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Keratinocyte nicotinic acetylcholine receptor activation modulates early TLR2-mediated wound healing responses

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Abstract

The cholinergic anti-inflammatory pathway spans several macro- and micro-environments to control inflammation *via* $\alpha 7$ nicotinic acetylcholine receptors (nAChRs). Physiologic inflammation is necessary for normal wound repair and is triggered, in part, *via* Toll-like receptors (TLRs). Here, we demonstrate that keratinocyte nAChR activation dampens TLR2-mediated migration and pro-inflammatory cytokine and antimicrobial peptide (AMP) production, which is restored by a $\alpha 7$ -selective nAChR antagonist. The mechanism of this response occurs by blocking the NF- κ B and Erk1/2 pathway during early and late wound healing. In a mouse model of *Staphylococcus aureus* wound infection, topical nAChR activation reduces wound AMP and TLR2 production to augment bacterial survival in wild-type mice. These findings suggest that aberrant $\alpha 7$ nAChR activation may impair normal wound healing responses, and that pharmacologic administration of topical nAChR antagonists may improve wound healing outcomes in wounds necessitating a more robust inflammatory response.

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AUTHOR CONTRIBUTIONS

M.K. performed all of the cell experiments, performed most mouse experiments, analyzed results and wrote the original version of the manuscript. T.M.G. performed mouse experiments and several subsequent analyses. K.A.R performed and executed several mouse experiments, supervised and designed experiments for other contributors, and edited the final versions of the manuscript. All authors were involved in the final editing of the manuscript and approval of submission.

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INTRODUCTION

Following skin injury or *S. aureus* infection, Toll-like Receptor 2 (TLR2) plays a crucial role in the induction of innate immune responses, promoting re-epithelialization and bacterial clearance^{1,2}. TLR2 recognizes components of gram-positive bacteria and forms heterodimers with TLR1 or TLR6 in a cluster of differentiation 14 (CD14)-dependent manner^{3,4}. This requires the recruitment of the intracellular anchor protein myeloid differentiation factor 88 (MyD88) which activates nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) pathway⁵ and leads to the production of pro-inflammatory cytokines and antimicrobial peptides (AMPs)^{1,2}. Stimulation of keratinocytes with the TLR2/6 ligand, MALP-2 (macrophage activating lipoprotein-2), induces robust expression of keratinocyte cathelicidin and β -defensin AMPs¹. Aberrant AMP expression and activity is one mechanism behind susceptibility to cutaneous microbial infections and pathologic inflammation, as AMPs provide direct microbicidal activity, stimulate inflammation, and aid in maintaining skin barrier function⁶⁻⁹.

The keratinocyte acetylcholine axis is comprised of enzymes for ACh synthesis and degradation to maintain epidermal homeostasis¹⁰. ACh stimulates epidermal cellular processes through ligand-gated ionotropic muscarinic ACh receptors (mAChRs) and nicotinic ACh receptors (nAChRs) expressed on keratinocytes classified by their respective agonists, muscarine and nicotine, respectively^{11,12,13}. We recently identified a novel mechanism for immunosuppression *via* the non-neuronal cholinergic system in the epidermis, by which keratinocyte $\alpha 7$ nAChR activation significantly reduced AMP responses during skin bacterial infection¹⁴.

It is unknown whether aberrant epidermal nAChR activation has the capacity to modulate cutaneous innate immune responses and bacterial survival during wound healing. In light of our previous reports that keratinocyte nAChR activation dampens AMP responses and influences the susceptibility to infection¹⁴, we hypothesize that epidermal nAChR activation would be detrimental to acute wound healing by suppressing TLR2-mediated wound healing responses upstream of AMP induction to delay healing and increase infection risk. In this study, we describe a mechanism by which altered keratinocyte nAChR activation can regulate TLR2-mediated wound healing responses. Our findings emphasize the potential benefit of topical nAChR agonists or antagonists to modulate wound healing outcomes.

