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Photosensitivity and type I IFN responses in cutaneous lupus are driven by epidermal derived interferon kappa

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Abstract

Objective: Skin inflammation and photosensitivity are common in cutaneous (CLE) and systemic lupus erythematosus (SLE) patients, yet little is known about the mechanisms that regulate these traits. Here we investigate the role of interferon kappa (IFN-κ) in regulation of type I IFN and photosensitive responses and examine its dysregulation in lupus skin.

Methods: mRNA expression of type I IFN genes was analyzed from microarray data of CLE lesions and healthy control skin. Similar expression in cultured primary keratinocytes, fibroblasts, and endothelial cells was analyzed via RNA-seq. *IFNK* KO keratinocytes were generated using CRISPR/Cas9. Keratinocytes stably overexpressing IFN-κ were created via G418 selection of transfected cells. IFN responses were assessed via phosphorylation of STAT1 and STAT2 and qRT-

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PCR for IFN-regulated genes. UVB-mediated apoptosis was analyzed via TUNEL staining. *in vivo* protein expression was assessed via immunofluorescent staining of normal and CLE lesional skin.

Results: *IFNK* is one of two type I IFNs significantly increased (1.5-fold change, FDR q<0.001) in lesional CLE skin. GO analysis showed that type I IFN responses were enriched (FDR=6.8×10^{-04}) in keratinocytes not in fibroblast and endothelial cells and this epithelial-derived IFN-κ is responsible for maintaining baseline type I IFN responses in healthy skin. Increased levels of IFN-κ, such as seen in SLE, amplify and accelerate responsiveness of epithelia to IFN-α and increase keratinocyte sensitivity to UV irradiation. Notably, knock-out of IFN-κ or inhibition of IFN signaling with baricitinib, abrogates UVB-induced apoptosis.

Conclusion: Collectively, our data identify IFN-κ as a critical IFN in CLE pathology via promotion of enhanced IFN responses and photosensitivity. IFN-κ is a potential novel target for UVB prophylaxis and CLE-directed therapy.

Keywords

Type I interferon; interferon kappa; cutaneous lupus; TYK2; photosensitivity

INTRODUCTION

Cutaneous lupus erythematosus (CLE) affects up to 70% of patients with systemic lupus erythematosus (SLE) and can also exist without the presence of systemic disease. CLE lesions can result in disfiguring scars, permanent hair loss, and significant loss of quality of life for patients[1]. There are no FDA approved therapies for CLE, largely related to a lack of pathogenic understanding of this disease.

A hallmark of CLE and SLE is the induction of skin lesions by ultraviolet (UV) light in up to 93% of patients[2]. These lesions are characterized by interface dermatitis and infiltration by both innate and adaptive immune cells. Patients with CLE exhibit increased type I interferon (IFN) signaling in their blood, which correlates with cutaneous disease activity[3], and increased expression of IFN responsive genes in lesional skin[4–6]. Cutaneous lesions in lupus are characterized by infiltration of plasmacytoid dendritic cells[7], and their production of IFN-α is a suggested source of type I IFN signaling in CLE[8,9]. However, the sources of IFN in cutaneous lupus have not been systematically evaluated.

IFN-κ is a member of the type I IFN family that is expressed primarily by keratinocytes[10] The chromosomal region encompassing *IFNK* has been suggested as a genetic risk locus for systemic lupus erythematosus, including some associations with cutaneous lupus erythematosus (CLE) phenotypes[11]. Intriguingly, overexpression of *IFNK* can induce autoimmune phenotypes in mice[12]. *IFNK* expression in keratinocytes is upregulated by ultraviolet light exposure[13], a well-known trigger of CLE[14], and IFN-κ can prime keratinocytes for inflammatory cytokine production. Importantly, we have shown that IFN-κ is required for overproduction of IL-6 by keratinocytes from SLE patients[13]. Despite this knowledge, little is known about the function of IFN-κ in the skin and its contribution to CLE and UV-sensitivity. We thus hypothesized that epidermal production of IFN-κ is

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elevated in CLE and that it is an essential contributor to cutaneous type I IFN responses and CLE lesions. Indeed, we found that IFN-κ is upregulated in CLE lesions and in keratinocytes from non-lesional SLE skin. IFN-κ is required for baseline expression of type I IFN-regulated genes in keratinocytes and drives enhanced responses to IFN-α. IFN-κ upregulates type I IFN-regulated gene expression in neighboring skin cells and stimulates activation of dendritic cells, important contributors to CLE pathogenesis[15]. Importantly, IFN-κ regulates the apoptotic response to UVB, and inhibition of IFN responses in lupus keratinocytes abrogates their enhanced apoptosis to UVB. Thus, we propose IFN-κ as a novel IFN critical for CLE pathology and a potentially important target for photoprophylaxis and specific CLE-directed therapy.

MATERIALS AND METHODS

Human Subjects:

According to the declaration of Helsinki, all patients and controls gave written, informed consent. The study protocol was approved by the Institutional Review Board of the University of Michigan Medical School. Systemic lupus erythematosus patients fulfilled ≥4 ACR criteria[16], had a documented history of cutaneous lesions, and were recruited from the University of Michigan Lupus Cohort. CLE patients used for microarray studies had both clinical and pathologic confirmation of diagnosis (online supplementary table S1). Normal controls were recruited by advertisement.

Cell Culture:

N/TERTs[17], an immortalized keratinocytes line, was used with the kind permission of Dr. James G. Rheinwald for generation of knock-out (KO) cell lines using non-homologous end joining via CRISPR/Cas9. N/TERTs were grown in Keratinocyte-SFM medium (ThermoFisher #17005-042) supplemented with 30 μg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, and 0.3 mM calcium chloride[18]. Primary human keratinocytes were established from healthy adults or lupus patients with a history of CLE as previously described[13,19]. Dermal fibroblasts and endothelial cells were isolated from normal human skin as previously described[20,21].

Generation of KO keratinocytes by CRISPR/cas9:

Guide RNAs were developed using a web interface for CRISPR design (http://crispr.mit.edu). The pSpCas9 (BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138) and used as cloning backbone. We followed the CRISPR/Cas9 protocol as previously discussed[18,22] to generate *TYK2* KO and *IFNK* KO cell lines. In short, for the *TYK2* knock-out, the following oligonucleotides were used for annealing: TYK2E3G1F: 5’-CACCGGACTCACTGAAAGTGACCCA-3’ and TYK2E3G1R: 5’-AAACTGGGTCACTTTCAGTGAGTCC-3’. For the *IFNK* knock-out, the following oligonucleotides were used for annealing: IFNKSGRNA1F1: 5’-CACCGGTTCAGTAAGTTACAGTCCA-3’ and IFNKSGRNA1R1: 5’-AAACTGGACTGTAACTTACTGAACC-3’. The annealed oligonucleotides were inserted into the cloning vector Px458 following the Ran et al., 2013[22] protocol. Ligated plasmids were transformed into competent *E. coli* (ThermoFisher # C737303) and then plated on LB-

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agar plate overnight. 12 colonies from each of the groups were selected and cultured in LB medium and plasmids were purified using Qiagen mini-prep kit (cat # 27106) and then the proper insertion of sgRNA target sequences were verified by Sanger sequencing. Purified plasmid was transfected into an immortalized keratinocyte line (N/TERTs) using the TransfeX transfection kit (ATCC # ACS4005). Single cells positive for GFP were sorted into 96 well plates using a MoFlo Astrios #1 cell sorter and grown up to ~50% confluence. Cells from 96 well plates were transferred into 12 well plates and grown to 50% confluence. DNA was extracted and PCR amplified using specific primers for *TYK2* and *IFNK: TYK2*: TYK2E3PCR-F: GTCTCTGGGCTGAGACTTGG, TYK2E3PCR-R: CCCCAGACTCACCAACTTTA and *IFNK*: IFNK1PCRF1: GTGTTTGTGGCTTGAGATCC, IFNK1PCRR1: GGTTGGGTGTATTGCAGAAA. Homozygous *TYK2* and *IFNK* mutations were verified by Sanger sequencing of the PCR product. For validation of findings a total of three independent CRISPR/Cas9 KO mutants were generated for *IFNK* and for *TYK2*.

