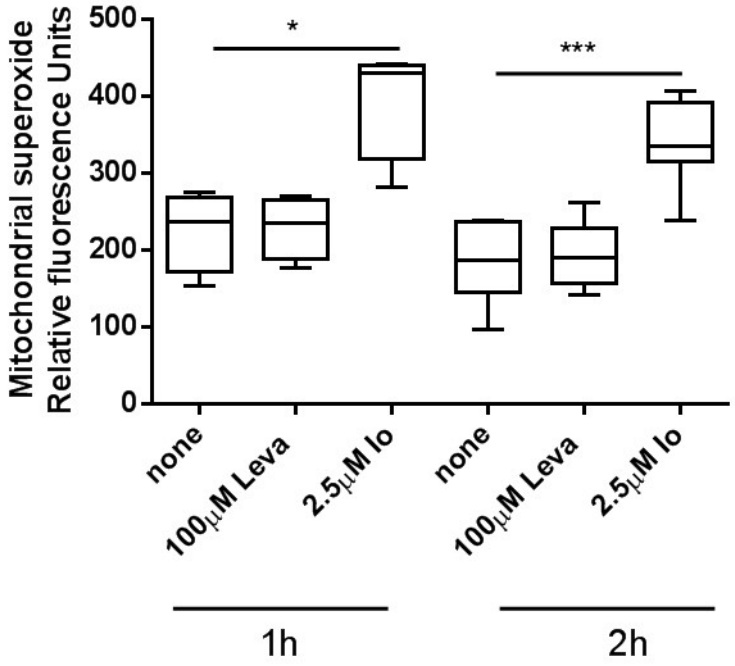
Supplementary Material

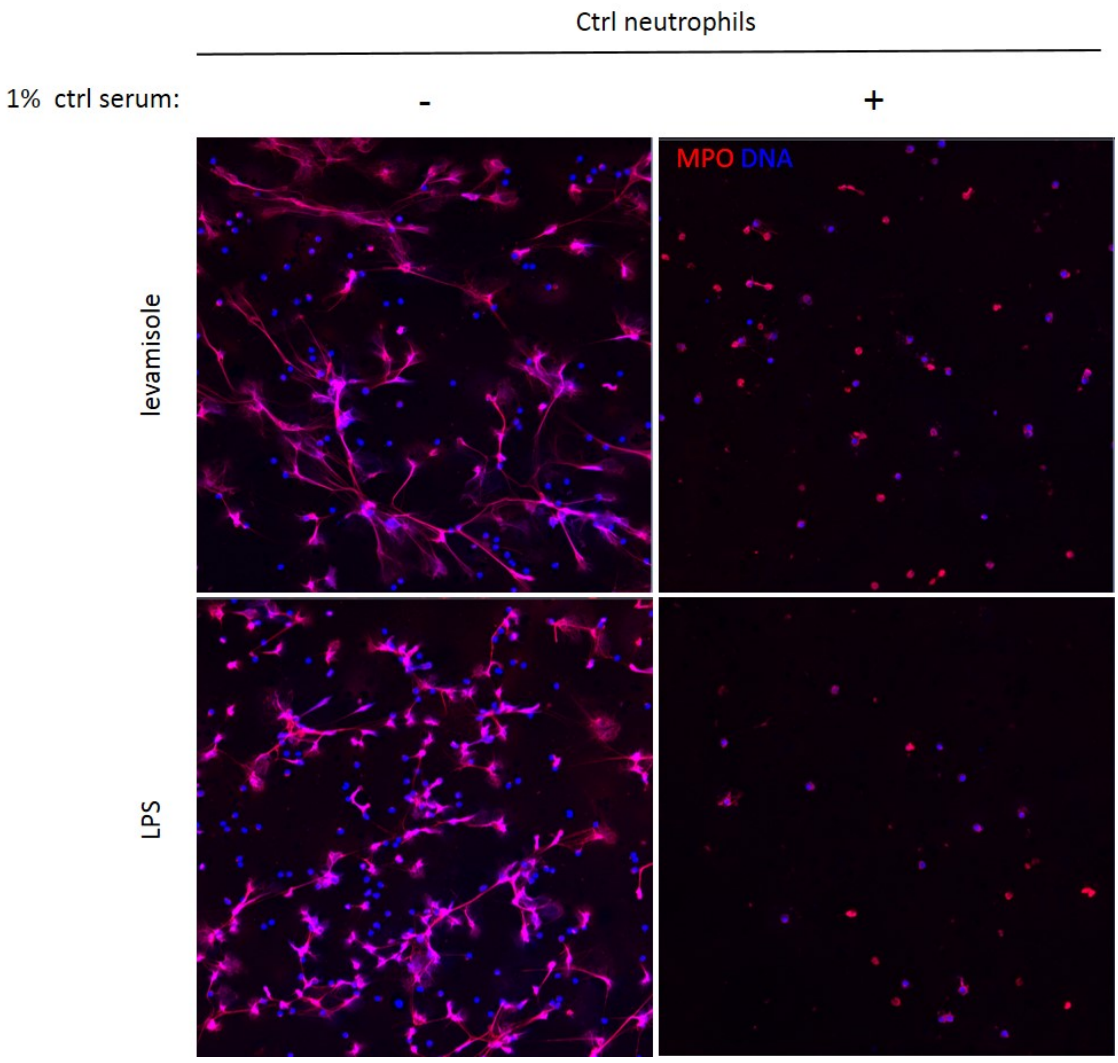
A role for muscarinic receptors in neutrophil extracellular trap formation and levamisole-induced autoimmunity