RESULTS

Keratinocyte nAChR activation dampens TLR2-mediated wound migration and AMP-dependent cytokine production

Activation of nAChRs in keratinocytes by nicotine and other agonists at supraphysiologic doses (μ M concentrations) was found to promote keratinocyte migration or wound closure by ionic- and kinase-dependent pathways^{15,16}. However, the capacity of physiologic levels (pM-nM concentrations) of nAChR agonists to influence TLR2-mediated keratinocyte migration has not been investigated. To first determine whether nAChR agonists impact TLR2-mediated keratinocyte migration, we analyzed the migration of epidermal keratinocytes following *in vitro* wounding using an *in vitro* scratch wound assay in

keratinocyte monolayers¹⁷. We immediately stimulated with nAChR agonists (ACh or nicotine) in the presence or absence of the TLR2/6 agonist, MALP-2. Although ACh is a natural agonist, the promiscuity of ACh on multiple receptor subtypes between mAChRs and nAChRs makes it difficult to distinguish direct and indirect effects¹³. Thus, nicotine is used to selectively assess direct effects on nAChR signaling, as it mimics ACh effects on nAChRs¹³. MALP-2 treatment significantly increased keratinocyte migration compared to vehicle treatment. Both ACh and nicotine significantly reduced TLR2-mediated migration by MALP-2 by >50%, indicating a direct role for nAChRs in this response (Fig. 1a). We then assessed whether the delay in migration is mediated by selective nAChRs; therefore, we employed two selective nAChR antagonists, an endogenous $\alpha 3\beta 4$ selective antagonist, Catestatin (Cst)¹⁸, and a pharmacological $\alpha 7$ receptor selective antagonist, α -Bungarotoxin (α -Bung)¹⁴. No changes in migration were observed with either nAChR antagonist applied alone. However, Cst restored MALP-2-mediated keratinocyte migration in the presence of 10^{-9} M ACh (31% vs. 43% migration) and nicotine (33% vs. 45% migration) compared to MALP-2 (72% migration) (Fig. 1a). α -Bung was more potent in restoring migration levels to that of MALP2 in the presence of 10^{-9} M ACh (31% vs. 52% migration) and nicotine (33% vs. 70% migration), indicating that TLR2-mediated keratinocyte migration was attenuated mostly through $\alpha 7$ nAChR activation. These results indicate that the presence or absence of a TLR2 ligand after wounding alters the sensitivity of keratinocyte migration to nAChR agonists. We next determined the maximal inhibitory dose of nAChR agonists on *in vitro* TLR2-mediated keratinocyte wound migration. Activation of nAChRs are known to mediate opposite effects depending upon the subtype involved and the concentration of agonist applied, which may lead to inhibitory effects of nAChRs^{13,15}. No significant differences in keratinocyte migration were observed in the absence of MALP-2 after stimulation with ACh (Supplementary Fig. 1a) or nicotine, although lower doses of nicotine (10^{-12} – 10^{-9}) appeared to exhibit a greater capacity to delay migration, as compared to ACh (Supplementary Fig. 1b). This suggests that different nAChR subtypes may be involved at concentrations below and above 10^{-9} M. In contrast, maximal delay of keratinocyte migration by ACh was observed at 10^{-9} M (50% decrease), as compared to MALP-2 alone (Supplementary Fig. 1c). The presence of nicotine was more potent in inhibiting TLR2-mediated keratinocyte migration, as a significant reduction in keratinocyte migration was observed at several concentrations, with maximal delay of migration observed at 10^{-9} M (65% reduction) (Supplementary Fig. 1d).

