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The Effect of Retinoic Acid on Neutrophil Innate Immune Interactions With Cutaneous Bacterial Pathogens

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Abstract

Vitamin A and its biologically active derivative, retinoic acid (RA), are important for many immune processes. RA, in particular, is essential for the development of immune cells, including neutrophils, which serve as a front-line defense against infection. While vitamin A deficiency has been linked to higher susceptibility to infections, the precise role of vitamin A/RA in host-pathogen interactions remains poorly understood. Here, we provided evidence that RA boosts neutrophil killing of methicillin-resistant *Staphylococcus aureus* (MRSA). RA treatment stimulated primary human neutrophils to produce reactive oxygen species, neutrophil extracellular traps, and the antimicrobial peptide cathelicidin (LL-37). Because RA treatment was insufficient to reduce MRSA burden in an in vivo murine model of skin infection, we expanded our analysis to other infectious agents. RA did not affect the growth of a number of common bacterial pathogens, including MRSA, *Escherichia coli* K1 and *Pseudomonas aeruginosa*; however, RA directly inhibited the growth of group A *Streptococcus* (GAS). This antimicrobial effect, likely in combination with RA-mediated neutrophil boosting, resulted in substantial GAS killing in neutrophil killing assays conducted in the presence of RA. Furthermore, in a murine model of GAS skin infection, topical RA treatment showed therapeutic potential by reducing both skin lesion size and bacterial burden. These findings suggest that RA may hold promise as a therapeutic agent against GAS and perhaps other clinically significant human pathogens.

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Ethics: This study was conducted according to the principles expressed in the Declaration of Helsinki. Blood was collected from healthy adult volunteers under written informed consent and approved by the Institutional Review Board of University of California San Diego (UCSD IRB Protocol #131002, 06/13/2023 to 06/12/2024). Animal experiments were approved by the UCSD Institutional Animal Care and Use Committee (IACUC Protocol # S00227M, Mouse Models of Bacterial Infection and Innate Immunity, 09/17/2021 to 09/16/2024).

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Keywords

retinoic acid; neutrophil; bacterial infection; *Staphylococcus aureus* ; group A *Streptococcus*; neutrophil extracellular traps; antimicrobial peptide; cathelicidin

Introduction

Vitamin A (retinol) and its metabolites play pivotal roles in numerous biological processes, including vision, growth and immune function.[1] Vitamin A deficiency (VAD) has been associated with increased susceptibility to infections and elevated mortality rates in both humans and rodents.[2–5] Multiple studies highlight the significance of vitamin A and its metabolites during infections. Low vitamin A levels in the liver are correlated with a higher risk of primary varicella,[6] and reduced serum retinol levels are linked to an increased incidence of acute respiratory infections.[7] Additionally, rats afflicted with VAD exhibit heightened vulnerability to *Staphylococcus aureus* infection, accompanied by impaired phagocyte function.[5] Furthermore, human studies and mice models provide compelling evidence supporting the therapeutic potential of vitamin A supplementation during treatment of various infectious diseases, including measles,[8–10] tuberculosis,[11] malaria[12] and non-typhoidal *Salmonella enterica*.[13] Despite the well-established connection between vitamin A and improved infection outcomes, the precise mechanistic underpinnings of this association are poorly understood.

One critical biologically active metabolite of vitamin A is retinoic acid (RA), a small lipophilic molecule that exerts its influence by binding to specific RA receptors and retinoid X receptors within the nucleus.[14–17] These receptors are linked to RA response elements found within the promoters of target genes.[18] Upon RA binding to its receptors, co-activator complexes are recruited, instigating the transcription of RA-responsive genes.[19,20] It is estimated that RA regulates more than 500 genes,[21] underscoring its wide-ranging impact on cellular functions. In the context of the immune system, RA signaling has been shown to regulate various immune cell types, including B and T lymphocytes,[22–24] and neutrophils. [22]

Neutrophils, the most abundant leukocytes in circulation, are a crucial first line of innate host defense against infection caused by various microorganisms. Generated from bone marrow precursors, neutrophils are released in large numbers into the circulation upon maturation.[25] Serving as proficient phagocytic cells, they have the ability to kill pathogens through various mechanisms, including the release of reactive oxygen species (ROS), proteinases and antimicrobial peptides such as cathelicidin LL-37 stored in pre-packaged granules.[26] Furthermore, neutrophils can ensnare and neutralize pathogens by releasing neutrophil extracellular traps (NETs) composed of DNA and other antimicrobial molecules, including LL-37.[27,28]

Multiple lines of evidence support a role of RA in neutrophil differentiation. Neutrophils possess RA response elements in their genome and express genes that respond to RA.[29] Additionally, studies have shown that leukemic cell lines undergo neutrophil maturation upon exposure to RA treatment.[29–31] Moreover, multipotent hematopoietic cells lacking functional retinoic acid receptors are unable to progress beyond the promyelocyte stage and

fail to differentiate into mature neutrophils.[32] Clinically, RA has demonstrated its efficacy by inducing remission in patients with acute promyelocytic leukemia through promotion of promyelocyte differentiation into granulocytes.[33,34]

While the requirement of retinoid signaling during neutrophil maturation is well-established, the precise role of RA in mature neutrophils during host-pathogen interactions remains enigmatic. Studies conducted in rats have linked vitamin A deficiency to impaired neutrophil function, resulting in diminished phagocytosis, chemotaxis and ROS production.[35] These findings suggest that retinoids, such as RA, may have the potential to augment the activity of mature neutrophils during infections. In this study, we aim to delineate the role of RA in host-pathogen interactions, focusing on both the neutrophil-mediated effects and the direct impact of RA on select bacterial pathogens. By investigating these aspects, we seek to shed light on the mechanisms through which RA influences immune responses and microbial clearance during infection.