Supplementary Figure 1.



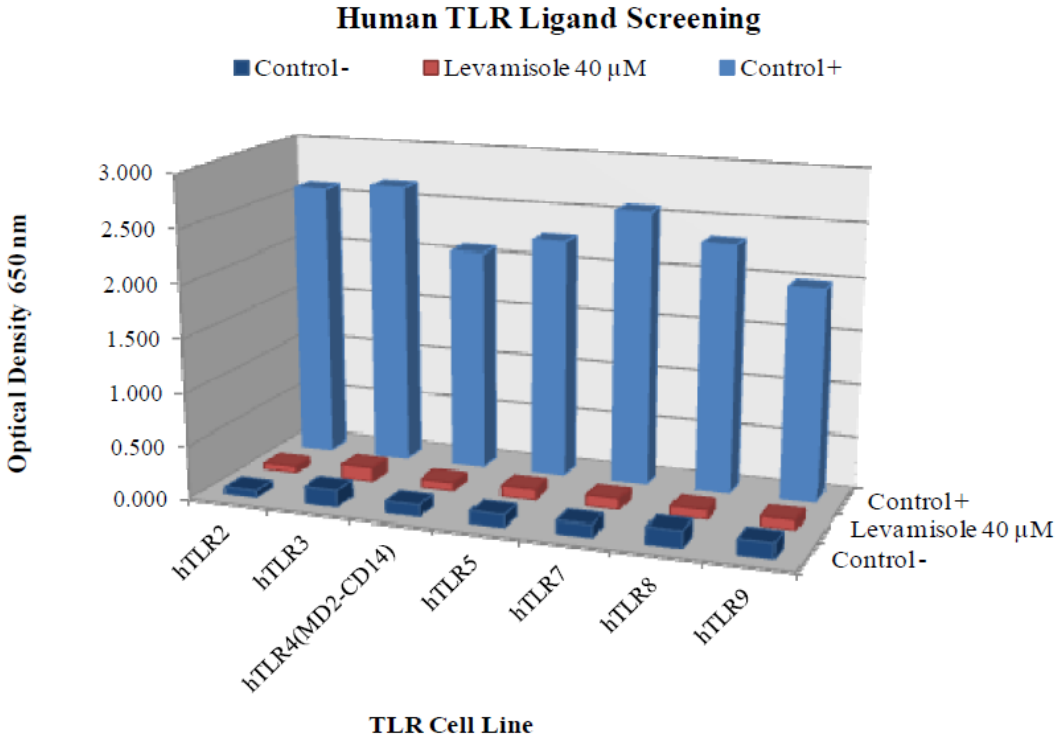
Supplementary Figure 1. Levamisole does not trigger mitochondrial ROS production during NET formation. Neutrophils from healthy donor (ctrl) were incubated with 100 μM levamisole (Leva) or 2.5 μM Ionophore (Io, positive control for mitochondrial ROS synthesis) for 2 h in the presence of mitoSOX. Plate was read at 1 and 2 h after induction. Results are expressed as relative fluorescence units and are the mean ± s.e.m. of 3 independent experiments. For statistical analyses Mann-Whitney U test was used; *P<0.05, ***P<0.001.

