Eosinophil Extracellular DNA Trap Cell Death (EETosis) occurs as a life-cycle stage-specific response to filariae

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Des Weiteren wurden Auszüge auf verschiedenen Konferenzen als Vortrag oder Poster präsentiert.

Table of Content

Abbreviations

Abbreviations

AAM	Alternatively activated macrophage
ABZ	Albendazole
Actb	Beta-actin
ADCC	Antibody-dependent cytotoxicity
AF	Alexa Fluor
Ag	Antigen
ALT	Abundant larval transcript
APOC	African Programme for Onchocerciasis Control
BmEos	Bone marrow-derived eosinophils
bp	Base pairs
BSA	Bovine serum albumin
BV	Brilliant violet
CAMs	Classically activated macrophages
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CLR	C-type lectin receptor
CNRS	Center National De La Recherche Scientifique
Cox1	Cytochrome oxidase c subunit 1
CpG	Cytidine and guanidine rich sequence
CRDs	Carbohydrate recognition domains
DALYs	Disability-adjusted life years
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
DEC	Diethylcarbamazine citrate
DNA	Deoxyribonucleic acid
DNDi	Drugs for Neglected Disease initiative
dNTP	Deoxynucleotide triphosphate
dpi	Days *post infectionem*
DPI	Diphenyleneiodonium
DTT	Dithiothreitol
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EET	Eosinophil extracellular DNA trap
EETosis	Eosinophil extracellular DNA trap cell death
Eos	Eosinophils
EPO	Eosinophil peroxidase
ES	Excretory-secretory product
EtOH	Ethanol
ETosis	Extracellular DNA trap cell death
ETs	Extracellular traps
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FBZ	Flubendazole
FcR	Fc receptor
FCS	Fetal calf serum

FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	Fluorescence minus one
FSC-A	Forward scatter area
FSC-H	Forward scatter height
FSC-W	Forward scatter width
Gapdh	Glycerinaldehyd-3-phosphat-dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPELF	Global Programme to Eliminate Lymphatic Filariasis
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HMDS	Hexamethyldisilazane
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IOP	Iso-osmotic percoll
IVM	Ivermectin
KO	Knock-out
L3 larvae	Third-stage larvae
L3Ag	L3 larval extract
LB	Lysogeny *broth*
LF	Lymphatic filariasis
LPS	Lipopolysaccharide
LsAg	*L. sigmodontis* adult worm extract
MACS	Magnetic activated cell sorting
MBP	Major basic protein
MDA	Mass drug administrations
MEM	Minimum essential medium
MF	Microfilariae
MFAg	Microfilarial extract
MPO	Myeloperoxidase
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
Nd1	NADH-ubiquinone oxidoreductase chain 1
NE	Neutrophil elastase
NET	Neutrophil extracellular DNA trap
NETosis	Neutrophil extracellular DNA trap cell death
NF-κB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells
NK cell	Natural killer cell
NOD	Nucleotide-binding oligomerization protein
o/n	Overnight
OCP	Onchocerciasis Control Programme
OD600	Optical density at 600 nm
PAD4	Peptidyl arginine deiminase 4
PAF	Platelet activating factor
Pam3Cys	Tripalmitoyl-S-glycerylcysteine
PAMPs	Pathogen-associated molecular pattern
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PFA	Paraformaldehyde

PGN	Peptidoglycan
PI	Propidium iodide
PMA	Phorbol-12-myristat-13-acetat
Poly (I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptor
qPCR	Quantitative PCR
RBC	Red blood cells
RELMα	Resistin-like molecule α
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SCF	Stem cell factor
SEM	Standard error of mean
SEM	Scanning electron microscopy
SSC-A	Side scatter area
TDB	Trehalose-6,6-dibehenate
TGF-β	Transforming growth factor-beta
Th cells	T helper cells
Th response	T helper response
TIRAP	Toll/IL-1R domain-containing adapter protein
TLR	Toll-like-receptor
TNF	Tumor necrosis factor
Tris-TE	Tris-EDTA buffer
TSLP	Thymic stromal lymphopoietin
TSLP	Thymic stromal lymphopoietin
U	Units
WHO	World Health Organization
***W*oLP**	Diacyl *Wolbachia* lipopeptide
***w*PAL**	*Wolbachia* surface peptidoglycan-associated lipoprotein
WT	Wild-type

Summary

Filarial nematodes represent a great burden of human morbidity causing diseases like onchocerciasis and lymphatic filariasis. The lack of a vaccination and a short-term therapy with a macrofilaricidal effect hampers the elimination of filarial diseases. To successfully eliminate filariasis, not only new therapies, but also a detailed knowledge of protective immunity is needed. To study the protective immunity against filarial parasites and to test potential new drug candidates, the *Litomosoides sigmodontis* mouse model is frequently used since it shares common immunological features with human filarial infections. Helminths typically induce a type 2 immune response in their host with a profound eosinophilia. Eosinophils are a pivotal cell type in controlling helminth infections and are of particular importance in eliminating microfilariae (MF), the progeny of the filariae. Next to the release of cytotoxic granules, a newly recognized defense mechanism of granulocytes is the so called extracellular DNA trap cell death (ETosis), which results in the release of intracellular DNA into the surrounding. Thereby, invading microorganisms are not only captured, but anti-microbial peptides are directly delivered to the microbes. There are several publications identifying the release of extracellular DNA by neutrophils in response to various stimuli, while only a few studies deal with eosinophil ETosis (EETosis) and simply one paper has been published on EETosis in response to helminths yet.

The results of this PhD thesis show for the first time that bone marrow-derived eosinophils (BmEos) as well as eosinophils isolated from infected animals are able to produce eosinophil extracellular DNA traps (EETs) in response to MF and inhibit MF motility in a DNA-trap- and cell contact-dependent manner *in vitro*. The origin of EETs was identified to be of nuclear and primarily of mitochondrial DNA. Eosinophils primed during *L. sigmodontis* infection and eosinophils from naïve animals showed comparable DNA-trap-dependent MF motility reduction. L3 larvae failed to induce EETosis and were only captured in the presence of plasma from infected animals. In contrast, eosinophils presented adult worm capturing capacity solely in the presence of plasma from naïve animals, which was DNA-trap-dependent. MF-induced ETosis was not based on shear forces, but rather on specific MF antigens recognized by eosinophils. Furthermore, EETosis triggered by MF was independent on antibody-coating as well as TLR-signaling and required no prior priming. Within this thesis the dectin-1 pathway was identified as underlying pathway in MF-induced ETosis. Results from this thesis further suggest the DNA-dependent inhibition of MF motility and MF-

induced DNA release by eosinophils as a conserved mechanism, since murine eosinophils responded to MF derived from the rodent filarial nematode *L. sigmodontis* and the canine heartworm *Dirofilaria immitis*. *In vivo* results showed an increase in local DNA concentration upon *L. sigmodontis* infection and raised systemic DNA concentrations upon intravenous MF injection, which was partly mediated by eosinophils, indicating a potential role of EETosis *in vivo* as well.

Furthermore, I was involved in the testing of several novel potential drug candidates targeting filariasis in pre-clinical studies using the *L. sigmodontis* rodent model. This includes the improvement of oral formulations for existing drugs like flubendazole, as well as new anti-*Wolbachia* drugs including AWZ1066S, ABBV-4083, boron-pleuromutilins and quinazolines.

In conclusion, the results of this thesis show for the first time that murine EETosis occurs as a life-cycle stage-specific response to filariae and identifies the underlying signaling pathway of MF-induced ETosis to be dectin-1-dependent. Furthermore, the results contribute to the knowledge about eosinophil-mediated protective immunity and show that the EETosis occurs as a universal and conserved mechanism against a variety of pathogens. Finally, new potential drug candidates were tested, which present strong candidates in fighting human filariasis.

Zusammenfassung

Filariosen, wie Lymphatische Filariose und Onchozerkose, zählen zu den vernachlässigten Tropenkrankheiten und sind ein großes Gesundheitsproblem in vielen tropischen und subtropischen Ländern. Die betroffenen Menschen leiden nicht nur unter den gesundheitlichen Folgen, sondern auch an der finanziellen und stigmatisierenden Belastung. Daher rief die WHO Initiativen, welche Massenbehandlungen mit Ivermectin und Bekämpfungen von den Krankheitsüberträgern beinhaltet, ins Leben. Die Eliminierung von Lymphatischer Filariose ist demnach bis 2020 und die Eliminierung von Onchozerkose bis 2025 angestrebt. Allerdings wird die Eliminierung dieser Krankheiten durch ein Mangel an effektiven Impfstoffen oder Medikamente zur Massenbehandlung, welche adulte Würme vollständig eliminieren, stark beeinträchtigt. Um den Kampf gegen Filarien weiter voranzutreiben, ist die Untersuchung der Immunantwort gegen Filarien unablässig. Protektive Immunantworten gegen Filarien, filarien-bedingte Immunmodulation und präklinische Studien, welche die Testung von neuen Filarienmedikamenten beinhaltet, kann anhand des gängigen *Litomosoides sigmodontis* Mausmodells erforscht werden. Es ist bereits bekannt, dass Filarieninfektionen eine Typ 2 Immunantwort (Th2), mit typischen Th2-assoziirten Zytokinen und Antikörpern, so wie bestimmten Zelltypen hervorrufen kann. Von großer Bedeutung sind hier die Eosinophilen Granulozyten, welche besonders zu der Bekämpfung der Mikrofilarien (MF), die Nachkommen der Würmer, beitragen. Eosinophile sind in der Lage mit ihrer Granular und den dort gespeicherten zytotoxischen Proteinen, Helminthen anzugreifen und zu töten. Des Weiteren wurde ein neuer Mechanismus bei Granulozyten entdeckt, der durch die Ausschüttung von extrazellulärer DNA pathogene Erreger einfangen kann und damit deren Eliminierung unterstützen kann. Der Vorgang wird als ETosis (extracellular DNA trap cell death) bezeichnet und Ergebnisse dieser Dissertation zeigen, dass knochenmarksgenerierte Eosinophile und Eosinophile, die aus infizierten und naiven Tieren isoliert wurden, DNA Netze ausschütten, wenn sie in Kontakt mit MF kommen. Die ausgeschüttete DNA, dessen Ursprung aus dem Nukleus und vorwiegend den Mitochondrien zurückzuführen ist, sorgt dafür, dass die MF in ihrer Beweglichkeit eingeschränkt werden. Im Vergleich zu knochenmarksgenerierte Eosinophilen, weisen Darm und Pleura Eosinophile eine erhöhte Fähigkeit auf MF einzufangen. Dieses geschieht unabhängig davon ob die Eosinophilen einen vorherigen Kontakt mit dem Erreger

hatten oder nicht. L3 Larven hingegen sind nicht in der Lage die DNA-Ausschüttung durch Eosinophile auszulösen und Eosinophile fangen L3 Larven nur dann ein, wenn Maus-Plasma von infizierten Tieren hinzugeführt wird. Erwachsene Würmer werden erst nach Zugabe von naivem Maus-Plasma eingefangen, welches teilweise über DNA-traps geschieht. Die MF-induzierte DNA-Ausschüttung geschieht unabhängig von Scherkräften und basiert vielmehr auf die Erkennung von spezifischen Oberflächenproteinen der MF. Zusätzlich zeigen Ergebnisse dieser These, dass die MF-induzierte Eosinophil ETosis (EETosis) über Dectin-1 Erkennung ausgelöst wird und beweist daher als Erstes den zugrundeliegenden Signalweg der ETosis. *In vivo* Analysen belegen, dass eine *L. sigmodontis*-Infektion zu erhöhten lokalen DNA-Ausschüttungen führt, während MF systemische ETosis fördern. Die MF-induzierte EETosis basiert auf einem konservierten Mechanismus, da Maus-Eosinophile nicht nur auf Mausparasiten reagieren sondern auch nach dem Kontakt mit MF des Hundeherzwurmes (*Dirofilaria immitis*) DNA ausschütten.

Des Weiteren unterstützte ich eine Vielzahl von präklinischen Studien, in denen, mit Hilfe des *L. sigmodontis* Modells, neue potenzielle Medikamente gegen Filariosen identifiziert wurden. Dabei wurde nicht nur die orale Formulierung für bereits bestehende Medikamente, wie Flubendazol, verbessert, sondern auch neue Medikamente gegen die endosymbiontischen *Wolbachia*-Bakterien (AWZ1066S, ABBV-4083, Bor-Pleuromutiline und Quinazoline) entwickelt.

Die Ergebnisse dieser Arbeit zeigen daher zum ersten Mal, dass die EETosis abhängig vom Lebenszyklus der Filarien ist und, dass die MF-induzierte EETosis über den Dectin-1 Signalweg übertragen wird. Zudem tragen die Ergebnisse dieser Arbeit zu dem Wissen über Eosinophil-vermittelten protektiven Immunantworten bei und zeigen, dass EETosis ein universeller und konservierter Mechanismus als Antwort auf eine Vielzahl von pathogenen Erregern ist. Des Weiteren wurden mehrere neuen Medikamenten getestet, von denen einige vielversprechende Kandidaten zur Bekämpfung von Filariosen identifiziert wurden.

1. Introduction

Helminths are one of the major causes of human pathology since they infect around 1/5 of the world population (1). An important clade within the helminths are filarial nematodes, which represent a great burden of human morbidity causing diseases like onchocerciasis and lymphatic filariasis, which belong to the group of neglected tropical diseases (2).

1.1 Filariae

Helminths are multicellular invertebrates that comprise nemathelminths (nematodes/roundworms) and platyhelminths (flatworms). The latter one is subdivided into trematodes (flukes) and cestodes (tapeworms) and they are characterized by a flat leaf-like or flat ribbon-like body, respectively. Nematodes are members of the phylum Nematoda and are one of the most abundant invertebrates on earth and they have adapted to more habitats than any other animal on earth including an endoparasitic or ectoparasitic life style of flora and fauna. *Caenorhabditis elegans* is one of the most studied free-living nematodes (3, 4).

Of human clinical relevance is the order Spirurida with the family Onchocercidae, which includes human pathogenic filariae such as *Wuchereria bancrofti*, *Brugia* species, *Loa loa* and *Onchocerca volvulus* as well as the canine heartworm *Dirofilaria immitis*, which infects dogs (3-6).

1.2 Filarial life-cycle

Filariasis is a vector-born disease and the transmission of filariae belonging to the superfamily Filarioidea and the family Onchocercidae are transmitted to the mammalian host by blood-feeding insects. Different filarial species require specific hosts and intermediate hosts for their life-cycle (4). The common life-cycle pattern of filariae starts with the transmission of the infective third-stage larvae (L3 larvae) to their host through the bite of the vector. Within the host, the larvae migrate to their final location, where they molt into adult worms via the L4 larval stage. After mating, the adult female worms release microfilariae (MF) into the skin or blood stream, where they can be taken up through another blood meal by the vector. Within the vector, the life-cycle of the filariae is completed through the development from the MF (L1) stage into the infective third-stage larvae by two moltings (Figure 1)(7).

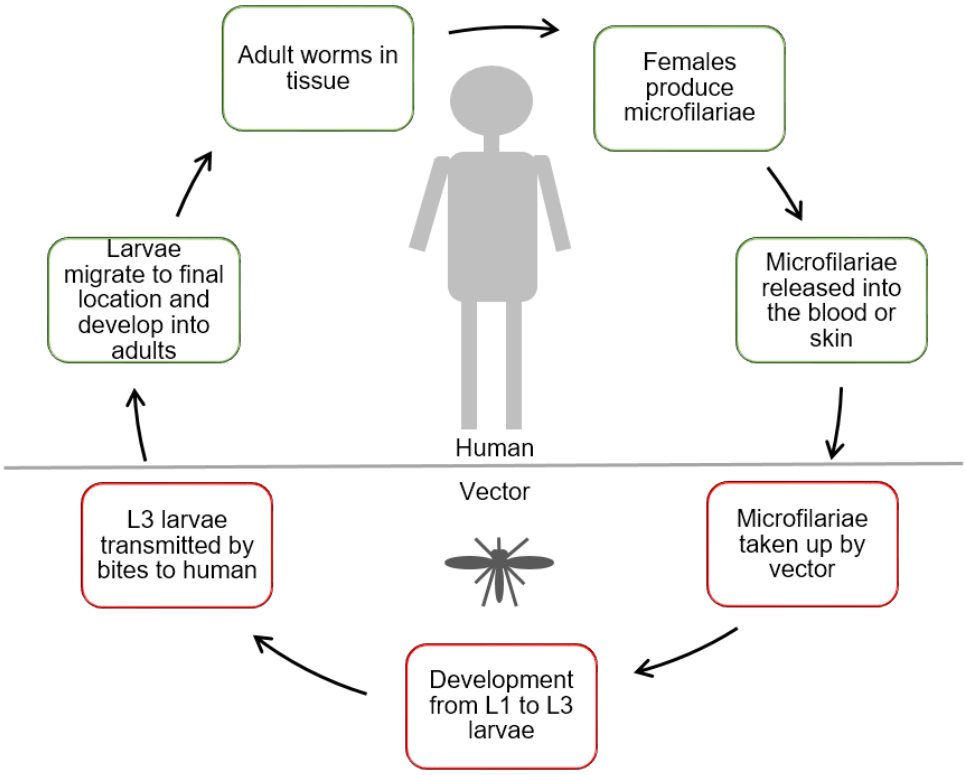

Figure 1. Filarial life-cycle. Adapted after Manson's Tropical medicine

1.3 Diseases: Onchocerciasis and lymphatic filariasis

1.3.1 Onchocerciasis

Onchocerciasis is also known as river blindness since severe immune reactions within the eye can cause vision impairment and blindness. The causative agent of onchocerciasis is the filarial nematode *O. volvulus,* which is transmitted through the bite of the black fly belonging to the *Simulium* species. Since these flies breed in rivers and streams, the incidence of the disease is restricted to regions close to river sites in sub-Saharan Africa and few areas in Yemen as well as the boarder of Venezuela and Brazil (7, 8).

Infective L3 larvae migrate to subcutaneous tissue and develop in skin nodules (onchocercomata) into adult worms. Adult male worms exhibit a size of 2-5 cm, while female adult worms can become 35 to 70 cm long. The adult worms mate and release MF around 10 to 15 months after the infection (7, 9). The MF are mainly found in the skin where they can be taken up again by the vector. Within the black fly, MF migrate from the midgut into the thoracic muscles via the hemocoel. Within the thoracic muscle, MF develop finally into third-stage larvae, which move to the blackfly's proboscis and can infected another human during a further blood meal. The life span of the adult worms can be up to 15 years, while MF can survive for 1 to 3 years (7, 8, 10, 11).

99 % of infected people live in 31 sub-Saharan African countries, while few infection spots in Latin America (border of Brazil and the Bolivarian Republic of Venezuela) and the Yemen are left (12-14).

It is estimated that in 2017, 20.9 million people were infected with onchocerciasis worldwide and that 14.6 million people presented skin diseases and 1.15 million people suffered from vision impairment and loss (14). Pathology due to onchocerciasis reduces agricultural productivity of local people and increases poverty leading to a large public health problem (14).

Pathology occurs due to immune reactions towards the *Wolbachia* endosymbionts that are present in most human-pathogenic filariae and are recognized by the immune system upon the death of MF and are mainly seen in people exposed to the parasite for a long period (15-18). *Wolbachia* from dead or dying MF can provoke inflammation in the skin leading to rashes, severe itching, and skin lesions or the cornea of the eye causing vision impairment and blindness over time. Neutrophils play a major role during this inflammation and together with eosinophils they are frequently found around dying MF in the skin and cornea (19). The *Wolbachia* surface peptidoglycan-associated lipoprotein (*w*PAL) was identified to induce neutrophil inflammation during ocular keratitis (20, 21). A further skin manifestation in onchocerciasis patients are the formation of nodules. These onchocercomata contain the adult worms and, depending on the immune status of the patient, the reaction surrounding the worms can differ among individuals. There are often neutrophils and fibrin in direct contact with the worms, which is surrounded by epithelioid cells and foamy macrophages. The last layer is commonly formed by lymphocytes and collagen deposits (22). Again, the release of *Wolbachia*-derived products within the onchocercomata drives neutrophil inflammation (23) as well as neutrophil extracellular DNA trap cell death (neutrophil ETosis/ NETosis) (24). ETosis is a type of cell death that causes the release of extracellular DNA into the surrounding environment, which mediates the entrapment of the foreign organism and can support its killing. Depletion of *Wolbachia* by antibiotics resolves the DNA traps within the nodules suggesting the specificity of the neutrophil ETs against *Wolbachia* and indicating the importance of the NETosis as part of the protective immunity against filariae (24).

1.3.2 Lymphatic filariasis

Lymphatic filariasis (LF) is caused by filarial nematodes belonging to the family of Filarioidea and depict a major health and economic burden in tropical countries (25).

Lymphatic filariasis, commonly known as elephantiasis, is caused by *Wuchereria bancroftii, Brugia malayi* and *Brugia timori,* which are transmitted by different mosquito species (7, 8, 26).

In the human host infective L3 larvae migrate from the bite wound to the lymphatics where they develop into adult worms and release MF into the peripheral blood after 8 months in case of *W. bancrofti* and 3 months in *B. malayi* infections (7). Circulating MF have nocturnal periodicity. MF taken up by the blood-feeding mosquito migrate from the midgut to the thoracic muscles. There they develop into third-stage infective larvae and migrate through the hemocoel to the mosquito's proboscis. Through another blood meal of the mosquito, the infective L3 larvae are transmitted again to another human host. Adult worms have a reproductive life-span of around 5 years were they can constantly produce MF that survive on average for 1 year (7, 8, 26).

LF is mainly found in sub-Saharan Africa and South-East Asia, while the distribution differs dependent on the parasite species. *W. bancrofti*, the main causative agent of lymphatic filariasis (90 % of the cases), is mainly found in sub-Saharan Africa, South-East Asia, India, Latin America and the Caribbean. *B. malayi* is primarily distributed in India, Malaysia, China, Indonesia, Philippines, and some Pacific islands, while *B. timori* occurs on the Timor Island of Indonesia (27).

It is estimated that more than 67.88 Million people are infected with LF, while 19.43 million people suffer from hydrocele and 16.68 million people from lymphedema, which leads to at least 2.8 million disability-adjusted life years (DALYs) (28-30).

In case of LF, immune reactions in response to adult worms residing in the lymphatics cause the disease symptoms. Clinical evident filariasis can develop through dilations of lymphatic vessels (lymphangiectasia) (31, 32). The vessel dilation is triggered through the release of host or worm proteins. Acute dermatolymphangioadenitis can impair lymphatic function supporting secondary bacterial and viral infections (31, 33-35). A second pathological pathway resulting in clinical evident filariasis is caused by the death of adult worms leading to acute inflammation in the lymphatic vessels and adjacent lymph nodes (32). The so called acute filarial lymphangitis can result in chronic lymphedema. Typical clinical manifestations include hydrocele, lymphedema, elephantiasis and acute adenolymphangitis. Hydroceles result from fluid retention in the tunica vaginalis leading to swelling around the testicles, while lymphedema and the progressing elephantiasis are often affecting lower extremities like the leg. These lymph swellings in the limbs and genitals lead to disabilities, physical impairment,

subsequent income loss and social stigma. While hydroceles are frequently observed for *Wuchereria* infection, *Brugia* infections do not show clinical signs of hydrocele and other genital lesions (36).

1.3.3 Treatment strategies

The first approach to control onchocerciasis has been done through the collaboration of the World Health Organization (WHO), the United Nations Development Programme and the Food and Agriculture Organization building the Onchocerciasis Control Programme (OCP) in 1974, which included vector control and, after the discovery of ivermectin, large-scale ivermectin treatment in West-Africa. Subsequently, in 1995 the WHO launched the African Programme for Onchocerciasis Control (APOC) and in 2000 the Global Programme to Eliminate Lymphatic Filariasis (GPELF) to target the control of these neglected tropical diseases. According to the latest update, defined in the Roadmap on Neglected Tropical Diseases 2021-2030, the main goal is the elimination of LF as public health problem and to stop the transmission of onchocerciasis by 2030 via mass drug administrations (MDA) and vector control (12, 37-39).

Standard drugs used for MDA include ivermectin (IVM), albendazole (ABZ) and diethylcarbamazine citrate (DEC) (25, 40). Ivermectin causes a microfilaricidal effect and temporary inhibition of the embryogenesis of adult female worms, which interrupts microfilaremia for up to 6-12 months (41). Its mode of action is the interaction with postsynaptic glutamate-gated chloride channels causing paralysis of the MF (42-44).

Similar, DEC targets the microfilarial stage and causes temporary inhibition of the embryogenesis of female worms. DEC is a piperazine derivative but the exact mode of action is still unclear. It has been suggested that DEC inhibits the cyclooxygenase pathway and the lipoxygenase pathway (42, 43, 45).

Another drug used in combination for MDA is ABZ, which is a carbamate benzimidazole and inhibits the polymerization of β-tubulin and microtubule formation in helminths. This broad-spectrum anti-helminthic drug is not only used to treat filariasis but also against other nematodes, flatworms and cestodes (42, 46). However, several reports show that ALZ alone or in combination with other microfilaricidal drugs has only little effect on MF and adult filariae (47).

The WHO recommends MDA of IVM annually or bi-annually for the control of onchocerciasis. LF is treated with a combination of IVM and ABZ in sub-Saharan Africa and recently DEC, IVM plus ABZ as triple therapy in countries non-endemic for

onchocerciasis and loiasis (48-50). Due to the MDA, LF prevalence has been reduced and fourteen countries have already achieved elimination, while 7 further countries, after stopping MDAs, are under surveillance of having eliminated LF. However, 52 countries are still endemic and require MDA treatments for LF (29).

Moreover, there are certain limitations for the current chemotherapy strategies. The triple therapy (IVM, ABZ and DEC) used to treat LF may cause serious adverse events in patients of loiasis and onchocerciasis (13, 51-55), impairing the implementation of the triple therapy in areas co-endemic of those diseases.

A further disadvantage of current treatment strategies is the restricted temporary inhibition of the embryogenesis and the lack of a macrofilaricidal, i.e. adult worm killing, efficacy. Since the adult worms have a reproductive life-span of up to 5 years for LF and up to 15 years for onchocerciasis, repeated annual or bi-annual drug administration is required for the reproductive life-span of the adult worms (25). However, in many countries like in Central-Africa, people live in conflict situations, poverty and rural communities inhibiting annual treatments. Furthermore, modeling studies suggest that the triple-drug therapy is in particular valid in clearing filariasis in areas with high prevalence while it loses its cost efficiency in areas with low prevalence (48). Thus, the expensive and labor-intensive treatment as well as the reduced usability of microfilaricidal drugs calls for the identification of an effective macrofilaricide, which can be administered over a short period of time (56, 57).

A validated good approach in identifying macrofilaricidal drugs has been done by screening drugs against the *Wolbachia* endosymbionts of filariae (58). Most human-pathogenic filariae (except for *Loa loa*) contain endosymbiotic *Wolbachia,* which are essential for growth, survival and reproduction of the filariae and can be targeted by antibiotics (59). Initial murine studies using the filarial nematode *Litomosoides sigmodontis* showed that the tetracycline doxycycline depletes *Wolbachia*-bacteria within the filariae, which leads to female worm sterility (60, 61). In first field studies administration of doxycycline together with ivermectin resulted in a long-lasting inhibition of filarial embryogenesis and accompanied MF clearance in onchocerciasis patients (17). Permanent sterility of female *O. volvulus* and *W. bancrofti* worms and the clearance of MF by doxycycline was further proven in additional clinical trials, where doxycycline was administered for 6 weeks (62-64). In contrast to existing anti-filarial drugs, doxycycline was shown to have a macrofilaricidal effect as well (65, 66). Therefore, doxycycline has been described as the first safe and well-tolerated

macrofilaricidal drug for filariasis. However, even though treatment duration could be reduced from 6 weeks to 4-5 weeks, a daily doxycycline treatment for such a period is not feasible for an MDA approach (67). Furthermore, doxycycline cannot be applied to children below the age of 8 years and pregnant as well as lactating women. Thus, doxycycline is a macrofilaricidal drug that can be used for individual therapy. Recent results from pre-clinical efficacy models suggest that combinations of different antibiotics allow a significant reduction of treatment time required to achieve the *Wolbachia* depletion in filariae. Thus, 4 daily oral treatments with a combination of moxidectin and rifapentine cleared >99 % of *Wolbachia* endosymbionts in *L. sigmodontis*-infected mice (68). Similarly, a combination of rifampicin and albendazole in pre-clinical studies has been proven to clear *Wolbachia* of *B. malayi* in SCID mice treated for one week (69).

Furthermore, additional drugs have been evaluated in pre-clinical studies and present promising candidates for filariasis. For example the tylosin derivative ABBV-4083 has an improved anti-*Wolbachia* activity. ABBV-4083 shows several advantages over doxycycline and tylosin due to its increased oral bioavailability and effectiveness after short oral dosing regimens in pre-clinical trials (70, 71). A first clinical trial investigated the safety of this drug, which presents a good candidate in eliminating human filariasis (72).

Further macrofilaricidal candidates under consideration for the treatment of filarial nematodes via the Drugs for Neglected Disease initiative (DNDi) are emodepside and oxfendazole (72). Oxfendazole is a methylcarbamate benzimidazole inhibiting microtubule formation and glucose uptake by nematodes. The broad spectrum anti-helminthic oxfendazole is used in veterinary settings treating nematodes like *Dictocaulus viviparous* and *Ascaris suum* in cattle, as well as *Muellerius capillaris* in sheep and horse (73). Its anti-filarial activity was shown in pre-clinical studies (74-78). Emodepside is also currently used in veterinary settings against helminth infections in dogs and cats. First-in-human studies assessed its safety and a phase II trial will analyze its safety and efficacy in onchocerciasis patients in sub-Saharan Africa (72).

1.4 *Litomosoides sigmodontis*

To study filariasis, its immune reaction, filarial immunomodulation and test potential drug candidates, a comparable animal model is required. Therefore, the rodent filarial nematode *L. sigmodontis* model was previously established. *L. sigmodontis* is found in North-, Central- and South-America and its natural host is the cotton rat (*Sigmodon*

hispidus). Mongolian gerbils (*Meriones unguiculatus*) as well as BALB/c mice are also susceptible for *L. sigmodontis* and the infection is permissive in these hosts since the parasite is able to complete its life-cycle (79, 80). Since many studies have shown similarities in the immune reaction occurring during a *L. sigmodontis* infection in mice and human filariasis, the *L. sigmodontis* infection is a validated model to study protective immunity and immunomodulation of chronic filarial infections as well as to pre-clinical test potential anti-filarial drug candidates (81, 82).

Infected blood feeding tropical rat mites (*Ornithonyssus bacoti*) transmit infective L3 larvae of *L. sigmodontis* via a blood meal. The larvae migrate from the penetration site in the skin through the lymphatics into the pleural cavity within 2 to 6 days after infection (days *post infectionem* (dpi)). It takes around 8 to 12 days for the L3 larvae to molt into L4 larvae followed by a second molting into adult worms 28 to 30 dpi. The adult worms sexually reproduce releasing MF (L1 larvae) into the peripheral blood around 56 dpi. By the next blood feeding, circulating MF can be taken up again by mites. Within the intermediate host, MF develop into infective L3 larvae via two molts (Figure 2) (83). Peak MF counts are observed in mice 70-77 dpi. Clearance of adult worms starts ~56 dpi and by 120 dpi most susceptible BALB/c mice have eliminated the infection. Semi-resistant C57BL/6 mice however eliminate the majority of worms after the molting into adult worms around 30 dpi (84).

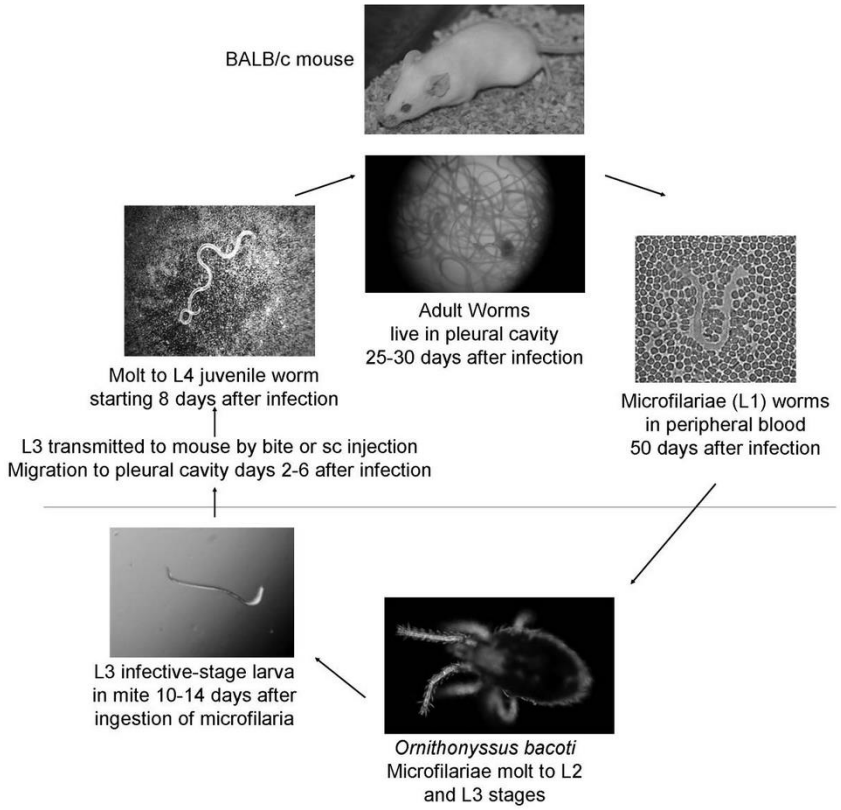

Figure 2. *Litomosoides sigmodontis* life-cycle. Hübner *et al.* (83)

1.5 Immune reactions

An immune response during an infection can be divided into different phases. The initial response occurs through the rather unspecific innate immunity serving as a first line response. Activation of complement, phagocytosing and antigen presenting cells are crucial in initiating adaptive immunity, which contributes to pathogen removal and clearance of the infection. Invading pathogens are immediately recognized by phagocytes like macrophages, dendritic cells and neutrophils. The recognition of the pathogens or tissue damage by the invading organisms occurs through different pattern recognition receptors (PRR) detecting pathogen-associated molecular pattern (PAMPs) or damage-associated molecular patterns (DAMPs), respectively. Important PRRs are toll-like receptors (TLRs), nucleotide-binding oligomerization (NOD) proteins and c-type-lectin receptors (CLRs), which are expressed on various innate immune cells (85-87). Fibroblasts, epithelial cells and keratinocytes are stimulated to produce various types of cytokines and chemokines attracting more neutrophils and macrophages. Together with complement activation, the release of antimicrobial peptides as well as opsonization by these phagocytes is supported. Activation of dendritic cells and macrophages at the site of infection leads to the migration into draining lymph nodes, where they activate naïve CD4+ T cells and thus initiate the adaptive immune response. Finally, effector CD4+ T cells interact with B cells to activate adaptive immunity and thus activate the humoral immune response (86).

Depending on the pathogen present, different cytokines are produced by the antigen presenting cells influencing the subsequent immune reaction (86, 88). Bacterial, viral and some protozoan infections trigger the type 1 immune response. Initiation of the type 1 immune response occurs in the presence of interferon gamma (IFNγ) and interleukin (IL) -12 cytokines produced by dendritic cells and natural killer (NK) cells. Important effector cells in the type 1 immune response are NK cells, NKT cells, CD4+ and CD8+ Th1 cells, classically activated macrophages (CAM) and neutrophils. The response includes high productions of IL-12, IL-18, IFNγ as well as immunoglobulin (Ig) G2b and IgG3 (86, 89).

