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A role for muscarinic receptors in neutrophil extracellular trap formation and levamisole-induced autoimmunity

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Levamisole, an anthelmintic drug with cholinergic properties, has been implicated in cases of drug-induced vasculitis when added to cocaine for profit purposes. Neutrophil extracellular trap (NET) formation is a cell death mechanism characterized by extrusion of chromatin decorated with granule proteins. Aberrant NET formation and degradation have been implicated in idiopathic autoimmune diseases that share features with levamisole-induced autoimmunity as well as in drug-induced autoimmunity. This study's objective was to determine how levamisole modulates neutrophil biology and its putative effects on the vasculature. Murine and human neutrophils exposed to levamisole demonstrated enhanced NET formation through engagement of muscarinic subtype 3 receptor. Levamisole-induced NETosis required activation of Akt and the RAF/MEK/ERK pathway, ROS induction through the nicotinamide adenine dinucleotide phosphate oxidase, and peptidylarginine deiminase activation. Sera from two cohorts of patients actively using levamisole-adulterated cocaine displayed autoantibodies against NET components. Cutaneous biopsy material obtained from individuals exposed to levamisole suggests that neutrophils produce NETs in areas of vasculitic inflammation and thrombosis. NETs generated by levamisole were toxic to endothelial cells and impaired endothelium-dependent vasorelaxation. Stimulation of muscarinic receptors on neutrophils by cholinergic agonists may contribute to the pathophysiology observed in drug-induced autoimmunity through the induction of inflammatory responses and neutrophil-induced vascular damage.

Introduction

Levamisole, a drug currently used to treat parasitic infections in veterinary medicine, has immunostimulatory properties (1). Cases of levamisole-induced autoimmunity in humans were initially reported in clinical trials of levamisole in pediatric populations (2). In 1999, levamisole was removed from the US market due to the risk of serious side effects, including agranulocytosis and drug-induced autoimmunity (3). Since 2008, numerous cases of autoimmune disease have been reported in people exposed to cocaine adulterated with levamisole to increase profit (4). Standard drug purity detection kits do not detect levamisole, and this compound may potentiate the effects of cocaine (5). In 2009, the US Drug Enforcement Agency estimated that 69% of seized cocaine coming into the US was contaminated with levamisole, making levamisole-induced autoimmunity a public health concern (6).

Susceptible subjects inadvertently exposed to levamisole via adulterated cocaine can develop a clinical syndrome of febrile illness, inflammatory arthritis, cutaneous vasculitis, microthrombotic disease, and agranulocytosis (7). In addition, production of high titers of multiple autoantibodies, including antineutrophil cytoplasmic antibodies (ANCA), antinuclear antibodies (ANA), and antiphospholipid antibodies, is a defining feature of levamisole-induced autoimmunity (8). Clinical recognition of cocaine use is of paramount importance, since the features of levamisole-induced autoimmunity overlap with features otherwise associated with ANCA-associated vasculitis (AAV), an idiopathic form of small vessel vasculitis, and with systemic lupus

erythematosus (SLE), a multisystemic autoimmune disease (9). Unlike these idiopathic autoimmune syndromes, which often require potent immunosuppressive therapies to achieve disease remission, symptoms of levamisole-induced autoimmunity typically resolve with cessation of the offending agent, emphasizing the need to recognize and understand the mechanisms underlying this unique clinical syndrome (10).

A short list of medications has been associated with cases of drug-induced autoimmunity, characterized by clinical features that mimic aspects of disease otherwise specific to AAV and/or SLE. These medications include propylthiouracil, methimazole, hydralazine, and procainamide, among others (11, 12). Although associations between certain medications and drug-induced vasculitis or drug-induced lupus have been known for decades, the underlying mechanisms by which levamisole and other medications induce autoimmunity remain incompletely characterized. Most of the prior investigations into the mechanisms of drug-induced autoimmunity have focused upon the effects of these medications on mononuclear cells. The potential contributions of neutrophils to drug-induced autoimmunity have not been extensively studied, yet neutrophil dysregulation plays an increasingly recognized role in the pathogenesis of certain autoimmune diseases including AAV and SLE (13).

Neutrophils stimulated by specific sterile and nonsterile stimuli can undergo a distinct form of cell death characterized by extrusion of granule proteins bound to a meshwork of chromatin and other nuclear material (14). These complexes of intermixed nuclear and cytoplasmic neutrophil contents have been termed neutrophil extracellular traps (NETs), and the process of NET formation has been termed NETosis. Recent studies suggest that NETs are implicated in both idiopathic and drug-induced autoimmunity (15). The presence of NETs in circulation and affected organs has been reported in association with SLE, AAV, and antiphospholipid antibody syndrome — three conditions that clinically resemble levamisole-induced autoimmunity (16–18). Molecules externalized in NETs typically include proteinase 3 (PR3) and myeloperoxidase (MPO), the major antigenic targets of ANCA in AAV, as well as double-stranded DNA and histones, antigenic targets of ANA in SLE and drug-induced lupus (19). Given the prevalence of ANCA and ANA in cases of levamisole-induced autoimmunity, NET formation may represent a mechanism whereby these intracellular antigenic targets are externalized and exposed to the adaptive immune system (15). A recent report characterized NET formation in association with levamisole, but the mechanisms underlying that association and the implications they may have in disease pathogenesis remain unclear (20).

While it is commonly speculated that genetic and environmental factors contribute to the development of various autoimmune diseases, opportunities to study a known environmental trigger of autoimmunity are rare. Given the clinical similarities between levamisole-induced autoimmunity and specific idiopathic autoimmune diseases in which neutrophils and NETs play a role in disease pathogenesis, we hypothesized that this drug triggers autoimmunity and vasculopathy in susceptible hosts by promoting NET formation and characterized the signaling pathways by which this process may occur. We describe a mechanism of muscarinic receptor agonism in neutrophils that modulates neutrophil function. We characterized dysregulation of NET formation and autoantibody synthesis against antigenic NET contents in two independent cohorts of patients exposed to levamisole-tainted cocaine, with and without overt clinical features of levamisole-induced autoimmunity. Finally, we studied the direct and indirect effects of levamisole on vascular tone and integrity. Novel mechanisms identified in the context of levamisole-induced autoimmunity may have application in a broader array of idiopathic autoimmune diseases, including AAV and SLE.

