

## Research Article

# The Influence of Adalimumab and Cyclosporine A on the Expression Profile of the Genes Related to TGF $\beta$ Signaling Pathways in Keratinocyte Cells Treated with Lipopolysaccharide A

Iwona Adwent <sup>1,2</sup>, Benjamin Oskar Grabarek <sup>1,3</sup>, Marta Kojs-Mrozkiewicz <sup>1</sup>,  
Ryszard Brus <sup>4</sup>, Rafał Staszkiwicz <sup>1,3</sup>, Andrzej Plewka <sup>5</sup>, Michał Stasiowski <sup>6</sup>,  
and Anita Lyssek-Boroń <sup>7,8</sup>

<sup>1</sup>Department of Histology, Cytophysiology and Embryology, Faculty of Medicine in Zabrze, University of Technology in Katowice, Poland

<sup>2</sup>Department of Dermatology, Andrzej Mielecki Memorial Independent Public Clinical Hospital, Medical University of Silesia, Katowice, Poland

<sup>3</sup>5th Military Clinical Hospital with the SP ZOZ Polyclinic, Krakow, Poland

<sup>4</sup>Department of Nurse, High School of Strategic Planning, Koscielna 6, 41-303 Dąbrowa Górnicza, Poland

<sup>5</sup>Institute of Health Sciences, University of Opole, Poland

<sup>6</sup>Department of Anaesthesiology and Intensive Therapy, SMDZ in Zabrze, Medical University of Silesia, Katowice, Poland

<sup>7</sup>Department of Ophthalmology with Paediatric Unit, St. Barbara Hospital, Trauma Center, Sosnowiec, Poland

<sup>8</sup>Department of Ophthalmology, Faculty of Medicine in Zabrze, University of Technology in Katowice, Poland

Correspondence should be addressed to Benjamin Oskar Grabarek; bgrabarek7@gmail.com

Received 2 June 2020; Accepted 13 July 2020; Published 26 July 2020

Academic Editor: Daniela Caccamo

Copyright © 2020 Iwona Adwent et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Background.** In the treatment of moderate to severe psoriasis, cyclosporine A (CsA) conventional therapy is used and biological, anti-cytokine treatment using, for example, anti-TNF drug—adalimumab. *Aim.* This study aimed at investigating the effect of CsA and adalimumab on the profile of mRNAs and protein expression associated with transforming growth factor  $\beta$  (TGF $\beta$ ) pathways in human keratinocyte (HaCaT) culture previously exposed to lipopolysaccharide (LPS). *Materials and Methods.* HaCaT culture was exposed to 1 ng/ml LPS for 8 hours+8  $\mu$ g/ml adalimumab for 2, 8, and 24 hours or 1 ng/ml LPS for 8 hours +100 ng/ml CsA for 2, 8, and 24 hours and compared to the control culture. Sulphorodamine B cytotoxicity assay was performed. The expression profile of mRNA related to TGF $\beta$  paths was indicated by microarray and RTqPCR analyses. The ELISA test was used to analyze changes on the proteome level. Statistical analysis consisted of ANOVA analysis and the post hoc Tukey test ( $p < 0.05$ ). *Results.* The cytotoxicity test showed that LPS, adalimumab, and cyclosporine in the concentration used in this experiment did not have any cytotoxicity effect on HaCaT cells. The largest fold changes (FC) in expression in ( $|FC| > 4.00$ ) was determined for *TGF $\beta$ 1-3*, *TGF $\beta$ R1-III*, *SKIL*, *SMURF2*, *SMAD3*, *BMP2*, *BMP6*, *JAK2*, *UBE2D1*, *SKP2*, *EDN1*, and *PRKAR2B* ( $p < 0.05$ ). In addition, on the protein level, the direct changes observed at mRNA were the same. *Conclusion.* Analysis of the microarray expression profile of genes associated with TGF $\beta$  signaling pathways has demonstrated the potential of cyclosporin A and adalimumab to induce changes in their transcriptional activity. The anti-TNF drug seems to affect TGF $\beta$  cascades to a greater extent than cyclosporin A. The obtained results suggest that the regularity of taking the drug is important for the efficacy of psoriasis therapy.

## 1. Introduction

The family of transforming growth factor beta (TGF $\beta$ ) is involved in the regulation of key cellular processes [1, 2]. The influence of TGF $\beta$  has been described and confirmed in the regulation of cell proliferation as well as the cell cycle [3, 4], cell differentiation [5], and programmed cell death [6–9].

There are 5 TGF $\beta$  isoforms; however, only the first three isoforms occur in humans [7, 10]. The biological effects exerted by this cytokine are the result of its interaction with TGF $\beta$ R1-III receptors. TGF $\beta$ 1 is characterized by the highest expression in human tissues among all cytokine isoforms. It plays an important role in the induction and pathogenesis of psoriasis, during which its elevated level is observed [11, 12]. Depending on the biological context, including changes in the level of secretion of interleukins (IL), IL-1, IL-6, and IL-8, TGF $\beta$  may inhibit or promote the proliferation of keratinocytes [13, 14]. Despite observations [15, 16], including ours [17, 18], that TGF $\beta$  may become a complementary molecular marker in the diagnosis of the severity of the psoriatic process [15–18], the exact mechanism of its action is still not fully understood.

It is worth noticing that according to the gene ontology, genes related to the TGF $\beta$  family are involved in the regulation of 52 different signaling pathways according to the PANTHER 14.1 program based on the gene ontology and the overrepresentation test [19]. The relationship of TGF $\beta$  is underlined, including the following signaling cascades: PI3K/Akt, Wnt, Hedgehog, Notch, tumor necrosis factor alpha (TNF- $\alpha$ ), JAK/STAT, interferon gamma (IFN- $\gamma$ ), SMAD, and nuclear factor kappa beta (NF $\kappa$ B) [20–23].

According to the recommendations of the Polish Dermatological Society dated 2018, cyclosporin A (CsA) and biological drugs, among others TNF- $\alpha$  inhibitors (adalimumab), are recommended for the treatment of moderate and severe cases of psoriasis [24, 25].

The molecular mechanism of action of CsA is associated with a decrease of IL-2 secretion. This is possible by immunostimulation of T lymphocytes, which is mediated by the cyclosporine-cyclophilin complex, which blocks the activity of calmodulin (CALN) [26–28]. TGF $\beta$  also inhibits IL-2 secretion via the SMAD-dependent pathway (TGF $\beta$ /SMAD3). Therefore, it seems important to identify changes in the expression of genes related to TGF $\beta$  under the influence of CsA [29–31]. In turn, adalimumab binds to the soluble and membrane-bound form of TNF- $\alpha$  and inhibits the interaction of this cytokine with the TNFR1 and TNFR2 receptors, consequently inhibiting the inflammatory response [32]. Beck indicates that between TNF- $\alpha$  and TGF- $\beta$ 1, there is a statistically significant positive correlation [33]. It is also indicated that TGF $\beta$  contributes to the increase in TNF- $\alpha$  expression; however, under the influence of CsA, the expression of TNF- $\alpha$  was suppressed [34]. However, under the influence of adalimumab therapy, the induction of the TGF $\beta$  pathway and the severity of its secretion by macrophages have been observed. This confirms the pleiotropic nature of cytokine activity and that the success of therapy depends on many factors [35].

The aim of this study was to evaluate the influence of adalimumab and cyclosporine A on the expression profile of genes associated with TGF $\beta$  signaling pathways in human keratinocyte cell line (HaCaT) after inducing an inflammatory process by lipopolysaccharide (LPS).

## 2. Material and Methods

**2.1. Cell Culture and Sulphorodamine B Cytotoxicity Assay.** Human keratinocyte cells (HaCaT; CLS Cell Lines Service, Eppelheim, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose–4500 mg/L (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and glutamine (2 mM) (Sigma-Aldrich, St. Louis, MO, USA) and maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

In the first stage of our research, the sulphorodamine B cytotoxicity assay (Sigma-Aldrich, St. Louis, MO, USA, catalog number 3520-42-1) was performed in accordance with the protocol provided by the manufacturer. The absorption was measured at a wavelength of 490–530 nm. The absorbance results observed from a control culture were described as 100%, and the percentage of viable cells in the culture exposed to any agents was revealed compared the control culture of untreated by the LPS/drug human keratinocyte cells.

