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Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs

María José Lapponi, Agostina Carestia, Verónica Inés Landoni, Leonardo Rivadeneyra, Julia Etulain, Soledad Negrotto, Roberto Gabriel Pozner and Mirta Schattner

Laboratory of Experimental Thrombosis (M.J.L., A.C., L.R., J.E., S.N., R.G.P., M.S.) and Laboratory of Physiology of Inflammatory Processes (V.I.L.), Institute of Experimental Medicine (IMEX), CONICET-National Academy of Medicine, Argentina**.**

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b) Address correspondence to: Dr. Mirta Schattner, Pacheco de Melo 3081 (1425) Buenos Aires, Argentina. Phone: +54-11-4805-5759 Ext 301, Fax: +54-11-4805-0712, E-mail: mschattner@hematologia.anm.edu.ar

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Abstract

The formation of neutrophil extracellular traps (NETs) is a newly described phenomenon that increases the bacteria killing ability and the inflammatory response of neutrophils. Because NET generation occurs in an inflammatory microenvironment, we examined its regulation by anti-inflammatory drugs. Treatment of neutrophils with dexamethasone had no effect while acetylsalicylic acid (ASA) treatment prevented NET formation. NETosis was also abrogated by the presence of BAY 11-7082 and Ro 106-9920, two structurally unrelated NF-κB inhibitors. The decrease in NET formation mediated by ASA, BAY-11-7082 and Ro 106-9920 was correlated with a significant reduction in the phosphorylation of NF-κB p65 subunit, indicating that the activation of this transcription factor is a relevant signaling pathway involved in the generation of DNA traps. The inhibitory effect of these drugs was also observed when NET generation was induced under acidic or hyperthermic conditions, two stress signals of the inflammatory microenvironment. In a mouse peritonitis model, while pretreatment of animals with ASA or BAY 11-7082 resulted in a marked suppression of NET formation along with increased bacteremia, dexamethasone had no effect. Our results show that NETs have an important role in the local control of infection and that ASA and NF-κB blockade could be useful therapies to avoid undesired effect of persistent neutrophil activation.

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Introduction

Neutrophils are the most abundant leukocytes in human blood and have an essential role in innate immunity as they are the first cells recruited to sites of infection and inflammation (Nathan, 2006). Neutrophils function to engulf microorganisms or opsonized particles and degrade them intracellularly as well as release microbicidal proteins and reactive oxygen species (ROS) (Segal, 2005). Recently, a novel pathogen killing mechanism (NETosis) has been described in these cells that involves the extracellular release of microbicidal proteins and their nuclear DNA upon activation by bacterial or fungal species (Brinkmann et al., 2004; Urban et al., 2006). These DNA structures named neutrophil extracellular traps (NETs) are composed of chromatin and are associated mainly with histones; many granular antimicrobial proteins including elastase, myeloperoxidase, calprotectin, lactoferrin and metalloprotease 9, as well as some cytoplasmic proteins that have potent antimicrobial effects, are also released (Brinkmann et al., 2004; Urban et al., 2009). Thus, NETs bind, disarm, and kill microbes extracellularly and independent of phagocytic uptake. In addition to their antimicrobial properties, NETs may serve as a physical barrier that prevents further spread of the pathogens. Furthermore, retaining granular proteins within the NETs may prevent potentially injurious proteins, such as proteases from diffusing away and inducing damage in tissue adjacent to the site of inflammation (Brinkmann and Zychlinsky, 2007). Although it was originally proposed that NETs are formed exclusively in tissues at sites of bacterial or yeast infection, NETs have also been found within blood vessels (specifically in the lung capillaries and liver sinusoids) where they ensnare bacteria in circulation during sepsis (Clark et al., 2007).

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In spite of the beneficial effects of this process in pathogen control, the removal of NETs must be regulated to ensure tissue homeostasis, as high amount of NETs may contribute to collateral damage within inflamed tissues. Excessive NET generation as a result of non-infectious inflammatory processes, as well as a lack of DNAses to degrade them, is associated with the pathogenesis of inflammatory and autoimmune diseases, including preeclampsia (Gupta et al., 2005), cystic fibrosis (Manzenreiter et al., 2012) and lupus (Hakkim et al., 2010). Moreover, the observation that NETs act as a scaffold for thrombus formation (Fuchs et al., 2010; Fuchs et al., 2012; von Bruhl et al., 2012) is increasingly being recognized as a critical phenomenon linking inflammation with venous thrombosis in both, infectious and non-infectious clinical settings. NETs are therefore similar to a double-edge sword, which functions not only as an effective antimicrobial first line defense mechanism but might also lead to organ failure and death if the process is uncontrolled.

Because an inflammatory microenvironment is an essential component of NET formation, we analyzed the effect of current anti-inflammatory drugs including dexamethasone and ASA on NET formation. We found that while dexamethasone had no effect, ASA significantly inhibited NET generation. In addition, two structurally unrelated NF-κB inhibitors also suppressed NET generation demonstrating the involvement of the transcription factor NF-κB in this process and the potential use of these drugs to control NETosis.

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Materials and Methods

Reagents

Phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI), evans blue dye, acetylsalicylic acid (ASA) and dexamethasone were purchased from Sigma Aldrich Inc. (St. Louis, MO). CELLSTAR culture microplates were purchased at Greiner Bio-One (Monroe, NC). Micrococcal nuclease (MNase) and the cell death detection ELISA^{PLUS} kit were purchased from Roche diagnostics (Mannheim, Germany). Ficoll-Hypaque (1.077 g/m density) and nitrocellulose membranes from GE Healthcare (Buckinghamshire, UK) and RPMI 1640 medium from Invitrogen (Carlsbad, CA) were used. The NF-κB inhibitors (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 11-7082) and 6-(phenylsulfinyl)tetrazolo[1,5-b]pyridazine (Ro 106-9920) were obtained from Biomol (Plymouth Meeting, PA) and Tocris (Ellisville, MO), respectively. Both drugs were dissolved in DMSO. TNF-α was purchased from Peprotech (Veracruz, Mexico). Rabbit anti-neutrophil elastase was obtained from Calbiochem-Merk Millipore (Darmstad, Germany) and anti-rabbit Alexa 488 was obtained from Invitrogen Molecular Probes (Eugene, OR). MacConkey agar was purchased from Britania (Buenos Aires, Argentina). RIPA lysis buffer and bicinchoninic acid protein assay were purchased from Pierce Chemical (Rockford, IL). Rabbit polyclonal anti-p-NFκB p65 (Ser 311), mouse monoclonal anti p-ERK (Tyr 204) and goat anti-rabbit HRP secondary antibody were purchased from Santa Cruz (Dallas, TX). Goat anti-mouse HRP was obtained from Dako (Glostrup, Denmark). (Mouse anti-actin antibody was obtained from BD Biosciences (San Jose, CA).

