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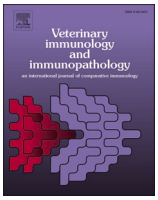

The effect of doxycycline on canine neutrophil functions

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ABSTRACT

Doxycycline is a broad-spectrum tetracycline-class antibiotic that is frequently used to treat bacterial infections. Its use has also been described in immune-mediated diseases due to its immunomodulatory properties. The aim of this study was to evaluate the immunomodulatory effect of doxycycline on canine neutrophil functions. Therefore, the release of reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETs) were determined after incubation of canine PMNs with doxycycline in three different concentrations (4 μg/mL, 20 μg/mL and 200 μg/mL) for one and three hours, respectively. Additionally, a neutrophil killing assay with a doxycycline-resistant *Staphylococcus aureus* was performed to determine the bactericidal effect of doxycycline treated PMNs in presence of plasma. Doxycycline significantly diminished the production of ROS. However, doxycycline concentrations of 4 μg/mL and 20 μg/mL significantly induced NETs. A synergistic bacteriostatic effect of PMNs and doxycycline on a doxycycline-resistant *Staphylococcus aureus* isolate was detectable. However, already PMNs and especially doxycycline alone inhibited the growth. In summary, doxycycline showed a concentration-dependent immunomodulatory property in canine PMNs with a reduced ROS production and increased NET-induction. This immunomodulatory effect resulted in a slightly increased elimination of a doxycycline-resistant *Staphylococcus aureus* by the doxycycline plasma concentrations achieved in dogs.

1. Introduction

Neutrophils represent a major cell type of the innate immune system. After an invasion of bacteria they are important cells in first line of defense to counteract them. They can perform different defense mechanisms: phagocytosis, degranulation, release of reactive oxygen species (ROS), and formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Fingerhut et al., 2020).

ROS play an important role in various physiologic and pathophysiologic processes. For a moderate ROS level positive effects especially on signalling, host defense and biosynthesis are described. Low ROS amount might be associated with decreased antibacterial capacity, whereas high levels of ROS can result in overshoot signalling, nonspecific damage to proteins, lipids and nucleic acid and increased risk of cardiovascular disease, neurologic disorders, cancer and chronic inflammation (Baien et al., 2020; Brieger et al., 2012; Santos et al., 2023; Steffensen et al., 2021).

NETs are formed and released by neutrophils to entrap, disarm and kill pathogens (Brinkmann et al., 2004). The process of NET formation can be triggered by different stimuli (Goggs et al., 2020) and is described in various species including mammals, fish and birds (Fingerhut et al., 2020). In dogs, NETs were described for example after contact with *Toxoplasma gondii* (Wei et al., 2020) and *Trypanosoma cruzi* (de Buhr et al., 2018) and in septic patients mainly caused by *Escherichia coli* and *Streptococcus canis* (Goggs et al., 2020; Letendre and Goggs, 2018). Furthermore, NETs are detected in immune-mediated diseases like immune-mediated hemolytic anemia (IMHA) in dogs (Jeffery et al., 2015).

Abbreviations: MOI, Multiplicity of infection; NET, Neutrophil extracellular trap; PMA, Phorbol 12-myristate 13-acetate; PMN, Polymorphonuclear leukocytes; ROS, Reactive oxygen species; RPMI, Roswell Park Memorial Institute; St., Staphylococcus.

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Several stimuli have been described to influence NET formation, either by stimulation or inhibition. These include physiologic and non-physiologic substances as drugs (methylprednisolone), natural substances (Gum arabic) (Baien et al., 2020; Jerjomiceva et al., 2014; Santos et al., 2023) and lipopolysaccharides, a major component of the outer membrane of gram-negative bacteria (Letendre and Goggs, 2018).

Doxycycline is frequently used as a broad-spectrum bacteriostatic antibiotic. Additionally, doxycycline has been described for the treatment of inflammatory diseases especially of the respiratory tract and the skin (Mueller et al., 2003; Navarro-Triviño et al., 2020; Stechmiller et al., 2010; Szatmári and van Geijlswijk, 2022). Furthermore, doxycycline is used to control immune-mediated diseases like symmetrical lupoid onychomadesis in dogs (Steimer et al., 2019) and it is the main therapy against systemic tick-borne diseases as ehrlichiosis and anaplasmosis (Sainz et al., 2015). Beside the clinical use the influence of tetracyclines on different cell lineages are described. Hume et al. showed that a doxycycline concentration of 6 μg/mL decreased canine lymphoma cell viability by 80% compared to untreated control cells (Hume et al., 2018). Furthermore, it was shown that the MHC II expression is decreased after doxycycline administration in canine peripheral blood lymphocytes and in microglia and macrophages by minocycline in rats, respectively (Nikodemova et al., 2007; Villaescusa et al., 2015). Additionally, treatment of dogs suffering from canine monocytic ehrlichiosis with doxycycline can increase platelet and eosinophil levels but may also increase IL-1ß and monocyte levels, exacerbating inflammation (Cardoso et al., 2023).

