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Vincent J. Maffei April 19, 2011

Tumor Necrosis Factor-alpha mediated transactivation of the Epidermal Growth Factor Receptor and Keratinocytes

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Abstract

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Tumor Necrosis Factor-alpha (TNF) has been implicated in a variety of signaling networks especially in the immune system. As such, TNF dysregulation has been linked to numerous autoimmune diseases particularly ones of chronic inflammation. TNF has been characterized historically as an inducer of nuclear factor kappa-B (NFkB) as well as an inducer of apoptosis. However, TNF in chronic inflammatory diseases such as psoriasis promotes hyper-proliferation of keratinocytes. The epidermal growth factor receptor family (EGFR) is a group of cell-surface proteins also involved in keratinocyte proliferation. This work reports that TNF induces EGFR activation in HaCaT keratinocytes through a matrix-metalloprotease (MMP) mechanism, which, in turn, leads to mitogen activated protein kinase activity (MAPK). MMP cleavage of EGFR-ligands is the proposed method of TNF-mediated EGFR activity. Moreover, TNF induction of the EGFR and NFkB pathways are independent and separate, which is contrary to current perspectives on TNF signaling. While TNF-induced EGFR activation is insufficient for cell cycle entry, it does protect keratinocytes from TNF-mediated apoptosis. Additionally, interleukin-8 expression, a marker of inflammation, by TNF occurs through mutually activated EGFR and NFκB pathways. Finally, neutralizing antibodies for the EGFR family were used to dissect individual contributions of receptor pairs towards a common signal output. The observations found for TNF-induced, EGFR-mediated IL-8 expression were plausible given findings in other studies. This work reveals TNF’s direct effects on keratinocytes that may contribute to inflammation pathophysiology.

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1. Introduction

1.1 Tumor Necrosis Factor-alpha

Tumor Necrosis Factor-alpha (TNF) participates in a number of signal transduction networks and autoimmune diseases. Originally "cachectin," TNF was renamed following reports that established it as a tumor necrosis, apoptotic agent from its hand in tumor degeneration. In these reports, cancer patients with co-morbid bacterial infections noticed a reduction in tumor size and density [1]. As a response to the infections, macrophages released TNF leading to early reports of its therapy against hyper-proliferation. TNF release is classically associated with immune response to pathogens, but it is equally involved in the manifestation of acute and chronic autoimmune inflammation. Commercial anti-TNF therapies such as Infliximab (Remicade) are highly effective therapeutic agents against inflammatory disorders. In particular, patients undergoing treatment for Crohn's disease, inflammatory bowel syndrome, psoriasis, rheumatory arthritis and ulcerative colitis generally respond well to anti-TNF therapy [2-6].

TNF is a trimeric cytokine that binds to either of two receptors: tumor necrosis factor receptor 1 or 2 (TNFR1 or 2). While both TNFR1 and TNFR2 are capable of binding to soluble TNF, TNFR2 has been reported to discriminate against soluble TNF in exchange for membrane-bound, juxtacrine signaling TNF. Studies revealed that soluble TNF has a greater affinity for TNFR1 (K_d = 19 pM) over TNFR2 (K_d = 42 nM) at 37°C leading to the assumption that exogenous, secreted TNF functions primarily through TNFR1 [7]. Upon binding to TNFR1, scaffolding proteins known as TNF receptor-associated factors (TRAFs) bind to the receptor and activate numerous downstream proteins such as the caspase family or nuclear factor-kappa B (NFκB) transcription factor. NFκB is comprised of at least two proteins p50/65 that translocate into the nucleus as transcription factors following ubiquitination of their inactivating protein

IκBα. TNF-induced caspase-8 is well known for its induction of apoptosis. In contrast, simultaneous NFκB translocation promotes cell survival, and together, these two pathways highlight the complexity of TNF signaling [8]. Along with TRAF2, TNF receptor-associated death domain (TRADD) and their fusion, TRAF2-TRADD, propagates this contradictory group of signals [9]. Consequently, it is held that TNF potentiates cell fate, which is predetermined by the balance of pro- and anti-apoptotic signaling environments. The diversity of signals instigated by TNF illustrates the situational dependence of TNFR activation; in some cases the cytokine may induce aberrant over-proliferation of tissue or in others its deterioration [10, 11]. TNF stimulation may also lead to no immediate cell fate unless the signaling environment is predisposed to an outcome through chemical inhibitors or protein agonists [11, 12]. Inhibitors, agonists and even other major signaling proteins such as the class I receptor tyrosine kinase, epidermal growth factor receptor (EGFR), are able to tip the scale of TNF signaling depending on their expression and constitution.

1.2 Epidermal Growth Factor Receptor

When it comes to cancer research and discovery, the epidermal growth factor receptor, also known as the erbb family of receptors, rarely goes overlooked. Decades of lab work and peer review have ensconced the EGFR so well into medical science culture that it has become a nexus for cancer biology and biochemical research [13-18]. Given its expression in epithelial, mesenchymal, neuronal and endothelial tissues, data outlining mechanisms behind the EGFR or erbb family may have global applications in the body.

The conventional wisdom surrounding EGFR activation involves three major steps: ligand binding, receptor dimerization and kinase phosphorylation leading to intra- and extra-cellular signal transduction. Ligands bind the EGFR in a manner that induces rapid

conformational changes of the receptor. This movement releases a "dimerization arm," a loop of amino acids that binds to other activated erbb receptors [19]. Once fused, the kinase region of one receptor activates the kinase region of its partner through phosphorylation of discrete tyrosine and serine residues. These residues for the most part are conserved across the erbb family with the exception of erbb-3, which is essentially deprived of an intact kinase region [20]. Tyrosine phosphorylation inevitably leads to GTP dependent signal transduction and second messenger production and release. Throughout the duration of stimulation, the EGFR may undergo endocytosis. While partially a regulatory event, this process activates proteins seated deeper in the cytoplasm or in the nucleus. Erbb-1 is particularly amenable to endocytosis; however, it has been shown that erbbs-2, -3 and -4 are relatively resistant to endocytotic regulation/signaling [21]. Once paired, the erbb receptors auto-phosphorylate cytoplasmic tyrosine residues in their respectable kinase regions. All four members of the EGFR or erbb family carry out these three steps such that erbbs 1, 2, 3 and 4 when ligand-bound must dimerize to become a relevant signaling entity [19]. This process has been elucidated biochemically by exhaustive crystal-computational studies, protein cross-linking, and even single molecule tracking on the plasma membrane [22-24].

Activation by direct ligand binding occurs through several soluble stimulants. Epidermal growth factor (EGF) is a strong inducer of EGFR activation. It is closely related in structure and function to transforming growth factor-alpha (TGFα), which is arguably the most natively potent ligand of the EGFR family. Heparin binding EGF (HB-EGF) and amphiregulin (AR) are also inducers of EGFR activation and players in constitutive EGFR signaling. In neuronal cells, neuregulin 1, 2, 3 and 4 are essential regulators of basal and induced EGFR activity. Finally, epiregulin, epigen and beta-cellulin have also demonstrated direct EGFR signaling capacity.

Studies have shown that the variety of direct inducers of erbb activation coincides with the diversity of erbb pairing and dimerization. Differences in ligand tertiary structure to some extent influence how an erbb receptor pairs with another and ultimately the overall pairing behavior [25-27].

While the EGFR is involved in a variety of processes, its capacity for tumorigenesis stems from roles in proliferation, cell survival, chemotaxis and differentiation. Direct activation of the EGFR has been shown to induce any one of these processes *de novo* showing the extent of control the EGFR has over cells as well as the necessity for effective regulation. EGFR dysregulation has been implicated in breast, colon, skin, lung, squamous-cell carcinoma, and pancreatic cancers, which prompted clinicians to develop methods that detect aberrant EGFR activity for the sake of early prognosis and preemptive treatment.

1.3 Inflammation and the EGFR

The EGFR family has a significant role in not only cancer but also TNF-mediated inflammation. TNF is by no means a direct activator of the EGFR; however, stimulation with the cytokine has been shown to upregulate phosphorylated EGFR *in vitro* and *ex vivo* [28, 29]. While inflammation is not immediately considered as related to cancer, their shared reliance on the EGFR and certain downstream proteins as well as their comparable physiologic manifestations make the connection some what intuitive. Inflammation is induced through any and all forms of stress whether mechanical, thermal, chemical or biological. For example, irritation in the skin leads to blood vessel dilation, cell proliferation, reorganization, ATP and cytokine release [30]. Incidentally, these iconic symptoms of inflammation are indistinguishable from symptoms of cancer; these are also features of hyper-proliferative tumors. Moreover, EGFR activation has been shown to upregulate transcription of inflammatory genes [31]. The

interleukin family of proteins is a target of EGFR-mediated inflammatory signaling, and interleukin-8, in particular, has been closely associated with the inflammatory response and may serve as a marker of inflammatory signaling [32-34].

This thesis asserts that EGFR activation has been under appreciated in TNF, inflammatory research. TNF has been linked to NFκB transcription factor activation. While NFκB is robustly induced by TNF stimulation, this association has produced a, perhaps, incomplete generalization in the literature that NFκB is the central and primary component of TNF-directed cell signaling, fate and disease. This work has set out to bolster the importance of TNF-induced EGFR activation in order to demonstrate its potential for central roles in inflammatory signal transduction and inflammatory disease irrespective of NFκB.

