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Unraveling the biological potential of skin fibroblast: responses to TNF-α, highlighting intracellular signaling pathways and secretome

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ABSTRACT

Objective: In this study, we examined the molecular response of human skin fibroblasts to an inflammatory cytokine to evaluate their suitability as models for immunopathology research.
Methods: Skin fibroblasts were stimulated with tumour necrosis factor (TNF)-α, and the transcriptome was profiled via RNA-Seq. The differentially expressed genes were screened to predict immunological pathways and interactions. The cytokines and signaling pathways were validated at protein level. Similarly to immune cells, TNF-α caused transcriptional and transductional changes in fibroblasts.
Results: Functional analysis revealed significant enrichment of TNF-α signaling and cell chemotaxis (normalized enrichment score = 2.59 and 3.42). We also detected enrichment of nuclear factor kappa B (NF-κB) target genes and NF-kB activation, confirmed by complete protein degradation of its inhibitor IκBa ($p = 0.0019$). The MAPK/ERK and p38 MAPK pathways were also activated. Finally, we observed significant secretion of proinflammatory cytokines and chemokines, such as interleukin 6 ($p = 0.02$), CXCL8 ($p = 0.027$), CCL2 ($p = 0.028$) and CCL5 ($p = 0.016$).
Conclusion: This study advances the biological understanding of skin fibroblast responses to TNF-α, revealing their intracellular pathways and secretome. It discloses techniques for leveraging fibroblasts' potential as *in vitro* models to identify inflammatory drivers, particularly when alternative models are inaccessible.

List of abbreviations

CCL	C-C motif chemokine ligand
CT	cycle threshold
CXCL	C-X-C motif chemokine ligand
DEGs	differentially expressed genes
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinase 1/2
FC	fold change
FDR	false discovery rate
GO	gene ontology
GSEA	gene set enrichment analysis
IL	interleukin
MAPK	mitogen-activated protein kinase
NES	normalized enrichment score
NF-κB	nuclear factor kappa B
NK	natural killer
PBS	Phosphate Buffer Saline
PC	principal component
RD	rare disease
TAK1	transforming growth factor b-activated kinase 1
TLRs	toll-like receptors
TNF(R)	tumour necrosis factor (receptor)

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1. Introduction

The immune response plays a crucial role in the pathogenesis and progression of many diseases. The analysis of immunological processes is essential for understanding of these diseases [1,2]. However, one major challenge to study various diseases is accessing tissue samples due to the low patient numbers and the complexity of sampling processes [3,4]. In fact, tissue sampling involves various professionals, technological, institutional and ethical factors to ensure that patients are spared from unnecessary procedures and healthcare visits [5]. Patient tissues are rationally available in biobanks [6,7], which constitute valuable opportunities for disease communities, alleviating issues related to sample access and facilitating research and drug development [8,9]. One of the most commonly collected samples are primary skin fibroblasts, which have the capacity to grow with minimal processing and yield information about an individual's health [10]. Fibroblasts, owing to their accessibility, reprogramming potential and applicability as disease models are invaluable resources for various diseases research [10–13].

Fibroblasts, the main component of connective tissue, exhibit varying morphologies and functions depending on their anatomical location and yet reflected in unique molecular and structural profiles that support vital functions, including trauma resistance, tissue repair, and extracellular matrix (ECM) reorganization [14–20]. Additionally, they secrete several mediators of local inflammation, such as cytokines and growth factors, allowing cell−cell communication with immune cells [21] express immune receptors essential for signaling pathway activation [22,23]. Among these receptors are the tumor necrosis factor receptors (TNFRs) [24] which respond to TNF-α (responsible for signaling pathways leading to necrosis or apoptosis) and play a critical role in activating inflammatory, immune-modulatory and apoptotic pathways [24,25]. TNFR activation induces the production of several key interleukins (ILs) and chemokines in fibroblasts and is involved in various biological processes, such as leukocyte differentiation and recruitment [23–26]. Thus, fibroblasts are considered sentinel cells for recognising pathological stimuli which contribute to immune response modulation to maintain homeostasis [22] and support and/or suppress immune responses related to infection [27,28], autoimmunity [29,30] and cancer [31].

Fibroblast features suggest that these cells can be used as *in vitro* models for studying molecular mechanisms underlying immunopathology, particularly when cellular and animal models are scarce. This approach is particularly important for patients with a genetic aetiology due to the reliance on limited single-centre samples such as PMM2-CDG, Coffin-Siris and periodontal Ehlers–Danlos syndrome, new models are urgently needed to understand immune defects [32–34].

This study aimed to describe the molecular response of skin fibroblasts to TNF-α and highlights their potential as reliable *in vitro* models for studying inflammatory drivers and responses when other models are unavailable.

2. Materials and methods

2.1. Cells, cell culture and TNF-α stimulation

Three primary human skin fibroblasts (GM00498 (3 years male), GM00969 (2 years female) and GM03349 (10 years male) from healthy donors were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. The cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 g/L glucose, l-glutamine (2 mM), penicillin-streptavidin (100 units/mL and 100 μg/mL) and 10 % (v/v) heat-inactivated foetal bovine serum at 37 °C in a 5 % CO_2 incubator. All cell culture reagents were obtained from Gibco. 5×10^5 cells were seeded in T75 flasks, and after 3 days, the cells were either stimulated with 10 ng/ml TNF-α for 5 h for gene expression analysis. 3×10^4 cells/200 μL/well were seeded in 96-well plate to perform resazurin assay. After 24 h, the cells were stimulated with 200 μL of tenfold serial dilutions of TNF-α in complete DMEM. To detect signaling proteins by western blotting, 2×10^5 cells were seeded in 6-well plates and stimulated with 10 ng/ml TNF-α for 0 to 120 min Fig. 1.

2.2. Cell viability and metabolic activity determined by the resazurin assay

Cell viability and metabolic activity were assessed by measuring the conversion of resazurin to resorufin [35]. After cell stimulation, the cells were incubated in complete DMEM containing 44 μM resazurin for 3.5 h and absorbance was taken at 600 and 570 nm using a UV−Vis microplate reader (SpectraMax190, Molecular Devices). Resorufin levels were estimated by calculating the difference between the absorbance values at 600 nm and 570 nm. The results of the stimulated samples were normalized against the non-stimulated conditions.

2.3. RNA extraction and quality control

Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instructions. The RNA purity and concentration were assessed using a NanoDrop (Thermo Fisher Scientific). RNA integrity was analysed using the High Sensitivity RNA Analysis Kit on a Fragment Analyser (Agilent Technologies, Inc.). RNA was deemed acceptable if the 280:260 nm and 260:230 nm ratios were greater than 1.9 and 1.5, respectively, and if the 28S:18S ratio was greater than 2.0.

2.4. RNA sequencing (RNA-seq)

cDNA libraries were prepared by the Genomics Unit of Instituto Gulbenkian de Ciência (Oeiras, Portugal) using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD (Lexogen Ghmb). RNA sequencing was performed on a NextSeq500 (Illumina) in 75-base single-end mode, with a minimum target coverage of 6 million reads per library.

2.5. Differential gene expression and correlation analyses of TNF-α-stimulated fibroblasts

The QuantSeq 3′ mRNA-Seq Integrated Data Analysis Pipeline (Lexogen GmbH) on the BlueBee® Genomics Platform was used to obtain the read counts for each gene of each sample. Specifically, the quality control of the RNA-Seq raw data was assessed using FASTQC (v 0.11.5) [36]. BBDuk software (v 35.92) was used to trim and remove the standard adapter sequences and poly(A) tails [37]. The resulting trimmed reads were aligned with the GRCh38 reference genome using STAR (v 2.5.2a) and counted via HTSeq [38,39].

The transcriptomic statistical analysis was done using the R (v 4.1.1) computer language as well as most graphical representations, including principal component (PC) analysis, boxplots, heatmaps and volcano plots [40]. The scripts were developed to conduct the analysis which are available at https://github.com/PGranjo/Model-of-inflammation.git. The quality-controlled read counts were assembled on a matrix, and the low number of read counts on each sample was filtered using the "edgeR" (v 3.36) package [41]. This package was also used to screen the differentially expressed genes (DEG) between TNF-α-stimulated and non-stimulated cells using a false discovery rate (FDR) ≤ 0.05 as the cut-off.

To explore similarities in gene expression patterns among fibroblasts and various immune cells in response to TNF-α, we analysed publicly available data from the CytoSig database [42]. Specifically, RNA-Seq transcriptomic profiles of dermal fibroblasts and immune cells upon TNF-α stimulation were downloaded (E-MTAB-5622 [43], SE109460 [44], GSE100382 [45], GSE95588 [46], GSE129210 [47], GSE98624 [48], GSE40548 [49] and GSE70068 [50]). The parametric Pearson correlation was used to examine the similarity of the transcriptome

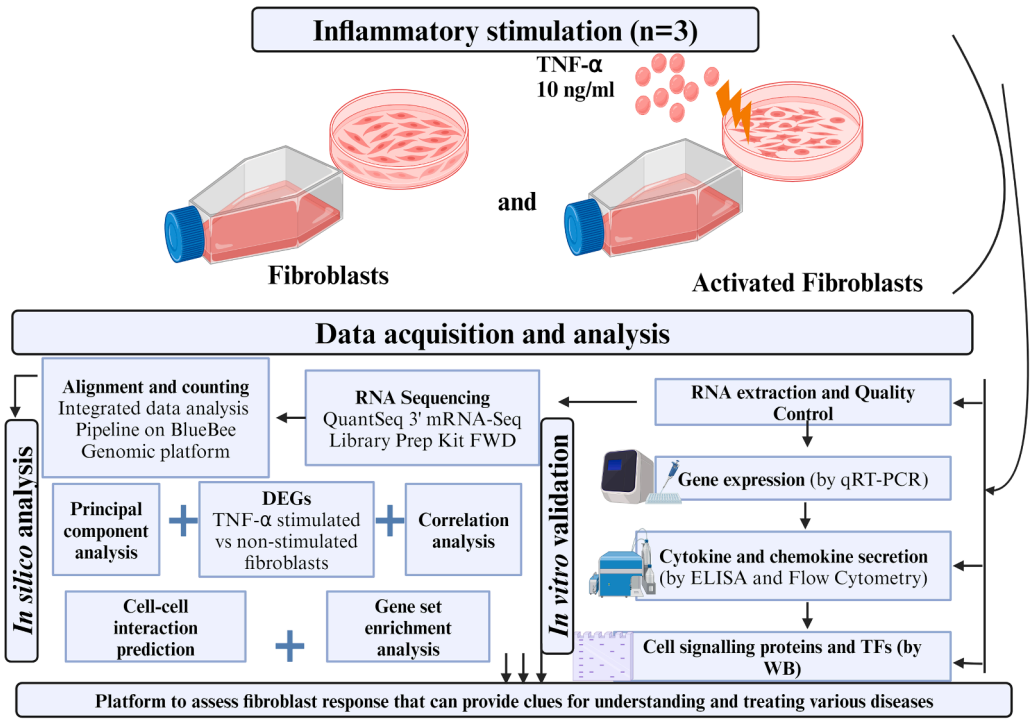

Fig. 1. Overview of the study design. A schematic representation of the preclinical research study design aimed at characterizing skin fibroblasts as a potential *in vitro* model for studying immunomodulation upon inflammatory stimuli, providing a platform to aid in the understanding and treatment of rare diseases. Created with BioRender. Legend: DEGs – Differentially expressed genes; TNF– Tumour necrosis factor; WB – Western blot.

