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EFFECTS OF TNF-α AND IL-1β ON THE ACTIVATION OF GENES RELATED TO INFLAMMATORY, IMMUNE RESPONSES AND CELL DEATH IN IMMORTALIZED HUMAN HaCat KERATINOCYTES

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The present experiments were designed to characterize by microarray analysis the transcriptional responses of human keratinocytes (HaCat) to TNF-α and IL-1β, given alone or in combination, in order to better understand the mechanisms underlying inflammatory, immune responses and cell death in which both cytokines play a pathophysiological role. Significant differences in the percentage and quality of genes dysregulated by TNF-α and IL-1β were shown. Both cytokines activated a series of genes involved in inflammatory, immune response as well as in cell death. In our experimental conditions, TNF-α, in contrast to IL-1β, did not induce a significant level of apoptosis in keratinocytes. However, given together both cytokines produced a significant decrease in apoptotic cells and synergistic transcriptional response which was due to the activation of several specific genes occurring after application of each cytokine. TNF-α and IL-1β evoked apoptotic effect and transcriptional responses were linked to the stimulation of their specific receptors since a pre-treatment with monoclonal antibodies *vs* TNF-α and/or IL-1β receptors was able to significantly reduce them.

The epidermis is a multilayer, stratified epithelium composed of keratinocytes that provide a physical barrier for the organism, protecting it from dehydration and a variety of environmental insults. It is implicated in allergic and severe skin adverse reactions to drugs such as toxic epidermal necrolysis (TEN; OMIM #608579) and Stevens–Johnson syndrome (SJS; OMIM #608579) both characterized morphologically by the rapid onset of keratinocyte cell death by apoptosis, a process that results in the separation of the epidermis from the dermis.

To date, the precise sequence of molecular and cellular events that lead to the development of SJS and TEN is only partially understood. The proposed pathogenesis has to take into account the rarity of these diseases and the involvement of specific types of drugs. A large body of evidence exists in the literature involving some cytokines of the

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tumor necrosis factor (TNF) family and interleukins (ILs) in the pathogenesis of cell death in epidermal keratinocytes (1). In particular, TNF-α, by binding to its specific cell-surface receptors, TNFR1 and TNFR2, has the ability to induce apoptosis (2) in several cells including keratinocytes (3), although other authors did not confirm such effect (4). By controlling gene expression, TNF-α orchestrates the cutaneous response to environmental damage and inflammation.

Interleukin 1 (IL-1) often referred to as prototypic inflammatory cytokine is a family of three closely-related proteins that are the products of separate genes. The agonists, IL-1α and IL-1β, are believed to exert identical actions via binding to a single 80kDa cell surface receptor (IL-1RI) (5). In particular, it has been reported that IL-1 treatment causes profound morphological changes of keratinocytes (6). IL-1β is believed to play an important role in some skin disorders such as psoriasis, contact dermatitis, and bullous diseases (7-9). In addition, IL-1β introduced into the rat brain has been reported to produce neurodegeneration (10). Several reports of microarray analyses investigated the transcriptional changes in cells induced by treatment with TNF-α and IL-1β (11-3). Important changes in gene expression profiles of human epidermal keratinocytes were reported with TNF-α (14), as well as with the treatment with IL-1α (6), showing that both cytokines are able to activate many genes involved in the inflammatory and immune responses as well as in cell death.

The present experiments were planned to investigate the apoptotic and transcriptional responses of TNF-α and IL-1β given alone or in combination in cell cultures (HaCat), to allow us to understand whether synergistic trascriptional and apoptotic responses occurred in comparison to the changes evoked by the single cytokine, and eventually to identify the contribution of each mono-component to the overall transcriptional response. To this aim, we applied microarray technology to characterize the modification of gene expression in HaCat cells treated with TNF-α, and IL-1β, given alone or in combination. As already shown, cellular genomic maps may represent a useful tool in understanding molecular alterations associated with cutaneous pathology (15).

In this work the attention was focused on gene expression changes evoked by both cytokines, specifically analyzing up- or down-regulation of genes related to inflammation, immune response, and cell death as well as cell differentiation, growth and repair mechanisms, in order to disclose specific genomic markers of pathological responses to inflammatory injuries.

Finally, the aim of the present experiments is to ascertain whether apoptotic and transcriptional responses to TNF-α and IL-1β were due to stimulation of their specific receptors (TNFRI, TNFRII and IL-1R).

MATERIALS AND METHODS

We investigated the genomic response of HaCat cells (human keratinocytes), using G4112F Whole Human Genome (4x44 K) oligo microarray Agilent platform, after 24- hour treatment with TNF-α and IL-1β alone and combined (TNF-α+IL-1β). IL-1β (Roche) and TNF-α (Invitrogen) were dissolved in phosphate buffered saline PBS (GIBCO), stored at −20°C, and further diluted in medium before the experiments.

Human keratinocyte cultures and cytokine treatment

Experiments were performed with the HaCat cell lines, the first permanent epithelial cell line for human skin, similar to normal keratinocytes, that exibits normal differentation and provides a resistant and promising tool to study keratinization reaction in human cells (16). Cells were propagated routinely in 75 cm^2 flasks using MEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin at 37°C in 95% air and 5% CO_2. The culture medium was changed every 2-3 days. The cells were grown in the flask up to 80-90% confluence and then Recombinant IL-1β (50 ng/ml) and/or TNF-α (50 ng/ml) were added in the fresh medium. Concentrations of the stimulators were selected on the basis of the dose-response relation of both cytokines regarding the HaCat response. In our experimental conditions the dose of 50 ng/ml resulted as the critical threshold for significant effect according to the data present in literature (6, 14, 17).

Cells staining

To determine whether the treatment with the cytokines given alone or in combination induced cell death, we used DAPI for cell staining. HaCat cells were grown on Lab-Tek chamber slides (Nunc, Roskilde, Denmark)

up to 80-90% confluence and then TNF-α (50 ng/ml) and/or recombinant IL-1β (50 ng/ml) were added to the fresh medium for 24 h. The same technique was applied to investigate the effects induced by cytokines (TNF-α; IL-1β) after pre-treatment (at 2, 8 and 24 h) with human monoclonal antibodies directed against their receptors (TNFRI, TNFRII and IL-1βR). The cells were rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 mins. The stained cells with DAPI (Sigma-Alcrich), were mounted, observed under the microscope (Nikon eclipse 80i) and photographed. The same protocol was applied to the untreated sample.

The dye-exclusion test (trypan blue) was used to determine the number of viable and mortality of HaCat cells after treatment with cytokines (18). Cells were trypsinized and a volume of 1 mL cell suspension was mixed with 2 mL of phosphate-buffered saline. 10 µL of cell suspensions was added to 90 µL of 0.4% trypan blue solution. HaCat cell viability was determined microspically (400× magnification) and calculated as the number of living cells (i.e., cells not stained with trypan blue) *vs* the number of dead cells (i.e., cells stained with trypan blue). The same protocol was applied to untreated samples.

The data are expressed as mean values ± SE (not stained) living cells counted in each treatment (TNF-α, IL-1β, TNF-α+IL-1β) and in untreated sample. Data were evaluated statistically for difference by using Student's *t*-test; $p < 0.001$ living cells *vs* untreated sample was considered statistically significant.