This study began as an assessment of TNF-induced EGFR in HaCaT keratinocytes, an immortalized keratinocyte cell line. While HaCaT keratinocytes are not primary keratinocytes, studies find this difference insignificant [35]. Direct activation of the EGFR in keratinocytes leads to increased cell proliferation [36]. TNF has also been shown to induce proliferation in EGFR expressing tissues [37]. However, keratinocytes have been previously reported to undergo apoptosis in the presence of TNF [38]. The accuracy of older cell counting methods to quantify let alone determine a system's proliferative state was suspect and motivated this work, in part, to assess TNF's ability to induce entry into cell cycle. It was hypothesized that TNF in HaCaT cells might induce proliferation through an EGFR-dependent mechanism. This study then focused on the mechanism by which TNF induces EGFR activation. TNF activates the EGFR through an indirect pathway, and since EGFR activation requires ligand release and binding, this study addressed the role of extracellular matrix-metalloprotease cleavage of tethered autocrine or paracrine ligands. TNF-mediated NFκB activation has been associated with pro-proliferative and

pro-survival mechanisms. Thus, it was hypothesized that TNF induction of the EGFR and promotion of NFκB translocation may be to some degree interdependent. The EGFR has also been implicated in TNF-mediated inflammation. TNF upregulation of interleukin-8 mRNA was assessed for EGFR and NFκB dependence. Finally, the EGFR family consists of four receptors. While erbb-4 is not expressed in HaCaT keratinocytes, erbbs-1, -2 and -3 are all significantly expressed [24]. This affords six distinguishable receptor pairs that may or may not have individualized, distinguishable downstream signaling behavior. This thesis proposes a method of decompiling the aggregate contributions of all possible dimer combinations using variable erbb inhibition and linear systems of equations. This method is applicable to any inhibitable, digitized output that bears some degree of EGFR dependence. For the purposes of a proof of concept, TNF-mediated, EGFR dependent IL-8 upregulation was examined given its intensity of induction and minimal basal expression.

2. Materials and Methods

2.1 Cell Culture

This work utilized epithelial, immortalized (HaCaT) keratinocytes. These cells in growth phase were incubated in 10% Fetal Bovine Serum/DMEM supplied by Thermo Scientific (HyClone) at 37°C and 5% ambient CO_2 until 90-100% confluence. All experiments were serum starved in .5% FBS/DMEM for 24 hours prior to treatments. All cellular passaging used HyClone trypsin in EDTA.

2.2 RNA Extraction & Real-time PCR

HaCaT keratinocytes were processed with the QIAShredder homogenization and RNeasy RNA extraction kits, which included a 15-minute DNase treatment. The kits and DNase were purchased from QIAGEN. Extracted RNA was then added to Super Script III Reverse

Transcriptase (along with necessary buffers/reagents) distributed by Invitrogen. The cDNA product underwent polymerase chain-reaction with SYBR Green PCR Master-mix (Applied Biosystems) and primers targeting Egr-1, IL-8 and/or A20 normalized by GAPDH or 18S baseline reaction. Comparative threshold counts ($\Delta\Delta C_T$) were performed by Applied Biosystems 7500 Fast Systems or Bio-Rad CFX96 real-time analysis of fluorescence. All procedures were carried out in line with manufacturer's instructions.

2.3 Western Blotting

Cellular cytoplasmic and nuclear protein fractionalization was conducted by PIERCE differential lysis buffer extraction (Thermo Scientific). Phosphatase and protease inhibitors supplemented the PIERCE kit solutions, and all steps were performed on ice or at 4°C. Samples were assayed for protein concentration, and 7.5 – 15ug of protein ran on Criterion 7.5/10% polyacrylamide gels purchased from Bio-Rad. The resultant protein separations underwent vertical column transfer onto nitrocellulose membranes. Membranes were stained with Ponceau S solution for visual consistency of protein transfer and blocked with 5% non-fat dairy milk. The blocked membranes were gently agitated overnight in primary antibody/5% NFDM solution at 4°C as instructed by antibody manufacturers. Phospho-p44/p42 and non-phosphorylated p44/p42 antibodies were purchased from Cell Signaling Technology; antibodies for egr-1 and p65 protein were purchased from Santa Cruz Biotechnology. Secondary antibody probing with HRP-conjugated goat, anti-rabbit or anti-mouse antibodies supplied by Bio-Rad was conducted at room temperature for durations and concentrations outlined by the manufacturer's suggestions. Immuno-blotting utilized Immun-Star HRP Chemiluminescent kit supplied by Bio-Rad, and film (Blue-Devil) exposure assayed illuminated membranes for protein content. In the absence of

non-phosphorylated Erk control, β-actin primary antibodies controlled for variations in loaded protein.

2.4 Cytokines & Pharmacologic Inhibitors

Purified, recombinant-human tumor necrosis factor-α and transforming growth factor-α were purchased from PeproTech. TNF units/milliliter (U/mL) concentrations were used in this work, and these conversions from mg/mL were calculated as instructed by PeproTech using a conversion equation for specific activity. The SA used for TNF was to 2 x 10^7 units/mg. Inhibiting pharmacologic small-molecules were utilized extensively in this work. These include the matrix metallo-protease inhibitor marimastat, the EGFR inhibitor PD 168393, the MEKII inhibitor PD 98059 and the proteasome inhibitor MG-132. Cells were pretreated for 30 minutes at 37°C and 5% CO_2 with the inhibitors prior to stimulation with cytokines. Inhibitors MG-132 and PD 98059 were reconstituted in dimethyl sulfoxide (DMSO). Using DMSO concentrations identical to those in reconstituted MG-132 and PD 98059, the DMSO vehicle shows no adverse or stimulatory effect on HaCaTs.

Neutralizing antibodies for erbb proteins were used in this work. Bristol-Myers Squibb manufactures the erbb-1 neutralizing antibody Erbitux. Genentech manufactures Herceptin, the erbb-2 neutralizing antibody. Millipore manufactures the erbb-3 neutralizing antibody H3.105.5. Studies have shown that all three agents are highly effective inhibitors at saturating concentrations (10 μg/mL) and highly discriminative against erbb proteins other than their target.

2.5 Annexin-V staining and Fluorescence Assisted Cell Sorting

HaCaT keratinocytes were starved for 72 hours in serum-free DMEM prior to treatment. Adherent cells were dissociated with 2.2 mM EDTA supplemented trypsin. The cells were washed with calcium chloride supplemented PBS to replenish free calcium, which is required for

proper Annexin-V binding to phosphatidylserine. Annexin-V conjugated to PE (BD Biosciences) and 7-AAD (BD-Biosciences) were added to unfixed cells and analyzed within an hour of staining. FACS analysis of apoptosis and necrosis was carried out in a FACSCalibur cell sorter (BD Biosciences). Raw data was analyzed by FlowJo flow cytometry analysis software (Ashland, OR). Histograms were gated for intact HaCaT keratinocytes by means of forward scatter, 7-AAD analysis. At least 7000 cells were analyzed in each histogram.

2.6 Inverse matrix evaluation of linear systems

All linear systems in this work were evaluated using the inverse matrix method. Linear systems of equations may be generalized by matrix multiplication of a coefficient matrix and a variable matrix, which is equated to a solution matrix. The coefficient matrix is an array of values wherein columns represent single variables and their coefficient in each equation by row. Its counterpart, the variable matrix, is a single column or row of corresponding variables to the row equations in the coefficient matrix. When multiplied together, the result is a solution matrix comprised of a vector matrix of solutions to the individual linear equations. For example, the following linear system may be broken down into three components:

$$4A+3B-2C = 12$$
$$B+4C = 3$$
$$6A = 19$$

$$\text{if } a = \begin{matrix} 4 & 3 & -2 \\ 0 & 1 & 4 \\ 6 & 0 & 0 \end{matrix}\; ; x = \begin{matrix} A \\ B \\ C \end{matrix}; d = \begin{matrix} 12 \\ 3 \\ 19 \end{matrix}, \text{ then } ax = d \text{ (eq. 1).}$$

Equation 1 describes the linear system as a multiplication of the coefficient matrix, a, by the variable matrix, x, which equates to the solution matrix, d. The goal of this process is to derive values for the variable matrix, x, that satisfy A, B and C when substituted into their parent linear equations. This study used the inverse matrix multiplication method:

$$\text{If } ax = d,$$
$$\text{then } a^{-1}ax = d\,a^{-1}.$$
$$\text{Since } a\,a^{-1} = I,$$
$$Ix = d\,a^{-1}.$$

I is the identity matrix and may be considered equivalent to 1. In effect, multiplying both sides by the inverse matrix, a^{-1}, will generate solutions for *x*, the variable matrix. This process was carried out using the Microsoft Excel command **=MMULT(MINVERSE(*a*),*d*)**, where *a* and *d* are the coefficient and solution matrices, respectively.

Dividing each variable solution by their corresponding uninhibited mRNA output generated percent contributions. This was performed to normalize the results across replicates.

2.7 Statistics

Two-tailed, paired Student's *t* tests were performed using Microsoft Excel where p-values are indicated.

3. Results

3.1 TNF induces expression of egr-1 in keratinocytes through an autocrine or paracrine, EGFR-dependent pathway

Keratinocyte stimulation by TNF activates several pathways including the NFκB and mitogen activated protein kinase (MAPK) pathways. Ultimately, activation of these pathways alters gene transcription. Many genes are activated exclusively by single pathway mechanisms, but several others rely on the contributions of more than one pathway. The degree of each contribution is particular to the cell-signaling network. TNF has been reported to upregulate egr-1 expression in mesenchymal and epithelial cells exclusively via an extracellular release kinase (Erk) MAPK dependent pathway [39, 40]. Therefore, this study assessed the role of Erk MAPK phosphorylation in TNF-induced, HaCaT egr-1 expression.

