Science Advances
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advances.sciencemag.org/cgi/content/full/6/30/eabb6049/DC1

Supplementary Materials for

Treatment of psoriasis with NFKBIZ siRNA using topical ionic liquid formulations

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Published 22 July 2020, *Sci. Adv.* **6**, eabb6049 (2020)
DOI: 10.1126/sciadv.abb6049

This PDF file includes:

Supplementary Materials

Materials and Methods

Circular dichroism

Circular dichroism measurements of dialyzed RNA samples were recorded at 15°C using a 1 cm pathlength quartz cell (Hellma 100-10-40, style 100-QS), in the Jasco J-815 spectropolarimeter equipped with a PFD-425S thermal controller unit at the Center for Macromolecular Interactions (CMI), Harvard Medical School. RNA concentrations were normalized in 10mM sodium phosphate buffer and incubated for 30mins at RT to ensure reduction and equilibrium, and then loaded into quartz cuvettes. Near-UV spectra were recorded from 200 nm to 310 nm at 20°C by averaging 5 scans at 0.1 nm intervals for each sample. Spectrum Manager 2 was used to subtract the baseline and the spectra were plotted as molar ellipticity, [θ] (deg·cm2·dmol-1).

Nuclear magnetic resonance

All NMR experiments were performed at 0 to 50 ºC on an Agilent DD2-600 NMR equipped with 5mm inverse triple-resonance nanoprobe with 1200 (H), 100 (C), 2000 (H) sensitivity at the Harvard CCB Laukien-Purcell Instrumentation Center, Magnetic Resonance Laboratory. Each IL formulation was characterized by 1H NMR by placing dried IL into an NMR tube containing a co-axial insert filled with DMSO – d6. NMR data were processed and analyzed using Mnova qNMR v1.0.

Gel Electrophoresis

In order to determine the stability of RNA in IL solutions, the dialyzed RNA samples were separated in 1% Agarose gels (containing 0.01% v/v 10,000X GelRed Nucleic Acid Stain, in 1X TBE). Agarose solution was prepared by dissolving in 1X TBE and heated in microwave at 60°C for 10min until dissolved completely. The agarose solution was poured into the casting stand and a 10-well comb was placed to generate wells that are 1.5mm deep. The melted agarose was allowed to cool for 30mins at RT for polymerization. The chamber was filled with 1X TBE buffer solution to a height of 1.5 cm above the gel surface. RNA samples were premixed with the agarose gel loading dye (6X) prior to loading 2 µL of samples into the wells from left to the right. The power supply was activated as soon as all wells were filled, to avoid initial diffusion of the dye into the gel. The samples were run at 100V for 40mins and were imaged using Azure c300 (Azure Biosystems) with cSeries Capture software at the Bauer Core Facility, Harvard University.

Porcine skin penetration ex-vivo studies

Porcine skin studies were carried out in Franz diffusion cells (FDC) with penetration area of 1.77cm^2. The porcine skin was obtained from Lampire Biological Laboratories, Pipersville, PA, USA. Briefly skins were thawed, hairs were trimmed, and washed with phosphate buffer saline (PBS, pH 7.4). A 36mm punch was used to cut out a disc of the skin and a scalpel was used to get rid of the connective tissues and subcutaneous fat layers. The skin (roughly 0.5mm thick) was placed on the diffusion cell with the stratum corneum (SC) layer facing upwards. The acceptor component of the cell was filled with PBS (~12mL) and equipped with a magnetic stirrer bar. 1mL PBS was added to the donor chamber and the conductivity was measured using a waveform generator (Agilent 33120) and voltmeter (Fluke 87 True RMS Multimeter) at a frequency of 100 Hz and amplitude,100 mV. Only skin samples with a measured transepidermal conductivity of less than 10 µA were used for further studies. The cells were kept in an oven at 37ºC to warm up. The donor compartment was left for 5mins before applying 20µL of Cy5-labelled siRNA-IL (siRNA 50µM) solution on top of the skin ensuring full coverage. The donor chamber and side-arm of the

cells were sealed with parafilm/foil and eppendorf respectively to reduce evaporation and were incubated at 37 °C for 24 h on a stirrer plate. Following incubation, the skin was removed from the cell, washed gently with PBS and were further analyzed using tape-stripping and confocal microscopy.

Cryosectioning

After removing the skin from the cell and washing in PBS, skin tissues were flash frozen (up to 2.0 cm in diameter) in OCT (Sakura Finetek, USA) using a suitable tissue mold. Thin sections of the skin (15-20 μm) corresponding to the application area were cut using a Leica Cryostat CM1850 (Leica, Buffalo Grove, IL) at -20ºC. The cut sections were transferred immediately to glass slides (kept at RT) by touching the slides to the sections and were further analyzed.

Confocal microscopy

Following sectioning in the cryostat, skin sections were covered with cover slips. Microscopy was performed on Zeiss 710 Confocal system equipped with Zeiss Axio Imager Z2 microscope with Colibri FL Illumination and CoolSnapnHQ2 camera. The sections were imaged with 40x air 1.2 numerical aperture objective and Ar laser 633nm for red fluorescent Cy5. Images were processed using a java-based image processing program, ImageJ/FIJI. All image acquisition and processing were executed under identical conditions for control and test samples.

