Genomic control of inflammation in experimental atopic dermatitis

Yan Liu1,2,6, Jozef Zienkiewicz1,2,6, Huan Qiao1, Katherine N. Gibson-Corley3, Kelli L. Boyd3, Ruth Ann Veach4 & Jacek Hawiger1,2,5,*

Atopic Dermatitis (AD) or eczema, a recurrent allergic inflammation of the skin, afflicts 10–20% of children and 5% of adults of all racial and ethnic groups globally. We report a new topical treatment of AD by a Nuclear Transport Checkpoint Inhibitor (NTCI), which targets two nuclear transport shuttles, importin α5 and importin β1. In the preclinical model of AD, induced by the active vitamin D₃ analog MC903 (calcipotriol), NTCI suppressed the expression of keratinocyte-derived cytokine, Thymic Stromal Lymphopoietin (TSLP), the key gene in AD development. Moreover, the genes encoding mediators of TH2 response, IL-4 and its receptor IL-4Rα were also silenced together with the genes encoding cytokines IL-1β, IL-6, IL-13, IL-23α, IL-33, IFN-γ, GM-CSF, VEGF A, the chemokines RANTES and IL-8, and intracellular signal transducers COX-2 and iNOS. Consequently, NTCI suppressed skin infiltration by inflammatory cells (eosinophils, macrophages, and CD4+ T lymphocytes), and reduced MC903-evoked proliferation of Ki-67-positive cells. Thus, we highlight the mechanism of action and the potential utility of topical NTCI for treatment of AD undergoing Phase 1/2 clinical trial (AMTX-100 CF, NCT04313400).

Atopic Dermatitis (AD), also known as eczema, is the most common, recurrent inflammatory skin disorder afflicting an estimated 10–20% of children and 5% of adults in all racial and ethnic groups in the US and abroad1,2, with the highest incidence among African Americans3. Therefore, the impact of AD on the well-being of a diverse population is significant and indicative of a need for effective and safe therapies. AD, caused by allergic insults, is mediated by inflammation and manifested by intense itch, recurrent eczematous lesions, and a fluctuating course4.

Treatment of AD mediated by a plethora of genes encoding the mediators of skin inflammation poses a significant challenge. Currently used broad spectrum therapy with glucocorticoids (e.g., triamcinolone), and targeted therapies with monoclonal antibodies (e.g., dupilumab, tralokinumab) blocking IL-4 receptor, or small molecule inhibitors targeting intracellular signaling intermediates, such as Janus Kinases (JAKs; e.g., upadacitinib and abrocitinib), Phosphodiesterase 4 (PDE4; e.g., crisaborole), and calcineurin (e.g., tacrolimus, pimecrolimus), only partially inhibit inflammatory signaling responsible for AD development and persistence2,5–7. Targeting one or two mediators of skin inflammation by inhibitors of JAKs, PDE4, calcineurin, as well as the receptor for IL-4 and IL-13 by monoclonal antibodies, has advanced the treatment of AD and other skin inflammatory diseases2. However, other untargeted mediators contribute to the refractoriness or relapse of AD (see Suppl. Fig. S1). In turn, a broad-spectrum therapy with glucocorticoids through their binding to the cognate nuclear receptor reprograms the inflammatory regulome in the cell's nucleus while simultaneously enhancing the expression of metabolic genes causing hyperglycemia, hyperlipidemia, osteoporosis, and skin atrophy2,4.

An alternative to glucocorticoids therapy is topical treatment with Nuclear Transport Checkpoint Inhibitor (NTCI). This anti-inflammatory cell-penetrating peptide targets two nuclear transport shuttles, cytoplasmic adaptor proteins termed importin α5 and importin β18,9. Targeting these two proteins that comprise the nuclear transport checkpoint (see Suppl. Fig. S1) produces a broad-spectrum anti-inflammatory activity through silencing of at least 50 genes encoding mediators of inflammation and metabolism10. Their expression depends on the nuclear transport/translocation of an entire set of proinflammatory Stress-Responsive Transcription Factors (SRTFs), which include NF-κB, cFos/cJun, STAT1, and NFATs. Most recently, we found that the nuclear import of STAT3, the transcription factor involved in allergic inflammation, is inhibited by targeting importin α5 with...
NTCI in primary human keratinocytes derived from adult skin (unpublished data). In parallel, Metabolic Transcription Factors (MTFs) are also translocated to the cell’s nucleus. MTFs comprise Sterol Regulatory Elements-Binding Proteins (SREBPs) translocated solely by importin β1, and Carbohydrate Response Element-Binding Proteins (ChREBPs) shuttled either by importin α5 that recognizes the NLS zip code or the importin β1 that binds to the Helix-Loop-Helix Leucine Zipper-binding site (see Suppl. Fig. S1). NTCI, the cSN50.1 peptide, stopped not only SRTFs and SREBPs but also ChREBPs on their path to nucleus by targeting the two components of the nuclear transport checkpoint, importin α5 and importin β1. Hence, the inflammatory regulome in the genome comprising the multiple genes that encode the mediators of allergic, autoimmune, microbial, and metabolic inflammation, cannot be activated. We postulated that AD can be treated by NTCI through inhibition of nuclear signaling, thereby suppressing inflammatory injury to the skin.