The aim of this study was to analyze the effect of doxycycline on neutrophil functions by determining the amount of ROS release and NET formation as well as the influence on the killing capacity of PMN on doxycycline-resistant bacteria, namely *Stapyhlococcus aureus*.

2. Materials and methods

2.1. Enrolment and sample collection

Seven adult clinically healthy dogs (Table 1) were included in this study and from each dog 10–14 mL blood was taken from the *Vena saphena lateralis* and collected with anticoagulant EDTA. A blood cell count was determined by the Advia®120 System (Siemens Healthineers, Germany) to exclude white blood cell abnormalities. For NET induction four dogs were used and blood of another three dogs were taken for analysis of ROS production and performing the PMN killing assay. Details are presented in Table 1.

The sample collection was conducted in line with the recommendations of the German Society for Laboratory Animal Science and the German Veterinary Association for the Protection of Animals (http://www.gv-solas.de). The blood taking procedure was registered at the Lower Saxonian State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, No. 33.9–42502-05–18A250 and 33.9–42502-05–21A609) and conducted with written consent from the dog owners.

2.2. PMN isolation

Canine PMNs were purified using a density gradient centrifugation with Histopaque 1.119 g/mL (Sigma-Aldrich, Germany) and human Pancoll 1.077 g/mL (Pan Biotech, Germany) followed by three times of hypotonic lysis of erythrocytes as previously described (Steffensen et al., 2021). Finally, the cells were resuspended in 1 mL Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher, no phenol red, Carlsbad, USA), stained with trypan blue (Merck; Germany) and counted in a Neubauer chamber.

2.3. NET induction in isolated PMNss

Cover slips (8 mm; Thermo Fisher Scientific (Bremen) GmbH) were placed in 48 well plates (Greiner Bio-One, Kremsmünster, Austria) and coated with poly-L-lysine (0.01% solution Sigma Aldrich, Munich, Germany) in accordance with the manufacturer's instructions and handled afterwards as described previously (de Buhr et al., 2019; Steffensen et al., 2021). Briefly, 2×10^5 PMNs/100 μL were seeded in each well with a final volume of 200 μL. The cells were stimulated with doxycycline (Doxycyclin-ratiopharm®SF 100 mg/5 mL, ratiopharm GmbH, Ulm, Germany) in three different concentrations 200 μg/mL, 20 μg/mL and 4 μg/mL to mimic in vivo properties of a maximum plasma concentration between 2.88 and 27.86 μg/mL within three hours after administration (Gutiérrez et al., 2014; Papich, 2021) and to evaluate the effect of a high concentration due to described doxycycline accumulation in PMNs (Davis et al., 2006). As a positive control methyl--β-cyclodextrin (CD, final concentration 10 mM; Sigma Aldrich, Munich, Germany) was added and RPMI medium served as negative control. The plates were centrifuged (5 min, 370 x *g*) and incubated for 3 h (37 °C; 5% CO_2). Cells were fixed with paraformaldehyde (4% final concentration) and stored at 4 °C until further analysis.

2.4. NET staining

NET staining was performed as previously described (Steffensen et al., 2021). After permeabilization and blocking of the samples, these were incubated with the first antibodies: mouse monoclonal-antibody against DNA/histone 1 (1:1000 diluted in blocking buffer; Millipore MAB3864; 0.55 mg/mL, Billerica, MA, USA) and a rabbit polyclonal-antibody against human myeloperoxidase (1:337.5 diluted in blocking buffer; A039829–2 Agilent, Santa Clara, CA, USA, 3.2 mg). For the isotype controls murine IgG2a (from murine myeloma, M5409–0.2 mg/mL, 1:364 Sigma Aldrich, Munich, Germany) and rabbit IgG (from rabbit serum, Sigma Aldrich, Munich, Germany, I5006, 1.16 mg, 1:108.75) were used. As secondary antibodies, goat anti-mouse Alexa 488Plus-conjugated antibody (1:500 in blocking buffer, Invitrogen, Carlsbad, CA, USA) and a goat anti-rabbit Alexa 633-conjugated antibody (1:500 in blocking buffer; Thermo Fisher Scientific 2 mg, Waltham, MA, USA) were used. After washing steps, staining with aqueous Hoechst 33342 (1:1000, stock 50 mg/mL, Sigma Aldrich, Munich, Germany) was conducted and the coverslips embedded in

Table 1
Study population of included dogs with white blood cell counts.

