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2-Undecanone derived from *Pseudomonas aeruginosa* modulates the neutrophil activity

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Running title: Immune modulation by 2-undecanone

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is a well-known Gram-negative opportunistic pathogen. Neutrophils play key roles in mediating host defense against *P. aeruginosa* infection. In this study, we identified a metabolite derived from *P. aeruginosa* that regulates neutrophil activities. Using gas chromatography-mass spectrometry, a markedly increased level of 2-undecanone was identified in the peritoneal fluid of *P. aeruginosa*-infected mice. 2-Undecanone elicited the activation of neutrophils in a $G_{\alpha i}$-phospholipase C pathway. However, 2-undecanone strongly inhibited responses to lipopolysaccharide and bactericidal activity of neutrophils against *P. aeruginosa* by inducing apoptosis. Our results demonstrate that 2-undecanone from *P. aeruginosa* limits the innate defense activity of neutrophils, suggesting that the production of inhibitory metabolites is a strategy of *P. aeruginosa* for escaping the host immune system.

Keywords

Pseudomonas aeruginosa, Bacterial metabolite, Volatile organic compound, 2-undecanone, Neutrophil

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic Gram-negative bacterium and is a major cause of mortality in immunocompromised patients (1). *P. aeruginosa* is also one of the six major bacteria detected in septic patients in intensive care units (2) and is a representative nosocomial pathogen carrying resistance to diverse antibiotics. *P. aeruginosa* is ubiquitous due to its large genome size which allows the bacteria to metabolize 70-80 different organic substrates as carbon sources and to have drug resistance (3). Also, *P. aeruginosa* has host immune escaping strategies including conformational change and releasing various virulence factors to avoid immune cell attack (1). Therefore, understanding the pathophysiology of *P. aeruginosa* is crucial to controlling the extent of bacterial infection.

Bacteria have specific metabolite profiles, including volatile organic compounds (VOCs), due to their distinct metabolism (2). Various bacterial metabolites interact with the host nervous and immune systems via receptor activation (4). The interaction between bacterial metabolites and the immune system have only recently started to be investigated, in comparison to the physical components of bacteria which represent pathogen-associated molecular patterns (5, 6). The relationship between bacterial metabolites and host immune responses has expanded as a new research field with a promise to establish strategies against bacterial infection.

Neutrophils are the most abundant leukocytes in the circulation and are the first immune cells that migrate to the sites of infection to kill pathogens through phagocytosis, as well as secretion of granules, reactive oxygen species (ROS), and neutrophil extracellular traps (NETs) (7). Immunocompromised and neutropenic patients are very susceptible to *P.*

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aeruginosa infections, and a 10^5-times smaller count of bacteria can lead to death in neutropenic mice compared to normal control mice (1). *P. aeruginosa* has several host immune system-modulating strategies, and some of them directly target neutrophils (8-10).

In this study, we identified 2-undecanone as a major metabolite in the peritoneal fluid of *P. aeruginosa*-infected mice. Although 2-undecanone alone activated neutrophils, it strongly suppressed several lipopolysaccharide (LPS)-induced immune responses. Our results show that 2-undecanone can act as an important molecular cue to escape the host immune system, thereby suggesting novel insights into the immunological function of VOCs.

Results

2-Undecanone is a major VOC in systemic infection of *P. aeruginosa*

We attempted to identify metabolic VOCs produced *in vivo* by systemic infection of *P. aeruginosa*. Mice were intraperitoneally injected with *P. aeruginosa* PAO1. For GC-MS analysis, peritoneal fluid was prepared with 2 ml of PBS at 12 h after the infection (Fig. 1A). According to the GC-MS analysis spectrum, 2-undecanone was detected in only PAO1-infected mice peritoneal fluid but not in the non-infected control mice (Fig. 1B, 1C, 1D). Also, we established a quantification method using GC-MS that can detect a range of 5 nM to 5 mM concentration (Fig. 1E). Based on this technique, the concentration of 2-undecanone upon *P. aeruginosa* infection was identified to be approximately 1.48 μM ± 1.17 μM (Fig. 1F). As this concentration was the result of dilution of peritoneal fluid with 2 ml of PBS, we calculated the *in vivo* concentration of 2-undecanone based on a previous work which reported that the volume of peritoneal fluid in steady-state male mice is 0.02 ± 0.01 ml (11).

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Collectively, our results suggest that 2-undecanone can reach a concentration of about 200 μM upon *P. aeruginosa* infection in mice.