The experimental model of AD caused by topical administration of MC903, the low calcemic analog of Vitamin D₃, was introduced by Pierre Chambon’s group after calcipotriol was found to cause the signs of AD in clinical trials for the treatment of skin psoriasis. MC903, applied topically to mouse ears, induces the characteristic signs of AD consisting of swelling, and T₃₁₂ type inflammation manifested in a dermal infiltrate. These cardinal criteria for MC903-induced AD were established by Gilhar and colleagues. The pathognomonic features of AD were linked to epidermal keratinocytes expressing Thymic Stromal Lymphopoietin (TSLP). Its genetic ablation prevented AD-like skin changes upon challenge with MC903. Moreover, TSLP role in promoting T₃₁₂ response and driving the pathogenesis is critical in the mechanism of AD.

Keratinocytes are strategically positioned in the outermost layer of the skin, the epidermis. They form a multilayered structure to maintain the functional integrity of the skin, which depends on three compartments within the epidermis: (i) the air–liquid barrier, (ii) the liquid–liquid barrier, and (iii) the immune defense barrier. Langerhans cells comprise the latter barrier. Their dendritic projections abut the keratinocytes, bridged by Tight Junctions (TJs), thereby demarcating the fluidic environment of the skin and favoring intercellular communication during the inflammatory response. Keratinocytes, also named ‘cytokinocytes’, contribute to immune responses in skin.

Keratinocytes sense and respond to proinflammatory insults by activating the signaling pathways to the nucleus, the site of the inflammatory regulome (see above). Therein, SRTFs and MTFs reprogram the inflammatory regulome and activate multiple genes that encode a myriad of inflammatory mediators. They encompass cytokines, chemokines, their receptors, cell adhesion molecules, as well as extracellular and intracellular signaling intermediates.

TSLP expressed by epidermal keratinocytes promotes T₃₁₂ response and drives the pathogenesis of AD. Therefore, we aimed to establish whether NTCI would suppress TSLP and other genes involved in the allergic skin inflammation underlying AD.

We show that NTCI effectively reduces the cardinal signs of AD in an experimental model. To our knowledge, this study is the first preclinical proof of concept for the genomic control of AD by NTCI at the nuclear transport level. It can be extended to other inflammatory skin diseases. Its translational value is being evaluated in the ongoing Phase 1/2 Clinical Trial for mild to moderate AD (AMTX-100 CF, NCT04313400).

**Results**

**Genomic control of experimental atopic dermatitis by a nuclear transport checkpoint inhibitor (NTCI).** AD was induced by a topical application of MC903 to both sides of the right ear for 23 days (Fig. 1).

Topical treatment with an aqueous solution of NTCI, cell-penetrating cSN50.1 peptide, 200 µg (66 nmoles, administered twice daily to the right ear) was initiated on day 20 and continued for 9 days. The right ear of MC903-challenged mice became progressively swollen until treatment with NTCI significantly reduced skin edema, one of the chief signs of inflammation (Fig. 2A). Moreover, skin lesions, prominently displayed in the ear during the MC903 induction, were healed after 9 days of treatment with NTCI (Fig. 2C).

Thus, treatment with NTCI effectively reduced existing skin inflammatory signs, such as swelling, discoloration, and scaling. A troubling side effect of AD induction with MC903 is body weight loss. Notably, the animals treated with NTCI maintained their body weight (Fig. 2B), indicating a lack of NTCI’s general toxicity. This is consistent with the lack of apparent toxicity associated with long-term NTCI treatment in previous preclinical studies. The lack of apparent toxicity of NTCI was also independently attested prior to the submission of the clinical trial protocol to the FDA.

Gene expression profiling of skin obtained from animals challenged with MC903 and left untreated was compared to skin obtained from animals challenged with MC903 and treated with saline, and animals challenged with MC903 and treated with NTCI. Gene expression in skin obtained from mock control animals, which were not challenged with MC903, is also shown for comparison (Fig. 3).

The development of skin lesions in MC903-challenged ears was accompanied by the induction of genes encoding the mediators of allergic skin inflammation, including TSLP, the inducer of the T₃₁₂ response, and its mediators, IL-4, IL-4Ra, IL-13, along with IL-6, IL-23α, IL-33, IFN-γ, and other cytokines, chemokines, growth factors, and intracellular signaling intermediates. TSLP activates Langerhans Cells, which initiate an adaptive TH2 immune response in skin.