Tape-stripping

After removal of the skin from the cell and washing in PBS, the SC was stripped from the epidermis using an adhesive tape up to ten layers (SC1, SC2-5, SC6-10). Following SC removal, the epidermis was separated from the dermis using a surgical sterile scalpel and a third of dermis (by area) was removed by punching with 4mm three times. Each layer was collected separately in glass vials containing 1mL of PBS/methanol (1:1) mixture and was left to shake overnight to extract the Cy5-siRNA from the skin layers, which was further analyzed using a plate reader (Tecan Safire, AG, Switzerland) on a 96-well plate at an excitation wavelength of 633 nm and emission wavelength of 665 nm.

Mice and treatments

Female SKH-1E hairless mice (6-8 weeks old) were purchased from Charles River Laboratories (MA, USA). The animals were kept in a controlled temperature (24 to 26 ºC), a daily 12:12 h light/dark cycle and food and water ad libitum. Experiments were performed according to the approved protocols by the Institutional Animal Care and Use Committee of the Faculty of Arts and Sciences, Harvard University. Healthy mice were treated with 25μL of GAPDH siRNA (50μM)-IL formulation each day for four consecutive days.

For the psoriasis model, mice were treated with a daily dose of freshly prepared 25μL of 50μM NFKBIZ siRNA-IL formulation to the dorsal skin in the morning and air dried. Six hours later, 62.5 mg 5% imiquimod cream (Aldara; Perrigo Co.) obtained from Patterson Veterinary, CO, USA was applied to the same region. Both the IL-siRNA and imiquimod treatments were continued for 4 days. The skin thickness of the dorsal skin was assessed daily by the double skin-fold thickness (DSFT) using an electronic digital Vernier caliper. Erythema and scaling were scored blindly using human Psoriasis Area and Severity Index (PASI) scoring system daily on a scale from 0 (no alteration) to 4 (very distinct alteration) as previously described. The single scores were combined, resulting in a theoretical maximal cumulative score of 8. On day 5, animals were euthanized in a CO2 chamber and the treated dorsal skins (skin area ~4cm^2) were harvested and collected for histology, and qPCR.

ELISA

For semi-quantitative measurement of GAPDH protein in mouse cells following GAPDH siRNA treatment, GAPDH SimpleStep ELISA Kit (ab176642, Abcam) was employed. Briefly, 200mg of harvested frozen skin was pulverized using mortar and pestle to form a powder and homogenized in chilled 0.5mL 1X cell extraction buffer. The lysates were incubated on ice for 20mins and centrifuged at 18000Xg for 20 min at 4°C.The supernatants were collected in clean tubes and the protein concentrations in each sample were quantified immediately using Nanodrop. The samples were diluted to 20mg/mL protein concentrations using 1X cell extraction buffer. The plate strips were prepared following manufacturer's protocol and protein levels were measured using a microplate reader (Biotec Synergy 2, USA) at 450nm.