Helminths on the other hand induce a type 2 immune response, which is associated with CD4+ Th2 cells, alternatively activated macrophages (AAMs) as well as the expansion, recruitment and activation of eosinophils, mast cells and basophils. High levels of IL-4, IL-5 and IL-13 as well as the isotypes IgG1 (mice)/ IgG4 (humans), IgA and IgE are observed (86, 90, 91).

Helminths have developed certain immunomodulatory methods downregulating parasite-specific immune responses and thereby ensuring parasite survival and causing a chronic infection. This was accomplished through the long co-evolution of parasites and their hosts enabling the parasites to modulate the immune system of their hosts. This however, serves certain benefits for the host as well, since a successful immunomodulation by the helminths limits immunopathology and avoids the development of clinical manifestations (92-96). The development of a more regulatory environment occurs through the generation of regulatory T cells and the production of transforming growth factor-beta (TGF-β) as well as IL-10. This causes the inhibition of T cell mediated immunity through a state of anergy. Furthermore, type 2 immune responses generated during helminth infections support the development of AAMs, which have mainly a regulatory role by suppressing T cell responses through arginase-1 production, TGF-β release and inhibiting the development of CAMs and thus suppressing pro-inflammatory immune responses (87, 97-99). An additional modification of the type 2 immune response includes antibody isotype switching from IgE to IgG4 in humans. The latter one exhibits non-inflammatory properties since it fails to crosslink receptors on basophils and mast cells as well as the activation of the complement system (31, 100-103).

1.6 Protective immunity against helminths

To overcome a helminth infection, the host typically generates a type 2 immune response, which is initiated by the production of alarmins, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 from epithelial cells in response to tissue damage caused by the multicellular parasites (90). As a consequence, type 2-releated cytokines like IL-4, IL-5, IL-9, IL-10, IL-13, IL-21, and IL-25 (104) as well as antibody isotypes IgG1 (mouse), IgG4 (human) and IgE are produced (105). Important cells associated with helminth infections are AAMs, Th2 cells, regulatory T cells, as well as granulocytes, which are pivotal cell types in controlling helminth infections (106).

Antigen presentation by dendritic cells and macrophages in the presence of IL-4 causes the priming of CD4+ T cells (86). These Th2 cells support the production of IL-4, 5 and 13 leading to the stimulation of mast cells and eosinophils. The Th2 cytokine IL-4 thereby causes B cells to switch to the isotype IgE and IgG1/IgG4, which further supports the Th2-immune response. IgE can either directly attack the parasite or can mediate cell degranulation by binding to the Fc epsilon receptor (FcεRI) on human eosinophils, mast cells and basophils (90). IgG1 binding to Fc gamma receptor (FcγR)

on mast cells, basophils, eosinophils as well as neutrophils and macrophages support the opsonization of pathogens and the activation of the respiratory burst (FcγRI) (86). Furthermore, the type 2 immune response occurring during helminth infections has an impact on the macrophage plasticity. Bacterial and viral infections evoke the production of the type 1 cytokine IFNγ inducing the production of CAMs. The type 2 cytokine IL-4 produced during helminth infections on the other hand causes a shift towards AAM development (87, 107). These macrophages produce arginase 1 and resistin-like molecule α (RELMα), which contributes to parasite expulsion as well as tissue repair and wound healing (87, 104, 108). RELMα is also involved in wound healing and tissue repair by stimulating collagen production in myofibroblasts (107). Thus, the massive tissue damage induced by the migrating larvae is limited and controlled through AAMs (109). On the other hand, AAM accumulating in the pleural cavity of *L. sigmodontis*-infected mice is thought to suppress Th2 immune responses facilitating the persistence of the filariae (108).

1.6.1 Granulocytes and helminth infections

1.6.1.1 Granulocytes

A major player in the innate immune response against several pathogens are granulocytes, which are a class of leukocytes of the innate immunity. They are comprised off neutrophils, eosinophils and basophils (86, 110).

1.6.1.1.1 Neutrophils

Neutrophils are the most abundant type of granulocytes and their differentiation is initiated through the release of IL-6, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (111-114). Inflammatory mediators like complement C5, Tumor necrosis factor (TNF), IL-8 and leukotriene B4 are also able to trigger neutrophilia and neutrophils are the first cell type recruited to the site of infections (115). Neutrophils are a crucial component of the innate immune response through phagocytosis, degranulation and the release of reactive oxygen species (ROS) and extracellular DNA traps. Pathogen recognition through CLR and complement receptors enables neutrophils to phagocytose and thereby remove the invading pathogens (116). Furthermore, the release of lytic enzymes and ROS can cause pathogen damage. For example neutrophil serine proteases like neutrophil elastase (NE), proteinase-3 and cathepsin G can directly damage and kill microbes (117). The same is true for several antimicrobial peptides

released by neutrophils like α-defensin and cathelicidins, which also serve as pro-inflammatory mediators (117, 118) .

Secretion of inflammatory mediators amplifies the initial immune response through the activation of resident cells, such as macrophages and mast cells as well as causing recruitment of additional effector cells like neutrophils, monocytes, lymphocytes, eosinophils and basophils (119, 120).

1.6.1.1.2 Basophils

Basophils are a comparatively rare type of granulocytes, which share common features with mast cells. Similar to mast cells, basophils also express the FcεRI and are able to secrete ROS as well as histamine. Furthermore, they are characterized by the presence of basophilic granules and the expression of high affinity FcεRI that is able to bind IgE. Activation of this receptor mediates the release of leukotriene C4 and histamine as well as IL-4 and IL-13 (119).

1.6.1.1.3 Eosinophils

Eosinophils are derived from the bone marrow and migrate quickly into the thymus and gastrointestinal tract. They are recruited, often in high numbers, to sites of tissue repair and to allergic inflammatory foci. Next to allergic responses, eosinophils are also associated with some viral infections and in particular with helminth infections since the high levels of IL-5 present during the helminth-induced type 2 immune response promotes the development of eosinophils. Eosinophil development is in addition supported by IL-3 and GM-CSF, while their recruitment to the site of infection occurs through the recognition of eotaxins (chemokine ligand 11 (CCL11/eotaxin 1) and CCL26/eotaxin 2) (119). Eosinophils are equipped with several FcRs, including FcαR, FcγRII, FcεRI and FcεRII as well as PRRs like TLRs and CLR. They are distinguished from the other granulocytes due to their high cationic protein content within their granules. The granule proteins eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) have all toxic activity (119, 121). For example ECP and EDN are ribonucleases with antiviral activity. ECP can also induce pore formation in target cells as well as suppression of T cell and B cell responses. MBP can stimulate mast cell and basophil degranulation, while EPO catalyzes the formation of ROS, which can kill target cells (122). All of these proteins may also directly damage parasitic helminths (119). Moreover, eosinophils can regulate important immune mechanisms by the rapid production of IL-4 and IL-13

and the ability to process and present antigens during helminth infections and thereby adding to host-protective immunity (119).

1.6.1.2 Granulocyte effector mechanisms

A pivotal effector mechanism of granulocytes is the release of their cytotoxic granules, which contain inflammatory mediators as well as anti-microbial peptides. This not only helps to eliminate invading pathogens but reinforces the ongoing inflammation. There are several mechanisms to activate the cells and to trigger the release of the intracellular granules.

Antibody-dependent cellular cytotoxicity (ADCC) occurs through the crosslinking of Fc receptors by antibodies on immune cells, which can cause cell degranulation. It is of particular importance for NK cells and granulocyte degranulation but can also occur in monocytes, macrophages and dendritic cells. The crosslinking of bound antibodies induces a signaling cascade within the cell that causes the release of cytoplasmic granules, phagocytosis and the upregulation/expression of surface activation molecules (119). FcγRII, FcγRIII and FcαRI are constitutively expressed by neutrophils and eosinophils. The crosslinking by IgG1, IgG3 and IgG2 as well as IgA antibodies, respectively can activate these cells to undergo different effector mechanisms like phagocytosis or granule release (86, 116). Human eosinophils are in addition equipped with FcεRI, which is also expressed on murine and human basophils and mast cells (119). Its crosslinking by IgE forces granular release as well (86).

Recognition of PAMPs and DAMPs by PRR is an additional method to activate granulocytes. The most extensively studied PRRs are TLRs, which not only detect PAMPs located in the extracellular environment but also PAMPs found within the cells. Their ligands can be peptides, nucleic acids or glycans (85). TLR-1, 2, 4, 5 and 6 are located at the plasma membrane and mainly recognize bacterial cell wall components, while TLR-3, 7, 8 and 9 can be found within the endosome and detect foreign nucleic acids. Most TLRs (except for TLR-3) signals via MyD88 inducing a signaling cascade that can lead to cytokine and chemokine transcription (85). Neutrophils and eosinophils are highly equipped with TLRs and constant expression of TLR-1, 2, 4, 5, 6, 7, 8 and 9 can be found on neutrophils (123), while eosinophils express TLR-1, 2, 3, 4, 5, 6, 7, 9 and 10 (121). Of particular importance in detecting bacteria are TLR-2, TLR-4 and TLR-6. TLR-4 for example recognizes LPS, while TLR-2 can form a heterodimer with TLR-1 or 6 recognizing peptidoglycans or lipopeptides, respectively (85). TLR activation is of particular importance for the recognition of *Wolbachia*. It was shown for

example that *Wolbachia* mediate an inflammatory response through TLR-2 and TLR-6 recognition and that this was dependent on the adaptor molecule MyD88 and Toll/IL-1R domain-containing adaptor protein (TIRAP) (124). The diacyl *Wolbachia* lipopeptide (*W*oLP) can directly induce neutrophil-mediated keratitis in mice as well as macrophage and dendritic cell activation. Primed dendritic cells in turn were able to polarize a mixed Th1 and Th2 response by CD4+ T cells and thus *Wolbachia* recognition by TLRs not only impacts the innate immunity but also has an effect on the adaptive immunity (20).

Furthermore, CLR are important PRRs expressed on various cells and are mainly known in anti-fungal responses (125). However, it becomes more and more evident that they also play a role in the recognition of bacterial, viral and parasitic infections (125). CLR are receptors that bind carbohydrates and they can be categorized into different groups according to their structure and phylogeny (126). The most important groups are group II, which are calcium-dependent lectins with single carbohydrate recognition domains (CRDs), group V, which are calcium-independent lectins with no CRDs and group VI, which are calcium-dependent and bearing multiple CRDs (126, 127). The second group includes the CLR dectin-2, which recognizes α-mannans (expressed on *Mycobacterium tuberculosis* (128)) and O-linked mannobiose-rich glycoproteins (*Schistosoma mansoni* (129)) as well as mincle, which binds α-mannose (*M. tuberculosis* (130)) and mannitol-linked glyceroglycolipid (*Candida albicans* (131)). Dectin-1 belongs to the group V and it recognizes β-glycans, which is for example expressed on *Leishmania infantum* (132), while group VI harbors the mannose receptor that binds high mannose, which can be expressed by soluble egg antigen (SEA) of *S. mansoni* (133) and Omega-1, produced by *Trichuris muris* (125, 134).

The binding of TLR and Dectin ligands activates the NF-κB pathway as well as interferon regulatory factors. As a consequence, exocytosis, which is the release of granules by fusion with the plasma membrane, and cytolysis, which is the rupture of the plasma membrane, occurs (135). Both mechanisms result in the release of granules containing cytotoxic cationic proteins as well as cytokines and chemokines into the surrounding tissue. This may not only lead to the killing of parasites, but also acts as an alert for other immune cells (119, 136).

1.6.1.2.1 Exocytosis/ cytolysis/ piecemeal degranulation

The release of the intracellular granules of granulocytes can occur through different mechanisms. The best known mechanisms are the classical exocytosis and compound

exocytosis, where the intracellular granules directly fuse with the plasma membrane or intracellular granules fuse together first and then with the plasma membrane, respectively. Granular release is often observed in interaction with large parasites. Another pathway in secreting the granular content into the surrounding environment is the piecemeal degranulation, which is characterized by the release of the granule content without fusion of the granules with the plasma membrane. This is accomplished through the shuttling of the granular content via secretory vesicles from the granules to the plasma membrane. The last method to release granular content is through cytolysis, which is a type of cell death that is distinct from apoptosis and necrosis. The loss of membrane integrity causes the release of the intracellular granules into the surrounding environment. However, no membrane blabbing and chromatin condensation and fragmentation occurs as it is seen for necrosis and apoptosis, respectively. In contrast, cytolytic cells can be associated with or without extracellular DNA, the latter being then described as extracellular DNA trap cell death (ETosis) (135).

1.6.1.2.2 Extracellular DNA trap cell death (ETosis)

A newly recognized defense mechanism of granulocytes is the so called extracellular DNA trap cell death (ETosis). It is a form of cell death, where intracellular DNA is explosively released into the surrounding (137). It is distinct from apoptosis and necrosis, where DNA stays condensed and compact (138). The mechanism was first recognized in neutrophils (NETosis/NETs) in response to microbes and their products. However, several publications show that also eosinophils (eosinophil ETosis/EETosis), basophils and mast cells are able to cast DNA traps (137, 139-141). The ETosis is initiated by a variety of foreign substances. These foreign substance trigger DNA decondensation, which causes the entangling of granules and cytosolic products with DNA. Finally, the cell membrane collapses, releasing the DNA, which then co-localizes with released granules containing anti-microbial peptides. This mechanism causes not only the capturing of invading organisms, but it also delivers anti-microbial peptides directly to the microbe, which inhibits its escape and promotes its killing (138, 142-145).

ETosis induction is not only triggered by several gram-positive and gram-negative bacteria, but also by fungi, viruses as well as parasites (141, 144-147). Furthermore, antibody-antigen complexes (148) and antibodies (149) can trigger DNA trap formation. However, ETosis induction is not only restricted to the stimulation by the

whole organism but can also be triggered through pathogen-derived products like lipopolysaccharide (LPS) as it was shown for EETosis and NETosis (141, 144). EETosis and NETosis can be induced by phorbol-12-myristat-13-acetat (PMA) and zymosan (150, 151), while calcium ionophore (152), IgG and IgA can trigger EETosis (137, 141). Additional factors can support the release of extracellular DNA by eosinophils. For example IL-5 and GM-CSF can enhance platelet activating factor (PAF)-induced EETosis (137) and IL-5 or IFNγ was required for human eosinophils ETosis in response to C5a, LPS and eotaxin (141).

The origin of the extracellular DNA is controversially discussed by researchers. There are some publications identifying the release of mitochondrial DNA from viable eosinophils and neutrophils (141, 153). However, there are also several publications stating the disintegration of the lobular structure of the neutrophil and eosinophil nuclei, chromatin decondensation and the nuclear envelop disaggregation, which leads finally to the rupture of the cell releasing histone-bearing nuclear DNA into the surrounding (137, 154). The release of mitochondrial DNA rather than nuclear DNA would require energy and it has been argued that DNA ejection may not be possible from a dead cell. Furthermore, viable cells can perform additional effector mechanisms like phagocytosis of the entrapped organism (155, 156). On the other hand, mitochondrial DNA is much rarer than nuclear DNA, which questions whether low mitochondrial DNA amounts may be able to entrap large multicellular pathogens such as helminths. In addition, it is believed that the high fluid resistance within the cytoplasm is too challenging for efficient ejection of mitochondrial DNA (138). Finally, mitochondrial DNA lack histones and is thus highly susceptible to damage. Moreover, histones from neutrophil traps have been shown to have additional antimicrobial activity (138, 145).

The pathway involved in DNA trap formation can occur via multiple distinct pathways and it appears to be dependent on the stimulus present. However, several publications have identified the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway to be involved in human eosinophil and neutrophil ETosis (137, 157, 158). While there is very little known about the pathway involved in EETosis, NETosis often involves NE and myeloperoxidase (MPO), which appear to be released from granules and contribute to histone degradation and thus to chromatin decondensation (159). Furthermore, the highly expressed peptidyl arginine deiminase 4 (PAD4) in neutrophils, which converts arginine into citrulline and thereby removes positive

charges from the histones, may be involved in chromatin decondensation during NETosis (155, 160).

Interestingly, there are distinct differences between neutrophil and eosinophil ETosis. Within the NETs, not only granules but also cytosolic proteins are found. Eosinophil DNA-traps on the other hand show mainly entangling of intact granules, which can still respond to cytokines leading to the release of their content (137, 154). Moreover, there are structural differences between neutrophil and eosinophil traps. Eosinophil traps are more diffuse, while neutrophil traps are thinner and present less globular structures (145). The stability of the DNA traps varies as well between the granulocytes. Eosinophil traps are less prone to DNA degradation, since they harbor less enzymes with protease activity, which can degrade the traps (145).

1.6.1.3 Granulocytes as effector cells in helminth infections

Due to the fast recruitment of neutrophils into the skin, they present the first line response against invading L3 larvae. Results from our group and colleagues showed that a delayed recruitment of neutrophils to the site of L3 infection and lack of neutrophils results in an increased worm burden in the *L. sigmodontis* mouse model (161-163). Furthermore, depletion of neutrophils from the granulomas, which are cell accumulations around the adult worms, resulted in a suppressed adult worm killing (164, 165).

The role of basophils during filarial infections is less clear. Depletion of basophils had an impact on eosinophilia and IgE production as well as numbers of parasite-specific T cells during *L. sigmodontis* infections (166). However, MF loads as well as adult worm burden remained unaffected by the depletion of basophils suggesting that basophils may primarily support the type 2 immune response than generating protective immunity against filariae during primary infection, although they are implemented in protective immune responses following vaccination (166, 167).

Eosinophil numbers on the other hand, increase during the course of helminth infection leading to an eosinophilia, a hallmark of parasitic helminth infections (105). A lack of eosinophils not only causes an increase in adult worm burden but also higher MF loads (168-170). This indicates a pivotal role of neutrophils in killing L3 larvae and adult worms, while eosinophils seem to be responsible for eliminating adult worms and MF during primary infections. Thus, granulocytes appear to have distinct roles and targets during filarial infections.

1.6.1.3.1 ETosis against parasites

Most studies focused on the ETosis in response to bacteria and their products, while so far and only few studies deal with ETosis in response to parasites.

1.6.1.3.1.1 Neutrophil ETosis

It is known that neutrophils are able to produce NETs in response to apicomplexan protozoan parasites like *Plasmodium falciparum,* the causative agent of malaria*. In vivo* results showed NET-entrapment and killing of trophozoite-infected erythrocytes in blood samples (171). For Sarcocystidae it was demonstrated that neutrophils as well as monocytes perform ETosis in response to tachyzoites of *Toxoplamsa gondii* (172). NET-formation in response to nematodes were proven for L3 larvae of *Haemonchus contortus* and *Strongyloides stercoralis*. *H. contortus,* known as barber's pole worm in ruminants are trapped by NETs (151), while L3 larvae of *S. stercoralis,* causative agent of the disease strongyloidiasis in humans, are not only trapped, but also killed by NETs (173). These studies only focused on the response towards L3 larvae. However, a recent publication by Muñoz-Caro *et al.* showed that canine neutrophils produce traps in response to L3 larvae as well as MF of *Dirofilaria immitis*, the canine heartworm. These traps promoted entrapment, but not killing of the filariae (174). In a similar manner, McCoy *et al.* demonstrated that neutrophils produce NETs in response to MF of *B. malayi,* but this NET-formation was not responsible for MF killing (175). Neutrophil traps were also observed around human onchocerciasis nodules. Stimulation of NETosis was induced by the endosymbiotic *Wolbachia* bacteria, since doxycycline treatment not only depleted the filarial endosymbionts but also abolished the NETs around the nodules (24).

1.6.1.3.1.2 Eosinophil ETosis

These studies indicate that neutrophils can mediate NETosis-dependent killing of L3 larvae and entrapment of MF, whereas the role of eosinophil extracellular DNA traps (EETs) is less well understood. Furthermore, NETs and EETs by neutrophils and eosinophils may have life-cycle stage-specific effects that either target L3 larvae, MF or adult worms. For eosinophils it is known that they are able to undergo ETosis in response to bacteria, immobilized immunoglobulins (IgG, IgA), cytokines with platelet activating factor, calcium ionophore, or phorbol myristate acetate, but almost no research has been done in response to parasites even though they present the main protective immune cell type against adult filaria and MFs (137, 175). One only

publication investigated the release of ETs by enriched bovine eosinophil fractions in response to *H. contortus* L3 larvae, which leaves the opportunity for a comprehensive study for the impact of eosinophil ETosis on filarial nematode and in particular on different life-cycle stages of filarial nematodes.

1.7 Aim

The aim of this study was to analyze parasite-specific eosinophil ETosis. Since granulocytes appear to have distinct roles against different parasitic life-cycle stages, the impact of different *L. sigmodontis* life-cycle stages on EETosis was to be investigated as well as the requirements of the viability of the larvae. Moreover, the EETosis capacity in response to different pathogens was to be analyzed. Therefore, not only *L. sigmodontis* was used to stimulate murine eosinophils, but also MF from the canine filariae *D. immitis* as well as *Escherichia coli* bacteria. Furthermore, the origin of the released DNA had to be determined and the functional attribution on larval entrapment had to be analyzed through larval motility assays. The ETosis capacity of bone marrow-derived eosinophils should be compared to eosinophils derived from infected and naïve animals. A further question was, if the DNA release by eosinophils occurs through an intrinsic effect or if prior priming impacts EETosis or is even required for it. Antibody-dependency on MF-induced eosinophil DNA release should be analyzed as well as the impact of plasma components itself or in combination with different larvae to determine the impact of EETosis on initial infections and re-infections. Moreover, the underlying pathway involved in eosinophil DNA release had to be examined. Therefore, the involvement of TLR and the adapter protein MyD88 and CLR signaling on EETosis was investigated. Finally, the *in vivo* relevance of ETosis and in particular the local and systemic release of eosinophil DNA should be analyzed during an *L. sigmodontis* infection itself and in response to MF.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

Mice strain	Origin	Headquarter of origin
µMT mice	A kind gift of Anne O'Garra, Francis Crick Institute	United Kingdom
BALB/c mice	Charles River Labs, Janvier	Erkrath, Germany Le Genest-St.-Isle, France
dblGATA mice	Jackson Laboratory	Bar Harbor, ME, USA
Gerbils (*Meriones unguiculatus*)	Charles River Labs, Janvier	Erkrath, Germany Le Genest-St.-Isle, France
Myd88 knock-out (KO) mice	Center National De La Recherche Scientifique (CNRS) Orléans	Orléans, France
TLR-2 KO mice	CNRS Orléans	Orléans, France
TLR-4 KO mice	CNRS Orléans	Orléans, France
FcγR KO (dectin-2) mice	A kind gift of Falk Nimmerjahn University Erlangen	University Erlangen, Germany

2.1.2 Materials

Material	Company	Headquarter of company
10x buffer for PCR	Qiagen	Hilden, Germany
30 gauge needle	Braun	Kronberg im Taunus, Germany
40 µm strainer	Greiner Bio-One	Kremsmünster, Austria
6x Tri-Track loading dye	Thermo Fisher Scientific Inc.	Waltham, MA, USA
70 µm cell strainer	Miltenyi Biotech	Bergisch Gladbach, Germany
Advanced RPMI 1640 medium	Gibco, Thermo Fisher Scientific Inc.	Carlsbad, CA, USA
Annexin V	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Bradford assay	Bio-Rad Laboratories, Inc	Hercules, CA, USA
BSA	PAA Laboratories, Inc., Thermo Fisher Scientific Inc.	Waltham, MA, USA
BSA standard	Thermo Fisher Scientific Inc.	Waltham, MA, USA
CASYton	Hoffmann-La Roche Ltd, AG	Basel, Switzerland
Compstatin	Tocris Bioscience, R & D Systems	Minneapolis, MN, USA
Compstatin control	Tocris Bioscience, R & D Systems	Minneapolis, MN, USA
Cytidine and guanidine rich sequence (CpG) ODN 1585	Invivogen	San Diego, CA, USA

Cytospin	Andrea Hettich GmbH & Co.KG	Tuttlingen, Germany
4’,6-diamidino-2-phenylindole (DAPI)	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Depleted zymosan	Invivogen	San Diego, CA, USA
DNA Quant-iT kit	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
DNase I	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Deoxynucleotide triphosphates (dNTPs)	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
E. coli ATCC25922/FDA strain Seattle 1946/DSM:1103/NCBI12210	American Type Culture Collection (ATCC)	Manassas, VA, USA
E. coli GFP ATCC	American Type Culture Collection (ATCC)	Manassas, VA, USA
Heat-treated fetal bovine serum (FBS) for eosinophil culture	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
FBS	PAN-Biotech GmbH	Aidenbach, Germany
FBS for neutrophil purification	PAA Laboratories, Inc., Thermo Fisher Scientific Inc.	Waltham, MA, US
Fetal calve serum (FCS)	PAN-Biotech GmbH	Aidenbach, Germany
FMS-like tyrosine kinase 3 ligand (FLT3L)	PeproTech	Rocky Hill, NJ, USA
Fluoro-gel	Electron Microscopy Sciences	Hatfield, PA, USA
Furfurman	Invivogen	San Diego, CA, USA
Gauze	Labomedic Medizin- und Labortechnik GmbH	Bonn, Germany
Gentamycin	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Giemsa solution	Merck Group	Darmstadt, Germany
Glutamax	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Heat-treated FBS for eosinophil culture	Gibco, Thermo Fisher Scientific Inc.	Carlsbad, CA, USA
Hydroxyethyle piperazineethanesulfonic acid (HEPES)	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
HotStar Taq	Qiagen	Hilden, Germany
Recombinant murine IL-5	PeproTech	Rocky Hill, NJ, USA
Isoflurane	AbbVie Deutschland GmbH & Co. KG	Wiesbaden, Germany
Kova slides	Fisher Scientific, Thermo Fisher Scientific Inc.	Waltham, MA, USA
L-glutamine	PAA Laboratories, Inc., Thermo Fisher Scientific Inc.	Waltham, MA, USA

Liberase	Hoffmann-La Roche Ltd	Basel, Switzerland
LPS Ultrapure	Invivogen	San Diego, CA, USA
Magnetic activated cell sorting (MACS) buffer	Miltenyi Biotech	Bergisch Gladbach, Germany
MACS columns	Miltenyi Biotech	Bergisch Gladbach, Germany
Minimal essential medium (MEM)	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Micrococcal nuclease	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Midori Green	Biozym Scientific GmbH	Hessisch Oldendorf, Germany
Mouse mitochondrial DNA copy number kit	Detroit R&D/antibodies on-line GmbH	Aachen, Germany
NanoView device	NanoView Diagnostics Inc.	Boston, MA, USA
Normal donkey serum	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Tripalmitoyl-S-glycerylcysteine (Pam3Cys)	Invivogen	San Diego, CA, USA
Polymerase chain reaction (PCR) primer	MicroSynth	Balgach, Switzerland
Penicillin/streptomycin	Gibco, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Percoll	GE Healthcare	Chicago, IL, USA
Paraformaldehyde (PFA)	Sigma Aldrich	St. Louis, MO, USA
Peptidoglycan (PGN)	Invivogen	San Diego, CA, USA
PMA	Sigma Aldrich	St. Louis, MO, USA
Polyinosinic:polycytidylic acid (Poly (I:C))	Invivogen	San Diego, CA, USA
Propidium iodide (PI)	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Qiagen QIAmp Mini DNA Prep kit	Qiagen	Hilden, Germany
Quick-load 100 bp ladder	BioLabs	Ipswich, MA, USA
R848	Invivogen	San Diego, CA, USA
Red blood cell (RBC) lysis buffer	eBioscience Inc., Thermo Fischer Scientific Inc.	Carlsbad, CA, USA
RPMI 1640 media	PAA, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Stem cell factor (SCF)	PeproTech	Rocky Hill, NJ, USA
Sterile phosphate saline buffer (PBS)	Gibco, Thermo Fisher Scientific Inc.	Carlsbad, CA, USA
Sucrose	Millipore, Sigma-Aldrich	St. Louis, MO, USA
Sytox orange	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Trehalose-6,6-dibehenate (TDB)	Invivogen	San Diego, CA, USA
Transwells	Corning, New York	NY, USA
Tris-TE buffer	Sigma Aldrich	St. Louis, MO, USA

Triton-X 100	Sigma Aldrich	St. Louis, MO, USA
Zymosan	Invivogen	San Diego, CA, USA

2.1.3 Devices and software

Device or software	Company	Headquarter of company
Adobe Photoshop CS2 version	Adobe	San José, CA, USA
Axio Observer 7	Carl Zeiss	Oberkochen, Germany
BD FACSCanto System	BD Biosciences	San Jose, CA, USA
Bio-Rad Gel Doc EZ Imaging system	Bio Rad Laboratories	Hercules, CA, USA
CASY TT	Hoffmann-La Roche Ltd, AG	Basel, Switzerland
Cell density meter model 40	Fisher Scientific International, Inc., Thermo Fisher Scientific	Pittsburgh, PA , USA
CR Corbett Research Rotor-Gene	Qiagen	Hilden, Germany
CytoFLEX S	Beckman Coulter	Brea, CA, USA
FACS Diva 5.1	BD Biosciences	San Jose, CA, USA
FACSAria III	BD Biosciences	San Jose, CA, USA
FlowJo 10.4.2 software	FlowJO, LLC	Ashland, OR, USA
GraphPad Prism 5.0, 6.0 or 8.0 version	GraphPad Software	San Diego, CA, USA
Hitachi SU 3500 SEM	Hitachi Hightech Technology	Tokyo, Japan
ImageLab software	Bio Rad Laboratories	Hercules, CA, USA
LSM 710	Carl Zeiss	Oberkochen, Germany
Perkin Elmar Wallac 1420 Victor2	PerkinElmer, Inc	Waltham , MA, USA
SoftMax Pro 7 software	Molecular Devices	San Jose, CA, USA
SpectraMax 190	Molecular Devices	San Jose, CA, USA
Tecan Infinite 200 Pro plate reader	Tecan Group	Männedorf, Switzerland
Zen2.0 software	Carl Zeiss	Oberkochen, Germany

2.1.4 Antibodies

Antibody	Clone/ order number #	Company	Headquarter of company
CCR3-PE	J073E5	Biolegend	San Diego, CA, USA
CD11b-AF700	M1/70	Biolegend	San Diego, CA, USA
CD11b-APC-Cy7	M1/70	Biolegend	San Diego, CA, USA
CD11c-BV605	N418	Biolegend	San Diego, CA, USA

CD369 (CLEC7A, dectin-1) PE	#bg1fpi	eBioscience Inc., Thermo Fischer Scientific Inc.	Carlsbad, CA, USA
CD45-PerCP-Cy5.5	30-F11	Biolegend	San Diego, CA, USA
Dectin-1/CLEC7A-750	#FAB17531S	R&D Systems, Inc.	Minneapolis, MN, USA
Goat anti-dectin-1/CLEC7A	#AF1756	R&D Systems, Inc.	Minneapolis, MN, USA
Goat anti-dectin-2/CLEC6A	#AF1525	R&D Systems, Inc.	Minneapolis, MN, USA
Rat anti-mincle-IgG	6G5	Invivogen	San Diego, CA, USA
Goat anti-murine Ig	#AB-108-C	R&D Systems, Inc.	Minneapolis, MN, USA
Goat anti-rabbit IgG (H+L) AF488 antibody	#A11008	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
Ly6C-AF488	HK1.4	Biolegend	San Diego, CA, USA
Ly6G-PE-Cy7	1A8	Invitrogen, Thermo Fisher Scientific Inc.	Waltham, MA, USA
PE MicroBeads	#130-048-801	Miltenyi Biotech	Bergisch Gladbach, Germany
Rabbit anti-murine eosinophil cationic protein (ECP)	#orb156688	BIOZOL Diagnostica Vertrieb GmbH	Eiching, Germany
SiglecF-AF647	E50-2440	BD Biosciences	San Jose, CA, USA
SiglecF-APC-Cy7	E50-2440	BD Biosciences	San Jose, CA, USA
SiglecF-BV421	E50-2440	BD Bioscience	San Jose, CA, USA
SiglecF-PE	E50-2440	BD Biosciences	San Jose, CA, USA
SiglecF-PE for MACS		Miltenyi Biotech	Bergisch Gladbach, Germany

2.1.5 Media

Purpose	Media composition
3 % medium for gut eosinophil isolation	Advanced RPMI 1640 medium with 3 % heat-treated FBS (Pan Biotech), 1 % penicillin/streptomycin, 2.5 % HEPES
Blocking buffer for confocal microscopy	10 % normal donkey serum containing 0.3 % Triton-X 100 in PBS with 0.1 % BSA
Bone marrow harvest for eosinophils, MF isolation	Advanced RPMI 1640 medium with 10 % heat-treated FBS (Pan Biotech), 1 % penicillin/streptomycin and 2 mM L-glutamine
Bone marrow-derived eosinophil medium	Advanced RPMI 1640 medium with 20 % heat-treated FBS (Gibco), 0.1 % gentamycin, 1 % penicillin/streptomycin, 2.5 % HEPES, and 1 % Glutamax
Digest medium for gut eosinophil isolation	Advanced RPMI 1640 medium supplemented with 1 % penicillin/streptomycin, 2 mM L-glutamine, 1 %

	sodium pyruvate, 2.5 % HEPES, 0.1 % β-mercaptoethanol and 0.05 % DNase and 0.1 mg/ml liberase
Fc-block	PBS/1 % BSA and 0.1 % rat IgG
Giemsa staining	19 ml microscopy buffer (Na_2HPO_4, $2H_2O$ 0.63 g/l, KH_2PO_4 0.13 g/l) pH 7.2 was mixed with 1 ml Giemsa azur-eosin-methylene-blue
L3 larval isolation	MEM medium with 10 % heat-treated FBS (Pan Biotech), 1 % penicillin/streptomycin and 2 mM L-glutamine
Shake medium for gut eosinophil isolation	Advanced RPMI 1640 medium with 1 % penicillin/streptomycin, 2.5 % HEPES, 2 mM EDTA
Staining buffer for confocal microscopy	0.3 M NaCl, 0.03 M sodium citrate, pH 7 o/n at 4 °C
Strip medium for gut eosinophil isolation	3 % medium with 5 mM EDTA and 0.145 mg/ml dithiothreitol (DTT)
Washing buffer for confocal microscopy	0.1 % BSA in 0.3 M NaCl, 0.03 M sodium citrate, pH 7 buffer

2.2 Methods

2.2.1 Animals and ethical statement

Experiments were conducted according to the Directive 2010/63/EU and animal protocols were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen).

BALB/c mice and gerbils (*Meriones unguiculatus*) were purchased from Charles River Labs or Janvier, dblGATA mice were originally purchased from the Jackson Laboratory, TLR-2 KO, TLR-4 KO and Myd88 knock-out (KO) mice were originally obtained from CNRS Orléans, France. µMT mice were a kind gift of PhD Francis Crick Institute, United Kingdom, while FcγR KO (dectin-2) mice were provided by Prof. Dr. Falk Nimmerjahn from the University Erlangen. All animals were housed at the animal facilities of the Institute for Medical Microbiology, Immunology and Parasitology. Animals were kept in individually ventilated cages with access to food and water *ad libitum.* Nestlets and wooden sticks were provided for an enriched environment. Animals were checked daily and infected animals were scored once per week with measurements of the body weight and analysis of the behaviour and appearance. Scores were assigned from A to C. A score of “A” refers to an animal with minor abnormalities and requires daily observations, while a score of “B” is given once the animal shows more severe signs of weight loss, abnormalities in social behaviours or injuries. These animals have to be presented to the supervisor. Animals scored for a

“C” present severe injuries, loss of more than 20 % of weight or sever pain and thus have to be eliminated immediately.