Evaluation of apoptosis and necrosis by flow cytometry

In order to characterize the type of cell death (apoptosis or necrosis) in samples untreated and treated with TNF-α, IL-1β, TNF-α+IL-1β given alone or after pretreatment with human monoclonal antibodies (TNFRI, TNFRII, IL-1R), we used flow cytofluorometry by Annexin V assay. Annexin V is an endogenous human protein that specifically binds to phosphatidylserine, a plasma membrane lipid that rapidly delocalizes from the inner leaflet to the outer leaflet in cells that are undergoing programmed cell death (apoptosis). Therefore, Annexin V is a sensitive marker of early to intermediate phases of apoptosis. Concomitantly, the extent of overall cytotopathogenicity was measured by the standard Propidium iodite (PI) staining. Co-staining with Annexin V and PI allows differentiation of viable cells (Annexin V^-, PI^-) from early stage apoptotic cells (Annexin V^+, PI^-) and late stage apoptotic cells (Annexin V^+, PI^+). Annexin V-FITC apoptosis detection kit (BioVision Research Products, Mountain View, CA USA) was used to detect the different phases of apoptosis.

After marking, cells were subjected to FACS analysis (FACSCalibur, Becton Dickinson) and analyzed by CellQuest software (BD Bioscience). At least 10,000 events were recorded and represented as dot plot.

RNA extraction, labelling, hybridization

For RNA extraction, labelling, hybridization cells were harvested 24 hours after treatment and total RNA was isolated by TRIZOL standard protocol (Invitrogen Corporation Carlsbad, USA). A small aliquot of RNA was then used for quantification and quality control using respectively a spectrophotometer (Nanodrop, Wilmington, USA) and an agarose gel electrophoresis.

Gene expression profile was analyzed in the whole human genome using Agilent's One-Color Microarray-based Gene Expression Analysis (Agilent technologies). This tool uses cyanine 3-labeled targets to measure gene expression in experimental and control samples. Agilent's Low RNA Input Linear Amplification Kit PLUS generates fluorescent cRNA (complementary RNA) with 1 µg of total RNA. The method uses T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3-labeled CTP.

Image analysis and processing

The acquired images were analyzed with Feature Extraction software 5.1. The Invariant Set Normalization method was used for the array data analysis (19). This method represents a useful tool to evaluate a selective gene expression pattern generated by a specific compound (20).

For analysis of the microarray data, we normalized the data using the Invariant Set method of dchip software (19). Raw data were filtered for genes that were significantly changed above factor 1.0 within the 95% confidence interval ($p < 0.05$) for each experiment subset-dependent dose. Data were logarithmically trasformed (log base 2), and directly used for cluster analysis (21).

In addition, enrichment of functional annotations was performed using the NIH Database for Annotation, Visualization, and Integrated Discovery (DAVID) and EASE online (http://david.abcc.ncifcr.gov/conversation.jsp) (22-23). Briefly, the lists of genes that had a locus link and t-test P value lower than 0.05 were created. In particular, two lists were created for up- and down-regulated genes for each treatment. These lists were analyzed using NIH DAVID and EASE online for enrichment of all GO annotations. Hierarchical clustering was performed using DAVID software.

Validation of relative gene expression by qRT-PCR analysis

To confirm the microarray results independently, we performed quantitative RT-PCR analysis (24) of 9

representative genes. We chose genes Hs 00171042_m1 (*CXCL10*), Hs 00171138_m1 (*CXCL11*), Hs 00957562_m1(*MMP9*), Hs00992441_m1 (*IL32*), Hs 00610058_m1 (*S100A9*), Hs 00374263_m1 (*S100A8*), Hs 00232088_m1 (*TP73*), Hs 00174086_m1 (*IL10*), Hs 00153350_m1 (*BCL2*). We used *CXCL11* and *MMP9* to confirm a direct activity of TNF-α on gene expression using monoclonal anti-human TNF receptor 1 and 2 antibodies (TNFR1; TNFR2).

The total RNA from treated and untreated keratinocytes at 24 h was reverse-transcribed to cDNA according to the protocol of the High Capacity cDNA Archive Kit (Applied Biosystem, Foster City, USA). The incubation conditions were the following: 10 min at 25°C and 2 h at 37°C. We performed QRT-PCR using the Taqman system (Applied Biosystem).

The expression levels of 9 genes and an internal reference Hypoxanthine-phopho-ribosyltransferase-1 (*HPRT1*), (25) were measured by multiplex PCR using Assay-on-Demand ™ gene expression products (Applied Biosystems, Foster City, CA, USA) labeled with six carboxyfluorescein FAM or VIC, (Applied Biosystems).

The simultaneous measurement of each gene-FAM and HPRT1-VIC made it possible to standardize the amount of cDNA added per sample. We performed PCRs using the Taqman Universal PCR Master Mix and the ABI PRISM 7000 Sequence Detection System. Each QRT-PCR experiment was performed in triplicate and repeated at least twice. A comparative threshold cycle (CT) was used to determine gene expression relative to a calibrator (RNA from untreated cells). Hence, steady state mRNA levels are expressed as n-fold difference relative to the calibrator. For each sample, our CT gene value is normalized using the formula $\Delta CT = Ctgene - Ct_{HPRT}$ To determinate the relative expression levels, the following formula was used: $\Delta\Delta CT = \Delta CTsample - \Delta CT$ calibrator and the value used to plot relative gene expression was calculated using the expression $2\Delta\Delta CT$.

Antibody pre-treatments to evaluate effects of cytokines

In order to confirm whether TNF-α- and IL-1β-induced effects were due to the stimulation of their specific receptors, HaCat cells were pre-treated with specific monoclonal antibodies. In particular, we used monoclonal anti-human tumor necrosis factor receptor type I (TNFR I) and/or monoclonal anti-human tumor necrosis factor receptor type II (TNFR II) (R&D Systems), for the TNF-α and anti-human monoclonal interleukine-1receptor (IL-1R), for the IL-1β treatments. Furthermore, we studied the effects of cytokines given simultaneously after blocking of their receptors (TNFR I, TNFR II, IL-1R) by use of the same monoclonal anti-human antibodies. The experiments were performed following specific time courses.

In particular, cells were grown in the flask up to 80-90% confluency and TNF RI (30 μg/ml) and/or TNF RII (30 μg /ml) were added in the fresh medium. After 2, 8 and 24 hours from antibody treatments, cells were washed with PBS, and TNF-α (50 ng/ml) was added in the fresh medium. The same protocol was applied for IL-1R (30 μg /ml) and for combined treatment, using the same concentration of antibodies and cytokines. After 24 hours of each specific treatment, total RNA was isolated following standard TRIZOL protocol (as specified above). *CXCL11* and *MMP9* expression patterns were assessed to evaluate the effects after TNFR receptor inactivation.

IL-10 and *SELP* expression levels were assessed to evaluated the effects after IL-1R receptor inactivation. For combined treatment, we evaluated the expression pattern of the *CXCL11*, *MMP9, IL-10* and *SELP.*

RESULTS

Effect of cytokines on HaCat cell viability

Trypan blue exclusion was used to detect cytotoxicity on the basis of the cell viability (data do not shown); in addition, the evaluation of viable and non-viable HaCat cells in samples treated with cytokines *in vitro* by fluorescent microscopic analysis (DAPI staining), shows that only after IL-1β application there was a significant ($p < 0.001$) increase in cell death which was also observed after combined (TNF-α + IL-1β) treatment, (Fig. 1 D). In contrast to IL-1β (Fig.1 C), TNF-α given alone produced only a moderate (Fig. 1 B), but not statistically significant, increase in cell death.

The apoptotic effects induced by TNF-α and by IL-1β were prevented by previous (2 and 8 h before) pre-treatment with human monoclonal antibodies *vs* IL-1R and TNFRI and TNFRII receptors, respectively. In fact, by pretreating culture cells with human monoclonal antibodies *vs* IL-1R and TNFRI and TNFRII receptors, it was possible to significantly reduce cell apoptosis induced by the combination of both cytokines (TNF-α 50 ng/ml and IL-1β 50 ng/ml). At least 5 experiments were performed for each human monoclonal antibody (Table I).