We next evaluated TLR2-mediated keratinocyte AMP and pro-inflammatory cytokine protein production by immunofluorescence and ELISA, respectively. MALP-2-induced cathelicidin expression was attenuated along the scratch wound edge by 10^{-9} M ACh and nicotine (Fig. 1b). At 3 hours post-wounding, ACh and nicotine significantly suppressed MALP-2-mediated IL-6 production by 28 % and 43 %, respectively (Fig. 1c), and IL-8 by 35 % and 26 %, respectively (Fig. 1d), albeit these were rather modest inhibitions. However, MALP-2-mediated TNF- α production was not significantly reduced (Fig. 1e). At 24 hours post-wounding, ACh, nicotine and Cst suppressed MALP-2-mediated IL-8 secretion by 76%, 69%, and 70% respectively (Fig. 1f). Only nicotine increased anti-inflammatory cytokine IL-10 production (~290%) (Fig. 1g). The effects of nAChR agonists on pro- and anti-inflammatory cytokines were blocked by both nAChR antagonists (Fig. 2f and g **and**

data not shown). These results suggest that $\alpha 7$ nAChR activation maximally suppresses TLR2-mediated innate immune inflammatory responses during keratinocyte wound healing.

Keratinocyte nAChR activation impairs TLR2 signaling

TLR2 stimulation induces the MAPK and NF- κ B pathways¹⁹ by promoting the nuclear translocation of NF- κ B and the degradation of inhibitor kappa B (I κ B), which positively correlates with the nuclear phosphorylated NF- κ B²⁰. Thus, we next evaluated if nicotine augments TLR2-mediated NF- κ B and MAPK signaling in keratinocytes post-wounding by Western Blot analyses. We determined the % change in protein abundance using semi-quantitative densitometry of target protein bands, and normalized the band densitometry to the densitometry of the respective housekeeping gene (TATA box or β -actin). The MALP-2-mediated increase in both I κ B degradation and nuclear p65 NF- κ B phosphorylation was observed from 5–30 minutes post-wounding (Fig. 2a), which was suppressed by 50% with nicotine exposure. Similarly, nicotine suppressed MALP-2-mediated Erk1/2 phosphorylation at three and six hours post-wounding by 60% (Fig. 2b). However, nAChR agonists did not alter the MALP-2-enhanced p38MAPK and STAT3 activation (Supplementary Fig. 2a and 2b). Collectively, nAChR activation inhibited TLR2 signaling by blocking the NF- κ B and Erk1/2 pathway at early and late time points of TLR2-mediated wound healing, respectively.

We next examined whether nAChR activation influences the expression of TLR2 and CD14 during normal and TLR2-mediated keratinocyte wound healing. Nicotine failed to alter the gene expression of TLR2 or CD14 without MALP-2 (Supplementary Fig. 3a and 3b). In the presence of MALP2, however, TLR2 protein levels were decreased by ~30% by both nicotine and ACh (Fig. 3a and 3b), although nicotine failed to suppress TLR2 gene expression. Furthermore, nicotine significantly suppressed MALP-2-mediated CD14 gene expression by ~50 %, which was consistent with changes in CD14 protein levels (Fig. 3a and 3b). In airway epithelial cells, nicotine was shown to suppress MyD88 and cytokine production as an anti-inflammatory mechanism²¹. Therefore, we next investigated whether nAChR activation in keratinocytes may utilize a similar pathway to block TLR2 responses. We observed that both nicotine and ACh reduced MALP-2-mediated MyD88 production by ~42% (Fig. 3b). These findings suggest that nAChR activation dampens keratinocyte TLR2 signaling during *in vitro* wound healing through several key intermediates at both the gene and protein level.

Epidermal nAChR activation decreases AMP responses, and increases bacterial survival in infected mouse wounds

To next assess whether altered epidermal activation would influence AMP responses during normal wounding, we employed our established model of topical nicotine treatment prior to murine excisional wounding. Wild-type mice treated topically with nicotine exhibited a significant reduction in the gene expression of the mouse cathelicidin *CRAMP*, but not β -defensin 3 (*DefB3*) and β -defensin 14 (*DefB14*) (Fig. 4a) after 24 hours in non-infected wounds. We then assessed the CRAMP protein abundance and localization in both uninfected and methicillin-resistant *S. aureus* (MRSA)-infected mouse wounds, as bacterial lipoproteins derived from *S. aureus* are known to stimulate TLR2-dependent AMP

