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DIFFERENTIAL INDUCTION OF TH2- AND TH1-ASSOCIATED RESPONSES BY FILARIAL ANTIGENS AND ENDOSYMBIOTIC *Wolbachia* IN A MURINE MODEL OF RIVER BLINDNESS

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Immune responses to filarial parasites like the river blindness inducing *Onchocerca volvulus* are obscured by combined reactions to the filarial nematodes themselves and their endosymbiont bacteria *Wolbachia*. Overall, infection with filarial nematodes induces a strong Th2 response characterized by IL-5 production and to a lesser degree a Th1 response and IFNγ production. Neutrophil and eosinophil infiltration into the corneal stroma are hallmark features of *Onchocerca volvulus* stimulation in a mouse model of river blindness. To determine the splenic and corneal response to filarial antigens in the absence of *Wolbachia*, C57BL/6 mice were immunized subcutaneously with either endosymbiotic *Wolbachia* alone, a soluble extract from the filaria *Acanthocheilonema viteae* that does not contain *Wolbachia*, or both, and injected into the corneal stroma. Neutrophil and eosinophil infiltration into the cornea was assessed by immunohistochemistry. In addition, Th1- and Th2-associated responses to filaria or *Wolbachia* were investigated by determining IL-5 and IFN-γ production by splenocytes. We found that *A. viteae* in the absence of *Wolbachia* induced IL-5 production and eosinophil infiltration, but not IFN-γ. Conversely, *Wolbachia* induced IFN-γ production and no migration of eosinophils. There was no difference in neutrophil infiltration. Together, these findings demonstrate a distinct Th-associated phenotype induced by filaria and *Wolbachia*.

Keywords: *Onchocerca*, filaria, river blindness, *Wolbachia*, *Acanthocheilonema*, cornea, IFNγ, IL-5, eosinophils, neutrophils

Introduction

Infection with the filarial nematode *Onchocerca volvulus* causes onchocerciasis, a disease characterized by ocular and dermal inflammation, leading to blindness and various forms of skin affection. WHO currently estimates about 37 million people to be infected with *O. volvulus*, mainly in sub-Saharan Africa, with more than 100 million people at risk of infection [1, 2]. There are two divergent types of immune responses: the hyperreactive sowda form with low worm burden due to an overwhelming immune response and strong symptoms, and the hyporesponsive generalized form with high worm burden but few symptoms [3].

Chronic infection with filarial nematodes induces production of IL-5 and IFNγ, and elevated blood eosinophilia. Generalized infection is associated with low levels of IL-5 while putatively immune patients show elevated levels of both IFNγ and mainly IL-5 [4]. Previous studies have demonstrated that IL-5 is predominantly induced during murine filarial infection. Similarly, dead and degenerating microfilariae in the skin of *O. volvulus*-infected individuals after chemotherapy are associated with increase of eosinophil, neutrophil, and macrophage numbers indicating a mixed Th1/Th2 response [4]. As filarial nematodes contain endosymbiotic *Wolbachia* bacteria that are associated with a pro-inflammatory host response, it has been difficult to discern the relative contribution of filarial and bacterial antigens to the host immune response.

We therefore utilized a murine model of ocular onchocerciasis in which mice are immunized subcutaneously and injected into the corneal stroma with either filarial antigens in the absence of *Wolbachia* or with isolated *Wolbachia*, or a combination of both. Previous studies showed that following immunization with *O. volvulus* extract (containing *Wolbachia* bacteria), mice develop an adaptive immune response characterized by high levels of IL-5 production and lower levels of IFNγ, and a predominant IgG1 antibody response. Injections of worm extract into the cornea then induce an early neutrophil infiltration at 24 h followed at 72 h by a predominant eosinophil infiltration [5]. In the current study, we used the *Wolbachia*-free filarial worm *Acanthocheilonema viteae* [6] as source for filarial extracts and insect-cell-derived *Wolbachia*.

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Methods

Parasites and bacteria

A. viteae worms were purchased from TRS laboratories (Athens, GA, United States), and worm extract was prepared as described previously [7]. Briefly, worms were washed extensively, kept in culture for 14 days in antibiotic-supplemented medium, and the absence of bacterial growth was monitored. Worms were then homogenized in HBSS, sonicated, the insoluble fraction was removed by centrifugation, and the soluble fraction was used for all studies.