Results

Levamisole induces NETs through nicotinamide adenine dinucleotide phosphate oxidase and peptidylarginine deiminase-dependent pathways. When peripheral blood neutrophils isolated from healthy human subjects were exposed to levamisole across a range of concentrations, including physiologic concentrations of levamisole reported in humans from previous clinical trials (21), significantly increased NET formation was observed compared with baseline levels when quantified by Sytox Green and by confocal microscopy (Figure 1, A and B). The canonical pathway described in NET formation includes translocation of granule proteins, such as neutrophil elastase and MPO, into the nucleus, followed by decondensation of DNA and disassembly of the nuclear envelop into lamin B–positive vesicles. We confirmed that levamisole induces these events in neutrophils. Immunofluorescence analysis of neutrophils stimulated with levamisole demonstrated that neutrophil elastase translocates into the nucleus and the nuclear envelope disintegrates into lamin B–positive compartments (Figure 1, C and D). For specific stimuli, ROS synthesis through the nicotinamide adenine dinucleotide phosphate oxidase (NOX) pathway as well as histone citrullination by peptidylarginine deiminase-4 (PAD4) have been implicated in the
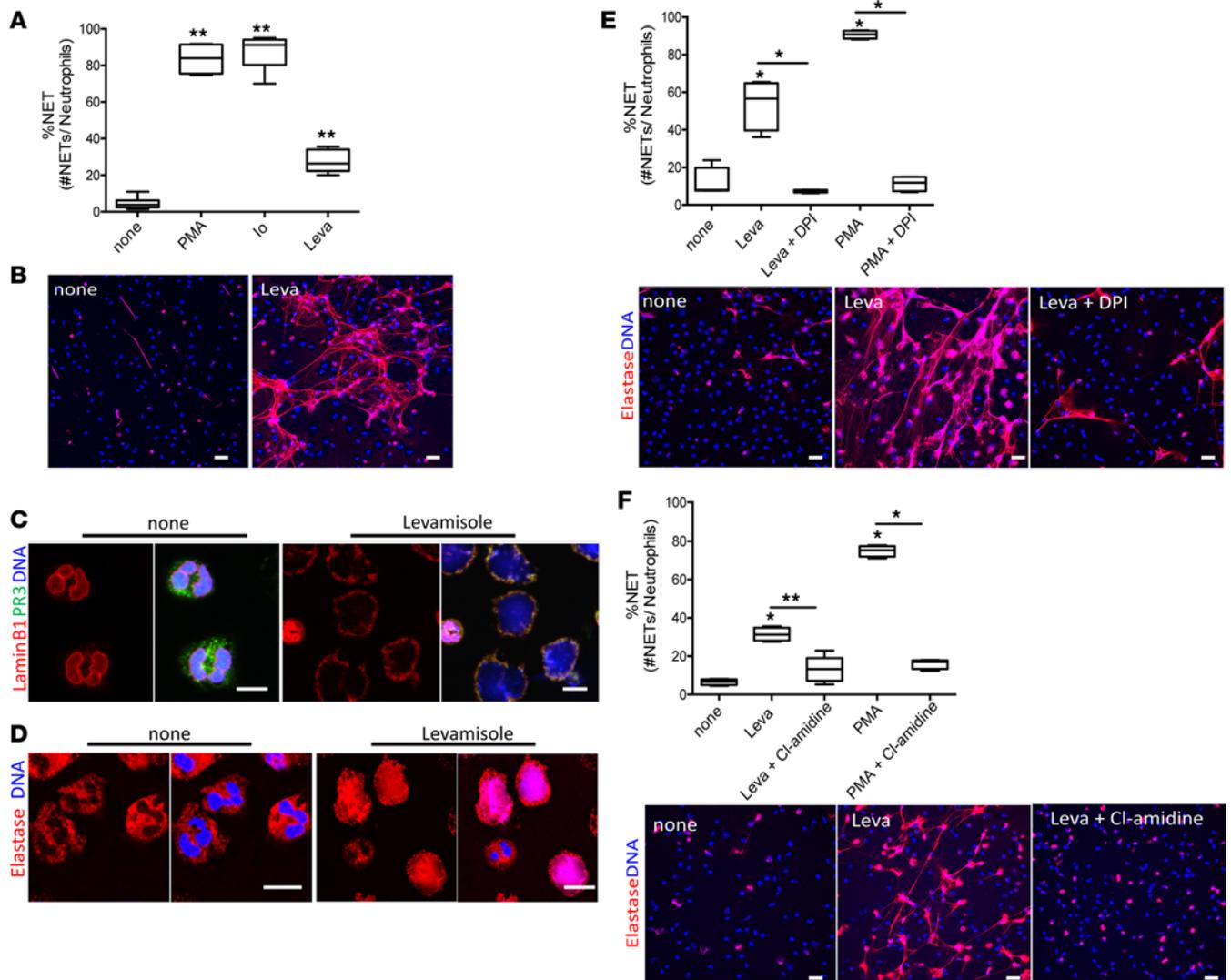
Figure 1. Levamisole induces NET formation in a nicotinamide adenine dinucleotide phosphate oxidase– (NOX-) and peptidylarginine deiminase–dependent (PAD-dependent) manner. Neutrophils from healthy controls were incubated with 100 μM levamisole or 2.5 μM calcium ionophore A23187 (Io) for 3 hours. (**A**) Sytox green assay was used to quantify neutrophil extracellular DNA release after stimulation. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Kruskal-Wallis with post-hoc Dunn's test was used. (**B**) Immunofluorescence analysis quantifies NET formation after 3 hours of incubation with levamisole (neutrophil elastase [red] and DNA [blue]). Scale bar: 20 μm. (**C**) Nuclear envelope marker lamin B (red) depicts areas of neutrophil nuclear membrane disintegration after 3 hours of incubation with levamisole. Scale bar: 10 μm. (**D**) Neutrophil elastase (red) translocates from the cytoplasm into the nucleus after 3 hours incubation with levamisole. Scale bar: 10 μm. (**E** and **F**) Treatment with (**E**) 5 μM diphenylene iodonium (DPI; NOX inhibitor) or (**F**) 200 μM Cl-amidine (PAD inhibitor) significantly decreases the percentage of netting neutrophils in response to levamisole. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Mann-Whitney U test was used; *$P <0.05$, **$P < 0.01$. Scale bar: 20 μm.

molecular mechanisms leading to NET formation (22–24). We assessed if levamisole induced NETs through this previously described pathway. Pharmacologic inhibition of PAD activity with Cl-amidine and of ROS generation with diphenylene iodonium, at doses that inhibit NOX but not mitochondrial ROS (5 μM), showed significant decreases in NET formation following levamisole stimulation (Figure 1, E and F). In addition, levamisole did not induce mitochondrial ROS synthesis, suggesting that this pathway is not activated following exposure to this drug (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.89780DS1). Previous studies had suggested that some drugs, like propylthiouracil, promote autoimmunity by modifying NET structure and interfering with NET degradation mediated by nucleases (25). In contrast, the NETs generated by levamisole did not display resistance to degradation by nucleases (Supplemental Figure 2). These results suggest that levamisole enhances NET formation in control neutrophils in a NOX- and PAD-dependent manner but does not interfere with NET degradation.
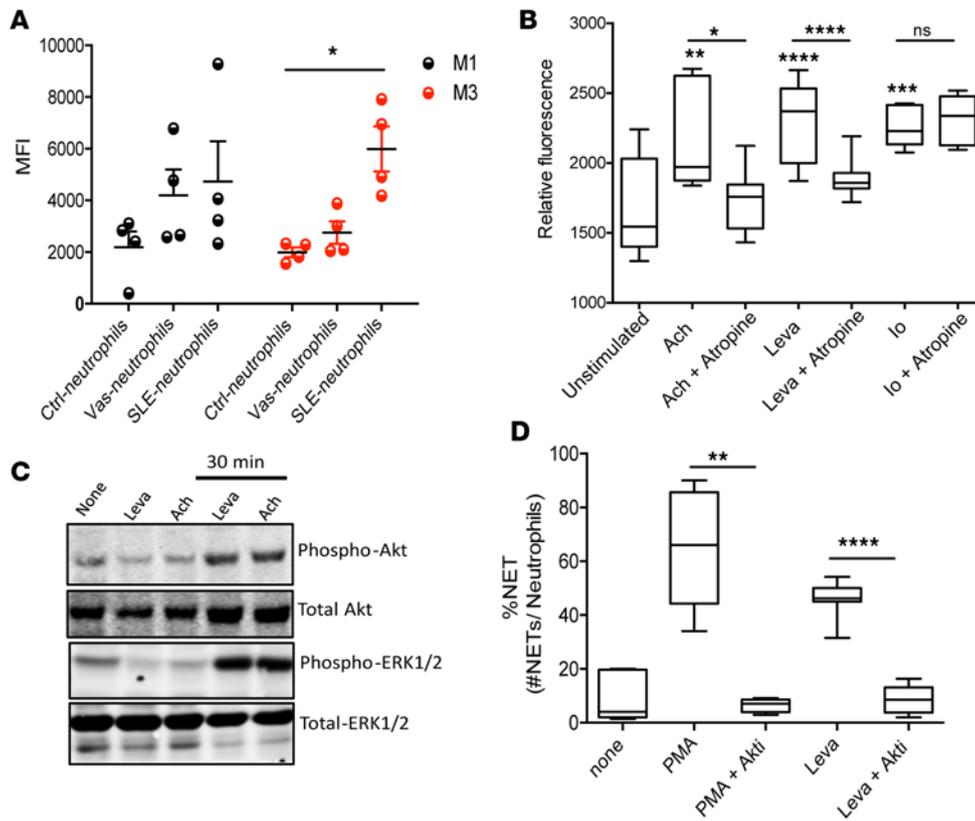
Figure 2. Levamisole and acetylcholine induce NETs through Akt and ERK pathways. (A) Quantification of M1 and M3 expression by flow cytometry in neutrophils from nonstimulated healthy controls (n = 5) and AAV (Vas) (n = 5) and SLE subjects (n = 5). Results are the mean ± SEM of 5 independent experiments. For statistical analyses, Kruskal-Wallis with post-hoc Dunn's test was used; *P < 0.05. **(B)** Quantification of neutrophil DNA released following 3-hour stimulation with levamisole (leva) or acetylcholine (Ach) in presence or absence of the muscarinic receptor inhibitor atropine. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Mann-Whitney U test was used; *P < 0.05, **P < 0.01,***P < 0.001,****P < 0.0001. **(C)** Phosphorylation status of Akt and ERK1/2 assessed by Western blot in neutrophils from healthy controls stimulated with acetylcholine or levamisole for 30 minutes. Representative Western blot image from 2 independent experiments. **(D)** Quantification of neutrophil DNA released in presence or absence of PMA or levamisole in the presence or absence of an Akt inhibitor for 3 to 4 hours. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Mann-Whitney U test was used; **P < 0.01,****P < 0.0001.