Supplementary Figure 2.



Supplementary Figure 2. Levamisole does not impair NET degradation by serum DNAse1. Neutrophils from healthy donor (ctrl) were incubated with 100 μM levamisole or 5μg/mL LPS for 3 h. After induction, netting neutrophils were incubated for 16h in presence or absence of 1% serum from healthy donor, as source of endogenous DNAse1. No impairment of NET degradation was detected in neutrophils treated with levamisole, suggesting that levamisole does not impair NET degradation by DNAse I. Red is MPO and blue is DNA. Original magnification 20x

Supplementary Figure 3.

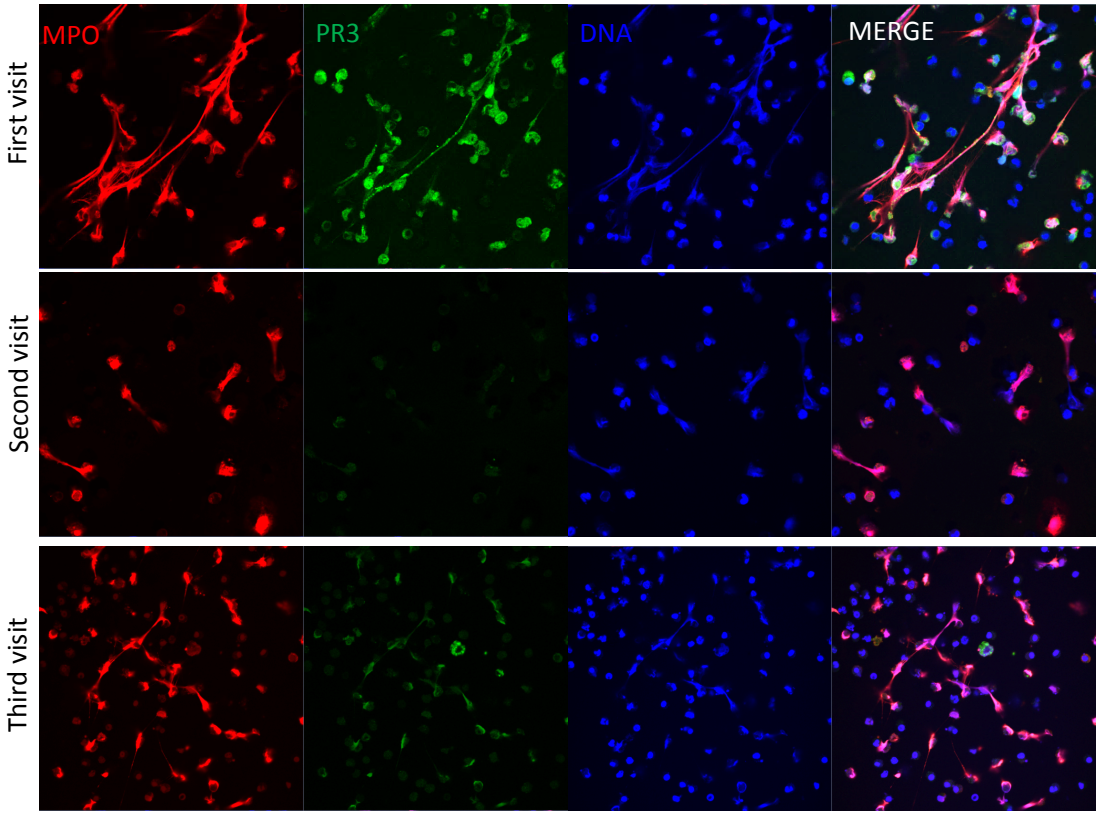


Control Ligands

hTLR2: HKLM (heat-killed *Listeria monocytogenes*) at 10^8 cells/mL
hTLR3: Poly(I:C) HMW at 1 µg/mL
hTLR4: *E. coli* K12 LPS at 100 ng/mL
hTLR5: *S. typhimurium* flagellin at 100 ng/mL
hTLR7: CL097 at 1 µg/mL
hTLR8: CL075 at 1 µg/mL
hTLR9: CpG ODN 2006 at 100 ng/mL

Supplementary Figure 3. Levamisole does not exhibit a stimulatory effect on human Toll-like receptor s(TLR) 2, 3, 4, 5, 7, 8, or 9. TLR 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, San Diego, CA).