2-Undecanone stimulates mouse neutrophils in a $G_{\alpha i}$-phospholipase C (PLC) pathway

We identified 2-undecanone as a major VOC during *P. aeruginosa* infection, and this finding is consistent with that of the previous results (12, 13). Our finding led us to examine the effects of the VOC on neutrophils, the most important innate immune cells in *P. aeruginosa* defense (1). Neutrophil migration into the infected area is the initial step for the initiation of a proper immune response against infection. Stimulation of neutrophils with 2-undecanone elicited significant chemotactic migration of the cells (Fig. 2A). Administration of 2-undecanone into the peritoneal cavity also elicited neutrophil recruitment *in vivo* (Fig. 2B). The results suggest that 2-undecanone acts as a chemoattractant for neutrophils both *in vitro* and *in vivo*. The secretion of cytosolic granules and ROS generation are other responses to neutrophil activation and mechanisms of bacterial killing (7). 2-Undecanone treatment significantly increased the level of degranulation and ROS generation in neutrophils (Fig. 2C, 2D). Our results indicate that 2-undecanone activates neutrophils leading to chemotactic migration, degranulation, and ROS generation.

Many extracellular stimuli that induce neutrophil migration have been reported to use the $G_{\alpha i}$-type G protein-coupled receptors (GPCRs) (14). Preincubation of neutrophils with pertussis-toxin (PTX), which inhibits ADP-ribosylation of the $G_{\alpha i}$ protein, significantly decreased migration towards 2-undecanone (Fig. 2E). Many extracellular stimulants that bind to PTX-sensitive GPCRs also stimulate PLC activity and increase the level of intracellular calcium (14). 2-Undecanone strongly elicited intracellular calcium increase in neutrophils,

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which was almost completely blocked by a PLC inhibitor (U-73122) but not by its inactive analog (U-73343) (Fig. 2F). The activation of several kinases such as ERK and Akt accompanies neutrophil activation by extracellular stimuli (15) and it was observed that 2-undecanone induced transient activation of ERK and Akt (Fig. 2G). Collectively, the results suggest that 2-undecanone stimulates neutrophils in a $G_{\alpha i}$-mediated signaling pathway and PLC-dependent calcium increase.

2-Undecanone limits LPS-induced immune responses in neutrophils and inhibits the bactericidal activity

Since 2-undecanone is a representative VOC produced by *P. aeruginosa*, we examined the effects of 2-undecanone on neutrophil functions with the bacteria and *P. aeruginosa*-derived LPS. When mouse neutrophils were stimulated with 2-undecanone in the presence of *P. aeruginosa*, the bactericidal activity of neutrophils was significantly decreased (Fig. 3A). Phagocytosis is known as a classical way of eliminating *P. aeruginosa* in a short period (1). However, phagocytic activity was not affected by 2-undecanone compared to the vehicle (Fig. 3B).

NET is one of the mechanisms of neutrophil-mediated bacterial killing (16). *P. aeruginosa*-derived LPS induced suicidal-NETs (Fig. 3C), the findings being consistent with that of a previous report (17), and the addition of 2-undecanone significantly decreased NET formation (Fig. 3C). Because LPS-induces NET formation in a ROS-dependent manner, we examined whether 2-undecanone affects ROS generation induced by LPS in neutrophils. 2-Undecanone significantly inhibited the LPS-induced ROS generation in neutrophils (Fig. 3D).

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These results suggest that 2-undecanone limits LPS-induced NET formation by decreasing ROS generation in neutrophils. Also, 2-undecanone significantly decreased LPS-induced cytokine (TNF-α, IL-6, IL-1β, IL-10) secretion (Fig. 3E). In separate experiments, we found that 2-undecanone alone did not induce cytokine production (data not shown). LPS is known to stimulate immune cells through the nuclear factor-κB (NF-κB) signaling pathway (18). We found that 2-undecanone alone induced p65 phosphorylation. On the contrary, it slightly decreased the level of p65 phosphorylation, and IκB degradation under LPS stimulation, whereas it did not change the level of TLR4 (Fig. 3F). These results suggest that 2-undecanone attenuates LPS-induced responses by partly interfering with TLR4 downstream of the NF-κB pathway.

2-Undecanone reduces neutrophil cell survival under LPS stimulation

We found that the phosphorylation of ERK and Akt was increased by 2-undecanone stimulation in neutrophils (Fig. 2G). As both ERK and Akt are important signaling molecules in LPS downstream signaling (19), we checked whether 2-undecanone affects the phosphorylation of ERK and Akt in response to LPS. 2-Undecanone decreased the phosphorylation induced by LPS (Fig. 4A). A previous report demonstrated that Akt acts as a switch between ROS-mediated NETosis and apoptosis. Blocking Akt activity induces cells to enter the apoptosis pathway, instead of entering NET formation, in a caspase-3 dependent manner (20). We found that LPS decreased the level of cleaved caspase-3, and increased an anti-apoptotic marker Bcl-2, which significantly showed the opposite effect in 2-undecanone treatment in neutrophils (Fig. 4B).

Next, we examined whether 2-undecanone affects cell death in the presence of LPS. It was

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observed that the viability of neutrophils was significantly decreased by 2-undecanone in the presence of LPS at two different time points of 2 h and 12 h after stimulation; however, 2-undecanone alone did not affect the viability of neutrophils (Fig. 4C).