The *L. sigmodontis* life-cycle was maintained as previously described (83). Briefly, patent-infected cotton rats were placed on bedding with *O. bacoti* mites overnight (o/n). On the next day, bedding and mites were collected, added to an Erlenmeyer flask and placed in an incubator for 12 to 14 days at 27 °C and 80 % humidity. For the infection of mice and jirds, 6–8 week old mice and 10-week old jirds were used (176). Infection of mice and jirds was done by placing naïve animals in metal cages on the bedding with mites containing infective *L. sigmodontis* L3 larvae o/n. To ensure equal infection of all groups, mice of one experiment were exposed to the same batch of mites. On the next day, the bedding was removed and animals were placed over water for 24 h before they were placed in housing cages again. Removal of remaining mites was accomplished by daily bedding exchange for 7 days.

2.2.2 Bone marrow-derived eosinophil cell culture

For the generation of bone marrow-derived eosinophils (BmEos), BALB/c mice, TLR-2 KO, TLR-4 KO, MyD88 KO and dectin-2 KO BALB/c mice were euthanized using an overdose of Isoflurane and hind legs were collected, flesh was removed and the bone marrow was harvested by cutting open the bones at the far ends and flushing them with 20 ml of Advanced RPMI 1640 medium with 10 % heat-treated fetal cave serum (FCS), 1 % penicillin/streptomycin and 2 mM L-glutamine using a 27 gauge needle and a 10 ml syringe. The collected bone marrow was filtered through a 70 µm cell strainer and cells were centrifuged at 400 g, 10 min at 4 °C. The cell pellet was re-suspended in 1 ml red blood cell (RBC) lysis buffer and incubated for 5 min at room temperature (RT). The reaction was stopped using 9 ml RPMI medium. Cells were counted using a CASY TT. To that end, 10 µl of the cell suspension were added to 10 ml CASYton and mixed before analysis. Following settings were used: the chosen capillary was 150 µm in size and the volume acquired was 400 µl. The dilution factor was $1*10^3$. The evaluation curser was set to 7.5 to 20 µm and the normalization curser to 5.5 to 20 µm. Bone marrow cells were seeded at a density of $1*10^6$ cells/ml in Advanced RPMI 1640 medium with 20 % fetal bovine serum (FBS), 0.1 % gentamycin, 1 % penicillin/streptomycin, 2.5 % hydroxyethyl piperazineethanesulfonic acid (HEPES), and 1 % Glutamax. First, cells were cultured with 100 ng/ml FMS-like tyrosine kinase 3 ligand (FLT3L) and stem cell factor (SCF) for 4 days and then the medium was supplemented with 20 ng/ml IL-5. Every other day half of the medium was exchanged

and on day 8 all cells were transferred into a new cell culture flask. Cell densities were determined using the CASY TT as described before.

After 12 days of culture, cells were harvested and checked for purity using flow cytometry. To check eosinophil purity, $1*10^6$ cells were blocked with 500 µl of Fc-block (PBS/1 % BSA and 0.1 % rat IgG) for 1 h at 4 °C, followed by staining with anti-SiglecF-PE, -Alexa Fluor 647 (AF647), -APC-Cy7 or –Brilliant Violet 421 (BV421) antibodies (1:800 dilution) in PBS for 30 min at 4 °C in the dark. Afterwards cells were washed with phosphate buffer saline (PBS) and flow cytometry was performed using the BD FACSCanto System or the CytoFLEX S and data was subsequently analysed using the FACS Diva 5.1 or the FlowJo 10.4.2 software. The gating procedure is displayed in Figure 3. First lymphocytes were gated using the forward (FSC-A) and side scatter (SSC-A). Duplets were excluded using SSC-A and the width forward scatter (FSC-W). Eosinophils were gated as SiglecF positive. Unstained controls were used for the gating. Eosinophil purity was on average 92.2 % and always above 84.4 %.

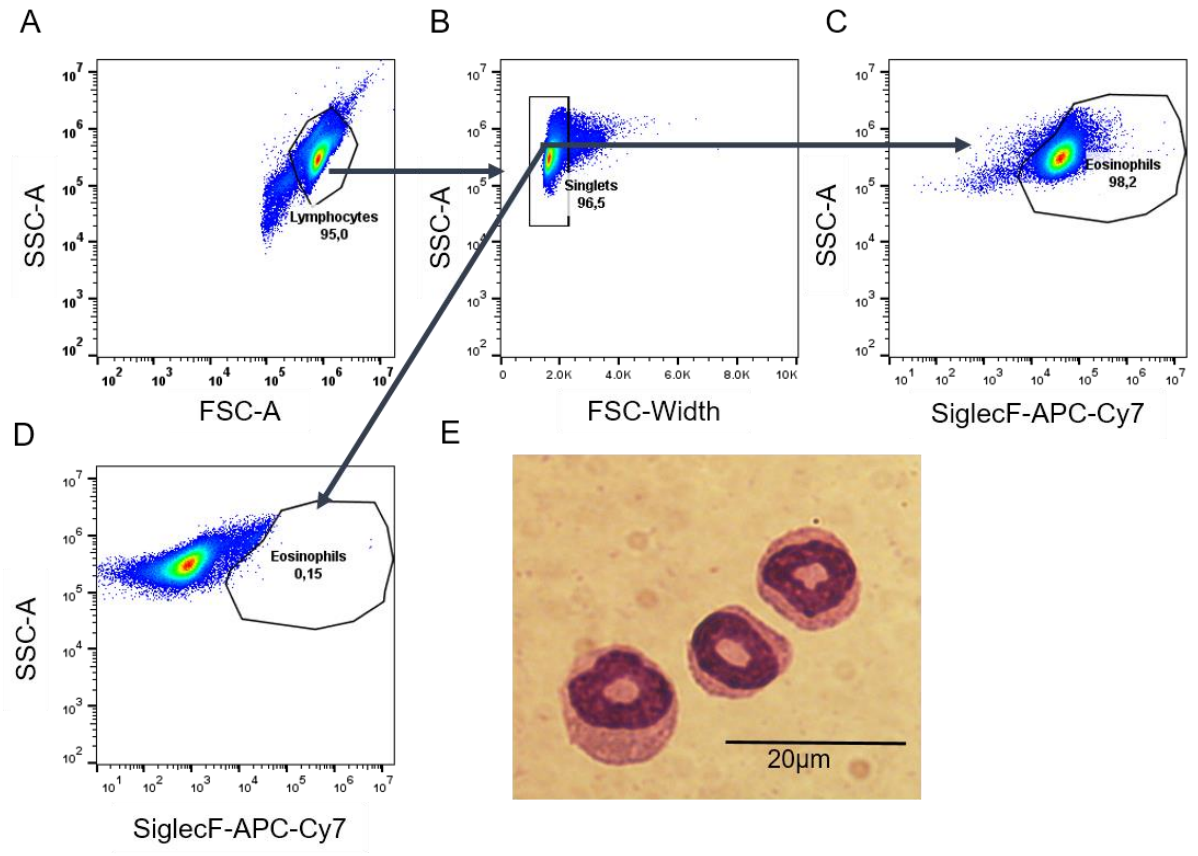

Figure 3. Bone marrow-derived eosinophils exceed a purity of 92 %. Gating strategy to determine purity of bone marrow-derived murine eosinophils by flow cytometry (A-D). Lymphocytes were gated (A), singlet cells chosen by SSC-A vs FSC-W characteristics (B), and SiglecF positive eosinophils determined (C). D: shows unstained controls. E: Giemsa staining of purified eosinophils.

Eosinophils were stained using Giemsa staining (Figure 1 E). Therefore, purified eosinophils were added to the funnel of a cytospin, centrifuged at 27 g for 3 min, air dried, fixed with methanol and stained with Giemsa solution for 20 min at RT. The Giemsa staining solution was prepared as followed: 19 ml microscopy buffer (Na_2HPO_4, $2H_2O$ 0.63 g/l, KH_2PO_4 0.13 g/l) pH 7.2 was mixed with 1 ml Giemsa azur-eosin-methylene-blue.

2.2.3 Neutrophil isolation from bone marrow

Wild-type (WT) BALB/c mice were euthanized with an overdose of Isoflurane. The femora and tibiae were removed and flesh was removed from the bones. The bone was cut open at the far ends and flushed with 5 ml of sterile PBS per bone using a 27 gauge needle and a 10 ml syringe. The collected bone marrow was centrifuged at 350 g, 4 min at 4 °C and re-suspended in 3 ml PBS. Percoll was diluted 1:10 with 10x PBS and 72 %, 64 % and 52 % percoll gradients were generated by diluting the percoll-PBS mix with RPMI 1640 (PAA Laboratories, Inc.) or PBS. For the neutrophil purification, 3 layers of a Percoll with RPMI were generated. The 64 % percoll gradient was layered over the 72 % gradient. This was followed by the 52 % percoll gradient and the cell solution on top. The gradient was centrifuged at 1500 g for 30 min without brake. The neutrophils were isolated between the 64 % and 72 % layer. Recovered cells were washed with 10 ml PBS and centrifuged at 350 g for 4 min at 4 °C. RBC lysis was performed using 1 ml 0.2 % sodium chloride (NaCl) solution followed by the addition of further 4 ml 0.2 % NaCl. Addition of 5 ml of 1.6 % NaCl stopped the reaction. Cells were centrifuged at 350 g, 4 min at 4 °C and re-suspended in 1 ml PBS. Cells were counted using Kova slides.

2.2.4 *Litomosoides sigmodontis* L3 larvae isolation

L. sigmodontis L3 larvae were either isolated from 5-day-infected jirds or directly picked from 12-week-infected mites. For the first approach, jirds were euthanized with an overdose of Isoflurane and the pleural cavity was flushed with 25 ml of minimum essential medium (MEM medium) with 10 % FBS, 1 % penicillin/streptomycin and 1 % L-glutamine. Using a binocular microscope with a 4x magnification, L3 larvae were picked. To avoid pleura cell contaminations, L3 larvae were added to fresh MEM medium. From this fresh medium, 10 L3 larvae were picked in 50 µl medium and added to a 96-well plate.

In the second approach L3 larvae were picked directly from infected mites. Therefore, 12-day-infected mites were dissected in MEM medium releasing the L3 larvae. Afterwards, L3 larvae were directly picked using a binocular microscope with a 4x magnification.

2.2.5 *Litomosoides sigmodontis* microfilariae isolation

L. sigmodontis MF were isolated from the blood of patent infected cotton rats. Therefore, cotton rats were narcotized with Isoflurane and blood was collected from the saphenous vein. The blood was placed in an incubator at 37 °C until prior use. The MF were purified using a sucrose-percoll gradient. From a 2.5 M sucrose stock solution, a 0.25 M sucrose solution was prepared as well as an iso-osmotic percoll (IOP) solution containing 0.25 M sucrose in percoll. The IOP was diluted with the 0.25 M sucrose solution to generate 30 % and 25 % sucrose-percoll solutions. 3 ml of the 25 % sucrose-percoll solution was layered on top of the 30 % sucrose-percoll solution, followed by 3 ml of 1:2 diluted blood (diluted in Advanced RPMI 1640 medium, 10 % heat-treated FBS, 1 % L-glutamine, 1 % penicillin/streptomycin). The gradient was centrifuged at 340 g, at RT for 30 min without break and the MF were collected from the layer between the 25 % and 30 % solution. MF were washed 3 times with medium and then diluted in 1 ml medium and 10 µl were transferred to a Neubauer chamber to determine the MF number.

2.2.6 Litomosoides sigmodontis adult worm isolation

Adult *L. sigmodontis* filariae from patent- and pre-patent-infected cotton rats or from patent-infected jirds were isolated from the pleural cavity. Therefore, the animals were euthanized with an overdose Isoflurane, the pleural cavity was opened and flushed with sterile PBS. Filariae were collected in a petri dish containing sterile PBS. Afterwards the pleural cavity was opened and remaining worms were collected. For crude extract preparation, worms were collected and further prepared as described below. For co-cultures, worms were separated with small brushes and placed in PBS in 6-well plates.

2.2.7 Escherichia coli culture

20 ml pre-heated lysogeny *broth* (LB) medium was inoculated with the tip of an inoculation loop containing parts of a frozen permanent *E. coli* ATCC25922/FDA strain stock, which is stored at -80 °C. Erlenmeyer flasks containing the LB medium and *E. coli* were incubated at 37 °C and 300 rpm o/n. On the next day, optical density at

600 nm (OD600) was determined from 1 ml medium control and bacterial solution using the cell density meter model 40. An OD600 of 1 of the culture was considered to contain $8*10^8$ bacteria/ml. Bacteria were harvested and centrifuged at 400 g, 15 min at RT and the cell pellet was re-suspended in eosinophil medium with supplements lacking antibiotics at a concentration of $2*10^7$ bacteria/ml.

2.2.8 Eosinophil purification from the small intestine and pleural cavity

Blood was drawn into ethylenediaminetetraacetic acid (EDTA) tubes from mice via the facial vein and 30 or 50 µl was added to 1 ml RBC lysis for MF counts. The remaining blood was centrifuged at 4602 g, RT for 5 min and plasma was collected. The plasma was diluted 1:10 with Tris-EDTA (Tris-TE) buffer and frozen at -20 °C for DNA quantification. Afterwards, naïve mice and 76 days after infection (dpi) wild-type and µMT mice were sacrificed with an overdose of Isoflurane and the abdomen was opened. To perform the pleural lavage, a little incision was made in the right upper part of the diaphragm. The pleural cavity was washed first with 1 ml PBS followed by additional 5 ml PBS. 50 µl of the first ml were added to 1 ml RBC lysis and used for MF counts. The remaining pleura lavage was filtered through gauze and the supernatant of the first ml was frozen at -20°C for DNA quantification while the cell pellet was combined with the remaining 5 ml pleura lavage. Afterwards, the pleural cavity was completely opened and the worms were collected in a petri dish containing PBS.

The small intestine was placed on ice in PBS and 3 % medium (Advanced RPMI 1640 medium with 3 % FCS, 1 % penicillin/streptomycin, 2.5 % HEPES) and the Peyer's patches were removed and the intestine was cut longitudinally. The faeces was removed by washing the intestine in the PBS with 3 % medium first, followed by a washing step in PBS only. The intestine was added into an Erlenmeyer flask containing 20 ml of 3 % medium with 5 mM EDTA and 0.145 mg/ml dithiothreitol (DTT) and shaken at 37 °C for 20 min. Afterwards the intestine was washed 3 times with 10 ml of shake medium (Advanced RPMI 1640 medium with 1 % penicillin/streptomycin, 2.5 % HEPES, 2 mM EDTA). The intestine was placed into a fresh beaker with 2 ml digest medium (Advanced RPMI 1640 medium supplemented with 1 % penicillin/streptomycin, 1 % L-glutamine, 1 % sodium pyruvate, 2.5 % HEPES, 0.1 % β-mercaptoethanol) and 0.05 % DNase and 0.1 mg/ml liberase and was minced with fine scissors. Additional 8 ml of digest medium was added and the digestion of the intestine by DNase and liberase was performed for 29 min at 37 °C while shaking.

Addition of 20 ml of 3 % medium terminated the reaction, which was followed by filtering the intestinal cells through a 70 µm strainer and centrifugation at 400 g for 5 min at 8 °C. The supernatant was removed, the cell suspension was re-suspended in fresh shake medium and filtered through a 40 µm strainer. After an additional centrifugation, cells were collected, counted using the CASY TT and further processed for magnetic activated cell sorting (MACS), cell sorting and flow cytometry.

2.2.9 Magnetic activated cell sorting of pleural and gut eosinophils

Eosinophils from wild-type- and µMT-infected mice were positively selected and isolated from the pleura and small intestine by MACS according to the manufacturer's protocol. To ensure increased survival of gut cells, reagents used for gut cell purifications always contained 5 % FCS. For MACS isolation, cells were incubated with 500 µl Fc block (MACS buffer, 0.1 % rat IgG) for 30 min on ice. Cells were centrifuged for 10 min at 400 g and 4 °C and re-suspended in MACS buffer with 1 % anti-SiglecF-PE antibody. The reaction was incubated for 20 min on ice, washed with 2 ml MACS buffer and re-suspended in 240 µl MACS buffer with 60 µl anti-PE beads, following a 15 min incubation period on ice. After washing of the cells, fresh MACS buffer was added and applied to equilibrated MACS columns, which were placed in a magnet. Columns were washed 3 times with MACS buffer, removed from the magnet and labelled cells were flushed out and collected in a fresh 50 ml tube. Cell counts were determined using CASY TT. Following settings were used: the chosen capillary was 150 µm in size and the volume acquired was 400 µl. The dilution factor was $1*10^3$. The evaluation curser was set to 7.5 to 20 µm and the normalization curser to 5.5 to 20 µm.

2.2.10 Flow cytometric analysis of pleural and gut eosinophils after magnetic activated cell sorting

Since the eosinophils were already positively labelled after the MACS procedure with anti-SiglecF-PE antibodies, the purity was determined using the BD FACS Canto system and data was subsequently analysed using the FACS Diva 5.1 software. Isolated pleura eosinophils achieved a mean purity of 98.4 %, while gut eosinophils only had a purity of around 30 % and were thus only enriched by MACS.

2.2.11 Cell sorting of pleural and gut eosinophils

In an additional approach, to achieve higher purities of gut and pleura eosinophils, cells from infected wild-type mice as well as gut cells from naïve wild-type mice were sorted

using the FACSAria III. Therefore, cells were blocked for 1 h at 4 °C with Fc-block and stained with Ly6C-AF488 (dilution 1:200), CD45-PerCP-Cy5.5 (dilution 1:100), CCR3-PE (dilution 1:200), Ly6G-PE-Cy7 (dilution 1:200), SiglecF-AF647 (dilution 1:200), CD11b-APC-Cy7/-AF700 (dilution 1:300), and CD11c-BV605 (dilution 1:200) antibodies for 30 min in the dark at 4 °C. After the staining, cells were washed with PBS and sorted at the BD FACSAria III. First, all lymphocytes were gated (SSC-A vs FSC-A), followed by single cell gating using FSC-H vs FSC-W followed by SSC-A vs SSC-W. CD45 positive cells and DAPI negative cells were further gated for CD11b expression. For the gut cells, CD11b positive cells were gated while for the pleura, CD11b intermediate cells were gated and checked for Ly6C and Ly6G expression. Ly6G and Ly6C double negative cells were then analyzed for their CCR3 and SiglecF expression and double positive cells and SiglecF single positive cells were sorted into PBS/1 % BSA with 10 % FCS.

2.2.12 Scanning electron microscopy

For scanning electron microscopy, glass slides were coated with 120 µl poly-lysine for 5 min at RT, washed 3 times with water and air dried. $0.5*10^6$ eosinophils were placed on the glass slides in 24-well plates (eosinophils alone and when cultured with *E. coli*) or in the upper compartment of transwells placed in 24-well plates (for co-cultures) and co-cultured with 50 L3 larvae, 50,000 MF or $5*10^8$ *E. coli* for 0 or 1 h at 37 °C. $3*10^5$ neutrophils were co-cultured with 50 L3 larvae or 10,000 MF for 0 and 3 h in transwells. Plates were centrifuged at 400 g, 5 min at RT, supernatant was removed and cells were fixed in 2.5 % glutaraldehyde for 30 min at RT. The samples were washed after the incubation period for 5 times with PBS for 5 min, followed by a dehydration process using an ethanol (EtOH) series. Therefore, glass slides or transwells were incubated for 10 min in 50 % EtOH, for 10 min in 70 % EtOH, for 10 min in 90 % EtOH and twice for 10 min in 100 % EtOH. EtOH was removed and plates were incubated twice for 5 min in hexamethyldisilazane (HMDS) and then air dried o/n. The mesh from the transwells was cut out from the plastic part and all samples were metalized with gold particles and viewed under a Hitachi SU 3500 SEM.

2.2.13 Confocal microscopy

To visualize extracellular DNA confocal microscopy was performed. Therefore, $5*10^5$ BmEos were co-cultured with 50 *L. sigmodontis* L3 larvae, $1.25*10^4$ *L. sigmodontis* MF, 5 % plasma or $6*10^6$ *E. coli*-green fluorescence protein (GFP) on 0.17 mm thick

cover glass slides in 24-well plates for 3 and 24 h. Furthermore, $5*10^5$ BmEos were co-cultured with $1.25*10^4$ *D. immitis* MF for 24 h. After incubation 2 % paraformaldehyde (PFA) was added for 10 min and cells were pelleted by centrifuging plates at 400 g, 4 °C for 10 min. Supernatant was carefully removed and fresh 4 % PFA was added and incubated for 20 min at RT. Afterwards, glass slides were washed with PBS and 0.1 % bovine serum albumin (BSA). Then blocking buffer (10 % normal donkey serum containing 0.3 % Triton-X 100 in PBS with 0.1 % BSA) was added. Blocking was incubated o/n at 4 °C. The next day, cells were stained with the unconjugated primary antibody against eosinophil cationic protein (ECP) (rabbit anti-murine) in 0.3 M NaCl, 0.03 M sodium citrate, pH 7 o/n at 4 °C (no ECP antibody for *E. coli* cultures). After washing twice with 0.1 % BSA in 0.3 M NaCl, 0.03 M sodium citrate, pH 7 buffer, the conjugated secondary goat anti-rabbit IgG (H+L)-AF488 antibody (1:1000 diluted) and anti-SiglecF-AF647 (1:400 diluted) were added in the 0.3 M NaCl, 0.03 M sodium citrate, pH 7 buffer and incubated for 3 h at 4 °C in the dark. After staining, the slides were washed with 0.1 % BSA in 0.3 M NaCl, 0.03 M sodium citrate, pH 7 buffer twice. All washing and buffer exchange steps were performed after centrifugation steps of 10 min at 400 g and 4 °C. For DNA visualization slides were stained with 5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI), washed twice with PBS and mounted using Fluoro-gel. Samples were analyzed using a LSM 710 or the Axio Observer 7 with the Zen2.0 software.

2.2.13.1 Fluorescence image editing

Confocal microscopy pictures were edited with the Adobe Photoshop CS2 version. Therefore, overlay pictures taken with the LSM 710 were exported from the Zen2.0 software and single stains were extracted and separated with Photoshop CS2. Each single stain was created as a new layer. DAPI stained pictures were duplicated and displayed in gray shades. The gray shading of the DAPI stained pictures was added as luminescent overlay on the blue layer with 20 % coverage. Single stained pictures were overlaid using negative multiplication. Scale bars were generated with the Zen2.0 software.

Single stained pictures taken with the Axio Observer 7 were exported separately from the Zen2.0 software and combined with the Photoshop CS2 version. DAPI stained pictures were duplicated and displayed in gray shades. The gray shading of the DAPI stained pictures was added as luminescent overlay with 20 % coverage. Single stained

pictures were overlaid using negative multiplication. Scale bars were generated with the Zen2.0 software.

2.2.14 *Litomosoides sigmodontis* crude extract preparation

Preparation of crude extract (antigen) was performed as previously described (176). Briefly, worms from the different life-cycle stages were collected (MF from blood of patent infected cotton rats (MFAg), adult worms from the pleural cavity of patent infected jirds (LsAg), L3 larvae from the pleural cavity of 5-day-infected jirds (L3Ag)), added to a potter with PBS, smashed at 4 °C until homogenized, centrifuged at 4000 g, 4 °C for 15 min and supernatant was collected. Protein concentration was determined using a Bradford assay with a BSA standard. Therefore, 3 µl of sample and BSA standard were added to a 96-well plate and 300 µl of 1x diluted Bradford assay was added. Absorbance was measured at 590 nm using the SpectraMax 190 with the SoftMax Pro 7 software.

2.2.15 Eosinophil stimulation assay and DNA quantification

2.2.15.1 Stimulation with different filarial life-cycle stages and extracts from those life-cycle stages

DNA released by BmEos was quantified after 24 h and 48 h of stimulation. Therefore, $1*10^5$ BmEos were plated in a 96-well plate and stimulated with 1250, 2500 and 5000 *L. sigmodontis* MF or 10 L3 larvae. $3*10^5$ gut and pleura cells were seeded in 96-well plates with $7.5*10^3$ MF.

For the transwell assay, $7.5*10^4$ MF were plated in a 24-well plate and $1*10^6$ BmEos were either added directly to the MF or in the upper compartment of a transwell. Adult filariae from patent or pre-patent cotton rats were co-cultured with BmEos in 24-well plates. Therefore, worms were collected and separated in a 6-well dish as described above. $5*10^5$ BmEos were plated in a 24-well plate with either 1 adult female worms or 1 adult male worms. For heat-treatment, larvae were heated to 60 °C for 60 min before *in vitro* co-culture. Crude extract (L3Ag, MFAg, LsAg) was used at a concentration of 25 µg/ml to stimulate BmEos.

BmEos were stimulated with the supernatant of 24 h cultures containing viable or heat-treated MF ($5*10^4$ MF) or L3 larvae (10 L3 larvae) or medium control. Therefore, L3 larvae and MF were isolated, half of them were heat-treated and cultured in 96-well plates for 24 h at 37 °C and 5 % CO_2. After 24 h, plates were centrifuged and supernatant was removed and frozen at -20 °C until further use. $1*10^5$ BmEos were

plated in 96-well plates together with the supernatant of MF, heat-treated MF, L3 larvae, heat-treated L3 larvae or medium control in the absence or presence of $5*10^4$ MF.

Furthermore, 2,500 MF, 2,500 heat-treated MF, 10 L3 larvae, 10 heat-treated L3 larvae or medium alone were cultured in 50 µl eosinophil medium for 24 h without eosinophils, plates were centrifuged at 400 g for 10 min at 4 °C and supernatant was collected and frozen at -20 °C until further use. BmEos were then cultured without or 2,500 MF for 24 h in the presence of the different supernatants collected.

In addition, $1*10^5$ BmEos were stimulated with $2.5*10^4$ *D. immitis* MF.

2.2.15.2 Erythrocytes, plasma, serum and whole blood stimulation

Blood was withdrawn from the vena facialis from naïve and infected mice. Blood was collected in EDTA or uncoated plastic tubes. Either whole blood was used or tubes were centrifuged at 376 g for 5 min at RT and serum or plasma was collected as well as remaining erythrocytes. Whole blood and erythrocytes were washed with RPMI medium and then counted using the CASY TT. Following settings were used: the chosen capillary was 150 µm in size and the volume acquired was 400 µl. The dilution factor was $4*10^5$. The evaluation curser was set to 3 to 10.01 µm and the normalization curser to 6 to 10.01 µm.

For the stimulation $1*10^5$ BmEos were plated in a 96-well plate in eosinophil medium with either $1*10^7$ erythrocytes, whole blood, 5 % serum, or plasma. Heat-treatment of plasma and serum was done at 55 °C for 30 min. Plasma was depleted in C3 convertase using 100 µM compstatin, control plasma was treated with compstatin control for 30 min at 37 °C.

2.2.15.3 TLR and CLR stimulation

TLR and CLR stimulation was performed in 96-well plates using $1*10^5$ BmEos, which were stimulated with 250 ng/ml tripalmitoyl-S-glycerylcysteine (Pam3Cys), 5 µg/ml polyinosinic:polycytidylic acid (Poly (I:C)), 100 ng/ml lipopolysaccharide (LPS), 500 ng/ml R848, 5 µM Cytidine and guanidine rich sequence (CpG) ODN 1585, or 50 ng/ml phorbol-12-myristat-13-acetat (PMA), 0.5 mg/ml zymosan and depleted zymosan, 100 µg/ml furfurman, 100 µg/ml peptidoglycan (PGN), and 100 µg/ml trehalose-6,6-dibehenate (TDB).

2.2.15.4 *Escherichia coli* stimulation

$3*10^5$ BmEos were plated in a 48-well plate and stimulated with $6*10^6$ *E. coli* in a total volume of 600 µl. Cells and bacteria were cultured in RPMI medium with supplement lacking antibiotics.

2.2.15.5 Dectin and mincle depletion

Dectin and mincle signaling was inhibited by the addition of depletion antibodies (goat anti-murine dectin-1 and -2 or rat anti-murine mincle antibodies) prior to MF stimulation. For the signaling inhibition, $1*10^5$ BmEos were cultured in 96-well plates without depletion antibodies or with 0.25 µg/ml goat anti-murine dectin-1 and -2 antibodies, rat anti-mincle, or goat anti-murine IgG as an isotype control. Afterwards, eosinophils were either incubated with medium control or $7.5*10^3$ MF for 24 or 48 h.

2.2.15.6 DNA quantification

Stimulation assays were performed for 24 and 48 h. As controls, DNA traps were degraded by the addition of 20 U/ml DNase I to half of the culture wells. After stimulation, extracellular DNA was detached from the cell culture bottom by the addition of 5 U micrococcal nuclease while culturing for 15 min at 37 °C with 5 % CO_2. The reaction was terminated by the addition of 1 mM EDTA, pH 8 and the cell culture plates were centrifuged at 400 g, 4 °C for 10 min. The supernatant was carefully removed and used for the DNA quantification assay. DNA amounts were determined with the DNA Quant-iT kit from Thermo Fischer. Therefore, 10 µl of the provided standard and 20 µl of supernatant were added to a 96-well plate and 100 µl of the DNA Quant-iT dsDNA HS reagent (1:200 diluted) was added. The readout was performed with the Perkin Elmar Wallac 1420 Victor2 or the Tecan Infinite 200 Pro plate reader using excitation/emission wavelengths of 485/535 nm. DNA amounts were calculated by generating a standard curve using the obtained values from the standard and the volume of each sample.

2.2.16 Eosinophil co-cultures and motility score

For co-cultures, $1*10^5$ BmEos were cultured in a 96-well plate in eosinophil medium and stimulated with $2.5*10^3$ MF or 10 L3 larvae of *L. sigmodontis* or with $1.25*10^3$ *D. immitis* MF. For transwell assays $1*10^6$ BmEos were placed in a 24-well plate in a transwell on top of $7.5*10^4$ *L. sigmodontis* MF (in the bottom of the well). Adult *L. sigmodontis* filariae were cultured in a 24-well plate with $5*10^5$ eosinophils. Therefore, 5 female or 5 male filariae were incubated with BmEos.

In co-cultures with gut and pleura eosinophils, $6*10^4$ eosinophils were cultured in a 48-well plate with $7.5*10^3$ *L. sigmodontis* MF (MACS) or $3*10^4$ cells were cultured in a 96-well plate with $3.75*10^3$ MF (sorting).

The impact of 5 % plasma on *L. sigmodontis* larval motility was analysed in eosinophil co-cultures. Pleura and gut eosinophils from infected µMT and WT animals (64 dpi) were co-cultured with *L. sigmodontis* MF in the presence or absence of the corresponding plasma. BmEos co-cultured with *L. sigmodontis* L3 larvae were supplemented with plasma or heat-treated plasma from wild-type naïve or plasma from *L. sigmodontis*-infected wild-type and µMT mice (64 dpi). Adult worms were co-cultured with BmEos in the presence of plasma from naïve or infected wild-type mice.

For L3 larval cultures, the number of larvae that were attached by eosinophils was accessed after 8 days of co-culture and the percentage was calculated.

Removal of DNA traps was accomplished by the addition of 20 U/ml DNase I to half of the culture wells. MF and L3 larval motility was scored daily for up to 5 days using a 10x and 4x magnification, respectively. Adult worm motility was analysed without magnification. The motility score ranged from 0 to 4. If no movement was detected the larva was scored as 0, while worms showing discontinuous movements at the far ends were scored with 1. Larvae having a score of 2 showed slower and discontinuous movements, while a score of 3 indicated slow, but continuous movements. Worms with a score of 4 showed fast and continuous movements.

2.2.17 Polymerase chain reaction and quantitative polymerase chain reaction

The origin of the extracellular DNA was determined using polymerase chain reaction (PCR) detecting mitochondrial and nuclear genes. Therefore, co-cultures of $2.5*10^5$ BmEos and $2.5*10^4$ MF or $6*10^6$ *E. coli* were generated and cultured for 48 h at 37 °C with 5 % CO_2. After stimulation, 2.5 U micrococcal nuclease was used to detach extracellular DNA from the cell culture bottom. 1 mM EDTA, pH 8 was used to stop the reaction. Cells and supernatant were collected and centrifuged at 400 g, 4 °C for 10 min. Supernatant and cell pellets were separated and cells were washed with PBS and re-suspended in 200 µl PBS. Both supernatants and cell pellets were stored at -20 °C until use. DNA extraction was done with the Qiagen QIAmp Mini DNA Prep kit according to the manufacture's description. Briefly, the cells were thawed and equilibrated to RT, 20 µl of proteinase K and 200 µl lysis buffer were added and incubated for 10 min at 56 °C. Afterwards 200 µl of 100 % EtOH was added, mixed

and everything was loaded onto spin columns. The spin columns were centrifuged at 6000 g for 1 min and washed twice. DNA was eluted in 50 µl AE buffer. The supernatant was supplemented with 1 µl of carrier RNA as well as an equal amount of lysis buffer and 100 % EtOH. Everything was loaded onto a spin column, centrifuge at 6000 g for 1 min, washed twice and eluted in 30 µl AE buffer. Concentrations and DNA purity were determined using the NanoView device. A PCR master mix was prepared containing 10x buffer, 10 µM forward and reverse primer, HotStar Taq, 10 mM dNTPs, and water. 18 µl of master mix was added to 2 µl of DNA purified from the supernatant, water as negative control or purified DNA from cell lysates as positive controls. Primers to detect the mitochondrial genes were cytochrome oxidase c subunit 1 (*Cox1*) and NADH-ubiquinone oxidoreductase chain 1 (*Nd1*) and primers to detect the nuclear genes were beta-actin (*Actb)* and Glycerinaldehyd-3-phosphat-Dehydrogenase (*Gapdh*). The primer sequences are as followed described in table 1.

Table 1. Primer sequences used to determine origin of extracellular DNA released by eosinophils in response to MF.

Primer	Forward primer	Reverse primer
Cox1	5´-CATCTGTTCTGATTCTTTGGGCACC-3´	5´-ATTACCTCCGTGTAGGGTTGC-3´
Nd1	5´-CCCTAGCAGAAACTAACCGGG-3´	5´-AGTTGGTCGTAGCGGAAGC-3´
Actb	5´-GCTCAGTAACAGTCCGCCTAGAA-3´	5´-ATCCTTAGCTTGGTGAGGGTG-3´
Gapd	5´-AGCCATCAGCTATGCACGTA-3´	5´-GAACGAGCCCTGGGATTAGG-3´

The PCR cycling program was used as followed: 15 min at 95 °C, followed by 35 cycles of 30 sec 95 °C, 1 min 56 °C and 30 sec 72 °C. Finally, an elongation step for 10 min at 72 °C was performed. 6x Tri-Track loading dye was added to the PCR products and applied to a 1.5 % agarose gel in 0.5 % TBE buffer containing Midori Green. The gel ran for 40 min at 120 V and 300 mA. Visualization and analysis was done using the Bio-Rad Gel Doc EZ Imaging system and the Image Lab software. The PCR product size was determined using the Quick-load 100 bp ladder. Expected sizes are: *Cox1*: 287 base pairs (bp), *Nd1*: 298 bp, *Actb*: 275 bp and *Gapdh*: 593 bp.

For qPCR, DNA purified from the supernatant of unstimulated eosinophils (control group) and eosinophils stimulated with MF (sample group) were used. Therefore, the mitochondrial copy number kit was used according to manufactures protocol. 10 µl of the real-time master mix was added to 1 µl of forward and 1 µl reverse primer together with 8 µl of sample, water as negative control or total C57BL/6 mouse liver DNA as positive control. For the qPCR, following program for the rotor gene was used: 95 °C for 10 min, following 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. The threshold

was set to 0.03 and the Ct values were used for the calculation of the mt/nt fold change ratio. Samples without Ct values were set to 40. The fold change was calculated as followed:

$$Ct(control) = Ct(mt\ control) - Ct(nt\ control)$$
$$Ct(sample) = Ct(mt\ sample) - Ct(nt\ sample)$$
$$\Delta Ct = Ct(sample\ or\ control) - average\ Ct(control)$$
$$mt/nt\ DNA\ fold\ change = 2^{-\Delta Ct}$$

First, the Ct values were calculated by subtracting the Ct values from the nuclear genes with the Ct values of the mitochondrial genes for the control samples (eosinophils without stimulation= Ct(control)) and the samples (eosinophils stimulated with MF= Ct(sample)). The average Ct(control) value was calculated by determining the mean of the Ct(control) values (eosinophils without stimulation). ΔCt values were determined by subtracting the average Ct(control) value from all Ct vales calculated (Ct control as well as Ct sample values). Finally, the mt/nt DNA fold change was determined by calculating 2 to the power of the minus ΔCt value, which gives the mitochondrial and nuclear ratio DNA fold change.