A time course was performed in order to characterize the role of TNF and egr-1 induction. HaCaT keratinocytes were stimulated with TNF for different durations that demonstrate a time-dependent relationship with expression magnitude. TNF upregulated Egr-1 protein levels as early as 45 minutes and decreased thereafter (Figure 1A). Likewise, egr-1 mRNA levels were upregulated 30 minutes post-treatment and decreased with time (Figure 1B).

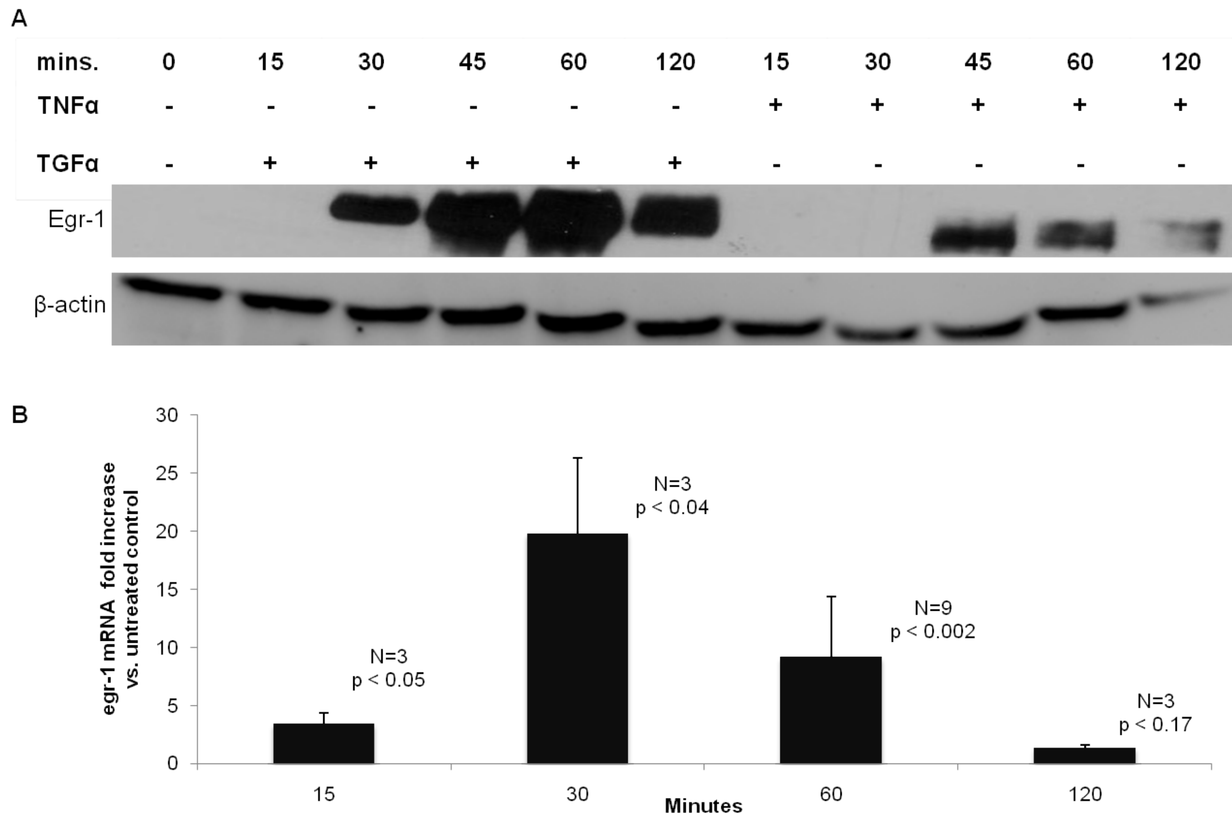


Fig. 1 TNF upregulates egr-1 protein and mRNA in HaCaT cells in a time dependent manner. HaCaT keratinocytes were starved for 24 hours in reduced serum and then treated with TNF (250 U/mL) or TGFα (5 ng/mL) as indicated. Nuclear cell extractions were immuno-probed for egr-1 and β-actin protein by western blot (A). Egr-1 mRNA fold increases were assessed by real-time RT-PCR at similar time points (B).

Egr-1 induction has been associated with EGFR activation [41, 42]. HaCaTs were treated with TNF along with small molecule inhibitors for the EGFR. Inhibition of egr-1 protein expression occurred following inhibition of the EGFR with the inhibitors AG 1478 (not shown) and PD 168393 (Figure 2B). Consistent with a central role of the EGFR, TNF induction of egr-1 mRNA

was also inhibited by PD 168393 (Figure 2A). In addition, neutralizing antibodies for erbb-1 (Erbitux) blocked egr-1 induction by TNF (data not shown).

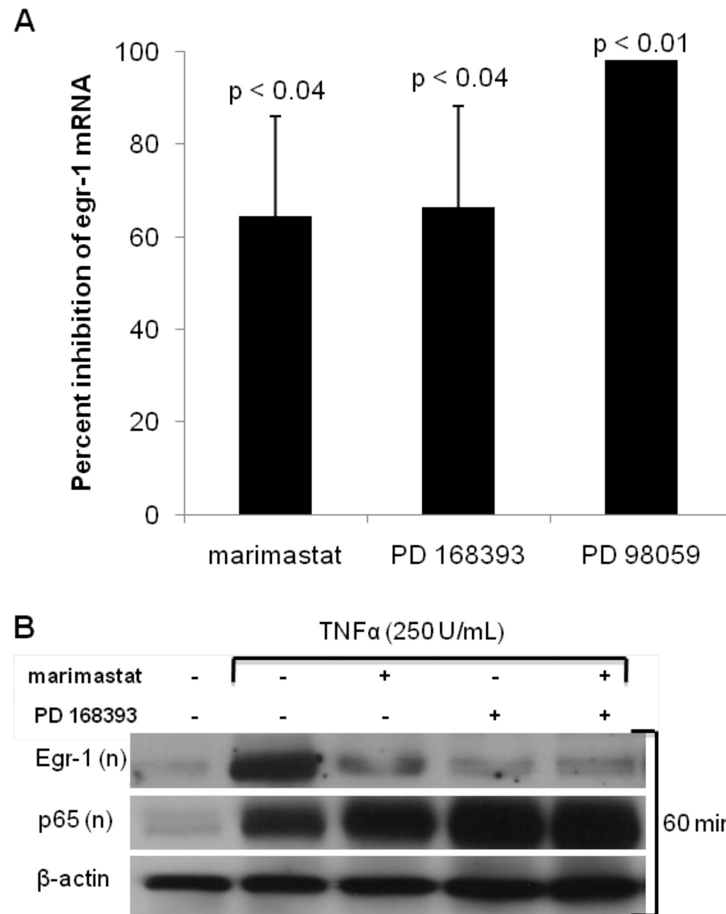


Fig. 2 Egr-1 protein and mRNA induction by TNF in HaCaT cells is dependent on MMP and EGFR signaling. HaCaT keratinocytes were stimulated with TNF (250 U/mL) for 1 hour following a 24-hour starvation in reduced serum and a 30-minute pre-treatment with either marimastat (10 μM), PD 168393 (62.5 nM) or PD 98059 (10 μM). Egr-1 mRNA upregulation was assayed by real-time RT-PCR analysis of egr-1 mRNA relative fold increases to an untreated control (A). Nuclear egr-1 and p65 protein upregulation was analyzed by western blot with a β-actin loading control (B); data are representative of three independent trials. mRNA data are the mean of three independent trials with error bars indicating standard deviation.

TNF is not a direct activator of the EGFR. Thus, the manner by which the cytokine activates the EGFR is of interest. Ligand-inducers of EGFR activity are limited in number and specific for the EGFR family. TNF must induce the release of these ligands in the extracellular space if it is to activate the EGFR. Based on observations in other cell systems [43], this study assessed the

dependence of TNF-induced egr-1 expression on matrix-metalloprotease (MMP) activity. This model would suggest that signal transduction through TNFR1 leads to an extracellular signaling cascade where MMPs might cleave EGFR-inducing ligands from the membrane and initiate autocrine or paracrine EGFR activation. MMP blockade was achieved by treatment with marimastat, a broad-spectrum MMP inhibitor. Consequently, marimastat reduced the expression of TNF-mediated egr-1 expression in both protein and mRNA transcription (Figures 2B and 2A). Near complete inhibition of egr-1 protein and mRNA was noticeable following inhibition of the EGFR and matrix-metalloproteases.

3.2 TNF induction of Erk MAPK phosphorylation is conveyed exclusively by an EGFR- and metalloprotease-dependent pathway

Prior studies have demonstrated the dependence of EGFR-mediated Erk phosphorylation on egr-1 upregulation [44]. This study examined TNF's capacity for Erk phosphorylation, and found this activity to be time-dependent. Phosphorylation following TNF treatment peaked at 15 minutes and cleared by 30 minutes (data not shown). The role of EGFR activation was again addressed by treatment of HaCaTs with PD 168393, which led to complete inhibition of TNF-induced Erk phosphorylation (Figure 3A).