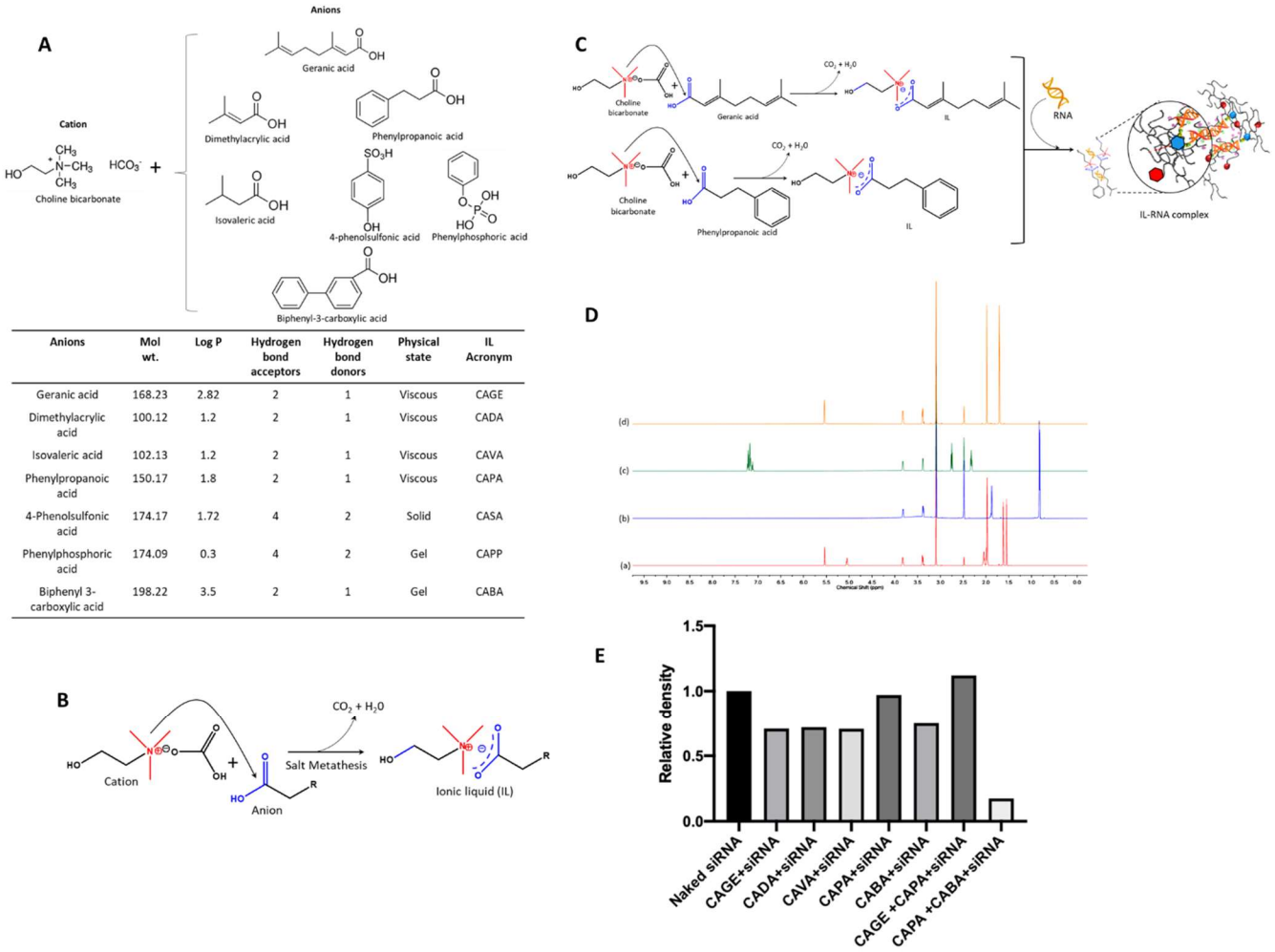
qPCR

After frozen tissues were pulverized to form a powder, tissue lysates were homogenized in 700 µL QIAzol Lysis Reagent and the total RNA was extracted using Qiagen miRNeasy Mini Kit (217004) according to the manufacturer's protocol. The mRNA levels were normalized and was reverse transcribed using Biorad iScript™ Reverse Transcription Supermix (1708841) to yield cDNA. Real-time reverse-transcription PCR was performed on the obtained cDNA with SsoFast EvaGreen Supermix (172-5211). Triplicate reactions for the gene of interest and the endogenous control (β-Actin) were performed separately on the same cDNA samples on a Biorad CFX 96 instrument. The following primer sequences of the mouse NFKBIZ, TNF-α, IL-17A, IL-17C, IL-19, IL-22, IL-23A, IL-36A, IL-36G, CCL20, S100A9, LCN2, and DEFB4 genes were used: GAPDH (Forward: 5'-ACCACAGTCCATGCCATCAC-3'; Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'), NFKBIZ (Forward: 5'-TATCGGGTGACACAGTTGGA-3'; Reverse: 5'-TGAATGGACTTCCCCTTCAG-3'), TNF-α (Forward: 5'-GGCAGGTTCTGTCCCTTTCAC-3'; Reverse: 5'-TTCTGTGCTCATGGTGTCTTTTCT-3'), IL-17A (Forward: 5'-ATGAGTGCCGACAAACAACG-3'; Reverse: 5'-GTGACGTGGAACGGTTGAGG-3'), IL-17C (Forward: 5'- CTGGAAGCTGACACTCACGA-3'; Reverse: 5'-GGTAGCGGTTCTCATCTGTG-3'), IL-19 (Forward: 5'-TTCCACGAGATCAAGAGAGC-3'; Reverse: 5'-TCTACACCTGTTCCGCTGAG-3'), IL-22 (Forward: 5'-TTGAGGTGTCCAACTTCCAGCA-3'; Reverse: 5'-GCATAGGTAGCCAGAGCCAG-3'), IL-23A (Forward: 5'-TGGCATCGAGAAACTGTGAGA-3'; Reverse: 5'-TCAGTTCGTATTGGTAGTCCTGTTA-3'), IL-36A (Forward: 5'-AGTGGGTGTAGTTCTGTAGTGTGC-3'; Reverse: 5'-GTTCGTCTCAAGAGTGTCCAGATAT-3'), IL-36G (Forward: 5'-CACAGATGAGAACCGCTACCC-3'; Reverse: 5'-GCGGATGAACTCGGTGTGGAA-3'), CCL20 (Forward: 5'-GTGGGTTTCACAAGACAGATG-3'; Reverse: 5'-TTTTCACCCAGTTCTGCTTTG-3'), S100A9 (Forward: 5'-CCTTCTCAGATGGAGCGCAG-3'; Reverse: 5'-TGTCCAGGTCCTCCATGATG-3'), LCN2 (Forward: 5'-GGACCAGGGCTGTCGCTACT-3'; Reverse: 5'-GGATCCCGATGGCTAGAGCA-3') and DEFB4/mBD4 (Forward: 5'-AGGGAAGGATGAGATTAAGACTGG-3'; Reverse: 5'-CTTGCTGGTTCTTCGTCTTTT-3'. Primers for the housekeeping gene, β-Actin (Forward: 5'-CGGTTCCGATGCCCTGAGGCTCTT-3'; Reverse: 5'-CGTCACACTTCATGATGGAATTGA-3'). The specificities of the primers were verified, and amplicon specificity was monitored by melting curve analysis. For each genomic sequence evaluated, a ΔCt value was calculated for each sample by subtracting the Ct value of the treated sample from the Ct value obtained for the untreated/control group. Calculating 2^ΔΔCt yielded the relative amount of PCR product (relative enrichment).

Histopathology and Immunohistochemistry

Sections (15-20 μm) from OCT embedded tissues were stained with hematoxylin and eosin and evaluated by light microscopy. For Ki67 immunohistochemistry, sections were heated at 100 in citrate buffer (pH 6.0) for 30 mins for antigen retrieval. Sections were incubated with anti-ki67 primary antibody (rabbit anti-mouse monoclonal; 1:1000 dilution; ab16667, Abcam, Cambridge, UK) overnight at 4°C and later with peroxidase coupled anti-rabbit IgG secondary antibody for 30 mins. Sections were stained with DAB, counterstained with hematoxylin and evaluated using light microscopy (Olympus BX53 microscope with Olympus camera). Epidermal thicknesses were measured for control and test samples using ImageJ/FIJI software.