Discussion

This study provides an example of the immunoregulatory effects of a microbial metabolite on host immune cells. We identified 2-undecanone as a major VOC in systemic infection of *P. aeruginosa* and revealed its effects on neutrophil responses. 2-Undecanone stimulated neutrophil chemotaxis; however, it blocked LPS-induced immune reactions by inducing neutrophil apoptosis. Our results suggest that 2-undecanone may act as an important strategic molecule for *P. aeruginosa* to escape the host immune system.

As the research of microbiota actively progresses, the interest in microorganism-derived metabolites has greatly increased. Recently, extensive effort has been expended on the identification of pathogen-derived molecules that affect the host immune system or host physiology. For example, 1-undecene, a VOC from *P. aeruginosa*, was reported as a pathogen-associated molecular pattern recognized by *C. elegans* that regulates the immune responses of worms (5). We also reported that 2-pentylfuran, a pneumococcal secreted metabolite, reacts with microglial odorant receptors and is involved in microglial activation (6). These studies suggest that VOCs can modulate immune responses during bacterial infection.

The growing interest in bacterial VOCs is derived from their roles in intra-kingdom to inter-kingdom and long-distance communication with other organisms (21). 2-Undecanone

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is a *Pseudomonas* genus-specific VOC detected both *in vitro* (13) and *in vivo* (12) and is already known for inducing the death of other microorganisms (22, 23). In the present study, it was observed that 2-undecanone dramatically decreased LPS-induced responses, eventually inducing neutrophil apoptosis whereas 2-undecanone alone activated neutrophils. This led us to hypothesize the relationship between 2-undecanone and neutrophils based on the original role of VOCs. The classical knowledge of bacterial VOCs is that they are not only byproducts of metabolism but also act as signals at low concentrations and long distances (21). Considering this feature, it appears that 2-undecanone alone can activate neutrophils as a signaling molecule; however, when it co-exists with LPS, it appears to turn into a virulence factor to promote cell death similarly to the cases of *Agrobacterium* (22) and nematodes (23). Such characteristics of VOC were also observed in the regulation of ROS production by 2-undecanone. 2-Undecanone alone stimulated ROS generation, whereas it decreased LPS-induced ROS generation in neutrophils. The interesting feature of 2-undecanone is that it functions as a virulence factor only in the presence of LPS, unlike other virulence factors such as pyocyanin, which acts alone to escape immune responses by inducing neutrophil apoptosis (10). Previous reports also demonstrated that 2-undecanone shows anti-inflammatory effect against fine particle-induced kidney inflammation or LPS stimulation (24-26). The mechanism behind the functioning of 2-undecanone as a virulent factor on neutrophils in the presence of LPS remains to be elucidated.

In conclusion, we demonstrated that 2-undecanone is the major VOC produced from *P. aeruginosa* in *P. aeruginosa*-infected mice. Neutrophil activities were dynamically modulated by 2-undecanone. It is hypothesized that 2- undecanone from *P. aeruginosa* can be used by the bacteria to escape the host immune system. Our study suggests that a VOC

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from pathogenic bacteria may serve as a virulence factor. In futuristic studies, investigation of the pathophysiological effects of VOCs from pathogens should continue to improve our understanding of the interaction between pathogens and the host immune system.

Materials and methods

Mice and enrichment of mouse neutrophils

8- to 10-weeks-old C57BL/6 male mice were purchased from Orient Bio Inc. (Seongnam, Korea). All experiments involving animals were carried out after receiving approval from the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University (Suwon, Korea). Mouse bone marrow neutrophil enrichment was conducted according to a previous report (27). Isolated cells were over 95% Ly6G-positive by flow cytometry (BD FACSCanto II, Franklin Lakes, NJ, USA).

GC-MS analysis

P. aeruginosa cultures were grown for 14 h at 37°C in tryptic soy broth (BD Biosciences, San Jose, CA, USA). Mice were infected with *P. aeruginosa* (3×10^7 CFUs/head) by intraperitoneal injection and mouse peritoneal fluid was collected at 12 h after infection. Extraction of VOCs from the peritoneal fluid was carried out by solid-phase microextraction method using an Agilent 65 μm DVB/PDMS SPME fiber. An Agilent 7890B-5977B GC/MSD system was utilized for the GC-MS analysis of all samples. Identical quantities (1 ml each) of all samples were used for each GC-MS analysis. The GC-MS spectrum data were analyzed using OpenChrom software. Peak identification was carried out with the database

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of the National Institute of Standard and Technology. The identity of 2-undecanone in biological samples was further confirmed by the retention time and mass spectrum produced from the working standards of 2-undecanone. Variation in retention time was less than ±1% relative to the average of the working standard samples

Chemotaxis assay

Chemotaxis assays were carried out according to a previous report (27). In the case of PTX treatment, neutrophils were incubated with PTX (100 ng/ml) in 2% FBS containing RPMI 1640 medium (Welgene, Gyeongsan, Korea) at 37℃ and 5% CO_2 for 4 h before the vehicle or 2-undecanone treatment.