Results

RA enhances neutrophil effector functions

Given the central role of RA signaling in developing neutrophils,[30,31] our initial investigation focused on the impact of RA treatment on neutrophil effector functions. To assess the bactericidal capacity of RA-stimulated neutrophils, we conducted primary human neutrophil killing assays using a well-characterized isolate of community-acquired methicillin-resistant *S. aureus* (MRSA) belonging to the USA300 lineage (USA300-TCH1516). Notably, RA treatment led to a significant enhancement of MRSA killing, and this effect was reversed upon the introduction of RA receptor antagonist BMS 493 (Figure 1A). To unravel the mechanisms underlying RA-induced neutrophil activation, we first examined the production of ROS and observed dose-dependent ROS production by these cells in response to RA treatment. ROS increases were detected at concentrations of RA as low as 5 μM, with higher RA concentrations (>50 μM) eliciting ROS levels comparable to those induced by the potent stimulant phorbol 12-myristate 13-acetate (PMA). Interestingly, when low-dose RA was combined with PMA, it resulted in an additive effect on ROS production, yielding even higher levels than either treatment alone (Figure 1B). In line with previous findings,[36] inhibiting PI3K prevented RA-induced ROS production (Figure 1C). However, PI3K inhibition had no discernible impact on PMA-induced ROS production, primarily mediated by protein kinase C.[37] To confirm the significance of ROS production in neutrophil killing of MRSA, we treated neutrophils with butylated hydroxyanisole (BHA), a ROS inhibitor, observing that ROS inhibition was indeed associated with increased MRSA survival (Figure 1D).

To assess a broader spectrum of neutrophil functions potentially modulated by RA, we investigated chemotaxis and phagocytosis. RA treatment did not affect chemotaxis, as demonstrated in a transwell assay (Figure 1E), similar to what has been described previously.[36] Additionally, RA did not have any discernible impact on the phagocytosis of MRSA by primary human neutrophils (Figure 1F). Importantly, our controls confirmed that RA, even at concentrations up to 50 μM, did not exert toxicity on neutrophils (Figure 1G), nor did it impair growth of MRSA in culture (Figure 1H).

Neutrophils serve another vital role in the formation of NETs, intricate structures composed of DNA and antibacterial proteins that trap and neutralize pathogens.[28] To determine whether RA treatment induces NET formation, we quantified released DNA using PicoGreen and visualized the process through immunostaining. High-dose RA stimulation significantly increased NET formation, while low-dose RA, in combination with PMA, displayed a synergistic effect, as quantified by PicoGreen (Figure 2A) and visualized by fluorescence microscopy (Figure 2B). Furthermore, we observed that RA-induced NETs correlated with enhanced MRSA killing (Figure 2C). Addition of DNase to eliminate NET integrity resulted in increased survival of MRSA in both RA-treated and -untreated samples in the neutrophil killing assay (MOI = 1) (Figure 2D). NETs are known to sequester various proteins, including cathelicidin LL-37, a cationic alpha-helical antimicrobial peptide that disrupts bacterial membranes and plays a crucial role in NET formation and stability.[38,39] Since LL-37 is produced by several cell types, including neutrophils,[40] we investigated whether RA could induce its expression in neutrophils. Western blot analysis revealed that RA treatment of neutrophils led to increased expression of the LL-37 in cellular lysates (Figure 2E), corresponding to increased release of LL-37 into the supernatant by dot blot analysis (Figure 2F). Collectively, these findings strongly indicate that RA can effectively modulate neutrophil activation during host-pathogen interactions, influencing NET formation and LL-37 expression at higher RA concentrations (50 μM or higher).

Retin-A treatment is insufficient to reduce lesion size or bacterial burden in a murine model of MRSA skin infection

Finding that RA augmented neutrophil microbicidal function, we explored the therapeutic potential of exogenous RA administration in vivo using mouse infection models, employing the commonly prescribed retinoic acid–based skin cream, Retin-A (tretinoin). Retin-A cream is recognized for its efficacy in treating various skin conditions, including acne vulgaris, through complex mechanisms believed to involve inflammation modulation and stimulation of new collagen synthesis.[41] In our study, mice were subcutaneously infected with 2×10^7 CFU of MRSA and subsequently treated with Retin-A every 24 hours for a duration of 5 days. Notably, Retin-A treatment did not influence the surface area of MRSA lesions (Figure 3A and B). Additionally, the analysis of CFU collected from homogenized lesions revealed no discernible difference between Retin-A–treated lesions and control lesions (Figure 3C). Consequently, the earlier findings regarding enhanced MRSA killing by RA-treated neutrophils did not translate into therapeutic benefits with Retin-A treatment in this murine skin infection model.

RA inhibits GAS growth in vitro

Our in vivo studies demonstrated that RA-mediated enhancement of neutrophil function was not sufficient to provide therapeutic benefit in a murine model of MRSA skin infection. However, some studies have suggested an impact of RA treatment on skin microbiota.[42] Consequently, we conducted assessments to determine the direct antimicrobial activity of RA on a selection of bacterial species. Various gram-positive and gram-negative pathogens were screened for RA-mediated inhibition of bacterial growth. Notably, RA had no discernible impact on the growth of gram-negative *Pseudomonas aeruginosa* and *Escherichia coli* or the gram-positive *Lactococcus lactis* (Figure 4A). While RA treatment had a minor

effect on the growth of group B *Streptococcus* (GBS), it caused decreased growth of two different strains of GAS. To further characterize the anti-GAS activity of RA, we conducted tests across a broader range of doses to determine the MIC. The MIC was determined to be 64 μM through a plate-based resazurin assay (Figure 4B). These data indicate that an RA concentration as low as 5 μM begins to slow the growth of GAS, while a concentration of 64 μM is necessary to completely inhibit growth over 12 h.

RA treatment enhances GAS clearance by neutrophils

Our discovery that RA has direct antibacterial activity against GAS led us to test the therapeutic benefit of RA on GAS clearance in the context of host-pathogen interactions. We hypothesized that combined effects of antibacterial action and enhancement of neutrophil function would lead to significantly increased killing of GAS by neutrophils in vitro and decreased bacterial burden in vivo. Indeed, we observed that neutrophil-mediated killing of GAS was more pronounced compared to what was previously found with MRSA (Figure 5A). To assess whether these effects translate to significant outcomes in vivo, we infected mice subcutaneously with 2×10^8 CFUs of GAS. The treated mice received Retin-A for 5 consecutive days, following the same regimen as in the MRSA skin infection model. Notably, we observed reduced skin lesions (Figure 5B and C) and lower bacterial burdens (Figure 5D) in the Retin-A–treated mice compared to the untreated controls, providing evidence for a significant therapeutic benefit of RA in the context of GAS infection in vivo.

