Effects of Tumor Necrosis Factor-α (TNFα) in Epidermal Keratinocytes Revealed Using Global Transcriptional Profiling*

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The abbreviations used are: TNFα, tumor necrosis factor-α; PBS, phosphate-buffered saline; MMP, matrix metalloproteinase; FACS, fluorescence-activated cell sorter.

Identification of tumor necrosis factor-α (TNFα) as the key agent in inflammatory disorders, e.g. rheumatoid arthritis, Crohn’s disease, and psoriasis, led to TNFα-targeting therapies, which, although avoiding many of the side-effects of previous drugs, nonetheless cause other side-effects, including secondary infections and cancer. By controlling gene expression, TNFα orchestrates the cutaneous responses to environmental damage and inflammation. To define TNFα action in epidermis, we compared the transcriptional profiles of normal human keratinocytes untreated and treated with TNFα for 1, 4, 24, and 48 h by using oligonucleotide microarrays. We found that TNFα regulates not only immune and inflammatory responses but also tissue remodeling, cell motility, cell cycle, and apoptosis. Specifically, TNFα regulates innate immunity and inflammation by inducing a characteristic large set of chemokines, including newly identified TNFα targets, that attract neutrophils, macrophages, and skin-specific memory T-cells. This implicates TNFα in the pathogenesis of psoriasis, fixed drug eruption, atopic and allergic contact dermatitis. TNFα promotes tissue repair by inducing basement membrane components and collagen-degrading proteases. Unexpectedly, TNFα induces actin cytoskeleton regulators and integrins, enhancing keratinocyte motility and attachment, effects not previously associated with TNFα. Also unanticipated was the influence of TNFα upon keratinocyte cell fate by regulating cell-cycle and apoptosis-associated genes. Therefore, TNFα initiates not only the initiation of inflammation and responses to injury, but also the subsequent epidermal repair. The results provide new insights into the harmful and beneficial TNFα effects and define the mechanisms and genes that achieve these outcomes, both of which are important for TNFα-targeted therapies.

Tumor necrosis factor-α (TNFα) is a multifunctional cytokine that mediates inflammation, immune response, and apoptosis (1). Inappropriate production or persistent activation of TNFα participates in a wide spectrum of diseases, including septic shock, diabetes, cancer, graft rejection, rheumatoid arthritis, and Crohn’s disease (2). Accumulating evidence indicates that TNFα also has a significant role in normal development and homeostasis of several organs. Mice deficient in TNFα lack germinal centers and show increased susceptibility to microbial pathogens due to incomplete inflammatory responses (3). TNFR1-mutant mice show similar abnormalities, in addition to defective formation of Peyer’s patches (4). In skin, TNFα is the master cytokine regulator in inflammatory diseases, such as psoriasis, contact dermatitis, drug eruptions, cutaneous T-cell lymphoma, etc. (5–7). TNFα is found in skin after injury (8–10) and is considered essential for angiogenesis during wound healing (11).

Agents targeting TNFα are now in clinical trials for treatment of inflammatory diseases, including psoriasis (12). However, our understanding of the TNFα action has not been sufficient to predict the full effects and side effects of such therapies because TNFαs have a remarkable variety of functions and the anti-TNFα treatments can simultaneously affect many cellular processes in both abnormal and normal tissues (13).

TNFα is a homodimer of 157 amino acid subunits produced primarily by activated macrophages but also by other cell types, including epidermal keratinocytes (14). A low level of TNFα is present in the upper layer of the healthy epidermis, but its synthesis and release from keratinocytes are greatly augmented by injury, infection, UV irradiation, and contact sensitizers (8–10). Of the two distinct cell-surface receptors for TNFα, TNFR1 and TNFR2, keratinocytes mainly express TNFR1 (16). The binding of TNFα to TNFR1 triggers a series of intracellular events resulting in the activation of transcription factors, including NFκB, AP-1, CCAAT enhancer-binding protein β, and others (17), 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 (14). TNFα activates the immune responses through inducing the production of additional signals, such as interleukin 1 (IL-1) and IL-8, transforming growth factor type β (TGF-β), intercellular adhesion molecule 1 (ICAM-1), etc. (18).