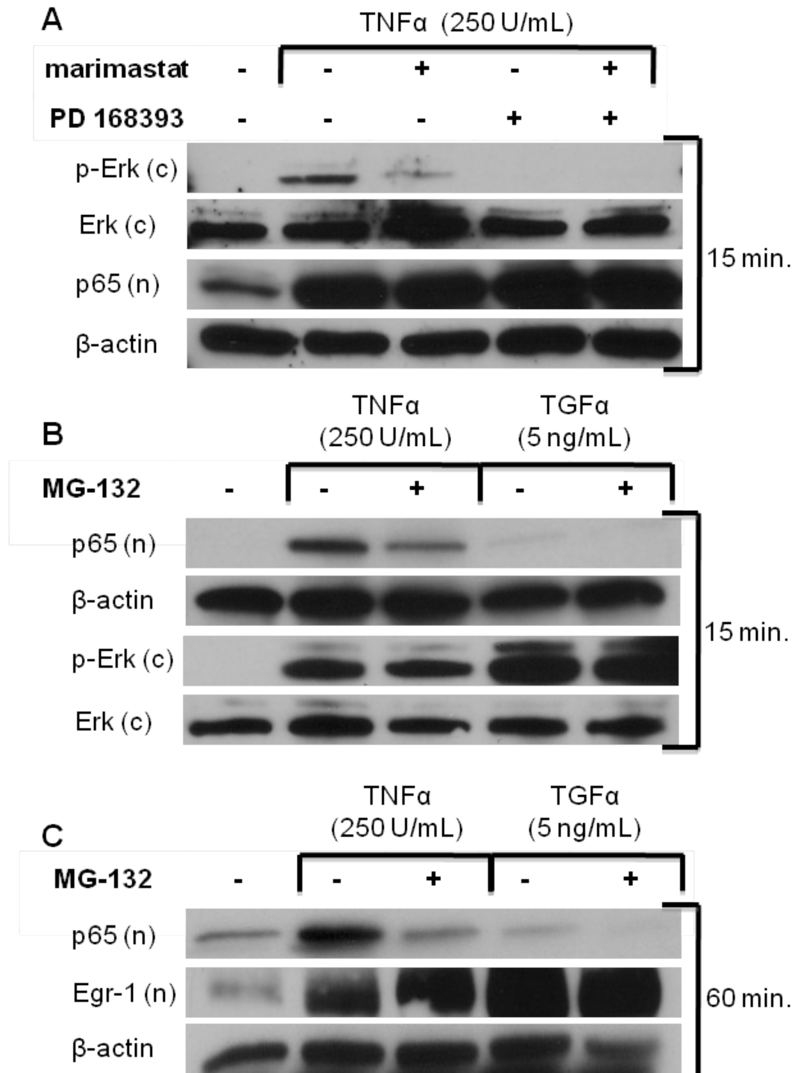


Fig. 3 TNF induces Erk phosphorylation in HaCaT cells-mediated by EGFR and MMP activity while Egr-1 induction and Erk phosphorylation are both independent of NF-κB activation. (A) HaCaT keratinocytes were starved for 24 hours in reduced serum, pre-treated with marimastat (10 μM) and/or PD 168393 (62.5 nM) for 30 minutes and then stimulated with TNF (250 U/mL). Cytoplasmic extracts were immuno-probed for phospho-Erk and non-phosphorylated Erk by western blot, and the complementary nuclear extracts were probed with anti-p65 and β-actin. (B) 24-hour serum starved HaCaT keratinocytes were pretreated with MG-132 (50 μM) for 30 minutes prior to a 15 minute (B) or 60 minute (C) stimulation by TNF (250 U/mL) or TGFα (5 ng/mL). Extracts stimulated for 60 minutes were probed for p65, egr-1, and β-actin nuclear protein by western blot (C). Extracts stimulated for 15 minutes were probed for phospho-Erk and non-phosphorylated Erk cytoplasmic protein along with p65 and β-actin nuclear protein by western blot (B).

The data involving egr-1 protein upregulation was consistent with a dependence on extra-cellular, matrix-metalloprotease activity. Treatment with marimastat prior to stimulation with TNF also led to a decrease in detectable phospho-Erk (Figure 3A). As a control, TGFα upregulation of phospho-Erk was attenuated solely by PD 168393 but not by marimastat (data not shown).

3.3 TNF-mediated induction of the EGFR/Erk/egr-1 pathway and NFκB activation occurs irrespective of each other

NFκB is known to influence pro-proliferative and pro-survival mechanisms [45, 46]. The EGFR/Erk/egr-1 pathway shows similar mitogenic capabilities, so it was logical to address whether or not concomitant activation of NFκB proteins and the EGFR were interdependent. TNF treated HaCaTs were pretreated with MG-132, a proteasome inhibitor known to block NFκB translocation of p65/50 proteins into the nucleus. Erk phosphorylation as well as egr-1 expression remained unaltered despite clear inhibition of p65 translocation (Figure 3A and 3B). Moreover, inhibition of TNF-induced EGFR/Erk/egr-1 expression by PD 168393 had no effect on p65 translocation (Figure 2B). MMP blockade also showed no effect on p65 translocation (Figure 2B). These data suggest that TNF signaling through NFκB and the EGFR/Erk/Egr-1 pathways in HaCaT keratinocytes are concomitant but diverge at a point prior to NFκB complex activation.

3.4.1 TNF-induced EGFR activity and inflammatory signaling

The EGFR has been implicated in acute and chronic inflammation. TNF is also closely linked to numerous chronic inflammatory diseases. Given the model proposed thus far, environments with concentrated TNF are likely to display EGFR activity. This study addressed

two markers of inflammatory signaling: cellular proliferation and induction of interleukin-8 mRNA.

3.4.2 TNF-mediated, EGFR-dependent proliferation and apoptosis

The chronic inflammatory disease psoriasis manifests in patients by thickened, scaly, hyper-proliferative keratinocytes that are often uncomfortable. These plaques on the skin are impactful and lead to significant reduction in patient quality of life [47]. Anti-TNF therapy has been shown to reduce the severity of these plaques and abolish almost all signs and symptoms of psoriasis [48]. It is possible that EGFR activation, which is closely linked to mitogenic pathways where in some cases direct EGFR activation, may lead to quantifiable increases in cell proliferation in patients with psoriasis. This study addressed whether or not TNF-induction of the EGFR/Erk pathway was sufficient for HaCaT cell cycle activation. HaCaT keratinocytes treated with TGFα were stained for the presence of nuclear ki-67, a nuclear marker of active proliferation. These EGFR active cells demonstrated clear upregulation of ki-67 by FACS analysis (Figure 5.1C, F).

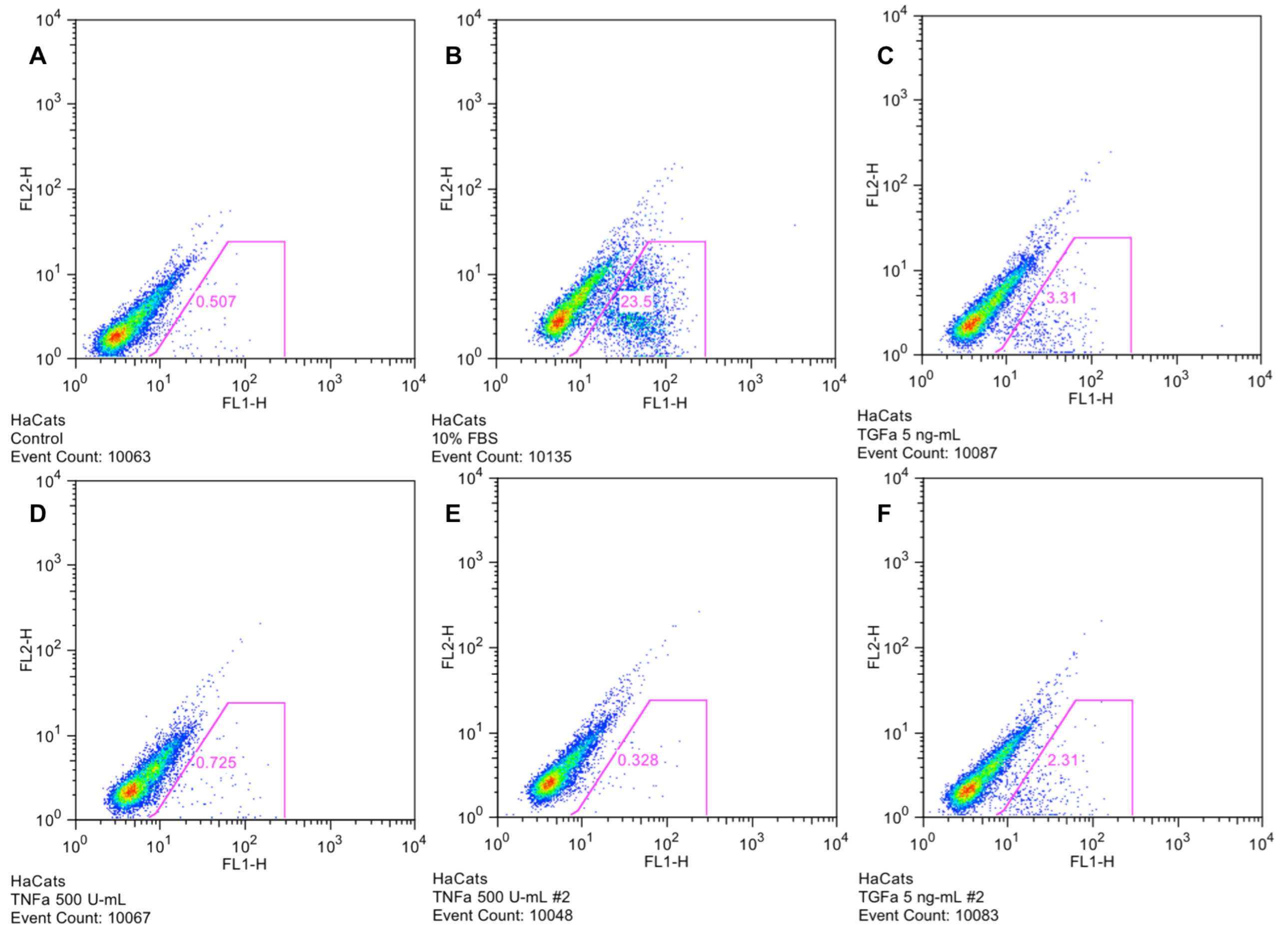


Fig. 5.1 TNF induced EGFR activation does not induce onset of cell cycle. HaCaT keratinocytes were starved in serum-free media for 72 hours before treatment with for 48 hours. Cells were harvested with trypsin, fixed in 75% ethanol at -20°C and then stained with ki-67/Alexaflour488. The cells were subjected to FACS analysis. Positive ki-67/Alexaflour488 signal corresponds to FL-1. (A) Untreated control; (B) 10% FBS treated; (C) TGFα treated (5 ng/mL); (D) TNF treated (500 U/mL); (E) TNF (500 U/mL) duplicate; (F) TGF (5 ng/mL) duplicate. Figure is representative of three trials.