Discussion

As the leading cause of bacterial pharyngitis, GAS infection is responsible for approximately 3 million antibiotic prescriptions and 5 million outpatient visits in the United States annually.[43] More severe manifestations of GAS infection include invasive diseases and immune-mediated complications such as rheumatic heart disease.[44] GAS can also cause skin infections, leading to conditions like impetigo and cellulitis, which affect as many as 111 million children worldwide.[44] In this study, we have demonstrated that RA enhances GAS clearance in a murine skin infection model. Our in vitro data suggest two mechanisms for GAS clearance. First, RA enhances neutrophil function, likely by increasing the production of ROS, NETs and the antimicrobial peptide LL-37. Of these three neutrophil boosting mechanisms, enhancement of ROS production is likely the predominant effect leading to enhanced neutrophil killing, as increases of ROS occur at RA concentrations as low as 5 μM. The second effect of RA is direct inhibition of GAS growth. The direct action of RA against GAS complements the enhanced neutrophil response induced by RA, resulting in improved GAS clearance. GAS incubated with 10 μM RA grew in culture for up to 4 hours, albeit with a slowed growth pattern due to the direct inhibitory effect of RA. However, when added to neutrophils, a striking >90% of RA-treated GAS was killed during the 30-minute incubation period (Figure 5A). We therefore conclude that RA sensitizes GAS to neutrophil killing.

This work aligns with other studies demonstrating RA-mediated immune modulation. Similar to our findings, RA-treated neutrophils have shown increased NET formation and cytotoxicity against various tumor cells.[45] Besides enhancing neutrophil function,

RA has been shown to induce production of the antimicrobial peptide cathelicidin in adipocytes,[46] keratinocytes[47] and bone marrow progenitor cells.[48] In this study, we extend this knowledge by demonstrating that RA can also induce the expression of human cathelicidin (LL-37) in primary human neutrophils. In addition to cathelicidin, independent studies have demonstrated that RA induces expression of other antimicrobial compounds, including bactericidal/permeability-increasing protein (BPI) in neutrophil cell culture[49,50] and resistin-like molecule α (RELMα) on murine skin.[51] Further investigation has indicated that RELMα can directly kill GAS by disrupting the bacterial membrane.[51] Moreover, retinoids have pleiotropic effects on other immune cells, including macrophages,[52–54] $CD4^+$ T cells[23,55] and dendritic cells on the skin,[56] all of which are likely to contribute to the RA-mediated immune response against GAS in vivo. These additional RA-mediated immune responses are likely to occur in conjunction with the mechanisms discussed in this study.

In comparison to RA-mediated immune effects, the direct antibacterial activity of vitamin A-–related compounds is less understood. Early studies have focused on the impact of oral RA administration on skin flora, revealing a reduction in *Cutibacterium acnes*, a decrease in gram-negative pathogens, and an increase in *S. aureus*.[57–60] These changes occurred alongside an RA-mediated decrease in sebum,[61] but the mechanisms are not fully understood. In line with our present findings, a recent study demonstrated that administration of isotretinoin to acne patients or RA to mice induced cathelicidin expression in the dermis, and resolution of *C. acnes* infection in mice was dependent on the expression of cathelicidin.[62] An area for future research is to investigate why retinoids have varying activities against different pathogens. In addition to our present work, some studies have shown that RA does not have activity against certain bacteria, including *S. aureus*, *E. coli* and *P. aeruginosa*.[63,64] However, another vitamin A derivative, retinaldehyde, has direct activity against *P. acnes* and *S. aureus*.[64,65] Furthermore, the growth of mycobacteria in culture was inhibited by three vitamin A derivatives, including RA.[66] Recently, a small molecule screen identified the therapeutic potential of vitamin A analogs against MRSA, as these synthetic retinoids induced MRSA killing through cytoplasmic membrane disruption.[67] We have expanded upon studies of retinoid-induced antimicrobial activity by demonstrating a dose-dependent inhibition of GAS by RA.

Our findings represent a first step in elucidating a potential clinical benefit of RA during GAS infection. RA offers a unique strategy to combat GAS infection by both boosting innate immune function and inhibiting bacterial growth. A limitation of this study is the challenge of isolating the specific contribution of neutrophil-boosting activity against GAS from the direct sensitivity of the bacteria to RA. Experiments involving MRSA, which is not sensitive to RA, highlight the ability of RA-induced neutrophil activation to enhance bacterial killing in vitro. Additionally, the precise mechanism underlying GAS susceptibility to RA remains unexplored in this study. Future investigations would benefit from identifying the specific bacterial genes involved in this interaction. Understanding the molecular and chemical basis of direct GAS inhibition by RA can inform the development of new antimicrobial therapeutics. Collectively, the data presented here merit further investigation into the antimicrobial potential of vitamin A–related compounds for GAS infections.

Materials and methods

Bacterial strains

S. aureus (strains Newman and methicillin-resistant USA300-TCH1516), GAS (serotype M1 strain 5448 and serotype M49 strain NZ131), GBS (serotype III strain COH1), *L. lactis* (strain NZ9000), *E. coli* (strain K1-RS218) and *P. aeruginosa* (strain PAO1) were used in this study. *S. aureus*, GAS, GBS and *L. lactis*, were propagated in Todd-Hewitt broth/agar, while *E. coli* and *P. aeruginosa* strains were propagated in Luria-Bertani broth/agar.

Neutrophil isolation

Human venous blood from healthy volunteers was collected (with heparin as a coagulant) according to University of California San Diego Human Research Protections Program-approved protocols. Neutrophils were isolated by layering 20 mL of blood on Polymorphprep (Axis Shield) as specified by manufacturer's instructions, followed by centrifugation for 30 minutes at 600 × *g* (without brakes) and one wash in PBS. Erythrocytes were lysed by mixing the pellet in 5 mL tissue culture–grade H_2O for 30 seconds, which was followed by PBS addition to 50 mL and centrifugation. Neutrophils, resuspended in 1 mL of PBS, were counted and set at a concentration of 10^7 cells/mL at room temperature for experimental use.