TNFα affects epidermis in cutaneous inflammatory diseases, and therefore we set out to identify and comprehensively analyze the TNFα-regulated genes in epidermal keratinocytes by using DNA microarrays. Several reports of microarray analyses of TNFα-regulated genes focused on cell lines or pathological conditions (19–21). However, we decided to use primary cultures of healthy human epidermal keratinocytes and profile the transcriptional changes 1, 4, 24, and 48 h after TNFα treatment. The gene expression patterns were compared with the corresponding untreated control at each time point, which allowed us to describe novel responses not previously associated with TNFα action or cutaneous inflammation. We found that TNFα works fast, with many genes regulated already after 1 h.
Among the regulated genes, we found those involved in immune and inflammatory responses, and although TNFα was known to regulate some of these, many are newly identified targets. Unexpectedly, TNFα regulates genes that affect the cell motility and cytoskeleton, which activates keratinocyte migration. TNFα also regulates genes involved in cell cycle and apoptosis, as well as basement membrane components and matrix metalloproteases. Because these genes and processes comprise important elements of wound-healing, we predicted that impaired wound-healing will be recognized as a side-effect of TNFα-targeted therapies.

The results indicate that TNFα affects a wide range of processes to integrate the epidermal responses to injury and suggest that TNFα is responsible not only for the initiation of inflammation, but also for the subsequent repair and regenerative processes. This finding has significant implications for the development of TNFα-targeted treatments.

**EXPERIMENTAL PROCEDURES**

**Human Keratinocyte Cultures and Cytokine Treatment**—We used the approach described before (22, 23). Briefly, normal human neonatal foreskin epidermal keratinocytes were obtained from Dr. M. Simon (Laboratory of Skin Biology, Unit SUNY, Stony Brook, NY). The cultures were initiated using 3T3 feeder layers and then frozen in liquid nitrogen until used. Once thawed, the keratinocytes were grown without feeder cells in a defined serum-free keratinocyte growth medium (KGM) supplemented with 2.5 ng/ml epidermal growth factor and 0.05 ng/ml bovine pituitary extract (keratinocyte-SFM, Invitrogen) at 37°C in 5% CO2. We avoid using serum because it can promote certain aspects of keratinocyte differentiation. The medium was replaced every 2 days, and the cells were expanded through three passages for the experiments. They were trypsinized with 0.025% trypsin, which was neutralized with 0.5 mg/ml of trypsin inhibitor. For all experiments, third-passage keratinocytes were used at 50–70% confluence. These primary cultures provide a more appropriate target than immortalized, aneuploid cell lines, and by using a single large batch of cells, we avoided variability due to growth conditions. We changed the medium from KGM to keratinocyte basal medium without supplements 24 h before treatment to avoid the effects of the supplements in growth medium. Keratinocytes were treated with human recombinant TNFα (50 ng/ml, Sigma) and harvested by scraping at 1, 4, 24, and 48 h after treatment. To avoid effects of changes in keratinocyte physiology during their growth in cultures, each time point had a TNFα-treated and a corresponding untreated, control sample. The entire experiment was performed twice, each time by a different experimenter.

**In situ Fluorescence Staining**—Keratinocytes were grown on Lab-Tek chamber slides (Nunc, Roskilde, Denmark) and incubated with KGM. The medium was changed to keratinocyte basal medium 24 h before cytokine treatment, as described above. The cells were treated with TNFα (50 ng/ml). At several time points after treatment, the cells were rinsed with phosphate-buffered saline (PBS) and then immediately fixed with 70% methanol in PBS for 2 h at 4°C. The stained cells were observed under the microscope (Zeiss, Axiophot), and images were captured with a digital photo camera (CoolPix, Nikon).

**Preparation of Labeled cRNA and GeneChip™ Hybridization**—We isolated total RNA from the cells with RNeasy kits (Qiagen) according to the manufacturer's instructions. Approximately 5–8 µg of total RNA was reverse-transcribed, amplified, and labeled as described (22). Fifteen micrograms of labeled cRNA was hybridized to HG-U95Av2 arrays (Affymetrix, Santa Clara, CA). Arrays were washed, stained with anti-biotin streptavidin-phyceroerythrin-labeled antibody, and scanned using the Agilent GeneArray™ scanner system (Hewlett-Packard).