In addition, experimental AD was associated with increased expression of genes encoding the chemokine RANTES, as well as two intracellular signaling intermediates, COX-2, and iNOS, responsible for the synthesis of prostaglandins and nitric oxide (NO), respectively. The latter, together with VEGF A, are significant factors in the microvascular endothelial injury associated with skin inflammation manifested by swelling and itching.

MC903-induced AD was manifested by infiltration of the skin with macrophages, eosinophils, and T lymphocytes, including CD4 + T cells. Inflammatory skin lesions consisted predominantly of macrophages and eosinophils, combined with an influx of CD4 + T cells, and significant proliferation of Ki67-positive cells in the basal
cell layers of the epidermis (Fig. 4A). Immunohistochemical analysis also reflected NTCI control of the cellular effectors of AD. Thus, the major criteria for the experimental AD were controlled by NTCI. Consequently, we recorded a striking reduction of experimental AD manifested by redness, swelling, and infiltration of immune cells involved in skin inflammation.

Skin infiltration by eosinophils, macrophages, and T cells in response to MC903 was suppressed by NTCI treatment as documented by a semiquantitative analysis of immunohistochemical (IHC) staining (Fig. 4B). T cell infiltrates consisted primarily of CD4+ T cells with a paucity of CD8+ T cells. Strikingly, the proliferation of Ki67-positive cells in the basal zone of the epidermis was also attenuated. Therefore, we suggest that the cellular immunopathology of experimental AD is tightly linked to the genomic burst of the key mediators of skin inflammation. Taken together, these cellular mediators were suppressed by NTCI treatment thereby demonstrating a profound attenuation of the skin inflammatory response.

Significantly, NTCI also suppressed skin inflammation induced by another agent, Phorbol-12-Myristate-13-Acetate (PMA), which produces severe thickening of the pinna (Fig. 5). Topically-applied PMA causes chronic skin inflammation mediated by Kit, a receptor tyrosine kinase required for mast cell accumulation20. The resolution of inflammation in this second model of experimental AD controlled by NTCI, is consistent with our prior discovery of its effectiveness in suppressing the PMA-induced nuclear transport of SRTFs comprising NF-κB, cFos/cJun, NFAT, and STAT1 in human T cells21. Thus, NTCI suppression of cardinal manifestations of MC903-induced AD (skin swelling, eczematous lesions, and dermal infiltrate), and PMA-induced skin inflammation attests to an effective and broad-range action of NTCI in these two preclinical settings.

**Discussion**

We wish to report the NTCI, cell-penetrating cSN50.1 peptide, as a novel agent for treatment of AD, a recurrent inflammatory skin disease afflicting a sizable population of children and adults worldwide. A major step in understanding the mechanism of AD is the outcome of NTCI topical treatment of skin lesions caused by repeated challenge with MC903 (calcipotriol) for 23 days in an experimental model of AD. This vitamin D3 analog was found to induce the signs of AD during its clinical trial in patients with psoriasis5. Notably, TSLP-deficient mice did not develop AD when challenged with MC903 thereby assigning a major role to the gene encoding TSLP in the mechanism of AD13.

NTCI suppresses the gene encoding TSLP in mice challenged with MC903 (see Fig. 3), thereby providing a new line of evidence for the key role of TSLP in the mechanism of AD. Moreover, success of NTCI treatment also lies in the control of genes encoding other significant mediators of the T112 response in AD, such as IL-4, its receptor, IL-4Ra, and IL-13. In addition, follicular helper T cells (Tfh) are essential for humoral immunity
and T-cell memory in atopic diseases. The migration of T<sub>FH</sub> cells depends on the chemokine receptor CXCR5 (CD185) regulated by SRTFs whose nuclear translocation is controlled by NTCI. Furthermore, silencing of the gene encoding IL-23α, is significant as this cytokine induces AD-like inflammation in CCR2-deficient mice. The gene encoding VEGF A, known as vascular permeability factor, was also suppressed by NTCI in the MC903-challenged skin explaining at least in part its reduction of swelling. Of note, the VEGF also contributes to the microvascular endothelial injury underlying skin inflammation in psoriasis and other skin disorders. Taken together, this outcome presents NTCI as a potentially effective and safe topical treatment of eczema and other inflammatory skin diseases.

Neuropathic itch is a significant sign of neurogenic inflammation in AD and other chronic skin inflammatory diseases. The migration of T<sub>H</sub> cells depends on the chemokine receptor CXCR5 (CD185) regulated by SRTFs whose nuclear translocation is controlled by NTCI. Furthermore, silencing of the gene encoding IL-23α, is significant as this cytokine induces AD-like inflammation in CCR2-deficient mice. The gene encoding VEGF A, known as vascular permeability factor, was also suppressed by NTCI in the MC903-challenged skin explaining at least in part its reduction of swelling. Of note, the VEGF also contributes to the microvascular endothelial injury underlying skin inflammation in psoriasis and other skin disorders. Taken together, this outcome presents NTCI as a potentially effective and safe topical treatment of eczema and other inflammatory skin diseases.

