

Fas Ligand-induced Proinflammatory Transcriptional Responses in Reconstructed Human Epidermis

RECRUITMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR AND ACTIVATION OF MAP KINASES^{*[5]}

Received for publication, July 17, 2007, and in revised form, September 14, 2007 Published, JBC Papers in Press, October 31, 2007, DOI 10.1074/jbc.M705852200

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Fas ligand (FasL) exerts potent proapoptotic and proinflammatory actions on epidermal keratinocytes and has been implicated in the pathogenesis of eczema, toxic epidermal necrolysis, and drug-induced skin eruptions. We used reconstructed human epidermis to investigate the mechanisms of FasL-induced inflammatory responses and their relationships with FasL-triggered caspase activity. Caspase activity was a potent antagonist of the pro-inflammatory gene expression triggered by FasL prior to the onset of cell death. Furthermore, we found that FasL-stimulated autocrine production of epidermal growth factor receptor (EGFR) ligands, and the subsequent activation of EGFR and ERK1 and ERK2 mitogen-activated protein kinases, were obligatory extracellular steps for the FasL-induced expression of a subset of inflammatory mediators, including CXCL8/interleukin (IL)-8, ICAM-1, IL-1 α , IL-1 β , CCL20/MIP-3 α , and thymic stromal lymphopoietin. These results expand the known physiological role of EGFR and its ligands from promoting keratinocyte mitogenesis and survival to mediating FasL-induced epidermal inflammation.

FasL,³ a member of the tumor necrosis factor family of cytokines (reviewed in Ref. 1), signals apoptotic cell death by

engagement of its cognate receptor, Fas (2, 3). FasL/Fas triggers apoptosis by activating the caspase family of proteases (reviewed in Refs. 4 and 5). FasL plays an important role in the effector function of cytotoxic T lymphocytes and also regulates their homeostasis (3). Deficiencies in either FasL or Fas are associated with autoimmune lymphoproliferative syndrome (a hereditary condition characterized by the accumulation of atypical lymphocytes and autoimmune manifestations) and systemic lupus erythematosus (6, 7). FasL expression enables the immunoprivileged state of the cornea and the testis (8, 9), thus further underscoring the important role of this cytokine in restricting autoimmune inflammation under normal homeostatic conditions.

On the other hand, several laboratories, including ours, have revealed the importance of FasL/Fas as a positive regulator of pathological inflammation as well, particularly in inflammatory diseases of the skin. Keratinocytes in the healthy human express Fas, but not FasL (10). However, abnormal expression of lytically active FasL was found in keratinocytes of patients with toxic epidermal necrolysis, suggesting that a suicidal keratinocyte reaction contributes to the pathogenesis of toxic epidermal necrolysis (10). FasL/Fas signaling was implicated in acute cutaneous graft *versus* host disease (11). More recently, FasL was found to be involved in the pathogenesis of eczematous dermatitides or eczemas (such as atopic dermatitis and allergic contact dermatitis) (12–15). A common histopathological feature of eczemas is the formation of exudative epidermal vesicles that are disruptive to the normal barrier function of the skin. Although vesicle formation in eczemas has been largely attributed to rupturing of keratinocyte attachments as a result of intercellular edema (spongiosis) (Ref. 12 and references therein), recent findings suggest that keratinocyte death plays a major role in vesicle formation (12, 13). This keratinocyte death appears to be apoptotic and to be mediated by FasL, delivered to the epidermis by infiltrating T lymphocytes and acting on Fas expressed on the surface of keratinocytes (13). Recently, expression of both FasL and Fas by epidermal keratinocytes was proposed to characterize fixed drug eruptions (16) and drug-induced maculopapular rashes (17). Finally, the Fas/FasL apoptotic pathway was proposed to contribute to the *Leishmania major*-induced cutaneous ulceration (18).

These findings clearly demonstrated the important role of FasL in epidermal destruction in inflammatory skin diseases.

^{*} This work was supported by National Institutes of Health Grants AI059335 and DK066439 (to B. E. M.) and CA-93718 (to M. S. I.) and grants from the Swiss National Science Foundation (to P. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1 and supplemental Tables S1–S6.

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³ The abbreviations used are: FasL, Fas ligand; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; RHE, reconstructed human epidermis; MAPK, mitogen-activated protein kinase; IL, interleukin; TSLP, thymic stromal lymphopoietin; ICAM-1, intercellular adhesion molecule 1; HEKn, human epidermal keratinocytes (neonatal); siRNA, small interfering RNA; HKGS, human keratinocyte growth supplement; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; RT, reverse transcription; qRT-PCR, quantitative real time RT-PCR; CXCL14, C-X-C motif ligand 14; AREG, amphiregulin; EREG, epiregulin; HB, heparin-binding; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase.

However, whether FasL could directly trigger the inflammatory process was not known. We demonstrated recently that FasL elicits a pro-inflammatory reaction in human HaCaT keratinocytes and reconstructed human epidermis (RHE) by triggering the expression of stress-responsive transcription factors, inflammatory cytokines, chemokines, and the adhesion molecule ICAM-1 (19). We demonstrated that oligomerization of Fas was required both for apoptosis and for the inflammatory gene expression and that caspase activity was essential for apoptosis but dispensable for the inflammatory gene expression (19). These results suggested that FasL was a pro-inflammatory cytokine in eczemas. FasL stimulated the activity of the NF- κ B transcription factor in HaCaT cells, and this activity was required for the FasL-induced activation of inflammatory genes (19). The requirement for NF- κ B suggested that the pro-inflammatory actions of FasL were dependent on transcriptional gene activation.

In this work, we employed RHE to elucidate three novel aspects of FasL-induced inflammation in the epidermis, namely: (i) the importance of the proper organotypic stratification, (ii) the inhibitory role of caspases, and (iii) the unexpected involvement of the EGFR-ERK axis in the transcriptional inflammatory responses to FasL in the epidermis.

EXPERIMENTAL PROCEDURES

RHE: Primary Human Epidermal Keratinocytes (Neonatal) (HEKn) and Their Immortalized Derivatives—HEKn-E6/E7 were established and propagated as described (20, 21). For RHE, we employed the method of Poumay *et al.* (22), with modifications as described in Ref. 19. RHEs were fixed in freshly made 4% *p*-formaldehyde, embedded in paraffin, and after sectioning were processed for immunohistochemistry following the specific protocols recommended by the respective manufacturers for each antibody.

siRNA-mediated Knockdown of ERK1 and ERK2 in RHE—The siRNA oligonucleotides were synthesized by the Department of Molecular Microbiology and Immunology Research Core Facility (Oregon Health & Science University, Portland, OR). Equimolar amounts of the complementary oligonucleotides were annealed in annealing buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl) by denaturing at 90 °C for 2 min followed by incubation at 37 °C for 1 h. The siRNA sequences were as follows: nonspecific control, sense 5'-CGAGUAGGCUUCGUG-ACUUDtT-3', antisense 5'-AAGUCACGAAGCCUACUC-GdTdT-3'; ERK1, sense 5'-GCCAUGAGAGAUGUCUACAdTdT-3', antisense 5'-UGUAGACAUCUCUCAUGGCdTdT-3', derived from Ref. 23; and ERK2, sense 5'-GCUAGGAACU-AUUUGCUUUDtT-3', antisense 5'-AAAGCAAUAGUCCUAGCdTdT-3'.

RNA interference was performed as outlined schematically in Fig. 8A and as described below. On Day (−5), HEKn-E6/E7 were cultured in 10 ml of EpiLife® medium supplemented with human keratinocyte growth supplement (HKGS) as per the manufacturer's instructions (Cascade Biologics, Portland, OR) in 100-mm tissue culture dishes (Sarstedt, Newton, NC) to a final confluency of ~40% after ~18 h of incubation at 37 °C/5%CO₂. On Day (−4), the medium was replaced with 8 ml of EpiLife®-HKGS. Lipofectamine®2000 reagent (Invitrogen) was

diluted to 20 μ g/ml in 1 ml of OPTIMEM®-reduced serum medium (Invitrogen) and incubated for 5 min at room temperature. Annealed siRNA was diluted to 500 nM in 1 ml OPTIMEM® medium and then mixed with 1 ml of dilute Lipofectamine™2000 for 20 min at room temperature. Two ml of siRNA/reagent mixture was added to the 8 ml of EpiLife®-HKGS medium/plate. The cells were transfected with siRNA (50 nM final concentration of each annealed siRNA) for 48 h. On Day (−2), transfected cells were trypsinized and counted. The cells were diluted in EpiLife®-HKGS medium to a concentration of 1×10^6 cells/ml, and 0.5 ml was plated per Millicell-PCF 0.4- μ m culture plate insert (Millipore, Billerica, MA). Inserts were placed in 6-well plates containing 2.5 ml of EpiLife®-HKGS medium supplemented with 1.5 mM CaCl₂, 10 ng/ml human recombinant keratinocyte growth factor (PeproTech, Inc., Rocky Hill, NJ), and 50 μ g/ml ascorbic acid. On Day 0, the RHEs were switched to differentiation as described previously in Ref. 19 and used typically at Day 4 as depicted in Fig. 8.

Chemicals, Fc:FasL, Blocking Reagents, Growth Factors, and Cytokines—All of the commonly used chemicals and EGF were from Sigma. Caspase inhibitors (z-VAD-fmk and z-IETD-fmk), UO126, SB203580, SP600125, and AG1478 were from Calbiochem. Fc:FasL has been described previously (24). The LA-1 antibody was from R & D Systems (Minneapolis, MN).

RNA Isolation, Affymetrix Microarray, and Quantitative Real Time RT-PCR (qRT-PCR)—These techniques were performed as previously described in Ref. 19. The following additional primers were employed here: CCL20/MIP-3 α mature mRNA forward, 5'-CGAATCAGAAGCAGCAAGCA-3'; CCL20/MIP-3 α mature mRNA reverse, 5'-AGCATTGATGTCACAGCCTTCA-3'; CCL20/MIP-3 α primary transcript forward, 5'-CCACCTCTGCGGCGAATCAGAAG-3'; CCL20/MIP-3 α primary transcript reverse, 5'-CTTAGGGACCCCCAGTTGAGCTC-3'; ICAM-1 primary transcript forward, 5'-CATCTACAGTAAGAAGGGGCAGGG-3'; ICAM-1 primary transcript reverse, 5'-TCGTGAGAATCACGTTGGGC-3'; TSLP mature mRNA forward, 5'-CCGTCTCTGTAGCAATCGGC-3'; and TSLP mature mRNA reverse, 5'-GGCAGCCTTAGTTTTCATGGC-3'. For the detection of primary transcripts by qRT-PCR, 3 μ g of total RNA were reverse-transcribed in the presence of SuperScript III and random hexamer primers (Invitrogen).