Dog	Breed	Age (year)	Sex	WBC x10^3/μL (6-12)	PMNs x10^3/μL (3-10)	Blood sample mL	Isolated PMNs x10^6/mL	NET induction	ROS production	Killing assay
1	Magyer Viszla	1	MI	10.97	7.26	13	24.6	x		
2	Golden Retriever	8	MN	6.27	3.81	10	14.9	x		
3	Malinois	1	MI	10.63	6.73	10	19.9	x		
4	Mixed breed dog	4	FN	7.91	5.02	10	19.6	x		
5	Magyar Viszla	5	MN	6.94	3.95	14	14.1		x	x
6	Mixed breed dog	1	FI	12.91	6.02	14	34.6		x	x
7	Mixed breed dog	1	FI	13.58	6.65	14	27.1		x	x

WBC = white blood cell count, PMN = polymorphonuclear cells, M = male, F = female, N = neutered, I = intact, NET = neutrophil extracellular trap, ROS = reactive oxygen species

ProLong®Gold antifade reagent (without DAPI, Invitrogen, Carlsbad, CA, USA).

2.5. NET quantification by immunofluorescence microscopy

For each sample six pictures were acquired randomly by using a Leica TCS SP5 confocal inverted-base fluorescence microscope with an HCX PL APO 40 × 0.75–1.25 oil immersion objective. Respective isotype controls were used for setting adjustment. The quantification was manually conducted as described previously using ImageJ software (version 1.52q, National Institute of Health, USA) (Steffensen et al., 2021).

2.6. Analysis of ROS production

Measurement of intracellular ROS production was performed by flow cytometry (Attune® NxT Acoustic Focusing Flow Cytometer, Invitrogen) as described previously (Bonilla et al., 2020; Steffensen et al., 2021). Briefly, isolated canine PMNs were adjusted to 2×10^5 cells/100 µL with a final volume of 200 µL and incubated with doxycycline in three different concentrations as described above. Incubation of PMNs with and without phorbol 12-myristate 13-acetate (PMA, final concentration of 25 nM; Sigma-Aldrich, Munich, Germany) served as positive and negative control, respectively. After 15 min of incubation (37 °C, 5% CO_2) 2′− 7′-dichlorofluorescein -diacetate (DCFH-DA; Sigma Aldrich, Munich, Germany) with a final concentration of 10 µM was added to each sample and all samples were incubated for further 30 min. All samples were analyzed in duplicates. A total of 20,000 events were recorded and the mean green fluorescence intensity of all cells (X-Mean of BL-1) was determined as a relative measurement of ROS production. For data analysis FlowJoTM10.8.1 software (Ashland, OR, USA) was used.

2.7. Bacterial strain and growth condition

A livestock-associated methicillin- resistant *Staphylococcus aureus* (*St. aureus,* LA-MRSA (Fessler et al., 2010)) was used. A streak out was made on blood agar plates (Columbia Agar with 7% sheep blood; Thermo Scientific, Waltham, USA) at 37 °C for 20–24 h. An overnight culture with one colony in 14 mL Simport tubes (Carl Roth, Karlsruhe, Germany) with 10 mL Mueller Hinton Broth (MHB) with Ca^{2+} 3.158 mg/L and Mg^{2+} 6.143 mg/L was prepared and incubated for around 18 h (200 rpm, 37 °C). A 1:10 dilution from the overnight culture was made in pre-warmed MHB (total 50 mL) and incubated at 37 °C at 200 rpm until it reached the late exponential growth phase with an optical density ($OD_{600\ nm}$) of 0.50 ± 0.01. The bacterial suspension was immediately put on ice, and then centrifuged twice (10 min at 3736 g, 4 °C). After each centrifugation the pellet was washed with 1x PBS (sterile, ice cold) and finally aliquoted a 500 µL. Aliquots were immediately frozen in liquid nitrogen and stored at − 80 °C until usage. Each cryostock was used only once.