**Northern Blotting**—For Northern blot analysis, 5 µg of total RNA was electrophoresed on 1% formaldehyde-agarose gels and transferred to Hybond nylon membrane (Amersham Biosciences). The probes were synthesized using keratinocyte RNA and the RT-PCR kit (Promega) with the following primers: EFNA1, 5'-CTGAGAAGAGGAC-ACAGGCAC-3' and 5'-TGGCACACGTGGTTCTTTTGGG-3'; NinI, 5'-A-ACGTTGACATCAGCGC-3' and 5'-GTGGTTAAGGTTGCTGAGAC-3'; SO2, 5'-GTGGCCAGGGAGAGATTCA-3' and 5'-CTGGGTTGAGCCACGAG-3'; MM2, 5'-GGCGCTAAGCTAGCAGC-3' and 5'-TGCAGGTTTATAGGAAACGC-3'; CND2, 5'-GGTAGCCGAGGGATTCC-3' and 5'-ACGGTCCCAGCTCCGG-3'; NF-B1, 5'-ACAATGGGTCA- CACCGAGA-3' and 5'-TGGAGGCTGTGGTATCCAGG-3'; TNFAIP5, 5'-CACATTGCGTGGACAGTTT-3' and 5'-ATATATGCTAGTGCCC-I10 Mg/ml RNase (Ambion); cells were analyzed with FACScan (BD Biosciences). We did not consider either genes deemed absent from all 16 microarrays or genes expressed at levels sufficiently low that expression values remained below 50 arbitrary units in all 16 microarrays, because we consider these values insufficiently reliable. To improve reliability, we checked individually the absolute expression levels and p values among all four time points. Genes were considered regulated if both criteria were met: (i) at least one statistical test must find the gene regulated, and (ii) the average expression level difference in the duplicate experiments must be more than 2-fold relative to untreated control at any time point.

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip. The table is based primarily on the Gene Ontology Consortium data and the data by J. M. Rouillard.3

**Flow Cytometry**—Approximately 5 × 10^6 trypsinized keratinocytes were fixed in 70% ethanol in PBS for 2 h at 4°C. After washing in cold PBS, we stained the cells with 37.5 µg/ml propidium iodide (Sigma) and 1 µg/ml RNase (Ambion); cells were analyzed with FACScan (BD Biosciences).

**RESULTS**

The immediate effects of TNFα cause the activation of transcriptional factors, including NFκB. As expected (24), the activation of NFκB leads to its nuclear translocation (Fig. 1, A and B). The consequent transcriptional induction of the NFκB-responsive genes results in profound morphological changes: the regular, polygonal “cobblestone” appearance of untreated keratinocytes is replaced by uneven arrangement, prominent cell-cell boundaries, and irregularly shaped cells 48 h after the TNFα treatment.
time points were very similar to each other, forming a treatment. A, in untreated keratinocytes, NF-kB was found in the cytoplasm. B, in the TNF-α-treated cells, NF-kB translocated to the nucleus. C, untreated keratinocytes have a regular, tightly packed, polygonal cobblestone appearance. D, TNF-α treatment caused irregular shape and uneven arrangement of keratinocytes after 48 h.

Microarray Data Mining—To obtain a comprehensive picture of the transcriptional changes caused by TNF-α, we grew primary cultures of untransformed, normal human epidermal keratinocytes and treated them with 50 ng/ml of TNF-α, which was followed by RNA isolation for microarray experiments. Of the ~12,000 genes present on the chips, some 7,300 were found expressed in keratinocytes; of these, some 230 genes were regulated by TNF-α, according to our criteria. Of the 230 genes, 183 were induced (80%), 43 were suppressed, and 4 were both up-regulated and down-regulated at different time points.

We used several well established data mining tools to analyze the microarray data. The scatter plots (in Fig. 2, A and B) representing log-transformed expression values of TNF-α-treated keratinocytes (ordinate) versus untreated corresponding controls (abscissa) show the distribution of expressed genes during the experiment, and indicate that the number of regulated genes increased with the time lapsed. TNF-α works quickly: more than 30 genes were induced at 1 h. Another feature of TNF-α was followed by RNA isolation for microarray experiments. Of the 230 genes, 183 were induced (80%), 43 were suppressed, and 4 were both up-regulated and down-regulated at different time points.