Isolation of human neutrophils

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Neutrophils were isolated from peripheral blood drawn from healthy donors by Ficoll Hypaque gradient centrifugation and dextran sedimentation, as described previously (Negrotto et al., 2006). This study was conducted according to the principles expressed in the Declaration of Helsinki. All patients provided written informed consent for the collection of samples and subsequent analysis. Cell suspensions contained > 96% neutrophils, as determined by May Grunwald-Giemsa stained cytopreps, and the levels of monocyte contamination were always < 0.2%, as evaluated by CD14 staining and flow cytometry. The cells ($4x10^5$/ml) were resuspended in RPMI 1640 medium supplemented with BSA (2%).

NET formation assay

Neutrophils ($2x10^5$) were seeded in 24-well flat-bottom-plates with coverslips, stimulated with PMA or TNF-α at pH 7.4, and placed in a humidified incubator at 37°C with CO_2 (5%) for 180 min. For quantification purposes, NETs were then digested with MNase (500 mU/ml) for 10 min at 37°C.

To study NET formation at hyperthermic conditions, neutrophils were placed in 3 different incubators with temperatures of 37, 40 and 42ºC.

To induce acidosis, the pH of the culture medium was adjusted to 7.4, 7.0 or 6.5 by the addition of isotonic HCl (1N) solution before the stimulation with PMA as described above. Neutralization prior to treatment with MNase was achieved by adding a precalculated volume of isotonic NaOH (1N) solution.

In selected experiments, neutrophils were first incubated for 30 min with ASA, dexamethasone, BAY 11-7082, Ro 106-9920 or vehicle (controls). ASA and dexamethasone were dissolved in RPMI. Stock solutions of BAY 11-7082 and Ro 106-9920 were prepared in DMSO and were further diluted in RPMI 1640 medium. The final

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DMSO concentration (0.1% v/v) did not have a toxic effect. All drugs were freshly prepared for each experiment.

Immunofluorescence assays

After PMA or TNF-α stimulation, cells were fixed with PFA (4%), permeabilized with triton (0.25%), stained with PI (2 μg/ml) washed and mounted on slides with PolyMount. In selected experiments, the generation of NETs was confirmed by labeling the cells with PI and antibody against elastase. Images for NET evaluation were taken and analyzed by confocal fluorescence microscopy, using an Olympus FV- 1000 (Tokyo, Japan) equipped with a Plapon 60 x / NA1·42 objective. *In-vivo*, the NET contribution to the bacterial count in the blood was determined by i.p. inoculation of MNase (100 U/mouse or 1 mg/kg, prepared in RPMI).

Quantification of extracellular DNA

DNA released from neutrophils during NET formation was digested with MNAse (500 mU/ml). EDTA (5 mM) was added to stop nuclease activity; supernatants were collected and stored at -20ºC until assayed. Nucleosomal DNA or complexes of DNA bound to histones were measured in the supernatants using commercially available cell death detection ELISA^{PLUS} kit according to the manufacturer's instructions. This test is a sandwich ELISA utilizing two monoclonal antibodies against histones and DNA that specifically detects mono and oligo-nucleosomes derived from eukaryotic cells, as a result of the MNase treatment of NET. The calibration curve was constructed using a standard of nucleosomal DNA of a known concentration.

Quantitation of apoptosis by fluorescence microscopy

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Neutrophils were treated with or without BAY 11-7082 (2.5 μM), Ro 106-9920 (2.5 μM) and ASA (5mM) for 180 min, washed and then apoptosis was evaluated 17 h later by fluorescence microscopy. Cells were labeled with a mixture of the fluorescent DNA-binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells (Negrotto et al., 2006). At least 300 cells were scored in each experiment.

Immunoblotting

Neutrophils were preincubated or not with the anti-inflammatory drugs and stimulated for 40 min with PMA (50 nM). Lysates were prepared using RIPA lysis buffer with a protease inhibitor cocktail. Cell debris was separated by centrifugation and protein in the supernatant was quantified using the bicinchoninic acid protein assay. Samples were then frozen at -80°C until use. Equal amounts of proteins (40 μg/sample) were separated by electrophoresis (12% SDS-polyacrylamide gels). Proteins were then electro-transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with a rabbit polyclonal anti-p-NFκB p65 (Ser 311), mouse monoclonal anti-p-ERK 1/2 (Tyr 204) and mouse monoclonal anti-actin antibody followed by a goat anti-rabbit HRP or anti-mouse HRP secondary antibody. Protein bands were visualized by enhanced chemiluminescence. Immunoblotting results were quantified using Gel-Pro Analyzer 3.1 software and the values from blot reprobes were used for monitoring equal protein loads.

Mice studies

BALB/c mice were bred in the animal facility of the Institute of Experimental Medicine. The mice studies were conducted according to principles set forth in the Guide

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for the Care and Use of Laboratory Animals (1985). Female mice aged 9–16 weeks and weighing 20–25 g were used throughout the experiments. They were maintained under a 12-h light–dark cycle at 22 ± 2°C and fed with standard diet and water, *ad libitum*.

Polymicrobial challenge

The caecum of an untreated mouse was removed and the intestinal content was left at 37°C for 24 h in sterile saline solution supplemented with 10% fetal calf serum (FCS) in aerobic conditions. Routine bacteriological identification revealed that the bacteria present in the inoculum belonged to *Enterococcus and Streptococcus spp* (Landoni et al., 2012). Dosage measurements of bacteria were performed in agar MacConkey plates. Aerobic enriched bacteria (5×10^7) were injected intraperitoneally (i.p.) and 3 h later, peritoneal lavage was performed with sterile saline solution as described below. In selected experiments, mice were injected i.p. with 1 dose (60 min) of ASA (100 mg/kg, dissolved in RPMI) or dexamethasone (2.5 mg/kg, dissolved in PBS) prior the polymicrobial challenge, 2 doses of BAY 11-708 (15 min prior and 90 min after, 5 mg/kg, dissolved in saline solution/DMSO (25%)) or MNase (15 min prior injection with bacteria, 1 mg/kg, dissolved in RPMI,).

Peritoneal lavage

Animals were euthanized and the peritoneal content was collected by peritoneal lavage, as previously described (Negrotto et al., 2006). Briefly, the skin of the abdomen was cut open at the midline after thorough disinfection and without injuring the muscle. Sterile saline solution (2 ml) was injected into and aspirated out of the peritoneal cavity twice, using a sterile syringe and needle, to rinse out the content from the peritoneal cavity. The number of neutrophils was determined microscopically after staining with

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Turk's solution and the peritoneal liquid was used for determination of bacterial clearance as described below.

Bacterial cultures

Blood and peritoneal lavage fluid were obtained; the cells were lysed with sterile distilled water. Aliquots of serial log dilutions of peritoneal fluid and blood were plated onto MacConkey agar which is a selective growth medium for intestinal bacteria. Plates were grown under aerobic conditions at 37°C and colony-forming units (CFU) were counted after an overnight incubation. Bacterial counts are expressed as CFU per milliliter of peritoneal lavage fluid or blood.

In-vivo evaluation of NET formation

In-vivo evaluation of NET formation was performed using a mouse model of peritonitis as previously described (Landoni et al., 2012). NETs were induced with i.p. bacterial injection and after 3 h peritoneal cells were carefully collected and gently seeded on coverslides. Immunostaining was performed as described above.