alterations upon TNF-α stimulation (log_2 fold change (FC)) between immune cells and skin fibroblasts, using FDR $\leq$ 0.05 as the cut-off, and was calculated using the corrplot package (v 0.92) [51].

2.6. Gene set enrichment analysis and cell–cell interaction analysis of TNF-α-stimulated fibroblasts

Based on the ranked gene list obtained using the sign(log_2(FC))-log_{10}(FDR) as a metric, gene set enrichment analysis (GSEA) was carried out with the WEB-based GEne SeT AnaLysis Toolkit 2019 [52] with an FDR$\leq$0.05 set as the cut-off and a minimum of 5 and a maximum of 2000 genes per category. The enriched GO terms were further visualized through Cytoscape's Enrichment Map Plugin [53,54]. For each group, the terms with links with >5 nodes were selected using the WordCloud plugin and encompassed by a circle [55].

The fibroblast and immune cell interactions were analysed from receptor–ligand pairs and repertoires of 144 primary cell types from the FANTOM5 project [56]. The corresponding ligand/receptor pairs were analysed for their gene expression in different immune cell lines (Supplementary Material 1). A threshold of 10 transcripts/million (~3 transcript copies/cell) was used to identify expressed receptors and ligands in immune cells, as previously described [56,57].

2.7. Gene expression analysis by real-time quantitative polymerase chain reaction (RT–qPCR)

cDNA was obtained with a high-capacity cDNA transcription kit (Applied Biosystems) as described previously [58]. RT–qPCR was performed using TaqMan probes and primers (Thermo Fisher Scientific), namely, IL-6 (Hs00174131_m1), as well as the endogenous controls β-actin (4352935E) and GAPDH (4333764F). The reaction was carried out on a Rotor-Gene 6000 Series (Corbett Research Ltd.). Gene expression was assessed by adapting the $2^{-\Delta\Delta}$ cycle threshold (CT) method [59]. The ΔCT was calculated by the difference between the CT values for the gene of interest and the endogenous control genes under a particular condition. The ΔΔCT was obtained to compare the stimulated *versus* the non-stimulated condition by subtracting the corresponding ΔCT.

2.8. Cytokine and chemokine secretion quantification by ELISA and LegendPlex technologies

Cell supernatants were analysed by sandwich ELISA to determine IL-1β, IL-4, IL-6, IL12 (p40), IL-15, and IFN-γ concentrations using Immunotools kits. The absorbance was measured at 450 nm in a UV–Vis microplate reader (SpectraMax190, Molecular Devices). A custom LEGENDplex™ Human Proinflammatory Chemokine Panel 1 (BioLegend) kit targeting C-X-C motif chemokine ligand (CXCL) 1, CXCL5, CXCL8, CCL2 and CCL5 was used [60]. The signal quantification was performed with an LSRFortessa™ X-20 Cell Analyser (BD Biosciences). The cytokine/chemokine concentrations were calculated using specific standard curves and normalized to the protein concentration quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2.9. Cell signaling protein detection by Western blot

Following stimulation, the cells were washed with cold PBS and lysed in Pierce IP lysis buffer (Thermo Fisher Scientific) supplemented with protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche) and phosphatase (1 mM sodium orthovanadate, Sigma–Aldrich) inhibitors. The protein concentration was determined using the Pierce BCA Protein Assay Kit. For nuclear factor kappa B (NF-κB) p65 detection, 25 μg of protein was separated on a 12.5 % SDS–PAGE acrylamide gel (Bio-Rad) for 4 h and subsequently transferred to PVDF membranes (Millipore) for 2.5 h at 500 mA. For the remaining proteins, 30 μg of protein extract was separated on a 10 % SDS–PAGE gel. The membranes were blocked with Carbofree blocking solution (VectorLabs). Immunoblotting was performed using antibodies against NF-κB p65 (1:500, sc-372), Iκ-Bα (1:600, sc-371), p38 (1:1000, sc-728) (Santa Cruz Biotechnology), P-p38 (1:1000, #9211), extracellular signal-regulated kinase

1/2 (ERK1/2, 1:1000, #9102) and P-ERK1/2 (1:1000, #9101) (Cell Signaling Technology) overnight, followed by 1 h of incubation with the secondary antibodies peroxidase AffiniPure donkey anti-rabbit IgG (*H + L*) (1:10000, #711-035-152, Jackson Laboratories) or anti-mouse-IgG-HRP (1:2500, #554002, BD Biosciences). The signal was visualized using the Lumi-Light Western Blotting Substrate (Roche) and X-ray films (Amersham Hyperfilm™ ECL). For the loading control, the membranes were stripped using ReStore® Western Blot stripping buffer (Thermo Fisher Scientific) and reprobed using a mouse monoclonal anti-α-tubulin antibody (T6074, 1:50000; Sigma–Aldrich). The band density was quantitated using ImageJ v.1.43 software and normalized to that of α-tubulin [61].

2.10. Statistical analysis

The normality of the data was assessed by the Shapiro–Wilk test. The remaining data was presented as the mean ± standard deviation. For comparisons of means between two groups, a two-tailed unpaired Student's t-test was performed. For comparisons of means between three or more groups, one-way ANOVA with Dunnett's multiple comparison test was performed. In multiple testing p values were adjusted using the FDR method and the statistical significance of the differences was analysed using GraphPad Prism (v.8.4.0, GraphPad LLC). Differences were considered statistically significant if p value/FDR≤0.05 or marginally significant if 0.05<*p* value/FDR≤0.1. The statistical analysis of the transcriptome data is described in the Transcriptome Data Analysis section.