The evidence that the observed cell death consisted mainly of apoptosis came from FACS analysis after staining with annexin V-FITC and PI which represents a useful tool for identifing the early and late programmed cell death. In particular, in comparison to control living cells, IL-1β produced a significant ($p < 0.001$) increase of early apoptotic

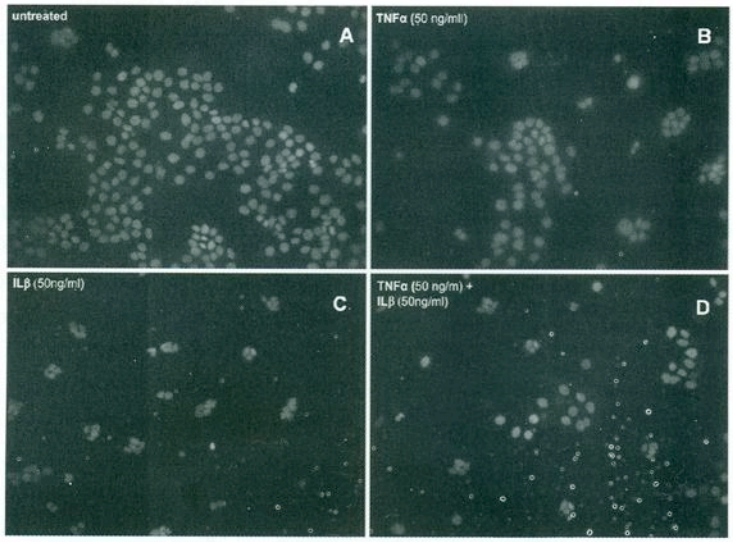


Fig. 1. *Photomicrographs of nuclei of HaCat cells stained by DAPI. The treatment with IL-1β (**C**) and TNF-α + IL-1β (**D**) reduces the number of nuclei stained by DAPI respect to the untreated cells (**A**). The reduction of the nuclei stained by DAPI, in the sample treated by TNF-α given alone (**B**) is not significant respect to the number of nuclei in untreated sample (A). Micrographs are typical example out of 5 different experiments.*

cells (8.37% Annexin V^+/PI^-). Similar percentages of apoptopic cells were obtained with a combined treatment (TNF-α + IL-1β; 8.10% Annexin V^+/PI^-) (see Table I).

Microarray data mining: genomic responses of HaCat cells

A comprehensive picture of the transcriptional changes after exogenous application of cytokines was obtained by using a G4112F Whole Human Genome (4x44 K) oligo microarray Agilent platform. Of the 41,000 genes present on the chip, about 3200 genes showed a differential expression induced by TNF-α (absolute Fold Change > 2): 1700 (4.3%) were up-regulated and 1500 (3.6%) were down-regulated. Expression changing was observed for 1100 genes after treatment with IL-1β (absolute Fold Change > 2): 600 (1.5%) were down-regulated and 500 (1.3%) were up-regulated. Combined treatment revealed a higher number of dysregulated genes (approx. 4000), (absolute Fold Change > 2): 2200 (5.3%) were up-regulated and 1800 (4.4%) were down-regulated Therefore, genomic response of HaCat cells to the IL-1β treatment was quantitatively lower in comparison to both TNF-α alone treatment and combined treatment (TNF-α+ IL-1β). Functional annotation tool NIH DAVID was used to characterize the enriched biological categories of genes affected by treatment with the two cytokines and the combined one (26). In silico analysis revealed that cell death, immune response and inflammatory response were the main affected pathways activated by each treatment (Table II).

Characterization of the TNFα- regulated genes

To understand the cellular processes affected in keratinocytes by TNF-α stimulation, we focused on a specific set of TNF-α-regulated genes involved in cell death, inflammatory response and immunity. We arranged the regulated genes in a hierarchical table of their cellular functions related to the 3 pathways analyzed (Fig. 3).

Cell death

TNF-α did not produce prominent cell death although this was reported in some tumour cell lines (27). Microarray analysis revealed that TNF-α altered the expression of both pro-apoptotic (*TP53* and *TP73*) and anti-apoptotic genes (*BIRC3, BIRC5* and *BIRC7*), suggesting a charged balance for the upcoming cell-fate decision. The TNF-α treatment induced many genes belonging to the TNF family

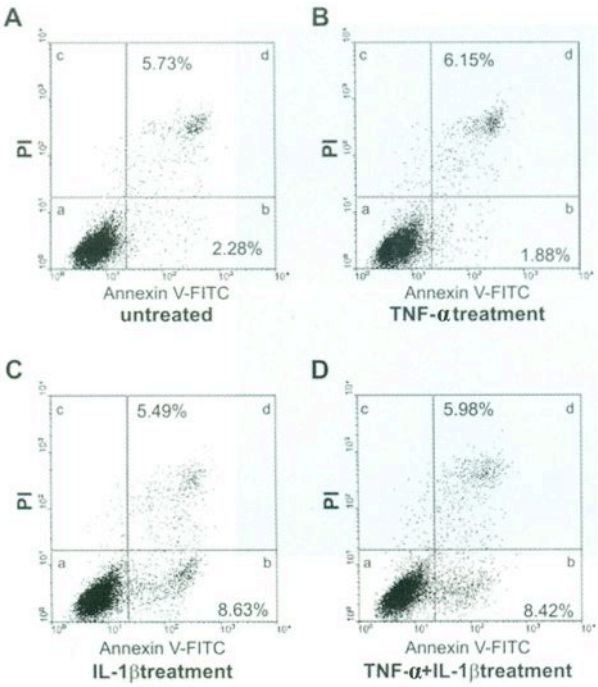


Fig. 2. *Cell death analysis as show by flow cytometry of nuclei from HaCat cells (stained PI and Annexin V-FITC). The dual parametric dot plots combining Annexin V-FITC an PI fluorescence show the viable cell population in the lower left quadrant (a; Annexin V⁻ PI⁻), the early apoptotic cells in the lower right quadrant (b; Annexin V⁺ PI⁻), the late apoptotic cells in the upper right quadrant (c; annexin V⁺ PI⁺), and the necrotic cell population in the upper left quadrant (d; (annexin V⁻ PI⁺). The number in each quadrant represents the percentages of cells.* ***A)*** *untreated HaCat.* ***B)*** *HaCat treated with TNF-α (50 ng/ml).* ***C)*** *HaCat treated with IL-1β (50 ng/ml).* ***D)*** *HaCat treated with TNF-α (50 ng/ml) and IL-1β (50 ng/ml). Results are representative of 5 independent experiments.*

such as *TNFRSF10C, TNFAIP3, TNFRSF1A, TNFRSF11B, TNFRSF6B, TNFRSF1B, TNF, TNFR1, NFKB1A, TRAF3.*

Inflammation and immune responses

TNF-α induced many inflammatory chemokines: *CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL10* and *CXCL11,* for the family of CXC, *CCL2, CCL3, CCL5, CCL7, CCL17, CCL20, CCL23* and *CCL25* for the family of CC chemokines. Furthermore, TNF-α induced members of the TGF-β family (*BMP6* and *BMP2*) as well as genes involved in the regulation of the complement cascade like *C1R, C1S, CR1, CD55, CD83, CFB, C1QC* and suppressed *CD86.* TNF-α also dysregulated many interleukin genes; in particular, this treatment induces IL-1α, *IL-1β, IL-1F5, IL-1 F6, IL-1F7, IL-1F8, IL-1F9, IL-1R2, IL-1RN, IL-6, IL-7R, IL-8, IL-18R1, IL-23A, IL-24, IL-29, IL-32,* on the contrary suppressed other genes of *IL* family (*IL1-R1, IL-10* and *IL-17α).*

TNF-α treatment induced genes of the S100 family, such as *S100A8, S100A9* and *S100A12*, which play an important role in epidermal differentiation. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. In addition, we found that TNF-α induced matrix metallo- proteinase *MMP9* which is the major gelatinase able to degrade collagen IV.