Neuropathic itch is a significant sign of neurogenic inflammation in AD and other chronic skin inflammatory diseases. In addition to triggering T<sub>H</sub>-mediated allergic response, keratinocyte-derived TSLP activates cutaneous sensory neurons to induce itch. Striking suppression of TSLP expression in the skin challenged by MC903 (see Fig. 3) explains the itch-reducing potential of NTCI. Moreover, its inhibition of nuclear translocation of STAT3 in human keratinocytes (unpublished results), and by extension, in sensory neurons, may also contribute to the itch-reducing action of NTCI. Both STAT3 and importin α5, which is the target of NTCI, are involved in lesioned peripheral nerve, as reported by Ben-Yaakov and colleagues.

The suppression of two other genes expressed in inflamed skin, IL-1β, and IL-6 (see Fig. 3) is also significant. These cytokines are responsible for the generalized inflammatory response known as the “acute phase protein response” associated with Endoplasmic Reticulum (ER) stress. Thus, NTCI counteracts both the localized and, potentially, systemic inflammatory response in AD. Moreover, the uncontrolled action of IL-1β is linked
Figure 3. The expression of genes encoding the mediators of skin inflammation is suppressed by NTCI. The expression of genes encoding the mediators of allergic inflammation in the ear samples (see Methods and Fig. 1 for a depiction of AD-like phenotype induction and treatment protocol) was determined using a real-time quantitative reverse transcription PCR (qRT PCR). The relative levels of expression were established using Livak's methods ($2^{-\Delta\Delta C_t}$) with the 18S gene as a reference and the Mock Control (unchallenged ear) group as the calibrator. Data is presented as a mean ± S.E.M. (n = 3 for Mock Control, n = 5 for all other conditions). A statistical analysis was performed using an ordinary one-way ANOVA with an uncorrected Fisher’s LSD test for a multiple comparison, *p < 0.05, **p < 0.005, ***p < 0.0005. The significance levels displayed over the bars of MC903 + Saline and MC903 + cSN50.1 columns represent the statistical difference of each compared to MC903 Untreated samples.

To an inflammatory disorder of the skin and bones caused by the loss-of-function mutation of IL-1 receptor antagonist50. IL-1β also mediates ILC2-independent "spontaneous AD" in mice with defective skin barrier31.

The discovery of the nuclear transport checkpoint inhibitor’s role in the control of the key genes underlying the mechanism of AD is of particular significance. We submit that AD is chiefly mediated by the proinflammatory transcription factors (SRTFs) (Fig. 6). These transcription factors encompass NF-kB, AP1 (cFos and cJun), STAT1, and NFATs as well as STAT3, the transcription factor involved in AD and other diseases mediated by allergic inflammation15.

In contrast to SRTFs and MTFs (MW > 45 kDa), the smaller transcription factors (MW < 45 kDa), some of which are essential to cell survival and maintenance, have “a free pass” to the nucleus, providing homeostatic control of the cell’s function and lifespan33. To this end, the smaller transcription factors cross the nuclear envelope...
Figure 4. The attenuation of MC903-induced skin inflammation in the murine pinna by NTCI (cSN50.1 peptide) treatment. (A) Exposure to MC903 without treatment or treated with a saline control leads to cellular infiltration comprising predominately macrophages (Iba-1) and eosinophils (major basic protein or MBP) combined with an influx of CD4+ T cells (CD4), leading to the thickening of the pinna as compared to the mock control. Moreover, the significant immunodetection of Ki67-positive cells indicated increased cellular proliferation in the basal cell layers of the epidermis in response to MC903. Treatment with NTCI (cSN50.1 peptide) reduced the cellular mediators of the inflammatory response. NTCI reduced skin edema (see also Fig. 2A). Shown are representative images of pinna samples with immunohistochemistry for key inflammatory cell markers (× 20 magnification). (B) A semiquantitative analysis of immunohistochemical (IHC) staining presented in panel A. Scoring was conducted according to following formulas: IHC scoring (all inflammatory cell markers): 0—No or almost undetectable immunoreactivity (IR); 1—Scattered and rare IR in the dermis; 2—Multifocal and barely coalescing IR in the dermis; 3—Diffuse IR in the dermis. IHC scoring for Ki67 in epidermis: 0—None; 1—Single cell layer IR at basal zone of epidermis; 2—IR 2—5 layers thick within the basal zone of epidermis; 3—IR > 5 cell layers thick within the basal zone of epidermis. The data is presented as a mean + S.E.M. (n = 5). The statistical analysis was performed using ordinary one-way ANOVA with an uncorrected Fisher’s LSD test for a multiple comparison, *p < 0.05, **p < 0.005.
while bypassing the nuclear transport checkpoint targeted by NTCI. Accordingly, we found that NTCI treatment did not alter the expression of the five housekeeping genes (Gusb, Hprt1, Hsp90ab1, Gapdh, and Actb). Our findings of significant suppression of the genes encoding TSLP and IL-1β have potential therapeutic implications for other human skin diseases caused by an inborn overproduction of these cytokines. The uncontrollable TSLP signaling plays a key role in patients with Netherton Syndrome, a rare hereditary skin disorder manifested by a susceptibility to AD, scaling skin, and hair abnormalities. The uncontrolled action of IL-1β is linked to an inflammatory disorder of the skin and bones caused by the loss-of-function mutation of IL-1 receptor antagonist. These and other autoinflammatory diseases of the skin and other organs are mediated by constitutive inflammation. The afflicted patients may benefit from NTCI-based therapy suppressing TSLP, IL-1β, and other relevant and excessively acting mediators.

