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The impact of Influenza A virus triggered neutrophil
extracellular traps on bacterial co-infections

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Explore as one

~Perseverance

Meiner Familie

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List of Abbreviations

%	Percent
AMP	Antimicrobial peptide
AM	Alveolar macrophage
<i>A.pp</i>	<i>Actinobacillus pleuropneumoniae</i>
ApX	<i>A.pp</i> -repeats-in-toxin
ARDS	acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
CD	Cluster of differentiation
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CRAMP	Cathelicidin-related antimicrobial peptide
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNase1L3	Deoxyribonuclease 1 like 3
dpi	Days post infection
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	Latin: <i>exempli gratia</i> (for example)
EM	Electron microscopy
ET	Extracellular trap
et al.	Latin: <i>et alii</i> (and others)
<i>G. parasuis</i>	<i>Glaesserella parasuis</i>
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
<i>H. influenzae</i>	<i>Haemophilus influenzae</i>
H ₂ O ₂	Hydrogen peroxide
HOCl	Hypochlorous acid
H3Cit	Citrullinated histone H3
HA	Hemagglutinin

Hib	<i>Haemophilus influenzae</i> type b
HOCl	Hypochlorous acid
IAV	Influenza A virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mg	Milligram
mL	Milliliter
MPO	Myeloperoxidase
NA	Neuraminidase
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NanA	<i>N</i> -acetylneuraminic acid lyase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NK	Natural killer
NP	Nucleoprotein
NSAID	Nonsteroidal anti-inflammatory drug
NTHi	Non-typable <i>Haemophilus influenzae</i>
O ₂ ⁻	Superoxide
ohr	Organic hydroperoxide reductase
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PCV2	Porcine circovirus 2
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cell
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
rhDNase	Recombinant human DNase 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RTX	Repeats-in-toxins
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
<i>S. suis</i>	<i>Streptococcus suis</i>
<i>ssp.</i>	Subspecies
St	Serotype
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
TRAP	tripartite ATP-independent periplasmic
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

1. Summary

Simon Lassnig

The impact of Influenza A virus triggered neutrophil extracellular traps on bacterial co-infections

Influenza A virus (IAV) is a pathogen that infects the respiratory tract of birds, and mammals especially humans and pigs. It causes a disease with respiratory symptoms and fever. The infection and the induced immune response severely increase the susceptibility of the host to a secondary bacterial pneumonia. Due to this, the majority of the deaths in seasonal epidemics and the pandemics of the past century were attributed to secondary bacterial pneumonia. Bacteria of the *Pasteurellaceae* belong to the most frequent co-infecting agents in IAV infections. The most important species are *Haemophilus (H.) influenzae* in humans and *Glaesserella (G.) parasuis* and *Actinobacillus pleuropneumoniae (A.pp)* in the pig. During an infection with IAV, a massive infiltration of neutrophil granulocytes is reported. Neutrophils are a part of the innate immune system and have a broad range of effector function to combat invading pathogens. One of these functions is the formation of neutrophil extracellular traps (NETs). Neutrophils expel their chromatin to entrap and kill pathogens. *H. influenzae* and *A.pp* can survive the antimicrobial effect of NETs and feed on released growth factors that are released upon degradation of NETs. In this study, the interaction between human and porcine *Pasteurellaceae* bacteria, namely *H. influenzae*, *G. parasuis* and *A.pp* and NETs was investigated. The NETs were either directly induced by the bacteria or by an ongoing IAV infection. Therefore, a combination of *in vitro*, *ex vivo* and *in vivo* methods was used, in combination with immunofluorescence microscopy and colorimetric assays to detect NETs.

In the first part of this study the induction of NETs by *G. parasuis* was shown for the first time. Additionally, it could be shown that *G. parasuis* survives NET-mediated killing and is able to use DNA and nicotinamide adenine dinucleotide (NAD) that is released by the degradation of NETs. This increased the growth of *G. parasuis*. In the second chapter of this thesis bronchoalveolar lavage fluid (BALF) of pigs naturally infected with IAV was investigated for NETs. For the first time, vesicular NET formation was described during an IAV infection. *In vitro* experiments showed that the BALF from IAV infected pigs had a growth-enhancing effect on lung pathogenic bacteria of the pig. As responsible substances, free DNA, NAD and sialic acids could be identified. The inflammatory milieu in the BALF samples did not enable porcine neutrophils to kill *A.pp*. In the third part of this study an experimental co-infection of a human IAV isolate and *H. influenzae* was conducted in the mouse model. The timeline of the infection

was characterized, and it could be shown that the preceding IAV infection aggravated the bacterial co-infection. During the course of the disease, the amounts of NETs increased and correlated strongly with the observed lung lesions. Additionally, the activity of the host DNase1 gradually increased. The results of this study allow future studies to test therapeutic drugs that target the formation of NETs. Especially DNase is interesting, to clarify if the beneficial effects of degraded NETs outweigh the detrimental effects of an increased release of nutrients for bacteria.

The results of this project contribute to a better understanding of the complex processes of the host-pathogen-interaction of respiratory disease and which impact NETs have.

2. Zusammenfassung

Simon Lassnig

Der Einfluss von durch Influenza A Virus ausgelösten neutrophilen extrazellulären Netzen auf bakterielle Ko-Infektionen

Das Influenza A virus (IAV) ist ein Infektionserreger, der den Respirationstrakt von Vögeln und Säugetieren, insbesondere von Menschen und Schweinen infiziert. Die Erkrankung geht für gewöhnlich mit Fieber und respiratorischen Symptomen einher. Durch die Infektion und die erzeugte Immunantwort des Wirtes laufen in der Lunge Prozesse ab, die die Anfälligkeit des Wirtes für eine bakterielle Sekundärinfektion stark erhöhen. Dadurch sind bei epidemischen Wellen und den pandemischen Ausbrüchen des 20. Jahrhunderts ein Großteil der Todesfälle bakteriell ausgelösten Lungenentzündungen zuzurechnen. Vertreter der *Pasteurellaceae* gehören zu den am häufigsten ko-infizierenden Bakterien bei IAV-Infektionen. Die wichtigsten Arten sind *Haemophilus (H.) influenzae* im Menschen und *Glaesserella (G.) parasuis* sowie *Actinobacillus pleuropneumoniae (A.pp)* beim Schwein. Während einer IAV-Infektion kommt es zu einer starken Infiltration von Neutrophilen Granulozyten, einem Bestandteil des angeborenen Immunsystems. Neben mehreren potenten Effektorfunktionen zur Bekämpfung von Krankheitserregern, können Neutrophile ihr Chromatin als neutrophile extrazelluläre Netze (NETs) ausschleusen, um Erreger zu fangen und zu töten. Vertreter der *Pasteurellaceae* können die Wirkung von NETs überleben und sich nach deren Degradierung von deren Abbauprodukten ernähren. In dieser Studie wurde die Interaktion von *Pasteurellaceae* des Menschen und des Schweins, namentlich *H. influenzae*, *G. parasuis* und *A.pp* mit NETs untersucht, die selbst oder durch eine vorherige IAV-Infektion erzeugt wurden.

Hierfür wurden *in vitro*, *ex vivo* und *in vivo* Methoden in Kombination mit Immunfluoreszenzmikroskopie und kolorimetrischen Nachweismethoden für NETs verwendet. Im ersten Teil dieser Studie wurde zum ersten Mal nachgewiesen, dass *G. parasuis in vitro* NETs in Neutrophilen des Schweins induziert, die antimikrobielle Wirkung übersteht und sich nach deren Degradierung von der frei werden DNA und Nicotinamidadenindinukleotid (NAD) ernährt und somit einen Wachstumsschub erfährt. Im zweiten Teil dieser These wurden Lungenspülproben (BALF) von Schweinen mit einer natürlichen IAV-Infektion auf NETs untersucht und zum ersten Mal die Bildung von vesikulären NETs bei IAV beschrieben. In *in vitro* Experimenten wurde gezeigt, dass die BALF-Proben von IAV infizierten Schweinen einen wachstumsfördernden Effekt auf bakterielle Lungenerreger des Schweins haben. Als auslösende Inhaltstoffe konnten freie DNA, NAD und Sialinsäuren ausgemacht werden. Das

inflammatorische Milieu in der BALF konnte porcine Neutrophile nicht bei der Eliminierung von *A.pp* unterstützen. Im dritten Abschnitt dieser Arbeit wurde in einem Tierversuch eine experimentelle Ko-Infektion mit einem humanen IAV-Isolat und *H. influenzae* in der Maus durchgeführt. Hierbei wurde der zeitliche Verlauf der Infektion charakterisiert und es konnte gezeigt werden, dass durch die vorangegangene IAV-Infektion die bakterielle Ko-Infektion verstärkt wurde. Im zeitlichen Verlauf stieg die Menge an NETs in der Lunge an und korrelierte stark mit den histologischen Schäden im Lungengewebe. Ebenso stieg die Aktivität der DNase1 kontinuierlich an. Die in diesem Studienabschnitt gewonnenen Daten ermöglichen es in zukünftigen Tierversuchen Therapeutika zu testen, die die Bildung von NETs beeinflussen. Der Fokus liegt hier auf DNase, um den Vorteil der Degradierung der NETs mit dem nachteiligen Effekt der erhöhten Nährstoffverfügbarkeit abzuschätzen.

Die Ergebnisse aus dieser Arbeit tragen dazu bei, die komplizierten Prozesse in der Wirt-Erreger-Interaktion bei respiratorischen Erkrankungen besser zu verstehen und den Einfluss von NETs einzuschätzen.

3. General Introduction

The Influenza A virus (IAV) is a viral pathogen that infects the respiratory tract of humans, pigs, and other mammals as well as birds. Due to its high mutation rate, it re-occurs in seasonal epidemics and occasionally causes pandemics with severe consequences for humans and pigs. As it can be transmitted from humans to animals it is classified as a zoonotic pathogen (**Figure 1**).

About 60 % of the pathogens known in human medicine are zoonotic pathogens(Taylor et al., 2001). Because of their natural reservoirs in animals, zoonotic diseases are nearly impossible to eradicate(Baum, 2008). The pig husbandry is an important economic sector, as pork is a major part of meat production for human consumption. The close biological proximity regarding aspects of the anatomy and physiology between humans and pigs makes it ideal as an animal model for human medical research or xenotransplantation but bears the constant risk of disease spillovers(Glud et al., 2021; Lunney et al., 2021; McGlone, 2013). In these spillover events, the pig can function as a mixing vessel for diseases from different animals, as already seen in recent IAV pandemics (**Figure 1**)(Ma et al., 2009).

Infections with IAV have been shown to increase the risk for severe bacterial co-infections. Until today, a high number of factors have been described that are involved in the susceptibility for a bacterial co-infection(B. Lee et al., 2015). One of them is the neutrophil granulocyte, a leukocyte of the innate immune system with a broad range of effector functions. In 2004, a new effector function has been described: the formation of neutrophil extracellular traps (NETs). They consist of decondensed chromatin of the neutrophil spiked with antimicrobial substances(Brinkmann et al., 2004). NETs entrap and kill invading pathogens, contributing to their clearance, but an increasing number of studies report detrimental effects of NETs(Fuchs et al., 2010; Khandpur et al., 2013; Zhu et al., 2018).

This study aimed to investigate the interaction of IAV with co-infecting bacteria and NETs in the lung.

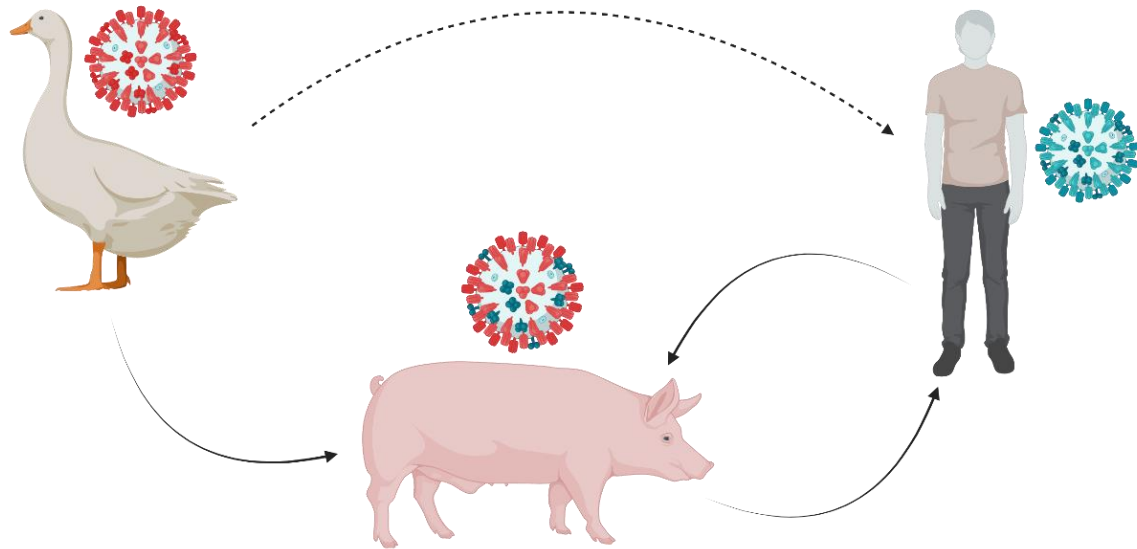


Figure 1 The direct transmission of avian IAV from birds to humans is possible but very rare (Uyeki, 2008). The pig is susceptible to IAV strains from humans and birds. As a “mixing vessel” reassortment processes in co-infected pigs can create new recombinant strains that are more adapted to humans and can cause pandemic outbreaks (prepared under license with BioRender.com).

3.1 Neutrophil Granulocytes

Neutrophil granulocytes belong to the innate immune system, as well as macrophages, monocytes, natural killer cells, dendritic cells, and the complement system(Alberts et al., 2002). They are the most abundant cell type in the leukocytes of many mammalian species. The group of leukocytes can be differentiated into granulocytes and agranulocytes. Granulocytes are neutrophils, eosinophils, and basophils, whereas monocytes and lymphocytes belong to the agranulocytes(Glenn & Armstrong, 2019). Granulocytes can be differentiated by their segmented nucleus and the presence of granules in the cytosol. The nucleus of neutrophils may vary in shape as well as the intensity of lobulation in different species, resulting in the name polymorphonuclear cells (PMN)(Fingerhut et al., 2020). In humans, neutrophils make up about 57-75 % of the leukocyte population, whereas in pigs the average is between 10-47 % neutrophils. Common animal models in infectious research as mice or hamster have around 20-30 % neutrophils, as it was reviewed by Fingerhut et al.(Breves et al., 2022; Fingerhut et al., 2020). Depending on the percentages within the white blood cells, it can be differentiated between a lymphocytic and a granulocytic hemogram. A lymphocytic hemogram has over 50 % of lymphocytes, while in a granulocytic hemogram, granulocytes outnumber lymphocytes(Breves et al., 2022). So, humans have a granulocytic hemogram, whereas pigs have a lymphocytic hemogram.

Neutrophils derive from the hematopoietic stem cell and do not divide anymore after maturation(Görgens et al., 2013; Tizard, 2017). Mature neutrophils are then released into the blood to circulate. A significant part of the mature neutrophils is not circulating but adheres to the endothelium of small vessels which is called the marginated pool. From here, the neutrophils can be recruited into circulation in a very short time during stress or upon infection(Breves et al., 2022).

A small fraction of neutrophils is resident in the lung and contributes to an anti-inflammatory state during health, by the production of Interleukin-6 (IL)(Bae et al., 2022). The majority of neutrophils is recruited upon pathogen sensing(Wengner et al., 2008). During acute infections, immature neutrophils can be released from the bone marrow to support the clearance of pathogens(Marsh et al., 1967).

Usually, neutrophils have a very short half-life span of about 19 hours (h) in circulation, until they undergo apoptosis(Lahoz-Beneytez et al., 2016). The life span of neutrophils is a topic of intensive scientific discussion(Bonilla et al., 2020). However, under inflammatory conditions, for example (e.g.) in the presence of pro-inflammatory cytokines as tumor necrosis factor- α

(TNF- α), antimicrobial peptides (AMPs) as LL-37 or β -defensins, and enzymes as myeloperoxidase (MPO) neutrophil apoptosis is suppressed (El Kebir et al., 2008; McGettrick et al., 2006; Nagaoka et al., 2006, 2008). These pro-inflammatory stimuli elongate the life span of neutrophils and prevent an early resolution of the inflammation (Bonilla et al., 2020).

Circulating neutrophils follow the chemotactic gradient of IL-8 migrating to the site of infection. They enter the tissue by transmigrating through the endothelial barrier using the binding of junctional adhesion molecules and cluster of differentiation (CD) 31 (Godaly et al., 1997; Woodfin et al., 2007). Inside the tissue, neutrophils crawl alongside the extracellular matrix of cells to the site of infection (Lämmermann et al., 2008). Neutrophils identify pathogens via pathogen recognition receptors. The pathogen-associated-molecular-patterns (PAMPs) of the microbes are sensed by Toll-like-receptors (TLRs) expressed on the surface of the neutrophil and result in their activation and the production of pro-inflammatory cytokines and chemokines, which attract more immune cells (Hayashi et al., 2003). This is supported by a broad range of cytokines produced by neutrophils to modulate the inflammation (Tamassia et al., 2018). Via IL-6, the recruitment of neutrophils is reduced to terminate the initial inflammation (Fielding et al., 2008). In case of an unregulated recruitment of neutrophils, an excessive infiltration of neutrophils into the infected tissue can occur, resulting in more harm than help (Kolaczowska & Kubes, 2013).

Neutrophils are part of the first line of defense after a pathogen has entered the body, for example via a skin lesion or by aspiration. To counteract pathogens, they exert a broad range of effector functions that will be described more detailed in the following chapter.

3.1.1 Antimicrobial effector functions

The antimicrobial effector functions of neutrophils include among others the degranulation of granular content, the generation of reactive oxygen species (ROS), phagocytosis and the formation of NETs (**Figure 2**).

As neutrophils have a short response time, they have antimicrobial substances packed in vesicles called granules, which they can secrete upon contact with pathogens. They have four groups of granules with distinct protein contents. Primary or azurophilic granules contain hydrolytic enzymes as neutrophil elastase (NE) and MPO. The secondary granules contain iron-binding lactoferrin and cathelicidins (as LL-37 in humans, PR-39 in swine or cathelicidin-related antimicrobial peptide (CRAMP) in mice). The third group is called tertiary or gelatinase

granules with matrix metalloproteinases (Udby & Borregaard, 2007). The fourth type of granules, named secretory granules, contains transmembrane receptors e.g. for TNF- α and the cationic antimicrobial peptide CAP37, as reviewed by Stock (Stock et al., 2018). The secretion is highly organized and well controlled, as the contents are highly cytotoxic. Therefore, the order of secretion is secretory granules first, then tertiary, secondary, and primary granules (Sengeløv et al., 1993).

Another powerful tool of neutrophils is the production and secretion of ROS. The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase reduces oxygen to superoxide anion (O_2^-). MPO oxidizes a wide range of substrates using hydrogen peroxide (H_2O_2), creating a broad range of ROS, e.g. hypochlorous acid (HOCl), which is the strongest ROS described so far (Winterbourn et al., 2016). A detrimental side effect of ROS production is the damage of surrounding tissue as collateral damage (Guzik et al., 2008; Prosperini et al., 2013). ROS are subsequently involved in other neutrophil effector functions, as phagocytosis.

Phagocytosis is a process of internalization of a particle into a vacuole, by extending the cell membrane around the pathogen. The resulting vacuole is called the phagosome. Phagocytosis is induced via C-type lectins receptors binding PAMPs or antibody-receptors binding opsonized pathogens. The vacuole then fuses with vesicles and the lysosome creating the phagolysosome, which acidifies the vacuole and kills the pathogen, as reviewed by Uribe-Quero and Rosales (Uribe-Quero & Rosales, 2017).

During the evolutionary arms race, pathogens have developed a broad range of mechanisms to interfere with neutrophil effector functions, to avoid their clearance, as reviewed by Urban (Urban et al., 2006). The specific mechanisms of immune evasion of the pathogens involved in this study will be addressed at later points.

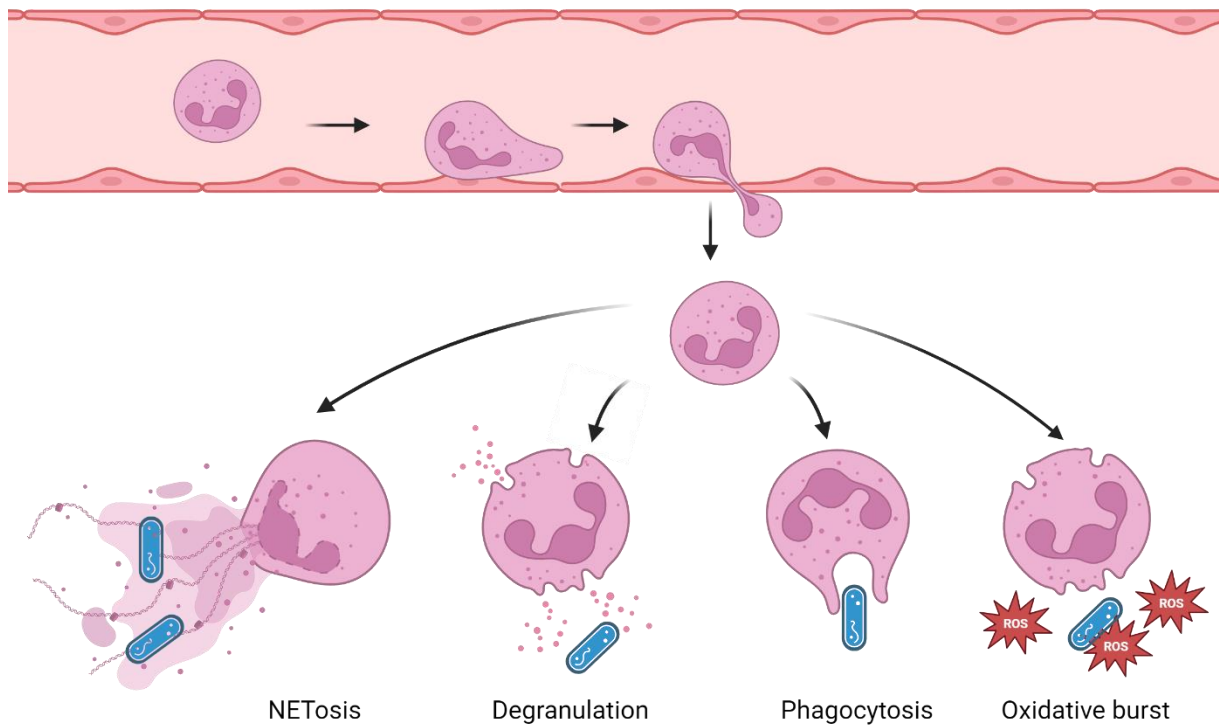


Figure 2 Neutrophil effector functions contribute to the clearance of pathogens. NETosis is a neologism of NET and apoptosis (prepared under license with BioRender.com).

