.1833.

Zheng, H., Gu, L., Wang, Z., Zhou, H., Zhang, C., Teng, X., Hu, Z., Wei, X., Liu, X., Zeng, F., Zhao, Q., Hao, Y., Hu, Y., Wang, X., Hu, J., Yu, J., Wu, W., Zhou, Y., Cui, K., Li, J., 2021. Establishing transcription profile of psoriasiform cutaneous in vitro using HaCaT cells stimulated with combination of cytokines. J. Visualized Exp. 169 https://doi.org/10.3791/61537.

Zhou, Y., Chen, X., Cao, Z., Li, J., Long, H., Wu, Y., Zhang, Z., Sun, Y., 2021. R848 is involved in the antibacterial immune response of Golden Pompano (Trachinotus ovatus) through TLR7/8-MyD88-NF-κB-signaling pathway. Front. Immunol. 11 https://doi.org/10.3389/fimmu.2020.617522.

Zhou, Z., Sun, L., 2015. Immune effects of R848: evidences that suggest an essential role of TLR7/8-induced, Myd88- and NF-κB-dependent signaling in the antiviral immunity of Japanese flounder (Paralichthys olivaceus). Dev. Compar. Immunol. 49 (1), 113–120. https://doi.org/10.1016/j.dci.2014.11.018.