Pathway-specific Profiling Identifies the NF-κB-dependent Tumor Necrosis Factor α-regulated Genes in Epidermal Keratinocytes*

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Identification of tumor necrosis factor α (TNFα) as the key agent in inflammatory disorders led to new therapies specifically targeting TNFα and avoiding many side effects of earlier anti-inflammatory drugs. However, because of the wide spectrum of systems affected by TNFα, drugs targeting TNFα have a potential risk of delaying wound healing, secondary infections, and cancer. Indeed, increased risks of tuberculosis and carcinogenesis have been reported as side effects after anti-TNFα therapy. TNFα regulates many processes (e.g. immune response, cell cycle, and apoptosis) through several signal transduction pathways that convey the TNFα signals to the nucleus. Hypothesizing that specific TNFα-dependent pathways control specific processes and that inhibition of a specific pathway may yield even more precisely targeted therapies, we used oligonucleotide microarrays and panthenolide, an NF-κB-specific inhibitor, to identify the NF-κB-dependent set of the TNFα-regulated genes in human epidermal keratinocytes. Expression of ~40% of all TNFα-regulated genes depends on NF-κB; 17% are regulated early (1–4 h post-treatment), and 23% are regulated late (24–48 h). Cytokines and apoptosis-related and cornification proteins belong to the “early” NF-κB-dependent group, and antigen presentation proteins belong to the “late” group, whereas most cell cycle, RNA-processing, and metabolic enzymes are not NF-κB-dependent. Therefore, inflammation, immunomodulation, apoptosis, and differentiation are on the NF-κB pathway, and cell cycle, metabolism, and RNA processing are not. Most early genes contain consensus NF-κB binding sites in their promoter DNA and are, presumably, directly regulated by NF-κB, except, curiously, the cornification markers. Using siRNA silencing, we identified cFLIP/CFLAR as an essential NF-κB-dependent antiapoptotic gene. The results confirm our hypothesis, suggesting that inhibiting a specific TNFα-dependent signaling pathway may inhibit a specific TNFα-regulated process, leaving others unaffected. This could lead to more specific anti-inflammatory agents that are both more effective and safer.

TNFα is the key initiator of inflammation; however, its deregulation causes many disorders, including toxic shock syndrome, rheumatoid arthritis, inflammatory bowel disease, psoriasis, etc. (1). Therefore, drugs targeting TNFα have been developed for inflammatory diseases, with expectations that these drugs would be more specific and avoid many side effects of the previous drugs, glucocorticoids and nonsteroidal anti-inflammatory drugs (2). However, the wide spectrum of processes affected by TNFα precludes our understanding and predicting fully the effects and side effects of the TNFα-targeted therapies. Overinhibiting the TNFα signals has a potential risk of delaying wound healing, secondary infections, and cancer; indeed, increased risks of tuberculosis and carcinogenesis have been reported as side effects after anti-TNFα therapy (3, 4). We believe that the dissection and comprehensive characterization of the pathways and genes regulated by TNFα will identify even more specific and finely tuned targets, which affect only a particular, defined subset of the TNFα-regulated genes. Inhibiting such could lead to anti-inflammatory treatments that are both more effective and safer. Therefore, we decided to dissect the epidermal responses to TNFα using pathway-specific profiling. Here, we identify the NF-κB-dependent TNFα-regulated genes in epidermal keratinocytes.

The binding of TNFα to its receptor triggers a series of intracellular events, resulting in the activation of transcription factors, including NF-κB, CEBPβ and AP-1 (5, 6). Different cellular processes seem to be differentially regulated by specific signal transduction pathways. For example, NF-κB activation impedes TNFα-induced apoptosis, whereas AP1 activation does not (7), in epidermis; activated NF-κB inhibits, whereas c-Jun N-terminal kinase promotes, TNFα-induced hyperproliferation (8–11), etc. TNFα causes activation of IKKs, kinases that phosphorylate IκB and induce its degradation, which results in activation of NF-κB. Importantly, knock-out of IκKα has a severe cutaneous phenotype with incomplete epidermal differentiation, which suggests a fundamental role for NF-κB in skin (12, 13). A knock-out of IKKβ is defective in signaling from TNFα to NF-κB, whereas deletion mutations of the IKKβ/NEMO account for most cases of incontinentia pigmienti, a dominantly inherited X-linked genodermatosis (14, 15). NF-κB is present in all epidermal layers but is nuclear only in the suprabasal ones, which further implicates NF-κB in epidermal differentiation (8). Constitutive activation of NF-κB in IκB-knock-out mice results in a widespread and lethal dermatitis in the first few days of life (16), and overexpression of NF-κB inhibits keratinocyte proliferation (8). Conversely, overexpres-