RESULTS

Identification of FasL-regulated Genes in RHE—To identify genes activated by FasL in an epidermotypic context, we performed Affymetrix microarray analyses on RNA extracted from RHE 4 h after exposure to FasL. The analyses were performed on HG-U133 Plus 2.0 GeneChip arrays, which interrogate over 47,000 human transcripts. Subsequent analysis identified 571 genes that were up-regulated at least 2-fold and 186 genes that were up-regulated at least 3-fold after FasL (available online at: [www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_\(2007\).xls](http://www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_(2007).xls)). The data were analyzed with both EASE (25) and L2L (26) software, which automate the process of biological theme determination by analyzing the overrepresentation of genes that belong to categories that are functionally and structurally defined. Supplemental Tables S1 and S2 display the L2L-generated hierarchical analyses of the genes whose mRNA

abundance was increased at least 3-fold after FasL. Overrepresented genes in the Biological Process categories were associated with wound, chemotaxis, inflammatory, immune, and stress responses (supplemental Table S1). The Molecular Function categories associated with these genes were identified as cytokine, chemokine, and growth factor activities (supplemental Table S2). EASE analysis produced similar results (not shown). A selection of 37 FasL-induced genes in the organotypic model is shown in supplemental Table S3.

We also analyzed the genes that were down-regulated in response to FasL (supplemental Tables S4–S6). For this purpose, we first excluded from the Affymetrix data all genes that were categorized as “absent” or “marginally present” in the control (untreated) RHE group. We excluded, additionally, all genes from the control group that displayed basal levels of expression below 100 arbitrary Affymetrix units. Of the remaining 985 genes, we selected those that were down-regulated at least 2-fold by the FasL treatment (a total of 442 Affymetrix probe IDs; *i.e.* 45%). Of these, 287 were annotated in the public databases Entrez Gene (www.ncbi.nlm.nih.gov/sites/entrez?db=gene) or Gene Cards (www.genecards.org). The L2L overrepresentation analysis of these genes by biological process and molecular function is shown in supplemental Tables S4 and S5, respectively. In the biological process categories, FasL-repressed genes appear to be overrepresented in the categories related to metabolic processes (including DNA, RNA, and protein metabolism). By molecular function, genes belonging to the categories peptidase, helicase, and kinase activities were down-regulated by FasL. Supplemental Table S6 contains the 68 genes that were down-regulated at least 2-fold by FasL and whose basal expression in the control RHE was the highest (*i.e.* at least 1000 arbitrary Affymetrix units). The top ranking FasL-repressed gene was the chemokine C-X-C motif ligand 14 (CXCL14), whose expression was down-regulated ~26-fold in the presence of FasL (see “Discussion”). Interestingly, the ability of FasL to trigger the repression of CXCL14 was significantly attenuated in the presence of the pan-caspase inhibitor z-VAD-fmk.

qRT-PCR was employed to investigate the temporal appearance of a variety of FasL-up-regulated gene transcripts that belonged to the molecular function categories identified as overrepresented in our Affymetrix array analyses. The mRNAs encoding transcription factors c-Fos, c-Jun, Egr1, and ATF3 displayed maximum increases in abundance 4 h after FasL treatment; their levels declined thereafter (Fig. 1A). The mRNAs encoding proinflammatory cytokines and chemokines and the adhesion molecule ICAM-1 were activated maximally between 2 and 6 h post FasL (specifically, 2 h for tumor necrosis factor- α , 4 h for ICAM-1, IL-1 α , CCL20/MIP-3 α , CXCL3/GRO γ , CXCL1/GRO α , and CXCL8/IL-8, and 6 h for TSLP), with the exception of IL-1 β (maximum at 12 h) (Figs. 1B and 2A). Previous studies from our laboratory have demonstrated that exposure of monolayers of keratinocytes to FasL resulted in the activation of the EGFR via the secretion of EGFR-binding ligands (21). Whereas the mRNA encoding amphiregulin (AREG) demonstrated a single transient peak of accumulation (at 4 h), mRNAs encoding epiregulin (EREG) and heparin-binding EGF (HB-EGF) displayed a prominent transient peak at 4 h

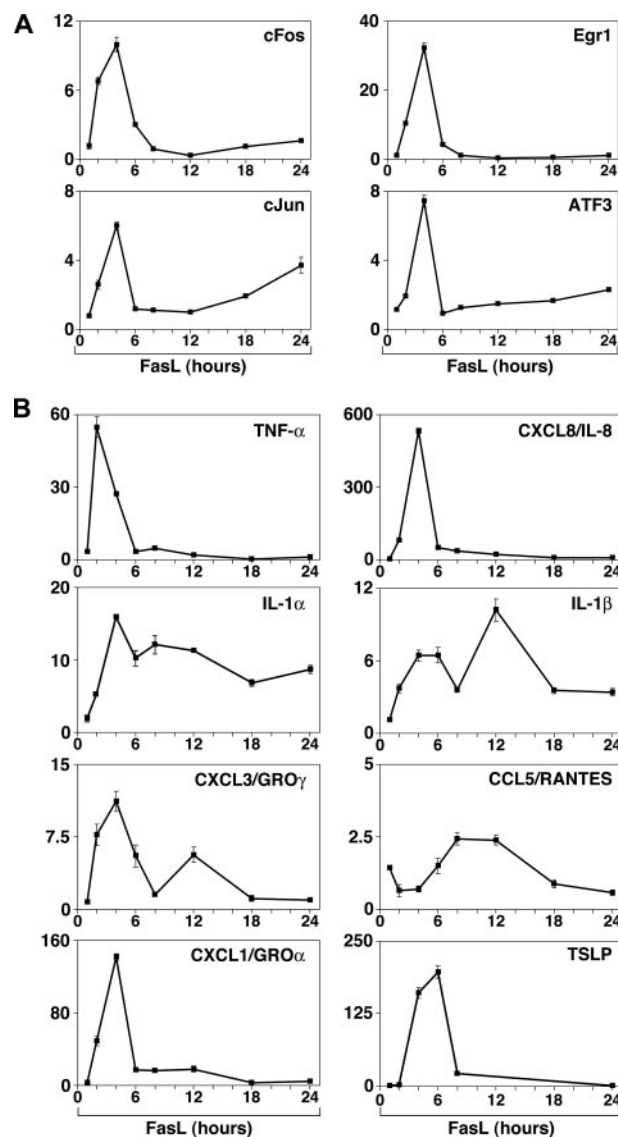


FIGURE 1. Time course of FasL-induced accumulation of mRNAs encoding transcription factors and inflammatory mediators in RHE. RHEs (14 days after the differentiation switch) were treated with FasL (250 ng/ml) for the indicated times as previously described (19) and then interrogated, using qRT-PCR, for the levels of specific transcripts at 1, 2, 4, 6, 12, 18, and 24 h of exposure to FasL. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicate qRT-PCRs. A, mRNAs encoding transcription factors. B, mRNAs encoding inflammatory mediators.

and a delayed second wave of activation that occurred between 18 and 24 h post FasL (Fig. 2B). In contrast to mRNA encoding the former EGFR-binding ligands, the mRNA for transforming growth factor- α was not induced more than 2-fold within the first 12 h after FasL stimulation but became elevated at 18 and 24 h post FasL.

Epidermal Organotypic Context Is Essential for the Inflammatory Responses of Human Keratinocytes to FasL—Surprisingly, treatment with FasL of either primary HEKn or immortalized (HEKn-E6/E7) keratinocytes in two-dimensional tissue culture conditions resulted in the strong activation of the immediate-early genes encoding transcription factors (c-Jun, c-Fos, ATF3, and Egr1), as in Fig. 1A, but failed completely to trigger the activation of genes encoding inflammatory media-

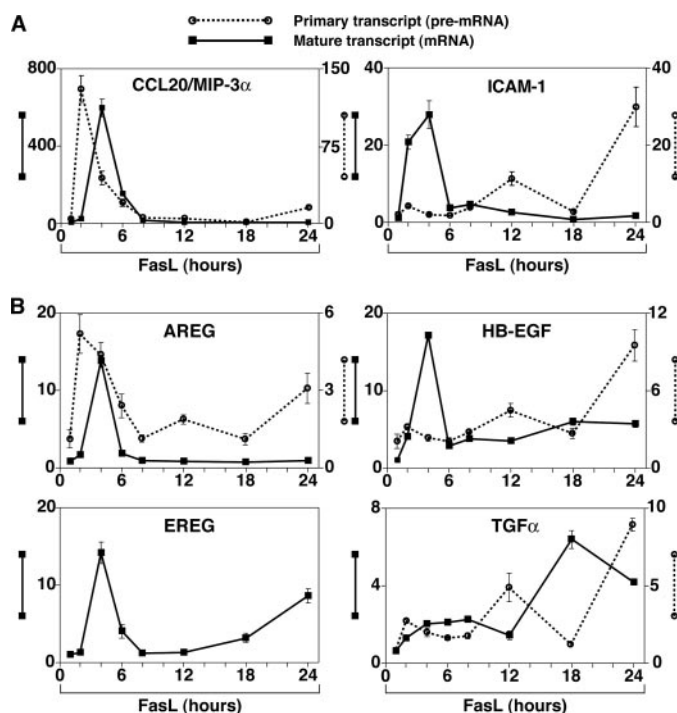


FIGURE 2. Time course of FasL-induced accumulation of primary transcripts versus their corresponding mature mRNAs. Treatment and analyses performed as for Fig. 1. Primary transcripts (dotted lines, y axes on the right-hand side of the graphs) and mature mRNAs (filled lines, y axes on the left-hand side of the graphs) encoding CCL20/MIP-3 α and ICAM-1 (A) or EGFR-binding ligands (B). EREG appeared not to have introns and therefore was not suitable for primary transcript analyses.

tors (such as cytokines and chemokines, as well as ICAM-1) or EGFR-binding ligands (not shown). These experiments have been repeated a number of times with identical outcomes. This lack of proinflammatory gene response to FasL in cultured keratinocytes was not affected by variations of Ca^{2+} levels or choice of growth medium (e.g. Dulbecco's modified Eagle's medium, with or without fetal calf serum) (not shown), suggesting that the proper epidermotypic context (and not growth conditions) was absolutely essential for the proinflammatory response to FasL.

