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Eosinophil proteins and their role during filarial infection

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Table of Contents

1. Introduction 9
1.1. Filariae …….. 9
1.1.1. Onchocerciasis and Lymphatic filariasis ……………..... 12
1.1.2. *Litomosoides sigmodontis* ………………….................... 16
1.2. Protective immunity against filarial infections 18
1.2.1. Type 2 immunity ….. 22
1.2.2. Eosinophils and their effector mechanisms 27
1.2.3. ETosis 30
1.2.4. Eosinophile Peroxidase and Major Basic Protein 34
1.2.5. The role of eosinophils and their effector mechanisms in filarial infections ………………………………………….... 34
2. Original Publications 39
2.1. Publication No 1: Eosinophils are an endogenous source of IL-4 during filarial infections and contribute to the development of an optimal T helper 2 response 40
2.2. Publication No 2: Major basic protein and eosinophil peroxidase are required for microfilariae motility inhibition by eosinophil ETosis mice in an eosinophil-dependent manner.. 58
3. Discussion ………………………………………………… 73
4. Zusammenfassung ………………………………………… 90
5. Summary ……………………………………………….... 93
6. Reference 96
7. Acknowledgement 114
8. Curriculum Vitae 117

9. Declaration ………………………………………………….. 119
10. Funding ...…………………………………..……… 120

List of Abbreviations

AAMs ……..……………….… alternatively activated macrophages

ADCC …...…….. antibody-dependent cell-mediated cytotoxicity

DEC ………………………………………….. diethylcarbamazine

dH2O …………………………………………….. distilled water

disability-adjusted life years ……..……………………… DALYs

DNases ……………………………….…. deoxyribonucleases

dpi ……………...……………………………… days post infection

ECP ……………………………… eosinophilic cationic protein

EDN ……..………………………. eosinophil-Derived Neurotoxin

EDTA …...…...………………… ethylenediaminetetraacetic acid

ET ……………………………………………….. extracellular trap

EET ……..………………………….. eosinophil extracellular trap

EPO …………………………….……. eosinophil peroxidase

FBS ……………………………….……….... fetal bovine serum

Fig …..………………………………………………….. figure

IFN-γ ……………………………………………… interferon-gamma

IgG ...………………………………………... immunoglobulin G

IgE ………………………………………….immunoglobulin E

IL ………..……………………………...………… interleukin

ILC2s ……………………………… group 2 innate lymphoid cells

IMMIP………………………………………………… Institute
for Medical Microbiology, Immunology and Parasitology

KO …..…………………………………………………… knock-out

L3 larvae ………..……………………………….. third larval stage

LF ……..…………………………………… Lymphatic Filariasis

LsAg …………………………………… *L. sigmodontis* antigen

MBP …..……………………………………… major basic protein

MF ………...…………………………………………….. microfilariae

MHC ………………………….. major histocompatibility complex

MPO ……………………………………………… myeloperoxidase

NE ……………………………………………….. neutrophil elastase

NTD …………………………...…… neglected tropical disease

PAD4 ……...………………………… peptidylarginine deiminase 4

PBS …..……………………………… phosphate buffered saline

RELMα …..……………………………… resistin-like molecule α

ROS …………………………………….. reactive oxygen species

RT ……………………………………...……. room temperature

spp …………………….………………..……… species pluralis

STH …...……………………………… Soil-transmitted helminths

TGF-β ………………………….. transforming growth factor-beta

Th2 …….……………………………………. T helper cell subset 2

TNF-α ……………………………… tumor necrosis factor-alpha

Tregs ……………………………………………. regulatory T cells

TSLP …..………………………… thymic stromal lymphopoietin

WHO ………………………………… World Health Organization

WT ………………..……………………………….… wild type

1. Introduction

1.1. Filariae

Filariae belong to the group of helminths, a collective term for multicellular parasitic organisms, mostly endoparasitic, that have at least two different cell types arranged in at least two layers. They are divided into flatworms (trematodes), tapeworms (cestodes) and roundworms (nematodes). According to World Health Organization (WHO) estimations, around two billion people were infected with helminths in 2020. Infections with soil-transmitted helminths (STH), such as *Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale* and *Necator americanus*, and certain parasitic filariae, like *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, are among the neglected tropical diseases (NTDs) which mainly affect people in sub-Saharan Africa. Filariae belong to the group of nematodes and the Onchocercidae family. Filariae are transmitted by vectors and can cause various diseases in humans. The most common infectious agents of the Onchocercidae family are the genera *Ochocerca volvulus*, *Brugia malayi*, *Wuchereria bancrofti*, *Loa loa* and *Mansonella perstans*, although the latter two are not currently classified as NTDs. Human-pathogenic filariae are large thread-like worms, several centimeters long, that can cause severe pathologies or asymptomatic infections. The life cycles of the different human pathogenic parasitic filarial nematodes share several similarities. Figure 1 (Fig. 1)

exemplary illustrates the life cycle of *Brugia malayi*. The infective third larval stage (L3) is transmitted by an arthropod vector, a mosquito, through its bite into the skin or blood vessels of the host. The type of vector, the L3 migration route and the location of the adult worms differ depending on the filarial species.

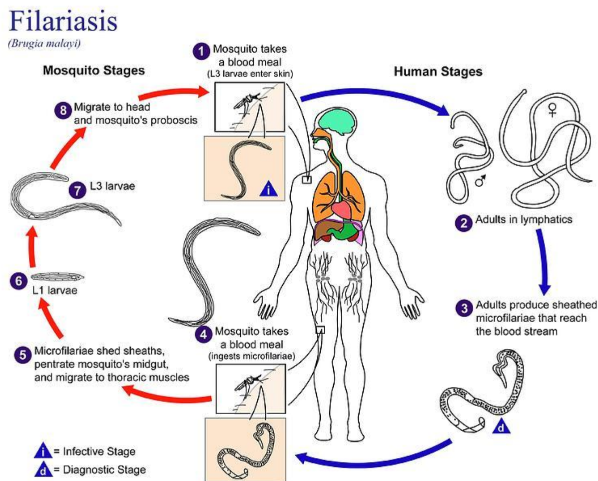

Fig. 1: Life cycle of Brugia malayi as an example for human filariasis (1)

For example, *Simulium* spp. transmit L3 larvae of *O. volvulus*. These migrate into the subcutaneous tissue, where they mature into adult worms which live in nodules under the skin for 9-11 years. This is where male and female worms mate. After mating, the female releases the progeny, first larval stages (so-called microfilariae, MF), into the skin. MF are taken up by the

next simulid blood meal and develop into infective L3 in the vector's midgut, thus completing the cycle. In 2017, an estimated 21 million people were infected with *O. volvulus*. Onchocerciasis, also known as river blindness, can lead to dermatitis, visual impairment and vision loss, resulting in significant disability-adjusted life years (DALYs). *M. perstans*, on the other hand, is transmitted by *Culicoides spp*. and can colonize various parts of the body. An estimated 120 million people are infected, but mostly subclinical. Remarkably, both *M. perstans* and *O. volvulus* harbor symbiotic *Wolbachia* bacteria, which are crucial for their development, fertility and survival. These *Wolbachia* specimen are targeted and killed by treatment with antibiotics, such as doxycycline, to ensure a long-term reduction in the filarial population (2–5). However, this treatment is rather long-lasting with 4-6 weeks. Of note, infections with *L. loa*, the causative agent of loiasis are not druggable by antibiotics since this filarial species does not contain *Wolbachia* specimen. It is transmitted by horseflies of the genus *Chrysops*. The adult *L. loa* worms live in the subcutaneous and connective tissue and release MF into the blood during the day. The disease is mainly endemic in sub-Saharan Africa. In 2011, around 14 million people were infected (6), and recent studies have shown that the mortality rate among infected people is considerably high (7). Lymphatic filariasis (LF), caused by *Brugia spp*. and *W. bancrofti*, with an estimated 51.4 million cases in 2018, is transmitted by

mosquitoes (8). The adult worms live in lymphatic vessels, while MF are circulating in the blood at night. The consequences of the disease can include hydrocele, lymphoedema and elephantiasis. The distribution of different filarial infection varies by species, with onchocerciasis, loiasis and mansonellosis occurring mainly in sub-Saharan Africa, while LF has a wider distribution and is also actively transmitted in South America and Southeast Asia.

1.1.1. Onchocerciasis and Lymphatic filariasis

Onchocerciasis is an NTD, commonly known as river blindness, caused by the filarial nematode *Onchocerca volvulus*. The disease is transmitted through bites of infected black flies of the genus *Simulium*, which breed in fast-flowing rivers and streams. When an infected black fly bites a human, it injects *O. volvulus* third larvae into the skin. These larvae migrate and mature into adult worms, which can live for years in subcutaneous tissues, forming nodules. The adult worms produce thousands of MF, which migrate throughout subcutaneous tissues and eventually through the cornea and other parts of the eye, causing a range of symptoms. The hallmark symptom of onchocerciasis is severe itching, particularly of the skin. Over time, MF death can lead to dermatitis in 47.8 % of patients (9), accompanied by skin alterations including thickening, depigmentation and nodules. In extreme cases, skin lesions can develop into large wounds

causing scars. This clinical picture is known as Sowda (Fig. 2) and occurs in a small number of infected patients (10). In some cases, MF migrate to the eyes, causing inflammation of the cornea and other ocular structures, which can ultimately lead to visual impairment or blindness (11).

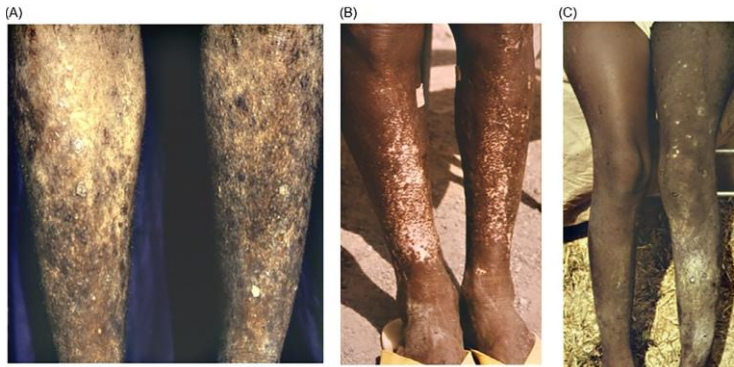

Fig. 2: *Papular dermatitis during onchocerciasis. (A) Hyperpigmentation and lichenification, (B) leopard skin and (C) unilateral hyperreactive onchodermatitis (left leg), termed sowda. (12)*

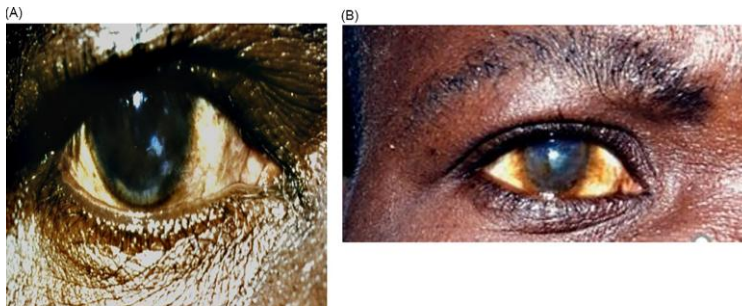

Fig. 3: *Onchocerciasis-driven sclerosis of the cornea. (A) Onset of cornea sclerosis and (B) advanced sclerosing keratitis leading to vision loss (12)*

LF, also known as elephantiasis, is a NTD caused by the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. LF is transmitted by mosquitoes - particularly species of the genera *Culex*, *Anopheles*, *Aedes*, and *Mansonia* - depending on their regional distribution (13).

LF is endemic in more than 70 tropical and subtropical countries. Globally, over 51.4 million people are currently infected, and approximately 657 million people are at risk of infection (14,15). Of the causative agents, *W. bancrofti* is responsible for approximately 90% of all cases, while *B. malayi* and *B. timori* account for the remainder, primarily in South and Southeast Asia (16).

When an infected mosquito bites a person, it injects L3 larvae. These larvae enter the lymphatic vessels, where they mature into adult worms within a period of 6 to 12 months. Adult worms can survive for 6–8 years (*Brugia spp.*) or up to 10 years (*W. bancrofti*) and produce millions of MF that circulate in the peripheral blood, often showing nocturnal periodicity (17).

The presence of adult worms causes lymphatic vessel dilatation, inflammation, and eventually obstruction, leading to lymphoedema, swelling of the limbs (Fig. 4), and, in males, hydrocele (Fig. 5) - a fluid accumulation in the male scrotum.

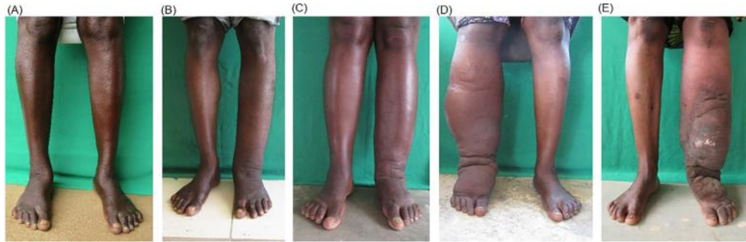

Fig 4: Different clinical stages of lymphedema of LF patients. *Staging was performed according to Dreyer et al. (2002), based on the presence of swelling, skin folds, knobs and mossy lesions. (A) Stage 2 right leg, (B) stage 3 left leg, (C) stage 4 left leg, (D) stage 5 right leg and (E) stage 6 left leg. (12).*

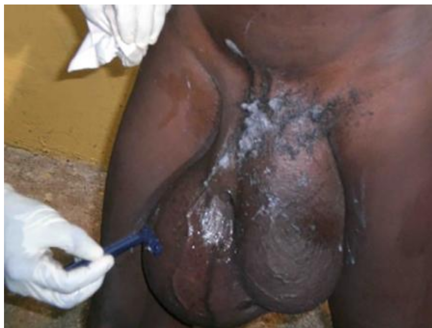

Fig. 5: Hydrocele of a W. bancrofti-infected man being prepared for hydrocelectomy *(circa 25 million people infected) (12)*

In advanced cases, chronic inflammation and fibrosis lead to elephantiasis, characterized by severe swelling and thickening of the skin (18).

The immune response during LF is dynamic and highly regulated. During acute infection, the immune system mounts a type 1-mediated inflammatory response with elevated levels of distinct cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF). However, the chronic presence of adult

worms leads to a downregulation of immune responses to avoid excessive tissue damage. This is achieved by the activation of regulatory T cells (Tregs) and the release of anti-inflammatory cytokines, particularly interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). These molecules suppress pro-inflammatory responses and help to prevent acute pathology but also to allow the parasites to evade host immunity and to persist for years (19). In some cases, the immune system overreacts - especially in response to dying worms – thereby causing chronic inflammation, lymphatic damage, and tissue fibrosis, which are hallmarks of the clinical disease (20).

Both onchocerciasis and LF are considered NTDs that disproportionally affect impoverished communities in tropical and subtropical regions. Control and prevention strategies for these diseases include mass drug administration with antiparasitic medications, vector control measures, and efforts to improve sanitation and access to healthcare.

1.1.2. *Litomosoides sigmodontis*

Litomosoides sigmodontis is a filarial nematode that naturally infects cotton rats (*Sigmodon hispidus*) and, like the human pathogenic nematodes described above, belongs to the Onchocercidae family. This species serves as a model organism for the study of human filarial infections, such as onchocerciasis and LF. *L. sigmodontis* is transmitted by the

tropical rat mite *Ornithonyssus bacoti* (for the life cycle please refer to Fig. 6). After the bite of the mite, infective L3 larvae of *L. sigmodontis* migrate through the skin and lymphatic system into the pleural cavity of its rodent host. In other susceptible rodents, such as mice or jirds, the larvae of *L. sigmodontis* develop into adult worms approximately 25-30 days after infection. Adult worms mate and produce MF, which can be found in the peripheral blood of the final hosts from ~ 50 days after infection. For transmission, MF are taken up during the blood meal by another rat mite, where they develop into infective L3 larvae. This model system allows researchers to study host-parasite interactions throughout the entire life cycle. This helps to study immune responses and mechanisms of filarial infections in more detail. Studies on *L. sigmodontis* infections have contributed significantly to our understanding of helminth-induced type 2 immune responses, immune regulation, parasite biology and potential therapeutic interventions against filarial diseases (21–26).

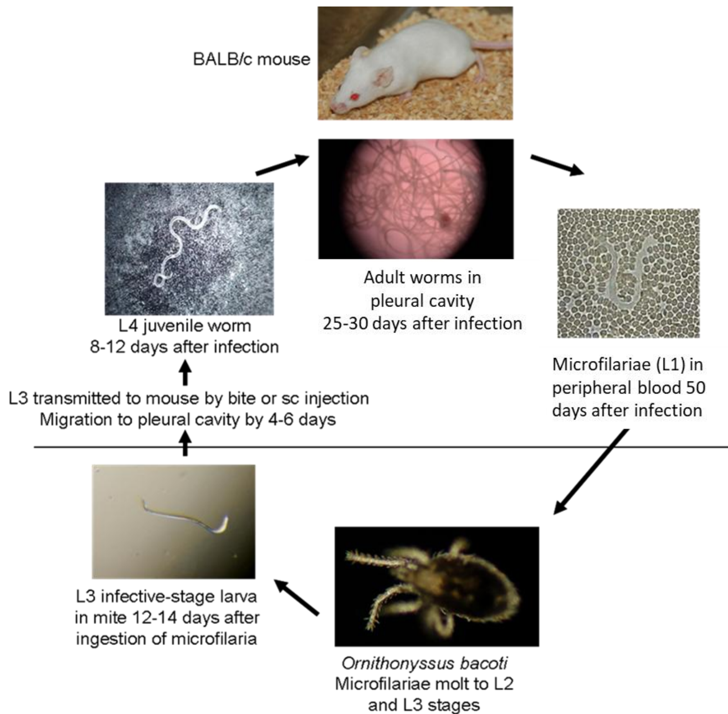

Fig. 6: Life cycle of Litomosoides sigmodontis *(26)*

1.2. Protective immunity against filarial infections

Protective immunity against filarial infections is primarily mediated by a type 2 immune response, which plays a central role in host defense against helminths (27,28). The immune profile is characterized by the activation of T helper 2 (Th2) cells and the release of cytokines like interleukin-4 (IL-4), IL-5, and IL-13. These cytokines orchestrate a complex immune cascade involving eosinophils, mast cells, and the production of

immunoglobulin E (IgE), all of which contribute to the defense against parasitic worms (29,30). IL-5 promotes the differentiation, recruitment, and activation of eosinophils, which release cytotoxic granules like major basic protein (MBP) and eosinophil cationic protein (ECP), damaging the cuticle of the parasites (31,32). IL-4 and IL-13 stimulate B cells to produce IgE and contribute to tissue remodeling and increased mucus production, which may assist in mechanical clearance of parasites in the gastrointestinal tract (33–36). Although such responses are effective in reducing worm burden, protective immunity is often insufficient, and reinfection remains a common outcome, particularly in endemic areas (27).

In addition to Th2 cells, group 2 innate lymphoid cells (ILC2s) have emerged as key players in the early phase of helminth infections (37–39). These cells rapidly produce IL-5 and IL-13 in response to epithelial-derived alarmins, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), released during tissue damage caused by migrating larvae. ILC2s are particularly important in mucosal tissues, where they contribute to the recruitment of eosinophils, goblet cell hyperplasia, and smooth muscle contraction. This innate response not only assists in the early containment of the parasite but also supports the development of adaptive Th2 immunity (37,40,41).

Filarial parasites, however, are especially adept in modulating host immune responses to promote chronic infection. Unlike gastrointestinal helminths which are expelled relatively quickly,

filarial nematodes like *W. bancrofti*, *O. volvulus*, and *L. loa* can persist in the human host for years, often with minimal pathology (10,20,42,43). This persistence is partly based on the parasites' ability to induce regulatory immune responses. Filariae stimulate the expansion of Tregs and the production of anti-inflammatory cytokines, such as IL-10 and TGF-β. These mediators suppress type 2 effector functions and limit inflammation, thereby allowing the parasite to evade immune reactions and killing. The dampened immune activation reduces tissue pathology, but at the cost of ineffective parasite clearance (10).