Generation of IFN-κ overexpressing N/TERTs

N/TERT keratinocytes stably overexpressing IFN-κ were generated using 4D-Nucleofector™ X Unit (Lonza Cologne, Germany). Cells were prepared using standard protocol for Normal Human Epidermal Keratinocyte™ X Unit kit (4D Nucleofector™ Solution, supplement, and 100μl single nucleocuvette) obtained from Lonza. For each electroporation, 3 μg pCMV6-AC-GFP-IFNκ plasmid (Origene, Rockville, MD) was used. Unit X program used was DS-138 for stable keratinocytes. Following transfection, keratinocytes were grown in a 48-well plate using fully supplemented Keratinocyte-SFM medium, penicillin streptomycin, and 500μg/ml G418 (Geneticin® by Thermo Fisher Scientific (Waltham, MA)) (for selection) followed by expansion for approximately 60 days. IFNK-GFP over-expression was validated using western blotting.

Evaluation of IFN response in N/TERT-IFN-κ cells

IFN-κ overexpressing N/TERTs were grown in parallel to wild-type N/TERTs with 2 μg/ml anti-IFN-κ goat polyclonal neutralizing antibody (Santa Cruz Biotechnology, Dallas, TX) or 2μg/ml isotype normal goat IgG (R&D Systems, Minneapolis, MN) for three days followed by stimulation with 5 ng/mL human recombinant IFNα2 (Schering Corporation, Kenilworth, NJ) for 1, 4, or 12 h. Cells were harvested in Tripure (Sigma-Aldrich, St. Louis, MO). RNA was isolated following protocol for Direct-zol™ RNA MiniPrep (Zageno Inc., Berlin, Germany) and cDNA was made. RT-PCR for *MX1* (normalized to *RPLP0)* was performed using ABI PRISM 7900HT (Applied Biosystems) using SYBR Green at the University of Michigan DNA-sequencing core.

Dendritic Cell Culture

Human dendritic cells (DCs) were generated *in vitro* as previously described[23]. Briefly, monocytes were isolated from PBMC using MACS negative selection beads (Miltenyi) and cultured in RPMI containing 10% FCS supplemented with GM-CSF (100ng/ml, R&D Systems # 215-GM-050) and IL-4 (20ng/ml, R&D Systems # 204-IL-010). Cultures were fed on day 4. On day 8, DC were seeded into poly (2-hydroxyethyl-methacrylate) coated 12-well culture plates (Fisher Scientific # 0720082) at a density of 1 million cells/well and

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stimulated for 6 hours (PCR) or 2 days (flow cytometry) with conditioned mediated generated from control or SLE KC exposed to 50 mJ/cm2 UVB. The DCs were treated in the presence/absence of baricitinib. DC phenotype was analyzed by flow cytometry using an LSR2 flow cytometer (BD) using antibodies against CD80 (Biolegend # 305218) and appropriate isotype control antibodies. Data analysis was performed using FCS Express (v4) software.

Microarray

Biopsies of CLE cases (n=90) were identified through the University of Michigan Pathology Database using the search terms “lupus” and “cutaneous lupus”. Control blocks were obtained from healthy volunteers. Patients who met both clinical and histologic criteria for DLE or sCLE were included in the study. Validation of clinical and pathologic CLE diagnosis was made via review of dermatology notes for each case. Detailed clinical information was obtained for each sample (Table S1). RNA was isolated from five 10μm sections of formalin-fixed paraffin embedded blocks of identified skin biopsies. As previously described[24], RNA was extracted using the E.N.Z.A. FFPE RNA Kit (Omega Bio-tek). Complementary DNA was prepared and biotinylated using the NuGEN Encore Biotin Module (Encore Biotin Module Manual, P/N M01111 v6). Labeled cDNA was hybridized at 48°C to Affymetrix Human Gene ST 2.1 array plates, which were then washed, stained and scanned using the Affymetrix GeneTitan system (software version 3.2.4.1515) with the assistance of the University of Michigan DNA Sequencing Core. Quality control and RMA (Robust Multi-array Average)[25] normalization of CEL files were performed in R software version 3.1.3 using custom CDF version 19 and modified Affymetrix_1.44.1 package from BrainArray (http://brainarray.mbni.med.umich.edu/brainarray/default.asp). Log_2 expression values were batch corrected using Combat implemented into GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/). The baseline expression was defined as minimum plus one standard deviation of the median of all genes. A variance filter of 80% was then applied. Of the 25,582 unique genes represented on the Human ST2.1 chip, a total of 20,410 genes passed the defined criteria. The normalized data file was uploaded to the Gene Expression Omnibus (GEO) Web site (http://www.ncibi.nlm.nih.gov/geo/) under accession number GSE81071 and will be available upon acceptance of this manuscript.

RNA extraction, qRT-PCR and RNA-Sequencing

RNAs were isolated from cell cultures using Qiagen RNeasy plus kit (Cat # 74136). qRT-PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems) with TaqMan Universal PCR Master Mix (ThermoFisher Scientific). Libraries for RNA-seq were generated from polyadenylated RNA and sequenced at six libraries per lane on the Illumina Genome Analyzer IIx. We used Tophat2[26] to align RNA-seq reads to the human genome, using annotations of GENCODE as gene model[27]. HTSeq was used to quantify gene expression levels[28]; normalization and differential expression analysis were performed by DESeq2[29]. The RNA-seq data will be made available to the Gene Expression Omnibus upon the acceptance of the manuscript.

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Immunostaining

According to our previous protocol[30], formalin fixed, paraffin-embedded tissue slides obtained from patients with cutaneous lupus were heated for 30 min at 60°C, rehydrated, and epitope retrieved with Tris-EDTA, pH 9. Slides were blocked, incubated with primary antibody (IFN-κ, Abnova #: H00056832-M01; IFN-α, Santa Cruz # sc-80996; IFN-β, Biolegend # 514004; MX1, Abcam # ab95926; Cytokeratin 14, Abcam # ab51054; p-STAT1, Cell Signaling # 9167; p-STAT2, Cell Signaling # 4441; CD11c, Abcam # ab52632; CD80, Life Span Biosciences # LS-C115529; CD3, Dako # A0452; CD123, Sigma-Aldrich# HPA003539; Mouse IgG2a, kappa isotype control (for IFN-κ), ThermoFisher # 14-4724-81; Mouse IgG2a Isotype Control (for CD80), ThermoFisher # 02-6200; Mouse IgG 1, kappa isotype control (for IFN-α, IFN-β), ThermoFisher #14-4714-81; Rabbit IgG Isotype Control (for Mx1, cytokeratin 14, p-STAT1, p-STAT2, CD11c,), LSBio # LS-C149375) overnight at 4 °C. Slides were incubated with biotinylated secondary antibody (biotinylated goat anti-rabbit IgG Antibody, Vector Laboratories # BA1000; biotinylated horse anti-mouse IgG Antibody, Vector Laboratories # BA2000) and then incubated with fluorochrome-conjugated streptavidin. Slides were prepared in mounting medium with DAPI. Images were acquired using Zeiss Axioskop 2 microscope and analyzed by SPOT software 5.1. Images presented are representative of three experiments.

Western Blot

Total protein was isolated from cultured cells using Pierce RIPA buffer (ThermoFisher # 89900) and run on precast gel (Bio-Rad # 456-1094S). The membrane was blocked and then probed by a primary antibody (IFN-κ, Abnova, Catalog #: H00056832-M01; Tyk2, Cell Signaling # 9312s; STAT1, Cell Signaling # 9172; STAT2, Cell Signaling # 4594; pSTAT1, Cell Signaling # 9167; pSTAT2, Cell Signaling # 4441; β-Actin, sigma # A5441), followed by a secondary antibody (anti-mouse IgG, AP-linked Antibody, Cell Signaling # 7056S), then washed 5 times, and substrate added (Fisher Scientific # 45-000-947). Membrane was scanned on Molecular Dynamics STORM 860 PhosphorImager (GE Health Care, STORM 860).

UVB irradiation and TUNEL assays.