Moreover, treatment with 10% growth media markedly increased ki-67 nuclear expression (Figure 5.1B). TNF, however, failed to induce significant ki-67 expression suggesting that activation of the EGFR/Erk pathway by TNF is insufficient for cell cycle activation (Figure 5.1D-E). TNF is known to potentiate both proliferation and apoptosis; therefore, this study assessed the apoptotic state of cells treated with TNF. Treated cells were stained with conjugated

Annexin-V-PE and revealed a significant degree of apoptosis over unstimulated cells (Figure 5.2A and 5.2B).

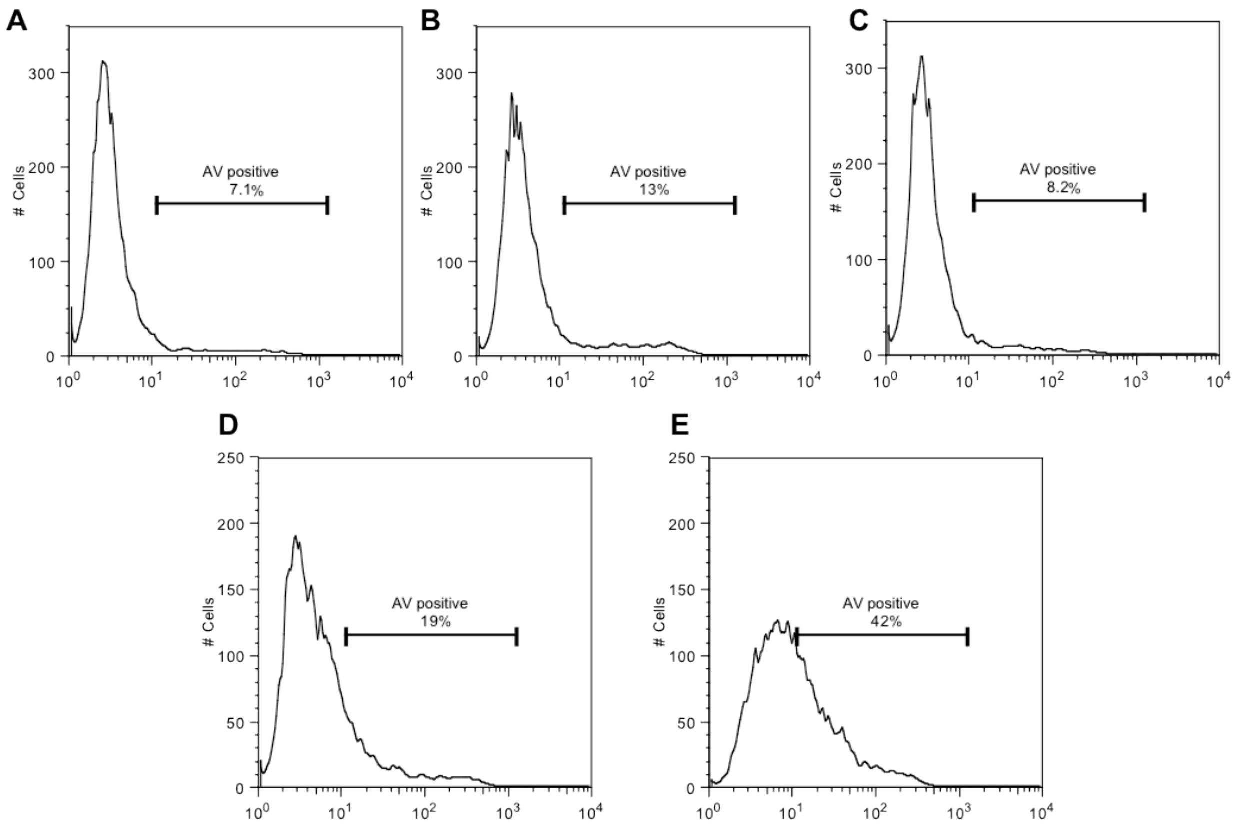


Fig. 5.2 EGFR blockade augments TNF-induced apoptosis whereas EGFR activation rescues cells from TNF-induced apoptosis. HaCaT keratinocytes were starved for 72 hours without serum, pretreated with 10 µg/mL of Erbitux EGFR/erbb-1 blocking antibodies for 30 minutes and then treated for 48 hours with TNF (500 U/mL) or TGFα (5 ng/mL). Samples were trypsinized, washed, and stained with Annexin-V-PE. Positive controls for AV-PE were used for compensation. Positive FL-2 (AV) represents apoptotic populations. (A) Untreated control showing basal levels of apoptosis; (B) Apoptosis following TNF treatment for 24 hours; (C) Combined TNF and TGFα treatments for 24 hours; (D) 24-hour treatment of Erbitux alone; (E) Erbitux combined with TNF for 24 hours. Figure is representative of four independent trials. Each histogram contains >7000 cells.

Pretreatment of cells with Erbitux inhibited TNF-induced EGFR activation; these cells showed a dramatic increase in apoptosis over control, TNF-free cells (Figure 5.2D and 5.2E). These data suggest that while TNF induction of the EGFR is incapable of instigating mitosis, it appears, nonetheless, protective from TNF-mediated apoptosis in HaCaT keratinocytes. Moreover,

treatment of cells with both TNF and TGFα repressed TNF-induced apoptosis and reduced the Annexin-V-PE signal to control levels (Figure 5.2C). Thus, additional EGFR activation by exogenous addition of TGFα to TNF-stimulated HaCaTs blocked impending apoptosis.

3.4.3 TNF induction of the EGFR is required for optimal interleukin-8 mRNA upregulation

Using PD 168393, it was shown that TNF and TGFα-induced egr-1 mRNA upregulation is conducted virtually exclusively through an EGFR/Erk mechanism. Moreover, egr-1 expression was unaffected by altered NFκB signaling. In contrast, TNF but not TGFα significantly-induced TNFIP3 (A20), an inducible, NFκB dependent gene linked to apoptosis and psoriasis pathophysiology by genome wide association studies (GWAS) [49]. Moreover, MG-132 specifically blocked TNF-induced A20 expression and left egr-1 expression unaltered (Figure 6B and 6C). PD 168393 pretreatment had no effect on TNF-mediated A20 mRNA upregulation (Figure 6C).

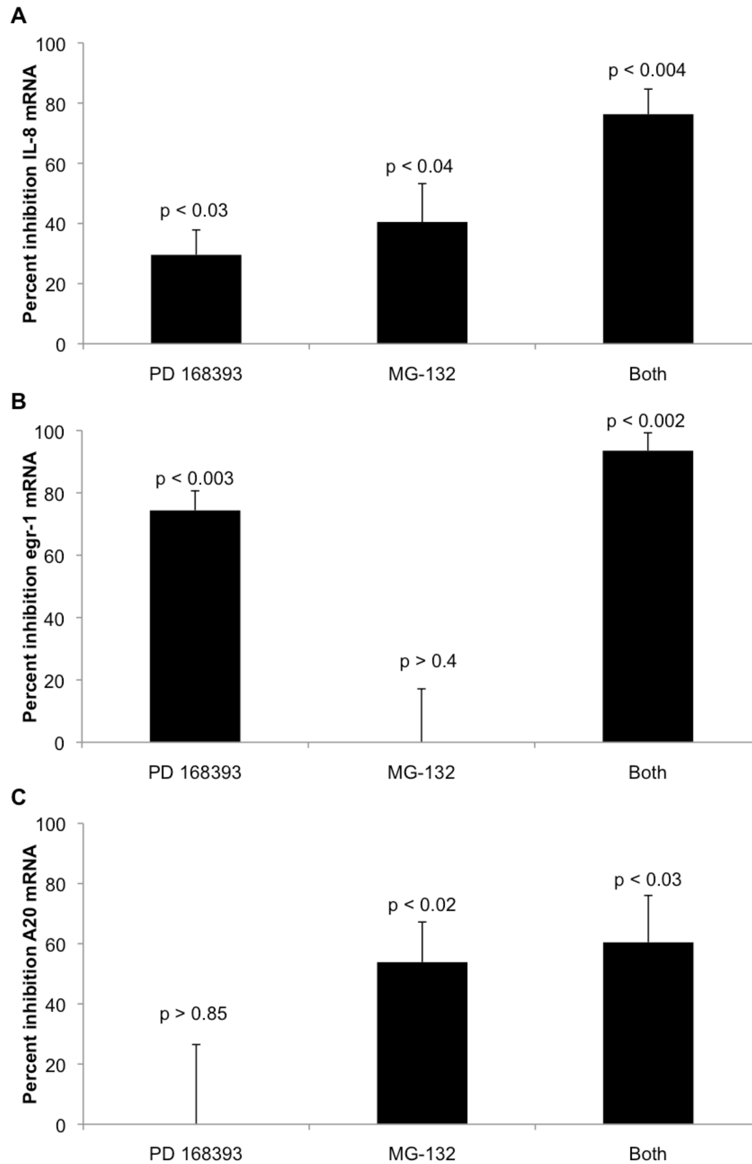


Fig. 6 TNF induces IL-8 mRNA transcription in HaCaT cells with contributions from both NF-κB and EGFR pathways. 24-hour serum starved HaCaT Keratinocytes were pretreated with PD 168393 (62.5 nM) and/or MG-132 (50 μM) for 30 minutes prior to a 1-hour treatment with TNF (250 U/mL). Human primers specific for IL-8 (A), egr-1 (B), and TNFAIP3 (A20) (C) cDNA measured mRNA upregulation relative to an untreated control. Each figure represents the mean of three independent trials with error bars for standard deviation.