3.1.2 Neutrophil extracellular traps (NETs)

Another important effector function of neutrophils is the formation of NETs. This effector function consists of decondensed nuclear chromatin that is spiked with antimicrobial peptides and is expelled in a suicidal manner. As the NET formation ends with the demise of the cells, this process is called NETosis which is a neologism from “NET” and “apoptosis”. This function was firstly detected in human neutrophils in 2004 by Brinkmann et al. as a mechanism that entraps and kills bacteria (Brinkmann et al., 2004). In the following years, several studies have identified various stimuli capable of inducing NETs. These include all types of pathogens and their surface structures, immunoglobulin (Ig) A immune complexes, pro-inflammatory cytokines, other cells, TLRs and chemical stimuli (Alemán et al., 2016; Brinkmann et al., 2004; Cadrillier et al., 2012; Guimarães-Costa et al., 2009; Keshari et al., 2012; Saitoh et al., 2012; Stacey et al., 2021; Urban et al., 2006). Further studies have shown that NETs do not only kill bacteria effectively, but also fungi, viruses, and parasites (Guimarães-Costa et al., 2009; Stacey et al., 2021; Urban et al., 2006). Neutrophils are not the only cell type that is able to release extracellular traps (ETs). They have also been found in cells as macrophages, mast cells,

eosinophils, and T-cells(Costanza et al., 2019; Von Köckritz-Blickwede et al., 2008; Wong & Jacobs, 2013; Yousefi et al., 2008).

NETs and ETs have been detected in various species besides humans, as in other mammalian species, non-mammalian vertebrates, and invertebrates, as well as in plants(Bonilla et al., 2022; Cubillo-Martínez et al., 2022; Jeffery et al., 2015; Palić et al., 2007; F. Wen et al., 2009).

The process of NET formation is a complex pathway with the involvement of several cellular components which roles are not yet fully understood. The formation of NETs is induced by the activation of surface receptors via a stimulus. The NADPH oxidase 2 produces ROS causing the migration of NE to the nucleus, in an MPO-dependent manner(Metzler et al., 2014). NE and the peptidyl arginine deiminase 4 (PAD4) are both causing decondensation of the chromatin. PAD4 causes the citrullination of histone H3(Leshner et al., 2012; Papayannopoulos et al., 2010). The nuclear membrane degrades, as the chromatin decondenses and is released into the cytosol(Y. Li et al., 2020; Neubert et al., 2018). The chromatin is decorated with different cellular components that act antimicrobial e.g. defensins, NE, lactoferrin, proteinase 3, cathepsin G or the ROS metabolizing enzyme MPO(Morán et al., 2022; Urban et al., 2009).

The first described pathway of NETosis which results in the death of the neutrophil has been renamed to suicidal NETosis, after a second pathway of NET formation had been detected. The second pathway is called vital or vesicular NETosis. In contrast to suicidal NETosis, the cell remains intact and is able to perform effector functions as phagocytosis, after the NETs are formed(Yipp et al., 2012). During vital NETosis, the chromatin is transported to the extracellular space in vesicles and only minimal lysis of neutrophils occurs(Pilsczek et al., 2010). The origin of the chromatin differs in two variants of vesicular NETosis. On the one hand, the chromatin derives from the mitochondria, on the other hand from the nucleus. The mitochondrial NET formation is a fast process that is dependent on ROS and can occur after stimulation with the complement system or granulocyte-macrophage colony-stimulating factor (GM-CSF) and lipopolysaccharide (LPS)(Yousefi et al., 2009). Vital NETosis with nuclear chromatin has been described as independent of ROS(Pilsczek et al., 2010).

NETs have two major principles how they act against pathogens. The first one is to entrap the pathogens and prevent their further spread into the tissue. Brinkmann et al. showed via electron microscopy that bacteria are getting trapped in NET fibers(Brinkmann et al., 2004). Saitoh et al. have shown that virus particles are electrostatically bound to the positively charged histones, as viral envelopes are negatively charged(Saitoh et al., 2012). The second task is to kill or at least weaken the pathogens. Therefore, several antimicrobial acting substances are attached to

the NETs, which are present in high concentrations. For example, NE can degrade virulence factors of gram-negative bacteria and α -defensins act bactericidal and virucidal (Salvatore et al., 2007; Weinrauch et al., 2002). MPO is bound to NET fibers and produces HOCl that contributes to pathogen killing (Parker, Albrett, et al., 2012). With these functions, NETs are able to kill and more importantly, trap pathogens until other components of the immune system are activated (Azzouz et al., 2018).

Bacterial pathogens do not surrender to their fate and have evolved several virulence factors which raise their survival by either inhibiting the release of NETs, degrading the NET fibers, or evading the bactericidal components, as reviewed by Schultz et al. (Schultz et al., 2022). Viral pathogens have also developed NET evading strategies. Herpesviruses encode a protein with nuclease function that could degrade NET fibers after the release from the cell (Martinez et al., 1996). Some viruses interfere with the host's cytokine production. E.g., the Kaposi's sarcoma-associated herpesvirus can cause the inhibition of the NET-inducing IL-8 (X. Li et al., 2011; Schönrich & Raftery, 2016). Parasites and arthropod-borne viruses may benefit from NET-inhibiting substances in the saliva of mosquitoes and ticks (Chagas et al., 2014).

Besides the mentioned beneficial effects of NETs, an increasing number of studies describe their detrimental effects. The most important are damaging of epithelial tissue, the formation of autoantibodies by the presentation of cellular components and the formation of immunothrombi consisting of blood cells and NET fibers (Fuchs et al., 2010; Khandpur et al., 2013; Saffarzadeh et al., 2012). To prevent uncontrolled NET formation and the associated consequences, the host secretes DNases which mediate the degradation of the NETs to resolve the inflammation (Hakkim et al., 2010). The degraded NETs and the entrapped pathogens are then phagocytosed by macrophages (Farrera & Fadeel, 2013).

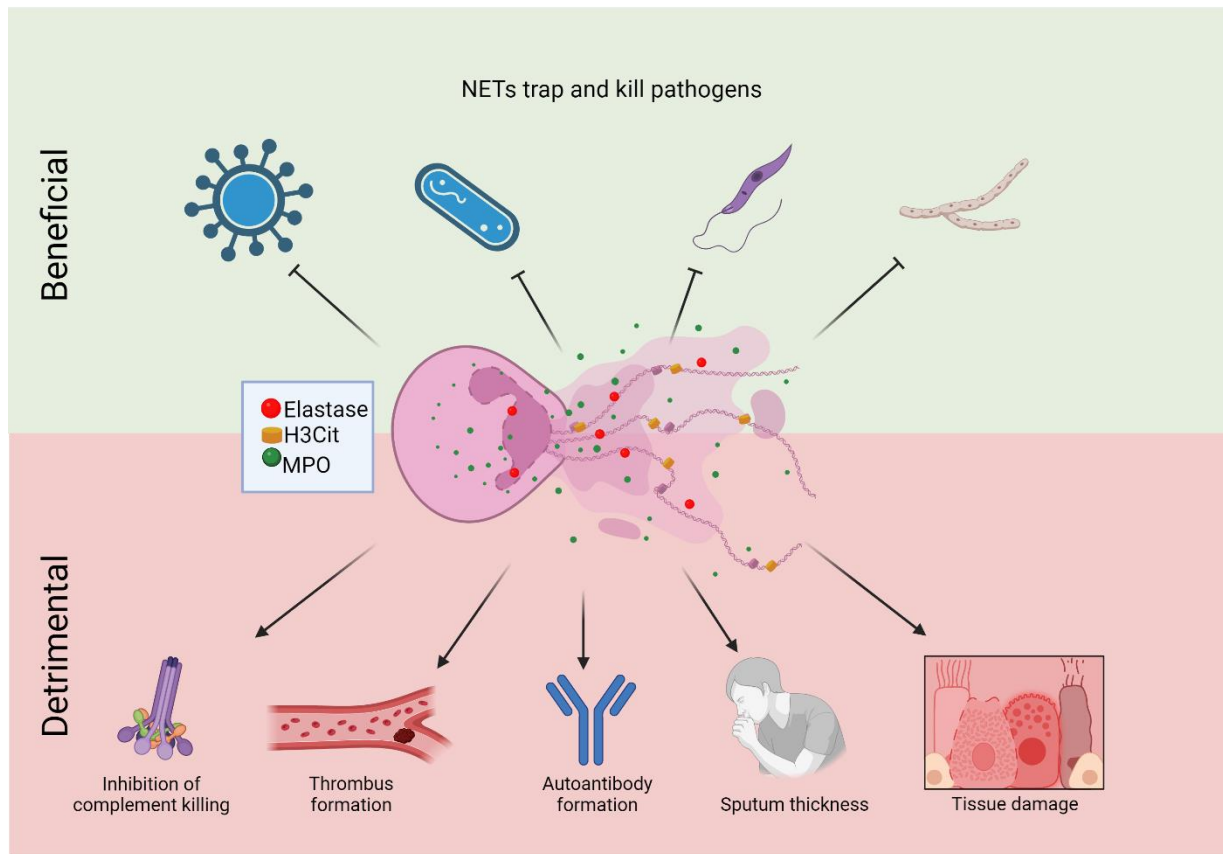


Figure 3 NETs incorporate substances with antimicrobial effects and entrap and kill pathogens. Detrimental effects of NETs contribute to morbidities (prepared under license with BioRender.com).

3.2 DNases in health and disease

DNases are a group of enzymes digesting double-stranded DNA by catalyzing the hydrolysis of phosphodiester bonds of the DNA backbone. There are three main groups of DNases, DNaseI, DNase1L3 and DNaseII(Baker et al., 1998). The first two are mainly involved in the digestions of extracellular DNA, e.g. the clearance of NETs and are dependent on cations (e.g. magnesium²⁺ and calcium²⁺)(Napirei et al., 2005). DNases contribute to physiological functions of the body. DNaseI is for example present in the blood and in the digestive tract to clear cell-free DNA(Napirei et al., 2000a). Free DNA has several origins that are not associated with diseases, as the digestion of food or exercise(Aucamp et al., 2018). The regulation of the activity of neutrophils by degrading NETs is an important task of DNases. By uncontrolled NET formation, the detrimental effects may overwhelm the beneficial effect, as for example by acute lung injury(Lefrançois et al., 2018). It is therefore conclusive that impaired DNase function is involved in several diseases(Keyel, 2017)

In diseases that involve insufficient DNase function, external admission of DNase can be used as a treatment. Recombinant human DNaseI (rhDNaseI) is an approved drug (Dornase alpha) used in the treatment of cystic fibrosis (CF) to degrade DNA present in the mucus and by this reduces its viscosity. This enhances the mucociliary clearance, alleviates the symptoms and reduces the risk of bacterial infections in the weakened lung (Suri, 2005). In different other diseases, a treatment with DNases shows promising results. For example, in the treatment of coronavirus disease 2019 (COVID-19) it has been used as an experimental drug and reduced the required oxygen ventilation in severely ill patients(Weber et al., 2020). In a mouse model with IAV and *Staphylococcus (S.) aureus* the administration of DNaseI showed beneficial effects on the severity on pneumonia(Yi et al., 2022).

Several clinical trials work on approving recombinant DNases as drugs for different disease complexes, predominantly for COVID-19 (NCT0435536) and CF (NCT02722122). However, the use of DNase treatment in COVID-19 or other respiratory infections is a topic that is under discussion in regard of potential bacterial co-infections profiting from degraded NETs, as it was reviewed by (de Buhr & von Köckritz-Blickwede, 2021)

The use of DNases to degrade NETs is not only a beneficial process for the host, but also a strategy of pathogens. A wide range of bacteria express proteins with nuclease activity that function as virulence factors by degrading DNA, as e.g. group A Streptococcus, *Streptococcus (S.) suis* or *Haemophilus (H.) influenzae*(Cho et al., 2015; de Buhr et al., 2014; Sumbly et al., 2005). Additionally, nuclease activity has also been shown for a viral protein of a herpes virus(X. Li et al., 2011). This enables the pathogens to survive the killing of the NETs and to further spread inside the tissue.

3.3 Lung infections in humans and pigs

Lung infections display a threat and a burden to healthcare in humans and animals. According to the World Health Organization (WHO), lower respiratory tract infections are the fourth leading cause of death in humans and cause the most fatalities of all transmittable diseases. In children pneumonia is the leading cause of death. This is particularly severe in developing countries with inadequate healthcare systems(Wardlaw et al., 2006; WHO, 2020). Recent pandemics showed the impact respiratory diseases have on human lives. The most important respiratory viruses in humans are next to influenza viruses, are coronaviruses, respiratory syncytial virus, rhinoviruses, adenoviruses and parainfluenzaviruses. Important bacterial

pathogens in humans are *Streptococcus (S.) pneumoniae*, *S. aureus*, *H. influenzae*, *Acinetobacter baumannii*, as reviewed by Blyth et al. and Santella et al. (Blyth et al., 2013a; Santella et al., 2021). To prevent respiratory infections, the vaccination of children against *H. influenzae* type b, *Bordetella pertussis*, and pneumococci is highly recommended in Germany. Additionally, adults are appealed to get vaccinated against influenza viruses and the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) (German Federal Ministry of Health, 2023).

Lung infections in pigs, also called porcine respiratory disease complex (PRDC), lead to economic losses in swine industry as they are the main cause of death in weaner and finisher pigs (Bush, 2015). The most important viral pathogens of the PRDC are the porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2) and swine influenza virus. Infections with PRRSV alone cause annual costs of over \$600 million in the United States, as estimated by Holtkamp et al. (Holtkamp et al., 2013). Important bacterial pathogens in pigs are *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae (A.pp)*, *S. suis*, *Pasteurella multocida* and *Bordetella bronchiseptica* as reviewed by (Boeters et al., 2023). To prevent losses, vaccination and the use of antibiotics are important, as well as improvements in housing conditions (Nielsen et al., 2021; Temple et al., 2020). The wide use of antibiotics in fattening farms, bears the risk of emerging antibiotic resistances (Lekagul et al., 2019).

3.4 Influenza A virus

The Influenza A virus (IAV) is an enveloped, single stranded, negative sense RNA virus with a genome segmented in eight parts. It belongs to the family *Orthomyxoviridae* and the genus *Alphainfluenzavirus*. The family *Orthomyxoviridae* has several other genera, including the influenza viruses B, C and D, which show differences in their antigenicity and host range. The enveloped capsid has a size of 80-120 nm and is spiked with the two glycoproteins hemagglutinin (HA) and neuraminidase (NA) and the M2 protein (Palese & Shaw, 2007). The genome segments are packed with the nucleoprotein (NP) and the polymerase to viral ribonucleoproteins (vRNP) (Eisfeld et al., 2015).

IAV infects mammals and birds, especially humans and pigs. In humans an IAV infection causes respiratory symptoms with fever, chills, and coughing (Dool et al., 2008). Annually, 300.000-600.000 fatal cases in humans during seasonal epidemics are estimated worldwide (Iuliano et al., 2018). The H1N1 pandemic from 1918 caused 20-50 million deaths

worldwide(Taubenberger & Morens, 2006). Up to 20 % of infected humans do not show clinical symptoms but can shed virus to uninfected(Ip et al., 2017). In pigs, IAV infections cause symptoms including fever, coughing, sneezing and diarrhea with relatively low mortality, but with high economic losses in livestock farming(Fouchier et al., 2003; Jo et al., 2007; Lange et al., 2009).

The transmission of IAV can occur via aerosols, droplets or contact transmission, from which aerosols are estimated to be the most important route(Cowling et al., 2013). After inhalation, the virus particles infect the epithelial cell line of the entire respiratory tract(Janke, 2014). The two glycoproteins hemagglutinin (HA) and neuraminidase (NA) are incorporated in the membrane of the viral particle, they recognize sialic acid(Sieben et al., 2020). The cell entry is mediated by binding of the viral particle via HA to sialic acid receptors causing the endocytosis into the endosome. The endosome acidifies and releases the vRNP. The vRNP is transported to the nucleus where transcription, replication, and export of vRNPs take place. The assembly of the new viral particles is located at the plasma membrane. The viral particle is released from the cell by cleavage of sialic acid by the NA, as reviewed by Samji (Samji, 2009).

Sialic acids appear in two major forms and differ by the position of the carbon atom they are bound to the subterminal galactose. Therefore, they are named α -2,3 or α -2,6 sialic acid receptors. The binding affinity of influenza viruses determines the host range, by the distribution of sialic acid variants in the tissue of host species(Zhao & Pu, 2022). Human as well as pig IAV isolates preferentially bind to α -2,6 sialic acids. This allows an interspecies transmission from human to swine and vice versa(Zhao & Pu, 2022). Chicken isolates also have α -2,6 among other subtypes, avian influenza may be transmitted to humans(Shi et al., 2013; Zhao & Pu, 2022). As pigs are susceptible to both human and avian influenza, they are considered as a “mixing-vessel” for pandemic influenza variants(Scholtissek et al., 1985).

Due to close contact of humans and pigs in rural areas and in swine industry, there is a risk of transmission of novel reassortants with epidemic or pandemic potential(Cheung et al., 2022; Jhung et al., 2013; G. J. D. Smith et al., 2009). Especially workers in the swine industry have an increased risk to transmit or to be infected(Myers et al., 2006).

Due to a strong immune pressure which applies on the glycoproteins, a broad range of antigenic subtypes have evolved. Until 2023 19 subtypes of HA and 11 subtypes of NA are known, whereas H17 and H18 and N10 and N11 are only found in bat influenza viruses(Fereidouni et al., 2023; Tong et al., 2012, 2013; Y. Wu et al., 2014).

Convalescent patients are provided with a lifelong immunity to this particular viral strain, but due to antigenic shift and drift, IAV can evade the developed immunity and re-infect a host (J. C. De Jong et al., 2000; Earn et al., 2002). Because of this, influenza vaccines have to be reevaluated and rebuild every flu season covering the likeliest lineages (Hayati et al., 2023).

Independent of an existing adaptive immune memory, an infection with IAV encounters the innate immune system and causes the secretion of IL-8, an important chemokine inducing chemotaxis of neutrophils (Henkels et al., 2011; Kowalczyk et al., 2014). IAV also induces the secretion of IL-8 in neutrophils and by this further increases the recruitment of neutrophils into the lung (J. P. Wang et al., 2008). It is well described that neutrophils rapidly undergo NET formation after contact with IAV infected tissue. The induction of NETs has furthermore been shown *in vitro* (Narasaraju et al., 2011; Stacey et al., 2021). The antiviral capability of NET components as histones, MPO and LL-37 has been shown (Hoeksema et al., 2015; Tripathi et al., 2014; K. Yamamoto et al., 1991). However, NETs are considered to be a double-edged sword during IAV. Zhu et al, described a correlation of high amounts of NETs and a poor disease outcome (Zhu et al., 2018). An exaggerated NET formation is causing acute lung injury in severely ill patients causing more harm than help (Narasaraju et al., 2011). It has been described that ROS produced by MPO cause tissue damage and promote the spread of the virus during IAV infections (Sugamata et al., 2012).

3.5 *Pasteurellaceae* in pigs and humans

3.5.1 *Actinobacillus pleuropneumoniae* (*A.pp*)

A.pp is a gram-negative, encapsulated rod-shaped bacterium of the family *Pasteurellaceae*. It is one of the most important bacterial pathogens in pigs and is distributed worldwide. It is highly contagious and in peracute infections, it can cause fatal pleuropneumonia within 24 h with hemorrhagic, fibrinous, and necrotic lung lesions (Gottschalk & Broes, 2019). *A.pp* is an asymptomatic colonizer of the tonsils from where it migrates to the lower respiratory tract upon disease onset (Müllebnner et al., 2018). Several factors causing a disease outbreak are described, e.g. stress, adverse climatic conditions, or previous infections, with other pathogens as swine influenza virus (Chiers et al., 2010; Gottschalk & Broes, 2019). This pathogen has a very high economic impact due to costs that are needed for treatment and caused by reduced growth and mortality of fattening pigs (Gottschalk & Broes, 2019). Two biotypes of *A.pp* are known and they differ by the need of an external nicotinamide adenine dinucleotide (NAD) source (V-factor).

Biotype 1 is NAD-dependent, whereas the growth of biotype 2 is independent of NAD(Pohl. et al., 1983). Until today 19 serotypes of *A.pp* have been detected and the majority belongs to biotype 1, only serotype 13 und 14 belong to biotype 2(Stringer et al., 2021). The distribution of serotypes in Europe is very heterogeneous with different prevalences in every region of Europe, but serotype 2 is overall the most prevalent serotype, followed by serotype 7 and 9(Dubreuil et al., 2000). Serotypes 5 and 7 are the most prevalent serotypes in North America(Gottschalk, 2015).

All known serotypes have varying virulence from avirulent to highly pathogenic, which influences the severity of the caused disease. Additionally, the virulence differs within a serotype regarding the region. Generally, serotypes 2, 9 and 11 in Europe and serotypes 1, 5 and 7 are highly virulent, influenced by their toxin profile(Frey, 2003; Gottschalk & Broes, 2019). The main virulence factors of *A.pp* are the *A.pp*-repeats-in-toxins (ApX). The four known toxins are named ApXI-ApXIV(Chiers et al., 2010; J. Liu et al., 2009). ApX1 is the strongest toxin with high hemolytic and cytotoxic properties, but combinations of the different toxins determine the virulence of the serotype(Frey et al., 1993).

Chronically infected animals soon become asymptomatic carriers and act as a natural reservoir infecting other animals of the herd(Chiers et al., 2010). The transmission of *A.pp* from an infected to a naïve animal happens via inhaled aerosols or via direct contact(Tobias et al., 2014). Upon infection, *A.pp* induces the production of pro-inflammatory cytokines in macrophages of the host(Z. W. Chen et al., 2011). This leads to a massive infiltration of neutrophils into the infected lung(Sassu et al., 2017). Toxins of *A.pp* damage porcine neutrophils, induce cell death and prevent phagocytosis of *A.pp*(Udeze & Kadis, 1992). *A.pp* strongly induced NETs upon contact to porcine neutrophils *in vitro* and *in vivo*(Bonilla et al., 2022; de Buhr et al., 2019). Also, the induction of vital NET formation was observed in *A.pp* infection *in vitro*(Bonilla et al., 2022). In a study from de Buhr et al, it was reported that NETs are ineffective against *A.pp* as well as the other effector functions and the bacteria are even receiving a growth boost in the presence of NETs that were degraded by DNase(de Buhr et al., 2019).

A.pp does not have an own DNase, so it is reliant on DNases of co-infecting bacteria as for example *Streptococcus (S.) suis* or on DNases provided by the host(de Buhr et al., 2019). The degradation of NETs releases the NET components, which enhance the growth of *A.pp*. NAD and adenosine were identified as growth factors. The 5'-nucleotidase enzymes were identified in *A.pp* and hydrolyze deoxyribonucleoside adenosine monophosphates to facilitate NET

components and are crucial for the NET-mediated growth boost (Bogan & Brenner, 2010; de Buhr et al., 2019).