Aa23 insect cells containing *Wolbachia* were provided by Dr. Mark Taylor (Liverpool School of Tropical Medicine, Great Britain). Cells were grown at 27°C with no CO_2 in 50% Mitsuhashi Maramorosh/50% Schneider's Insect Cell Medium, supplemented with 10% insect cell grade FBS and 1% Pen/Strep. Cells were harvested and sonicated twice for 1 min. Cells were then centrifuged at 600 *g* for 5 min. Supernatants were pooled and centrifuged again for a total of five times. Finally, the supernatants were centrifuged at 12,100 *g* for 10 min, and the pellet containing *Wolbachia* was resuspended in HBSS.

Mice

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, United States) and kept under pathogen-free conditions at the local animal facility. Mice were immunized subcutaneously three times at weekly intervals with either 10 μg *A. viteae* in squalene adjuvant [7], *Wolbachia*, or with *A. viteae* and *Wolbachia*. Squalene adjuvant contained 10% squalene (Sigma Aldrich, St. Louis, MO, United States), 0.4% Tween 80 (Sigma Aldrich, St. Louis, United States) and 1% Pluronic L (BASF, Ludwigshafen, Germany) in PBS. One week after the last immunization, corneas were injected with *A. viteae*, *Wolbachia*, or a mixture of both [8, 9].

Immunohistochemistry

After 24 h, mice were sacrificed, eyes were snap frozen in OCT, and 5 μm sections were stained for neutrophils using NIMP/R14 or for eosinophils using anti-murine MBP (JJ Lee, Mayo Clinic AZ, United States). Cells were directly counted by fluorescence microscopy.

ELISA

Spleens from immunized mice were removed following three subcutaneous immunizations in weekly intervals. Single-cell suspensions were prepared and stimulated in triplicate with *A. viteae*, *Wolbachia*, *A. viteae* + *Wolbachia*, or anti-CD3 as positive control for 72 h at 37 °C. IL-5 and IFNγ production was measured by ELISA (R&D Systems). Limit of detection was 30 pg/ml for IL-5 and 10 pg/ml for IFNγ.

Statistics

GraphPad Prism™ was used to analyze the data by Student's *t*-test, and $p < 0.05$ was considered significant.

Results

Eosinophilia is a hallmark feature of helminth infection, and these cells migrate to the corneal stroma in a murine model of ocular onchocerciasis using *O. volvulus* soluble extracts containing *Wolbachia* [5]. To determine if *Wolbachia* or filaria are essential for eosinophil infiltration, mice were immunized subcutaneously and injected intrastromally with extract from *A. viteae* worms that naturally do not harbor *Wolbachia*. Additionally, corneas were injected with *Wolbachia* alone or a combination of *A. viteae* and *Wolbachia*. As shown in *Figure 1a*, injection of *A. viteae* and *A. viteae*/*Wolbachia* induces eosinophil infiltration while injection of *Wolbachia* alone does not. When mice were immunized with *Wolbachia* alone, only the intrastromal challenge with *A. viteae*/*Wolbachia* could induce eosinophil infiltration, indicating that *A. viteae* is required for eosinophil migration, but *Wolbachia* is required for generation of an adaptive immune response that allows *A. viteae*-induced eosinophil migration *(Fig. 1b)*. When mice were immunized with a combination of *A. viteae* and *Wolbachia*, filaria and *Wolbachia* could induce eosinophil migration separately, although filariae were required for efficient eosinophil infiltration *(Fig. 1c)*.

Previous studies in our lab showed neutrophil infiltration after intrastromal injection of filarial extract even in the absence of an adaptive immune response. After immunization with *A. viteae*, *Wolbachia*, or *A. viteae* and *Wolbachia*, we show neutrophil infiltration after challenge with any extract. This demonstrates that neutrophil infiltration is indeed independent of immunization status. Neutrophil infiltration is also observed in the presence of filarial extract only as well as in the presence of *Wolbachia* only *(Fig. 1d–f)*.

As eosinophil release from the bone marrow is largely dependent on IL-5, and we found that *A. viteae*, but not *Wolbachia*, is important for eosinophil migration into the corneal stroma, we next examined if *A. viteae* induces IL-5 production by splenocytes in the absence of *Wolbachia*.