Transcriptional Regulation of FasL-induced mRNAs—To investigate the FasL-induced accumulation of mRNAs encoding inflammatory mediators at a level mechanistically and temporally closer to true transcriptional activation of the corresponding genes, we determined the accumulation of selected primary transcripts following FasL administration. To this end we employed a random hexamer-primed method of cDNA synthesis and exon-intron border-specific primers for qRT-PCR. As shown in Fig. 2, some of the primary transcripts tested displayed kinetics of increase that preceded those of the corresponding mature mRNAs (best exemplified by CCL20/MIP-3 α , Fig. 2A; and AREG, Fig. 2B, RNAs). These examples suggest that FasL-induced elevation of the mRNAs encoding inflammatory mediators and EGFR-binding ligands in RHEs involves true transcriptional activation of the corresponding genes. These analyses further permitted us to identify increased levels of primary FasL-induced transcripts at later times (24 h) after the addition of FasL (e.g. ICAM-1, Fig. 2A; and AREG and HB-EGF, Fig. 2B). Interestingly, this delayed accumulation of

primary transcripts was not mirrored by a corresponding increase in the levels of their mature mRNAs (Fig. 2), suggesting a level of regulation of these genes that escapes detection when RT-PCR is employed to detect levels of mature mRNA transcripts.

Caspase Activity Is a Potent Antagonist of the Pro-inflammatory Action of FasL in RHE—Because FasL is known to activate proapoptotic pathways in keratinocytes and other cell types, we set out to investigate the potential role of FasL-activated caspases in regulating the expression of proinflammatory mRNAs following exposure of RHEs to FasL. First, we asked whether caspases were activated within the time frame of onset and optimal expression of FasL-induced genes. RHEs treated with FasL displayed processing of procaspase 8 into the p43/p41 intermediate fragments and the p18 mature large subunit as early as 2 h (Fig. 3A). The processing of caspase 8 was inhibited by the caspase 8-specific inhibitor z-IETD-fmk (27, 28) (Fig. 3A). The activation of caspase 8 was accompanied by a small, but detectable, processing of the effector procaspase 3, first to the p20/p19 intermediates and subsequently to the p17 mature large subunit of the active caspase 3 (Fig. 3A). Apoptotic nuclei were not detectable at 4 h after FasL but became evident by 24 h in the basal layers of FasL-treated RHEs (as shown by hematoxylin and eosin staining; Fig. 3B). Basal layers of RHEs at 24 h (but not 4 h) post FasL also revealed positive immunoreactivity for active caspase 3 (Fig. 3B). These data demonstrate that FasL engaged the apoptotic cascade as early as 2 h post addition but that the morphological manifestation of apoptosis was not yet evident within the time frame of onset and optimal expression of FasL-induced genes (2–4 h).

We then employed either z-IETD-fmk or the pan-caspase blocker z-VAD-fmk to investigate whether the engagement of apoptotic cascades by FasL would modify the accumulation of proinflammatory transcripts in response to FasL. Fig. 4 demonstrates that the application of either z-IETD-fmk or z-VAD-fmk resulted in a dramatic increase in expression of a variety of transcripts that encode cytokines and chemokines. For example, in the absence of caspase inhibitors, FasL triggered a 475-fold (± 58) increase in the expression of TSLP mRNA at 4 h post addition. However, in the presence of either z-IETD-fmk or z-VAD-fmk, the FasL increased the abundance of TSLP mRNA to 20,000- and 17,000-fold, respectively (Fig. 4A). Similarly, in the absence of caspase inhibitors, FasL triggered a 11-fold (± 2.6) increase in the expression of IL-6 mRNA at 4 h post addition. In the presence of either z-IETD-fmk or z-VAD-fmk, the IL-6 mRNA was increased by FasL to 1,721-fold (± 130) and 622-fold (± 21), respectively (Fig. 4A). In addition to these quantitative differences, inhibition of caspases affected the responsiveness to FasL qualitatively as well. For instance, FasL alone failed to induce the expression of CCL5/RANTES mRNA, but FasL in the presence of either z-IETD-fmk or z-VAD-fmk caused a 15–25-fold increase in expression of the CCL5/RANTES mRNA (Fig. 4A and Ref. 19). The potentiating effect of caspase inhibitors on FasL-induced gene expression patterns appeared biased in favor of inflammatory mediators. For example, in the presence of z-IETD-fmk, FasL effected a >40-fold increase in TSLP mRNA and >160-fold increase in IL-6 mRNA (Fig. 4A). By comparison, the addition of FasL

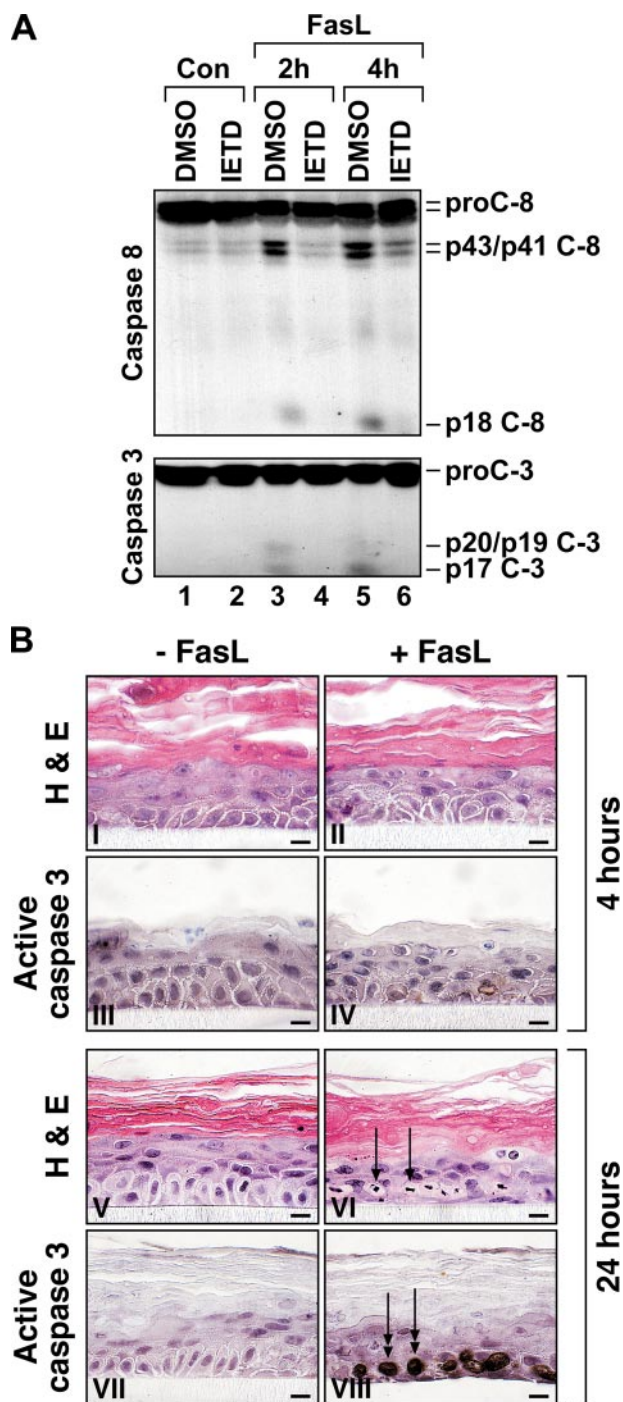


FIGURE 3. Activation of caspases by FasL in RHE. A, RHEs were treated with FasL as in Fig. 1 in the presence of either Me₂SO (vehicle) or z-IETD-fmk (IETD, 50 μ M), both administered 30 min prior to FasL. Activation of caspases 8 and 3 was determined in immunoblot analyses. B, hematoxylin and eosin (H & E) staining or immunohistochemical detection of active caspase 3 at either 4 or 24 h after treatment with FasL. The experiments were performed as described in Ref. 19. Examples of apoptotic cells, determined by morphology (hematoxylin and eosin staining) or the presence of active caspase 3, are identified by single- or double-headed arrows, respectively. DMSO, dimethyl sulfoxide.

increased the expression of mRNA encoding EGFR-binding ligands (AREG, HB-EGF, and EREG) only ~3-fold in the presence of z-IETD-fmk and even less in the presence of z-VAD-fmk (Fig. 4B). Furthermore, FasL-induced transforming growth factor- α mRNA expression was not substantially increased by

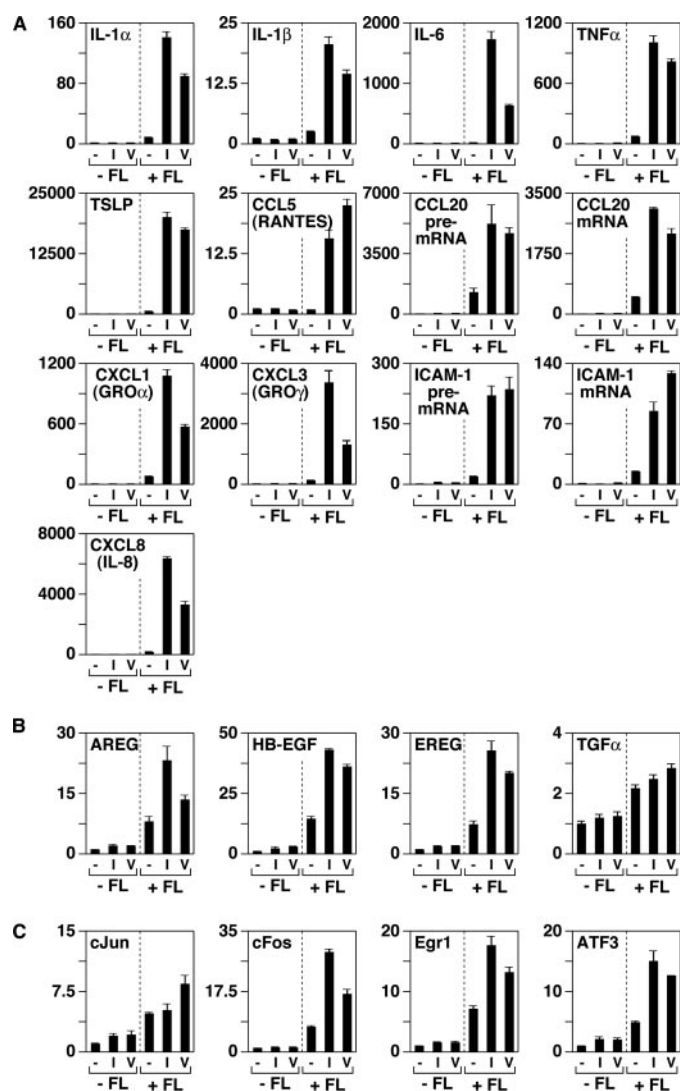


FIGURE 4. Effects of caspase inhibitors on the FasL-induced expression of inflammatory mediators (A), EGFR-binding ligands (B), or transcription factors (C). RHEs were treated with FasL as in Fig. 1 in the presence of a 30-min pretreatment with Me₂SO (vehicle, lanes -) or 50 μ M of either z-IETD-fmk (lanes I) or z-VAD-fmk (lanes V). qRT-PCR analyses were performed 4 h after FasL. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. Note that the qRT-PCR analyses of CCL20/MIP-3 α and ICAM-1 RNA expression were performed both for the primary transcripts and the mature mRNAs.

either caspase inhibitor (Fig. 4B). In a similar fashion, the potentiation of FasL-induced expression of the mRNAs encoding the transcription factors c-Fos, c-Jun, Egr1, and ATF3 was within the range of ~3-fold (Fig. 4C). The measurement of primary transcripts encoding CCL20/MIP-3 α and ICAM-1 suggested that the potentiating effect of caspase inhibitors on the FasL-induced proinflammatory gene expression occurred at the level of transcription (Fig. 4A).