In many filarial species, the presence of intracellular bacterial endosymbionts of the genus *Wolbachia* plays a critical role in immune modulation and disease progression. *Wolbachia* endosymbionts are essential for the survival and reproduction of several filarial nematodes, including *W. bancrofti* and *O. volvulus*. Antigens derived from *Wolbachia* can trigger strong innate immune responses and are believed to contribute to the inflammatory pathology seen in diseases like lymphatic filariasis and onchocerciasis (43–45). Notably, depletion of *Wolbachia* through antibiotic treatment - most effectively by doxycycline - leads to sterility in adult female worms and a gradual reduction in microfilarial load. This approach not only targets the parasite indirectly but also appears to shift the host immune response toward a more effective profile for parasite elimination (43).

Despite these immune mechanisms, natural immunity to filarial parasites is often insufficient for complete protection, and individuals in endemic areas may harbor adult worms and MF for decades without developing overt disease. This coexistence is a hallmark of helminth-induced immunoregulation and presents a significant challenge for vaccine development. An effective vaccine would need to enhance protective type 2 responses while circumventing the regulatory environment established by the parasite. Understanding the fine balance between immune activation and suppression in filarial infections is crucial for developing targeted therapies and for designing immunomodulatory interventions that can achieve long-term protection.

In conclusion, protective immunity against filarial infections involves a multifaceted interplay of innate and adaptive type 2 immune responses, with critical contributions by Th2 cells, ILC2s, eosinophils, and IgE-mediated mechanisms. However, the capacity of filarial parasites to manipulate host immunity through several regulatory pathways and by symbiotic bacteria underscores the complexity of achieving sterile immunity. Continued investigation of these host-parasite interactions is essential for the development of vaccines and immunotherapies that can break the cycle of chronic filarial disease.

1.2.1. Type 2 immunity

To combat helminth infections, the host typically mounts a type 2 immune response, which is initiated by alarmins, such as TSLP, IL-25, and IL-33, released by epithelial cells in response to tissue damage or parasite components (27). Distinct cell types of the immune system, like dendritic cells, alternatively activated macrophages (AAMs) and epithelial cells, recognize invading pathogens and trigger the release of alarmins (46–48). The second phase of the immune response involves Th2 cells activation. Therefore, dendritic cells process and present helminth antigens to naive T cells in the lymph nodes, which then differentiate into Th2 cells (46–48). These Th2 cells produce key type 2 cytokines, such as IL-4, IL-5, IL-9, IL-10, IL-13, IL-21, and IL-25, which regulate various immune functions (49–51). IL-4 promotes the differentiation of B cells and induces the production of IgE, immunoglobulin G (IgG1) (in mice), and IgG4 (in humans). IgE binds to parasites and to the surface of basophils and mast cells, which degranulate upon antigen recognition, releasing inflammatory mediators like histamine and leukotrienes (42). This leads to local inflammation, aiding in the defense against the parasites. IL-5 activates eosinophils, which release cytotoxic substances like MBP, eosinophil peroxidase (EPO) and ECP, all of which can directly damage and kill helminths (52). IL-13 promotes mucus production by goblet cells and increases intestinal peristalsis, aiding the mechanical expulsion of intestinal worms. IL-9 and IL-10 help to

maintain a balanced Th2 response thereby circumventing excessive inflammation, while IL-21 promotes antibody production (53–56). Chemokines, such as eotaxines, orchestrate the recruitment and activation of granulocytes like eosinophils, mast cells, and basophils, all key players in anti-helminth immune responses. In an IL-4-dominated environment, macrophages shift towards an alternatively activated phenotype, producing factors like arginase 1 and resistin-like molecule α (RELMα) (57–59). These AAMs play a critical role in tissue repair, wound healing, and parasite expulsion. RELMα also induces collagen production, which can limit tissue damage induced by migrating larvae (28,58). Additionally, IgG1 and IgG4 antibodies enhance immune responses by opsonizing parasites for phagocytosis and triggering respiratory burst mechanisms in immune cells through FcγR binding (60). The immune system balances inflammation and healing through IL-10 and TGF-β, which promote tissue remodeling, collagen deposition, and suppression of inflammation (61,62). This ensures that - while the parasites are being eliminated - tissue damage is minimized, and healing is promoted. Finally, the development of memory T and B cells ensures a faster and more effective immune response during re-infections, providing long-term protection (28).

The specific manifestation of type 2 immunity can vary depending on the host-parasite combination. During a variety of

filarial infections, an increase in eosinophils was observed, which are thought to support protective immune responses.

In the *Litomosoides sigmodontis*-infected mouse model, a robust type 2 immune response is observed. Eosinophils are prominently recruited to infection sites (63), where they release cytotoxic granules containing MBP, EPO, and ECP, contributing to parasite clearance (64,65). Additionally, eosinophils have been identified as a significant source of IL-4 during the early phases of infection, thereby further amplifying the Th2 response (63). This cytokine milieu promotes the differentiation of AAMs, which express markers like arginase-1 and RELMα, aiding in tissue repair and parasite expulsion (63).

Human infections with *W. bancrofti*, the causative agent of lymphatic filariasis, also elicit a type 2 immune response. Patients exhibit elevated levels of IL-4, IL-5, IL-10, and IL-13, along with increased IgE and IgG4 antibodies. Eosinophilia is a common feature, and eosinophils contribute to parasite control through degranulation. Interestingly, studies have identified two subsets of Th2 cells in infected individuals: one subset that produces IL-4 and IL-13, and another one which releases IL-4, IL-5, and IL-13. The latter subset is associated with higher eosinophil counts, suggesting a more potent effector function (66).

Human infections with *B. malayi*, another filarial parasite causing lymphatic filariasis, similarly induce a type 2 immune response. Patients exhibit elevated IL-4, IL-5, and IL-13 levels,

along with increased IgE and eosinophil counts (67). These immune components work synergistically and are suggested to control parasite burden and mediate tissue repair mechanisms.
In *O. volvulus* infections of humans, responsible for onchocerciasis / river blindness, a type 2 immune response is also predominant. Affected individuals display increased levels of IL-4, IL-5 and IL-13, along with elevated IgE concentrations. Eosinophils infiltrate nodular lesions, releasing cytotoxic granules, such as MBP, ECP, EPO, and EDN (68), that were shown to damage both parasites and host tissues, contributing to the pathology observed in chronic infections (21).
In the veterinary field, infections with *Dirofilaria repens* in dogs also elicit a pronounced type 2 immune response. In naturally infected dogs, an elevated expression of Th2-associated genes, including GATA3, STAT6, and IL-13 was found (69). These markers indicate a skewing towards a type 2-dominated response, characterized by increased eosinophil activity and IgE production. Notably, the expression of IL-10, a regulatory cytokine, was also observed, suggesting a mechanism to balance the immune response and prevent excessive inflammation. This Th2-biased immunity is suggested to play a crucial role in controlling the parasite while mitigating tissue damage (70).
Dirofilaria immitis, the causative agent of canine heartworm disease, equally induces a type 2 immune response. Previous studies have demonstrated that dogs with patent infections

exhibit higher levels of type 2 cytokines, such as IL-4 and IL-10, compared to uninfected controls. Additionally, the presence of the endosymbiont bacterium *Wolbachia* within *D. immitis* may further modulate the host's immune response and facilitate parasite survival (71–73).

In cattle, *Onchocerca ochengi* infections naturally occur and serve as a model for human onchocerciasis. Infected cattle develop a type 2 immune response, characterized by elevated levels of IL-4, IL-5, and IL-13, along with increased eosinophil counts. These cytokines and effector cells contribute to the containment of the parasite and limit tissue damage. Interestingly, the immune response in cattle also includes regulatory components, such as IL-10, which help to modulate inflammation and prevent excessive tissue pathology (74).

Taken together, type 2 immunity represents a conserved and essential defense mechanism across diverse host–filaria systems. By the activation of Th2 cells and cytokine-mediated recruitment of effector cells like eosinophils, this response facilitates parasite control and tissue repair. Its effectiveness, however, varies by host–parasite combination and depends on a well-regulated balance between immune activation and regulation to avoid tissue damage.

1.2.2. Eosinophils and their effector mechanisms

Eosinophils are specialized white blood cells that play a crucial role in immune responses, particularly during parasitic helminth infections and allergic reactions. These cells contain granules in the cytoplasm, which store cytotoxic enzymes, toxins, and proteins essential for their effector functions (21). When eosinophils are activated, e. g. by IL-5, they release granule contents, leading to cytotoxic effects on pathogens and contributing to immune defense mechanisms (75,76).

Eosinophils combat parasitic infections through various mechanisms that involve the release of toxic proteins. MBP disrupts the membranes of parasites, leading to their destruction (77). EPO generates reactive oxygen species (ROS), which pose significant damage on pathogens (31). Additionally, ECP and Eosinophil-Derived Neurotoxin (EDN) further contribute to membrane damage and impair parasite viability (31,78,79). Apart from releasing toxic proteins, eosinophils also play a role in antibody-dependent cell-mediated cytotoxicity (ADCC). By binding to parasites coated with IgE and IgG, eosinophils release their granules, causing damage or even death of the parasites. Furthermore, the production of ROS enhances their ability to disrupt the structural integrity of pathogens (80–82).

Eosinophils show notable numerical and functional changes during filarial infections. In patients with Tropical Pulmonary Eosinophilia (TPE) caused by *W. bancrofti* or *B. malayi*,

peripheral eosinophil counts can exceed 3,000/µL and may reach up to 80,000/µL during acute phases (83,84). Interestingly, these counts often exhibit diurnal variation, being lower at night when symptoms like coughing are more severe, possibly due to eosinophil sequestration in lung tissue (85).

Post-treatment dynamics also highlight the immunological role of eosinophils. Following diethylcarbamazine (DEC) therapy against *W. bancrofti*, eosinophil counts initially drop due to tissue migration, then rebound within 24 hours, correlating with inflammatory responses (86).

In *M. perstans* infections, eosinophil responses appear more modest. A case study reported eosinophil counts of 3% pre-treatment, decreasing to 1% post-therapy, indicating a mild but present eosinophilic response (87).

Beyond their direct cytotoxic functions, eosinophils also play a regulatory role in immune responses by producing and releasing cytokines, such as IL-4, IL-5, IL-13, and TGF-β. These cytokines promote a type 2 immune response, which is essential for combating helminth infections (28). Additionally, eosinophils express class II major histocompatibility complex (MHC) and co-stimulatory molecules, enabling them to present antigens to T cells, thereby linking innate and adaptive immune responses (88). Furthermore, eosinophils interact with other immune cells, such as mast cells (89), basophils (90), and macrophages by releasing mediators that modulate immune responses (91). These interactions contribute to the

coordination and amplification of immune defense mechanisms, ensuring a comprehensive response to parasitic infections.

The interaction between eosinophils and filariae, including microfilarial stages, plays a significant role in disease progression. These interactions can have both protective and pathological consequences (21). On the one hand, eosinophils contribute to parasite elimination through ADCC-mediated killing and ROS-induced damage (80–82,92). Eosinophil-mediated activation of Type 2-driven immune responses also aids in controlling parasite survival. On the other hand, excessive eosinophil activation and degranulation can lead to harmful effects, such as inflammation and tissue damage (21). This may contribute to clinical manifestations like lymphedema and skin lesions (93). Additionally, chronic eosinophil activity promotes fibrosis through collagen deposition, leading to long-term tissue dysfunction and structural damage (94).

Overall, eosinophils play a dual role in immune defense, providing protection against parasitic infections while also contributing to immune-mediated tissue damage when excessively activated. Their involvement in filarial infections underscores the complexity of their interactions with both pathogens and the host immune system. A more detailed understanding of these mechanisms is essential for developing targeted therapies that optimize the protective functions of eosinophils while minimizing their harmful effects.

1.2.3. ETosis

Granulocytes, including eosinophils, possess an effector mechanism known as ETosis — short for extracellular trap-mediated cell death, which is distinct from apoptosis and necrosis (95). Initially observed in neutrophils, this process entails the explosive release of intracellular DNA into the extracellular environment, thereby entangling pathogens and delivering antimicrobial peptides. As triggered by various pathogens and stimuli, the process of ETosis involves DNA decondensation, membrane collapse, and the release of DNA mixed with antimicrobial peptides, aiding in trapping and killing invading organisms (96–98). The mechanism of ETosis follows a structured sequence of cellular events. Upon cell activation, chromatin within the eosinophil nucleus begins to decondense, driven by intracellular signaling cascades initiated by specific triggers. This step is crucial for the unraveling of nuclear DNA, making it available for extracellular release. Triggering signals include adhesion of various stimuli, pathogens or mediators to substrates, as ETosis is strongly enhanced by integrin-mediated adhesion, and stimulation through cytokines (IL-5, IL-33), immune complexes (IgG, IgA), bacterial or fungal components (e.g., LPS, zymosan), and pharmacological agents like PMA and calcium ionophore ionomycin. Recent insights into ETosis mechanisms have highlighted two distinct but potentially overlapping pathways: the NADPH oxidase (NOX)-dependent and the Ca^{2+}-dependent route (99). The

NOX-dependent pathway is characterized by ROS production at the plasma membrane via NADPH oxidase activation. In neutrophils, this leads to the release and nuclear translocation of neutrophil elastase, which facilitates histone cleavage and chromatin unfolding (100,101).

In eosinophils, ROS similarly support PAD4 activation, which catalyzes histone citrullination, weakening DNA-histone interactions and promoting chromatin decondensation (102,103). Conversely, Ca^{2+}-ionophores such as ionomycin trigger mitochondrial ROS generation and PAD4 activation independently of NOX, underscoring the flexibility of the ETosis process in response to different stimuli (99).

Furthermore, recent findings demonstrate that viable microfilariae induce eosinophil ETosis (EETosis) through pattern recognition receptor signaling and inflammasome activation (104,105). Specifically, dectin-1 recognition of microfilarial components leads to activation of the AIM2-canonical inflammasome in a pathway dependent on caspase-1, ASC, and gasdermin D (106). This mechanism, analogous to inflammasome-driven NETosis in neutrophils, highlights a convergent inflammatory platform that facilitates chromatin release in response to helminth infection. Following chromatin decondensation, the nuclear envelope disintegrates in a controlled manner, distinct from necrosis, allowing the expanded chromatin to mix with cytoplasmic components. At this stage, the cell undergoes plasma membrane rupture, and

the decondensed DNA, often decorated with intact granules, is extruded into the extracellular space. In eosinophils, this process may include both nuclear and mitochondrial DNA release. Mitochondrial ETosis (mtETosis) is thought to enable a faster, ROS-dependent extracellular DNA response without causing immediate cell death, maintaining some cytoplasmic integrity (99). Eosinophils can release mitochondrial DNA in response to specific stimuli, often without undergoing cell death, a process referred to as ‘vital ETosis’. While this phenomenon is characteristic of eosinophils, it has also been observed in neutrophils under certain conditions, though less commonly (99,100). Different cell types like eosinophils, basophils, and mast cells, respond via ETosis to pathogens, antibodies, and pathogen-derived products. Triggers like PMA, zymosan, calcium ionophore, IgG, IgA, and cytokines (e. g. IL-5, IL-33, IFNγ) induce ETosis in eosinophils (95,101–103). The source of ETosis-derived extracellular DNA remains debated, with some suggesting mitochondrial DNA release and others proposing chromatin decondensation and nuclear envelope disaggregation (104,105). Neutrophil and eosinophil extracellular traps differ structurally and in content. NETs consist of decondensed nuclear chromatin coated with granule-derived and cytosolic proteins like neutrophil elastase (NE) and myeloperoxidase (MPO) (98,106). This loose, protein-decorated DNA matrix remains accessible to nucleases and is rapidly degraded in vivo, a process that aids

in resolving inflammation. In contrast, eosinophil extracellular traps (EETs) are structurally more compact and are enriched with intact eosinophilic granules. These granules contain cytotoxic proteins like EPO, MBP, and ECP, enhancing the antimicrobial activity. Importantly, EETs show significantly lower proteolytic activity and higher resistance to nucleases than NETs, allowing them to persist longer in tissue environments (103). The low protease content and dense chromatin packing in EETs — along with limited endogenous DNase activity — contribute to their increased stability and prolonged presence in inflamed tissues. This can be beneficial for pathogen control but may also perpetuate inflammation in chronic diseases such as asthma (99,107). Additionally, Eosinophil-released, mitochondria-derived DNA tends to be more condensed and resistant to enzymatic degradation. Because EETs contain fewer endogenous nucleases and lack the aggressive proteolytic remodeling found in NETs, they are more persistent and less easily degraded by DNases (99,108). These differences contribute to the prolonged stability and pathogen-trapping function of eosinophil extracellular traps, even though EETs may also exacerbate tissue inflammation in a chronic disease context.

1.2.4. Eosinophile Peroxidase and Major Basic Protein

The EPO and MBP, along with EDN and ECP, are important granular components that can be released during ETosis. EPO generates ROS by catalyzing the oxidation of halides and pseudohalides in the presence of hydrogen peroxide (75). This process produces hypohalogenous acids which own antimicrobial properties and can damage pathogens. In allergic reactions and inflammatory conditions, EPO can lead to tissue damage by promoting oxidative stress and inflammation (109). MBP makes up about half of the granules of eosinophils (110). It plays a crucial role in the defence of parasitic infections, especially helminths. MBP is toxic to parasites and can affect membranes, leading to their destruction (77). In addition to its antiparasitic effect, MBP is also involved in inflammatory reactions and tissue damage associated with allergic diseases, such as asthma and allergic rhinitis (68). MBP can activate mast cells and basophils, promoting the release of histamine and other inflammatory mediators, thereby contributing to allergic reactions (111,112). Both EPO and MBP are essential components of the eosinophil arsenal against pathogens.

1.2.5. The role of eosinophils and their effector mechanisms in filarial infections

In the context of filarial infections, eosinophils contribute to immune defense not only through cytotoxic granule release and

cytokine production, but also via the formation of extracellular traps. ETosis is nowadays recognized as a common effector mechanism of innate immune cells that also is utilized by eosinophils in response to parasites. EETs have been shown to entrap MF, limiting their movement and facilitating immune-mediated killing. These traps are composed of DNA fibers decorated with granule proteins such as EPO, MBP, and ROS, which can damage both MF and adult filariae (68,95,103,113).
Studies in the *L. sigmodontis* mouse model demonstrated that mice lacking EPO or MBP show significantly higher worm burdens, highlighting the crucial role of these granule proteins in parasite control (64). Similarly, eosinophil-mediated cytotoxicity has been documented in *B. malayi* infections, where eosinophils contribute to parasite clearance through both granule release and ADCC. However, the absence of a single granule component in these models often only partially impairs parasite killing, suggesting that eosinophils employ multiple mechanisms (114).
Eosinophils also play a crucial role in *O. volvulus* infections, where the death of MF in the skin and cornea can lead to dermatitis and vision loss, respectively. Although neutrophils are primarily associated with ocular pathology in onchocerciasis, eosinophils have been shown to infiltrate infection sites following stimulation by *Wolbachia*-derived products (115,116). In vitro studies revealed that eosinophil products like MBP and ECP can damage corneal

cells, suggesting a pathological role in vision loss (117). Interestingly, hyper-reactive onchocerciasis patients, who exhibit strong immune responses against MF, display significantly higher ECP levels and suffer from severe dermatitis, despite having low or absent MF counts in the skin (51). This indicates a pivotal role of eosinophil-driven inflammatory responses in these individuals.
Moreover, during treatment with the microfilaricidal drug DEC, patients often experience inflammatory reactions known as Mazzotti reactions, characterized by extensive eosinophil infiltration and coating of dead MF with MBP (118). In contrast, ivermectin treatment leads to eosinophil accumulation in regional lymph nodes, along with elevated levels of ECP and EPO, highlighting the dynamic response of eosinophils to different therapeutic agents (119).
In *Loa loa* infections, eosinophils are implicated in both protective and pathological responses (120). Treatment with ivermectin and DEC is associated with increased eosinophil activation and infiltration into affected tissues, including the brain, where severe complications like encephalitis can occur. Studies in hyper-microfilaremic baboons showed that eosinophils and macrophages block capillaries in the brain parenchyma, causing microvascular damage (121). Interestingly, temporary residents in endemic regions exhibit stronger eosinophilic responses compared to locals, correlating with symptoms like Calabar swelling (122). Furthermore, in *W.*

bancrofti infections, eosinophils are key players in the pathogenesis of TPE, a hypersensitivity reaction directed against MF trapped in the lungs. Here, eosinophil accumulation and degranulation contribute to airway inflammation, tissue damage, and elevated eosinophil counts - sometimes exceeding 80,000 eosinophils/µL in peripheral blood (83). The detrimental role of eosinophils is further supported by murine models that mimic TPE, where MF entrapment in lung tissue leads to pronounced eosinophilic infiltration and exacerbating inflammation (123,124). Eosinophils also regulate immune responses through the release of cytokines such as IL-4, IL-5, and IL-13, which drive type 2 immunity (28). Additionally, eosinophils function as antigen-presenting cells by expressing MHC class II and co-stimulatory molecules bridge innate and adaptive immune responses. Their interactions with mast cells, macrophages, and other leukocytes further shape the local immune environment during infection (112,113).