***In vivo* migration assay**

2-Undecanone (4 mg/kg) and vehicle were injected to mice intraperitoneally. After 2 h of injection, mouse peritoneal fluid was collected, and peritoneal cells were stained with antibodies of CD11b and Ly6G (Thermo Fisher Scientific). Stained cells were analyzed by flow cytometry (FACSCanto II).

Degranulation assay

Degranulation was measured using a β-hexosaminidase assay, as previously reported (27). Briefly, isolated neutrophils were incubated with vehicle or 2-undecanone for 30 min. Both supernatants and lysates were incubated with the substrate solution at 37℃ and 5% CO_2 for 2 h. O.D. was measured at 405 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

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Detection of cellular ROS using DCF-DA

Mouse neutrophils stimulated with 2-undecanone were treated with 5 μM DCF-DA reagent (Thermo Fisher Scientific) in serum-free RPMI 1640 medium at 37℃ for 30 min. The levels of cellular ROS were measured with flow cytometry (FACSCanto II).

Calcium measurement

Intracellular calcium measurement was carried out as previously reported (28). Briefly, fura-2 loaded neutrophils were stimulated with 2-undecanone or vehicle in the absence or presence of U-73122 or U-73343. The changes in fluorescence ratios (340:380 nm) were monitored using a spectrofluorophotometer (RF5301PC, SHIMADZU, Tokyo, Japan).

Measurement of cytokines

Mouse neutrophils were stimulated with *P. aeruginosa*-derived LPS (100 ng/ml) at 37℃ and 5% CO_2 for 24 h with or without stimulation of 2-undecanone. The cell-free supernatants were obtained and measured by ELISA according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA).

Measurement of NET formation

Mouse neutrophils (2×10^5 cells/ml) were seeded on 0.01% poly-L-lysine-coated 48-well plates. Neutrophils were incubated with 50 μg/ml of *P. aeruginosa*-derived LPS (Sigma Aldrich) at 37℃ and 5% CO_2 for 4 h. Stimulated neutrophils were fixed with 4% of formaldehyde in PBS for 15 min at room temperature. After washing the samples with RPMI 1640 medium, extracellular DNA was stained with 100 nM SYTOX Orange (Thermo Fisher

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Scientific) in RPMI 1640 medium for 15 min at room temperature and quantified by fluorescence microscope (Optinity, Korea Lab Tech, Seongnam, Korea).

Measurement of phagocytosis

Mouse neutrophils were resuspended in RPMI 1640 medium (5×10^6 cells/ml) and incubated with GFP-labeled, serum-opsonized *P. aeruginosa* PAO1 with a multiplicity of infection (MOI) of 25 at 37℃ for 1 h. Phagocytosis was stopped with ice and samples were fixed with ice-cold 4% paraformaldehyde in PBS. Samples were analyzed for bacterial uptake by flow cytometer (FACSCanto II).

Measurement of bacteria-killing activity

Mouse neutrophils were resuspended in RPMI 1640 medium (5×10^6 cells/ml) and incubated with serum-opsonized *P. aeruginosa* PAO1 at an MOI of 25 at 37℃ for 1 h. Neutrophils were lysed with 1 μg/ml BSA in water, diluted to 1:200, and plated on tryptic soy agar dishes.

Analysis of neutrophil apoptosis by flow cytometry

Mouse neutrophils were stimulated with 2-undecanone for the indicated times. Neutrophil apoptosis was determined by the Annexin-V/PI staining kit (BD). The staining procedure was performed according to the manufacturer's instructions. Co-staining was analyzed on a flow cytometer (FACSCanto II).

Western blot analysis

Cell lysates were prepared using RIPA sample buffer (iNtRON Biotechnology, Seongnam,

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Korea). Western blot analysis was conducted according to a previous report (6). Primary antibodies used were anti-phospho-ERK, anti-phospho-Akt, anti-total-Akt, anti-IκB, anti-p-p65, anti-p65, anti-cleaved caspase3, anti-Bcl-2, and anti-β-actin from Cell Signaling Technology (Beverly, MA, USA). The anti-TLR4 and anti-total-ERK were from Santa Cruz Biotechnology (Dallas, TX, USA). Secondary horseradish peroxidase-conjugated antibodies used were anti-mouse IgG and anti-rabbit IgG from Enzo Lifesciences (Farmingdale, NY, USA).

Statistical analysis

GraphPad Prism software was used to evaluate the results. All results are expressed as the mean ± SEM for the data obtained from the indicated number of experiments. Statistical analysis was performed using the Student's *t*-test or two-way analysis of variance (ANOVA). A P value ≤ 0.05 was considered statistically significant.

Acknowledgements

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Figure legends

Figure 1. Identification of 2-undecanone in *P. aeruginosa*-infected mice peritoneal fluid.