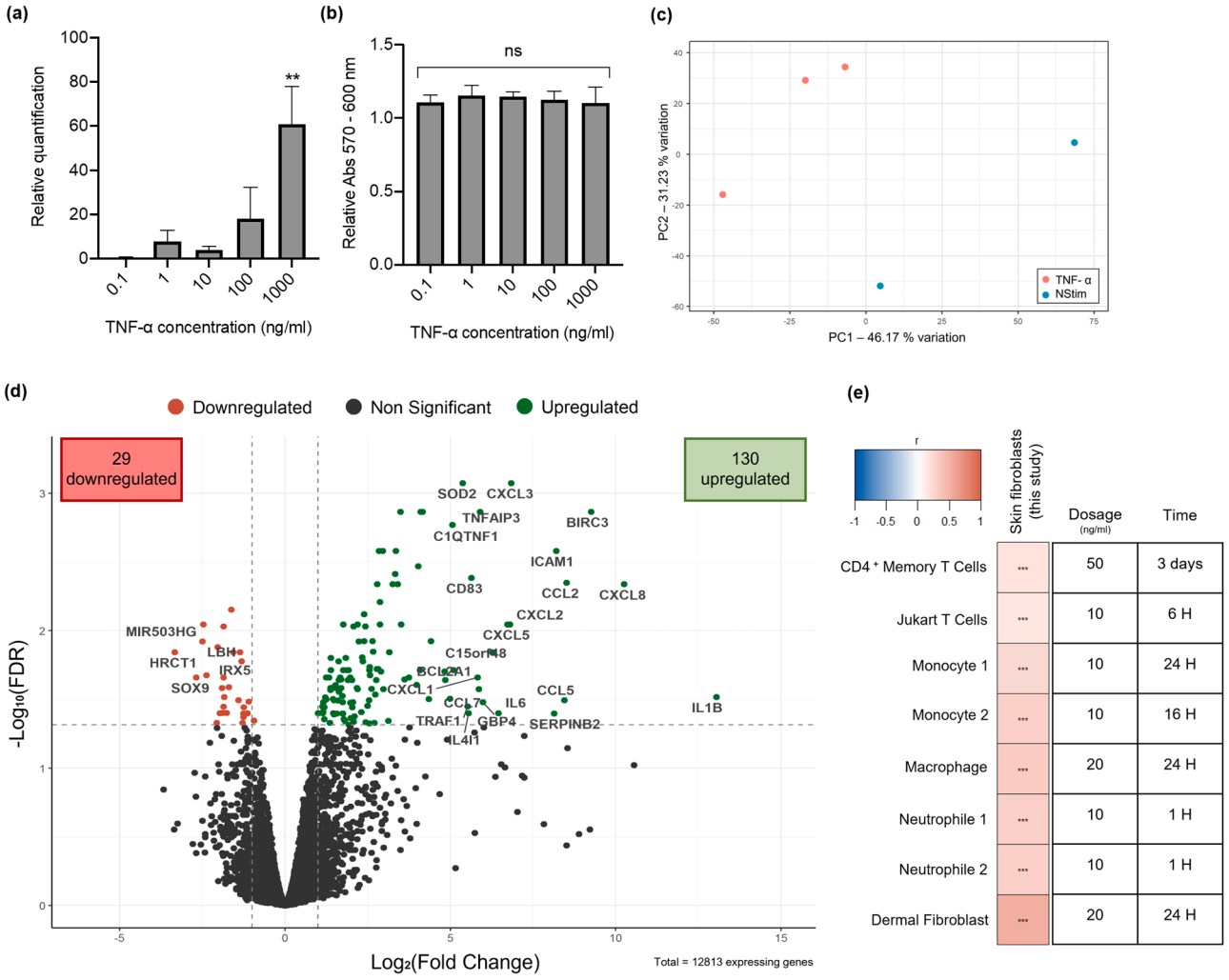

Fig. 2. TNF-α causes a shift in the fibroblast transcriptional profile, which is correlated with the immune cell response. (A) Relative IL6 gene expression in response to TNF-α stimulation ($t = 5$ h) at various concentrations (0.1, 1, 10, 100 and 1000 ng/ml) quantified by RT–qPCR. IL6 mRNA levels were normalized to those of β-actin and GAPDH, which were used as endogenous controls, and the relative quantification was performed according to the nonstimulated conditions ($2^{-\Delta\Delta CT}$ method). The data are presented as the means ± SDs ($n = 3$). Statistical significance was assessed by one-way ANOVA with Dunnett's multiple comparisons (FDR < 0.01 (**)); (B) Cell viability and metabolic activity of fibroblasts after TNF-α stimulation ($t = 24$ h) at different concentrations (0.1, 1, 10, 100 and 1000 ng/ml) quantified by the resazurin assay. The data are presented as the means ± SDs ($n = 3$). Relative values are related to the nonstimulated conditions. (C) Principal component (PC) analysis biplots of human skin fibroblasts before and upon TNF-α stimulation (10 ng/ml, $t = 5$ h). Stimulated (red dots, $n = 3$) and nonstimulated (NStim, blue dots, $n = 2$) samples were distinguished by two factors, PC1 and PC2, which accounted for 46.17 % and 31.23 %, respectively, of the data variability. The nonstimulated condition of one of the samples was excluded from this analysis due to an RNA sequencing technical issue. (D) Volcano plot of all expressed genes. The DEG cut-off criterion was an FDR < 0.05. The red dots represent the downregulated DEGs, the green dots represent the upregulated genes, and the black dots represent all genes not differentially expressed upon stimulation (FDR > 0.05). (E) The whole transcriptomic differential expression profile of stimulated fibroblasts positively correlated with all TNF-α-stimulated immune cell transcriptomic profiles in the CytoSig database (FDR < 0.001 (***) based on parametric Pearson correlation).

3. Results

3.1. The effects of TNF-α stimulation on skin fibroblasts mimic the transcriptome alterations observed in immune cell lines

In the obtained results, the TNF-α induced the expression of the *IL6* gene in skin fibroblasts, with higher doses tending to lead to increased gene expression of *IL6* (Fig. 2A). Additionally, TNF-α treatment led to a 10 to 15 % increase in cell metabolic activity without causing a decrease in viability (Fig. 2B, Supplementary Material 2–Supplementary Fig. 1). The fibroblasts expressed both TNF-α receptors (TNFR1 and TNFR2) Supplementary Material 2–Supplementary Fig. 2).

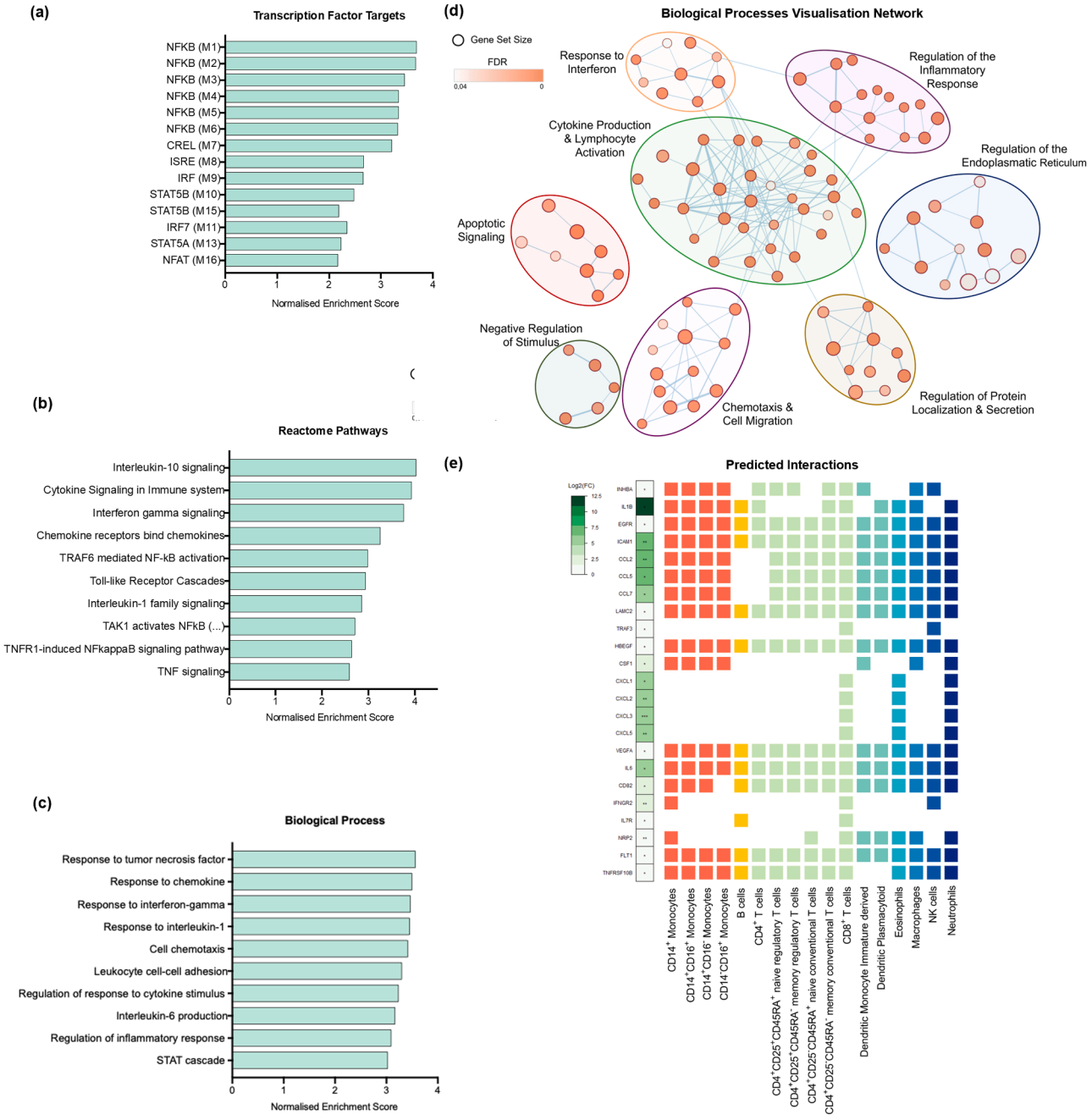

Fig. 3. Gene Set Enrichment Analysis (GSEA) plots of patient-derived fibroblasts in response to TNF-α. GSEA of fibroblasts treated for 5 h was used to identify enriched transcription factors, pathways, and biological processes and to predict fibroblast-immune cell interactions based on the DEGs. (A) Normalized enrichment score (NES) of the top significantly enriched transcription factor targets. Motifs for which the transcription factors are not annotated (unknown) are not represented but can be found in Supplementary material 2–Supplementary Table 1; (B) NES from immune-related pathways (from Reactome). The complete list of pathways is found in Supplementary material 4. (C) NESs from nonredundant inflammatory-related biological process terms. All GSEA analyses were performed using the WebGestalt platform, and all displayed terms were statistically significant (false discovery rate (FDR) < 0.05). (D) Enrichment map of TNF-α stimulation of skin fibroblasts. The visualization network displays the enriched biological process gene sets of stimulated fibroblasts. The size of the nodes characterizes the number of genes inserted in each dataset, and the orange gradient represents the significance of each term. Light orange is less statistically significant than strong orange is. Clusters of functionally related gene sets were manually circled and labelled with the help of the WordCloud plugin. Links with fewer than 5 nodes were excluded. (E) Log2-fold fold change (FC) of differentially expressed receptors and ligands upon TNF-α stimulation in skin fibroblasts annotated with a prediction of cell–cell interactions with immune cells. These Log2FCs are statistically significant, with FDR < 0.05 (*), FDR < 0.01 (**) or FDR < 0.001 (***).

To further investigate the response to TNF-α stimulation, we analysed the overall transcriptomic profile of the fibroblasts, which showed a consistent gene expression distribution between the cell lines (Supplementary Material 2-Supplementary Fig. 3). Following RNA sequencing, a PC analysis showed that TNF-α-stimulated and non-stimulated samples clustered separately, indicating that all the stimulated samples responded to TNF-α in the same direction (as indicated by PC1 explaining 46.17 % of the sample variance) and the two groups of samples had distinct transcription profiles (Fig. 2C). Further, the differential gene expression investigated the presence of 159 DEGs (Supplementary Material 3) which involves 130 up regulated and 29 down regulated DEGs in the stimulated condition as compared to the non-stimulated condition (Fig. 2D).

The fibroblast response similarity with other cells was explored by resorting to the CytoSig database, which contains transcriptomic profiles of different cell lines generated in response to different stimuli, including TNF-α. Therefore, the correlation between the transcriptomic alterations in TNF-α-stimulated fibroblasts and the profiles of T cells, monocytes, macrophages, astrocytes, and other dermal fibroblasts was investigated. Correlations with other dermal fibroblasts ($r = 0.51$), macrophages ($r = 0.32$), neutrophils ($r = 0.30$ and $r = 0.29$) and monocytes ($r = 0.28$ and $r = 0.24$) as well as with T cells ($r = 0.17$ and $r = 0.14$ for $CD4^+$ memory T and Jurkat T cells, respectively) showed that (FDR≤0.05) the response of stimulated fibroblasts not only was significantly correlated with dermal fibroblasts but also with the TNF-α response of immune effector cells, including neutrophils, monocytes, macrophages and T cells (Fig. 2E).