In contrast to this, two studies claim the opposite, that NETs do act antimicrobial against *A.pp*, as their inhibition reduces bacterial killing (Bao et al., 2021; P. Chen et al., 2022). In the study of de Buhr and colleagues, serotype 2 was used, in the studies of Chen and Bao, serotype 5b was used. Considering this, a serotype dependent phenotype is conceivable and would require further investigation. A potential factor of immune evasion against NET-mediated killing is the sap transporter, which showed to grant resistance against the neutrophilic AMP PR-39 *in vitro* and in a mouse model (Xie et al., 2017a). PR-39 is a part of porcine NETs (de Buhr et al., 2019). Another mechanism would be antioxidant systems as the organic hydroperoxide reductase (ohr) that is involved in oxidative stress management (Shea & Mulks, 2002).

Next to preventive measures, antibiotics are the main therapeutic measurement, but antibiotic resistances are increasing. Effective antibiotic drugs with low frequencies of resistances are for example enrofloxacin or florfenicol (A. de Jong et al., 2023; Hennig-Pauka et al., 2022). Several effective inactivated vaccines are available, which contain combinations of prevalent serotypes. However, the vaccines are not sufficient to completely prevent *A.pp* infections and spread, as they are not protective against heterologous serovars (Kruse et al., 2017; Loera-Muro & Angulo, 2018).

3.5.2 *Glaesserella parasuis*

Glaesserella (G.) parasuis is a gram-negative, pleomorphic bacterium in the family *Pasteurellaceae*. It requires NAD (V-factor) from an external source for its growth. In 2019, it has been renamed from *Haemophilus parasuis* to *Glaesserella parasuis* (Dickerman et al., 2020). This bacterium is distributed worldwide and one of the most important pathogens in pigs. It is a common colonizer of the upper respiratory tract and causes Glässer's disease and lower respiratory tract infections with mortality rates of 5-10% (Moller & Kilian, 1990). Thus, it has a high economic impact for farmers (Costa-Hurtado et al., 2020).

Until today, 15 serotypes of *G. parasuis* have been described with their virulence ranging from avirulent to highly virulent. The strains 1, 5, 10, 12, 13, 14 are considered to be highly virulent, as they cause severe symptoms and mortality. The strains 2, 4, 8, 15 are considered to be moderately virulent, as they cause severe symptoms, but no mortality. The strains 3, 6, 7, 9, and 11 cause no clinical symptoms and are classified as avirulent, as reviewed by Kielstein and

Rapp-Gabrielson, as well as Zhang et al.(Kielstein & Rapp-Gabrielson, 1992; P. Zhang et al., 2019). Additionally, a high percentage of isolates is non-typable(Kielstein & Rapp-Gabrielson, 1992). The serotypes 4 and 5 are the most prominent serotypes in different countries, followed by 2 and 12-15(Aragon et al., 2019; Castilla et al., 2012; J. Zhang et al., 2012).

The upper respiratory tract of piglets is colonized by *G. parasuis* early in their live. During this time, maternal antibodies prevent an immediate disease outbreak(Moller & Kilian, 1990; Oliveira et al., 2004). Transmission occurs via direct contact from an infected animal to an uninfected. Asymptomatic animals often are carriers of *G. parasuis* and form a reservoir that can infect naïve animals around them. Outbreaks are often induced by stressors like transport, previous infections, or adverse air quality in the stable(Aragon et al., 2019).

Glässer's disease is a systemic infection with severe symptoms as polyserositis, meningitis and arthritis(Vanier et al., 2006). Besides this, *G. parasuis* causes lower respiratory tract infections with purulent, fibrinous pneumonia, without systemic spread(Aragon et al., 2019). Peracute infections lead to death within 48 h accompanied by high fever, coughing and other symptoms(Peet et al., 1983; Vahle et al., 1995). Animals that survive an outbreak often stay chronically infected with impaired growth and lameness(Aragon et al., 2019). After infection a protective antibody response is build that protects against homologous challenge, whereas cross-protection is limited and hard to predict(de la Fuente et al., 2009).

As soon as a stressor is applied and *G. parasuis* starts migrating, it encounters the innate immune system in the lung and avirulent strains are quickly cleared by alveolar macrophages (AM)(Olvera et al., 2009). Virulent strains need opsonization before they can be phagocytosed and cleared(Costa-Hurtado et al., 2012). Invasion of endothelial cells causes stimulation of pro-inflammatory cytokines as IL-6 (acute phase response) and IL-8 (chemotaxis of leukocytes). Virulent strains survive complement killing and can thereby cause systemic infection(Cerdà-Cuellar & Aragon, 2008a). Virulence factors involved in the evasion of complement killing in the serum are the outer membrane protein P2 and the polysaccharide biosynthesis protein CapD(X. Wang et al., 2013; B. Zhang et al., 2012).

Although *G. parasuis* is a very important porcine pathogen, it is not known if or to what extent NETs are involved in the immune response of a *G. parasuis* infection. Considering the literature that is available about *A.pp*, another important porcine *Pasteurellaceae* species, it can be assumed that *G. parasuis* is able to induce NETs and is as well resisting their function, but further research in this direction is needed to answer this open question(de Buhr et al., 2019).

Antibiotic therapy against *G. parasuis* remains an important therapy aspect. Commonly used drugs are penicillin, enrofloxacin, or florfenicol(A. de Jong et al., 2023). Resistances against antibiotics are widely detected but differ vastly between countries and high resistance frequencies against antibiotics as tetracycline or cephalothin can be detected. This is a matter of concerns(Aragon et al., 2019; Silva et al., 2022; Wiencek et al., 2022).

Since the European antibiotic reduction strategy, antibiotic usage has significantly decreased and more focus has been laid on preventive measures. This has shown positive effects on the frequencies of antibiotic resistances(Wiencek et al., 2022). Prevention of *G. parasuis* outbreaks can either be done by reducing stressors for the animals or by inducing immunity by vaccines. There are several vaccines available, but failures are very commonly caused by low cross protection of inactivated vaccines as an animal cannot be vaccinated against every serotype(H. Liu et al., 2016; Macedo et al., 2015).

3.5.3 *Haemophilus influenzae*

H. influenzae is a gram-negative, pleomorphic coccobacilli of the family *Pasteurellaceae*. Like many other members of the *Pasteurellaceae*, it is dependent on the V-factor (NAD) and additionally on the X-factor, which is hemin(Van Eldere et al., 2014). It is a part of the normal flora of the human nasopharynx and is present in the majority of healthy adults' upper airways(P. King, 2012). The colonization usually happens during the first years of life and is often acquired in places of close contact, e.g. in daycare centers. *H. influenzae* is transmitted via respiratory droplets during coughing or sneezing(Faden et al., 1995; Farjo et al., 2004).

By the composition of the polysaccharide capsule *H. influenzae* can be typed into six serotypes named from "a" to "f", additionally non-typable *H. influenzae* (NTHi) isolates without a capsule are described(Van Eldere et al., 2014). Type b has been the predominant serotype while other serotypes were very rare(McElligott et al., 2020). Since the vaccine against *H. influenzae* type b (Hib) overall cases are declining, but the proportion of NTHi cases is rising, as the vaccine is not cross protective against NTHi(Gessner & Adegbola, 2008; Takla et al., 2020). Against NTHi, no approved vaccine is available, but promising vaccine candidates using the outer membrane proteins (OMPs) are being investigated, as it was reviewed by Behrouzi(Behrouzi et al., 2017).

Important virulence factors of NTHi are the fimbriae, OMPs and lipooligosaccharides (LOS), involved in cell adherence and an IgA protease, cleaving IgA-antibodies as a method of immune evasion, (Anderson et al., 2011; Krasan et al., 1999; Swords et al., 2000; Vitovski et al., 2002).

NTHi are involved in several disease scenarios in and around of the respiratory tract. NTHi is the leading cause of conjunctivitis and middle ear infections causing 25-35 % of otitis media infections in young children(Broides et al., 2009; Patel et al., 2007). In chronic obstructive pulmonary disease (COPD), NTHi is present in the majority of cases(Wilkinson et al., 2017). It causes pneumonia with symptoms as fever, cough, and systemic upset. Risk groups for severe NTHi infections are children below five years of age and elderly persons, as well as immunocompromised (P. King, 2012; Van Wessel et al., 2011). Invasive infections with NTHi may also lead to meningitis with a case fatality rate of 10 %, but convalescent patients may suffer from significant long-term complications as hemiparesis or seizures(Collins et al., 2015).

NTHi are known to be potent NET inducer, both *in vitro* and *in vivo*. In an *in vivo* study, high loads of NETs have been found in the sputum of an NTHi patient(Hamaguchi et al., 2012). Juneau and colleagues found that the part of the bacterium that was most potent to induce NETs is the LOS. Interestingly, only ROS-dependent NET formation was observed(Juneau et al., 2011). Like *A.pp*, NTHi can resist the NET-mediated killing *in vitro* and *in vivo* and can also feed on the NETs after their degradation by DNases(Bing Pang, 2013; de Buhr et al., 2019). The inhibition of 5'-nucleotidase reduced the growth-enhancing effect, indicating that the mechanism is the same as in *A.pp*(de Buhr et al., 2019).

Two *in vitro* studies detected LOS as the main agent, which is protecting NTHi from neutrophil mediated killing, phagocytosis as well as NETs(Hong et al., 2009; Juneau et al., 2011). The *sap* transporter provides immune evasion against AMPs, which are often incorporated in NETs(Shelton et al., 2011). Additionally, antioxidative systems as catalase and peroxidorexin-glutaredoxin increase the resistance against oxidative stress and the survival in NETs(Juneau et al., 2015a).

Infections with *H. influenzae* can be treated successfully with antibiotics, as cefotaxime, ceftriaxone, and ciprofloxacin(Ibar-Bariain et al., 2021). However, the presence and emergence of antibiotic resistances in *H. influenzae* is a cause for concerns(Kiedrowska et al., 2017; Tristram et al., 2007)

3.6 Co-Infections during Influenza

IAV is a major respiratory pathogen and co-infections with other viruses, as PCV and PRRSV, in the pig, or adenoviruses, respiratory syncytial virus or SARS-CoV-2 in humans, are common(Chrun et al., 2023; Goka et al., 2013; Yan et al., 2023). In this chapter of the thesis

although, the focus shall be laid on bacterial co-infections during IAV infection. Bacterial co-infections are an important risk factor during IAV infections. During the H1N1 IAV pandemic from 1918/19, known as the Spanish flu, 95 % of the human fatalities were attributed to a bacterial co-infection with common bacteria from the upper respiratory tract (Morens et al., 2008). The major co-infecting pathogens were identified as *H. influenzae*, *S. pneumoniae*, *Streptococcus pyogenes* and *S. aureus* (Blyth et al., 2013b). In the following pandemics in 1957/58 and 1968/69 far less fatalities were reported, but again most deaths were attributed to bacterial co-infections (Morens et al., 2008; Oswald et al., 1958). In the 2009 H1N1 pandemic, about 25 % of patients administered to intensive care unit had a bacterial co-infection and *S. aureus*, *S. pneumoniae* and *H. influenzae* were the most frequent agents (Blyth et al., 2013b).

In the pig, the frequencies of bacterial co-infections after a viral infection are not as well described as in humans, but several studies describe the occurrence and severity of bacterial co-infections in IAV infected pigs (Kowalczyk et al., 2014; Pomorska-Mól, Dors, Kwit, Czyżewska-Dors, et al., 2017; Pomorska-Mól, Dors, Kwit, Kowalczyk, et al., 2017; Salogni et al., 2020; Unterweger et al., 2016; Vereecke et al., 2023). In a study about natural infections in pig breeding facilities in Italy, IAV was the most abundant first infection that was co-infected by *G. parasuis* (Salogni et al., 2020). 17 of 25 tested pig farms in Germany were infected with IAV and in the co-infected animals, *Glaesserella ssp.* made up nearly 40% of the co-infecting bacteria and significantly contributed to the severity of the clinical signs sneezing and coughing (Vereecke et al., 2023). A preceding IAV infection increased the bacterial titer, lung lesions and cytokine response of a secondary *G. parasuis* infection, in comparison to a single infection (Pomorska-Mól, Dors, Kwit, Czyżewska-Dors, et al., 2017). Comparable results have been published concerning the co-infection with IAV and *A.pp* or *Bordetella bronchiseptica* (Loving et al., 2010; Pomorska-Mól, Dors, Kwit, Kowalczyk, et al., 2017).

The processes ongoing during a respiratory infection in the lung are very complex and difficult to fully understand, as a broad range of cell types, cytokines and signal cascades are involved. Therefore, it is not surprising that a lot of parameters have been described by which an infection with IAV is preparing or benefiting a bacterial co-infection. They can roughly be classified into three groups: (1) structural damage and exhaustion effects from the preceding viral infection, (2) increased availability of nutrients and (3) a shift in the cytokine profile adapted to a viral infection.

Firstly, tissue damage is caused during the IAV infection, e.g. by an overshooting NET formation (Loosli et al., 1975; Narasaraju et al., 2011; Yi et al., 2022). The NA enzyme of IAV

can degrade mucin, damaging the mucosal barrier(Wheeler & Nungester, 1942). The damaged tissue reveals entry sites for the bacteria to adhere, enabling their invasion into the lung(Meng et al., 2019; Plotkowski et al., 1986). IAV also affects the mucociliary apparatus of the lung and thereby reduced the clearance rate of pathogens invading the lung(Pittet et al., 2010). AMs resident in the lung are depleted and need several days to recover(Ghoneim et al., 2013).

Secondarily, the previously mentioned cleavage of mucin by NA causes an increase in free sialic acid which can be utilized as a carbon source by streptococcus bacteria displaying a valuable resource in nutrient-poor tissues(Siegel et al., 2014). Genes used to metabolize sialic acids as a nutrient source have been found in a broad range of bacteria phyla, including the *Proteobacteria*, with the family *Pasteurellaceae*(McDonald et al., 2016). The utilization of sialic acids has already been shown for *H. influenzae*(Severi et al., 2005).

The third and most multifaceted step is the modulation of the immune system by the production of virus induced cytokines. Type I interferon (IFN1) and type III interferon, including IFN- α , IFN- β and IFN- λ are the most important interferons in influenza infections. They are produced immediately following the infection by airway epithelial cells, macrophages and plasmacytoid dendritic cells(Ioannidis et al., 2013). IFNs bind to cell surface receptors and induce the upregulation of hundreds of IFN-stimulated genes, shifting the immune system to an antiviral state(Randall & Goodbourn, 2008). This limits viral replication but increases the susceptibility for bacterial co-infections.

An increase in IFN1 inhibits the recruitment of neutrophils into the lung by AMs(Shahangian et al., 2009). A similar effect can be seen on the recruitment of monocytes into the upper respiratory tract(Nakamura et al., 2011). IFN1 act inhibitory on the production of IL-17 which has influence on antibacterial immunity. A lack of IL-17 reduced the production of the AMP lipocalin 2 by bronchial epithelial cells(B. Lee et al., 2015). The induced reduction in IL-1 β , IL-17, IL-22, and IL-23 reduced the maturation of Th-17 cells, resulting in a lack of bacterial clearance in the lung(Kudva et al., 2011; Z. Zhang et al., 2009).

The type II interferon Interferon- γ (IFN- γ) is produced by T lymphocytes and natural killer (NK) cells during the IAV infection. IFN- γ causes an inhibition in macrophage phagocytosis. This process which occurs late in the IAV infection, mediated by T-cells, is suspected to increase the formation of influenza-specific immunity via dendritic cells. During bacterial co-infections, this reduces bacterial clearance(Bot et al., 1998; Holt et al., 1993; Sun & Metzger, 2008). NET formation by neutrophils has been shown to be dependent on IFN- γ *in vivo*(Gomez et al.,

2015). However, NETs that are formed during an IAV infection fail to effectively clear bacterial pathogens during co-infections(Moorthy et al., 2013).

4. Aims of the study

The overall aim of this study was, to investigate the host-pathogen interaction of *Pasteurellaceae* bacteria of human and porcine origin with regard to NETs and a previous infection with IAV. Therefore, this study was subdivided into three parts which addressed the following aims.

Aim 1: How is the host-pathogen interaction between *G. parasuis* and porcine neutrophils characterized and is there a serotype dependent difference? (Chapter 5.1)

G. parasuis is an important porcine pathogen, causing severe suffering and economic losses. The formation of NETs is a powerful antimicrobial effector function but has been shown to be ineffective and even detrimental in *Pasteurellaceae* infection. Therefore, the aim of this part of the study was to investigate NET induction by *G. parasuis* with varying virulence and the antimicrobial effect of NETs *in vitro*.

Aim 2: Does an infection with IAV induce NETs in the lungs of pigs that provide growth factors for bacterial co-infection with *Pasteurellaceae* species? (Chapter 5.2)

Bacterial co-infections during an IAV infection are known to be an important risk factor for fatal disease outcome. The formation of NETs during IAV infection is described, as well as the presence of growth factors from degraded NETs. Based on this knowledge, this study aimed to analyze the milieu in the porcine lung via bronchoalveolar lavage fluids (BALF). We wanted to know, if the BALF has growth-enhancing effects on porcine *Pasteurellaceae* species and if the BALF modulates the immune response of porcine neutrophils *in vitro*.

Aim 3: How is the temporal course of the formation of NETs during a co-infection of IAV and *H. influenzae* in mice characterized? (Chapter 5.3)

Overshooting NET formation is a known parameter of NETs that is detrimental during severe IAV cases. The degradation of NETs has shown promising benefits on the disease outcome after IAV infection, but the *in vitro* described growth benefit of *Pasteurellaceae* has never been taken into account. For this reason, this study aimed to determine the time point during a

co-infection of IAV and *H. influenzae in vivo*, where the highest amount of NETs are present, to test the influence of a DNase therapy on the disease outcome in the future.

5. Results

5.1 How does *Glaesserella parasuis* interact with porcine neutrophil effector functions?

Studying the interaction of neutrophils and *Glaesserella parasuis* indicates a serotype independent benefit from degradation of NETs

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5.2 How does the milieu in an influenza A virus infected porcine lung act on lung pathogenic bacteria and porcine neutrophil functions?

Impact of bronchoalveolar lavage from Influenza-A-virus diseased pigs on neutrophil functions and growth of co-infecting pathogenic bacteria

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5.3 What is the timeline of a co-infection with influenza A virus and *Haemophilus influenzae* in a mouse model?

Temporal development of neutrophil extracellular traps during co-infection with influenza A virus and *Haemophilus influenzae*

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Abstract

Secondary bacterial infections following infection with influenza A virus (IAV) display a severe threat for patients. Neutrophil extracellular traps (NETs) are extensively produced during IAV infections and are described to have beneficial or detrimental effects. Recent studies demonstrated detrimental effects of degraded NETs during infection with *Pasteurellaceae* by providing growth factors. This study aimed to determine the amount of NETs and the DNase activity in lung lavage and serum during single and co-infection of IAV and non-typeable *Haemophilus influenzae* (NTHi). Mice were infected with the pathogens and NET-formation in the lung quantified over four days post-infection. An increased NET-formation was observed in the lungs of IAV-infected and co-infected mice. However, no decrease in bacterial load was detectable during co-infection with IAV, indicating that NETs did not act bactericidal against NTHi or antiviral against IAV. The DNase activity increased in the IAV-infected groups within the lung, whereas in serum measured at the same time it decreased. As DNase treatment of affected patients is widely discussed to reduce detrimental effects by NETs, it can be hypothesized that a measurement of serum DNase activity could therefore serve as disease marker enabling a risk assessment for the decision to treat patients with DNase.

Keywords: Influenza A virus, *Haemophilus influenzae* (NTHi), neutrophil extracellular traps, mouse, co-infection, DNase activity

Introduction

The Influenza A virus (IAV) is a zoonotic pathogen that infects various mammals as humans and pigs. It can cause a severe respiratory disease with febrile symptoms. Seasonal waves with up to 600.000 deaths worldwide occur annually(1,2). Bacterial co-infections are an important factor for disease complications. During the IAV pandemic in 1918 about 95% of the fatalities were attributed to a bacterial co-infection(3). Several pathways describe how an IAV infection is facilitating a bacterial co-infection, e.g. the destruction of lung tissue and the shift of the cytokine profile(4–6). In humans and pigs, bacteria from the family of *Pasteurellaceae* can be found as co-infecting agents of IAV infections(7,8).

Haemophilus (H.) influenzae belongs to this family and is a human pathogenic species that colonizes the upper respiratory tract. Non-typable *H. influenzae* (NTHi) causes otitis media, conjunctivitis, meningitis, and pneumonia(9,10). It migrates to the lower respiratory tract to cause illness which is favored by stress or previous illness(10).

During lung infections, neutrophil granulocytes transmigrate as one of the first cells to the infection site. Neutrophils have a broad range of effector functions, including the formation of neutrophil extracellular traps (NETs)(11). NETs consist of chromatin spiked with antimicrobial substances and are released extracellular to entrap and kill pathogens(12). The induction of NETs during IAV infection has been shown *in vitro* and *in vivo*(13,14). During co-infections NETs induced by IAV were described as ineffective in clearing bacteria(15).

DNases of the host degrade NETs routinely to prevent detrimental effects. *In vivo* therapy with DNase during severe influenza and COVID-19 alleviated symptoms and raised survival rates(16,17). Bacteria from the family of *Pasteurellaceae* have been reported to be able to survive the killing of NETs *in vitro*(18). However, the degradation of NETs liberates NET components, as NAD or adenosine, which can be taken up by *Pasteurellaceae* bacteria as growth factors and enhance the severity of the infection(18). This leads to the question if DNase therapy ends up beneficial or detrimental for the patient in case of bacterial co-infections(19).

In this study we have investigated the timeline of a bacterial co-infection with IAV as first infection and NTHi as secondary infection in mice regarding NET-formation and host DNase activity. The aim of this study was to determine the time point at which highest NET-formation occurs which might have negative effects on the course of the disease and to correlate those values with additional blood markers or histological lung damage. The data could help, to identify the period when DNase therapy could potentially have a protective therapeutic effect.

Material and Methods

Ethics statement

The infection experiment with mice was approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Hannover and the local permitting authorities in the Lower Saxony State Office for Consumer Protection and Food Safety (approval number: 33.8-42502-04-23-00425)

Preparation of viral and bacterial inoculum

The Influenza strain A/Hamburg/NY1580/09 (hereafter HH15) was thawed and diluted in sterile 1x PBS until $3,3 \times 10^4$ plaque forming units (PFU)/mL, resulting in an inoculum of 1×10^3 PFU in 30 μ L(24).

The bacterial inoculum of NTHi strain “ATCC 49766” was prepared by growing the bacteria until the exponential growth phase on the day of infection. Details are described in supplemental material.

Animals and experimental infection

Female C57BL/6J mice of 6 weeks were purchased from Janvier Labs, France. All animals lived in individually ventilated cages in groups of five in a BSL-2 stable facility with unlimited access to water and food. The animals were held two weeks prior to the start of the experiment to habituate to the new conditions.