Keratinocyte cultures were grown to 80% confluence and were irradiated in PBS with 50mJ/cm^2 UVB (310nm) via a UV-2 irradiator (Tyler Industries, Alberta, Canada). After media replacement, cells were grown for 8 hours followed by staining for TUNEL according to manufacturer's instructions (Sigma) and counterstaining with DAPI. Percent TUNEL+ cells were quantified using CellC (# cells positive for Red (TUNEL) and DAPI staining/# cells DAPI positive). For CLE biopsies, TUNEL staining was performed according to manufacturer protocol (Roche # 12156792910). In short paraffin embeded CLE and control slides were dewaxed and rehydrated according to statdard protocol and then treated with proteinase K solution. Slides were then treated with TUNEL reaction mixture in a humidified chamber followed by PBS washing. Finally slides were mounted by vectashield mounting medium with DAPI. Images were acquired using Zeiss Axioskop 2 microscope and analyzed by SPOT software 5.1. Images presented are representative of three experiments.

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Statistical Analysis

Calculations were made using GraphPad Prism v.6. For *in vitro* studies, comparisons of the means between experimental variables was made via unpaired two-sided Student's t-test for normally distributed variables and via Mann-Whitney for non-normally distributed variables. For both microarray and RNA-seq, false discovery rate (FDR) was used to control for multiple testing.

RESULTS:

IFN-κ is increased in lesional and non-lesional lupus skin

Type I IFNs have been purported to play a role in cutaneous lupus erythematous (CLE); however, which IFNs are present in CLE are unknown. To address this, we investigated the expression of type I IFN family members in microarray data obtained from CLE lesions (n=90) and healthy control skin (n=13). 10 type I IFN members passed cut-off values for detectible expression, and of those, only *IFNA10* (1.7-fold change, FDR $q<0.05$) and *IFNK* (1.5-fold change, FDR $q<0.001$) had significantly increased expression in lesional CLE skin (figure 1A). This finding held true when subtypes of CLE (discoid lupus erythematosus (DLE) vs. subacute cutaneous lupus erythematosus (SCLE) were considered or whether or not patients had CLE only or CLE associated with SLE (online supplementary figure S1A). No elevations of IFN-λ, a type III IFN, were detected by microarray (online supplementary figure S1C). To validate the microarray data, we examined expression of IFN-α, IFN-β, and IFN-κ in CLE lesions by immunofluorescence. While IFN-α and I FN-β1 were exclusively found to be localized to the dermal inflammatory infiltrate, IFN-κ expression was localized primarily to the epidermis and co-stained with epidermal cytokeratin 14 (figure 1B and online supplementary figure S2, S3). Some IFN-κ staining was also noted in the dermis which may reflect IFN-κ binding to dermal mucin or possible secretion by infiltrative plasmacytoid dendritic cells (pDCs) (Supplementary figure S4) or myeloid cells (mDCs), which has been reported[1031]. To confirm increased IFN signaling in CLE, we confirmed expression of *MX1* and *OASL*, two type I IFN regulated genes, to be increased in CLE skin (8.6-, 2.4-fold change respectively, FDR $p<0.001$) (figure 1C and online supplementary figure S1B), and this was paralleled by increased STAT1 and STAT2 expression (online supplementary figure S1D) and pSTAT1 and pSTAT2 activation in lesional skin (figure 1D). IFN-κ demonstrated co-localization with MX1 in the epidermis, whereas no co-staining between IFN-κ and MX1 was seen in the inflammatory infiltrate (figure 1E and online supplementary figure S3), suggesting epidermal IFN-κ along with dermal IFN-α and IFN-β likely contribute to the overall type I IFN signaling in CLE.

Keratinocyte-produced IFNK is the source of basal type I IFN activity in healthy skin.

Little is known about the production of type I IFNs in the skin. To understand this, we started by examining healthy cultured keratinocyte (KC) global gene expression using RNA-seq (n=3), fibroblasts (n=3) and endothelial cells (n=4) from healthy controls. Gene ontology analysis of transcripts differentially expressed uniquely to each cell population demonstrated that "type I interferon responses" were enriched in KCs (False Discovery Rate (FDR)=6.8E-04) but not in fibroblasts or endothelial cells. Consistent with this observation, type I IFN response genes including *MX1, OASL and OAS1–3*, and *IRF5–7* were

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predominantly expressed in keratinocytes (figure 2A). To address the source of the type I IFN response in KCs we analyzed the expression of all type I IFN family members in those three cell types. Of the 17 known type I IFN family members, *IFNK* had higher expression in KCs and was the most elevated IFN when keratinocytes, fibroblasts and endothelial cells were compared (figure 2B). These observations were confirmed by immunofluorescence of healthy control skin, which demonstrated low-level expression of IFN-κ in the epidermis, co-localizing with the epidermal marker; cytokeratin 14, but no expression of IFN-α or I FN-β1 was detected in the epidermis (figure 2C and online line supplementary figure S2, S3). Similarly, the interferon response protein MX1 was expressed throughout the epidermis and showed patchy co-localization with IFN-κ, particularly in the basal epidermal layers (online supplementary Figure S5). To confirm whether production of IFN-κ from keratinocytes was functional, we collected the conditioned media of cultured human keratinocytes and transferred that to primary human fibroblast cultures. After 6 hours in the conditioned KC medium, fibroblasts had increased mRNA expression of type I IFN response genes including *MX1* ($p<0.001$), *IRF7* ($p<0.01$), and *OASL* ($p<0.05$) (n=3 for all), but no increase was seen in fibroblasts treated with non-conditioned keratinocyte medium or conditioned fibroblast medium (figure 2D). This increase in interferon response genes was driven by IFN-κ as *MX1, IRF7*, and *OASL* expression could be blocked by addition of a neutralizing antibody against IFN-κ (10μg/ml), but not with isotype, anti-IFN-α, or I FN-β1 antibodies (10μg/ml).

To further study the role of IFN-κ in keratinocyte biology, we knocked out (KO) *IFNK* using non-homologous end joining (NHEJ) repair via CRISPR/Cas9 in a human keratinocyte line. Guide RNA was designed for the *IFNK* gene and homozygous mutation was confirmed by Sanger sequencing (figure 3A). Absence of the IFN-κ protein was confirmed by western blotting of KC lysates (online supplementary Figure S6A), and ELISA from conditioned KC medium (figure 3B). KO of *IFNK* in unstimulated KCs reduced basal mRNA expression of type I IFN response genes compared to WT, including *MX1* ($p<0.001$), *IRF7* ($p<0.01$), *IRF9* ($p<0.01$) and *OASL* ($p<0.001$) (n=3 for all) (figure 3C and online supplementary figure S6A). Collectively, these data indicate that human keratinocytes express and secrete functionally active IFN-κ that is required to maintain basal expression of interferon response genes in keratinocytes.

Type I IFN activity in keratinocytes is dependent upon Tyk2

Type I IFNs signal through the type I IFN receptor (IFNAR1 and IFNAR2) followed by activation of the non-receptor tyrosine kinase 2 (TYK2). To determine if IFN-κ utilized TYK2 to maintain basal type I IFN activity in keratinocytes, we used CRISPR/Cas9 to knock-out the *TYK2* gene in N/TERT KCs (online supplementary figure S6B). Insertion of a frameshift mutation was confirmed by Sanger sequencing (online supplementary figure S6B). Knock-out of *TYK2* in unstimulated KCs led to decreased basal expression of type I IFN response genes including *MX1* ($p<0.001$), *IRF7* ($p<0.001$), *IRF9* ($p<0.001$), *OASL* ($p<0.01$) (n=3 for all) (figure 3D). In addition, KO of *TYK2* led to an inability of KCs to respond to exogenous IFN-α (50ng/ml) or IFN-κ (figure 3D). Notably IFN-κ was induced by IFN-α or IFN-κ exposure, and this induction was abolished in *TYK2* KO ($p<0.001$ for both, n=3) (figure 3D).

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IFN-κ is required for rapid triggering of IFN responses in keratinocytes

The ability of cells to generate a rapid interferon response can depend on proper priming of the interferon pathway. For example, in PBMCs, basal IFN-β production is required to confer "IFN readiness" for responses to commensal organisms[32]. Similarly, we hypothesized that IFN-κ may provide a similar "IFN readiness" in keratinocytes. Consistent with this hypothesis, baseline pSTAT1 and pSTAT2 levels were markedly suppressed in *IFNK* KO KCs compared to WT (p<0.001, n=3 for each) (figure 4A, B). Similar findings were noted in unstimulated *TYK2* cells, which serve as an IFN-signaling KO control. With stimulation by exogenous IFN-α (50ng/ml), pSTAT1 and pSTAT2 levels in *IFNK* KO displayed a small lag in activation but approached WT levels after 20min of stimulation. In contrast, pSTAT1 and pSTAT2 in *TYK2* KO remained suppressed at all time points (10, 20 and 30 min) (figure 4A, B), consistent with its role in transmitting all type I IFN signals. Notably, *IFNK* KO KCs had both a decreased and a delayed response to added IFN-α (as measured by mRNA expression of *MX1* and *OASL*), which was most notable at lower doses of IFN-α stimulation (1 and 5 ng/ml), and approximated WT levels of expression at higher concentrations (50ng/ml) (n=3 for all) (figure 4C).