TNF treatment also revealed an up-regulation of histocompatibility sets of genes (*HLA-C, HLA-DOA, HLA-DQ1)* and a down-regulation of other genes (*HLA-DMA, HLA-DMB* and *HLA-DRA).*

Characterization of the IL-1β regulated genes

To understand the cellular processes affected in keratinocytes by stimulation with IL-1β, we focused our attention on a specific set of IL-1β regulated genes which are involved in the control of cell death, inflammatory response and immunity. Below we report the regulated genes in a hierarchical table of their cellular functions related to the 3 pathways analyzed (Fig. 4).

Cell death

IL-1β produced a down-regulation of FAIM2, IL-10, DPF1, GHRL, involved in anti-apoptotic function. Surprisingly, we did not find any up-regulated genes related to cell death.

Inflammation and immune responses

In the present experiments we found significant activation of just two genes, *SELP* and *IL-10,* which were up- and down-regulated respectively. In addition, we found an up-regulation of some genes of the complement family (*C1QB, C1QC, CR1, C4BPB),* whereas other genes *(C4BPA, C5AR1*) were down-regulated. Finally, we found an up-regulated gene known as *PGLYRP1* of the

Table I. *Data are the mean values (±SEM) of at least 5 experiments of apoptotic rate of HaCat cells detected through Annexin V-FITC/PI staining and analyzed by FACS; after treatment with TNF-α,IL-1β, TNF-α + IL-1β given alone or after pretreatment with human monoclonal antibodies TNFRI, TNFRII and IL-1R.*

Sample	Early apoptotic cells (%) (annexin V^+/PI^-)	Advanced apoptotic cells (%) (annexin V^+/PI^+)
untreated	2.81±0.26	5.16±0.02
TNF-α	2.45±0.31	5.31±0.12
IL-1β	8.37±0.20 [a]	5.60±0.21
TNF-α + IL-1β	8.10±0.26 [b]	6.02±0.13
TNF-α + TNFRs	2.09±0.06	5.17±0.18
IL-1β + IL-1R	2.66±0.08	5.48±0.05
TNF-α + IL-1β + + TNFRs + IL-1R	3.39±0.03	5.72±0.07

[a] *P< 0.0001 vs pretreatment with human monoclonal antibody (IL-1R).* [b] *P< 0.0001 vs pretreatment with human monoclonal antibodies (TNFRI, TNFRII, IL-1R).*

Epidermal Differentiation Complex (EDC), located on chromosome 1q21 .

Characterization of the (TNF-α + IL-1β) regulated genes

A combined treatment with both cytokines (TNF-α and IL-1β) revealed a larger number of differentially expressed genes in comparison to those observed in single treatments. Interestingly, a synergic effect of the two cytokines on the genomic response of the HaCat cells was suggested by greater folding changes (FC) in comparison to genes dysregulated after TNF-α treatment.

To understand the cellular processes affected in keratinocytes by stimulation with TNF-α + IL-1β, we focused our attention on specific sets of genes which are involved in the control of cell death, inflammatory response and immunity. We arranged the regulated genes in a hierarchical table of their cellular functions related to the 3 pathways analyzed (Fig. 5).

Cell death

Microarray data analysis revealed that the combined treatment influenced the gene expression profiles of many cytokine family genes involved not only in the inflammatory response but also in cell apoptosis. We observed a dysregulation both in pro-apoptotic genes (*IL-1α, IL1-β, IL-24)* and anti-apoptotic gene (*IL-6)*.

A deeper investigation of the anti-apoptotic genes revealed a marked upregulation of *BIRC3,* while *BIRC1* and *BIRC5* were down-regulated. The BIR motifs region interacts with TNF receptor-associated factors 1 and 2 (TRAF1 and TRAF2) to form a heteromeric complex, which is then recruited to the tumor necrosis factor receptor 2 (TNFR2) (28-29).

Another gene implicated in the anti-apoptotic process which resulted as down-regulated in our microarray experiments was *CASP8* which is involved in the programmed cell death induced by Fas and in various apoptotic stimuli and in activating *CASP3, CASP4, CASP6, CASP7, CASP9* and *CASP10*.

We also found the down-regulation of TP family genes which are involved in the apoptotic processes, such as TP53 and TP73. TP53 acts as a tumour suppressor in many tumour types and induces growth arrest or apoptosis, depending on the physiological circumstances and cell type (30).

Inflammatory and immune response

In the same way as that observed for treatment with TNF-α ginev alone, combined treatment disregulates many genes involved in the inflammatory and immune response pathways: CXC chemokine

Table II. *Transcriptional effects of different modules of treatment with TNF-α and IL-1β on 10 most significant enriched biological categories using DAVID bioinformatic tool.*

Category (Biological Process)	No. of genes	*P* value
TNF-α up-regulated genes		
defense response	108	6.90E-34
inflammatory response	69	6.90E-27
immune system process	140	1.90E-23
death	75	7.00E-07
regulation of apoptosis	53	3.00E-06
acute inflammatory response	14	7.20E-05
positive regulation of immune response	14	2.40E-04
neutrophil chemotaxis	6	2.70E-04
leukocyte activation	23	7.50E-04
complement activation	8	1.40E-03
TNF-α down-regulated genes		
induction of apoptosis by intracellular signals	8	1.20E-03
cell death	53	4.60E-03
regulation of apoptosis	37	5.90E-03
inflammatory response	22	2.10E-02
adaptive immune response	9	2.10E-02
humoral immune response	8	3.70E-02
negative regulation of cytokine production	3	5.40E-02
regulation of cytokine biosynthetic process	7	6.40E-02
negative regulation of apoptosis	16	7.20E-02
lymphocyte mediated immunity	7	9.90E-02
IL1-β up-regulated genes		
B cell mediated immunity	6	1.40E-03
immune effector process	8	1.50E-03
inflammatory response	13	1.80E-03
humoral immune response	6	4.60E-03
lymphocyte mediated immunity	6	4.80E-03
leukocyte mediated immunity	6	6.70E-03
adaptive immune response	6	6.70E-03
complement activation, classical pathway	4	7.30E-03
acute inflammatory response	5	2.50E-02
innate immune response	5	6.70E-02

Category (Biological Process)	No. of genes	*P* value
IL1-β down-regulated genes		
immune system process	35	4.70E-03
T cell activation	8	5.70E-03
negative regulation of cytokine production	3	1.10E-02
cellular defense response	6	1.20E-02
cytokine metabolic process	6	1.30E-02
T cell proliferation	5	1.30E-02
immune response	28	1.30E-02
leukocyte differentiation	7	1.60E-02
regulation of lymphocyte activation	6	1.80E-02
apoptosis	21	8.40E-02
(TNF-α+IL1-β) up-regulated genes		
inflammatory response	21	2.30E-04
defense response	30	1.20E-03
immune system process	50	1.60E-03
immune response	41	3.40E-03
acute inflammatory response	7	1.60E-02
programmed cell death	32	2.70E-02
leukocyte activation	12	2.90E-02
lymphocyte activation	11	3.10E-02
activation of immune response	6	3.80E-02
cell death	32	4.90E-02
(TNF-α+IL1-β) down-regulated genes		
regulation of apoptosis	43	1.50E-02
induction of apoptosis by intracellular signals	7	1.90E-02
inflammatory response	26	2.70E-02
humoral immune response	9	4.70E-02
death	58	5.50E-02
adaptive immune response	9	7.40E-02
regulation of lymphocyte differentiation	4	8.40E-02
regulation of immune response	10	9.60E-02

genes (*CXCL2, CXCL3, CXCL5, CXCL10, CXCL11)* and CC chemokine genes (*CCL2, CCL3, CCL16, CCL20* and *CCL25).*

Confirmation of microarray results

To confirm the array results, we performed quantitative RT-PCR analysis for each treatment considering nine of the more representative genes. We selected genes which are markedly up- or down-regulated and which are representative of the three pathways of our interest (cell death, immunity and inflammatory response). Notably, we confirmed that the TNF-α treatment given alone or in combination with IL-1β enhanced the expression levels of *CXCL11, CXCL10, IL-32, MMP9, S100A8* and *S100A9* and reduced the expression levels of *TP73, IL-10, BCL2* (Fig. 6 A, B, C, D).

