Anti-neutrophil properties of natural gingerols in models of lupus

Ramadan A. Ali¹, Alex A. Gandhi¹, Lipeng Dai², Julia Weiner¹, Shanea K. Estes¹, Srilakshmi Yalavarthi¹, Kelsey Gockman¹, Duxin Sun², and Jason S. Knight¹

¹ Division of Rheumatology, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA
² Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan, USA

Correspondence:
Jason S. Knight, MD, PhD
1150 W Medical Center Drive, Ann Arbor, MI 48109-5678
734-936-3257
jsknight@umich.edu

Running title: Gingerols in lupus and APS

Conflict of interest: None of the authors has any financial conflict of interest to disclose.
ABSTRACT

Ginger is known to have anti-inflammatory and anti-oxidative effects, and has traditionally been used as an herbal supplement in the treatment of various chronic diseases. Here, we report anti-neutrophil properties of 6-gingerol, the most abundant bioactive compound of ginger root, in models of lupus and antiphospholipid syndrome (APS). Specifically, we demonstrate that 6-gingerol attenuates neutrophil extracellular trap (NET) release in response to lupus- and APS-relevant stimuli through a mechanism that at least partially dependent on inhibition of phosphodiesterases. At the same time, administration of 6-gingerol to mice reduces NET release in various models of lupus and APS, while also improving other disease-relevant endpoints such as autoantibody formation and large-vein thrombosis. In summary, this study is the first to demonstrate a protective role for ginger-derived compounds in the context of lupus, and importantly provides a potential mechanism for these effects via phosphodiesterase inhibition and attenuation of neutrophil hyperactivity.
INTRODUCTION

Systemic lupus erythematosus (lupus) is the prototypical systemic autoimmune disease, characterized by antinuclear autoantibodies and circulating immune complexes. Lupus has the potential to impact essentially any organ in the body. Antiphospholipid syndrome (APS) is a closely related thromboinflammatory disease defined by the presence of circulating antiphospholipid antibodies (aPL), as detected by anticardiolipin anti-beta-2-glycoprotein I (anti-β2GPI) immunoassays or the functional lupus anticoagulant screen (1, 2). APS is a leading acquired cause of thrombosis and pregnancy loss (3) and can occur as a standalone syndrome (primary APS) or in association with lupus (4). In fact, aPL are present in one-third of lupus patients, amplifying the risk of the thromboembolic events that are among the leading causes of morbidity and mortality in lupus.

Neutrophils release neutrophil extracellular traps (NETs)—tangles of chromatin and microbicidal proteins expelled from neutrophils in response to both infectious and sterile stimuli (5, 6)—which have garnered much recent attention as amplifiers of inflammation and thrombosis in autoimmune diseases such as lupus and APS (7-10). For example, rising levels of blood neutrophils predict glomerulonephritis in lupus (11), while recent studies by our group and others have revealed a key role for neutrophils and NETs in the thrombotic manifestation inherent to lupus and APS. We have shown that APS patients have higher levels of cell-free DNA and NETs in plasma and that their neutrophils are prone to spontaneous NETosis as compared with healthy controls (12). Furthermore, aPL isolated from APS patients can trigger healthy control neutrophils to release NETs (12), while also potentiating thrombosis in vivo in neutrophil- and NET-dependent fashion (13). In lupus and APS, NETs promote type I interferon production (14, 15) and autoantibody formation (16, 17). At the same time, lupus-associated autoantibodies including anti-ribonucleoprotein/RNP (14), aPL (12), and anti-double-stranded DNA/dsDNA (18) accelerate NETosis, thereby setting up a vicious cycle.
Ginger has long been perceived to have anti-inflammatory and anti-oxidative properties (19, 20), and has been used traditionally as herbal medicine for the treatment of many ailments including chronic conditions such as asthma and arthritis. The health-promoting properties of ginger have been attributed to its richness in phenolic phytochemicals (21) such as gingerols and shogaols (22, 23). Of these bioactive compounds, 6-gingerol is the most abundant in fresh ginger, with concentrations up to 2,100 µg g⁻¹ (24). It has been suggested that ginger may mediate its anti-inflammatory effects by reducing levels of pro-inflammatory cytokines, as well as tempering synthesis and secretion of chemokines at sites of inflammation. For example, whole ginger extract can inhibit IL-12, TNF-α, IL-1β, RANTES, and MCP-1 production by lipopolysaccharide (LPS)-stimulated macrophages (25). Similar effects can be achieved using 6-gingerol alone (26), which also extend to human synoviocytes and chondrocytes (27, 28). In vivo, 6-gingerol blocks activation of NF-κB in phorbol ester-stimulated mouse skin (29) and suppresses the inflammatory response to carrageenan-induced paw edema in rats (30). However, none of these studies have provided a detailed explanation of mechanism.