Clearly, the NTCI reported in this study represents a new class of broad-spectrum anti-inflammatory agents for topical (localized) and, if needed, systemic therapy. We are not aware of any published study of AD, which applied a similar method of controlling gene transcription by the inhibition of nuclear transport to suppress the expression of the key genes involved in the development of AD through the TH2 response. Among the other known broad-spectrum anti-inflammatory therapies, the glucocorticoids suppress inflammatory regulome via the action of their cognate nuclear receptor, which functions as transcription factor. However, glucocorticoids cause hyperglycemia, hyperlipidemia, osteoporosis, skin atrophy, and immunosuppression. In striking contrast, NTCI reduces blood glucose, cholesterol, and triglycerides, and increases the innate immunity-mediated clearance of bacteria.
These points are relevant for the link between AD and microbial inflammation or metabolic inflammation. First, AD predisposes skin to infections with *Staphylococcus aureus*. The staphylococcal immunotoxin, *Staphylococcal Enterotoxin B* (SEB), also known as superantigen, stimulates IL-13 production, which is viewed as a potential etiologic factor in infants with AD. NTCI suppressed the expression of IL-13 in the skin in our study (see Fig. 3). We anticipate that NTCI would suppress staphylococcal immunotoxin-associated flare ups of AD consistent with our previous studies demonstrating NTCI control of SEB-induced toxic shock and acute respiratory distress syndrome. Second, AD is associated with Metabolic Syndrome in pediatric and adult patients. Its spectrum encompasses obesity, hyperglycemia, hyperlipidemia, fatty liver, atherosclerosis, and hypertension. In the preclinical model of Metabolic Syndrome, due to the genetic ablation of the LDL receptor (*ldlr*-/-), combined with the Western Diet, NTCI controlled the metabolic hypersensitivity to a sublethal dose of LPS, a well-known inducer of microbial inflammation. Thus, NTCI can also control AD, which is associated with inflammation caused by autoimmune, microbial, and metabolic inducers (see Fig. 6).

In summary, we successfully enabled NTCI to control the proinflammatory transcriptional mechanism of AD, the most common skin disease in the world. Our findings are of significant relevance to millions of individuals worldwide displaying signs of AD as well as other skin disorders that depend on the nuclear transport of transcription factors responding to inflammatory stress in human keratinocytes and immune cells. Our results provide a mechanistic blueprint for the translational application of NTCI (AMTX-100 CF), which is undergoing Phase 1/2 clinical trial for mild to moderate AD (NCT04313400).

Materials and methods
The synthesis and purification of cell-penetrating nuclear transport checkpoint inhibitor, cSN50.1 peptide. Our cell-penetrating NTCI peptide, cSN50.1 (AAVALLPAILALLAP-CVQRKRQKLMPC, 2986 Da) was synthesized as described elsewhere. Briefly, the peptide chain was assem-
bled through Solid Phase Peptide Synthesis (SPPS) according to standard Fmoc chemistry protocols using an automated peptide synthesizer FOCUS XC (APPTec, Louisville, KY). Crude peptides were removed from the resin with a TEA cleavage cocktail and purified by dialysis against double-distilled water in 1 KDa membrane (Spectra/Por 7; Spectrum Laboratories, Rancho Dominguez, CA). The purity and structure of the final products were verified respectively by an analytical C18 RP HPLC (Beckman Coulter GOLD System, Brea, CA) and MALDI mass spectroscopy (Voyager Elite; PerSeptive Biosystems, Framingham, MA).

**Animal studies.** Animal experiments were carried out in compliance with the ARRIVE guidelines and in strict accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health, and submitted protocols were approved by the Vanderbilt University Institutional Animal Care and Use Ethics Committee (Permit Number: A3227-01). Mice were closely monitored during the course of experiments and euthanized by Isoflurane inhalation followed by cervical dislocation at the experimental endpoint.