To understand the cellular processes affected in keratinocytes by TNF-α stimulation, we arranged the regulated genes in a hierarchical table of their cellular functions (Fig. 4). The first level of hierarchy indicates the major function or category of each gene; the second integrates these functions into cellular tasks or activities; and the third assigns them to a global process affected by TNF-α. Below, we focus on the specific sets of TNF-α-regulated genes, those that are involved in the innate immunity, inflammation, tissue repair and remodeling, cytoskeletal rearrangements, epidermal differentiation, cell-cycle regulation, and apoptosis.

Innate Immunity and Inflammation—Because TNF-α is well known to be an activator of innate immunity and inflammation in skin (9, 16), these effects will not be described in great detail here. TNF-α induces members of its own family (TNF-α, LTβ), the TGF-β family (INHBA, BMP2), angiogenic cytokines (ECGF1, VEGFC, EFN A1), and others (NK4, TGFA, SEMA3C, BST2). The IL-8 family proteins (GRO1, GRO2, GRO3, and IL-8) have a potential for attracting the neutrophils, chemokines (CXCL10, CXCL11, CCL5, and CCL20) attract monocytes and macrophages, whereas CCL27 attracts the skin-associate memory T-cells. TNF-α induced chemokine receptor (HM74), interleukin receptors and their antagonists (IL-4R, OSMR, and IL-1R1), interferon receptors (IFNAR2, IFNGR2), and ICAM-1. Importantly, TNF-α also induced antigen-processing proteases (PSMB9, PSMB10, PSME2), a putative antigen transporter (ABCB2), and major histocompatibility complex class I (MHC1) antigens, which permits effective antigen presentation on keratinocytes. Complement components (BF) and enzymes of arachidonic acid metabolism (ALOX15B) and endopeptidase (ADAM9) were also induced. Taken together, these results suggest that TNF-α induces chemokines, cytokines, growth factors, and cell-surface receptors that could in vivo invite hematopoietic cells, neutrophils, memory T cells, monocytes, and macrophages, all of which contribute to the innate immunity and inflammation.

Effects upon Tissue Remodeling, Cytoskeleton, and Cell Migration—the effects of TNF-α upon tissue remodeling, cytoskeleton, and cell migration have not been extensively studied. The reconstruction of the extracellular matrix (ECM) might be an important factor for tissue repair during and after inflammation. The induction of matrix metalloproteinase (MMP)-9 was particularly dramatic (see Fig. 3). MMP9 is the major gelatinase able to degrade collagen IV, and it is actively involved in tissue degradation, wound healing, and tumor metastasis (25). Surprisingly, it appears that MMP inhibitor TIMP3 was suppressed by TNF-α, which could result in a synergistic facilitation of tissue degradation by the MMPs in vivo. In addition, the induction of laminins LAMA3, LAMB3, and LAMC2, as well as HSPG2, a major heparan sulfate proteoglycan of basement membranes with adhesive and growth regulatory properties, strongly suggests an in vivo role for TNF-α in the production of basement membrane (26). Fibril-associated collagen 1A1 and mucin-related membrane protein TIA-2 were also induced by TNF-α. TNF-α regulates the coagulation system through the induction of plasminogen activator and its receptor (PLAU, PLAUR) and serum amyloid A (SAA1).

TNF-α induced both adhesion and cytoskeleton-associated proteins in keratinocytes, suggesting a characteristic regulation of cell attachment and migration. Specifically, certain integrins (ITGAV5, ITGAV, and ITGB6) and adhesion-related genes (EFNA1, CEP4, MLP, NCK1, and ARHE) were highly regulated by TNF-α. Integrins are transmembrane receptors that mediate the dynamic linkages between the cytoskeleton and the extracellular matrix, as well as transduce signals to and
from the cell interior (27). Additionally, TNFα induced several adhesion molecules (NINJ1, HXB, and HEF1). This is the first report of NINJ1 induction by TNFα; this induction peaks at 4 h after TNFα stimulation, indicating a rapid regulation (also shown in Fig. 3).