The cytotoxicity assay was made for cell culture treated with lipopolysaccharide (LPS; Sigma Aldrich, Poznań, Poland) in three different concentrations: 1  $\mu$ g/ml, 2  $\mu$ g/ml, and 10  $\mu$ g/ml for, cells exposed to adalimumab (0.8  $\mu$ g/ml, 8  $\mu$ g/ml, and 80  $\mu$ g/ml), cyclosporine A (1 ng/ml, 10 ng/ml, and 100 ng/ml) in comparison with the untreated cells (control of the experiment).

In next step, based on our previous observation, human keratinocyte cells were exposed to LPS in concentration 1  $\mu$ g/ml for 8 hours [36] and next, 8  $\mu$ g/ml adalimumab and 100 ng/ml cyclosporine A was added (concentration corresponding to the average therapeutic concentration in blood plasma in patients with psoriasis) for 2, 8, and 24 hours. Untreated cells were a control culture [37, 38].

**2.2. RNA Isolation.** The first step of molecular analysis was isolation of complete RNA from cell cultures using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA, catalog number 15596026) according to the manufacturer's recommendation. Extracts of ribonucleic acid were evaluated qualitatively and quantitatively and, next, classified to the molecular analysis.

**2.3. Microarray Profile of Genes Expression.** The second step was associated with evaluation of the expression pattern of mRNAs related to TGF $\beta$  paths using expression microarray HG-U133A 2.0 (Affymetrix, Santa Clara, CA) and GeneChip™ 3' IVT PLUS Reagent Kit and GeneChip™ HT 3' IVTPLUS Reagent Kit (Thermo Fisher, catalog number 902416) according to the manufacturer's protocol.

The names of probes and number of their ID were found from the Affymetrix NetAffx™ Analysis Center database after

entering the phrase “TGF beta signaling pathways” (<http://www.affymetrix.com/analysis/index.affx>; accessed on 10<sup>th</sup> March 2020).

The microarray analysis consisted of synthesizing cDNA from RNA extracts, adding poly-A control, synthesizing of biotinyl aRNA and hybridization aRNA of specimens with microarray probes and data analysis. The detailed protocol for molecular analysis was also described in our previous studies [37, 38].

**2.4. Quantitative Reverse-Transcription Polymerase Chain Reaction.** In the second stage of molecular analysis, the RTqPCR reaction was carried out to confirm the changes in the transcriptional activity of the genes for which the differences in expression compared with the control (FC, fold change, the  $2^{-\Delta\Delta Ct}$  method) obtained from microarray data were  $|FC| > 4.00$  and corresponding with *TGFβ1-3*, *TGFβRI-III*, *SKIL*, *SMURF2*, *SMAD3*, *BMP2*, *BMP6*, *JAK2*, *UBE2D1*, *SKP2*, *EDN1*, and *PRKAR2B*. Endogenous control was β-actin (*ACTB*). Table 1 presents nucleotide sequences of primers used in RTqPCR. The thermal condition of RTqPCR was as follows: reverse transcription (45°C for 10 min), activation of the polymerase (95°C for 2 min), and 40 cycles including denaturation (95°C for 5 s), annealing (60°C for 10 s), and elongation (72°C for 5 s).

This was conducted with the use of the SensiFAST™ SYBR No-ROX One-Step Kit, (Bioline, London, UK) according to the protocol.

**2.5. ELISA Assay.** The next stage of the molecular analysis was associated with determining changes in the level of selected proteins related to TGFβ paths. Enzyme-linked immunosorbent assay (ELISA) reaction was carried out in this part of the study as recommended in the protocol. All ELISA kits were purchased from Thermo Fisher Scientific Company (Table 2).

**2.6. Statistical Analysis.** The statistical analysis of the obtained microarray data was done using the Transcriptome Analysis Console (Thermo Fisher, USA) and STATISTICA 13 PL (Cracow, Poland) software in order to analyze the results we obtained from the RTqPCR, ELISA reaction. The first stage of statistical analysis was to determine the normality of the data distribution. For this purpose, the Shapiro-Wilk test was performed; the result of which ( $p > 0.05$ ) indicated that further analysis should be carried out using parametric methods. ANOVA variance analysis was performed, followed by Tukey’s post-hoc test when expression changes were statistically significant ( $p < 0.05$ ).

### 3. Results

The performed sulphorodamine B cytotoxic test did not showed that LPS, adalimumab, and cyclosporine in concentration used to determinate expression profile of TGFβ-related genes affect the vitality of human keratinocyte ( $p > 0.05$ , one-way ANOVA test). However, when adalimumab in concentration of 80 μg/ml was added to the culture, it was observed that 41.07% cells are viable compared to

TABLE 1: The nucleotide sequence of primers used to amplify selected genes by RTqPCR reaction.

mRNA	Primers sequence
<i>TGFβ1</i>	Forward: 5' TGAACCGGCCTTTCCTGCTTC TCATG3'
	Reverse: 5' GCGGAAGTCAATGTACAGCTG CCGC3'
<i>TGFβ2</i>	Forward: 5' TACTACGCCAAGGAGGTTTAC AAA3'
	Reverse: 5' TTGTTTCAGGCACTCTGGCTTT3'
<i>TGFβ3</i>	Forward: 5' CTGGATTGTGGTTCCATGCA3'
	Reverse: 5' TCCCCGAATGCCTCACAT3'
<i>TGFβRI</i>	Forward: 5' -ACTGGCAGCTGTCATTGCTGG ACCAG-3'
	Reverse: 5' -CTGAGCCAGAACCTGACGTTG TCATATCA-3'
<i>TGFβR2</i>	Forward: 5' -GGCTCAACCACCAGGGCATCC AGAT-3'
	Reverse: 5' -CTCCCCGAGAGCCTGTCCAGA TGCT-3'
<i>TGFβR3</i>	Forward: 5' -ACCGTGATGGCATTGCGTTT GCA-3'
	Reverse: 5' -GTGCTCTGCGTGCTGCCGATG CTGT-3'
<i>SKIL</i>	Forward: 5' -AGAGACTCTGTTTGCCCAA-3'
	Reverse: 5' -CAGGATGGGGCATTGAATGG-3'
<i>SMURF2</i>	Forward: 5' -CGGCAAGAACTTCCCAACA-3'
	Reverse: 5' -GCAACGCCTCCATAGTCAAG-3'
<i>SMAD3</i>	Forward: 5' -CTCTGGGTGCTTGGGAACTA-3'
	Reverse: 5' -ATCCAAATGCAGCCAAACGT-3'
<i>BMP2</i>	Forward: 5' -AATGCAAGCAGGTGGGAAAG-3'
	Reverse: 5' -GCTGTGTTTCATCTTGGTGCA-3'
<i>BMP6</i>	Forward: 5' -AGAAGAAGGCTGGCTGGAAT-3'
	Reverse: 5' -GAAGGGCTGCTGTGCTGAAG-3'
<i>JAK2</i>	Forward: 5' -AGTAAAAGTCCACCAGCGGA-3'
	Reverse: 5' -AGGAGGGGCGTTGATTTACA-3'
<i>UBE2D1</i>	Forward: 5' -ATCCACCTGCTCACTGTTCA-3'
	Reverse: 5' -GTGACCATTGTGACCTCAGA-3'
<i>SKP2</i>	Forward: 5' -TTCACGTCATTCTCCTGCCT-3'
	Reverse: 5' -CATGCCTGTAATCCCAGCAC-3'
<i>EDN1</i>	Forward: 5' -ATCCTCTGCTGGTTCTGAC-3'
	Reverse: 5' -AGGTCCATTGTCATCCCCAG-3'
<i>PRKAR2B</i>	Forward: 5' -CTCAGGGAGATTCGGCTGAT-3'
	Reverse: 5' -ATTGACAGTCCCAATGGCG-3'
<i>ACTB</i>	Forward: 5' -GAAGGTGAAGGTCGGAGTC-3'
	Reverse: 5' -GAAGATGGTGATGGGATTC-3'

TABLE 2: Names of kit reagents used to determinate the level of selected protein by ELISA assay.