Antagonism at TNF-α and IL-1β receptors

An up-regulation of *CXCL11* and *MMP9* was observed with microarray experiments after TNF-α treatment. In cultured cells pre-treated with monoclonal antibodies *vs* TNFR1 and TNFR2 receptors at 2 and 8 hours before cytokine treatment, we observed a significant decrease of the *CXCL11* and *MMP9* expression levels in respect to the levels revealed after 24 h (Fig. 7A). In the same way, in cultured cells pre-treated with monoclonal antibodies *vs* IL1R receptor, at 2 and 8 hours after IL-1β addition, we observed a significant decrease in the *SELP* and *IL-10* expression levels. (Fig. 7B). In cultured cells pre-treated with monoclonal antibodies *vs* TNFR1,TNFR2 and IL1R receptors at 2 and 8 hours, after a combined treatment (TNF-α +IL-1β) we observed a significant decrease of

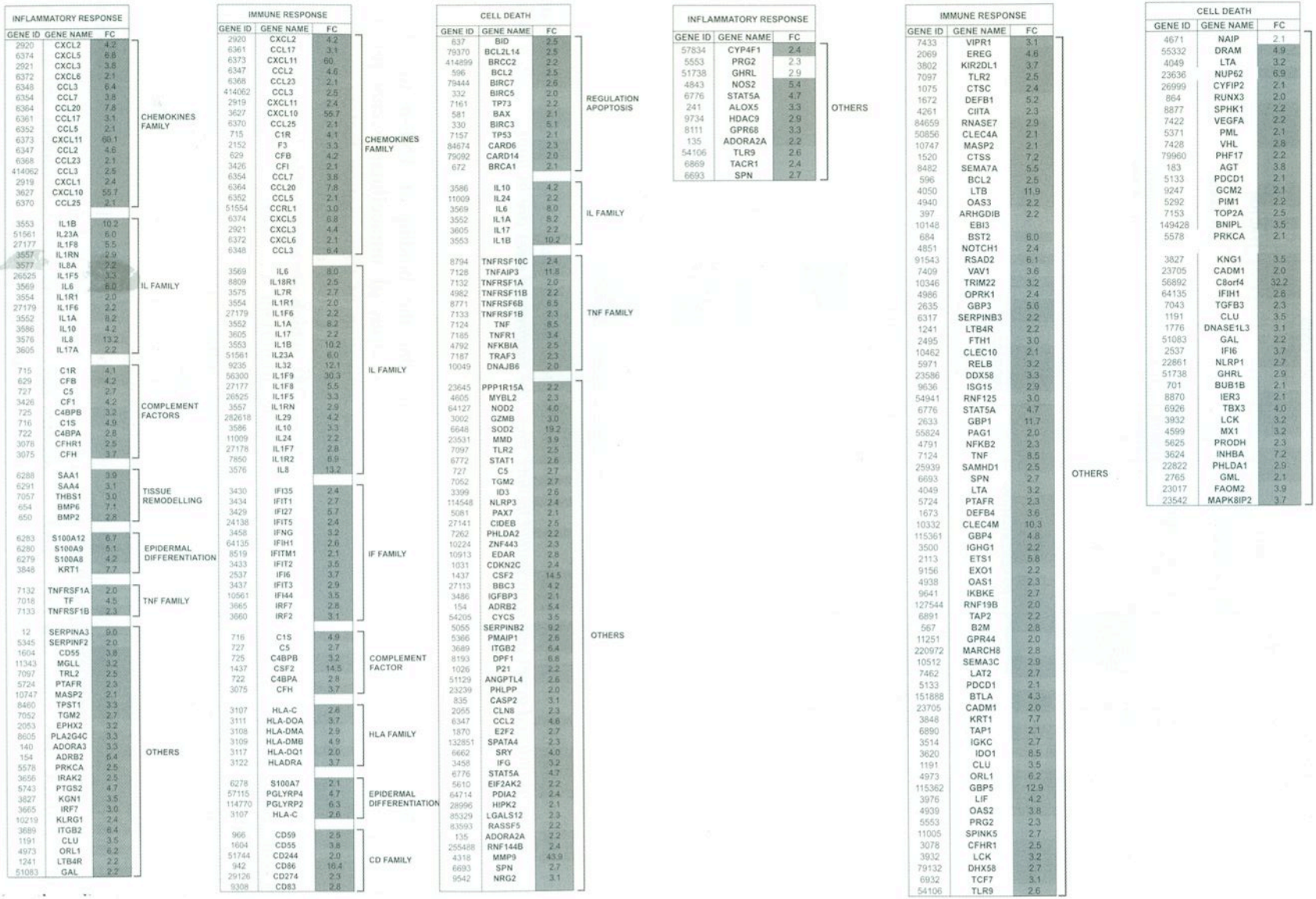


Fig. 3. *List of genes regulated by TNF-α. Genes are arranged according to a hierarchical tree of related molecular and cellular functions. TNF-α induced genes are represented in dark grey; the suppressed ones are in light grey. Results are representative of 3 independent experiments.*