Supplementary Figures



Anions	Mol wt.	Log P	Hydrogen bond acceptors	Hydrogen bond donors	Physical state	IL Acronym
Geranic acid	168.23	2.82	2	1	Viscous	CAGE
Dimethylacrylic acid	100.12	1.2	2	1	Viscous	CADA
Isovaleric acid	102.13	1.2	2	1	Viscous	CAVA
Phenylpropanoic acid	150.17	1.8	2	1	Viscous	CAPA
4-Phenolsulfonic acid	174.17	1.72	4	2	Solid	CASA
Phenylphosphoric acid	174.09	0.3	4	2	Gel	CAPP
Biphenyl 3-carboxylic acid	198.22	3.5	2	1	Gel	CABA

Figure S1. Design and synthesis of an in-house cholinium-based IL library for improved biocompatibility and interaction with RNA. (A) Cholinium-based IL library constituting various anions that are synthesized with CAGE as the reference IL. (B) General synthetic scheme for salt metathesis employed in the synthesis of ILs. (C) Synthetic scheme of the optimized IL combination (CAGE + CAPA) for the delivery of siRNA. (D) 1H-NMR spectra of the synthesized ILs that remain viscous at RT, (a) CAGE, (b) CAVA, (c) CAPA and (d) CADA. (E) Relative density of the siRNA band following IL incubation measured with Image J software.