Non-lesional lupus keratinocytes express increased IFN-κ, which contributes to dendritic cell activation, rapid IFN-regulated gene transcription, and photosensitivity

Patients with lupus have abnormal responses to UV light which lead to CLE development. In order to determine whether "normal" appearing keratinocytes from lupus patients also demonstrated dysregulated IFN-κ expression, we cultured keratinocytes from non-lesional, non-sun-exposed skin biopsies from patients with systemic lupus erythematosus and a history of CLE lesions or healthy controls. These unstimulated keratinocytes had increased baseline protein expression of IFN-κ and demonstrated constitutively higher baseline pSTAT1 and pSTAT2 activity (n=3, 2 examples shown) (figure 5A). In contrast, keratinocytes from uninvolved and lesional psoriatic skin did not show increased IFN-κ mRNA expression (n=4) (online supplementary figure S7). As further validation of the increased basal IFN-κ production in SLE KCs, we were able to induce a greater IFN response in fibroblasts by transfer of conditioned media from SLE KCs than of conditioned media from healthy control KCs (figure 5B). To study the impact of increased IFN-κ in KCs, we generated N/TERT keratinocytes overexpressing IFN-κ at about twice the amount of endogenous IFN-κ (figure 5C). Similar to lupus-derived KCs, these cells had higher baseline expression of type I IFN response genes (figure 5D). IFN-κ over-expressing KCs had accelerated and increased early responses to IFN-α compared to non-overexpressing N/TERTs (as shown by *MX1* mRNA expression at 1 h, figure 5E). Importantly, the early upregulation of *MX1* was abrogated by addition of neutralizing IFN-κ antibody (figure 5E). After 4 hours of IFN-α stimulation, N/TERT control KCs had a robust type I IFN response that was also dependent on IFN-κ as neutralizing antibodies also diminished *MX1* expression (figure 5E). These data support a role for IFN-κ in CLE pathogenesis in which chronic elevation of IFN-κ amplifies basal IFN responses and promotes rapid response to other type I IFNs.

UVB induction of keratinocyte apoptosis is thought to be an essential trigger for CLE lesions[33]. Indeed, consistent with reported literature[34], we detected increased apoptosis in

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CLE lesions via TUNEL staining (figure 6A). We next examined if increased UVB-mediated apoptosis was a feature of SLE keratinocytes. As shown in figure 6B, non-lesional SLE keratinocytes exhibited increased UVB-mediated apoptosis compared to control keratinocytes (p=0.0013). We thus examined the impact of IFN-κ overexpression on UVB-mediated apoptosis in keratinocytes. As shown in figure 6C, overexpression of IFN-κ resulted in enhanced basal and UVB-mediated increases in TUNEL staining, whereas UVB-induced apoptosis was nearly completely abrogated in IFN-κ KO keratinocytes (figure 6D). Similarly, UVB-induced apoptosis could be inhibited by the Jak inhibitor, baricitinib, in both normal and SLE keratinocytes (p<0.05, and p<0.01, respectively) (figure 6E).

One mechanism for enhanced immune activation in SLE is type I IFN activation of dendritic cells[35]. Previously, we had identified increased IFN-κ secretion from SLE vs. control keratinocytes following UVB stimulation[13]. To determine whether conditioned medium from UV irradiated keratinocytes was sufficient to induce CD80 expression on dendritic cells and elicit a type I IFN response (via *MX1* mRNA expression), SLE and healthy control keratinocytes were treated with UVB and the supernatants were collected and added to primary dendritic cell cultures. Consistent with heightened type I IFN responses in SLE keratinocytes, all 3 SLE cultures resulted in induction of MX1 and CD80 after UVB exposure (figure 6F). Notably, the responses from UVB conditioned keratinocyte media were completely inhibited by IFN blockade by baricitinib (p<0.001) (figure 6F). Consistent with these findings we observed increased expression of CD80 on CD11c+ dendritic cells in CLE skin lesions (figure 6G). These data support the upregulation of IFN-κ in lupus keratinocytes as a key regulator of heightened inflammatory and apoptotic responses in SLE skin, which leads to a priming of SLE skin for enhanced keratinocyte apoptosis and immune activation following UVB stimulation (figure 6H).

DISCUSSION

In this study, we have identified IFN-κ as the most prominent type I IFN produced by keratinocytes and characterized its role in regulation of basal type I IFN responses in skin. Further, we have shown that this cytokine can amplify exogenous IFN signals and drive enhanced type I IFN responses and photosensitivity in SLE keratinocytes. Importantly, we have identified IFN-κ as one of two type I IFNs significantly increased in lesional CLE, and have implicated it in the activation of dendritic cells, an important pathogenic cell population in CLE skin.

Regulation of the type I IFN response in the skin is important for antiviral defenses, and consistent with this, IFN-κ has recently been found to be important for protection against human papillomavirus infections[36]. Using high resolution RNA-seq data, we found baseline type I IFN activity in keratinocytes but minimal activity in dermal fibroblasts or endothelial cells. Others have also identified basal IFN-κ activity in healthy control cultured KCs through multiple methods of detection; however, using chromogenic immunohistochemistry, they were unable to detect IFN-κ *in vivo*. Our staining, using a different antibody and immunofluorescent technique with high resolution microscopy, was able to show the presence of IFN-κ in basal KC *in vivo*. While IFN-κ was not one of the most dysregulated genes in healthy control KCs, the unstimulated IFN signature in epithelial cells was

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prominent. This is consistent with new data which suggests very low levels (attomolar) of type I IFNs are sufficient for generating IFN responses[37]. Importantly, the IFN activity was transferable to fibroblasts and endothelial cells in an IFN-K-dependent manner. This suggests that IFN-κ has pleotropic functions in healthy skin: protecting keratinocytes against intracellular pathogens and "sounding the alarm" to other immune and non-immune cell populations in the skin.

The effects of IFN-κ on upregulation of IFN response genes were dependent on the expression of Tyk2, and neutralized by anti-IFN-κ antibody, suggesting that IFN-κ is secreted by keratinocytes and uses classical IFN signaling pathways to activate cellular responses. Both *TYK2* and *IFNK* gene loci have been implicated as susceptibility factors for systemic lupus erythematosus and cutaneous manifestations of lupus[11,38]. Our data suggest that one mechanism by which *TYK2* might contribute to CLE susceptibility is through regulation of autocrine IFN-κ activity. Further studies will test this hypothesis.

Our data support a role for IFN-κ as a rheostat for IFN responses in keratinocytes. For example, we found that phosphorylation of STAT1 and STAT2 was delayed by the absence of IFN-κ and that transcriptional responses to small amounts of IFN-α were delayed in the absence of IFN-κ. This could be overcome by higher doses of IFN-α. These data suggest that baseline IFN-κ expression primes keratinocytes for responses to low grade signaling through the type I IFN receptor. This is similar to data that support a parallel role for IFN-β, which primes for IFN responses in myeloid cells in response to gut flora[32]. In contrast, overexpression of IFN-κ has pathologic consequences. Indeed, we found that "normal" keratinocytes from SLE patients produce more IFN-κ at baseline and that overexpression of IFN-κ can amplify responses to other type I IFNs in an IFN-κ dependent manner.

The heightened expression of type I IFN response genes in CLE has been known for some time[7]. However, which type I IFNs contribute to cutaneous IFN signatures has not been examined until now. Intriguingly, we find that IFN-κ is one of only two type I IFNs upregulated in CLE lesions. Our data support keratinocytes as a source of this cytokine, but does not exclude production by myeloid populations, which have also been cited to produce IFN-κ[39,40]. IFN-α10 is also detected in CLE lesions, and we would surmise that this cytokine stems from recruited pDC populations in CLE skin. Importantly, given our data on the role of IFN-κ in fine-tuning the IFN response, we propose that an important step in CLE pathogenesis is the amplification of IFN signaling as lupus keratinocytes make more IFN-κ at baseline and have skewed IFN responses as a result. Paracrine IFN-α thus can further amplify this loop, priming for inflammatory responses as we have recently shown[13] and upregulating IFN-κ (figure 3D) (summarized in figure 6H). Paracrine IFN-κ produced by pDCs (Supplementary Figure 4) may also contribute to this loop. Indeed, our data may also explain the recent clinical trial results in which blockade of IFN-α, which doesn't block IFN-κ, results in only mild improvements in CLE lesions[41], whereas blockade of the type I IFN receptor, which blocks IFN-κ signaling as well as IFN-α, appears to result in more robust clinical improvement[42].