3.2. Fibroblast response to TNF-α activates signaling pathways congruent with predicted fibroblast-immune cell interactions

Following the analysis of the expression profiles of fibroblasts stimulated with or without TNF-α, GSEA was performed to assess the enriched targets of transcription factors, pathways, and biological processes (Supplementary Material 4). The changes in the expression of the top enriched targets after TNF-α stimulation suggested activation of the NF-κB pathway (Fig. 3A, Supplementary Material 2-Supplementary Table 1). Indeed, 7 different NF-κB binding motifs (M1-M7) constitute the top of this list. Other targets include the binding motifs of interferon regulatory factors, signal transducers and activators of transcription family members, and nuclear factors in activated T cells. Moreover, a total of 150 pathway-related genes, mainly involved in various cytokine and chemokine signaling pathways as well as the TNF receptor and transforming growth factor b-activated kinase 1 (TAK1)-induced activation of NF-κB signaling, were significantly enriched following TNF-α stimulation (Normalized enrichment score (NES)=2.59); these findings are consistent with the identified targets of transcription factors (Fig. 3B).

The cytokine/chemokine signaling was associated with the immune processes such as chemotaxis, leukocyte adhesion, IL-6 production and regulation of the inflammatory responses (Fig. 3C). A significant overlap was observed between the enriched biological processes (Fig. 3D, Supplementary Material 4), where the main clusters corresponded to cytokine production and lymphocyte activation and were directly linked to the regulation of protein localisation and secretion; the regulation of the inflammatory response, chemotaxis, and cell migration; and the response to interferon. Other clusters included the regulation of the endoplasmic reticulum, which includes several enzymatic reactions, the exportation of the proteins to the cytoplasm and subsequent exocytosis, apoptotic signaling and negative regulation of stimuli (Fig. 3D).

Fig. 3E showed the changes in the expression of the DEGs that encode receptors and ligands in fibroblasts and the possible crosstalk with their pairs in immune cells. As shown in Fig. 3E, the upregulation of *ICAM1* may potentiate the interaction between skin fibroblasts and immune cells (macrophages, T cells, monocytes, B cells, natural killer (NK) cells, dendritic cells, neutrophils, and eosinophils) since these cells express one or more receptors known to ligate ICAM1 (*ITGB2, ITGAL, ITGAX, ITGAM, IL2RA* and *ILR2G*) (Supplementary Material 1). In the stimulated fibroblasts, the upregulation of *CCL2, CCL5* and *CCL7* may influence the recruitment of immune cells expressing corresponding chemokine receptors (macrophages, naive and memory regulatory/conventional T cells, monocytes, NK cells, dendritic cells, neutrophils and eosinophils). On the other hand, the *CXCL1, CXCL2, CXCL3* and *CXCL5* gene products can potentially attract cytotoxic $CD8^+$T cells, eosinophils, and neutrophils according to their CCR/CXCR expression.

3.3. Experimental validation of the TNF-α-induced pathways through NF-κB and MAPK signaling activation

The gene expression associated with cytokines, chemokines, signaling proteins and transcription factors was analysed. According to Fig. 4A, genes that encode cytokines, *IL1B, IL6* and *IL15*, upregulate their expression upon TNF-α stimulation, suggesting potential in affecting both the acute phase and adaptive immune responses. Other transcripts related to well-described proinflammatory and anti-inflammatory cytokines were not detected (*IL4, IL10, IL12* and *IFNG*). The *CXCL1, CXCL5, CXCL8, CCL2* and *CCL5* chemokine genes were also upregulated in stimulated fibroblasts. The expression of other proteins, specifically those encoding transcription factors, namely, *NFKB1, NFKB2* and *NFKBIA,* also increased following inflammatory triggering (Fig. 4A).

At the protein level, we confirmed significant changes in the expression of IL-6 ($p = 0.02$) while the expression of IL-1β or IL-15 was negligible (Fig. 4B). Also the secretion of IL-4, IL-10, IL-12 and IFN-γ was very low or not detected. Fibroblasts secreted chemokines, with notable overexpression of the proinflammatory chemokines CXCL8 ($p = 0.027$), CCL2 ($p = 0.028$) and CCL5 ($p = 0.016$), upon TNF-α stimulation (Fig. 4C). Additionally, CXCL5 and CXCL1 exhibited marginal overexpression ($p = 0.055$ and $p = 0.082$, respectively). These protein changes were consistent across all the cells and validated the above mentioned gene expression changes (Supplementary Material 2-Supplementary Table 2).

The analysis of proteins involved in TNF-α receptor downstream signaling, namely, the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways was done. Considering the high enrichment of NF-κB targets, the protein expression of the p65 subunit was analysed which was not changed with increasing stimulation time from 0 to 120 min (Supplementary Material 2–Supplementary Fig. 4). However, the expression of the endogenous inhibitor IκBα was completely abrogated upon TNF-α stimulation ($p = 0.0019$; Fig. 4D; Supplementary Material 2–Supplementary Fig. 5), revealing the activation of the NF-κB pathway. Analysis of the other downstream proteins tested also revealed significant differences upon TNF-α stimulation, namely, the ERK1/2 (FDR = 0.017), p38 (FDR = 0.011) and p-p38 (FDR=0.0003) proteins (Fig. 4D, Supplementary Material 2–Supplementary Fig. 5). Further analysis also revealed the activation of both the MAPK/ERK and p38 MAPK pathways since the p-ERK1/2:ERK1/2 and p-p38:p38 ratios significantly increased upon TNF-α stimulation (FDR = 0.017 and FDR = 0.011, respectively).

Overall, our data showed that TNF-α activates several pathways in skin fibroblasts, namely, the NF-κB MAPK/ERK and p38 MAPK signaling pathways, which, individually or collectively, are responsible for increased IL-6 and chemokine expression.

4. Discussion

The new European regulations that restrict the use of animal models in research, prioritizing the combination of *in vitro* cellular models and computational analysis is crucial for addressing immune physiology and pathology [62]. The scarcity of *in vivo, in vitro* models and the difficulty in obtaining patient samples underscore the importance of the Euro-BioBank, the Telethon Network of Genetic Biobanks and the NIGMS Human Genetic Cell Repository, which are critical for supporting basic,