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sion of IkB causes hyperplasia and inflammation and leads to squamous cell carcinomas (9, 10). Therefore, we decided to use pathway-specific transcriptional profiling and identify comprehensively the NF-κB-dependent, TNFα-regulated genes in epidermal keratinocytes.

Many environmental stimuli, such as microbial infections, chemicals, and UV light, can activate the NF-κB pathways (17, 18). We have recently characterized the complete spectrum of TNFα-regulated genes in the epidermis, using large DNA microarrays (19). To identify the NF-κB-dependent subset of TNFα-regulated genes, we used parthenolide, which specifically blocks the degradation of IκBα, resulting in immediate and persistent inhibition of activation of NF-κB, without affecting other pathways (20). We chose parthenolide, a pharmacologic agent, over generic inhibitor (e.g. transfection with a dominant inhibitor of the pathway) to avoid the need for long term cultivation of keratinocytes in the absence of functional NF-κB, which could affect additional pathways (21). Using pathway-specific gene expression profiling, we identified the NF-κB-dependent genes in normal human keratinocytes and revealed that NF-κB is responsible for innate immunity, inflammation, cytoskeletal organization, and cellular survival against apoptotic signals.

**EXPERIMENTAL PROCEDURES**

**Human Keratinocyte Cultures and Cytokine Treatment**—Human neonatal foreskin epidermal keratinocytes were first grown in a defined growth medium, KGM, supplemented with 2.5 ng/ml epidermal growth factor and 0.05 mg/ml bovine pituitary extract (keratinocyte-SFM; Invitrogen), as described (22, 23). Third-passage keratinocytes were used at 50–70% confluence, at which point we switched to KGM, the same medium but unsupplemented. Keratinocytes were treated 24 h later with 50 ng/ml human recombinant TNFs (Sigma), 10 μM parthenolide (BIO-MOL), or both. We first optimized the parthenolide concentration and found that the 20 μM level is lethal to the cells.2 At each time point, we harvested the treated and a corresponding, matched, untreated control sample.

**Immunofluorescence and TUNEL Staining**—Keratinocytes were grown on Lab-Tek chamber slides (Nunc) in KGM and then incubated in KGM 24 h before cytokine treatments. The cells were fixed with 70% methanol, rinsed with PBS, and incubated first with primary antibodies (rabbit polyclonal anti-NF-κB; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 1% bovine serum albumin and then with fluorescein isothiocyanate-labeled anti-IgG (Sigma). To detect apoptotic nuclei, we used the DeadEnd Fluorometric TUNEL System (Promega). Nuclei were counterstained with propidium iodide (Vector Laboratories, Inc., Burlingame, CA) in PBS containing 1% bovine serum albumin and then with fluorescein isothiocyanate-microcopy (Zeiss, Aixisphot), and images were captured with a digital photo camera (DKC-5000; Sony). For the quantitative determination of TUNEL-positive cells, for each experimental condition we selected 10 different medium power views at random and counted the total number of cells and the TUNEL-positive cells in each view.

**NF-κB Motif Binding Assay**—We used 10 μg of cell extract in a TransAM NF-κB kit (Active Motif), which can measure the binding of activated NF-κB to its consensus sequence attached to a microwell plate, according to the manufacturer’s instructions.

**Preparation of Labeled mRNA and GeneChip Hybridization**—We isolated total RNA from the cells using RNeasy kits (Qiagen) according to the manufacturer’s instructions. Approximately 5–8 μg of total RNA was transcribed, amplified, and labeled as described (22, 23). Fifteen micrograms of labeled cRNA was hybridized to HGU95Av2 arrays (Affymetrix). Arrays were washed, stained with anti-biotin streptavidin-phycocerythrin labeled antibody, and scanned using the Agilent GeneArray Scanner system (Hewlett-Packard).