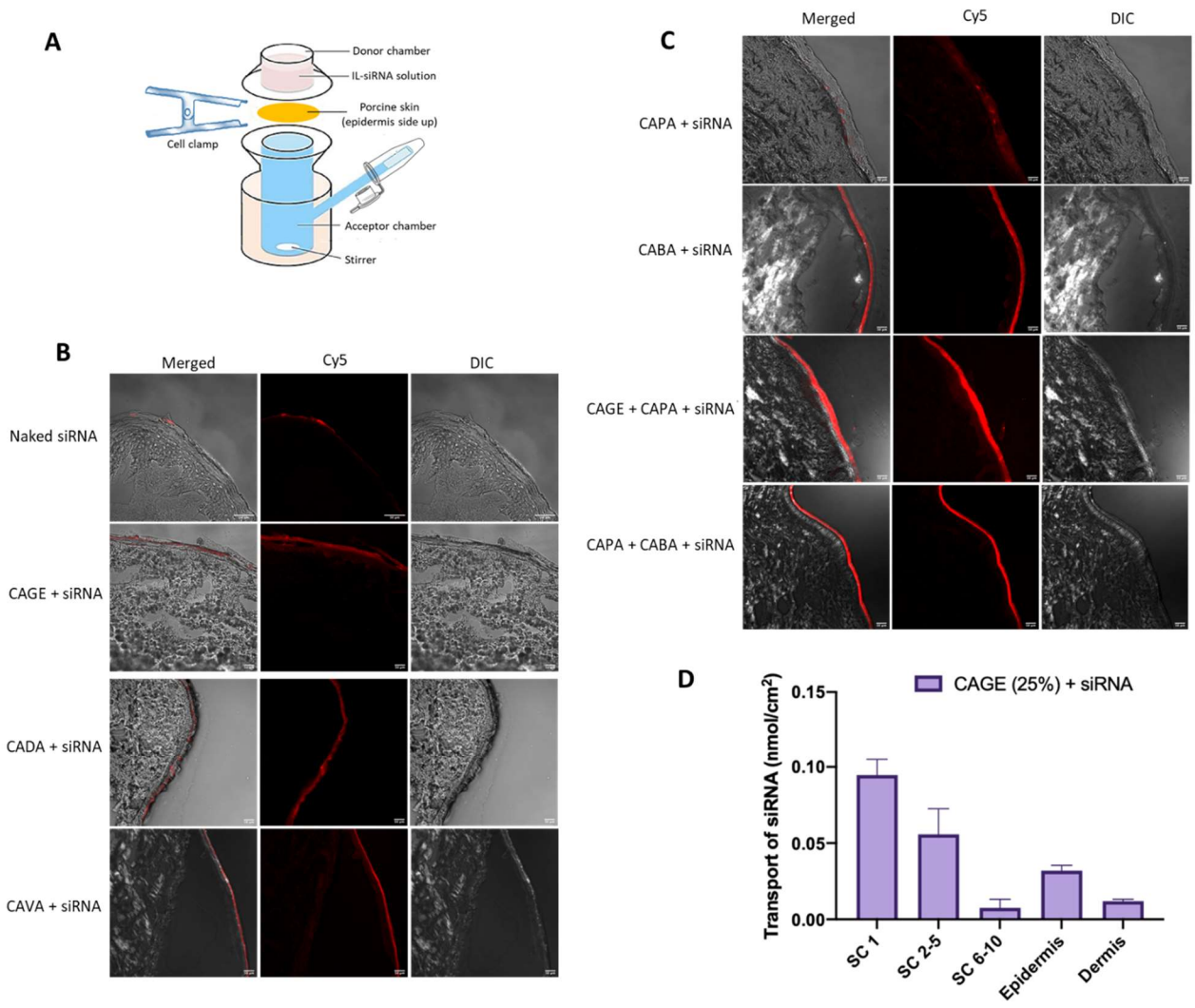


Figure S2. Improved epidermal accumulation of Cy5-labelled siRNA in presence of ILs. (A) Schematic representation of the Franz diffusion cell (FDC) setup for the ex-vivo porcine skin permeation studies. (B) Representative confocal images of the controls, naked siRNA and siRNA in presence of CAGE. (C) Epidermal accumulation of Cy5-siRNA in the presence of newly synthesized cholinium-based ILs and combinations at a ratio 1:1 following 24hrs incubation of porcine skin. Left to right: merged, Cy5, differential interference contrast (DIC). Scale bars, 50µm. (D) Transport of Cy5-labelled siRNA into the different layers of skin determined by tape-stripping method (n=3). Data are averages ± SEM, was determined to be non-parametric by normality test and statistics by Kruskal-Wallis test.

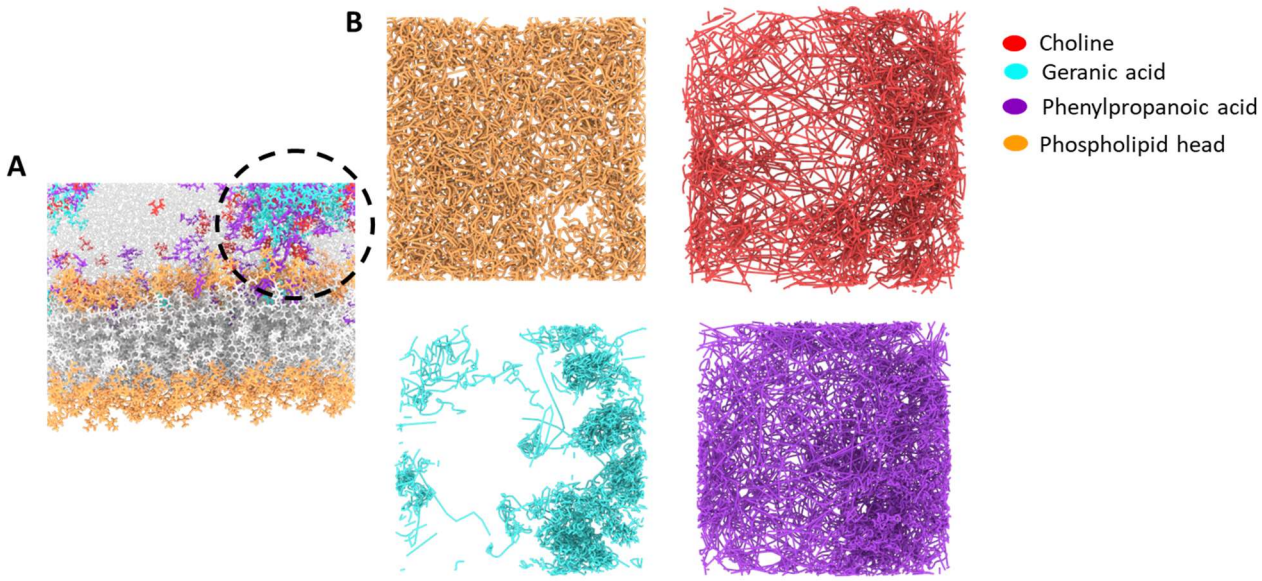


Figure S3. Major contribution of IL species mobility in IL-lipid bilayer interaction and permeation. (A) Lipid bilayer simulation in presence of the IL combination (highlighted with the circle) (B) Trajectories of the individual ionic species within the IL combination, CAGE + CAPA simulation using the python library MDAnalysis.