For intranasal (i.n.) infections, mice were anesthetized with a mixture of 75 mg/kg bodyweight (bw) ketamine hydrochloride (Ursotamin 100 mg/mL, PZN-12727581 Serumwerk, Bernburg, Germany) and 5 mg/kg bw xylazine hydrochloride (Xylazin 20 mg/mL, REF: 01809, PZN-14022086, Serumwerk, Bernburg, Germany). The primary infection was done with 1×10^3 PFU HH15 in 30 μ L PBS i.n.. Three days later, the secondary infection was performed with 0.64 - 1.58×10^5 CFU NTHi in 30 μ L PBS. The control animals received 30 μ L PBS instead of the viral, bacterial or both infections. The bodyweight and clinical score of the animals was recorded once daily, until an increased clinical score would demand an observation interval of two times per day. At day 4, 5, 6 and 7 after the first infection, 5 animals per group were euthanized during an anesthesia with a mixture of 112.5 mg/kg bw ketamine and 7.5 mg/kg bw

xylazine after inhalation of 3 % isoflurane (1000 mg/g Baxter vet., LOT: 21/20A31, Baxter International, Deerfield, Illinois, United States). The euthanasia was done by final blood taking by incision of the aorta. Following, liver and spleen were removed and the trachea was exposed for bronchoalveolar lavage. The lavage was performed by an injection and aspiration of 1 mL cold PBS into the trachea with a cannula. Afterwards, the lung and heart were removed and prepared for further analysis. In the lab, a section of the right lung lobe was homogenized using a homogenizer (FastPrep24™, MP Biomedicals, Santa Ana, California, United States) with the settings: 4 m/s for 30 sec in a Lysing Matrix M (MP Biomedical, REF: 116923050-CF, Santa Ana, California, United States). The homogenate was centrifuged at 9600 x g for 10 min at 4 °C and the supernatant was taken off and aliquoted. Analysis of bacterial load in lung, BALF, blood, liver and spleen and NET formation in BALF (cells) was performed immediately, all other samples were stored at -80 °C until processing.

Bacterial and viral titer determination

The HH15 titer was determined by performing plaque assay with 40 µL of BALF as described above and then calculated as described previously(20).

The bacterial load of BALF and lung homogenate was determined by plating 20 µL of a ten-fold dilutions. 50 µL of BALF were added to 5 mL of a liquid broth. On the next day, a streak-out for single colonies was performed on agar plates and NTHi colonies were detected by typical colony morphology by two trained examiners.

The bacterial titer of liver and spleen was determined by cutting the organ half, rubbing the cut surface on an agar plate and performing a single streak-out with an inoculation loop.

The results of the bacterial load were presented as “score total bacterial load” which was calculated as follows: per test, 0-5 points were assigned to organs and BALF and total of 15 points could be reached. 0 points = negative, 1 point = detected via enrichment culture, 2 points = low grade detected on plate, 3 points = medium grade detected on plate, 4 points = high grade detected on plate and 5 points = CFU/mL counted for lung and BALF and detected in blood.

Histology of the lung

The entire left lung was instilled and immersion-fixed with 10 % neutral-buffered formalin, embedded in paraffin and longitudinal sections of 3 μm thickness were stained with hematoxylin and eosin(21,22). Extent and severity of inflammatory infiltrates, degeneration and necrosis in alveoli, airways and vessels were assessed with a semi-quantitative scoring system as previously described(23). Histopathological evaluations were performed by two board certified veterinary pathologists.

NET staining of lung section

The paraffin embedded lung tissue sections (3 μm thickness) were analyzed in batches of 10 lung slices at the same time. Sections were stained for immunofluorescence analysis as described previously 22. Details are described in supplemental data.

ELISA and DNase activity measurement in BALF and Serum

DNase I Activity Assay Kit (Abcam, REF: ab234056, Cambridge, United Kingdom) was used to determine the DNase I activity in the BALF and serum samples.

The following assays were conducted: the Citrullinated Histon H3 (Clone 11D3) ELISA Kit (REF: Cay501620-96, Cayman Chemical Company, Ann Arbor, Michigan, United States); the Interleukin-6 ELISA Kit (REF:MBS730957-96, MyBioSource, San Diego, California, United States); the C-reactive antimicrobial peptide (CRAMP) ELISA (MyBioSource, REF: MBS285843-96, San Diego, California, United States). The assays were performed following the manufacturer's instructions.

NETs scoring in histological slices from the lung

The NETs score of the lung was determined by one trained person as follows: 1. Determination of histone score: no signal = 0, histone signal normal = 1; histone signal 10 % higher = 2; histone signal 25 % higher = 3; histone signal 50 % higher = 4; histone signal 75 % higher = 5. 2. Determination of H3cit score: no signal = 0, H3cit signal 5 % = 1; H3cit signal 10 % higher = 2; H3cit signal 25 % higher = 3; H3cit signal 50 % higher = 4; H3cit signal 75 % higher = 5. 3. Total NETs lung score: for each animal three images were taken and analyzed. The sum of the histone and the H3cit score per image was calculated and finally the mean for all three images calculated.

Statistical analysis

Data were analyzed using Excel (Microsoft Office 365) and GraphPad Prism version 10.0.2 (232) (GraphPad Software, San Diego, CA, USA). Normal distribution was tested using the D'Agostino & Pearson K2 normality test. Differences and correlations between groups were tested as indicated in the figure legends.

Results and Discussion

In this study we were interested to determine NET-formation in co-infections of IAV and NTHi. We infected mice i.n. with 10^3 PFU IAV (HH15). This dose range was described to induce severe but not lethal IAV disease in C57BL/6J mice(24). Co-infection was conducted 3 dpi with 10^5 CFU NTHi (ATCC49766) i.n., causing high lethality in a co-infection study with Influenza A/Puerto Rico/8/34, H1N1 virus (PR8) after 8 dpi(25). Therefore, the last day of the experiment was scheduled to 7 dpi (**Supplementary Fig.1**). IAV infected and co-infected mice developed weight loss up to 20-25 %, calculated relative to 0 dpi and severe clinical signs of respiratory disease (**Fig.1A-B**). We have measured an increase in IFN- γ in the BALF of IAV infected mice (**Supplementary Fig.2**). Postmortem, the pathogen titers were determined in BALF. The HH15 titers reached 10^5 to 10^6 PFU/mL and no significant difference was detected comparing single or co-infected mice (**Fig.1C**). However, a slight reduction in viral titer over the timeline of the experiment could be seen in the co-infected mice. Previous studies describe that a bacterial co-infection does not influence the replication or clearance of an IAV or SARS-CoV-2 infection (6,25,26). However, one study showed an influence of secondary pneumococcal infection on IAV starting at 5 dpi (15). The bacterial infection was cleared in the single infected mice after 6 dpi, whereas the co-infected animals were still infected on 7 dpi (**Fig.1D**). The preceding IAV infection reduced the bacterial clearance and thereby enhanced the bacterial infection. Promotion of bacterial co-infection by ongoing IAV infections are well studied and well described(6,25). One reason for bacterial co-infections after IAV infection is the tissue damage inside the lung. Indeed, the histological analysis of the lungs in this study revealed severe lung lesions in the groups infected with IAV that are significant higher compared to the uninfected group (**Fig.1E, Supplementary Fig.3A**). Lung lesions were observed in the group of mice infected only with NTHi, however the score was not significantly different from the uninfected animals. One reason for tissue damage in the lung during IAV infection could be the release of NETs, as for example histones are described to be detrimental. This phenomenon was described in mice infected with a lethal dose of PR8(27). Furthermore, in this study they showed increased levels of histones inside nasal washes from influenza infected humans. This indicates that comparable mechanisms occur in mice and humans.

As NET-formation has not been investigated in the context of IAV and NTHi co-infection *in vivo*, we firstly analyzed NET-formation inside lung sections by confocal immunofluorescence microscopy and visualized DNA-histone-1 complex and citrullinated histone 3 (H3cit). During NET release histone 3 is extensively post-translational modified and a citrullination takes place

which leads to the NET marker H3cit(28,29). Mice infected with IAV showed massive levels of NETs. It was especially remarkable that NETs covered the surface of the alveoli in IAV and IAV + NTHi infected animals (**Fig.2A**). Previous studies have already shown NETs in lung tissue of IAV infected animals. However, as of the best to our knowledge, no quantification was conducted(30). We could show that the NETs lung score significantly increases in the IAV infected animals over time and were already high and significantly increased at 4 dpi in co-infected animals (**Fig. 2B, Supplementary Fig. 3B**). Interestingly, we recently performed quantification of NETs in SARS-CoV-2 infected hamsters and showed that a similar NET phenotype was observed in the lungs at 3 and 6 dpi, but with lower expression(22). However, the hamsters showed severe lung symptoms as well. This indicates that viral infections in general can massively induce NETs inside the lung, which does not lead to a reduction of clinical symptoms. At the same time, there is no decrease of bacterial load during co-infection compared to mono-infections. These data assume that IAV induced NETs are not acting bactericidal against NTHi *in vivo* during co-infection with IAV (**Fig.1D**). This goes in line with previous findings that NTHi survives *in vivo* in biofilms and is resistant to killing within NETs(31).

The strong phenotype of NET-formation was not observed in NTHi single infected animals or uninfected animals. However, NET-formation was detected in the lung tissue of NTHi infected mice which gradually increased during the infection, but not to an extent that is significantly different from the baseline in uninfected mice (**Fig.2B**). This is due to an unexpected increase of H3cit signal in some of the uninfected animals at day 6 and 7 dpi, which increased the total NETs lung score. NTHi is known to induce NETs *in vitro* and to form NET containing biofilms in the airways(31–33). However, an increased NET-formation *in vivo* has not been described yet.

Interestingly, a strong significant positive correlation between the NETs lung score and the total lung lesion score was observed (**Fig.2C**), underscoring the described observations in IAV infections for the first time with NETs quantification inside lung tissue. Excessive NET-formation was described to correlate with increased lung tissue damage in IAV infection(30). We did not detect a significant difference in lung lesion score between IAV and IAV+NTHi mice. From studies working with the same infection model, we would expect a significant increase in the lung lesions in the co-infected groups on 8 and 9 dpi(6,25).

Next to NET quantification using a semi-quantitative detection method in lung tissue, we measured specific NET markers in BALF (**Fig.3**). Levels of the NET marker H3cit in BALF

were only increased in the mice first infected with IAV, but not in the uninfected or NTHi infected mice (**Fig.3A Supplementary Fig.3C**). Furthermore, a strong positive and significant correlation was observed between H3cit and total lung lesion score as well as NETs score inside the lung. In serum, H3cit was not detectable at all. This could be due to the reason that only little H3cit is detectable in serum of healthy mice and therefore values are maybe below the detection limit range of the used commercial H3cit ELISA. However, neither the IAV infection nor the NTHi infection increased H3cit inside the blood.

Host DNases have an important function in degrading NETs during infections to prevent or reduce detrimental effects of NETs(34). It is largely unknown, how DNase activity develops during a respiratory infection. Therefore, we have measured the DNase activity in BALF and serum over the entire period of this infection study. DNase activity in BALF was significantly increased at 4 dpi and further increased until 7 dpi in IAV infected mice (**Fig.3D, Supplementary Fig.3D**). In co-infected mice, DNase activity was significantly increased on 4 and 7 dpi. NTHi single infected mice showed no increase in DNase activity compared to uninfected mice. DNase activity in the lung strongly correlated with lung lesions, the NETs score and the amount of H3cit in BALF (**Fig.3 E-G**). Therefore, the detected DNase activity is reflecting the response of the host to the massive NET-formation inside the lung. Comparable phenotypes are described in SARS-CoV-2 infected humans in blood, where an increased amount of NETs and DNase activity was detected(35). However, studies measuring DNase activity in BALF during infections are rare. Recently, we showed a decreased DNase activity in BALF from IAV infected pigs. However, the analyzed animals were all from commercial farms and had an unknown time of IAV infection. It cannot be excluded that at one point of longer infection an exhaustion of DNase activity occurs(36).

One treatment approach for lung diseases associated with massive mucus production and necrotic tissue in the alveoli is the inhalation of Dornase alpha (rhDNase), which is also known to support the degradation of NETs. This treatment for lung diseases is already used, under investigation or widely discussed, as for example in cystic fibrosis, respiratory syncytial virus infection or SARS-CoV-2 infections(37–40). When discussing the beneficial effect of NET degradation in lung diseases by application of external DNase, at least two points need to be considered. Firstly, it needs to be considered that NET degradation releases growth factors for *Pasteurellaceae* and thereby their growth may be boosted and the host-pathogen interaction of bacteria and neutrophils is shifted to an advantage of the pathogen(18,36). This could lead to an unexpected co-infection as several pathogens in the respiratory tract belong to this bacterial family. Secondly, there are only limited data available about the DNase activity inside the host

and therefore the status quo is that an application is conducted without any quantified parameter either inside the lung or, even better, inside the blood.

Intuitively, DNase administration is recommended to be started as early as possible to prevent lung tissue damage, as NETs contain various substances that cause tissue damage(41,42). The amount of NETs and the lung lesion score gradually increased (**Fig.1E, 2B**) and daily DNase administration in IAV + *Staphylococcus aureus* co-infected mice significantly reduced lung lesions(17). Considering the release of growth factors by degradation of NETs, an early DNase therapy with less NETs present results in a reduced release of NAD compared to later application. Additionally, degradation of NETs resolved inflammation(17). However, in a sepsis model, a single dose of DNase two hours after infection resulted in increased tissue damage(43).

Therefore, knowledge about the DNase activity inside the host is urgently needed. Interestingly, serum DNase activity in co-infected mice significantly decreased on 7 dpi, whereas it increased in BALF (**Fig.4A, Supplementary Fig.3E**). Dropping serum DNase activity may therefore be an indicator for increasing DNase activity and high NET-formation in the lung. However, the serum DNase activity did not correlate with the lung lesion score or the NETs lung score. Therefore, this requires further investigations with either higher numbers of tested individuals or later time points. In addition, there is no range of normal values of DNase activity described in humans. As one study described the difference of DNase activity depending on the sex in mice, further studies are needed in this regard(44).

Next to the characterization of the timeline of the co-infection and the NET-formation in the lung, we aimed to explore an easily accessible marker when DNase therapy may be indicated during infection. Blood derived markers are generally easier accessible than lung derived markers. On the other hand, lung derived markers are more significant and correlate better with the lung status. As above discussed, serum DNase activity is an interesting candidate, but in this study, it was not a clear indicator. Therefore, we additionally investigated CRAMP as it is a potential marker for NETs and is incorporated into murine NETs, but is most probably not secreted extracellularly upon bacterial stimuli(45,46). However, no differences in CRAMP levels in the serum were detected between the infection groups, although we observed a weak correlation to the NETs lung score (**Fig.4E-J, Supplementary Fig.3F**).

While we could exclude CRAMP and H3cit as a useful serum derived marker, serum DNase activity could be a topic of further investigation. However, future studies need to include more blood derived markers of inflammation to identify more significant candidates.

One other candidate could be IL-6. We measured IL-6 inside BALF and could see a significant decrease in IAV single infected animals and a tendency in co-infected animals (**Supplementary Fig.4A**). Furthermore, IL-6 correlates negatively with several of the other parameter including total lung lesion score and NETs lung score. Due to limitation of serum in this study, we were not able to measure IL-6 in serum. However, future studies could investigate this more in detail to clarify if maybe a combined measurement of DNase activity and IL-6 could be an approach to identify an optimal treatment time point of DNase in individuals suffering from lung disease.

Ultimately, future studies are needed to clarify, if the beneficial effects of degradation of NETs mediated by external DNase outweigh the potential detrimental effect of released growth factors for *Pasteurellaceae*. If so, DNase therapy can be a valuable tool in the treatment of respiratory viral infections. Additionally, diagnostics need to exclude a *Pasteurellaceae* colonization before DNase therapy may be considered.

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Figure captions

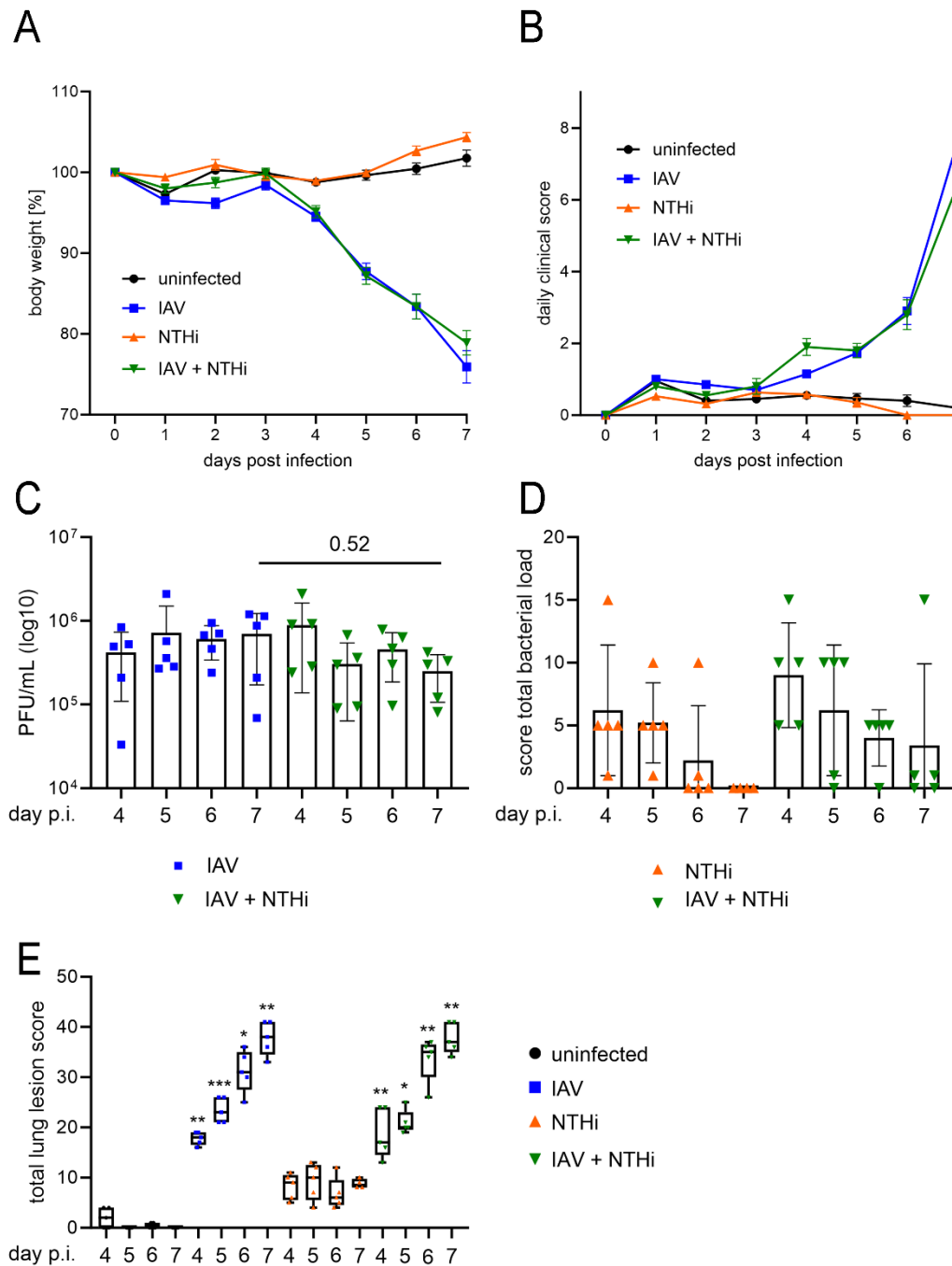


Figure 1 Pathogenicity and microbiology of IAV and *H. influenzae* (NTHi) co-infection in C57BL/6J mice over time. Eight weeks old mice were infected with 10^3 PFU HH15 or PBS on 0 dpi and co-infected with 10^5 CFU NTHi or PBS on 3 dpi. Uninfected animals received PBS on both infections ($n=20$ each group). (A) The weight loss in percent, relative to 0 dpi is depicted as mean \pm SEM. (B) The development of the clinical score is presented as mean \pm SEM. (C) Titer of HH15 in the BALF of mice in PFU/mL ($n=5$ per day and group) determined by plaque assay and presented as mean

\pm SD. No virus was detected in uninfected or bacteria single infected animals. The statistical difference was calculated with unpaired, one-tailed Student's t-test between the days. **(D)** The total score of bacterial loads in BALF, lung, liver, spleen, and blood is presented as mean \pm SD (n=5 per day and group, NTHi 7 dpi n = 4). No NTHi was detected in uninfected or IAV single infected animals. The statistical difference was calculated with unpaired, one-tailed Student's t-test between the days. **(E)** The total lung lesion score including airway, vascular and alveolar lesions in left lung lobe is significantly increased in IAV and IAV+NTHi infected mice. For each group a Box and Whisker plot with the minimum and maximum values and the individual points is presented (n=5 per day and group, NTHi 7 dpi = 4). The statistical analysis was calculated for each day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (*p < 0.05; **p < 0.01; ***p < 0.001).