Vascular permeability assay

Evans blue dye (30 mg/kg in PBS) was injected intravenously (i.v.) and 20 min later, mice were euthanized and peritoneal lavage was performed with 1 ml of sterile saline solution. The Evans blue dye collected from the peritoneal cavity was measured spectrophotometrically at 560 nm (Kanaoka et al., 2001).

Statistical analysis

Data are expressed as the means ± SEM. The ANOVA plus the Newman-Keuls or Dunnet's (for mice studies) test were employed to determine significant differences

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between the groups. A *p* value lower than 0.05 was considered to be statistically significant.

Results

Effects of anti-inflammatory drugs on NET formation

In the first experiments we determined the formation of NETs by stimulation of neutrophils with PMA or the pro-inflammatory cytokine TNF-α. Double labeling of the cells with PI to identify DNA, and with an antibody against elastase, a neutrophil granular-derived protein that decorates the DNA strands, demonstrated that both stimuli triggered the formation of NETs (Figure 1). Albeit NETs are usually generated during inflammatory clinical settings, how anti-inflammatory drugs affect this process is not completely understood at present. Hence, we analyzed the effects of the most commonly used anti-inflammatory drugs, ASA and dexamethasone, on NET formation triggered by PMA or TNF-α. We found that while PMA-induced NET formation was not modified by ASA (1mM), it was markedly inhibited by higher ASA concentrations (5 mM) (Figure 2 A and B). The amount of NETs was similar in controls and in dexamethasone-treated neutrophils. To further corroborate the fluorescence microscopy studies, NET formation was measured by ELISA. The quantification of the released DNA confirmed that the formation of extracellular traps was significantly diminished by ASA (Figure 2 B). The effect of both, ASA and dexamethasone was similar when NETs were triggered by stimulation of neutrophils with TNF-α (Figure 2 A and B).

The transcription factor NF-κB is a key regulator of inflammation and therefore plays a pivotal role in a wide range of inflammatory diseases (Manzenreiter et al., 2012). Since the effects of high ASA concentrations are not only related to cyclo-oxygenase acetylation and include, among others, inhibition of NF-κB activation (Yin et al., 1998), we next investigated the role of NF-κB blockade on NET formation. Neutrophils were incubated with BAY 11-7082 or Ro 106-9920, two structurally unrelated specific inhibitors

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of IκBα phosphorylation (Pierce et al., 1997) and IκBα ubiquitination (Swinney et al., 2002), respectively. Figure 2 A and B shows that the release of DNA traps was markedly impaired by these drugs. Western blot analysis confirmed that phosphorylation of NF-κB p65 subunit triggered by PMA was significantly inhibited by pretreatment of the neutrophils with BAY 11-7082 or Ro 106-9920 in a concentration-dependent manner (Figure 2 C). In addition they showed that while 1 mM of ASA did not modify activation of NF-κB, the phosphorylation of p65 subunit of NF-κB was markedly impaired in PMN preincubated with 5 mM of ASA. On the other hand, dexamethasone even at 10 μM had no effect. ERK phosphorylation, a pathway recently described to be implicated in NET formation (Figure 2 C) (Hakkim et al., 2011) was not modified by any of the drugs employed.

The absence of nuclear changes determined in morphological studies indicated that inhibition of NET formation mediated by either ASA or NF-κB inhibitors was not associated to significant drug-induced neutrophil apoptosis and therefore did not account for the observed inhibition (*% apoptosis: Control*: 55±3; Bay 2.5μM: 62±4; Ro 2.5μM: 64±4; ASA 5mM: 55±4).

Effect of anti-inflammatory drugs on NET formation under acidosis or hyperthermic conditions

Because NET generation occurs in an inflammatory microenvironment, we next examined whether the inhibitory effect of the anti-inflammatory drugs was still evident when NETs are generated under characteristic stress signals of this *millieu* such as hyperthermia or acidosis. Interestingly, ELISA studies showed that the increase in temperature from the physiologic 37 to 42ºC as well as the decrease in pH values, markedly augmented NET formation (Figure 3 A). Despite this increase, ASA or NF-κB

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inhibitors were still effective as both exerted a significant suppression of NETosis under these stressful conditions (Figure 3 B).

ASA and BAY 11-7082 impair *in-vivo* NET formation

Having demonstrated that ASA and the NF-κB inhibitors had profound inhibitory effects on NET formation *in-vitro*, we next explored the effects of these drugs in an *in-vivo* model of peritonitis (Landoni et al., 2012). Consistent with our *in-vitro* results, we found that in mice pretreated with ASA or BAY 11-7082, NET formation was significantly inhibited in the peritoneal cavity and also that PMN migration towards the infection site was decreased (Figure 4 A and B). To further elucidate the role of NETosis in the clearance of bacterial infections, we also quantified the bacteria load in the peritoneal cavity and blood of animals that were treated with ASA, BAY 11-7082, dexamethasone or not treated. While peritoneal bacterial counts (expressed as colony forming units per milliliter, CFU/ml) were significantly lower in ASA-treated mice compared to control animals, the CFUs were similar to mice infused with BAY 11-7082 (Figure 4 C). Interestingly, the treatment of mice with either anti-inflammatory drug prior to the bacterial challenge, resulted in a marked increase in bacteremia (Figure 4 D), indicating that NETs contribute towards limiting bacterial dissemination. Dexamethasone treatment did not alter PMN migration, NET formation or bacterial loads in peritoneum or blood, further supporting our *in-vitro* results. To analyze whether increased bacteremia was due to an augmented vascular permeability mediated by the anti-inflammatory drugs, we next evaluated the presence of evans blue dye in the peritoneal cavity, which indicates dye migration from the vasculature to the tissues. Spectrophotometrical analysis of peritoneal lavage fluid collected from infected and non-infected mice treated with ASA, BAY 11-7082 **or**

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dexamethasone, indicated that infection by itself markedly increased vascular permeability; however, this effect was not further increased by any of the employed anti-inflammatory drugs (Figure 4 E). Additionally, bacteremia was evaluated after MNase treatment, which disrupts NETs, but does not alter the number of neutrophils that migrate to the peritoneum. Figure 4 D shows that bacteremia was significantly increased when NETs were disrupted enzymatically. Together, these data indicate that in this experimental model of peritonitis, inhibition of NETosis failed to locally restrict the infection.

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Discussion

Recently, NETs were identified as an alternative mechanism of pathogen killing. Although NET formation may have a beneficial effect for the host in terms of isolating and preventing the spread of invading bacteria, the persistent activation of neutrophils or deficient NET degradation by DNAses can also promote injury to the host (Hakkim et al., 2010; Caudrillier et al., 2012). Thus, although there are beneficial effects of this process in pathogen control, NET removal must be regulated to ensure tissue homeostasis. The mechanisms or molecules involved in the regulation of NET formation are not yet completely known. Although still a matter of debate, the release of DNA to the extracellular *milieu* is considered a cell death program (NETosis) distinct from apoptosis and necrosis (Fuchs et al., 2007). Moreover, it has been recently suggested that the interplay between autophagy, ROS formation and PAD4-dependent histone citrullination promotes the collapse of both nuclear and granular neutrophil membranes and mediates intracellular chromatin decondensation, while inhibiting the apoptotic machinery (Remijsen et al., 2011).