Figure 2a shows that IL-5 was produced by splenocytes from *A. viteae*-immunized mice stimulated with *A. viteae* or *A. viteae*/*Wolbachia*. This finding indicates that filarial antigens induce IL-5, and is consistent with *Figure 1a* showing eosinophils in the corneas of these mice. Surprisingly, we found that spleen cells from *Wolbachia*-immunized mice that were stimulated *in vitro* with *Wolbachia* only also produced IL-5.

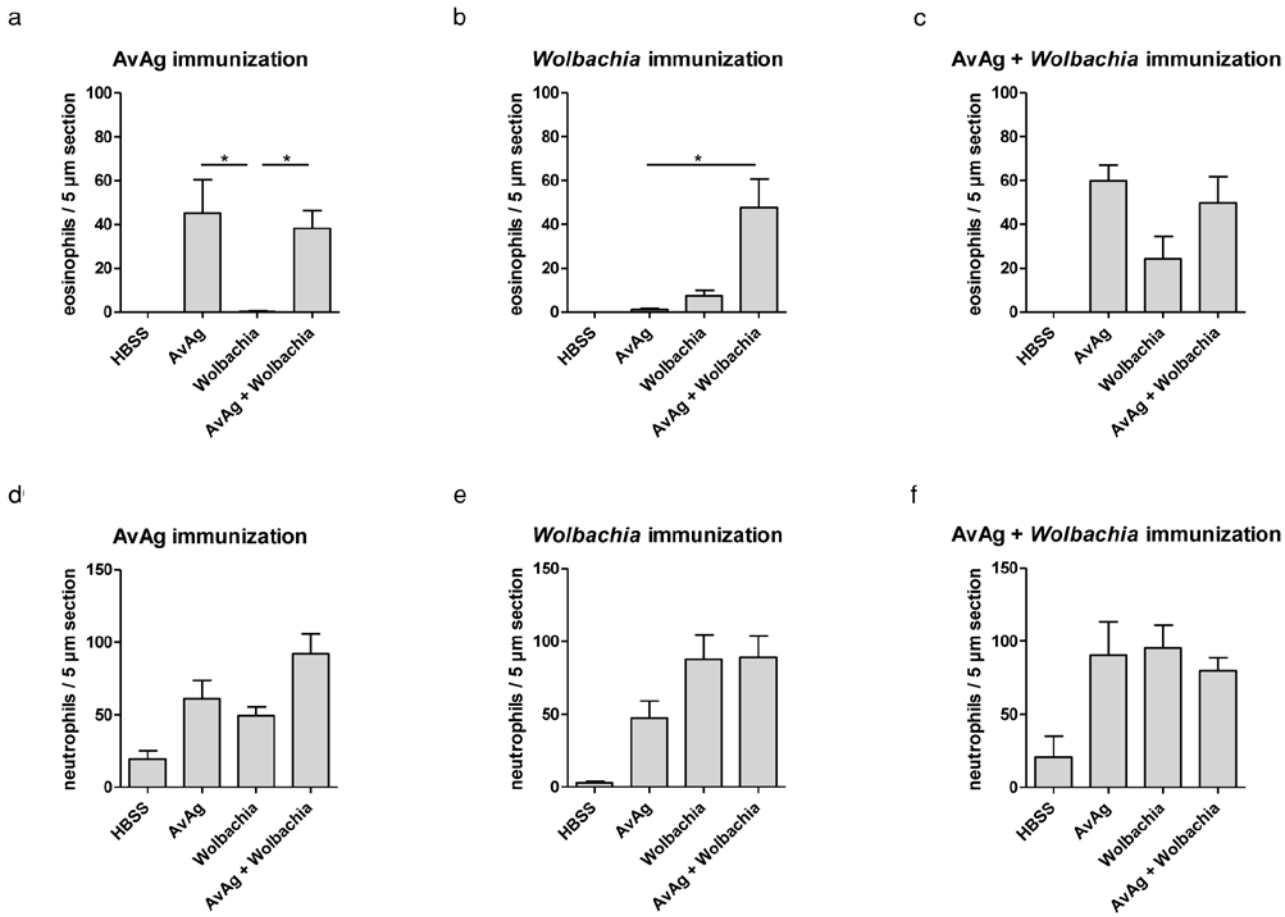

Fig. 1. C57BL/6 mice were immunized with AvAg (a, d), *Wolbachia* (b, e), or a mixture of AvAg and *Wolbachia* (c, f). Corneas were injected with HBSS as negative control, AvAg, *Wolbachia*, or AvAg + *Wolbachia*. Eosinophil (a–c) and neutrophil (d–f) infiltrations were determined 24 h post injection. The *X*-axis indicates the agent used for intrastromal injections. Data shown are combined results of two experiments as mean ± SEM with two corneas for HBSS injection, six corneas for AvAg and *Wolbachia* injection, and seven corneas for AvAg + *Wolbachia*. Statistical testing used the Kruskal-Wallis test followed by Dunn's multiple comparison test. * indicates $p<0.05$

Mice co-immunized with *A. viteae*/*Wolbachia* produced IL-5 after *in vitro* stimulation with either *A. viteae* or *Wolbachia* alone, or both. Together, these results demonstrate that for the *in vitro* production of IL-5, the presence of the same antigens during priming and challenge is required.

In contrast to IL-5 production by *A. viteae*-immunized and stimulated samples, these cells did not produce IFNγ *(Fig. 2b)*. Also, IFNγ was not induced by *A. viteae* stimulation in splenocytes from *Wolbachia* immunized mice. IFNγ production was not induced in the converse experiment, with *A. viteae*-immunized and *Wolbachia*-stimulated cells. Even splenocytes from double-immunized mice did not induce IFNγ when *A. viteae* only was used as *in vitro* stimulus. These findings indicate that *A. viteae* does not induce IFNγ when used in either priming or recall stimulation. Conversely, *Wolbachia* induced IFNγ when used for priming and stimulation. We conclude that *Wolbachia* is primarily required for IFNγ production by splenocytes, whereas *A. viteae* induces IL-5 but not IFNγ production.

Of note, IFNγ levels in these experiments were higher than IL-5 levels following splenocyte stimulation. We hypothesize that this is due to adjuvant-mediated Th1 induction during the immunization phase.

Discussion