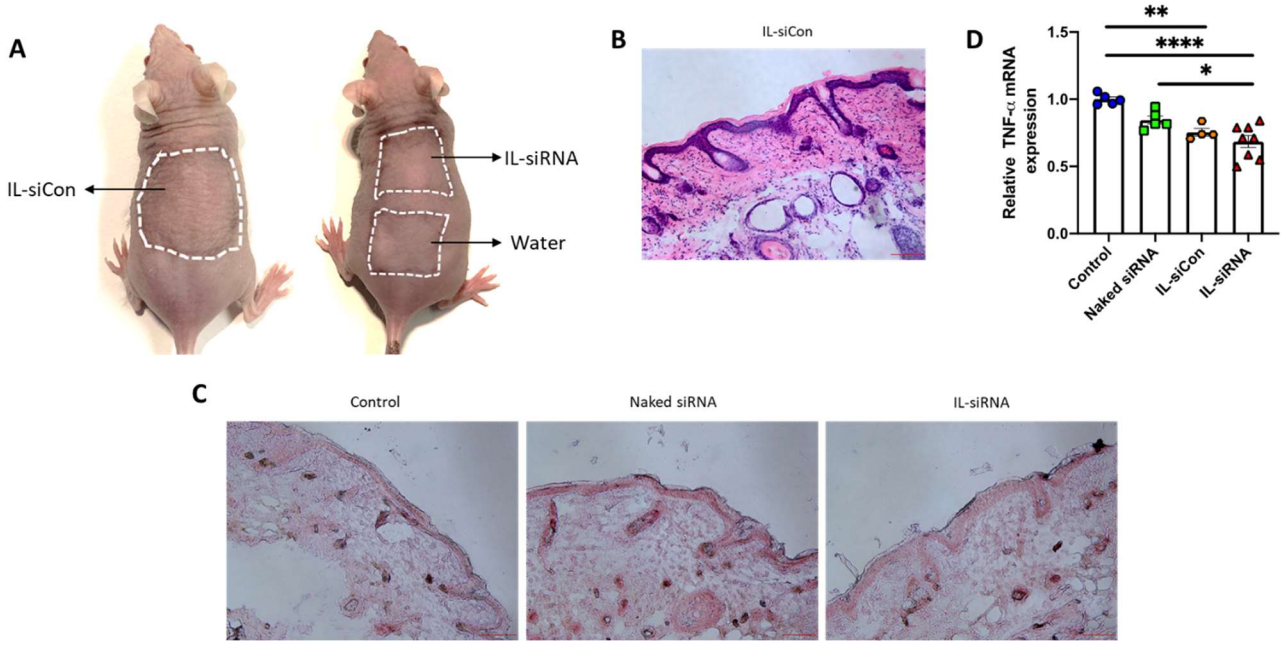


Figure S4. Highly biocompatible IL formulation without toxicity and irritation upon topical application. (A) Application sites of healthy mice treated topically with IL-GAPDH siRNA and were compared with water- and IL-siCon groups. (B) H&E staining of the skin section from the healthy mice treated topically with IL-siCon for 4 consecutive days. Scale bars, 100μm, Magnification, 10x. (C) Skin sections from the healthy mice were analyzed for hyper-proliferation by staining with the proliferation marker, Ki67. Scale bars, 100μm. Quantitative analysis for IHC was not performed since no proliferated regions were observed. (D) TNF-α mRNA expression was measured by qPCR and β-actin mRNA expression was used for normalization. Data are averages ± SEM, statistics by one-way ANOVA with Tukey HSD post-test. *P<0.05, **P < 0.01, ****P < 0.0001. (control, n=5; naked siRNA, n=5; IL-siCon, n=4; IL-siRNA, n=8). Photo credit: Fig S4A: Abhirup Mandal, Harvard University, Figs. S4B, C: Ninad Kumbhojkar, Harvard University.

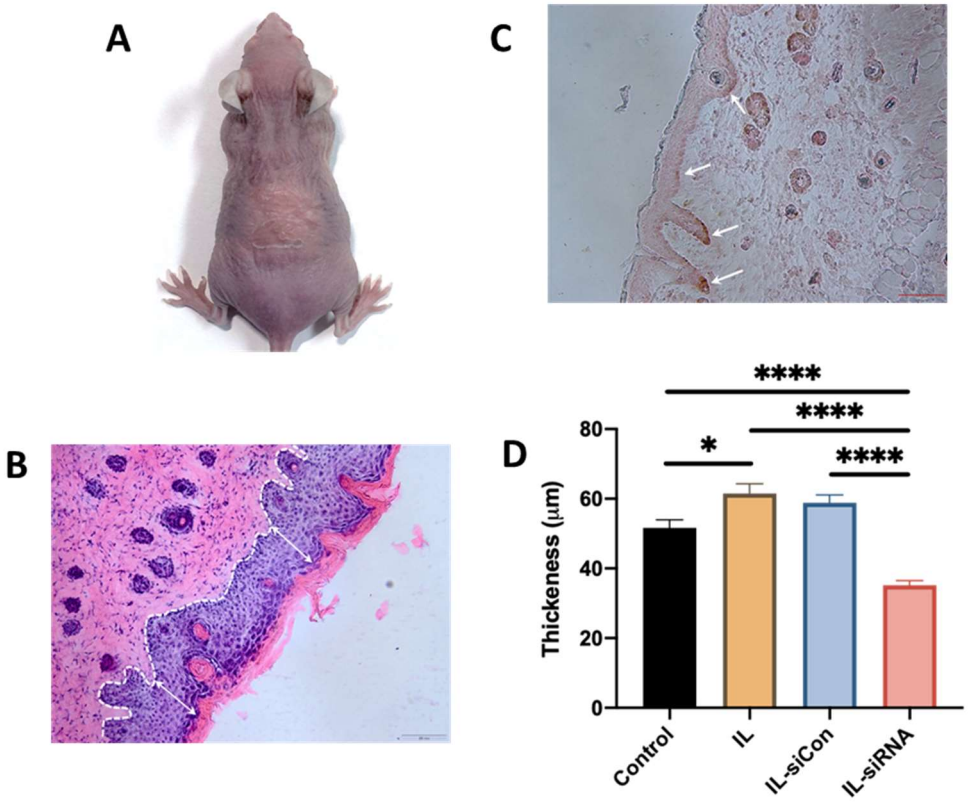


Figure S5. Characterization of IL-siCon effects in imiquimod-induced psoriatic mice. (A) Psoriasis-induced mice were treated topically with IL-siCon. For 4 consecutive days. (B) H&E staining of the skin section from the imiquimod-induced psoriatic mice treated topically with IL-siCon. Scale bars, 50µm, Magnification, 10x. (C) Skin sections from the psoriatic mice were analyzed for hyper-proliferation by staining with the proliferation marker, Ki67. Scale bars, 100µm. (D) Epidermal thickness; means of epidermal thickness calculated based on 10–15 random site measurements with Image J software. Data are averages ± SEM, statistics by one-way ANOVA with Tukey HSD post-test. *P<0.05, ****P < 0.0001. Photo credit: Fig S5A: Abhirup Mandal, Harvard University, Figs. S5B, C: Ninad Kumbhojkar, Harvard University.