(A) Schematic figure of sample preparation for GC-MS analysis. (B) A representative GC-MS sample spectrum of 2-undecanone (2-Und) from the PAO1-infected sample and (C) library spectrum. (D) The structure of 2-undecanone and a representative extracted ion chromatogram overlay of the 2-undecanone peak. 2-Undecanone was only identified in the PAO1-infected sample (red line). (E) GC-MS standard curve based on 10-fold series of 2-undecanone. (F) The quantified levels of 2-undecanone in PAO1-infected mouse peritoneal fluid.

Figure 2. 2-Undecanone stimulates neutrophils in a $G_{\alpha i}$ and PLC pathway.

(A) Mouse neutrophils were applied to multi-well Boyden chambers containing different concentrations of 2-undecanone for 90 min to check *in vitro* chemotaxis. (B) *In vivo* migration of neutrophils into the peritoneum of vehicle and 2-undecanone (4 mg/kg). After 2 h of intraperitoneal injection, peritoneal cells were analyzed by flow cytometry. (C) 2-Undecanone (200 μM) was administrated to neutrophils for the degranulation assay. (D) Cellular ROS was measured by DCF-DA for 1 h. (E) *In vitro* chemotaxis with PTX treatment towards 2-undecanone. (F) Relative cytosolic Ca^{2+} concentrations were expressed as fluorescence ratios (340:380 nm). (G) Neutrophils were stimulated with 2-undecanone (200 μM) for various lengths of time. The levels of p-ERK, t-ERK, p-Akt, t-Akt, and β-actin were measured using Western blot analysis. The data are representative of three independent experiments. Data are expressed as the mean ± SEM $*P < 0.05$ and $**P < 0.01$ by Student's

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t-test (A, B right, C, D right) and by two-way ANOVA (E).

Figure 3. 2-Undecanone inhibits bactericidal activity and LPS-induced immune responses of neutrophils.

(A) Mouse neutrophils were co-incubated with serum-opsonized PAO1 with MOI of 25 at 37℃ for 1 h and bacterial killing activity was measured. (B) Neutrophils were co-incubated with serum-opsonized green fluorescent protein-tagged PAO1 with an MOI of 25 at 37℃ for 1 h. Phagocytic cells with engulfed bacteria were analyzed by flow cytometry. (C) Neutrophils were stimulated with LPS (50 μg/ml) and vehicle or 2-undecanone (200 μM) for 4 h. NET formation was visualized by staining the cells with SYTOX Orange. (D) Intracellular ROS levels were measured by DCF-DA. Neutrophils were stimulated with LPS (1 μg/ml) and vehicle or 2-undecanone (200 μM) for 2 h. (E) Levels of cytokines were measured by ELISA. Neutrophils were incubated with LPS (100 ng/ml) and vehicle or 2-undecanone (200 μM) for 24 h. (F) Neutrophils were stimulated with LPS (1 μg/ml) and vehicle or 2-undecanone (200 μM) for 2 h. The data are representative of three independent experiments. Data are expressed as the mean ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P<0.0001$ by Student's *t*-test (A, B right, C right, D right, E).

Figure 4. 2-Undecanone decreases neutrophil cell survival under LPS stimulation.

(A and B) Mouse neutrophils were stimulated with LPS (1 μg/ml) and vehicle or 2-undecanone (200 μM) for 2 h. (A) The levels of p-ERK, t-ERK, p-Akt, t-Akt, and β-actin, (B) cleaved caspase-3, Bcl-2, and β-actin were measured using Western blot analysis. (C) Apoptosis of neutrophils was determined by anti-Annexin V antibody and PI staining.

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Neutrophils were stimulated with or without LPS (1 μg/ml) and vehicle or 2-undecanone (200 μM) for 2 h and 12 h. The data are representative of three independent experiments. Data are expressed as the mean ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by two-way ANOVA (C bottom).