Photosensitivity is a hallmark of SLE patients and is strong contributor to poor quality of life[1]. Photosensitive lesions are characterized by increased epidermal apoptosis and

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increased inflammatory infiltrates, including dendritic and plasmacytoid dendritic cells, in the dermis[43]. Until now, we have not understood *why* SLE skin is predisposed to this reaction. Here, we have identified that chronic exposure to IFN-κ is sufficient to increase apoptosis of keratinocytes after UVB exposure and that SLE keratinocytes, which overexpress IFN-κ, display increased sensitivity to UVB in an IFN-signaling dependent manner. In addition, keratinocyte-produced IFN-κ is able to activate DCs, which further amplifies inflammatory responses after UVB exposure. This identifies regulation of IFN-κ as a key to controlling photosensitivity. Further research will explore the mechanisms by which IFN-κ predisposes to photosensitivity.

Other groups have identified IFNλ, a type III IFN with importance for anti-viral responses as dysregulated in CLE keratinocytes. IFNλ1 levels are increased via immunohistochemistry in the epidermis of DLE and SCLE lesions and were identified as increased in the serum of patients[44]. Serum IFNλ3 levels (IFNλ1 and IFNλ2 levels were undetectable in this study) may be higher in patients with anti-Ro antibodies, which is a common feature of patients with SCLE[45]. Our microarray data did not support increased IFNλ 2 or IFNλ3 in CLE biopsies. Unfortunately, IFNλ1 did not meet cutoff thresholds for detection. However, our *in vitro* data which evaluated the effects of IFNAR or IFNκ neutralization on baseline IFN gene expression in keratinocytes does not support a role for IFNλ in maintaining basal IFN signatures in keratinocytes. It is possible that elevated IFNλ production may contribute stimulated IFN responses, and this can be addressed in future studies.

In summary, we have identified IFN-κ as a critical regulator of basal type I IFN responses in keratinocytes and as a dysregulated cytokine in non-lesional SLE skin which contributes to amplification of IFN responses, activation of dendritic cells, and photosensitive apoptosis. Further research into the role of IFN-κ in systemic lupus erythematosus and the targeting of IFN-κ in CLE lesions should be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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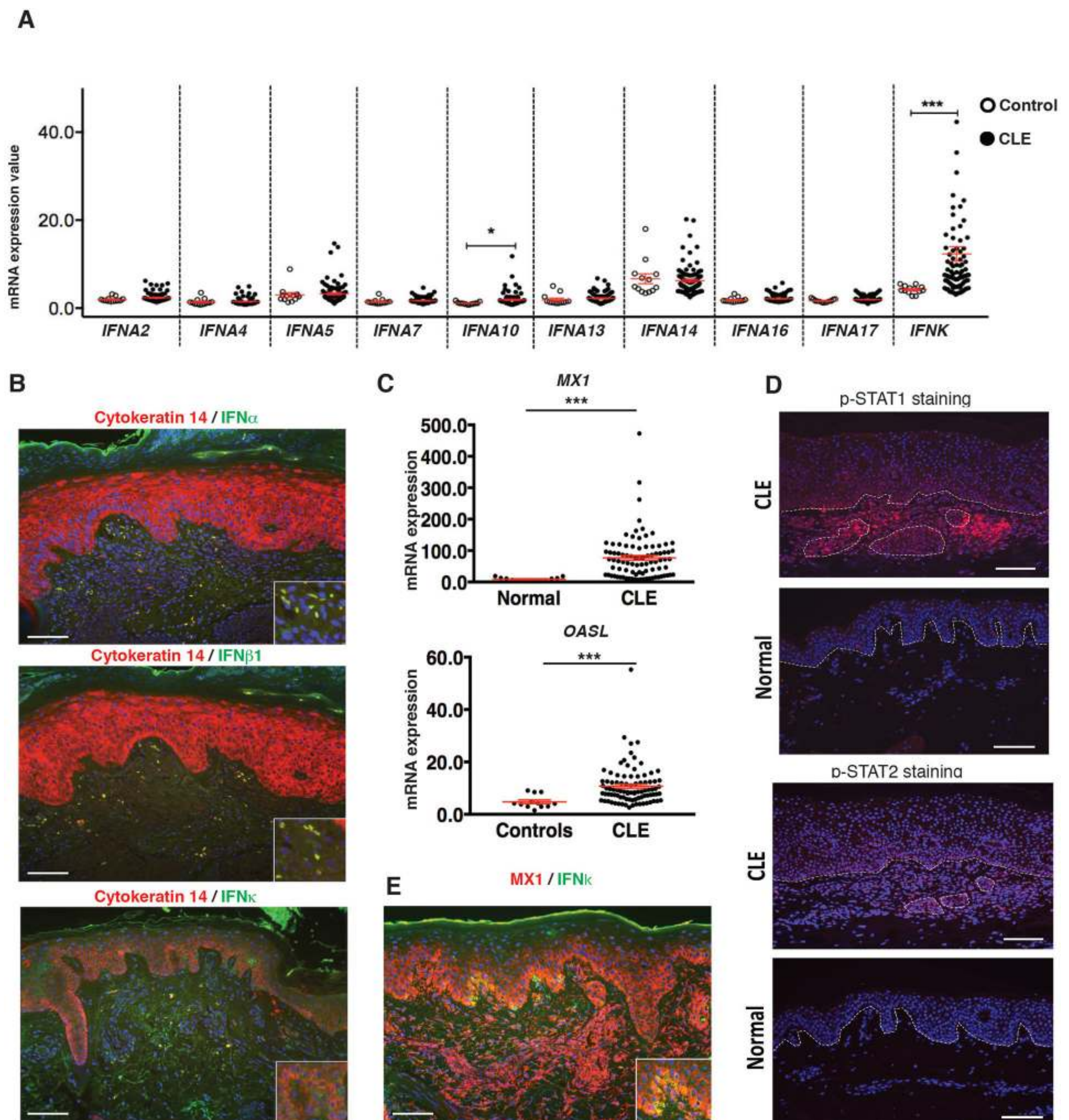

Figure 1. IFN-κ is upregulated in CLE lesions and lupus keratinocytes.

(**A**) mRNA expression of type I interferon genes in cutaneous lupus (n=90 CLE, n=13 control). Of the 10 type I family members that had detectable mRNA expression on Affymetrix ST 2.1 array, only *IFNK* (FDR q<0.001) and *IFNA10* (FDR q<0.05) had significantly increased expression in cutaneous lupus (CLE) compared to healthy skin *(IFNB* was not detectable). (**B**) Immunofluorescence of CLE lesions revealed IFN-α and IFN-β1 staining in the dermis near the inflammatory infiltrate, whereas IFN-κ expression was seen in the epidermis and dermis of CLE lesions (epidermis is indicated by cytokeratin 14 staining). Representative staining of 3 CLE and 3 controls are shown. (**C**) Type I IFN

response genes, including *MX1* and *OASL* are increased in CLE lesional biopsies (FDR q<0.001) (n=90 CLE, n=13 control). (**D**) In skin lesions of CLE compared to healthy skin, there is increased phosphorylation of both STAT1 (pSTAT1; red) and STAT2 (pSTAT2; red). Representative staining of 3 CLE and 3 controls are shown. (**E**) The IFN response protein MX1 was prominently expressed in CLE skin and exhibited co-localization with IFN-κ in the epidermis (as evidenced by yellow color).