Interestingly, in *O. ochengi* natural infections, cattle show a dominance of neutrophils within nodules, while eosinophils only infiltrate these sites upon the depletion of *Wolbachia* endosymbionts (125,126). This suggests that *Wolbachia* may play a protective role for adult worms by preventing eosinophil-mediated damage. In *O. lienalis* mouse models, eosinophils are essential for protective immunity upon secondary exposure to MF, demonstrating their role in adaptive immune responses (127).

Although EETs are efficient in trapping and damaging parasites, their stability and resistance to DNase-mediated degradation - due to the presence of intact granules and low proteolytic activity - may also prolong local inflammation (95). This persistence can exacerbate tissue fibrosis, particularly in chronic infections. Thus, eosinophils play a dual role: they contribute to protective immunity against filariae but also to the development of filarial pathology when dysregulated (21).

2. Original Publications

2.1. Major basic protein and eosinophil peroxidase support microfilariae motility inhibition by eosinophil ETosis

Schumacher PP, Ajendra J, Lenz B, Risch F, Ehrens A, Nieto-Pérez C, Koschel M, Aden T, Hoerauf A, Hübner M

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Own part in the publication:

- Project planning: 70 %, together with co-authors and supervisor
- Conduction of experiments: 90 %, together with co-authors
- Evaluation of experiments: 90 %, mainly independent
- Writing of the manuscript: 80 %, together with supervisor

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Major basic protein and eosinophil peroxidase support microfilariae motility inhibition by eosinophil ETosis

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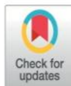

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Abstract

Eosinophils are a hallmark of filarial infections. They are primary effector cells and can attack filariae by releasing extracellular traps that contain toxic cationic proteins, such as eosinophil peroxidase and major basic protein. Previous studies demonstrated that the extracellular traps of eosinophils are induced by the microfilariae of *Litomosoides sigmodontis* and that they inhibit their motility. In this project, we aimed to investigate the role of these cationic proteins during the extracellular trap-mediated immobilization of microfilariae. Our results indicate that extracellular DNA traps from knockout mice that lack eosinophil peroxidase or major basic protein are significantly less able to immobilize and kill microfilariae. Accordingly, the addition of these cationic proteins to *in vitro* cultures inhibited microfilariae motility in a dose-dependent manner. Moreover, we examined eosinophils from the natural host, the cotton rat *Sigmodon hispidus*. While eosinophils of cotton rats release DNA after stimulation with PMA and zymosan, microfilariae did not trigger this effector function. Our work shows that eosinophil granule proteins impair the motility of microfilariae and indicate significant differences in the effector functions of eosinophils between the mouse model and the natural host. We hypothesize that the absence of DNA nets released by cotton rat eosinophils in response to microfilariae may explain the higher microfilarial load and longer patency of the natural host.

Author summary

Filarial nematodes are thread-like worms that can cause debilitating diseases such as river blindness (onchocerciasis) or elephantiasis (lymphatic filariasis). Similar to most helminth infections, filarial infections lead to eosinophilia, an increase of eosinophil granulocyte numbers in the peripheral blood. Although eosinophils have been shown to contribute actively to the protective immune responses, the underlying mechanisms are not yet fully understood. In the present study, we investigated the protective effect of the eosinophil granule proteins major basic protein (MBP) und eosinophil peroxidase (EPO) against the filarial progeny, the microfilariae, of the rodent filarial nematode *Litomosoides*

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sigmodontis. Eosinophils lacking MBP or EPO inhibited microfilariae motility *in vitro* to a lesser extent than wild-type eosinophils. Consistent with these results, the addition of MBP and EPO inhibited microfilariae motility *in vitro*. Additionally, we demonstrate that eosinophils from the natural host of *L. sigmodontis*, the cotton rat, did not inhibit microfilariae motility in the same way, indicating either intrinsic differences among cotton rat and mouse eosinophils or an adaptation of the parasite to its natural host.

Introduction

Eosinophils are a hallmark of helminth infections, exhibiting both protective and pathological functions in the immune response to filarial infections [1–3]. Eosinophils produce crystalloid granules that contain major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO), which are associated with the killing of the filarial progeny (microfilariae (MF)) and drug-induced adverse events in filariasis patients.

During onchocerciasis (aka river blindness), the death of *Onchocerca volvulus* MF in the skin and the cornea can lead to dermatitis and vision loss, respectively. While the results on the role of eosinophils and their granules in the development of keratitis are controversial [4–6], their impact on dermatitis is more clear. Increased inflammatory immune responses against the MF, as observed in hyper-responsive onchocerciasis patients or following DEC treatment, are associated with eosinophil responses and significantly elevated ECP, EDN and MBP levels [7–9]. Such an increased eosinophil recruitment and activation was also observed in humans and primate animal models following treatment-induced killing of MF of the African eye worm *Loa loa* [10,11]. Similarly, temporary residents, who more frequently develop clinical symptoms during infections with *L. loa* and following DEC treatment are associated with higher eosinophil counts and eosinophil granule protein levels in comparison to endemic individuals [12,13]. Despite these findings, treatment with anti-IL-5 antibodies to reduce eosinophil responses following DEC treatment in loiasis patients did not reduce the adverse events [14]. Thus, more work is required to decipher the role of eosinophils and their granule proteins during filarial pathology. In the case of tropical pulmonary eosinophilia (TPE), a severe disease that affects some patients with lymphatic filariasis, eosinophil responses to MF are the cause of severe lung inflammation associated with increased levels of EDN and MBP [15–18].

With regard to protective immune responses to filariae, studies on diverse filarial species and animal models have demonstrated that eosinophils support the clearance of adult worms [1–3,19,20]. Interestingly, in nodules containing adult *Onchocerca* filariae, it was shown in cattle as well as humans that neutrophils primarily surround viable adult worms and release extracellular DNA traps. Only after antibiotic clearance of the *Wolbachia* endosymbionts of filariae did eosinophils infiltrate the nodules and degranulate, supporting a role in worm killing [21–23]. Eosinophils also contribute to the clearance of MF, as was shown by injection of *Brugia malayi* MF in naïve wildtype (WT) and eosinophil-deficient PHIL mice [24]. Of note, lack of MBP and EPO did not impact the survival of *B. malayi* MF after injection in naïve mice in comparison to WT controls [24]. This is in contrast to *in vitro* findings, where MBP, ECP, EDN, and EPO were shown to kill *B. malayi* and *Brugia pahangi* MF [25]. Furthermore, data from a mouse model with experimentally injected *Onchocerca lienalis* MF indicated that mice develop resistance to a re-injection with MF, which was dependent on the presence of eosinophils [26].

In line with the results described above, *Litomosoides sigmodontis* infections in eosinophil-deficient dblGATA mice and IL-5 deficient mice resulted in a higher worm

burden, prolonged worm survival and increased MF load [27,28]. In line, eosinophilic IL-5 transgenic mice had a significantly reduced *L. sigmodontis* adult worm burden [29] suggesting an essential role of eosinophils in host protection. The cytotoxic granule proteins MBP and EPO have been linked to susceptibility to infection with *L. sigmodontis* before [30], as mice on the "resistant" 129/SvJ background deficient for either EPO or MBP developed a significantly higher worm burden than 129/SvJ WT mice [30]. More recently, our group demonstrated that eosinophils release extracellular DNA traps (EETosis) during filarial infections [31,32]. *In vitro,* eosinophils undergo ETosis upon MF stimulation, which inhibits MF motility in a contact-dependent manner. EETosis has also been shown to facilitate MF clearance *in vivo* [31].

Next to citrullinated histones, eosinophil extracellular traps contain the granule proteins ECP, EDN, MBP, and EPO [1]. In this study, we investigated whether the co-localized eosinophil granule proteins MBP and EPO are required for the EETosis-mediated immobilization and potential killing of MF. By utilizing eosinophils of MBP- and EPO-deficient mice and co-cultures with MBP and EPO, we found that these granule proteins indeed contribute to eosinophil-mediated MF immobilization and may play a role in MF killing. Interestingly, studies with the natural cotton rat host *Sigmodon hispidus* revealed that their eosinophils are less able to immobilize MF, suggesting a better adaptation of *L. sigmodontis* to its natural host compared to laboratory mouse strains or intrinsic differences in eosinophil effector functions of both host species. In conclusion, our study provides a deeper insight into the effector functions of eosinophil and the phenomenon of EETosis during filarial infections.

Methods

Ethics statement

All experiments were performed according to EU directive 2010/63/EU and approved by the appropriate authority Landesamt für Natur-, Umwelt- und Verbraucherschutz (LANUV, Recklinghausen, Germany; AZ 81-02.04.2020.A103; 81-02.04.40.2022.VG029).

Mice/ Cotton rats

129/SvJ wildtype (WT) mice were purchased from Charles River Laboratories. EPO and MBP-knock-out (KO) mice were originally obtained from Nancy A. Lee and James J. Lee, Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, Scottsdale, Arizona, USA, and were bred at the "Haus für Experimentelle Therapie" of the University Hospital Bonn, Germany. Mice of both sexes were used.

Cotton rats (*Sigmodon hispidus*) were bred at the Institute for Medical Microbiology, Immunology and Parasitology. Female cotton rats were used. Mice and cotton rats were housed in individually ventilated cages with unlimited access to food and water and a 12-h day/night cycle. Knockout of MBP and EPO was confirmed by PCR.

(EPO-KO: P1: 5′-TGAAACCCCCAAACTGACGG-3′, P2: 5′-ACAGAGCTAAGC GGGACGTG-3′, P3: 5′-CATCGAGCGAGCACGTACTC-3′; MBP-KO: P1: 5′-ATGC CACTGTGAGACAGGGTAAGG-3′, P2: 5′-CAGATGAAGAGCAGACGCTC-3´, P3: 5′-GAACCAGCTGGGGCTCGAG-3′)

Microfilariae isolation

MF were isolated as described before [33]. In brief, cotton rats infected with *L. sigmodontis* were anaesthetized with isoflurane and blood was collected. The collected blood was then diluted 1:2 with Advanced RPMI 1640 medium (Gibco, ThermoFisher Scientific,

California, USA) containing 10% FBS (Gibco, ThermoFisher Scientific, California, USA), 1% L-glutamine (Gibco, ThermoFisher Scientific, California, USA) and 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific, California, USA). A sucrose-percoll gradient was used for MF purification as previously described [33]. Therefore, a gradient of 30% and 25% percoll-sucrose-solution (Cytiva, Global Life Sciences Solutions USA, Marlborough USA) was added to a 15 mL tube and the diluted blood was layered on top. The gradient was centrifuged without breaks at 340 g for 30 min at room temperature and MF were collected from the layer between the 25 and 30% solution. The purified MF were washed 3 times with Advanced RPMI medium and then diluted in 1 mL medium and quantified with a Neubauer counting chamber.

Eosinophil generation

Bone marrow from naïve SvJ/129, EPO-KO and MBP-KO mice and naïve cotton rats was used to generate bone marrow-derived eosinophils as previously described [31,32]. After red blood cell lysis (Invitrogen, eBiosience ThermoFisher Scientific, California, USA) for 5 min with 1 mL of RBC lysis buffer diluted 1/10 in dH_2O, bone marrow cells were counted using the CASY TT- cell counter system. 1 x 10^6 cells/mL were seeded in pre-warmed Advanced RPMI medium supplemented with 20% FBS, 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific, California, USA), 0.1% gentamycin (Gibco, ThermoFisher Scientific, California, USA), 2.5% HEPES (Gibco, ThermoFisher Scientific, California, USA) and 1% Glutamax (Thermo Fisher Scientific GmbH, Germany). The cells were cultured for the first 4 days with stem cell factor (SCF, 100 ng/mL) (Peprotech, Rocky Hill, USA) and FMS-like tyrosine kinase 3 ligand (FLT3L, 100 ng/mL) (Peprotech, Rocky Hill, USA). The growth factors were then exchanged with IL-5 (20 ng/mL) (Peprotech, Rocky Hill, USA). Half of the medium was replaced every other day. The cell culture flask was replaced on day 6 for cotton rat and day 8 for mouse eosinophils. Cells were harvested after 12 days for mouse eosinophils and 8-10 days for cotton rat eosinophils.

The cells were then checked for purity by flow cytometry for mouse eosinophils with the surface marker SiglecF (APC-Cy 7 rat anti-mouse Siglec-F, Dye: APC-Cy7, Clone: E50-2440, BD Biosciences, Franklin Lakes, USA) or Diff Quick (RAL Diff-Quick kit, RAL DIAGNOSTICS, Martillac, France) staining for cotton rat eosinophils as there are no cross-reactive antibodies for cotton rats, which could be used for flow cytometry. The resulting purities of the cell cultures were 91.0 - 97.3% for WT SvJ eosinophils, 90.3 - 92.5% for MBP-deficient eosinophils, 90.7 – 97.6% for EPO-deficient eosinophils and 96.4 - 97.7% for the cotton rat eosinophils.

A separate experiment was performed to identify additional cell populations that were present alongside the bone marrow-derived eosinophils. The following antibodies were used (antibodies were sourced from Biolegend, San Diego, USA unless stated otherwise): αCD11b-Al700 (clone: M1/70), αLy6G-PE-Cy7 (clone: 1A8), αF4/80-PerCP-Cy5.5 (clone: BM8), αCD4-Al488 (clone: RM4-5), αCD19-APC (clone: 6D5), αCD8-APC-Cy7 (clone: 53-6.7), αCD1-BV510 (clone: 145-2C11), αCD11c-BV605 (clone: N418). αCD3-BV510 (clone: 145-2C11), αI-A/I-E-BV421 (clone: M5/114.15.2) and αSiglecF-PE (E50-2440, obtained from BD Biosciences, Franklin Lakes, USA). In this experiment, eosinophils constituted 95.9% of cells, neutrophils constituted 1.46% of cells and the remaining cell population frequencies ranged between 0.02% and 0.56% and were made up of DCs and monocytes (S1 Fig). Antibodies to differentiate mature from immature granulocytes were not included in the analysis. Diff Quick staining of cotton rat eosinophils revealed a proportion of ~ 2% non-eosinophils. These non-eosinophil cells represented neutrophil granulocytes, lymphocytes, plasma cells and other cell populations (S2A Fig).

Establishment of co-culture

Co-cultures were prepared in F-bottom 96-well plates with 1 x 10^5 eosinophils as well as 2.5 x 10^3 MF or 1 x 10^4 MF per well in Advanced RPMI medium. The medium was supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific, California, USA), 0.1% gentamycin (Gibco, ThermoFisher Scientific, California, USA), 2.5% HEPES (Gibco, ThermoFisher Scientific, California, USA) and 1% Glutamax (Thermo Fisher Scientific GmbH, Germany). As positive control, eosinophils were stimulated with phorbol-12-myristat-13-acetat (PMA, 0.5 μg/mL) (Cayman Chemical, Michigan, USA) and zymosan (0.5 mg/mL) (InvivoGen, Toulouse, France). In a subset of experiments, eosinophils were co-cultured with 0.1, 1 or 10 ng/mL MBP or EPO (EPO: LS Bio, Lynwood Australia, MBP: My Bio Source, San Diego, USA). The co-cultures were incubated at 37°C for either 24 hours (DNA release) or 72 hours (motility assays). After 72 hours, eosinophil viability dropped to 86-90%, preventing further analysis. As an internal control, a subset of samples were treated with DNase (20 U/mL) to ensure that the measured release was indeed DNA.

Motility assay

For the motility assay, MF were scored after 4, 24, 48, and 72 hours. The motility of 20 MF per well were scored from three separate wells using a bright-field microscope (Leica Model DMIL LED, Leica Microsystems CMS GmbH, Wetzlar, Germany, magnification 10x and 20x). The motility score ranged from 0 to 4 with 0 meaning no movement and 4 representing continuous and fast motility. MF with a score of 3 showed slower but continuous movements, while a score of 2 indicated slow and discontinuous movements. MF with a score of 1 only showed sporadic movements at the ends. The assessment was done in a blinded manner.

DNA quantification

For DNA quantification, the DNA Quant-iT kit (Invitrogen by Thermo Fisher Scientific GmbH, Germany) was used as previously described [31,32]. Therefore, the samples were treated with micrococcal nuclease for 15 min at 37°C to break up the released DNA networks for better measurement. This process was stopped with 1 mM EDTA. After centrifugation at 400 g for 10 minutes at 4°C, 20 μl of sample and 10 μl of the supplied standard were added to a 96-well F-bottom plate and 100 μl of the 1/200 diluted DNA Quant-iT-dsDNA HS reagent was added. The absorbance was measured using a Tecan Infinite 200 Pro plate reader (Tecan Trading AG, Switzerland) at excitation/emission wavelengths of 485/535 nm. DNA was quantified via the absorbance values of the standard.

Fluorescence microscopy

1 x 10^5 murine bone marrow-derived eosinophils were co-cultured in Advanced RPMI 1640 medium containing 1% penicillin, 1% streptomycin, 0.1% gentamycin, 1% L-glutamine, 10% FBS and 20 ng/mL of murine recombinant IL-5 with 1 x 10^4 MF in 96 well plates for 24 and 72 h. After incubation, plates were centrifuged for 10 minutes with 400 g at RT and the supernatant was removed. 200 μl PBS including 0.002% propidium iodide was added to each well and incubated for 20 min at 37°C with 5% CO_2. The plates were then centrifuged at 400 g for 6 min at room temperature and 50 μl were replaced with fresh PBS. For DAPI staining, 15 mm coverslips were coated in a 24-well plate with 400 μl poly-L-lysine/well overnight at 4°C. The coverslips were then washed three times and the supernatant was discarded. The slides were then sterilized for 20 min under UV light and air-dried. For each well, 1 x 10^5 bone marrow-derived eosinophils of the cotton rat were added and incubated with PMA (Cayman

Chemical, Michigan, USA), zymosan (InvivoGen, Toulouse, France) or MF for 24 hours at 37°C. Advanced RPMI medium supplemented with 2.5% FBS, 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific, California, USA), 0.1% gentamycin (Gibco, ThermoFisher Scientific, California, USA), 2.5% HEPES (Gibco, ThermoFisher Scientific, California, USA) and 1% Glutamax (Thermo Fisher Scientific GmbH, Germany) was used. Afterwards the cells were fixed with 4% paraformaldehyde (ThermoFisher Scientific, California, USA) and washed with H_2O. Eosinophils were then permeabilized with 2% Bovine Serum Albumin (PAN Biotech, Passau, Germany) and 0.1% Triton-X (ThermoFisher Scientific, California, USA) overnight at 4°C. After washing with PBS, the cells were stained with 1/1000 diluted DAPI (300 nm, ThermoFisher Scientific, California, USA) with or without 5 µg/ml wheat-germ-agglutinin in PBS (ThermoFisher Scientific, California, USA) for 20 min at room temperature in the dark. The reaction was stopped through the addition of H_2O and the supernatant was discarded. The cells were analyzed using the Axio Observer 7 with the Zen2.6 software (Carl Zeiss Microscopy Deutschland GmbH, Germany).