Interestingly, TNFα induced many proteinase inhibitors (HE4, CST6, PI3, SLPI, SPINK5, SERPINB1, SERPINB8, SERPINB2, SERPINB3), perhaps to protect keratinocytes from excess inflammation. Additional proteinase inhibitors, such as SPINK5, may have a role in epidermal barrier function (28) and protease inhibitor 3 (PI3) is an epithelial host-defense protein against microbial infections (29).
Taken together, these TNFα-induced changes in expression of integrins, adhesion molecules, and actin-related genes imply profound changes of the organization of the actin cytoskeleton. Indeed, we found major differences in the actin skeleton: in the treated cells, actin filaments assembled into perinuclear cages and extended into filopodia and lamellipodia. In contrast to the tight packing of control keratinocytes, the TNFα-treated ones withdrew from their neighbors and spread out (Fig. 5A).

We expected that the cytoskeletal changes caused by TNFα would affect cell behavior, in particular, cell migration and cell-cell interactions. To examine the effect of TNFα on keratinocyte migration, we performed real-time recording of cell cultures. Fig. 5B shows the comparison of the growing pattern of keratinocyte cultures with or without TNFα. Whereas the untreated keratinocytes simply fill out the empty spaces, the TNFα-treated keratinocytes extensively migrate and completely change the shapes of the empty spaces by moving in and out of them. This suggests that TNFα stimulates the motility of keratinocytes. We traced the migration of keratinocytes using images from real-time recordings of cultured keratinocytes during the 8 h after TNFα treatment. We found that the TNFα-treated keratinocytes migrate much more than the untreated ones, they move in random directions, and they form many short-lived cell-to-cell contacts (Fig. 5C). Therefore, the TNFα-induced changes of expression of cytoskeleton-associated proteins significantly enhance keratinocyte motility, an essential component of the wound-healing process not previously associated with TNFα.

Control of Cell Growth and Cell Death—Because TNFα only mildly affects the cell proliferation rate in culture (see below), its effects upon growth and apoptosis have remained largely unknown. However, our data clearly show that TNFα profoundly affects these processes. TNFα specifically affects the cell cycle of keratinocytes. The G1/S and G2/M cell cycle stages were blocked during the 8 h after TNFα treatment. We found that the TNFα-treated keratinocytes migrate much more than the untreated ones, they move in random directions, and they form many short-lived cell-to-cell contacts (Fig. 5C). Therefore, the TNFα-induced changes of expression of cytoskeleton-associated proteins significantly enhance keratinocyte motility, an essential component of the wound-healing process not previously associated with TNFα.

Taken together, these TNFα-induced changes of gene expression have profound effects upon the cell fate, i.e. differentiation, cell growth, and cell death, by inducing the expression of differentiation markers, proteins that block keratinocytes in the G1 phase, pro- and anti-apoptotic proteins.

Transcription and Signaling Pathways—TNFα induces the expression of many transcription factors. Interestingly, TNFα seems to induce both the NFκB and the FasL apoptotic signaling pathways.

IRF1 and SOX4 were especially remarkable, whereas NFκB1 and IκB (p105/p50), NFκB2 (p49/p100), RELA (p65), and RELB are the components of NFκB. Two interferon regulatory factor (IRF) family (IRF1, IRF5) and two Sry HMG box (SOX) family (SOX4, SOX9) proteins were induced, whereas MYC, a multifunctional transcriptional factor, was suppressed. The induction of IRF1 and SOX4 were especially remarkable, because IRF1 regulates cell growth and apoptosis (33), whereas SOX4 is required for the development of lymphocytes and thy- 

mocytes (34). Although SOX transcription factors perform a variety of important roles in vertebrate development, little is known about their function in adult tissues. We suspect that

**Fig. 3. Northern blot analysis confirms the results of microarrays.** A, seven representatives were selected from the TNFα-regulated genes and their relative mRNA levels examined using Northern blotting. B, the microarray data showing the fold differences between the treated cells and their corresponding untreated controls for each of the nine genes at each time point. For easy observation, the induced genes are represented in red and orange; the suppressed ones are represented in green. C, the microarray data showing signal intensities for each gene at all time points. The values in the TNFα-treated conditions are highlighted in beige. The expression patterns of each gene show excellent agreement between the two different methods, the Northern blotting and the microarrays.
**Fig. 4. List of TNF-α-regulated genes.** The genes are arranged according to a hierarchical tree of related molecular and cellular functions. The TNF-α-induced genes are represented in red; the suppressed ones are represented in green. Shades of blue (*) indicate the maximum signal intensities, which represent the absolute expression levels of mRNA ranging from low (light blue) to high (dark blue). FC, fold change.
the induction of SOX4 and SOX9 by TNFα has an important role in epidermal tissue repair and morphogenesis.