2.8. PMN killing assay

The assay was performed as described previously (Bonilla et al., 2020; Steffensen et al., 2021). Briefly, 2×10^5 isolated PMNs were seeded in 48 well plates with a final volume of 200 µL and infected with *St. aureus* (multiplicity of infection (MOI) = 1). Additionally, doxycycline in three different concentrations (200 µg/mL, 20 µg/mL, 10 µg/mL) was added and incubated with 10% of endogenous canine plasma for 1 and 3 h (37 °C, 5% CO_2) after centrifugation of the plates (5 min, 370 g). As untreated growth control one sample without doxycycline was incubated with RPMI instead of plasma. As further growth controls, all samples were additionally incubated without PMNs, but plasma and doxycycline were added. To determine the colony forming units (CFU)/mL, serial dilutions at the time points 0, 1 and 3 h were plated on blood agar plates and incubated for 20 h at 37 °C. The survival factor (SF) was calculated with the formulas $SF = CFU_{1\ h}/CFU_{0\ h}$, $SF = CFU_{3\ h}/CFU_{0\ h}$ and $SF = CFU_{3\ h}/CFU_{1\ h}$.

2.9. Statistical analysis

Data were analyzed using Excel (Microsoft 365) and GraphPad Prism 9.0.2 (GraphPad Prism Software, San Diego, USA). Test for normal distribution was performed with Shapiro-Wilk test. Data were analyzed with one-tailed paired Student's t-test or a one-way ANOVA and are presented with mean ± SD. Probabilities lower than 0.05 were considered as statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results and discussion

3.1. Doxycycline reduces ROS release from canine PMNs

Due to the fact that ROS can be used as a parameter for neutrophil activation (Fingerhut et al., 2020), we analyzed ROS production after incubation of canine PMNs with three different doxycycline concentrations (200 µg/mL, 20 µg/mL, 4 µg/mL) and without further stimulation. These concentrations were chosen, as the plasma concentration in dogs after 5 mg/kg i.v. application reaches a value of 11.56 ± 1.09 µg/mL and this value is reflected in the lower selected concentration range (Riond et al., 1990). There was dose-dependent significant lower ROS release after 45 min of incubation with doxycycline (Fig. 1).

Consistent with our findings, an insight of antioxidant effects of doxycycline has been described previously among other things in a cell-free system. Doxycycline inhibited the formation of redox-mediated malondialdehyde-acetaldehyde (MAA) protein adducts (Clemens et al., 2018). In Adenovirus infected mice, acute lung injury was reduced after receiving doxycycline by ameliorating inflammatory signs of viral pneumonia, independent of presence and amount of detected virus titer. Additionally, decreased activity of specific toxic products secreted by neutrophils, like matrix metalloproteinases, were only detected in infected animals compared to control (Ng et al., 2012). Due to the fact that an accelerated production of ROS can be involved in the pathogenesis of different diseases (Brieger et al., 2012), the inhibition of ROS by doxycycline might be one of the mechanisms contributing to its immunomodulating character in dogs. Some studies suggested that administration of low dosages of doxycycline might be even more effective in suppressing inflammatory responses than high dosages (Di Caprio et al., 2015). This is in contrast to the results of the present study, as the highest dosage (200 µg/mL) resulted in the highest decrease of ROS release by canine PMNs (Fig. 1D). Nevertheless, the lowest dosage (4 µg/mL) was still effective in achieving a significant reduction of ROS release compared to the untreated control. Interestingly, a dog with bronchiectasia, a disease characterised by chronic lung inflammation, was treated successfully with low dosages (1.5 mg/kg/day) of doxycycline (Szatmári and van Geijlswijk, 2022) and low dosages have also been described for the treatment of inflammatory skin diseases (Mueller et al., 2003). Serum levels below 1 µg/mL were achieved by dosages of 1–2 mg/kg/day doxycycline in the dog (Kim et al., 2013).

However, a limitation of this study is that the ROS inhibition was only analysed comparing unstimulated neutrophils and doxycycline treated neutrophils. Future studies should focus on the effect of doxycycline on neutrophils that are treated to release ROS e.g. by adding bacteria or PMA to induce ROS.

3.2. Doxycycline induces NETs

In the next step we analyzed another defence mechanism of PMNs after doxycycline treatment, the NET formation. There was a significant increase of NET releasing cells after incubation with doxycycline at concentrations of 4 µg/mL and 20 µg/mL (Fig. 2).