Onchocerciasis patients experience severe skin disease and visual impairment, often resulting in blindness. Studying human onchocerciasis is mostly limited to the analysis of activation of peripheral blood mononuclear cells or skin responsiveness, and the analysis of onchocercal nodules. Ocular studies in human patients are scarce. Our laboratory previously established a mouse model of *Onchocerca* keratitis where repeat injections of filarial antigens induce the generation of an adaptive immune response similar to that of patients with generalized onchocerciasis [10]. The subsequent injection of filarial antigens into the corneal stroma induces migration of neutrophils and eosinophils, with lower numbers of macrophages and T cells infiltrating the corneal stroma. Homologies to human onchocercal keratitis may only be hypothesized as human corneal samples for the investigation of pathology are scarce for obvious reasons.

Previous studies using this murine model of *Onchocerca* keratitis demonstrated that (1) there is not only a predominant Th2 response, with elevated IL-4, IL-5, IgE, IgG1, and eosinophilia, but also an induction of IFNγ [10]; (2) the presence of endosymbiotic *Wolbachia* is essential for neutrophil recruitment to the corneal stroma and development of corneal haze [7]; (3) *Wolbachia*-induced corneal inflammation is TLR2 and MyD88 dependent [11, 12]; and (4) dendritic cell activation and IFNγ production are dependent on TLR2, whereas Th2-associated responses (IL-5 production, eosinophil infiltration into the cornea) are TLR2 independent [13]. These findings are consistent with the hypothesis that *Wolbachia* induce Th1-associated responses and neutrophil recruitment through TLR2/MyD88-dependent responses, whereas Th2 responses are *Wolbachia* independent and may be induced directly by filarial antigens. The relative contribution of filaria and *Wolbachia* in the generation of adaptive immune responses was investigated in this study. To address the relative role of bacterial vs. filarial antigens in the generation of immune responses, we immunized wildtype C57BL/6 mice subcutaneously and injected the corneal stroma with *Wolbachia* alone, filarial antigens in the absence of *Wolbachia* (*A. viteae*), or a combination of both antigens.

Results from the current study show that production of the Th1 cytokine IFNγ completely depends on the presence of *Wolbachia*. *Wolbachia* are required during the priming and challenging phase. Stimulation with filarial proteins alone (*A. viteae* extract) was not sufficient for IFNγ induction. This confirms the hypothesis that Th1 responses are primarily *Wolbachia* and thus TLR2 dependent [13].