Additional TNFα-induced transcription factors and transcription regulators include MBOX1, HOXA9, MAFF, HDAC7B, HIVEP1, and ZNF267. MAFF is a stress-responsive transcription inducer (35). Histone deacetylase alters chromosome structure and affects transcription factor access to DNA (36). Thus, many transcription factors and regulators are mobilized for the immune response, cell-cell interaction, and cell fate control.

The Time Course of the TNFα Effects—The time course analysis of gene expression pattern reflects the sequence of the biological events responding to TNFα stimulation. We estimated the weighted earliest and median times of regulation for each functional category of regulated genes (Fig. 7). Judging from these results, the earliest effects of TNFα seem to assemble the immunocompetent cells at the site of the wound, and then initiate the immune and inflammatory responses. Subsequently, TNFα modulates adhesion, motility, and cell-cell interactions, which are followed by the induction of matrix metalloproteases that participate in ECM degradation. Finally, TNFα influences the cell fate, i.e. survival or cell death, and the rebuilding of the extracellular matrix.

DISCUSSION

TNFα is known as the principal regulator of diverse inflammatory and immune processes in human skin diseases, but the breadth of its action is not known. We have described here the global gene expression changes in normal human epidermal keratinocytes treated with TNFα. We have identified comprehensively the set of TNFα-regulated genes that play significant roles in skin innate immunity and inflammation. Furthermore, we have described novel functions of TNFα and discovered a wide spectrum of genes affecting many additional cellular processes that have not been associated previously with TNFα action or cutaneous inflammation. The result obtained with cultured keratinocytes may not be entirely reproduced in inflammatory skin diseases, although similar processes are likely under TNFα control in vivo as well. These processes include tissue repair and ECM remodeling, cytoskeletal changes, cell migration, keratinocyte differentiation, and cell-fate control. Our results describe fully an orchestrated program of the epidermal transcriptional responses controlled by TNFα in response to skin injury and inflammation. Although certain aspects of the TNFα responses can be damaging and destructive, others are clearly beneficial, and TNFα-targeting therapies must take both into account.

Specifically, TNFα, as the regulator of innate immunity and inflammation, induces specialized chemokines to attract neutrophils and macrophages to the skin in the early phase of inflammation. These results explain the pathological findings in acute bacterial infection, where the increased production of TNFα and massive infiltrations of phagocytic cells can be seen. Interestingly, TNFα also induced CCL27, a skin-specific memory T-cell attractant, suggesting the participation of TNFα in fixed drug eruption and other T-cell-mediated skin inflammation processes, such as psoriasis and atopic and allergic contact dermatitis (37, 38). We show here that TNFα also induces the expression of antigen presentation-related molecules in keratinocytes.

In addition to initiating innate immunity and inflammation processes, TNFα also contributes to the repair of damaged skin. For example, TNFα-induced angiogenesis is considered essential for wound healing, and TGFβ has an important role in wound repair, whereas TGFα is mitogenic after skin injury (11, 9, 39, 40). TNFα induces plasminogen, which is required for the normal repair of skin wounds and plays a central role in cancer invasion, and SAA1, an acute-phase reactant modulating platelet adhesion at injury site, which initiates coagulation via circulating micro-vesicles and platelets (41–43). This implies that, on the one hand, TNFα triggers the initiation of inflammation, but on the other hand, provides effective means for the attenuation of inflammation and its resolution as well.

MMP9 was among the molecules most dramatically induced by TNFα, possibly causing tissue degradation. Excessive MMP9 activity is associated with non-healing chronic wounds (44), and deficient levels of MMP9 were found in hypertrophic scars where too much collagen is deposited (45). Additionally, MMP9 may be responsible for detaching the basal keratinocytes from the basement membrane, promoting their migration to cover the exposed connective tissue (46). Apparently, the TNFα-regulated MMP9 plays a role both in tissue degradation and in repair from damage.