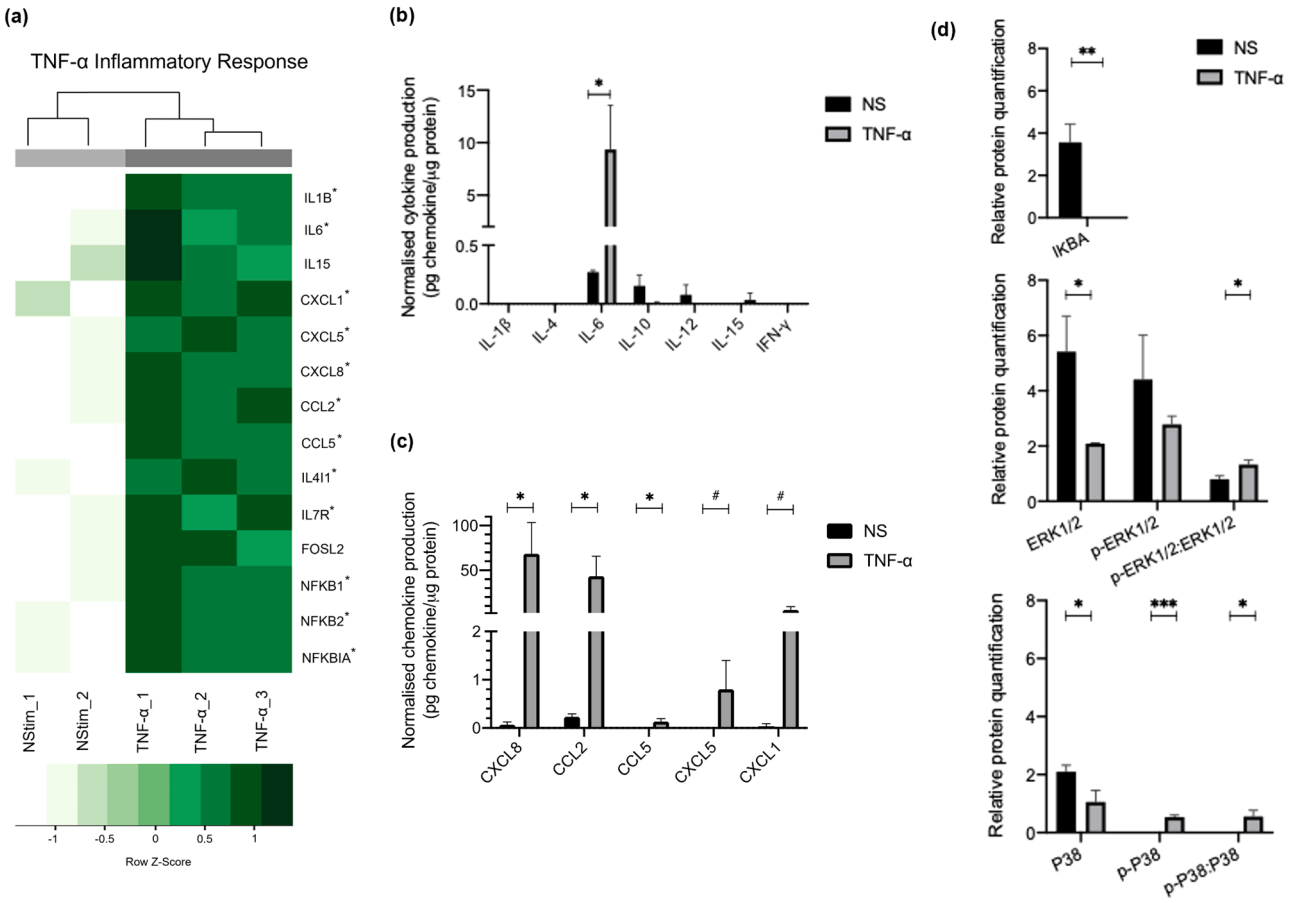

Fig. 4. Gene and protein expression of TNF-α downstream signaling proteins and analysis of inducible cytokines. (A) Heatmap of immune-related gene expression in non-stimulated (NS) (light grey) and TNF-α-stimulated (dark grey) fibroblasts ($t = 5$ h). The green gradient represents the expression of each gene in each sample and is presented as a z score, with lighter green indicating lower levels of gene expression and darker green indicating higher levels of gene expression. Genes marked with '*' are differentially expressed (false discovery rate (FDR) < 0.05); (B) Cytokine production in NS- and TNF-α-stimulated samples ($t = 24$ h) measured via ELISA. The data are presented as the means ± SDs ($n = 3$). Statistically significant values were assessed by two-tailed unpaired Student's t tests ($p < 0.05$ (*)). Normalized values were obtained by dividing the concentration of cytokines (pg/ml) by the concentration of total protein (mg/ml). (C) Chemokine production in the NS- and TNF-α-stimulated samples ($t = 24$ h) quantified using a Mix and Match LegendPlex Kit. The data are presented as the means ± SDs ($n = 3$). Statistically significant values were assessed by two-tailed unpaired Student's t tests ($p < 0.05$ (*) and $0.1 < p < 0.05$ (#)). Normalized values were obtained by dividing the concentration of chemokines (pg/ml) by the concentration of total protein (mg/ml). (D) Relative protein quantification of cell signaling proteins and transcription factors (namely, IκBα, p38, p-p38, extracellular signal-regulated kinase (ERK)1/2 and p-ERK1/2) in NS- and TNF-α-stimulated fibroblasts ($t = 30$ min) via western blotting ($n = 3$). α-Tubulin bands were used as a loading control and housekeeping protein normalization method. Statistically significant values were assessed by two-tailed unpaired Student's t tests for IκBα ($p < 0.01$ (**) and by one-way ANOVA with Dunnett's multiple comparisons for ERK1/2, p-ERK1/2, p38, p-p38 and the respective ratios (FDR < 0.05 (*), FDR < 0.001 (***)).

translational research and maximizing outcomes with limited resources [7,63–65].

Fibroblast cultures were traditionally used for biochemical studies and are now part of established biobanks containing genetically stable models [66]. They have been used to study immunopathology in the context of autoimmune and inflammatory conditions such as psoriasis, rheumatoid arthritis, and Crohn's disease [67–69]. Since pathogenic variants can systematically impact molecular mechanisms, irrespective of cell type or tissue, our hypothesis is that fibroblasts can serve as a valuable tool for studying immune defects. Hence, for many diseases, especially those with poorly understood underlying mechanisms, skin fibroblasts provide a valuable model for investigating disease pathogenesis.

To characterize how the skin fibroblasts respond to inflammatory stimuli at the molecular and functional levels, the pro-inflammatory cytokine TNF-α was selected. It has already been reported that TNF-α causes transcriptional changes in fibroblasts related to cytokine signaling, which are controlled by anti-TNF-α therapy [70]. Badran et al. performed RNA sequencing of TNF-α-stimulated dermal fibroblasts to analyse gene expression disparities in patients with *RELA* haploinsufficiency [71]. However, the study primarily emphasized pathogenic alterations, providing limited insights into the full fibroblast response to this cytokine. To the best of our knowledge, this is the first study to use RNA sequencing to reveal the transcriptome in response to TNF-α. Here, we provide comprehensive evidence that fibroblasts respond to TNF-α through signaling pathways similar to those of immune cell lines (Fig. 2E). These findings highlight the immunological role of fibroblasts; however, future research efforts should aim to determine which pathways are similarly activated between fibroblasts and each immune cell type to investigate their potential as immune cell models. The results of this study also bring important insights into skin inflammatory diseases. Not only the response of skin fibroblasts to TNF-α was described at the transcriptomic level, but also secreted proteins and possible interactions with a plethora of immune cells that can be further studied. This is particularly relevant in diseases in which the pathogenesis is based on or includes exacerbated levels of TNF-α (*e.g.* psoriasis or atopic dermatitis). Besides, in skin inflammatory conditions, mechanisms such as alteration in protein levels and cell-cell interactions enhance the fibroblast proliferation activity and interaction with immune cells. These lead to the activation of different signaling pathways,

regulating chemokines, melanocyte migration and T cell differentiation which results in exacerbating the disease [72]. Thus, our research provides a strong foundation for using fibroblasts in the analysis and treatment of skin inflammatory diseases.

Our functional analysis of the transcriptome confirmed the role of skin fibroblasts as supporters of an immune response. The results showed the activation of immune-related pathways, the production of effector cytokines, their chemo-attractant role, and their adhesion to leukocytes, which were previously reported to some extent [23]. This finding is consistent with the observed enrichment of several motifs in the NF-κB, which is well known to be involved in immune and inflammatory processes [73]. Additionally, the induction of genes related to apoptosis was also detected, a response extensively described following the engagement of TNFR1 and its death domain [74]. Surprisingly, several transcription factors, pathways and biological processes related to TLRs and interferon signaling were also enriched. Since TLRs are activated after recognition of pathogen-associated molecular patterns, enrichment of this pathway was not expected after the TNF-α challenge. However, it is well established that TNFR and TLRs share downstream signal transduction mechanisms, mainly through the formation of TAK1-TAK1-binding protein complexes, which lead to the activation of NF-κB, c-Jun N-terminal kinase and p38 signaling [75]. Although TLRs are different from TNFR in terms of the usage of intracellular adaptors, they ultimately lead to the downstream recruitment of TAK1-binding proteins to form a complex, leading to the activation of the same pathways as those involving TNF-α [76]. The enrichment of interferon signaling results from its counteracting and anti-inflammatory effects on TNF-α, which are required for the control and fine-tuning of the magnitude and extent of the inflammatory reaction, ensuring homeostasis and preventing a deleterious response [77]. Accordingly, two of the main biological process clusters, interferon signaling and regulation of the inflammatory response, were associated (Fig. 3D).

After TNF-α stimulation of fibroblasts, we detected high secretion of IL-6, which is in agreement with previous reports on dermal and other fibroblast types [78–81], highlighting the pleiotropic functions of IL-6, which include roles in innate and adaptive immune responses as well as cell proliferation and differentiation [82]. We could not detect significantly secreted IL-1β or IL-15 even though the expression of these genes was upregulated nearly 13- and 3-fold, respectively, upon treatment with TNF-α. IL-1β is produced in an inactive pro-form that needs to be cleaved by the caspase-1 inflammasome before it is released and has biological activity [83]. It is possible that the mechanism that controls this posttranslational modification and other mechanisms involved in its regulation depend on the duration and intensity of the stimuli [84,85]. It has been reported that very few cells secrete detectable levels of IL-15 due to the presence of many initiation codons in the 5′ untranslated regions that compromise the efficiency of protein translation [86]. Therefore, IL-15 expression analysed via different translation-oriented approaches would be great advancement in the present knowledge.

For the investigation of fibroblasts role in innate immunity we detected the synthesis of chemokines upon inflammatory stimulus, which were previously shown to be produced by skin fibroblasts following other biological or chemical challenges [87–91]. The expression of these chemokines with IL-6 and the signaling pathways previously discussed confirms the inflammatory response of the fibroblasts under study given their association with inflammatory diseases [12,82,92,93]. Under inflammatory conditions, CXCL1, CXCL5 and CXCL8 have major chemotactic effects on neutrophils, but the migration of T cells and basophils have also been reported for CXCL8. Additionally, CCL5 attracts monocytes, eosinophils and subsets of lymphocytes, while CCL2 regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells [94]. However, our predictive analysis of receptor–ligand interactions between fibroblasts and immune cells suggested that several additional interactions may occur. Therefore, demonstrating the specificity and functional impact of stimulated skin fibroblast response products on a range of immune cells would be interesting. For example, the role of IL-6 (which potentially interacts with all representative immune cell subsets) is complex and context dependent, leading to different functional outcomes depending on the target cell. Secreted IL-6 can induce the differentiation and activation of immune cells, such as monocytes and T and B cells [95–97], stimulate antibody production by B cells [98] and activate innate immune cells, such as macrophages and neutrophils, enhancing their phagocytosis and cytokine secretion [99,100] among other functions. Although these studies suggest that skin fibroblast-produced IL-6 has important implications for skin immunity, it is important to note that IL-6 function may be influenced by other cytokines and cells present in the skin microenvironment [96,98]. Therefore, further research is needed to fully elucidate the mechanisms underlying the effects of skin fibroblast-secreted IL-6 on different immune cells *in vitro* and *in vivo*. The analysis of cell signaling molecules and transcription factors confirmed the activation of the NF-κB, MAPK/ERK and p38 MAPK signaling pathways, which have been previously described as TNF-α-induced pathways [101,102]. This is not surprising given the well-described roles of these proteins in inflammation-related processes [73,103]. In future studies, a bigger sample size of primary fibroblasts from different genders and age groups can be examined to validate and further support the results obtained so far. In the present research, the results obtained emphasize the distinct intracellular immunological pathways involving fibroblasts, which will contribute to exploring immunotherapeutic agents for multiple diseases.

5. Conclusion

This study answers the needs and priorities of the disease community by increasing the use of biobanks and available cell models to determine the mechanisms and facilitate basic and translational research. Fibroblasts not only further contribute to widening the body of knowledge on skin fibroblast immunity, specifically the inflammatory response to TNF-α but also show that even when conventional immune cells are unavailable, fibroblasts can be reliable models for studying intra and intercellular immunomodulation mechanisms. This approach can reduce the impact of challenging sample access in immunopathology research on various diseases. Fig 1

Ethics approval and consent to participate

Approval was obtained from the ethics committees of the NOVA Science and Technology School (FCT-NOVA) and the CEIC Fundació Sant Joan de Déu (PIC-136-19), and the procedures used in this study adhered to the tenets of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available for download by authorized investigators in the dbGaP repository at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs003313.v1.p1. The code(s) developed to conduct the analysis are available at https://github.com/PGranjo/Model-of-inflammation.git.