The major transcription factor regulating the generation of IFNγ-producing Th1 cells is T-box expressed in T cells (T-bet) [14] whereas Th1 induction is strongly down-regulated by the expression of GATA-3 [15, 16]. It was found in filaria-infected patients that T-bet expression in T cells is down-regulated following stimulation with filarial proteins and subsequently decreased IFNγ production [17]. In contrast, stimulation with live larvae decreased the expression of both T-bet and GATA-3 as well as production of Th1 and Th2 cytokines [18]. Expression of T-bet also negatively regulates generation of Th17 cells as shown by overwhelming IL-17 production in T-bet$^{-/-}$ mice following immunization with soluble schistosome egg antigen [19]. IL-17 expression was also found increased in filarial-infected patients compared with uninfected patients [20]; however, the role of IL17 was not subject of this study.

TLRs and T-cell responses are also linked by thymic stromal lymphopoietin (TSLP). Activation of dendritic cells via TLRs usually results in IL-12 production and induction of Th1 responses by T cells [21]. However, if dendritic cells are activated by TSLP, Th2 responses are induced as indicated by the production of Eotaxin-2, TARC, and MDC, and by the lack of IFNγ and IL-12 production [22], although the authors refer to those Th2 cells as inflammatory Th2 cells due to their production of TNFα and their lack in producing IL-10. TSLP can be induced

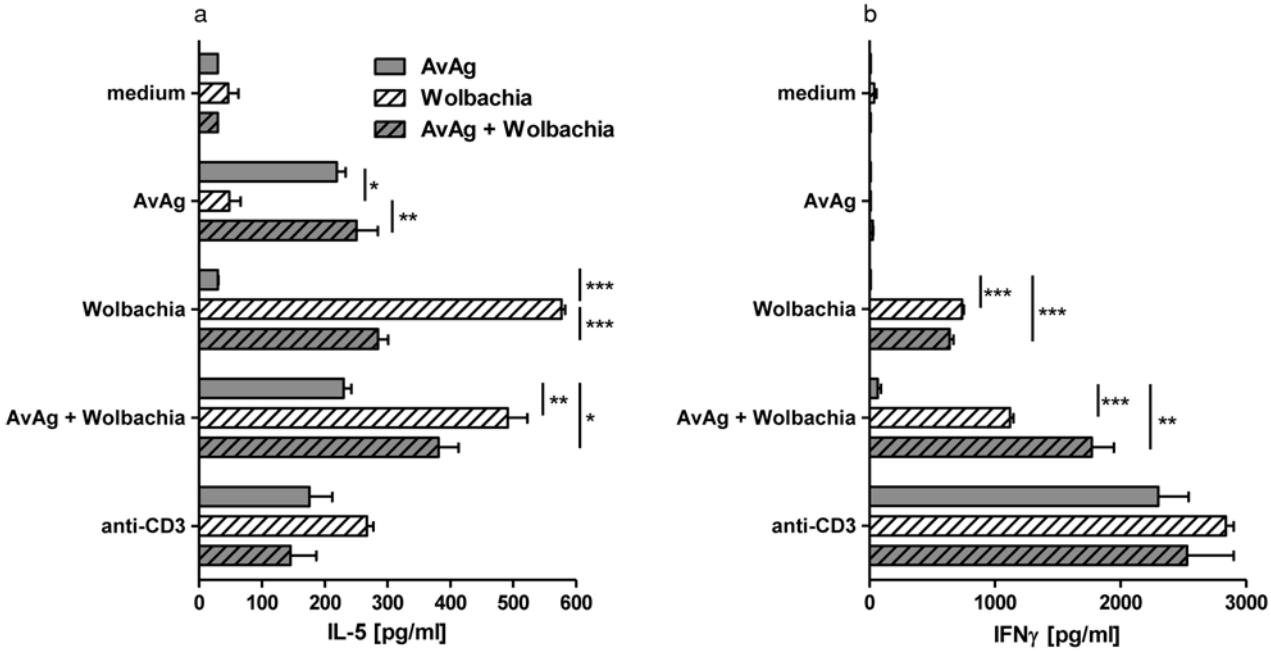

Fig. 2. C57BL/6 mice were immunized with AvAg, *Wolbachia*, or a mixture of AvAg and *Wolbachia*, and single cell splenocyte cultures were stimulated with medium, AvAg, *Wolbachia*, AvAg + *Wolbachia*, or anti-CD3. IL-5 and IFNγ levels were determined by ELISA. Limit of detection was 10 pg/ml for IFNγ and 30 pg/ml for IL-5. Data shown are representative of two experiments with three mice per group and is shown as mean ± SEM. *, **, *** represent statistical significance as calculated by the student's t-test corresponding to $p<0.05$, $p<0.01$ and $p<0.0001$, respectively