Recently the direct effect of methylprednisolone on canine

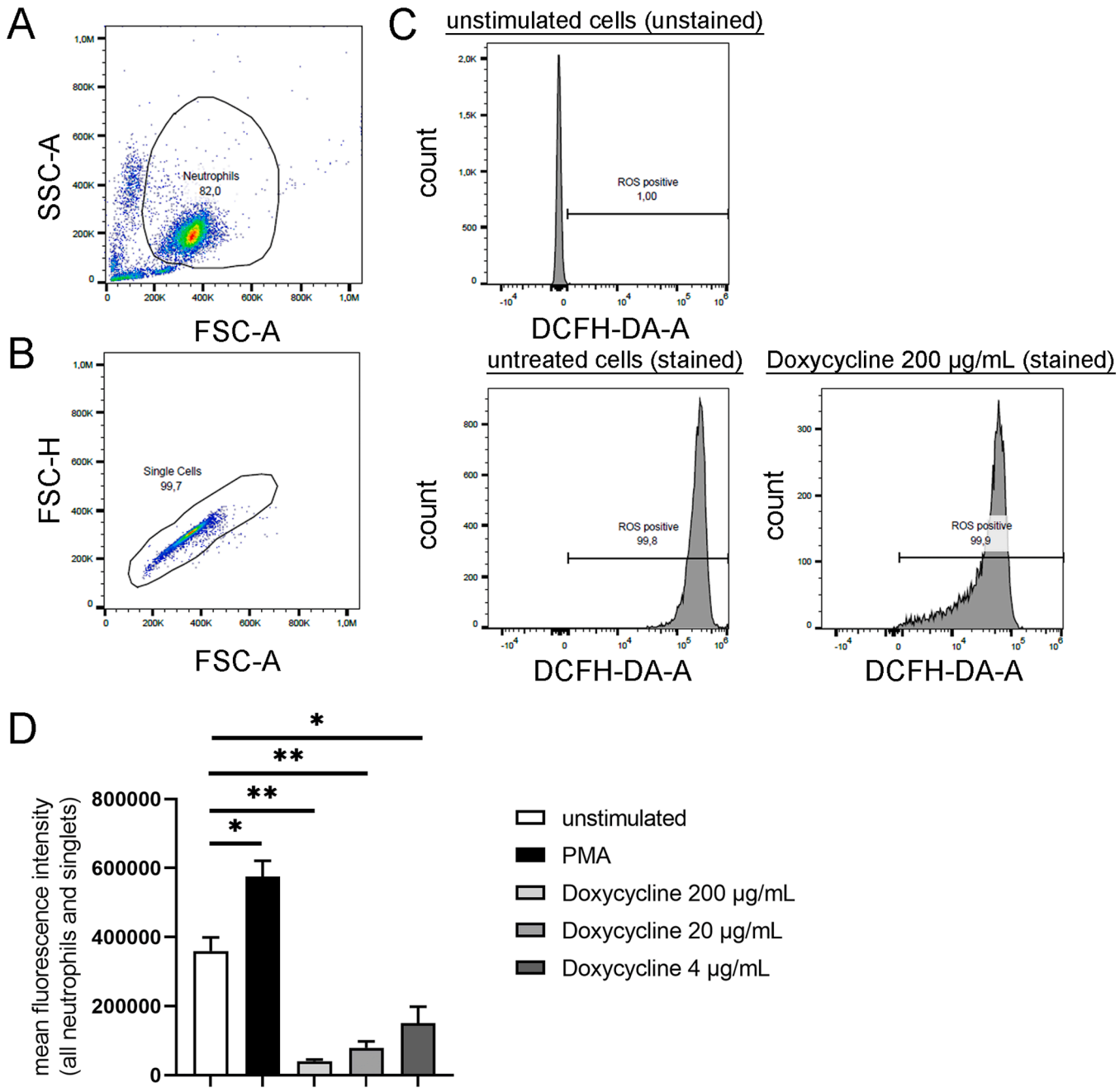

Fig. 1. Doxycycline significantly diminishes ROS release in canine PMNs. Isolated PMNs produced reduced amounts of ROS upon incubation with different concentrations of doxycycline. PMA was used as positive control. (A–C) The intracellular ROS production was determined by adding 2′– 7′-dichlorofluorescein diacetate (DCFH-DA) to unstimulated, PMA-stimulated and doxycycline stimulated cells. The gating strategy for the DCF-positive cells (ROS oxidases DCFH-DA, resulting in fluorescence of 2′–7′-dichlorofluoresein) by flow cytometry is presented. (A) Based on FSC-A and SSC-A, the PMN population was gated. (B) Based on FSC-A and FSC-H, all singlets were gated from the PMN population. (C) The population of ROS-positive cells was gated according to the unstained control, as also unstimulated PMNs produce ROS. The shift of the peak indicates an alteration of ROS production. Example histograms are presented. (D) The mean fluorescence intensity is presented. Concentration-dependent, after 45 min, Doxycycline caused a significant reduction of ROS production by PMNs. The mean fluorescence intensity is presented. The untreated and stained cells were used as a control to determine a change in ROS production during the stimulation with doxycycline. Data were analyzed with one-tailed paired Student's t-test (*: $p < 0.05$) and are presented with mean ± SD ($n = 3$ experiments with the mean of duplicates are presented).

neutrophils, including NET formation, has been shown (Steffensen et al., 2021). Furthermore, several studies showed an effect of antibiotics on neutrophils regarding NET formation. Whereas clindamycin did not induce NETs in human neutrophils, incubation with amoxicillin resulted in a significant increase of NET release in human neutrophils (Bystrzycka et al., 2016). Enrofloxacin was shown to enhance NET formation in bovine neutrophils (Jerjomiceva et al., 2014).