We were intrigued by recent studies that reported a role for ginger extract and specifically 6-gingerol as inhibitors of cyclic AMP (cAMP)-specific phosphodiesterase (PDE) activity. For example, both ginger extract and 6-gingerol appear to inhibit PDE4 activity in vitro as tested in human cell lines expressing predominantly the PDE4 isoform (31). In addition, an in silico study identified three candidates (including 6-gingerol) from ginger as potential PDE4 inhibitors capable of inhibiting cAMP binding and hydrolysis by PDE4 (32). PDEs (and especially PDE4, the predominant isotype expressed by leukocytes) are attractive therapeutic targets for chronic inflammatory diseases. Theophylline, a nonspecific PDE inhibitor, is leveraged for its anti-inflammatory and vasodilatory properties in the treatment of asthma and chronic obstructive pulmonary disease (33). There has also been targeted drug development including the PDE4
inhibitor apremilast, which is currently used in the clinic in the context of psoriasis, psoriatic arthritis, and Behçet's disease (34).

The anti-inflammatory effects of ginger have yet to be investigated in the context of lupus and APS. Of particular note, no attention has been given to the effects of gingerols on activation and function of neutrophils as thromboinflammatory mediators in lupus and APS. Given evidence that gingerols may similarly exploit cAMP-regulated pathways that we recently characterized in lupus and APS neutrophils (35), we here sought to determine the extent to which ginger-derived compounds might function as a natural suppressor of aberrant neutrophil hyperactivity.
RESULTS

Gingerols inhibit NETosis elicited by *E. coli* LPS. We first tested the efficacy of three related compounds, 6-gingerol, 8-gingerol, and 10-gingerol (differing only in length of aliphatic side chain), for their ability to suppress NETosis by control neutrophils. We found that both 6- and 8-gingerol completely neutralized LPS-triggered NETosis at concentrations as low as 10 micromolar (Figure 1A-C). We then asked whether inhibition would extend to NETosis activated by phorbol 12-myristate 13-acetate (PMA). Indeed, PMA-mediated NETosis was also suppressed by all gingerols (Figure 1D).

Gingerols inhibit NETosis elicited by lupus and APS autoantibodies. Neutrophils are activated by various lupus-relevant stimuli including RNP-containing immune complexes (ICs) and aPL to release NETs. We tested the efficacy of 6-gingerol, 8-gingerol, and 10-gingerol for their ability to suppress NETosis when control neutrophils were activated by either RNP ICs or aPL. All three gingerols suppressed RNP IC-induced NETosis at the 10-micromolar concentration, while 6- and 8-gingerol neutralized aPL-mediated NETosis at the same dose (Figure 1E-F). The impact of 6-gingerol on NETosis was also assessed by immunofluorescence microscopy with similar results (Figure 1G). The 10 micromolar concentration is the lowest dose that prevents NETosis in response to LPS (Figure 1A), PMA, and APS IgG (Supplemental Figure 1A-B). At the same time, we found that neutrophils appear healthy over 3 hours even at concentrations as high as 1 mM 6-gingerol (Supplemental Figure 1C). In summary, these data demonstrate that gingerols have broad anti-NETosis properties that extend to lupus-relevant stimuli such as RNP ICs and aPL.

Gingerols inhibit ROS formation by neutrophils. Ginger has been reported to have anti-oxidative properties. Thus, we reasoned that gingerols might suppress NETosis by preventing the neutrophil oxidative burst, as reactive oxygen species (ROS) are required for most forms of
NETosis. All gingerols suppressed formation of H$_2$O$_2$ in neutrophils, whether stimulated by LPS, PMA, RNP ICs, or aPL (Figure 2A-D). Taken together, these data suggest a potential mechanism by which gingerols mitigate NETosis, namely by suppressing ROS formation.

**6-gingerol inhibits cAMP-specific PDE activity.** Ginger extracts and specifically 6-gingerol have been suggested to function as PDE inhibitors. Here, we reasoned that 6-gingerol might suppress NETosis through modulation of cAMP levels and downstream pathways. We first tested the effect of 6-gingerol on PDE activity in neutrophils. We found that 6-gingerol reduced PDE activity by 40%, as compared to a 50% reduction by the synthetic PDE4 inhibitor rolipram (Figure 3A). We also measured intracellular concentrations of cAMP upon stimulation of neutrophils with the adenylate cyclase activator forskolin. Interestingly, both 6-gingerol and IBMX (another synthetic PDE inhibitor) significantly potentiated intracellular cAMP concentrations as compared with untreated samples (Figure 3B). Having documented a gingerol-mediated increase in intracellular concentrations of cAMP, we considered that activity of the key downstream cAMP-dependent kinase, protein kinase A (PKA), might also increase in neutrophils. Indeed, 6-gingerol significantly enhanced neutrophil PKA activity (Figure 3C-D). Furthermore, the suppressive effects of 6-gingerol on NETosis could be mitigated by blocking PKA activity (Figure 3E). In summary, these data demonstrate that 6-gingerol attenuates NETosis *in vitro* through a mechanism that at least partially depends on inhibition of PDE activity, potentiation of cAMP levels, and resultant activation of PKA.