ERK, JNK, and p38 MAP Kinases Contribute Nonredundantly to the Inflammatory Responses of Human Keratinocytes to FasL—We have previously found that FasL activated JNK, p38, and ERK MAP kinases in monolayer cultures of human keratinocytes (21). To investigate the contribution of MAPKs in the inflammatory response triggered by FasL in RHE, we applied specific inhibitors, singly and in combination, that are

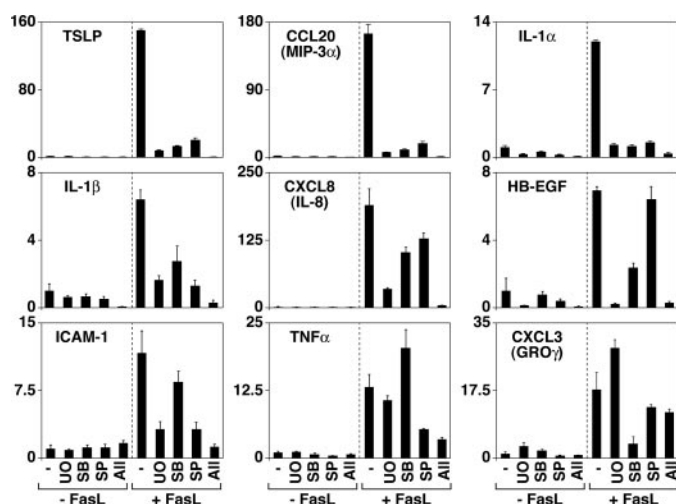


FIGURE 5. Effects of MAP kinase inhibitors on the expression of FasL-induced genes. RHEs were treated with FasL as in Fig. 1 in the presence of 30-min pretreatments with either Me₂SO (vehicle, —) or various antagonists as labeled. UO, UO126, 10 μ M; SB, SB203580, 10 μ M; SP, SP600125, 40 μ M; All, all three inhibitors together. qRT-PCR analyses were performed 4 h after FasL. The data are presented as fold change relative to the corresponding vehicle-treated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs.

known to interfere with effective transduction of the ERK, JNK, and p38 MAPK cascades: UO126, an inhibitor of MEK, the upstream activator kinase for ERK; SP600125, a direct inhibitor of JNK; and SB203580, a direct inhibitor of p38 MAPK. The effectiveness and specificity of these inhibitors was confirmed in immunoblot analyses using phospho-specific antibodies against these kinases and/or their downstream phosphorylated targets (data not shown). We investigated the effects of inhibiting MAPKs on the FasL-stimulated expression of various mRNAs encoding proinflammatory mediators and EGFR-binding ligands. The FasL-induced expression of all genes tested was abolished in the presence of all three MAPK inhibitors, suggesting that MAPK activity in general is essential for FasL-induced mRNA expression (Fig. 5). Several FasL-induced mRNAs (e.g. IL-1 α , IL-1 β , CCL20/MIP-3 α , and TSLP) were strongly inhibited by each of the three MAPK inhibitors when applied singly (Fig. 5), whereas other FasL-induced mRNAs were dependent on one or two of the MAPK members for their activation (Fig. 5). Taken together, these results suggest that the ERK, JNK, and p38 MAPKs are instrumental in a nonredundant fashion in orchestrating the proinflammatory responses to FasL in RHE. Furthermore, HB-EGF (Fig. 5), AREG, EREG (not shown here), IL-1 α , IL-1 β , CCL20/MIP-3 α , TSLP, CXCL8/IL-8, and ICAM-1 (Fig. 5) were identified as FasL-induced mRNAs that displayed a strong dependence on the ERK family of MAPKs.

Recruitment of EGFR to Mediate the ERK-dependent Inflammatory Responses to FasL—We further investigated the subset of FasL-induced genes encoding growth factors and inflammatory mediators whose induction by FasL displayed strong dependence on the ERK family of MAPKs. We previously reported that FasL triggers the activation of ERK MAPKs in cultured human keratinocytes through stimulation of the auto-crine secretion of soluble EGFR-binding ligands, one of which we identified as AREG (21). To determine whether EGFR and

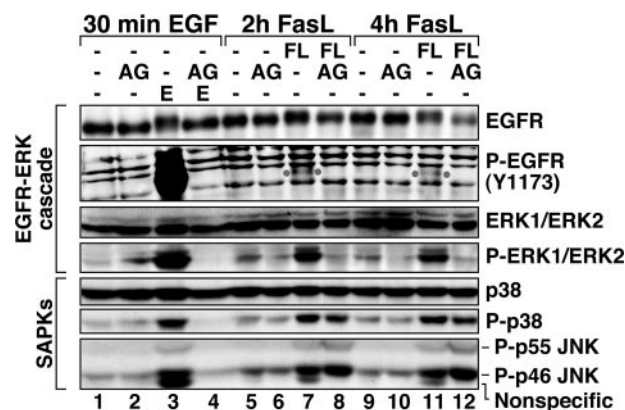


FIGURE 6. Activation of EGFR and MAP kinases in RHE. RHEs were treated with FasL (FL) as in Fig. 1 in the presence of 30-min pretreatments with either vehicle (Me₂SO, lanes —) or AG1478 (10 μ M, lanes AG). The phosphorylated states of EGFR and MAP kinases were assessed using phospho-specific antibodies at the indicated times. Treatment with EGF (lanes E, 100 ng/ml) served as positive control. Immunoblot detections of total ERK1/2 and p38 MAPK were used to monitor for expression levels and equal loading.

its ligands mediate FasL-induced pro-inflammatory responses in RHEs, we first investigated whether FasL treatment would trigger EGFR activation in the reconstructed epidermis in a manner similar to the one observed in cultured keratinocytes. Indeed, RHEs treated with FasL for 2 or 4 h displayed increased phosphorylation of EGFR (Fig. 6, lanes 7 and 11). FasL-induced EGFR phosphorylation was abolished in the presence of AG1478, a small molecule inhibitor of the kinase activity of EGFR (29) (Fig. 6, lanes 8 and 12). FasL also triggered the phosphorylation of p44 ERK1 and p42 ERK2 (Fig. 6, lanes 7 and 11), and this phosphorylation was abrogated by AG1478 (Fig. 6, lanes 8 and 12). FasL stimulated the phosphorylation of JNK and p38 α MAP kinases (Fig. 6, lanes 7 and 11), but in contrast to the phosphorylation of ERK, the phosphorylation of JNK and p38 α was not dependent on EGFR activity (Fig. 6, lanes 8 and 12).

We next employed either AG1478 (at a low dose, 100 nM, to minimize possible “off target” effects; Fig. 7A) or LA1, an EGFR-neutralizing antibody (30–32) (Fig. 7B) to investigate the potential contribution of EGFR to FasL-induced gene expression. Either mode of EGFR inhibition substantially suppressed the FasL-induced activation of HB-EGF, IL-1 α , IL-1 β , CCL20/MIP-3 α , CXCL8/IL-8, TSLP, ICAM-1 (Fig. 7), and AREG (not shown) but not that of tumor necrosis factor- α mRNA (Fig. 7). We concluded, therefore, that part of the inflammatory action of FasL was mediated through activation of the EGFR.

Contribution of Both ERK1 and ERK2 to the FasL-induced Gene Expression—To assess the relative contributions of ERK1 and ERK2 to the FasL-induced pro-inflammatory gene expression, we developed a technique for siRNA-mediated gene knockdown in RHE preparations (outlined schematically in Fig. 8A). In the optimization phase, it appeared that a sustained siRNA-mediated knockdown of various gene products could be maintained for ~4 days after the switch to organotypic differentiation (Fig. 8A). To assess how early organotypic reconstructions develop epidermal characteristics, we measured barrier function and the expression of differentiation markers. RHEs established proper barrier function by day 3 after the switch to

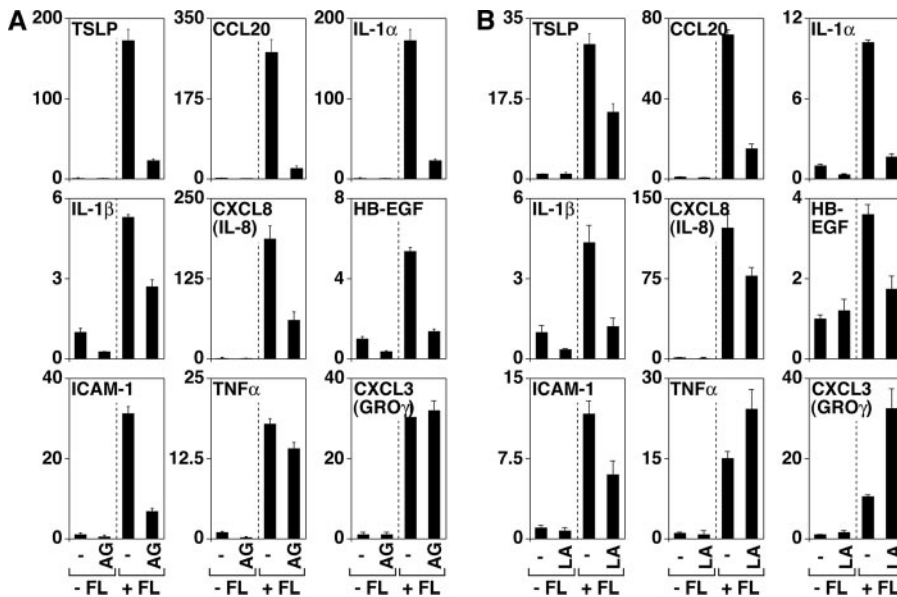


FIGURE 7. Effects of EGFR antagonists on the expression of FasL-induced genes. RHEs were treated with FasL as in Fig. 5 in the presence of 30 min pretreatments with either AG1478 (A, lanes AG, 100 nM) or AG1 (B, lanes AG, 10 μ g/ml). qRT-PCR analyses were performed 4 h post FasL. The data are presented as fold change relative to the corresponding vehicle-treated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. FL, FasL.