production in keratinocytes¹. We observed a robust reduction in CRAMP along the wound edge in uninfected mice, which was more pronounced in MRSA-infected wounds (Fig. 4b). This effect was not due to non-specific bacterial antibody absorption, as our IgG controls were negative for immunofluorescence. CRAMP is known to be chemotactic for neutrophils²², and we observed a robust reduction in wound neutrophils via anti-GR1 immunofluorescence in nicotine-treated mice as compared to controls (Fig. 4b). Finally, we assessed whether the reduction in AMP production would result in a functional defect following nicotine treatment. We observed a significant increase in *S. aureus* burden in Day 1 wounds (Fig. 4c), and increased bacterial dissemination into the kidney at day 7 post-wounding (Fig. 4d).

DISCUSSION

TLR2 activation leads to the production of several immunoregulatory molecules, including pro-inflammatory cytokine and AMP production, to initiate cutaneous wound healing¹. Consequently, dampened TLR2 responses after wounding would be detrimental to the tissue repair process. Our previous studies identified a novel mechanism for AMP regulation *via* the nAChR activation in response to skin infection¹⁴. Here, we hypothesized that altered states of nAChR activation modulate TLR2 activation, upstream of AMP regulation, to diminish TLR2-dependent wound repair responses.

Our results confirmed these hypotheses while also revealing that TLR2 is a major target of nAChR activation after wounding, and that the presence of a TLR2 ligand alters the response to nAChR activation in keratinocytes. The mechanism of this response occurs by blocking the NF- κ B and Erk1/2 pathway during early and late wound healing. Inhibition of this response is achieved by the presence of nAChR antagonist, with maximal restoration of TLR2-dependent wound healing responses observed with a $\alpha 7$ nAChR antagonist. In MRSA infected wounds in normal mice, nAChR activation dampens TLR2-mediated pro-inflammatory cytokine and AMP production, which likely contributes to greater bacterial survival and dissemination into the kidney observed in nicotine treated mice.

Although keratinocytes express both nAChRs and mAChRs²³, muscarine (10^{-9} M) did not disrupt TLR2-mediated keratinocyte migration in our studies (data not shown). This suggests that ACh attenuated TLR2-mediated keratinocyte migration selectively through nAChRs, specifically through the $\alpha 7$ nAChR, as this nAChR subtype has been shown to impair cutaneous innate immunity¹⁴. Because the $\alpha 7$ selective nAChR antagonist, α -Bung, reversed the nAChR agonist effect on keratinocyte responses more potently than the $\alpha 3\beta 4$ -selective nAChR antagonist, Cst, we concluded that nAChR activation suppresses TLR2-mediated inflammation and migration primarily through the $\alpha 7$ nAChR in keratinocytes.

We also demonstrate for the first time that nAChR activation reduced TLR2-dependent AMP responses through a selective reduction of pro-inflammatory cytokines and cathelicidin, along with the suppression of NF- κ B and Erk1/2 pathways in a keratinocyte model of wound infection. Furthermore, nicotine significantly reduced CD14 gene expression, but not TLR2, suggesting that nAChR activation may exert a greater effect on CD14. Since TLR2 recognizes ligands transferred by CD36 in CD14-dependent fashion²⁴,

the nAChR-mediated down-regulation of CD14 likely diminishes the ability of TLR2 to recognize bacterial components. MyD88 plays an essential role in TLR2-mediated NF- κ B activation²⁵, and nicotine was found to suppress MyD88 expression via $\alpha 7$ nAChR in airway epithelial cells²⁵. Although the mechanism for MyD88 suppression in keratinocytes remains to be determined, nAChR activation may directly suppress MyD88 through the inhibition of NF- κ B activation²⁶ and/or the induction of IL-10 secretion²⁷. Thus, nAChR activation may attenuate the binding of exogenous or endogenous ligands to TLR2 by suppressing co-factors and adaptor protein expression.

Our study reveals not only the inhibitory effect of nAChR activation on TLR2-mediated wound responses, but also new insights and potential drug targets to improve wound healing outcomes in non-healing wounds.