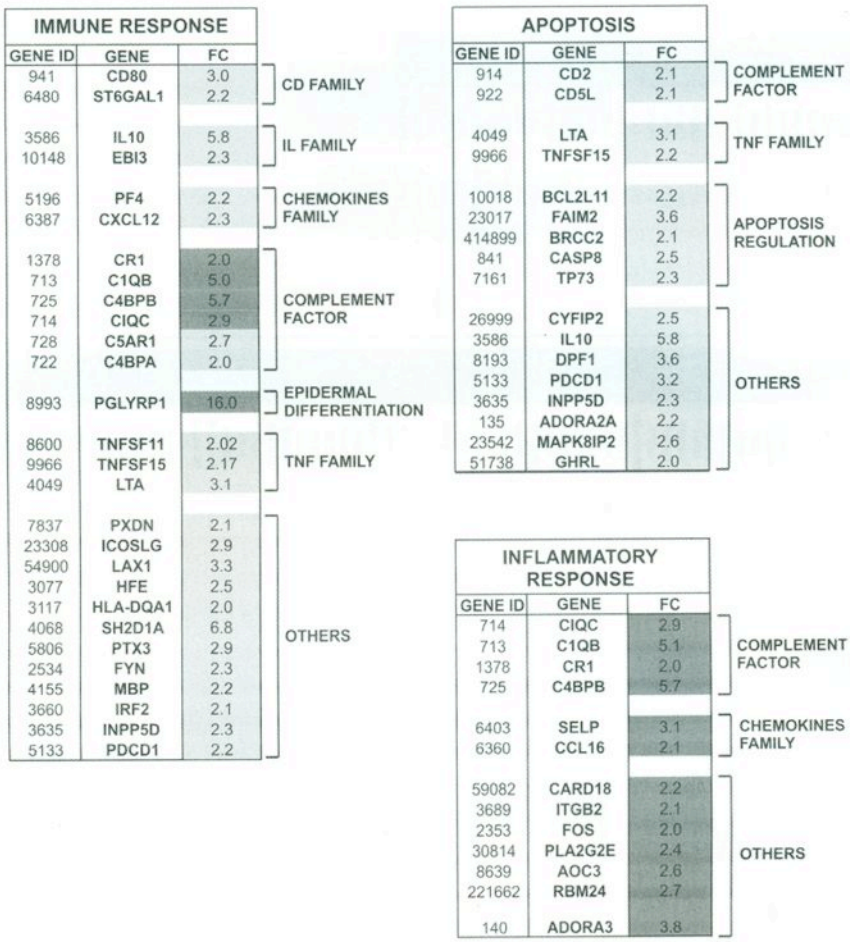


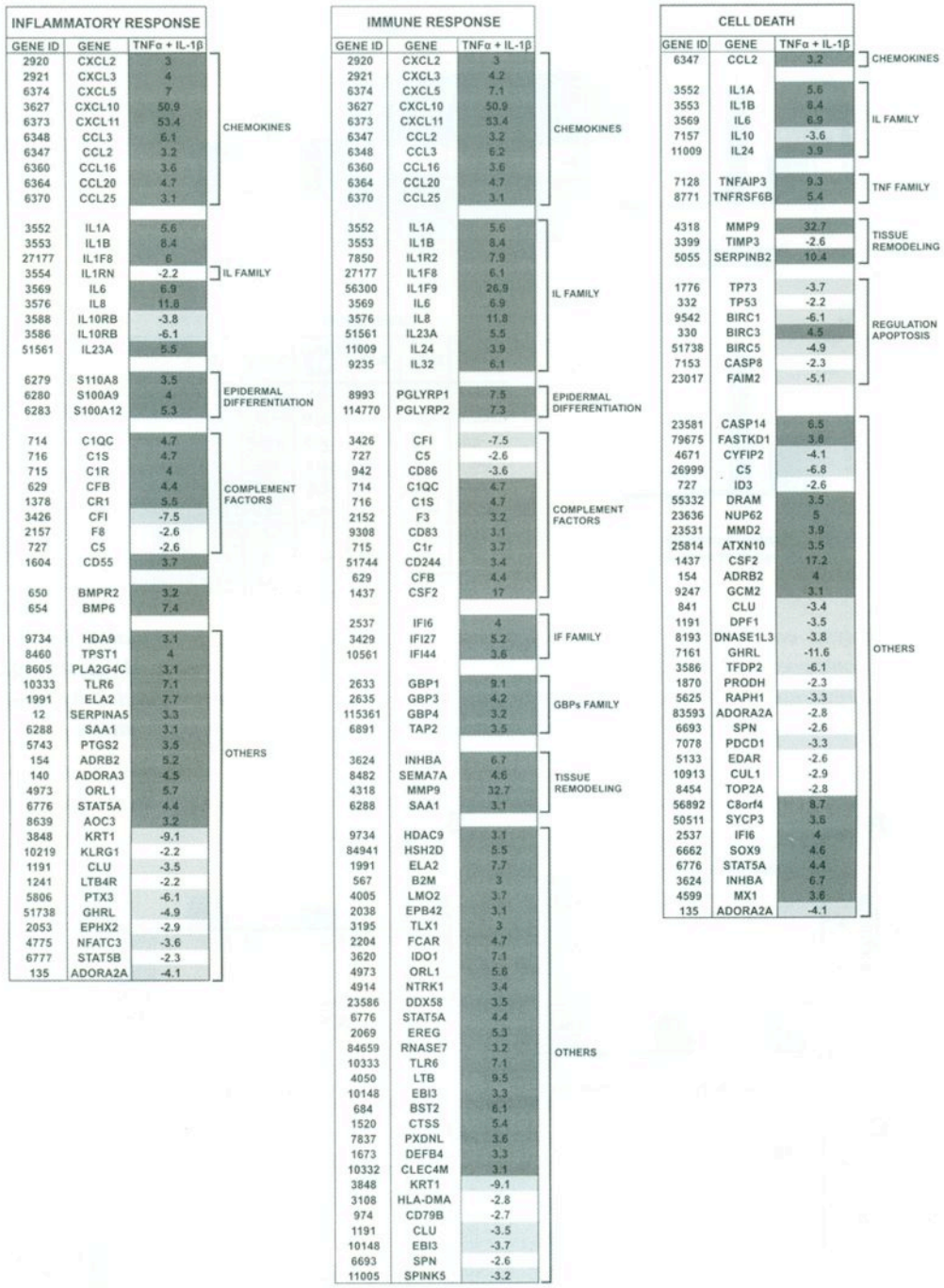
Fig. 4. *List of genes regulated by IL-1β. Genes are arranged according to a hierarchical tree of related molecular and cellular functions. IL-1β induced genes are represented in dark grey; the suppressed ones are in light grey. Results are representative of 3 independent experiments.*

CXCL11, MMP9, SELP and *IL-10* expression levels (Fig. 7 C, D).

DISCUSSION

The present experiments confirm that both TNF-α and IL-1β are two fundamental cytokines regulating different sets of genes mainly involved in inflammatory, immune responses and cell death (14).

As far as TNF-α is concerned, we found activation of many genes belonging to the TNF family, such as *TNFRSF10C, TNFAIP3, TNFRSF1A, TNFRSF11B, TNFRSF6B, TNFRSF1B, TNF, TNFR1, NFKB1A, TRAF3*. Data exist in literature showing that the binding of TNF-α to *TNFR1* triggers a series of intracellular events resulting in the activation of transcription factors including NFκB, AP-1, CCAAT enhancer-binding protein and others (28), which are responsible for the induction of genes important for diverse biological processes, including cell growth and death, oncogenesis, and immune, inflammatory and stress responses (29). In our experimental conditions TNF-α induced the expression of many genes involved in the activation of inflammatory cytokines which is in agreement with their role in the regulation of cell trafficking of various types of leukocytes. However, recent studies have reported that neutrophils are involved in the production of chemokines in response to a



INFLAMMATORY RESPONSE

GENE ID	GENE	TNFα + IL-1β	
2920	CXCL2	3	CHEMOKINES
2921	CXCL3	4	
6374	CXCL5	7	
3627	CXCL10	50.9	
6373	CXCL11	53.4	
6348	CCL3	6.1	
6347	CCL2	3.2	
6360	CCL16	3.6	
6364	CCL20	4.7	
6370	CCL25	3.1	
3552	IL1A	5.6	IL FAMILY
3553	IL1B	8.4	
27177	IL1F8	6	
3554	IL1RN	-2.2	
3569	IL6	6.9	
3576	IL8	11.8	
3588	IL10RB	-3.8	
3586	IL10RB	-6.1	
51561	IL23A	5.5	
6279	S110A8	3.5	EPIDERMAL DIFFERENTIATION
6280	S100A9	4	
6283	S100A12	5.3	
714	C1QC	4.7	COMPLEMENT FACTORS
716	C1S	4.7	
715	C1R	4	
629	CFB	4.4	
1378	CR1	5.5	
3426	CFI	-7.5	
2157	F8	-2.6	
727	C5	-2.6	
1604	CD55	3.7	
650	BMPR2	3.2	OTHERS
654	BMP6	7.4	
9734	HDA9	3.1	
8460	TPST1	4	
8605	PLA2G4C	3.1	
10333	TLR6	7.1	
1991	ELA2	7.7	
12	SERPINA5	3.3	
6288	SAA1	3.1	
5743	PTGS2	3.5	
154	ADRB2	5.2	
140	ADORA3	4.5	
4973	ORL1	5.7	
6776	STAT5A	4.4	
8639	AOC3	3.2	
3848	KRT1	-9.1	
10219	KLRG1	-2.2	
1191	CLU	-3.5	
1241	LTB4R	-2.2	
5806	PTX3	-6.1	
51738	GHRL	-4.9	
2053	EPHX2	-2.9	
4775	NFATC3	-3.6	
6777	STAT5B	-2.3	
135	ADORA2A	-4.1	