Because NETs and inflammation are concomitant events, we explored the effects of the most commonly used anti-inflammatory drugs worldwide, ASA (5 mM) and dexamethasone. We found that in contrast to dexamethasone, ASA markedly inhibited NET formation. Interestingly, the anti-inflammatory effects of the ASA concentration (5 mM) used in this study are generally attributed to its action on cyclo-oxygenase independent targets including the inhibition of NF-κB (Yin et al., 1998; Negrotto et al., 2006). Thus, our findings suggested that this major transcription factor involved in the inflammatory response could be a key regulatory factor of NET formation. In fact, the profound inhibition of NETs by two structurally different inhibitors of NF-κB activation together with previous studies showing that PMA or TNF-α stimulation of neutrophils

induces both the nuclear accumulation of NF-κB/Rel proteins and the concomitant degradation of cytoplasmic IkB-α (McDonald et al., 1997), strongly support a role of NF-κB in the biogenesis of NETs. Furthermore, Western blot studies showed that while treatment of neutrophils with ASA 5 mM or 2.5 μM of Ro 106-9920 or BAY 11-7082, resulted in a significant impairment of the PMA-induced phosphorylation of NF-κB p65 subunit, dexamethasone or 1mM ASA had no effect. In addition, none of the drugs used interfered with ERK pathway. Altogether, these data strongly indicate that activation of NF-κB is a signaling pathway involved in the generation of NETs.

Extracellular acidosis, a hallmark of the inflammatory *milieu*, may intensify acute inflammatory responses by inducing neutrophil activation, as well as by delaying spontaneous apoptosis and extending neutrophil functional lifespan (Trevani et al., 1999). Similar to these findings, our data showing that extracellular acidosis fosters the formation of NETs strongly suggests that low pH values may contribute to the amplification of neutrophil-mediated inflammatory responses, but also promote neutrophil bactericidal responses through the induction of NETosis and the inhibition of apoptosis.

It has been previously shown that acute or long-term concurrent exposure to febrile range hyperthermia exerts multiple actions that profoundly increase neutrophil recruitment, enhance pathogen clearance and increase lethal lung injury in mouse models of pneumonia and hyperoxia (Hasday et al., 2003). Furthermore, these consequences to innate immune function and regulation of inflammation were also observed after exposure to long-term hyperthermia (Tulapurkar et al., 2011). Interestingly, because the bacterial proliferation rate appears to not be influenced by high temperatures (Jiang et al., 2000), it was suggested that the reduced pathogen burden could have been achieved through effects on host bacterial clearance mechanisms. Our present findings suggest that an increase of NET generation could be one of the mechanisms responsible for both, efficient

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pathogen clearance and the lung injury observed under experimental hyperthermia conditions. We found that the increased NET generation triggered by either, low pH values or heat, is susceptible to inhibition by ASA or NF-κB inhibitors suggesting that a potential use of these drugs is to prevent undesired tissue damage due to an uncontrolled inflammatory response. This hypothesis is further supported by our *in-vivo* experiments showing that pretreating mice with both, ASA or BAY 11-7082 but not dexamethasone, markedly impaired NET formation in the peritoneum of infected mice. It was recently reported that ASA ingestion may be beneficial for the treatment of transfusion-related acute lung syndrome (Caudrillier et al., 2012). Interestingly, the effect of ASA in this study was not associated with an interference of ASA with the ability of neutrophils to induce NETs, but rather its inhibitory effect on platelet activation, which is a phenomenon involved in NET formation. Because the doses of ASA used in both studies were the same, our data further suggest that the mechanistic bases for ASA treatment would include not only the suppression of platelet activation but also a blockade of neutrophil NF-κB activity. While the use of aspirin as an anti-inflammatory therapy is widely accepted, the use of NF-κB inhibitors is just emerging. In this sense, experimental studies in animals showed that alteration of the NF-κB pathway using BAY 11-7082, yielded beneficial results in functional, sensorimotor and biochemical deficits associated with diabetic neuropathy (Kumar et al., 2012) as well as a decrease in the risk of myocardial injury associated to ischemia-reperfusion (Kim et al., 2010). Furthermore, it has been shown that selective inhibition of NF-κB activation in inflammatory cells could be a treatment option for patients with inflammatory bowel disease patients (Dijkstra et al., 2002), patients at early stage of ankylosing spondylitis (Chen and Liu, 2006) or rheumatoid arthritis (Mucke, 2012).

We observed that while ASA significantly decreased the number of CFU in peritoneum, BAY 11-7082 showed no effect. The differences between the CFU counts

observed between ASA and BAY 11-7082 treatment could be explained by the reported ASA-mediated anti-inflammatory effects not associated to inhibition of the NF-κB activation. In this sense, ASA through the aspirin-triggered lipoxins and resolvins promote phagocytosis enhancing bacterial clearance (Serhan et al., 2000; Scannell and Maderna, 2006; Prescott and McKay, 2011; Dalli et al., 2013). In contrast, NF-κB inhibition by BAY 11-7082 has been reported to impair phagocytosis (Giraldo et al., 2010). In addition, the observation that during the antimicrobial response neutrophils first kill bacteria by phagocytosis and then by NETs (Fuchs et al., 2007) could also contribute to the differences found between ASA and BAY 11-7082 in the bacterial count. Although NETosis suppression appears to be beneficial in some sterile inflammatory diseases, we also found that inhibition of NET formation by ASA and BAY 11-7082 leads to bacterial dissemination toward the blood stream. This effect was not due to an increase in vascular permeability mediated by the anti-inflammatory drugs. It was also observed in the animals treated with DNase, suggesting that, at least in this mouse model of peritonitis, NET formation helps to localize the infection and prevents bacterial dissemination**.**

Increased bacteremia mediated by salicylate treatment was previously reported in experimental peritonitis (Spagnuolo and Ellner, 1979). In this early study, the ability of neutrophils to generate NETs was still unknown. Thus, the increased bacteremia was linked to a decreased influx of peritoneal granulocytes early in the course of infection, which allowed pathogen multiplication. While extrapolation of mouse data to humans is not direct, the adverse influence of NET inhibition in the outcome of infections should also be considered in the development of new therapies.

Neutrophils are short-lived inflammatory cells that are exposed to cytokines, acidosis and high temperatures during systemic fever and at sites of local inflammation in several infectious and non-infectious diseases including cancer. Our results indicate that

NF-κB activation is a relevant mediator in the genesis of these extracellular DNA traps, both *in-vitro* and *in-vivo*. Although limiting NET formation appears to be helpful in sterile inflammatory diseases, more research is needed to understand if this applies to infectious diseases as well.

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Autorship Contributions

Participated in research design: Schattner, Lapponi.

Conducted experiments: Lapponi, Carestia, Landoni, Rivadeneyra, Etulain, Negrotto and Pozner.

Contributed new reagents or analytic tools: Landoni.