2.2.18 Eosinophil and microfilariae viability analysis via flow cytometry

For determining eosinophil and MF viability after co-culture, $2*10^6$ BmEos were co-cultured with $5*10^5$ MF in the presence or absence of 20 U/ml DNase I for 5 days. After incubation, cells were harvested and centrifuged at 400 g, 10 min at 4 °C. Cell pellets were re-suspended in 500 µl Fc-block and incubated for 1 h at 4 °C. After centrifugation at 400 g, 10 min at 4 °C, supernatant was removed and eosinophils were stained with SiglecF-AF647 (1:400 diluted) for 30 min at 4 °C in the dark. After cells were washed, Annexin V and propidium iodide (PI) staining was performed according to the manufacturer's protocol. Briefly, $1*10^5$ cells were re-suspended in 100 µl 1x Annexin V binding buffer, 5 µl Annexin V and 1 µl PI were added and incubated for 15 min at RT in the dark. The reaction was stopped after the addition of 400 µl Annexin V buffer. Flow cytometric analysis was performed at the FACS Canto I. FSC-A and SSC-A were used to gate all cells and MF. Singlets of eosinophils were gated using the SSC-A and FSC-W. Singlets were gated as FSC-W low, duplets as FSC-W intermediate and MF as FSC-W high. Eosinophils were gated as SiglecF positive cells. Eosinophils and MF were analyzed for PI and Annexin V staining. Annexin V binds phosphatidylserine, which is bound to the cell membrane and flips to the outside of a cell that is dying while

propidium iodide binds DNA. Thus, Annexin V single positive cells can be considered as apoptotic cells, Annexin V and propidium iodide PI double positive as necrotic cells and propidium iodide single positive as dead cells (177). Thus, Annexin V and PI double negative cells were considered as viable, while Annexin V single positive cells were considered as apoptotic cells and Annexin V and PI double positive and PI single positive cells were marked as dead and necrotic cells, respectively. Single stains and fluorescence minus one (FMOs) were used for the gating strategy.

2.2.19 Dectin expression on bone marrow-derived eosinophils

For the detection of dectin expression on BmEos, cells were seeded at a density of $2.5*10^5$ cells/ml in eosinophil medium and stimulated with 25,000 MF, 0.1 mg/ml zymosan or 50 ng/ml PMA for 48 h. Afterwards, cells were harvested and blocked in 500 µl Fc-block for 1 h at 4 °C. Cells were centrifuged, supernatant was removed and cells were stained with anti-SiglecF-BV421 (1/800 dilution) and anti-dectin-1 PE (1/400 dilution) in PBS/ 1 % BSA for 30 min at 4 °C in the dark. Afterwards, cells were washed in PBS and measured at the CytoFLEX S. Appropriated FMOs were prepared.

2.2.20 Intravenous microfilariae injection, blood microfilariae count and DNA quantification of pleural lavage and plasma

BALB/c wild-type and dblGATA mice were infected with *L. sigmodontis* as described above. To analyze extracellular DNA released upon infection, naïve and infected BALB/c mice were euthanized 75 dpi with an overdose of Isoflurane and the pleural cavity was flushed with 1 ml PBS. The DNA within the pleural cavity was quantified from the undiluted pleural lavage using the DNA Quant-iT kit. Analysis of DNA release in response to MF and the impact of eosinophils on DNA release was analyzed in wild-type and dblGATA mice 41 dpi, which were intravenously injected with $1*10^5$ MF. Before and 24 h after the injection, blood was drawn from the vena facialis, collected in EDTA tubes, centrifuged at 4602 g for 5 min and plasma was collected. The plasma was diluted 1:5 in Tris-EDTA and frozen at -20 °C until DNA quantification. DNA quantification was performed as described above using the DNA Quant-iT kit.

2.2.21 *Dirofilaria immitis* microfilariae co-cultures

Purified *D. immitis* microfilariae were kindly provided by Dr. Daniel Kulke from Bayer Animal Health GmbH (Bayer Animal Health GmbH, Leverkusen, Germany). *D. immitis* MF were purified from the blood of patent-infected beagles. Therefore, blood was withdrawn from the cephalic vein. MF purification was done according to the protocol

provided by the NIH/NIAID Filariasis Research Reagent Resource Center (FR3; College of Veterinary Medicine, University of Georgia, Athens, GA, USA) (http://www.filariasiscenter.org/protocols/Protocols/ purification-of-microfilariae-by-filtration). Co-cultures with BmEos and *L. sigmodontis* or *D. immitis* MF were generated and extracellular DNA was quantified, MF motility was assessed and confocal microscopy pictures were generated. For the DNA quantification, $1*10^5$ BmEos were stimulated with $5*10^3$ *L. sigmodontis* or $2.5*10^3$ *D. immitis* MF for 24 h in 96-well plates. After incubation, DNA traps were detached from the bottom of the well as described before and extracellular DNA was quantified using the DNA Quant-iT kit. The motility was analysed daily for a total of 3 days from cultures with $1*10^5$ BmEos and $2.5*10^3$ *L. sigmodontis* or $1.25*10^3$ *D. immitis* MF.

2.2.22 Statistical analysis

All data were analysed using the GraphPad Prism 5.0 or 8.0 version. Spearman's test for heteroscedasticity was performed for data from repeated experiments and pooling of data was only done for data not passing the heteroscedasticity test. Furthermore, data were analysed for normal distribution using the Kolmogorov-Smirnov normality test. For normally distributed data, the 1-way ANOVA test with Bonferroni post-hoc test was used for multiple comparison and selected pairs of columns were compared. Non-parametric data were analysed with the Kruskal-Wallis test and the Dunn's post-hoc test. Motility scores were tested with the 2-way ANOVA and the Bonferroni post-hoc test for multiple comparison. Extracellular DNA released by pleura and gut eosinophils were analysed using the Friedman test with the Dunn's post-hoc test, while extracellular DNA within plasma and the pleural cavity of animals were analysed using the Mann-Whitney t-test. In all cases, p-values smaller than 0.05 were considered as statistically significant ($p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***). Cell purities were measured with flow cytometry and analysed using the FlowJo 10.4.2 version.

3. Results

In a first attempt to analyze the impact of a life-cycle stage-specific ETosis response on granulocytes, neutrophils and eosinophils were isolated from bone marrow of naïve animals and co-cultured with L3 larvae and MF of *L. sigmodontis*. Using different techniques such as SEM, confocal microscopy and DNA quantification, ETosis in response to the different parasite life-cycle stages was examined.

3.1 Bone marrow-derived neutrophils produce DNA-like nets in response to L3 and MF of *L. sigmodontis*.

To analyze neutrophil ETosis in response to helminths, neutrophils were purified from bone marrow of naïve mice and co-cultured for 3 h with *L. sigmodontis* L3 larvae or MF. Scanning electron microscopy revealed that neutrophils produce DNA-like structures in response to L3 larvae as well as MF (Figure 4).

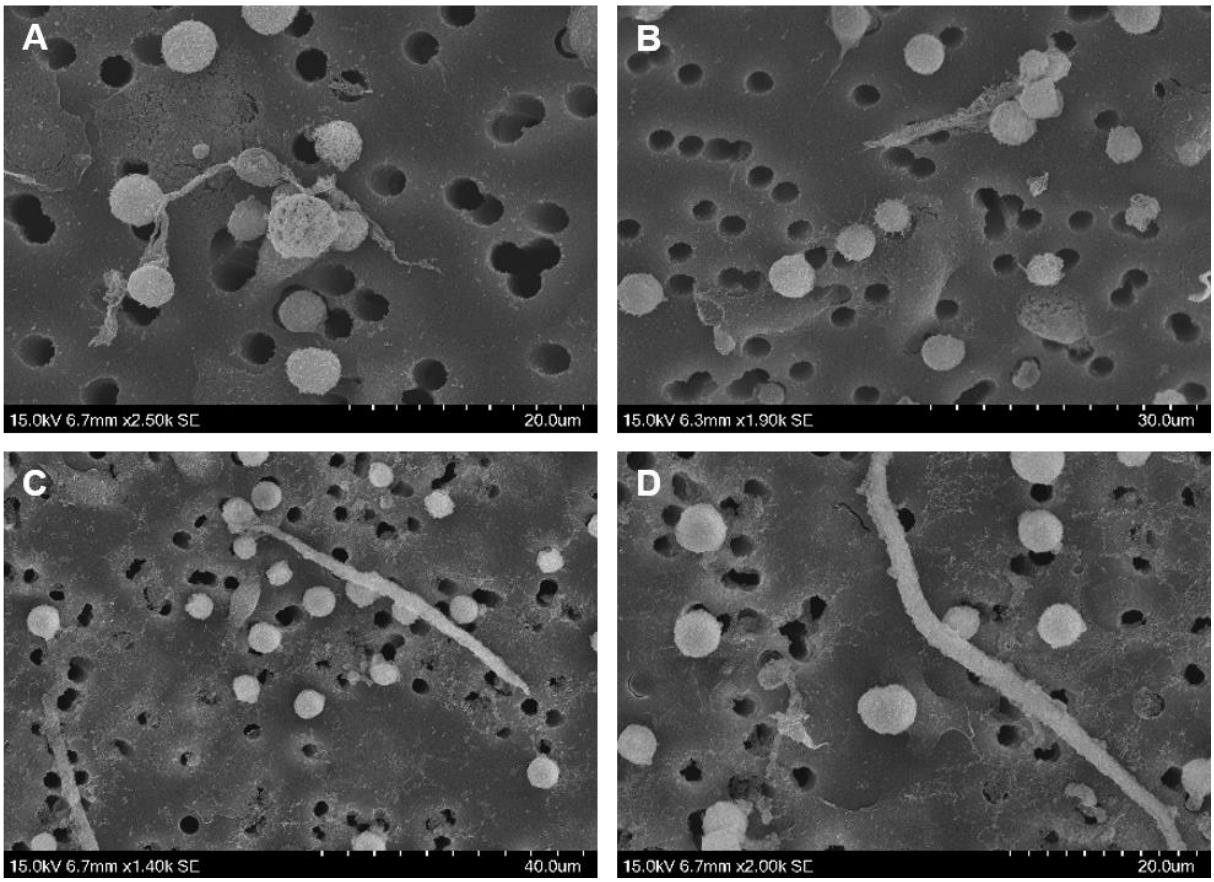

Figure 4. Bone marrow-derived neutrophils produce DNA-like nets in response to L3 and MF of *Litomosoides sigmodontis*. Representative scanning electron microscopy pictures of bone marrow-derived neutrophils after *in vitro* culture stimulation with *L. sigmodontis* L3 larvae (A and B) and *L. sigmodontis* microfilariae (C and D).

3.2 Bone marrow-derived eosinophils produce DNA-like nets in response to MF but not L3 larvae of *Litomosoides sigmodontis*.

Eosinophil ETosis in response to the different life-cycle stages of *L. sigmodontis* was investigated with the help of eosinophils derived from murine bone marrow (BmEos). Therefore, eosinophils were generated from bone marrow of naïve animals by IL-5 induced differentiation. After 12 days of culture, eosinophils exceeded a purity above 92 % and showed the typical characteristic O-shaped nucleus and no neutrophil contamination when stained with Giemsa (Figure 3).

Eosinophils were stimulated with *L. sigmodontis* L3 larvae and MF for 1 h and SEM pictures were taken. Eosinophils without stimulation presented a roundish appearance (Figure 5 A and B), while eosinophils co-cultured with L3 larvae showed a distorted surface suggesting degranulation (Figure 5 C and D). However, no DNA-like structures were observed. In contrast, MF stimulation caused the release of massive DNA-like structures (Figure 5 E and F).

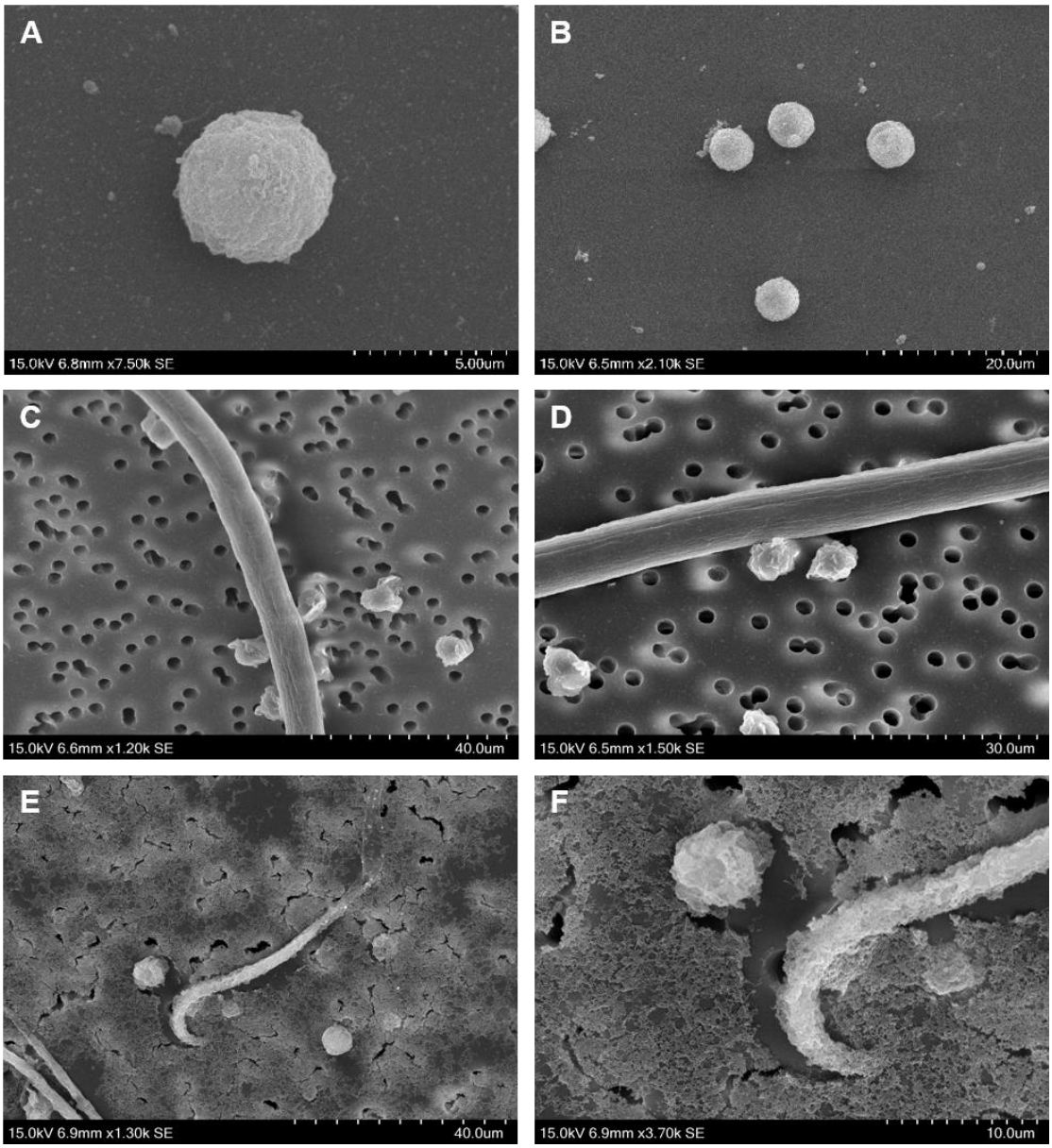

Figure 5. Bone marrow-derived eosinophils produce DNA-like nets in response to MF, but not L3 larvae of *Litomosoides sigmodontis*. Representative scanning electron microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture without stimulation (A and B), stimulation with *L. sigmodontis* L3 larvae (C and D) and *L. sigmodontis* microfilariae (E and F).

To analyze if these structures consist of extracellular DNA, confocal microscopy was performed. BmEos showed staining for eosinophil cationic protein (ECP) (Figure 6 A and E) and SiglecF (Figure 6 B and F). Unstimulated cells as well as cells co-cultured with L3 larvae did not show extracellular-DAPI-stained DNA and the nucleus appeared intact (Figure 6 C and G). Figure 6 D and H show the overlay. On the other hand, eosinophils incubated with MF showed DAPI-stained extracellular DNA with ECP attached to the extracellular DNA (Figure 7 D, H, L and P). The O-shaped nuclear structure of the eosinophils was lost and the shape of the extracellular DNA varied from long filamentous structures (Figure 7 C, G and O) to concentrated roundish structures

(Figure 7 K). MF were often attached by eosinophils (Figure 7 D and L). In Figure 8 A – C the DAPI-staining of the extracellular DNA is displayed in gray shades for better visualization.

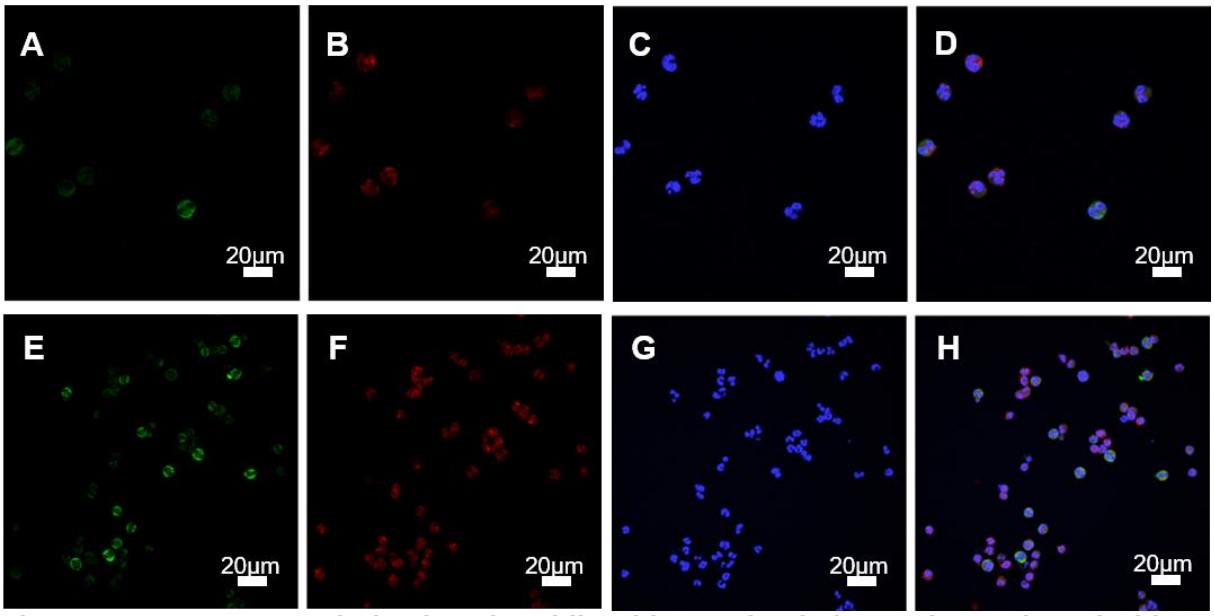

Figure 6. Bone marrow-derived eosinophils without stimulation and co-cultured with L3 larvae do not produce extracellular DNA traps. Representative confocal microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture without stimulation (A-D) and co-cultured for 24 h with L3 larvae (E-H) and stained for ECP (A+E) (green), SiglecF (B+F) (red), DAPI (C+G) (blue). D+H shows the overlay.

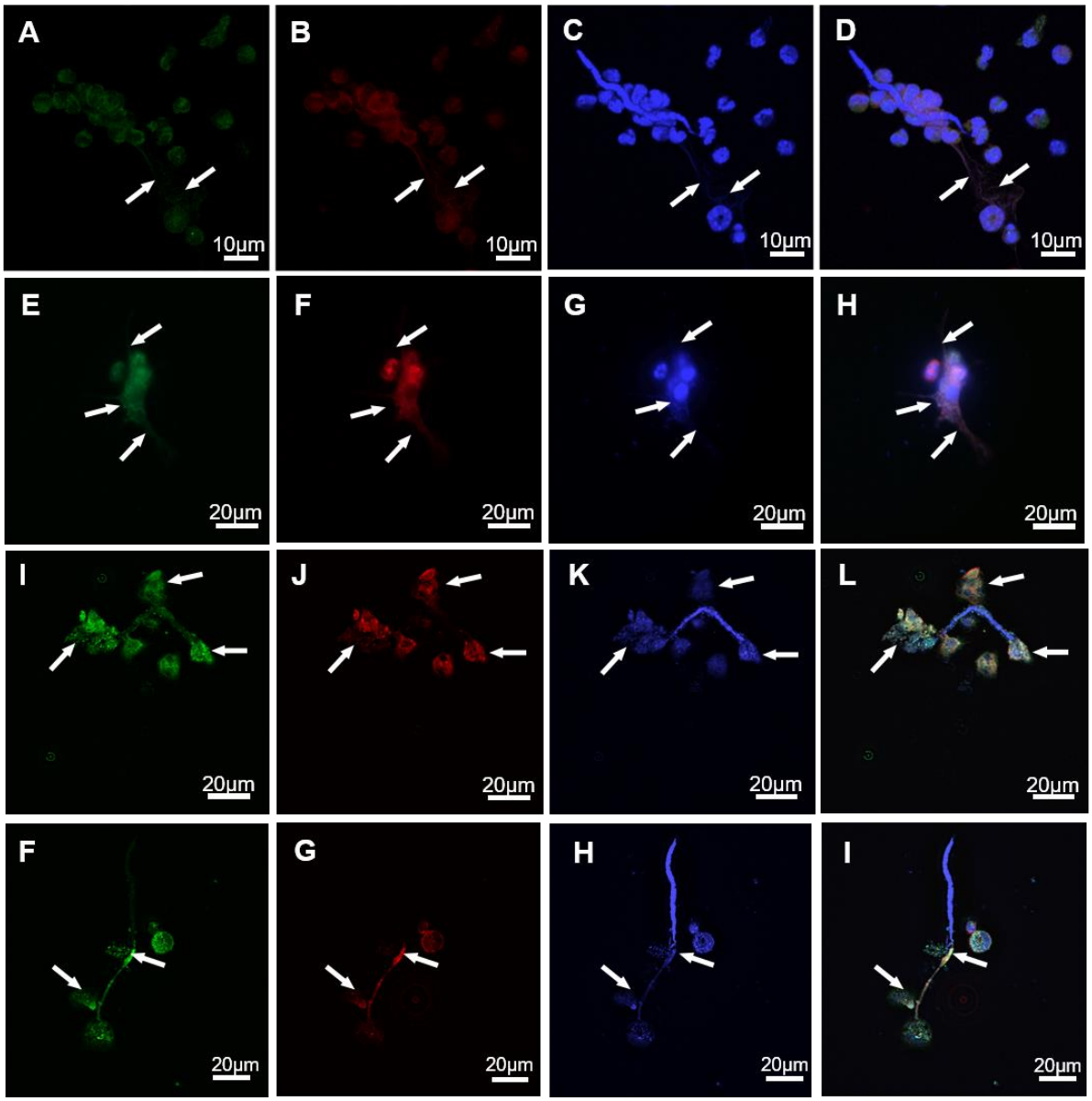

Figure 7. Bone marrow-derived eosinophils produce extracellular DNA traps in response to microfilariae of *Litomosoides sigmodontis*. Representative confocal microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture with *L. sigmodontis* microfilariae stained for ECP (A, E, I, M) (green), SiglecF (B, F, J, N) (red), DAPI (C, G, K, O) (blue). D, H, L, P shows the overlay. Arrows indicate extracellular DNA.

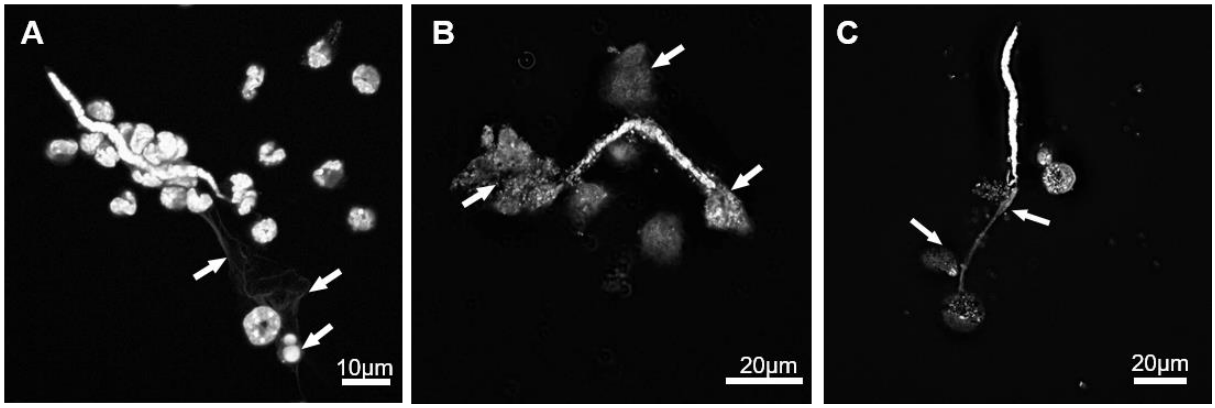

Figure 8. Bone marrow-derived eosinophils produce extracellular DNA traps in response to microfilariae of *Litomosoides sigmodontis*. Representative confocal microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture with *L. sigmodontis* microfilariae stained for DAPI (white). Arrows indicate extracellular DNA.

Furthermore, extracellular DNA released by eosinophils were quantified within the supernatant of the cell cultures. As a positive control zymosan was used since it is a known inducer of neutrophil and monocyte ETosis (151, 178). Figure 9 A shows that zymosan increased extracellular DNA release by BmEos, which was reduced in the presence of DNase. Eosinophils released DNA in response to MF as well, which increased with the number of MF (Figure 9 B), while L3 larvae failed to trigger extracellular DNA release (Figure 9 C). Transwell-assays where MF were separated from eosinophils revealed that direct contact is required to stimulate DNA release (Figure 9 D).

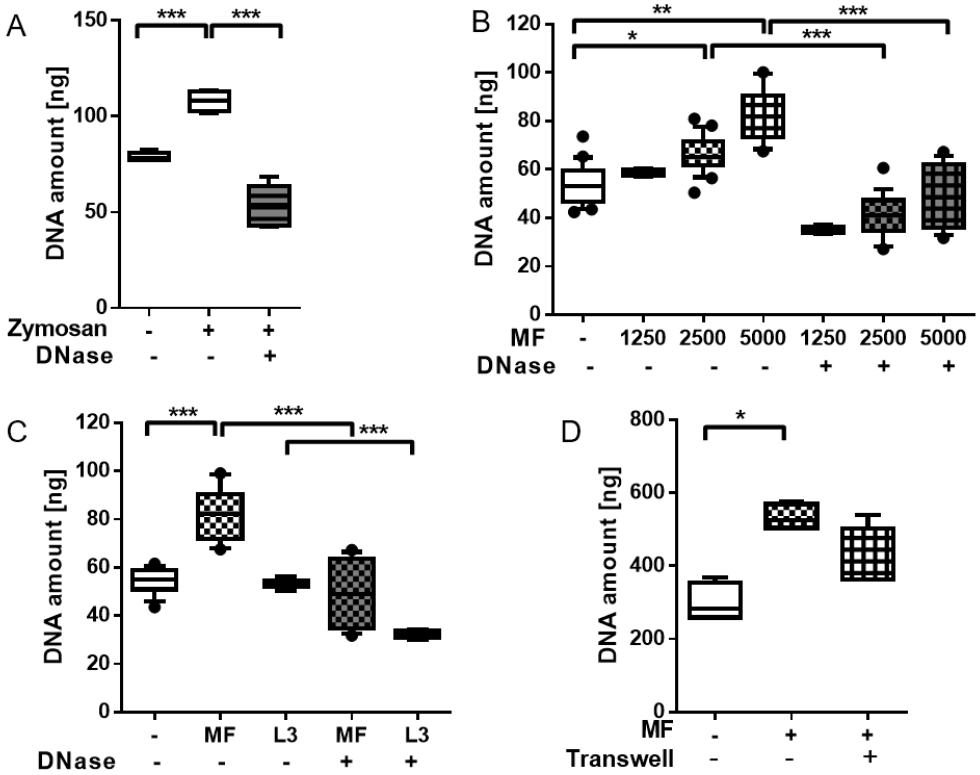

Figure 9. Extracellular DNA is released by bone marrow-derived eosinophils in response to *Litomosoides sigmodontis* microfilariae but not to L3 larvae. Released DNA from bone marrow-derived eosinophils stimulated in the presence or absence of DNase for 24 h with (A) 500 µg/ml zymosan, (B) 1250, 2500 or 5000 *L. sigmodontis* microfilariae (MF), (C) 5000 *L. sigmodontis* MF or 10 L3 larvae, and (D) released DNA from bone marrow-derived eosinophils that were in direct contact or separated via transwell from MF for 24 h. Shown are box plots with 10-90 percentile. Data shown in (A) is representative of one of three independent experiments with n= 5, in (B) pooled date from 3 independent experiments with n= 20 for unstimulated and 2500 MF; n= 4 for 1250 MF; and n= 16 for 5000 MF, in (C) representative data from one of two independent experiments with n= 16 for unstimulated; n= 12 for MF; n= 8 for L3, and (D) one independent experiment with n= 4. Normal distribution was tested by Kolmogorov-Smirnov normality test. Spearman's rank correlation test for heteroscedasticity was performed and only data failing the heteroscedasticity were pooled. Statistical analysis was done using 1-way ANOVA with Bonferroni post-hoc test for parametric data (A, C) and Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data (B and D). $p<0.05$*, $p<0.01$**, $p<0.001$***.

To further investigate if shear forces created by the movement of the MF trigger DNA release, heat-treated, dead MF were used to stimulate eosinophils. Figure 10 A shows that viable as well as heat-treated, dead MF induced eosinophil ETosis. To analyze if MF proteins induce the DNA release, crude extract of MF (MFAg), as well as from adult worms (LsAg) and L3 larvae (L3Ag) were prepared. In agreement with the results observed for the viable larvae, only MF crude extract triggered eosinophils to release DNA after 24 h (Figure 10 B), while adult worm crude extract slightly increased DNA release by eosinophils after 48 h (Figure 10 C). L3 larval crude extract did not trigger DNA release after 24 h and even reduced DNA release after 48 h of stimulation

compared to unstimulated controls (Figure 10 B and C). Even though viable L3 larvae failed to trigger ETosis, heat-treated, dead L3 larvae stimulated eosinophils to release extracellular DNA (Figure 10 D). To further analyze this, viable and heat-treated L3 larvae and MF were cultured without cells for 24 h and the supernatant was used to stimulate eosinophils alone or eosinophils cultured with MF. When BmEos were only cultured with the collected supernatant and without MF, the different supernatants did not impact DNA release, although supernatant collected from heat-treated L3 larvae slightly increased the DNA release by trend (Figure 10 E). In the presence of MF however, the supernatant of MF, heat-treated MF (MF Heat), L3 larvae (L3) and heat-treated L3 larvae (L3 Heat) significantly increased the DNA release by BmEos compared to BmEos cultured with only MF in the absence of supernatant (Figure 10 F). MF, heat-treated MF and L3 larval supernatant induced comparable DNA release by BmEos in the presence of MF, while supernatant collected from heat-treated L3 larvae induced a higher DNA release. Supernatant collected from 24 h culture containing only medium (m) did not further increase DNA release by BmEos and thus, the additional ETosis-boost must come from substances released from the larvae (Figure 10 F).

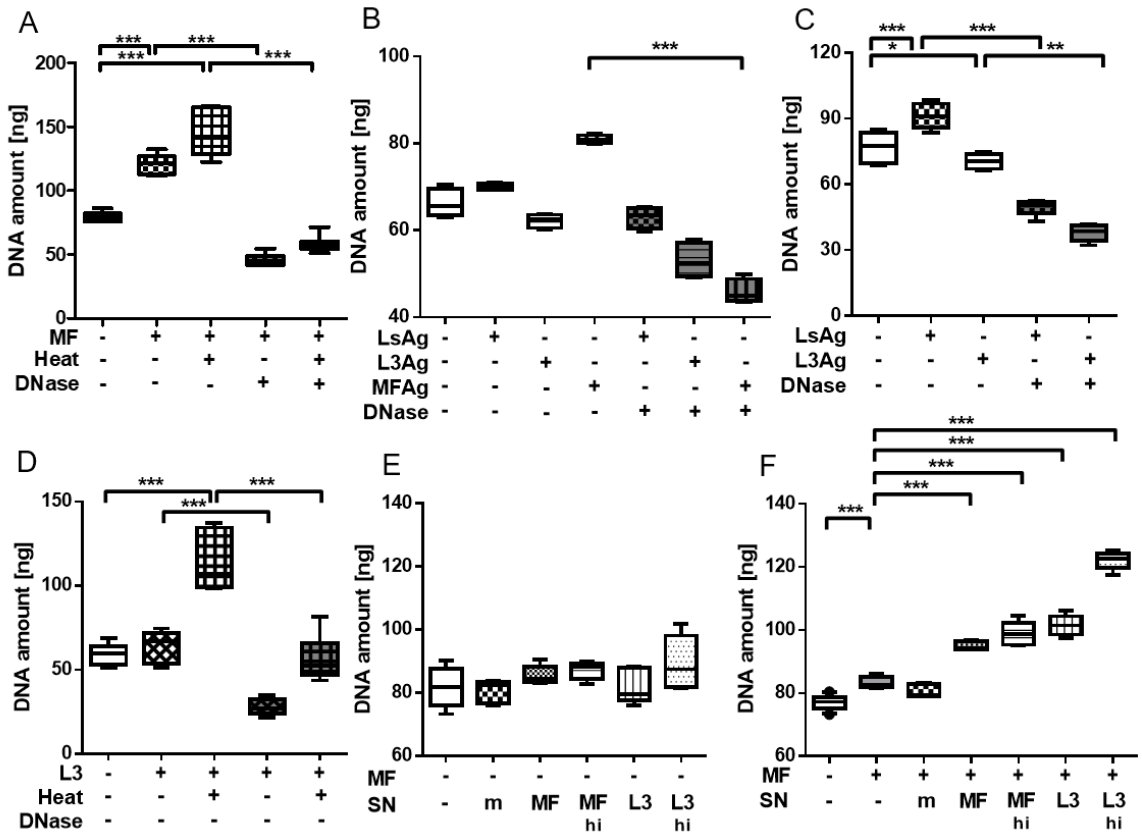

Figure 10. Extracellular DNA is released by bone marrow-derived eosinophils in response to *Litomosoides sigmodontis* microfilariae, but not to L3 larvae. Released DNA from bone marrow-derived eosinophils stimulated in the presence or absence of DNase with viable or heat-treated (Heat) microfilariae (MF) (after 48 h of culture) (A), 25 µg/ml crude extract from adult worms (LsAg), L3 larvae (L3Ag) or MF (MFAg) for 24 h (B) or 48 h (C), viable or heat-treated (Heat) L3 larvae (after 48 h of culture) (D). Released DNA from bone marrow-derived eosinophils stimulated with medium from cultures containing viable or heat-treated (Heat) MF or L3 larvae alone (E) or together with MF (F). Shown are box plots with 10-90 percentile. Data shown in (A) are pooled data from two independent experiments with n= 6 for - DNase, n= 7 for + DNase, in (D) pooled data from two independent experiments with n= 6 for - DNase and n= 7 for + DNase. B and C are one representative experiment from three independent experiments with n= 4, E and F are one out of two independent experiment with n= 5. Normal distribution was tested by Kolmogorov-Smirnov normality test. Spearman's rank correlation test for heteroscedasticity was performed and only data failing the heteroscedasticity were pooled. Statistical analysis was done by using Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data (B) and 1-way ANOVA with Bonferroni post-hoc test for parametric data (A, C-F). $p<0.05$*, $p<0.01$**, $p<0.001$***.