Levamisole engages neutrophil muscarinic receptors to trigger NETs. NET formation can be initiated by stimulation of various membrane and intracellular receptors expressed by neutrophils (e.g., TLRs, Fc γ receptors, etc.) or by engagement of ion channels (e.g., calcium ionophores). To gain insight into putative receptors that are engaged by levamisole, we first assessed whether this compound induced TLR activation. Screening analysis showed that levamisole does not activate TLRs (Supplemental Figure 3). Since levamisole belongs to the ionotropic cholinergic agonist group of anthelmintics, we hypothesized that it may engage muscarinic receptors expressed in neutrophils to induce NET formation (26–28). There are 5 known muscarinic subtype receptors (M1–M5) (29). M1, M3, and M5 bind Gq proteins, whereas M2 and M4 bind Gi proteins. There are known pharmacologic antagonists with varying selectivity for the M1–M4 muscarinic subtype receptors (30). Since signaling through M1 and M3 leads to increases in intracellular calcium and PAD4 is a calcium-dependent enzyme implicated in NET formation, we hypothesized that levamisole

likely engaged either M1 or M3. Flow cytometry analysis confirmed that neutrophils from healthy controls express muscarinic receptors (Figure 2A). Furthermore, levels of M3 were significantly higher in neutrophils obtained from SLE subjects when compared with neutrophils from healthy donors, suggesting that some of these muscarinic receptors may be upregulated in neutrophils in association with autoimmune diseases (Figure 2A and Supplemental Table 1). To test whether levamisole engages muscarinic receptors to trigger NET formation, control neutrophils were preincubated with atropine, a nonselective muscarinic receptor inhibitor, and then stimulated either with levamisole or acetylcholine, a muscarinic agonist. Acetylcholine significantly enhanced NET formation (Figure 2B), while incubation with atropine significantly decreased NET formation induced by either levamisole or acetylcholine but not that of NETs generated by stimulation with a calcium ionophore (Figure 2B). These results indicate that neutrophils express muscarinic receptors and that their engagement by agonists, including levamisole, can promote NET formation. Furthermore, neutrophils from patients with certain autoimmune diseases may be more prone to undergo NETosis when exposed to muscarinic receptor agonists, given their upregulation of these receptors on the cell surface.

Akt has been described as a bona fide molecular switch that regulates the type of cell death that neutrophils develop, as activation (phosphorylation) of this molecule shifts cell death from apoptosis to NETosis (31). Other kinases such as ERK are activated during NETosis (32). Indeed, the RAF/MEK/ERK pathway has been implicated in NET formation through activation of NOX and upregulation of antiapoptotic
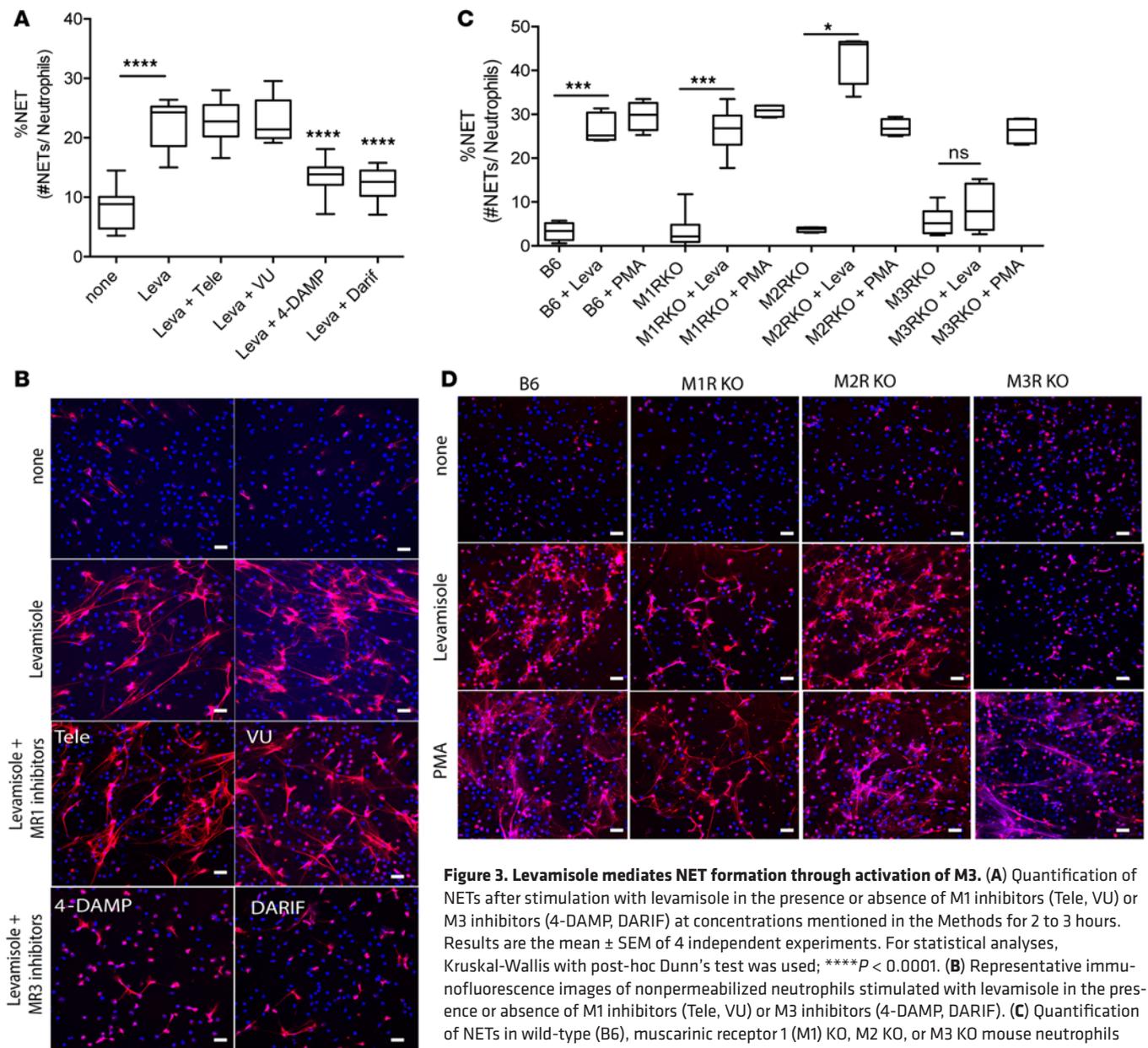
Figure 3. Levamisole mediates NET formation through activation of M3. (A) Quantification of NETs after stimulation with levamisole in the presence or absence of M1 inhibitors (Tele, VU) or M3 inhibitors (4-DAMP, DARIF) at concentrations mentioned in the Methods for 2 to 3 hours. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Kruskal-Wallis with post-hoc Dunn's test was used; ****$P < 0.0001$. **(B)** Representative immunofluorescence images of nonpermeabilized neutrophils stimulated with levamisole in the presence or absence of M1 inhibitors (Tele, VU) or M3 inhibitors (4-DAMP, DARIF). **(C)** Quantification of NETs in wild-type (B6), muscarinic receptor 1 (M1) KO, M2 KO, or M3 KO mouse neutrophils after stimulation with levamisole or PMA for 4 hours. Results are the mean ± SEM of 4 independent experiments. For statistical analyses, Kruskal-Wallis with post-hoc Dunn's test was used; *$P < 0.05$, ***$P < 0.001$. **(D)** Representative immunofluorescence images of nonpermeabilized mouse neutrophils incubated with levamisole or PMA for 4 hours. Scale bar: 20 μm.