*The experimental model of atopic dermatitis (Eczema).* Atopic dermatitis (AD)-like phenotype was induced in 8-week-old C57BL/6 female mice (The Jackson Laboratory) by topical application of the Vitamin D$_3$ analog, calcipotriol (MC903)$^{11,41}$. The experimental groups (Mock Control, n = 3; MC903 Untreated, n = 5; MC903 + Saline, n = 5; and MC903 + cSN50.1, n = 5) were selected using a double blinded randomization method$^{17}$.

We used saline as a control representing a vehicle for all the NTCI peptide used in this study. This vehicle control is used in FDA-approved clinical studies of human subjects in which the tested new drug is administered, including therapeutic peptides. Each experiment was performed twice to assure statistical significance and experimental reproducibility.

The Treatment Protocol (see Fig. 1 for a graphic representation of the model and treatment protocol): the initiation of AD-like phenotype induction began with a bolus dose of 6 nmoles MC903 (Millipore-Sigma #C4369) applied topically to the right ear (3 nmoles on each side). Repeated exposure to the irritant (once-a-day, Q.D.) was initiated on day 3 with 2 nmoles of MC903 (1 nmoles on each side) and the challenge was continued for 21 days. NTCI treatment was introduced on day 20 as a twice daily (B.I.D.) topical application of its saline solution (10 µl of 10 mg/ml on each side) and was continued for 9 days. The control group of mice received a topical saline treatment (10 µl) on both sides of the right ear. On day 29, all mice were euthanized by over inhalation of isoflurane followed by cervical dislocation. 3–4 samples of the right ear (3 mm in diameter) were collected postmortem from auricula using a sterile disposable biopsy punch. One sample was fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry studies, remaining samples were snap frozen and stored at ~80 °C for total RNA extraction.

*PMA-induced skin inflammation.* Skin inflammation was induced in 8-week-old C57BL/6 female mice (The Jackson Laboratory) by topical application of the Phorbol-12-Myristate-13-Acetate (PMA, Millipore-Sigma #524,400). The experimental groups (Vehicle, n = 5; PMA, n = 5; and PMA + cSN50.1, n = 5) were selected using a double blinded randomization method as described before$^{17}$. A single dose of PMA (2 nmoles in 10 µl EtOH) was administered topically on each side to the right ear. Topical treatment with NTCI, cSN50.1 peptide (16.7 nmoles in 10 µl EtOH on each side of the right ear) or vehicle (10 µl EtOH on each side) was administered 30 min before and 3, 6, and 8 h. after PMA challenge. The unchallenged control group of mice was treated topically with vehicle only (10 µl EtOH) following the same treatment schedule. Ear thickness was measured at 30 min before ("0 h" timepoint) and 3, 6, 8 and 24 h. after PMA challenge. 3 mm ear punch biopsies were collected at 8 h. after PMA challenge from treated area under mild anesthesia (5% isoflurane). Ear samples were fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry studies. 24 h. after PMA challenge, all mice were euthanized by over inhalation of isoflurane followed by cervical dislocation.

**Gene expression assay by real-time quantitative reverse transcription PCR.** The mouse ear punch biopsies were disrupted in lysis buffer on ice using a Dounce hand homogenizer. Total RNA was isolated using NucleoSpin RNA Plus kit (Macherey–Nagel, Germany) according to the manufacturer’s instructions. RNA concentration and purity were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). 1 µg of the obtained RNA was reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad, CA). A real-time quantitative reverse transcript PCR was carried out in a 96-well plate on a QuantStudio 3 instrument using the Taqman Fast Advanced Master Mix (Thermo Fisher Scientific) according to the manufacturer’s protocol. The FAM-labeled probes for mouse genes were obtained from Thermo Fisher Scientific. The raw Ct values were converted into relative expression levels using Livak’s methods (2$^{-ΔΔCt}$) with the 18S gene as a reference, and the Mock Control group as a calibrator (control). Converted Ct values were used for statistical analysis.

**Histology and immunohistochemistry.** Pinna samples were collected by the postmortem punch biopsy of the skin (see above) and fixed overnight in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 µm and stained with Hematoxylin and Eosin (H&E). Immunohistochemistry analyses with antibodies against macrophages (Iba-1; FujiFilm, 019-19,741), eosinophils (MBP; Mayo Clinic, MR-14.7), CD4 (SYSC, HS-360 017), CD8 (SYSC HS-361 003), and Ki67 (Abcam, ab16667) were performed on the Leica Bond Max (Leica Biosystems Inc. Buffalo Grove, IL) following standard protocols in the Translational Pathology Shared Resource at Vanderbilt University Medical Center. Briefly, all assays were antigen retrieved using an Epitope Retrieval 2 solution for 20 min except MBP (Epitope Retrieval 1 solution for 15 min). Leica polymer was used as a secondary antibody for Iba-1, CD8, and Ki67 while a rat secondary antibody (Vector BA-4001) was used for MBP and CD4. The Bond Polymer Refine Detection system was used for visualization.
chemical stains were evaluated by a veterinary pathologist (K.N.G-C and K.L.B) blinded to the composition of the groups.