**Array Data Analysis**—We used the same data analysis approach for data extraction as before (19, 22), including Microsuite 5.0 (Affymetrix). Differences in expression of transcripts were determined by calculating the fold change. To compare data from multiple arrays, the signal of each probe set was scaled to the same target intensity value. Genes were considered regulated if the expression levels differed more than 2-fold relative to untreated control at any time point. To improve reliability, we checked individually the absolute expression levels and p values among all four time points. To reduce false positive regulated genes, we eliminated the genes that show a “zigzag” pattern of changes during the time course studied. To determine the NF-κB-dependent genes, we compared the values from the TNFα-treated and parthenolide + TNFα-treated cultures. We removed all genes deemed absent in the samples with a higher expression and eliminated duplicates of the genes. The hierarchical clustering was performed using TIGR Multi-Experiment Viewer algorithms available on the World Wide Web at www.tigr.org/software/mtm (24).

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip, primarily based on data from J. M. Ruillard and the Gene Ontology Consortium Data (available on the World Wide Web at cpg.niwh.gov/Genes/GOBrowser and dot.ped.med.umich.edu:2000/ourimage/pub/shared/JMR_pub_affymnot.html). The genes were annotated according to this table.

**Identification of NF-κB Binding Sites in the Promoters of Regulated Genes**—The upstream sequence for each gene was obtained from the Human Genome Browser Gateway (available on the World Wide Web at genome.cse.ucsc.edu/cgi-bin/hgGateway?org=human). We searched for a NF-κB binding motif in the 2 kb upstream from the coding region, using MOTIF (available on the World Wide Web at motif.genome.ad.jp) and mwscan (available on the World Wide Web at www.marcus.br/cbc.jp/hin/hph-tfsearch) with the cut-off threshold of 85%. We classified the identified sites into “perfect match” sequences identical to the κB consensus GGGNNYNYCC and into one nucleotide “mismatch” sequences.

**Real Time PCR**—To confirm the microarray results, quantitative real time PCR was performed using LightCycler-RNA Amplification Kit SYBR Green I (Roche Applied Science). The PCR primers for each gene were designed using software (Roche Applied Science), with a target melting temperature at 60 °C. 300 ng of total RNA was applied for RT-PCR. The primers used for PCR analysis were as follows: EFNA1 (GAGGTGCAGGGTCCTCAC, GCTAGGTGATGTTAGCC), CEP4 (CAGCCGGTCGTGAATTT, GCCTGGTTAACTCAGACT), SX4 (GTTGTAAAAGGTAGATAC, ACAGCGGTTAATGAC), NINJ1 (AAGCCTGCGATCCAG CGACGT, GGGTTGAGTTTCTAGAGG), NINJ2 (ATGCTACCTGCGACGT, GGGTTGAGTTTCTAGAGG), NIPHS (CGCACTGCTTCTCC, CTGAGCTTGCAACGACCAA), NFκB1 (CCCAACACCTCGTGA, CAATGAGGTGAGCGGCAA), KRT15 (CTTGGAGGCAATGGTTA, CTTGGAGGCAATGGTTA), and GAPDH (GGTGGTACACGGAT, CACACGTACAGGC). The relative changes of gene expression were estimated and normalized to GAPDH by using the 2ΔΔCt method (25).

**siRNA**—The 21-mer small interfering RNA duplexes (siRNA) with 3’ overhang dimers of uridine were synthesized using a Silencer siRNA Construction Kit, according to the manufacturer’s instructions (Ambion). The oligonucleotide sequences were as follows: NFκB1 (AA GTAGTACATATAAAGGTGGAATC, TTTCGCTAAAATATGGAATC) and cFLIP (AAGAAGCACTGATACAGTTCTCT, GCGTTGTTAAGGTC GTACTTGAC). The transfec-