Protein	Name of the kit	Catalog number
TGF $\beta$ 1	TGF beta-1 Human ELISA Kit	BMS249-4
TGF $\beta$ 2	TGF beta-2 Human ELISA Kit	BMS254
JAK2	JAK2 (total) Human ELISA Kit	KHO5521
BMP2	BMP-2 Human ELISA Kit	EHBMP2
BMP6	BMP-6 Human ELISA Kit	EHBMP6
EDN1	Endothelin-1 (ET-1) Human ELISA Kit	EIAET1

untreated cells ( $p < 0.05$ , one-way ANOVA test, post-hoc Tukey test). Figure 1 shows cytotoxicity assay results.

The second stage of our study was focused on analyzing microarray profile of TGF $\beta$ -related genes.

Out of 22,277 mRNA IDs presented in HG-U133A\_2, 2777 mRNA were associated with TGF $\beta$  signaling pathways (Affymetrix NetAffx Analysis Center database after entering the phrase: “tgf beta signaling pathways”, data obtained on 28<sup>th</sup> February 2020).

The one-way ANOVA test indicated that from 999 mRNA on the significant level  $p < 0.05$ , 351 mRNA were differentiating keratinocyte culture exposed to LPS in comparison to a control, and for 16 of them, ( $|FC| > 4.00$ ). In turn, changes in expression of 307 mRNA were statistically significant in cell culture with LPS and adalimumab compared to the control, and 214 mRNA-differentiated HaCaT culture were exposed to LPS and cyclosporine A from the control.

Then, a post-hoc Tukey test was carried out. It allowed indicating the number of mRNA differentiating between the different groups compared. The analysis showed that the number of mRNAs differentiating HACAT cultures exposed to LPS and adalimumab compared with the control for each time of incubation is as follows: H-2 vs C-106 mRNA, H-8 vs C-84 mRNA, and H-24 vs C-11 mRNA. For cultures with LPS and cyclosporine A, the following number of differentiating mRNA may be observed compared to the control: H-2 vs C-111 mRNA, H-8 vs C-57 mRNA, and H-24 vs C-42 mRNA.

The next step was associated with determining the number of mRNA characteristic for 2, 8, and 24 hours exposition to the drug in comparison with the control.

With reference to keratinocytes exposed to LPS and adalimumab, it could be observed that 23 of 106 differentiating mRNA IDs were characteristic for 2-hour long incubation, 12 of 84 after 8 h exposition of the cells to anti-TNF drug, and only 2 of 11 mRNA specifically differentiating after 24 h. It is worth to see that 3 mRNA IDs corresponding to genes: TGF $\beta$ 1, BMP6, and JAK2 were differentiated genes regardless of the incubation time.

In turn, for keratinocyte culture treated with LPS and cyclosporine A, it indicated the following number of differentiating mRNA, 12 mRNA IDs of 111 mRNA IDs after 2 h compared to control and 9 mRNA IDs of 57 mRNA IDs after 8 h exposure of cells to drug, and prolonged incubation time to 24 h showed that 2 mRNA IDs of 42 mRNA IDs were char-

acteristic. In turn, 4 mRNA IDs changed their expression regardless of time: TGF $\beta$ 1, TGF $\beta$ R1, BMP2, and SMAD3.

Table 3 presents the results of microarray expression profile of genes related to TGF $\beta$  pathways ( $|FC| > 4.00$  from culture exposed to LPS in comparison with the control).

In turn, Figures 2 and 3 show the results of transcriptional activity of analysing genes obtained from RTqPCR as a validation of the microarray experiment. The obtained results confirmed differences in microarray expression pattern of evaluating genes.

Next, we assess variances in the level of TGF $\beta$ 1, TGF $\beta$ 2, JAK2, BMP2, BMP6, SMAD3, and EDN1 on the proteome level in the HaCaT culture treated with LPS and either adalimumab or cyclosporine (Table 4).

It can be observed that the direct changes were similar as observed on the transcriptome level. The statistical analysis indicated that variances in the expression pattern of all analyzed proteins in the HaCaT culture treated with any agent with comparison with the control, untreated cells were statistically significance ( $p < 0.05$ ).

The last step of analysis was carried out using the overrepresentation test with the Bonferroni correction to determine TGF $\beta$ -dependent signaling pathways associated with 16 differentiating keratinocyte genes, in both cultures. The analysis showed that the genes analyzed most closely associated with the TGF beta signaling pathway, oxidative stress response, G2/M transition of mitotic cell cycle, SCF-dependent proteasomal ubiquitin-dependent protein catabolic process, JAK/STAT signaling pathway, and interferon-gamma signaling pathway.

## 4. Discussion

There were several reasons for us to study the microarray gene expression profile associated with the signaling pathways induced by TGF $\beta$ . Firstly, in our previous studies, we observed that under the influence of cyclosporin A therapy in patients with psoriasis, there is a change in the transcriptional activity of TGF $\beta$ 1-3 and TGF $\beta$ R1-III [17, 18]. Studies have indicated that CsA therapy significantly increases the risk of skin cancer [39, 40]. Therefore, it seems reasonable to better understand how CsA also affects other transcripts of TGF $\beta$ -dependent path genes. Secondly, we also found that adalimumab influences the expression pattern of the BIRC5 gene [38], encodes survivin, which is not present under physiological conditions. Its expression is found in the case of the cancerous process [41–43]. Like ciclosporin, adalimumab therapy might be associated with an increased risk of skin cancers [44–46], which confirms the need for further research not only on the efficacy but also the safety profile of anti-TNF therapies. Changes in the expression of TGF $\beta$ s and signaling pathways induced by it are observed, e.g., in psoriasis [15–18], in renal fibrosis [47], in bone formation [48], and in the course of tumors [49, 50].

Therefore, in this study, we evaluated not only the effect of cyclosporin A and adalimumab on the expression of TGF $\beta$ -induced pathways but also compared the effect of both drugs on them. Comparing the microarray expression profile of analysed transcripts in HaCaT culture, it can be

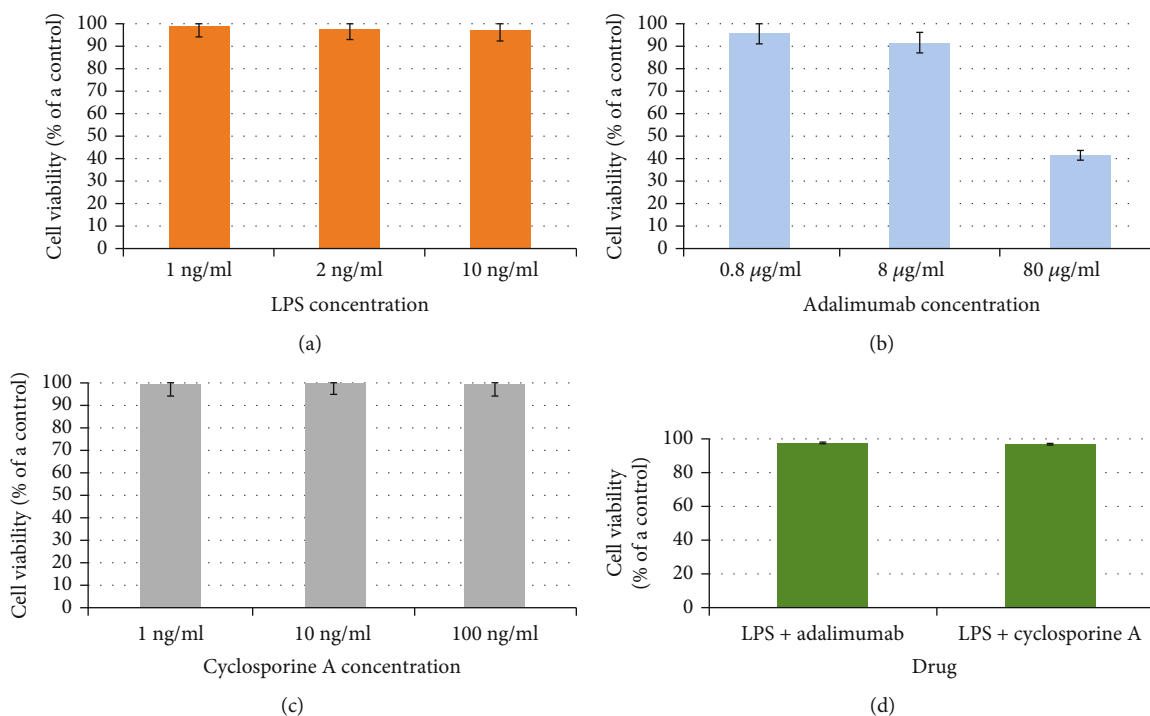


FIGURE 1: Results of cytotoxicity assay ((a) HaCaT with LPS, (b) HaCaT with adalimumab, (c) HaCaT with cyclosporine A, and (d) HaCaT with LPS and adalimumab or cyclosporine A).