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Figure 1

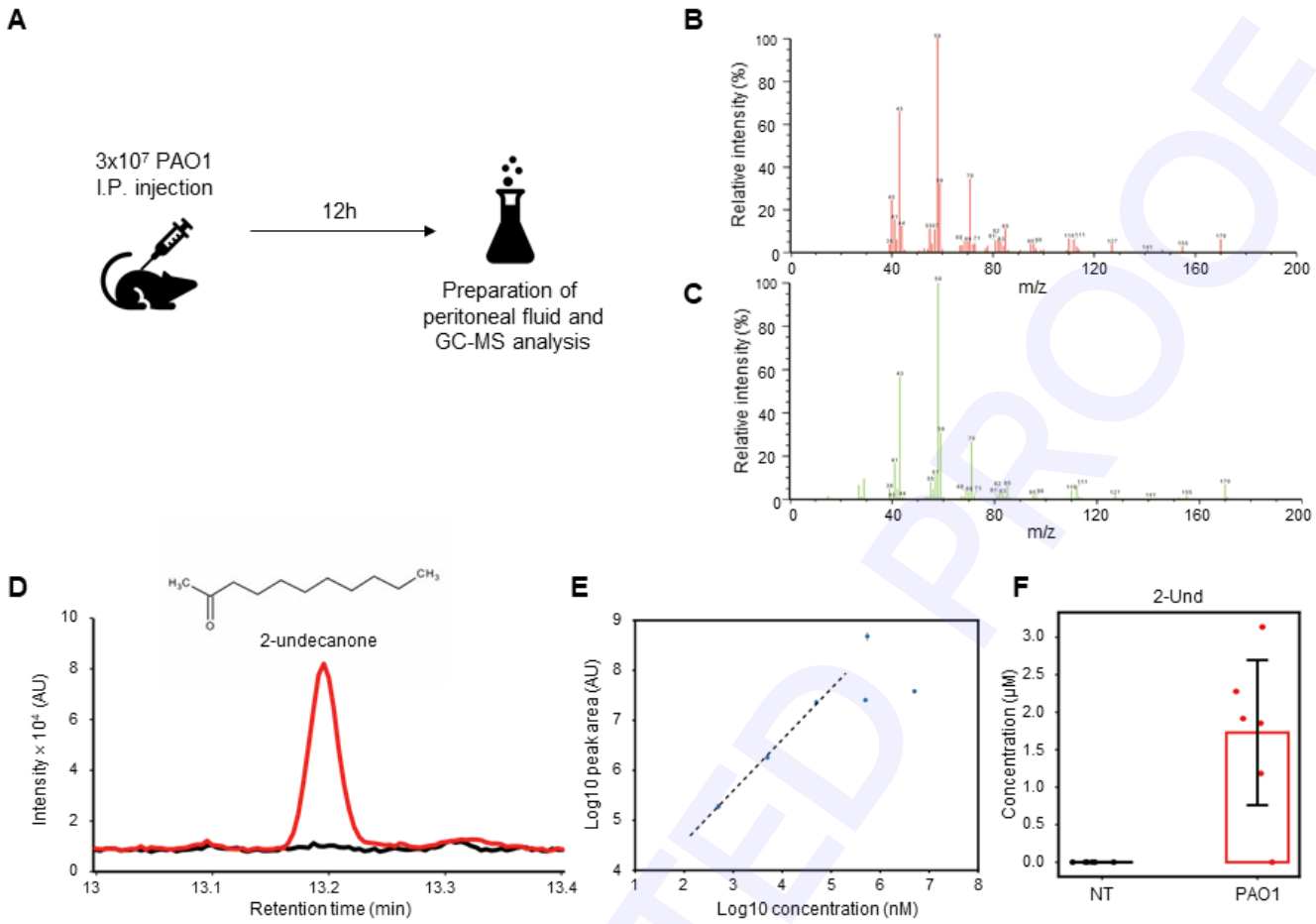

Fig. 1. Figure 1