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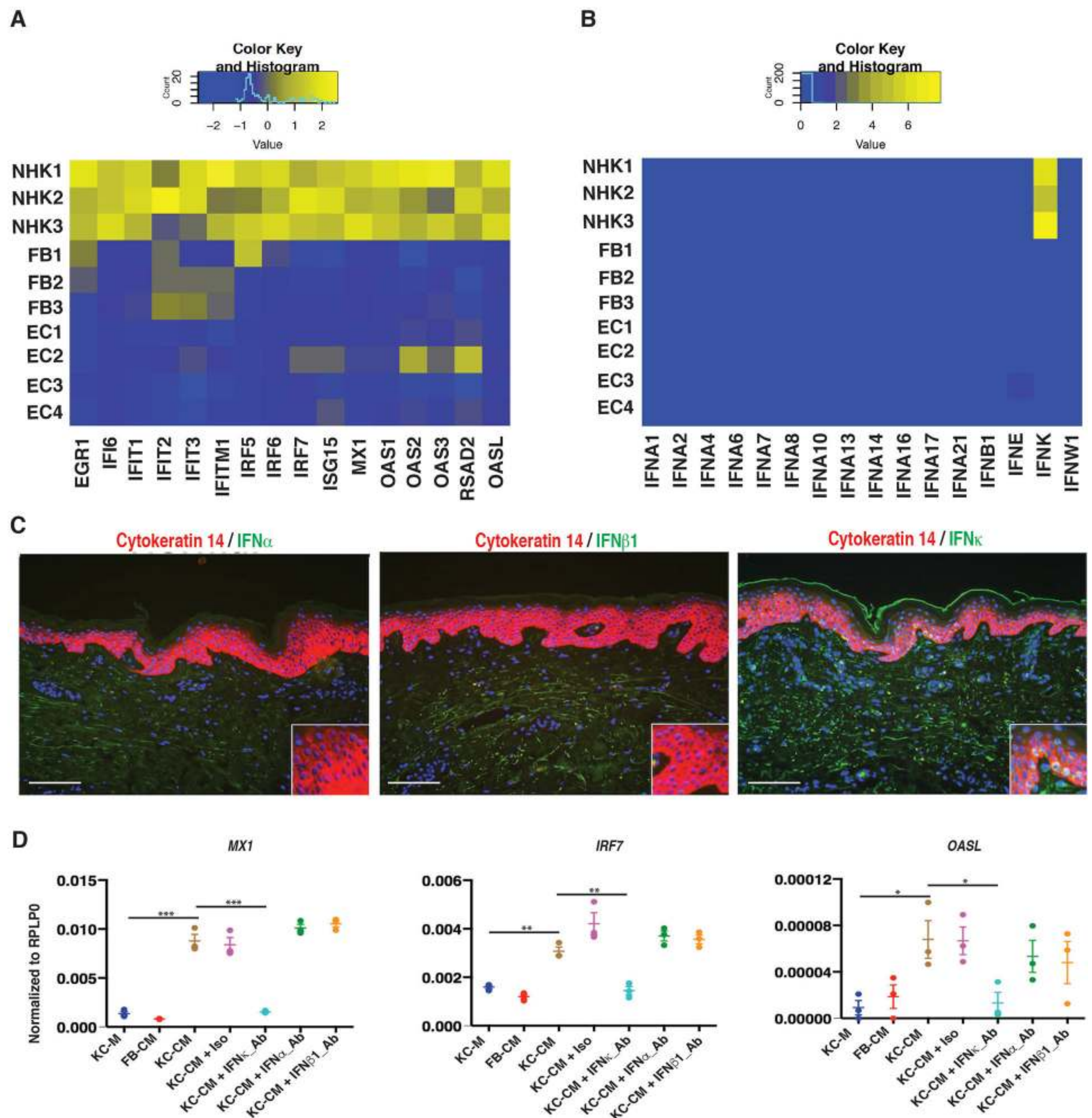

Figure 2. Keratinocytes exhibit baseline type I interferon activity and express IFN-κ.

(**A**) Unstimulated normal human keratinocytes (NHK1–3) exhibit heightened expression of type I IFN target genes compared to dermal fibroblasts (FB1–3) and dermal endothelial cells (EC1–4) as measured by RNA-seq (FDR=6.8E-04). (**B**) RNA-seq reveals *IFNK* as the only type I IFN detectable in the three skin-derived cell types, and its expression was limited to keratinocytes (**C**) Immunofluorescent co-localization with the epidermal marker cytokeratin 14 showed co-localization with IFN-κ in healthy epidermis, but no expression of IFN-α, or IFN-β1. Representative of 3 slides is shown (**D**) The type I IFN response could be transferred to fibroblasts following exposure to keratinocyte conditioned medium for 24hrs (KC-CM), but not non-conditioned keratinocyte medium (KC-M) or conditioned fibroblast

medium (FB-CM), as determined by expression of *MX1, IRF7*, and *OASL*. This response could be effectively inhibited by addition of neutralizing anti-IFN-κ antibody (10μg/ml), but not with isotype (KC-CM+Iso), anti-IFN-α (KC-CM-IFN-α_Ab) or anti-IFkN-β1(KC-CM-IFNβ1_Ab) antibodies (10μg/ml) (n=3 for all). Data shown with SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$ (unpaired two-sided Student's t-test). n=3 for each experiment.

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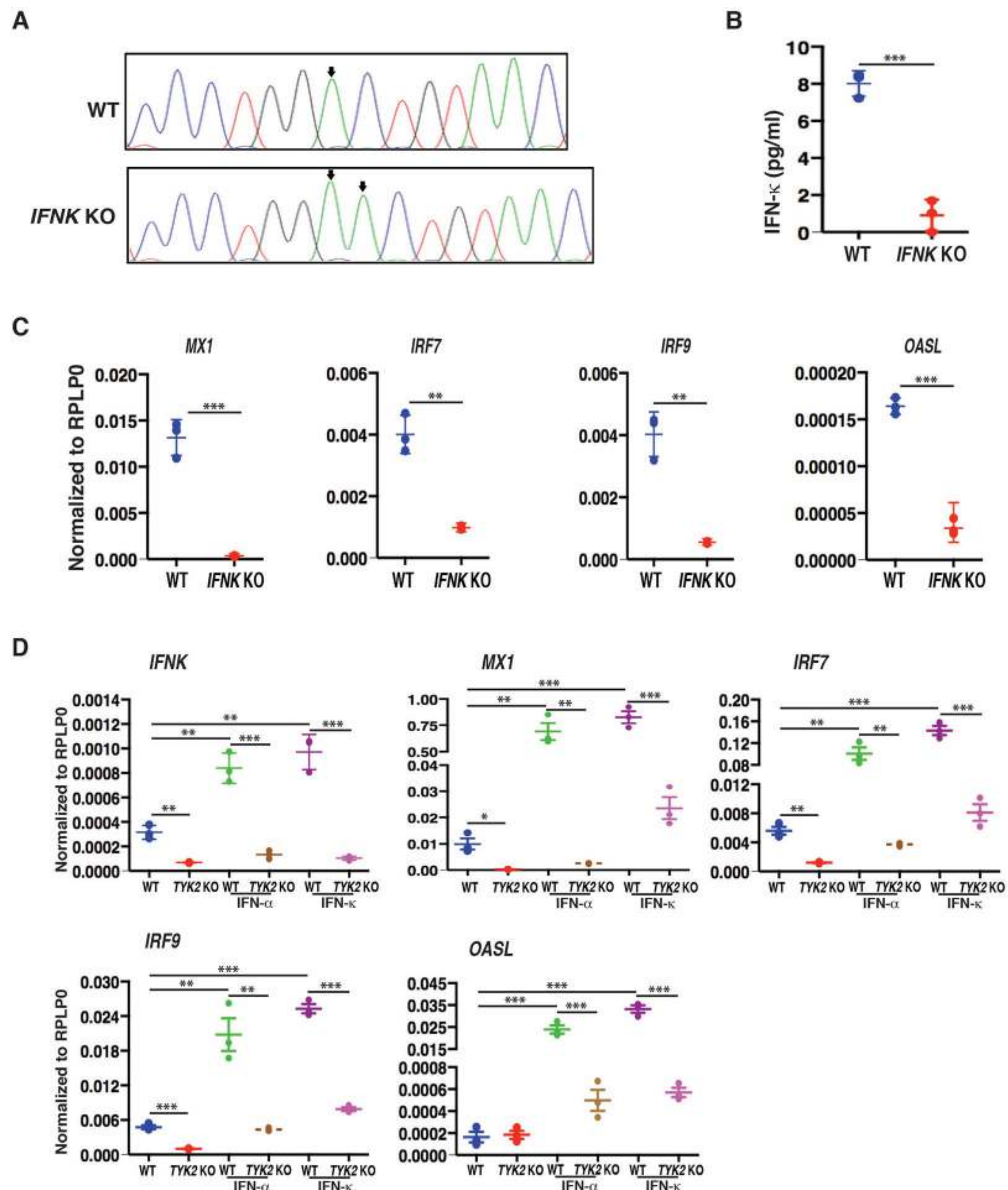

Figure 3. *IFNK* knock-out abolishes type I interferon activity in keratinocytes.