The NET induction by doxycycline is especially important because therapeutic approaches of doxycycline e. g. in immune-mediated hemolytic anemia are associated with thromboembolic events (Carr et al., 2002; Kidd and Mackman, 2013) and thromboembolic events are associated with NET induction. However, microvascular thrombosis is also a useful tool to prevent pathogens to enter into systemic circulation (Goggs et al., 2020). Consequently, it would be interesting to evaluate if the treatment with doxycycline is associated with a higher risk of thromboembolism, which warrants the need for antithrombotic drugs.

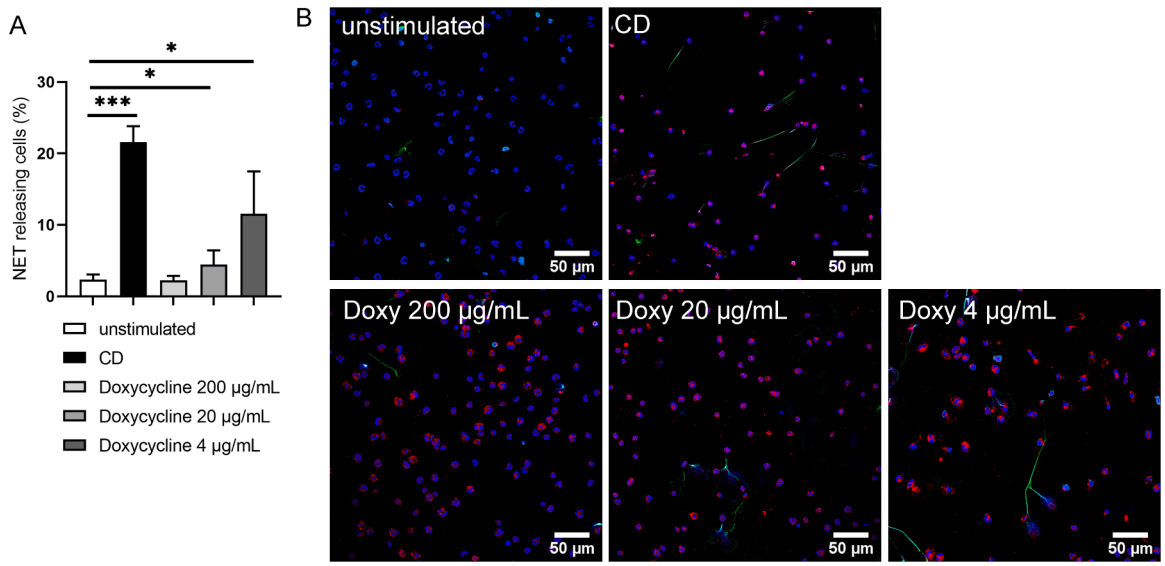

Fig. 2. Doxycycline induces dose-dependent NETs in canine PMNs after 3 h of incubation. Canine blood-derived PMNs were incubated with doxycycline. Samples were analyzed for NETs by confocal immunofluorescence microscopy. A) For each sample, six randomly taken pictures from two individual slides were analyzed. All cells were counted and the mean of activated cells per sample was calculated. Data were analyzed with one-tailed paired Student's t-Test calculated always to negative control (n = 7; mean ± SD * $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$). (B) Representative images (overlay) of all samples incubated with doxycycline, a negative control (RPMI) and a positive control Methly-β-cyclodextrin (CD) are presented (blue = DNA, green = DNA/histone-1-complexes, red = myeloperoxidase).

NETs might also be beneficial in diseases associated with overactivated immune system. It was shown that NETs induce a more anti-inflammatory cytokine and chemokine profile (downregulation of IL-6, upregulation IL-10) in patients with rheumatoid arthritis and gout (Ribon et al., 2019; Schauer et al., 2014). This effect was also described for doxycycline alone (Bostanci et al., 2011). The release of NETs might be another immunomodulatory mechanism complementing the anti-inflammatory effect of doxycycline.

A limitation of this study part is a missing time-kinetic.