Performed data analysis: Schattner, Lapponi, Carestia and Pozner.

Wrote or contributed to the writing of the manuscript: Schattner.

References

(1985) *Guide for the care and use of laboratory animals*. US Department of Health and Human Services.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y and Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**:1532-1535.

Brinkmann V and Zychlinsky A (2007) Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* **5**:577-582.

Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, Toy P, Werb Z and Looney MR (2012) Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest* **122**:2661-2671.

Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH and Kubes P (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**:463-469.

Chen J and Liu C (2006) Is sulfasalazine effective in ankylosing spondylitis? A systematic review of randomized controlled trials. *J Rheumatol* **33**:722-731.

Dalli J, Winkler JW, Colas RA, Arnardottir H, Cheng CY, Chiang N, Petasis NA and Serhan CN (2013) Resolvin d3 and aspirin-triggered resolvin d3 are potent immunoresolvents. *Chem Biol* **20**:188-201.

Dijkstra G, Moshage H and Jansen PL (2002) Blockade of NF-kappaB activation and donation of nitric oxide: new treatment options in inflammatory bowel disease? *Scand J Gastroenterol Suppl*:37-41.

Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V and Zychlinsky A (2007) Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**:231-241.

Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., Wrobleski SK, Wakefield TW, Hartwig JH and Wagner DD (2010) Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* **107**:15880-15885.

Fuchs TA, Brill A and Wagner DD (2012) Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol* **32**:1777-1783.

Giraldo E, Martin-Cordero L, Hinchado MD, Garcia JJ and Ortega E (2010) Role of phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and nuclear transcription factor kappa beta (NF-k beta) on neutrophil phagocytic process of Candida albicans. *Mol Cell Biochem* **333**:115-120.

Gupta AK, Hasler P, Holzgreve W, Gebhardt S and Hahn S (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol* **66**:1146-1154.

Hakkim A, Fuchs TA, Martinez NE, Hess S, Prinz H, Zychlinsky A and Waldmann H (2011) Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol* **7**:75-77.

Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, Herrmann M, Voll RE and Zychlinsky A (2010) Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* **107**:9813-9818.

Hasday JD, Garrison A, Singh IS, Standiford T, Ellis GS, Rao S, He JR, Rice P, Frank M, Goldblum SE and Viscardi RM (2003) Febrile-range hyperthermia augments pulmonary neutrophil recruitment and amplifies pulmonary oxygen toxicity. *Am J Pathol* **162**:2005-2017.

Jiang Q, Cross AS, Singh IS, Chen TT, Viscardi RM and Hasday JD (2000) Febrile core temperature is essential for optimal host defense in bacterial peritonitis. *Infect Immun* **68**:1265-1270.

Kanaoka Y, Maekawa A, Penrose JF, Austen KF and Lam BK (2001) Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis in mice lacking leukotriene C4 synthase. *J Biol Chem* **276**:22608-22613.

Kim YS, Kim JS, Kwon JS, Jeong MH, Cho JG, Park JC, Kang JC and Ahn Y (2010) BAY 11-7082, a nuclear factor-kappaB inhibitor, reduces inflammation and apoptosis in a rat cardiac ischemia-reperfusion injury model. *Int Heart J* **51**:348-353.

Kumar A, Negi G and Sharma SS (2012) Suppression of NF-kappaB and NF-kappaB regulated oxidative stress and neuroinflammation by BAY 11-7082 (IkappaB phosphorylation inhibitor) in experimental diabetic neuropathy. *Biochimie* **94**:1158-1165.

Landoni VI, Chiarella P, Martire-Greco D, Schierloh P, van-Rooijen N, Rearte B, Palermo MS, Isturiz MA and Fernandez GC (2012) Tolerance to lipopolysaccharide promotes an enhanced neutrophil extracellular traps formation leading to a more efficient bacterial clearance in mice. *Clin Exp Immunol* **168**:153-163.

Manzenreiter R, Kienberger F, Marcos V, Schilcher K, Krautgartner WD, Obermayer A, Huml M, Stoiber W, Hector A, Griese M, Hannig M, Studnicka M, Vitkov L and Hartl D (2012) Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. *J Cyst Fibros* **11**:84-92.

McDonald PP, Bald A and Cassatella MA (1997) Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* **89**:3421-3433.

Mucke HA (2012) Iguratimod: a new disease-modifying antirheumatic drug. *Drugs Today (Barc)* **48**:577-586.

Nathan C (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**:173-182.

Negrotto S, Malaver E, Alvarez ME, Pacienza N, D'Atri LP, Pozner RG, Gomez RM and Schattner M (2006) Aspirin and salicylate suppress polymorphonuclear apoptosis delay mediated by proinflammatory stimuli. *J Pharmacol Exp Ther* **319**:972-979.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T and Gerritsen ME (1997) Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* **272**:21096-21103.

Prescott D and McKay DM (2011) Aspirin-triggered lipoxin enhances macrophage phagocytosis of bacteria while inhibiting inflammatory cytokine production. *Am J Physiol Gastrointest Liver Physiol* **301**:G487-497.

Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P and Vanden Berghe T (2011) Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ* **18**:581-588.

Scannell M and Maderna P (2006) Lipoxins and annexin-1: resolution of inflammation and regulation of phagocytosis of apoptotic cells. *ScientificWorldJournal* **6**:1555-1573.

Segal AW (2005) How neutrophils kill microbes. *Annu Rev Immunol* **23**:197-223.

Serhan CN, Takano T, Chiang N, Gronert K and Clish CB (2000) Formation of endogenous "antiinflammatory" lipid mediators by transcellular biosynthesis. Lipoxins and aspirin-triggered lipoxins inhibit neutrophil recruitment and vascular permeability. *Am J Respir Crit Care Med* **161**:S95-S101.

Spagnuolo PJ and Ellner JJ (1979) Salicylate blockade of granulocyte adherence and the inflammatory response to experimental peritonitis. *Blood* **53**:1018-1022.

Swinney DC, Xu YZ, Scarafia LE, Lee I, Mak AY, Gan QF, Ramesha CS, Mulkins MA, Dunn J, So OY, Biegel T, Dinh M, Volkel P, Barnett J, Dalrymple SA, Lee S and Huber M (2002) A small molecule ubiquitination inhibitor blocks NF-kappa B-dependent cytokine expression in cells and rats. *J Biol Chem* **277**:23573-23581.

Trevani AS, Andonegui G, Giordano M, Lopez DH, Gamberale R, Minucci F and Geffner JR (1999) Extracellular acidification induces human neutrophil activation. *J Immunol* **162**:4849-4857.

Tulapurkar ME, Hasday JD and Singh IS (2011) Prolonged exposure to hyperthermic stress augments neutrophil recruitment to lung during the post-exposure recovery period. *Int J Hyperthermia* **27**:717-725.

Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, Brinkmann V, Jungblut PR and Zychlinsky A (2009) Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog* **5**:e1000639.

Urban CF, Reichard U, Brinkmann V and Zychlinsky A (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol* **8**:668-676.

von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Kollnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B and Massberg S (2012) Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* **209**:819-835.

Yin MJ, Yamamoto Y and Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* **396**:77-80.