METHODS

Cell culture

Normal epidermal human keratinocytes (NHEKs) were purchased from Life Technologies (catalog no. C-001-5C) and were grown in serum-free EpiLife[®] medium (Gibco) containing 0.06 mM Ca²⁺ and EpiLife Defined Growth Supplement (Gibco), L-glutamine (HyClone Laboratory) with the addition of 100 U ml⁻¹ of penicillin and 100 μ g ml⁻¹ of streptomycin at 37 °C under standard tissue culture conditions. Culture dishes or plates were coated with Coating Matrix Kit (Life Technologies). The cultures were maintained for up to five passages in this media. Cells were treated at 90–100% confluence.

In vitro assay of epidermal keratinocyte wound healing

For scratch assay, NHEKs were washed with PBS and pretreated with 10 μ g mL⁻¹ mitomycin C (Enzo Life Sciences) for 2 hours to prevent cellular proliferation before wounding. After 2 hours, the mitomycin C was removed and cells were washed three times with PBS. Cells were wounded following *in vitro* wound healing assay, as described above. To standardize the position of the wound for photography, small lines were made on the underside of the well using a thin permanent marker. Cells were incubated with nine different culture conditions in triplicate wells. Cells were stimulated with 100ng mL⁻¹ macrophage-activating lipopeptide-2 (MALP-2) (Enzo Life Sciences), 10⁻¹² – 10⁻⁶ M Acetylcholine (ACh) (Sigma-Aldrich) or Nicotine (Nic) (Sigma-Aldrich), 10⁻⁸ M Catestatin (Cst) (GenScript), 10⁻⁷ M α -Bungarotoxin (Bung) (MP Biomedicals), or their combination. PBS was used as a negative control (vehicle treated cells). The same fields were photographed after 24 hours and the area migrated by keratinocytes was calculated using the Image J Software program (NIH Image). The percent migration at 24 hours was calculated by subtracting the total area of the scratch at 24 hours from the total area of the scratch at 0 hours, divided by the total area at 0 hours multiplied by 100. Images of the areas were collected with an EVOS[®] FL Digital Microscope (Electron Microscopy Sciences).

For ELISA, western blot, and quantitative RT-PCR, NHEKs were scratched once per well vertically with a sterile P200 pipette tip to create an artificial wound. Cells were then washed twice to remove cellular debris, and then incubated with PBS, 100ng mL⁻¹ MALP-2

(Enzo Life Sciences), 10^{-10} or 10^{-9} M ACh (Sigma-Aldrich), 10^{-9} M Nic (Sigma-Aldrich), or their combination for 15, 30, 60 minutes and 3, 6, and 24 hours. Cells were washed with PBS two times. Culture supernatants were collected after spin down at 500G for 5 minutes at 4 °C. Cells and culture supernatant were kept at -80°C until use.

Murine MRSA wound infection

Wild-type C57BL/6 mice were purchased from Jackson Laboratories. All animal experiments were performed with the approval of the Loyola University Chicago, Health Sciences Campus IACUC. Three days prior to topical vehicle or nicotine treatment, dorsal mouse fur was plucked under isoflurane anesthesia and immediately depilated. Vehicle (1:1000 ethanol: water) or 10^{-9} M nicotine (Sigma) were topically applied to dorsal skin twice a day for 3 days. After nicotine treatment, skin wounds were made with 3mm biopsy punch at 6 sites, and then 10 μ l of MRSA USA300 (10^8 CFU/ml) was applied topically to each wound at final concentration of 10^6 CFU/wound. On day 1 post-wounding, wounds were harvested and prepared for subsequent analyses. One wound per mouse was homogenized in sterile PBS with 1 mm zirconia beads in a mini bead-beater (BioSpec Products), diluted and plated on THA to quantify the CFU/ml.

Enzyme-linked immunosorbent assay

The levels of IL-6, IL-8, IL-10, and TNF- α in culture supernatant of NHEKs and IL-6, MIP-2, IL-10, and IL-17A in mouse skin wound homogenates were determined using an ELISA kit in accordance with the manufacturer's recommended protocols (PepProTech).