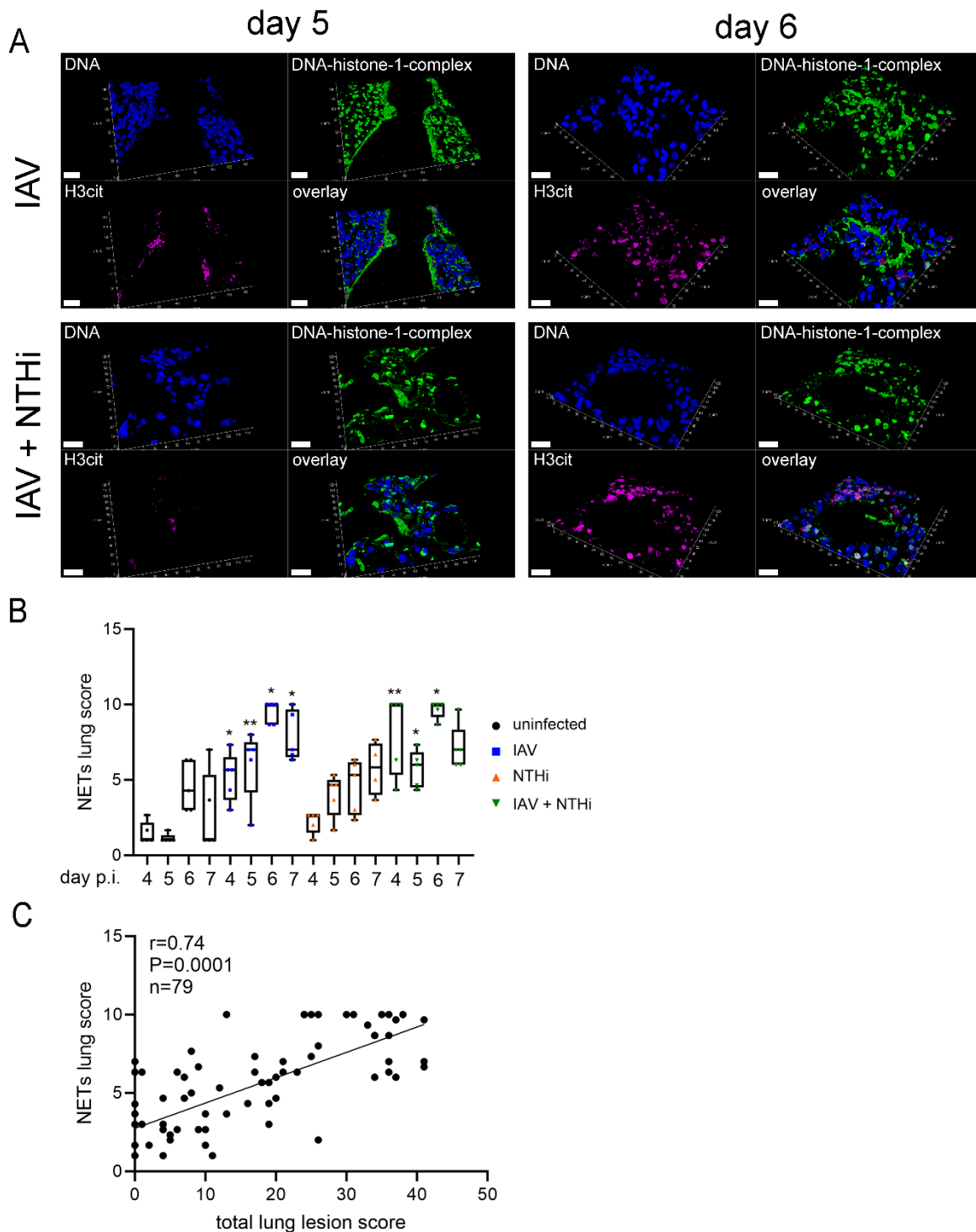


Figure 2 NET-formation is increasing over time in IAV infected and co-infected animals and correlates with the lung damage.

NETs were detected in lungs of mice infected with IAV and NTHi by confocal immunofluorescence microscopy and correlate with the total lung lesion score. (A) NETs were detected in the lungs of mice infected with IAV or NTHi and co-infected with IAV and NTHi. Representative 3D images of IAV single infection and IAV+NTHi co-infection 5 dpi and 6 dpi are shown. The 3D images of z-stacks were constructed with LAS X 3D Version 3.1.0 software (Leica);(blue = DNA, green = DNA-histone-1-

complex, magenta = H3Cit) scale bar = 20 μm . **(B)** The daily NETs lung score was calculated and is significantly increased in IAV infected and IAV+NTHi co-infected mice (n=5 per day and group; NTHi 7 dpi=4). The daily NETs lung score includes three images per animal, scored for H3cit and DNA-histone-1 complexes as described in material and methods in detail. The statistical analysis was calculated for each day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (*p < 0.05; **p < 0.01). **(C)** The NETs lung score strongly correlates with the total lung lesion score. The correlation analysis was performed by calculating Spearman r . A simple linear regression is depicted in the graph and the values r , p and n are given.

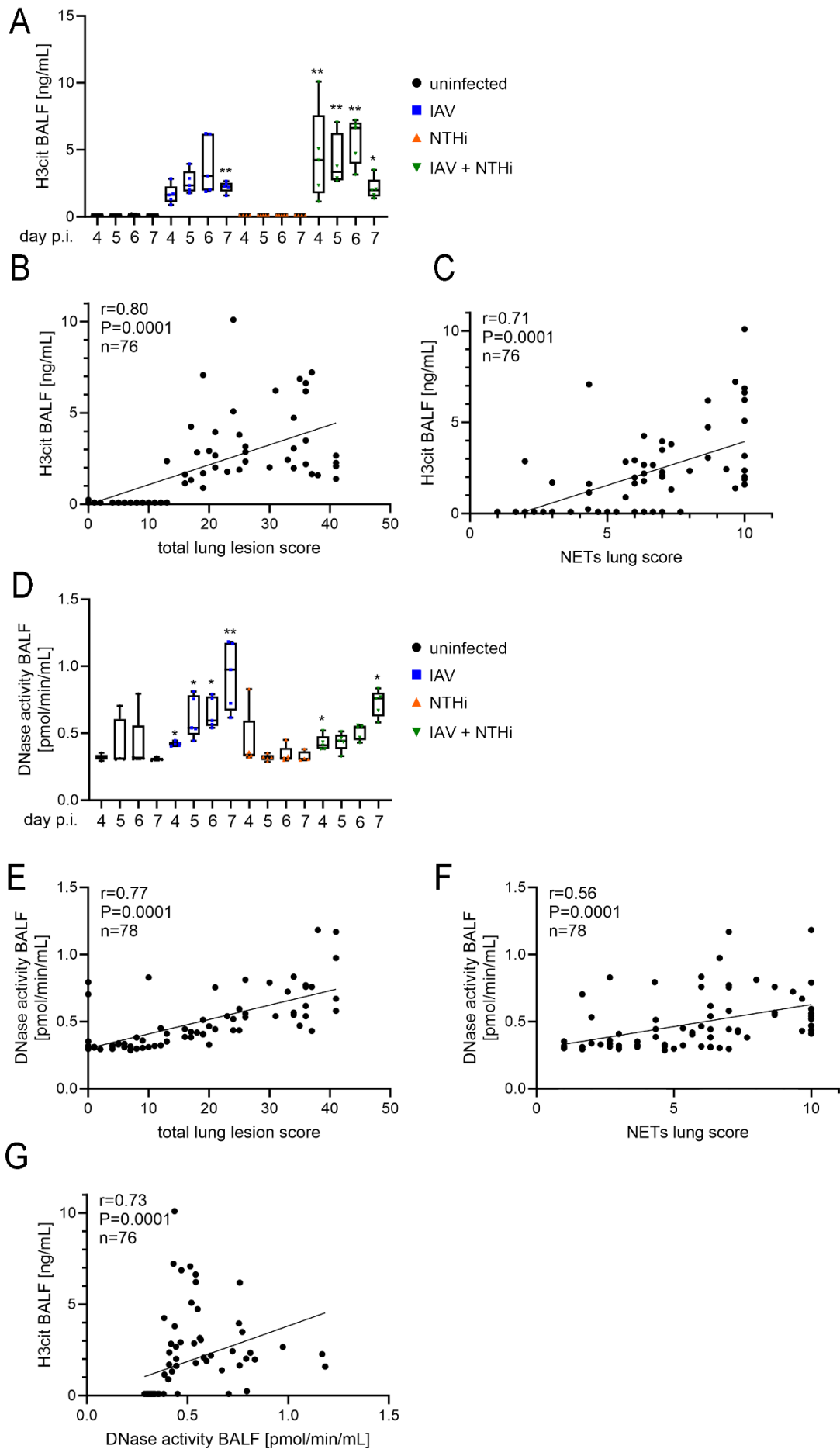


Figure 3 H3cit levels and DNase activity were measured in the BALF of mice correlated to other markers. (A) H3cit was significantly increased in the lungs of mice infected with IAV and co-infected with IAV+NTHi. For each group, a Box and Whisker plot with the minimum and maximum value and the individual points is presented. (B,C) H3cit in BALF correlated strongly with the total lung lesion score (B) and the NETs lung score (C). (D) DNase1 activity is significantly increased in the BALF of mice infected with IAV and co-infected with IAV+NTHi. For each group, a Box and Whisker plot with the minimum and maximum value and the individual points is presented. (E, F, G) DNase1 activity correlated strongly with the total lung lesion score (E), NETs lung score (F) and H3cit (G).

The statistical analysis in (A) and (D) was calculated for each day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (* $p < 0.05$; ** $p < 0.01$). The correlation analysis was performed by calculating Spearman r . A simple linear regression is depicted in the graph and the values r , p and n are given.

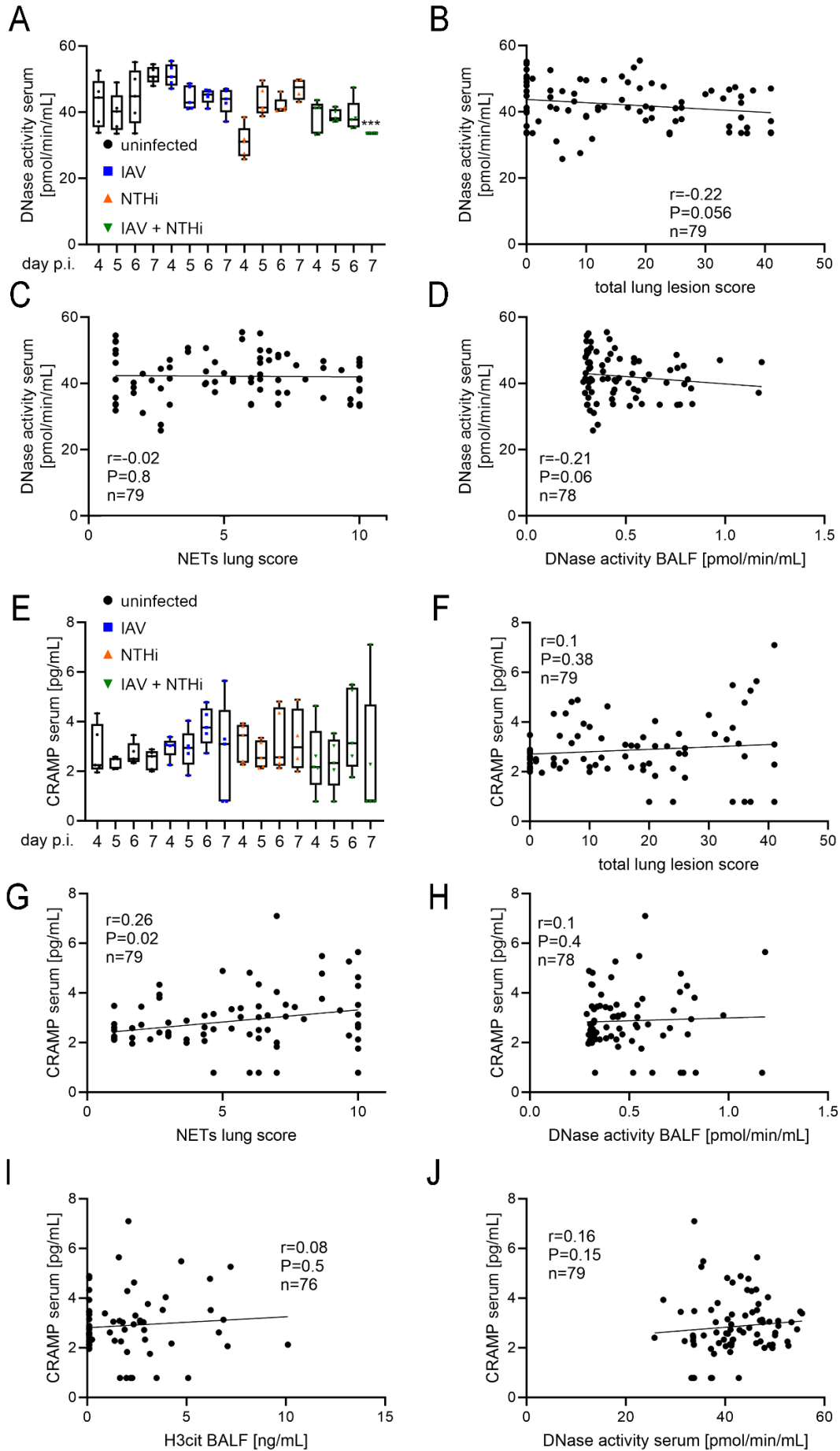


Figure 4 DNase activity and CRAMP levels were measured in the serum and correlated to other markers. (A) DNase activity is significantly reduced in the serum of mice co-infected with IAV and NTHi at 7 dpi. For each group, a Box and Whisker plot with the minimum and maximum value and the individual points is presented. (B, C, D) Serum DNase activity did not significantly correlate with the total lung lesion score (B), NETs lung score (C) and the DNase activity in the BALF (D). (E) Serum CRAMP levels have an increased range during late IAV infections but do not differ significantly. For each group, a Box and Whisker plot with the minimum and maximum value and the individual points is presented. (F, G, H, I, J) Serum CRAMP levels correlate weakly with the NETs lung score (G), but not with the total lung lesion score (F), the DNase activity in BALF (H), the H3cit levels in BALF (I) and the DNase activity in serum (J).

The statistical analysis in (A) and (E) was calculated for each day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (**p < 0.001). The correlation analysis was performed by calculating Spearman r . A simple linear regression is depicted in the graph and the values r , p and n are given.

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Supplemental Material and Methods

Preparation of viral stock

The Influenza strain A/Hamburg/NY1580/09 (hereafter HH15) was kindly provided by Professor Dr. Gülsah Gabriel and grown in Madin-Darby canine kidney (MDCK-II) cells at 37 °C and 5 % CO₂ for 48 hours until a cytopathic effect of 50-60 % was detected. The medium was taken off, centrifuged at 2000 x g for 5 min, sterile filtered and frozen at -80 °C in aliquots. The viral titer was determined by plaque assay using MDCK-II cells.

Plaque Assay IAV

MDCK-II cells were seeded to full confluency in a 6-well flat bottom plate. After washing with phosphate buffered saline (PBS) 333 µL of a ten-fold dilution series was added to each well and PBS to the last well as control. The infection was incubated at 37 °C, 5 % CO₂ and the plate was shaken every 10 minutes. After 30 min, 3 mL of the low viscosity overlay, prepared as described previously, was added to each well(1). Briefly, 2.5 % Avicel ® RC-591 (Dupont, REF: RC591-NFDR0801, Wilmington, Delaware, United States) was dissolved in ddH₂O, sterilized and on the day of infection mixed 1:1 with 2x Modified Eagle Medium (2xMEM) (ThermoFisher, REF: 21935-028, Waltham, Massachusetts, United States) and supplemented with 1 % Penicillin/Streptomycin (Sigma, REF: P433, St. Louis, Missouri, United States), 1 % L-Glutamine (Gibco, ThermoScientific, REF: 35050-038, Waltham, Massachusetts, United States) and 0.2 % bovine serum albumin (PAN Biotech, REF: P06-138810, Aidenbach Germany) and 0.1 % tosyl phenylalanyl chloromethyl ketone-trypsin (Sigma, REF: T1426-100mg, St. Louis, Missouri, United States). The plate was incubated for 72 h without moving. The cells were fixed by adding 1 mL 16 % paraformaldehyde (PFA), resulting in a PFA concentration of 4 % for at least 30 min. To stain the virus infected cells, the overlay was discarded and 1 mL 0.3 % tergitol 15-S-9 (Roth, REF: 9975.1, Roth, Karlsruhe, Germany) in PBS was added to each well for 30 min at room temperature (RT) on an orbital shaker at 60 rpm. The tergitol was removed and 400 µL of the primary antibody anti-Influenza A virus Nucleoprotein (Abcam, REF: ab43821, Cambridge, United Kingdom), diluted 1:1000 in Superblock (ThermoScientific, REF: 37536, Waltham, Massachusetts, United States) was added for 1 h, at RT and shaken on an orbital shaker at 60 rpm. The antibody was removed and the plate was washed 3 times with washing buffer (0.05 % Tween20 (Sigma, REF:P1379-100ml, St. Louis, Missouri, United States) in PBS). The secondary staining was performed with

400 μ L rabbit anti-mouse IgG-HRP, (Biozol, REF: 6170-05, Eching, Germany) (diluted 1:1000 in Superblock) for 1 h at RT on an orbital shaker at 60 rpm. The plate was washed twice with washing buffer. 350 μ L KPL TrueBlue™ Peroxidase Substrate (SeraCare, REF: KPLI50-78-02, Milford, Massachusetts, United States) were added and incubated at RT until blue color became visible. The plates were washed with tap water, dried and the blue plaques were counted. The counted plaques were calculated into plaque forming units (PFU)/mL.

Preparation of bacterial stock

NTHi strain “ATCC 49766” was grown on a Columbia agar plate with chocolated horse blood (Oxoid, ThermoFisher, REF: PB0124A, Waltham, Massachusetts, United States) for 24 h at 37 °C and 5 % CO₂. To grow NTHi in liquid culture, a colony of NTHi was inoculated in 10 mL pleuropneumoniae-like organisms medium (PPLO) (ThermoFisher, REF: 11738862, Waltham, Massachusetts, United States) supplemented with 1 % Tween80 (Roth, REF: 9139.1, Karlsruhe, Germany) (10 % in ddH₂O), 1 % IsoVitaleX substitute and 5 % laked horse blood (Oxoid, ThermoFisher, REF: 11464149, Waltham, Massachusetts, United States) in a Simport tube, shaking at 200 rpm at 37 °C and 5 % CO₂. The supplement solution (IsoVitaleX) was prepared as described previously(2). IsoVitaleX enrichment is needed for an optimal growth of NTHi and supplies the V-factor nicotinamide adenine dinucleotide (NAD) and chemically defined substances for the cultivation of nutritionally fastidious microorganisms.

The NTHi ATCC 49766 strain was kindly provided from Professor Dr. Barbara Kahl and was recovered from Columbia agar plate with chocolated horse blood and grown in liquid culture to an OD_{600nm} of 0.7, mixed with 15 % glycerol and frozen in aliquots at -80 °C.

Growth conditions of NTHi to prepare the infection inoculum

NTHi was grown on Columbia agar plate with chocolated horse blood overnight. The next day, one single colony was transferred into liquid culture and grown for approximately 3 h until reaching an OD_{600nm} of 0.7. The culture was then cooled on ice and washed three times with PBS, by centrifugation for 5 min at 2000 x g, taking off supernatant and adding 10 mL PBS. After the last centrifugation step, the pellet was resuspended in 3 mL PBS and the OD_{600nm} was adjusted to 0.1, which equals $\sim 1.7 \times 10^8$ colony forming units (CFU)/mL. Dilution by factor 1:50 results in an inoculum of 3.3×10^6 CFU/mL.

NET staining of lung section

For NET detection paraffin sections of lungs from all animals were analyzed. Samples were deparaffinized and handled as previously described(3). NETs in these samples were stained as described previously with minor changes(4). Briefly, the H3cit and DNA-histone-1 complex was stained using as primary antibodies: a mouse anti DNA/histone 1 (Sigma Aldrich, Millipore, IgG2a; REF: MAB3864; 1.5 mg/mL, diluted 1:273, Billerica, Massachusetts, USA) and a rabbit anti-human H3cit (Abcam, REF: ab5103, 1mg/mL diluted 1:25, Cambridge, United Kingdom) in blocking buffer. A respective isotype control was included in each staining batch. Therefore, murine IgG2a (Sigma Aldrich, from murine myeloma, REF: M5409-1mg, 0.2 mg/mL, diluted 1:36.4 Munich, Germany) and rabbit IgG (Sigma Aldrich, from rabbit serum, REF: I5006, 1.16 mg/mL, diluted 1:29, Munich, Germany) were included. All antibodies were incubated overnight at 4 °C.

As secondary antibodies goat anti-mouse Alexa 488Plus (Invitrogen, REF: A32723, 2mg/mL, Carlsbad, CA, USA) and goat anti-rabbit Alexa 568 (Invitrogen, REF: A11011, 2mg/mL, Carlsbad, CA, USA), both diluted 1:500 in blocking buffer, were used. All samples were processed using the TrueVIEW autofluorescence quenching kit (Vector Laboratories, REF: SP-8400-15, Newark, California, United States) following the manufacturer's instructions and counterstained using Hoechst 33342 (Sigma Aldrich, REF: 14533-100MG, diluted 1:1000, stock 50 mg/mL, Munich, Germany).

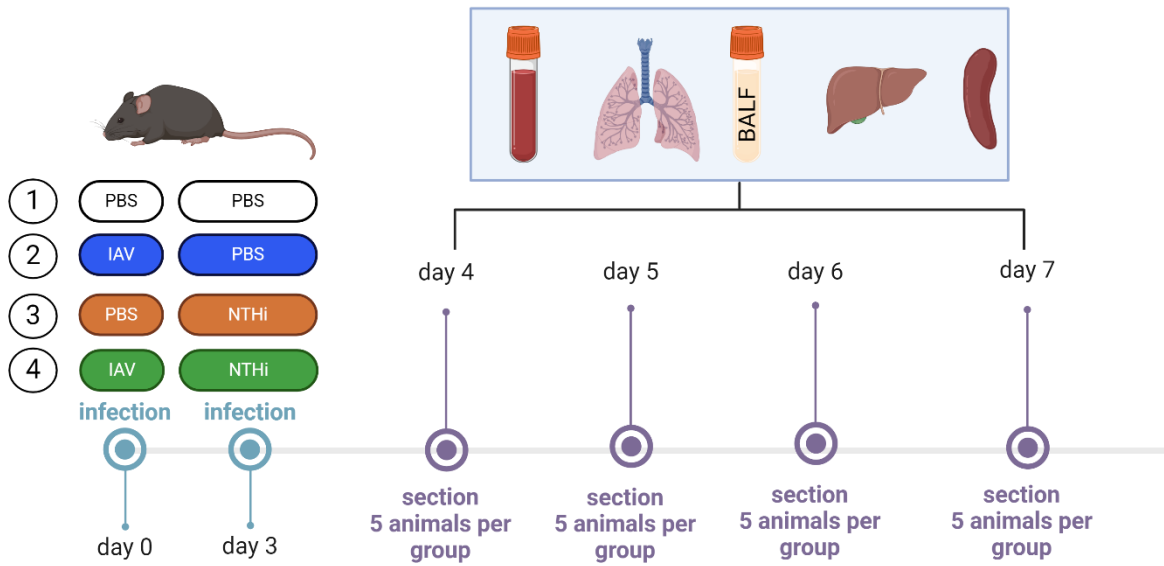
Microscopy of NET-stained lung sections

The sections were imaged using a Leica TCS SP5 AOBS confocal inverted-base fluorescence microscope with an HCX PL APO $\times 40$ 0.75–1.25 oil immersion objective. The settings for each batch were adjusted to their respective isotype controls. Per lung slice 3 images were randomly taken without overlap. The 3D images were conducted using 3D reconstruction of z-stacks with LAS X 3D Version 3.1.0 software (Leica).