Statistics

Prism 7.0 (version 7.0c, GraphPad Software) was used for statistical analysis. The data were tested for normality using the Shapiro-Wilks test. Differences between experimental groups for non-parametric data were determined using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. The differences between the experimental groups for parametric data were assessed by one-way-ANOVA followed by Sidak's post-test or two-way-ANOVA followed by Bonferroni post-test. The data are presented as scatter dot plot with mean and SEM.

Results

Inhibition of microfilariae motility by eosinophils is impaired in the absence of MBP and EPO

To investigate the contribution of MBP and EPO in immobilizing MF, bone marrow-derived eosinophils of MBP-KO, EPO-KO, and 129/SvJ WT control mice were co-cultured with isolated MF from cotton rats for 72 hours and MF motility was monitored over time.

Already after 4 hours of culture, MF cultured with eosinophils from WT mice showed significantly decreased motility (mean score of 2.99, continuous slow movement) compared to MF cultured with eosinophils from mice lacking MBP or EPO, or MF alone. MF from the latter groups still showed a continuous fast movement (EPO-KO mean score of 3.42; MBP-KO mean score of 3.45; MF alone mean score of 3.56; Fig 1).

At later time points (24 h, 48 h, and 72 h), MF motility decreased under all conditions compared to MF-only controls. However, WT eosinophils reduced MF motility significantly more than eosinophils from MBP- and EPO-KO mice (Fig 1). In line, completely immobile MF as well as MF staining positive for propidium iodide were more frequent in co-cultures with WT eosinophils compared to MBP-KO and EPO-KO eosinophil co-cultures, indicating a potential impact of MBP and EPO on MF killing (S3, S4A-C and S5A-B Figs). Taken together, this data demonstrates that both MBP and EPO are important for the eosinophil-dependent immobilization of MF.

DNA release in mice lacking EPO or MBP is similar to WT controls

To confirm that the reduced ability of eosinophils from mice lacking EPO or MBP to inhibit MF motility was not due to an impaired ability to undergo EETosis, we next examined the presence of free extracellular DNA in the culture supernatants from the experiments shown in Fig 1. Flow cytometric analysis of the eosinophils revealed a purity of >90%, which was

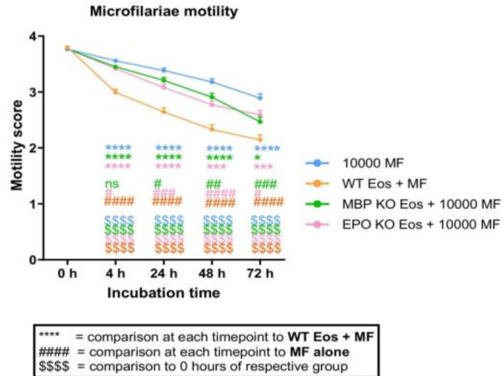

Fig 1. MBP and EPO contribute to inhibition of MF motility. Motility of 10000 MF that were co-cultured with bone marrow-derived eosinophils from MBP-KO, EPO-KO, and SvJ wildype (WT) control mice for up to 72 h. Pooled data from 5 independent experiments with a total of n = 300 microfilariae (MF) (3 wells per experiment with 20 MF each). Motility was documented per condition, not per well. Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparisons test (A-B). *,# p<0.05, ## p<0.01, ***,### p<0.001, ****,$$$$,#### p<0.0001, ns = not significant.

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comparable for all mouse strains (Figs 2A and S1). The eosinophils were cultured for 24 h with MF and the known EETosis inducers zymosan and PMA as positive controls. As shown in Fig 2B, MF lead to DNA release, while stimulation with PMA and zymosan induced the highest DNA release in this setup. As expected, DNase treatment reduced the DNA amount (Fig 2B). When comparing extracellular DNA release by eosinophils derived from EPO-KO and MBP-KO mice with 129/SvJ WT control eosinophils, similar though not identical patterns for MF-induced DNA release were observed for eosinophils from all three-mouse strains after 24 h (Fig 2C). After 72h, DNA was also released after stimulation with MF from eosinophils of all three mouse strains (Fig 2D). Notably, MBP-KO eosinophils released significantly lower amounts of DNA compared to WT and EPO-KO eosinophils after stimulation with MF (Fig 2D). No statistical significant difference in DNA release in response to stimuli like PMA and zymosan was observed among the three groups after 24 or 72 hours (Fig 2E-F). In general, MBP-deficient eosinophils showed the lowest DNA release at both time points, although this difference did not reach statistical significance. ELISAs to detect EPO and MBP were performed from cell culture supernatants from eosinophils stimulated with PMA, zymosan and MF, but the levels were below the detection limit.

In summary, these results show that stimulation with MF leads to DNA release in both WT as well as EPO and MBP KO mice, although MBP KO eosinophils showed slightly lower DNA

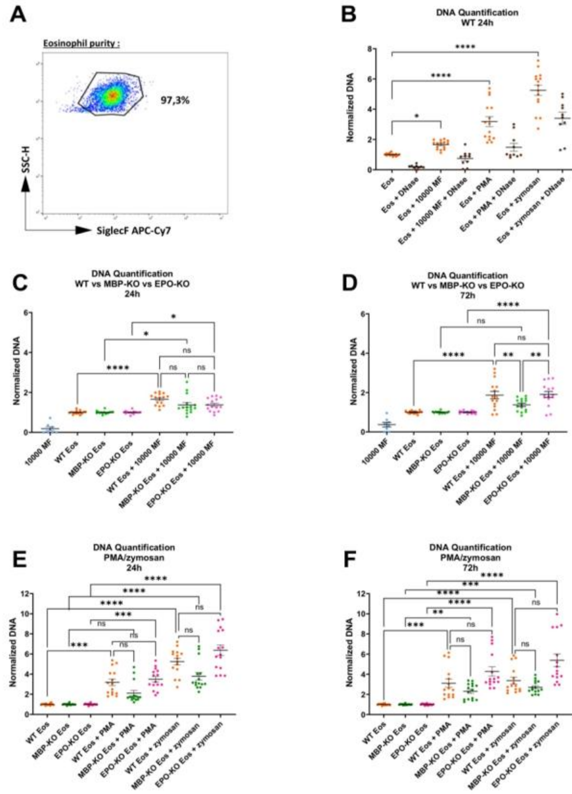

Fig 2. Similar DNA release of eosinophils of mice lacking EPO or MBP and WT controls. Representative flow cytometry-plot displaying the purity of bone marrow-derived eosinophils. Eosinophils were identified as SiglecF+ and SSC-H high (A). Normalized DNA in

supernatant of bone marrow-derived eosinophils from SvJ wildtype (WT) mice stimulated for 24 h with 10000 microfilariae (MF), PMA, or zymosan (B). Pooled data from 5 independent experiments with a total of n = 300 MF (3 wells per experiment with 20 MF each). Normalized DNA in supernatant of bone marrow-derived eosinophils from SvJ WT, MBP-KO and EPO-KO mice stimulated for 24 h (C,E) and 72 h (D,F) with 10000 MF or PMA and zymosan. Pooled data from 5 independent experiments with a total of n = 300 MF/ 15 wells. Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparisons test for selected groups for non-parametric data (B-F). Data is shown as median with interquartile range (B) and Min to Max (C-F). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = non-significant.

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release in some conditions. One possible explanation for this observation is the sheer amount of MBP that is present in eosinophils under normal circumstances. MBP usually makes up about half of all proteins in eosinophils and, as a result, eosinophils from MBP KO mice might require less DNA to transport their granule proteins out of the cell during EETosis. Nevertheless, both granule proteins contributed to the reduction of MF motility, confirming our hypothesis that both proteins contribute to MF immobilization in mice.

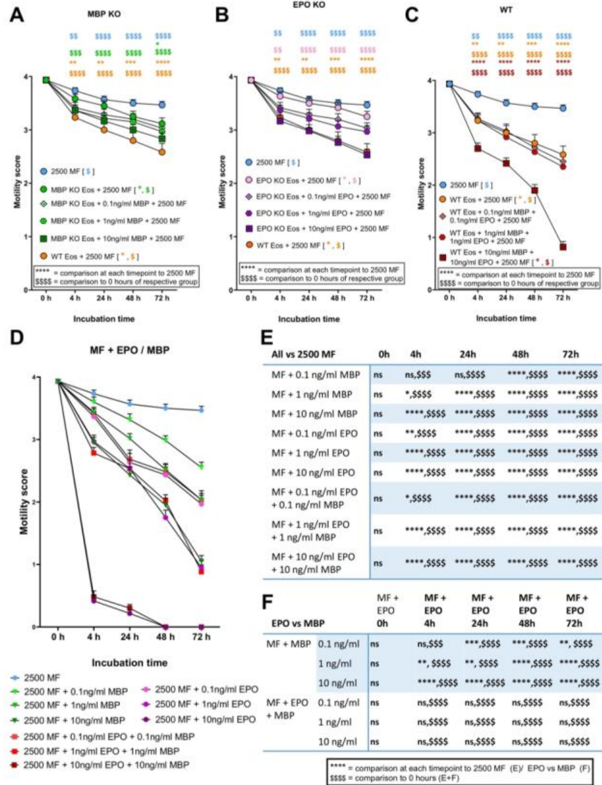
MBP and EPO inhibit the motility of microfilariae *in vitro* in a concentration dependent manner

The results of the previous experiments indicated that the DNA inside the DNA traps is not solely responsible for the immobilization of MF. Instead, it seems likely that a significant function of the released DNA is dedicated to the guided transportation of granule proteins directly to the immobilized pathogen. To investigate the direct influence of MBP and EPO on MF motility, co-cultures with different concentrations of both proteins were performed in the absence or presence of WT and KO eosinophils.

Both MBP (Fig 3A) and EPO (Fig 3B) enhanced the MF motility inhibition in the presence of the respective MBP- and EPO-KO eosinophils in a dose-dependent manner. MBP- and EPO-KO eosinophils achieved a similar MF motility inhibition with 10 ng/ml MBP and EPO (slow continuous to discontinuous MF movements, mean score of 2.83 and 2.53, respectively) as the co-culture with WT eosinophils (mean score of 2.58) after 72h. The addition of MBP and EPO to WT eosinophils further reduced the MF motility in a dose dependent manner, reaching a MF motility mean score of 0.82 after 72 hours of co-culture with 10 ng/ml of both proteins, indicating sporadic movement at the distal end of the MF (Fig. 3C). Finally, we examined whether MBP and EPO inhibit MF motility in the absence of eosinophils. Indeed, MBP and EPO inhibited MF more efficiently in the absence of eosinophils, resulting in complete inhibition of MF motility after 72 h of co-culture with 10 ng/ml EPO plus 10 ng/mL MBP or 10 ng/ml EPO alone (Fig 3D-F). The addition of 10 ng/ml MBP led to sporadic, distal movements of MF (mean score 1.05). The comparison of MF motility inhibition by MBP and EPO indicate that EPO inhibited MF motility significantly more than MBP (Fig. 3F). These results show that EPO and, to a lesser extent, MBP inhibit the motility of MF *in vitro* by themselves even in the absence of eosinophils.

Eosinophils of the natural host are impaired in their response to microfilariae

The natural host of *L. sigmodontis* is the cotton rat (*Sigmodon hispidus*). In contrast to the BALB/c mouse strain, in which the infection lasts up to 100 days and approximately 50% of the animals develop microfilaremia (S6A Fig) [34–36], almost all cotton rats develop microfilaremia with high MF levels and remain infected for more than one year (S6B Fig) [37]. To investigate whether the higher susceptibility of cotton rats is related to changes in eosinophil functions in response to MF, we examined whether eosinophils from cotton rats form



All vs 2500 MF	0h	4h	24h	48h	72h
MF + 0.1 ng/ml MBP	ns	ns,$$$	ns,$$$$	****,$$$$	****,$$$$
MF + 1 ng/ml MBP	ns	*,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 10 ng/ml MBP	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 0.1 ng/ml EPO	ns	**,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 1 ng/ml EPO	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 10 ng/ml EPO	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 0.1 ng/ml EPO + 0.1 ng/ml MBP	ns	*,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 1 ng/ml EPO + 1 ng/ml MBP	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + 10 ng/ml EPO + 10 ng/ml MBP	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$

EPO vs MBP		MF + EPO 0h	MF + EPO 4h	MF + EPO 24h	MF + EPO 48h	MF + EPO 72h
MF + MBP	0.1 ng/ml	ns	ns,$$$	***,$$$$	***,$$$$	**, $$$$
	1 ng/ml	ns	**, $$$$	**, $$$$	****,$$$$	****,$$$$
	10 ng/ml	ns	****,$$$$	****,$$$$	****,$$$$	****,$$$$
MF + EPO + MBP	0.1 ng/ml	ns	ns,$$$$	ns,$$$$	ns,$$$$	ns,$$$$
	1 ng/ml	ns	ns,$$$$	ns,$$$$	ns,$$$$	ns,$$$$
	10 ng/ml	ns	ns,$$$$	ns,$$$$	ns,$$$$	ns,$$$$

Fig 3. MBP and EPO inhibit microfilariae motility in a concentration-dependent manner *in vitro*. Motility of microfilariae (MF) that were co-cultured with bone marrow-derived eosinophils from MBP-KO (A), EPO-KO (B), and SvJ wildtype (WT) controls (C) and treated with

recombinant MBP and EPO at indicated concentration for 4 h, 24 h, 48 h, and 72 h with 2500 MF (D). Statistical results of the data shown in Fig 3 D (E-F). Data from a single experiment with a total of n = 60 MF/ 3 wells. Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparisons test. (A-D). * $p<0.05$, **,$$ $p<0.01$, ***,$$$ $p<0.001$, ****,$$$$ $p<0.0001$, ns = non-significant.

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extracellular traps and affect MF motility in a similar manner to eosinophils from BALB/c mice. Eosinophils were generated from the bone marrow of cotton rats and the purity, as determined by Giemsa staining, was >90%. Importantly, eosinophils from mice and cotton rats had granules in the majority of cells (100% of mouse eosinophils, 88% of cotton rat eosinophils) and a distinct circular or segmented nucleus indicating that the cells used were equal in maturity (S2B-C Fig). A representative picture of the generated eosinophils is shown in Fig 4A. Eosinophils from cotton rats reduced the MF motility within the first 48 hours of co-culture. However, after 72 hours, the movements of the MF were still continuous (mean score 3.23) and higher than of MF cultured in the absence of eosinophils (mean score 2.97; Fig 4B). Importantly, MF co-cultured with eosinophils from cotton rats showed no PI staining, indicating their viability (S4D Fig). To investigate whether eosinophils of naïve cotton rats are capable of undergoing EETosis, we next measured the presence of free extracellular DNA in the culture supernatants 24h after stimulation with different numbers of MF and concentrations of zymosan and PMA as positive controls. As shown in Figs 4C and S7, eosinophils from cotton rats are capable of undergoing EETosis, as stimulation with PMA and zymosan resulted in an increased release of extracellular DNA, which was reduced after DNase treatment. This observation was confirmed by DAPI staining and fluorescence microscopy (S8 Fig). In contrast, stimulation with 2500, 10,000, 20,000 and 40,000 MF did not result in extracellular DNA release after 24 h and 72 h of stimulation (Fig 4D-E). These results indicate that MF do not induce extracellular trap formations by eosinophils of the natural host. We hypothesize that this could present a parasite-host adaptation that explains the higher susceptibility of cotton rats to infections with *L. sigmodontis* or intrinsic differences in cotton rat and mouse eosinophils.

Discussion

ETosis has gained prominence in the study of the effector functions of eosinophils. This particular form of cell death involves the release of decondensed nuclear or mitochondrial DNA into the surrounding environment, with the primary purpose of trapping pathogens to promote their elimination [1,38–40]. Studies focusing on eosinophil ETosis in the context of helminth infections are limited as most studies have focused on neutrophil extracellular traps, which have been shown to play a role in various helminth species such as *Dirofilaria immitis* [41], *Haemonchus contortus* [42], *Strongyloides stercoralis* [43], *Nippostrongylus brasiliensis* [44,45] and *L. sigmodontis* [46]. These studies indicate that neutrophils mediate NETosis-dependent trapping of L3s, preventing successful migration to the site of infection. The role of eosinophils in this context, however, remains understudied. Eosinophilia and increased levels of their granule proteins were primarily observed after treatment of onchocerciasis and loiasis patients with microfilaricidal drugs as well as during acute filarial pathology associated with the death of MF, such as dermatitis in hyper-responsive onchocerciasis patients or patients with tropical pulmonary eosinophilia [1–3,8–10,15–17]. However, the role of EETosis and the effect of specific eosinophil granule proteins on MF viability and development of pathology requires further research.

Previously, Ehrens et al. demonstrated that MF from both *L. sigmodontis* and *D. immitis* trigger EETosis and *L. sigmodontis* MF-induced ETosis is dependent on dectin-1-, AIM2-,

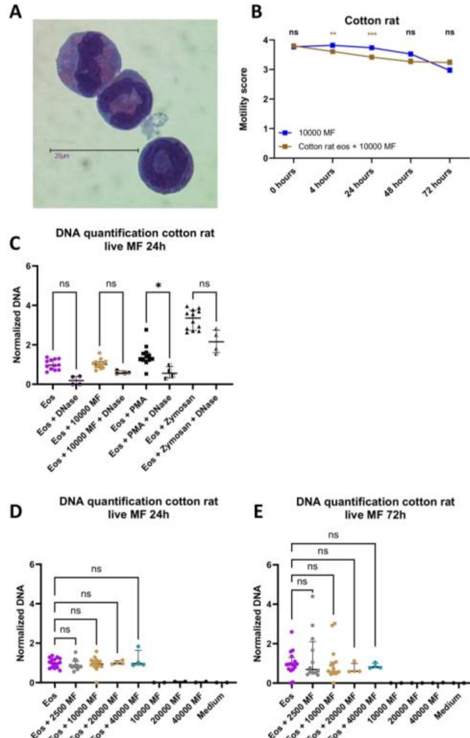

Fig 4. Eosinophils of the natural host do not release DNA traps in response to microfilariae and do not reduce microfilariae motility. Quick Diff staining of bone marrow-derived eosinophils from a cotton rat (A). Motility of 10,000 microfilariae (MF) co-cultured with bone marrow-derived cotton rat eosinophils (Eos) at 4 h, 24 h, 48 h, and 72 h (B). Free DNA in supernatant of bone marrow-derived cotton rat eosinophils stimulated for 24 h with 10,000 MF, PMA, or zymosan (C). Representative data from 2 experiments. Free DNA in supernatant of bone marrow-derived

cotton rat eosinophils stimulated for 24 h (D) or 72 h (E) with 2500, 10,000, 20,000 or 40,000 MF, eosinophils or both. Pooled data from 1-3 independent experiments with a total of n= 80-320 MF/ 4-16 wells (D-E). Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparisons test (B). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparisons test for selected groups for non-parametric data (C-E). Data is shown as median with interquartile range (C-E). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = non-significant.

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caspase-1-, ASC-, and GSDMD [31,32]. MF covered in eosinophil DNA traps were immobilized and therefore removed more efficiently *in vivo* [31]. Since eosinophil traps are known to also contain cytotoxic granule proteins, we investigated whether the two major granule proteins of eosinophils, MBP and EPO, support the killing of MF after they are immobilized by DNA traps. Previous studies with 129/SvJ WT mice lacking either MBP or EPO showed that deficiency of these granule proteins leads to a significantly increased *L. sigmodontis* adult worm burden after 28 dpi [30]. However, worms were cleared from the EPO-KO, MBP-KO and WT 129/SvJ mice prior to the onset of microfilaremia, preventing the analysis of the effects of both granule proteins on the MF stage.