Immunofluorescence for cells and mouse skin

For cell staining, NHEKs were seeded on chamber slides (Electron Microscopy Sciences). Cells were fixed with methanol and then incubated with anti-cathelicidin (Abcam) at 1:500. Primary antibody was detected with FITC--conjugated secondary antibody (Jackson ImmunoResearch). For mouse wounds, fresh frozen sections (8 μ m) were incubated with rabbit anti-CRAMP antibody (Abcam) diluted at 1:500, or rat anti-GR-1 antibody (BD biosciences) diluted at 1:1000 overnight at 4 °C. Primary antibody was detected using a FITC- or Cy3-conjugated secondary antibody (Jackson ImmunoResearch) overnight at 4 °C. Rabbit IgG (Abcam) or rat IgG (Abcam) were used as negative controls. For cells and mouse wounds, nuclei were stained using Prolong Antifade Gold with DAPI (Life technologies). Images were captured with an EVOS® FL Digital Microscope (Electron Microscopy Sciences).

Western blot

For keratinocytes, cytosol and nuclear extracts were isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) per the manufacturers protocol. Total skin lysates were flash frozen and then pulverized using a liquid nitrogen cooled biopulverizer (Biospec). Powdered tissues was then dissolved in in RIPA buffer containing 10u/mL Collagenase (Sigma-Aldrich) and incubated for 1hr at 37°C. Afterward, the homogenates were sonicated on ice for 10 sec (Fischer Scientific), spun as 17,000 $\times$ g for 5 minutes. Supernatants were transferred to new tubes and stored at -80°C until use. HALT®

phosphatase and protease inhibitor cocktail (Pierce Biotechnology) was present in all reagents. The protein samples were heated at 90 °C for 5 minutes prior to separation in a 4–20% gradient minigel (Bio Rad). Proteins in gels were transferred to PVDF membranes and immunoblotted with; mouse monoclonal anti-human I κ B α at 1:1000 (Cell Signaling Technology), rabbit polyclonal anti-human STAT3 at 1:2000 (Cell Signaling Technology), rabbit polyclonal anti-human phospho-STAT3 at 1:2000 (Cell Signaling Technology), rabbit polyclonal anti-human p38 MAPK at 1:1000 (Cell Signaling Technology), rabbit polyclonal anti-human phospho- p38 MAPK at 1:1000 (Cell Signaling Technology), rabbit polyclonal anti-human Erk1/2 at 1:1000 (Cell Signaling Technology), rabbit polyclonal anti-human phospho- Erk1/2 at 1:1000 (Cell Signaling Technology), rabbit polyclonal anti-human β 2 microglobulin at 1:1000 (Pierce Biotechnology), and mouse monoclonal anti-human β actin at 1:2000 (Santa Cruz Biotechnology) for cytosol extraction; rabbit polyclonal anti-human NF- κ B p65 at 1:1000 (Cell Signaling Technology), rabbit monoclonal anti-human phospho-Ser536 NF- κ B p65 at 1:1000 (93H1; Cell Signaling Technology), and mouse monoclonal anti-human TATA box (Millipore) at 1:1000 for nuclear extraction; rabbit polyclonal anti-TLR2 (Abcam) at 1:1000, mouse monoclonal anti-CD14 (Santa Cruz Biotechnology) at 1:1000, rabbit polyclonal anti-MyD88 (Cell Signaling) at 1:1000, and mouse monoclonal anti- β -Actin at 1:1000 for cell culture. Goat anti-rabbit HRP or goat anti-mouse HRP (Vector Laboratories) at 1:5000 was used as the secondary antibody. Visualization of the immunoreactive bands was performed by enhanced chemiluminescence (Pierce Biotechnology). Densitometry of target bands was normalized to the respective housekeeping gene (TATA box or β -actin) and analyzed using Image J software (NIH Image).