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CRediT authorship contribution statement

Carlota Pascoal: Writing – original draft, Visualization, Software, Methodology, Conceptualization. **Pedro Granjo:** Writing – original draft, Visualization, Software, Methodology. **Patrícia Mexia:** Writing – original draft, Methodology. **Diana Gallego:** Methodology. **Rita Adubeiro Lourenço:** Methodology. **Shally Sharma:** Methodology, Writing – review & editing, Writing – original draft. **Bélen Pérez:** Visualization, Supervision. **Margarida Castro-Caldas:** Visualization, Methodology. **Ana Rita Grosso:** Visualization, Supervision. **Vanessa dos Reis Ferreira:** Supervision, Conceptualization. **Paula Alexandra Videira:** Visualization, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2025.107057.

References

[1] K. Cemile, V.B. Christine, V.D.Z. Julie, How network-based approaches can complement gene identification studies in frontotemporal dementia, Trends Genet. 38 (2022) 944–955, https://doi.org/10.1016/J.NEUROBIOLAGING.2022.03.018.

[2] A. Hanaford, S.C. Johnson, The immune system as a driver of mitochondrial disease pathogenesis: a review of evidence, Orphanet J. Rare Dis. 17 (2022) 335, https://doi.org/10.1186/S13023-022-02495-3.

[3] M. Lucia, C. Marco, W.C. Mary, The challenge for a European network of biobanks for rare diseases taken up by RD-Connect, Pathobiol 81 (2015) 231–236, https://doi.org/10.1159/000358492.

[4] V. Boulanger, M. Schlemmer, S. Rossov, A. Seebald, P. Gavin, Establishing patient registries for rare diseases: rationale and challenge, Pharmaceut. Med 34 (2020) 185–190, https://doi.org/10.1007/S40290-020-00332-1/METRICS.

[5] D. Mascalzoni, E.S. Dove, Y. Rubinstein, H.J. Dawkins, A. Kole, P. McCormack, S. Woods, O. Riess, F. Schaefer, H. Lochmüller, B.M. Knoppers, M. Hansson, International Charter of principles for sharing bio-specimens and data, Eur. J. Hum. Genet. 23 (2015) 721–728, https://doi.org/10.1038/ejhg.2014.197.

[6] M. Garcia, J. Downs, A. Russell, W. Wang, Impact of biobanks on research outcomes in rare diseases: a systematic review, Orphanet. J. Rare Dis. 13 (2018) 1–13, https://doi.org/10.1186/S13023-018-0942-Z/FIGURES/2.

[7] H. Lochmüller, S. Aymé, F. Pampinella, B. Melegh, K.A. Kuhn, S.E. Antonarakis, T. Meitinger, The role of biobanking in rare diseases: european consensus expert group report, Biopreserv. Biobank. 7 (2009) 155–156, https://doi.org/10.1089/BIO.2010.7302.

[8] R. Francisco, S. Brasil, C. Pascoal, A.C. Edmondson, J. Jaeken, P.A. Videira, C. de Freitas, V.R. Ferreira, D. Marques-da-Silva, A community-led approach as a guide to overcome challenges for therapy research in congenital disorders of glycosylation, Int J. Environ. Res. Public Health 19 (2022) 6829, https://doi.org/10.3390/IJERPH19116829.

[9] M. Monticelli, R. Francisco, S. Brasil, D. Marques-da-Silva, T. Rijoff, C. Pascoal, J. Jaeken, P.A. Videira, V.R. Ferreira, Stakeholders' views on drug development: the congenital disorders of glycosylation community perspective, Orphanet. J. Rare Dis. 17 (2022), https://doi.org/10.1186/S13023-022-02460-0.

[10] Y. Li, U. Polak, A.D. Clark, A.D. Bhalla, Y.Y. Chen, J. Li, D.L L.Seyer, J. S.Butler, M.N. Napierala, Establishment and maintenance of primary fibroblast repositories for rare diseases—Friedreich's ataxia example, Biopreserv. Biobank. 14 (2016) 324–329, https://doi.org/10.1089/BIO.2015.0117.

[11] J. Villegas, M. McPhaul, Establishment and culture of human skin fibroblasts, Curr. Protoc. Mol. Biol. 71 (2005), https://doi.org/10.1002/0471142727.MB2803S71.

[12] F. Mizoguchi, K. Slowikowski, K. Wei, J.L. Marshall, D.A. Rao, S.K. Chang, H. N. Nguyen, E.H. Noss, J.D. Turner, B.E. Earp, P.E. Blazar, J. Wright, B. P. Simmons, L.T. Donlin, G.D. Kalliolias, S.M. Goodman, V.P. Bykerk, L. B. Ivashkiv, J.A. Lederer, N. Hacohen, P.A. Nigrovic, A. Filer, C.D. Buckley, S. Raychaudhuri, M.B. Brenner, Functionally distinct disease-associated fibroblast subsets in rheumatoid arthritis, Nat. Commun. 9 (2018) 789, https://doi.org/10.1038/s41467-018-02892-y.

[13] G. Auburger, M. Klinkenberg, J. Drost, K. Marcus, B. Morales-Gordo, W.S. Kunz, M. Jendrach, Primary skin fibroblasts as a model of Parkinson's disease, Mol. Neurobiol. 46 (2012) 20–27, https://doi.org/10.1007/S12035-012-8245-1.

[14] M.V. Plikus, X. Wang, S. Sinha, E. Forte, S.M. Thompson, E.L. Herzog, R. R. Driskell, N. Rosenthal, J. Biernaskie, V. Horsley, Fibroblasts: origins, definitions, and functions in health and disease, Cell 184 (2021) 3852–3872, https://doi.org/10.1016/J.CELL.2021.06.024.

[15] M.B. Buechler, S.J. Turley, A short field guide to fibroblast function in immunity, Semin. Immunol. 35 (2018) 48–58, https://doi.org/10.1016/J.SMIM.2017.11.001.

[16] J.M. Sorrell, A.I. Caplan AI, Chapter 4 fibroblasts—a diverse population at the center of it all, Int. Rev. Cell Mol. Biol. 276 (2009) 161–214, https://doi.org/10.1016/S1937-6448(09)76004-6.

[17] G. Parsonage, F. Falciani, A. Burman, A. Filer, E. Ross, M. Bofill, S. Martin, M. Salmon, C.D. Buckley, Global gene expression profiles in fibroblasts from synovial, skin and lymphoid tissue reveals distinct cytokine and chemokine expression patterns, Thromb. Haemost. 90 (2003) 688–697, https://doi.org/10.1160/TH03-04-0208.

[18] T. Krausgruber, N. Fortelny, V. Fife-Gernedl, M. Senekowitsch, L.C. Schuster, A. Lercher, A. Nemc, C. Schmidl, A.F. Rendeiro, A. Bergthaler, C. Bock, Structural cells are key regulators of organ-specific immune responses, Nature 583 (2020) 296–302, https://doi.org/10.1038/s41586-020-2424-4.

[19] M. Ravikanth, P. Soujanya, K. Manjunath, T.R. Saraswathi, C.R. Ramachandran, Heterogenecity of fibroblasts, J. Oral. Maxillofac Pathol. 15 (2011) 247, https://doi.org/10.4103/0973-029X.84516.

[20] D. Lindner, C. Zietsch, P.M. Becher, K. Schulze, H.P. Schultheiss, C. Tschöpe, D. Westermann, Differential expression of matrix metalloproteases in human fibroblasts with different origins, Biochem. Res. Int. 1 (2012) 875742, https://doi.org/10.1155/2012/875742.

[21] S. Davidson, M. Coles, T. Thomas, G. Kollias, B. Ludewig, S. Turley, M. Brenner, C.D. Buckley, Fibroblasts as immune regulators in infection, inflammation and cancer, Nat. Rev. Immunol. 21 (2021) 704–717, https://doi.org/10.1038/s41577-021-00540-z.

[22] L.A. Bautista-Hernández, J.L. Gómez-Olivares, B. Buentello-Volante, V.M.B. de Lucio, Fibroblasts: the unknown sentinels eliciting immune responses against microorganisms, Eur. J. Microbiol. Immunol. (Bp) 7 (2017) 151, https://doi.org/10.1556/1886.2017.00009.

[23] K.J. Cavagnero, R.L. Gallo, Essential immune functions of fibroblasts in innate host defense, Front. Immunol. 13 (2022), https://doi.org/10.3389/FIMMU.2022.1058862.

[24] H. Wajant, D. Siegmund, TNFR1 and TNFR2 in the control of the life and death balance of macrophages, Front. Cell Dev. Biol. 7 (2019) 91, https://doi.org/10.3389/FCELL.2019.00091/BIBTEX.

[25] D.J. MacEwan, TNF ligands and receptors – a matter of life and death, Br. J. Pharmacol. 13 (2002) 855, https://doi.org/10.1038/SJ.BJP.0704549.