**RESULTS**

**Parthenolide Inhibits NF-κB Activation by TNFα in Keratinocytes**—Activation of NF-κB by TNFα leads to its nuclear translocation and subsequent binding to the NF-κB sequence motifs in DNA; parthenolide blocks both processes in the TNFα-treated keratinocytes (Fig. 1, a and b). We expect, therefore, that the NF-κB-dependent genes will not be regulated by TNFα in the parthenolide-pretreated cells. Prevention of apoptosis is, arguably, the best known NF-κB-specific TNFα-regulated process (7). To demonstrate this phenomenon in epidermal keratinocytes, we used the TUNEL assay. The TNFα treatment caused degenerative changes indicative of apoptosis in only a few cells; however, the inhibition of NF-κB with parthenolide caused massive cell death after the TNFα treatment (Fig. 1c). Parthenolide by itself does not affect the cell viability. Quantitative analysis of apoptotic cells confirmed these results (Fig. 1d). Thus, the activation of the NF-κB pathway prevents apoptosis in the TNFα-treated keratinocytes, presumably because of the antiapoptotic genes induced by the NF-κB-dependent pathway.
Identification of the NF-κB-dependent TNFα-regulated Genes—In our previous work, we described 293 genes regulated by TNFα (19); of these, 118 (40%) are NF-κB-dependent. Of the 118, 51 (17%) belong to the early genes (i.e. regulated 1–4 h after the TNFα treatment), whereas 67 (23%) are late, regulated 24 and 48 h post-treatment (Table I). To confirm the array results independently, we performed quantitative real time RT-PCR analysis of seven representative genes, six induced by TNFα and one suppressed; GAPDH, which is not regulated by TNFα, served as the control. To our satisfaction, all genes tested showed equivalent regulation in the two methods, microarrays and RT-PCR (Fig. 2).

Statistical analysis of the functional categories shows a very specific, nonrandom segregation into early, late and NF-κB-independent categories (Table II). The most prominent among the early genes are the secreted proteins (i.e. chemokines, cytokines, and growth factors (Tables IA and II). Thus, signaling to the surrounding cells is one of the most important functions of the NF-κB-dependent responses to TNFα. NF-κB is known to play an essential role in innate immunity and the inflammatory host response in skin (26). All nine of the TNFα-regulated chemokines are NF-κB-dependent, as are 10 of 11 of the genes associated with antigen presentation. These genes tend to have multiple NF-κB motifs, and the chemokine family genes tend to have the NF-κB binding motifs at short distances from the TATA box (Table II), which indicates direct transcriptional regulation by NF-κB. Moreover, the TNFα-induced gene expression of the chemokine gene family is very rapid but transient, also suggesting high dependence on direct transcriptional regulation.

Although the NF-κB pathway has been implicated in the differentiation of epidermal cells, IKK knock-outs having conspicuous epidermal phenotype (8), little is known about the NF-κB-associated regulation of the epidermal differentiation genes. Curiously, although the induction of cornified envelope markers is an early NF-κB-dependent event (Table IA), we find that these genes lack the perfect NF-κB consensus motifs (Table II), which suggests a different regulatory mechanism of transcriptional regulation by NF-κB.

Two secreted metalloproteases, MMP9 and MMP10 (gelatinase B and stomelysin-2), were also in the early NF-κB-dependent group, as were the transcription factors TNFAIP3, SOX4, and IRF1; these may be responsible, in part, for the indirect NF-κB-dependent late transcriptional effects.

The late NF-κB-dependent genes comprise very different functional categories from the early ones (Table IA). Prominent among these are the antigen presentation-related proteins, components of the complement, CD58, and HLA markers. This implicates the NF-κB pathway in the immunomodulatory effects of TNFα. Extracellular matrix proteins collagen-XVI and chondroitin sulfate proteoglycan 4, which play roles in the attachment of keratinocytes to the basement membrane and in the cadherin-mediated adhesion (27, 28), belong to the late genes, as does MMP13 (collagenase 3). Therefore, remodeling...
TABLE I
List of NF-κB-dependent TNFα-regulated genes