TABLE 3: Microarray profile of TGFβ-dependent genes in HaCaT culture exposed to LPS or LPS+adalimumab/cyclosporine A (|FC| >4.00 in the first comparison).

Group ID	MRna	LPS		LPS+adalimumab		LPS+cyclosporine A		
		H-8 vs C	H-2 vs C	H-8 vs C	H-24 vs C	H-2 vs C	H-8 vs C	H-24 vs C
203085_s_at	TGFβ1	12.11	4.16	-8.36	-11.07	8.66	-2.33	-1.33
220407_s_at	TGFβ2	4.14	-3.28	-4.45	-3.02	1.01	-3.66	-4.06
209747_at	TGFβ3	-4.99	-1.22	3.16	3.22	2.22	2.18	3.01
206943_at	TGFβRI	4.03	-2.99	-3.05	-4.01	-2.1	-2.62	-2.66
207334_s_at	TGFβRII	4.15	-3.21	-3.33	-2.96	-4.36	-4.69	-5.02
204731_at	TGFβRIII	4.18	1.85	-1.74	-1.72	-1.11	-1.22	-1.31
206675_s_at	SKIL	-7.26	-4.02	2.23	3.11	-5.22	1.11	2.01
205596_s_a	SMURF2	7.09	-2.11	-9.14	-9.19	-3.39	-10.21	12.01
205397_x_at	SMAD3	18.96	-7.01	-11.99	-15.03	-8.13	19.11	-20.94
205289_at	BMP2	-11.55	8.01	7.96	8.12	3.62	7.05	7.06
206176_at	BMP6	-14.02	+1.01	19.06	22.05	-2.24	2.09	6.33
205842_s_at	JAK2	4.36	-1.25	-1.98	-2.06	-2.22	-4.08	-4.01
214590_s_at	UBE2D1	-3.88	-1.11	3.08	3.14	-2	1.11	-1.33
210567_s_at	SKP2	5.02	2.62	-3.42	-3.22	1.79	-2.33	-1.17
218995_s_at	EDN1	4.14	-1.48	-1.95	-2.07	-2.03	-1.99	-2.74
203680_at	PRKAR2B	4.29	1.22	-1.5	-1.11	-1.22	-1.32	-1.18

+ indicates overexpression of gene (increased level of mRNAs); - indicates suppressed gene expression (decreased level of mRNAs); ID: ID of the probe on a microarray; FC: fold change; C: control culture; H-2, H-8, and H-24: time of exposure to the medicine.

concluded that in adalimumab, statistically significant changes in transcriptional activity of a larger number of genes than cyclosporin A were made. Regarding the keratinocytes exposed to adalimumab, this study was focused on

genes that, based on the gene ontology, were classified directly on the TGFβ pathway [51].

SKIL encodes the Ski-related novel protein N (SnoN) which is associated with the TGFβ/SMAD pathway and, as

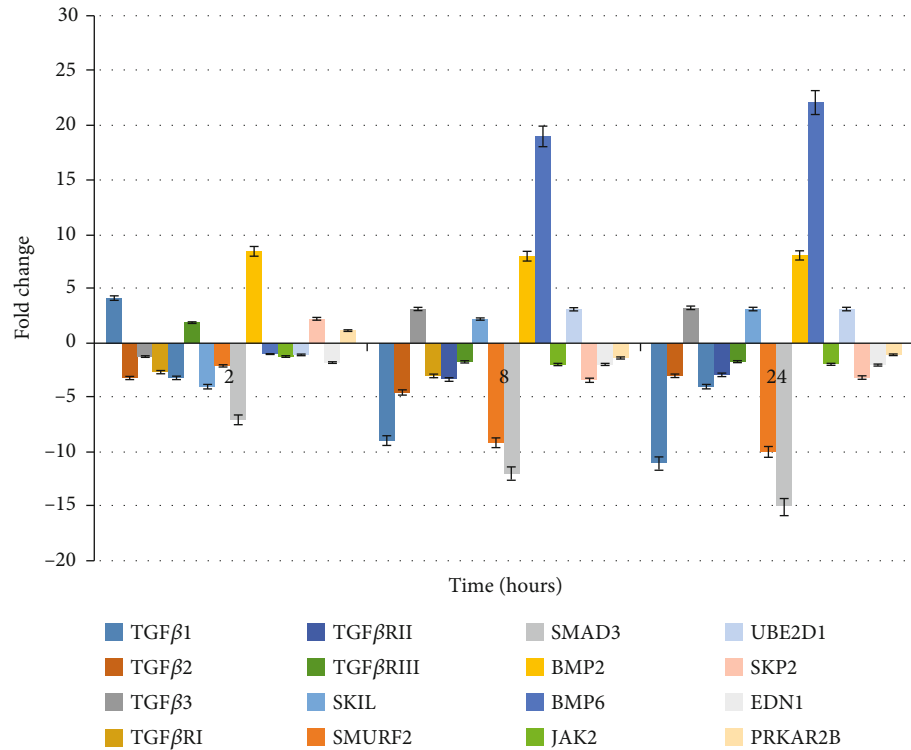


FIGURE 2: Expression of 14 mRNA in the HaCaT culture treated with LPS+adalimumab (RTqPCR results) presented as fold change in expression when compared to a control culture.

the studies show, also with the pathway dependent on the p53 gene, which plays a key role in carcinogenesis [52, 53]. Cao et al. showed a strong correlation between the expression of SKIL and TGFβ1 of hepatocellular carcinoma; the level of expression of which was significantly elevated compared to controls [54]. On the other hand, the observations made by Longerich et al. showed no significant role of SKIL in hepatocellular carcinoma [55], which is most probably associated with a smaller number of the study group than in the studies of Cao et al. [54]. In our studies, we found an increase in SKIL expression in keratinocytes exposed to adalimumab compared to controls, which may suggest changes associated with neoplastic transformation. The effect of silencing the SKIL gene expression can also be caused directly, activated by TGFβ [56] and SMAD3 [57–60], overexpressed in our study. However, with respect to the *SMURF2* gene belonging to the HECT family of E3 ubiquitin ligases, Smad ubiquitination regulators, the negative regulator of the TGFβ pathway [61–63], we observed a decrease in its level after administration of the anti-TNF drug, confirming the therapeutic effect of adalimumab. Zhao et al. demonstrated that TNF-α overexpresses SMURF2 while reducing transcripts encoding E-cadherin promoting epithelial-mesenchymal transition (EMT). [64]. It has also been confirmed that biological treatment restores the balance between SMURF2 and TNF-α expression [65]. Thus, considering the simultaneous expression of these three genes and the time from the introduction of the drug into the culture, it can be concluded that the observed changes may also result from the activation of adaptation mechanisms associated with the changing TNF-

α concentration in the presence of adalimumab and the aspiration of the homeostatic system. In turn, after 8 hours of HACAT incubation with the drug, we found overexpression of *BMP2* and *BMP6* (bone morphogenetic protein 2, 6) with simultaneous silencing of TGFβRIII expression; GDF5 (cartilage-derived morphogenetic protein 1) was involved in the differentiation of joint forming cells and interstitial spaces [66]. Chen et al. and Joo et al. emphasise, respectively, that the increase in the expression of BMP2 and BMP6 positively correlates with the severity of changes observed in ankylosing spondylitis [67, 68]. In addition, studies by Chen et al. indicated that the change in the expression profile of BMP2 resulted directly from the exposure of PBMCs to TNF-α [67]. Therefore, it seems that the observation of nearly 7- and 3-fold increase in the BMP2 and BMP6 transcriptional activities suggests that adalimumab did not adequately neutralize TNF-α. One should also not forget that cytokines are characterized by pleiotropic activity [69]. However, the study by Eguchi et al. should be taken into account, who also noted the overexpression of BMP2 under the influence of anti-TNF therapy (etanercept), which considered the beneficial effect of therapy [70]. The decrease in the *GDF5* (growth differentiation factor-5) transcriptional activity observed by us is convergent with the observations of Liu et al. who observed the silencing of its expression in diseases occurring with peripheral joint degradation. They emphasize that the observed expression profile of GDF5 is the result of the action of TNF-α and IL-1β, which inhibit the secretion of GDF5 [71, 72]. This is further evidence of the gradual disappearance of adalimumab and loss of keratinocytes