3.3. Indications that doxycycline enhances neutrophil killing of doxycycline-resistant St. aureus over time

To investigate the influence of immunomodulating effects by doxycycline, we finally analyzed the influence on a doxycycline-resistant *St. aureus* in presence of doxycycline treated canine neutrophils (Fig. 3). To mimic the in vivo situation, canine plasma from the respective donor was added to the assay. Therefore, neutrophils could act against *S. aureus* in an environment comparable found in vivo, as only other blood cells are missing. As Riond and colleagues measured in dogs a plasma concentration of 11.56 ± 1.09 µg/mL after i.v. application of 5 mg/kg doxycycline, we decided to include 10 µg/mL in this assay with plasma, to analyse an effect that reflects a situation directly after injection in vivo (Riond et al., 1990). A survival factor (SF) of the bacteria was calculated and a value below one indicate a killing of bacteria for the chosen time frame, whereas a value higher one indicates growth of bacteria. After one hour independent of the treatment, all samples showed a SF below 0.5, indicating that the bacteria had to adjust to the experimental conditions (Fig. 3A). Therefore, identified significant differences should be carefully interpreted, as high standard deviations are observed. After three hours compared to the start inoculum the doxycycline-untreated growth controls (RPMI – plasma and plasma - doxycycline) showed high SFs, indicating that bacteria can grow under this assay conditions (Fig. 3B). Furthermore, a doxycycline treatment with the chosen concentrations resulted again in a SF lower than one. Therefore, doxycycline alone was already killing *S. aureus*. Nevertheless, the lowest SF was observed in samples with doxycycline treated PMNs. Even there was no significant difference detected inside the group with PMNs and different doxycycline concentrations, the SF was significantly reduced in samples with neutrophils treated with 200 and 20 µg/mL compared to samples without neutrophils. Therefore, a tendency for a synergistic effect was observed. By analysing the SF 3 h/1 h, and therefore conducting an analysis of bacteria that are adapted after 1 h to the assay conditions, a significant lower SF was observed in samples with PMNs treated with 10 µg/mL doxycycline. Nevertheless, future studies should include other bacteria and maybe conduct all analysis with either a higher starting multiplicity of infection (MOI) or fresh grown bacteria. However, higher MOI can lead to artificial cytotoxic effects and fresh grown bacteria have the limitation to result in higher standard deviations. Although the isolate is resistant to doxycycline, a direct inhibiting influence on growth was observed. The discrepancy between a determined MIC (minimum inhibitory concentration) and the final mode of action in presence of a body fluid (here plasma), was observed in regard of cathelicidins. The MIC value of cathelicidins was different comparing cerebrospinal fluid and cell culture media(Meurer et al., 2020). The growth reduction in the presence of doxycycline and PMNs could be due to the fact that the bacteria are directly affected by doxycycline and in addition indirectly by the PMNs. Previously, it was shown that methylprednisolone augments the killing capacity of PMNs in gram positive bacteria (Steffensen et al., 2021). Further studies are needed, to investigate if a comparable effect on gram negative bacteria is seen. Moreover, a limitation of this study part is that lower concentrations of doxycycline (4 µg/mL) are not included in this study part.

Finally as concentrations of 4, 10 and 20 µg/mL were associated with valuable plasma concentrations in vivo, one limitation of this study is that a clear cut off value for doxycycline induced cytotoxicity is missing. As cytotoxicity of doxycycline was found to be dose-dependent in tumor cell lines (Song et al., 2014) further studies are needed to detect the influence of high plasma concentrations (200 µg/mL) of doxycycline on canine neutrophils.

4. Conclusion

By using a doxycycline concentration that can be achieved in the plasma of dogs, a tendency of a synergistic bactericidal effect of canine PMNs and doxycycline was observed. Furthermore, this study shows in vitro an immunomodulatory effect on canine neutrophils using concentrations of doxycycline that are in vivo achieved. Since ROS