Footnotes

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Figure legends

Figure 1. NET induction by PMA and TNF-α. Neutrophils were stimulated or not with PMA (50 nM) or TNF-α (20 ng/ml) for 180 min. Cells were then fixed and stained with PI for DNA (red) and the specific marker anti-neutrophil elastase (green) and were visualized by confocal fluorescence microscopy (n=3). Original magnification, 60x (scale bar: 20 μm).

Figure 2. Effect of anti-inflammatory drugs on the ability of neutrophils to release NETs. Neutrophils were pretreated or not (vehicle) for 30 min with ASA, dexamethasone (Dex), BAY 11-7082 or Ro 106-9920, followed by a 180 min activation with PMA or TNF-α. A) DNA was stained with PI and visualized by confocal fluorescence microscopy (n=3). Original magnification, 60x (scale bar: 20 μm). B) NET-associated DNA was quantified in the supernatants by ELISA (n=4, ***p<0.001 *vs.* unstimulated; †††p<0.001 *vs.* PMA or TNF-α-stimulated neutrophils). C) Western Blot analysis of PMA stimulated neutrophils treated with various concentrations of anti-inflammatory drugs. Briefly, neutrophils were preincubated at 37ºC for 30 min with anti-inflamatory drugs, then were stimulated for 40 min with PMA (50 nM) and then lysates were immunoblotted with anti-p-NFκB p65, pERK1/2. Each membrane was reprobed with anti-actin antibody to calculate the relative IOD using GEL-PRO software (n = 3, ***p < 0.001; **p<0.01 *vs*. PMA stimulated neutrophils).

Figure 3. ASA and NFκB inhibitors also effectively suppressed the increased NETosis under hyperthermic or acidic conditions. A) Neutrophils were stimulated or not with PMA at different temperatures or pH values for 180 min and NET-associated DNA was determined by ELISA (n=4, ***p<0.001 *vs.* unstimulated, †p<0.05 *vs.* PMA-stimulated neutrophils at 37ºC or pH 7.4). B) Neutrophils set at hypertermic or acidic conditions were

pretreated or not (vehicle) for 30 min with ASA, dexamethasone, BAY 11-7082 or Ro 106-9920, followed by a 180 min activation with PMA. NET-associated DNA was quantified in the supernatants by ELISA (n=4, ***p<0.001 *vs.* PMA-stimulated neutrophils at each condition).

Figure 4. *In-vivo* NETosis inhibition mediated by ASA or BAY 11-7082. Mice were treated with ASA (100 mg/kg), BAY 11-7082 (5 mg/kg), Dex (2.5 mg/kg) or MNase (1 mg/kg) and then injected i.p. with bacteria. After 180 min, peritoneal PMN were carefully collected and seeded onto poly L-lysine coated coverslides. DNA was stained with PI and NETs were visualized by confocal microscopy. A) Representative microphotographs from one independent experiment are shown (n=12, scale bar: 20 μm), from one control mouse, one mouse treated with ASA, one mouse treated with BAY 11-7082, one mouse treated with Dex and one mouse treated with MNase. B) Neutrophil migration to the peritoneal cavity was determined by microscopy after nuclei staining with Turk`s solution. C) and D) Colony-forming units (CFU) were evaluated 180 min after polymicrobial challenge in C) peritoneal lavage fluid and in D) peripheral blood using Mac Conkey agar (n = 18, * p<0.05 *vs.* vehicle, n.s.: non-significant). E) Mice were treated with ASA (100 mg/kg), BAY 11-7082 (5 mg/kg) or Dex (2.5 mg/kg) and then injected or not with bacteria. After 180 min, Evans blue dye (30 mg/kg) was injected i.v. and the OD at 560 nm in the peritoneal fluid was determined 20 min later. The results are expressed as fold change relative to the uninfected control (n = 7, ***p < 0.001 *vs*. uninfected control).