[26] L. Mueller, L. von Seggern, J. Schumacher, F. Goumas, C. Wilms, F. Braun, D. C. Broering, TNF-α similarly induces IL-6 and MCP-1 in fibroblasts from colorectal liver metastases and normal liver fibroblasts, Biochem. Biophys. Res. Commun. 397 (2010) 586–591, https://doi.org/10.1016/J.BBRC.2010.05.163.
[27] A.E. Montes-Gomez, J. Garcıa-Cordero, E. Marcial-Juarez, G. Shrivastava, G. Visoso-Carvajal, F.J. Juárez-Delgado, L. Flores-Romo, M.C. Sanchez-Torres, L. Santos-Argumedo, J. Bustos-Arriaga, L. Cedillo-Barron, Crosstalk between dermal fibroblasts and dendritic cells during Dengue Virus infection, Front. Immunol. 11 (2020), https://doi.org/10.3389/FIMMU.2020.538240.
[28] J.Y. Jang, I.S. Song, K.J. Baek, Y. Choi, S. Ji, Immunologic characteristics of human gingival fibroblasts in response to oral bacteria, J. Periodontal Res. 52 (2017) 447–457, https://doi.org/10.1111/JRE.12410.
[29] A. Berroth, D. Ing, J. Kuhnl, N. Kurschat, A. Schwarz, F. Stab, T. Schwarz, H. Wenck, R.F. Holst, G. Neufang, Role of fibroblasts in the pathogenesis of atopic dermatitis, J. Allergy Clin. Immunol. 131 (2013) 1547–1554, https://doi.org/10.1016/j.jaci.2013.02.029, e6.
[30] J. Pritchard, S. Tsui, N. Horst, W.W. Cruikshank, T.J. Smith, Synovial fibroblasts from patients with rheumatoid arthritis, like fibroblasts from Graves' disease, express high levels of IL-16 when treated with Igs against insulin-like growth factor-1 receptor, J. Immunol. 173 (2004) 3564–3569, https://doi.org/10.4049/JIMMUNOL.173.5.3564.
[31] L. Monteran, N. Erez, The dark side of fibroblasts: cancer-associated fibroblasts as mediators of immunosuppression in the tumor microenvironment, Front. Immunol. 10 (2019), https://doi.org/10.3389/FIMMU.2019.01835.
[32] R. Francisco, C. Pascoal, D.M. da-Silva, S. Brasil, F.M.P. Santos, R. Altassan, J. Jaeken, AR. Grosso, V.R. Ferreira, P.A. Videira, New insights into immunological involvement in congenital disorders of glycosylation (CDG) from a people-centric approach, J. Clin. Med. 9 (2020) 2092, https://doi.org/10.3390/JCM9072092.
[33] N. Hollander, G.W. Tusscher, Coffin–Siris syndrome: clinical description of two cases, Clin. Case Rep. 10 (2022), https://doi.org/10.1002/CCR3.6598.
[34] F. Malfait, C. Francomano, P. Byers, J. Belmont, B. Berglund, J. Black, L. Bloom, J. M. Bowen, A.F. Brady, N.P. Burrows, M. Castori, H. Cohen, M. Colombi, S. Demirdas, J.D. Backer, A.D. Paepe, S. Fournel-Gigleux, M. Frank, N. Ghali, C. Giunta, R. Grahame, A. Hakim, X. Jeunemaitre, D. Johnson, B. Juul-Kristensen, I. Kapferer-Seebacher, H. Kazkaz, T. Kosho, M.E. Lavallee, H. Levy, R. Mendoza-Londono, M. Pepin, F.M. Pope, E. Reinstein, L. Robert, M. Rohrbach, L. Sanders, G.J. Sobey, T.V. Damme, A. Vandersteen, C.V. Mourik, N. Voermans, N. Wheeldon, J. Zschocke, B. Tinkle, The 2017 international classification of the Ehlers–Danlos syndromes, Am. J. Med. Genet. C Semin. Med. Genet. 175 (2017) 8–26, https://doi.org/10.1002/AJMG.C.31552.
[35] R.P.S. Patrício, P.A. Videira, F. Pereira, A computer-aided drug design approach to discover tumour suppressor p53 protein activators for colorectal cancer therapy, Bioorg. Med. Chem. 53 (2022) 116530, https://doi.org/10.1016/J.BMC.2021.116530.
[36] Babraham bioinformatics - FastQC A quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, 2023.
[37] BBDuk Guide - DOE Joint Genome Institute. https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/, 2023.
[38] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T.R. Gingeras, STAR: ultrafast universal RNA-seq aligner, Bioinform 29 (2013) 15–21, https://doi.org/10.1093/BIOINFORMATICS/BTS635.
[39] S. Anders, P.T. Pyl, W. Huber, HTSeq–a Python framework to work with high-throughput sequencing data, Bioinform 31 (2015) 166–169, https://doi.org/10.1093/BIOINFORMATICS/BTU638.
[40] R Core Team, R: A Language and Environment For Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2022. https://www.r-project.org/.
[41] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a bioconductor package for differential expression analysis of digital gene expression data, Bioinform 26 (2010) 139–140, https://doi.org/10.1093/BIOINFORMATICS/BTP616.
[42] P. Jiang, Y. Zhang, B. Ru, Y. Yang, T. Vu, R. Paul, A. Mirza, G. Altan-Bonnet, L. Liu, E. Ruppin, L. Wakefield, K.W. Wucherpfennig, Systematic investigation of cytokine signaling activity at the tissue and single-cell levels, Nat. Methods 18 (2021) 1181–1191, https://doi.org/10.1038/s41592-021-01274-5.
[43] E-MTAB-5622. https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-5622, 2023.
[44] GSE109460. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109460, 2023.
[45] GSE100382. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100382, 2023.
[46] GSE95588. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95588, 2023.
[47] GSE129210. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129210, 2023.
[48] GSE98624. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98624, 2023.
[49] GSE40548. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40548, 2023.
[50] GSE70068. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70068, 2023.
[51] T. Wei, V. Simko, R. package "corrplot": visualization of a Correlation Matrix, (2021) Version 0.92 https://github.com/taiyun/corrplot.
[52] Y. Liao, J. Wang, E.J. Jaehnig, Z. Shi, B. Zhang, WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs, Nucleic Acids Res. 47 (2019) W199–W205, https://doi.org/10.1093/NAR/GKZ401.
[53] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2013) 2498–2504, https://doi.org/10.1101/GR.1239303.
[54] D. Merico, R. Isserlin, O. Stueker, A. Emili, G.D. Bader, Enrichment map: a network-based method for gene-set enrichment visualization and interpretation, PLoS One 5 (2010) e13984, https://doi.org/10.1371/JOURNAL.PONE.0013984.
[55] L. Oesper, D. Merico, R. Isserlin, G.D. Bader, WordCloud: a Cytoscape plugin to create a visual semantic summary of networks, Source Code Biol. Med. 6 (2011) 1–4, https://doi.org/10.1186/1751-0473-6-7/FIGURES/2.
[56] J.A. Ramilowski, T. Goldberg, J. Harshbarger, E. Kloppmann, M. Lizio, V. P. Satagopam, M. Itoh, H. Kawaji, P. Carninci, B. Rost, A.R.R. Forrest, A draft network of ligand–receptor-mediated multicellular signalling in human, Nat. Commun. 6 (2015) 1–12, https://doi.org/10.1038/ncomms8866.
[57] V.E. Velculescu, S.L. Madden, L. Zhang, A.E. Lash, J. Yu, C. Rago, A. Lal, C. J. Wang, G.A. Beaudry, K.M. Ciriello, B.P. Cook, M.R. Dufault, A.T. Ferguson, Y. Gao, T. He, H. Hermeking, S.K. Hiraldo, P.M. Hwang, M.A. Lopez, H. F. Luderer, B. Mathews, J.M. Petroziello, K. Polyak, L. Zawel, K.W. Kinzler, Analysis of human transcriptomes, Nat. Genet. 23 (1999) 387–388, https://doi.org/10.1038/70487.
[58] Z. Silva, T. Ferro, D. Almeida, H. Soares, J.A. Ferreira, F.M. Deschepper, P. J. Hensbergen, M. Pirro, S.J.V. Vliet, S. Springer, P.A. Videira, Class I stability is modulated by cell surface sialylation in human dendritic cells, Pharm 12 (2020) 249, https://doi.org/10.3390/PHARMACEUTICS12030249.
[59] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408, https://doi.org/10.1006/METH.2001.1262.
[60] J.S. Lehmann, P. Rughwani, M. Kolenovic, S. Ji, B. Sun, LEGENDplex™: bead-assisted multiplex cytokine profiling by flow cytometry, Methods Enzymol. 629 (2019) 151–176, https://doi.org/10.1016/BS.MIE.2019.06.001.
[61] J. Schindelin, C.T. Rueden, M.C. Hiner, K.W. Eliceiri, The ImageJ ecosystem: An open platform for biomedical image analysis, Mol. Reprod. Dev. 82 (2015) 518–529, https://doi.org/10.1002/mrd.22489.
[62] Commission - directorate-general environment E. towards replacement of animals for scientific purposes, 2021. doi:10.2779/461562.
[63] M. Mora, C. Angelini, F. Bignami, A.M. Bodin, M. Crimi, J.H.D. Donato, A. Felice, C. Jaeger, V. Karcagi, Y. LeCam, S. Lynn, M. Meznaric, M. Moggio, L. Monaco, L. Politano, M.P. de la Paz, S. Saker, P. Schneiderat, M. Ensini, B. Garavaglia, D. Gurwitz, D. Johnson, F. Muntoni, J. Puymirat, H. Lochmüller, The EuroBioBank Network: 10 years of hands-on experience of collaborative, transnational biobanking for rare diseases, Eur. J. Hum. Genet. 23 (2015) 1116–1123, https://doi.org/10.1038/ejhg.2014.272.
[64] C. Baldo, L. Casareto, A. Renieri, G. Merla, B. Garavaglia, S. Goldwurm, E. Pegoraro, M. Moggio, M. Mora, L. Politano, L. Sangiorgi, R. Mazzotti, V. Viotti, I. Meloni, M.T. Pellico, C. Barzaghi, C.M. Wang, L. Monaco, M. Filocamo, The alliance between genetic biobanks and patient organisations: the experience of the telethon network of genetic biobanks, Orphanet. J. Rare Dis. 11 (2016) 1–8, https://doi.org/10.1186/S13023-016-0527-7/FIGURES/2.
[65] R. Francisco, S. Brasil, C. Pascoal, A.C. Edmondson, J. Jaeken, P.A. Videira, C. de Freitas, V.R. Ferreira, D.M. Da Silva, A community-led approach as a guide to overcome challenges for therapy research in congenital disorders of glycosylation, Int. J. Environ. Res. Public Health 19 (2022) 6829, https://doi.org/10.3390/IJERPH19116829/S1.
[66] C. Lindvall, M. Hou, T. Komurasaki, C. Zheng, M. Henriksson, J.M. Sedivy, M. Björkholm, B.T. Teh, M. Nordenskjöld, D. Xu, Molecular characterization of Human telomerase reverse transcriptase-immortalized Human fibroblasts by gene expression profiling | Cancer Research | American Association for Cancer Research, Cancer Res. 63 (2003) 1743–1747.
[67] S. Lefevre, F.M.P. Meier, E. Neumann, U.M. Ladner, Role of synovial fibroblasts in rheumatoid arthritis, Curr. Pharm. Des. 21 (2015) 130–141, https://doi.org/10.2174/1381612820666140825122036.
[68] A. Gęgotek, P. Domingues, A. Wroński, E. Skrzydlewska, Changes in proteome of fibroblasts isolated from psoriatic skin lesions, Int. J. Mol. Sci. 21 (2020) 1–17, https://doi.org/10.3390/IJMS21155363.
[69] J.C. Martin, C. Chang, G. Boschetti, Single-cell analysis of Crohn's disease lesions identifies a pathogenic cellular module associated with resistance to anti-TNF therapy, Cell 178 (2019) 1493–1508, https://doi.org/10.1016/J.CELL.2019.08.008, e20.
[70] D.W. Dziadecka, J. Gola, B. Grabarek, U. Mazurek, L.B. Wcisło, E.J. Kucharz, Effect of adalimumab on the expression of genes encoding TNF-α signal paths in skin fibroblasts *in vitro*, Postepy Dermatol. Alergol. 35 (2018) 413–422, https://doi.org/10.5114/ADA.2018.77673.
[71] Y.R. Badran, F. Dedeoglu, J.M.L. Castillo, W. Bainter, T.K. Ohsumi, A. Bousvaros, J.D. Goldsmith, R.S. Geha, J. Chou, Human RELA haploinsufficiency results in autosomal-dominant chronic mucocutaneous ulceration, J. Exp. Med. 214 (2017) 1937–1947, https://doi.org/10.1084/JEM.20160724.
[72] Y. He, Z. Han, Q. Zhang, L. Liu, J. Chang, Role of fibroblasts in nonfibrotic autoimmune skin diseases, Mol. Med. 30 (2024) 178, https://doi.org/10.1186/s10020-024-00949-x.
[73] T. Liu, L. Zhang, D. Joo, S.C. Sun, NF-κB signaling in inflammation, Signal Transduct. Target Ther. 2 (2017) 17023, https://doi.org/10.1038/SIGTRANS.2017.23.