In summary, these results prove a life-cycle stage-specific response with eosinophils only undergoing ETosis in response to MF, but not viable L3 larvae.

3.3 Extracellular DNA released in response to microfilariae are of nuclear and primarily of mitochondrial origin.

The confocal microscopy pictures showed distorted nuclei in eosinophils exposed to MF and thus indicate that the released DNA probably consists of nuclear DNA. To further conform this, PCR of the purified DNA from the supernatant of eosinophil and

MF co-cultures were performed. The presence of mitochondrial DNA was analyzed using primers, which detect *Nd1* and *Cox1*, while nuclear DNA was measured with specific primers for *Gapdh* and *Actb*. Figure 11 A shows that the cell pellet, which was used as a positive control, contained, as expected, nuclear and mitochondrial DNA. The extracellular DNA within the supernatant consisted of mitochondrial and nuclear DNA as well, with the mitochondrial DNA being the more predominant DNA type. To further analyze the distribution of the mitochondrial and nuclear DNA ratio, qPCR of supernatant from eosinophils stimulated with MF and without stimulation was performed. Eosinophils stimulated with MF released DNA with a significantly higher mitochondrial to nuclear DNA ratio.

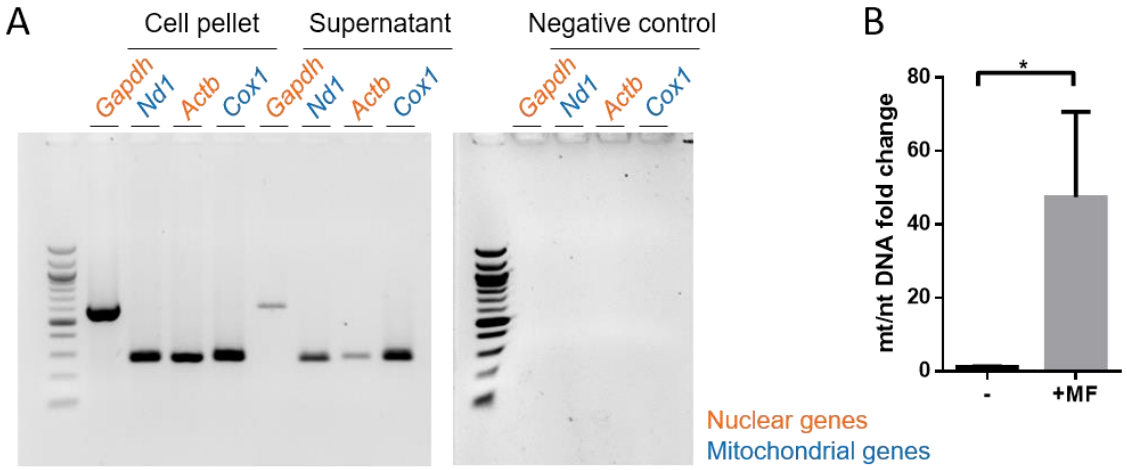

Figure 11. Extracellular DNA released in response to microfilariae are of nuclear and primarily of mitochondrial origin. A: PCR products of mitochondrial genes (*Nd1, Cox1*) (in blue) and nuclear genes (*Gapdh, Actb*) (in orange) in the supernatant and cell pellet of bone marrow-derived eosinophils that were stimulated *in vitro* with *Litomosoides sigmodontis* microfilariae. Negative controls consisted of PCR samples without DNA. Results are representatives form a total of three independent experiments with triplicates. B: qPCR of supernatant from bone marrow-derived eosinophils without stimulation (-) and stimulated with microfilariae (MF). Shown is the mitochondrial (mt)/ nuclear (nt) DNA fold change comparing the mt fold change of DNA traps in the supernatant to the average mt fold change of eosinophils alone. Shown is the mean with SEM. Pooled data from two independent experiments with n= 7. Analysis was done using an unpaired one-tailed t-test. p>0.05*

Thus, these data suggest that the eosinophil extracellular DNA released in response to MF consists of nuclear and primarily mitochondrial DNA.

3.4 Eosinophils inhibit microfilariae motility in an extracellular DNA trap-dependent manner.

It has been suggested that the function of extracellular DNA is to entrap microorganisms, enabling their escape and promoting its killing (137, 179, 180). To investigate if eosinophil extracellular DNA enables MF entrapment, eosinophil and MF co-cultures were generated and the MF motility was assessed daily. Therefore, a 5-

point score ranging from 0 to 4 with 0 meaning complete absence of larval movement and 4 equaling fast and continuous larval movement was used. MF cultured without cells showed a reduced movement over 72 h (black line), which was significantly reduced in the presence of BmEos (green line) (Figure 12 A). The MF motility reduction by eosinophils was restored upon DNase addition (blue dotted line) (Figure 12 A). The MF motility reduction was contact dependent, since MF motility reduction was not observed when the MF were separated from the eosinophils by a transwell (Figure 12 B). Furthermore, eosinophils from the gut of naïve and infected animals as well as from the pleura of infected animals were used for co-cultures with MF and compared to the MF-reducing capacity of BmEos. This not only allows the comparison of the e*x vivo* generated BmEos with *in vivo* generated eosinophils, but also shows the potency of eosinophils, which were in direct contact with the infection (pleural cavity) and eosinophils, which are derived from a distant site of the infection (gut). In addition, studying eosinophils from the gut of naïve animals and infected animals will show, if potential priming of circulating antigens from infected animals may impact eosinophil ETosis. Eosinophils isolated from the gut and the pleural cavity of *L. sigmodontis*-infected animals were much more potent in reducing the MF motility compared to BmEos from naïve mice (Figure 12 C). Already after the first day of co-culture, MF cultured with eosinophils from the pleura or gut of infected animals showed a significantly lower motility (pleura mean= 1.4, gut mean= 0.88) than MF cultured with BmEos (mean= 3.68) ($p<0.0001$). Therefore, lower numbers of eosinophils were used for co-cultures with gut and pleural cavity eosinophils for subsequent experiments. Figure 12 D reveals that even though gut eosinophils from naïve and infected animals were able to reduce MF motility in a comparable manner, pleura eosinophils from *L. sigmodontis*-infected animals were slightly but significantly more potent in reducing MF motility (day 1: pleura mean= 2.35, gut mean= 2.85; $p=0.0001$, day 2: pleura mean= 1.3, gut mean= 1.75; $p= 0.0003$, day 3: pleura mean= 1.1, gut mean= 1.42; $p= 0.0279$). DNA quantification demonstrates that the eosinophils from the different origins were all able to release extracellular DNA in response to zymosan (Figure 12 E-G). In contrast, pleura eosinophils from infected animals released DNA in response to MF, though not statistical significant (Figure 12 E), and no increase in DNA release was observed from gut-derived eosinophils from infected and naïve animals in response to MF (Figure 12 F, G). However, pleura eosinophils from infected animals as well as gut eosinophils from infected and naïve animals reduced MF motility in a DNA-trap-

dependent manner, since addition of DNase to the co-culture improved MF motility again (Figure 12 H-J). Since the eosinophils from different origins were all able to trap MF in a DNA-trap-dependent manner, ETosis in response to MF may occur via an intrinsic effect.

Next to eosinophils derived from wild-type mice, eosinophils were isolated from the gut and pleura of µMT mice, which lack B cells and thus a humoral response. Therefore, eosinophils isolated from µMT mice lack antibody-coating on their surface, which will show, if MF-induced ETosis is antibody-dependent. In co-cultures with MF, pleura and gut eosinophils derived from µMT mice were also able to reduce MF motility in a DNA-trap-dependent manner (Figure 12 K and L), indicating that MF entrapment is antibody-independent.

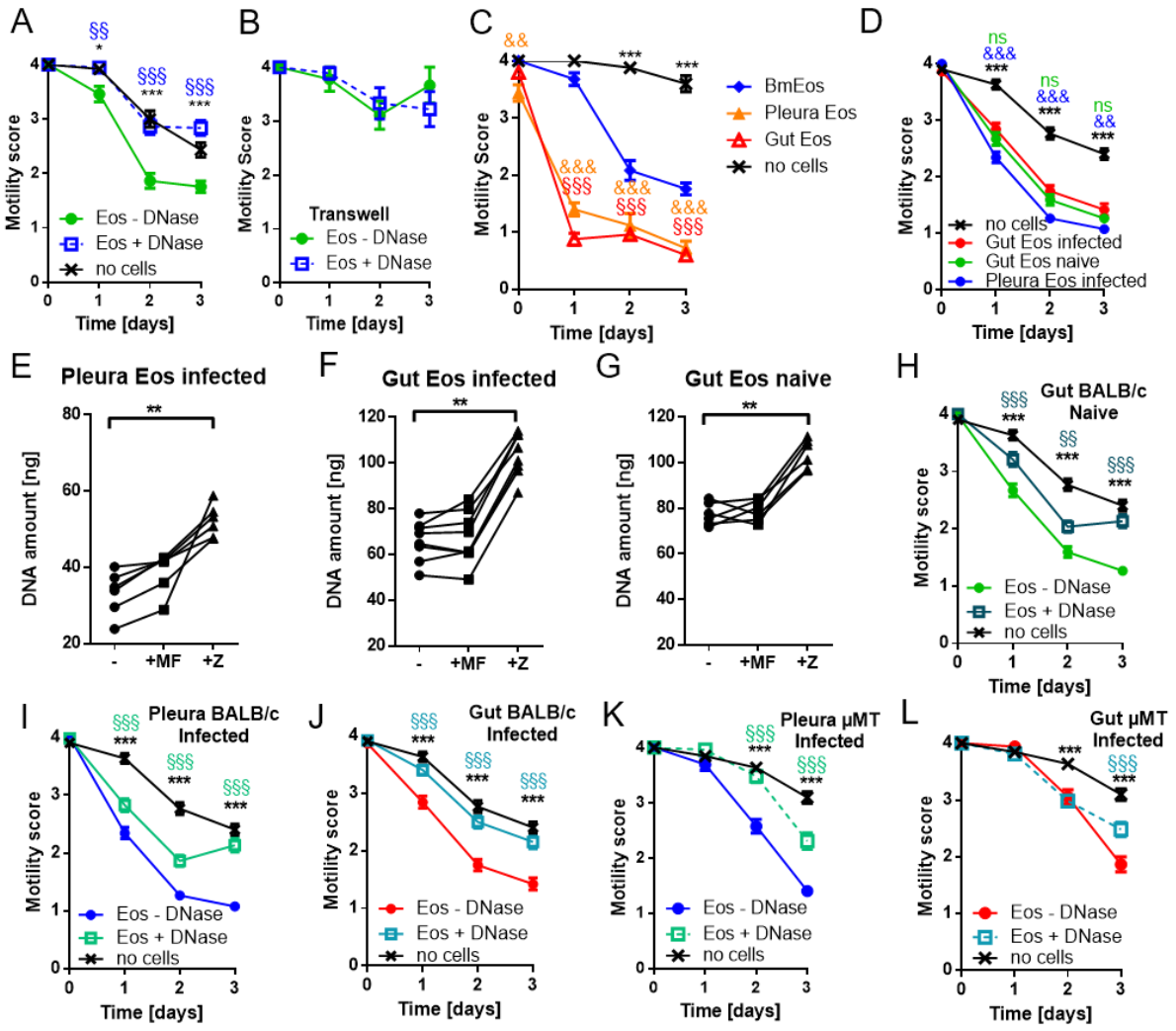

Figure 12. Eosinophils inhibit microfilariae motility in an extracellular DNA trap-dependent manner. Motility of microfilariae that were cultured alone (no cells: ✖), in direct contact (A) or separated by a transwell (B) with bone marrow-derived eosinophils in the absence (●) or presence (◘) of DNase. C: Comparison of microfilariae motility in the absence (✖) or presence of bone marrow-derived eosinophils (BmEos, ◆), pleura eosinophils (Pleura Eos, ▲) and gut eosinophils (Gut Eos, △) from *Litomosoides sigmodontis*-infected mice.

Motility of microfilariae co-cultured with gut eosinophils from naïve and *L. sigmodontis*-infected mice and thoracic cavity eosinophils from *L. sigmodontis*-infected mice (D), gut eosinophils (J) and pleural eosinophils (I) from *L. sigmodontis*-infected mice or gut eosinophils from naïve mice (H) in the presence and absence of DNase. Motility of microfilariae co-cultured with pleura eosinophils (K) and gut eosinophils (L) from *L. sigmodontis*-infected µMT mice in the presence and absence of DNase. E-G: Released DNA from pleura eosinophils from infected animals (E) and gut eosinophils from infected (F) and naïve animals (G) stimulated with *L. sigmodontis* microfilariae (MF) or zymosan (Z). Shown is the mean with SEM (A-D+H-L) or repeated measurements (E-G). Data shown are one representative experiment out of 16 independent experiments with n= 37 (A)/ one representative experiment out of 2 independent experiments with n= 9 (B), one experiment with n= 25 (C)/ one representative experiment out of 4 independent experiments n= 52 (D)/ pooled date from two independent experiments with n= 7 (unstimulated and +MF), n= 6 (+zymosan) (E), n= 8 (F), n= 6 (G)/ one representative experiment with n= 52 (H-L). Spearman's rank correlation test for heteroscedasticity was performed and only data failing the heteroscedasticity were pooled. Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done using 2-way ANOVA with Bonferroni post-hoc test. A, B, H-L: comparing groups to Eos – DNase (§= Eos + DNase, *= no cells), C: comparing groups to BmEos (&= Pleura eos, §= Gut eos, *= no cells), D: comparing groups to gut eosinophils from infected mice (&= pleura eosinophils, *= no cells). E-G: Statistical analysis was done by Friedman test with Dunn's post-hoc test for non-parametric data. $p<0.05$*, $p<0.01$**, $p<0.001$***.

In summary, these results show that eosinophils are able to reduce MF motility in a DNA-trap-dependent manner, which is antibody-independent and occurs via an intrinsic effect.

3.5 Microfilariae are killed by eosinophils.

Since the MF motility assays revealed entrapment of MF by eosinophils, apoptosis staining of eosinophils and MF were performed using Annexin V and PI staining. Flow cytometry analysis revealed that most eosinophils cultured for 5 days alone or together with MF stained negatively for Annexin V and PI (viable). However, eosinophils cultured with MF showed a significant reduction in Annexin V negative cells (with MF mean= 91.05, without MF mean= 95.3, p= 0.0292) and a significant increase in Annexin V single positive cells (with MF mean= 6.7, without MF mean= 1.35, p= 0.0127) compared to eosinophils cultured without MF (Figure 13 A). In contrast, most MF cultured with eosinophils stained positively for Annexin V and PI (dead) and no differences were observed for MF in co-cultures in the presence or absence of DNase (with DNase mean= 93.0, without DNase mean= 90.95) (Figure 13 B). MF motility of MF cultured without cells reduced over time. Similar, these MF show Annexin V and PI double positive staining as well as Annexin V single staining (apoptotic). However, MF without cells showed less Annexin V and PI double staining (MF without cells mean= 81.25, MF with cells mean= 90.95) and more Annexin V only staining (MF

without cells mean= 18.75, MF with cells mean= 7) compared to MF in co-culture suggesting that eosinophils support the killing of MF (Figure 13 B).

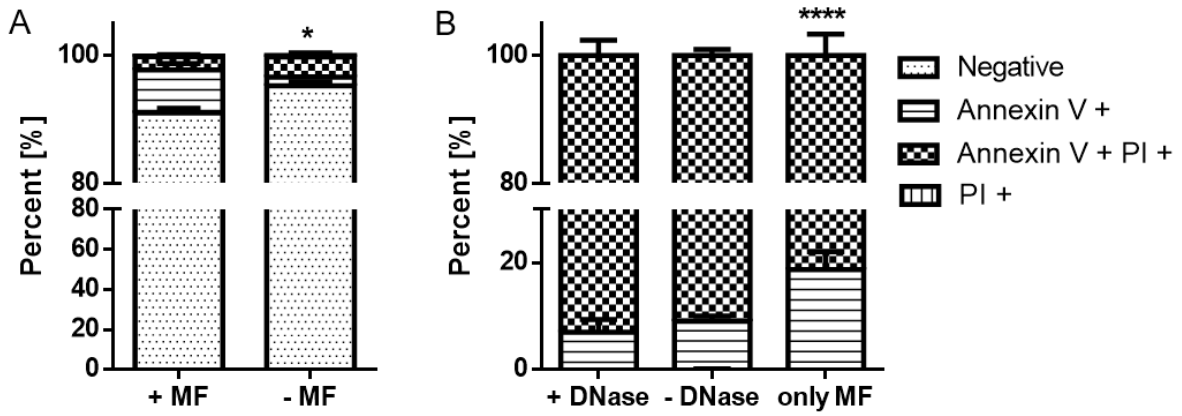

Figure 13. Microfilariae are killed by eosinophils. Percentage of viable eosinophils (A) and MF (B) in co-cultures assessed by Annexin V and PI positivity after 5 days of culture. Percentage of dead (PI + and Annexin V + PI +), dying (Annexin V +) and viable (negative) (A) bone marrow-derived eosinophils co-cultured in the presence or absence of MF and (B) MF co-cultured with eosinophils in the presence or absence of DNase or MF cultured alone (MF only). Shown is the mean with SEM. Data represent one independent experiment with n= 2. Analysis was done using 2-way ANOVA with Bonferroni post-hoc test comparing A: + MF with – MF or B: to + DNase. Statistical significance was reached for A: negative ($p= 0.0292$) and Annexin V + ($p= 0.0127$) and B: Annexin V + (+ DNase vs only MF: $p<0.0001$) and Annexin V + PI + (+ DNase vs only MF: $p<0.0001$). $p<0.05$*, $p<0.0001$****.

Thus, these results suggests that more eosinophils stain for Annexin V, which is an indicator for cytolysis, in MF co-culture and that eosinophils can kill MF in co-cultures, which is however independent on DNA traps.

3.6 Eosinophils release extracellular DNA in response to plasma but not serum from mice, which is independent on the complementary system.

After maturation, granulocytes are released into the peripheral blood circulation and enter the tissues upon stimulation. Therefore, the impact of blood and blood components on DNA release by eosinophils was investigated. For neutrophils it was shown that serum as well as serum albumin inhibits NETosis, which may serve the physiological purpose to prevent blood clots and thus thrombi (181). The contribution of coagulation factors was investigated by the use of blood or blood components in the presence or absence of EDTA. Erythrocytes with and without coagulation factors as well as whole blood failed to trigger DNA release by BmEos (Figure 14 A). The same was true for serum from naïve animals with coagulation factors, while plasma from naïve animals without coagulation factors triggered eosinophil DNA release (Figure 14

B and D-H). Heat-treatment of plasma from naïve animals reduced the DNA release again (Figure 14 B and C), while compstatin treatment, which inhibits C3 cleavage, had no impact on DNA release.

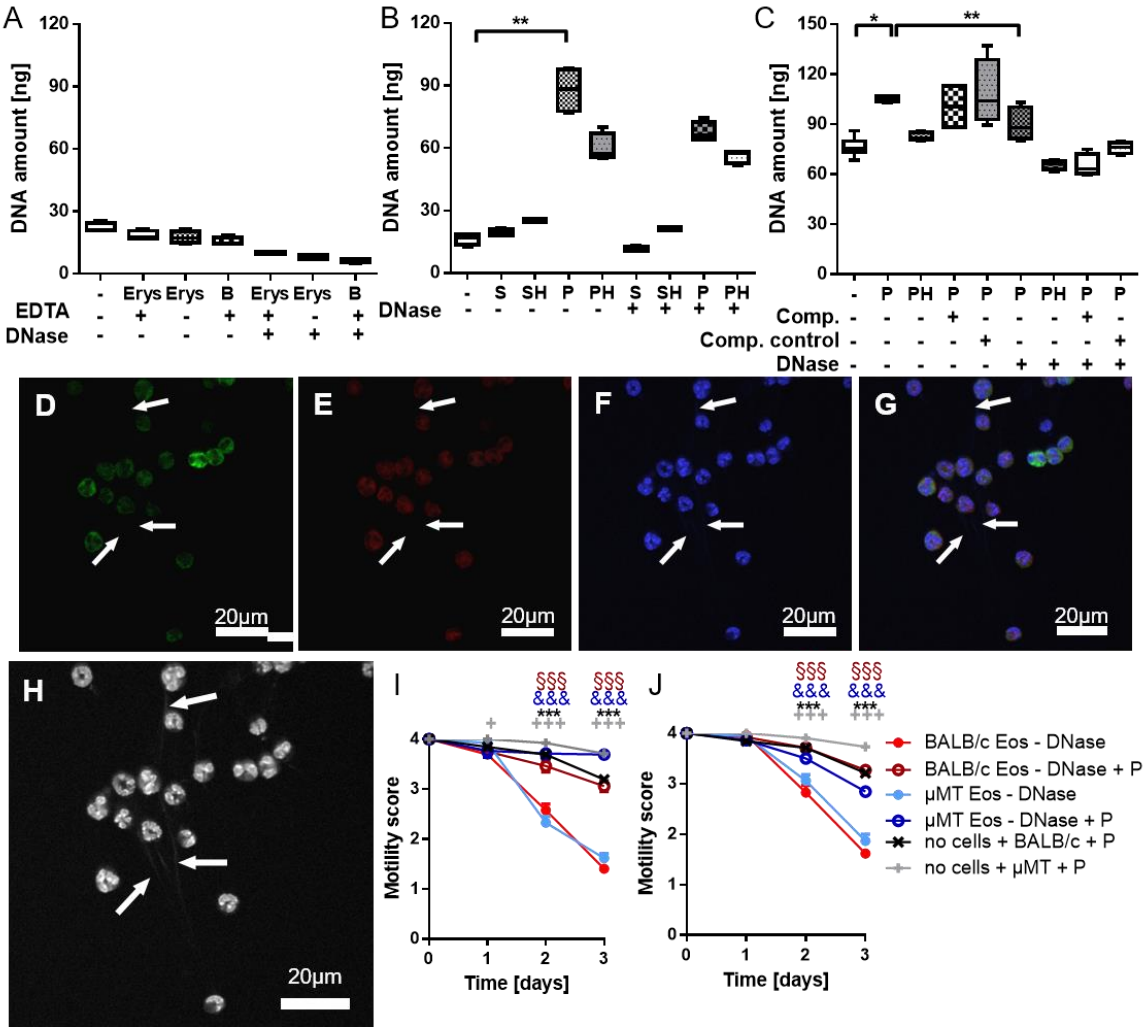

Figure. 14. Bone marrow-derived eosinophils release extracellular DNA in response to plasma, but not serum from mice, which is independent on the complement system. Released DNA from bone marrow-derived eosinophils from wild-type BALB/c mice stimulated in the presence or absence of DNase for 24 h with A: $1*10^7$ erythrocytes (Erys) with and without coagulation factors (-/+ EDTA), and whole blood without coagulation factors (+ EDTA), B: 5 % serum (S), heat-treated serum (SH), plasma (P), and heat-treated plasma (PH), C: plasma (P), which was either heat-treated (PH) or depleted in the complementary system (compstatin or compstatin control). D-H: confocal microscopy pictures of bone marrow-derived eosinophils co-cultured with plasma for 3 h and stained for ECP (D) (green), SiglecF (E) (red), DAPI (F) (blue). G shows the overlay and H shows the DAPI staining in gray shades. I+J: Microfilariae motility scores in co-cultures with pleura (I) and gut (J) eosinophils from wild-type (BALB/c) or µMT mice in the presence or absence of the corresponding plasma (P). A-C: Shown are box plots with 10-90 percentile. Data are from one out of 2 (A)/ 3 (B)/ 1 (C) independent experiment with A+B n= 4 and C: n= 8 (eosinophils without stimulation) or n= 4 (eosinophils with stimulation). I+J: mean with SEM of a single experiment with n= 52. A-C: Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data. I+J: Statistical analysis was done by 2-way ANOVA followed by Bonferroni post-hoc test comparing groups to BALB/c Eos - DNase (§= BALB/c Eos – DNase + Plasma, &= µMT Eos – DNase + Plasma *= no cells + BALB/c Plasma, += no cells + µMT Plasma). $p<0.05$*, $p<0.01$**, $p<0.001$***.

This indicates that plasma components can trigger eosinophils to release DNA, which is not the case for serum, containing coagulation factors, or erythrocytes. Furthermore, the DNA release triggered by plasma is independent on the complement system, but partly dependent on heat-labile plasma components.
Interestingly, addition of plasma to eosinophil and MF co-cultures prevented MF motility reduction by pleura (Figure 14 I) and gut eosinophils (Figure 14 J), suggesting that even though plasma triggers DNA release, further plasma components support MF motility and prevent MF entrapment (Figure 14 I and J).

3.7 Eosinophils only inhibit L3 larval motility in the presence of plasma from infected animals in an extracellular DNA trap-dependent manner.

Eosinophils are known to contribute to protective immunity against adult filariae and MF, but not against primary L3 larval infections (182-184). Nevertheless, eosinophils show protective immunity against L3 larvae during secondary infections and vaccinations (185, 186). Thus, the impact of eosinophils on DNA release by BmEos and L3 larval motility in the presence or absence of different plasmas was investigated. The previous results have shown that eosinophils failed to release extracellular DNA in response to L3 larvae analyzed by SEM, confocal microscopy and DNA quantification (Figure 4 C and D, figure 5 E-H, figure 8 C). In accordance, eosinophils did not inhibit L3 larval motility in co-cultures regardless if the L3 larvae were isolated from the pleural cavity of jirds or picked from mites (Figure 15 B and C) proving that eosinophils do not contribute to protective immunity against primary L3 larval infections. Addition of plasma from naïve and infected animals raised the DNA release by BmEos in co-culture with L3 larvae. This increase was even higher than for BmEos stimulated with plasma alone (Figure 15 A). However, addition of plasma from naïve animals did also not impede L3 larval motility (Figure 15 D). Plasma from WT animals infected for 64 days reduced third-stage larval motility significantly (red line), which was partly dependent on DNA-traps, as addition of DNase partially restored L3 motility again (orange dotted line) (Figure 15 E). Interestingly, plasma from µMT mice, lacking B cells and thus a humoral immune response, did not reduce L3 larval motility by eosinophils (Figure 15 F), suggesting that antibodies enable L3 larval entrapment by eosinophils in a partly DNA-trap-dependent manner. Heat-treatment of the plasma from infected WT animals still enabled eosinophils to reduce L3 motility (purple line) (Figure 15 H), while heat-treatment of plasma from naïve and µMT mice had no impact on L3 larval motility (Figure 15 G and I).

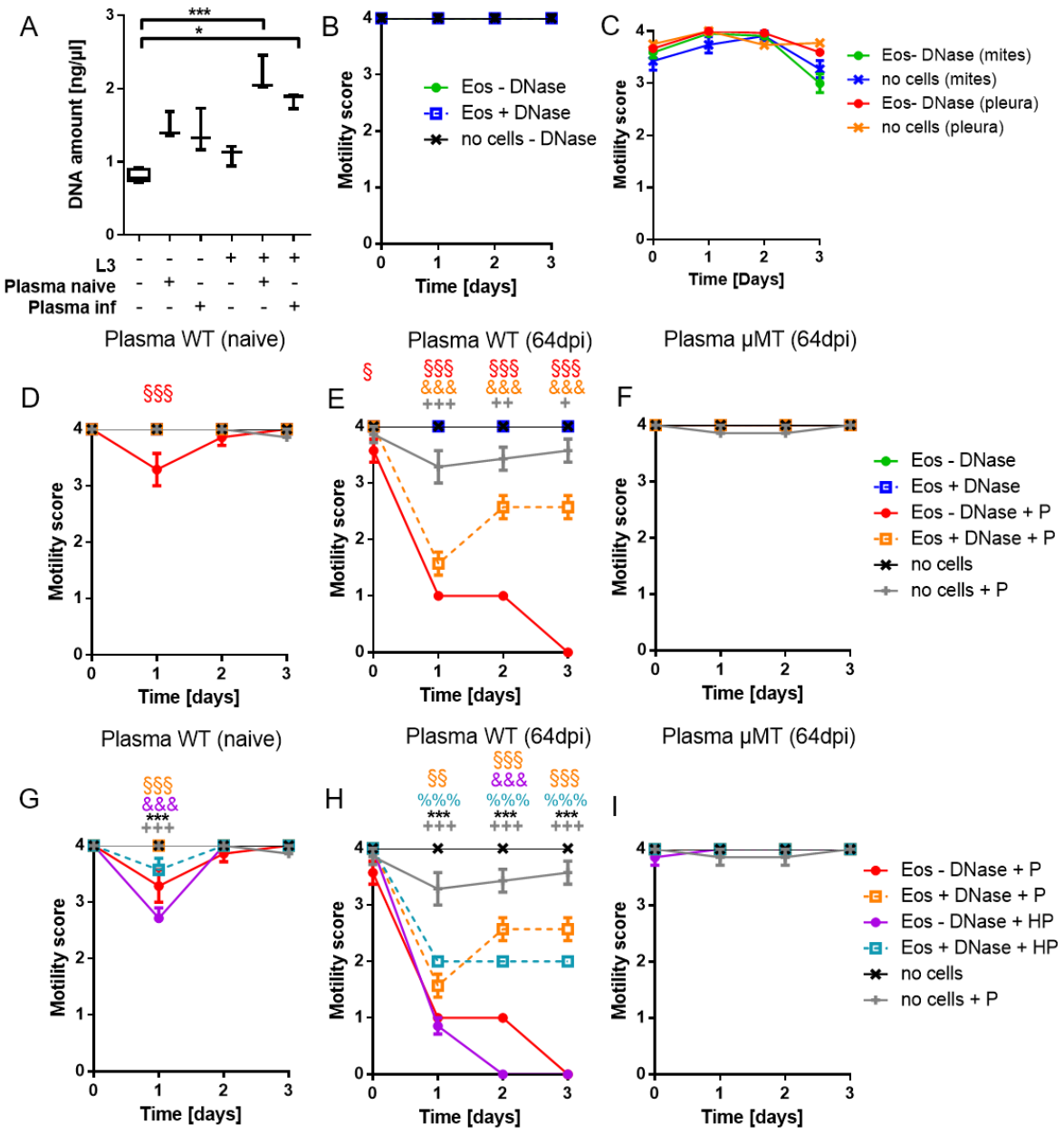

Figure 15. Eosinophils only inhibit L3 larval motility in the presence of plasma from wild-type infected animals, which is partly dependent on extracellular DNA traps. DNA release by bone marrow-derive eosinophils in response to L3 larvae with or without plasma and motility of L3 larvae that were cultured alone (no cells: ✖) or with bone marrow-derived eosinophils in the absence (●) or presence (◘) of DNase. A: Measured DNA release by BmEos after stimulation with plasma from naïve or infected (inf) animals in the presence or absence of L3 larvae for 24 h. Motility score of L3 larvae B: from pleural cavity of jirds or C: from mites. D-I: L3 larval motility co-cultured with BmEos in the presence of plasma (P) (D-F) or heat-treated plasma (HP) (G-I) from naïve (D and G), wild-type infected (E and H) or µMT infected (F and I). Shown are box plots with 10-90 percentile (A) or the mean with SEM (B-I) and data are from one out of two independent experiments with A: n= 7 (unstimulated) and n= 3 (stimulated) and B-I: n= 7. Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data (A) or 2-Way ANOVA with Bonferroni post-hoc test (B-I). A: comparing groups to Eos unstimulated, B, D, E and F: comparing groups to Eos – DNase (§= Eos + DNase + P, &= Eos + DNase + P *= no cells, += no cells + P), C: comparing groups to Eos – DNase (mites), G-I: comparing groups to Eos – DNase + P (§= Eos + DNase + P, &= Eos – DNase + HP, %= Eos + DNase + HP, *= no cells, += no cells + P). p<0.05*, p<0.01**, p<0.001***.

In summary, these results support that eosinophils do not contribute to protective immunity in primary L3 larval infection, but rather require antigen-specific antibodies to entrap third-stage larvae, which occurs partly in a DNA-trap-dependent manner.

3.8 Eosinophils only inhibit adult worm motility in the presence of plasma from naïve animals in an extracellular DNA trap-dependent manner.

Next to MF and L3 larvae, the impact of EETosis on adult worms was analyzed by BmEos and adult worm co-cultures. DNA quantification of extracellular DNA in the supernatant of co-cultures reveal that eosinophils released DNA when cultured with adult female worms only, while male worms failed to induce DNA release (Figure 16 A and B). Heat-treatment of the female worms abolished the DNA release by eosinophils (Figure 16 A). This could suggest that the shear forces created by the worm movement induce the DNA release with larger forces for female worms than for smaller male worms, heat-labile ES products of the female worms induce EETosis or released MF from the female uteri trigger the DNA release.

Furthermore, adult worm motility in co-cultures was assessed. Eosinophils mediated no inhibition of adult worm motility in co-cultures (Figure 16 C), while addition of plasma from naïve animals enabled eosinophils to entrap adult worms in a DNA-trap-dependent manner (Figure 16 D). Plasma from infected animals (64 dpi) only slightly reduced adult worm motility after 7 days of co-culture regardless on the presence or absence of DNase (Figure 16 E).