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Figure 2

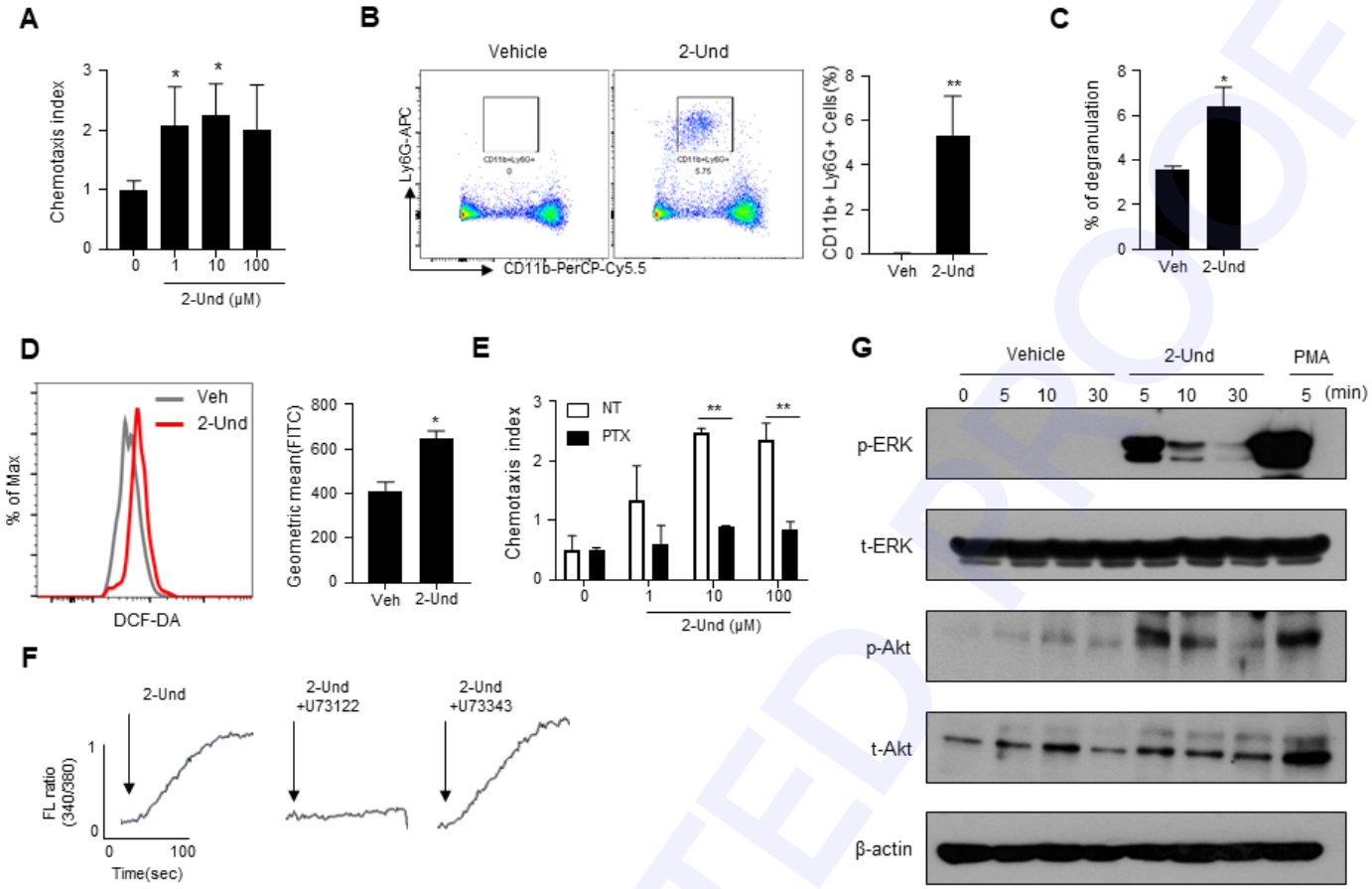

Fig. 2. Figure 2

UNCORRECTED PROOF

Figure 3