Supplementary Figure 4.



Supplementary Figure 4. Fluctuating degree of spontaneous NET formation by peripheral blood neutrophils in association with levamisole use. A patient inadvertently exposed to levamisole via contaminated cocaine was seen over three separate visits. The first visit occurred during a period of daily cocaine use for > 20 years with the most recent use within 24 hours. Spontaneous NETs containing myeloperoxidase (MPO, red), proteinase 3 (PR3, green) and DNA (blue) were abundant. The second visit occurred during a period of relative abstinence and there were fewer NETs. The third visit occurred during a period of relapse with contaminated cocaine and demonstrated moderate spontaneous NETs. Original magnification 40x See *Supplementary Table 3* for further information.

Supplementary Table 1. Clinical and demographic characteristics of patients with ANCA-Associated Vasculitis and Systemic Lupus Erythematosus in whom abundance of muscarinic surface receptors was quantified

Subject	Age	Gender	Disease Activity	Prednisone (mg daily)	Other Medications
SLE 1	45	Female	Remission	5	HCQ
SLE 2	49	Female	Remission	5	AZA/HCQ
SLE 3	42	Female	Remission	0	HCQ
SLE 4	45	Female	Remission	15	HCQ
SLE 5	39	Female	Remission	5	HCQ
AAV 1	65	Male	Remission	20	None
AAV 2	64	Male	Remission	3	RTX
AAV 3	59	Female	Remission	0	RTX
AAV 4	63	Female	Remission	0	RTX
AAV 5	79	Female	Remission	0	AZA/RTX

SLE = systemic lupus erythematosus. AAV = ANCA-associated vasculitis. HCQ = hydroxychloroquine. AZA = azathioprine. RTX = rituximab.

Supplementary Table 2. Serum Autoantibodies in Two Cohorts of Cocaine Users Exposed to Levamisole via Adulterated Cocaine

Autoantibody	**NIDA Cohort** *Cocaine Positive* N = 52	**NIDA Cohort** *Cocaine Negative* N = 20	**P value** (Cocaine Positive vs. Cocaine Negative)	**UCSF Cohort** N=6	**P value** (NIDA Cocaine Positive vs. UCSF)
Levamisole Detected	100%	0%	<0.01	100%	1.00
ANA	8%	5%	1.00	83%	<0.01
PR3 **OR** MPO - ANCA	4%	0%	1.00	83%	<0.01
PR3 **AND** MPO -ANCA	2%	0%	1.00	67%	<0.01

NIDA = National Institute of Drug Abuse. UCSF = University of San Francisco. ANA = antinuclear antibody. PR3 = proteinase 3. MPO = myeloperoxidase. ANCA = antineutrophil cytoplasmic antibody. NIDA cohort = community dwellers in high-risk neighborhood for illicit drug use, none of whom have overt clinical features of levamisole-induced autoimmunity. UCSF cohort = patients with clinically apparent levamisole-induced autoimmunity. ANCA and ANA testing by enzyme Linked immunosorbent assay (Mayo Clinic Medical Laboratories, Rochester, MN). Cocaine positivity determined by urine drug toxicology screening test. Levamisole detected by mass spectrometry. Fisher's exact test used for statistical comparison between groups.

Supplementary Table 3. Correlation of Clinical and Serologic Parameters in a Patient with Intermittent Ongoing Use of Levamisole-Adulterated Cocaine and Membranous Glomerulonephritis

Visit	Patient Reported Cocaine Use	Most Recent Cocaine Use	Urine Levamisole (ng/mL)	Benzoyl-ecgonine (ng/mL)	ANCA Level	Protein Creatinine Ratio, Urine	Degree of Spontaneous NETosis (Supp Fig 4)
Baseline	Daily for 20 years	< 24 hours	331	1,908	MPO-ANCA 63 PR3-ANCA 27	7.67	Prominent
2 Month	1 time since last visit	2 days prior	33	200	MPO-ANCA 76 PR3-ANCA 25	6.42	Few
3 Month	3 times since last visit	4 days prior	67	376	MPO-ANCA 70 PR3-ANCA 24	7.77	Moderate
1 Year	1 time over the last month	2 days prior	Not done	Not done	MPO-ANCA 85 PR3-ANCA 28	4.67	Not Done