**Statistical analysis.** Normal distribution of data sets was verified using normal probability plot (q-q) and Kolmogorov–Smirnov Normality Test. A statistical analysis was performed using tools built-in Prism 6 software (GraphPad). Gene expression profile in ear skin samples of C57Bl/6 mice as well as semiquantitative IHC scores were analyzed by ordinary one-way ANOVA with an uncorrected Fisher's LSD test for a multiple comparison. The data is presented as a mean ± SEM. p values of <0.05 were considered significant.

**Data availability**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Received: 12 May 2022; Accepted: 25 October 2022

Published online: 07 November 2022

**References**

1. Nutten, S. Atopic dermatitis: Global epidemiology and risk factors. *Ann. Nutr. Metab.* 66(Suppl 1), 8–16 (2015).
2. Brunner, P. M., Guttmann-Yassky, E. & Leung, D. Y. The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. *J. Allergy Clin. Immunol.* 139(4S), S65–S76 (2017).
3. Kaufman, B. P., Guttmann-Yassky, E. & Alexia, A. F. Atopic dermatitis in diverse racial and ethnic groups—Variations in epidemiology, genetics, clinical presentation and treatment. *Exp. Dermatol.* 27(4), 340–357 (2018).
4. Weidinger, S., Beck, L. A., Bieber, T., Kabashima, K. & Irvine, A. D. Atopic dermatitis. *Nat. Rev. Dis. Primers.* 4(1), 1 (2018).
5. Gottlieb, A. B. Therapeutic options in the treatment of psoriasis and atopic dermatitis. *J. Am. Acad. Dermatol.* 53(1 Suppl 1), S3-16 (2005).
6. Beck, L. A. et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. *N. Engl. J. Med.* 371(2), 130–139 (2014).
7. Hawiger, J. & Zienkiewicz, J. Decoding inflammation, its causes, genomic responses, and emerging countermeasures. *Scand. J. Immunol.* 90(6), e12819 (2019).
8. Schoepf, S., Schacke, H., May, E. & Asadullah, K. Glucocorticoid therapy-induced skin atrophy. *Exp. Dermatol.* 15(6), 406–420 (2006).
9. Zienkiewicz, J., Armitage, A. & Hawiger, J. Targeting nuclear import shuttles, importins/karyopherins alpha by a peptide mimicking the NFkappaB1/p50 nuclear localization sequence. *J. Am. Heart Assoc.* 2(5), e00386 (2013).
10. Liu, Y. et al. Nuclear transport modulation reduces hypercholesterolemia, atherosclerosis, and fatty liver. *J. Am. Heart Assoc.* 2(2), e00093 (2013).
11. Li, M. et al. Topical vitamin D3 and low-calcemic analogues induce thymic stromal lymphopoietin in mouse keratinocytes and trigger atopic dermatitis. *Proc. Natl. Acad. Sci. USA.* 103(31), 11736–11741 (2006).
12. Gilhar, A. et al. Mouse models of atopic dermatitis: A critical reappraisal. *Exp. Dermatol.* 30(3), 319–336 (2021).
13. Li, M. et al. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue M903 on mouse skin. *J. Invest. Dermatol.* 129(2), 498–502 (2009).
14. Marschall, P. et al. Dual function of Langerhans cells in skin TSLP-differentiated CD4+ T-helper T cells. *Allergy Clin. Immunol.* 147(9), 1778–1794 (2021).
15. Kubo, A., Nagao, K. & Amagai, M. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. *J. Clin. Invest.* 122(2), 440–447 (2012).
16. Jiang, Y. et al. Cytokinocytes: the diverse contribution of keratinocytes to immune responses in skin. *JCI Insight.* 5(20) (2020).
17. Liu, Y. et al. Hyperlpidemic hypersensitivity to lethal microbial inflammation and its reversal by selective targeting of nuclear transport shuttles. *Sci. Rep.* 11(1), 19097 (2021).
18. Eber, S. et al. Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. *J. Allergy Clin. Immunol.* 119(4), 982–990 (2007).
19. Theoharides, T. C. et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc. Natl. Acad. Sci. USA.* 107(9), 4448–4453 (2010).
20. Waskow, C., Bartels, S., Schlenner, S. M., Costa, C. & Rodewald, H. R. Kit is essential for PMA-inflammation-induced mast-cell accumulation in the skin. *Blood* 109(12), 3563–3570 (2007).
21. Torgerson, T. R., Colosia, A. D., Donahue, J. P., Lin, Y. Z. & Hawiger, J. Regulation of NF-kappa B, AP-1, NFAT, and STAT1 nuclear accumulation in the skin. *J. Allergy Clin. Immunol.* 90(6), e12819 (2019).
22. DiGiandomenico, A. et al. The “genomic storm” induced by bacterial endotoxin is calmed by a nuclear transport modifier that attenuates localized and systemic inflammation. *PLoS ONE* 9(10), e110183 (2014).
23. Bromley, S. K., Larson, R. P., Ziegler, S. F. & Luster, A. D. IL-23 induces atopic dermatitis-like inflammation instead of psoriasis-like inflammation in CCR2-deficient mice. *PLoS ONE* 8(3), e58196 (2013).
24. Steinhoff, M., Schmelz, M., Szabo, I. L. & Oaklander, A. L. Clinical presentation, management, and pathophysiology of neuropathic itch. *Lancet Neurol.* 17(8), 709–720 (2018).
25. Siiskonen, H. & Harvima, I. Mast cells and sensory nerves contribute to neurogenic inflammation and pruritus in chronic skin inflammation. *Front Cell Neurosci.* 13, 422 (2019).
26. Wilson, S. R. et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. *Cell* 155(2), 285–295 (2013).
27. Ben-Yaakov, K. et al. Axonal transcription factors signal retrogradely in lesioned peripheral nerve. *EMBO J.* 31(6), 1350–1365 (2012).
28. Poli, V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J. Biol. Chem.* 273(45), 29279–29282 (1998).
29. Gabay, C. & Kushner, I. Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* 340(6), 448–454 (1999).
30. Aksentijevich, I. et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N. Engl. J. Med.* 360(23), 2426–2437 (2009).
31. Schwartz, C. et al. Spontaneous atopic dermatitis in mice with a defective skin barrier is independent of IL22 and mediated by IL-1β. *Allergy* 74(10), 1920–1933 (2019).
32. Furue, M. Regulation of skin barrier function via competition between AHR Axis versus IL-13/IL-4/JAKSTAT6/STAT3 Axis: pathogenic and therapeutic implications in atopic dermatitis. *J. Clin. Med.* 9(11), 3741 (2020).
33. Hawiger, J. & Zienkiewicz, J. Endothelial cytoprotection in metabolic and microbial inflammation underlying cardiovascular diseases. *Cardiovasc. Res.* (2022) - under review