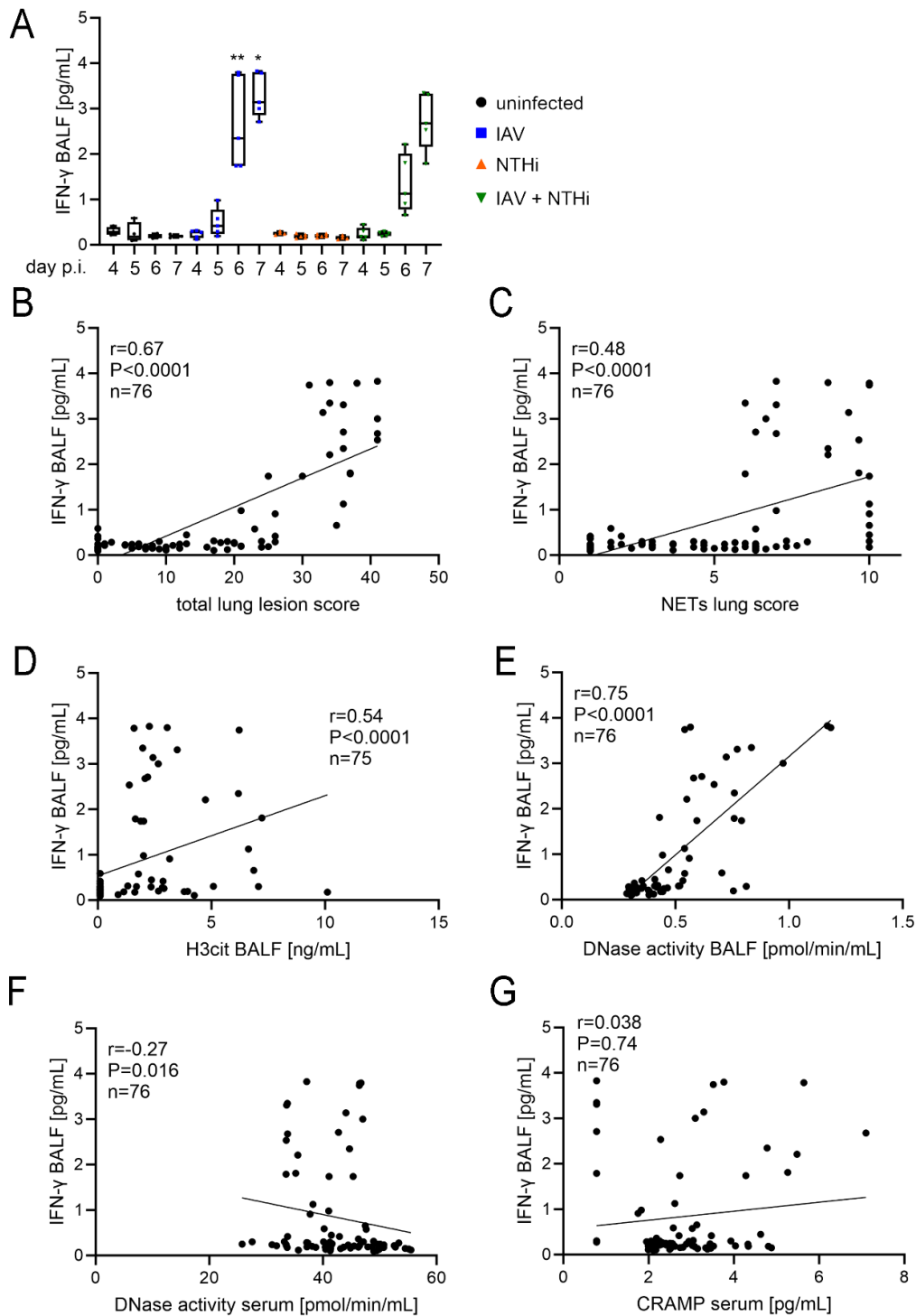
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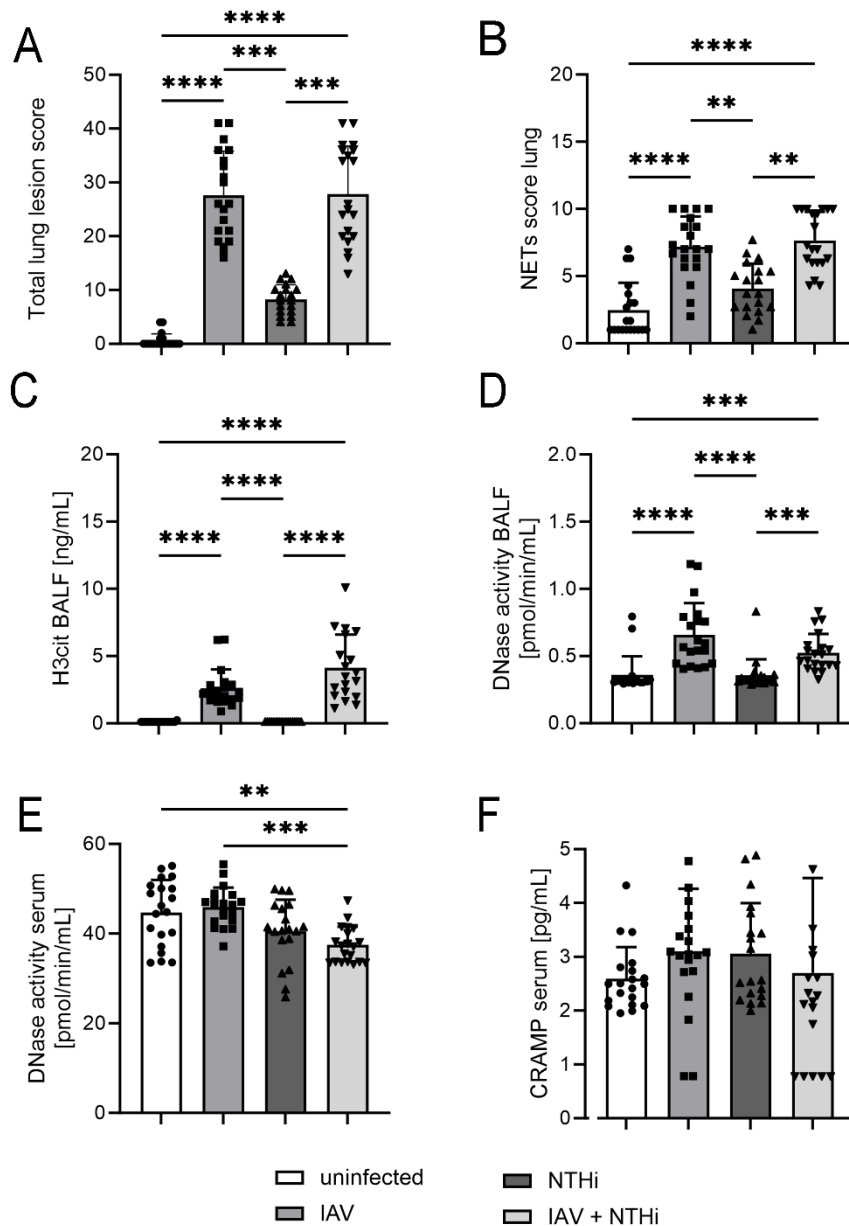
Supplemental figures



Supplementary Figure 1 The course of the mouse experiment is shown. Infection with IAV or PBS was conducted on day 0. Infection with NTHi or PBS occurred on day 3. On days 4-7 post infection, 5 animals from each of the four infection groups were euthanized and serum, lung, bronchoalveolar lavage fluid (BALF), liver and spleen samples were taken for further analysis.

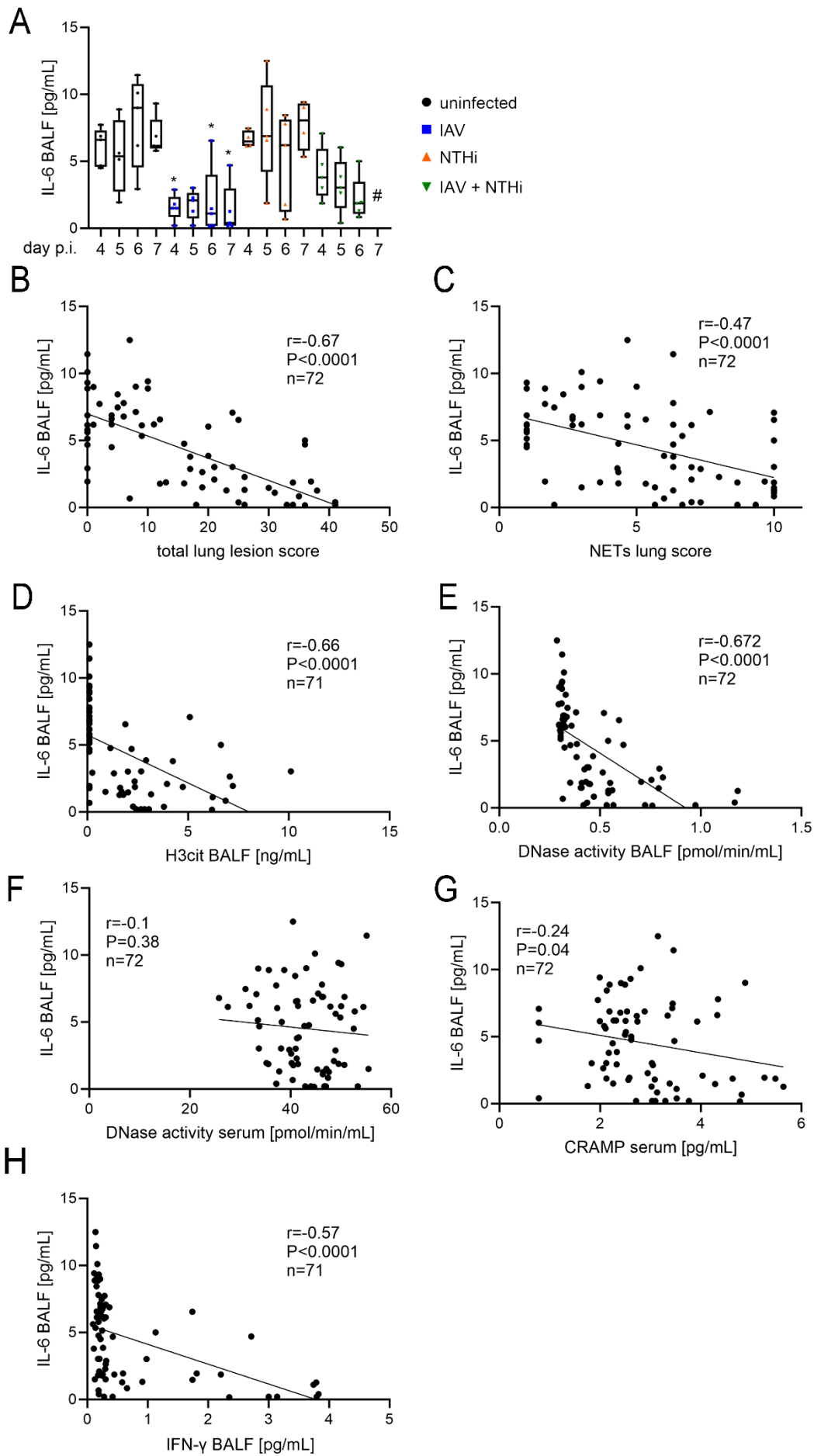


Supplementary Figure 2 The amount of IFN- γ in BALF is increasing at day 6 post infection in the IAV infected animals and correlates with several markers and scores. (A) Values of IFN- γ in BALF are presented as box and whiskers with individual values for each animal. The statistical analysis was calculated for each section day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (*p < 0.05; **p < 0.01). (B-G) Correlation analysis of IFN- γ in BALF with all detected scores and measured markers in BALF serum were calculated. The values were calculated with a nonparametric Spearman correlation (two-tailed) and for orientation a simple linear regression line was added.



Supplementary Figure 3 The graphs show the results from all animals from all section days in one column per infection group. Each sign reflects one animal. (A) Total lung lesion score, details are presented in **Figure 1E** (uninfected n= 20, IAV n=20, NTHi n=19, IAV + NTHi n=20). (B) NETs score lung, details are presented in **Figure 2** (uninfected n= 20, IAV n=20, NTHi n=19, IAV + NTHi n=20). (C) Amount of H3cit measured as NET marker in BALF, details are presented in **Figure 3A** (uninfected n= 19, IAV n=20, NTHi n=18, IAV + NTHi n=19). (D) DNase activity in BALF measured as NET regulating factor, details are presented in **Figure 3D** (uninfected n= 19, IAV n=20, NTHi n=19, IAV + NTHi n=20). (E) DNase activity in serum measured as NET regulating factor, details are presented in **Figure 4a** (uninfected n= 20, IAV n=20, NTHi n=19, IAV + NTHi n=20). (F) Amount of CRAMP in serum measured as NET factor, details are presented in **Figure 4E** (uninfected n= 20, IAV n=20, NTHi

n=19, IAV + NTHi n=20). The statistical analysis was calculated with one-way ANOVA using Dunn's multiple comparisons test (**p < 0.01; ***p < 0.001; ****p < 0.0001). All data are presented as mean \pm SD.



Supplementary Figure 4 The amount of IL-6 in BALF is lower at all days post infection in the IAV infected animals and correlates negative with several markers and scores. (A) Values of IL-6 in BALF are presented as box and whiskers with individual values for each animal. The statistical analysis was calculated for each section day with one-way ANOVA using multiple comparisons test calculating differences from all infection groups to the uninfected group (* $p < 0.01$). Sign # shown at day 7 post infection marks that in the coinfection group no values could be measured due to a technical error. **(B-H)** Correlation analysis of IL-6 in BALF with all detected scores and measured markers in BALF serum were calculated. The values were calculated with a nonparametric Spearman correlation (two-tailed) and for orientation a simple linear regression line was added.

6. Discussion

Neutrophils are an important part of the innate immune system with powerful effector functions to combat invading pathogens. However, neutrophils have often been described as a double-edged sword that can harm its wielder under certain circumstances. Overshooting formation of NETs has been described to be detrimental in various diseases. It is important to investigate the influence of NETs in the context of viral-bacterial co-infections to develop better treatments and to reduce the burden of infectious diseases, as for example of the respiratory tract.

This study investigated the interaction of the porcine pathogen *G. parasuis* with neutrophils and the effectivity of their effector functions to control an infection [Chapter 5.1]. Furthermore, the effect that IAV has on the inflammatory milieu of the porcine lung was investigated in naturally infected pigs. Parts of this study focused on how an IAV infection affects neutrophil effector functions [Chapter 5.2]. Based on these results, we investigated the co-infection of a human IAV strain and the bacterium *H. influenzae* in mice, regarding the formation of NETs [Chapter 5.3]. Taken together, these results have improved our understanding of the interaction of *Pasteurellaceae* bacteria and NETs and allow us to find targets for potential therapeutics.

6.1 *Pasteurellaceae* induce NETs

In 2004 the induction of NETs by bacteria has been shown by Brinkmann et al. (Brinkmann et al., 2004). Since then, many studies have demonstrated the induction and the effectivity of NETs against various bacterial pathogens but also viruses, parasites, and fungi (Brinkmann et al., 2004; Guimarães-Costa et al., 2009; Stacey et al., 2021; Urban et al., 2006).

For *G. parasuis* the induction of NET formation was not known. Therefore, we analyzed NET formation in porcine neutrophils by three serotypes of *G. parasuis* using immunofluorescence microscopy. We have detected that *G. parasuis* induced NETs to a lesser extent [Chapter 5.1. Figure 2] than *A.pp* did in porcine neutrophils in the study from de Buhr et al. (de Buhr et al., 2019). NTHi induced NETs in human blood derived neutrophils to an extent of 40 %, which is less than *A.pp* but more than *G. parasuis* (P. T. King et al., 2015). It is important to note that in the study of King et al. a MOI of 100 was used, whereas in the studies with *A.pp* and *G. parasuis* a MOI of 0.1-2 was used. Juneau et al. used an MOI of 0.01 of NTHi and detected comparable amounts of NETs in human neutrophils (Juneau et al., 2011). Therefore, it is unlikely that the increased NET induction in comparison to *G. parasuis* is owed to a higher MOI. During severe bacteremia, the ratio of neutrophils to bacteria in the blood does not fall below 100:1 neutrophils versus bacteria, which would represent an MOI of 0.01 (Y. Li et al., 2002). So, MOIs of 100

produce results that are hardly transferable to *in vivo* situations. Additionally, during interspecies comparison of NET-formation, it is necessary to keep in mind that a stimulus may have a different effect on neutrophils of another species. For example, PMA is a potent inducer in human neutrophils, causing NET-positive cells of 80-100 %, whereas in porcine neutrophils, only about 20 % NET-positive cells are observed (Bonilla et al., 2022; García-Bengoa et al., 2023).

We have discussed a possible cause for the difference in NET induction by *G. parasuis* and *A.pp* in porcine neutrophils. The first possible factor is the production of toxins. *A.pp* is producing the RTX exotoxins ApXI-IV, which are an important virulence factor. RTX toxins are pore forming toxins causing membrane damage and are potently causing activation and cell death in porcine neutrophils (Jansen et al., 1995; Welch, 1991). ApX toxins are described to bind to CD18-receptors and NET induction by *A.pp* was significantly reduced by blocking CD18 by Bonilla et al. (Bonilla et al., 2022; S.-C. Li et al., 2021). *Mannheimia haemolytica* is a bovine respiratory pathogen of the *Pasteurellaceae* that produces RTX toxins and induced NETs in bovine neutrophils via its RTX leukotoxin in a CD-18 dependent manner (Aulik et al., 2010). As an opportunistic pathogen, *Mannheimia haemolytica* does also infect pigs, causing pneumonia (Angen et al., 1999). So, the induction of NET formation via Apx toxins is conceivable. In *G. parasuis* however, no comparable toxins are known, which leaves the assumption that *G. parasuis* is not as strongly activating neutrophils as it is described for *A.pp*.

A second possible cause for reduced amounts of NETs could be a degradation by nucleases, as the nuclease SsnA from the porcine pathogen *S. suis* is used for NET evasion (de Buhr et al., 2014). In [Chapter 5.1. Figure 3] we have demonstrated that *G. parasuis* shows nuclease activity. However, visual comparison of the NET morphology indicates that the nuclease activities of *G. parasuis* and *H. influenzae* are not primarily contributing to the low numbers of NETs visible in *in vitro* assays [Chapter 5.1. Figure 2] (Cho et al., 2015; P. T. King et al., 2015). In *H. influenzae*, which is not secreting toxins, the LOS were identified as the most potent NET inducing factor (Juneau et al., 2011). Therefore, the LPS of *G. parasuis* displays an interesting target to investigate which component of the bacterial cell is the main NET inducing factor of *G. parasuis*.

From studies with *A.pp* and *H. influenzae* we know that they are inducing NETs to a high extent, but that these NETs are not effective in clearing the pathogens (de Buhr et al., 2019; Juneau et al., 2011). In [Chapter 5.1, Figure 4] we have seen that *G. parasuis* is not being killed by present porcine neutrophils, but rather grows to a higher extent. In contrast, to *G. parasuis* and

A.pp although, the before mentioned *Mannheimia haemolytica* is entrapped and killed by bovine neutrophils and NETs(Aulik et al., 2010). This indicates that the resistance against NET-mediated killing is not related to the RTX toxins and is not a general ability of the *Pasteurellaceae*.

As the enhanced growth of *G. parasuis* in the presence of neutrophils looked familiar to the phenotype seen in neutrophil mediated killing of *A.pp* and *H. influenzae*, we were interested if the molecular pathway is the same. In [Chapter 5.1, Figure 5] we have confirmed that the growth-enhancement, caused by neutrophils is following the same biochemical pathway that is dependent on the 5'-nucleotidase as in the other V-factor dependent *Pasteurellaceae* species *A.pp* and *H. influenzae*(de Buhr et al., 2019). These insights may display a potential target for future therapeutics against V-factor dependent bacteria to counteract increasing antibiotic resistances, as reviewed by (Gerlach & Reidl, 2006).

6.2 NET evasion of *Pasteurellaceae*

NETs consist of decondensed chromatin of the nucleus or the mitochondria of neutrophils and are decorated with antimicrobial substances to entrap and kill invading pathogens(Brinkmann et al., 2004). The incorporated substances can either act antimicrobial directly or indirectly via the production of ROS. The resistance against NETs may be mediated by a resistance against one or more components of NETs.

The first potential way of NET evasion would be to avoid the formation of NETs in the first hand. Several strategies have evolved for this. The secretion or induction of IL-10 inhibits the formation of NETs via inhibition of ROS. This is described for example for group B streptococci, but also *A.pp* is inducing IL-10 during the commensal colonization of the tonsils(Carlin et al., 2009; Müllebnner et al., 2018). Another potential way of inhibiting NET formation is the inhibition of oxidative burst, as one pathway for NET formation is ROS-dependent(Kirchner et al., 2012). *Bordetella pertussis* uses an adenylate cyclase toxin to inhibit oxidative burst in human neutrophils and subsequently the formation of NETs(Eby et al., 2014). *Lactobacillus rhamnosus* is significantly decreasing oxidative burst in neutrophils and thereby inhibits NET formation by further stimuli(Vong et al., 2014). *In vitro* however, *A.pp*, *G. parasuis* and *H. influenzae* have been shown to be potent NET inducer [Chapter 5.1, Figure 2] so avoiding the formation of NETs is not the primary evasion strategy of the bacteria involved in this study(de Buhr et al., 2019; Juneau et al., 2011). In NTHi infected mice

[Chapter 5.3, Figure 2] only insignificant amounts of NETs were formed in comparison to the uninfected animals. Although the *in vitro* NET formation of NTHi was quantified, no data quantifying the *in vivo* NET formation by NTHi has been available in the literature (P. T. King et al., 2015).

After the formation of NETs, the immediate degradation of the NET fibers is a strategy to evade the antimicrobial properties and the subsequent killing by macrophages phagocytosing the NETs. Several bacteria as *S. suis*, *S. aureus*, or *Mycoplasma pneumoniae* have developed nucleases that were described to degrade NETs (de Buhr et al., 2014, 2015; Thammavongsa et al., 2013; T. Yamamoto et al., 2017). *A.pp* does not produce a nuclease and is thereby dependent on nucleases from co-infecting bacteria or the host (de Buhr et al., 2019). For *H. influenzae* the nuclease NTHi Nuc and for *G. parasuis* the cytolethal distending toxin subunit B (CdtB) are described to have nuclease activity **[Chapter 5.1, Figure 3]** (Cho et al., 2015; G. Li et al., 2017). For *H. influenzae* it has been demonstrated that the nuclease is involved in remodeling of biofilms *in vivo*, but a direct NET degrading effect outside of biofilms has not yet been shown (Cho et al., 2015). However, it is conceivable that the nuclease activity of *H. influenzae* has contributed to the low amount of NETs that were observable in the lungs of NTHi infected mice **[Chapter 5.2, Figure 2]**. To clarify, to which extent these two proteins contribute to the evasion of NETs, further investigations are needed.

If bacteria cannot degrade NETs by nucleases, they need abilities to resist the antimicrobial properties of the NET components. Next to the entrapment and the prevention of the further spread of the pathogen, the killing of the NETs is a part of their function. The most important antimicrobial parts of NETs are the production of ROS and the incorporation of AMP s with direct antimicrobial activities (Papayannopoulos & Zychlinsky, 2009).

The MPO is incorporated into the NET fibers, as well as the NADPH oxidase to produce local zones with high concentrations of ROS (Munafo et al., 2009; Parker, Albrett, et al., 2012). In a study by Munafo et al., it was shown that DNase degradation of NET fibers inactivated the ROS production of attached NADPH oxidase and MPO (Munafo et al., 2009). It is conceivable that nuclease activity of *H. influenzae* and *G. parasuis* inhibits extracellular ROS production and increases their survival.