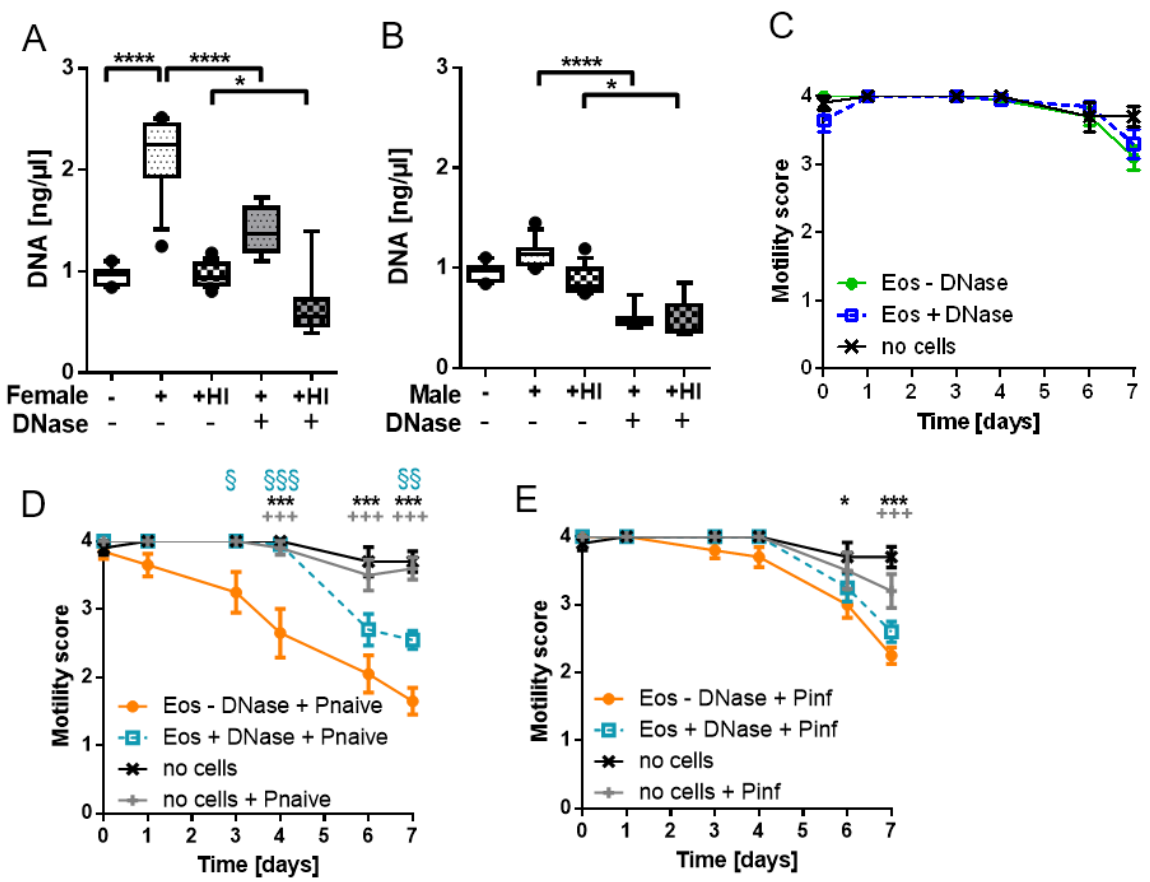

Figure 16. Eosinophils only inhibit adult worm motility in the presence of plasma from naïve animals in an extracellular DNA trap-dependent manner. Released DNA from bone marrow-derived eosinophils from wild-type BALB/c mice without stimulation and with female (A) and male (B) adult *Litomosoides sigmodontis* worms isolated from patent–infected jirds. Worms were either viable or heat-treated (HI). C-E: Motility of adult worms that were cultured alone (no cells: ✖) or with bone marrow-derived eosinophils in the absence (●) or presence (◘) of DNase. C: adult worms cultured without plasma, D: with plasma (P) from naïve animals or plasma from infected wild-type mice (E). A-B: shown are box plots with 10-90 percentile. Data are from one out of three independent experiment with n=5 (unstimulated and n=7 (+ worms). C-E: shown is the mean with SEM. Data are from one independent experiment with n=20. A-B: Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done using 1-way ANOVA with Bonferroni post-hoc test for parametric data (A) or Kruskal-Wallis test with Dunn's post-hoc test for non-parametric data (B). C-E: Statistical analysis was done using 2-way ANOVA with Bonferroni post-hoc test. C: comparing groups to Eos – DNase, D and E: comparing groups to Eos – DNase + P (§= Eos + DNase+ P, *= no cells, += no cells + P). p<0.05*, p<0.01**, p<0.001***, p<0.0001****.

This indicates, that eosinophils alone are not able to entrap adult worms *in vitro*, while The addition of plasma supports DNA release and adult worm entrapment. Plasma from infected animals however, may contain immunomodulatory components alleviating adult worm entrapment.

3.9 *Escherichia coli* trigger DNA release by eosinophils.

Next to protective immunity against helminths, eosinophils contribute to the elimination of bacterial infections as well. Eosinophils are able to phagocytose bacteria and

activate the adaptive immunity through antigen presentation (121). However, phagocytosis by neutrophils occurs much more frequently than eosinophil phagocytosis (187, 188). A more important effector mechanism by eosinophils is the release of cationic proteins including MBP, ECP and EPO, which have known antibacterial properties (121). Publications have also shown that eosinophils release extracellular DNA in response to bacteria, enabling their binding and thus contributing to their elimination (141).

In accordance, scanning electron microscopy pictures showed the uptake of *E. coli* by phagocytosing BmEos (Figure 17 A and B). Moreover, BmEos released net-like structures in response to *E. coli,* which were proven to be DNA by confocal microscopy (Figure 17 E-H) and fluorescence assays (Figure 17 I). PCR revealed similar to the DNA released by eosinophils in response to MF that BmEos extracellular DNA in response to *E. coli* consists out of nuclear and primarily mitochondrial DNA (Figure 17 J).

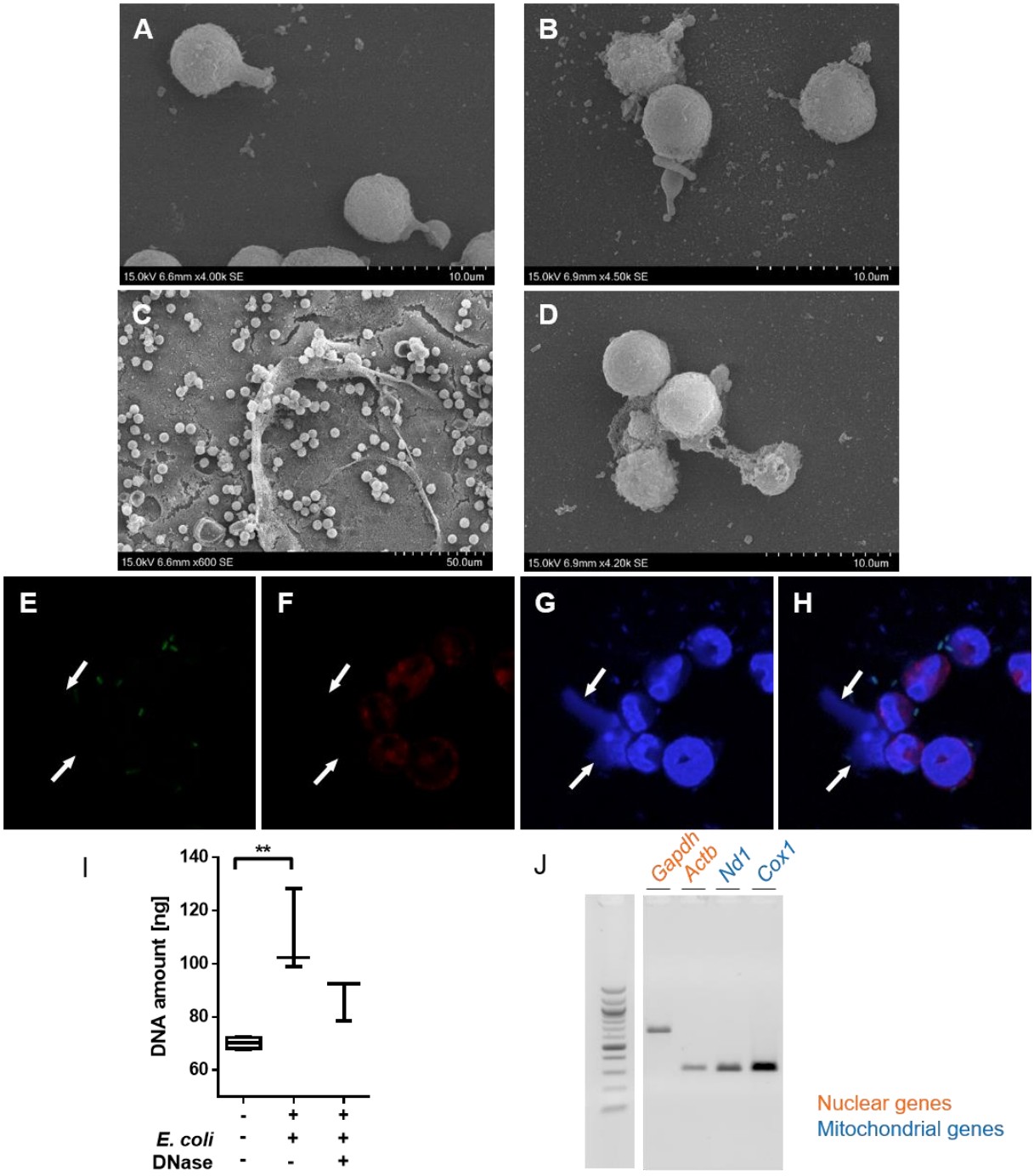

Figure 17. *Escherichia coli* triggers DNA release by eosinophils. A-D: Representative scanning electron microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture stimulation with *Escherichia coli*. E-H: confocal microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture with *E. coli*-GFP stimulation stained for E: *E. coli*-GFP (green), F: SiglecF (red), G: DAPI (blue). H shows the overlay. I: DNA quantification from supernatant of bone marrow-derived eosinophils stimulated with *E. coli* (n= 6 (medium control and n= 3 *E. coli*) in the presence or absence of DNase. J: PCR products of mitochondrial genes (*Nd1, Cox1*) (in blue) and nuclear genes (*Gapdh, Actb*) (in orange) in the supernatant of bone marrow-derived eosinophils that were stimulated *in vitro* with *E. coli*. Shown is in I box plots with 10-90 percentile. Data are from one out of two independent experiments with n= 6 (unstimulated) or n= 3 (with *E. coli*). Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis with Dunn's post-hoc test for non-parametric data. p<0.01**.

These results indicate, that eosinophil ETosis occurs as a universal defense mechanism against MF as well as bacteria.

3.10 BmEos produce extracellular DNA-traps in response to MF, PMA and zymosan in a TLR2-, TLR4- and MyD88-independent manner.

E. coli recognition by immune cells can occur via different TLRs and NODs. An important PAMP of *E. coli* is LPS, which is detected by TLR-4 and partly by TLR-2 (189). Furthermore, it was proven that *Wolbachia* endosymbionts of filariae are recognized via TLR-2 and TLR-6 and neutrophils produce extracellular DNA traps *in vivo* to the endosymbiotic *Wolbachia* bacteria of *O. volvulus*, suggesting, that bacterial- and MF-induced ETosis may be triggered through the recognition by TLRs (24).

In order to test whether EETosis is induced via TLRs, BmEos were stimulated with Pam3Cys (TLR-1+2), Poly (I:C) (TLR-3), LPS (TLR-4) and R848 (TLR-7+8). However, only a slight increase in extracellular DNA release was observed for TLR-3 and -4 stimulation (Figure 18 A). CpG, a TLR-9 agonist on the other hand, was able to significantly increase extracellular DNA in eosinophil cultures (Figure 18 B). However, this increased DNA recognition was due to the CpG detection by the DNA quantification assay, since CpG cultures without cells showed similar DNA concentrations than CpG-stimulated eosinophil cultures (Figure 18 B).

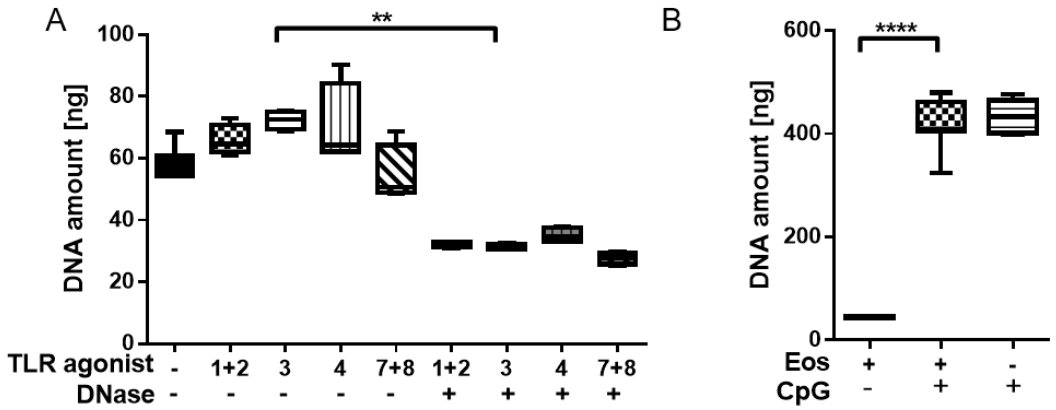

Figure 18. Bone marrow-derived eosinophils do not produce extracellular DNA-traps in response to TLR-stimuli. Released DNA from bone marrow-derived eosinophils from wild-type BALB/c mice without stimulation and with Pam3Cys (TLR-1+2 agonist), Poly (I:C) (TLR-3 agonist), LPS (TLR-4 agonist) and R848 (TLR-7+8 agonist) (A) and CpG (TLR-9 agonist) (B). Shown are box plots with 10-90 percentile. Data are from one independent experiment with n= 4 (TLR-3, 7+8) or from one representative experiment out of 2 (TLR-1+2 and 4) (A) and one experiment out of 4 independent experiments (TLR-9) with n= 5 (unstimulated) and n= 7 (+CpG and CpG alone). Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data (A) and 1-way ANOVA for parametric data (B). $p<0.01$**, $p<0.0001$****.

To test, if MF trigger the DNA release through TLR recognition, BmEos from TLR-2, TLR-4 and MyD88 KO mice were generated and with MF, zymosan and PMA stimulated.

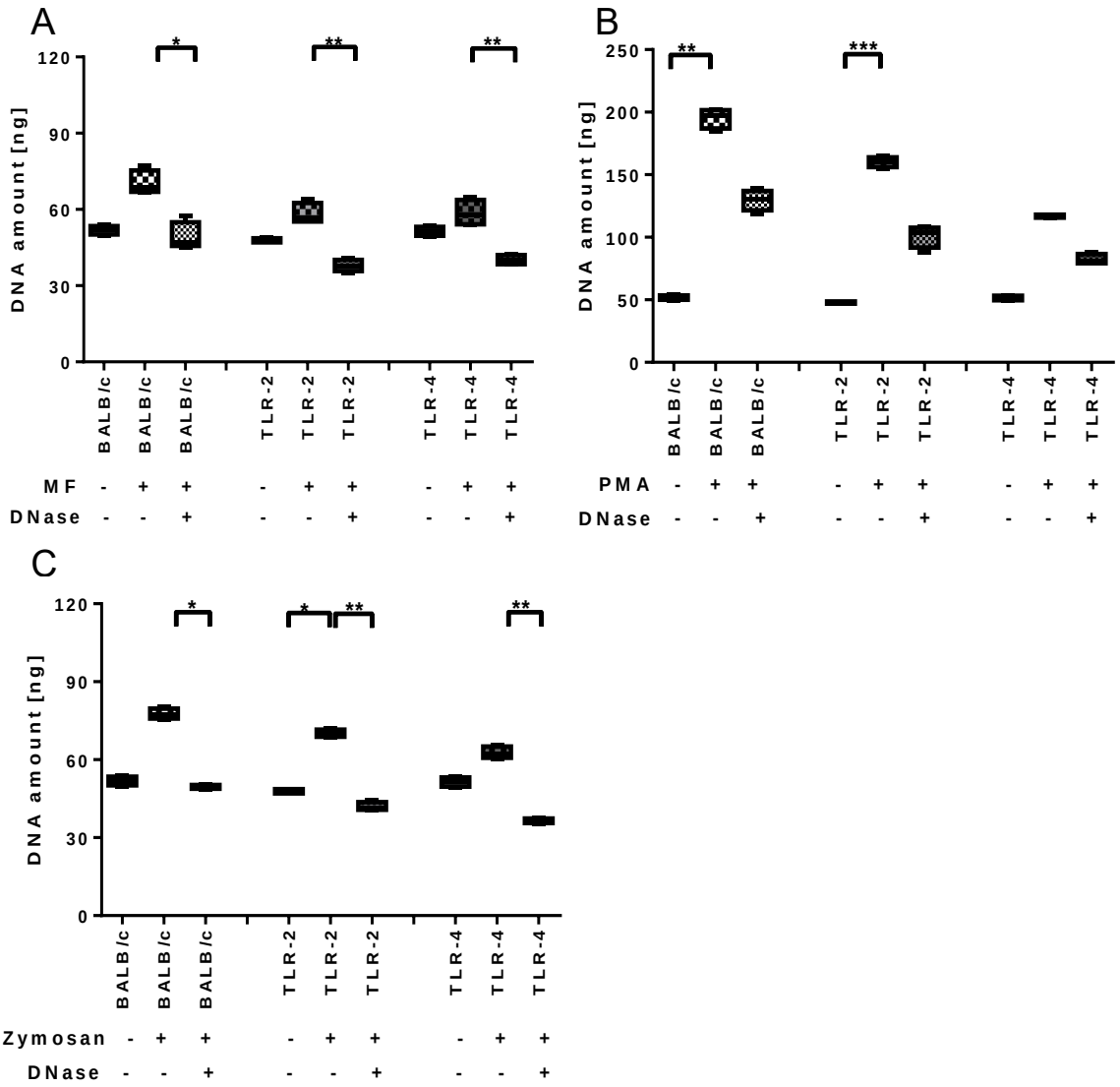

Figure 19. Bone marrow-derived eosinophils produce extracellular DNA-traps in response to microfilariae, PMA and zymosan in a TLR-2- and TLR-4-independent manner. Released DNA from bone marrow-derived eosinophils from wild-type BALB/c mice, TLR-2 and TLR-4 KO BALB/c mice stimulated in the presence or absence of DNase for 24 h with (A) *Litomosoides sigmodontis* microfilariae (MF), (B) PMA, and (C) zymosan. Shown are box plots with 10-90 percentile. Data are from one independent experiment with n= 4. Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data. $p<0.05$*, $p<0.01$**, $p<0.001$***.

Figure 19 A shows that the MF-induced DNA release by eosinophils is independent on TLR-2 and -4 recognition, since MF were able to trigger EETosis in KO mice as well. As expected, stimulation of PMA and zymosan, which are known to signal via protein kinase C and the dectin receptor, respectively, induced DNA release by TLR-2 and

TLR-4 deficient BmEos (Figure 19 B, C). As signaling of most TLRs (except for TLR-3) occurs via MyD88, BmEos lacking MyD88 signaling pathways were also investigated. MyD88-deficient eosinophils were still able to undergo MF-, PMA- and zymosan-induced ETosis (Figure 20 A-C).

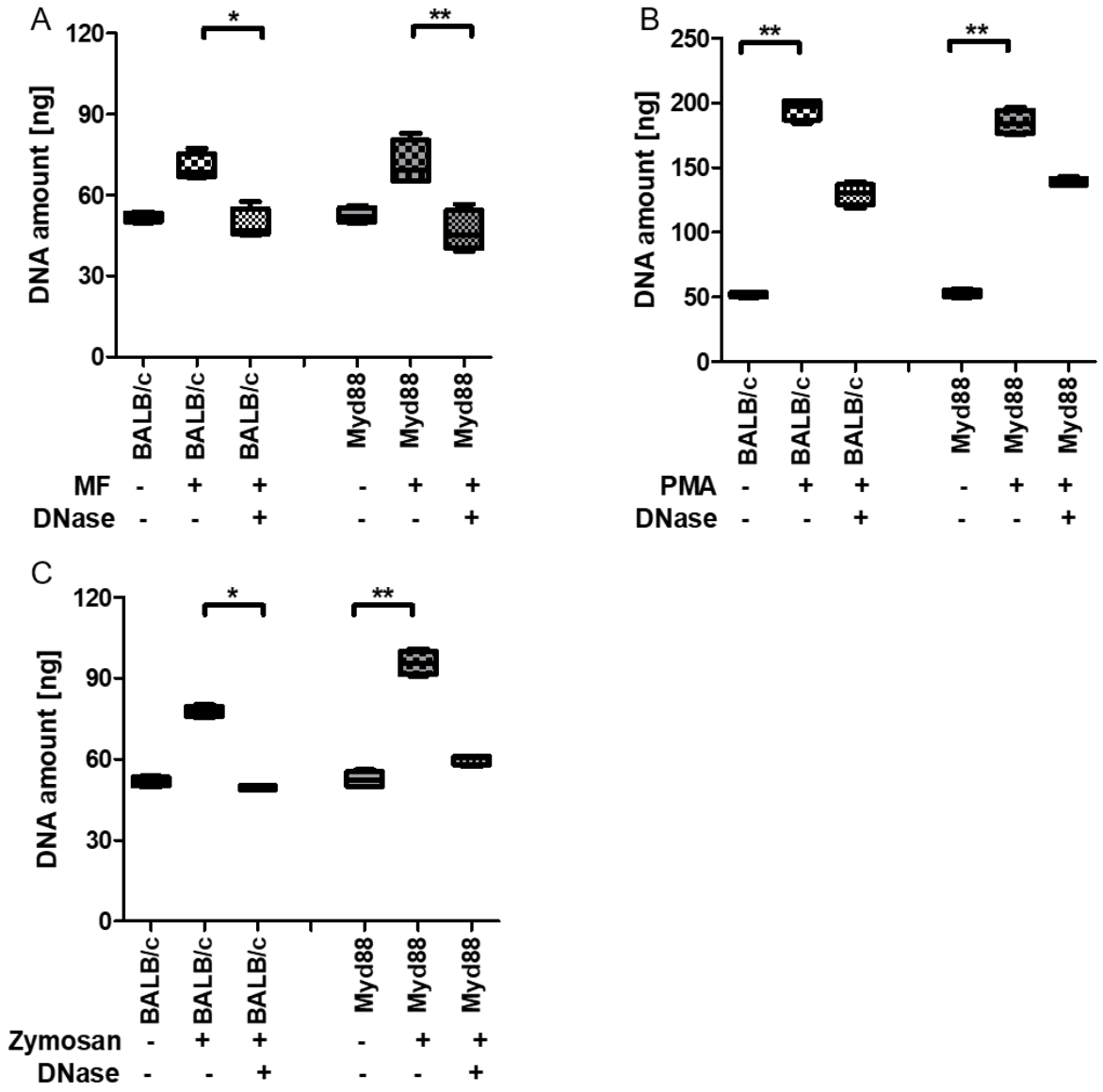

Figure 20. Bone marrow-derived eosinophils produce extracellular DNA-traps in response to microfilariae, PMA and zymosan in a MyD88-independent manner. Released DNA from bone marrow-derived eosinophils from wild-type BALB/c and MyD88 KO BALB/c mice stimulated in the presence or absence of DNase for 24 h with (A) *Litomosoides sigmodontis* microfilariae (MF), (B) PMA, (C) and zymosan. Shown are box plots with 10-90 percentile. Data are from one out of two independent experiment with n= 4. Normal distribution was tested by Kolmogorov-Smirnov normality test. Statistical analysis was done by Kruskal-Wallis test followed by Dunn's post-hoc test for non-parametric data. $p<0.05$*, $p<0.01$**, $p<0.001$***.

These results suggest that the recognition of MF leading to the eosinophil ETosis does not occur via TLRs.

3.11 Microfilariae induce ETosis in bone marrow-derived eosinophils via the dectin-1 signaling pathway.

Since TLR signaling did not result in DNA release by eosinophils, the impact of MF-induced activation of C-type lectin receptors (CLRs) on ETosis was analyzed. CLRs are another type of PRRs, which bind carbohydrates (190). Thus, they are mainly involved in recognizing and adding innate immune responses to fungi (125). However, several publications indicate that helminths are recognized through CLRs as well (129, 134, 191). Furthermore, zymosan, a well-known ETosis inducer, is a cell wall preparation from *Saccharomyces cerevisiae* activating TLR-2 as well as the CLR dectin-1 (192). To distinguish, which component triggers the DNA release by eosinophils, depleted zymosan, which solely activates dectin-1, and peptidoglycan (PGN), which only binds TLR-2, were used to stimulate eosinophils. Quantification of the released DNA by eosinophils revealed that the active component of zymosan in inducing ETosis occurs via dectin-1 recognition since zymosan and depleted zymosan were both able to stimulate the release of DNA in equal amounts, while PGN failed to trigger DNA release (Figure 21 A). Furfurman, a dectin-2 agonist, only slightly increased DNA release by eosinophils, while trehalose-6,6-dibehenate (TDB), a mincle agonist, induced a significant increase in DNA release by eosinophils (Figure 21 A).

This suggests, that ETosis induction can be triggered through the recognition by certain CLR. To analyze if MF-induced ETosis occurs via the signaling of CLRs, depletion antibodies were used in eosinophil and MF co-cultures. Addition of dectin-1 and -2 depletion antibodies prevented eosinophils to release extracellular DNA in response to MF. Depletion with solely dectin-1 antibodies resulted in similar inhibition of the MF-induced ETosis, whereas dectin-2 and mincle depletion as well as the isotype controls failed to inhibit the DNA release triggered by MF (Figure 21 B).

Moreover, BmEos were derived from WT and dectin-2 knockout mice and stimulated with MF and zymosan. Figure 21 C shows that BmEos derived from dectin-2 KO mice present a higher baseline DNA release than WT BmEos (unstimulated eosinophils). Stimulation with zymosan significantly and with MF partly increased the DNA release by both eosinophil-types in a comparable manner compared to baseline level. MF motility inhibition furthermore, is independent on the dectin-2 receptor as MF show the same motility in BmEos cultures generated from WT and KO mice (Figure 21 D).

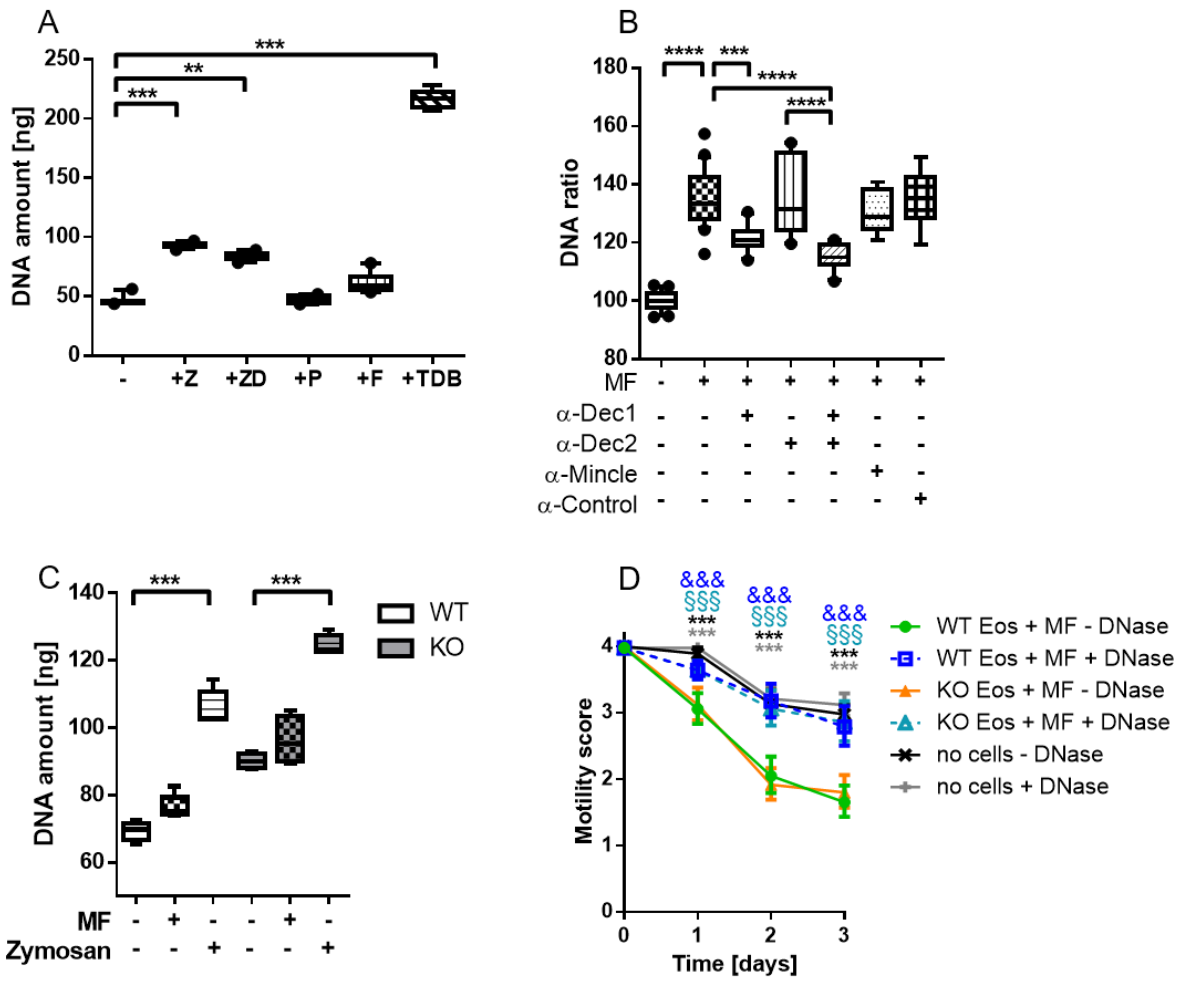

Figure 21. Microfilariae induce ETosis in bone marrow-derived eosinophils via the dectin-1 signaling pathway. Released DNA from bone marrow-derived eosinophils that were stimulated (A) for 48 h with zymosan (Z, dectin-1 and TLR-2 agonist), zymosan depleted (ZD, dectin-1 agonist), peptidoglycan (P, TLR-2 agonist), furfurman (F, dectin-2 agonist), trehalose-6,6-dibehenate (TDB, Mincle agonist) and (B) for 24 h with microfilariae (MF) in combination with dectin-1, -2 and mincle depleting antibodies or isotype control. C: DNA release after 24 h measured from bone marrow-derived eosinophils isolated from wild-type (WT) mice (white boxes) or dectin-2 knock-out mice (KO, grey boxes) stimulated with 5,000 MF or zymosan. D: MF motility co-cultured with wild-type (WT) eosinophils or eosinophils from dectin-2 KO mice in the presence or absence of DNase. A-C: Shown are box plots with 10-90 percentile or D: mean with SEM. Data shown in (A) are from one out of 3 independent experiments with n= 10, in (B) pooled data from two independent experiments with n= 15 (unstimulated and +MF), n= 10 (+ MF and antibodies), in (C) data from one independent experiment with n= 5 (WT) or n= 4 (KO) and in (D) data from one independent experiment with n= 52. A-C: Normal distribution was tested by Kolmogorov-Smirnov normality test. B: The ratio was calculated by dividing the DNA amount released by the stimulated cells by the mean DNA amount released by the unstimulated cells. Spearman's rank correlation test for heteroscedasticity was performed and only data failing the heteroscedasticity were pooled. A-C: Statistical analysis was done using 1-way ANOVA (comparing eosinophils alone with MF, eosinophils with MF with eosinophils with MF and antibodies, and the different stimuli) with Bonferroni post-hoc test for parametric data and D: 2-way ANOVA with Bonferroni post-hoc test comparing groups to WT Eos + MF – DNase (&= WT Eos + MF + DNase, $= KO Eos + MF – DNase, §= KO Eos + MF + DNase, *= no cells). $p<0.05$*, $p<0.01$**, $p<0.001$***.

However, the literature suggest that murine eosinophils do not express dectin-1 compared to human eosinophils (193). To investigate whether murine eosinophils are able to express dectin-1, flow cytometry was performed staining for dectin-1, MHCII and CD86. Indeed, BmEos expressed low levels of dectin-1 when unstimulated (Figure 22 D and G). However, the same is true for MHCII (Figure 22 E and H) as well as CD86 (Figure 22 F and I) expression. Stimulation of BmEos with PMA induced dectin-1, MHCII and CD86 expression by BmEos (Figure 22 D-I). MF and zymosan on the other hand did not increase dectin-1 expression on BmEos (Figure 22 G), while MHCII and CD86 expression is slightly but not significantly upregulated after MF and zymosan stimulation (Figure 22 H and I).

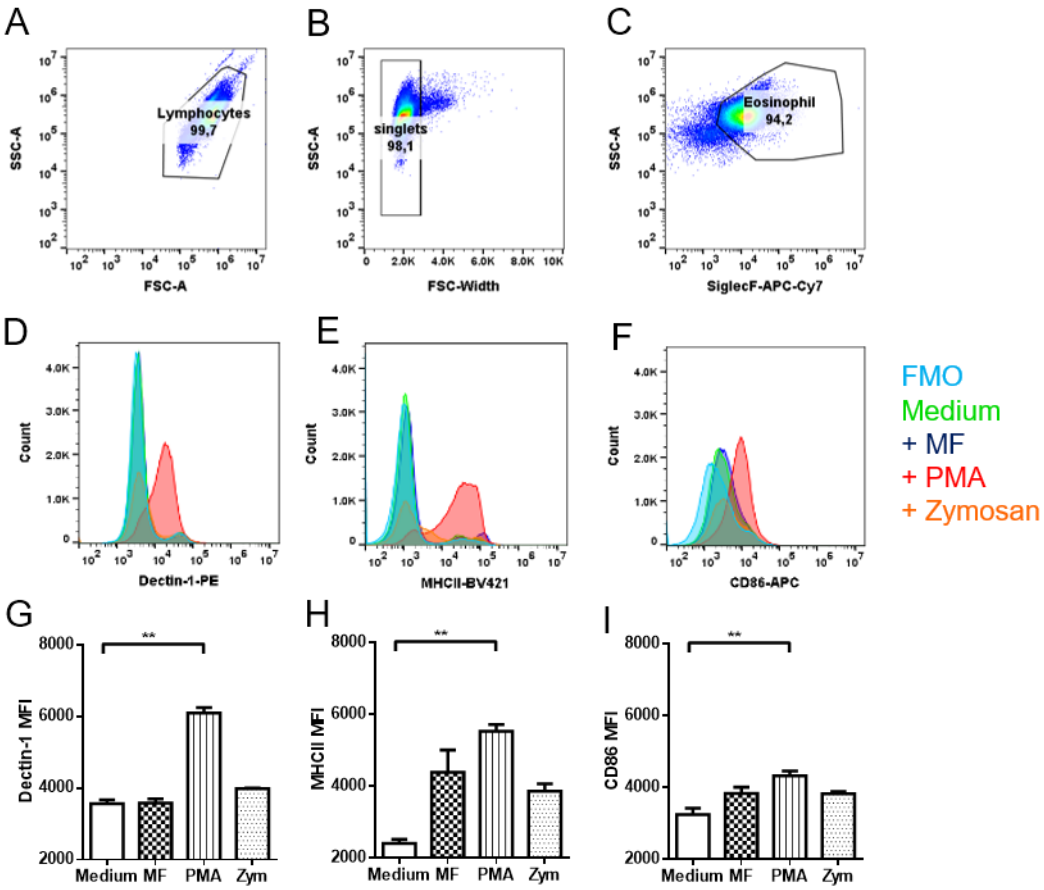

Figure 22. Murine eosinophils can upregulate their dectin-1 expression. Flow cytometry analysis of dectin-1, MHCII and CD86 expression on bone marrow-derived eosinophils. A-F: gating strategy for dectin-1, MHCII and CD86 expression of bone marrow-derived eosinophils without stimulation (medium) or after stimulation with 5,000 MF, 50 ng/ml PMA or 0.1 mg/ml zymosan for 48 h. Lymphocytes were gated (A), singlet cells chosen by SSC-A vs FSC-W characteristics (B), and SiglecF positive eosinophils determined (C). D-F: mean fluorescence intensity (MFI) of dectin-1 (D), MHCII (E) and CD86 (F) of eosinophils stimulated with MF (dark blue), PMA (red), zymosan (orange) and without stimulation (medium (green)). FMO for the respective antibody are shown in light blue. G-I: MFI of dectin-1 (G), MHCII (H) and CD86 (I) expression of eosinophils without stimulation, or stimulated with MF, PMA or zymosan for 48 h. Shown are mean with SEM. Data shown are from one out of two independent experiment with n= 3. Statistical analysis was done using Kruskal-Wallis with Dunn's post hoc test for non-parametric data. $p<0.01$**.

Thus, eosinophil ETosis can be triggered by the activation of dectin-1 and mincle receptor, while MF-induced ETosis occurs via the recognition of the dectin-1 receptor.

3.12 *Litomosoides sigmodontis* infection and microfilariae injection increases free DNA *in vivo*, which is partly mediated by eosinophils.

To investigate the *in vivo* impact of eosinophil ETosis in response to filarial infections and in particular the impact of eosinophil ETosis in response to MF, mice were infected with *L. sigmodontis*. Mice infected for 75 days with *L. sigmodontis* had significantly increased local DNA concentrations in the thoracic cavity, the site of adult worm residence, compared to age- and sex-matched naïve controls (Figure 23 A). Moreover, even though the DNA concentrations within the pleural cavity and the worm numbers did not correlate, it was still statistically significant (Figure 23 B).