Activated macrophages and keratinocytes produce TNFα and subsequently, TNFα induces production of secondary pro-inflammatory cytokines. However, over-production of these inflammatory molecules may exaggerate the inflammation and potentially lead to life-threatening conditions, such as septic shock. Interestingly, we find that TNFα also provides mechanisms for negative feedback. For example, TNFα induced negative regulators of NFκB pathway and anti-inflammatory molecules, including NAF1, NFKBIA, NFKBIE, SSI-1, TNFAIP3, SOD2, and many proteinase inhibitors.

We recognize that some of the TNFα-regulated molecules have multiple functions, thus confounding simple interpretations. One example is ALOX: its metabolites are important in inflammatory skin diseases, such as psoriasis (47), but have also been involved in the establishment of the epidermal lipid barrier (48).
TNFα regulates two antithetical processes, adhesion and migration, by inducing integrins and proteins that affect both actin polymerization and disassembly. The coordinated response is important because cell migration requires both a formation of cell-matrix adhesions at the leading edge and their dissolution at the rear end of the moving cell (49). In addition to integrins, TNFα induced actin-binding proteins, G-proteins, protein kinases, and phosphatases that affect the cytoskeletal organization (50). Thus, TNFα regulates both extracellular and cytoskeletal proteins, causing their rearrangements, which are essential for the keratinocyte attachment and migration.

TNFα induces NINJ1, which was originally identified as an adhesion molecule induced by nerve injury (51). CD47 initiates G-protein signaling and modulates cell adhesion and migration through its association with integrins (52). Ephrin A1 is a secreted ligand with a significant role in inflammatory angiogenesis and in actin re-organization through its receptor and Rho proteins as well as a role in the inflammatory angiogenesis.
induced by TNFα (53, 54). CEP4 is a member of the CDC42-binding protein family and regulates the organization of the actin cytoskeleton through Rho-GTPase, leading to cell shape changes (55). NCK1 has been suggested to function in the organization of the lamellipodia actin network (56). Smoothelin (SMTN) contains an actin localization sequence similar to tropomycin T (57). Little is known about the function of MLP, a protein that binds calmodulin and filamentous actin (58). The protein that binds calmodulin and filamentous actin (58).

The quickest response to TNF is proliferation or apoptosis. This hypothesis will have to be proven directly in our future experiments. Anti-TNF therapy has been proposed for the treatment of many inflammatory diseases (65, 66). However, over-inhibiting the TNF signals has a potential risk of causing delayed wound healing, secondary infections, and cancer due to suppression of the immune and inflammatory processes and defects of proper tissue repair. In fact, tuberculosis and carcinogenesis have been reported as side effects after long-term anti-TNF therapy (66). Anti-TNF treatments, therefore, must be finely tuned for each individual case. We believe that a comprehensive knowledge about the broad spectrum of gene expression regulated by TNFα, which has been shown here, will contribute to make these treatments both more effective and safer.

In summary, this work presents a complete description of the transcriptional changes caused by TNFα in epidermal keratinocytes and we advance two significant and novel concepts: (i) TNFα is essential not just for the initial inflammation and response to injury, but also for the subsequent repair and recovery phase, and (ii) TNFα primes the keratinocytes to anticipate additional extracellular stimuli and then to react quickly and efficiently through proliferation or apoptosis. TNFα activates a series of immune responses and triggers inflammation, which is followed by tissue degradation and repair (Fig. 7). Keratinocytes migrate to repair damaged epidermis, contacting with and detaching from each other and the basement membrane. Some keratinocytes will die by apoptosis as a result of the inflammatory response, whereas some will survive on the newly regenerated basement membrane and proliferate, depending upon additional factors. Keratinocytes must also initiate differentiation to produce a stronger stratified epidermal shield against further tissue damage. These results demonstrate a unique aspect of TNFα as the key organizer of responses to skin injury, regulating a wide scope of biological processes ranging from immune response and inflammation to cell migration, epidermal differentiation, and tissue repair. We find it amazing that the regulation of gene expression for all of these processes can be achieved by a single cytokine, TNFα, in a single cell type, the epidermal keratinocytes.

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