In NETs, extracellular ROS production damages bacteria by unfolding proteins (Parker, Albrett, et al., 2012). To counteract the damaging effect of ROS, antioxidant systems have evolved in bacteria. OxyR is a transcription factor which has been detected in many bacterial species (Christman et al., 1989; Rocha et al., 2000). It is oxidized during oxidative stress and

induces the expression of antioxidant enzymes as hydrogen peroxidase I or glutathione reductase (Zheng et al., 1998). OxyR has been described in all three *Pasteurellaceae* species that are included in this study (Guo et al., 2023; Harrison et al., 2007; Y. Wen et al., 2018). It neutralizes H₂O₂, the substrate of MPO which is incorporated into NET fibers to produce extracellular ROS (Parker, Albrett, et al., 2012). Mutants lacking OxyR show impaired growth *in vitro* and for mutants of *A.pp* and *H. influenzae* reduced virulence *in vivo* has been demonstrated (Guo et al., 2023; Honn et al., 2017; Y. Wen et al., 2018; Whitby et al., 2012).

In *Francisella tularensis*, a zoonotic, intracellular bacterial pathogen of the rabbit that infects leucocytes and may cause pneumonia, oxidative resistance by OxyR has been described (Honn et al., 2017). This bacterium does also induce NETs *in vitro* and *in vivo*. The NET formation was independent of NADPH oxidase, but dependent on MPO and addition of H₂O₂ significantly increased NET formation. This is a hint that OxyR dependent scavenging of H₂O₂ interferes with NET formation and increases survival. Comparable to *Pasteurellaceae*, NETs fail to kill the bacteria and ultimately contribute to disease progression and enhanced tissue damage (Honn et al., 2017; Pulavendran et al., 2020).

Additionally, the survival of *H. influenzae* in NETs *in vivo* and *in vitro* has been demonstrated to be mediated by a catalase as well as a peroxiredoxin-glutaredoxin independent from OxyR (Juneau et al., 2015b). This indicates that the resistance against ROS which contributes to survival in NETs is based on redundant systems and does not rely on one factor.

The resistance against ROS mediated the survival of *A.pp* in porcine AMs which could contribute to the survival of *A.pp* after phagocytosis by NET clearing AMs (Crujisen et al., 1992). The ROS defense from OxyR seems to be a crucial defense tool and testing a knockout mutant in a NET killing assay as performed in [Chapter 5.1, Figure 4] could proof its importance in NET survival.

In NETs, several components as AMPs, e.g. LL-37 in humans and β -defensin or enzymes as cathepsin G or NE are incorporated to increase the antimicrobial activities of NETs (Papayannopoulos & Zychlinsky, 2009). However, it has been shown that AMPs are not equally effective against bacteria and several resistances could contribute to the survival of bacteria in NETs.

Two mechanisms are known how resistance against AMPs is achieved by *Pasteurellaceae*, the first being transporter systems that prevent cathelicidins from attacking the bacterial cell. The

second mechanism are attachments and modifications altering the charge of the LPS to gain resistance against cationic AMPs(Ramjeet et al., 2005; Xie et al., 2017b).

PR-39 is an AMP that is incorporated into porcine NET fibers and acts antimicrobial by interfering with the DNA and protein biosynthesis of the bacterium(Boman et al., 1993). A study by Hennig-Pauka et al. has demonstrated that *A.pp* shows a resistance against PR-39 and the mode of resistance has later been attributed to the products of the *sapA* operon known in several bacteria(Hennig-Pauka et al., 2006; Xie et al., 2017b).

The *sapA* operon encodes for a periplasmatic binding protein which is part of the *sap* transporter system and is important for AMP resistance in gram-negative bacteria(Mount et al., 2010; Ondari et al., 2017; Rivera et al., 2023). In *H. influenzae*, *sapA* mediates resistance against LL-37 and β -defensins(Rivera et al., 2023). Other transporter systems have also been described in gram-negative bacteria as for example the multiple transferable resistance transporter in *Haemophilus ducreyi*(Rinker et al., 2011).

Cationic AMPs use electrostatic attraction to adhere to bacterial cells to act bactericidal(Harder et al., 2001). Gram-negative bacteria can alter the charge of their LPS structure to evade the innate immune system by different chemical modifications(Simpson & Trent, 2019). *H. influenzae* mutants that lack the acylation of the LOS have a stronger negative charge and are therefore significantly more susceptible to cationic AMPs like β -defensins(Nizet, 2006; Starner et al., 2002). A study by Ramjeet et al. demonstrated that a truncation of the outer core of the LPS of *A.pp* substantially reduced the resistance against several porcine AMPs(Ramjeet et al., 2005). Interestingly, the strongest effect was observed in protegin-1, an AMP of the porcine neutrophil. Wildtype strains of *A.pp* show resistance against protegrin-1(Zeng et al., 2020). This indicates that the resistances of *Pasteurellaceae* are multifaced.

Data on *G. parasuis* resistances against porcine AMPs are scarce. The *sapA* gene, which has been described in *A.pp* and *H. influenzae* as well as other gram-negative bacteria has not yet been investigated in *G. parasuis*, but it was detected via blast analysis using uniprot.org and the *G. parasuis* strain SH0165 and the gene name “*sapA*”. Interestingly, the resistance of *G. parasuis* against a synthetic AMP that derived from the cecropia moth as a novel drug has been described and investigated(C. Wang et al., 2014).

In conclusion, the resistance of *Pasteurellaceae* bacteria against ROS and AMPs, in combination with the formation of biofilms and nuclease activity in some species of this family, are likely contributing to the survival in NETs. The evasion of NET-mediated killing, in

combination with the metabolic exploitation of NET components presumably leads to the detrimental effects of NETs during infections with *Pasteurellaceae* bacteria. In general, more data about the human pathogens in terms of resistances against neutrophils functions is available, but many of the mechanisms were found or can be assumed in porcine representatives.

6.3 Bacterial growth benefits in lungs with IAV and NETs

In [Chapter 5.2, Figure 4] and [Chapter 5.3, Figure 1] we saw that all three *Pasteurellaceae* species involved in this study grew effectively in lungs with NETs *in vivo* or in BALF with NETs *in vitro/ex vivo*, either directly induced or via a previous infection. From [Chapter 5.1, Figure 5] and a study by de Buhr and colleagues, we know that *A.pp*, *G. parasuis* and *H. influenzae* can metabolize NAD that is present in NETs(de Buhr et al., 2019). In [Chapter 5.2, Figure S1] we saw that the BALF samples of the animals that were infected with *Pasteurellaceae* species had significantly reduced amounts of NAD. Most likely, the NAD was consumed by present *Pasteurellaceae A.pp*, *G. parasuis* and *Pasteurella multocida*.

V-factor dependent *Pasteurellaceae* species have lost the ability to synthesize NAD *de novo*. This is a process that might have happened during adaptation to the host in pathogens that are specialized on one host, as discussed by Gerlach et al.(Gerlach & Reidl, 2006). Therefore, it is mandatory to add a source of NAD or a precursor to the growth medium in *in vitro* experiments.

NAD is an important component of the respiratory chain and a coenzyme for several enzymes(Brand & Murphy, 1987). It is present in every cell of the eucaryotic body and can be detected in the serum of humans and in plasma, laryngeal-, tracheal-, and lung-washes, as well as in cerebrospinal fluid of pigs in concentrations of 0.18 to 1.52 μM (Gerlach & Reidl, 2006; O'Reilly & Niven, 2003). De Buhr et al. have demonstrated that NAD is present in the supernatant of neutrophils that have undergone NETosis(de Buhr et al., 2019). NAD is present in the cytosol, although a significant amount, ranging from 30-70 % depending on the cell type, is stored in the mitochondria, as reviewed by Stein et Imai(Stein & Imai, 2012). Therefore, it is likely that the NAD released during NET formation has derived from the cytoplasm of neutrophils and the mitochondria, if mitochondrial chromatin is involved during vital NETosis(Yousefi et al., 2009).

In addition to the metabolism of NAD or a direct precursor as nicotinamide riboside, de Buhr et al. have shown that *A.pp* can use external DNA as a source to generate NAD(de Buhr et al.,

2019). In [Chapter 5.2, Figure 5] we showed that the content of cell-free DNA correlated with the growth speed of *A.pp.* However, this phenotype could not be observed in *G. parasuis* [Chapter 5.2, Figure S1]. During the replication of DNA, two phosphate groups are cleaved from adenosine triphosphate and the resulting adenosine monophosphate is incorporated into the DNA strand. After the degradation of DNA, adenosine monophosphate can be used as a substrate for NAD synthesis (Heppel & Hilmoe, 1953; Walt et al., 1980). This allows the bacteria to recycle the DNA of deceased bacteria or degraded NETs.

Interestingly, the utilization of DNA as a nutrient source is not limited to *Pasteurellaceae*. In seawater bacteria which live in nutrient scarce ecosystems, the utilization of DNA as a nutrient source has evolved. Dissolved DNA is rapidly taken up by bacteria and resulted in an enhanced growth. The authors discussed that in marine bacteria, DNA may provide up to 10 % of the bacterial nitrogen and carbon demand and is a valuable source for phosphorus (Jørgensen & Jacobsena, 1996).

Besides the dependency on an NAD source, bacteria are in a constant fight about nutritious resources with the host and other commensal or pathogenic bacteria. Therefore, mechanisms to acquire nutrients and trace elements are an important factor of fitness as reviewed by Murdoch and Skaar (Murdoch & Skaar, 2022). The host in return uses mechanisms to reduce the availability of nutrients which is named nutritional immunity (Sakamoto et al., 2017). However, the lung produces vast amounts of nutrient rich mucus which production is additionally increased during respiratory infections as a defense tool, as reviewed by Li et Tang (Y. Li & Tang, 2021; Siegel et al., 2014). Mucus is a sialic acid rich substance and during an infection, IAV particles cleave the sialic acid to reach and infect the cells below the mucus (Cohen et al., 2013). This results in an increased amount of available sialic acid and displays an attractive nutrient source for bacteria that are able to utilize it (Siegel et al., 2014). It is described that the content of sialic acid in the nasopharynx of mice is increased during an infection with IAV (Siegel et al., 2014). The BALF of the IAV infected animals in [Chapter 5.2, Figure 3] had increased sialic acid contents. In the study by Siegel et al., a direct growth-enhancing effect of sialic acid was described for *S. pneumoniae* (Siegel et al., 2014). This enhanced replication enables the bacteria to spread to the lower respiratory tract. This is interesting, as infections with pneumococci start with an asymptomatic colonization of the nasopharynx/upper respiratory tract (Bogaert et al., 2004). The three *Pasteurellaceae* species addressed in this study are colonizers of the upper respiratory tract which initiate the disease by migration to the lower respiratory tract (P. King, 2012; Moller & Kilian, 1990; Müllebnner et al., 2018). The genes encoding for the *N*-acetylneuraminic acid lyase (NanA) as an enzyme to utilize sialic acid from

the host have been detected in the *Pasteurellaceae* (McDonald et al., 2016). These genes allow the conversion of sialic acid to fructose-6-phosphate and pyruvate, which are metabolized during glycolysis and the citric acid cycle, as reviewed by Vimr et al. (Vimr et al., 2004). So, metabolizing sialic acids as an energy source may be an underestimated factor that favors the co-infection of *Pasteurellaceae* during an IAV infection.

The use of sialic acids although is not only limited as a nutrient source, but as an active component of immune evasion. The tripartite ATP-independent periplasmic (TRAP) transporter enables *H. influenzae* to scavenge and incorporate available sialic acid into the LOS. This masks its antigens and creates resistance against complement killing in serum, by reducing the binding of C-reactive proteins, IgG, and IgM (Oerlemans et al., 2019). This displays an important virulence factor and without the TRAP transporter or available sialic acids, *H. influenzae* strains cannot cause systemic infections (Allen et al., 2005; Bouchet et al., 2003; Oerlemans et al., 2019). Virulent strains of *G. parasuis* can cause systemic infections which requires serum resistance. Interestingly, in the virulent reference strain Nagasaki, which can cause systemic infections, sialylation of the LOS was reported (Cerdà-Cuéllar & Aragon, 2008b). The sialylation of the LPS/LOS is described in more *Pasteurellaceae* species as *Haemophilus somnus* and in other bacteria as *Neisseria gonorrhoe* (Inzana et al., 2002; Ram et al., 1998).

Ultimately, *S. aureus*, *S. pneumoniae* and *H. influenzae*, the main co-infecting bacteria in human IAV are known to metabolize sialic acid sugars (Siegel et al., 2014). *G. parasuis*, the main co-infecting agent in swine IAV is also described being able to metabolize sialic acids (Lichtensteiger & Vimr, 1997; Vereecke et al., 2023). This emphasizes that the ability to metabolize sialic acids is an important benefit during co-infection with IAV. However, in **[Chapter 5.2, Figure S3]** we did not observe a correlation of bacterial growth of *G. parasuis* and the content of sialic acids. It is known that a change of growth conditions impacts the gene expression of bacteria and therefore it is conceivable that the incubation in BALF influenced the expression of sialic acid metabolizing genes (Lone et al., 2009).

Co-infections during IAV are often initiated days after the symptom onset of IAV (L. N. Lee et al., 2010). During this time, the milieu in the respiratory tract is dominated by pro-inflammatory stimuli from cytokines **[Chapter 5.3, Figure S2]** (Hennet et al., 1992; Turlewicz-Podbielska et al., 2021). Cytokines are proteins with a low molecular weight that mediate the communication between cells and are involved in many processes, as cell growth and differentiation as well as inflammation and immunity. They are short living proteins and bind to cells via high affinity

receptors(Nicod, 1993). An interesting study by Merduri et al. has shown that the human pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α were increasing the growth speed of three nosocomial pathogens of the acute respiratory distress syndrome, namely *S. aureus*, *Acinetobacter spp.* and *Pseudomonas aeruginosa*(Merduri et al., 1999). Importantly, the cytokines only affected the growth speed and not the cell count at the stationary phase. During IAV infections, the secretion of IL-1 β , IL-6, and TNF- α is significantly increased, contributing to inflammation(Bawazeer et al., 2021; Dienz et al., 2012; Seo & Webster, 2002). In [Chapter 5.3, Figure S3] we saw that the IL-6 was significantly reduced in the BALF of mice infected with IAV compared to the healthy control or the mice infected with NTHi. Usually, IL-6 is increasing in mice in IAV infections until day seven and resolves afterwards(Dienz et al., 2012).

Hogan et al. reported that recombinant bovine IFN- γ increased the growth speed of *Escherichia (E.) coli in vitro*(Hogan et al., 1993). Comparable results have been described for IL-2 and GM-CSF(Denis et al., 1991). In [Chapter 5.3, Figure S2] we have shown that IFN- γ is significantly increased in the BALF of IAV and IAV and NTHi co-infected mice and in [Chapter 5.2, Figure 3] that BALF of IAV-positive pigs had significantly increased amounts of IFN- γ compared to IAV-negative and healthy pigs. Although, most of the animals in [Study 5.2] were below the detection limit. It has been shown that *E. coli* is able to deplete IL-2 *in vitro*, although the activity of proteases was excluded(Denis et al., 1991). Merduri and colleagues have discussed that gram-negative bacteria have receptors for IL-1 β and TNF- α on their cell surface and that it potentially alters metabolic processes(G. Luo et al., 1993; Merduri et al., 1999; Porat et al., 1991). In the host, cytokines are internalized after binding to the receptor(Nicod, 1993). It is conceivable, that the reported depletion of cytokines by bacteria is also due to receptor mediated internalization.

The direct effect of cytokines on bacteria is a poorly investigated topic. The available data was only generated *in vitro* and with a small range of bacterial species. However, this could be an underestimated factor during IAV co-infections. Especially if bacterial uptake of cytokines could influence the immune response *in vivo*.

6.4 Comparison of respiratory co-infections in other disease constellations

The co-infection of IAV and NTHi did not significantly alter the viral titer in the BALF [Chapter 5.3, Figure 1], this was also demonstrated in two studies with IAV and *H. influenzae*.

The first study by Wu et al. co-infected IAV with NTHi and the second one by Lee et al. with *H. influenzae* type b. In both studies an increase in bacterial titers and disease severity was observed, whereas the viral titers were not influenced (L. N. Lee et al., 2010; X. Wu et al., 2021). In an experimental co-infection of SARS-CoV-2 and *S. pneumoniae* in mice, an increase in bacterial titer but no influence on the viral titer was observed (A. P. Smith et al., 2022). However, on seven days post infection (dpi) a slight decrease in the viral titer in the co-infected animal could be observed. Comparably, a study by Moorthy and colleagues described a significantly increased clearance of IAV in the late stage of a co-infection with *S. pneumoniae* in mice (Moorthy et al., 2013). Interestingly, two studies by Pomorska-Mól et al. that co-infected pigs with IAV and *A.pp* or *G. parasuis* respectively, showed that the virus shedding was increased in the co-infected animals. In contrast to the virus, no effect on the bacterial titer was observed (Pomorska-Mól, Dors, Kwit, Czyżewska-Dors, et al., 2017; Pomorska-Mól, Dors, Kwit, Kowalczyk, et al., 2017). In the pig studies although the inoculation of both pathogens was performed simultaneously, whereas the co-infection with *H. influenzae* was performed three dpi with IAV. Lee et al. has investigated the optimal timepoint of the co-infection with the strongest effect on the bacterial co-infection. An interval of three to four days between IAV infection and bacterial co-infection caused the highest lethality in mice. In accordance with the studies of Pomorska-Mól, a simultaneous infection resulted in minor lethality. Interestingly, if the bacterial infection is administered first and three dpi the viral infection occurred, no lethality was observed in mice (L. N. Lee et al., 2010). These results underline that the enhancing effect of IAV develops during the infection.

The time intervals between first and second infection are dependent on the investigated pathogen and its individual kinetics. For example, van Dixhoorn and colleagues studied the co-infections with PRRSV and *A.pp* in piglets and administered the infections eight days apart (Van Dixhoorn et al., 2016). Two studies investigating the co-infection of IAV and *S. pneumoniae* used an interval of seven days between the first and second infection (Moorthy et al., 2013; Siegel et al., 2014). In the study on SARS-CoV-2, the timeframe for co-infection was investigated and five to seven dpi resulted in the most severe symptoms and lethality (A. P. Smith et al., 2022).

In the study by Moorthy and colleagues, the preceding IAV infection increased the bacterial load of *S. pneumoniae* in the lung and delayed the clearance. The total histology score of the lung of a co-infection of IAV and *S. pneumoniae* shows a minor, not significant increase of the lung lesions in co-infected animals compared to the animals single infected with IAV (Moorthy et al., 2013). This shows the same phenotype as in our animal experiment in [Chapter 5.3,

Figure 1]. In the study by Lee et al. however, a significant increase in lung lesions was detected in the co-infected animals. The histology in this study was performed on nine dpi, the sixth day of the co-infection. In our study, the last animal was euthanized at seven dpi [**Chapter 5.3, Figure S1**], due to the described increase in lethality at eight and nine dpi (L. N. Lee et al., 2010; X. Wu et al., 2021). Therefore, a further, remarkable increase in lung lesions in the co-infected animals would have to be expected in the next days.

The presented animal trial demonstrated a co-infection during a severe influenza disease, including high weight loss and lung lesions. A study from Zhang et al. demonstrated that a mild influenza disease with reduced weight loss and lung lesions is sufficient to induce a fatal co-infection with NTHi (X. Zhang et al., 2023). Common cold viruses are usually associated with a mild flu like disease or just respiratory symptoms as nasal discharge and sore throat. Rhinoviruses are the most common viruses causing common cold (Mäkelä et al., 1998). During a common cold, bacterial co-infections are frequent and complicate the recovery or develop into severe infection, predominantly in young children. In a large study with young children from China infected with the common cold viruses, rhinoviruses and metapneumoviruses, bacterial co-infections were rare but a significant risk factor for severe symptoms and intensive care unit admission (Wan et al., 2023). Infants administered to intensive care unit with respiratory syncytial virus infection had a rate of bacterial co-infection of up to 40 %. However, studies investigating the molecular processes during common cold infections that lead to exacerbated co-infections are scarce. Rhinoviruses may reduce the clearance of NTHi in the lung by attenuating the induction of IL-8 by NTHi. The authors state that the reduced infiltration of neutrophils resulted in the bacterial survival, although they did not deliver any evidence of neutrophil killing of NTHi (Unger et al., 2012). This has to be viewed critically, as Juneau et al. have reported that human neutrophils do not kill NTHi, neither via NETs nor via phagocytosis (Juneau et al., 2011).

These data show that respiratory, bacterial co-infections are a threat largely independent of the first infecting virus and have the potential to severely worsen a supposedly harmless infection.

6.5 Potential therapeutic approaches against NETs in IAV infections

The aim of study part 3 [**Chapter 5.3**] was to characterize the NET formation during a co-infection with IAV and NTHi in mice. Based on these data, future studies may test potential therapeutics to alleviate the severity of a bacterial co-infection and NET-related morbidities.

There is a need of alternative treatment strategies than antibiotics, as the development of vaccines is insufficient and antibiotic resistances have been increasing fast (Aslam et al., 2018; Kruse et al., 2017; Teuber, 2001). The European strategy to reduce the use of antibiotics in animal husbandry showed beneficial effect on the frequencies of antibiotic resistances in porcine bacterial communities (More, 2020; Wiencek et al., 2022). This shows that alternative prevention and treatment mechanisms need to be further developed and show beneficial results. An important aspect of disease prevention is to increase the housing quality of farm animals, as it has shown positive effects on disease susceptibility (Van Dixhoorn et al., 2016). Applied to humans, during the COVID-19 pandemic, increased hygienic standards and strict rules have reduced hospital-acquired infections and the transmission of other pathogens (Cerulli Irelli et al., 2020; Seid et al., 2022). However, as it is impossible to fully prevent infections and especially zoonotic pathogens are impossible to eradicate, therapeutic approaches independent of antibiotics need to be developed.

The use of rhDNase is an approved drug in the treatment of CF and has shown beneficial effects in various respiratory disease scenarios and NET-associated morbidities (Jarrahi et al., 2023; Suri, 2005). DNases are produced by the host to perform physiological functions and their dysfunction is involved in several diseases. Impaired DNase activity which leads to an insufficient NET degradation is described to play a role in autoimmune diseases as for example lupus nephritis or rheumatoid arthritis (Hakim et al., 2010; Khandpur et al., 2013). The respiratory tract is also affected by uncontrolled NET formation, as it is described for several viral and bacterial lung infections and COPD, resulting in increased tissue damage and reduced clearance of pathogens (Grabcanovic-Musija et al., 2015; Lefrançois et al., 2018; Zhu et al., 2018). In [**Chapter 5.2, Figure 2**] we have measured reduced activity of DNase I in the BALF of lung infected pigs. Initially discussed as an effect of exhaustion due to prolonged disease, the reduced DNase activity may contribute to insufficient NET degradation and therefore to impaired bacterial clearance.