**6-gingerol attenuates lupus-relevant disease activity in mice.** We next tested the efficacy of 6-gingerol on disease activity in a lupus mouse model. Administration of 6-gingerol to TLR7 agonist-treated mice (Figure 4A) resulted in a marked reduction in plasma NET levels as evident by reductions in both cell-free DNA and myeloperoxidase (MPO)-DNA complexes (Figure 4B-C). Key antibodies including anti-dsDNA, anti-β$_2$GPI, and total IgG were also...
reduced (Figure 4D-F). Spleen size, total leukocytes, lymphocytes, neutrophils, and platelets were not significantly altered by 6-gingerol (Supplemental Figure 2A-E). Given that 6-gingerol inhibited cAMP-specific PDE activity in vitro, we reasoned that it would also increase cAMP concentration in vivo and thereby inhibit proinflammatory cytokines—similar to synthetic PDE inhibitors (36). As expected, 6-gingerol treated mice showed significantly lower levels of both IFN-γ and TNF-α (Supplemental Figure 3A-B). We next investigated the efficacy of 6-gingerol treatment after disease onset in the same lupus model. To do this, 6-gingerol treatment was delayed for four weeks relative to the initiation of the TLR7 agonist; both TLR7 agonist and 6-gingerol were then administered together for the final two weeks of the experiment (Figure 5A). While vehicle-treated mice accrued higher levels of NETs and autoantibodies between weeks 4 and 6, these increases were markedly blunted in the mice receiving 6-gingerol (Figure 5B-E and Supplemental Figure 4A-D). Taken together, these data suggest that 6-gingerol reduces lupus-relevant NET release and autoantibody formation in vivo.

6-gingerol attenuates aPL-mediated venous thrombosis. The pathologic role of neutrophils and NETs in thrombosis has been observed in both human (37-39) and mouse studies (13, 40, 41). Since 6-gingerol suppressed aPL-mediated NETosis in vitro, we reasoned that it might also mitigate aPL-accelerated NETosis and thrombosis in vivo. We induced large-vein thrombosis utilizing an electrolytic inferior vena cava (IVC) model that we have described previously (35, 42) (Figure 6A). Administration of aPL increased serum NET levels, which returned to baseline when mice were treated with 6-gingerol (Figure 6B). As expected, administration of aPL, but not control IgG, increased thrombus length and weight, which again returned to control levels upon administration of 6-gingerol (Figure 6C-D). We also examined the effect of the synthetic PDE4 inhibitor rolipram on NETosis and venous thrombosis. Similar to 6-gingerol, rolipram suppressed aPL-mediated NETosis in vitro in dose-dependent fashion (Figure 7A). In the aforementioned model of aPL-accelerated large-vein thrombosis, administration of rolipram to
the APS mice returned circulating MPO-DNA (Figure 7B) and thrombus size (Figure 7C-D) to levels seen in control mice. In summary, these data demonstrate that 6-gingerol suppresses NETosis and venous thrombosis in vivo and provide further support that 6-gingerol functions as a PDE4 inhibitor given that its effects are phenocopied by a synthetic PDE4 inhibitor.

Kinetics of 6-gingerol in plasma and neutrophils
Finally, we performed pharmacokinetic studies of 6-gingerol. In particular, we focused on the distribution of 6-gingerol in neutrophils, the major theme of the above experiments. Interestingly, we observed accumulation of 6-gingerol in neutrophils at the same time levels were dropping in plasma (Figure 8A-B). These results are in line with previous work suggesting that ginger biophenolics are rapidly cleared from plasma by conversion into glucuronide conjugates, but then reconverted into their free forms in tissues by enzymes such as β-glucuronidases (43).
DISCUSSION

Here, we have revealed anti-neutrophil properties of 6-gingerol that may have protective effects in disease states such as lupus and APS. In vivo, we characterized two lupus-relevant inflammatory models and found that 6-gingerol reduced NETosis in both. Beyond inhibition of NETosis, we also saw positive effects of disease phenotypes such as autoantibody formation and thrombosis, and observed that 6-gingerol behaved very similarly to a synthetic PDE4 inhibitor.

Mechanistically, we found that the effects of 6-gingerol on neutrophils are at least partially attributable to its ability to inhibit PDE activity (Supplemental Figure 5). In vitro data demonstrate that 6-gingerol increases intracellular concentrations of cAMP and enhances PKA activity in neutrophils. Furthermore, 6-gingerol suppressed the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ, similar to PDE4 inhibitors (36), therefore exerting an overall anti-inflammatory effect in TLR7 agonist-induced lupus. The activation of cAMP/PKA by 6-gingerol would seem to support this pathway as a potential therapeutic target in lupus and APS with drugs such as PDE4 inhibitors (44). This is in agreement with our previous work demonstrating the potential role of adenosine receptors, cAMP, and PKA in suppressing APS-mediated NETosis and thrombosis (35).

Ginger has previously been reported to have anti-inflammatory (45), antioxidant (46), and antithrombotic (47) effects. Regarding the latter, gingerol-related compounds including 6-gingerol were shown to prevent platelet aggregation (48), although this finding has not been reproduced by all groups (49). Here, it is certainly possible that both anti-platelet and anti-neutrophil properties are contributing to protection against venous thrombosis. Beyond thrombosis, ginger has also been considered as a treatment for rheumatic diseases. Several clinical studies have suggested beneficial effects of ginger for the treatment of arthritis. For
example, ginger extract was found to alleviate pain and decrease joint swelling (50, 51); to our knowledge, neutrophil phenotypes have not been considered in any of these clinical studies. In mice, there is evidence that ginger may modulate inflammatory cell trafficking with decreased recruitment of neutrophils, eosinophils, and monocytes into inflamed airways (52). Here, there was a trend toward reduced neutrophil numbers in circulation (Supplemental Figure 2A), and future studies should endeavor to look specifically at the impact of 6-gingerol on neutrophil trafficking in models of autoimmunity.