[74] G. Chen, D.V. Goeddel, TNF-R1 signaling: a beautiful pathway, Science 296 (2002) 1634–1635, https://doi.org/10.1126/SCIENCE.1071924.
[75] J.H. Shi, S.C. Sun, Tumor necrosis factor receptor-associated factor regulation of nuclear factor κB and mitogen-activated protein kinase pathways, Front. Immunol. 9 (2018) 1849, https://doi.org/10.3389/FIMMU.2018.01849/BIBTEX.
[76] Y.R. Xu, C.Q. Lei, TAK1-TABs complex: a central signalosome in inflammatory responses, Front. Immunol. 11 (2021) 3208, https://doi.org/10.3389/FIMMU.2020.608976/BIBTEX.
[77] T. Cantaert, D. Baeten, P.P. Tak, L.G.M. van Baarsen, Type I IFN and TNFα cross-regulation in immune-mediated inflammatory disease: basic concepts and clinical relevance, Arthritis Res. Ther. 12 (2010) 1–10, https://doi.org/10.1186/AR3150/TABLES/2.
[78] H.N. Nguyen, E.H. Noss, F. Mizoguchi, C. Huppertz, K.S. Wei, G.F.M. Watts, M. B. Brenner, Autocrine loop involving IL-6 family member LIF, LIF receptor, and STAT4 drives sustained fibroblast production of inflammatory mediators, Immunity 46 (2017) 220–232, https://doi.org/10.1016/J.IMMUNI.2017.01.004.
[79] H.J. Chae, J.O. Byun, S.W. Chae, p38 MAPK and NF-kappaB on IL-6 release in human gingival fibroblasts, Immunopharmacol. Immunotoxicol. 27 (2005) 631–646, https://doi.org/10.1080/08923970500418851.
[80] L. Yin, Y.Y. Hu, J.L. Xu, J. Guo, J. Tu, Z.Q. Yin, Ultraviolet B inhibits IL-17A/TNF-α-stimulated activation of Human dermal fibroblasts by decreasing the expression of IL-17RA and IL-17RC on fibroblasts, Front. Immunol. 8 (2017) 91, https://doi.org/10.3389/FIMMU.2017.00091.
[81] B. Chen, S. Tsui, T.J. Smith, IL-1 beta induces IL-6 expression in human orbital fibroblasts: identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy, J. Immunol. 175 (2005) 1310–1319, https://doi.org/10.4049/JIMMUNOL.175.2.1310.
[82] T. Tanaka, M. Narazaki, T. Kishimoto, IL-6 in inflammation, immunity, and disease, Cold Spring Harb. Perspect. Biol. 6 (2014) a016295, https://doi.org/10.1101/cshperspect.a016295.
[83] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β, Mol. Cell 10 (2002) 417–426, https://doi.org/10.1016/S1097-2765(02)00599-3.
[84] K. Yamato, Z. El-Hajjaoui, H.P. Koeffler, Regulation of levels of IL-1 mRNA in human fibroblasts, J. Cell Physiol. 139 (1989) 610–616, https://doi.org/10.1002/JCP.1041390322.
[85] G. Lopez-Castejon, D. Brough, Understanding the mechanism of IL-1β secretion, Cytokine Growth Factor Rev. 22 (2011) 189, https://doi.org/10.1016/J.CYTOGFR.2011.10.001.
[86] P.Y. Perera, J.H. Lichy, T.A. Waldmann, L.P. Perera, The role of Interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use, Microbes Infect. 14 (2012) 247, https://doi.org/10.1016/J.MICINF.2011.10.006.
[87] P. Fallahi, R. Foddis, G. Elia, F. Ragusa, A. Patrizio, S. Benvenga, A. Cristaudo, A. Antonelli, S.M. Ferrari, CXCL8 and CXCL11 chemokine secretion in dermal fibroblasts is differentially modulated by vanadium pentoxide, Mol. Med. Rep. 18 (2018) 1798–1803, https://doi.org/10.3892/MMR.2018.9121.
[88] J.M. Rojas, T. Arán-Sekul, E. Cortés, R. Jaldín, K. Ordenes, P.R. Orrego, J. González, J.E. Araya, A. Catalán, Phospholipase D from Loxosceles laeta spider venom induces IL-6, IL-8, CXCL1/GRO-α, and CCL2/MCP-1 production in Human skin fibroblasts and stimulates monocytes migration, Toxins (Basel) 9 (2017), https://doi.org/10.3390/TOXINS9040125.
[89] O. Reichert, L. Kolbe, L. Terstegen, F. Staeb, H. Wenck, M. Schmelz, H. Genth, V. Kaever, D. Roggenkamp, G. Neufang, UV radiation induces CXCL5 expression in human skin, Exp. Dermatol. 24 (2015) 309–312, https://doi.org/10.1111/EXD.12652.
[90] M. Schürmann, F. Oppel, S. Shao, V. Volland-Thurn, C. Kaltschmidt, B. Kaltschmidt, L.U. Scholtz, H. Sudhoff, Chronic inflammation of middle ear cholesteatoma promotes its recurrence via a paracrine mechanism, Cell Commun. Signal. 19 (2021), https://doi.org/10.1186/S12964-020-00690-Y.
[91] N. Noso, M. Sticherling, J. Bartels, A.I. Mallet, E.S.J. Christophers, Identification of an N-terminally truncated form of the chemokine RANTES and granulocyte-macrophage colony-stimulating factor as major eosinophil attractants released by cytokine-stimulated dermal fibroblasts, J. Immunol. 156 (1996) 1946–1953, https://doi.org/10.4049/jimmunol.156.5.1946.
[92] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, J. Interferon Cytokine Res. 29 (2009) 313–326, https://doi.org/10.1089/jir.2008.0027.
[93] E. Gremese, B. Tolusso, D. Bruno, S. Perniola, G. Ferraccioli, S. Alivernini, The forgotten key players in rheumatoid arthritis: IL-8 and IL-17 - Unmet needs and therapeutic perspectives, Front. Med. (Lausanne) 10 (2023) 956127, https://doi.org/10.3389/fmed.2023.956127.
[94] R.S. Smith, T.J. Smith, T.M. Blieden, R.P. Phipps, Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation, Am. J. Pathol. 151 (1997) 317.
[95] I. Sebina, L.G. Fogg, K.R. James, M.S.F. Soon, J. Akter, B.S. Thomas, G.R. Hill, C. R. Engwerda, A. Haque, IL-6 promotes CD4+ T-cell and B-cell activation during Plasmodium infection, Parasite Immunol. 39 (2017) e12455, https://doi.org/10.1111/PIM.12455.
[96] T. Korn, E. Bettelli, M. Oukka, V.K. Kuchroo, IL-17 and Th17 cells, Annu. Rev. Immunol. 27 (2009) 485–517, https://doi.org/10.1146/ANNUREV.IMMUNOL.021908.132710.
[97] P. Chomarat, J. Banchereau, J. Davoust, A.K. Palucka, IL-6 switches the differentiation of monocytes from dendritic cells to macrophages, Nat. Immunol. 1 (2000) 510–514, https://doi.org/10.1038/82763.
[98] O. Dienz, S.M. Eaton, J.P. Bond, W. Neveu, D. Moquin, R. Noubade, E.M. Briso, C. Charland, W.J. Leonard, G. Ciliberto, C. Teuscher, L. Haynes, M. Rincon, The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells, J. Exp. Med. 206 (2009) 69, https://doi.org/10.1084/JEM.20081571.
[99] M.R. Fernando, J.L. Reyes, J. Iannuzzi, G. Leung, D.M. McKay, The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages, PLoS One 9 (2014), https://doi.org/10.1371/JOURNAL.PONE.0094188.
[100] P.G. Mullen, A.C.J. Windson, C.J. Walsh, A.A. Fowler, H.J. Sugerman, Tumor necrosis factor-alpha and interleukin-6 selectively regulate neutrophil function *in vitro*, J. Surg. Res. 58 (1995) 124–130, https://doi.org/10.1006/JSRE.1995.1020.
[101] M.S. Hayden, S. Ghosh, Regulation of NF-κb by TNF family cytokines, Semin Immunol. 26 (2014) 253, https://doi.org/10.1016/J.SMIM.2014.05.004.
[102] G. Sabio, R.J. Davis, TNF and MAP kinase signaling pathways, Semin. Immunol. 26 (2014) 237, https://doi.org/10.1016/J.SMIM.2014.02.009.
[103] U. Moens, S. Kostenko, B. Sveinbjørnsson, The role of mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) in inflammation, Genes (Basel) 4 (2013) 101, https://doi.org/10.3390/GENES4020101.