While A20 and egr-1 expression rely on single pathway mechanisms following TNF stimulation, interleukin-8 (IL-8) has a shared reliance on both the NFκB and EGFR/Erk pathway. Stimulation of HaCaTs with TNF induces marked levels of IL-8 mRNA transcription, which is inhibited in part by PD 168393 (Figure 6A). In addition, MG-132 pretreatments attenuated IL-8 mRNA

transcription, and the combination of PD 168393 and MG-132 resulted in additive IL-8 mRNA inhibition (Figure 6A). Treatment with PD 98059, a direct inhibitor of Erk phosphorylation, showed near total inhibition of TNF-mediated IL-8 induction. These data suggest that TNF-induced IL-8 mRNA upregulation is reliant on both the NFκB and EGFR/Erk pathways.

3.5 TNF activation of the erbb receptor network may have individual and distinguishable contributions towards IL-8 mRNA transcription

The EGFR family consists of four erbb receptors: 1, 2, 3 and 4. Upon activation, these receptors must dimerize. No inherent restrictions in pairing have been reported, and many studies stress the plurality and freedom of possible erbb receptor pairing combinations. The necessity of erbb proteins to dimerize coupled with the EGFR dependence of IL-8 mRNA transcription prompted this study to pursue the complexity of this element.

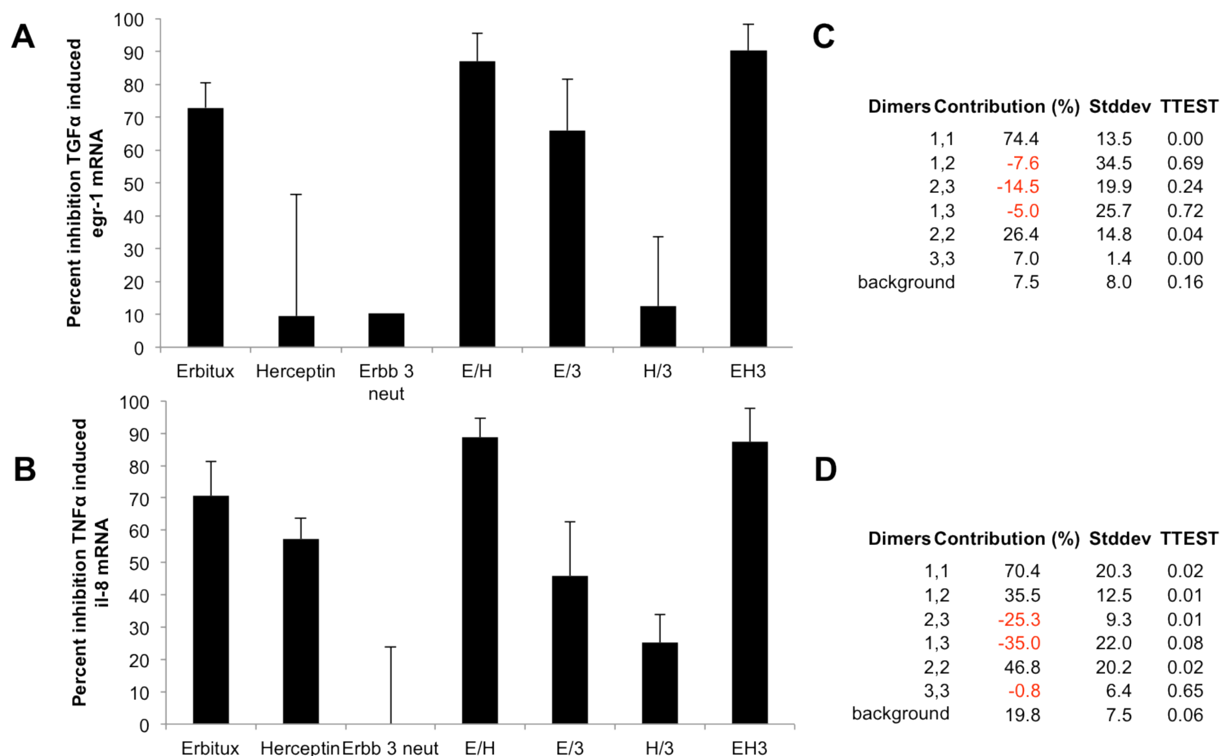
Four possible receptors may dimerize following reception of EGFR inducing ligand. This affords 10 different receptor pairs in the fully expressed erbb environment. HaCaT keratinocytes, however, lack erbb-4 expression [50], and thus, erbb-4 was removed from consideration leaving 6 possible pairs. The remaining pairs are 1,1; 1,2; 2,2; 1,3; 2,3; and 3,3. Inhibition of any one of these receptor pairs will generate a change in output mRNA levels to a degree consistent with their capacity to induce IL-8 mRNA induction and their concentration. Inhibition was achieved with monoclonal antibodies that target individual erbb proteins. Upon stimulation, some amount of IL-8 mRNA will be transcribed. This output may be equated to a linear combination of all receptor pairs that individually contribute to some degree. For this purpose, the equation $A + B + C + D + E + F + G = T$, where T represents the uninhibited, TNF-induced IL-8 mRNA transcription output. A-F are variables for the individual contributions of 1,1; 1,2; 2,2; 1,3; 2,3; and 3,3 receptor pairs that may or may not contribute to the IL-8 readout. G is included as a

measure of background, EGFR-independent induction of IL-8 mRNA transcription. If the system is treated with Erbitux, an erbb-1 specific neutralizing antibody, erbb-1 is unable to receive a ligand, unable to dimerize, and is, consequently, removed from the signaling economy. Thus a new equation: C + E + F + G = Erb, where Erb represents the erbb-1 inhibited IL-8 mRNA output. Commercial therapeutics are available for erbb-2; Herceptin or (Trastuzumab commercially) is an erbb-2 specific neutralizing antibody. Treatment with Herceptin removes erbb-2 from the signaling environment much like Erbitux and erbb-1. A + D + F + G = Her, where Her represents the erbb-2 inhibited mRNA output. Treatment with an erbb-3 neutralizing antibody produces the analogous equation A + B + C + G = 3, where 3 represents the erbb-3 inhibited output. These first four equations (T, Erb, Her and 3) are the precursors to a linear system of equations. A solvable linear system matrix with seven variables requires seven equations and, consequently, seven inhibitory conditions. The remaining three equations may be produced by double inhibition. A + G = Her/3; C + G = Erb/3; F + G = Erb/Her and G = Erb/Her/3 make up the remaining possible inhibitory conditions and corresponding equations. While eight equations are available, only seven are necessary, so “3” was omitted from initial analysis; however, permutational swaps with “3” for each equation all produced the same qualitative results. In accordance with the process described in the methods section, coefficient, variable and solution matrices were produced (Figure 7).

	1,1	1,2	2,3	1,3	2,2	3,3	Back			
No Inhibitor	1	1	1	1	1	1	1	19999.30	11210.03851	**A**
Erbitux	0	0	1	0	1	1	1	5964.71	6713.624098	**B**
Herceptin	1	0	0	1	0	1	1	10375.38	-2925.99633	**C**
E/H	0	0	0	0	0	1	1	3054.41	-3889.071753	**D**
E/3	0	0	0	0	1	0	1	8728.32	5836.300166	**E**
H/3	1	0	0	0	0	0	1	14102.05	162.3917924	**F**
EH3	0	0	0	0	0	0	1	2892.02	2892.016462	**G**
	Coefficient matrix, *a*							Solution matrix, *d*	Variable matrix, *x* in bold with accompanying solutions	

Fig. 7 Variable inhibition and inverse matrix multiplication decompile the "no inhibitor" signal into individual erbb pair contributions. Example above is a replicate of matrix-inhibited TNF-induced IL-8 expression. Solution matrix, *d*, is the raw data corresponding to the inhibitory conditions specified on the far left. Binary coefficients in *a* were used to indicate the presence or absence of a variable for a given inhibitory condition. The resulting solution of the variable matrix is a breakdown of the uninhibited raw signal; summation of each solution will equate to 19999.30, the uninhibited signal. Replacement of any one equation with "3" does not alter the provided observations. mRNA signal is in units of fold increase over unstimulated control.

Solving for the variable matrix by means of the inverse matrix method produced contributions for each receptor pair (Figure 8).