We found that 6-gingerol concentrations as low as 10 µM were effective in mitigating NETosis. This is a concentration that provides no cellular toxicity over 24 hours (53). In fact, for the duration of our assay (3 hours), we did not detect toxic effects even at molar concentration of 6-gingerol. Ginger extracts are generally regarded as safe (54) with 2 grams daily (approximately 25 mg kg⁻¹), demonstrating low levels of toxicity and high levels of tolerability in humans (55). Given the faster metabolic rate of mice as compared with humans (and the allometric scaling factor of 10), 250 mg kg⁻¹ of ginger extract has been a common dose for murine in vivo studies. As ginger extracts typically consist of 5% active phenolic compounds (mostly 6-gingerol), the dose chosen here (10 mg kg⁻¹ 6-gingerol) lines up well with previous work.

Free forms of ginger biophenolics including 6-gingerol can be detected in human plasma upon oral administration of ginger extracts (56, 57). These compounds are rapidly cleared from plasma and converted into glucuronide conjugates. A recent study proposed a model whereby these conjugated forms are reconverted into their free forms in tissues by enzymes such as β-glucuronides (43), which are known to be abundantly expressed by neutrophils (58). Consistent with this model, we appreciated accumulation of 6-gingerol in neutrophils at the same time levels were dropping in plasma.
While it is unlikely that ginger extract or 6-gingerol would find a role as a primary therapeutic in individuals with active disease, one wonders if future studies might administer ginger supplements to individuals at high risk for autoimmune conditions and/or cardiovascular disease (for example, individuals with autoantibodies who have yet to have clinical events, or patients with cardiovascular risk factors). In such scenarios, its anti-neutrophil properties might prove protective against disease emergence. Based on the data presented here, we would argue that such studies should have not only clinical endpoints, but also mechanistic endpoints focusing on neutrophil activity.
MATERIALS AND METHODS

Purification of patient IgG. IgG was purified from APS or control sera with a Protein G Agarose Kit following the manufacturer’s instructions (Pierce) as previously described (35). Briefly, serum was diluted in IgG binding buffer and passed through a Protein G Agarose column at least five times. IgG was then eluted with 0.1 M glycine and neutralized with 1 M Tris. This was followed by overnight dialysis against PBS at 4 °C. IgG purity was verified with Coomassie staining, and concentrations were determined by BCA protein assay (Pierce) according to manufacturer’s instructions. All IgG samples were determined to be free of detectable endotoxin by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (88282) according to manufacturer’s instructions.

Human neutrophil purification and NETosis assays.

Blood from healthy volunteers was collected into heparin tubes by standard phlebotomy techniques. The anticoagulated blood was then fractionated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). Neutrophils were further purified by dextran sedimentation of the red blood cell layer, before lysing residual red blood cells with 0.2% sodium chloride. Neutrophil preparations were at least 95% pure as confirmed by both flow cytometry and nuclear morphology.

To assess NETosis, neutrophils were resuspended in RPMI media (Gibco) supplemented with 0.5% bovine serum albumin (BSA, Sigma) and 0.5% fetal bovine serum (Gibco), which had been heat-inactivated at 56°C. Neutrophils (1x10^5/well) were then cultured in 96-well plates at 37°C with 100 nM PMA (Sigma), 2 µg ml⁻¹ LPS (Escherichia coli O26:B6, L2654, Sigma), 10 µg ml⁻¹ APS IgG, or 10 µg ml⁻¹ RNP ICs. RNP ICs were formed by mixing IgG purified from three individuals with lupus and anti-RNP positivity with SmRNP (purchased from Arotec, New Zealand). In some cases, cultures were also supplemented with 6-gingerol, 8-gingerol, and 10-
gingerol (Cayman Chemical). After three hours in culture, NET-associated MPO activity was measured as follows. The culture media was discarded (to remove any soluble MPO) and replaced with 100 µL of RPMI supplemented with 10 U ml⁻¹ Micrococcal nuclease (Thermo Fischer Scientific). After 10 minutes at 37°C, digestion of NETs was stopped with 10 mM EDTA. Supernatants were transferred to a v-shaped 96-well plate, and centrifuged at 350xg for 5 minutes to remove debris. Supernatants were then transferred into a new plate. To measure for MPO activity, an equal volume of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (1 mg ml⁻¹, Thermo Fischer Scientific) was added to each well. After 10 minutes of incubation in the dark, the reaction was stopped by the addition of 50 µL of 1 mM sulfuric acid. Absorbance was measured at 450 nm using a Cytation 5 Cell Imaging Multi-Mode Reader.

**Immunofluorescence microscopy.** For immunofluorescence microscopy, 1.5×10⁵ neutrophils were seeded onto coverslips coated with 0.001% poly-L-lysine (Sigma) and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Blocking was with 1% BSA overnight at 4°C. The primary antibody was against neutrophil elastase (Abcam 21595, diluted 1:100), and the FITC-conjugated secondary antibody was from Southern Biotech (4052-02, diluted 1:250). DNA was stained with Hoechst 33342 (Invitrogen). Images were collected with a Cytation 5 Cell Imaging Multi-Mode Reader.