Figure 1

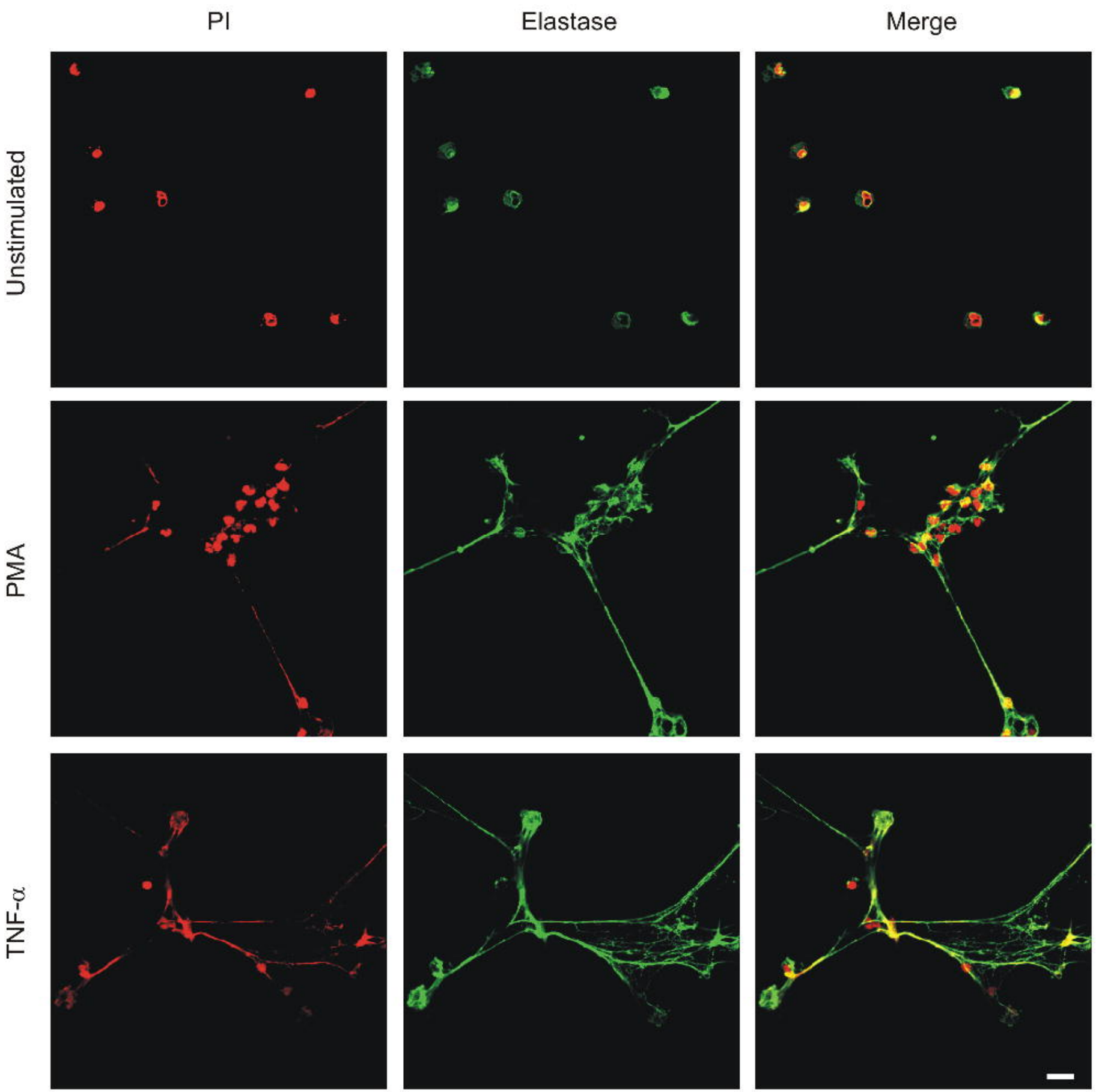


Figure 2

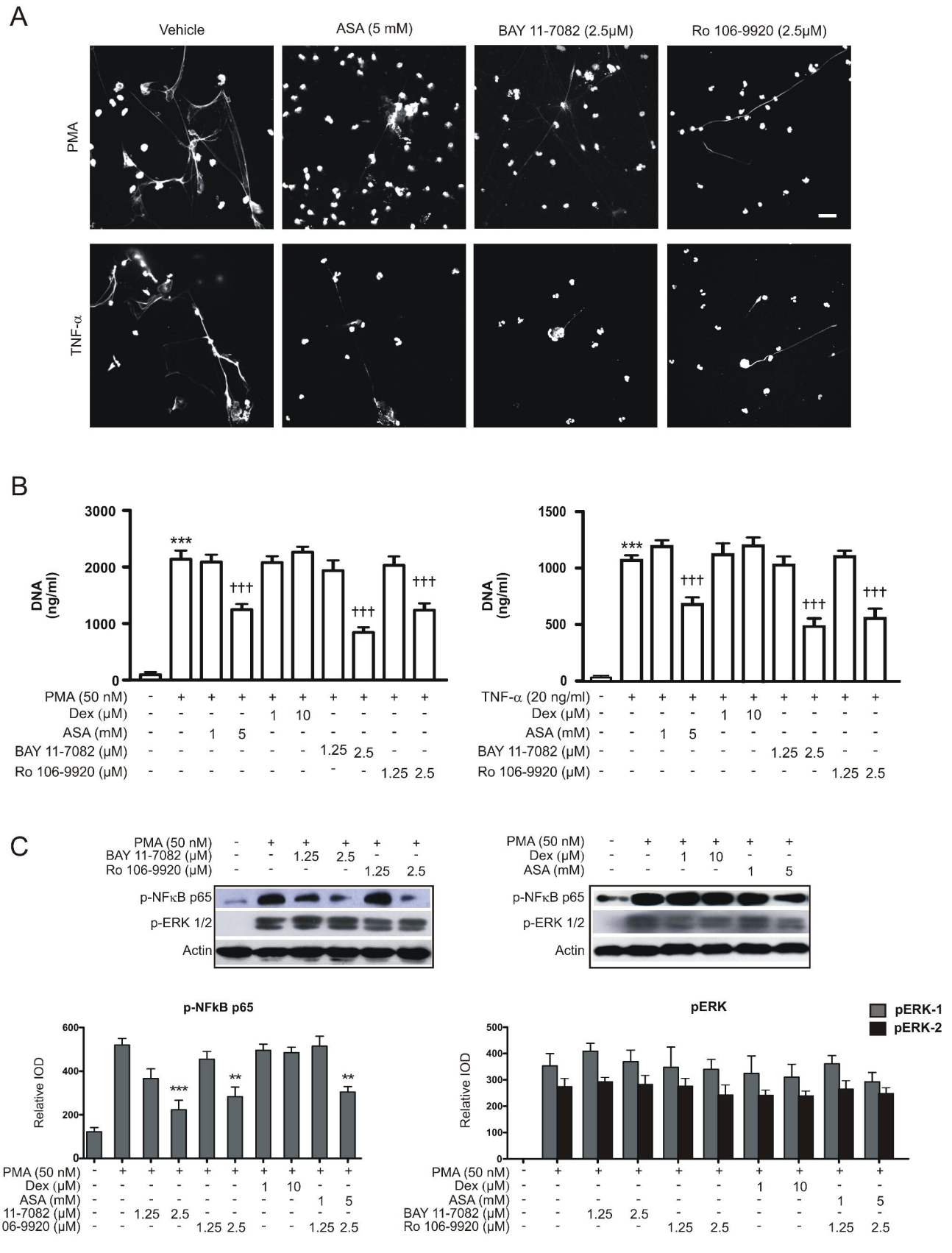

A
Vehicle
ASA (5 mM)
BAY 11-7082 (2.5μM)
Ro 106-9920 (2.5μM)
PMA
TNF-α
B
DNA (ng/ml)
3000
2000
1000
0
***
†††
PMA (50 nM) - + + + + + + + + +
Dex (μM) - - - - 1 10 - - - -
ASA (mM) - - 1 5 - - - - - -
BAY 11-7082 (μM) - - - - - - 1.25 2.5 - -
Ro 106-9920 (μM) - - - - - - - - 1.25 2.5
1500
1000
500
0
TNF-α (20 ng/ml) - + + + + + + + + +
Dex (μM) - - - - 1 10 - - - -
ASA (mM) - - 1 5 - - - - - -
BAY 11-7082 (μM) - - - - - - 1.25 2.5 - -
Ro 106-9920 (μM) - - - - - - - - 1.25 2.5
C
PMA (50 nM) - + + + + +
BAY 11-7082 (μM) - - 1.25 2.5 - -
Ro 106-9920 (μM) - - - - 1.25 2.5
p-NFκB p65
p-ERK 1/2
Actin
PMA (50 nM) - + + + + +
Dex (μM) - - 1 10 - -
ASA (mM) - - - - 1 5
p-NFκB p65
p-ERK 1/2
Actin
p-NFkB p65
Relative IOD
600
400
200
0
***
**
PMA (50 nM) - + + + + + + + + +
Dex (μM) - - - - - - 1 10 - -
ASA (mM) - - - - - - - - 1 5
BAY 11-7082 (μM) - - 1.25 2.5 - - - - - -
Ro 106-9920 (μM) - - - - 1.25 2.5 - - 1.25 2.5
pERK
pERK-1
pERK-2
Relative IOD
500
400
300
200
100
0
PMA (50 nM) - + + + + + + + + +
Dex (μM) - - - - - - 1 10 - -
ASA (mM) - - - - - - - - 1 5
BAY 11-7082 (μM) - - 1.25 2.5 - - - - - -
Ro 106-9920 (μM) - - - - 1.25 2.5 - - 1.25 2.5