proteins. We assessed the activation status of Akt and ERK during levamisole-induced NETosis. Western blot analysis demonstrated that levamisole and acetylcholine promote Akt phosphorylation (Figure 2C). Furthermore, inhibition of Akt significantly decreased levamisole-induced NET formation (Figure 2D). Incubation of neutrophils with levamisole induced ERK1 and ERK2 phosphorylation. These results indicate that engagement of muscarinic receptors in neutrophils induces the NOX-dependent NETosis pathway through the activation of the Akt and RAF/MEK/ERK pathways.

M3 mediates levamisole-induced NETosis. To gain insight into the possible muscarinic receptor subtypes that mediate NETosis induced by levamisole, we pretreated neutrophils with M1 receptor antagonists (telenzepine, VU0255035) or M3 receptor antagonists (4-DAMP, darifenacin) prior to levamisole stimulation. With this pharmacologic approach, we found that NET formation induced by levamisole was significantly abrogated in the presence of M3, but not M1, antagonists (Figure 3, A and B), suggesting that levamisole preferentially uses M3 to trigger NET formation. To confirm this observation using a genetic
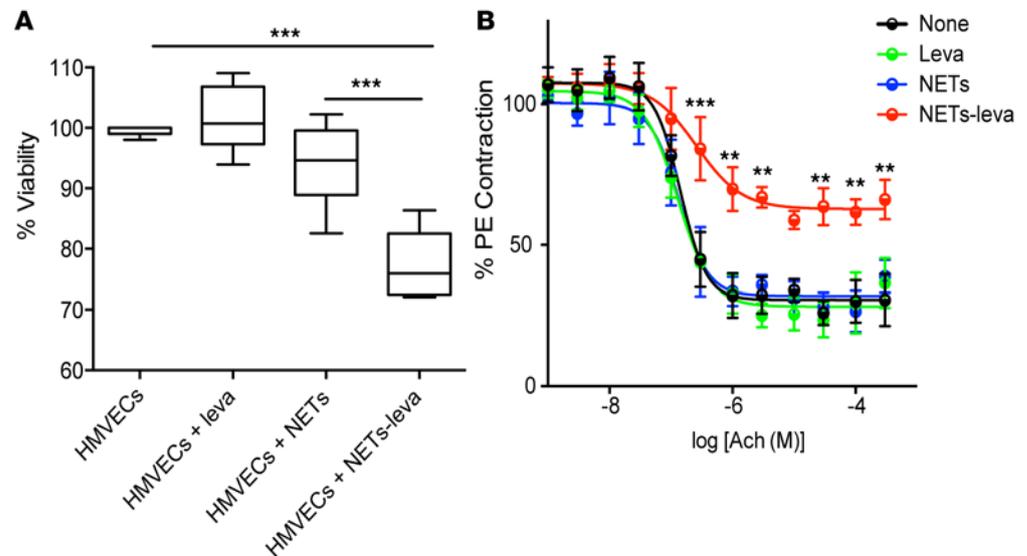
Figure 4. Levamisole-induced NETs promote endothelial cell cytotoxicity and endothelial dysfunction. Human microvascular endothelial cells (HMVECs) were incubated in presence of levamisole (leva), spontaneously generated NETs (NETs), or levamisole-induced NETs (NETs-leva) for 24 hours. (**A**) Cytotoxicity assay was performed after 24 hours of incubation by colorimetric plate assay, as described in Methods. Results are the mean ± SEM of 6 independent experiments. For statistical analyses, Mann-Whitney *U* test was used; ***$P < 0.001$. (**B**) NETs generated by levamisole impair endothelium-dependent vasorelaxation in response to acetylcholine (Ach). Results are the mean ± SEM of 3 independent experiments. For statistical analyses, 2-way ANOVA with post-hoc Tukey's test was used; **$P < 0.01$, ***$P < 0.001$.

approach, purified bone marrow–derived neutrophils from M1, M2, or M3 receptor KO mice were incubated with levamisole (33). Levamisole induced NETosis in wild-type neutrophils, and this induction was markedly inhibited in neutrophils from M3 KO mice but not in cells isolated from M1 or M2 KO mice (Figure 3, C and D). These results indicate that levamisole induces NETosis through engagement of M3 receptor in neutrophils.

Levamisole-generated NETs promote endothelial dysfunction. Levamisole-induced autoimmunity is characterized in many cases by cutaneous lesions with thrombotic or leukocytoclastic vasculitis, with or without vascular occlusion and subsequent necrosis, implicating that damage to the vasculature plays a prominent role in the disease (34). NETs generated in specific diseases promote endothelial damage and activate cell death pathways in the vasculature (35, 36). We assessed whether NETs generated by levamisole promote enhanced vasculopathic effects. Human microvascular endothelial cells (HMVECs) were incubated alone or in the presence of levamisole, spontaneously generated NETs isolated from human neutrophils, or in vitro–generated levamisole-induced NETs. Endothelial cells incubated with levamisole-induced NETs displayed significantly enhanced levels of apoptosis when compared with cells incubated with levamisole or with spontaneously generated NETs (Figure 4A). These results suggest that endothelial cytotoxicity triggered by levamisole is mediated by the generation of NETs with vasculopathic effects. Endothelium-dependent vasorelaxation is a surrogate of nitric oxide–dependent vascular homeostasis, and perturbations in this process promote endothelial dysfunction. We assessed if levamisole-generated NETs promoted endothelial dysfunction. Endothelium-dependent vasorelaxation of murine aortic rings was significantly impaired in response to levamisole-induced NETs, when compared with levamisole alone or with spontaneously generated NETs (Figure 4B). These observations support the hypothesis that NETs generated by levamisole induce endothelial cell death and vascular dysfunction and that this phenomenon may play a role in levamisole-induced vasculitis.