The four leftmost columns show the comparison of control, untreated, and TNFα-treated cultures at 1, 4, 24, and 48 h post-treatment; the next four columns show the comparison of TNFα-treated cultures with and without pretreatment with parthenolide. The red numbers show-fold induction by TNFα, whereas the green ones show-fold suppression of the TNFα-induced levels by parthenolide. Unigene accession numbers are provided for easy reference. A, genes regulated in the early time points, 1 and 4 h after the addition of TNFα. B, genes regulated in the late time points, 24 and 48 h after treatment. The double line separates the TNFα-induced genes from the suppressed ones. The presence of the perfect NF-κB motif in the 2-kb upstream sequence is marked with PM, a single base mismatch with SBM, and its absence with “none.” The column showing the number of NF-κB motifs in 2 kb upstream from each gene is marked with a number symbol, and the position of a motif in the upstream sequence is given in the last column.
of the extracellular matrix and interaction with the neighboring cells seems to be an important component of the late NF-κB-dependent responses to TNFα. Confirming this, we find proteolysis inhibitors, adhesion and junctional proteins in this group as well.

Interestingly, the late NF-κB-dependent group also contains MADH1 and MADHIP, which form a transcription factor complex and provide a nexus between the TNFα/NF-κB and the transforming growth factor β/SMAD signaling pathways.

Several TNFα-suppressed genes were identified in the NF-κB-dependent group. However, using microarray approaches, we cannot determine with certainty the timing of their regulation, early versus late, because this depends on the mRNA degradation rates and other factors.

Importantly, the TNFα-regulated cell cycle proteins are statistically overrepresented in the non-NF-κB-dependent category. TNFα inhibits keratinocyte proliferation and arrests the cells in G2 phase (19). The arrest seems to be wholly dependent on TNFα-regulated pathways other than NF-κB (Table II). Cell surface receptors and metabolic enzyme also belong to the TNFα-regulated genes that are independent from the NF-κB.

Whereas most early genes are directly regulated by NF-κB, the late genes, in turn, may depend on the transcription factors regulated by the early genes. If so, we expect the promoters of the early genes to have more NF-κB binding sites than those of the late genes. Therefore, we used promoter analysis software to determine the presence of the NF-κB binding sequences in the 200-bp segments of the 2-kb sequences upstream from the coding region of each NF-κB-dependent gene (Table III). We calculated the average number of NF-κB sites per 200-bp interval and the S.D. of these numbers. The results indicate that only the promoter-proximal 200 bp of the early regulated genes have a statistically significant, greater than 2-S.D., increase in the number of the NF-κB motifs.

Correlation of the NF-κB sites in the promoters with the functional analysis of the regulated genes indicates that the distribution of the NF-κB sites is not random (Table II). Chemokines and cytokines as a group have the highest accumulation of the NF-κB motifs, especially in the first 200 bp of their upstream sequences. Integrins and apoptosis-related proteins also have an overabundance of NF-κB sites. In contrast, keratins, cell cycle, and proteolysis genes generally lack NF-κB motifs. Curiously, although the induction of cornified envelope markers is NF-κB-dependent, we found that these genes lack the perfect NF-κB consensus motifs but contain single base mismatches; this suggests a different regulatory mechanism of their transcriptional regulation by NF-κB (Table II).

Identification of cFLIP as an Essential NF-κB-dependent Antiapoptotic Gene—Prevention of apoptosis is, arguably, the best known NF-κB-specific TNFα-regulated process (7). In other systems, NF-κB was shown to regulate several genes encoding proteins with antiapoptotic properties, such as cFLIP, A20, cIAP, TRAF1, and Bcl-XL (29–31). Perhaps unexpectedly, only one such antiapoptotic gene was found in the early NF-κB-dependent category, cFLIP (31). A caveat in such conclusions is the fact that some genes have multiple, interacting functions. For example, TNFAIP3 and SOX4 are transcription factors with imputed antiapoptotic roles (32, 33).

Because massive apoptosis can be seen in TNFα-treated cells with disrupted NF-κB activity, we were particularly interested in the NF-κB-dependent antiapoptotic genes. We focused on cFLIP, because the protein product of this gene is an inactive caspase that dimerizes with and thereby inactivates caspase-8 as well as binds to TNFα receptor-associated proteins TRAF-1, TRAF-2, and FADD, thereby blocking the apoptotic signals from the receptor (34). We argued that if cFLIP is an essential component of the NF-κB pathway that rescues the keratino-
As expected, the NF-κB-targeted mRNAs was monitored using Northern blots (Fig. 3). The effective reduction of NF-κB in the control, NF-κB-asterisk, and the even distribution of the NF-κB in the promoter-proximal 200 bp of the early genes, marked with the asterisk, and the even distribution of the NF-κB motifs in the absence of the cytokine, the siRNAs have no visible effects.