**H₂O₂ assay.** The generation of H₂O₂ was quantified as described previously (17). Briefly, H₂O₂ production was detected by a colorimetric assay, with 50 µM Amplex Red reagent (Invitrogen) and 10 U ml⁻¹ horseradish peroxidase (Sigma) added to the culture medium. Absorbance was measured at 560 nm and linearity was assured with an H₂O₂ standard curve.

**Measurement of PDE activity.** Human neutrophils (1x10⁷) were washed twice with cold PBS and pelleted by centrifugation at 2500xg for five minutes. The cell pellet was re-suspended in
100 µL of RIPA buffer (Sigma, R0278) supplemented with protease inhibitors (Roche Diagnostics GmbH, 35440400) for 15 minutes on ice. The mixture was centrifuge at 14,000xg for five minutes to clear cell debris. The activity of PDE was measured using the Bridge-It cAMP-PDE assay kit (Mediomics, St. Louis, MO, USA, Cat # PD-1016). The supernatant was mixed with the reaction mixture according to manufacturer’s instruction in the presence or absence of 10 µM 6-gingerol or 0.1 µM PDE4 inhibitor rolipram and allowed to proceed for one hour at 37 °C. The reaction was stopped and assay solution was added. After 30 minutes at 37 °C, fluorescence was measured with a Synergy HT Multi-Mode Microplate Reader (BioTek) at excitation 480 and emission 520. Relative activity was calculated and normalized to mean control values.

**Measurement of intracellular cAMP.** cAMP levels in human neutrophils were measured using the Bridge-It cAMP Designer fluorescence assay kit (Mediomics, Cat # 122934). Briefly, neutrophils (1x10⁵) were washed twice with PBS and re-suspended in 100 µL Krebs-Ringer Bicarbonate Buffer (KRB) (without IBMX). To investigate the effect of 6-gingerol on the cAMP level, neutrophils were incubated at room temperature for 30 minutes in the presence or absence of 10 µM 6-gingerol. Neutrophils were then stimulated with 100 µM forskolin for 10 minutes. Samples were centrifuged at 12,000xg for two minutes and supernatants were discarded. The cAMP designer assay solution was then added to the cell pellet and carefully transferred to 96-well black side clear bottom plates. The plate was incubated at room temperature for 30 minutes before measuring fluorescence with a Synergy HT Multi-Mode Microplate Reader (BioTek) at excitation 480 with band pass 20 and emission 540 with band pass 40.

**Measurement of PKA activity.** Neutrophils (1x10⁷) were pre-incubated with 10 µM 6-gingerol or 0.1 µM PDE4 inhibitor rolipram and then treated with 100 µM forskolin or 1µM cAMP. The
activity of PKA was then measured using the PKA activity assay kit (Arbor Assays, Cat # K027-H1). Briefly, neutrophils were washed twice with cold PBS and pelleted by centrifugation at 2500xg for five minutes. The cell pellet was re-suspended in 1 mL of activated cell lysis buffer for 30 minutes on ice. The mixture was centrifuge at 10,600xg at 4°C for 10 minutes to pellet the cell debris. In a 96-well plate, the supernatant was mixed with the kinase assay buffer followed by addition of ATP and the plate was incubated at 30°C for 90 minutes. The plate was washed 4 times followed by the addition of donkey anti-rabbit IgG HRP conjugate and the rabbit phosphor PKA substrate antibody and then incubated at room temperature for 60 minutes. The plate was washed four times, followed by the addition of TMB substrate and incubation at room temperature for 30 minutes. Stop solution was added and the optical density was read at 450 nm using a Cytation 5 Cell Imaging Multi-Mode Reader.

**Animal housing and treatments.** Mice were purchased from The Jackson Laboratory and housed in a specific pathogen-free barrier facility, and fed standard chow. Male C57BL/6 mice were used for venous thrombosis experiments and 6-gingerol (AdooQ Bioscience) was administered by intraperitoneal injection, 10 mg kg\(^{-1}\) daily. Therapy was always started one day before IVC surgery, and continued through the duration of the experiment. Female BALB/c mice were used for lupus-induced R848 and 6-gingerol was administered by intraperitoneal injection 20 mg kg\(^{-1}\) three times per week.

**In vivo venous thrombosis.** To model large-vein thrombosis, we employed an electrolytic model that has been used previously by our group and others (35, 42). Briefly, after exposure of the IVC, any lateral branches were ligated using 7-0 Prolene suture (back branches remained patent). A 30-gauge silver-coated copper wire (KY-30-1-GRN, Electrospec) with exposed copper wire at the end was placed inside a 25-gauge needle, and inserted into the IVC where it was positioned against the anterior wall and functioned as the anode. Another needle was implanted subcutaneously, completing the circuit (cathode). A constant current of 250 μA was
applied for 15 minutes. The current was supplied by a voltage-to-current converter that we described in detail previously (42). After removal of the needle, the abdomen was closed. Before recovery from anesthesia, mice received a single intravenous injection of either control or APS IgG (500 µg). 24 hours later, mice were humanely euthanized, blood was collected, and thrombus size was measured.