Neutrophil total killing assay

Freshly isolated neutrophils were stimulated with 25 nM PMA (Sigma) for 20 minutes when indicated. Prior to adding bacteria to the assay, neutrophils were treated with 5 μM RA with or without 10 μM BMS493 or 25 μM BHA for 20 minutes. Log-phase bacteria were then introduced to the cells at an MOI of 1 bacterium per cell, followed by centrifugation for 5 minutes at 500 × *g* to enhance bacterial contact. Bacteria were incubated with neutrophils for 30 minutes at 37°C in 5% CO_2. Neutrophils were lysed with 0.025% Triton X-100, and bacteria were counted by serial dilution on Todd Hewitt (TH) agar plates. Bacterial survival was reported as a percentage of the initial CFU per well. Neutrophil killing experiments with ROS inhibition involved pre-incubation of neutrophils with or without 25 μM BHA (ROS inhibitor) for 20 minutes before infection. Neutrophil killing assays with DNase were done with 30-minute pre-incubation of DNase at 100 U/mL.

Neutrophil oxidative-burst assay

Human neutrophils at 2×10^6/mL in calcium- and magnesium-free Hanks balanced salt solution (HBSS) were incubated with 100 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 20 min. After washing and resuspension at 5×10^6/mL in HBSS with added calcium and magnesium, neutrophils (5×10^5/well) were treated with 12.5 nM PMA, 1-500 μM RA, or a combination of both. Neutrophils were incubated at 37°C and 5% CO_2, and fluorescence (485-nm excitation, 530-nm emission) was measured after 2 hours using a SpectraMax M3 plate reader. For ROS experiments with PI3K inhibition, neutrophils were pre-treated for 15 minutes with 10 μM wortmannin.

Quantification of neutrophil chemotaxis

Neutrophil migration was evaluated using the transwell method.[68] Lower wells of a Corning 24-well Transwell plate with 3 μm pores received 500 nM *N*-formyl methionyl-leucyl phenylalanine (fMLP, Sigma Aldrich) at a final volume of 650 μL. Upper chambers, containing 1×10^6 neutrophils in 150 μL HBSS at varying RA concentrations, were incubated at 37°C for 45 minutes with 5% CO_2. After removing upper chambers, migrated neutrophils in lower chambers were lysed with 0.1% Triton X-100 (pH = 7.4; 64 μL) for 10 minutes at room temperature. Elastase activity, indicating relative cell migration, was quantified by adding 1 mM p-nitroanilide, and absorbance at 405 nm was measured after 30 minutes using a SpectraMax M3 plate reader (Molecular Devices).

Neutrophil phagocytosis assay

Neutrophils were stimulated with 10-100 μM RA for 20 minutes in RPMI medium with 2% FBS, followed by infection with bacteria at MOI = 1 bacterium per cell with log-phase MRSA carrying a plasmid encoding eGFP. After incubation with or without 10 g/mL cytochalasin D for 20 minutes, cells were treated with 100 U/mL penicillin and 100 g/mL streptomycin for 10 minutes to eliminate any bacteria not phagocytosed by neutrophils. After three washes in PBS, cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry. Fluorescence intensity was measured for 10,000 cells per condition. Uninfected polymorphonuclear leukocytes/neutrophils served as a negative control. Flow cytometry data were analyzed with FlowJo v. 9.4.10 (Tree Star, Inc.).

Neutrophil viability assay

Neutrophils that were treated with 5, 50 or 500 μM RA, 10 μM BMS, or 25 μM BHA for 2 hours were mixed with 2% propidium iodide stain for a final concentration of 0.4% propidium iodide. Stained cells were transferred to a FACS tube and run on a BD FACSCalibur flow cytometer. Data were analyzed using FlowJo v. 9.4.10 (Tree Star, Inc.).

NET induction assays

Each well of a 96-well plate was seeded with 2×10^5 neutrophils, stimulated with or without 12.5 nM PMA (positive control) and 0-500 μM RA. After 4 hours of incubation at 37°C, cells were centrifuged and washed, and NET DNA was extracted with 500 mU/mL MNase. MNase was inactivated with 5 mM EDTA after 10-minutes incubation. Released NETs were measured by incubation of supernatant with Quant-iT PicoGreen reagent (Invitrogen) for 5 minutes at room temperature, and fluorescence was measured on a SpectraMax M3 plate reader (480-nm excitation, 520-nm emission) using SoftMax Pro software.

Neutrophil extracellular trap imaging

NETs were induced as aforementioned with or without 25 nM PMA (positive control) and 0-500 μM RA for 4 hours at 37°C on 0.01% poly-L-lysine (Sigma, #P4707) coated chambered well slides. NETs were fixed with 4% paraformaldehyde and blocked with 2% BSA-PBS + 0.3% Triton X-100 for 45 minutes. Mouse anti-human myeloperoxidase (1:500, Marine Biotech, MAB208P) was added as primary antibody to detect NETs and slides were incubated overnight. Slides were washed three times with PBS and incubated with

AlexaFluor 488 goat anti-mouse (1:1000) secondary antibody for 1 hour in the dark. After four washes with PBS, chambers were removed, and mounting medium containing DAPI was utilized.

NET-based bacterial killing assay

Neutrophils in RPMI + 2% 70°C heat-inactivated FBS were added to a 24-well plate at a density of 4×10^5 cells/well with or without 25 nM PMA and 500 μM RA. After a 4-hour incubation at 37°C (with 5% CO_2), 4×10^4 bacteria were added to each well (MOI = 0.1), and plates were centrifuged at 1600 rpm for 5 minutes. After an additional 30 minutes incubation at 37°C (with 5% CO_2), samples were serially diluted in 96-well plates containing sterile H_2O and plated on THA plates to determine percentage survival relative to inoculum.