Results from the present *in vitro* study demonstrate that EPO and MBP inhibit MF motility in a dose-dependent manner. Using comparable concentrations of EPO and MBP, EPO was significantly more potent in inhibiting MF motility in comparison to MBP. Thus, EPO may be, at comparable concentrations to MBP, more toxic for MF. This is supported by a previous study by Hamann et al., who observed a more efficient *in vitro* killing of *Brugia pahangi* and *B. malayi* MF by purified EPO in comparison to purified MBP or ECP [25]. While these findings indicate a protective effect of eosinophil granules against MF per se, they do not consider that MBP is more abundant in eosinophils and comprises almost half of the mass of secondary granules in eosinophils [47] and lack the interaction with other eosinophil effector functions. To address this limitation, the present study used co-cultures of MF and bone marrow-derived eosinophils of EPO KO, MBP KO and WT mice. Eosinophils from both KO mouse strains had the capacity to undergo EETosis and form DNA traps, but were less efficient at immobilizing MF compared to WT eosinophils. In addition, potential killing of MF, as assessed by PI staining after 72 hours of co-culture and quantification of the frequency of completely immobilized MF, was more prominent in co-cultures with WT eosinophils than KO eosinophils. Of note, MF motility inhibition and potential killing of MF was comparable for EPO KO and MBP KO eosinophils, indicating a comparable impact of both granule proteins upon release by eosinophils. Given that the lack of MBP or EPO did not completely prevent the motility inhibition and potential killing of MF, it can be hypothesized that redundancy of both granule proteins and potentially other eosinophil granules, such as ECP and EDN, exists and determines the microfilaricidal effect. Future studies should investigate differences in the induction of EETosis in response to MF of different filarial species, e.g., *L. loa*, *Mansonella perstans* and *O. volvulus*, as well as patients with or without filarial pathology, to determine whether EETosis is associated with the development of filarial pathology.

Our experiments on the stimulation of eosinophils from the bone marrow of cotton rats indicate that differences and potential host-specific adaptations concerning the induction of EETosis exist. We demonstrate that cotton rat eosinophils are generally capable of undergoing EETosis. Although, based on the normalized DNA, cotton rat eosinophils release less DNA in response to zymosan and PMA compared to mouse eosinophils. Importantly, MF-induced EETosis was not observed in cotton rat eosinophils in contrast to our findings with mouse eosinophils. This may indicate either intrinsic differences in eosinophil responses from cotton

rats and mice or a distinct adaptation of the parasite to its natural host. Thus, *L. sigmodontis* MF may counteract the effects of eosinophils in its natural host, which may increase the susceptibility of cotton rats, resulting in a significantly higher MF load and a longer patency period than in mice. One such potential mechanism was identified by Bouchery et al. who demonstrated that L3 larvae of the murine nematode *N. brasiliensis* secrete DNase to degrade traps formed by neutrophils in mice [45]. Our observation that murine eosinophils are more reactive toward *L. sigmodontis* than comparable eosinophils from the natural host is in line to observations with *B. malayi* and *O. lienalis* that naturally occur in humans and cattle, respectively, and elicit significantly stronger immune responses in the rodent model [48,49]. These differences highlight the potential adaptation of the natural host to chronic filarial infections and the wide variety of immune evasion strategies that were discovered for filariae and helminths in general [50].

These findings demonstrate the need for further research on the natural host to gain a better understanding on the host-parasite relationship, particularly with regard to human disease. It is conceivable that studies on surrogate models of the natural host, such as *O. ochengi* [51], could clarify the question of why some human-pathogenic filarial species lead to the development of filarial pathology, e.g., in onchocerciasis and lymphatic filariasis, whereas others lead mainly to asymptomatic infections, e.g., in *Mansonella perstans* infections [52].

Supporting information

S1 Fig. Purity of murine eosinophils. Representative flow cytometry-plot displaying the purity of murine bone marrow-derived eosinophils. Eosinophils were identified as SiglecF+ and CD11b +. Additional cell populations in the bone marrow-derived eosinophil culture are shown in the table below.
(TIF)

S2 Fig. Purity of cotton rat eosinophils. Cell composition of the cotton rat eosinophil culture as determined by Diff Quick staining (A). Cotton rat and mouse eosinophils were stained with wheat-germ-agglutinin (WGA) to identify the granules and DAPI to depict the morphology of the nucleus (B). White stars indicate cotton rat eosinophils that do not display granules (10 cells out of 117 total, 11.7%). (C) Zoomed in image of a comparison between cells that show granules and those that do not (white stars).
(TIF)

S3 Fig. Percentage of completely immobile microfilariae. Percentage of completely immobile (score 0) microfilariae (MF) of co-cultures with 10000 MF (A). Pooled data from 5 independent experiments with a total of n = 300 MF (3 wells per experiment with 20 MF each). Motility was documented per condition, not per well. Data were analyzed using two-way ANOVA followed by Bonferroni multiple comparisons test. * $p<0.05$, ** $p<0.01$, ns = non-significant.
(TIF)

S4 Fig. Mice deficient for EPO or MBP, and the natural host are less capable of killing microfilariae. Propidium iodide (PI) staining of bone marrow-derived eosinophils co-cultured with microfilariae (MF) for 72h. Microscopic pictures (magnification 100x) of SvJ wildtype **(A)**, MBP KO **(B)**, EPO KO **(C)** and cotton rat eosinophils **(D)** after 72 hours of incubation with MF (Transmitted Light left, PI middle, overlay right). The pictures are representative for five experiments.
(TIF)

S5 Fig. MBP KO and EPO KO mouse eosinophils are less effective in killing microfilariae than WT eosinophils. Percentage of propidium iodide positive stained microfilariae (MF) after 24h (A) and 72h (B) of co-culture with bone marrow-derived eosinophils. Data is shown as median with interquartile range. The data were pooled from 3 (A, n=9 images) and 2 (B, n=6 images) independent experiments. Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparisons test for selected groups for non-parametric data (A-B). *$p<0.05$, ns = non-significant.
(TIF)

S6 Fig. Comparison of microfilariae counts over time between BALB/c mice and cotton rats. Blood microfilariae counts in BALB/c mice from day 58 to day 113 post infection **(A)**. Blood microfilariae counts in cotton rats from 3 months post infection to 19 months post infection **(B)**. Data is shown as mean and SEM (A-B). Pooled data n = 5-16 mice (A) and n = 1-76 cotton rats (B) per data point.
(TIF)

S7 Fig. Eosinophils of the natural host are able to perform ETosis in response to PMA and zymosan. Free DNA in supernatant of bone marrow-derived cotton rat eosinophils stimulated for 24h or 72h with increasing concentrations of PMA or zymosan. Single experiment with n= 80 microfilariae/ 4 wells. Data is shown as median with interquartile range.
(TIF)

S8 Fig. Eosinophils of the natural host can perform EETosis, but not in response to microfilariae. DAPI staining of bone marrow-derived eosinophils co-cultured with PMA (A), zymosan (B) or 2500 microfilariae (MF) (C) for 24h. The pictures are representative of two experiments. 200x magnification.
(TIF)

S1 Table. Original data sets shown in Fig 1, Fig 2B-F , Fig 3A-D , Fig 4B-E, S2A Fig, S3 Fig, S5 Fig A-B, S6A-B Fig, S7A-D Fig.
(XLSX)

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2.2. Eosinophils Are an Endogenous Source of Interleukin-4 during Filarial Infections and Contribute to the Development of an Optimal T Helper 2 Response

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Eosinophils Are an Endogenous Source of Interleukin-4 during Filarial Infections and Contribute to the Development of an Optimal T Helper 2 Response

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Keywords

Eosinophils · Interleukin-4 · Nematode infection · CD4$^+$ T cells · Macrophages

Abstract

Introduction: Interleukin-4 (IL-4) is a central regulator of type 2 immunity, crucial for the defense against multicellular parasites like helminths. This study focuses on its roles and cellular sources during *Litomosoides sigmodontis* infection, a model for human filarial infections. ***Methods:*** Utilizing an IL-4 secretion assay, investigation into the sources of IL-4 during the progression of *L. sigmodontis* infection was conducted. The impact of eosinophils on the Th2 response was investigated through experiments involving dblGATA mice, which lack eosinophils and, consequently, eosinophil-derived IL-4. ***Results:*** The absence of eosinophils notably influenced Th2 polarization, leading to impaired production of type 2 cytokines. Interestingly, despite this eosinophil deficiency, macrophage polarization, proliferation, and antibody production remained unaffected. ***Conclusion:*** Our research uncovers eosinophils as a major source of IL-4, especially during the early phase of filarial infection. Consequently, these findings shed new light on IL-4 dynamics and eosinophil effector functions in filarial infections

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Plain Language Summary

Filarial nematodes can cause severe diseases like onchocerciasis and lymphatic filariasis, posing a significant public health challenge in tropical regions, putting over a billion people at risk. The WHO categorizes these infections as neglected tropical diseases and aims to eliminate onchocerciasis transmission and lymphatic filariasis as a public health issue by 2030. Filarial nematodes modulate the immune system of their host, and the induction of protective immune responses still requires a better understanding. Eosinophils have been identified as a key immune cell type in the well-established murine model for filarial infection, *Litomosoides sigmodontis*. However, their precise roles and interactions with other components of the type 2 immune

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response remain unclear. Our study reveals that eosinophils play a crucial role as a primary source of interleukin-4, the central cytokine in type 2 immunity. By using dblGATA mice, we found that the absence of eosinophils resulted in a reduced T helper 2 response but did not impact the alternative activation of macrophages or antibody production. In summary, our research uncovers an underappreciated function of eosinophils and their significant influence on type 2 immune responses. © 2024 The Author(s).
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Introduction

Interleukin-4 (IL-4) plays a pivotal role in orchestrating the immune response associated with type 2 immunity, primarily aimed at defending against large multicellular parasites like helminths [1]. IL-4 exerts its influence across a spectrum of innate and adaptive immune cells. One of its critical functions is to guide the differentiation of T cells into T helper 2 (Th2) cells [2]. IL-4 activates the transcription factor STAT6 leading to the expression and activation of GATA3, the master regulator of Th2 cells. These Th2 cells then produce not only IL-4 but also the other signature cytokines IL-5 and IL-13 [2]. Together with IL-13, IL-4 engages the IL-4 receptor, triggering an alternate activation pathway in macrophages [3]. This activation is a cornerstone of wound healing and tissue repair during type 2 immune responses [4]. IL-4 also plays a crucial role in B cell activities, including class switching and the production of immunoglobulin E (IgE) [5]. Furthermore, it dampens inflammatory responses, acting as a regulatory element in the immune system [4]. However, it is important to note that IL-4 has a dual nature. On the flip side, it can contribute to fibrosis and exacerbate the pathogenesis of various allergic diseases [6]. While CD4$^+$ T cells have long been recognized as a primary source of IL-4, particularly during helminth infections, innate immune cells like basophils and mast cells have been described as significant producers of IL-4 [7–9]. *Litomosoides sigmodontis* is a rodent filarial nematode which serves as a model for human filarial infections, recapitulating immune responses as they occur, e.g., in onchocerciasis and lymphatic filariasis patients [10, 11]. This filarial nematode gets transmitted via the tropical rat mite *Ornithonyssus bacoti* and migrates through skin and lymphatics to the pleural cavity by 5–8 days post-infection (dpi) [12]. In susceptible BALB/c mice, these parasites develop to sexual maturity by 35 dpi and produce their offspring, the microfilariae, which can be found in the peripheral blood by 57 dpi onward. Similar to other helminth infections, *L. sigmodontis* induces a type 2 immune response [13, 14]. However, the immunomodulatory capacity of this parasite leads to a strong regulatory immune milieu, facilitating the parasites' long-term survival in the host. Studies using IL-4 and IL-4R-KO mice have demonstrated a critical role for IL-4 and signaling via the IL-4 receptor in microfilariae control but not adult worm burden [15, 16]. Fertility and length of female adult worms were found to be enhanced in the absence of both IL-4 and IL-4R. Additionally, both studies found reduced eosinophil numbers in IL-4 and IL-4R-KO mice and an absence of IgE in infected IL-4 KO mice [15]. In contrast to the susceptible BALB/c strain, C57BL/6 mice eliminate *L. sigmodontis* infection before patency [14, 17]. However, in the absence of IL-4, C57BL/6 mice become as susceptible as BALB/c mice [18]. Furthermore, studies have demonstrated the requirement for IL-4 to induce an alternative macrophage phenotype [19–21], which in turn orchestrates eosinophil-mediated immunity to filarial nematodes [22].

While many functions for IL-4 have been shown for *L. sigmodontis* infection [11, 23], the contribution of different cellular sources of IL-4 to the infection process has remained unclear. To address this knowledge gap, we employed an IL-4 secretion assay to investigate both innate and adaptive sources of IL-4 throughout the course of *L. sigmodontis* infection. While our experiments confirmed a central role for CD4$^+$ T cell-derived IL-4, eosinophils emerged as a major source of IL-4, particularly during the early phase of infection before worms reach sexual maturity. To investigate the impact of eosinophils and their IL-4 contribution on the type 2 response, we utilized dblGATA mice, which lack eosinophils and therefore exhibit eosinophil-derived IL-4 deficiency. Strikingly, while eosinophil deficiency had no discernible impact on macrophage proliferation and polarization, it significantly curtailed the presence of Th2 cells at the site of infection, particularly during the middle phase of infection. These findings shed new light on the intricate dynamics of IL-4 sources and on an underappreciated function of eosinophils during *L. sigmodontis* infection.

Methods

Mice

A total of 6–8-week-old male and female BALB/c J wild-type (WT) mice were purchased from Janvier Labs (Saint-Berthevin, France). BALB/c *ΔdblGata1* mice were originally obtained from

Jackson Laboratory (Bar Harbor, USA), and eotaxin-KO and IL-5-KO mice were originally obtained from Prof. Dr. Klaus Matthaei (Stem Cell & Gene Targeting Laboratory, Australian National University, Canberra, Australia). All mice were bred at the "Haus für Experimentelle Therapie" of the University Hospital Bonn. Mice were housed in individually ventilated cages with unlimited access to food and water and a 12-h day/night cycle. All experiments were performed according to EU directive 2010/63/EU and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV, Recklinghausen, Germany) under license AZ 81.02.04.2020.A103.

Natural Infection

Mice were naturally infected with infective *L. sigmodontis* third-stage larvae via exposure to the tropical rat mite *O. bacoti* as described before [13]. In short, mice were placed over night in cages with bedding material containing infected mites. The next day, bedding material with the mites was removed and after another 24 h mice were moved back to new cages. To ensure comparable infections of both groups, mice from both groups were exposed simultaneously to the same batch of mites containing the infective larvae.

Parasite Recovery

Mice were sacrificed via an overdose of isoflurane (AbbVie, Wiesbaden, Germany) by 10, 15, 35/37, 57/60, 72, and 90 dpi. To determine adult worm burden, the pleural cavity of individual mice was flushed with 1 mL of PBS (PAA Laboratories, Pasching, Austria) which then contained adult worms. Remaining adult worms in the pleural cavity and the peritoneum were isolated with a dissection probe.

Isolation of Pleural Cavity Cells

Pleural cavity cells were obtained following lavage with PBS (PAA Laboratories). The first ml of the lavage was collected, worms removed, and cells separated by centrifugation and the supernatant stored at −20°C for cytokine measurements at a later time point. The cells were combined with the cells of a following lavage with 4 mL of PBS.

Preparation of L. sigmodontis *Antigen*

For the preparation of *L. sigmodontis* adult worm extract (LsAg), freshly isolated adult worms were rinsed in sterile PBS before being mechanically homogenized under sterile conditions. Insoluble material was removed by centrifugation at 300 *g* for 10 min at 4°C. Protein concentrations of crude extracts were determined using the Advanced Protein Assay (Cytoskeleton, Denver, USA).

Measurement of Cytokines and Antibodies by ELISA

Cytokine concentrations were determined within the first ml of pleural cavity lavage by ELISA. IL-4, IL-5, and IL-13 (Invitrogen, MA, USA) were all measured according to kit protocols. To determine parasite-specific immunoglobulin levels in plasma, plates were coated with 10 μg/mL LsAg overnight. After blocking with PBS/1% BSA, plasma was added in serial dilutions. Following incubation, plates were washed and secondary biotinylated antibodies against IgE, IgG1, or IgG2a/2b (BD Biosciences, NJ, USA) were added. After washing and incubation with Streptavidin-HRP, plates were washed, TMB substrate added, and the enzymatic reaction stopped with sulfuric acid. Optical density (OD) was measured at 450 nm (Spectramax 240pc Molecular Devices, CA, USA).

Flow Cytometric Analyses of Pleural Cavity Cells

Pleural cavity cells were counted using the Countess Cell Counter (Thermo Fisher, MA, USA). Cells were then stained for live/dead (Life Technologies) and subsequently incubated with Fc-block (1:500 CD16/CD32 and 1:50 mouse serum) and were then stained with fluorescence-conjugated antibodies. For staining of intracellular cytokines, cells were stimulated for 4 h at 37°C with cell stimulation cocktail containing protein transport inhibitor (eBioscience) and then stained with live/dead. After surface antibody staining, cells were fixed for 1 h at RT using IC fixation/permeabilization buffer (BioLegend) and cells were then incubated for 20 min at RT in permeabilization buffer (BioLegend). Intracellular staining was performed for cytokines using antibodies for IL-5, IL-13, as well as for GATA3, RELMα, arginase-1, and Ki67. Samples were analyzed by flow cytometry with LSRFortessa (BD Biosciences), and data were analyzed using FlowJo v10.2 software.

Eosinophil Generation for in vitro Studies

Bone marrow from naïve mice was used to generate bone marrow-derived eosinophils as previously described [24]. Bone marrow cells were counted using CASY® TT-cell counter system (OMNI Life Science GmbH & Co. KG), and cells were seeded in Advanced RPMI medium with 20% FBS, 1% penicillin/streptomycin, 0.1% gentamycin, 2.5% HEPES, and 1% GlutaMAX (Thermo Fisher Scientific GmbH, Germany). Cells were cultured with stem cell factor and FMS-like tyrosine kinase 3 ligand (PeproTech, Rocky Hill, USA) for the first 4 days. Afterward, the growth factors were exchanged with IL-5 (PeproTech, Rocky Hill, USA). Half of the medium was exchanged every other day, and on day 8, the cell culture flask was exchanged. After 12 days, cells were harvested and checked for the eosinophil purity using flow cytometry (>95%). Eosinophils were then seeded in 24-well plates and stimulated with LsAg for 24, 48, and 72 h. Supernatant was taken at these time points and stored at −20°C for further use.

IL-4 Secretion Assay

To identify IL-4 sources within the pleural cavity, the IL-4 secretion assay kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was employed. Pleural cavity single-cell preparations were stimulated for 3 h at 37°C with cell stimulation cocktail containing protein transport inhibitor (eBioscience). Subsequently, an IL-4-specific catch reagent was attached to the cell surface of all immune cells. The cells were then incubated for 45 min at 37°C to allow cytokine secretion. The secreted IL-4 bound to the IL-4 catch reagent on the secreting cells. These cells were subsequently labeled with a second IL-4-specific antibody, the phycoerythrin-conjugated IL-4 detection antibody for sensitive detection by flow cytometry for 10 min on ice. Further surface markers for different immune cell populations were added parallel to the IL-4 detection antibody. Cells were then washed and analyzed on an LSRFortessa flow cytometer.

Statistics

Prism 7.0 (version 7.0c, GraphPad Software) was used for statistical analysis. Differences between two experimental groups were assessed by Mann-Whitney test, while differences between

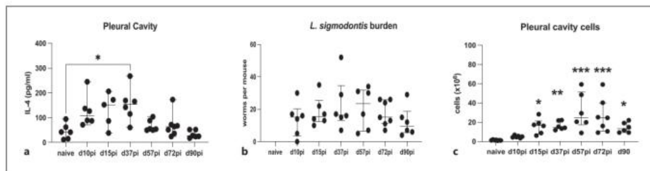


Fig. 1. IL-4 is a signature cytokine of *L. sigmodontis* infection. **a** IL-4 protein levels in the pleural lavage of naïve and *L. sigmodontis*-infected BALB/c mice. *L. sigmodontis* worm burden (**b**) and total pleural cell counts (**c**) during the course of infection. Data are representative for 3 independent experiments. Data are shown as median with interquartile range and tested for significances using Kruskal-Wallis test and Dunn's posttest for nonparametric data comparing each time point to the naïve control (**a**). $^{*}p < 0.05$.

three or more experimental groups were assessed using Kruskal-Wallis test for nonparametric data, followed by Dunn's multiple comparison test. For differences between experimental groups across different time points, two-way ANOVA with Tukey's posttest for nonparametric data and with Sidak's posttest for parametric data was utilized. Data were tested for correlation using Spearman test. Data are shown as median with interquartile range.