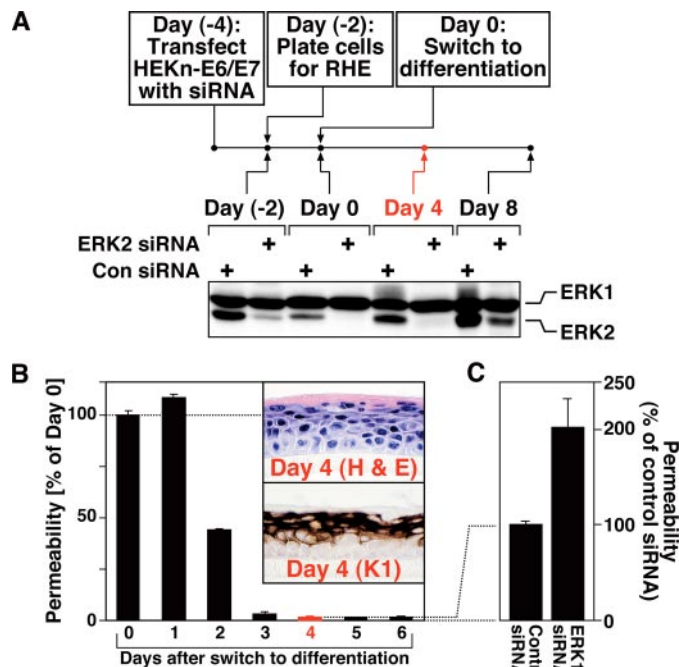


FIGURE 8. A, efficient siRNA-mediated knockdown of ERK2 at day 4 after the switch to differentiation. HEK293-E6/E7 were transfected with the indicated siRNAs, plated, and differentiated into RHEs as depicted in the experimental scheme. Immunoblot analysis with an antibody against ERK1 and ERK2 (pan-ERK antibody). ERK1 serves as a loading and specificity control. B, proper barrier function, morphology, and keratin 1 expression of RHEs at day 4 after the switch to differentiation. "Outside-In" barrier function was assessed by Toluene Blue O dye penetration assay, and it is presented as a percentage of permeability relative to the permeability of the constructs immediately after the initiation of differentiation (Day 0). Insets, epidermal morphology (hematoxylin and eosin staining, H & E) and detection of keratin 1 (K1) expression. C, effect of double knockdown of ERK1 and ERK2 on the barrier function. Note that the decrease of barrier function (i.e. increase in permeability) after the knockdown of ERK is small compared with the range of permeability decrease occurring during normal epidermal differentiation (dotted lines connecting B and C).

differentiation (Fig. 8B), and when examined at day 4, they displayed mature epidermal stratification, including the presence of *stratum corneum* and proper expression of keratin 1 (Fig. 8B, inset). For these reasons, the experiments described below were performed using RHEs at day 4 after the initiation of differentiation. RHEs that were subjected to a double knockdown of ERK1 and ERK2 (Fig. 9) showed a pattern of epidermal differentiation that was similar to control RHEs (supplemental Fig. S1). However, the double knockdown cultures exhibited a slightly delayed onset of differentiation, manifested by the decreased thickness of the *stratum corneum* (supplemental Fig. S1), a weaker (but properly stratified) keratin 1 immunoreactivity (supplemental Fig. S1), and a 2-fold decrease in barrier function (Fig. 8C).

Fig. 9 shows the effect of knocking down ERK MAPKs on the FasL-induced expression of CXCL8/IL-8. As expected from the previous findings using UO126 (Fig. 5), reducing the levels of both ERK1 and ERK2 in two independent experiments inhibited the responsiveness of CXCL8/IL-8 to FasL by 87 and 90%, respectively. It appeared that both ERK1 and ERK2 contributed to the activation of CXCL8/IL-8 by FasL, because single knockdowns of either kinase caused only partial inhibition of CXCL8/IL-8 expression (Fig. 9). Similar outcomes were observed for CCL20/MIP-3a, ICAM-1, and TSLP (not shown). The degree of siRNA-mediated effects on FasL-induced gene expression appeared to correlate with the achieved efficiency of knockdown of a targeted protein (compare, for instance, the knockdown of ERK1 in the two experiments presented in Fig. 9). These results strongly support the notion that both ERK1 and ERK2 contribute to the genomic responses to FasL.

DISCUSSION

Apoptosis and Inflammation in the Skin—The studies presented here and recently (12–14, 21, 33) have been directed toward uncovering the possible roles of FasL and apoptosis in human eczema. Eczema is traditionally thought of as disease characterized primarily by inflammatory phenomena. However, apoptosis of keratinocytes is a key hallmark of the acute eczematous epidermis and is thought to play an important role in spongiosis and vesicle formation in dermatitis (12–14, 21, 33). FasL appears to be the most prominent candidate for the mediation of keratinocyte apoptosis in dermatitis (12–14, 21, 33). Neither apoptosis nor spongiosis characterizes the epidermal lesion in psoriasis, another skin disease that is inflammatory, but not eczematous (34). Thus, apoptosis is a defining feature specifically of the eczematous type of tissue inflammation. One important question from a clinical point of view is whether attempts to suppress keratinocyte apoptosis in the epi-

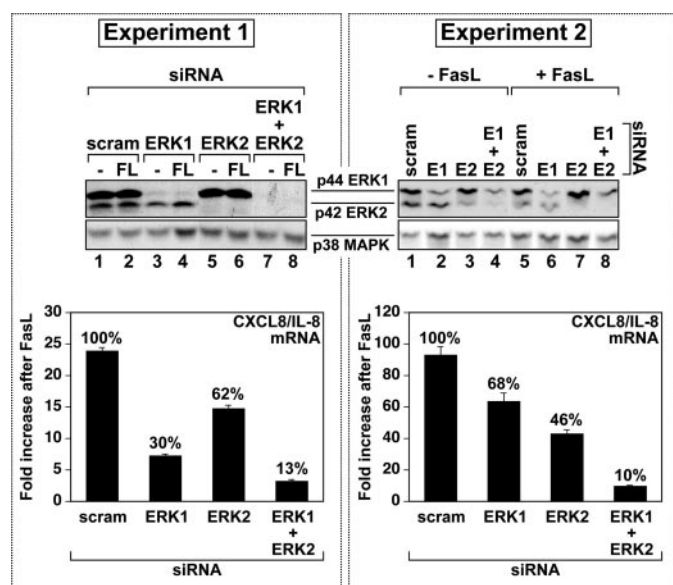


FIGURE 9. siRNA-mediated knockdown of ERK1, ERK2, or both, in RHE and the effect of the knockdown on the expression of CXCL8/IL-8. RHEs were transfected in two independent experiments with the indicated siRNAs (scram, "scrambled" control siRNA; for Experiment 2, E1 denotes ERK1, and E2 denotes ERK2) and analyzed at day 4 post the differentiation switch as described in the text and in Fig. 8A. The top panels depict immunoblot detections of ERK1, ERK2, and p38 MAP kinases. p38 MAPK is used as control for siRNA specificity and loading control. The bottom panels depict the expression of CXCL8/IL-8 mRNA 4 h after FasL treatment was determined by qRT-PCR at day 4 after the differentiation switch. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. The percentage of change relative to scrambled siRNA is indicated.

dermis would be beneficial to dermatitis patients. Alternatively, apoptosis could be part of the "healing" process that serves the purpose to restrict uncontrolled inflammation, even at the price of temporary tissue damage. Our findings presented here suggest that therapeutic strategies aimed at epidermal apoptosis suppression at the level of caspase activity may prove detrimental by aggravating inflammation (see below).

Positive and Negative Regulation of Gene Expression by FasL—The unbiased approach to gene expression analysis offered by microarray technologies allows for the identification of both positively and negatively regulated genes in experimental systems of interest. Although the main body of work presented here deals with genes that were induced by FasL in RHE, the Affymetrix microarray analysis revealed that a substantial number of RNA transcripts (as high as ~45%, see "Results") was reduced in abundance 4 h after FasL administration. A minority (12%) of the 287 annotated repressed genes considered in our analysis was affected more than 3-fold. Of the 68 FasL-down-regulated transcripts selected for their high basal expression in untreated RHE (supplemental Table S6), also 12% (*i.e.* the eight transcripts labeled in red in supplemental Table S6) were down-regulated more than 3-fold. The chemokine CXCL14, whose expression was reduced ~26-fold after FasL, may be of particular interest for further investigation. The relatively high levels of CXCL14 mRNA expression in our RHE (1182 arbitrary Affymetrix units, see primary data available online at [www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_\(2007\).xls](http://www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_(2007).xls)) are in agreement with the findings of Moser and co-workers

(35, 36), who reported high constitutive levels of CXCL14 expression in healthy human epidermis and in RHE. Recently, the same research group reported evidence strongly supporting a critical role for cutaneous CXCL14 in targeting blood precursors of dendritic cells to the epidermis for their differentiation as Langerhans cells (36). Because CXCL14 appears to act in a constitutive manner under non-inflammatory conditions, the authors suggested that CXCL14 might play a role in the steady-state functions of dendritic cells such as presentation of self-antigens for the purpose of elimination of self-reacting T cells and/or induction of suppressor/regulatory T cells (36). It is therefore tempting to speculate that FasL affects the genomic responses of epidermal keratinocytes by both inducing pro-inflammatory chemokines (*e.g.* CCL20, CXCL1–3, and CXCL8) and repressing, simultaneously, the expression of a potentially "anti-inflammatory" chemokine, CXCL14.