Autoantibodies to NET proteins develop in individuals exposed to levamisole. The prevalence of autoantibodies in cocaine users exposed to levamisole is unknown. Since levamisole induces formation of NETs that externalize various autoantigens, we hypothesized that a percentage of individuals exposed to levamisole would develop autoantibodies recognizing molecules present in NETs. We studied two cohorts of cocaine users with confirmed exposure to levamisole. The NIDA cohort consisted of cocaine users in the Baltimore, Maryland, USA, region, none of whom had overt clinical symptoms of levamisole-induced autoimmunity.
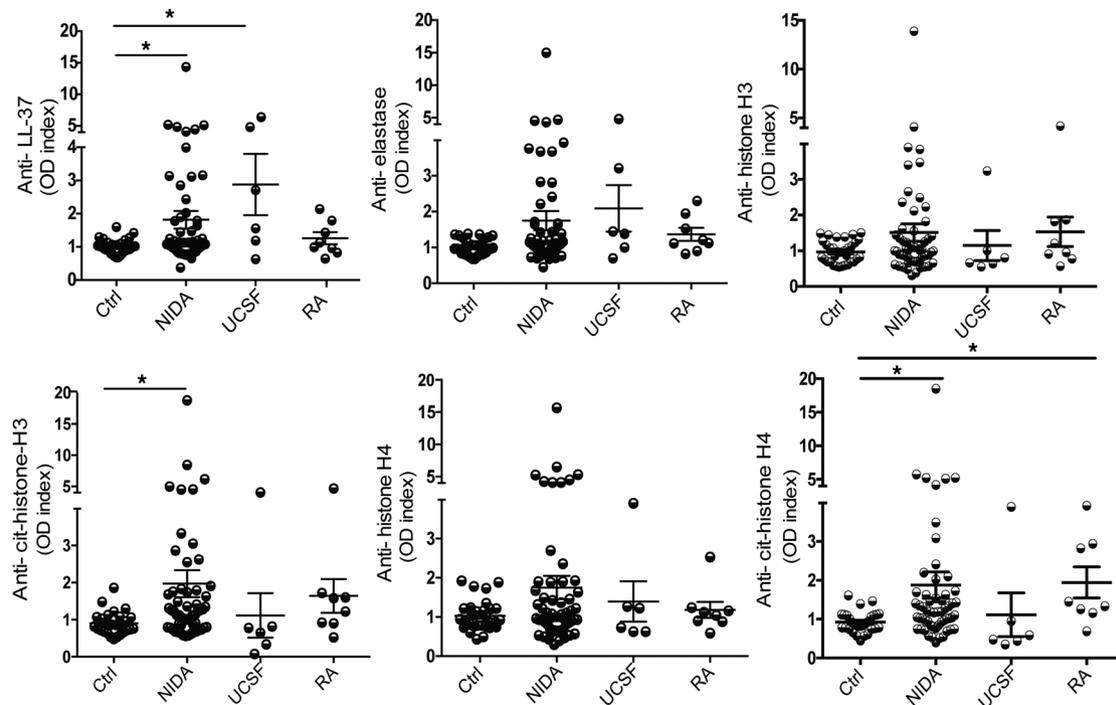
Figure 5. Autoantibodies to NET proteins are present in sera of users of levamisole-tainted cocaine. Quantification of anti-LL37, anti-elastase, anti-histone H3, anti-citrullinated histone H3, anti-histone H4, and anti-citrullinated (cit) histone H4 in RA (*n* = 8), NIDA (asymptomatic active users of levamisole-tainted cocaine, *n* = 52), UCSF (levamisole-induced vasculitis, *n* = 6), or healthy donor (*n* = 20) sera. Results are expressed as OD index (OD in patient serum/mean OD in control sera). Results are displayed as the mean ± SEM. For statistical analyses, Kruskal-Wallis with post-hoc Dunn's test was used; *P < 0.05.

The UCSF cohort consisted of subjects with ongoing clinical symptoms of levamisole-induced autoimmunity, as previously described (8).

Of 52 subjects in the NIDA cohort with a positive urine toxicology screening test for cocaine, 100% had detectable levels of levamisole and cocaine metabolites in the urine. The average concentration of levamisole detectable in urine was 11.9 μM, and the maximum observed concentration was 1,085 μM. There were no differences in the prevalence of ANA and ANCA between cocaine users and noncocaine users in the NIDA cohort (8% versus 5% for ANA and 4% versus 0% for ANCA, P = 1.0). In contrast, 83% of subjects with overt levamisole-induced autoimmunity from the UCSF cohort had detectable ANA and ANCA (Supplemental Table 2). Among cocaine users in the NIDA cohort, there was no correlation between autoantibody titers and urinary levels of levamisole (r = –0.03 for PR3-ANCA; r = 0.02 for MPO-ANCA; r = –0.02 for ANA) or urinary levels of cocaine metabolites (r = 0.02 for PR3-ANCA; r = 0.05 for MPO-ANCA; r = –0.03 for ANA).

Despite the low prevalence of ANA and ANCA in the NIDA cohort, sera from these subjects demonstrated increased levels of antibodies that recognize other NET-related proteins. Antibodies against the neutrophil granule proteins cathelicidin (LL-37) and neutrophil elastase were detected in many patients exposed to levamisole-tainted cocaine in the NIDA and UCSF cohorts. Antibodies against citrullinated histones H3 and H4, but not native H3 and H4, were detectable in the majority of patients in the NIDA cohort (Figure 5), at levels comparable to those observed in patients with rheumatoid arthritis (RA), a condition with well-characterized development of autoantibodies recognizing citrullinated histones (19).

These results demonstrate that autoantibodies to several proteins commonly found within NETs are detectable in sera from humans exposed to levamisole via contaminated cocaine, even in absence of overt clinical features of autoimmune disease. Differences in the prevalence of antibodies directed against NET-related proteins between the NIDA and UCSF cohorts suggest that clinically apparent levamisole-induced autoimmunity is associated with the development of specific autoantibodies.

NETs are identified in skin lesions of individuals with levamisole-induced vasculitis. To identify in vivo evidence of NET formation in tissue and confirmation of a putative vasculopathic role of these lattices, we analyzed skin biopsies from 5 subjects diagnosed with levamisole-induced vasculitis. Indeed, histologic evaluation confirmed the presence of both vasculitic and microthrombotic disease in affected tissue, often
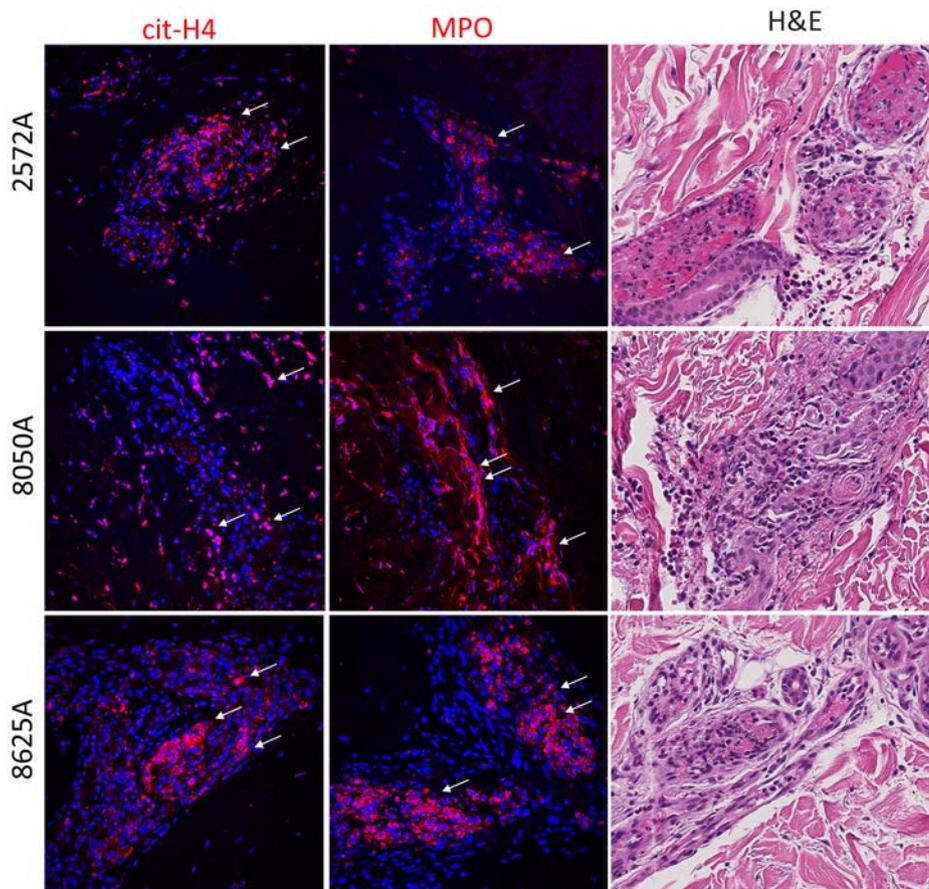
Figure 6. NETs are present in skin lesions from patients with levamisole-induced vasculitis. Formalin-fixed, paraffin-embedded cutaneous biopsy material obtained from 5 patients with confirmed levamisole-induced autoimmunity was stained for citrullinated-histone H4 (cit-H4), myeloperoxidase (MPO), or H&E. Representative images are displayed. Biopsy from patient 2572A demonstrated leukocytoclastic vasculitis in the small capillaries with karyorrhexis and intravascular thrombi with dense neutrophilic infiltrate in the deeper dermis. Biopsy from patient 8050A demonstrated perivascular and perieccrine neutrophilic infiltrates with areas of intravascular thrombosis. Biopsy from patient 8625A demonstrated a large, organized thrombus within a deep dermal artery. NETs (pink) were observed in associated areas of neutrophilic infiltrate in each of these specimens (arrows). Original magnification, ×40.