(**A**) A guide RNA targeting a 20nt sequence in exon 1 of the *IFNK* gene was designed and knock-out was performed via non-homologous end-joining repair (NHEJ) resulting in the insertion of a single nucleotide (A) in the *IFNK* gene. (**B**) Absence of the secreted IFN-κ protein was confirmed by ELISA. (**C**) *IFNK* KO suppressed baseline type I IFN activity in unstimulated keratinocytes as determined by expression of *MX1, IRF7, IRF9* and *OASL*. Data shown with SEM, **p<0.01, ***p<0.001, n=3 for all). (**D**) Baseline type I IFN activity was measured in unstimulated WT and *TYK2* KO keratinocytes as determined by expression of IFNK, *MX1, IRF7, IRF9* and *OASL via real-time PCR*. Similar expression changes were measured after treatment with either IFN-α (5 ng/ml) or IFN-κ (5 ng/ml). Data shown with SEM, **p<0.01, ***p<0.001, n=3 for all (unpaired two-sided Student's t-test). WT: N/

TERTs (immortalized human keratinocytes); *IFNK* KO: *IFNK* knock-out keratinocytes; *TYK2* KO: *TYK2* knock-out keratinocytes.

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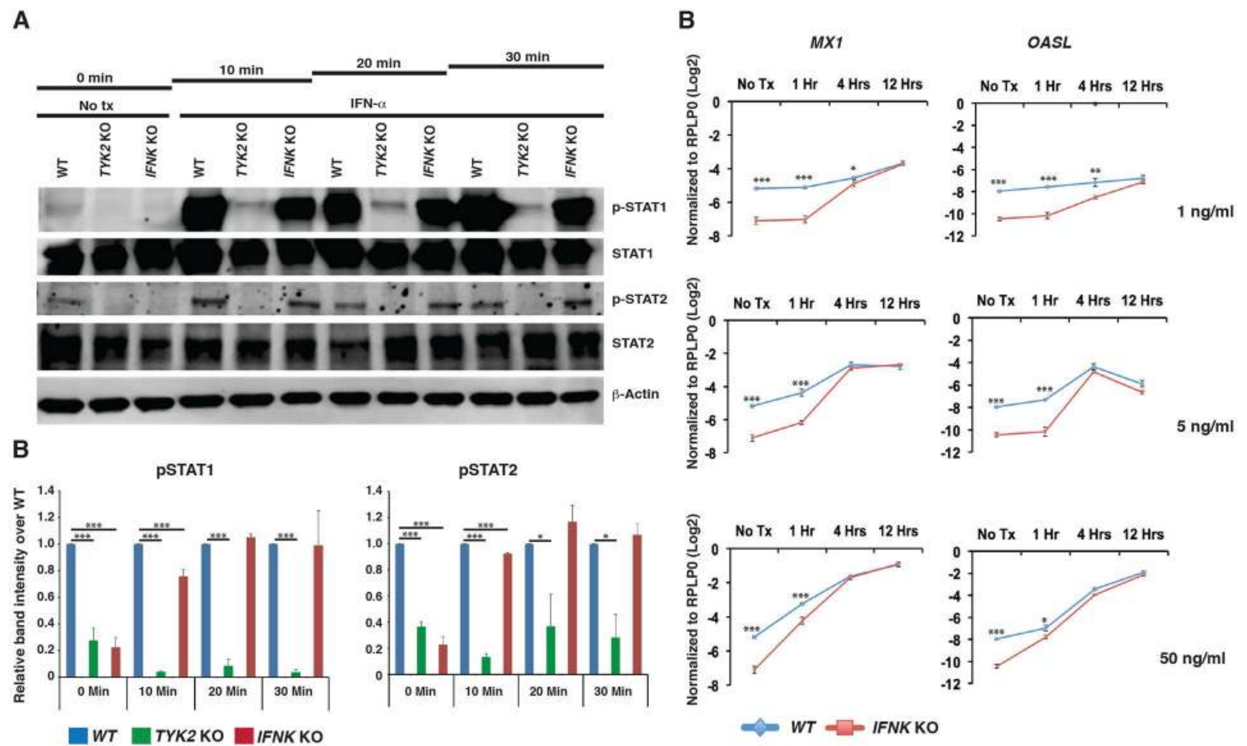

Figure 4. IFN-κ is required for rapid keratinocyte responses to exogenous IFN-α.

(**A, B**) WT, *IFNK* KO or *TYK2* KO KCs were treated with or without IFN-α for the indicated time points followed by Western blot for phosphorylated and total STAT1 and STAT2. Quantification of phosphorylation as compared to WT is shown in (**b**). (**C**) WT or *IFNK* KO KCs were treated with the indicated concentrations of IFN-α followed by qRT-PCR for the indicated genes. Data shown with SEM, *p<0.05, **p<0.01, ***p<0.001, n=3 for all (unpaired two-sided Student's t-test). WT: N/TERTs (immortalized human keratinocytes); *IFNK* KO: *IFNK* knock-out keratinocytes; *TYK2* KO: *TYK2* knock-out keratinocytes.

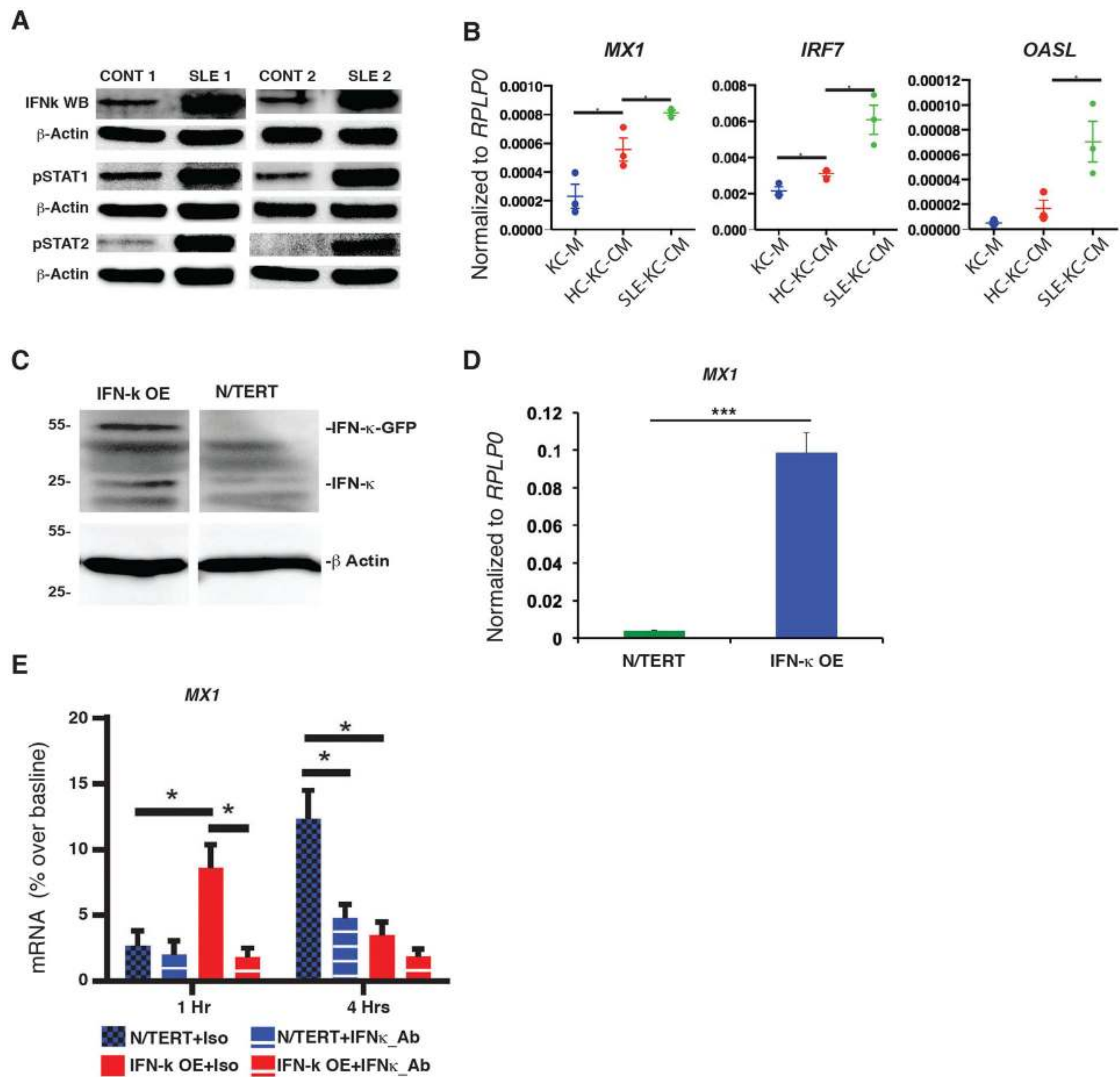

Figure 5. CLE keratinocytes have heightened basal IFN-κ expression and type I IFN activity.