Pro-inflammatory Actions of FasL in Human Keratinocytes: Importance of the Organotypic Context—As described here and in Ref. 19, a single trigger, FasL, reproduces key clinical and molecular hallmarks of the eczematous epidermis in RHE. However, the inflammatory genomic responses to FasL observed in RHEs are in stark contrast to the lack of the same responses under two-dimensional tissue culture conditions. *In vitro* tissue culture has been employed previously to study the behavior of the eczematous keratinocyte. For instance, cultured keratinocytes from patients with atopic dermatitis display abnormal responses to T cell-derived cytokines (37, 38). However, the inappropriate genomic responses of cultured keratinocytes to FasL observed by us underscore the limitations of the tissue culture in studying eczemas. Ironically, the pro-inflammatory potential of FasL in human keratinocytes was initially discovered in the keratinocyte cell line HaCaT (19). We were therefore surprised to observe the complete lack of inflammatory gene expression in FasL-treated primary keratinocyte explants (HEKn) or in their immortalized derivatives, HEKn-E6/E7. However, the same HEKn or HEKn-E6/E7 when grown in an epidermotypic context (RHE) "regained" the ability to respond to FasL with increased expression of inflammatory mediators. As far as we have been able to determine, the main reason for this different behavior is the inability of HEKn or HEKn-E6/E7 in tissue culture to respond to FasL with activation of NF- κ B (not shown).⁴ In contrast, HaCaT cells in tissue culture potently activate NF- κ B in response to FasL (19). Indeed, siRNA-mediated knockdown of the p65/RelA subunit in HaCaT (19) or in HEKn-E6/E7 RHEs (not shown)⁴ abrogated the FasL-triggered expression of inflammatory mediators. It should be emphasized that HEKn and HEKn-E6/E7 in tissue culture display normal NF- κ B activation in response to cytokines other than FasL (*e.g.* tumor necrosis factor- α , or IL-1 β ; not shown).⁴ Furthermore, HEKn and HEKn-E6/E7 in tissue culture display an efficient response to FasL as determined by apoptosis (21) or expression of NF- κ B-independent genes (such as *c-fos* or *c-jun*; not shown).⁴ Therefore, it is reasonable to conclude that HEKn and HEKn-E6/E7 in tissue cul-

⁴ S. M. Farley, D. E. Purdy, O. P. Ryabinina, P. Schneider, B. E. Magun, and M. S. Iordanov, manuscript in preparation.

ture are specifically deficient in the proinflammatory genomic responses to FasL and that epidermotypic context (RHE) restores these responses. Because keratinocytes in tissue culture most resemble the undifferentiated transit-amplifying cells in the basal layer of the epidermis, it is tempting to speculate that the cells responding to the pro-inflammatory actions of FasL in RHEs are not the basal keratinocytes but rather keratinocytes within the *strata spinosum* or *granulosum*. Presumably, HaCaT cells differ from HEKn and HEKn-E6/E7 by having either gained a positive, or having lost a negative, FasL-triggered regulator of NF- κ B.

Possible Anti-inflammatory Functions of Caspases—Our results using the caspase inhibitors z-IETD-fmk and z-VAD-fmk (Fig. 4) strongly support the notion that caspase activity may have the physiological role of limiting the extent of FasL-triggered inflammation in the skin. Initially, we considered the simplest explanation, namely that caspases limit the inflammatory response by promoting cell death. By means of rapid elimination of FasL-challenged keratinocytes, the extent of transcriptional activation and release of inflammatory mediators by the same cells would be restricted. Although this explanation cannot be ruled out, it does not fully concur with the experimental observations. As Figs. 1 and 2 demonstrate, the peak of inflammatory gene expression occurs within 4 h after the administration of FasL. At this time, the caspase activity, although clearly detectable (Fig. 3), is well below the levels observed at later time points (Fig. 3B). Importantly, at 4 h after FasL, keratinocytes in the RHEs display normal morphological characteristics (Fig. 3B, panels I and II). Nuclear pyknosis, karyorrhexis, and cell swelling were only observable at later times (e.g. 24 h; Fig. 3B, panel VI). We have also reported previously that HaCaT cells, treated with FasL for up to 4 h and displaying $\geq 75\%$ cleaved poly(ADP-ribose) polymerase, nevertheless maintain high levels of biosynthetic activity, as determined by incorporation of [3 H]leucine into newly synthesized proteins (19). Taken together, these findings argue against the conclusion that caspases limit FasL-triggered inflammation by simply eliminating inflammatory cytokine-producing cells. We propose an alternative hypothesis, i.e. that the initial activation of caspases triggers an active anti-inflammatory program *before* the onset of cell death. Such an early action of caspases would, therefore, define a novel function for these proteases, a function that is kinetically separable from their cytotoxic properties. The anti-inflammatory functions of caspases may be effected, for instance, through suppression of the FasL-triggered activation of NF- κ B, a possibility being currently investigated.

One clinically relevant conclusion from these findings might be that attempts to suppress keratinocyte apoptosis by means of caspase inhibition in acute dermatitis may have the unintended consequences of aggravated inflammatory response and, ultimately, a worse outcome for the patient. An important unanswered question that remains is whether the anti-inflammatory signals associated with activated caspases are effected directly by the apical caspases 8 and 10 or further downstream by effector caspases such as caspases 3 and 7. Should the anti-inflammatory functions be specific for the apical caspases only, strategies aimed at inhibiting effector caspases may be beneficial in limiting apoptosis, yet avoiding aggravated inflammation.

A Novel Role of the EGFR-Ras-ERK Cascade in Mediating Inflammation in the Skin—We have previously reported that the basal levels of ERK activity in HEKn or HEKn-E6/E7 in tissue culture were determined by the autocrine secretion of ligands that bind to the EGFR (20). Interfering with either the extracellular ligand-binding portion of EGFR by means of the LA-1 antibody or the intracellular kinase domain of EGFR reduced the basal phosphorylation of ERK1 and ERK2 to undetectable levels (20). We have also reported that FasL-induced ERK activation of HEKn or HEKn-E6/E7 in culture is mediated by FasL-simulated secretion of EGFR ligands (one of which was identified as amphiregulin) and the subsequent activation of EGFR (21). FasL also activated EGFR in RHEs (Fig. 6), and interfering with the activity of EGFR prevented FasL-triggered activation of ERK (Fig. 6). A subset of FasL-induced inflammatory mediators in RHEs was found to require ERK activity (Fig. 6). Taken together, these findings had allowed us to predict that EGFR would be recruited to mediate some of the inflammatory gene expression in response to FasL in the RHE. This was indeed found to be the case (Fig. 7). However, it should be emphasized that the biological roles of EGFR in human skin are likely to be much more complex than can be appreciated with the available tools for experimentation. For instance, CXCL8/IL-8, an EGFR-dependent, FasL-induced, potent inflammatory mediator, is also a suspected keratinocyte mitogen (39). Bearing in mind that FasL triggers the production of mitogenic EGFR ligands in human keratinocytes (21), it is likely that the physiological outcomes of the presence of FasL in the human epidermis may be determined by the interplay of three seemingly disparate phenomena: apoptosis, inflammation, and mitogenesis (the last two being, at least partially, dependent on EGFR). With the exception of the findings that anti-EGFR-directed therapies of cancer produce cutaneous acne-like rashes as side effects (40), our understanding of the roles of EGFR in the human skin is still very limited.

The Roles of ERK1 and ERK2 in the Genomic Responses of Keratinocytes to FasL—We report here that both ERK1 and ERK2 are involved in mediating the proinflammatory gene expression in response to FasL (Fig. 9). A single siRNA-mediated knockdown of either ERK1 or ERK2 reduced FasL-induced CXCL8/IL-8 expression significantly, but a double knockdown appeared necessary to reduce CXCL8/IL-8 expression by more than 85% (Fig. 9). Recently, Vantaggiato *et al.* (41) presented evidence that ERK1 and ERK2 play opposing roles in mediating the proliferative effects of mitogenic growth factors and Ras-driven oncogenic transformation. According to this model, ERK2 mediates the proliferative and transforming effects of activated Ras, whereas ERK1 inhibits these effects by competing with ERK2 for their mutual upstream activator MEK (41, 42). Interestingly, CXCL8/IL-8 expression was recently reported to be a transcriptional target of Ras signaling (43), and based on these findings, our initial expectations were that we would observe opposing roles of ERK1 and ERK2 in mediating the expression of CXCL8/IL-8 in FasL-treated RHEs. The fact that this was not the case (Fig. 9) underscores the complexity of MAPK regulation and function and strongly emphasizes the need to investigate MAPK signaling pathways in the context of specific tissues and organs. In conclusion, the RHE-based orga-

notypic model described here could be applicable to study pharmacologic manipulations of the FasL-driven genomic responses as novel therapeutic options for the treatment of dermatitis and other inflammatory skin conditions.

Acknowledgments—We thank Thanh-Hoai Dinh and Anjali Dotson for the excellent technical assistance.

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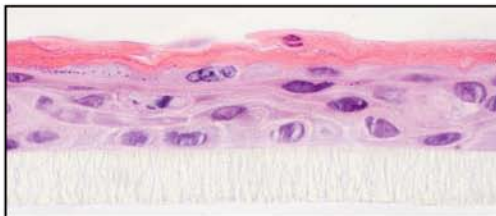
SUPPLEMENTAL FIGURE LEGENDS:

Figure S1. Effect of double ERK1 and ERK2 knockdown on epidermotypic morphology (H&E) and keratin 1 expression of RHEs at day 4 after the switch to differentiation.

H & E

Keratin 1

**Control
siRNA**



**ERK1/2
siRNA**

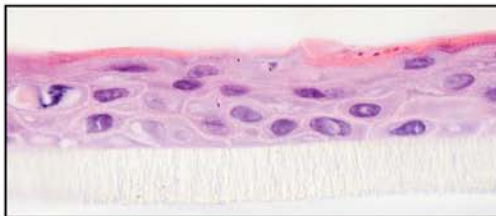


Figure S1

TableS1. FasL-Induced Genes Categorized by Biological Process

Category name	Total probes	Expected matches	Actual matches	Fold enrichment	Binomial p-value
response to external stimulus	971	2.03	17	8.39	6.16E-11
cell-cell signaling	1115	2.33	17	7.3	4.97E-10
organismal physiological process	3749	7.83	30	3.83	1.21E-09
cell communication	7004	14.62	43	2.94	1.30E-09
response to wounding	767	1.6	14	8.74	1.72E-09
chemotaxis	203	0.42	8	18.88	1.56E-08
taxis	203	0.42	8	18.88	1.56E-08
locomotory behavior	223	0.47	8	17.18	3.24E-08
signal transduction	6405	13.37	37	2.77	7.76E-08
behavior	366	0.76	9	11.78	1.14E-07
response to abiotic stimulus	792	1.65	12	7.26	1.79E-07
response to chemical stimulus	667	1.39	11	7.9	2.52E-07
response to pest, pathogen or parasite	983	2.05	13	6.33	2.61E-07
immune response	1560	3.26	16	4.91	3.46E-07
response to other organism	1017	2.12	13	6.12	3.82E-07
response to biotic stimulus	1808	3.77	17	4.5	4.98E-07
response to stimulus	4047	8.45	26	3.08	9.25E-07
neurophysiological process	1320	2.76	14	5.08	1.24E-06
defense response	1731	3.61	16	4.43	1.32E-06
inflammatory response	388	0.81	8	9.88	2.12E-06
cell surface receptor linked signal transduction	2629	5.49	19	3.46	5.05E-06
response to stress	2180	4.55	17	3.74	5.89E-06