within the same biopsy specimen. Vascular pathology included extensive perivascular neutrophilic inflammation (2 patients), leukocytoclastic vasculitis with fibrinoid necrosis and karyorrhexis (2 patients), and thrombosis (3 patients). Areas of dense neutrophilic infiltrate were observed within the deep dermis (3 patients). By immunofluorescence microscopy, we quantified infiltrating netting neutrophils (MPO positive and cit-H4 positive) in skin lesions. Immunofluorescence studies suggested the presence of NETs in affected skin lesions in 4 of 5 patients with levamisole-induced autoimmunity (Figure 6). NETs were visualized in areas in which there was vasculitic or thrombotic involvement and in areas of neutrophilic infiltration within the deeper dermis. In the one skin biopsy without detectable NETs, histologic characterization demonstrated few infiltrating neutrophils and recanalization of an arterial thrombus in a single affected dermal artery, findings consistent with a chronic lesion. These results demonstrate that infiltrating netting neutrophils are seen in areas of cutaneous vasculitis and microthrombi in tissue from patients with levamisole-induced autoimmunity, implicating NET formation in disease pathogenesis.

Levels of NETosis fluctuate in response to changes in levamisole exposure. NET formation was studied longitudinally in a patient with intermittent ongoing use of levamisole-tainted cocaine. The patient presented with membranous glomerulonephritis, which has been described as an atypical feature of levamisole-induced autoimmunity (37). Serologic evaluation demonstrated the characteristic pattern of autoantibodies associated with levamisole ingestion, including presence of ANCA directed against both MPO and PR3, ANA, anti-double-stranded DNA, and antiphospholipid antibodies (lupus anticoagulant and IgM anticardiolipin). On longitudinal evaluation, urinary levamisole levels correlated with the degree of reported cocaine use and the quantity of cocaine metabolites in the urine but did not correlate with ANCA status, which remained persistently positive at low levels over 1 year of observation (Supplemental Table 3). The patient was able to minimize cocaine intake, and the degree of proteinuria improved during follow-up. Neutrophils from the patient showed prominent spontaneous NETosis during periods of heavy levamisole-tainted cocaine use compared with periods of relative abstinence, and these NETs externalized MPO, PR3, and nuclear material in association with high levels of autoantibodies (Supplemental Figure 4). These observations provide in vitro and in vivo evidence that levamisole promotes neutrophils to undergo NET formation and highlight that the degree of spontaneous NETosis correlates with the degree of use of levamisole-tainted cocaine. Further, NETs triggered by levamisole contain the major autoantigenic targets of autoantibodies that are commonly associated with levamisole-induced autoimmunity.

Discussion

Levamisole-induced autoimmunity, AAV, and SLE share many clinical and laboratory features, including the presence of autoantibodies directed against neutrophil proteins and nucleic acids. Because neutrophils and NETs likely play important roles in the pathogenesis of AAV and SLE, we hypothesized that levamisole may stimulate neutrophils to undergo NET formation, with subsequent externalization of autoantigens. While a previous publication reported that levamisole can trigger NET formation (20), to our knowledge our study is the first of its kind to characterize the specific mechanisms of levamisole-mediated NETosis, unraveling an association between M3 muscarinic receptors on the surface of neutrophils and drug-induced autoimmunity. The subcellular events that constitute levamisole-mediated NET formation were found to be dependent on activation of NOX, Akt, and the RAF/MEK/ERK pathway and are dependent on histone citrullination by PADs. This study also provides the first in vivo evidence to our knowledge that NETosis occurs in humans inadvertently exposed to levamisole via contaminated cocaine. Autoantibodies to NET products were identified in subjects exposed to levamisole-tainted cocaine, and immunofluorescence studies were consistent with the presence of netting neutrophils in affected skin from patients with levamisole-induced autoimmunity. Furthermore, we demonstrate increased expression of M3 muscarinic receptors on neutrophils from patients with SLE, suggesting that cholinergic receptor profiles on the surface of neutrophils may play a pathogenic role in the broader spectrum of systemic autoimmune diseases. These findings highlight that pathogenic mechanisms of drug-induced autoimmunity can also be identified in counterpart idiopathic autoimmune syndromes, and, by extension, insight gained from the study of drug-induced autoimmunity may in turn improve understanding of more complex, idiopathic autoimmune diseases.

The discovery that muscarinic receptors can modulate neutrophil function adds to an expanding literature focused on mechanisms by which neurotransmitters may interact with immune cells and alter their function. Cholinergic stimulation of the nicotinic acetylcholine receptor a7 subunit on the surface of macrophages can inhibit synthesis of proinflammatory cytokines and prevent immune-mediated damage in models of sepsis, colitis, and arthritis (38–42). This process, dubbed the "cholinergic antiinflammatory pathway," suggests that neural circuits may reflexively modulate immune responses (43). The traditional view that acetylcholine acts solely as a neurotransmitter is challenged by the observation that it can also be synthesized by nonneuronal cells, including epithelial, endothelial, mesenchymal, and immune cells (44). Indeed, findings from our study reveal that the interaction between the cholinergic system and innate immunity may be more complex than previously considered. We observed that stimulation of muscarinic receptors on neutrophils by acetylcholine or levamisole triggers NET formation and that this phenomenon is inhibited by specific anticholinergic therapies. Very recently, nicotine was described to have the capacity to trigger NETs by engagement of the nicotinic acetylcholine receptor through an Akt-dependent but NOX-independent pathway (45). These results suggest that neuroimmunomodulation in the neutrophil is complex and dependent on which type of receptor is engaged on the cell surface and on the type of stimulus that leads to the formation of NETs.

Functional studies demonstrated that levamisole-induced NETs, but not levamisole alone, are directly toxic to vascular endothelium and potentially contribute to the characteristic vascular pathology seen in cases of levamisole-induced autoimmunity. Potential toxic intermediates generated in the interaction between levamisole and NET proteins may explain this observation. Activation of M3 receptors on vascular endothelial cells by acetylcholine promotes arterial vasodilation via nitric oxide synthesis (46). However, direct stimulation of M3 receptors on vascular smooth muscle cells promotes vasoconstriction in pathologies in which the vascular endothelium is disrupted (47). In keeping with these observations, endothelium-dependent vasorelaxation of murine aortas in response to acetylcholine was impaired in the presence of levamisole-induced NETs but was unaffected by levamisole alone. Neutrophil and endothelial cell interactions are critical components in the pathogenesis of vasculopathy observed in vasculitic and thrombotic diseases, the major histologic features of levamisole-induced vascular disease. Our data suggest that vascular pathology could in part be mediated by not previously described interactions among the nonneuronal cholinergic system of the vascular endothelium, cholinergic receptors on the surface of neutrophils, NETs, and levamisole.

We observed that levamisole triggers NET formation by engaging M3 receptors on neutrophils. Previously, M3 receptors have been implicated in airway diseases in which their activation causes bronchoconstriction (48). Anticholinergics that selectively target the M3 muscarinic receptor are effective

bronchodilators for patients with chronic obstructive pulmonary disease. Evidence from M3 muscarinic receptor–deficient mice indicates that this receptor regulates neutrophilic inflammation in response to cigarette smoke. In murine models of asthma, antagonism of M3 alleviates airway hyperresponsiveness, neutrophil-mediated inflammation, and airway remodeling (49). As mentioned above, M3 receptor signaling may also play a causal role in specific idiopathic autoimmune diseases. Relative to neutrophils from healthy controls, an increased abundance of M3 muscarinic receptors was observed in neutrophils from patients with SLE in clinical remission at the time of sampling, suggesting that upregulation of M3 muscarinic receptors may not simply reflect the activation state of the neutrophil. We did not observe significant differences in the abundance of M3 muscarinic receptors on neutrophils from patients with AAV relative to healthy controls. These differences may reflect biologically relevant differences between neutrophil biology in SLE versus AAV or, alternatively, the fact that patients with AAV in our cohort were receiving more potent background immunosuppressive medications for remission maintenance compared with the patients with SLE (Supplemental Table 1).