IMMUNE RESPONSE

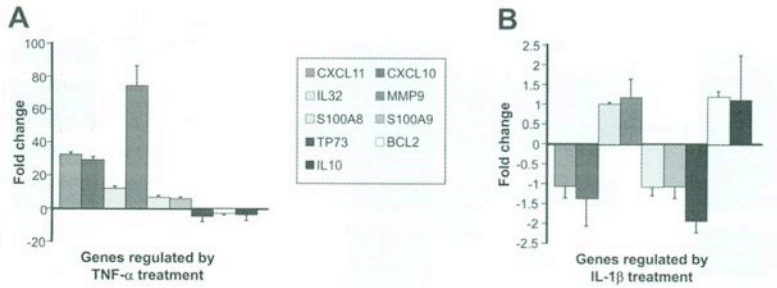
GENE ID	GENE	TNFα + IL-1β	
2920	CXCL2	3	CHEMOKINES
2921	CXCL3	4.2	
6374	CXCL5	7.1	
3627	CXCL10	50.9	
6373	CXCL11	53.4	
6347	CCL2	3.2	
6348	CCL3	6.2	
6360	CCL16	3.6	
6364	CCL20	4.7	
6370	CCL25	3.1	
3552	IL1A	5.6	IL FAMILY
3553	IL1B	8.4	
7850	IL1R2	7.9	
27177	IL1F8	6.1	
56300	IL1F9	26.9	
3569	IL6	6.9	
3576	IL8	11.8	
51561	IL23A	5.5	
11009	IL24	3.9	
9235	IL32	6.1	
8993	PGLYRP1	7.5	EPIDERMAL DIFFERENTIATION
114770	PGLYRP2	7.3	
3426	CFI	-7.5	COMPLEMENT FACTORS
727	C5	-2.6	
942	CD86	-3.6	
714	C1QC	4.7	
716	C1S	4.7	
2152	F3	3.2	
9308	CD83	3.1	
715	C1r	3.7	
51744	CD244	3.4	
629	CFB	4.4	
1437	CSF2	17	
2537	IFI6	4	IF FAMILY
3429	IFI27	5.2	
10561	IFI44	3.6	
2633	GBP1	9.1	GBPs FAMILY
2635	GBP3	4.2	
115361	GBP4	3.2	
6891	TAP2	3.5	
3624	INHBA	6.7	TISSUE REMODELING
8482	SEMA7A	4.6	
4318	MMP9	32.7	
6288	SAA1	3.1	
9734	HDAC9	3.1	OTHERS
84941	HSH2D	5.5	
1991	ELA2	7.7	
567	B2M	3	
4005	LMO2	3.7	
2038	EPB42	3.1	
3195	TLX1	3	
2204	FCAR	4.7	
3620	IDO1	7.1	
4973	ORL1	5.6	
4914	NTRK1	3.4	
23586	DDX58	3.5	
6776	STAT5A	4.4	
2069	EREG	5.3	
84659	RNASE7	3.2	
10333	TLR6	7.1	
4050	LTB	9.5	
10148	EBI3	3.3	
684	BST2	6.1	
1520	CTSS	5.4	
7837	PXDNL	3.6	
1673	DEFB4	3.3	
10332	CLEC4M	3.1	
3848	KRT1	-9.1	
3108	HLA-DMA	-2.8	
974	CD79B	-2.7	
1191	CLU	-3.5	
10148	EBI3	-3.7	
6693	SPN	-2.6	
11005	SPINK5	-3.2	

CELL DEATH

GENE ID	GENE	TNFα + IL-1β	
6347	CCL2	3.2	CHEMOKINES
3552	IL1A	5.6	IL FAMILY
3553	IL1B	8.4	
3569	IL6	6.9	
7157	IL10	-3.6	
11009	IL24	3.9	
7128	TNFAIP3	9.3	TNF FAMILY
8771	TNFRSF6B	5.4	
4318	MMP9	32.7	TISSUE REMODELING
3399	TIMP3	-2.6	
5055	SERPINB2	10.4	
1776	TP73	-3.7	REGULATION APOPTOSIS
332	TP53	-2.2	
9542	BIRC1	-6.1	
330	BIRC3	4.5	
51738	BIRC5	-4.9	
7153	CASP8	-2.3	
23017	FAIM2	-5.1	
23581	CASP14	6.5	OTHERS
79675	FASTKD1	3.8	
4671	CYFIP2	-4.1	
26999	C5	-6.8	
727	ID3	-2.6	
55332	DRAM	3.5	
23636	NUP62	5	
23531	MMD2	3.9	
25814	ATXN10	3.5	
1437	CSF2	17.2	
154	ADRB2	4	
9247	GCM2	3.1	
841	CLU	-3.4	
1191	DPF1	-3.5	
8193	DNASE1L3	-3.8	
7161	GHRL	-11.6	
3586	TFDP2	-6.1	
1870	PRODH	-2.3	
5625	RAPH1	-3.3	
83593	ADORA2A	-2.8	
6693	SPN	-2.6	
7078	PDCD1	-3.3	
5133	EDAR	-2.6	
10913	CUL1	-2.9	
8454	TOP2A	-2.8	
56892	C8orf4	8.7	
50511	SYCP3	3.6	
2537	IFI6	4	
6662	SOX9	4.6	
6776	STAT5A	4.4	
3624	INHBA	6.7	
4599	MX1	3.8	
135	ADORA2A	-4.1	

Fig. 5. *List of genes regulated by a combined treatment with TNF-α + IL-1β. Genes are arranged according to a hierarchical tree of related molecular and cellular functions. TNF-α + IL-1β induced genes are represented in dark grey; the suppressed ones are in light grey. Results are representative of 3 independent experiments.*

variety of stimulants including LPS, TNF-α, and IFN-γ, thereby contributing to immunomodulation (31). Furthermore, TNF-α induced members of the TGF-β family (*BMP6* and *BMPR2*) as well as genes involved in the regulation of the complement cascade like *C1R, C1S, CR1, CD55, CD83, CFB, C1QC* and suppressed *CD86* and *CD79B*. TNF-α dysregulated also many interleukin genes with



D

	GENE ARRAY			REAL TIME PCR		
GENE	TNFα	IL1β	TNFα+ IL1β	TNFα	IL1β	TNFα+ IL1β
CXCL11	+60	+1.8	+53	+32	-1	+34
CXCL10	+50	+1.3	+50.9	+30	-1	+28
IL32	+12	+1.5	+10.9	+13.5	+1	+13.8
MMP9	+28	+1.3	+32.7	+75	+1	+90.7
S100A8	+4.2	+1.6	+3.5	+7.3	-1	+2.4
S100A9	+4.7	+1.5	+3.8	+6.7	-1	+5.1
TP73	-5.8	-1.7	-11	-3	-1	-3
IL10	-4.1	-5.76	-6.14	-2	+1.1	-2.6
BCL2	+1.08	+1.07	+1.1	-1.4	+1.2	-1

Fig. 6. *RT-PCR analysis confirms the results of microarray. The mRNA levels of 9 regulated genes were examined using RT-PCR and compared with HPRT control.* ***A)*** *genes regulated by TNF-α treatment;* ***B)*** *genes regulated by IL-1β treatment;* ***C)*** *genes regulated by TNF-α+IL-1β treatment;* ***D)*** *Fold change of genes regulated by cytokines results by Whole Genome Array and Realtime PCR. Results are representative of 3 independent experiments.*