Importantly, the cFLIP-targeted siRNA also induced apoptosis in the TNFα-treated keratinocytes. The TUNEL assay confirmed the cell death by apoptosis.

| Functional class                  | Total | Early | Late | E + L | Neither | NFκB sites | No NFκB sites |
|-----------------------------------|-------|-------|------|-------|---------|------------|---------------|
| Secreted, chemokine               | 9     | 9     | 0    | 0     | 9       | 10         | 10            |
| Secreted, cytokine                | 16    | 8     | 50   | 2     | 13      | 10         | 63            |
| Receptor                          | 5     | 0     | 0    | 20    | 1       | 20         | 4             |
| Antigen presentation              | 11    | 2     | 18   | 8     | 73      | 10         | 91            |
| Inflammation                      | 20    | 4     | 20   | 5     | 9       | 45         | 11            |
| ECM                               | 7     | 0     | 0    | 3     | 43      | 33         | 4             |
| Integrin                          | 5     | 0     | 0    | 2     | 40      | 20         | 40            |
| Adhesion, junctional              | 6     | 1     | 17   | 3     | 50      | 40         | 67            |
| Actin-related                     | 8     | 3     | 38   | 1     | 13      | 50         | 40            |
| Cytoskeleton                      | 7     | 5     | 71   | 0     | 5       | 71         | 29            |
| Keratin                           | 7     | 3     | 43   | 1     | 14      | 43         | 57            |
| Cell cycle                        | 8     | 0     | 0    | 1     | 13      | 13         | 13            |
| Apoptosis-related                 | 8     | 3     | 38   | 1     | 13      | 50         | 40            |
| Tumor suppressor                  | 6     | 0     | 0    | 3     | 50      | 50         | 50            |
| Transcription                     | 28    | 4     | 14   | 8     | 21      | 10         | 38            |
| Signal transduction               | 26    | 2     | 8    | 10    | 38      | 12         | 44            |
| Proteasome-related                | 11    | 1     | 9    | 4     | 36      | 55         | 65            |
| Mitochondrial                     | 5     | 1     | 20   | 2     | 40      | 3       | 60            |
| Metabolism                        | 32    | 1     | 3    | 7     | 22      | 8         | 25            |

Average ± SD: 22+/−27 27+/−19 50+/−22 50+/−22 37+/−22 39+/−20 67+/−19 33+/−19

**Table III**

Distribution of NF-κB motifs in the promoters of regulated genes

Distribution of the motifs upstream from the genes is plotted for 200-bp intervals. Note the statistically significant cluster of NF-κB motifs in the promoters of regulated genes. The total number of genes in each category is given; only categories with five or more members are represented, smaller ones being statistically unreliable. We determined the percentage of genes in each category that are early, late, or neither, as well as those containing a perfect match PM NF-κB sites, a single base mismatch SBM, or none. Having calculated the averages and S.D. values of these percentages (bottom row), we identify the functional categories that are more than one S.D. different from the average. Those overrepresented are boxed; those underrepresented are underlined. The last column lists the average number of NF-κB motifs per gene in each functional category.

cytoskeleton processes after the TNFα stimulation, suggesting cytoskeletal reorganization. Therefore, although both the antiapoptotic and the cytokine-dependent effects of TNFα are mediated by NF-κB, only the antiapoptotic ones depend on cFLIP (Fig. 4).

![Fig. 3. siRNAs targeting NF-κB or cFLIP induce apoptosis in the TNFα-treated keratinocytes.](image)

a. Northern blots demonstrate that the siRNAs targeting NF-κB or cFLIP cause reduced levels of corresponding mRNAs in keratinocyte cultures 48 h after the treatment. GAPDH-targeted siRNA has no effect on the cell morphology. NF-κB-targeted siRNA and parthenolide greatly affect the morphology of the treated cells. The morphology of the surviving cFLIP-targeted siRNA cells does not change drastically. Note that TNFα is absolutely necessary for all of the changes observed.