Findings from this study also provide insight into the prevalence of autoantibodies in people inadvertently exposed to levamisole via contaminated cocaine. In accordance with reports that suggest the high prevalence of levamisole-tainted cocaine in the US, we observed detectable levels of this drug in the urine in 100% of cocaine users we studied, emphasizing that contamination of cocaine with levamisole is a potential major public health concern. In the NIDA cohort, a model of levamisole exposure in the preclinical disease state, we observed a low prevalence of ANA and ANCA measured by clinical tests and a significant increase in other NET-specific autoantibodies. In contrast, ANA and ANCA were observed in the majority of patients with clinically evident levamisole-induced autoimmunity in the UCSF cohort, along with increases in anti-NET antibodies. Longitudinal studies would be required to determine whether or not presence of anti-NET antibodies precedes the development of ANA or ANCA and predicts the development of clinical autoimmunity. Furthermore, it will be important to identify in future studies how the genetic makeup of a particular individual promotes the evolution from NET-specific autoantibodies and neutrophil dysregulation to well-established autoimmune disease when exposed to levamisole-tainted cocaine.

It is important to consider that cocaine itself has been clinically associated with drug-induced vasculitis and ANCA (50). We did not directly characterize the effects of cocaine on neutrophils; however, cocaine and levamisole independently have been reported to induce NET formation (20). Potential synergistic effects of cocaine and levamisole and potential drug-protein adduct effects should be explored in future studies.

Despite decades of research on the immunostimulatory properties of levamisole, no instances of levamisole-induced vasculitis have been reported in animal models. This in part may be explained by the fact that murine neutrophil biology appears to critically differ from that of humans, limiting the potential utility of mouse models of disease (51). Nevertheless, it would be informative in the future to explore in vivo whether modulation of cholinergic pathways may abrogate drug-induced immune dysregulation.

In summary, these observations demonstrate that levamisole can modulate neutrophil responses through engagement of the cholinergic system. NETs generated by engagement of the neuronal-immune axis may play a critical role in drug-induced vasculitis and vascular dysfunction and, perhaps, in the pathogenesis of certain idiopathic autoimmune diseases. Modulation of cholinergic receptors on neutrophils and inhibition of specific muscarinic receptors may constitute novel therapeutic targets in a spectrum of inflammatory and autoimmune diseases.

Methods

Human subjects

NIDA cohort. This cohort consisted of 500 community-dwelling individuals in a neighborhood at high risk for illicit drug abuse in Baltimore, Maryland, USA. Sera and urine collected from a random subset of this cohort were used for this study. Subjects were screened by questionnaire for history of illicit drug use and for presence of symptoms related to levamisole-induced autoimmunity. Levamisole, cocaine, and cocaine metabolite testing of urine were used to confirm current exposure to levamisole/cocaine. Subjects who reported a history of cocaine use within the last 30 days without confirmation of cocaine metabolites in the urine were excluded from study.

UCSF cohort. This cohort consisted of 6 patients with confirmed levamisole-induced autoimmunity as previously reported (8). Stored serum and plasma samples were used for autoantibody profiling experiments. Paraffin-embedded tissue from skin biopsies was used to test for the presence of NETs and netting neutrophils in affected tissue, as mentioned below.

NIAMS cohort. Subjects with AAV, SLE, or RA and healthy controls were recruited from respective cohorts for these diseases within NIAMS. Patients with AAV fulfilled the 1990 American College of Rheumatology (ACR) criteria for granulomatosis with polyangiitis (Wegener's) (52). Patients with SLE fulfilled the revised ACR criteria for the disease (53). Patients with RA met the 1987 revised ACR classification criteria (54). Remission for AAV or SLE was defined by a Birmingham Vasculitis Activity Score or SLE disease activity index score of 0, respectively (55, 56).

Mice

Breeding pairs of muscarinic receptor KO mice (M1, M2, and M3) on C57/B6 background were obtained from Jurgen Wess, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health. Mice were bred and housed in a pathogen-free facility. Only females were used in the experiments described here. C57/B6 controls were purchased from The Jackson laboratory.

Cell isolation and culture

Normal-density neutrophils were isolated from the red blood cell layer via dextran sedimentation as previously described (19). HMVECs were maintained in endothelial cell basal medium-2 (EBM-2) (Lonza), supplemented with the microvascular endothelial growth factor-2 (without hydrocortisone) bullet kit (Lonza) in 0.2% gelatin-coated 96-well plates. Cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

Reagents

Drugs were used at the following concentrations: 2.5 μM calcium ionophore A23187 (Sigma-Aldrich), 40 or 100 μM levamisole (Sigma-Aldrich), 100 nM telenzepine (TOCRIS), 10 μM 4-DAMP (TOCRIS), 10 μM VU0255035 (TOCRIS), 10 μM darifenacin (Selleckchem), 100 nM atropine (Sigma-Aldrich), 10 μM acetylcholine (Sigma-Aldrich), 200 μM Cl-amidine (gift from Paul Thompson and Venkataraman Subramaniam, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts, Worcester, Massachusetts, USA), 5 μM diphenylene iodonium (Sigma-Aldrich), and 10 μM Akt inhibitor (EMD Millipore). Hoechst, Mitosox, and Sytox Green were from Life Technologies. The following antibodies were used for flow or immunofluorescence microscopy: rabbit polyclonal antibodies against human MPO (DAKO, catalog A0398), citrullinated-histone H4 (Millipore, catalog 07-596), muscarinic receptor 1 clone H-120 (Santa Cruz, catalog sc-9106), human elastase (Abcam, catalog ab21595), lamin B1 (Abcam, catalog ab16048), phospho-p44/42 MAPK (Erk1/2) (Cell Signaling, catalog 4370), phosphor-Akt (Cell Signaling, catalog 9271), mouse monoclonal antibody against PR3 clone D-1 (Santa Cruz, catalog sc74534), p44/42 MAPK (Erk1/2) clone 3A7 (Cell Signaling, catalog 9107), Akt clone 40D4 (Cell Signaling, catalog 2920), and goat polyclonal antimuscarinic receptor 3 clone H-20 (Santa Cruz, catalog sc-31486). Human recombinant histone H3 and histone H4 were purchased from BioLabs. Recombinant neutrophil elastase was from Lee Bio solutions, and recombinant cathelicidin (LL-37) was from Ana Spec Inc. Secondary antibodies coupled to fluorophores were purchased from Life Technologies.

NET quantification

Plate assay. NET quantification was performed as previously described (19). Briefly, neutrophils were resuspended in RPMI without phenol red containing 0.2 μM Sytox green. Neutrophils (2×10^5) were incubated in the presence or absence of 100 μM levamisole, 2.5 μM calcium ionophore, 10 μM acetylcholine, or 100 nM atropine in 96-well black plates for 2 to 3 hours at 37°C. Fluorescence was measured in a Biotek Synergy H1 Hybrid Reader (Biotek). Results were reported as DNA relative fluorescence units.

Fluorescence microscopy. After stimulation, neutrophils were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Neutrophils were permeabilized with 0.2% (wt/vol) Triton X-100 in PBS for 10 minutes at room temperature. Cells were blocked with 0.2% porcine skin gelatin (Sigma-Aldrich) in PBS for 30 minutes and incubated in a humid chamber for 1 hour at 37°C with primary antibody diluted in blocking buffer. Coverslips were washed 3 times and incubated with secondary antibodies coupled to Alexa Fluor

488 or Alexa Fluor 555 fluorophores for 30 minutes at 37°C. DNA was stained with 1:1,000 fluorescence dye (Hoechst) for 10 minutes. Samples were washed 3 more times with PBS and mounted on glass slide using Prolong-gold solution. Images were acquired on a Zeiss LSM780 confocal laser-scanner microscope.