Quantification of MPO-DNA complexes. MPO-DNA complexes were quantified similarly to what has been previously described (59). This protocol used several reagents from the Cell Death Detection ELISA kit (Roche). First, a high-binding EIA/RIA 96-well plate (Costar) was coated overnight at 4°C with anti-human MPO antibody (Bio-Rad0400-0002), diluted to a concentration of 0.5 µg ml⁻¹ in coating buffer (Cell Death kit). The plate was washed three times with wash buffer (0.05% Tween 20 in PBS), and then blocked with 1% BSA in PBS for one hour at room temperature. The plate was again washed three times, before incubating for one hour at room temperature with 1:500 mouse serum in the aforementioned blocking buffer. The plate was washed five times, and then incubated for one hour at room temperature with 1x anti-DNA antibody (HRP-conjugated; Cell Death kit) diluted 1:100 in blocking buffer. After 5 more washes, the plate was developed with 3,3',5,5'-TMB substrate (Invitrogen) followed by a 2N sulfuric acid stop solution. Absorbance was measured at a wavelength of 450 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek). Data was normalized to an in vitro-prepared NET standard, included on every plate.

Resiquimod treatment. Female BALB/c mice were treated with TLR7 agonist resiquimod (R848, Enzo Life Science) as previously described (60) with slight modifications. Epicutaneous application was to the ear three times per week with 100 µg of resiquimod dissolved in 8 µL DMSO. For 6-gingerol treatment, some mice were injected intraperitoneally with 20µg kg⁻¹ of 6-
gingerol on the same days as R848 treatment. Serum and tissues were collected after six weeks of treatment.

**Quantification of anti-dsDNA and total IgG.** Kits for mouse anti-dsDNA (5120) and mouse total IgG (6320) were purchased from Alpha Diagnostic International and performed according to manufacturer’s instructions.

**Quantification of anti-β2GPI.** High-binding EIA/RIA plates were coated overnight at 4°C with 1 µg ml⁻¹ mouse β2GPI (R&D 6575-AH) diluted in coating buffer from a Cell Death Detection ELISA kit. Plates were then washed with 0.05% Tween 20 in PBS, and blocked with 1% BSA in PBS for one hour at room temperature. The plate was again washed, before incubating for one hour at room temperature with 1:500 mouse serum in the aforementioned blocking buffer. The plate was washed, and then incubated for one hour at room temperature with anti-mouse IgG HRP (Jackson ImmunoResearch 115-035-068) diluted 1:20,000 in blocking buffer. The plate was washed and developed with 3,3',5,5'-TMB substrate (Invitrogen) followed by a 2N sulfuric acid stop solution. Absorbance was measured at a wavelength of 450 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek).

**Quantification of cell-free DNA.** Cell-free DNA was quantified in mouse serum using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen) according to the manufacturer’s instructions.

**Complete blood counts.** Peripheral leukocyte and platelet counts were determined with an automated Hemavet 950 counter (Drew Scientific).
**Quantification of IFN gamma and TNF alpha.** Kits for quantitative detection of mouse IFN gamma (88-7314) and TNF alpha (88-7324) were purchased from Invitrogen and performed according to manufacturer’s instructions.

**Pharmacokinetic studies of 6-gingerol in neutrophils.** Male C57BL/6 mice were treated with 6-gingerol 20 mg kg⁻¹ by intraperitoneal injection. Peripheral blood was collected after 0.5, 2, 4, and 24 hours after 6-gingerol treatment. Plasma was collected and neutrophils were isolated from peripheral blood using the EasySep negative selection kit Cat# 19762 according to the manufacturer's instructions.

**Bioanalysis.** Neutrophil samples were suspended with 100 μL of 20% acetonitrile and then subjected to 3 freeze-thaw cycles. Samples were sonicated twice (3 seconds each) to breakdown neutrophil pellets, with cooling on ice for 15 seconds between sonications. A stock solution of 6-gingerol was diluted to 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng mL⁻¹ in methanol, as working solutions. To prepare calibration curves, 30 μL blank cell suspension was spiked with 30 μL of the various working solutions above respectively; whereas 30 μL of tested samples were spiked with 30 μL methanol. All samples, were spiked with 30 μL internal standard solution. All the samples were vortexed for extraction, and centrifuged at 12500 rpm at 4°C for 15 minutes. Finally, isolated supernatants from each tube were submitted for subsequent LC-MS analysis. Plasma samples were prepared in a similar way.

**LC-MS analysis**

The mass spectrometer, AB Sciex QTRAP® 5500, coupled with Shimazu LC20A liquid chromatography system was operated in multiple reaction monitoring (MRM), positive mode for determination of 6-gingerol in neutrophil pellets and plasma samples. Chromatographic separation was accomplished with the application of a Waters XBridge® C18, 2.1X50mm, 5-μm
column. The mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of 100% acetonitrile supplemented with 0.1% formic acid. The HPLC was subjected to gradient elution, at 0.4 mL/minute, and positive ion mode was adopted in the mass spectrometer. The mobile phase B was maintained at 1% during the initial 0.5 minutes, and then increased to 99% from 0.5 minutes to 2.5 minutes. The composition of mobile phase B was decreased to 1% at 4.5 minutes, and maintained there until the end of the run time at 6.6 minutes. Mass/charge (m/z) transitions 295.1-137.2 and 295.1-177.0 were monitored for 6-gingerol quantification in biological samples while mass/charge (m/z) transition 455.2-425.2 was monitored for internal standard.