We have measured high amounts of NETs in the lungs of mice infected with IAV and the amount of NETs correlated strongly with the lung lesion score [**Chapter 5.2, Figure 2**]. In other studies with IAV infections in mice, high loads of NETs have been correlated with fatal outcomes in severe IAV and with enhanced tissue damage (Narasaraju et al., 2011; Zhu et al., 2018). Additionally, NETs induced during IAV infections do not act antimicrobial during a secondary bacterial infection but contribute to tissue damage and compromised lung functions (Moorthy et al., 2013). Therefore, treatment with DNase to control NET formation has been investigated in different disease scenarios. In CF, treatment with rhDNase is an

approved therapy that resolves symptoms by reducing the viscosity of the sputum and improves the clearance (Shak et al., 1990; Suri, 2005). In IAV co-infection with *S. aureus*, NET degradation mediated by DNase administration showed promising results and reduced inflammation, weight loss and lung injury of the mice (Yi et al., 2022). This applied also for mice single infected with methicillin-resistant *S. aureus* (MRSA), where constant DNase administration reduced the mortality rate of infected individuals (Lefrançois et al., 2018). As discussed earlier, degradation of NETs reduced the activity of the NADPH oxidase and MPO which reduced oxidative stress and tissue damage induced by NETs (Munafo et al., 2009). In the recent COVID-19 pandemic, DNase treatment was tested and gave promising results in severe SARS-CoV-2 infections by alleviating symptoms and reducing the need of increased oxygen concentration during mechanical ventilation (Weber et al., 2020). The beneficial effects of DNase have also been described in a mouse model of SARS-CoV-2 induced acute respiratory distress syndrome (ARDS) (Jarrahi et al., 2023).

So, these results are very promising indicating that DNase administration could display a beneficial treatment option for patients of severe IAV infections. However, with regard to potential co-infections with bacteria from the *Pasteurellaceae* that can utilize NAD present in degraded NETs, the use of DNase has to be critically reviewed (de Buhr et al., 2019; de Buhr & von Köckritz-Blickwede, 2021). The authors emphasized that the effect of DNase mediated NET degradation on potentially co-infecting *Pasteurellaceae* is understudied and could lead to severe, unexpected consequences. In [Chapter 5.3, Figure 3], the DNase activity in the BALF of mice infected with IAV and of the co-infected mice was increased and the increase correlated with the NETs detected in the lungs. This is a potential indicator for an increased degradation of NETs, resulting in the release of growth factors. However, there was no difference detectable in the severity of the lung lesions in IAV and IAV+NTHi mice by seven dpi [Chapter 5.3, Figure 3]. As discussed in the previous chapter, it is highly expected that an increase in lung lesions and mortality would occur on eight and nine dpi, as it was reported in the study from Lee et al. (L. N. Lee et al., 2010; X. Wu et al., 2021). According to our hypothesis, the increasing DNase activity results in the degradation of the NETs present in the lung, releasing growth factors for NTHi, which are able to grow better, causing more lung lesions. This is supported by a study from de Buhr and colleagues, reporting that DNase activity measured in the BALF of pigs infected with *A. pp* correlated with the detected lung lesions (de Buhr et al., 2019).

On the one hand, DNase mediated NET degradation significantly improved the disease outcome, on the other hand *in vitro* data and observations of an animal trial suggest that degradation of NETs may benefit the co-infecting *Pasteurellaceae*. Until today, no study was

performed to test DNase treatment during an *in vivo* infection with *Pasteurellaceae*. For this reason, [Chapter 5.3] designed an infection scenario to test DNase as a potential treatment in future studies and to clarify if the beneficial or detrimental effect of DNase outweighs the other. In addition, other therapeutic approaches may be tested using this model.

Other potential targets for the inhibition and regulation of NET formation are enzymes and signaling cascades that are involved in the activation process and the decondensation of the chromatin. As a key enzyme of the neutrophil, NE plays an important role in the formation of NETs. Upon induction, NE migrates from the azurophilic granules to the nucleus and promotes the decondensation of the chromatin (Papayannopoulos et al., 2010). According to Papayannopoulos et al., NE is crucial for the formation of NETs, as its inhibition or knockout causes a complete inhibition of NET formation in response to infectious agents in mice (Papayannopoulos et al., 2010). In contrast to this, Martinod et al. reported that no or only minor reduction in NET formation was observed in NE knockout mice (Martinod et al., 2016). Using inhibitors of NE has been investigated as a potential treatment for NET-mediated diseases.

Sivelestat is an inhibitor of NE and has been used in clinical studies with ARDS, pneumonia, or bypass surgery (Imokawa et al., 2006; Kawabata et al., 1991; Pan et al., 2023; Y. Wang et al., 2022). In the ARDS study with children, it could reduce mortality and significantly alleviated disease parameter (Y. Wang et al., 2022). A NET-inhibiting effect of sivelestat was shown in an ischemia-reperfusion injury model, where it significantly reduced NET markers and improved recovery (C. L. Wang et al., 2023). In a co-infection study with IAV and MRSA, where high levels of NETs were reported, a treatment with sivelestat was performed. The treated mice showed less body weight loss and reduced pro-inflammatory cytokines. Additionally, the formation of NETs was inhibited in the lung which was demonstrated by immunohistochemistry (Yi et al., 2022). The treatment of human patients with IAV with sivelestat has only been done in single cases and broader studies are lacking (Itoh et al., 2011; Y. Li et al., 2024). During the COVID-19 pandemic, a treatment with sivelestat has been suggested for vulnerable patients with severe COVID-19 (Laforge et al., 2020). Two studies from Chinese hospitals tested a treatment with sivelestat and one study reported no effect on disease parameters and survival, whereas a second study by Li and colleagues, which is currently available as a preprint, reported reduced time in the intensive care unit and an increased survival of treated patients (Y. Li et al., 2024; M. Luo et al., 2023).

These data indicate that NE inhibitors as sivelestat are promising candidates for future therapies in severe IAV infections, but further research is needed. The fact that the formation of NETs is inhibited instead of that formed NETs are degraded could display a benefit in infections with *Pasteurellaceae* in comparison to DNases.

In contrast to NE, which is discussed to be mandatory for NET formation, ROS is involved in, but not crucial for the formation of NETs(Papayannopoulos et al., 2010). So, NET formation can either occur via an ROS-dependent or an ROS-independent pathway(Pilsczek et al., 2010). The first one may be addressed with antioxidant drugs which reduces the amount of formed NETs(Parker, Dragunow, et al., 2012).

The production of excessive amounts of ROS is an important aspect of the pathogenesis of IAV infections and other NET-associated diseases as rheumatoid arthritis, as it contributes to tissue damage(Akaike et al., 1996; Datta et al., 2014). Therapeutic drugs that inhibit or scavenge ROS contribute to the survival in severe infection and reduce the formation of NETs via the ROS-dependent pathway. In 1989, before the discovery of NETs, ROS produced, among others, by the xanthine oxidase were detected as harmful in IAV infections and administration of the ROS scavenger superoxide dismutase alleviated a potentially lethal IAV infection in mice(Oda et al., 1989). Years later, ROS produced by xanthine oxidase has been described to induce NET formation(Al-Khafaji et al., 2016).

N-acetyl-L-cysteine is a medication for acetaminophen poisoning and a precursor of the antioxidant glutathione and has shown antioxidant properties(Lewis et al., 2022; Sen et al., 1994). Therefore, it was tested on its effect on NET formation and *in vitro* assays showed significant reduction in NETs in human neutrophils(Muñoz-Sánchez et al., 2023). Due to its antioxidant properties, it was also proposed as a therapeutic approach for vulnerable patients in COVID-19 by Laforge and colleagues(Laforge et al., 2020). However, *in vivo* studies proving NET-inhibitory effects at *in vivo* relevant concentrations are missing.

Diphenyleneiodonium (DPI) is an NADPH oxidase inhibitor and is used to investigate ROS-independent pathways of NET formation. By blocking NADPH oxidase function, ROS-dependent NETosis is inhibited(Buck et al., 2019). Leung et al. tested the NET-inhibiting effect of DPI *in vivo* and described a reduced NET formation by NADPH oxidase inhibition in experimental thrombocytopenia in mice(Leung et al., 2021). Aswell as for *N*-acetyl-L-cysteine, further *in vivo* studies in infected animal models or human patients are lacking. It needs to be considered that only ROS-dependent NET formation is inhibited upon administration(Y. Liu et al., 2021). A beneficial side effect of ROS-targeting drugs would be, a reduction in ROS-

mediated tissue damage which would reduce the susceptibility of the host for a bacterial co-infection(Loosli et al., 1975).

In addition to immunomodulatory drugs as ROS inhibitors, drugs that directly target the infectious agent, antibiotics or antivirals may target the formation of NETs. NA inhibitors as zanamivir or oseltamivir are the most frequently used drug against IAV and target the replication of the viral particles by blocking NA activity(Świerczyńska et al., 2022). The NA inhibitor oseltamivir administered in combination with SCH527123 an antagonist of the CXC chemokine receptor 2 resulted in reduced lung pathology and formed NETs in mice and piglets *in vivo*. This increased the survival of mice infected with a lethal dose of IAV(Ashar et al., 2021). The combination therapy with the antagonist against the neutrophil receptor CXCR2 that binds chemokines including IL-8 increased the effects of both single therapies(Addison et al., 2000; Ashar et al., 2021).

A study by Formiga and colleagues reported that the NA inhibitors oseltamivir and zanamivir significantly reduced ROS production and NET formation in human neutrophils. Interestingly, the effect of oseltamivir increased the survival of septic mice without the involvement of an influenza virus infection. The authors have discussed that hosts NA activity is involved in neutrophil functions and thereby is a target to reduce overshooting neutrophil responses(de Oliveira Formiga et al., 2023). These data, although preliminary data are involved, shed light on previously unknown mechanisms of neutrophil regulations and may be a valuable topic of future studies.

If during an IAV infection a bacterial co-infection is detected and the infecting species has been determined, antibiotics are described to improve the disease outcome. Antibiotics are drugs that inhibit the growth of bacteria by interfering with their metabolism in diverse pathways. In a recent study, we have detected that doxycycline has a modulatory effect on canine neutrophils. *In vivo* relevant concentrations of doxycycline did on the one hand, significantly decrease the production of ROS, but on the other hand induced the formation of NETs *in vitro*. Interestingly, increasing concentration of doxycycline increased the ROS-inhibiting effect, but reduced the induction of NETs(Rieder et al., 2024). Other studies have detected that some antibiotics act on neutrophils *in vitro*, by inhibiting the formation of NETs. For example, a pre-treatment of neutrophils with 1 µg/mL gentamicin significantly reduced the amount of NETs induced by phorbol myristate acetate (PMA) or calcium ionophore(Manda-Handzlik et al., 2017). As the inhibitory effect was induced by pre-treating the neutrophils, serum levels of the antibiotic might play an important role in the transferability of this finding into an *in vivo* situation and a

study by Barza and Lauermann reported a therapeutic serum concentration of gentamicin of 5-8 $\mu\text{g/mL}$ (Barza & Lauermann, 1978). Recommended antibiotics in the treatment of *Pasteurellaceae* are e.g. cefotaxime, ceftriaxone, ciprofloxacin for *H. influenzae* and enrofloxacin and florfenicol for *G. parasuis* and *A.pp*(A. de Jong et al., 2023; Hennig-Pauka et al., 2022; Ibar-Bariain et al., 2021). For ciprofloxacin and ceftriaxone, a NET-inhibiting effect was described in *in vitro* as well as *in vivo* studies(Duan et al., 2021; Pulavendran et al., 2020). Some antibiotics do not act modulating on NET formation, as it was reported for cefotaxime(Manda-Handzlik et al., 2017). Enrofloxacin however, induces NET formation in bovine neutrophils *in vitro* at an *in vivo* relevant concentration, but therefore acted inhibiting on the phagocytosis activity(Jerjomiceva et al., 2014).

Generally, antibiotics reduce the bacterial titer and by this contribute to a reduction of the inflammation which thereby further reduces the amount of newly formed NETs. If the co-infecting agent has been determined with diagnostic methods and its resistance profile is known, the choice of the antibiotic and the dose administered may be adapted to influence NET formation and other effector functions of neutrophils.

In conclusion, due to the complex pathways that are involved in the formation of NETs, a broad variety of therapeutic approaches can be seized. It is especially interesting that drugs that initially target a pathogen that is not involved in the infection scenario may be repurposed as they show beneficial effects in other diseases. The drugs discussed in this chapter should be investigated in future studies to increase the knowledge about regulation of NETs in infectious diseases. Thereby, future therapeutics may be developed which contribute to a reduction of the suffering of patients, either human or animal, with severe IAV and bacterial co-infection.

7. Concluding Remarks

The host-pathogen interaction is a complex process which involves a broad range of cells of the immune system of the host. In this study, the host-pathogen interaction of neutrophils and a co-infection of IAV and *Pasteurellaceae* of porcine and human origin was studied. Special emphasis was placed on their effector function NETs. In complex scenarios it can be helpful to isolate one distinct aspect and investigate it *in vitro*.

In study part 1 [**Chapter 5.1**] *in vitro* assays were performed and showed that *G. parasuis* induces NETs in porcine neutrophils and survives their antimicrobial properties. Furthermore, *G. parasuis* does even receive a growth benefit from growth factors present in the NETs. One of these factors was identified as NAD, the crucial growth factor of the *Pasteurellaceae*. The detection of nuclease activity in *G. parasuis* supernatants and only little ROS production from neutrophils upon contact with *G. parasuis* started to unravel how *Pasteurellaceae* resist NETs.

After the first insights were gained in *in vitro* assays, more and more aspects may be added to better mimic the *in vivo* situation. Therefore, in study part 2 [**Chapter 5.2**], BALF from IAV infected pigs were taken and their influence on porcine neutrophils and bacteria were investigated. The growth of *G. parasuis* and *A.pp* was promoted by substances that were present in the IAV infected lung, including NET components and sialic acids. Neutrophils isolated from the BALF were undergoing vesicular NET formation. The milieu in the BALF did not enable porcine neutrophils to kill *A.pp* *in vitro*.

Ultimately, the knowledge gained in *in vitro* assays can be transferred into an *in vivo* experiment. In study part 3 [**Chapter 5.3**], a co-infection with a human IAV strain and the human *Pasteurellaceae* *H. influenzae* was performed. The timeline of the infection was characterized, and massive NET formation and severe lung lesions were detected in the IAV infected mice. The preceding IAV infection benefited the *H. influenzae* infection. Comparable to the previous *in vitro* assays, NETs failed to kill *H. influenzae* and the degradation by an increasing activity of DNase promoted its growth.

In conclusion, this study further investigated a new risk factor during bacterial co-infections in IAV infected hosts. NETs that are formed in response to IAV do not act antimicrobial against co-infecting *Pasteurellaceae* bacteria. The host that is already weakened by the previous infection is then confronted with bacteria that receive growth factors from NETs that are degraded by host DNases. Therefore, future research is needed to investigate if DNase therapy, a promising therapeutic approach in NET-associated diseases, may be more harm than help. An

overview about the processes ongoing in this scenario are depicted in the figure below (**Figure 4**) and further questions, to be answered in future studies are described in the following section.

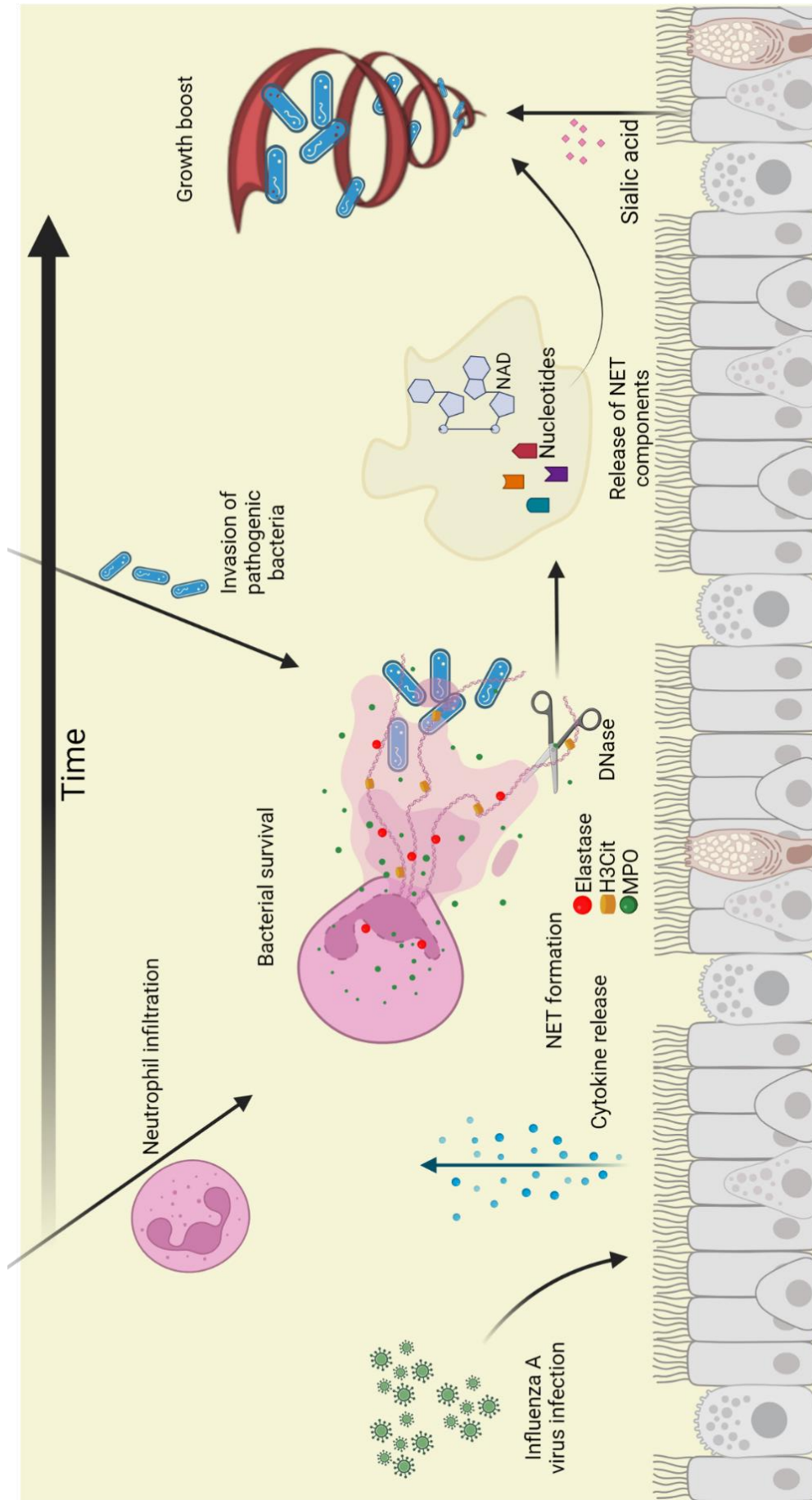


Figure 4 The processes during a co-infection of IAV and *Pseudomonas aeruginosa* bacteria. IAV infects the lung tissue and induces the production of pro-inflammatory cytokines, causing the infiltration of neutrophils. Neutrophils are stimulated to produce massive amounts of NETs and *Pseudomonas aeruginosa* bacteria migrate into the lung. NETs fail to kill the bacteria. Degradation of NETs by host DNases causing the release of the NET components NAD and DNA. In combination with sialic acids released by the IAV infection, NET components enhance the growth of the co-infecting bacteria.

8. Future Outlook

What are the NET inducing components of *G. parasuis*?

In this study, we have shown that *G. parasuis* induces NETs in porcine neutrophils. However, the exact components of the bacterium that are involved in the induction remain unknown. Juneau and colleagues have tested isolated components of NTHi and detected that the LOS is the most potent NET-inducing factor in NTHi (Juneau et al., 2011). It would be interesting, to test the LPS and OMPs of *G. parasuis* in a NET induction assay like it was performed in [Chapter 5.1, Figure 2]. Therefore, the targeted structure can be isolated using the method described by Juneau and colleagues (Juneau et al., 2011). An interesting candidate of the OMPs of *G. parasuis* would be the protein HPS_06257, which is used in subunit vaccines because of its immunogenic and protective potential (X. Chen et al., 2022).

Bonilla et al. have tested if the formation of NETs in response to *A.pp* is ROS-dependent or independent using DPI as a NADPH oxidase inhibitor. Testing the NET formation in response to *G. parasuis* in combination with DPI would be particularly interesting because of the antioxidant system OxyR that is reported in *G. parasuis* (Y. Wen et al., 2018).

What components of *Pasteurellaceae* are involved in the resistance against NETs?

In this study several potential structures were discussed that may enable the survival of *Pasteurellaceae*. In a future study, mutant strains of *A.pp*, *G. parasuis* and *H. influenzae* that are lacking one enzyme or surface protein could be tested in response to NET-mediated killing. Interesting targets would be, among others, a truncation of the LPS or LOS, as described for *A.pp* and *H. influenzae* (Nizet, 2006; Ramjeet et al., 2005). This knockout of the resistance against AMPs would generate valuable insights in the importance of AMPs for the antimicrobial effect of NETs.

Additionally, it would be interesting to use mutant strains of *H. influenzae* and *G. parasuis* that are lacking the nuclease (NTHi Nuc in *H. influenzae* and Cdt in *G. parasuis*) and test their survival in the neutrophil killing assay presented in [Chapter 5.1, Figure 4] (Cho et al., 2015; G. Li et al., 2017).

How do drugs that interfere with the formation of NETs influence the co-infection of IAV and NTHi?

In the third part of this study [**Chapter 5.3**] we have characterized the formation of NETs and the activity of DNase during the co-infection of IAV and NTHi in mice. This infection model may be used to test the effect of a DNase treatment that degrades NETs. In future studies other drugs that have been discussed in this study may be tested regarding their influence on the bacterial co-infection. In addition to the testing of substances, it could be of interest to use knockout mice that are deficient for NE and therefore incapable of formation of NETs(Papayannopoulos et al., 2010). Alternatively, mice that are deficient for PAD4, another knockout mutant that has severely impaired NET formation could be included in this research(P. Li et al., 2010). In contrast to NE and PAD4 knockout mice, mice deficient for DNase are incapable of degrading NETs(Napirei et al., 2000b). This would allow even more detailed insights into the effects of DNase therapies.

9. Reference

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10. Affidavit

I herewith affirm that I am the sole author of the thesis titled “The impact of Influenza A virus triggered neutrophil extracellular traps on bacterial co-infections”, which was written according to the principles of good scientific practice.

I did not make use of any paid dissertation services or other consultants. Nor did anyone receive unpaid services from me for work related to the contents of the submitted thesis.

The thesis was completed at the following institutions: Department of Biochemistry and Research Center for Emerging Infections and Zoonoses (RIZ), University of Veterinary Medicine Hannover, Germany.

This thesis has not been previously submitted for evaluation for admission to an examination, doctoral graduation, or any such purpose.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

[22.02.2024], signature

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