by IL-1β and TNFα [23], and the production of those cytokines following stimulation of murine corneal epithelial cells with filarial extracts has been demonstrated [24]. Interestingly, corneal epithelial cells were also shown to produce TSLP in response to the TLR2/6 ligand FSL1 [25]. Thus, filaria-dependent IL-5 induction might occur in a TLR2-independent pathway requiring TSLP; however, *Wolbachia*-induced IL-5 production is more likely to involve TLR2 as this is the major known receptor for these bacteria. To confirm TLR2-dependent IL-5 production, future experiments could use $TLR2^{-/-}$ and $MyD88^{-/-}$ mice in comparison to C57BL/6 mice. The role of TSLP in the induction of Th2 responses might be further investigated by determining TSLP concentration in murine corneal lysates and by blocking TSLP in the cornea by injection of anti-TSLP.

In contrast to IFNγ production only being induced by *Wolbachia*, IL-5 production did require priming, but could be induced by both filaria and *Wolbachia*. The presence of identical antigens was required during priming and challenge stimulation. This is unexpected as the production of IL-5 was previously found to be TLR2 independent and thus thought to be *Wolbachia* independent [13]. This finding might indicate that *Wolbachia* is not only activating the immune system via TLR2, but other Toll-like receptors like TLR4 that have been previously implicated in the pathogenesis of filariasis might also be involved [7, 26].

In addition to this generalized immune response, we also analyzed the local infiltration of neutrophils and eosinophils into the corneal stroma of immunized mice. We found, as has been demonstrated previously, that neutrophil migration does not require immunization [11]. Filarial antigens and *Wolbachia* both efficiently induced neutrophil migration into the corneal stroma, independent of the immunization status. It has been previously shown that not only are *Wolbachia* sufficient for induction of neutrophil migration but also that wolbachial lipoproteins can induce neutrophil infiltration into the corneal stroma [27, 28].

Eosinophil infiltration into the mouse cornea was analyzed following the immunization of mice with different antigens. In contrast to neutrophil infiltration that does not require a filarial-specific adaptive immune response, eosinophil infiltration into the mouse cornea has been shown to only occur following immunization with filarial extracts [10]. This has been demonstrated for *O. volvulus* and *B. malayi* that both contain *Wolbachia* bacteria. It is presumed that the injection of AvAg+ *Wolbachia* induces a similar cellular infiltration into the corneal stroma, thus there is no expected eosinophil infiltration into naive mouse corneas following AvAg, *Wolbachia* or AvAg+ *Wolbachia* injection.

Presence of filarial antigens during priming and challenge sufficed to induce eosinophil migration whereas *Wolbachia* stimulation during priming and challenge did not result in eosinophil infiltration into the corneal stroma. However, when we immunized with *Wolbachia*, there appeared to be initiation of the adaptive immune response, and AvAg + *Wolbachia* injection into the corneal stroma was sufficient to induce eosinophil migration. Even though eosinophil numbers are usually increased during filarial infection, the mechanism by which eosinophils affect parasite survival remains unclear. Eosinophils and IgE antibodies have been implicated in the generation of protective immunity against *O. volvulus* [29]. Similarly, it has been shown in jirds that immunization with *A. viteae* induces eosinophil-mediated protective immunity [30]. This suggests that immunization using wolbachial products is probably much less efficient as it does not affect eosinophils as strongly as filarial proteins do.

Overall, we confirm that filarial worms themselves are important for the induction of an adaptive Th2 immune response characterized by IL-5 production and eosinophil migration whereas *Wolbachia* is required for IFNγ production. Surprisingly, *Wolbachia* could also induce IL-5 production, however, without subsequent eosinophil migration into the cornea. Both filaria and *Wolbachia* suffice to induce neutrophil migration.

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