Table S2. FasL-Induced Genes Categorized by Molecular Function

Category name	Total probes	Expected matches	Actual matches	Fold enrichment	Binomial p-value
cytokine activity	355	0.74	10	13.49	6.31E-09
chemokine activity	65	0.14	5	36.85	2.95E-07
chemokine receptor binding	65	0.14	5	36.85	2.95E-07
G-protein-coupled receptor binding	78	0.16	5	30.7	7.38E-07
signal transducer activity	5219	10.9	30	2.75	1.35E-06
receptor binding	1226	2.56	13	5.08	2.94E-06
glycosaminoglycan binding	220	0.46	5	10.89	1.12E-04
polysaccharide binding	227	0.47	5	10.55	1.30E-04
GABA receptor activity	48	0.1	3	29.94	1.47E-04
pattern binding	238	0.5	5	10.06	1.62E-04
transmembrane receptor activity	1881	3.93	13	3.31	2.26E-04
heparin binding	159	0.33	4	12.05	3.76E-04
growth factor activity	321	0.67	5	7.46	6.33E-04
protein binding	10104	21.09	37	1.75	1.06E-03
receptor activity	3095	6.46	16	2.48	1.08E-03
transmembrane receptor protein tyrosine kinase	246	0.51	4	7.79	1.89E-03
extracellular ligand-gated ion channel activity	132	0.28	3	10.89	2.79E-03
GABA-A receptor activity	38	0.08	2	25.21	2.91E-03
tumor necrosis factor receptor binding	40	0.08	2	23.95	3.23E-03
transmembrane receptor protein kinase activity	290	0.61	4	6.61	3.42E-03
carbohydrate binding	479	1	5	5	3.61E-03
enzyme regulator activity	1655	3.46	9	2.6	9.06E-03

Table S3. Selection of FasL-Induced Genes in the Human Organotypic Model of Eczematous Dermatitis

ProbeSetID	Gene Symbol	Gene Bank #	Protein Name/Function	Fold Change			
				zVAD	FasL	zVAD+FasL	zVAD+FasL/ FasL
205180_S_AT	ADAM8	NM_001109.1	ADAM metalloproteinase domain 8	-2.46	3.73	2.46	-1.41
226228_AT	AQP4	T15657	aquaporin 4	9.19	39.40	315.17	9.85
205476_AT	CCL20	NM_004591.1	chemokine (C-C motif) ligand 20	-1.32	10.56	18.38	2.00
204470_AT	CXCL1	NM_001511.1	chemokine (C-X-C motif) ligand 1	1.00	24.25	73.52	2.46
209774_X_AT	CXCL2	M57731.1	chemokine (C-X-C motif) ligand 2	-1.15	13.93	24.25	1.62
207850_AT	CXCL3	NM_002090.1	chemokine (C-X-C motif) ligand 3	3.73	8.00	21.11	4.92
208891_AT	DUSP6	BC003143.1	dual specificity phosphatase 6 (MKP-3)	1.62	3.48	2.30	-1.52
201693_S_AT	EGR1	AV733950	early growth response 1	1.15	3.73	3.25	-1.07
203499_AT	EPHA2	NM_004431.1	EPH receptor A2	1.32	3.25	3.25	1.07
209189_AT	FOS	BC004490.1	v-fos FBJ murine osteosarcoma viral oncogene homolog	1.62	3.73	4.92	1.23
204420_AT	FOSL1	BG251266	FOS-like antigen 1	-1.41	5.66	3.03	-1.62
223541_AT	HAS3	AF232772.1	hyaluronan synthase 3	-1.32	3.73	4.92	1.41
203821_AT	HBEGF	NM_001945.1	heparin-binding EGF-like growth factor	1.00	3.25	2.83	-1.23
202637_S_AT	ICAM1	AI608725	intercellular adhesion molecule 1 (CD54)	-1.15	7.46	34.30	3.73
230735_AT	IFNAR2	AI653318	interferon (alpha, beta and omega) receptor 2	7.46	14.93	19.70	1.41
202859_X_AT	IL8	NM_000584.1	interleukin 8	-1.23	22.63	64.00	3.25
210511_S_AT	INHBA	M13436.1	inhibin, beta A (activin A)	-1.32	6.06	14.93	2.46
217173_S_AT	LDLR	S70123.1	low density lipoprotein receptor	1.00	3.03	1.87	-1.62
207339_S_AT	LTB	NM_002341.1	lymphotoxin beta (TNF superfamily, member 3)	1.07	3.48	3.48	1.41
209072_AT	MBP	M13577.1	myelin basic protein	2.00	3.03	157.59	42.22
200796_S_AT	MCL1	BF594446	myeloid cell leukemia sequence 1 (BCL2-related)	2.14	3.03	1.74	-1.52
205828_AT	MMP3	NM_002422.2	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	-3.25	4.29	4.00	-1.15
203936_S_AT	MMP9	NM_004994.1	matrix metalloproteinase 9 (gelatinase B)	-1.07	3.03	3.48	1.07
243807_AT	NCOA7	BG432498	nuclear receptor coactivator 7	1.00	3.25	1.62	-1.62
243296_AT	PBEF1	AA873350	pre-B-cell colony enhancing factor 1	1.23	3.25	3.48	1.07
205479_S_AT	PLAU	NM_002658.1	plasminogen activator, urokinase	1.00	3.25	6.50	2.14
204897_AT	PTGER4	AA897516	prostaglandin E receptor 4 (subtype EP4)	-1.23	4.29	2.14	-2.83
1554997_A_AT	PTGS2	AY151286.1	prostaglandin-endoperoxide synthase 2 (COX2)	1.00	6.06	12.13	2.14
215078_AT	SOD2	AL050388.1	superoxide dismutase 2, mitochondrial	1.87	4.00	13.00	2.83
203438_AT	STC2	AI435828	stanniocalcin 2	4.00	7.46	3.48	-2.00
203998_S_AT	SYT1	AV723167	synaptotagmin I	1.00	6.96	238.86	48.50
203167_AT	TIMP2	NM_003255.2	TIMP metalloproteinase inhibitor 2	1.52	3.03	1.32	-1.87
216005_AT	TNC	BF434846	tenascin C (hexabrachion)	-4.29	3.03	22.63	7.46
207113_S_AT	TNF	NM_000594.1	tumor necrosis factor (TNF superfamily, member 2)	-2.00	7.46	25.99	3.48
202643_S_AT	TNFAIP3	AI738896	tumor necrosis factor, alpha-induced protein 3	-1.41	6.50	14.93	2.46
206026_S_AT	TNFAIP6	NM_007115.1	tumor necrosis factor, alpha-induced protein 6	2.30	9.19	73.52	13.93
210512_S_AT	VEGF	AF022375.1	vascular endothelial growth factor	1.41	3.48	2.30	-1.52

Table S4. FasL-Downregulated Genes Categorized by Biological Process

Category name	Total probes	Expected matches	Actual matches	Fold enrichment	Binomial p-value
cellular metabolic process	13698	109.94	171	1.56	3.74E-08
primary metabolic process	13734	110.23	171	1.55	4.42E-08
metabolic process	14899	119.58	180	1.51	1.41E-07
macromolecule metabolic process	12057	96.77	150	1.55	2.90E-07
cellular process	21119	169.5	236	1.39	7.26E-07
biopolymer metabolic process	9246	74.21	118	1.59	1.53E-06
cellular macromolecule metabolic process	5926	47.56	79	1.66	1.74E-05
cellular protein metabolic process	5844	46.9	78	1.66	1.88E-05
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6799	54.57	86	1.58	4.77E-05
protein metabolic process	6234	50.04	80	1.6	5.38E-05
regulation of cellular process	7923	63.59	95	1.49	1.32E-04
RNA metabolic process	5276	42.35	68	1.61	1.62E-04
regulation of metabolic process	5126	41.14	66	1.6	2.05E-04
response to biotic stimulus	375	3.01	11	3.65	2.78E-04
regulation of biological process	8552	68.64	99	1.44	3.18E-04
biological regulation	9210	73.92	105	1.42	3.63E-04
DNA repair	465	3.73	12	3.22	4.73E-04
regulation of cellular metabolic process	4928	39.55	62	1.57	5.49E-04
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4577	36.74	58	1.58	6.83E-04
RNA splicing	489	3.92	12	3.06	7.33E-04
response to virus	138	1.11	6	5.42	9.32E-04
response to endogenous stimulus	649	5.21	14	2.69	9.68E-04
regulation of transcription	4454	35.75	56	1.57	9.90E-04
response to DNA damage stimulus	594	4.77	13	2.73	1.27E-03
transcription	4634	37.19	57	1.53	1.45E-03
DNA metabolic process	1328	10.66	22	2.06	1.47E-03
RNA biosynthetic process	4285	34.39	53	1.54	1.85E-03
mRNA processing	549	4.41	12	2.72	1.93E-03
post-translational protein modification	2858	22.94	38	1.66	2.35E-03
mRNA metabolic process	643	5.16	13	2.52	2.52E-03
RNA splicing, via transesterification reactions	168	1.35	6	4.45	2.53E-03
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	168	1.35	6	4.45	2.53E-03
nuclear mRNA splicing, via spliceosome	168	1.35	6	4.45	2.53E-03
protein modification process	3373	27.07	43	1.59	2.74E-03
regulation of transcription, DNA-dependent	4177	33.53	51	1.52	2.85E-03
transcription, DNA-dependent	4278	34.34	52	1.51	2.85E-03
proteolysis	1242	9.97	20	2.01	3.21E-03
spliceosome assembly	80	0.64	4	6.23	4.04E-03
biopolymer modification	3464	27.8	43	1.55	4.33E-03
ubiquitin cycle	938	7.53	16	2.13	4.60E-03
cellular macromolecule catabolic process	621	4.98	12	2.41	5.13E-03
protein folding	471	3.78	10	2.65	5.40E-03
protein sumoylation	15	0.12	2	16.61	6.31E-03
cellular component organization and biogenesis	5086	40.82	58	1.42	6.33E-03
ribonucleoprotein complex biogenesis and assembly	351	2.82	8	2.84	8.14E-03
response to stimulus	3706	29.75	44	1.48	8.27E-03
organelle organization and biogenesis	2117	16.99	28	1.65	8.53E-03
cytoskeleton organization and biogenesis	1007	8.08	16	1.98	8.73E-03
RNA processing	922	7.4	15	2.03	8.91E-03
S phase of mitotic cell cycle	18	0.14	2	13.84	9.05E-03
positive regulation of gene-specific transcription	18	0.14	2	13.84	9.05E-03
negative regulation of progression through mitotic cell cycle	18	0.14	2	13.84	9.05E-03
S phase	18	0.14	2	13.84	9.05E-03
keratinocyte differentiation	55	0.44	3	6.8	9.94E-03