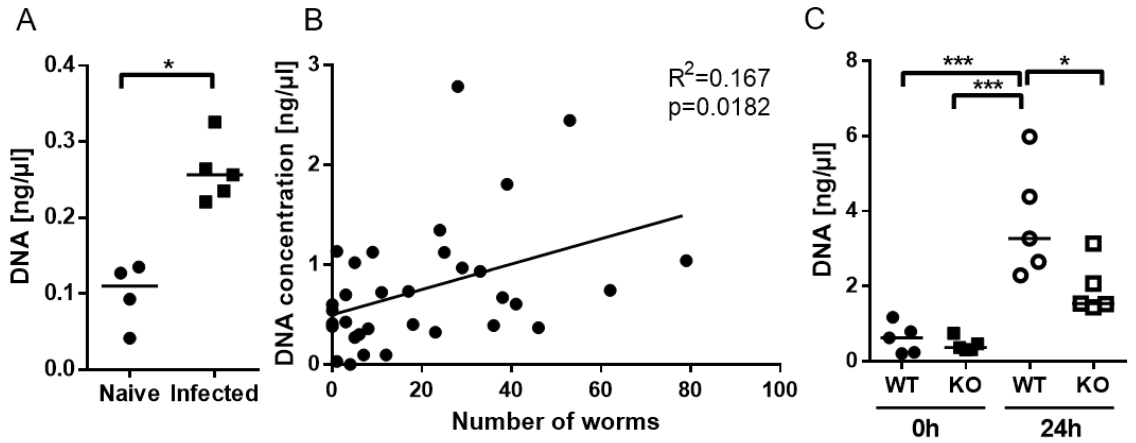

Figure 23. *Litomosoides sigmodontis* infection and microfilariae injection increases free DNA *in vivo*, which is partly mediated by eosinophils. A: Free DNA from the thoracic cavity lavage of naïve and *L. sigmodontis*-infected mice 75 days after infection (n= 4-5). C: Correlation of worm number and DNA concentration within the pleural cavity of infected animals. C: Free DNA in the peripheral blood of pre-patently *L. sigmodontis*-infected BALB/c wild-type (WT) and dblGATA (KO) mice before and 24 h after intravenous injection with microfilariae**.** Shown is the median (A and C). Data are from one out of 5 independent experiments (n= 4 (naïve) and n= 5 (infected) (A)/ pooled data from 3 independent experiments (n= 33) (B)/ 1 independent experiment (n= 5) (C). Normal distribution was tested by Kolmogorov-Smirnov normality test. Spearman's rank correlation test for heteroscedasticity was performed and only data failing the heteroscedasticity were pooled. Statistical analysis was done by Mann-Whitney test for non-parametric data (A), linear regression analysis (B) and 1-way ANOVA with Dunn's post-hoc test for parametric data (C).

Furthermore, WT and dblGATA mice, lacking eosinophils, were infected with *L. sigmodontis* for 41 days. At that time point, adult worms have developed and an eosinophilia has been established, but no MF are present yet. To analyze the systemic impact of MF on DNA release, MF were intravenously injected in these mice and DNA concentrations were measured in the plasma. 24 h after MF injection, WT and KO mice showed increased DNA concentrations within the plasma. However, the increase in

plasma DNA was significantly higher for WT animals than for eosinophil-deficient mice (Figure 23 C), indicating that MF trigger a systemic DNA release *in vivo,* which is partly mediated by eosinophils.

Taken together, this suggests that even though MF trigger the release of DNA by immune cells, including eosinophils, the actual *in vivo* relevance of DNA traps has still to be tested.

3.13 *Dirofilaria immitis* microfilariae trigger DNA release by eosinophils.

ETosis has been described for various immune cells including eosinophils, neutrophils and monocytes from different species in response to distinct pathogens suggesting that ETosis is a conserved mechanism among different species. This raises the question, if ETosis is species restricted or if immune cells can recognize and undergo ETosis in response to different foreign species. Thus, murine eosinophil ETosis was analyzed in response to MF of the canine heartworm *D. immitis*. Confocal microscopy shows that murine eosinophils attached to the canine MF and released extracellular DNA (Figure 24 A-E). This was confirmed by DNA quantification. BmEos released comparable amounts of DNA in response to *D. immitis* MF and *L. sigmodontis* MF (Figure 24 F). Furthermore, BmEos reduced *D. immitis* MF motility in a comparable manner than *L. sigmodontis* MF motility (Figure 24 G). In accordance, addition of DNase restored *D. immitis* MF motility again, indicating that BmEos entrap *D. immitis* MF in a DNA-trap-dependent manner as well (Figure 24 H).

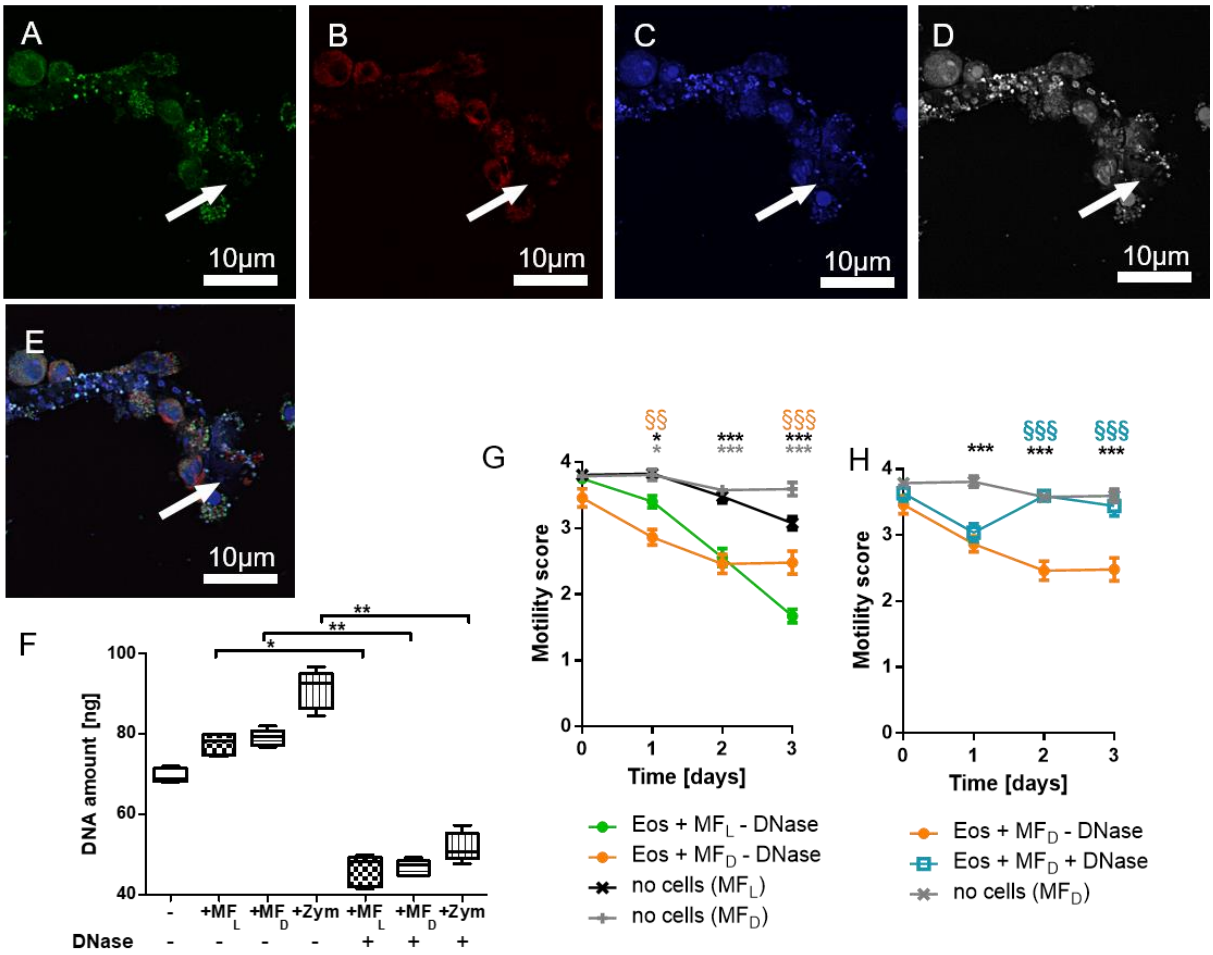

Figure 24. *Dirofilaria immitis* microfilariae trigger DNA release by eosinophils. Representative confocal microscopy pictures of bone marrow-derived eosinophils after *in vitro* culture with *D. immitis* MF stained for A: ECP (green), B: SiglecF (red), C+D: DAPI (blue (C) and white (D)). E shows the overlay. F: DNA quantification from supernatant of murine bone marrow-derived eosinophils stimulated with zymosan (Zym), microfilariae (MF) from *D. immitis* (MF_D) or *L. sigmodontis* (MF_L) (n=5) in the presence or absence of DNase. G+H: MF motility score of MF_D and MF_L cultured alone (no cells) or co-cultured with eosinophils in the absence (●) or presence (◘) of DNase. G: comparison of *L. sigmodontis* MF and *D. immitis* MF motility in the presence or absence of eosinophils. H: *D. immitis* MF motility cultured alone or co-cultured with eosinophils in the presence or absence of DNase. Shown is I: box plots with 10-90 percentile. G+H: mean with SEM. Representative Data from 1 out of 3 independent experiments with n=5 (A) and n=52 (B and C). Normal distribution was tested by Kolmogorov-Smirnov normality test. F: Data were analyzed by Kruskal-Wallis with Dunn's post-hoc test for non-parametric data and G+H: Statistical analysis was done using a 2-way ANOVA with Bonferroni post-hoc test (J: comparing groups to MF_L – DNase, K: comparing groups to Eos + MF_D -DNase (§= MF_D, *= no cells). p>0.05*, p>0.01**, p>0.001***.

Thus, eosinophil ETosis in response to MF is not species restricted and presents a universal mechanism enabling the recognition and defense of different species.

4. Discussion

The results of this thesis show for the first time that eosinophil ETosis occurs as a life-cycle stage-specific response against MF but not against the L3 larval stage of filariae. The release of nuclear and in particular mitochondrial DNA-traps contribute to a reduction in MF motility, while L3 larvae were only entrapped in the presence of plasma from infected animals. DNA released by eosinophils in response to MF was independent on shear forces and did not require prior priming or antibody-coating. Instead, the underlying signaling pathway of the MF-induced ETosis was identified to be dectin-1-dependent. Furthermore, the release of DNA by eosinophils in response to MF and the DNA-dependent inhibition of MF motility seems to be a conserved mechanism, since murine eosinophils responded to MF derived from the rodent filarial nematode *L. sigmodontis* and the canine heartworm *D. immitis*. Finally, *in vivo* studies showed that an *L. sigmodontis* infection caused an increase in local DNA concentration, while intravenous MF injection raised systemic DNA concentrations, which were partly mediated by eosinophils.

From murine studies, it is known, that neutrophils are an important cell type during the early phase of infection (194). A delayed neutrophil recruitment into the skin, enables more *L. sigmodontis* L3 larvae to migrate into the pleural cavity (161) and the majority of invading L3 larvae are killed through neutrophils when the humoral immune response is missing (162, 195). Furthermore, a depletion of neutrophils from granuloma results in a delayed adult worm killing (164, 165). Neutrophils also mediate protection against MF as it was shown for *Acanthocheilonema viteae*, *O. volvulus* and *B. malayi* MF (175, 196, 197).

However, the main effector cells against MF are eosinophils. For example, a lack of eosinophils results in increased MF and adult worm burden in *L. sigmodontis*-infected mice (170). However, in primary infections, IL-5 depletion has no impact on L3 larvae (198), while the overexpression of IL-5 causes eosinophils to target larvae starting 10 dpi, which is after the molting from L3 into L4 larvae, and they accumulate around young adult worms forming granulomas (169, 199). Thus, in primary infections, eosinophils appear to mediate protective immunity against adult worms and MF rather than against L3 larvae. However, during re-infection and vaccination eosinophils mediate protection against L3 larvae, which is dependent on antibodies (169, 195).

This suggests, that granulocytes serve different roles during a helminth infection with distinct effector mechanisms against the different life-cycle stages of the filariae. It

appears that neutrophils are important first line responders against invading L3 larvae but also against adult worms and MF, while eosinophil numbers expand during the course of infection and mainly contribute to the elimination of adult worms and MF.
Granulocytes present different effector mechanisms in fighting of pathogens. A newly recognized mechanism is the so called ETosis, where intracellular DNA, with the toxic granules and proteins trapped inside the DNA, is explosively released into the surrounding environment in response to different pathogens and stimulating factors (137). So far, there are several publications on neutrophil ETosis in response to various nematodes (151, 173-175), while eosinophil ETosis is less well studied.

4.1 Life-cycle stage-specific EETosis response

Within my thesis, the impact of the different larval stages of the filarial nematode *L. sigmodontis* on eosinophil and neutrophil ETosis was investigated. Scanning electron microscopy reveal that neutrophils released DNA in response to MF and L3 larvae of *L. sigmodontis*. Similar results for neutrophil ETosis have been reported for L3 larvae and MF of different filarial species as well. Thus, NETosis is observed for L3 larvae of *H. contortus*, *S. stercoralis* and *D. immitis in vitro* as well as for *Nippostrongylus brasiliensis* L3 larvae *in vivo* (151, 174, 200, 201). Furthermore, neutrophils were shown to undergo NETosis in response to MF of *D. immitis* and *B. malayi in vitro* (174, 175). With regard to the filariae, it is suggested that *Wolbachia* endosymbionts are responsible for the release of the DNA traps by neutrophils. Thus, *O. volvulus* nodules contain DNA traps, which are not observed in patients that were treated with the *Wolbachia*-targeting drug doxycycline. Since the DNA traps co-localize with neutrophil-derived products, the source of the extracellular DNA is most likely of neutrophil origin (24). Similar to the established roles of neutrophils and eosinophils against the different life-cycle stages of filariae, eosinophils released extracellular DNA only in response to *L. sigmodontis* MF but not viable L3 larvae as observed by the SEM, confocal microscopy and DNA quantification methods. SEM revealed that neutrophil traps in response to L3 larvae appeared more thread-like, while MF rather triggered the release of more diffuse structures. In a similar manner, human neutrophil traps in response to *H. contortus* L3 larvae are described as aggregated (clusters of NET structures), spread (elongated web-like structures) and diffuse (globular compact form), while especially the spread and aggregated structures lead to larval entrapment (151). On the other hand, results from this thesis show that most of the eosinophil DNA traps

appeared to be diffuse and more extensive, while in a few cases thread like structures were observed. Ueki *et al.* also described distinct differences between eosinophil and neutrophil traps, with human neutrophil traps appearing thinner and more globular and eosinophil traps more diffuse (154). Furthermore, for neutrophil ETosis it was demonstrated that different stimuli require different pathways (202). These results show that similar to human granulocytes, murine eosinophils and neutrophils have distinct differences in their DNA trap structures. Since neutrophils present different appearances of DNA-like structures in response to the different larval stage there may also be different pathways involved in inducing ETosis as well as functional differences of the distinct DNA structures ejected.

Furthermore, the results from this thesis show that the DNA released by eosinophils in response to MF was dependent on the MF number and independent on shear forces since heat-treated MF also induced EETosis. Furthermore, also antigen prepared from MF induced DNA release, suggesting that antigens present on the MF are responsible for the recognition by the BmEos and trigger to release the DNA.

L3 larvae on the other hand did not induce DNA release by eosinophils (Figure 9 C), which raises the question if L3 larvae are either not recognized by eosinophils or L3 larvae may suppress eosinophil-mediated effector mechanisms. Such a suppressive mechanism may be the release of DNase, which is known for L3 larvae and adult worms of *Trichinella pseudospiralis* that secret DNase II (203) and which may contribute to the degradation of DNA traps and thus prevents the detection of extracellular DNA in co-cultures. Another possible mechanism of immune modulation can occur through the secretion of excretory-secretory (ES) products by nematodes. ES products from *Fasciola hepatica* can for example induce apoptosis of eosinophils and thereby limit eosinophil-mediated killing (204). The filarial products abundant larval transcript-1 (ALT-1) and -2, which are able to inhibit macrophage function *in vitro,* are highly expressed by the infective larval stage of *B. malayi* and may also have an immunomodulatory impact on other immune cells like eosinophils (205). Further support of the immunomodulatory influence of L3 larvae is given by the decreased DNA release by eosinophils after L3Ag stimulation (Figure 10 B). The immunomodulatory molecules secreted by the larvae may be contained in the antigen preparation, which could inhibit eosinophil ETosis. The results from this thesis show, that even though viable L3 larvae of *L. sigmodontis* failed to trigger EETosis, heat-treated L3 larvae were able to stimulate DNA release. Thus, heat-treated, dead L3

larvae may not be able to release immunomodulatory products, which enables eosinophil function and the release of DNA traps or prevents DNA trap-degradation. Another possibility is the release of danger molecules by the dying larvae, which could activate immune cells. In *O. volvulus* patients it is for example shown that DEC treatment, which kills MF, causes enhanced dermal infiltration of eosinophil and neutrophils, which attack the MF (206). The same could be true for damaged L3 larvae, which may mediate enhanced eosinophil ETosis. This is also supported by the results of the eosinophils, MF and supernatant co-culture experiment, where eosinophils were stimulated in the presence or absence of MF in combination with different supernatants collected from MF, heat-treated MF, L3 larvae and heat-treated L3 larvae (Figure 10 E and F). Addition of supernatant from heat-treated L3 larvae to the eosinophils in combination with MF did not only raise the DNA release compared to eosinophil-MF cultures lacking the supernatant, but also showed the highest increase in DNA release by eosinophils compared to the other supernatants and MF-stimulated eosinophils. The release of these molecules may give an additional activation signal to the eosinophils, which enhances EETosis induction. Interestingly, supernatant from viable L3 larvae did not prevent MF-induced EETosis. Thus, immunomodulation by viable L3 larvae may require the encountering of the immune cells. Since the supernatant from the viable L3 larvae was collected in the absence of cells, immunomodulatory molecules may not have been secreted. Another possibility could be that the MF stimulus is larger than the suppressive effect of the L3 larval supernatant. Comparable to the supernatant from viable and heat-treated MF, supernatant from dying L3 larvae may serve as an additional activation for the eosinophils to undergo ETosis when encountering MF. Similar findings have been observed for human neutrophils and eosinophils as well. For example it has been reported that human neutrophils release mitochondrial DNA in response to LPS or C5a when primed with GM-CSF (153), while IL-5 or GM-CSF can enhance PAF-induced human eosinophil ETosis (137).

Thus, viable L3 larvae may actively secrete compounds that suppress eosinophil ETosis or degrade DNA traps when encountering eosinophils. These molecules may also be present in the antigen preparation, which reduces the release of eosinophil DNA traps. In the absence of immune cells, no secretion of these molecules occurs and thus, secreted compounds rather serve as an additional stimulus for MF-induced EETosis. Heat-treated, dead L3 larvae on the other hand may secrete danger molecules, enhancing eosinophil ETosis, which enables DNA release by eosinophils

in response to the heat-treated L3 larvae themselves or further activate EETosis during MF-stimulation. Similar, released compounds from viable and heat-treated MF may also function as additional priming molecules for MF-induced EETosis.

Another explanation for the lack of DNA release by eosinophils in response to viable L3 larvae could be due to the fact that eosinophils do not recognize L3 larvae. Immunomodulation or the secretion of DNases by L3 larvae would also impact the DNA traps of neutrophils, which was not seen in the SEM pictures. Thus, since neutrophils are able to react and release DNA in response to L3 larvae, eosinophils may only be restricted in the recognition of adult worms and MF. Therefore, the identification of the recognition receptors involved in L3 larval sensing by neutrophils could explain the differences in the life-cycle stage-specific response observed for eosinophils and neutrophils.

So far, only one publication analyzed the impact of EETosis on helminths. Muñoz-Caro *et al.* identified that human neutrophils and eosinophils produce extracellular DNA traps in response to *H. contortus* L3 larvae and that these traps contribute to larval entrapment (151). The results of this paper are in contrast to the murine eosinophil results observed in this thesis, where eosinophils were only able to cast DNA traps in response to dead L3 larvae. However, the eosinophils used for the *H. contortus* co-culture did not exceed a purity of 30 % and thus contaminations with other cell types such as neutrophils could contribute to the observed results, which restrains the conclusions from the *H. contortus* study.

Thus, the results show that the parasite life-cycle stage-specific response of neutrophils and eosinophils towards MF and L3 larvae is also reflected in the cells ETosis behavior.

4.2 DNA trap origin

The origin of the extracellular DNA during ETosis is controversially discussed. Yousefi *et al.* supports the theory of mitochondrial DNA that is released from viable cells (141, 153), while Ueki *et al.* states the release of nuclear, histone-bearing DNA (137, 154). The results of this thesis show the release of nuclear and primarily mitochondrial DNA by BmEos. Confocal microscopy supports the presence of nuclear DNA, since in most cases the nucleus appeared disintegrated and a loss of the nuclear shape was evident. The discrepancy observed for the results of this thesis and the published data could be of multiple reasons. The ejection of only mitochondrial DNA has several challenges,

since eosinophils possess relatively low amounts of mitochondrial DNA and the entrapment of large, multicellular parasites by such low amounts appears negligible, which makes it unlikely that the release of mitochondrial DNA alone may be an effective mechanism in fighting multicellular pathogens in general and filariae in particular (138). Ueki *et al.* on the other hand claims that eosinophils only release nuclear DNA. However, the statement relies solely on the detection of histone proteins trapped in the DNA traps through confocal microscopy, while the analysis for the presence or absence of mitochondrial DNA is missing (137, 154). A further argument, pointed out by several authors, is the existence of the different DNA types as a result of different stimuli with parasites forcing the release of nuclear DNA, while bacteria induce mitochondrial DNA release. Indeed, the release of mitochondrial DNA is often associated with LPS and bacteria from eosinophils and neutrophils as well as CpG (141, 153, 207), which is a motif that is particularly high in bacterial DNA (208), while nuclear DNA release is frequently observed in response to parasites. For example *T. gondii* and *Neospora caninum* have been reported to induce nuclear DNA release by neutrophils (209), *Aspergillus fumigatus* causes eosinophils to eject nuclear DNA (210) and *Listeria monocytogenes* triggers mast cell trap formation, which are of nuclear origin as well (211). However, the results from this thesis show the ejection of mitochondrial and nuclear DNA not only in response to MF of *L. sigmodontis,* but also in response to *E. coli*, querying the suggested theory. A possible explanation could be that *Wolbachia* and bacteria trigger the release of mitochondrial and the filariae itself the ejection of nuclear DNA. However, the release of nuclear DNA by neutrophils is also reported for *S. aureus* (146). In fact several additional publications identified the release of mitochondrial and nuclear DNA from different cell types in response to diverse stimuli. For example, mitochondrial and nuclear DNA is released from neutrophils in response to PMA-mediated NETosis (212), while stimulation with *E. coli*, *C. albicans*, *Mycobacteriam massiliense* and *Entamoeba histolytica* causes the release of mitochondrial and nuclear DNA from macrophages (213-215). Thus, the identification of the exact origin of the DNA traps, the implication of the stimuli used as well as the underlying mechanism, which may impact the release of different DNA types has still to be further investigated. Despite the discrepancy of the origin of the DNA traps, the results of this thesis suggests that ETosis appears to be an universal defense mechanism against different pathogens.

4.3 Microfilariae entrapment by EETosis and ETosis function

To analyze the functional role of the DNA traps produced in response to MF, MF motility was analyzed. BmEos significantly inhibited MF motility over time, which was abolished in the presence of DNase. This suggests, that the motility reduction relies on the DNA traps. The question remains how the entrapment is accomplished. Ueki *et al.* could identify non-electrostatic interactions as a mechanism to entrap particles by the DNA (154). Additional experiments would be required to analyze if MF are trapped through this interaction as well.

Flow cytometry analysis of eosinophil cultures revealed that MF increased the number of Annexin V positive and reduced the number of Annexin V and PI double negative eosinophils compared to eosinophils without MF (Figure 13 A). Annexin V single staining can be an indication for increased cytolysis, with ETosis being a possible cytolytic mechanism (216). Thus, the results could indicate that MF increase cytolysis and thus may increase ETosis in eosinophils. However, already published results using confocal microscopy show that human eosinophil ETosis results in only low Annexin V binding (137). Therefore, an increase in Annexin V single positive cells with no increase in PI staining after MF incubation as observed by the results of this thesis could indicate an increase in apoptosis rather than ETosis. The increase in apoptosis could be a result of apoptosis induction by the MF as it was shown for ES products from *Fasciola hepatica* (204, 217). However, since flow cytometry instead of confocal microscopy was done, eosinophils undergoing ETosis may be lost due to the adhesive nature of the DNA traps, which stick to the plastic well. Furthermore, cells undergoing ETosis will lose their shape and thus may be detected as debris during flow cytometry. Distinguishing MF-induced eosinophil ETosis from apoptosis and necrosis will therefore require confocal microscopy analysis as well, which would allow the comparison of MF-induced cell death with Fas-induced apoptosis and heat-induced necrosis.

Interestingly, MF show higher staining for Annexin V and PI when co-cultured with eosinophils compared to MF cultured alone, suggesting eosinophil-mediated killing of the MF. However, no differences between MF cultured with eosinophils in the presence or absence of DNase was observed. This, suggests that eosinophil DNA traps mediate the entrapment of MF, which is further indicated by the reduced motility in the absence of DNase, but not the killing of MF. Similar, McCoy *et al.* showed that *B. malayi* MF mediate NETosis, but the killing of the MF is independent of the DNA-traps (175).

4.4 *Ex vivo* vs *in vivo* generated eosinophils

Since BmEos were generated *ex vivo*, it was tested whether they mimic eosinophils that were generated and primed *in vivo*. Therefore, eosinophils isolated from the pleura of infected animals and from the gut of infected and naïve animals were co-cultured with MF and the motility was assessed. Eosinophils isolated from the pleura and gut of animals were much more potent in reducing MF motility compared to BmEos suggesting a more naïve state of the *ex vivo* generated eosinophils. However, priming of the eosinophils through antigens appears not to be required for the enhanced MF motility reduction, since eosinophils isolated from the pleura of infected animals, which are in direct contact with the infection, reduced the MF motility in a comparable manner than eosinophils isolated from the gut of infected and naïve animals. Thus, priming of gut eosinophils through circulating antigens in the infected animals could be excluded as well. However, released DNA detected by the *in vivo* generated eosinophils did not increase upon stimulation with MF compared to gut and pleura eosinophils without stimulation. Only pleura eosinophils appeared to release more DNA in response to MF compared to unstimulated control pleura eosinophils, but this increase did not reach statistical significance. Several points could explain the lack of increased DNA release from *in vivo*-generated eosinophils in comparison to BmEos. Less numbers of cells were used for the pleura and gut eosinophil-MF co-cultures compared to the BmEos stimulation and thus, less DNA is ejected. Furthermore, DNA degradation by DNases present in the FBS used for cell culturing may prevent the detection of the scattered extracellular DNA.

Binding of antibodies through Fc receptors is a common mechanism of eosinophils enabling them to respond quickly to pathogens through antibody-dependent cellular cytotoxicity (ADCC) (119). Compared to BmEos, eosinophils isolated from infected and naïve animals are coated by antibodies. To test, if antibody-coating enhances the ETosis capacity, eosinophils were isolated from the pleura and gut from WT animals as well as from µMT mice, which lack B cells and thus a humoral immunity. Therefore, eosinophils derived from the µMT mice lack antibody-coating. However, motility assays showed no difference in the DNA-trap-dependent motility reduction of WT eosinophils compared to µMT eosinophils isolated from the gut and pleura. Thus, the trapping of the MF through ETosis is independent of antibody-coating. Interestingly, *in vivo* eosinophil- and neutrophil-mediated *O. volvulus* MF killing was shown to be dependent on IgG binding to the MF (197).

Therefore, ETosis may occur as a fast, intrinsic effect with no additional components required and be responsible for entrapment of the larvae, while other mechanisms like ADCC and the contribution of other immune cells account for the larval killing.

4.5 Blood components and EETosis

In this regard, the dependency of serum and other blood factors was investigated. The results of this thesis show that erythrocytes and whole blood (treated with EDTA) did not induce ETosis in eosinophils. The same was true for serum, while plasma triggered DNA release by eosinophils. This ETosis-induction was partly dependent on heat-labile components since heat-treatment of the plasma slightly reduced the DNA release by eosinophils compared to untreated plasma. However, complement factors seem not to be responsible for the drop in DNA release upon heat-treatment, since compstatin, which is an inhibitor of the C3 convertase, did not prevent the ETosis-induction by plasma. These results suggest that plasma factors are able to trigger EETosis, which is prevented by coagulation factors present in serum, but also by whole blood. Thus, multiple factors present in the blood may prevent spontaneous EETosis induction within blood vessels. For human eosinophils, it was shown that immobilized IgG and IgA can induce ETosis and may also be responsible for the plasma-triggered ETosis-induction by BmEos (137). This shows, that even though MF-induced ETosis occurs through an antibody-independent mechanism, Ig crosslinking can trigger ETosis suggesting that separate mechanisms can be responsible for provoking ETosis.

Several results dealing with blood components and ETosis are already published. As mentioned before, Ueki *et al.* identified that the binding of pathogens to the DNA traps most likely occurs through hydrophobic interactions. Experiments using sulfate beads confirmed however, that addition of serum factors like BSA or serum itself (FBS), which inhibit hydrophobic interactions, prevent the binding to DNA traps (154). Moreover, serum contains DNases, which could contribute to the destruction of the DNA traps (218). Thus, serum and blood components may not only inhibit ETosis-induction, but may also prevent the binding to the DNA traps and its destruction, which provides an additional safety mechanism in thrombus formation. In contrast, for neutrophils it was shown that platelets aggregate with NETs supporting thrombus formation (219). Thus, additional experiments have to be performed to identify the plasma components, which are responsible for the ETosis induction, and analyze the impact of the blood components like serum, whole blood, erythrocytes and platelets on ETosis that has

been induced through additional stimuli. Of note, EDTA, which was used to obtain the plasma, does not impact the binding of the particles to the DNA traps (154). However, the effect of EDTA on the ETosis induction has still to be determined to exclude that EDTA may be responsible for the DNA release by eosinophils.

Interestingly, even though plasma induced ETosis, the addition of plasma to eosinophil and MF co-cultures enhanced MF motility. Thus, plasma may indeed interfere with the binding capacity of the DNA traps to the MF or DNases enhances the destruction of the DNA traps. The enhanced motility is independent on antibodies since plasma from µMT mice supported MF motility in a comparable manner than plasma from WT mice. Moreover, MF motility in cultures with plasma alone was even higher than in MF cultures without plasma, suggesting that plasma factors support MF survival.

Of note, all experiments conducted for this thesis had to be performed in the presence of FBS, to prevent eosinophil-apoptosis. However, serum DNase may reduce released DNA concentrations while serum factors could diminish ETosis induction and thus may explain the reduced EETosis capacity compared to the NETosis described in the literature, where no FBS could be used (181).

Thus, some blood components may be ETosis inducers, while several simultaneous safety mechanisms within the blood prevent spontaneous and excessive ETosis. Furthermore, plasma factors may support MF survival.

4.6 L3 larval entrapment by EETosis

L3 larvae were not entrapped by eosinophils (Figure 15 B) supporting the theory of a life-cycle stage-specific response by eosinophils and neutrophils. This was regardless if the L3 larvae were isolated from the pleural cavity of infected jirds or directly from infected mites. Thus, modulation of L3 larvae through antibody- or complement-coating, which may occur during larval migration within the jird (L3 larvae isolated from pleura), has no impact on eosinophil-mediated entrapment. In contrast to the results observed for the eosinophil and MF co-culture, addition of plasma from infected animals to the eosinophil and L3 larvae co-culture enhanced the L3 motility reduction. Plasma from naïve animals on the other hand had no impact on L3 motility reduction. Thus, plasma components produced during the infection enhanced eosinophil-mediated larval entrapment. Several studies demonstrate that eosinophils mainly mediate protective immunity against adult worms and MF during a primary infection rather than against L3 larvae. However, re-infection and vaccination studies show that

eosinophils are important effector cells against L3 larvae during secondary infections and following vaccination, which was antibody-dependent (169, 195). This is also reflected in the observed results of this thesis. Under naïve conditions, eosinophils were unable to entrap L3 larvae, while plasma from infected animals enabled eosinophils to respond to L3 larvae. This is not achieved through complement factors, since heat-treatment of the plasma had no impact on larval entrapment. The enhanced activity is rather mediated by specific antibodies since plasma from infected µMT mice, which lack antibodies, did not support larval motility reduction by eosinophils and was comparable to the results observed for the plasma from naïve animals. Interestingly, addition of plasma from infected as well as naïve animals to eosinophil and L3 larval co-cultures raised DNA release by eosinophils, which was even higher than the DNA release observed for eosinophils stimulated by plasma alone. Furthermore, the motility reduction of L3 larvae by eosinophils in the presence of WT plasma from infected animals was only partly dependent on DNA traps, since addition of DNase restored larval motility to only some extent. Thus, even though DNA is released by the eosinophils in the presence of plasma, the binding of the larvae by the DNA traps is probably prevented through additional factors present in the plasma as it was observed for eosinophil-MF co-cultures in the presence of plasma.

Therefore, the eosinophil-mediated L3 larval entrapment during re-infection rather relies on multiple mechanisms with the humoral immunity of particular and the DNA traps of minor importance. Moreover, since L3 larvae motility reduction was partly dependent on DNA traps, while MF motility is enhanced after the addition of plasma, inhibition of larval binding through plasma components may be life-cycle stage-specific, suggesting that different binding mechanisms to the DNA traps may be in play.

4.7 Adult worm entrapment by EETosis

Finally, the impact of EETosis on adult worms was analyzed. The results show that antigen preparation of adult worms induced EETosis only after 48 h of stimulation. The adult worms used to generate the crude extract were derived from patent infected animals and thus contained MF, which may be responsible for the EETosis induction. The delayed ETosis response could be a result of a smaller MF/ adult worm ratio within the crude extract. For viable adult worms only female adult worms, but not male adult worms triggered EETosis. Heat-treatment of the female worms abolished the DNA release by eosinophils. Thus, heat-treatment may prevent the release of MF from the

female uteri, which could be responsible for the DNA release by the eosinophils. To further analyze this, additional experiments with female worms from pre-patent animals are required. Furthermore, shear forces of the large female worms may have induced cytolysis of the eosinophils with leakage of DNA into the surrounding, which would also explain why the smaller adult male worms did not induce DNA release by eosinophils. Another possibility is the release of ES products by the female worms, which triggers the DNA release, while heat-treatment kills the adult filariae and thus blocks ES release.

Even though eosinophils are known to mediate protective immunity against adult filariae, in co-cultures they did not impact adult worm motility (199). However, addition of plasma inhibited the motility in a DNA-trap-dependent manner. Thus, even though ETosis against adult female worms or their released MF occurs as an intrinsic effect, additional plasma components are required to enable eosinophils to harm adult worms. Interestingly, plasma from naïve animals caused enhanced motility reduction compared to plasma from infected animals, suggesting that the plasma from infected animals may contain immunomodulatory components suppressing eosinophil-mediated trapping.

In the *L. sigmodontis*-mouse model it was shown that especially neutrophils mediate adult worm killing. IL-5 neutralization prevents the accumulation of eosinophils and neutrophils at the site of infection, with neutrophils and not eosinophils being the important cell type in mediating adult worm killing (164). This study showed that eosinophils are rather essential to support macrophage-mediated cyto- and chemokine production, which results in neutrophil accumulation and inflammatory granuloma formation (164). Thus, eosinophils alone may not directly be responsible for adult worm killing but rather support neutrophils indirectly during this task.