**Statistics.** Data analysis was with GraphPad Prism software version 8. For continuous variables, group means were compared by either $t$-test (two groups) or one-way ANOVA (more than two groups); correction for multiple comparisons was by Dunnett’s test. Statistical significance was defined as $p < 0.05$.

**Study approval.** This study complied with all relevant ethical regulations and was approved by the University of Michigan Institutional Review Board; all participants provided informed consent for blood donation. Mice experimental protocols were approved by the University of Michigan Institutional Animal Care and Use Committee, and all relevant ethical regulations were followed.
AUTHOR CONTRIBUTIONS

R.A.A., A.A.G, L.D, J.W., S.K.E., S.Y, K.G., and D.S. conducted experiments and analyzed data. R.A.A and J.S.K. designed the study and analyzed data. All authors drafted the manuscript, and gave approval before submission.
ACKNOWLEDGEMENTS

R.A.A. was supported by NIH T32AR007080. J.S.K. was supported by grants from the NIH (R01HL134846), Lupus Research Alliance, and Burroughs Wellcome Fund. The work was supported by an administrative supplement to R01HL134846 from the NIH Office of Dietary Supplements.
REFERENCES


Figure 1: Gingerol suppresses NETosis in response to various stimuli. Human neutrophils were isolated from healthy volunteers and then treated with various stimuli for 3 hours in the presence of different gingerol analogs. NETosis was quantified by measuring the enzymatic activity of nuclease-liberated myeloperoxidase (MPO). Dose response to LPS-mediated NETosis upon treatment with 6-gingerol (A); 8-gingerol (B); and 10-gingerol (C). NETosis in response to PMA (D), RNP ICs (E), and APS IgG (F) was quantified in the presence of 10 µM
gingerols. NETosis was assessed by immunofluorescence microscopy (G). Neutrophils were treated with either LPS, PMA, RNP ICs or APS IgG in the presence or absence of 6-gingerol. 6-gingerol=10 µM, blue=DNA, green=extracellular neutrophil elastase, and scale bar=100 microns. For panels (A-F), mean and standard error of the mean (SEM) are presented for \( n = 3 \) independent experiments; \( *p < 0.05, **p < 0.01, \) and \( ***p < 0.001 \) as compared with the 0-µM gingerol group by one-way ANOVA corrected with Dunnett’s test.
**Figure 2**: Gingerols suppress reactive oxygen species (ROS). Human neutrophils were treated with various stimuli in the presence of different gingerol analoges for 1 hour. Hydrogen peroxide formation was measured by a colorimetric assay. Mean and standard error of the mean (SEM) are presented for $n = 3$ independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ as compared with the LPS-alone group (A), PMA-alone group (B), RNP-ICs-alone group (C) or APS-IgG-alone group (D) by one-way ANOVA corrected with Dunnett’s test.
Figure 3: 6-gingerol blocks PDE activity and raises cAMP levels. Human neutrophils were treated with 6-gingerol. Some samples were additionally treated with forskolin, cAMP, and synthetic PDE4 inhibitors (rolipram and IBMX) as indicated. PDE activity (A), cAMP levels (B), and PKA activity (C-D) were measured with kits as described in Methods. In panel E, neutrophils were treated with APS IgG in the presence or absence of 6-gingerol and/or PKA inhibitor. NETosis was quantified by measuring the enzymatic activity of nuclease- liberated myeloperoxidase (MPO). For all panels, mean and standard error of the mean (SEM) are presented for n=3-4 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by one-way ANOVA corrected with Dunnett’s test; NS=not significant.
Figure 4: 6-gingerol attenuates NET release and autoantibody formation in a lupus mouse model. BALB/c mice were treated topically with TLR7 agonist (R848) or vehicle DMSO for 6 weeks (3x per week). Some mice were additionally injected (IP) with 20 mg kg⁻¹ 6-gingerol (3x per week). Schematic of the TLR7 agonist (R848)-induced lupus model (A). NET levels in serum were assessed by measuring cell-free DNA (B), and MPO-DNA complexes (C). Anti-double stranded DNA (anti-dsDNA) (D), anti-beta-2 glycoprotein I (β₂GPI) IgG (E), and total IgG (F) levels in serum were assessed by ELISA. For all panels, mean is presented as a horizontal line; *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the R-848-alone group by one-way ANOVA corrected with Dunnett’s test.
Figure 5: Efficacy of 6-gingerol treatment after development of lupus phenotype in a lupus mouse model. BALB/c mice were treated topically with TLR7 agonist (R848) or vehicle DMSO for 6 weeks (3x per week). Starting at week 4 of treatment, some mice were additionally injected (IP) with 20 mg kg⁻¹ 6-gingerol (3x per week). Schematic the TLR7 agonist (R848)-induced lupus model by week 4 followed by 6-gingerol treatment (A). NET levels in serum were assessed before and after 6-gingerol treatment by measuring MPO-DNA complexes (B). Anti-double stranded DNA (anti-dsDNA) (C), anti-beta-2 glycoprotein I (β₂GPI) IgG (D), and total IgG (E) levels in serum were assessed by ELISA before and after 6-gingerol treatment. For all panels, mean is presented as a horizontal line; *p < 0.05, **p < 0.01, ***p < 0.001 by paired t test.
Figure 6: 6-gingerol prevents aPL Ab-mediated acceleration of venous thrombosis.