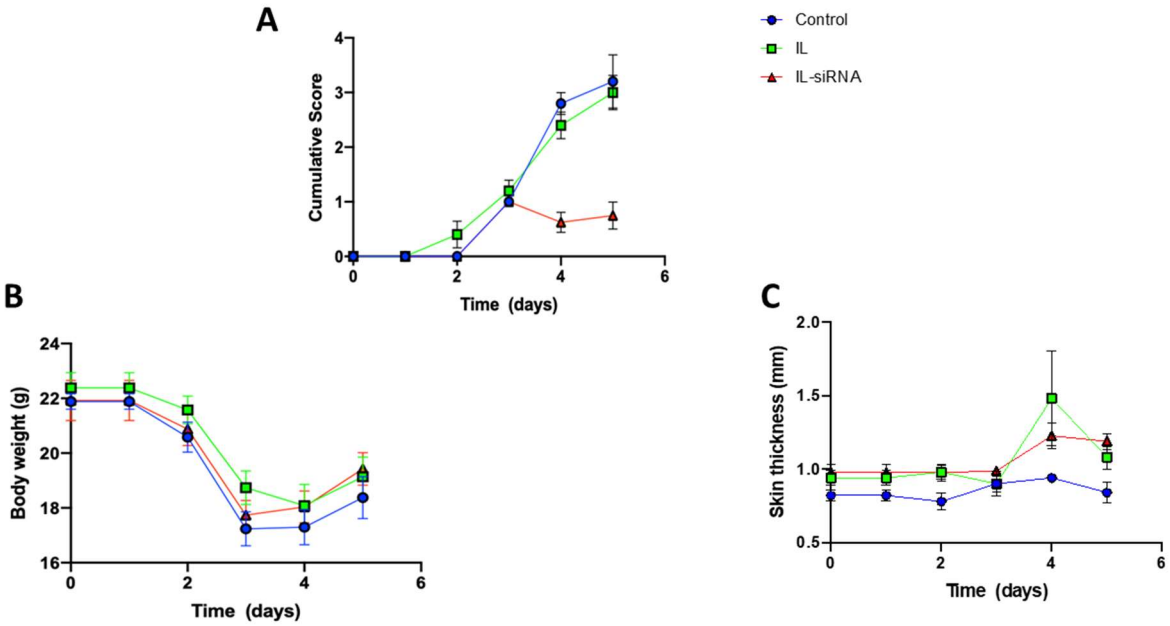


Figure S6. Effects of IL-NFKBIZ siRNA in imiquimod-induced psoriasis-like skin inflammation in mice. Imiquimod-induced psoriatic mice were analyzed for cumulative score (A), body weight (B) and skin thickness (C), monitored by the double skin-fold thickness (DSFT) over a period of 5 days of induction/application. Data are averages ± SEM. (control, n=4; IL, n=4; IL-siRNA, n=8).