Dimers	Contribution (%)	Stddev	TTEST
1,1	74.4	13.5	0.00
1,2	-7.6	34.5	0.69
2,3	-14.5	19.9	0.24
1,3	-5.0	25.7	0.72
2,2	26.4	14.8	0.04
3,3	7.0	1.4	0.00
background	7.5	8.0	0.16

Dimers	Contribution (%)	Stddev	TTEST
1,1	70.4	20.3	0.02
1,2	35.5	12.5	0.01
2,3	-25.3	9.3	0.01
1,3	-35.0	22.0	0.08
2,2	46.8	20.2	0.02
3,3	-0.8	6.4	0.65
background	19.8	7.5	0.06

Fig. 8 TNF and TGFα stimulate erbb pairing; variable erbb inhibition sheds light on subsequent downstream IL-8 and egr-1 transcription reliance on erbb dimerization behavior. 48-hour serum starved HaCaT Keratinocytes were treated with Erbitux, Herceptin, Erbb 3 neutralizing antibody or a combination thereof for 1 hour at 10 µg/mL prior to a 1-hour treatment with TNF (250 U/mL) or TGFα (5 ng/mL). Human primers specific for egr-1 (A) and IL-8 (B) cDNA reflect mRNA upregulation over an untreated control. Each figure represents the mean of three independent trials with error bars for standard deviation. "Erbb 3 neut" in (B) maintains a negative percent inhibition at -19%. "Erbb 3 neut" in (A) has yet to be replicated. Tables (C) and (D) contain the outputs of inverse matrix multiplication from (A) and (B) respectively. Contributions in (%) normalized data across three or more replicates. "TTEST" is the p-value from paired, two-tailed Student's *t* test against an array of zeros.

The predominating contributor to IL-8 mRNA upregulation was the erbb-1 homodimer followed by the erbb-2 homodimer and the 1,2 heterodimer. Heterodimers 1,3 and 2,3 produced largely negative contributions, and the erbb-3 homodimer was essentially non-contributive. Finally, the background variable, G, was consistently contributive.

4. Discussion

Historically, TNF was discovered for its ability to reduce tumor size and density. This effect relied on its ability to induce rapid apoptosis in amenable tissues, and it was popularized as

a Coley Toxin with respect to its discoverer Dr. William Coley [51]. TNF would be recognized solely as a pro-apoptotic factor for some time after. As a consequence, TNF was hardly regarded as a driver of hyper-proliferation in chronic inflammation. In fact, studies assessed the therapeutic value of TNF whereby the cytokine might perform as a Coley Toxin and abolish these tumor-like lesions. These patients responded terribly to the application of TNF to these growths [52], which solicited a recapitulation of both TNF as a tumor necrosis factor and the keratinocyte make-up of psoriatic plaques. This began with the observation that patients undergoing anti-TNF treatment for inflammatory bowel disease saw a rapid decrease in co-existent, psoriatic symptoms. This, in turn, necessitated a broadening of scope and re-characterization of TNF signaling especially in keratinocytes and chronic inflammatory disease.

TNF driven inflammatory disease and key cellular mediation have been studied extensively. TNF is highly involved in immune response and function. Consequently, induction of psoriasis is often attributed to macrophage and T-cell activity and in some studies fibroblasts and endothelial cells. Incidentally, psoriatic plaques are comprised of keratinocytes. Whether or not TNF has direct effects on keratinocytes, irrespective of immunocytes, and whether or not these effects are dependent on the EGFR and/or NFκB were essential questions of this thesis.

Egr-1 was used as a marker for EGFR activity given its purported linkage in prior studies. TNF upregulated Egr-1 protein and mRNA motivated a follow-up study addressing TNF-mediated EGFR activation. The EGFR's role in proliferation has been characterized exhaustively in other cellular contexts; thus, considerations were made to incorporate a model that includes EGFR-mediated proliferation as well as TNF-induced inflammation in keratinocytes. It was hypothesized that TNF-mediated, EGFR activity was sufficient to initiate cell cycle and to optimally induce expression of the inflammatory gene, interleukin-8. Subsequently, it was

determined that TNF does indeed have direct effects on keratinocytes that might contribute to inflammatory pathophysiology. Egr-1 protein accumulation and mRNA expression by TNF was highly reliant on the EGFR as determined by small molecule inhibition of the EGFR and neutralizing antibodies. Additionally, Egr-1 protein and mRNA were completely dependent on phosphorylation of Erk proteins, which furthered the necessity to address a role for the EGFR. Egr-1 was thus a veritable marker for EGFR activity in HaCaT cells dependent on an EGFR/Erk/egr-1 signaling paradigm.

TNF is not a direct activator of the EGFR, and thus, mediators such as extracellular metalloproteases might play a role. It was found that the inhibition of MMPs with small molecules implied a model whereby protease cleavage of direct inducers of the EGFR from the membrane would promulgate this cascade. This stresses an inside-out signaling dynamic. EGFR ligands in this event could return to the membrane and continue the signal (autocrine) or activate neighboring cells with expression of the EGFR (paracrine). Amplification of signal through a second-messenger (i.e. TGFα) illustrates TNF's potential to induce acute cellular stimulation and, in the context of invariant TNF concentration, chronic stimulation. EGFR ligands are essential for long-term keratinocyte survival [12]. This requires constitutive reconstitution of membrane bound TGFα to some degree providing for perpetual TNF signaling and dysregulation. The protease dependence demonstrated in this work is not wholly novel; nevertheless, this inside-out model has been appreciated as a G-protein dependent phenomenon and not one directly involving the EGFR which encouraged its reassessment in this study [53]. TNF has been shown to release TGFα from epithelial cells [54], and this observation reinforces the data provided herein of an indirect MMP-dependent mode of EGFR activation.

TNF activation of NFκB transcription factor proteins has been highly characterized. The co-evolution of TNF and NFκB research has produced an understanding that NFκB is a central component in the maintenance of TNF signaling. Given similar roles in proliferation and survival as well as TNF's reliance on NFκB, both the EGFR and NFκB proteins were hypothetically associated. However, small molecular inhibition of the EGFR did not affect NFκB translocation of activated proteins into the nucleus. Moreover, inhibition of NFκB by small molecules had no affect on the EGFR/Erk/egr-1 pathway. Contrary to previous assumptions, NFκB and the EGFR bear no interconnectivity aside from their shared responsiveness to TNF treatments. This prompts a reassessment of the importance and centrality of NFκB in TNF signaling. In the prior model, TNF induction of the EGFR would in some part be amenable to NFκB inhibition. These data, however, discount the universality of this paradigm, elevate the EGFR in inflammatory signaling, and stress the plurality of TNF-signaling as a better paradigm to replace the prior.

Since TNF-mediated NFκB activation and EGFR transactivation are distinct, independent pathways, the question of the EGFR's function in this signaling environment remained. Knowing that direct EGFR activation is sufficiently mitogenic, it was hypothesized that TNF stimulation of the EGFR might lead to cell proliferation. Ki-67 staining and analysis following addition of TNF revealed otherwise. On the contrary, TNF induces limited apoptosis in HaCaTs as assessed by Annexin-V staining. It was subsequently concluded that TNF-mediated, EGFR activity in keratinocytes is insufficient for induction of the cell cycle. While this observation does not entirely support the original hypothesis, the addition of TGFα to TNF treated cells rescued cells from apoptosis. Furthermore, targeted EGFR neutralization antibodies promoted a dramatic TNF-mediated apoptotic response. These data suggest a nuanced role for the EGFR particularly in the context of TNF signaling. Here, the EGFR is effectively recalibrated as an inducer of

survival and not proliferation. Studies in other cell lines support this re-characterization of the EGFR [29].

The question of the EGFR's function was also addressed with regards to upregulation of inflammatory genes. Interleukin-8 mRNA upregulation was chosen to examine a potential role for EGFR-mediated alterations in *de novo* transcription of inflammatory genes. Using both small molecules and neutralizing antibodies, it was determined that TNF-induced IL-8 expression is dependent on EGFR/Erk activity. Moreover, total erbb receptor inhibition by combining erbb-1, 2 and 3 antibodies reduced the IL-8 mRNA upregulation by 90%. On the other hand, NFκB inhibition by small molecules also inhibited IL-8 mRNA upregulation. Together, the NFκB inhibition data as well as the pan-erbb inhibition data imply the duality of the IL-8 signaling pathway. Incidentally, treatment with TGFα alone was fully incapable of inducing any IL-8 mRNA transcription despite the proposed EGFR connectivity. However, in this study as well as others combined treatment of TGFα with TNF synergistically augmented IL-8 mRNA content in cells over those treated with TNF alone [54]. While the NFκB transcription factor and the EGFR contribute to IL-8 expression, it is apparent that these pathways are mutually sufficient and individually insufficient for optimal IL-8 expression. Despite the lack of well-defined pathway overlap, these pathways share transcriptional endpoints to some extent; moreover, concomitant activation of NFκB and the EGFR is to an extent a significant event. The plausibility of these conclusions rests in data regarding the promoter region of IL-8. The IL-8 promoter bears two pertinent binding sites: an AP-1, EGFR-relevant one and an NFκB one; both must be occupied in order to recruit transcriptional proteins and induce optimal genetic activity [55, 56]. EGFR inhibition was shown to induce near total inhibition of IL-8 mRNA. Given the data concerning the IL-8 promoter, it is plausible that total NFκB inhibition would blunt IL-8 mRNA expression.

Lastly, this thesis proposed a method that elucidates individual erbb dimer contributions to IL-8 mRNA signaling. The matrix data suggests the following observations: erbb-1 homodimers are highly contributive; erbb-1,2 heterodimers are highly contributive; erbb-2 homodimers form and are capable of IL-8 signaling; erbb-3 homodimers are non-contributive; erbb-3 heterodimers were negatively contributive; and an EGFR independent mechanism is statistically significantly contributive. Without other forms of verification, it is difficult to appreciate the magnitude of individual contributions; nevertheless, qualitative conclusions may be drawn from these data and are supported by other studies.

Erbb-1 is highly expressed in EGFR bearing cells and is receptive to the four main EGFR binding ligands in keratinocytes: TGFα, EGF, HB-EGF and AR [27]. Since TNF fails to activate erbb receptors directly, it is likely that ligand shedding to some extent contributes to the IL-8 signal. Furthermore, IL-8 mRNA was synergistically upregulated with combinatorial stimulation by TGFα onto TNF, which suggests a ligand-dependent element to this signal.