LL-37 human cathelicidin expression analysis

Neutrophils in RPMI + 2% heat-inactivated FBS were incubated in a 96-well at 1×10^6 cells/well with DMSO (vehicle control), 5 μM, 50 μM or 500 μM RA for 3 hours at 37°C with 5% CO_2. Plates were then centrifuged at 500 × *g* for 5 minutes. Supernatant was collected for dot blot analysis, and the remaining cells were suspended in NuPAGE LDS Sample Buffer (Invitrogen #NP0007) and reducing agent (Li-Cor Biosciences, #926-68072) for western blot analysis. Neutrophil cellular lysates were incubated for 10 minutes at 95°C and centrifuged at full speed for 3 minutes. Supernatants were collected, and 20 μL was run on a 4%-12% NuPAGE Bis-Tris gel (Invitrogen, cat #NP0335BOX) followed by transfer to nitrocellulose membrane (Merck Millipore Ltd., cat# ISEQ00010). For dot blot analysis, 8 μL of neutrophil supernatants was spotted onto nitrocellulose and left to dry at room temperature for 1 hour. Western blot and dot blot membranes were blocked overnight in 10% BSA in TBST. Immunoblotting was done with primary antibody to LL-37 (Santa Cruz, #sc-166770) at 1:500 in TBST with 1% BSA for 1 hour. For western blot analysis, primary antibody to actin (Sigma, A5316) was added at 1:5000. After three washes with TBST, membranes were incubated for 1 hour with 1:10,000 secondary donkey anti-mouse antibody conjugated to red fluorescent dye for use in LI-COR system (LI-COR Biosciences, #926-68072). Blots were analyzed using the Odyssey imaging system and ImageStudio software (LI-COR).

Retinoic acid growth curves

Bacteria were cultured overnight and diluted in TH broth at a starting OD_{600} of 0.1. Cultures were incubated at 37°C, and OD_{600} measurements were taken every 30 minutes or 1 hour to assess growth until $OD_{600} = 0.8$ (stationary phase).

Retinoic acid MIC

Bacteria cultured in RPMI + 20% TH broth were grown to mid-logarithmic phase ($OD_{600} = 0.4$). Cultures were subsequently diluted 1:200 in RPMI + 20% THB, and 100 μL was added to each well of a 96-well plate. RA was added to final concentrations of 128 μM, 96 μM, 64 μM, 48 μM, 32 μM, 24 μM, 16 μM, 12 μM, 8 μM or 0 μM for incubation at 37°C. Resazurin was used to assess bacterial viability after 18 hours, and the MIC was the

lowest RA concentration that did not produce a color change from blue (resazurin) to pink (resorufin).

Retin-A skin infection model

All animal experiments and procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee. CD1 female mice (Charles River Laboratories) aged 8 to 10 weeks were subcutaneously infected with 2×10^8 CFU GAS or 2×10^7 CFU MRSA in 100 μL volume. Retin-A 0.1% cream was topically administered to skin lesions every 24 hours from 1 day post infection to 5 days post infection. On day 5 post infection, skin lesions were excised and homogenized in PBS with 1.5-mm zirconium beads. Lesion homogenates underwent serial dilution and plating on TH agar plates to determine bacterial loads. ImageJ software was used to determine lesion size as area in mm^2 units.

Statistical analysis

Experiments in this study were done a minimum of three times, each in triplicate. A minimum of three different healthy volunteers were used for assays involving primary human neutrophils. The combined data were normalized and presented as mean ±SEM, except where specified. Statistical analyses were performed using Student's *t* test, one-way ANOVA with Tukey's multiple-comparison posthoc test, or two-way ANOVA with the Bonferroni posthoc test. GraphPad Prism version 5.0 (GraphPad Software Inc.) was employed for all statistical tests, and significance was set at $P < 0.05$.

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Data availability:

All data generated or analyzed during this study are included in this published article. Additional data are available from the corresponding author on reasonable request.

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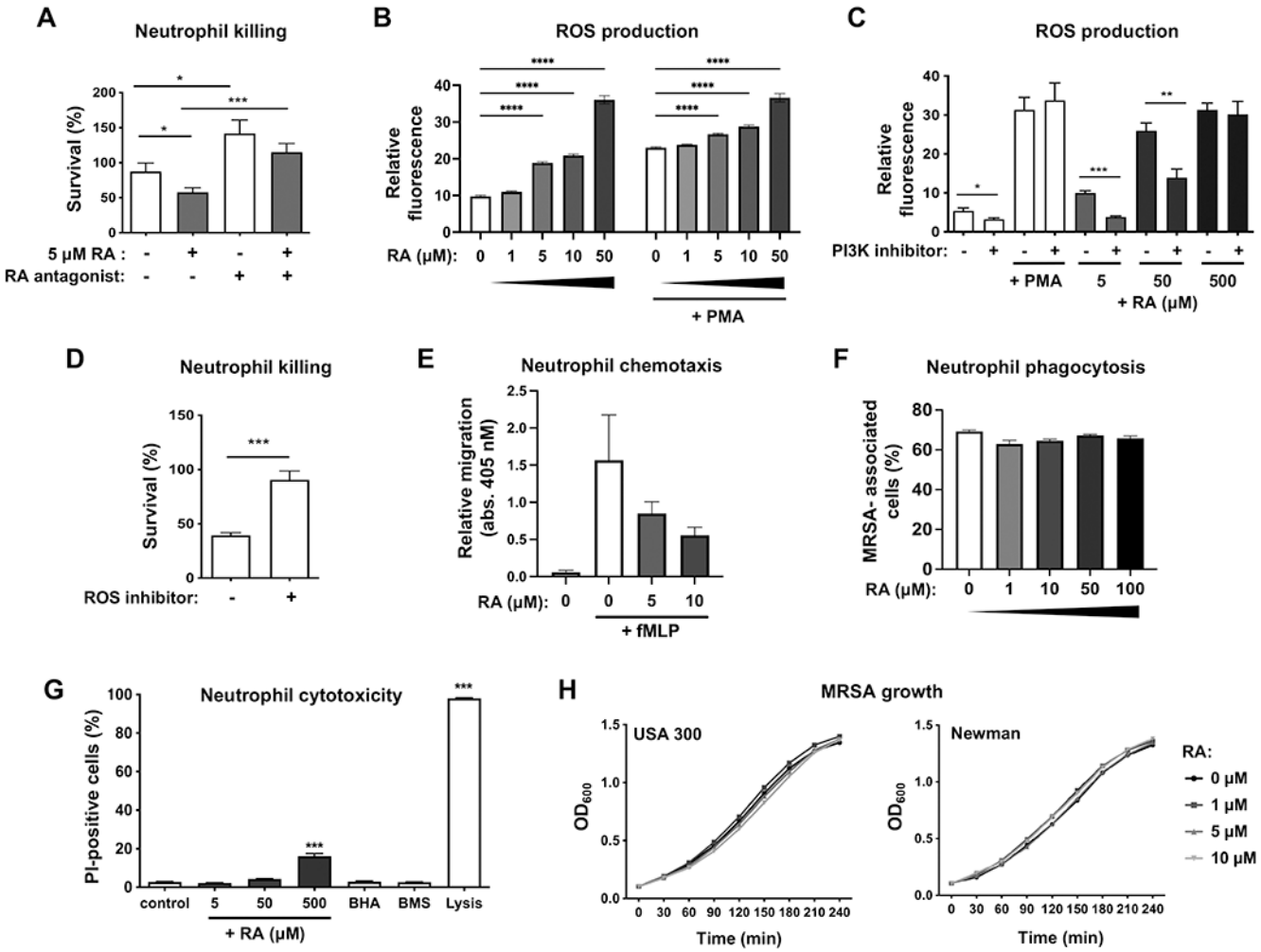