Importantly, although blocking cFLIP causes apoptosis of TNFα-treated cells, the blocking has no major effect on the morphology of the remaining, viable cells (Fig. 3c). This confirms the specific antiapoptotic property of cFLIP. In contrast, the parthenolide-treated and NF-κB-siRNA-treated cells demonstrate extensive morphological changes in surviving cells (Fig. 3c). The NF-κB-targeted keratinocytes show elongated processes after the TNFα stimulation, suggesting cytoskeletal reorganization. Therefore, although both the antiapoptotic and the cytokine-dependent effects of TNFα are mediated by NF-κB, only the antiapoptotic ones depend on cFLIP (Fig. 4).
with FADD and procaspase-8, interfering with the activation of procaspase-8 (37), as the essential NF-κB-dependent TNFα-induced antiapoptotic gene and confirmed its significant role in keratinocytes by using an siRNA silencing approach. We note that additional genes in Table I may have antiapoptotic properties as well. Overexpression of antiapoptotic genes is thought to interfere with death of cancer cells, resulting in the resistance against radiation therapy or chemotherapy (38). If cFLIP plays such a role in skin cancer, then drugs targeting this protein could make effective supplements for cancer therapy.

Third, inhibition of NF-κB resulted in disturbance of cytoskeletal organization and detachment of cell-to-cell contacts. We identified several actin regulators, integrins, and adhesion molecules as NF-κB-dependent, TNFα-regulated genes. For example, CEPA is a member of the CDC42-binding protein family and regulates the organization of the actin cytoskeleton through Rho-GTPases, leading to cell shape changes (39); ephrin A1 is a secreted ligand with a significant role in actin reorganization through its receptor and Rho proteins (40); CD47 initiates G-protein signaling and modulates cell adhesion and migration through its association with integrins (41); and HEF1, a focal adhesion-associated docking protein, has a unique function in response to cellular detachment (42). Taken together, these results suggest that NF-κB has a significant role in coordination of cellular processes associated with cell shape, adhesion, motility, and detachment.

Fourth, although the NF-κB pathway has been implicated in the differentiation of epidermal cells (IKK knock-outs having conspicuous epidermal phenotype (8)), little is known about the NF-κB-associated regulation of the epidermal differentiation genes. Interestingly, although a cluster of cornification markers falls in the category of early regulated NF-κB-dependent genes (Table I), we find that these genes do not have the perfect NF-κB motifs in their promoter regions (Table II). This agrees very well with the completely independently derived findings that NF-κB indirectly regulates keratin gene expression (i.e. without binding to the keratin gene promoter DNAs) (43). In the case of keratin K6, NF-κB and C/EBPβ form a complex that binds to the C/EBPβ consensus sequence motif in the promoter through the C/EBPβ DNA binding domain (43). This suggests indirect transcriptional regulation of epidermal markers by NF-κB-containing complexes of transcription factors. Perhaps significantly, the cornification marker genes do contain single base mismatch motifs, which may play a role in forming or stabilizing the NF-κB-containing complexes.

Finally, in contrast to the four categories described above, it appears that the TNFα-regulated cell cycle proteins do not require the activation of the NF-κB pathway. TNFα inhibits keratinocyte proliferation and arrests the cells in the G1 phase of the cycle (19). This process, unregulated by NF-κB, seems to be wholly dependent on the other TNFα-regulated pathways (Fig. 4). This is in good agreement with studies that identified the c-Jun N-terminal kinase pathway, rather than the NF-κB pathway, as the one critical for the proliferation effects of TNFα (44).

We note that the regulation of transcription of a given gene may depend on multiple pathways (e.g. both the NF-κB and the c-Jun N-terminal kinase pathway). Genes in this category would be identified by our approach as NF-κB-dependent, and we do not imply that additional pathways play no role in their regulation. However, our results indicate that more specific approaches to treating inflammatory disorders are possible and point to the pathway-specific transcriptional profiling as the right method to identify the best therapeutic targets.

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