However, treatment with the MMP inhibitor marimastat failed to produce significant inhibition of TNF-induced IL-8 expression. This can be potentially explained by the duplicitous modes of EGFR activation and IL-8. While a ligand dependent, erbb-1-driven mechanism is certainly a component, there also may be a concomitant ligand-independent component driven by erbb-2.

Studies have implicated the Src kinase in ligand-independent, TNF-driven activation of the erbb-2 kinase [37]. Erbb-2 lacks a ligand-binding region and maintains a perpetually open dimerization arm [19]. This affords great liberty in erbb-2 signaling as its activation occurs shortly after binding to similarly active receptors. In addition, this scenario suggests the potential for erbb-2 homodimers, which the matrix data suggests as well. Ultimately, it is probable that the

ligand-dependent component of IL-8 expression is merely unresponsive to marimastat, which is also suggested by data with a different inhibitor of matrix-metalloproteases, TAPI-2. TAPI-2-induced ~50% inhibition of IL-8 mRNA expression by TNF although this experiment has not yet been replicated. Marimastat may not be as broad of an inhibitor as assumed.

Erbb-3 heterodimers were negatively contributive. This may be explained by the tendency erbb-3 has in reducing optimal erbb pairing. It was shown that erbb-1, erbb-2 homodimers and erbb-1,2 heterodimers where highly contributive. Given erbb-3's lack of kinase, pairing with erbb-1 or erbb-2 would theoretically reduce the pool of optimal IL-8-signaling erbb pairs. This conclusion would benefit by matrix inhibition of cell lines that support TNF-mediated IL-8 expression and express greater amounts of erbb-3. Alternatively, over-expression assays of erbb-3 in HaCaT keratinocytes may augment this phenomenon.

The matrix data demonstrated statistically significant EGFR-independent signaling. While miniscule, this signal may be a result of imperfect inhibition, subtle NFκB signaling to both AP-1 and NFκB promoter regions or AP-1 independent regions.

If verified, this matrix presents a quantitative outlook on erbb pairing behavior. Inevitably, any conditions that accompany matrix inhibition will be linked to an EGFR receptor profile. Verifiable inhibition profiles allow a researcher to deduce and decompile the signaling profile of any system with unknown erbb receptor behavior. In the case of this thesis, TGFα-induced egr-1 mRNA upregulation was subject to matrix inhibition. While the data requires further replication to rule out statistical uncertainty, averaged results converge on reasonable and verifiable observations (Figure 8). TGFα-induced egr-1 mRNA upregulation was highly reliant on erbb-1 homodimers. This is consistent with many studies that demonstrate TGFα's capacity to activate erbb-1 directly. In one study, a cell-line natively EGFR-free was transfected by

Lenferink, *et al.* with erbb-1 DNA. Subsequent TGFα treatment greatly enhanced proliferation presumably through erbb-1 homodimers [26]. Co-transfection with erbb-2 DNA showed no significant increase in proliferation by TGFα; however, EGF augmented cell proliferation with erbb-1 alone and more so in co-transfected, erbb-1 and -2 cells. Lenferink's work suggests that TGFα is unable to facilitate erbb-1,2 heterodimerization. The matrix data provides that erbb-1,2 is non-contributive to TGFα stimulated egr-1 expression. The same study concluded that TGFα was incapable of erbb-2,3 heterodimerization, which is also consistent with matrix data.

Lastly, erbb-2 homodimers were contributive to TGFα-induced egr-1 expression according to the matrix; given erbb-2's inability to bind ligands, TGFα should not induce direct erbb-2 activation. This may best be explained by experimental error, statistical uncertainty and/or altered background egr-1 expression. Egr-1 maintains some amount of unstimulated, basal expression, which is likely the cause of experimental inconsistency. Single neutralizing antibody, stimulant-free controls were run in all matrix studies in order to assess background egr-1 expression. However, combined antibody controls have not been performed and may further improve these data. This data point will improve with additional replications.

In both matrix studies, erbb-3 homodimers were non-contributive. This is expected due to erbb-3's lack of an intact kinase. Nevertheless, an impaired kinase does not preclude homodimerization of erbb-3. This prompts an evaluation of matrix "contributions." At least two elements affect an erbb pair's contribution to downstream events: the quantity of receptor pair and the capacity to affect the output gene transcription. While erbb-3 homodimers may fail to contribute to gene expression given their impotency, the absence of contribution of other erbb pairs may be regarded for either their lack in quantity, signaling capacity or both.

5. Conclusions

TNF induces indirect, EGFR activation that upregulates Erk phosphorylation and egr-1 expression. This induction is metallo-protease dependent. TNF upregulates IL-8 expression, which is a product of simultaneous EGFR and NFκB activity. In HaCaT keratinocytes, the EGFR and NFκB bear no interconnectivity, which is contrary to current TNF-NFκB perspectives. Mutual activation, however, is required for certain alterations in transcriptional environment that are withheld in non-mutual activation scenarios. Figure 9 illustrates these observations.

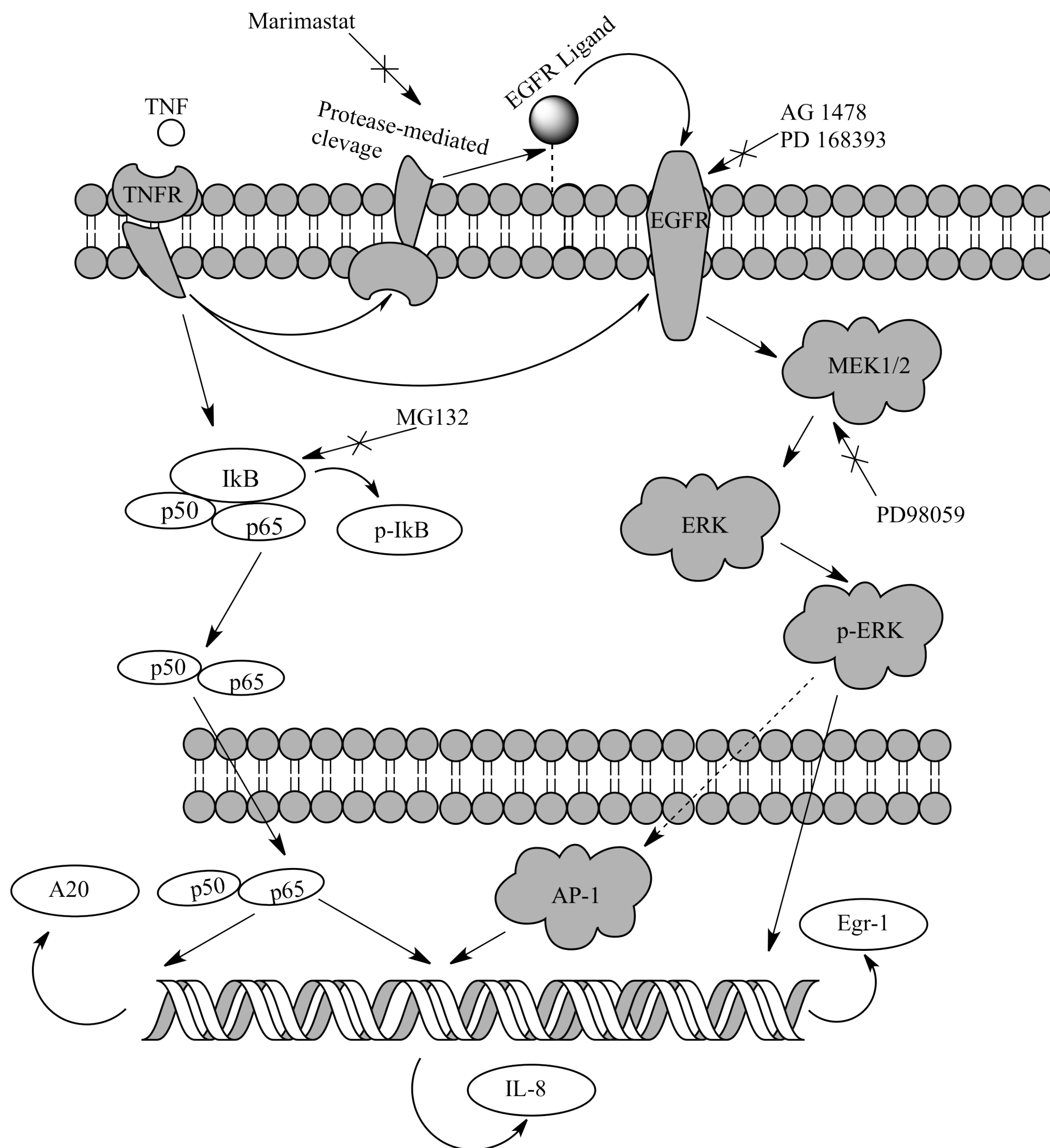


Fig. 9 Proposed TNF-stimulated NFκB and EGFR signaling pathways in HaCaT keratinocytes. Beginning with TNF reception, ligand dependent and independent EGFR/Erk activation leads to altered gene transcription. Likewise, TNF induces NFκB translocation into the nucleus providing additional alterations in genetic activity. These pathways are distinct and independent. Solid lines represent observed connections in signaling pathways while dotted lines represent presumed connections.

TNF-mediated EGFR activation in keratinocytes is insufficient for onset of cell cycle. However, EGFR activation counteracts TNF-induced apoptosis promoting cell survival. This provides a mechanism by which psoriatic plaques override TNF-induced apoptosis. Lastly, a method of erbb matrix inhibition suggested that ligand-dependent and ligand-independent, erbb-2-dependent pathways are involved in optimal IL-8 expression through the EGFR.

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