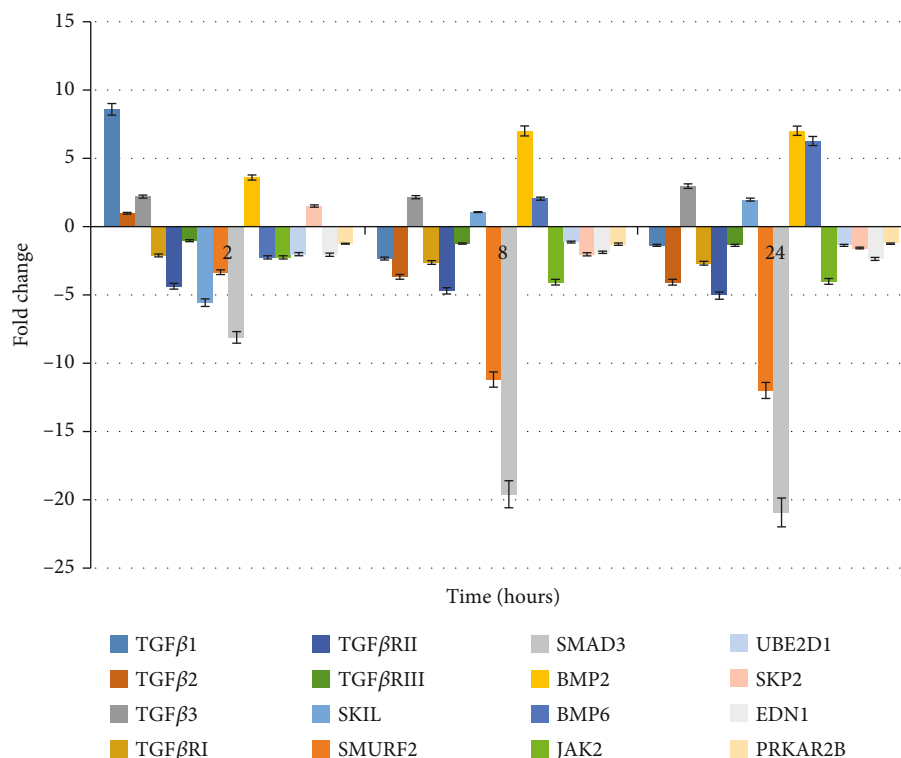


FIGURE 3: Expression of 14 mRNA in the HaCaT culture treated with LPS+cyclosporine A (RTqPCR results) presented as fold change in expression when compared to a control culture.

TABLE 4: Changes in the level of selected proteins in cell culture exposed to LPS, LPS+adalimumab, LP+cyclosporine A, and a control culture.

Protein	Control	LPS		LPS+adalimumab		LPS+cyclosporine A		
	H-0	H-8	H-2	H-8	H-24	H-2	H-8	H-24
TGF $\beta$ 1 (pg/ml)	396.85	1159.33	741.04	455.01	211.06	799.14	521.11	341.06
TGF $\beta$ 2 (pg/ml)	32.85	211.09	110.65	77.01	36.84	144.09	100.27	42.11
JAK2 (ng/ml)	2.22	27.11	12.44	8.01	3.12	11.02	6.37	3.18
BMP2 (pg/ml)	52.09	74.04	369.88	2104.44	2096.58	758.95	1985.09	2145.01
BMP6 (pg/ml)	36411.43	411.08	8856.02	10651.55	13751.06	2214.08	4648.12	10258.09
EDN1 (pg/ml)	12.67	33.25	22.47	21.01	18.25	19.24	18.78	18.96

susceptibility to anti-TNF. Therefore, regular, continuous intake of a TNF- $\alpha$  inhibitor is appropriate to maintain adequately low TNF- $\alpha$  levels. Comparing the level of *TGF $\beta$ RIII* in HACAT culture with adalimumab and in patients treated with cyclosporine A [17], the same direction of change and decrease in activity during the course of treatment can be seen.

JAK2 (Janus kinase 2) silencing during molecular-targeted therapy, including adalimumab has been observed in both Taylor et al. [73] and Vollenhoven et al. [74]. It should also be emphasized that the JAK2 expression profile indicates that adalimumab counteracts EMT [75], which is consistent with the observations in HaCaT culture exposed to the anti-TNF drug. *UBE2D1* is associated with the TGF $\beta$ /ubiquitin proteasome pathway [74]. Koné-Pau et al. indicated that ubiquitination and degradation in TNFR1 pro-

teasomes is one of the key factors of inhibition of the NF $\kappa$ B pathway, contributing to the reduction of inflammation [76]. Kawamoto et al. indicated that ubiquitination is a process controlled by NOTCH and TNF- $\alpha$  pathways and that infliximab (TNF- $\alpha$  inhibitor) therapy in the course of inflammatory bowel diseases results in a reduction of UBD at the protein level [77]. The *UBE2D1* expression profile that is different from our profile may result from the use of other anti-TNF drugs and their different construction and other research model.

Our analysis indicated that among the genes differentiating the culture exposed to cyclosporin A from control, the *SKP2* (S phase kinase-associated protein), *EDN1* (endothelin 1), and *PRKAR2B* (protein kinase cAMP-dependent type II regulatory subunit beta) genes showed association based on the analysis of the PANTHER 14.1 program. For all of these

transcripts, a decrease in activity was observed compared to untreated cells.

Changes in the transcriptional activity of the *SKP2* gene were analyzed most often in the context of carcinogenesis. It is indicated that this gene may become a new molecular marker of the carcinogenesis process, because its overexpression is associated with a worse prognosis [78–80]. Also in relation to *PRKR2B*, we found that CsA reduced the level of its expression, which is consistent with the observations of Zgheib et al. [81]. This gene encodes regulatory subunit of the cAMP-dependent protein kinases [82]; the increased expression of which is associated with the tumor cells obtaining a higher metastatic potential by induction of EMT [83, 84]. Therefore, the silencing of *SKP2* and *PRKR2B* after the addition of cyclosporin A may confirm its anti-inflammatory properties and efficacy. In addition, it can be assumed that in the described relationship between CsA therapy and an increased risk of cancer [39, 40], the *SKP2* and *PRKR2B* genes are not involved or their role is marginal.

When assessing the pattern of expression of *EDN1* under the influence of CsA, we found that it is different from that observed in HaCaT culture exposed to adalimumab [85]. Considering the observation by Chen et al. that hypoxia induces overexpression of *EDN1* [86] for 8 hours and that methotrexate inhibits endothelin activity [87], silencing the expression of *EDN1* confirms the anti-inflammatory properties of CsA and indirectly indicates the safety of its use [88]; the phenomenon of hypoxia is associated with the induction of neoplastic transformation [89, 90].

In summary, the regularity of taking the drug is important for the efficacy of psoriasis therapy. It should also take into account the regular observation of patients, monitoring the effectiveness of therapy and the possibility of adverse events during therapy with adalimumab and cyclosporin A.

## 5. Conclusions

Analysis of the expression profile of genes associated with TGF $\beta$  signaling pathways has demonstrated the potential of cyclosporin A and adalimumab to induce changes in their transcriptional activity. The anti-TNF drug seems to affect TGF $\beta$  cascades to a greater extent than cyclosporin A. The evaluation of the microarray profile indicated the efficacy and safety of both drugs being used in keratinocyte cell culture.

## Data Availability

The data used to support the findings of this study are included in the article. The data will not be shared due to the third-party rights and commercial confidentiality.

## Conflicts of Interest

The authors declare no conflict of interest, financial, or otherwise.