Figure 1. RA enhances neutrophil functions against MRSA.

A: Neutrophil killing of MRSA at MOI = 1 bacteria/cell for 30 minutes. Neutrophils were incubated for 20 minutes in the presence or absence of 5 μM RA and 10 μM BMS493 (RA antagonist) prior to addition of bacteria. B: ROS production by neutrophils with increasing concentrations of RA in the presence or absence of 12.5 nM PMA. Neutrophils were incubated with 100 μM 2,7- dichlorofluorescein diacetate for 20 minutes and fluorescence intensity was read every 15 minutes to determine ROS production over time. C: Neutrophil ROS production as in (B) with indicated concentration of RA in the presence or absence of 10 μM wortmannin (PI3K inhibitor). D: Neutrophil killing of MRSA during 30 minutes incubation at MOI = 1. Neutrophils were incubated in the presence or absence of 25 μM BHA (ROS inhibitor) for 20 minutes prior to infection. E: Neutrophil chemotaxis in response to 500 nM fMLP in the presence or absence of RA, as measured by relative migration for 45 minutes in a transwell assay. F: Flow cytometry phagocytosis assay of neutrophils incubated with increasing concentrations of RA for 20 minutes followed by infection with eGFP-expressing MRSA at MOI = 1. Neutrophils associated with eGFP (MRSA) are expressed as a percent of total neutrophils. G: Cytotoxicity assay by propidium iodide staining of neutrophils treated with increasing RA concentrations, 10

μM BMS493 or 25 μM BHA for 2 hours. H: Growth curves of USA 300 and Newman MRSA strains treated with 0, 1, 5 or 10 μM RA. OD_{600} measurements were taken every 30 minutes for 4 hours. Panels A-G show mean ± SEM; *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001; Student's *t* test. Abs, absorbance; fMLP, *N*-formyl-Met-Leu-Phe; MRSA, methicillin-resistant *Staphylococcus aureus*; OD_{600}, optical density at 600 nm; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PMA, phorbol myristate acetate; PMNs, polymorphonuclear leukocytes/neutrophils; RA, retinoic acid; ROS, reactive oxygen species.