Table S5. FasL-Downregulated Genes Categorized by Molecular Function

Category name	Total probes	Expected matches	Actual matches	Fold enrichment	Binomial p-value
binding	20792	166.88	239	1.43	7.99E-08
catalytic activity	9439	75.76	118	1.56	3.88E-06
protein binding	11895	95.47	141	1.48	7.17E-06
nucleic acid binding	6176	49.57	79	1.59	6.59E-05
nucleotide binding	4134	33.18	56	1.69	1.76E-04
transferase activity	3308	26.55	45	1.69	6.48E-04
transferase activity, transferring phosphorus-containing groups	1977	15.87	30	1.89	9.49E-04
DNA binding	4072	32.68	52	1.59	1.05E-03
purine nucleotide binding	3542	28.43	46	1.62	1.42E-03
ATP-dependent helicase activity	206	1.65	7	4.23	1.51E-03
hydrolase activity	3896	31.27	49	1.57	1.93E-03
cysteine-type peptidase activity	293	2.35	8	3.4	2.82E-03
oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	36	0.29	3	10.38	3.03E-03
oxidoreductase activity	1248	10.02	20	2	3.38E-03
adenyl nucleotide binding	2882	23.13	37	1.6	4.59E-03
peptidase activity	1028	8.25	17	2.06	4.82E-03
helicase activity	321	2.58	8	3.11	4.86E-03
pyrophosphatase activity	1210	9.71	19	1.96	5.18E-03
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	1212	9.73	19	1.95	5.27E-03
hydrolase activity, acting on acid anhydrides	1215	9.75	19	1.95	5.40E-03
aldehyde dehydrogenase (NAD) activity	14	0.11	2	17.8	5.50E-03
ATP binding	2723	21.86	35	1.6	5.60E-03
calcium-release channel activity	16	0.13	2	15.57	7.17E-03
insulin receptor substrate binding	16	0.13	2	15.57	7.17E-03
caspase activity	49	0.39	3	7.63	7.23E-03
nucleoside-triphosphatase activity	1164	9.34	18	1.93	7.39E-03
ATP-dependent DNA helicase activity	50	0.4	3	7.48	7.65E-03
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	96	0.77	4	5.19	7.68E-03
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	96	0.77	4	5.19	7.68E-03
dioxygenase activity	96	0.77	4	5.19	7.68E-03
kinase activity	1719	13.8	24	1.74	7.69E-03
DNA-dependent ATPase activity	97	0.78	4	5.14	7.96E-03
oxidoreductase activity, acting on the aldehyde or oxo group of donors	51	0.41	3	7.33	8.08E-03
nucleotidyltransferase activity	219	1.76	6	3.41	9.01E-03

Table S6. FasL-Downregulated Genes in Reconstructed Human Epidermis (≥2-fold change)						
(All 68 genes with ≥1000 arbitrary Affymetrix units of expression in untreated RHEs)				Fold Change		
ProbeSet ID	Gene Bank #	Gene Symbol	Protein Name/Function	zVAD	FasL	zVAD+FasL
237038_at	AI927990	CXCL14	chemokine (C-X-C motif) ligand 14	-1.52	-25.99	-2.30
201027_s_at	NM_015904.1	EIF5B	eukaryotic translation initiation factor 5B	-4.00	-4.00	-1.15
201650_at	NM_002276.1	KRT19	keratin 19	-1.52	-3.48	-2.46
221768_at	AV705803	SFPQ	splicing factor proline/ glutamine-rich	-4.29	-3.48	-1.07
204455_at	NM_001723.1	DST	dystonin, bullous pemphigoid antigen 1 (BPAG1)	-1.87	-3.25	-2.14
200751_s_at	BE898861	HNRPC	heterogeneous nuclear ribonucleoprotein C (C1/ C2)	-2.83	-3.03	1.00
1552619_a_at	NM_018685.1	ANLN	anillin, actin binding protein	-3.03	-3.03	-1.15
208950_s_at	BC002515.1	ALDH7A1	aldehyde dehydrogenase 7 family, member A1	-1.74	-3.03	-1.41
201008_s_at	AA812232	TXNIP	thioredoxin interacting protein	-2.46	-2.64	1.00
1557910_at	BG612458	HSP90AB1	heat shock protein 90kDa alpha (cytosolic), B1	-2.83	-2.64	-1.07
1554921_a_at	BC020726.1	SCEL	scellin	-2.83	-2.64	-1.32
208846_s_at	U90943.1	VDAC3	voltage-dependent anion channel 3	-2.14	-2.64	-1.41
201805_at	NM_002733.1	PRKAG1	protein kinase, AMP-activated, gamma 1 non-catalytic	-2.83	-2.64	-1.15
1553530_a_at	NM_033669.1	ITGB1	integrin, beta 1 (fibronectin receptor, beta 1)	-1.87	-2.64	1.07
219104_at	NM_016422.1	RNF141	ring finger protein 141	-2.83	-2.64	-1.32
1555278_a_at	BC035554.1	CKAP5	cytoskeleton associated protein 5	-2.30	-2.64	1.00
201507_at	NM_002622.2	PFDN1	prefoldin subunit 1	-1.62	-2.64	-1.23
206166_s_at	AF043977.1	CLCA2	chloride channel, calcium activated, family member 2	-2.64	-2.46	-1.15
201946_s_at	AL545982	CCT2	chaperonin containing TCP1, subunit 2 (beta)	-2.83	-2.46	-1.15
208631_s_at	U04627.1	HADHA	hydroxyacyl-Coenzyme A dehydrogenase, alpha	-2.14	-2.46	-1.07
218235_s_at	NM_016037.1	UTP11L	UTP11-like, U3 small nucleolar ribonucleoprotein	-1.87	-2.46	-1.23
210338_s_at	AB034951.1	HSPA8	heat shock 70kDa protein 8	-2.46	-2.30	-1.07
200883_at	NM_003366.1	UQCRC2	ubiquinol-cytochrome c reductase core protein II	-2.00	-2.30	-1.23
209684_at	AL136924.1	RIN2	Ras and Rab interactor 2	-1.15	-2.30	-2.00
204734_at	NM_002275.1	KRT15	keratin 15	-1.23	-2.30	-1.52
201829_at	AW263232	NET1	neuroepithelial cell transforming gene 1	-1.52	-2.30	-2.30
201711_x_at	AI681120	RANBP2	RAN binding protein 2	-3.25	-2.30	-1.23
200640_at	NM_003406.1	YWHAZ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta	-2.14	-2.14	-1.07
200046_at	NM_001344.1	DAD1	defender against cell death 1	-1.74	-2.14	-1.23
217356_s_at	S81916.1	PGK1	phosphoglycerate kinase 1	-2.14	-2.14	-1.23
200729_s_at	NM_005722.1	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	-2.00	-2.14	1.00
1555383_a_at	BC017500.1	POF1B	premature ovarian failure, 1B	-2.83	-2.14	-1.07
216032_s_at	AF091085.1	ERGIC3	ERGIC and golgi 3	-2.30	-2.14	-1.07
211090_s_at	Z25435.1	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	-2.46	-2.14	1.07
210125_s_at	AF044773.1	BANF1	barrier to autointegration factor 1	-1.87	-2.14	1.00
205363_at	NM_003986.1	BBOX1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1	-2.00	-2.14	-1.41
217815_at	NM_007192.1	SUPT16H	suppressor of Ty 16 homolog (S. cerevisiae)	-1.87	-2.14	1.00
222387_s_at	BG476669	VPS35	vacuolar protein sorting 35 homolog (S. cerevisiae)	-2.30	-2.14	-1.15
202119_s_at	NM_003909.1	CPNE3	copine III	-1.41	-2.14	1.00
213126_at	BG230758	MED8	mediator of RNA polymerase II transcription, subunit 8	-1.87	-2.14	-1.15
1557100_s_at	AL038005	HECTD1	HECT domain containing 1	-2.83	-2.14	-1.41
209316_s_at	BC001465.1	HBS1L	HBS1-like (S. cerevisiae)	-2.30	-2.14	1.00
200600_at	NM_002444.1	MSN	moesin	-1.87	-2.14	1.23
208907_s_at	BC005373.1	MRPS18B	mitochondrial ribosomal protein S18B	-1.87	-2.14	-1.23
218527_at	NM_017692.1	APTIX	apratxin	-1.41	-2.14	-1.15
212216_at	AW000954	PREPL	prolyl endopeptidase-like	-1.62	-2.14	-1.07
233878_s_at	BE536170	XRN2	5'-3' exoribonuclease 2	-2.14	-2.14	-1.07
207515_s_at	NM_004875.1	POLR1C	polymerase (RNA) I polypeptide C, 30kDa	-2.64	-2.14	-1.32
212379_at	BE966876	GART	phosphoribosylglycinamide formyltransferase	-3.25	-2.14	-1.32
222975_s_at	AI423180	CSDE1	cold shock domain containing E1, RNA-binding	-2.00	-2.00	-1.23
208642_s_at	AA205834	XRCC5	Ku autoantigen, 80kDa	-1.62	-2.00	-1.23
201241_at	NM_004939.1	DDX1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	-1.52	-2.00	-1.23
1554253_a_at	BC028703.1	LASS3	LAG1 homolog, ceramide synthase 3 (S. cerevisiae)	-2.64	-2.00	1.00
217850_at	NM_014366.1	GNL3	guanine nucleotide binding protein-like 3 (nucleolar)	-2.30	-2.00	-1.07
200953_s_at	NM_001759.1	CCND2	cyclin D2	-1.52	-2.00	-1.07
208761_s_at	U67122.1	SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	-2.00	-2.00	-1.41
212896_at	D29641.2	SKIV2L2	superkiller viralicidic activity 2-like 2 (S. cerevisiae)	-2.30	-2.00	-1.23
201476_s_at	AI692974	RRM1	ribonucleotide reductase M1 polypeptide	-2.30	-2.00	-1.15
201478_s_at	U59151.1	DKC1	dyskeratosis congenita 1, dyskerin	-2.14	-2.00	-1.15
201894_s_at	NM_001920.1	DCN	decorin	-1.62	-2.00	-1.15
208673_s_at	AF107405.1	SFRS3	splicing factor, arginine/ serine-rich 3	-1.87	-2.00	-1.74
205822_s_at	NM_002130.1	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	-2.30	-2.00	1.32
201635_s_at	AI990766	FXR1	fragile X mental retardation, autosomal homolog 1	-2.00	-2.00	-1.23
202614_at	NM_006345.2	SLC30A9	solute carrier family 30 (zinc transporter), member 9	-1.62	-2.00	-1.41
210317_s_at	U28936.1	YWHAE	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, epsilon	-2.30	-2.00	1.23
208803_s_at	AF069765.1	SRP72	signal recognition particle 72kDa	-1.87	-2.00	-1.15
201083_s_at	AA740754	BCLAF1	BCL2-associated transcription factor 1	-2.14	-2.00	-1.32
212428_at	AW001101	KIAA0368	KIAA0368	-2.64	-2.00	-1.07

Fas Ligand-induced Proinflammatory Transcriptional Responses in Reconstructed Human Epidermis: RECRUITMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR AND ACTIVATION OF MAP KINASES

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J. Biol. Chem. 2008, 283:919-928.

doi: 10.1074/jbc.M705852200 originally published online October 31, 2007

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