4.8 Microfilariae-induced EETosis pathway

An important question that was addressed in this thesis targeted the pathway involved in inducing ETosis. Several publications state that some cases of ETosis-induction in human cells are dependent on NADPH oxidase. Unpublished results from our group confirmed this for the MF-induced ETosis in human eosinophils (220), but the inhibition of the NADPH oxidase through diphenyleneiodonium (DPI) in murine eosinophils resulted in cell death. Therefore, the impact of the NADPH oxidase pathway involved in MF-induced ETosis by murine eosinophils could not be validated. Furthermore, in

some cases of neutrophil ETosis, NE and MPO seem to be supporting the release of nuclear DNA (159, 221). These molecules are mainly found in the granules of neutrophils and thus it is questionable if they contribute to eosinophil ETosis as well. Furthermore, PAD4 was proven to be important during neutrophil ETosis by contributing to histone modification and thus supporting chromatin decondensation (160). PAD4 is mainly found in macrophages and neutrophils, but is also expressed by eosinophils. Thus, its contribution to eosinophil ETosis has still to be determined (222). Despite these findings, the pathway involving the actual recognition of the ETosis-inducing stimuli remains less well understood. Eosinophils and neutrophils are equipped with FcRs, TLRs and CLRs, which enables them to respond quickly to invading pathogens (86, 119, 121). A link between the TLR recognition and NETosis was shown for the recognition of *Wolbachia* from *O. volvulus* via TLR-2 and TLR-6 (24). The findings of this thesis suggests that BmEos are not triggered through TLR-7/8 and -9 to release extracellular DNA. Even though the TLR-9 agonist CpG caused a significant increase in extracellular DNA by BmEos, the measured DNA is rather related to unspecific detection of the CpG motifs by the DNA quantification assay and thus needs further analysis. Interestingly, human leukocytes release DNA traps in response to CpG and non-CpG oligodeoxynucleotides of class C, which was analyzed through fluorescence microscopy rather than quantitative DNA measurement (207). Thus, CpG may still be able to trigger DNA release by BmEos, which could be superimposed by the false positive CpG signal detected. Additional experiments using confocal fluorescence microscopy are required to further investigate the impact of TLR-9 activation and EETosis induction. Since TLR-1/2, -3 as well as TLR-4 stimulation showed only a slight increase in DNA release by BmEos, the question arises how *E. coli* bacteria stimulated the EETosis induction. Commonly *E. coli* is recognized through LPS-induced TLR-4 signaling and for neutrophils it was shown that LPS triggers NETosis (181). However, the NETosis-induction was triggered by 5 µg/ml LPS while for this thesis 100 ng/ml LPS was used. Thus, the low DNA release upon LPS stimulation seen in the results could depend on the lower LPS concentration used. The induction of the different eosinophil effector mechanisms, like ETosis and phagocytosis, may depend on the conditions present. Therefore, low concentrations of LPS and bacteria may trigger phagocytosis while high concentrations may favor ETosis. Another explanation for the discrepancy of EETosis-induction by LPS *and E. coli*-stimulation could result from the fact, that eosinophil ETosis is only triggered by

the whole bacteria, which may be recognized through the NOD receptor, the mannose receptor or dectin-2 (223-225). Furthermore, *E. coli*-induced EETosis, similar as for *Wolbachia* bacteria, may also be triggered through TLR-6 recognition (21).

The results showed that MF still trigger DNA release in BmEos isolated from TLR-2, -4 and MyD88 knockout mice (Figure 19 and 20 A). Therefore, either the MF-induced ETosis-signaling occurs through different pathways or additional pathways may compensate for the lack of these receptors. Thus, other important PRR classes were investigated for triggering EETosis in general and MF-induced EETosis in particular. Zymosan is a potent inducer of ETosis and it stimulates through the dectin-1 and TLR-2 receptor (226). To extrapolate the part responsible for the ETosis induction, depleted zymosan, which only triggers the dectin-1 receptor, and PGN, which activates the TLR-2 receptor, were used to stimulate BmEos. Similar to the results observed for Pam3Cys, which activates TLR-1 and TLR-2, PGN did not induce DNA release by eosinophils. Depleted zymosan on the other hand, resulted in a similar DNA release by eosinophils as zymosan. Therefore, the ETosis-inducing capacity by zymosan seems to be specific for dectin-1 stimulation. Since zymosan is a potent inducer of ETosis, other CLR were explored for their ETosis-inducing potential. Furfurman, which is an agonist for the dectin-2 receptor, only increased DNA release by BmEos by trend, while TDB, a mincle agonist, significantly raised DNA ejection from BmEos. These results indicate that CLR recognition may be an important signaling pathway in inducing ETosis. To explore if MF-induced ETosis is triggered through CLR, depletion antibodies were used in co-cultures. Dectin-1 and -2 double depletion inhibited the MF-induced DNA release, which was due to the dectin-1 depletion since blocking of only dectin-1 and not dectin-2 prevented the DNA release as well. This suggests that the recognition of MF and the induction of the MF-triggered ETosis occurs through the dectin-1 receptor. In accordance, BmEos generated from dectin-2 KO mice were still able to release DNA in response to MF, even though the increase in DNA release was smaller compared to the release by WT BmEos. Furthermore, MF motility inhibition by BmEos derived from dectin-2 KO mice was comparable to the inhibition by BmEos from WT mice supporting the conclusion that the dectin-1 receptor is involved in the recognition and subsequent ETosis-induction in response to MF. Interestingly, it was shown for *L. sigmodontis* infection in mice that eosinophils attach to adult worms via carbohydrates (227). Thus, also MF may be bound by carbohydrates present on the surface of the filariae via the dectin-1 receptor triggering the DNA release. In contrast,

for neutrophils it was shown that *C. albicans*-induced DNA traps are independent of dectin-1 and ROS, but rather dependent on the recognition by CR3 (CD11b/CD18) (228), which could indicate that the recognition of pathogens and the subsequent casting of DNA traps can occur through multiple receptors. On the downside, the literature states that murine eosinophils do not express dectin-1 compared to human eosinophils (193), which are known to present dectin-2 and dectin-1 expression (229). Indeed, flow cytometric analysis of BmEos of this thesis confirmed a low dectin-1 expression at resting state. However, stimulation using PMA caused the upregulation of this receptor suggesting that murine eosinophils show a low constitutively expression of dectin-1, which can be upregulated upon activation. On the other hand, MF stimulation did not increase dectin-1 expression on BmEos, which could either suggest that the low expression of dectin-1 is sufficient for MF recognition or other receptors may be involved in the recognition as well. In case of NETosis it was shown that *Wolbachia* bacteria from *O. volvulus* adult worms induce the release of DNA traps through TLR-2 and TLR-6 recognition. Since the lack of MyD88, which is a downstream signaling molecule of TLR-2 and TLR-6, had no impact on the MF-induced EETosis, *Wolbachia* from MF may not be involved in EETosis or are recognized by other receptors that compensate the lack of MyD88.

Thus, the results show that CLR recognition is an important mechanism in inducing EETosis and the release of DNA traps triggered by MF occurs through the decin-1 receptor. However, the impact of further receptors involved in MF recognition and MF-triggered EETosis has still to be investigated.

4.9 *In vivo* ETosis

Moreover, the *in vivo* relevance of DNA traps and in particular of eosinophil DNA traps was analyzed within this thesis. The infection caused a significant increase in local DNA release as evident by the increased pleural DNA. However, several cell types including neutrophils (151), mast cells (140), macrophages (140) and lymphocytes (207) are able to undergo ETosis and since the infection causes an increase in several cell types (170, 176), the raised pleural DNA may be a result of extracellular DNA released by various cell types within the pleural cavity. To confirm that the DNA release is caused by eosinophils and to analyze the impact of MF on EETosis, dblGATA and WT mice were infected for 41 days. At that time point, adult worms and profound eosinophilia are already present, while MF have not been produced yet. Intravenous

MF injection resulted in a significant systemic increase in extracellular DNA in dblGATA and WT mice. However, the DNA increase in WT mice was significantly higher than for dblGATA mice, which lack eosinophils. Thus, the DNA release in response to MF is partly produced by eosinophils, while other can cells contribute to the increased DNA as well. The release of extracellular DNA in response to different pathogens has been shown in other *in vivo* studies indicating the functional attribution of the DNA traps *in vivo* as well. Murine neutrophils produce extracellular traps after administration of *T. gondii*, *Naegleria fowleri* and *A. fumigatus*, which was analyzed through histological staining of lung tissue (230-232). In septic mice, NETs and platelets promote intravascular coagulation and NET depletion results in a decreased survival (233, 234). However, the depletion of DNA occurred rather unspecific through DNase treatment and no direct link between the DNA traps and neutrophils were provided *in vivo* (234). Thus, DNA traps from other cells such as eosinophils may be depleted as well contributing to the reduced survival of these mice. The importance of the extracellular DNA-trap-formation *in vivo* is also depicted by the nuclease expression of *S. aureus*, which mediates the escape of neutrophil extracellular DNA traps *in vivo* (235). *In vivo* ETosis was not only shown for murine cells, but also for chicken immune cells, where heterophils, which are immune cells comparable to mammalian neutrophils (236), produce DNA traps in response to *Salmonella* species (237). In human disease settings NETs are observed in *O. volvulus* nodules in response to *Wolbachia* bacteria (24). Thus, ETosis is not only an important mechanism contributing to the control and elimination of pathogens, but is also a conserved mechanism, which is mediated by different cell types of the innate immune system as well as by different species.

4.10 ETosis as a conserved mechanism between species

To further investigate the versatile nature of ETosis, ETosis was analyzed across species. Therefore, murine eosinophils were co-cultured with canine MF of *D. immitis*. For canine neutrophils it was already shown that they produce extracellular traps in response to MF and L3 larvae of *D. immitis* (174). However, murine eosinophils were also able to release extracellular DNA in response to *D. immitis* MF and inhibit MF motility in a DNA-trap-dependent manner. Furthermore, massive attachment of eosinophils to *D. immitis* MF was observed during confocal microscopy. Thus, similar patterns from *L. sigmodontis* and *D. immitis* MF may be recognized by the eosinophils leading to the ETosis. Further investigations have to be done to analyze if *D. immitis*

MF-induced ETosis is mediated via the dectin-1 receptor as well. Nevertheless, the current results prove that ETosis can occur as an inter-species response (238).

4.11 Summary

Similar to neutrophils, eosinophils undergo a form of cell death by releasing extracellular DNA traps upon activation, in which intracellular DNA is explosively released. Despite the fact that eosinophils are critical mediators of protective immune responses during filarial infection, it is not known if and how EETosis operates during filarial infection. As eosinophils exert differential effects on each life-cycle stage of filarial infection, mediating the killing of adult worms and MF, but not infective L3 larvae during primary infection, we investigated EETosis in response to different life-cycle stages of the rodent filarial nematode *L. sigmodontis.* Using scanning electron and confocal microscopy as well as fluorescence DNA quantification, we demonstrate that *L. sigmodontis* MF, but not L3 larvae, trigger eosinophil DNA release. Only dead L3 larvae triggered eosinophils to release DNA, suggesting that viable L3 larvae may actively inhibit ETosis. L3 larval entrapment by eosinophils was also accomplished in the presence of plasma from infected animals, which was partly dependent on DNA traps, reflecting the previously shown importance of eosinophils against L3 larvae during re-infection and after vaccination rather than primary infections. Adult filariae were trapped in a DNA-trap dependent manner in the presence of plasma and in particular in the presence of plasma from naïve animals, which could indicate the secretion of immunomodulatory molecules by the adult filariae during infection impacting the EETosis-capacity. The MF-induced EETosis was demonstrated to be contact dependent, but independent on antibody-coating as well as prior priming through circulating antigens. MF-induced EETosis did not require TLR-2, TLR-4 and MyD88 signaling. In contrast to other reports stating solely mitochondrial or nuclear DNA release, leading to a controversial discussion regarding the origin of the released DNA, the origin of the released DNA in response to MF was partly nuclear and primarily mitochondrial. *In vitro*, EETosis inhibited MF motility in a DNA- and cell contact-dependent manner. In line with our *in vitro* findings, we also observed increased local DNA concentrations upon *L. sigmodontis* infection supporting a potential role of ETosis *in vivo*. Our study also delineates other aspects of EETosis-mediated MF killing as we show that in comparison to bone marrow-derived eosinophils, eosinophils from the pleura and gut of infected animals entrapped MF more efficiently. Furthermore,

irrespective of whether they were in direct (pleural) or indirect (gut) contact with *L. sigmodontis* or derived from naïve animals (gut), a similar inhibition of MF motility was observed, suggesting that a prior priming of eosinophils during filarial infection is not required. Moreover, experiments on antibody deficient µMT mice revealed that antibodies were not essential for MF motility inhibition via EETosis. On the other hand, *in vitro* depletion experiments identified that MF-induced EETosis is mediated via the dectin-1 signaling pathway. Finally, DNA-dependent inhibition of MF motility by eosinophils is shown to be a conserved mechanism, as MF from *L. sigmodontis* and the canine heartworm *D. immitis* induced ETosis in murine eosinophils. In conclusion, our study documents for the first time, the life-cycle stage-specific occurrence of EETosis as a protective mechanism against filariae and identified the involved signaling pathway via dectin-1. We also elucidate several unique features of EETosis-mediated MF-killing like its mitochondrial and nuclear origin, contact dependency, inessential prior priming, independence of antibodies, and evolutionary conservation across species.

5. Additional projects:

5.1 Pre-clinical studies

Lymphatic filariasis and onchocerciasis belong to the group of neglected tropical diseases and are caused by filarial nematodes (2). Existing treatment strategies include mass drug administration targeting the microfilarial stage and temporarily inhibit the embryogenesis of the adult worms and thus, prolonged and annual treatments are required for the reproductive life time of the adult worms (239). Successful elimination of these diseases could be significantly improved by the discovery of a macrofilaricidal agent.

A common feature of many filarial worms as well as their offspring, the microfilariae, are endosymbiotic *Wolbachia* bacteria (58). *Wolbachia* bacteria live in a mutual relationship with the filariae by providing certain supplements that enable filarial maturation, reproduction and survival (59). Using antibiotics, like doxycycline, targeting the endosymbiotic bacteria enables the growth retardation of the adult filariae as well as preventing their reproduction (62, 65). However, since doxycycline has to be administered daily for 4 to 5 weeks, it cannot be used for mass drug administration but rather for individual therapy (67). Thus, new anti-*Wolbachia* drugs with reduced regimen durations provide a good approach in successfully eliminating lymphatic filariasis and onchocerciasis. Several potential new drug candidates with effective anti-*Wolbachia* activity and short duration regiments have been identified during these pre-clinical studies including AWZ1066S, ABBV-4083, AN11251, CBR417 and CBR490. Furthermore, flubendazole as a direct acting macrofilaricidal drug has been evaluated for onchocerciasis in the *Litomosoides sigmodontis* jird model.

PNAS 2019 Jan

AWZ1066S, a highly specific anti-*Wolbachia* drug candidate for a short-course treatment of filariasis

W. David Hong, Farid Benayoud, Gemma L. Nixon, Louise Ford, Kelly L. Johnston, Rachel H. Clare, Andrew Cassidy, Darren A. N. Cook, Amy Siu, Motohiro Shiotani, Peter J. H. Webborn, Stefan Kavanagh, Ghaith Aljayyoussi, Emma Murphy, Andrew Steven, John Archer, Dominique Struever, Stefan J. Frohberger, **Alexandra Ehrens**, Marc P. Hübner, Achim Hoerauf, Adam P. Roberts, Alasdair T. M. Hubbard, Edward W. Tate, Remigiusz A. Serwa, Suet C. Leung, Li Qie, Neil G. Berry, Fabian Gusovsky,

Janet Hemingway, Joseph D. Turner, Mark J. Taylor, Stephen A. Ward, and Paul M. O'Neill

AWZ1066S was identified as a new pre-clinical anti-*Wolbachia* candidate for treating filarial diseases. *In vivo* results showed a good bioavailability with a high metabolic stability in the mouse PK profile. Furthermore, 7 day AWZ1066S treatment reduced 98 % of *Wolbachia* in *B. malayi* worms transplanted into SCID mice and more than 99 % of *Wolbachia* in the *L. sigmodontis* gerbil model. MF release was prevented in the SCID mice, while peripheral *L. sigmodontis* MF decline began at 6 weeks after treatment start, achieving a complete clearance of MF after 14 weeks after treatment start. AWZ1066S lead to 98-99 % *Wolbachia* reduction, the inhibition of embryogenesis and MF clearance after 7 days of treatment compared to 28-day treatments for doxycycline, and shows therefore an improved efficacy compared to doxycycline and is thus a potent pre-clinical candidate in filarial treatment.

PLOS NTD 2019 Jan

Macrofilaricidal efficacy of single and repeated oral and subcutaneous doses of flubendazole in *Litomosoides sigmodontis* infected jirds

Marc P. Hübner, **Alexandra Ehrens**, Marianne Koschel, Bettina Dubben, Franziska Lenz, Stefan J. Frohberger, Sabine Specht, Ludo Quirynen, Sophie Lachau-Durand, Fetene Tekle, Benny Baeten, Marc Engelen, Charles D. Mackenzie, Achim Hoerauf

Flubendazole (FBZ) is a methylcarbamate benzimidazole that binds β-tubulin and thereby inhibits microtubule polymerization. It is frequently used in veterinary settings as well as against human gastrointestinal nematodes (240). Subcutaneous administration was previously shown to have an excellent macrofilaricidal effect. However, extended exposure following subcutaneous dose of flubendazole can result in an undesired toxicological profile and abscess formation at the injection site (241). Furthermore, accepted target product profiles for a new macrofilaricide prefer oral doses over parental administrations and therefore, this study compares different oral regimens of flubendazole to subcutaneous applications. This study shows that repeated oral administrations of FBZ for 10 days clears up to 90 % of adult *L. sigmodontis* worms in gerbils. Furthermore, embryogenesis of remaining female adult worms was inhibited by an irreversible damage of the female uteri and the uterine content and thus, peripheral microfilaremia was cleared. However, follow up studies

showed unwanted toxicology profiles of oral flubendazole regimens, which makes it unsuitable for human clinical studies (242).

PLOS NTD 2019 Feb

Discovery of ABBV-4083, a novel analog of Tylosin A that has potent anti-*Wolbachia* and anti-filarial activity

Thomas W. von Geldern, Howard E. Morton, Rick F. Clark, Brian S. Brown, Kelly L. Johnston, Louise Ford, Sabine Specht, Robert A. Carr, Deanne F. Stolarik, Junli Ma, Matthew J. Rieser, Dominique Struever, Stefan J. Frohberger, Marianne Koschel, **Alexandra Ehrens**, Joseph D. Turner, Marc P. Hübner, Achim Hoerauf, Mark J. Taylor, Stephen A. Ward, Kennan Marsh, Dale J. Kempf

Tylosin A is a macrolide antibiotic and is mostly active against gram-positive bacteria and mycobacteria. It is usually applied in veterinary settings involving different bacterial infections in cattle and swine. Even though it was proven to deplete endosymbiotic *Wolbachia* in filariae, its poor bioavailability makes it unsuitable for clinical treatments. The present publication describes the medical chemistry to obtain an improved derivate of Tylosin A. Derivatization of the 4”-OH group of Tylosin A with a benzyl ether resulted in ABBV-4083, which had an improved oral absorption and anti-*Wolbachia* activity in comparison to Tylosin A. Pre-clinical testing using the *L. sigmodontis* gerbil model revealed a potent anti-*Wolbachia* effect and an inhibition in embryogenesis by ABBV-4083. Treatment for 14 days once a day not only reduced the *Wolbachia* content by two logs but also caused a clearance in microfilarial load 12 weeks post treatment. This was accomplished through profound loss of all embryonic stages in female worms. Due to its long term sterility effect and the maintenance of MF clearance ABBV-4083 may provide a potent anti-filarial drug with reduced treatment durations.

Science Translational Medicine 2019 Mar

Preclinical development of an oral anti-*Wolbachia* macrolide drug for the treatment of lymphatic filariasis and onchocerciasis

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Fanny F. Fombad, Robert Carr, Howard E. Morton, Ghaith Aljayyoussi, Achim Hoerauf, Samuel Wanji, Dale J. Kempf, Joseph D. Turner and Stephen A. Ward

Screening of a compound library identified the macrolide Tylosin A as an effective anti-*Wolbachia* agent. Derivations at the 4”-OH group improved bioavailability and enhanced anti-*Wolbachia* activity leading to the selection of two semisynthetic lead compounds (A-1535469 and A-1574083 (ABBV-4083)). The enhanced potency of the compounds was *Wolbachia* specific since the *in vitro* efficacy against other bacteria was comparable to the original Tylosin A compound. Both compounds showed a superior *Wolbachia* reduction against *B. malayi* larvae in susceptible IL-4Rα$^{-/-}$ BALB/c when administered orally for 14 days compared to doxycycline. *Wolbachia* depletion in adult *B. malayi* worms was also proven after a 7-day treatment with A-1535469 (50 to 250 mg/kg) in CCR3$^{-/-}$ immunodeficient mice and after a 14-day treatment with A-1574083 (10 and 50 mg/kg) in a gerbil model. Furthermore, 99.7 % *Wolbachia* depletion was shown in female *L. sigmodontis* worms after a 14-day regimen of A-1574083 (100 mg/kg), which was highly superior to a 14-day doxycycline treatment (0 % *Wolbachia* reduction). Adult male *O. ochengi, implanted into* immunodeficient mice or outbred gerbils, showed a 99.4 and 98.8 % median reduction in *Wolbachia* after a 14-day administration of A-1535469 (250 mg/kg) and A-1574083 (75 mg/kg), respectively.

Both compounds depleted more than 90 % of *Wolbachia* after 1 or 2 weeks of treatment in *B. malayi*-, *L. sigmodontis*- and *O. ochengi*-infected animals by blocking embryogenesis and clearing peripheral microfilaremia and thus being superior in their efficacy compared to doxycycline or minocycline treatment for 3 or 4 weeks of treatment. Treatment duration of 14 days with one of the identified compounds, ABBV-4083, achieved an anti-*Wolbachia* depletion of >99 % and cleared peripheral microfilaremia in *B. malayi*-infected SCID mice 12 weeks after treatment start. *L. sigmodontis*-infected gerbils showed a constant decline in peripheral MF loads after a 14-day-treatment with ABBV-4083, which was superior to doxycycline treatment for the same duration. The safety profile of ABBV-4083 was evaluated in rats and dogs revealing no adverse effects at effective doses. Based on those observations ABBV-4083 was selected for clinical trials as a promising novel drug candidate for lymphatic filariasis and onchocerciasis.

PLOS NTD 2019 Aug

In vivo kinetics of _Wolbachia_ depletion by ABBV-4083 in _L. sigmodontis_ adult worms and microfilariae

Marc P. Hübner, Marianne Koschel, Dominique Struever, Venilin Nikolov, Stefan J. Frohberger, **Alexandra Ehrens**, Martina Fendler, Illiana Johannes, Thomas W. von Geldern, Kennan Marsh, Joseph D. Turner Schultz, Mark Taylor, Stephen A. Ward, Kenneth Pfarr, Dale J. Kempf, Achim Hoerauf

ABBV-4083 was previously identified to be a potent anti-*Wolbachia* drug and thus a strong pre-clinical candidate in the treatment against filariasis. This publication investigated the *Wolbachia*-depletion kinetics in adult worms and microfilariae isolated from *L. sigmodontis*-infected mice and jirds of different ABBV-4083 regimens and tested the effect of missed doses and washout periods. The mouse experiments show that immediately after treatment end of a 7-day treatment with ABBV-4083 reduced the *Wolbachia* levels by 94 %, while a 10-day regimen resulted in a reduction of 98.7 %. However, during a washout period the *Wolbachia* ftsZ/actin ratio further declined and thus, a *Wolbachia* reduction of 91.5 % after 5-day-regimen was further reduced to 99.9 % following a 3-week the washout period. Furthermore, the results show that once per day treatments with 75 mg/kg ABBV-4083 was sufficient to reduce *Wolbachia* levels effectively with no further significant improvement when treatments are given twice a day. In accordance, *Wolbachia* depletion in MF correlated with the depletion in female adult worms. Thus, detection of *Wolbachia* levels in MF may present a potential surrogate marker for drug efficacy. Jird experiments showed that daily treatments of 100 mg/kg ABBV-4083 for 7 days caused a reduction in all embryonal stages starting 4 weeks after treatment onset and a total lack of all embryonal stages 14 weeks after treatment. The effect of missed doses on *Wolbachia*-depletion was investigated, as well. Skipping up to four daily treatments did not impact *Wolbachia*-depletion in adult worms and MF compared to continuous treatments, when the treatments were given subsequently at the end to complete the regimen.

Science Translational Medicine 2019, May

Discovery of short-course anti-_Wolbachial_ quinazolines for elimination of filarial worm infections

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Frédéric Landmann, Bettina Dubben, Franziska Lenz, Dominique Struever, **Alexandra Ehrens**, Stefan Frohberger, Hanna Sjoberg, Nicolas Pionnier, John Archer, Andrew Steven, Valerine C. Chunda, Fanny F. Fombad, Patrick W. Chounna, Abdel J. Njouendou, Haelly M. Metuge, Bertrand L. Ndzeshang, Emma Murphy, Narcisse V. Gandjui, Desmond N. Akumtoh, Tayong D. Kwenti, Ashley K. Woods, Mitchell V. Hull, Wen Xiong, Kelli L. Kuhen, Mark Taylor, Samuel Wanji, Joseph D. Turner Schultz, Marc P. Hübner, William Sullivan, Achim Hoerauf, Xin-Jie Chu, Jason Roland, H. Michael Petrassi, Matt S. Tremblay, Sean B. Joseph, Peter G., Case W. McNamara

This publication describes the discovery of new anti-*Wolbachia* antibiotics. Using a high-throughput screening assay, over 300,000 compounds were tested for their anti-*Wolbachia* activity in *Drosophila melanogaster* cells. Identified compounds were further screened for their *Wolbachia*-specificity. The quinazolines CBR417 and CBR490 showed a high anti-*Wolbachia* activity with no general activity against other gram-positive or gram-negative bacteria. *Ex vivo* assays showed an enhanced *Wolbachia* depletion by these two compounds from female B*. pahangi* ovaries compared to doxycycline. In the *in vivo L. sigmodontis* mouse model, the new identified quinazolines show rapid *Wolbachia* clearance from filarial nematodes. A 4-day regimen at 60 mg/kg once a day eliminated 99.80 % (CBR490) and 99.96 % (CBR417) of *Wolbachia* in *L. sigmodontis* adult female worms, which was superior to a bi-daily doxycycline treatment for 14 days. An even more drastically efficacious dosing regimen with one single dose or two doses within two weeks still resulted in a *Wolbachia* reduction of more than 99 %. Thus, these two new compounds may provide a single-dose cure to treat filarial infections.

Journal of Medical Chemistry 2019 Mar

Boron-Pleuromutilins as Anti-*Wolbachia* Agents with Potential for Treatment of Onchocerciasis and Lymphatic Filariasis

Robert T. Jacobs, Christopher S. Lunde, Yvonne R. Freund, Vincent Hernandez, Xianfeng Li, Yi Xia, David S. Carter, Pamela W. Berry, Jason Halladay, Fernando Rock, Rianna Stefanakis, Eric Easom, Jacob J. Plattner, Louise Ford, Kelly L. Johnston, Darren A. N. Cook, Rachel Clare, Andrew Cassidy, Laura Myhill, Hayley Tyrer, Joanne Gamble, Ana F. Guimaraes, Andrew Steven, Franziska Lenz, **Alexandra Ehrens**, Stefan J. Frohberger, Marianne Koschel, Achim Hoerauf, Marc P.

Hübner, Case W. McNamara, Malina A. Bakowski, Joseph D. Turner, Mark J. Taylor, and Stephen A. Ward

Pleuromutilins belong to an antibiotic class of ribosomal protein synthesis inhibitors. The current manuscript describes the medical chemistry, which resulted in the identification of AN11251. The modification of the boronpleuromutilin at the C(14) positions bearing a 7-fluorobenzoxaborole led to a good bioavailability as well as an attractive PK profile. The new drug candidate reduced *Wolbachia* from *B. malayi* L4 larvae in the SCID mouse model as well as from *L. sigmodontis* adult worms in the BALB/c mouse model. The reduction in the *L. sigmodontis* worms reached up to 99 % after a bi-daily treatment duration of 14 with 50 mg/kg.

Submitted manuscript

IN VIVO* efficacy of the boron-pleuromutilin AN11251 against *Wolbachia* of the rodent filarial nematode *Litomosoides Sigmodontis

Alexandra Ehrens*, Christopher S. Lunde*, Robert T. Jacobs, Dominique Struever, Marianne Koschel, Stefan J. Frohberger, Franziska Lenz, Martina Fendler, Yvonne R. Freund, Rianna Stefanakis, Eric Easom, Xianfeng Li, Jacob J. Plattner, Achim Hoerauf[#], Marc P. Hübner[#]

*, [#] equal contribution

The newly discovered boronpleuromutilin AN11251 was tested for its *in vivo* efficacy against *Wolbachia* in the *L. sigmodontis* mouse model and compared to doxycycline and rifampicin. Bi-daily oral treatments of 200 mg/kg AN11251 for 10 days resulted in a *Wolbachia* reduction of more than 99.9 %, which exceeded the *Wolbachia* reduction of doxycycline and was comparable to the reduction achieved by high-dose rifampicin administrations. Furthermore, combination therapies of AN11251 and doxycycline were conducted to reduce regimen duration. However, the combination therapy had no further impact on *Wolbachia* depletion. Thus, AN11251 is superior to doxycycline and with a treatment duration of 10 to 14 days, an excellent pre-clinical candidate in the elimination of lymphatic filariasis and onchocerciasis.

5.2 Protective immunity

Filarial nematodes are a major public health problem in tropical and sub-tropical countries. Due to their parasitic nature they are able to induce a chronic infection in their hosts through immunomodulatory mechanisms. Helminths typically induce a type 2 immune response, which is associated with AAMs, eosinophils, mast cells and basophils. A valid tool in assessing filarial infections, including protective immunity, immunomodulation and potential new drug candidates, is the *L. sigmodontis* mouse model since it closely resembles human filarial infections.

Parasites & Vectors 2019, May

Susceptibility to *L. sigmodontis* infection is highest in animals lacking IL-4R/IL-5 compared to single knockouts of IL-4R, IL-5 or eosinophils

Stefan J. Frohberger, Jesuthas Ajendra, Surendar Jayagopi, Wiebke Stamminger, **Alexandra, Ehrens**, Benedikt C. Buerfent, Achim Hoerauf, Marc P. Hübner

Using the *L. sigmodontis* mouse model, the impact of IL-5, eosinophils and the IL-4R on protective immune responses was directly compared in this publication using knock-out mice. A lack of eosinophils as shown with dblGATA mice and IL-$5^{-/-}$ mice resulted in an increased worm and MF burden. Lack of IL-4R resulted, similar as the absence of eosinophils to an earlier onset of microfilaremia. AAMs numbers positively correlated with the adult worm load, which was probably due to associated changes in eosinophils. A combined deficiency in IL-$4R^{-/-}$ and IL-$5^{-/-}$ resulted in the highest *L. sigmodontis* susceptibility with an earlier onset of microfilaremia, enhanced embryogenesis, MF loads and prolonged adult worm survival.

Submitted manuscript

S100A8/A9 deficiency increases neutrophil activation and protective immune responses within the lung against invading infective L3 larvae of the filarial nematode *Litomosoides sigmodontis*

Stefan J. Frohberger, Frederic Fercoq, Anna-Lena Neumann, Jayagopi Surendar, Wiebke Stamminger, Estelle Remion, **Alexandra Ehrens**, Thomas Vogl, Achim Hoerauf, Coralie Martin, Marc P. Hübner.

Neutrophils present the first line response against invading L3 larvae of filarial nematodes. A common protein mainly expressed by neutrophils is S100A9, which

forms the heterodimer calprotectin with S100A8 (243). This study investigated the impact of S100A9/A8 deficiency on *L. sigmodontis* infection using S100A9/A8-deficient mice. Knock-out mice presented a significantly reduced worm burden within the pleural cavity 12 days after natural infection using the vector or subcutaneous L3 larval inoculation compared to wild-type controls. Thus, immune responses within the skin, which were circumvented by the subcutaneous injection, were not responsible for the reduced worm burden. The bronchoalveolar lavage of S100A9/A8-deficient mice revealed an increase in macrophages, eosinophils and neutrophils as well as neutrophil-related chemokines and elastase, another prominent neutrophil molecule. Furthermore, neutrophils isolated from S100A9/A8-deficient mice were more potent in reducing L3 larval motility *in vitro* compared to neutrophils isolated from wild-type mice. Even though knock-out mice presented an increase in inflammatory cells and markers within the lung, lung pathology, which could be a result of migrating L3 larvae, was less prominent in the knock-out mice compared to wild-type controls. Taken together, these results suggest that the lung rather than the skin is involved in the protective immunity observed in the S100A9/A8-deficient mice. In accordance, neutrophil depletion from the lung of knock-out mice abolished the phenotype and resulted in an increased worm burden, supporting that the protective effect mediated by neutrophils of the S100A9/A8-deficient mice occurs within the lung. Similarly, intravenous injection of L3 larvae increased the worm burden in S100A9/A8 deficient mice indicating that the protective effect of the lung neutrophils is mediated by the successively entrance of the L3 larvae, while intravenous injection causes the arrival of the L3 larvae simultaneously and circumvents these protective immune responses within the lung. Thus, consecutively arrival of the L3 larvae of *L. sigmodontis* within the lung establishes a protective immunity, which is reduced by neutrophil-related calprotectin.

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Scientific contributions

Publications in peer-reviewed journals

Published Paper before PhD

1. Yefremova Y, Melder FTI, Danquah BD, Opuni KFM, Koy C, **Ehrens A**, Frommholz D, Illges H, Koelbel K, Sobott F, Glocker MO. **Apparent activation energies of protein-protein complex dissociation in the gas-phase determined by electrospray mass spectrometry.** Anal Bioanal Chem. 2017 Nov;409(28):6549-6558. doi: 10.1007/s00216-017-0603-4. Epub 2017 Sep 12.

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12. **Ehrens A**, Lenz B, Neumann A, Giarrizzo S, Frohberger SJ, Stamminger W, Bürfent BC, Fercoq F, Martin C, Kulke D, Hoerauf A, Hübner MP. **Microfilariae trigger eosinophil extracellular DNA traps in a Dectin-1-dependent manner.**

Conferences

Poster presentation at the 27th annual meeting of the German Society for Parasitology (DGP) in Göttingen, Germany, 9-12th March 2016 (Poster title: NK cells promote *Litomosoides sigmodontis* development and embryogenesis)

Oral presentation at the 28th annual meeting of the German Society for Parasitology (DGP) in Berlin, Germany, 21-24th March 2018 (Talk title: Murine eosinophils trap microfilariae of the rodent filarial nematode *Litomosoides sigmodontis* in an ETosis-dependent mechanism)

Oral presentation at the 67th annual meeting of the American Society of Tropical Medicine and Hygiene in New Orleans, USA, 28th October- 1st November 2018 (Talk title: Eosinophil extracellular traps mediate entrapping of microfilariae of the rodent filarial nematode *Litomosoides sigmodontis*)

Oral presentation at the Cluster Science Days and the 10th International Symposium of IFReC in Bonn, Germany, 5-6th November 2018 (Talk title: Murine Eosinophil Extracellular Traps mediate entrapping of Microfilariae of the Rodent Filarial Nematode *Litomosoides sigmodontis*)

Poster presentation (including a poster prize) at the “Parasitic Helminths - New Perspectives in Biology and Infection” conference in Hydra, Greece, 1-6th September 2019 (Poster title: Microfilariae trigger eosinophil extracellular traps in a Dectin-1-dependent manner)

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