NET isolation

NETs were isolated as previously described (57). Briefly, neutrophils were purified and seeded in 24-well tissue culture plates in RPMI without phenol or stimuli and incubated for 3 hours at 37°C. Supernatants were harvested and NETs were digested with 10 U/ml of micrococcal nuclease (Thermo) for 15 minutes at 37°C. Supernatants were collected and centrifuged at 300 g for 5 minutes at 4°C. NET supernatants were transferred to a fresh tube and stored at –80°C until used.

TLR ligand screening

A TLR ligand screening (InvivoGen) was performed as described by the manufacturer (http://www.invivogen.com/custom-tlr-screening) to assess whether levamisole (40 μM) activates TLR pathways. Screening was performed using a panel of HEK293-TLR-Blue clones engineered to express only a single specific TLR and a SEAP-reporter plasmid activated with NF-κB. Cells incubated with TLR-specific ligands were used as a positive control. Cell activation was evaluated as an increase in SEAP activity measured as absorbance at OD_{650} nm, using Quanti-Blue reagent according to the manufacturer's protocol (InvivoGen).

Western blot

Neutrophils were lysed as previously described (57). Proteins were separated in a 4%–12% gradient Bis-Tris gel (Invitrogen) transferred onto a nitrocellulose membrane and blocked with 10% BSA for 30 minutes at room temperature. After overnight incubation with primary antibodies, membranes were washed 3 times with PBS-Tween (PBS-T) and incubated with secondary antibody coupled to IRDye 800CW. Membrane was washed 3 times with PBS-T and developed using a Li-COR Odyssey Clx scanner (Li-COR Biosciences).

Detection of antibodies recognizing NET proteins by ELISA

A 96-well plate was coated with 100 ng/ml of recombinant LL37, elastase, histone H3, citrullinated H3, histone H4, or citrullinated H4 in PBS overnight. The plate was washed with 0.05% PBS-T and blocked with 10% FBS for 1 hour at room temperature. Diluted (1:1,000) sera from the NIDA cohort ($n = 52$), UCSF cohort ($n = 6$), patients with RA ($n = 8$), and healthy donors ($n = 20$) were incubated overnight at 4°C. Patients with RA were included as positive controls, given the known prevalence of antibodies directed against citrullinated histones in this disease. The plate was washed 3–5 times with 0.05% PBS-T. Horseradish-conjugated anti-human IgGs secondary antibody was incubated for 1 hour at room temperature, followed by 5–7 washes with PBS-T. Reactive protein was detected after incubation with TMB substrate for 5 minutes followed with 0.16 N sulfuric acid (stop solution). The plate was read at 450 nm in a Biotek Synergy H1 Hybrid Reader (Biotek). Results are presented as OD index, which is the ratio of the OD in the patient serum to the mean OD in healthy control sera.

Endothelium-dependent vasorelaxation quantification

Endothelium-dependent vasorelaxation was performed as previously described (58, 59). Female C57BL/6 mouse aortas were dissected and aortic rings were mounted in a myograph apparatus (DMT620M, DMT-USA). Aortas were incubated with 100 μg spontaneously generated NETs, 100 μg levamisole-generated NETs, or 100 μM levamisole for 15 minutes at room temperature. Aortic rings were precontracted with phenylephrine (PE) until reaching a stable plateau in the contraction. Then, graded concentrations of acetylcholine (10^{-9}– 10^{-5} moles/liter) were added cumulatively to test endothelium-dependent vasorelaxation. Results were reported as a percentage of PE contraction as previously described (59).

Endothelial cytotoxicity assay

Ten thousand HMVECs per well were incubated in the absence or presence of 100 μg spontaneously generated NETs, 100 μg levamisole-induced NETs, or 100 μM levamisole in 96-well plate for 24 hours at 37°C. Cytotoxicity was assessed using the Cell Cytotoxicity assay (Abcam) according to manufacturer's recommendations. Fluorescence was measured at 570 nm and 605 nm, and an OD_{570}/OD_{605} ratio was calculated for each sample. Percentage cell viability was calculated as a ratio of $OD_{sample}/OD_{ctrl} \times 100$.

Quantification of urinary levamisole, cocaine, and cocaine metabolites

Levamisole, cocaine, and cocaine metabolites in urine were analyzed using liquid chromatography–mass spectrometry. Standards of levamisole (Sigma-Aldrich), $^{13}C_6$-D-levamisole (AlsaChim), D_3-cocaine (Sigma-Aldrich), and 250 μg/ml certified reference material Cocaine Multi-Component Cocaine Mixture (Sigma-Aldrich, C-088-1ML) were obtained. The drugs and metabolites were extracted from urine by vortexing a 25-μl aliquot of urine and 475 μl water with 0.1% formic acid containing $^{13}C_6$-D-levamisole and D_3-cocaine internal standards for 5 minutes. The tube was then centrifuged at 20,817 g for 10 minutes. Supernatant (300 μl) was transferred to a HPLC vial and sealed. The chromatographic separation was performed using water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Initially, we used the following conditions: 400 μl/minute of 90% A for the first 0.5 minutes, then gradient to 75% A at 1.5 minutes, followed by column cleanup with 60% A at 2 minutes until 3.5 minutes, and then returning to initial conditions of 90% A at 4 minutes and equilibrating for 1 minute. Assays were performed with an Acquity I-Class UPLC (Waters Corp.) and a Waters Cortecs C18+ column (2.1 mm × 100 mm, 1.7 μm) coupled with a Q-Exactive high-resolution-accurate mass MS (Thermo Scientific) at 70,000 resolution with an HESI-II electrospray source. The quantitative analysis of levamisole, cocaine, and cocaine metabolites in positive ion mode was done based on the $[M+H]^+$ ions with levamisole at m/z 205.0794, cocaine at m/z 304.1543, and benzoylecgonine at m/z 290.1387. Calibration curves were determined for levamisole, cocaine, and benzoylecgonine.

Immunohistochemistry

Paraffin-embedded tissues were incubated with xylene (Sigma-Aldrich) for 20 minutes at room temperature. Tissues were dehydrated through a series of incubations with various ethanol dilutions (100%, 95%, 70%, and 50%). Slides were rinsed with deionized water and rehydrated with PBS for 10 minutes at room temperature. Antigen retrieval was performed for tissue stained with anti-citrullinated histone H4. Slides were then blocked with 10% BSA for 30 minutes at room temperature and incubated overnight with primary antibody anti-MPO (DAKO, 1:1,000) or anti-citrullinated histone H4 (Millipore, 1:100)) diluted in 5% BSA. Slides were washed 5 times with PBS and incubated with 1:200 donkey anti- rabbit Alexa Fluor 555 (Life technologies) secondary antibody for 1 hour at room temperature. After 5 washes with PBS, slides were stained with 1:1,000 Hoechst for 10 minutes. Images were acquired on a Zeiss LSM780 confocal laser-scanner microscope.

Statistics

Results are presented as the mean ± SEM. Data were analyzed using GraphPad Prism software. Pairwise differences in distributions were compared using a 2-tailed Student's t test or, in cases of non-Gaussian distributions, using the Mann-Whitney U test. Differences in distributions between multiple groups were compared using the Kruskal-Wallis 1-way ANOVA test with post-hoc Dunn's test. For the vasorelaxation experiments, 2-way ANOVA with post-hoc Tukey's test was used to compare differences across groups. A P value of less than 0.05 defined statistical significance.

Study approval

All human subjects included in this study provided written informed consent prior to study participation. Participating local IRB boards (NIAMS, NIDA, and UCSF) approved the research. Subjects were recruited from the NIAMS, NIDA, and UCSF cohorts.

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