Schematic of the electrolytic model of venous thrombosis (A). Direct current results in the release of free radicals within the inferior vena cava, which activate endothelial cells and initiate a thrombogenic environment in the presence of constant blood flow. MPO-DNA complexes (B) were measured in serum of mice treated with control IgG or APS IgG in the presence or absence of 6-gingerol. Thrombus formation was assessed at 24 h. Thrombus length (C) and thrombus weight (D) were measured. Representative thrombi (E). **p < 0.01, ***p < 0.001, and ****p < 0.0001 by one-way ANOVA corrected with Dunnett’s test.
Figure 7: The synthetic PDE4 inhibitor rolipram suppresses APS-mediated NETosis and venous thrombosis. Human neutrophils were stimulated with APS IgG for 3 hours. Some samples were additionally treated with the PDE4 inhibitor rolipram. NETosis was quantified by measuring the enzymatic activity of nuclease-liberated myeloperoxidase (MPO) (A). MPO-DNA complexes (B) were assessed for control IgG- or APS IgG-treated mice in the presence or absence of rolipram. Thrombus formation was assessed at 24 hours. Thrombus length (C) and thrombus weight (D) were measured; **p < 0.01, ***p < 0.001, and ****p < 0.0001 by one-way ANOVA corrected with Dunnett's test.
Figure 8: Kinetics of 6-gingerol in plasma and neutrophils following injection (IP) with 20 mg kg$^{-1}$ of 6-gingerol. Male C57BL/6 mice were injected (IP) with 6-gingerol 20 mg kg$^{-1}$ and peripheral blood was collected after 0.5, 2, 4, and 24 hours. 6-gingerol concentrations in plasma (A) and in neutrophils (B) were then quantitated. Values and error bars shown in the figure represent mean and standard error of the mean (SEM), respectively.
Supplemental Figure 1: *In vitro* efficacy and toxicity of 6-gingerol. Dose response to PMA- (A) and APS-mediated (B) NETosis upon treatment with 6-gingerol. For viability studies, neutrophils were cultured in the presence or absence of 1 mM 6-gingerol for 3 hours. Trypan blue was used to stain the dead cells, and viable cells were counted thereafter. For all panels, mean and standard error of the mean (SEM) are presented for $n = 3$ independent experiments; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared with the 0-μM gingerol group by one-way ANOVA corrected with Dunnett’s test; NS=not significant.
Supplemental Figure 2: BALB/c mice were treated topically with TLR7 agonist (R848) or vehicle DMSO (3x per week) as in Figure 4. Some mice were additionally injected (IP) with 20 mg kg⁻¹ of 6-gingerol (3x per week). Six weeks later, various end points were assessed. Spleen size (A) was measured. White blood cells (WBC) (B), lymphocytes (C), neutrophils (D), and platelets (E) were quantified in peripheral blood. Mean is presented as a horizontal line; *p < 0.05, **p < 0.01, by one-way ANOVA Tukey’s multiple comparison.
Supplemental Figure 3: 6-gingerol suppresses pro-inflammatory cytokine production in a lupus mouse model. BALB/c mice were treated topically with TLR7 agonist (R848) or vehicle DMSO (3x per week) as in Figure 4. Some mice were additionally injected (IP) with 20 mg kg\(^{-1}\) of 6-gingerol (3x per week). Six weeks later, pro-inflammatory cytokines IFN-γ (A) and TNF-α (B) were measured in serum by ELISA. For all panels, mean is presented as a horizontal line; *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) as compared with the R-848-alone group by one-way ANOVA corrected with Dunnett’s test.
Supplemental Figure 4: Slope of NETs and autoantibodies before and after 6-gingerol treatment in a lupus mouse model. BALB/c mice were treated topically with TLR7 agonist (R848) or vehicle DMSO for 6 weeks (3x per week). Starting at week 4, some mice were additionally injected (IP) with 20 mg kg⁻¹ 6-gingerol (3x per week). The change in NET levels (A), anti-double stranded DNA (anti-dsDNA) (B), anti-beta-2 glycoprotein I (anti-β₂GPI) IgG (C), and total IgG (D) in serum before and after 6-gingerol treatment by was calculated and the slope of each individual mouse was plotted (A). For all panels, mean is presented as a horizontal line; *p < 0.05, **p < 0.01, ***p < 0.001 by paired t test.
Supplemental Figure 5: Graphical presentation of 6-gingerol anti-neutrophil properties in models of lupus. 6-gingerol inhibits PDE4 thereby increasing intracellular concentrations of cAMP and enhancing PKA activity in neutrophils; thus, suppressing NET release in models of lupus, while also attenuating other disease-relevant activities such as autoantibody formation and large-vein thrombosis.