## Authors' Contributions

I.A. and B.O.G. should be considered joint first authors. I.A. is responsible for conceptualization, investigation, visualization, writing—original draft, review, and editing. B.O.G. performed conceptualization, data curation, given final approval of the version to be published, writing—review and editing, methodology, and revising it critically. M.K.M. is responsible for analysis and interpretation of data, writing—original draft. R.B. is responsible for data curation and writing—original draft. In turn, R.S. is responsible for project administration and statistical analysis. A.P. has given the final approval of the version to be published and performed acquisition of data. M.S. is responsible for project administration and statistical analysis. A.L.B. has given the final approval of the version to be published, revising it critically.

## Acknowledgments

We would like to thank Mister Oskar Ogloszka for improving the article by English checking and editing.

## References

- [1] G. E. Esebanmen and W. H. R. Langridge, "The role of TGF- $\beta$  signaling in dendritic cell tolerance," *Immunologic Research*, vol. 65, no. 5, pp. 987–994, 2017.
- [2] L. O. Roy, M. B. Poirier, and D. Fortin, "Differential expression and clinical significance of transforming growth factor- $\beta$  isoforms in GBM tumors," *International Journal of Molecular Sciences*, vol. 19, no. 4, p. 1113, 2018.
- [3] Y. Zhang, P. B. Alexander, and X.-F. Wang, "TGF- $\beta$  family signaling in the control of cell proliferation and survival," *Cold Spring Harbor Perspectives in Biology*, vol. 9, no. 4, p. a022145, 2017.
- [4] S. Colak and P. ten Dijke, "Targeting TGF- $\beta$  signaling in cancer," *Trends in Cancer*, vol. 3, no. 1, pp. 56–71, 2017.
- [5] I. Grafe, S. Alexander, J. R. Peterson et al., "TGF- $\beta$  family signaling in mesenchymal differentiation," *Cold Spring Harbor Perspectives in Biology*, vol. 10, no. 5, p. a022202, 2018.
- [6] D. Kajdaniuk, B. Marek, H. Borgiel-Marek, and B. Kos-Kudła, "Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) in physiology and pathology," *Endokrynologia Polska*, vol. 64, no. 5, pp. 384–396, 2013.
- [7] A. B. Roberts, "Molecular and cell biology of TGF- $\beta$ ," *Mineral and Electrolyte Metabolism*, vol. 24, no. 2-3, pp. 111–119, 1998.
- [8] R. Ganesan and N. Sivalingam, "Transforming growth factor beta 2 inhibits growth and proliferation potential of Smad4 and p53 mutated human colon adenocarcinoma cells," *Pathology Oncology Research*, vol. 25, no. 2, pp. 819–821, 2019.
- [9] N. Schmitt and H. Ueno, "Regulation of human helper T cell subset differentiation by cytokines," *Current Opinion in Immunology*, vol. 34, pp. 130–136, 2015.
- [10] H. Sabbineni, A. Verma, and P. R. Somanath, "Isoform-specific effects of transforming growth factor  $\beta$  on endothelial-to-mesenchymal transition," *Journal of Cellular Physiology*, vol. 233, no. 11, pp. 8418–8428, 2018.
- [11] C. Albanesi, "Immunology of psoriasis," in *Clinical Immunology*, pp. 871–878, Elsevier B.V., 2019, Content repository only!



- [12] C. Fotiadou, E. Lazaridou, E. Sotiriou, and D. Ioannides, "Targeting IL-23 in psoriasis: current perspectives," *Psoriasis: Targets and Therapy*, vol. Volume 8, pp. 1–5, 2018.
- [13] G. Han, C. A. Williams, K. Salter, P. J. Garl, A. G. Li, and X. J. Wang, "A role for TGFbeta signaling in the pathogenesis of psoriasis," *The Journal of Investigative Dermatology*, vol. 130, no. 2, pp. 371–377, 2010.
- [14] H. H. El-hadidi, A. S. Hassan, G. El-hanafy, K. S. Amr, S. F. Abdelmesih, and M. F. Abdelhamid, "Transforming growth factor- $\beta$ 1 gene polymorphism in psoriasis vulgaris," *Clinical, Cosmetic and Investigational Dermatology*, vol. 11, pp. 415–419, 2018.
- [15] I. Flisiak, P. Zaniewski, and B. Chodyncka, "Plasma TGF-beta1, TIMP-1, MMP-1 and IL-18 as a combined biomarker of psoriasis activity," *Biomarkers*, vol. 13, no. 5, pp. 549–556, 2008.
- [16] A.-R. M. A. Meki and H. Al-Shobaili, "Serum vascular endothelial growth factor, transforming growth factor  $\beta$ 1, and nitric oxide levels in patients with psoriasis vulgaris: their correlation to disease severity," *Journal of Clinical Laboratory Analysis*, vol. 28, no. 6, pp. 496–501, 2014.
- [17] A. Michalska-Bańkowska, D. Wcisło-Dziadecka, B. Grabarek et al., "Variances in the mRNA expression profile of TGF- $\beta$ 1-3 isoforms and its TGF- $\beta$ RI-III receptors during cyclosporin a treatment of psoriatic patients," *Advances in Dermatology and Allergology*, vol. 35, no. 5, pp. 502–509, 2018.
- [18] A. Michalska-Bańkowska, D. Wcisło-Dziadecka, B. Grabarek et al., "Quantitative analysis of transforming growth factor beta isoforms mRNA TGF- $\beta$ 1-3 in the patients with psoriasis," *Postępy Nauk Medycznych*, vol. 31, no. 1A, pp. 4–9, 2018.
- [19] H. Mi, B. Lazareva-Ulitsky, R. Loo et al., "The PANTHER database of protein families, subfamilies, functions and pathways," *Nucleic Acids Research*, vol. 33, suppl\_1, pp. 284–288, 2004.
- [20] B. S. Li, J. Y. Huang, J. Guan, and L. H. Chen, "Camptothecin inhibits the progression of NPC by regulating TGF- $\beta$ -induced activation of the PI3K/AKT signaling pathway," *Oncology Letters*, vol. 16, no. 1, pp. 552–558, 2018.
- [21] A. Di Stefano, C. Sangiorgi, I. Gnemmi et al., "TGF- $\beta$  signaling pathways in different compartments of the lower airways of patients with stable COPD," *Chest*, vol. 153, no. 4, pp. 851–862, 2018.
- [22] X. Guo and X. F. Wang, "Signaling cross-talk between TGF- $\beta$ /BMP and other pathways," *Cell Research*, vol. 19, no. 1, pp. 71–88, 2009.
- [23] K. Luo, "Signaling cross talk between TGF- $\beta$ /Smad and other signaling pathways," *Cold Spring Harbor Perspectives in Biology*, vol. 9, no. 1, p. a022137, 2017.
- [24] A. Reich, Z. Adamski, G. Chodorowska et al., "Psoriasis. Diagnostic and therapeutic recommendations of the Polish Dermatological Society. Part I: Mild psoriasis," *Dermatology Review*, vol. 105, no. 2, pp. 225–243, 2018.
- [25] A. Reich, J. Szepietowski, Z. Adamski et al., "Psoriasis Diagnostic and therapeutic recommendations of the Polish Dermatological Society Part II: moderate to severe psoriasis," *Dermatology Review*, vol. 105, no. 3, pp. 329–357, 2018.
- [26] M. Kovalik, K. L. Thoday, and A. H. M. van den Broek, "The use of ciclosporin A in veterinary dermatology," *The Veterinary Journal*, vol. 193, no. 2, pp. 317–325, 2012.
- [27] P. Forsythe and S. Paterson, "Ciclosporin 10 years on: indications and efficacy," *The Veterinary Record*, vol. 174, Suppl 2, pp. 13–21, 2014.
- [28] R. Diehl, F. Ferrara, C. Müller et al., "Immunosuppression for in vivo research: state-of-the-art protocols and experimental approaches," *Cellular & Molecular Immunology*, vol. 14, no. 2, pp. 146–179, 2017.
- [29] D. Krasowska, "Zastosowanie cyklosporyny w dermatologii w oparciu o najnowsze badania kliniczne," *Practical Dermatology*, vol. 5, no. 4, pp. 5–16, 2013.
- [30] S. C. McKarns, R. H. Schwartz, and N. E. Kaminski, "Smad3 Is Essential for TGF- $\beta$ 1 to suppress IL-2 production and TCR-induced Proliferation, but not IL-2-induced proliferation," *The Journal of Immunology*, vol. 172, no. 7, pp. 4275–4284, 2004.
- [31] B. H. Nelson, T. P. Martyak, L. J. Thompson, J. J. Moon, and T. Wang, "Uncoupling of prometogenic and antiapoptotic functions of IL-2 by Smad-dependent TGF-beta signaling," *The Journal of Immunology*, vol. 170, no. 11, pp. 5563–5570, 2003.
- [32] Y. Tone, K. Furuuchi, Y. Kojima, M. L. Tykocinski, M. I. Greene, and M. Tone, "Smad3 and NFAT Cooperate to induce Foxp3 expression through its enhancer," *Nature Immunology*, vol. 9, no. 2, pp. 194–202, 2008.
- [33] B. Beck, "Transforming growth factor- $\beta$ 1 and tumor necrosis factor- $\alpha$  concentration in serum during disturbed lymph flow from a liver in rats," *Journal of Physiology and Pharmacology*, vol. 68, no. 1, pp. 91–98, 2017.
- [34] Y. Q. Zhang, Y. Chen, Y. M. Ding, and T. H. Yu, "Protective effect of cyclosporine on inflammatory injury of renal tubular epithelial cells," *European Review for Medical and Pharmacological Sciences*, vol. 22, no. 19, pp. 6551–6559, 2018.
- [35] Z. Szondy and A. Pallai, "Transmembrane TNF-alpha reverse signaling leading to TGF-beta production is selectively activated by TNF targeting molecules: therapeutic implications," *Pharmacological Research*, vol. 115, pp. 124–132, 2017.
- [36] M. Schweizer, I. Adwent, B. O. Grabarek, and D. Boroń, "Analysis of the influence of adalimumab to the expression pattern of mRNA and protein of TGF- $\beta$ 1-3 in dermal fibroblast exposed to lipopolysaccharide," *Advances in Dermatology and Allergology*, vol. 37, no. 1, 2020.
- [37] B. Grabarek, D. Wcisło-Dziadecka, J. Gola et al., "Changes in the expression profile of Jak/Stat signaling pathway genes and miRNAs regulating their expression under the adalimumab therapy," *Current Pharmaceutical Biotechnology*, vol. 19, no. 7, pp. 556–565, 2018.
- [38] B. Grabarek, M. Schweizer, I. Adwent et al., "Differences in expression of genes related to drug resistance and miRNAs regulating their expression in skin keratinocytes exposed to adalimumab and cyclosporine A," *Advances in Dermatology and Allergology*, vol. 36, no. 1, 2019.
- [39] N. Skroza, N. Bernardini, I. Proietti, and C. Potenza, "Clinical utility of ingenol mebutate in the management of actinic keratosis: perspectives from clinical practice," *Therapeutics and Clinical Risk Management*, vol. Volume 14, pp. 1879–1885, 2018.
- [40] S. Geller, H. Xu, M. Leibold, B. Nardone, M. E. Lacouture, and M. Khetarpal, "Malignancy risk and recurrence with psoriasis and its treatments: a concise update," *American Journal of Clinical Dermatology*, vol. 19, no. 3, pp. 363–375, 2018.
- [41] W. H. Lim, G. R. Russ, G. Wong, H. Pilmore, J. Kanellis, and S. J. Chadban, "The risk of cancer in kidney transplant recipients may be reduced in those maintained on everolimus and reduced cyclosporine," *Kidney International*, vol. 91, no. 4, pp. 954–963, 2017.