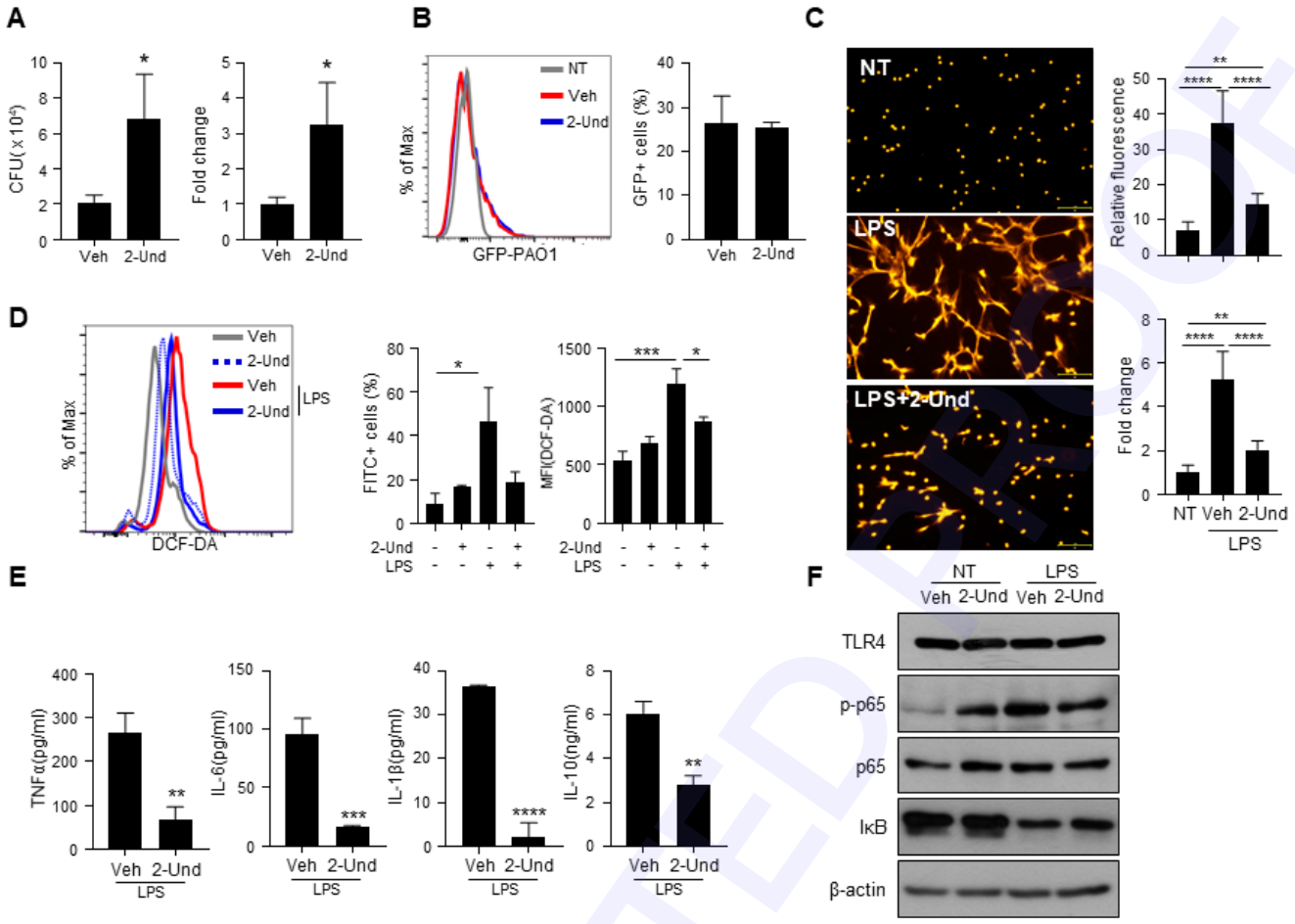

Fig. 3. Figure 3

UNCORRECTED PROOF

Figure 4

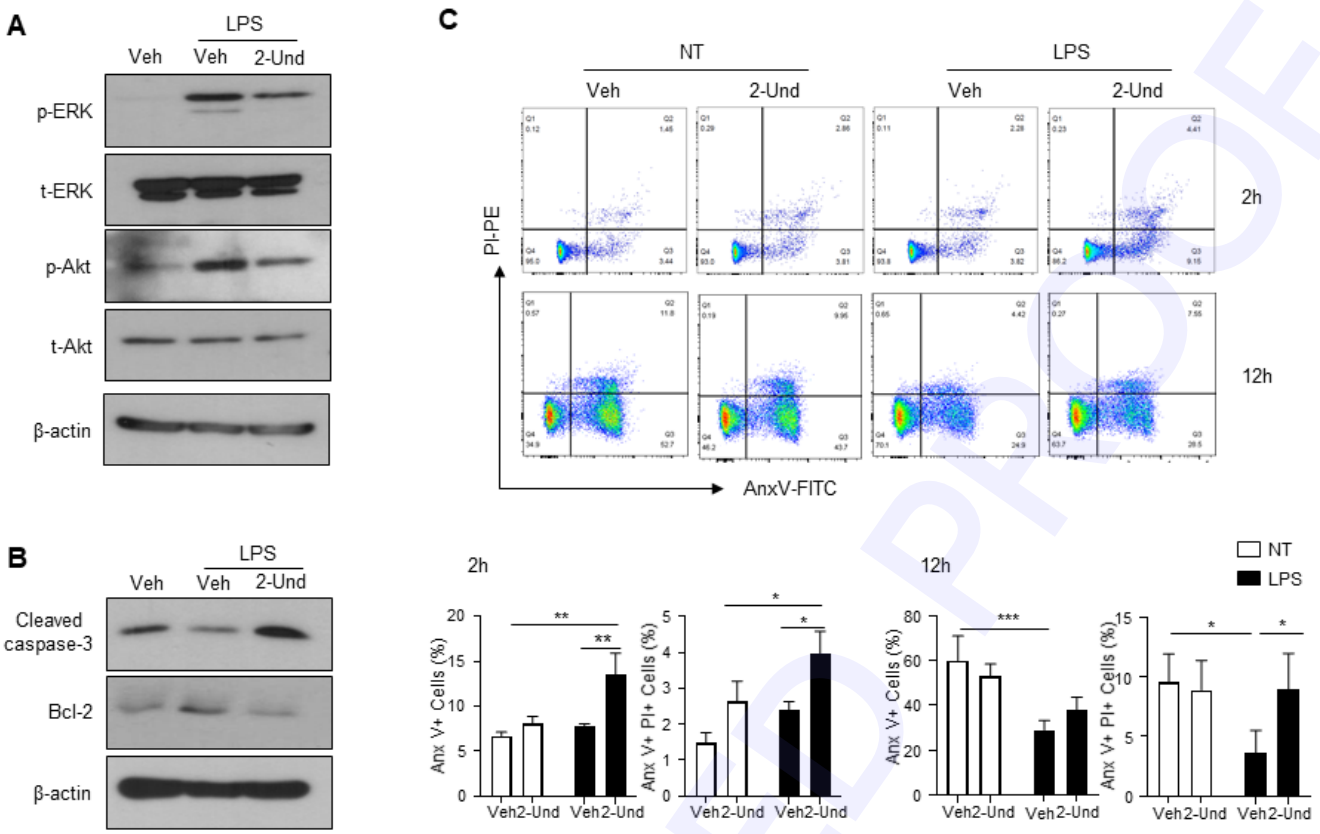

Fig. 4. Figure 4

UNCORRECTED PROOF