Quantitative real time RT-PCR

Total RNA was extracted from cells or mouse skin homogenates using TRIzol[®] reagent (Life Technologies) following manufacturer's protocol. Complementary DNA was synthesized using iScript[™] cDNA Synthesis Kit (Bio Rad). Quantitative real time RT-PCR was performed in triplicate using TaqMan[®] Universal PCR Master Mix (Life Technologies) on a StepOnePlus[™] Real-Time PCR System (Life Technologies). Fluorogenic probe and primers were used to detect TLR2 (Hs01872448_s1), CD14 (Hs02621496_s1), and β -Actin (4326315E-1004019) for human cell samples; CRAMP (Mm00438285_m1), DefB2 (Mm0065704_m1), DefB14 (Mm00806979_m1), IL-6 (Mm00446190_m1), MIP-2 (Mm00436450_m1), and TLR2 (Mm00442346_m1) for mice samples (Life Technologies). Expression of target gene was normalized with β -Actin. Expression was analyzed by the 2^{-C_t} method.

Statistical analysis

Values were expressed as means $\pm$ s.e.m. The unpaired t-test or unpaired Mann-Whitney test were applied to analyze the differences between two groups. One-way ANOVA with appropriate Dunnett's or Bonferroni's post-test were used for comparing control group or all groups, respectively. Analyses were performed using GraphPad Prism Version 5.02 software for Windows. $P < 0.05$ was considered to be significant. All statistical analyses were confirmed with Dr. Jim Sinacore, Ph.D., a biostatistician at Loyola University Chicago.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Keratinocyte nicotinic acetylcholine receptor (nAChR) activation dampens TLR2-mediated migration in a scratch wound assay
- Antagonism of the alpha 7 nAChR restores TLR2-mediated migration and defects in intracellular MAPK and NF κ B signaling
- Topical nicotine treatment reduces TLR2 and TLR2-dependent antimicrobial peptide (AMP) production in *S. aureus* infected murine wounds.
- Topical nAChR antagonists may be a novel therapeutic approach to improve TLR2-mediated inflammation in wounds requiring a more robust inflammatory response, ultimately improving wound healing outcomes.

Figure 1.

Nicotinic AChR activation dampens TLR2 agonist-mediated NHEKs migration during *in vitro* wound healing to suppress cathelicidin expression and pro-inflammatory cytokine production. (A) Scratch migration assay. MALP2-mediated keratinocytes migration at 24 hours post-wounding cultured with 10^{-9} M ACh or Nic in presence of nAChR antagonist 10^{-8} M Catestatin (Cst) or 10^{-7} M α -Bungarotoxin (Bung) ($n=3$, repeated at least twice). Representative data from one independent experiment are presented as the mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus MALP2 (One way ANOVA with Dunnett's post-

test). (B) Representative immunofluorescence staining for cathelicidin (green) during *in vitro* wound healing stimulated with 10^{-9} M ACh or 10^{-9} M Nic in presence of MALP2 as merged image together with DAPI (blue). Rabbit IgG is displayed as negative control on the right. White dotted lines demarcate wound edge. Scale bars, 200 μ m. (C–G) Production of pro- and anti-inflammatory cytokines following *in vitro* infection wound. The concentrations of (C) IL-6, (D) IL-8, (E) TNF- α protein at 3 hours post-wounding, (F) IL-8 at 24 hours post-wounding, and (G) IL-10 at 6 hours post-wounding from culture supernatants ($n=3$, repeated at least twice). Veh, Vehicle; M, MALP2 (100 ng ml^{-1}); ACh, Acetylcholine (10^{-9} M); Nic, Nicotine (10^{-9} M); Cst, Catestatin (10^{-8} M); Bung, α -Bungarotoxin (10^{-7} M). Representative data from one independent experiment are presented as the mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus MALP2 (One way ANOVA with Dunnett's post-test).

Figure 2.