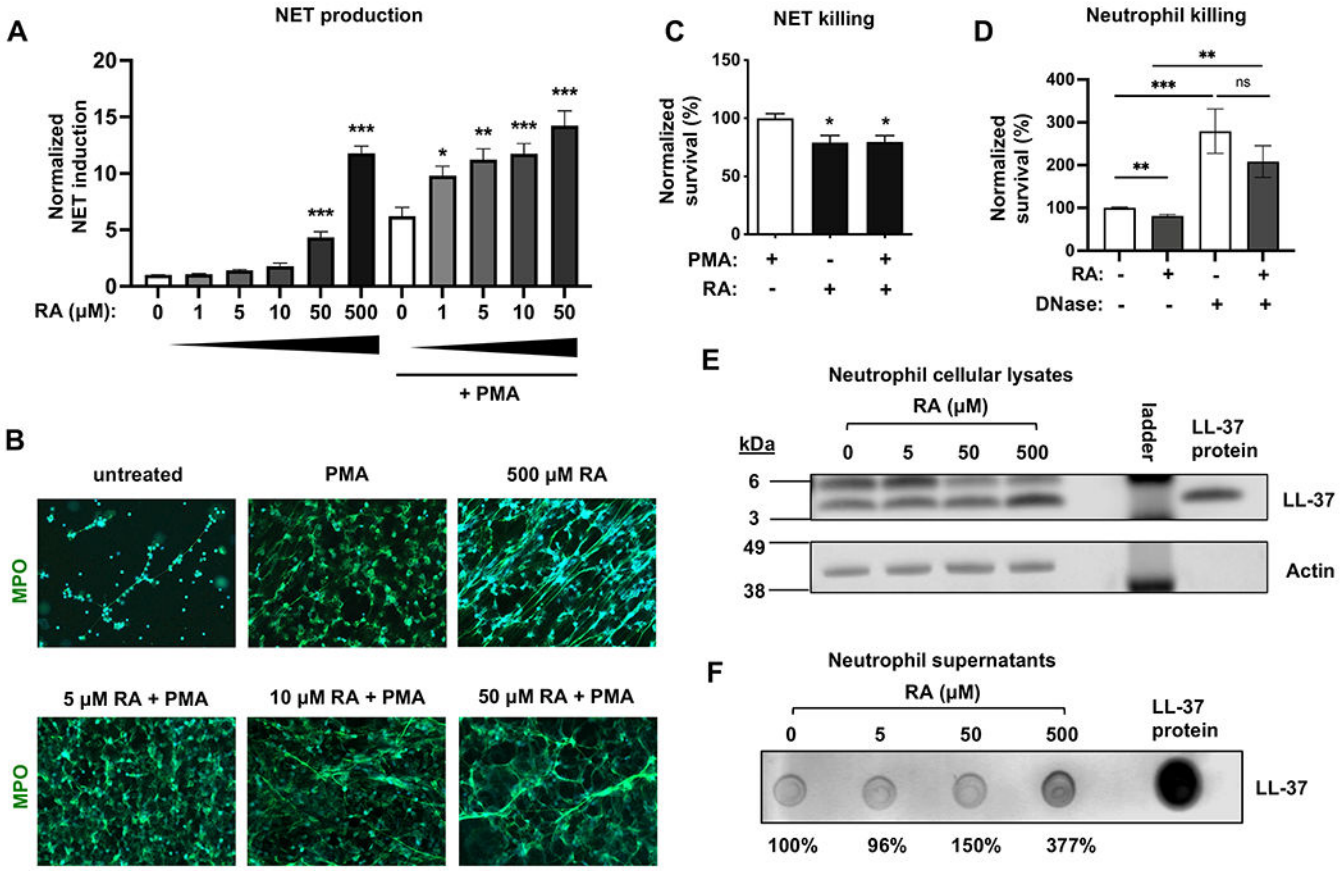

Figure 2. RA promotes NET formation.

A: Fold change in NETosis triggered by increasing concentrations of RA in the presence or absence of 12.5 nM PMA. NETs were quantified using PicoGreen dye staining for extracellular DNA. B: Immunocytochemical analysis in response to RA in the presence or absence of 25 nM PMA. Green: myeloperoxidase (NETs); blue: DAPI (nuclei). C: NET-mediated killing of MRSA after RA or PMA treatment. Neutrophils were treated for 4 hours with 25 nM PMA (NET activation) or 500 μM RA, followed by incubation with bacteria for 30 minutes at MOI = 0.1. Values are normalized to the mean of the PMA only condition (left bar). D: Neutrophil killing assay of MRSA (MOI =1) in the presence or absence of 10 μM RA and 100 U/ml DNase. E: Western blot of LL-37 expression in neutrophil lysates after incubation with increasing RA concentrations for 3 hours. Top panel: band corresponding with LL-37 as confirmed by recombinant LL-37 protein positive control; bottom panel: actin loading control. F: Dot blot analysis of LL-37 produced by neutrophil supernatants after a 3-hour incubation with increasing RA concentrations. Panels A, C, and D show mean ± SEM; *P<0.05; **P<0.01; ***P<0.001; Student's t test.
NET, neutrophil extracellular trap; PMA, phorbol myristate acetate; MPO, myeloperoxidase; DAPI, 4',6-diamidino-2-phenylindole; RA, retinoic acid.

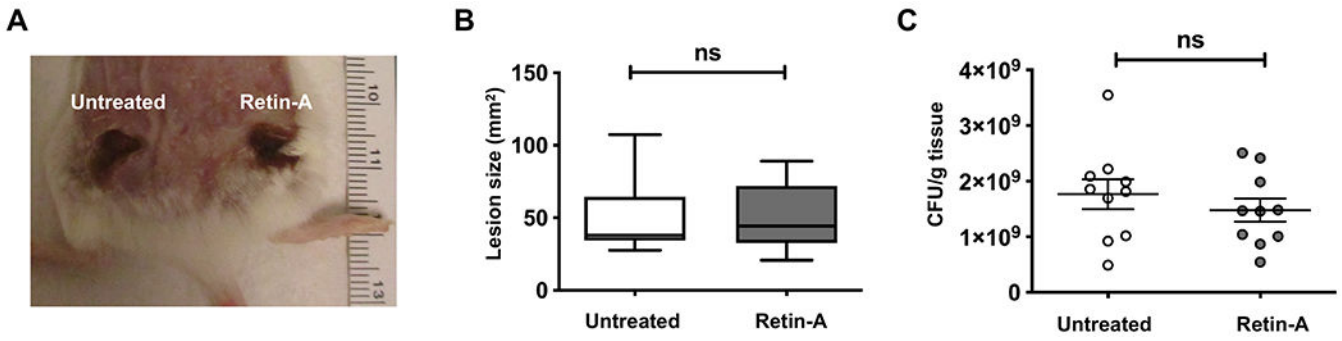

Figure 3. Retin-A treatment is insufficient to reduce lesion size or bacterial burden in a murine model of MRSA skin infection.

A: Representative image of MRSA skin infection after subcutaneous injection of 2×10^7 CFU MRSA and Retin-A treatment. Mice were treated topically with Retin-A (tretinoin) 0.1% cream every 24 hours for 5 days post infection. B and C: Lesion size (B) and Bacterial burden (C) after infection and RA administration. Figure B depicts a box and whisker plot demonstrating upper quartile, median and lower quartile with ranges. Figure C shows mean ± SEM. ns, non-significant; Student's *t* test. MRSA, methicillin-resistant *Staphylococcus aureus*; RA, retinoic acid.