- [42] G. Troiano, A. Guida, G. Aquino et al., "Integrative histologic and bioinformatics analysis of BIRC5/Survivin expression in oral squamous cell carcinoma," *International Journal of Molecular Sciences*, vol. 19, no. 9, p. 2664, 2018.
- [43] L. Wang, J. Huang, M. Jiang, and L. Sun, "Survivin (BIRC5) cell cycle computational network in human no-tumor hepatitis/cirrhosis and hepatocellular carcinoma transformation," *Journal of Cellular Biochemistry*, vol. 112, no. 5, pp. 1286–1294, 2011.
- [44] X. Luo, C. Deng, Y. Fei et al., "Malignancy development risk in psoriatic arthritis patients undergoing treatment: a systematic review and meta-analysis," *Seminars in Arthritis and Rheumatism*, vol. 48, no. 4, pp. 626–631, 2019.
- [45] M. M. Asgari, G. T. Ray, J. L. Geier, and C. P. Quesenberry, "Malignancy rates in a large cohort of patients with systemically treated psoriasis in a managed care population," *Journal of the American Academy of Dermatology*, vol. 76, no. 4, pp. 632–638, 2017.
- [46] F. Atzeni, A. Carletto, R. Foti et al., "Incidence of cancer in patients with spondyloarthritis treated with anti-TNF drugs," *Joint, Bone, Spine*, vol. 85, no. 4, pp. 455–459, 2018.
- [47] X. M. Meng, P. M. K. Tang, J. Li, and H. Y. Lan, "TGF- $\beta$ /Smad signaling in renal fibrosis," *Frontiers in Physiology*, vol. 6, p. 82, 2015.
- [48] M. Wu, G. Chen, and Y. P. Li, "TGF- $\beta$  and BMP signaling in osteoblast skeletal development and bone formation homeostasis and disease," *Bone Research*, vol. 4, no. 1, 2016.
- [49] V. Syed, "TGF- $\beta$  signaling in cancer," *Journal of Cellular Biochemistry*, vol. 117, no. 6, pp. 1279–1287, 2016.
- [50] C. J. David, Y. H. Huang, M. Chen et al., "TGF- $\beta$  tumor suppression through a lethal EMT," *Cell*, vol. 164, no. 5, pp. 1015–1030, 2016.
- [51] I. Fabregat, J. Moreno-Càceres, A. Sánchez et al., "TGF- $\beta$  signalling and liver disease," *The FEBS Journal*, vol. 283, no. 12, pp. 2219–2232, 2016.
- [52] J. Ning, Y. Zhao, Y. Ye, and J. Yu, "Opposing roles and potential antagonistic mechanism between TGF- $\beta$  and BMP pathways: implications for cancer progression," *eBioMedicine*, vol. 41, pp. 702–710, 2019.
- [53] D. Pan, Q. Zhu, M. J. Conboy, I. M. Conboy, and K. Luo, "SnoN activates p53 directly to regulate aging and tumorigenesis," *Aging Cell*, vol. 11, no. 5, pp. 902–911, 2012.
- [54] Y. Cao, R. Agarwal, F. Dituri et al., "NGS-based transcriptome profiling reveals biomarkers for companion diagnostics of the TGF- $\beta$  receptor blocker galunisertib in HCC," *Cell Death & Disease*, vol. 8, no. 2, article e2634, 2017.
- [55] T. Longerich, K. Breuhahn, M. Odenthal, K. Petmecky, and P. Schirmacher, "Factors of transforming growth factor beta signalling are co-regulated in human hepatocellular carcinoma," *Virchows Archiv*, vol. 445, no. 6, pp. 589–596, 2004.
- [56] N. G. Denissova, C. Pouponnot, J. Long, D. He, and F. Liu, "Transforming growth factor beta -inducible independent binding of SMAD to the Smad7 promoter," *Proceedings of the National Academy of Sciences*, vol. 97, no. 12, pp. 6397–6402, 2000.
- [57] S. L. Stroschein, W. Wang, S. Zhou, Q. Zhou, and K. Luo, "Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein," *Science*, vol. 286, no. 5440, pp. 771–774, 1999.
- [58] S. L. Stroschein, S. Bonni, J. L. Wrana, and K. Luo, "Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN," *Genes & Development*, vol. 15, no. 21, pp. 2822–2836, 2001.
- [59] S. Bonni, H. R. Wang, C. G. Causing et al., "TGF- $\beta$  induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation," *Nature Cell Biology*, vol. 3, no. 6, pp. 587–595, 2001.
- [60] Q. Zhu, S. Pearson-White, and K. Luo, "Requirement for the SnoN oncoprotein in transforming growth factor  $\beta$ -induced oncogenic transformation of fibroblast cells," *Molecular and Cellular Biology*, vol. 25, no. 24, pp. 10731–10744, 2005.
- [61] G. Murakami, T. Watabe, K. Takaoka, K. Miyazono, and T. Imamura, "Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads," *Molecular Biology of the Cell*, vol. 14, no. 7, pp. 2809–2817, 2003.
- [62] M. Z. Qureshi, S. Jabeen, G. Butt et al., "Tudor tells about new twists in the story tale of SMURFs," *Cellular and Molecular Biology*, vol. 62, no. 5, pp. 38–43, 2016.
- [63] P. Kavsak, R. K. Rasmussen, C. G. Causing et al., "Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation," *Molecular Cell*, vol. 6, no. 6, pp. 1365–1375, 2000.
- [64] C. Zhao, Z. Xu, Z. Wang et al., "Role of tumor necrosis factor- $\alpha$  in epithelial-to-mesenchymal transition in transplanted kidney cells in recipients with chronic allograft dysfunction," *Gene*, vol. 642, pp. 483–490, 2018.
- [65] C. Suo, J. Zhou, G. U. Min, and R. Tan, "Bortezomib suppresses renal tubular epithelial-mesenchymal transition induced by TNF- $\alpha$  through downregulation of Smurf2 in HK-2 cells," *Chinese Journal of Organ Transplantation*, vol. 39, no. 4, pp. 232–237, 2018.
- [66] G. Schett, R. Landewé, and D. van der Heijde, "Tumour necrosis factor blockers and structural remodelling in ankylosing spondylitis: what is reality and what is fiction?," *Annals of the Rheumatic Diseases*, vol. 66, no. 6, pp. 709–711, 2007.
- [67] M. H. Chen, H. A. Chen, W. S. Chen, M. H. Chen, C. Y. Tsai, and C. T. Chou, "Upregulation of BMP-2 expression in peripheral blood mononuclear cells by proinflammatory cytokines and radiographic progression in ankylosing spondylitis," *Modern Rheumatology*, vol. 25, no. 6, pp. 913–918, 2015.
- [68] Y. B. Joo, S. Y. Bang, T. H. Kim et al., "Bone morphogenetic protein 6 polymorphisms are associated with radiographic progression in ankylosing spondylitis," *PLoS One*, vol. 9, no. 8, article e104966, 2014.
- [69] E. Corsini and R. V. House, "Evaluating cytokines in immunotoxicity testing," *Methods in Molecular Biology*, vol. 1803, pp. 297–314, 2018.
- [70] Y. Eguchi, S. Wakitani, Y. Imai et al., "Antitumor necrotic factor agent promotes BMP-2-induced ectopic bone formation," *Journal of Bone and Mineral Metabolism*, vol. 28, no. 2, pp. 157–164, 2010.
- [71] F. L. Liu, L. H. Lin, H. K. Sytwu, and D. M. Chang, "GDF-5 is suppressed by IL-1 $\beta$  and enhances TGF- $\beta$ 3-mediated chondrogenic differentiation in human rheumatoid fibroblast-like synoviocytes," *Experimental and Molecular Pathology*, vol. 88, no. 1, pp. 163–170, 2010.
- [72] N. G. Juel, J. I. Brox, J. C. Hellund, K. B. Holte, and T. J. Berg, "The prevalence of radiological glenohumeral osteoarthritis in long-term type 1 diabetes: the Dialong shoulder study," *Scandinavian Journal of Rheumatology*, vol. 47, no. 4, pp. 325–330, 2018.