(**A**) Cultured keratinocytes from uninvolved skin of patients with SLE and a history of cutaneous lupus exhibited increased protein expression of IFN-κ along with increased pSTAT1 and pSTAT2 activity (representative data from two (out of 3) control and two (out of 3) SLE patients shown). (**B**) Fibroblasts were treated with KC media alone (KC-M), conditioned media from healthy control KCs (HC-KC-CM) or conditioned media from SLE-KCs (SLE-KC-CM) (n=3 each in triplicate) for 24 hours followed by qRT-PCR for IFN-regulated genes *MX1, IRF7*, and *OASL*. (**C**) KCs stably overexpressing IFN-κ were generated; representative Western Blot of IFN-κ overexpressing line (IFN-κ OE) (both native and GFP-tagged) is shown. (**D**) MX1 mRNA expression in IFN-κ OE compared to N/TERTs. (**E**) IFN-κ OE or N/TERTs were grown in the presence or absence of a neutralizing antibody to IFN-κ for 3 days followed by stimulation with 5 ng/mL IFN-α for the indicated

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time points. *MX1* expression was assessed via real-time PCR, normalized to *RPLP0* expression and expressed as percent change over unstimulated cells. IFN-K_Ab: IFN-κ antibody; Iso=Isotype control for IFN-κ antibody; Data shown with SEM, *p<0.05, **p<0.01, ***p<0.001, n=3 for all (unpaired two-sided Student's t-test).

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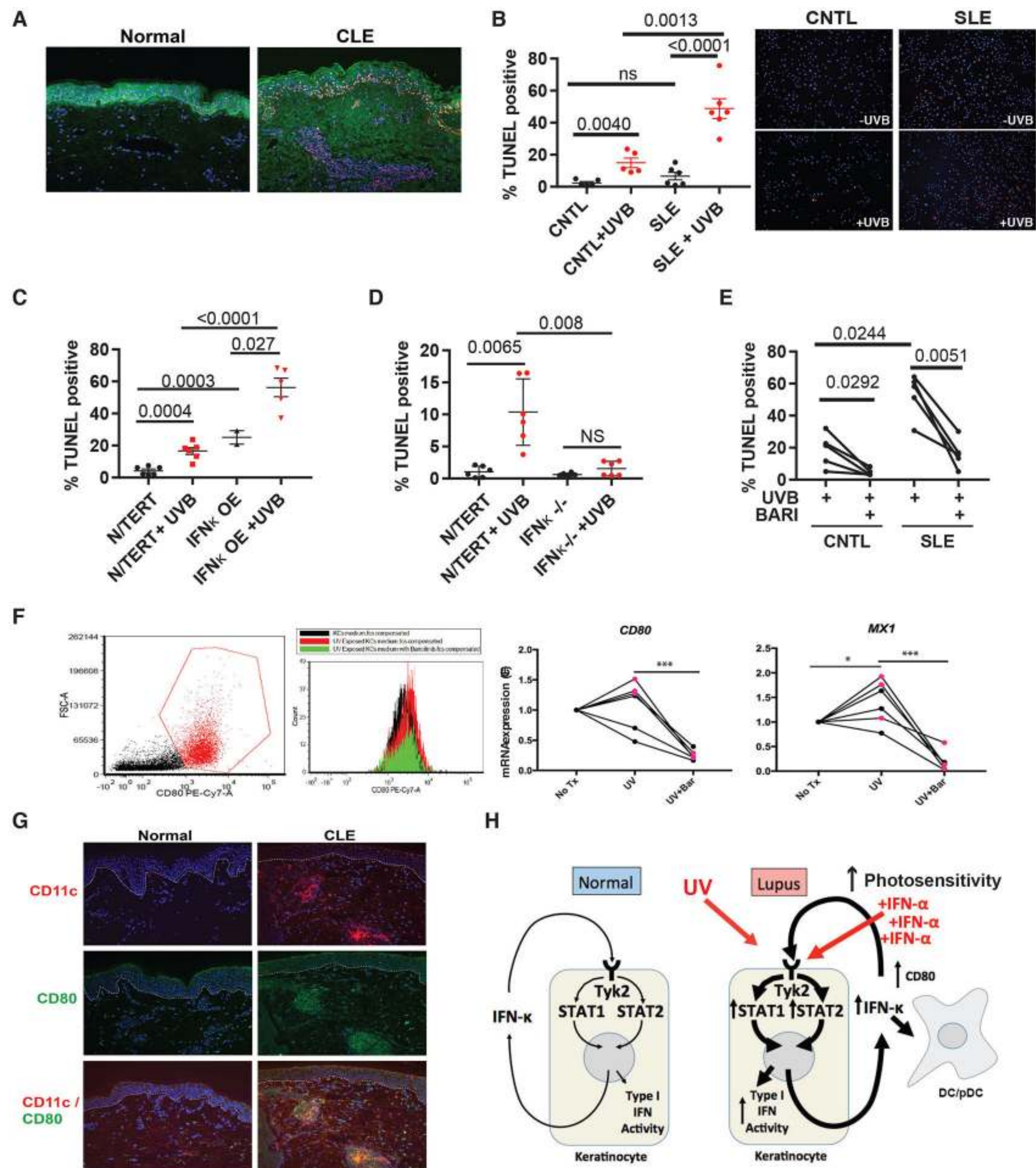

Figure 6. IFN-κ promotes UVB induced apoptosis in CLE.

(**A**) TUNEL staining of normal and CLE skin shows prominent apoptosis in CLE epidermis. Figure is representative of 3 samples. (**B**) Healthy control and non-lesional SLE KCs (n=6 each) were treated with or without 50mJ/cm^{2}UVB followed by TUNEL staining after 8 hours of culture. Representative photos are shown on the right. (**C**) IFNK-OE KCs or N/TERTs were treated with UVB followed by TUNEL staining as in (b). (n=5 each). (**D**) *IFNK* KO or N/TERT KCs were treated with UVB followed by TUNEL staining as in (b) (n=6 each). (**E**) Control or SLE KCs (n=5 each) were treated with or without baricitinib (BARI) followed by treatment with 50mJ/cm^{2}UVB followed by TUNEL staining after 8 hours of culture. (**F**) Condition medium from control or SLE UVB-irradiated KCs was added to monocyte-derived dendritic cell cultures in the presence or absence of baricitinib followed

by measurement of CD80 surface expression by flow cytometry and *CD80* and *MX1* mRNA expression via RT-PCR. Red dots=SLE keratinocyte conditioned media (three different patients), Black dots=control conditioned media (three different healthy controls). All statistical comparisons made via unpaired two-sided Student's t-test). (**G**) Immunofluorescent staining denotes increased expression of CD80 on CD11c+ dendritic cells in CLE skin lesions. (**H**) Schematic outline of the role of IFN-κ in SLE. In control KCs, a basal level of IFN-κ maintains tonic low-level IFN signaling and primes for IFN responses. In SLE KCs, increased autocrine IFN-κ expression drives an elevated tonic IFN response and primes for rapid, robust IFN responses and apoptosis to UVB. Secreted IFN-κ can activate DCs to stimulate immune responses in the skin.

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