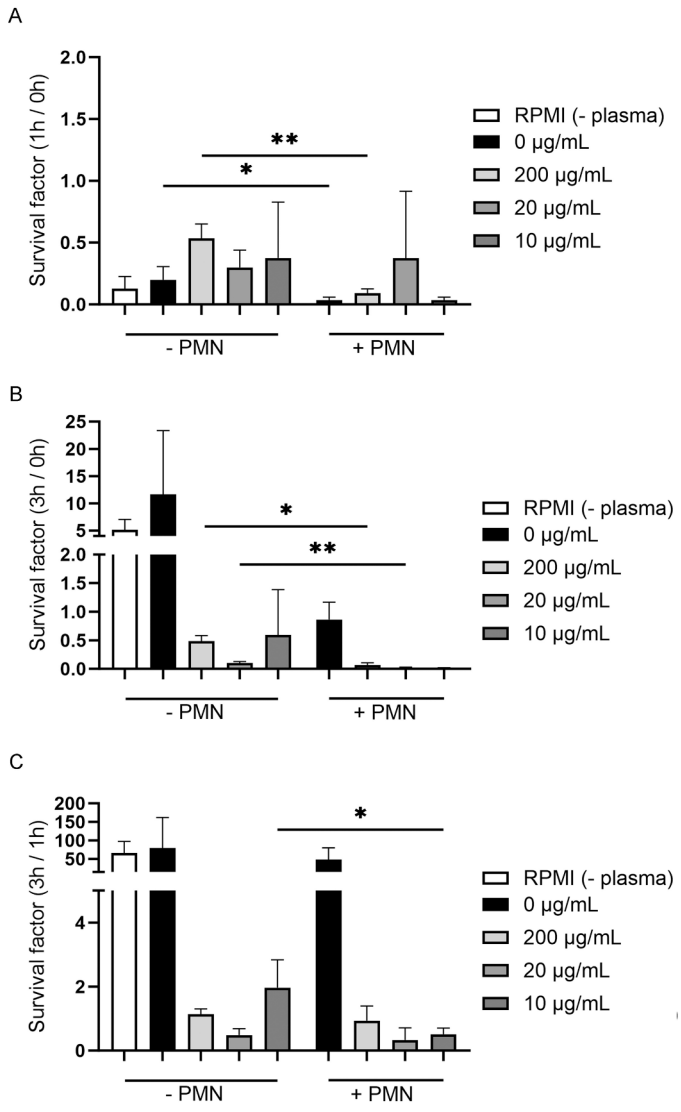

Fig. 3. The growth of doxycycline-resistant St. aureus is reduced in presence of doxycycline alone, PMNs alone and doxycycline treated PMNs. A doxycycline-resistant *St. aureus* was incubated for one and three hours in presence of canine plasma, doxycycline and PMNs. A growth control (RPMI) without plasma was included in the experiment. The colony forming units (CFU) were determined and the survival factor (SF) calculated with the respective formula ($SF = CFU_{1\,h}/CFU_{0\,h}$, $SF = CFU_{3\,h}/CFU_{0\,h}$ and $SF = CFU_{3\,h}/CFU_{1\,h}$). (A) After one hour the SF was in all samples, independent of the treatment, below one. The bacteria adapted to the assay conditions and therefore are reduced in CFU in comparison to the starting inoculum. In presence of PMNs alone and PMNs treated with doxycycline in a concentration of 200 µg/mL the SF of a doxycycline-resistant *St. aureus* was significantly reduced. (B) After three hours of incubation the *St. aureus* was growing in absence of PMNs and doxycycline. In presence of PMNs the SF in all samples was below one. Compared to *St. aureus* only treated with doxycycline, the SF was significantly decreased in samples incubated with doxycycline (200 µg/mL and 20 µg/mL) treated PMNs. (C) The SF 3 h / 1 h reflects the bacterial growth after the adaption of *St. aureus* to the experimental conditions. In absence of PMNs and doxycycline the SF was around 50, indicating a tremendously bacterial growth. In presence of doxycycline without PMNs the SF was decreased compared to samples without doxycycline. Compared to *St. aureus* only treated with doxycycline, the SF was decreased in samples incubated with doxycycline (significantly in 10 µg/mL) treated PMNs. All graphs show the mean ± SD of three independent experiments. Statistical differences were detected by one-tailed paired Student`s t-test between samples + /- PMN. Statistically differences are presented as following * $p < 0.05$, * * $p < 0.01$. Statistical differences in the groups -PMN and +PMN was analysed with ANOVA-table, however no significant difference was detected.

production was diminished by doxycycline, the detected NET formation is likely ROS independent induced by doxycycline. These findings help to understand more in detail how doxycycline influence immune cells, namely PMNs, and present therefore new insights how in vivo observed immunomodulatory effects of doxycycline could be explained.

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CRediT authorship contribution statement

Rieder Johanna Cornelia: Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Steffensen Nicole:** Data curation, Methodology, Writing – original draft, Writing – review & editing, Formal analysis, Investigation, Validation, Visualization. **Imker Rabea:** Writing – review & editing, Formal analysis, Investigation. **Lassnig Simon:** Writing – review & editing, Investigation. **de Buhr Nicole:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Investigation.

Declaration of Competing Interest

None.

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