Nicotinic nAChR activation impairs TLR2 signaling during TLR2-mediated keratinocyte wound healing. (A, B) Western blot analyses for TLR2 signaling pathway in cytosolic and nuclear extracts from wounded NHEKs stimulated with vehicle, MALP2 (100ng ml⁻¹), ACh (10⁻⁹ M), Nic (10⁻⁹ M) or their combination ($n=3$, repeated at least twice). (A) Cytosolic I κ B and nuclear pNF- κ B. (B) Cytosolic phosphorylated and total Erk1/2. Beta-2 microglobulin (β 2MG), TATA box, and β -Actin were used as a loading control. Representative data from one independent experiment are shown. The numbers represent the quantitative protein expression that was determined by densitometry of I κ B and pNF- κ B after normalization to the loading control. Phosphorylated Erk1/2 was normalized with total Erk1/2. Vehicle is given an arbitrary value of 1.0. Veh, Vehicle; M, MALP2; ACh, Acetylcholine; Nic, Nicotine.

Figure 3. Nicotinic nAChR activation suppresses CD14 and MyD88 expression in TLR2-mediated keratinocyte wound healing. (A) Quantitative RT-PCR analyses of TLR2 and CD14 in wounded NHEKs stimulated with nicotine in presence of MALP2 (100ng ml^{-1}) ($n=3$). Representative data from one independent experiment are shown as the mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$ versus MALP2 (one way ANOVA with Dunnett's post-test), ($n=3$, repeated at least twice). (B) Western blot analyses for TLR2, CD14, and MyD88 in total cell lysates from wounded NHEKs stimulated with vehicle, MALP2 (100ng ml^{-1}), ACh (10^{-9} M), Nic (10^{-9} M) or their combination ($n=3$, repeated at least twice). Beta-Actin was used as the loading control. The numbers represent the quantitative protein expression that was determined by densitometry normalization to loading control. Vehicle is given an arbitrary value of 1.0. Veh, Vehicle; M, MALP2; ACh, Acetylcholine; Nic, Nicotine.

Figure 4.

Epidermal nAChR activation decreases cathelicidin in wild-type mice and influences bacterial survival in diabetic mouse wounds. (A) Quantitative RT-PCR analysis of antimicrobial peptides in non-infected day 1 skin wounds from wild type (C57Bl/6) mice ($n=6$). Black bar represents nicotine treatment. Data are presented as the mean $\pm$ s.e.m. * $p<0.05$ (Student's t-test). (B) Representative immunofluorescence staining for mouse cathelicidin CRAMP (green) and Ly6G Gr-1 as neutrophil marker (red) in skin wounds from wild type (C57Bl/6) mice with vehicle or nicotine (10^{-9} M) treatment. Image merged

together with DAPI (blue). Rat IgG or rabbit IgG are displayed as negative controls on the right. White dotted lines demarcate epidermal-dermal junction. Scale bars, 200 μ m. (C, D) Vehicle or nicotine (10^{-9} M) was applied on dorsal skin for 3 days prior to excisional wounding and topical infection with 10^6 CFU/wound of MRSA. Bacterial survival in skin wound at day 1 post-wounding and MRSA dissemination into kidney at day 7 post-wounding in C57Bl/6 mice ($n=6-8$ per group, repeated twice) by bacterial plating assay. Representative data from one independent experiment are shown. Data are presented as the median $\pm$ range. Each dot indicates a single mouse. $*p < 0.05$ vs. vehicle for respective control or diabetic mouse (Unpaired one-tailed Mann-Whitney test). CRAMP, cathelicidin-related antimicrobial peptide; DefB3, mouse β -defensin 3; DefB14, mouse β -defensin 14; GR-1, granulocyte receptor-1.

Figure 5. Schematic representation of the mechanisms by which keratinocyte nAChR activation reduces TLR2-mediated wound healing responses

Keratinocyte nAChR activation by agonist (ACh or nicotine) blocks TLR2 and co-receptor production through the inhibition of NF- κ B and Erk1/2 activation, leading to the suppression of pro-inflammatory cytokine and cathelicidin production. This leads to an overall suppression of TLR2-mediated wound healing responses (e.g. keratinocyte migration) and greater susceptibility to wound pathogens (e.g. *S. aureus*).