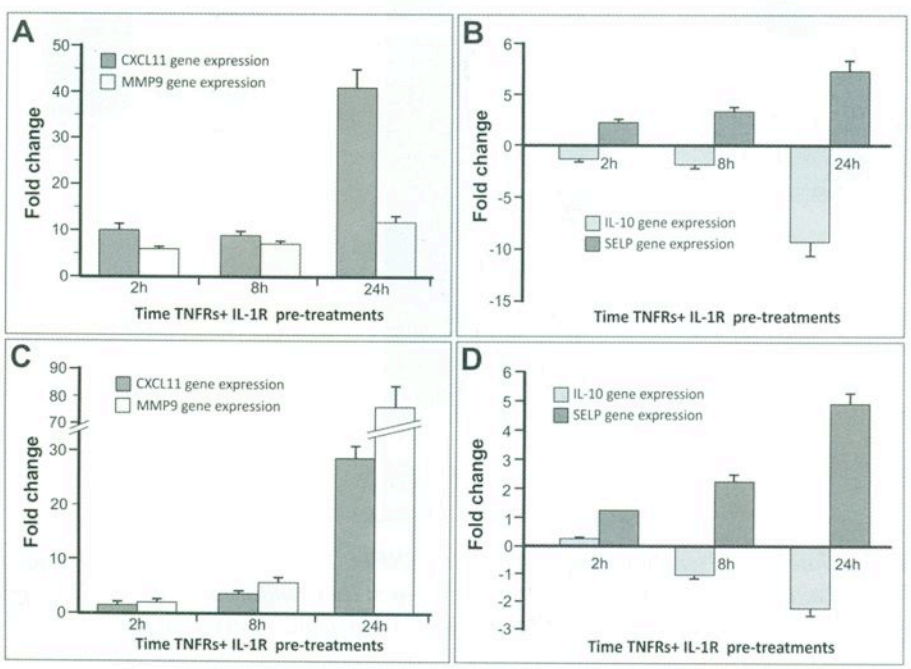


Fig. 7. *Time courses of RT-PCR analysis of genes regulated by cytokines TNF-α and IL-1β after blocking their receptors with monoclonal antibodies:* ***A)*** *regulated genes by TNF-α after pre-treatment with inhibitors of its receptors (TNFRI and TNFRII);* ***B)*** *regulated genes by IL-1β after pre-treatment with inhibitors of its receptor (IL-1R);* ***C)*** *and* ***D)*** *regulated genes by TNF-α and IL-1β given simultaneously, after pre-treatment with inhibitors of their receptors. The graphics are representative of 3 different experiments for each human monoclonal antibody used and for each time studied.*

pleiotropic effects on various immune responses, inflammatory processes, and haematopoiesis (28). In addition, it is known that interleukins elicit a wide array of biologic activities that initiate and promote the host response to injury or infection by activating a set of transcription factors, including *NFKB* and *AP1* which in turn induce production of effectors of the inflammatory response. TNF-α up-regulated two proteinase inhibitors, *SERPINA3, SERPINB3*, while down-regulated *SERPINF2* and *SPINK5* which may have a role in epidermal barrier function (32).

In addition, TNF-α treatment induced genes of the S100 family, like *S100A8, S100A9* and *S100A12*, which play an important role in epidermal differentiation. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21.

An induction of matrix metalloproteinase *MMP9* was particularly evident. This gene represents the major gelatinase able to degrade collagen IV, and it is actively involved in tissue degradation, wound healing, and tumor metastasis (33). This gene may play an essential role in local proteolysis of the extracellular matrix and in leukocyte migration.

Taken together, these results suggest that TNF-α induces chemokines, cytokines, growth factors, and cell-surface receptors that *in vivo* could attract hematopoietic cells, neutrophils, memory T cells, monocytes, and macrophages, all of which contribute to innate immunity and inflammation.

Regarding apoptosis, we have found that TNF-α induced both pro- (*TP53, TP73*) and anti- (*BIRC3, BIRC5, BIRC7*) apoptotic genes. However, we detected only a modest rate of apoptosis in the TNF-α treated cells, as well as in their gene expression and in their morphological changes. Similar results were found by Banno et al. (2004), and those suggest that TNF-α may induce in keratinocytes some signals which trigger cell death.

Regarding the trascriptional effects induced by IL-1β, our results are in line with those obtained by Yano et al. (2008) (6), and reveal the activation of genes involved in inflammation, differentiation, proteolysis and cell death in epidermal keratinocytes.

In particular, we found that IL-1β, although stimulating many genes overlapping TNF-α (6), specifically produces down-regulation in some antiapoptotic genes (*FAIM2, IL-10, DSPF1, GHRL).* As far as genes implicated in inflammatory and immune responses are concerned, IL-1β produced an up-regulation of *SELP* and a down-regulation of *IL-10.*

SELP gene encodes a 140 kDa protein that is stored in the alpha-granules of platelets and Weibel-Palade bodies of endothelial cells. This protein redistributes to the plasma membrane during platelet activation and degranulation and mediates the interaction of activated endothelial cells or platelets with leukocytes. The membrane protein is a calcium-dependent receptor that binds to sialylated forms of Lewis blood group carbohydrate antigens on neutrophils and monocytes (34), and its expression on platelet surface is highly correlated with serotonin transporter density in human subjects (35).

IL-10 is involved in regulating the intensity and duration of the immune response, and modulates matrix metalloproteinase expression (36); IL-10 signaling plays a non-critical role in suppression of inflammatory mediators, resolution of the inflammatory response, and fibrous tissue deposition following myocardial infarction (36).

The IL-1β-treated cells showed a marked increase in the number of apoptotic cells, as revealed by keratinocyte morphological changes. It is interesting that both apoptotic and transcriptional response induced by TNF-α and/or IL-1β given alone or in combination were significantly prevented by specific human monoclonal antibodies *vs* TNFRI, TNFRII and IL-1R receptors given alone or in combination. This suggests the existence of a link between gene dysregulation evoked by both cytokines and cell apoptotic effects. Concerning the other investigated cell pathways (immunity and inflammatory response), we did not find a great percentage of genes dysregulated by IL-1β treatment. This suggests that IL-1β transcriptional effects are more selectively addressed to apoptotic genes. In fact, IL-1β induced down-regulation of anti-apoptotic genes (like *FAIM2, IL10, DPF1,GRL1)* and this could be the mechanism by which IL-1β promotes apoptosis.

The simultaneous application of TNF-α and IL-1β to human keratinocytes produced synergistic

effects on gene expression which may very likely be due to additive or multiplicative effects of the single cytokines. These results confirm that the biological functions of IL1-β and TNF-α overlap and may complement each other in the systemic inflammatory response syndrome (6). Besides the dysregulation of genes that we obtained with TNF-α and IL-1β given alone, following the combined treatment we observed up-regulation of pro-apoptotic factors (*IL1A, IL1B, IL24*) and down-regulation of anti-apoptotic genes (*IL-6* and *CASP8)*. Despite the fact that our results indicate that the combined treatment should promote apoptosis to a larger extent than the single cytokines, we were unable to show this potentiation at least after 24 h exposure. Therefore, it would be interesting to know whether, at later stages, following repeated administration of the cytokines, an increased number of apoptotic cells occurs and whether TNF-α contributes to this effect.

In conclusion, the present experiments show significant differences between the three different modalities of treatment in terms of number and type of differentially expressed genes. The genomic response of HaCat cells to the IL-1β treatment is quantitatively less evident *vs* TNF-α alone, but qualitatively more addressed to cell death pathway.

TNF-α alone did not produce a significant increase in apoptotic cells revealing that 24 hours after its application there is a balance between the activation of apoptotic genes and anti-apoptotic ones. Although we observed a moderate overlapping between TNF-α and IL-1β dysregulated genes, their folding changes resulted greater in the combined treatment, indicating a synergistic effect of the two cytokines.

Similar results were also obtained by other authors by comparing the effects of each cytokine given alone (6, 14).

Our results show that the TNF-α- and IL-1β-induced transcriptional and apoptotic responses were due to stimulation of their specific receptors since a pre-treatment with anti- TNF-α and IL-1β monoclonal antibodies was able to prevent them.

Finally, since TNF-α and IL-1β are involved in cell damage, HaCat cells treated with both cytokines, could represent a good *in vitro* model to study the early genomic response that occurs in serious skin reactions (i.e. TEN and SJS), after treatment with several drugs.

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