Results

Eosinophils Are a Major Source of IL-4 during L. sigmodontis *Infection*

To quantify IL-4 protein levels at the site of infection, pleural lavage was obtained from naïve and infected mice at various time points during the course of *L. sigmodontis* infection, followed by an IL-4 ELISA. IL-4 increased in response to infection and reached a statistically significant elevation by 37 dpi (Fig. 1a). Interestingly, during the later stages of infection (57 dpi, 72 dpi, and 90 dpi), time points when microfilariae are present, IL-4 concentrations declined, eventually returning back to levels observed in naïve mice. However, this decline was not due to the clearance of adult worms, as mice still possessed viable worms at 90 dpi (Fig. 1b). Pleural cavity cells were increased throughout infection, reaching statistical significance from 15 dpi onward compared to naïve controls (Fig. 1c).

To decipher the sources of IL-4, a murine IL-4 secretion assay kit was employed. In this assay, pleural cavity cells were restimulated and subsequently incubated with an IL-4-specific catch reagent and detection antibody. Cells secreting IL-4 were detected using regular flow cytometric surface staining (Fig. 2a). Similar to IL-4 protein levels, the frequency of IL-4-producing cells increased during the early phase of infection (10, 15, and 37 dpi), followed by a decline back to baseline levels during the later stages of infection (Fig. 2b). Similar kinetics were observed for absolute numbers of IL-4-producing cells in the pleural cavity. Notably, the highest influx of immune cells into the pleural cavity occurred around 37 dpi, following the molt of the L4 stage to adult worms (Fig. 2b). To gain deeper insights into the cellular composition of the IL-4-positive subset among CD45^{+} cells, the immune cells within the IL-4^{+} proportion of CD45^{+} cells were further investigated. As illustrated in Figure 2c, our investigation demonstrates dynamic shifts in the dominant sources of IL-4 at different stages of infection. As expected, CD4^{+} T cells emerged as a consistent and prominent source of IL-4 throughout the course of the infection. However, during the early phases of infection, following the migration of the infective third-stage larvae into the pleural cavity and molt into fourth-stage larvae, Siglec F^{+}CD11b^{+} eosinophils accounted for the highest proportion of IL-4-secreting cells, displaying a decisive role in these initial stages. A surprising discovery was the substantial contribution of Ly6G^{+}CD11b^{+} neutrophils to IL-4 production, a role they assumed from their appearance in the pleural cavity at 37 dpi, after adult worms developed, persisting until 90 dpi, when the natural clearance of the adult worms occurs. In addition to these primary sources, the analysis identified CD11b^{+} cells, which may include basophils and mast cells, as well as CD11b^{-} cells, which may include ILC2, B cells, and NK T cells among others (Fig. 2c), cell types which have been described to be involved in *L. sigmodontis* infection. When quantifying the absolute numbers

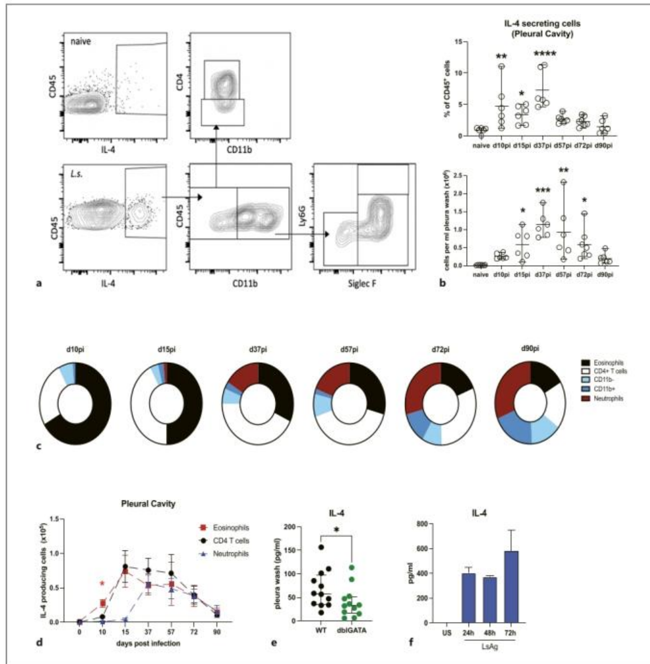


Fig. 2. Eosinophils are a major source of IL-4 during filarial infection. Representative FACS plots displaying the gating strategy for pleural cavity using the IL-4 secretion assay. Cells are derived from single, live cells. **a**, **b** Percentage and absolute number of IL-4⁺ immune cells within the pleural cavity of naïve and *L. sigmodontis*-infected BALB/c mice during course of infection. Pie charts representing frequencies of IL-4⁺ cells within the pleural cavity during the course of *L. sigmodontis* infection. **c** Each circle represents 100%. **d** Absolute numbers of IL-4⁺ eosinophils, IL-4⁺CD4⁺ T cells, and IL-4⁺ neutrophils over the course of infection. **e** IL-4 protein levels in the pleural lavage on 10 dpi with *L. sigmodontis* in BALB/c (WT) and dblGATA mice. **f** IL-4 protein levels measured from supernatants of bone marrow-derived eosinophils stimulated with LsAg for 24 h, 48 h, and 72 h as well as unstimulated controls. Data representative for two independent experiments (**a–d**) or pooled from two independent experiments with $n = 6$ (**e**, **f**). Data are shown as median with interquartile range (**b**, **d**, **e**) or as mean ± SEM (**f**) and tested for significances using Kruskal-Wallis test and Dunn's posttest for nonparametric data (**b**, **f**), two-way-ANOVA with Tukey's posttest (**d**) or Mann-Whitney test (**e**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

of cells producing IL-4, eosinophils were the most numerous producers of IL-4 10 dpi, followed by $CD4^+$ T cells. Again, neutrophils appear during the later stages of infection, when the IL-4 levels in the pleural cavity generally decreased (Fig. 2d). To further confirm that eosinophils are a major IL-4 producer during *L. sigmodontis* infection, ELISA was performed using pleural wash from infected eosinophil-deficient dblGATA mice and WT controls. As shown in Figure 2e, dblGATA mice secreted significantly less IL-4 compared to BALB/c WT controls at 10 dpi (Fig. 2e). Furthermore, bone marrow-derived eosinophils were stimulated with *L. sigmodontis* crude antigen (LsAg) for 24 h, 48 h, and 72 h and IL-4 was measured in the supernatants. IL-4 was detected at all tested time points with the highest production measured after 72 h restimulation, while unstimulated eosinophils did not secrete IL-4. These data confirm that eosinophils indeed produce IL-4 in response to filarial antigen in a time-dependent manner (Fig. 2f).

$IL\text{-}4^+$ Eosinophils Are Highly Activated and Express CCR3, CD125, and ST2 but Do Not Express CD101

Single-cell methodology and transcriptomic studies have largely contributed to uncover new cell subtypes and cell heterogeneity. As such, eosinophils have been characterized in more detail in health and disease in recent years [25–27]. In this study, we employed a flow cytometry approach to elucidate the characteristics of eosinophils on 10 dpi, which presented the time point where eosinophils were the dominant IL-4 producers. At this specific time point of infection, eosinophil numbers are significantly elevated in the bloodstream (Fig. 3a) and at site of infection, the pleural cavity (Fig. 3b). Notably, blood and pleural eosinophilias are dependent on the presence of IL-5 and, to some extent, reliant on eotaxin-mediated recruitment (online suppl. Fig. 1A, B; for all online suppl. material, see https://doi.org/10.1159/000536357). However, eosinophil numbers do not exhibit a direct correlation with the worm burden at this time point (Fig. 3c). Subsequently, we conducted a comparative analysis of eosinophils in the blood and the pleural cavity of infected mice, focusing on established markers of eosinophils and their activation status. Eosinophils are primarily produced in the bone marrow and are released into the bloodstream upon infection, subsequently infiltrating the pleural cavity, where the parasites reside. Upon entering the pleural cavity, eosinophils upregulate different proteins associated with an either regulatory or inflammatory phenotype as well as increased cell activation compared to their blood counterparts. CCR3 is the receptor for the eotaxins 1 and 2 and is upregulated in pleural eosinophils when compared to blood eosinophils. Similarly, the receptors for IL-33 (ST2) and IL-5 (CD125) were significantly upregulated on eosinophils upon entry into the pleural cavity (Fig. 3d), indicating a requirement for these two cytokines for full activation and induction of effector mechanisms. Recently, $CD101^+$ eosinophils were described during infection with the lung-migrating nematode *Nippostrongylus brasiliensis*, exhibiting a more inflammatory phenotype compared to resident $CD101^{lo}$ eosinophils [26, 28]. In our setting, a small portion of eosinophils were found to upregulate CD101 when entering the pleural cavity (4.99%; Fig. 3d). Moreover, classical activation markers, including CD69, CD86, and MHCII, exhibited a substantial upregulation specifically in pleural eosinophils when compared to their counterparts in the bloodstream within the same mouse. CD62L was found to be downregulated on pleural eosinophils compared to blood eosinophils. CD62L, CD69, ST2, MHCII, and CCR3 were also assessed on 35 dpi, exhibiting a similar expression profile on eosinophils as found on 10 dpi (online suppl. Fig. 1C). Additionally, these aforementioned markers were assessed and compared between pleural eosinophils from both naïve and *L. sigmodontis*-infected mice at 10 dpi and were found to be significantly upregulated in response to infection (online suppl. Fig. 1D). Recently, Gurtner et al. [25] presented a protocol to distinguish eosinophil subsets in different tissues using CD80 and PD-L1. However, in our model, eosinophils within the pleural cavity during *L. sigmodontis* infection did not express PD-L1 and exhibited low CD80 expression (data not shown). We subsequently investigated which markers exhibited upregulation specifically on IL-4-producing eosinophils. As shown in Figure 3e, f, eosinophils responsible for IL-4 production prominently expressed CCR3 and CD125, with 59.6% of eosinophils displaying ST2 expression. It is noteworthy that IL-4-negative but not IL-4-producing eosinophils express CD101 (Fig. 3e, f). CD101 was described to be a feature of an inflammatory phenotype of eosinophils. Additionally, $IL\text{-}4^+$ eosinophils exhibit a high level of activation, exemplified by their markedly increased expression of CD69 (75%), CD86 (93.8%), and MHCII (53.6%; Fig. 3f).

Eosinophil-Derived IL-4 Is Dispensable for Macrophage Proliferation and Polarization

Having established the significant contribution of eosinophils as a pivotal source of IL-4 during filarial infection, further immune parameters were assessed to understand the impact eosinophils have on the type 2 response. For this, eosinophil-deficient dblGATA mice

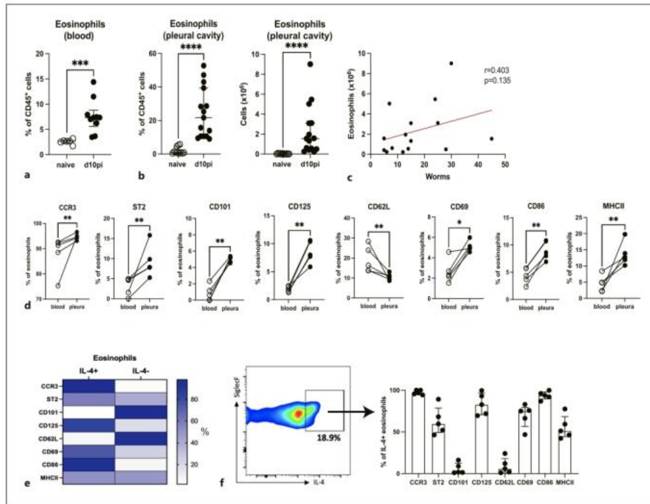
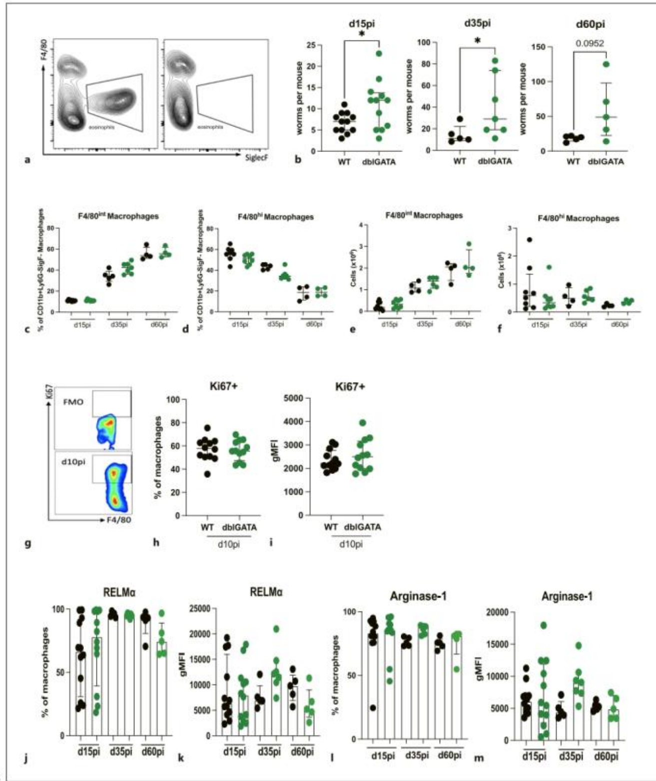


Fig. 3. IL-4-producing eosinophils are highly activated and do not express CD101. Eosinophil frequency in blood (**a**) and pleural cavity (**b**) as well as absolute eosinophil numbers of pleural cavity in naïve and infected mice on 10 dpi. **c** Linear regression of worm burden versus eosinophil percentage for 10 dpi. **d** Comparison of frequencies of CCR3, ST2, CD101, CD125, CD62L, CD69, CD86, and MHCII-positive eosinophils in blood and pleura on 10 dpi. **e** Heat map showing the percentage of IL-4$^+$ and IL-4$^-$ eosinophils expressing indicated activation markers, $n = 5$ per group and marker. **f** Representative FACS plot displaying gating for IL-4$^+$ eosinophils and graph showing percentage of expression of different markers on IL-4$^+$ eosinophils. Data shown in (**a–c**) are pooled from three independent experiments with 5 mice each. Data shown in (**d–f**) are representative for two independent experiments. Data are shown as median with interquartile range (**a**, **b**, **f**) and tested for significance using Mann-Whitney test (**a**, **b**, **d**) or tested for correlation using Spearman test (**c**). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

were used (Fig. 4a). These mice have a complete ablation of the eosinophil lineage, even under conditions, which normally stimulate eosinophil development. As shown in Figure 4b, these mice exhibit a significantly increased worm burden during the course of *L. sigmodontis* infection (Fig. 4b). Moreover, throughout the course of infection, dblGATA mice exhibited notably reduced pleural immune cell counts in comparison with BALB/c WT controls, primarily attributed to the absence of the major eosinophil population (online suppl. Fig. 2A–C). Pleural macrophages are a key cell type during *L. sigmodontis* infection, and impairments within the macrophage compartment can have a major effect on infection outcome [23]. The polarization of macrophages to an alternatively activated phenotype is IL-4R-dependent and required for successful elimination of the parasites [23, 29]. However, a study by Finlay et al. [23] demonstrated that *L. sigmodontis*-infected BALB/c mice fail to



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fully convert recruited monocytes efficiently into tissue-resident F4/80^{hi} macrophages, displaying an intermediate phenotype (F4/80^{int}). Efficient integration of monocytes into the resident macrophage pool requires Th2 cells and IL-4R signaling [23]. Therefore, we compared both F4/80^{int} and F4/80^{hi} macrophage populations in dblGATA mice and BALB/c WT controls. While the kinetics of these populations confirm previous findings, no differences were found in frequency and absolute numbers of both these macrophage stages in the pleural cavity between dblGATA mice and BALB/c WT controls during all tested time points (Fig. 4c–f). Macrophages undergo a proliferative burst in the pleural cavity on 10 dpi, which depends on the presence of IL-4 [29]. Therefore, the expression of the proliferation marker Ki67 was measured on pleural F4/80^{hi} macrophages on 10 dpi (Fig. 4g–i). Eosinophil-deficient mice did not show impaired macrophage proliferation, as frequency of Ki67^{+} macrophages as well as mean fluorescence intensity of Ki67 did not differ between dblGATA mice and WT controls (Fig. 4h, i). Intracellular staining for proteins RELMα (Fig. 4j, k) and arginase-1 (Fig. 4l, m), both associated with the alternatively activated, IL-4R-dependent phenotype of macrophages, did not reveal significant differences during the absence of eosinophils in both frequencies and MFI. A smaller proportion of RELMα+ macrophages was observed in dblGATA mice on 60 dpi; however, this did not reach statistical significance (Fig. 4j). Similar tendencies for reduced MFI for RELMα in dblGATA mice were observed as well (Fig. 4k). Taken together, these data demonstrate that eosinophils are dispensable for macrophage polarization and proliferation in the pleural cavity of *L. sigmodontis*-infected mice.

Eosinophils Are Required for an Optimal Development of the Th2 Response

CD4^{+} T cells have been previously described to be essential in controlling worm and microfilariae burden [30, 31]. Additionally, depletion of CD4^{+} T cells resulted in diminished Th2 cytokines, eosinophilia, and IgE levels, demonstrating the central role for CD4^{+} T cells during filarial infection [30]. The polarization of CD4^{+} T cells to Th2 cells requires IL-4. Therefore, differences in CD4^{+} T cell numbers, GATA3 expression – the master transcription factor of Th2 cells – as well as type 2 cytokine production of these T cells, were investigated in dblGATA mice and WT controls. CD4^{+} T cell frequency did not differ between both tested groups (Fig. 5a). However, on 35 dpi absolute CD4^{+} T cell numbers were significantly reduced in dblGATA mice compared to WT controls, indicating an eosinophil-dependent impact on T cell numbers (Fig. 5b). GATA3 expression in percentage and MFI were not changed throughout the infection due to eosinophil deficiency (Fig. 5c, d), but absolute numbers of GATA3^{+} CD4^{+} T cells were lower on 35 dpi (Fig. 5e). Next, we assessed the ability of the GATA3^{+}CD4^{+} T cells to produce signature Th2 cytokines. For this, GATA3^{+}CD4^{+} T cells were intracellularly stained for IL-5 and IL-13. Frequencies of these cytokines and absolute numbers of IL5^{+} and IL-13^{+} CD4^{+} T cells did not differ between dblGATA mice and BALB/c WT controls on 15 and 60 dpi; however, on 35 dpi, CD4^{+} T cells in dblGATA mice produced significantly less IL-5 and IL-13, and absolute numbers of both IL-5^{+} CD4^{+} T cells and IL-13^{+} CD4^{+} T cells were significantly reduced in the absence of eosinophils (Fig. 5e, f).

Absence of Eosinophils Has a Minimal Impact on L. sigmodontis*-Specific Antibody Production*

In line with its function in orchestrating the type 2 response, IL-4 is recognized for its ability for co-stimulating B cells and favoring IgE class switching. However, it remains unclear whether eosinophils and eosinophil-derived IL-4 have an impact on antibody production during filarial infections. To address this question, parasite-specific IgE, IgG2a/b, and IgG1 in the serum of infected dblGATA mice and WT controls were measured. Parasite-specific IgE, IgG2a/b, and

Fig. 4. Lack of eosinophils does not affect macrophage polarization and proliferation. **a** Representative FACS plots comparing WT and dblGATA mice for eosinophils, gated as Siglec F^{+}F4/80^{-}Ly6G^{-}CD11b^{+}. **b** Worm burden by 15 dpi, 35 dpi, and 60 dpi between dblGATA mice and BALB/c WT controls. Frequency and absolute number of F4/80^{int} (**c**, **e**) and F4/80^{hi} macrophages (**d**, **f**) in the pleural cavity at 15 dpi, 35 dpi, and 60 dpi of dblGATA mice and BALB/c WT controls. **g** Representative FACS plot displaying Ki67 staining on 10 dpi and FMO control for pleural cavity macrophages. Percentage (**h**) and gMFI (**i**) for intracellular Ki67 staining of pleural F4/80^{hi} macrophages of dblGATA mice and BALB/c WT controls on 10 dpi. Percentage (**j**) and gMFI (**k**) of intracellular RELMα on F4/80^{hi} macrophages between dblGATA mice (green) and BALB/c WT controls (black) for 15 dpi, 35 dpi, and 60 dpi. Percentage (**l**) and gMFI (**m**) of intracellular arginase-1 on F4/80^{hi} macrophages between dblGATA mice (green) and BALB/c WT controls (black) for 15 dpi, 35 dpi, and 60 dpi. Data in (**b**) are pooled from two independent experiments for 15 dpi, 35 dpi, and 60 dpi. Data in (**c–f**) and (**h–m**) are representative for two independent experiments. Data are shown as median with interquartile range (**b–f**, **h–m**). Data were tested for significance using Mann-Whitney test (**b**, **h**, **i**) or Kruskal-Wallis with Dunn's posttest (**c–f**, **j–m**). $^{*}p < 0.05$.