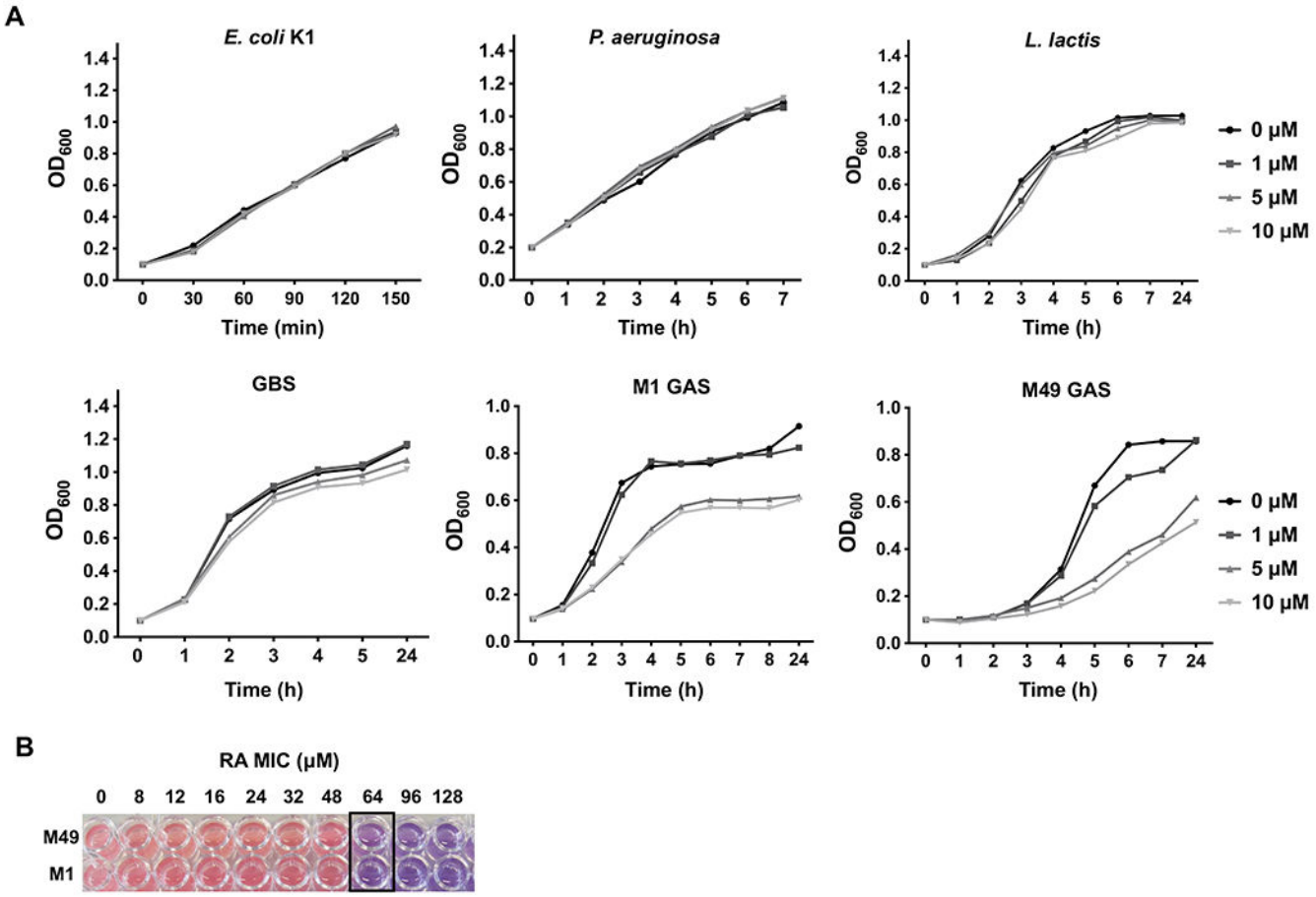

Figure 4. RA has specific antibacterial activity against group A *Streptococcus*.

A: Growth curves with hourly OD_{600} measurements of indicated bacterial strains treated with 0, 1, 5, or 10 μM RA. B: M49 and M1 GAS were grown in RPMI + 20% THB with increasing concentrations of RA for 18 h, and bacterial growth was measured by metabolic activity against resazurin. *E. coli*, *Escherichia coli*; GBS, group B *Streptococcus*; GAS, group A *Streptococcus*; *L. lactis*, *Lactococcus lactis*; OD_{600}, optical density at 600 nm; *P. aeruginosa*, *Pseudomonas aeruginosa*; RA, retinoic acid.

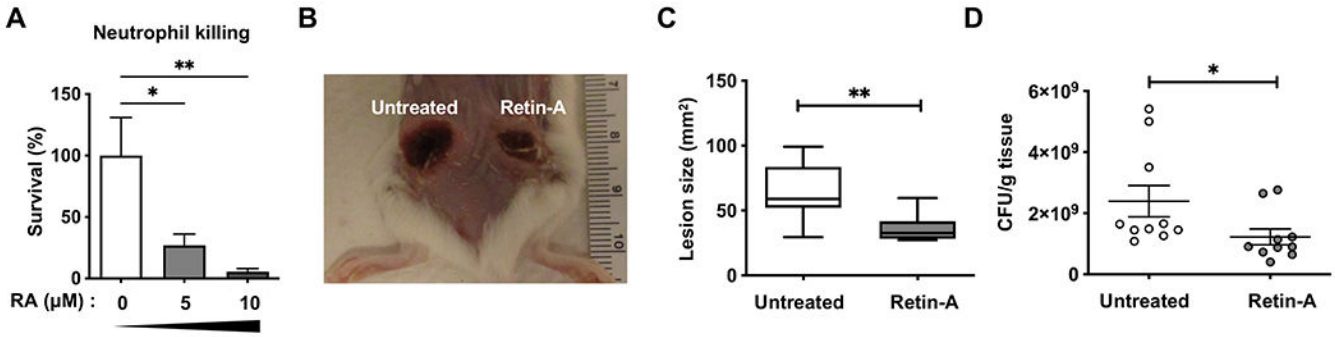

Figure 5. RA treatment enhances GAS clearance by neutrophils.

A: Neutrophil killing assay with GAS at MOI = 1 bacteria/cell. Prior to GAS addition, neutrophils were treated with 0, 5, or 10 μM RA. B: Representative image of lesions after subcutaneous injection of CD-1 mice with 2×10^8 CFU GAS followed by topical administration of Retin-A 0.1% cream every 24 hours for 5 days post infection. C and D: Lesion size (C) and bacterial burden (D) were assessed following infection and RA administration. Panels A and D display mean ± SEM; Panel C depicts a box and whisker plot demonstrating upper quartile, median and lower quartile with ranges; $*P < 0.05$; $**P < 0.01$; Student's *t* test. GAS, group A *Streptococcus*; RA, retinoic acid.