- [73] P. C. Taylor, E. C. Keystone, D. van der Heijde et al., “Baricitinib versus placebo or adalimumab in rheumatoid arthritis,” *The New England Journal of Medicine*, vol. 376, no. 7, pp. 652–662, 2017.
- [74] R. F. van Vollenhoven, R. Fleischmann, S. Cohen et al., “Tofacitinib or adalimumab versus placebo in rheumatoid arthritis,” *The New England Journal of Medicine*, vol. 367, no. 6, pp. 508–519, 2012.
- [75] S. Y. Kim and K. H. Baek, “TGF- $\beta$  signaling pathway mediated by deubiquitinating enzymes,” *Cellular and Molecular Life Sciences*, vol. 76, no. 4, pp. 653–665, 2019.
- [76] I. Kone-Paut, S. Georjin-Laviallec, C. Galeotti et al., “New data in causes of autoinflammatory diseases,” *Joint Bone Spine*, vol. 86, no. 5, pp. 554–561, 2019.
- [77] A. Kawamoto, S. Nagata, S. Anzai et al., “Ubiquitin D is upregulated by synergy of notch signalling and TNF- $\alpha$  in the inflamed intestinal epithelia of IBD patients,” *Journal of Crohn's & Colitis*, vol. 13, no. 4, pp. 495–509, 2019.
- [78] J. Jia, J. Ren, Y. He et al., “The prognostic value of Skp2 expression level in cancer: a meta-analysis,” *International Journal of Clinical and Experimental Medicine*, vol. 11, no. 2, pp. 431–444, 2018.
- [79] Y. Zhang, Y. S. Zvi, B. Batko et al., “Down-regulation of Skp2 expression inhibits invasion and lung metastasis in osteosarcoma,” *Scientific Reports*, vol. 8, no. 1, p. 14294, 2018.
- [80] Z. Li, D. L. Qi, H. P. Singh, Y. Zou, B. Shen, and D. Cobrinik, “A novel thyroid hormone receptor isoform TR $\beta$ 2-46 promotes SKP2 expression and retinoblastoma cell proliferation,” *The Journal of Biological Chemistry*, vol. 294, no. 8, pp. 2961–2969, 2019.
- [81] E. Zgheib, A. Limonciel, X. Jiang et al., “Investigation of Nrf2, AhR and ATF4 activation in toxicogenomic databases,” *Frontiers in Genetics*, vol. 9, 2018.
- [82] A. Wachter and T. Beißbarth, “Decoding cellular dynamics in epidermal growth factor signaling using a new pathway-based integration approach for proteomics and transcriptomics data,” *Frontiers in Genetics*, vol. 6, 2016.
- [83] J. Sha, Q. Han, C. Chi et al., “PRKAR2B promotes prostate cancer metastasis by activating Wnt/ $\beta$ -catenin and inducing epithelial-mesenchymal transition,” *Journal of Cellular Biochemistry*, vol. 119, no. 9, pp. 7319–7327, 2018.
- [84] J. Sha, W. Xue, B. Dong et al., “PRKAR2B plays an oncogenic role in the castration-resistant prostate cancer,” *Oncotarget*, vol. 8, no. 4, pp. 6114–6129, 2017.
- [85] D. Wcisło-Dziadecka, B. Grabarek, N. Zmarzły et al., “Influence of adalimumab on the expression profile of genes associated with the histaminergic system in the skin Fibroblasts in vitro,” *BioMed Research International*, vol. 2018, Article ID 1582173, 11 pages, 2018.
- [86] I. C. Chen, Y. T. Lin, J. S. Huang et al., “Decreased ambient oxygen tension alters the expression of endothelin-1 iNOS and cGMP in rat alveolar macrophages,” *International Journal of Medical Sciences*, vol. 16, no. 3, pp. 443–449, 2019.
- [87] Y. Koyama, A. Ukita, K. Abe et al., “Dexamethasone downregulates endothelin receptors and reduces endothelin-induced production of matrix metalloproteinases in cultured rat astrocytes,” *Molecular Pharmacology*, vol. 92, no. 1, pp. 57–66, 2017.
- [88] L. A. Nattkemper, H. L. Tey, R. Valdes-Rodriguez et al., “The genetics of chronic itch: gene expression in the skin of patients with atopic dermatitis and psoriasis with severe itch,” *Journal of Investigative Dermatology*, vol. 138, no. 6, pp. 1311–1317, 2018.
- [89] K. L. Eales, K. E. R. Hollinshead, and D. A. Tennant, “Hypoxia and metabolic adaptation of cancer cells,” *Oncogenesis*, vol. 5, no. 1, article e190, 2016.
- [90] I. Marchiq and J. Pouyssegur, “Hypoxia cancer metabolism and the therapeutic benefit of targeting lactate/H<sup>+</sup>v symporters,” *Journal of Molecular Medicine*, vol. 94, no. 2, pp. 155–171, 2016.