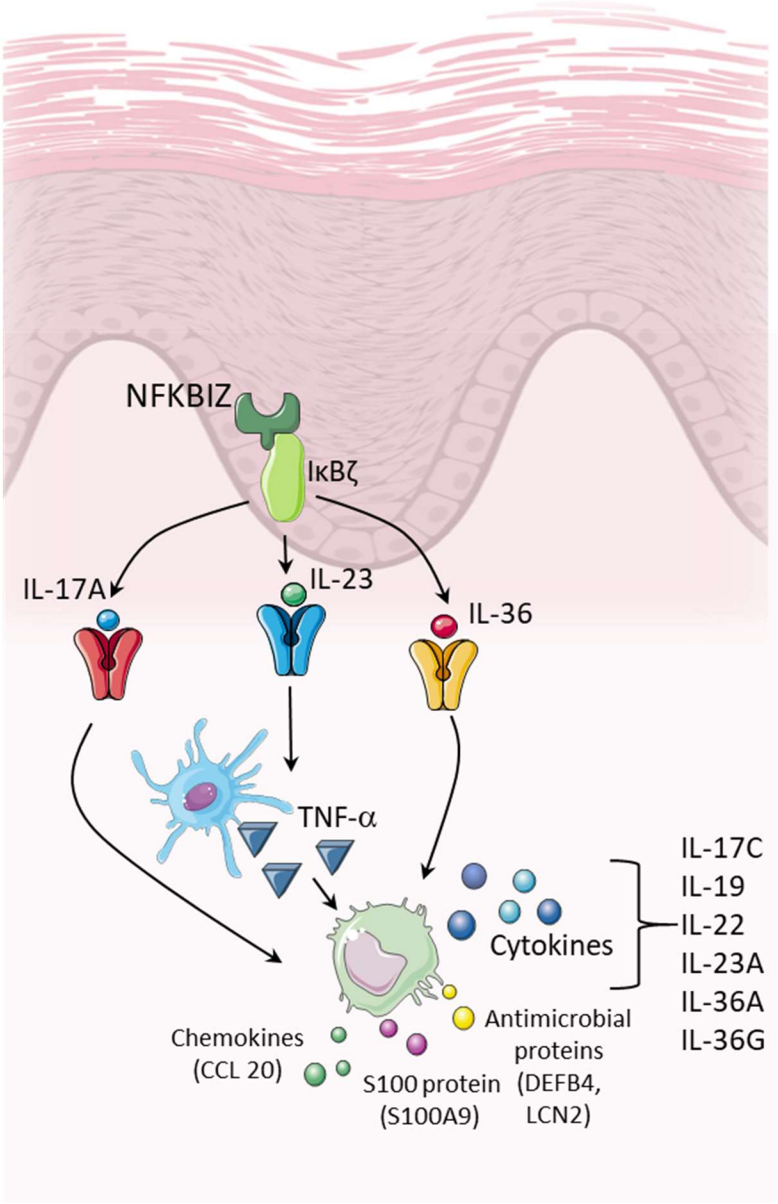


Figure S7. Schematic representation of NFKBIZ as a key mediator of proinflammatory-signaling pathways and psoriasis-associated downstream genes in keratinocytes. NFKBIZ encodes IκBζ which is critically involved in the pathogenesis of psoriasis by mediating downstream effects of IL-17A, IL-23 and IL-36. IκBζ also plays a crucial role in the transcriptional upregulation of antimicrobial proteins, S100 protein, cytokines and chemokines involved in the pathogenesis of psoriasis.

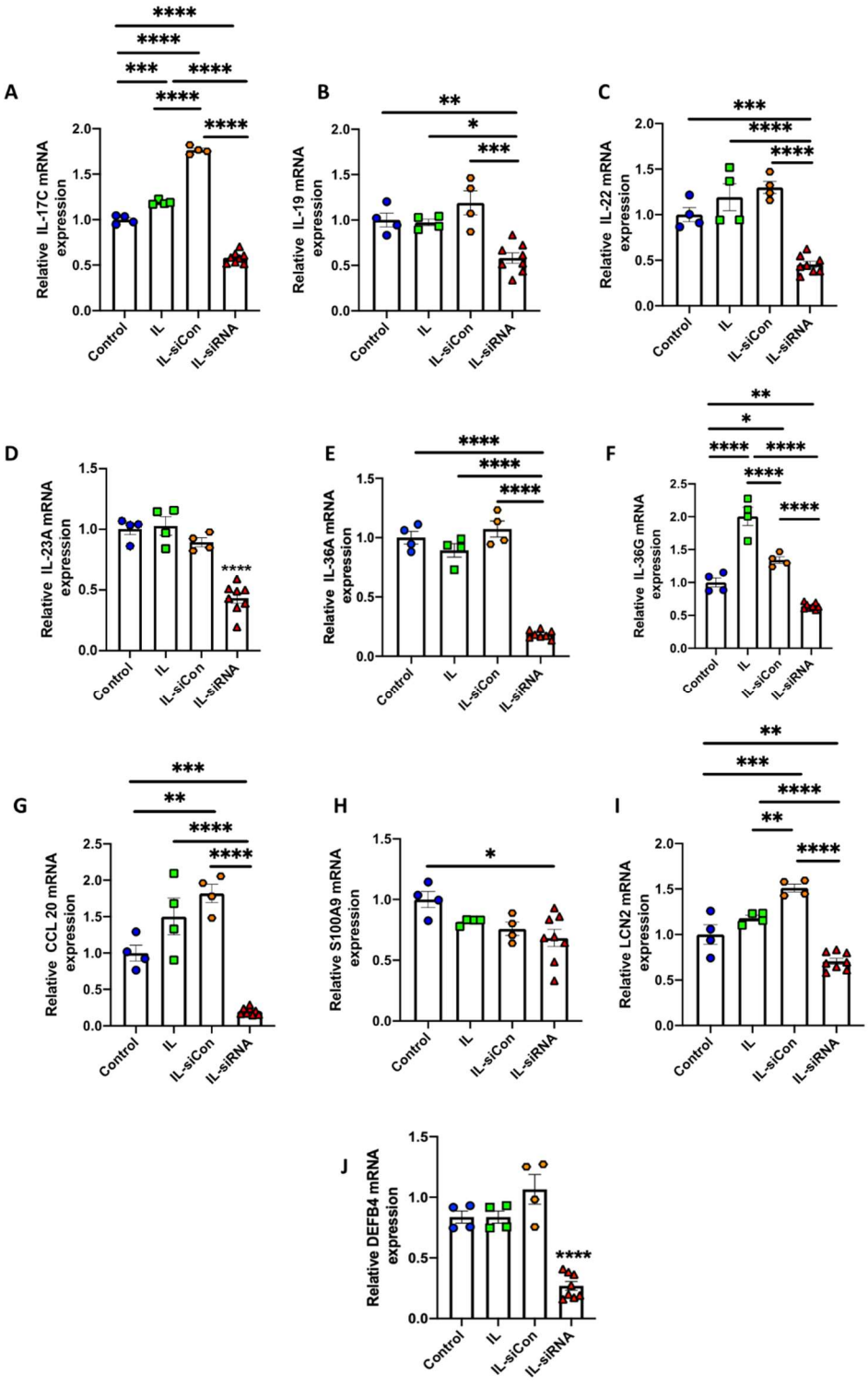


Figure S8. Downstream effect of silencing NFKBIZ on psoriasis-related gene products. (A-J) mRNA expression was measured by qPCR and β-actin mRNA expression was used for normalization for cytokines, IL-17C, IL-19, IL-22, IL-23A, IL-36A, IL-36G (A-F); chemokine, CCL 20 (G); S100 protein, S100A9 (H); antimicrobial protein, lipocalin-2, LCN2 and β-defensin-2, DEFB4 (J). Data are averages ± SEM, statistics by one-way ANOVA with Tukey HSD post-test. *P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (control, n=4; IL, n=4; IL-siCon, n=4; IL-siRNA, n=8).