Figure 3

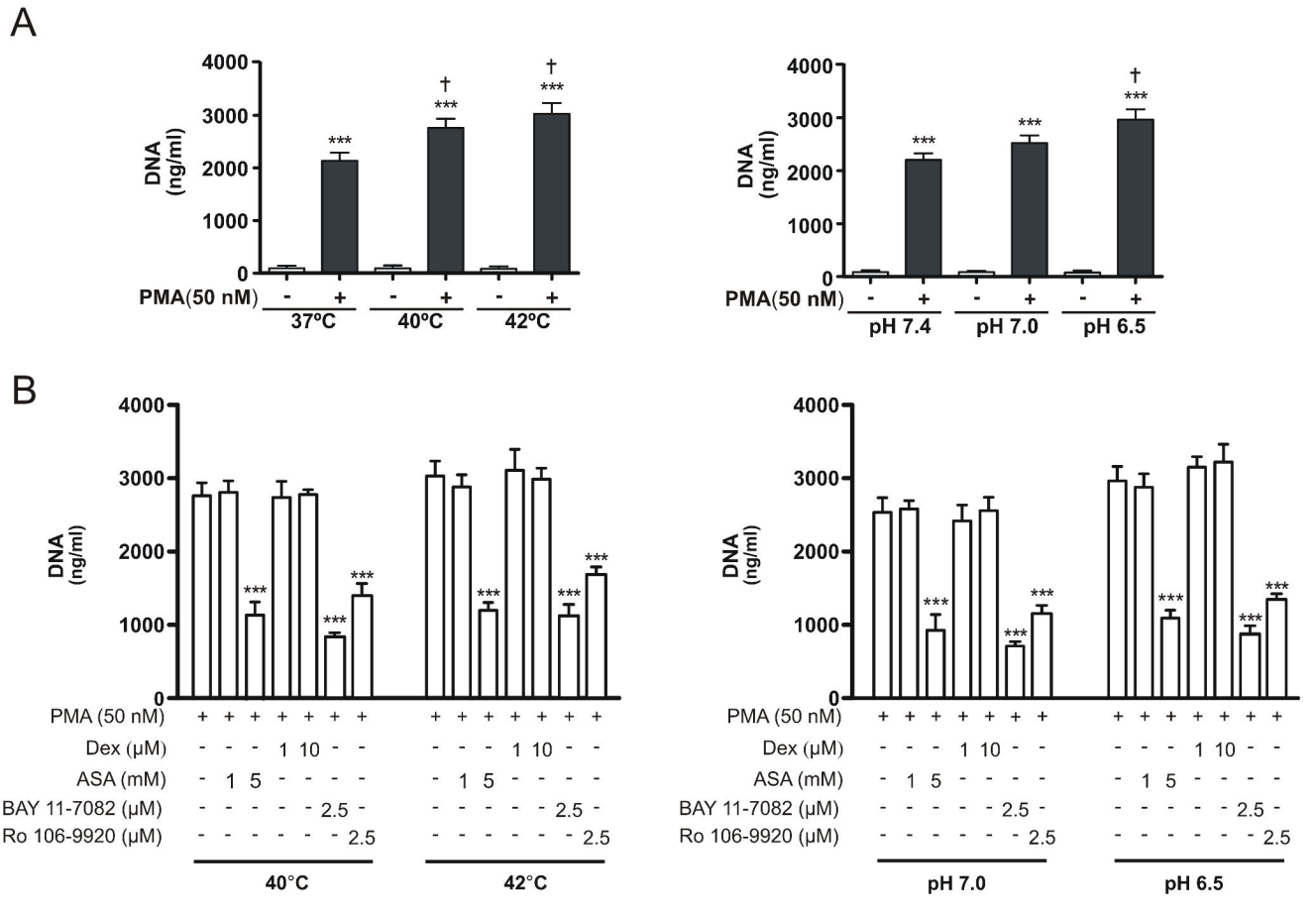
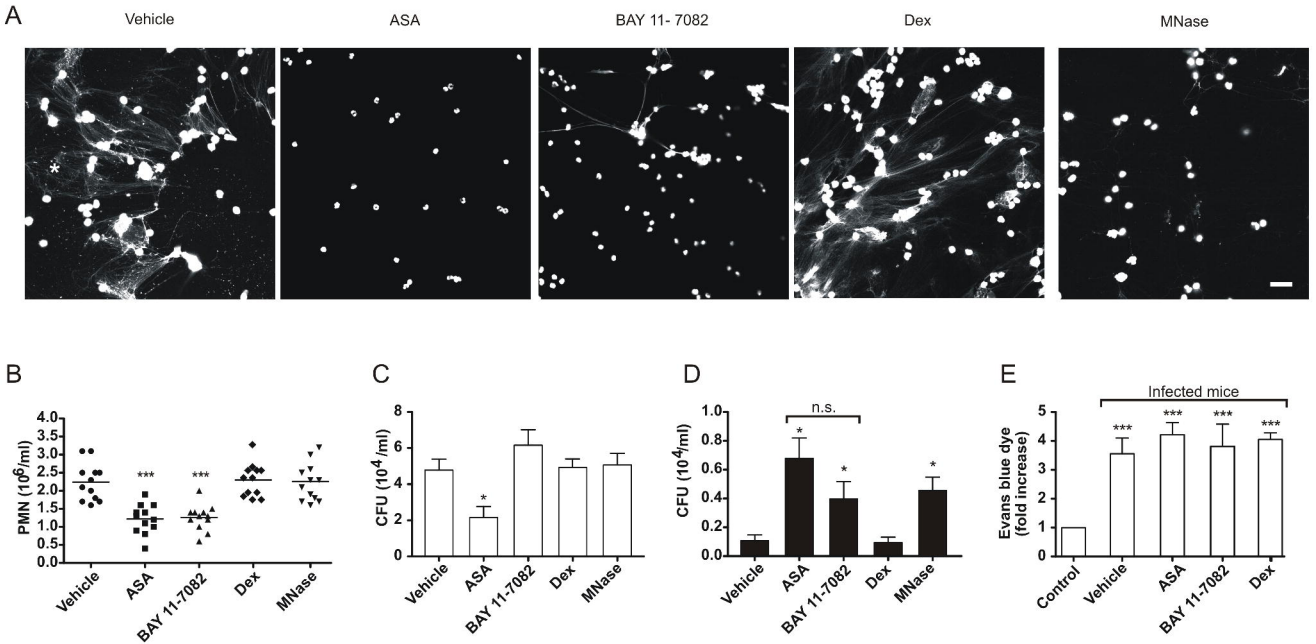

A
DNA (ng/ml)
4000
3000
2000
1000
0
***
†
PMA(50 nM) - + - + - +
37°C
40°C
42°C
pH 7.4
pH 7.0
pH 6.5
B
PMA (50 nM)
Dex (μM)
ASA (mM)
BAY 11-7082 (μM)
Ro 106-9920 (μM)
40°C
42°C
pH 7.0
pH 6.5

Figure 4


A
Vehicle
ASA
BAY 11- 7082
Dex
MNase
*
B
PMN (10^6/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Vehicle
ASA
BAY 11-7082
Dex
MNase
C
CFU (10^4/ml)
8
6
4
2
*
Vehicle
ASA
BAY 11-7082
Dex
MNase
D
CFU (10^4/ml)
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
*
*
*
Vehicle
ASA
BAY 11-7082
Dex
MNase
E
Infected mice
Evans blue dye (fold increase)
5
4
3
2
1
0
***
***
***
***
Control
Vehicle
ASA
BAY 11-7082
Dex