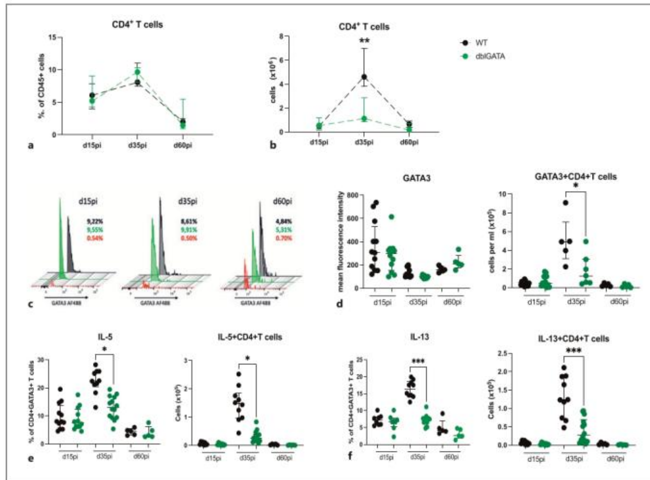

Fig. 5. Eosinophils are required for an optimal Th2 response. Frequency (**a**) and absolute numbers (**b**) of CD4⁺ T cells in the pleural cavity of dblGATA (green) and BALB/c WT mice (black) for 15 dpi, 35 dpi, and 60 dpi. **c** Histograms displaying GATA3 expression between dblGATA (green), WT (black), and FMO controls (red) on pleural CD4⁺ T cells for 15 dpi, 35 dpi, and 60 dpi. **d** GATA3 gMFI on CD4⁺ T cells and absolute numbers of GATA3⁺CD4⁺ T cells in the pleural cavity of dblGATA (green) and WT controls (black) on 15 dpi, 35 dpi, and 60 dpi. Frequency and absolute numbers of intracellular IL-5 (**e**) and IL-13 (**f**) on pleural CD4⁺ T cells on 15 dpi, 35 dpi, and 60 dpi in dblGATA and BALB/c WT controls. Data pooled from two independent experiments, shown as median with interquartile range (**a**, **b**, **d–f**). Data were tested for significance using Kruskal-Wallis with Dunn's posttest. $^{*}p < 0.05$, $^{**}p < 0.01$.

IgG1 were barely detectable at 15 dpi (online suppl. Fig. 3A–C), and no differences were observed between both tested groups. Similarly, no differences in antibody titers for tested Igs were found on 35 dpi (online suppl. Fig. 3D–F). On 60 dpi, OD for IgE at 1:2 dilution was significantly reduced in dblGATA mice compared to WT controls (Fig. 6a). However, with lower dilutions, no differences were detected. OD IgG2a/b was increased in dblGATA mice, but did not reach statistical significance (Fig. 6b). No differences were found for IgG1 (Fig. 6c).

Discussion

Eosinophils are a hallmark immune cell type of type 2 immunity and are highly increased during helminth infections. Eosinophils have been described to have both, protective functions and pathology-inducing features in filariasis [32]. Specifically, their ability to release granule proteins such as MBP or EPO [33] and the formation of extracellular traps have been investigated in the context of filarial infections [24]. Here, we show that eosinophils are an important source of IL-4, a cytokine central to type 2

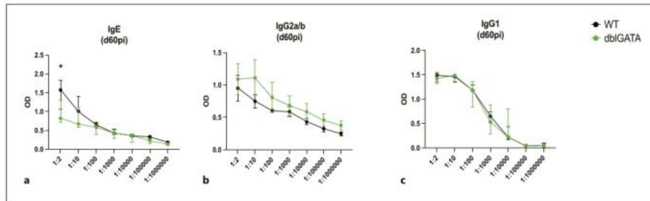

Fig. 6. Eosinophil deficiency has minimal affect on antibody production. OD of LsAg-specific IgE (**a**), IgG2a/b (**b**), and IgG1 (**c**) in serum of dblGATA mice and BALB/c WT control on 60 dpi. Data are pooled from two independent experiments with $n = 10$ per time point. Data are shown as median with interquartile range and were tested for significance with two-way ANOVA and Tukey's posttest. $^{*}p < 0.05$.

immunity. Eosinophils are recruited in high numbers during *L. sigmodontis* infection in the pleural cavity [23]. Our data indicate that eosinophils heavily contribute to the IL-4 environment within the site of infection. Eosinophils have been previously recognized as a significant source of IL-4 during various disease settings. They have been shown to be major IL-4-expressing cells in white adipose tissue, playing a pivotal role in inducing alternative activation of macrophages [34]. Eosinophils have also been linked to IL-4 production in contexts related to tissue repair, as exemplified in a study on liver regeneration [35]. In a model of a toll-like receptor 2-mediated paw inflammation, eosinophils were necessary for "M2 macrophage" polarization and eosinophil depletion resulted in reduced IL-4 levels and increased edema formation [36]. During pulmonary cryptococcosis, eosinophils contribute to IL-4 production and shape the Th2 cell cytokine profile [37]. In human allergic patients, eosinophils may be an important IL-4 source and enhance the allergic response through Th2 formation and inducing isotype switching to IgE [38]. Our data are in line with these studies and show for the first time that eosinophils secrete IL-4 during a filarial infection of the pleural cavity. The eosinophil-derived IL-4 has an impact on the Th2 response and type 2 cytokine production as numbers of CD4+ T cells and IL-5 and IL-13 levels were reduced in the absence of eosinophils. Similar findings were observed in a study of acute schistosomiasis, where lack of eosinophils resulted in lower concentrations of IL-5 and IL-13 in infected liver tissue, indicating that eosinophils participate in the establishment and amplification of Th2 responses [39]. Our data also indicate that the connection between Th2 cells and eosinophils is of major importance to infection outcome and resolution. The eosinophil-induced Th2 cells can in turn produce not only IL-4, but also IL-5, which is necessary for further eosinophil maturation and activation, leading to a positive feedback loop between these two hallmark cell types of type 2 immunity. Studies have demonstrated that Th2 cells in BALB/c mice infected with *L. sigmodontis* acquire features of hyporesponsiveness [40]. This happens around the time microfilariae appear in BALB/c mice, resulting in Th2 cells less efficient in type 2 cytokine production. We see in our study also lower Th2 cytokines at 60 dpi. A further study has provided evidence that T cell hyporesponsiveness is intrinsic to the Th2 cell and by 60 dpi, Th2 cells acquire a dysfunctional phenotype characterized by IL-21^{+}Egr2^{+}c-Maf^{+}Blimp-1^{+}IL-4^{lo}IL-5^{lo}T-bet^{+}IFNγ^{+} [41]. Eosinophils provide IL-4 for maintenance of Th2 cells during the middle phase of infection. However, long-term Th2 cells will still become hyporesponsive independent of eosinophil activity. Moreover, Th2 cells are needed for monocyte-to-macrophage conversion, which also fails in BALB/c mice [23]. All these BALB/c mice-specific impairments are resulting in failure to kill worms as observed in C57BL/6 mice. Therefore, experiments using eosinophil-deficient mice on a C57BL/6 background could provide more evidence on the impact eosinophils and their IL-4 production have on infection outcome. The absence of eosinophils led to an augmented adult worm burden, corroborating prior findings using dblGATA mice. Frohberger et al. [16] revealed a substantial increase in both adult worm count and microfilariae load in the absence of eosinophils, persisting up to 120 dpi. Notably, this deficiency resulted in a prolonged circulation of microfilariae at significantly higher levels.

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These observations underscore the profound impact of altered immune responses due to eosinophil deficiency and possibly eosinophil-derived IL-4 on the infection outcome. Eosinophils have often been implicated in driving pathology [32]. A recent study highlighted that the absence of eosinophils could potentially protect against visceral pleural hyperplasia in *L. sigmodontis* infection [42]. To gain deeper insights, future investigations should delve into the intricate relationship between eosinophils, tissue damage, and the influence of IL-4, paving the way for a more comprehensive understanding of their impact. While an impact of eosinophil deficiency was observed in the $CD4^+$ T cell populations, macrophages were not impacted in this study. Our data are hereby in line with two previous studies. In a study by Turner et al. [22], CCR3-KO mice and the *Brugia malayi* implant model were used to demonstrate that lack of CCR3-dependent eosinophilia did not impact alternative activation of macrophages as well as RELMα and arginase-1 expression. However, lack of eosinophils resulted in an impairment in RELMα secretion. Similarly, Jackson-Jones et al. [43] did not observe an eosinophil-dependent impairment in macrophage proliferation using dblGATA mice but saw a reduction in RELMα expression in their model of in vivo IL-33 delivery. However, both these studies have investigated the peritoneal cavity, so tissue-depending differences in RELMα production and secretion could be an explanation for the different study outcomes. The question remains why eosinophil-derived IL-4 does not impact macrophages, but $CD4^+$ T-cell-derived IL-4 does. Depleting $CD4^+$ T cells during *L. sigmodontis* infection resulted in failure of macrophages to proliferate in the absence of Th2 cells [23, 44]. Importantly, IL-13 is capable of inducing macrophage proliferation as well and may be taking over a more dominant, compensatory role during the absence of eosinophil-derived IL-4. Another intriguing observation of this study was the finding that neutrophils are also capable of producing IL-4 during a type 2 immune response. Neutrophils have been described to be involved in helminth infections in general [45] and in filarial infection in particular [46]. However, their precise role and how they influence various facets of the type 2 immune response remain elusive. Future studies will investigate the role neutrophils have in the context of type 2 responses.

A notable limitation of this study is the reliance on dblGATA mice as opposed to an eosinophil-specific IL-4 KO mouse model. To our knowledge, such a model does not presently exist, rendering dblGATA mice the most suitable option for investigating eosinophils and their absence in this context. Furthermore, while our study primarily focuses on IL-4, we cannot dismiss the possibility that eosinophils might exert their effects on the Th2 response through other means. Previous research has highlighted the T cell-modulating and T cell-regulating capabilities of neutrophil-derived granule proteins [47], implying a similar potential for eosinophils in shaping the immune response. These aspects warrant further investigation to comprehensively understand the mechanisms at play in eosinophil-mediated immune modulation. Though the potential targeting of eosinophils and their IL-4 production as a therapeutic strategy against human filarial diseases is an enticing prospect, reaching conclusive insights necessitates further comprehensive research and investigation. In summary, our study presents for the first time eosinophils as a major source of IL-4 during filarial infections and this IL-4 is necessary for Th2 cell development and cytokine production during the middle phase of infection.

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Statement of Ethics

All experiments were performed according to EU directive 2010/63/EU and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV, Recklinghausen, Germany) under license AZ 81.02.04.2020.A103.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization: Marc P. Hübner and Jesuthas Ajendra. Data curation: Cécile Guth and Jesuthas Ajendra. Experimentation: Cécile Guth, Pia Philippa Schumacher, Archena Vijayakumar, Hannah Borgmann, Helene Balles, Marianne Koschel, Frederic Risch, Benjamin Lenz, and Jesuthas Ajendra. Investigation: Cécile Guth, Pia Philippa Schumacher, Archena Vijayakumar, Hannah

Borgmann, Helene Balles, Marianne Koschel, and Jesuthas Ajendra. Resources: Achim Hoerauf and Marc P. Hübner. Supervision: Achim Hoerauf, Marc P. Hübner, and Jesuthas Ajendra. Writing – original draft: Cécile Guth, Marc P. Hübner, and Jesuthas Ajendra. Writing – review and editing: all authors.

Data Availability Statement

Data that support the interpretation of findings presented in this paper are openly available. Inquiries can be directed to the corresponding authors.

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Discussion

Eosinophils play a pivotal role in type 2 immune responses, particularly during helminth infections (27). Traditionally, they have been recognized for their dual capacity to mediate protection against pathogens while simultaneously contributing to immunopathology (21). In filarial infections, like those caused by *L. sigmodontis*, eosinophils are recruited in large numbers to infection sites, where they exert their functions by both cytokine production and direct effector mechanisms (63,128).

The results of my thesis underscore the centrality of eosinophils not merely as terminal effector cells but also as crucial initiators and modulators of type 2 immunity. Building on findings that eosinophils produce IL-4 upon interaction with helminth-derived stimuli, we extend the understanding of eosinophil-derived IL-4 as a pivotal endogenous signal for T helper 2 (Th2) polarization. In particular, Jacobsen et al. (2011) demonstrated that eosinophils are an endogenous source of IL-4 and modulate dendritic cell function, thereby promoting Th2 responses in a pulmonary allergy model (129). Their study showed that eosinophils can directly influence dendritic cell maturation and CD4+ T cell polarization via IL-4, acting as early amplifiers of Th2 immunity. Our data reinforce and expand upon this mechanism by demonstrating that in the absence of eosinophils, as seen in dblGATA mice, the Th2 environment is substantially impaired, highlighting a dependency on eosinophil-derived signals for the full development of a

competent type 2 response during filarial infection. Interestingly, eosinophil function also appears to be temporally dynamic, differing between early and late phases of helminth infection. In the early phase, eosinophils contribute to Th2 priming and cytokine amplification via IL-4 production and interactions with dendritic cells (89,129). In contrast, during the later stages of infection, eosinophils have been shown to participate more in tissue remodeling, regulation of alternatively activated macrophages, and parasite clearance via degranulation or ETosis (42,130). However, the exact mechanisms that initiate IL-4 production in eosinophils remain insufficiently explored. Emerging evidence suggests that eosinophils respond to helminth-derived antigens and alarmins such as IL-33, IL-25, and TSLP, which are released by epithelial cells during infection or tissue damage (89,131). These cytokines may prime eosinophils to produce IL-4 either directly or indirectly via activation of innate lymphoid cells or through interaction with dendritic cells (132,133). Additionally, pattern recognition receptors (PRRs) such as TLR7 and Dectin-1, which are expressed on eosinophils, could contribute to sensing of helminth components and trigger intracellular signaling cascades leading to IL-4 transcription (134). This is of specific interest for this study, as we previously demonstrated that microfilariae trigger eosinophil ETosis via Dectin-1 (75). The specific signaling pathways and transcription factors involved in this process—such as STAT6, GATA-1, or NFAT—remain to be

dissected in more detail and offer a promising direction for future research (135). It is demonstrated that eosinophils serve as a significant source of IL-4 during *L. sigmodontis* infection, contributing to the local type 2 immune milieu. This aligns with previous findings where eosinophils were identified as IL-4 producers in adipose tissue, sustaining alternatively activated macrophages and modulating tissue immune tone (136), indicating a broader immunomodulatory role across multiple disease contexts. In line, *L. sigmodontis* infection led to increased numbers of eosinophils and alternatively activated macrophages in epididymal adipose tissue (EAT), enhancing glucose tolerance in diet-induced obese mice in an eosinophil-dependent manner (126). While *L. sigmodontis* antigen (LsAg) administration did not affect body weight, adipose tissue mass, or adipocyte size, it promoted type 2 immune responses, increased eosinophils, alternatively activated macrophages, and type 2 innate lymphoid cells in EAT. Furthermore, eosinophils may exert distinct functional roles depending on the anatomical site of infection or inflammation. For example, in the lungs, eosinophils are key mediators of Th2 immunity and allergic inflammation, whereas in adipose tissue, they support metabolic homeostasis by maintaining alternatively activated macrophages and contributing to insulin sensitivity (136,137). Our findings in the epididymal adipose tissue (EAT) following *L. sigmodontis* infection confirm this metabolic relevance and

highlight eosinophils' multifaceted roles beyond classical immune defense.

These observations raise the intriguing possibility of therapeutically harnessing eosinophil functions in non-infectious human diseases. In metabolic syndrome or type 2 diabetes, strategies that restore or mimic eosinophil-derived IL-4 and type 2 immunity could promote adipose tissue homeostasis and improve glucose tolerance (137,138). Given the increasing burden of obesity-related inflammation, eosinophils may represent an underexplored therapeutic axis for metabolic reprogramming.

Interestingly, eosinophil-derived IL-4 appears to be non-redundant compared to other IL-4-producing cells, such as basophils, Th2 cells themselves, or ILC2s. Although all these cell types contribute to the type 2 cytokine milieu, eosinophils seem to be particularly important in the early amplification of the response. For instance, while ILC2s are potent producers of IL-5 and IL-13, they do not appear to compensate for the lack of eosinophil-derived IL-4 in dblGATA mice, underlining the unique contribution of eosinophils to Th2 polarization. Moreover, eosinophils' localization within tissues and their capacity to engage in ETosis might provide an additional microenvironmental context for their cytokine secretion, potentially creating localized cytokine gradients that direct T cell differentiation (133,139). PCR array analysis further showed that LsAg treatment reduced inflammatory responses and

enhanced the expression of genes involved in insulin signaling and fatty acid uptake. These findings suggest that *L. sigmodontis* infection and LsAg administration mitigate diet-induced EAT inflammation and improve glucose tolerance, indicating that helminth-derived products could represent novel strategies for enhancing eosinophil-driven insulin sensitivity (140,141).

The critical impact of eosinophil-derived IL-4 became evident in eosinophil-deficient dblGATA mice, where impaired Th2 polarization was observed, reflected by decreased numbers of CD4+ T cells and diminished IL-5 and IL-13 levels. This corroborates the findings of Jacobsen et al. (2011), who demonstrated that eosinophils are a physiologically relevant source of IL-4 during allergic immune responses and play a key role in shaping the Th2 environment essential for effective immunity. While previous studies, including own studies with *L. sigmodontis*, have shown that alternative activation of macrophages can be maintained independent of eosinophils (137,142,143), our recent results reveal that Th2 cell maintenance critically relies on eosinophil-derived cytokines during the middle phase of infection.

It remains unclear whether helminths actively suppress eosinophil IL-4 production as an immune evasion mechanism. While the secretion of DNases by helminths such as *N. brasiliensis* to degrade extracellular traps is well-documented, similar inhibitory strategies targeting IL-4 production pathways

could exist but remain unexplored. Parasite-derived immunomodulators may interfere with transcriptional regulation or epigenetic control of cytokine genes within eosinophils, or modulate key parts of the signaling cascade, such as the IL-33 receptor ST2 or TSLP receptor pathways. Understanding how helminths may manipulate eosinophil IL-4 production specifically could identify novel parasite-host checkpoints exploitable for therapy (144,145). This distinction is important as it underscores the eosinophils' role as "early Th2 amplifiers", supporting the establishment and stabilization of type 2 responses rather than simply participating in terminal effector functions. Notably, Jacobsen et al. (2011) demonstrated that eosinophil-derived IL-4 was active early in the immune cascade, indicating a priming role for eosinophils in Th2 commitment. Moreover, Wu et al. (2011) provided complementary evidence by showing that eosinophil-derived IL-4 in adipose tissue sustains alternatively activated macrophages and modulates immune homeostasis, reinforcing the notion of eosinophils as initiators of tissue-specific type 2 responses beyond infection contexts (136).

These insights emphasize that eosinophils act not only as cytokine sources but also as cellular platforms that facilitate durable Th2 programming during helminth infection.

Moreover, the current data emphasize the complexity of eosinophil-mediated immunity, particularly in relation to ETosis - the release of extracellular DNA traps. ETosis by eosinophils

shares mechanistic similarities with NETosis by neutrophils, involving nuclear decondensation and expulsion of DNA into the extracellular space (103,146). However, eosinophil ETosis is also distinct since it often incorporates mitochondrial DNA and is tightly associated with the concomitant release of toxic granule proteins (95). The release of granules contents, such as MBP, EPO, ECP, and EDN, onto these DNA traps enhances their cytotoxic potential, enabling efficient pathogen immobilization and killing.