34. Briot, A. et al. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. *J. Exp. Med.* **206**(5), 1135–1147 (2009).

35. Ong, P. Y. et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**(15), 1151–1160 (2002).

36. Lehmann, H. S., Heaton, T., Mallon, D. & Holt, P. G. Staphylococcal enterotoxin B-mediated stimulation of interleukin-13 production as a potential aetiologic factor in eczema in infants. *Int. Arch. Allergy Immunol.* **135**(4), 306–312 (2004).

37. Liu, D. et al. Suppression of staphylococcal enterotoxin B-induced toxicity by a nuclear import inhibitor. *J. Biol. Chem.* **279**(18), 19239–19246 (2004).

38. Liu, D., Zienkiewicz, J., DiGliomendico, A. & Hawiger, J. Suppression of acute lung inflammation by intracellular peptide delivery of a nuclear import inhibitor. *Mol. Ther.* **17**(5), 796–802 (2009).

39. Silverberg, J. I. & Paller, A. S. Association between eczema and stature in 9 US population-based studies. *J. Invest. Dermatol.* **139**(3), 984-990.e1 (2019).

Acknowledgements
We thank Ania Hawiger and Adam Hawiger for their assistance in the preparation of this manuscript. We thank Drs. Mary-Margaret Chren and Eric Tkaczyk, and their colleagues in the Department of Dermatology, Vanderbilt University, for their constructive comments about this study.

Author contributions
J.Z. and J.H. designed the study; Y.L., J.Z., H.Q., and R.A.V. performed experiments; Y.L., J.Z., H.Q., R.A.V., K.N.G-C, and K.L.B acquired and analyzed data; Y.L., J.Z., and J.H. drafted the manuscript; J.Z. prepared figures; All authors reviewed the manuscript; J.H. approved the submitted version.

Funding
This work was supported by an Endowed Louise B. McGavock Chair bestowed by Vanderbilt University, the Immunotherapy Program, Department of Medicine, Vanderbilt University Medical Center, and the U.S. Department of Veterans Affairs (Award#: 2 T01 BX002750). The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). The Translational Pathology Shared Resource is supported by NCI/NIH Cancer Center Support Grant P30CA068485 and the Shared Instrumentation Grant S10 OD023475-01A1 for the Leica Bond RX. Hawiger Laboratory received financial support from Amytrx Therapeutics, Inc. for the initial feasibility study.

Competing interests
Y.L., J.Z., R.A.V., and J.H. are coinventors of patents assigned to Vanderbilt University and the US Department of Veterans Affairs. J.H. cofounded Amytrx Therapeutics, Inc. Other authors have declared no conflict of interest.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-23042-x.

Correspondence and requests for materials should be addressed to J.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2022