In current experiments, the absence of MBP or EPO significantly impaired the ability of eosinophils to immobilize MF. MBP, a highly cationic protein, is known to disrupt cell membranes, while EPO catalyzes the formation of reactive oxygen species that are toxic for parasites (68). Interestingly, the observation that ETosis-mediated microfilarial killing still occurred—albeit at reduced efficiency—in the absence of either MBP or EPO, suggests that the cytotoxic activity of eosinophils does not rely on a single effector molecule. Instead, this points toward a functional redundancy among granule proteins, where other components such as eosinophil cationic protein (ECP) or eosinophil-derived neurotoxin (EDN), both of which possess cytotoxic and ribonucleolytic activity, may partially compensate for the loss of MBP or EPO (68).

The redundant activity among eosinophil granule proteins may reflect evolutionary pressures to ensure the robustness of eosinophil effector functions against a broad range of

pathogens. Such a multilayered defense system would prevent pathogens from evading eosinophil attacks through single-target mutations. Future studies dissecting the individual and combined roles of MBP, EPO, ECP, and EDN using knockout models could shed light on their relative contributions to parasite killing. It should be noted that in vitro assays were carried out in the current study, where only eosinophils were used, which does not reflect the in vivo situation with its complex interactions between various cell types in different local milieus. Obviously, in vivo, other immune cells would help to eliminate MF. For example, in blood, non-motile MF would be removed faster from the spleen. In the *L. sigmodontis* mouse model, it has already been shown that splenectomy leads to a significantly prolonged microfilaremia (147).
The functional importance of eosinophils is particularly evident when comparing the course of *L. sigmodontis* infection in laboratory mice and cotton rats. Laboratory mice exhibit an initial robust type 2 immune response with effective eosinophil-mediated microfilarial immobilization and killing. In contrast, in cotton rats, which serve as the natural host for *L. sigmodontis*, eosinophils seem to have a reduced capacity for ETosis and fail to effectively immobilize MF. This suggests that the parasite has evolved mechanisms to specifically counteract eosinophil effector functions in its natural host (113).
One leading hypothesis is that *L. sigmodontis* MF secrete DNases capable of degrading extracellular traps. Analogous

findings come from *Nippostrongylus brasiliensis*, where parasite-derived DNases were shown to digest neutrophil extracellular traps, thereby facilitating larval migration and evasion of host immunity (144). From a mechanistic perspective, the failure of cotton rat eosinophils to perform ETosis upon microfilarial exposure could result from either parasite-derived inhibitors of ETosis-related pathways or from intrinsic host species-specific differences in eosinophil activation thresholds. Potential parasite-derived factors include not only DNases but also secreted protease inhibitors, antioxidants, or other immunomodulatory proteins that interfere with eosinophil signaling cascades. Alternatively, cotton rat eosinophils may express lower levels of pattern recognition receptors (PRRs) involved in ETosis initiation, such as Dectin-1 or TLRs, which are known to participate in trap formation (146). Comparative analyses of eosinophil transcriptomes and proteomes between laboratory mice and cotton rats could provide insights into the molecular basis of the observed differences in ETosis capacity. However, they are technically demanding due to the lack of cotton rat specific antibodies and lack of cross-reactivity of existing antibodies.

Furthermore, functional assays assessing DNase activity in microfilarial excretory-secretory products from *L. sigmodontis* would be crucial to substantiate the hypothesis of parasite-mediated trap degradation.

If *L. sigmodontis* employs a similar strategy, this would represent a highly adapted immune evasion mechanism, specifically fine-tuned to its natural host environment. Supporting this hypothesis, patency duration and MF loads are markedly higher in cotton rats compared to mice. The longer persistence of adult worms and circulating MF implies that the parasite not only evades immediate immune destruction but may also actively manipulate the host immune environment to favor its survival and transmission. In turn, the host may have evolved tolerance mechanisms to limit immunopathology despite persistent infection, a phenomenon commonly observed in chronic helminth infections (42). Further comparative studies assessing PRR expression profiles, reactive oxygen species production, and degranulation capacity between murine and cotton rat eosinophils would be essential to elucidate the mechanisms underlying this functional disparity. Such investigations could uncover new aspects of host-parasite coevolution and provide insights into why certain hosts develop effective immunity while others tolerate chronic infection.

These findings mirror observations in other filarial infections. For instance, *B. malayi* infections in rodent models elicit stronger and more effective immune responses compared to natural infections in humans, where parasites often establish long-term persistence with minimal pathology (148). Similarly, *O. volvulus* infections in humans display a remarkable balance between host immune control and parasite survival, with

pathology often linked to immune-mediated responses against dying MF rather than live worms (149).

Alternatively, cotton rat eosinophils may have an altered intrinsic effect towards a defect, in terms of their response to MF. Thus, the cotton rat's eosinophils may have undergone host-specific adaptations that render them less responsive to MF, potentially as a trade-off to minimize immunopathology during chronic infection. Such adaptations could mirror evolutionary compromises seen in other chronic helminth infections, where tolerance rather than elimination is favored to prevent host mortality and ensure parasite transmission (42). However, given that cotton rat eosinophils responded to positive controls such as PMA and ionomcyin indicate that per se, they can undergo ETosis. Future studies should analyze whether other ETosis inducers, such as *Candida albicans* or bacteria, trigger ETosis in cotton rats to further clarify this aspect. Additional analysis of the signaling pathways of cotton rat eosinophils stimulated with MF could further improve our understanding of ETosis in natural and experimental hosts.

The broader implications of these findings are significant. They highlight that studying helminth-host interactions exclusively in laboratory models may overlook critical aspects of natural infection dynamics. Parasites that have co-evolved with their hosts likely possess sophisticated immune evasion strategies that are absent or ineffective in non-natural hosts. Indeed, discrepancies between experimental models and natural

infections have been noted for other filarial parasites, such as *B. malayi* and *O. volvulus* (150–152).

Understanding these differences is not merely academic but has direct translational relevance. If parasite-mediated suppression of ETosis contributes substantially to filarial persistence, then strategies aimed at enhancing extracellular trap formation or preventing their degradation could improve parasite clearance. For example, inhibitors targeting parasite DNases or selectively increasing host ROS production in eosinophils could enhance natural immune mechanisms without exacerbating the immunopathology that can be caused by eosinophil dysregulation. The concept of therapeutically influencing eosinophil functions must therefore be taken with a grain of salt, as otherwise eosinophils may not only contribute to parasite killing but also cause increased tissue pathology.

Furthermore, vaccines or therapies developed based on rodent models may fail if they do not account for the immune evasion strategies employed by parasites in their natural hosts. Thus, the cotton rat – *L. sigmodontis* system offers a unique opportunity to study immune evasion in a biologically relevant context, with potential lessons for human filarial diseases such as lymphatic filariasis and onchocerciasis.

In human onchocerciasis, eosinophil-driven inflammation is a major contributor to skin and ocular lesions caused by the death of MF (146), for example following DEC treatment. However, depletion of IL-5 by antibodies in humans, reduced eosinophil

numbers and their migration into tissues, but did not reduce the side effects of DEC treatment. This highlights the difficulties of therapeutic immunomodulation, which requires precise tuning to enhance protective responses while minimizing collateral tissue damage (153).

The main granule proteins involved in eosinophil responses are MBP, ECP, EDN, and EPO (21). MBP is a highly cationic protein known for its strong cytotoxic activity against parasites (154). However, it also triggers mast cell and basophil degranulation, amplifying local inflammation. In onchocerciasis, MBP has been detected on the surface of MF following DEC treatment, indicating its involvement in the inflammatory response to dying parasites (118). ECP, another granule protein, has ribonuclease activity and is capable of disrupting cell membranes, contributing to tissue damage (155). It is significantly elevated in hyperreactive onchocerciasis patients, who suffer from severe dermatitis due to the efficient removal of MF from the skin, suggesting a direct eosinophil-mediated inflammatory response (149). EDN, with its neurotoxic properties, also plays a role in tissue damage during eosinophil activation. Elevated levels of EDN have been observed in patients with tropical pulmonary eosinophilia, a severe manifestation of lymphatic filariasis patients marked by intense eosinophilic infiltration and inflammation in the lungs (85,156). Similarly, EPO, which catalyzes the formation of ROS, can inflict damage not only on MF but also on surrounding host tissues,

contributing to the pathology observed in chronic infections (21,31).

Future work should also investigate whether similar evasion mechanisms exist in human filarial infections such as *L. loa*, *M. perstans*, and *O. volvulus*. This topic is currently investigated in our EOSNET study in collaboration with Prof. Wanji in Cameroon. Here, we intend to analyze whether different filarial species induce different eosinophil responses in the field and whether these are dependent on *Wolbachia* specimen (not present in *Loa Loa*) or related to pathologic alterations in the patients (onchocerciasis patients with and without pathology). We also want to clarify whether *Mansonella* MF fail to induce ETosis, since mansonellosis patients normally do not develop any pathology and whether the death of MF species with pathologic impact (*O. volvulus*) induce stronger eosinophil responses than others, as we have already stated for *L. sigmodontis* (21).

Although direct evidence is currently limited, clinical observations suggest that MF of *Loa loa* and *M. perstans* can persist for years in the human host, thereby driving minimal inflammation, indicating effective immune modulation (157,158). Whether suppression of ETosis plays a role in this context remains an open and intriguing question.

In addition to eosinophils, neutrophils have emerged as important players during helminth infections. Recent studies show that neutrophils can produce IL-4 and contribute to type 2

immunity (144). However, their role appears distinct from that of eosinophils, being more prominent during early infection phases and more geared towards initial parasite containment rather than sustained Th2 amplification. Whether neutrophil-derived IL-4 can compensate for eosinophil-derived IL-4 in settings of eosinophil deficiency remains to be investigated, however, preliminary data suggest only partial redundancy (159).

In summary, our findings provide compelling evidence for the multifaceted roles of eosinophils during filarial infections. Beyond their classical function as cytotoxic effector cells, eosinophils emerge as critical immunoregulatory players, particularly through their ability to produce IL-4 and sustain type 2 immune responses. This dual role is essential for the initial control of filarial infections, as demonstrated by the impaired Th2 polarization and enhanced parasite burden observed in eosinophil-deficient models (63,65).

However, several limitations of the current study should be pointed out. First, while the use of dblGATA mice is invaluable for investigating the role of eosinophils, it does not allow for the targeted investigation of single specific eosinophil-derived factors, such as IL-4, nor does it allow for the investigation of a lack of eosinophils at a particular and restricted time point during infection. Future studies employing conditional knockout models targeting IL-4 specifically in eosinophils would provide a more nuanced understanding on the contributions of single

cytokines to infection outcome. Moreover, while in vitro assays of ETosis and microfilarial killing provide important mechanistic insights, in vivo confirmation of these processes is necessary to fully appreciate their relevance in the complex milieu of host-parasite interactions.

Another important consideration addresses the potential tissue-specific variation in eosinophil functions. While the current study primarily focused on the pleural cavity, eosinophil responses in other tissues like the skin, lungs, and lymphatics may differ significantly. Given the tissue tropism of different filarial species, investigating eosinophil functions across multiple anatomical sites will be critical for comprehensive understanding of their roles in filarial infections.

Looking forward, several promising avenues for future research emerge from our findings. Comparative transcriptomic and proteomic analyses of eosinophils from laboratory mice and cotton rats could elucidate the molecular basis of the observed functional differences in ETosis capacity. Furthermore, the identification and characterization of potential parasite-derived DNases or other ETosis-inhibiting molecules might reveal novel targets for therapeutic intervention. Moreover, exploring the induction and functionality of ETosis in human eosinophils during natural filarial infections could provide critical translational insights.

In the context of vaccine and immunotherapy development, strategies aimed at enhancing eosinophil-mediated effector

functions must carefully balance the promotion of parasite clearance with the risk of exacerbating immunopathology. Targeted modulation of eosinophil activation, for example by selectively enhancing their cytotoxic functions while limiting pro-fibrotic activities, could represent a promising approach. Similarly, interventions that preserve the protective aspects of type 2 immunity without tipping the balance toward chronic inflammation or tissue damage would be desirable.

Ultimately, the current study contributes to a growing body of evidence highlighting the complexity and sophistication of host-parasite interactions in filarial infections. Eosinophils, long regarded as simple effectors of helminth immunity, nowadays reveal as versatile and dynamic players whose functions are finely tuned by the evolutionary pressures exerted by chronic parasitic infections. A deeper understanding of these processes holds promise not only for improving therapeutic outcomes in filarial diseases but also for informing broader immunological paradigms of type 2 immunity, chronic infection, and immune regulation.

Zusammenfassung

In der vorliegenden Doktorarbeit wurde die Rolle von eosinophilen Effektorproteinen bei Filarieninfektionen untersucht. Dabei lag der Schwerpunkt darauf, wie Eosinophile zur Immobilisierung und Eliminierung von MF beitragen, und zwar sowohl durch die Bildung extrazellulärer DNA-Netze (ETosis) als auch durch die Freisetzung von Granulaproteinen – insbesondere MBP und EPO. Anhand von Mausmodellen, darunter KO-Mäuse für MBP und EPO, und Vergleichen mit natürlichen Wirten (Baumwollratten) konnte gezeigt werden, dass diese Proteine für die Hemmung der MF-Motilität von entscheidender Bedeutung sind und maßgeblich zur eosinophil-vermittelten Parasitenbekämpfung beitragen.

Ein weiterer Schwerpunkt meiner Arbeit lag auf der immunmodulatorischen Funktion von Eosinophilen, insbesondere durch die Produktion von IL-4. Die Studie zeigte, dass Eosinophile eine essentielle und frühe Quelle für IL-4 während der Infektion mit *L. sigmodontis* darstellen und somit als potente Initiatoren und Verstärker von Typ-2-Immunantworten fungieren. Zwar konnte die alternative Makrophagenaktivierung auch ohne Eosinophile aufrechterhalten werden, doch war ihre Anwesenheit entscheidend für eine effektive Th2-Polarisierung, wie die verringerte Zahl an $CD4^+$-T-Zellen und niedrigere Spiegel von IL-5 und IL-13 in eosinophilen-defizienten dblGATA-Mäusen belegen. Dies unterstreicht die nicht redundante Rolle von

durch Eosinophilen produziertem IL-4 bei der Etablierung eines funktionellen Th2-Milieus, die nicht durch andere IL-4-produzierende Zellen wie ILC2 oder Th2-Zellen kompensiert werden konnte. Darüber hinaus befasste sich diese Arbeit mit möglichen Mechanismen der IL-4-Induktion in Eosinophilen und wies auf epitheliale Alarmine (IL-33, IL-25, TSLP) und Helminth-Komponenten, die über PRRs (z. B. TLR7, Dectin-1) wahrgenommen werden, als potenzielle Auslöser hin. Transkriptionsregulatoren wie STAT6, GATA-1 und NFAT wurden als Kandidaten für die Regulation der IL-4-Produktion in Eosinophilen identifiziert, wobei diese Mechanismen noch weiter aufgeklärt werden müssen.

Ein interessanter Aspekt der Studie war der Vergleich zwischen Labormäusen und dem natürlichen Endwirt, der Baumwollratte (*Sigmodon hispidus*). Es wurde festgestellt, dass die Eosinophilen der Baumwollratte weniger effektiv bei der Immobilisierung von MF sind als die der Maus. Diese Beobachtung deutet auf mögliche wirtspezifische Anpassungen des Parasiten hin, die möglicherweise Immunausweichstrategien gegen die Funktion von Eosinophilen beinhalten, wie die Unterdrückung der IL-4-Produktion oder die Resistenz gegen ETosis. Schließlich wurden die translationalen Implikationen dieser Ergebnisse durch weitere Ergebnisse der Arbeitsgruppe untermauert, die zeigen, dass die durch Eosinophile gesteuerte Typ-2-Immunität positive metabolische Effekte haben kann. Eine Infektion mit *L.*

sigmodontis und die Verabreichung von LsAg verbesserten die Glukosetoleranz und Insulinsensitivität bei ernährungsbedingt fettleibigen Mäusen in einer Eosinophil-abhängigen Weise, insbesondere im Fettgewebe, wo Eosinophile die Aufrechterhaltung alternativ aktivierter Makrophagen unterstützen.

Zusammenfassend liefert die vorliegende Dissertation wichtige neue Erkenntnisse über die Doppelrolle der Eosinophilen: Einerseits fungieren sie als direkte Effektorzellen, die über ETosis und zytotoxische Granulatproteine (MBP, EPO) zur Parasitenbekämpfung beitragen, andererseits treten sie durch ihre frühe und lokalisierte Produktion von IL-4 als wichtige immunregulatorische Zellen in Erscheinung, die die adaptive Th2-Reaktion prägen. Diese Erkenntnisse tragen zu einem besseren Verständnis des komplexen Wechselspiels zwischen Wirt und Parasit bei und könnten in Zukunft neue therapeutische Ansätze gegen Filarieninfektionen ermöglichen.

Summary

In the current work, the role of eosinophil effector proteins in filarial infections was investigated. It focused on how eosinophils contribute to the immobilization and elimination of MF through both the formation of extracellular DNA traps (ETosis) and the release of granule proteins – in particular MBP and EPO. Using mouse models, including KO mice for MBP and EPO, and comparisons with natural hosts (cotton rats), it was shown that these proteins are crucial for inhibiting MF motility and contribute significantly to eosinophil-mediated parasite control.

Another focus of the current work was the immunomodulatory function of eosinophils, particularly through the production of IL-4. The study demonstrated that eosinophils represent an essential and early source of IL-4 during infection with *L. sigmodontis*, thereby acting as potent initiators and amplifiers of type 2 immune responses. Although alternative macrophage activation could be maintained without eosinophils, their presence was crucial for effective Th2 polarisation, as evidenced by the reduced number of $CD4^+$ T cells and lower levels of IL-5 and IL-13 in eosinophil-deficient dblGATA mice. This highlights the non-redundant role of eosinophil-derived IL-4 in the establishment of a functional Th2 milieu, which could not be compensated by other IL-4-producing cells such as ILC2s or Th2 cells.

In addition, this work addressed possible mechanisms of IL-4 induction in eosinophils, pointing to epithelial-derived alarmins (IL-33, IL-25, TSLP) and helminth components sensed via PRRs (e.g. TLR7, Dectin-1) as potential triggers. Transcriptional regulators such as STAT6, GATA-1 and NFAT were identified as candidates in the regulation of IL-4 production in eosinophils, although these mechanisms require further elucidation.

One interesting aspect of the study was the comparison of *L. sigmodontis* infections in a mouse model and the natural host, the cotton rat (*Sigmodon hispidus*). It was found that eosinophils of cotton rats are less effective in immobilizing MF than those of mice. This observation suggests host-specific adaptations of the parasite, potentially involving immune evasion strategies targeting eosinophil function, such as suppression of IL-4 production or resistance to ETosis.

Finally, the translational implications of these results were underpinned by further findings of the working group showing that eosinophil-driven type 2 immunity can exert beneficial metabolic effects. *L. sigmodontis* infection and LsAg administration improved glucose tolerance and insulin sensitivity in diet-induced obese mice in an eosinophil-dependent manner, especially in adipose tissue, where eosinophils support the maintenance of alternatively activated macrophages.

In summary, the present dissertation provides new insights into the dual role of eosinophils: on the one hand, they act as direct effector cells that contribute to parasite control via ETosis and cytotoxic granule proteins (MBP, EPO) and on the other hand, they emerge as critical immunoregulatory cells through their early and localized production of IL-4, shaping the adaptive Th2 response. These findings contribute to a better understanding of the complex interplay between host and parasite and could enable new therapeutic approaches against filarial infections in the future.

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

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Declaration

I, Pia Philippa Schumacher, hereby declare that I have completed the submitted doctoral thesis independently and without any unauthorized external assistance. The work received only the financial support explicitly mentioned within the thesis. All analyses presented in this doctoral thesis were conducted in accordance with the principles of good scientific practice, as outlined in the statute of Justus Liebig University Giessen for ensuring good scientific conduct. All passages taken directly or analogously from published or unpublished sources have been properly identified and referenced as such, in accordance with academic standards.

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